PROCEEDINGS

22-25
MAY 2022

EURO IX RESIDUE

Veterinary residue control, the beginning of a new era

Hotel **De Ruwenberg**
St. Michielsgestel, the Netherlands

RESIDUES OF VETERINARY DRUGS IN FOOD

Edited bij:
Leendert A. van Ginkel

Proceedings of the EuroResidue IX conference
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22-25 May, 2022

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PREFACE

Two years later than originally planned, the EuroResidue IX conference was organized in May 2022. These proceedings reflect the contributions that were made and will provide the reader with an extensive update on the scientific, technical and legislative developments with respect to the analyses of food of animal origin for the presence of residues of veterinary drugs. As such, this is the next extension of a series of proceedings which started in 1990 at the occasion of the first EuroResidue conference. These proceedings will be complemented by a special issue of the scientific journal: “Food Control” which will contain a selection of peer reviewed publications of manuscripts presented during EuroResidue IX.

As in 1990, the majority of papers is dedicated to analyses, about 65%. The nature of these papers, however, has shifted dramatically. Currently, the majority of methods is based on liquid chromatography combined with mass spectrometry, a technique virtually non-existent in 1990. Was the majority of methods presented in 1990 developed for the detection of one or a small group of compounds, the focus nowadays is on very broad “multi-class” methods frequently suitable for the detection of hundreds of compounds and methods based on omics type of approaches for a more effect-based identification of compounds. These, however, are not the most important changes.

The concern about the safety, wholesomeness and availability of food has increased over the passed decades. Also, the production of food, especially of animal origin, has become a topic of general concern due to its impact of the environment. But, most importantly, the problem of availability of safe and wholesome food for the entire world population has not been solved yet. This results in a more holistic approach to food production. Rightfully, this is also reflected in the different contributions presented during the conference and included in these proceedings. Important topics are the recirculation of residues in the environment and safety studies on new protein sources for future feed and food production.

Over the past decades there has been a close cooperation between the organizers of the EuroResidue conferences and the Belgium conferences on “Veterinary Drug Residues in Food”, resulting in an alternating series of bi-annual conferences in The Netherlands and Belgium. This cooperation was further extended by the merger of the respective Scientific Committees into a single SC responsible for the programming of both conferences.

For EuroResidue this conference was the last conference in its current format. However, an open scientific dialogue remains important for facing all the new challenges with respect to our food to which the analytical community can contribute. During the months to come the continuation using a new and modern format, will be further discussed and developed.

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The organizers of EuroResidue IX acknowledge gratefully the support given by:

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ABOUT THESE PROCEEDINGS

Dear reader,

Two years later than originally planned we are happy to present the proceedings of the EuroResidue IX conference. As in 2016, these proceedings are available as a PDF file exclusively.

In total 132 contributions were included in the scientific programma, 5 Keynote presentations, 34 Oral and 93 Poster presentations.

These proceedings contain a total of 84 Extended Abstracts. Additionally, for those oral and poster presentations for which no extended abstract was submitted, the original short abstracts are included.

We emphasize that the authors are responsible for the content and the quality of their published contribution. The manuscripts were only "mildly" edited so that they fit in the format and style of the rest of the book. The presentation style of the authors has remained untouched as much as possible.

After the conference only the Extended Abstract will be published on the EuroResidue webside.

As noted in the instructions, the authors have assigned the right to reproduce and distribute the manuscripts on a worldwide basis to the publisher, i.e. EuroResidue Conferences Foundation (Stichting Euroresidue Conferenties). It is assumed that, in individual cases, the authors have obtained permission to reproduce any figure, data or text from the respective copyright holders. The EuroResidue Conferences Foundation does not own the copyrights; the manuscripts remain the property of the authors.

These proceedings are published in two parts.

Part I: Introduction and programme
Part II: Keynote, Oral and poster presentations.

We express our gratitude to all members of the Scientific committee for their thorough review of all the submitted abstracts, and thank Sanne Biggelaar of the EuroResidue Secretariat for managing the entire process of around the initial abstracts and the full Manuscripts included in these proceedings.

We hope you find this book worthwhile to read and to archive for later use.

The editor and the EuroResidue Conferences Foundation.

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Programme

Sunday 22th of May 2022

15.00-17.00	Pre-conference workshop: “Residue analysis for dummies” organised by WFSR
16.00-20.00	Registration open
18.15-20.15	Get-together party in the bar of Hotel de Ruwenberg

Monday 23rd of May 2022

9.00-9.15	**Opening of the EuroResidue IX conference** Dr. A.A. Bergwerff EuroResidue foundation
First session	**Food of the future and residues**
Chairman:	B. Le Bizec
Co-chairman:	A. Cannavan
9.15-9.55	**Keynote lecture 1 (KN1)** Dr. J. Schefferlie CBG-MEB, Netherlands Past and future of MRL setting practices. EMA's perspective and experience
9.55-10.35	**Keynote lecture 2 (KN2)** Dr. Sara Stead FERA, York, United Kingdom New perspectives in the field of veterinary drug residue analysis in production animals and commodities
11.30-12.10	**Keynote lecture 3 (KN3)** Dr. F. Verstraete EC DG SANTE Understanding of the content and impact of the latest European regulation on veterinary substances (e.g. EU/2021/808)
12.10-12.50	**Keynote lecture 4 (KN4)** Dr. T. Kuuranne Swiss Doping Control Laboratory Food safety and anti-doping analyses in human sports: Inadvertent exposure to prohibited substances by nutrition

12.50-14.30 **Poster presentations**

13.15-14.00 Vendor Seminar: Agilent T
Title: Keeping our meat and fish products safe. A comprehensive, quantitative LC-MS/MS veterinary drug screening workflow from sample preparation to result
Presenter: Carola Damen, PhD

13.15-14.00 Vendor Seminar: Waters
Title: What's New and Exciting in 2022 from Waters!
Presenter: Dr. Simon Hird

Second session **Parallel session Advances in screening technologies**

Chairman: L. Bailly-Chourriberry
Co-chairman: Vincent Chabottaux

14.30-14.50 **Oral lecture 1 (O1)**
Dr. U. Mulow-Stollin
BVL Berlin, Germany
Proficiency tests as treasure troves of information – Inferences regarding the application of multi-residue screening methods throughout the European Union

14.50-15.10 **Oral lecture 2 (O2**)
Msc. Josha Jager
WFSR, Wageningen, Netherlands
New Opportunities for smart sampling using Coated Blade Spray Mass Spectrometry

15.10-15.30 **Oral lecture 3 (O3)**
Dr. Z.K. Kaabia
Laboratoire des Courses Hippiques (LCH), Verrieres-le-Buisson, France
Improved high throughput routine analysis of anabolic steroids and the comprehensive analysis of their free and conjugated forms in horseracing

16.30-16.50 **Oral lecture (O7)**
Dr. E. Jongedijk
WFSR, Wageningen, Netherlands
GC-HRMS for risk-based food monitoring on steroids

16.50-17.10 **Oral lecture 5 (O8)**
Dr. A. Arrizalaga-Larranaga
WFSR, Wageningen, Netherlands
Direct ionization mass spectrometry insights and its performance for the screening of growth promotors

Third session **Parallel session Advances in sample preparation**

Chairman: G. Biancotto
Co-chairman: T. Kuuranne

14.30-14.50 **Oral lecture 6 (O4)**
Dr. P.G. Garcia
Laboratoire des Courses Hippiques (LCH), Verrieres-le-Buisson, France
New approach for the detection of hydrophilic drugs in horse plasma for doping control by LC-MS

14.50-15.10 **Oral lecture 7 (O5**)
Dr. A. Kaufmann
Official food control authority of the canton of Zurich, Switzerland
Improving the QuEChERS Liquid/Liquid Extraction of Analytes with Widely Varying Physicochemical Properties: Example of 201 Veterinary Drugs in Milk

15.10-15.30 **Oral lecture 8 (O6)**
Dr. A.C. Huet
CER Groupe, Marloie, Belgium
Detection of natural steroid hormone abuse in cattle using a combination of immunoaffinity chromatography and gas chromatography/combustion/Isotope Ratio Mass Spectrometry

16.30-16.50 **Oral lecture (O9)**
Msc I. Bongers
WFSR, Wageningen, Netherlands
Dried blood spots: a new and efficient approach for monitoring prohibited substances.

16.50-17.10 **Oral lecture 10 (O10)**
Dr. G.R. Regan
Teagasc Food Research Centre, Foodsafety Department, Dublin, Ireland
Development and application of a multi-residue method for nitrofuran analysis, including new bound marker metabolites

Tuesday 24th May 2022

Fourth session **Confirmation technologies**

Chairman: S. Stead
Co-chairman: E. Daeseleire

9.00-9.20 **Oral lecture 11 (O11)**
Dr. P.J. Jedziniak
National Veterinary Research Institute, Pulawy, Poland
Headache with analysis of NSAIDs in foods of animal origin

9.20-9.40 **Oral lecture 12 (O12)**
Dr. I Barbu
WFSR, Wageningen, Netherlands
LC-MS methods for detection of inhibitor and growth promoter proteins

9.40-10.00 **Oral lecture 13 (O13)**
Dr. G. Dervilly
LABERCA, Nantes, France
Benefits of integrating traveling wave ion mobility spectrometry into liquid chronmatography and massspectrometry workflows for steroid analysis

11.15-11.35 **Oral lecture 14 (O14)**
Dr. V.D. Delcourt
Laboratoire des Courses Hippiques (LCH), Verrieres-le-Buisson, France
Innovative low-flow LC-HRMS approach opens new perspectives for high throughput doping control analyses: application to somatotropin detection

11.35-11.55 **Oral lecture 15 (O15)**
Dr. M. Nobile
University of Milan, Department of Veterinary Medicine and Animal Science, Milan, Italy
Occurrence of antibiotic residues in raw bovine milk and their relevance toward food safety and technological implications

11.55-12.15 **Oral lecture 16 (O16)**
Dr. Mokh
National Council for Scientific Research (CNRS) - Lebanese

Atomic Energy Commiss
Hormones residues in bovine matrices: sampling, analysis, and health risk assessment.

12.15-13.30 **Poster presentations**

12.30-13.15 Vendor Seminar: SCIEX
Title: ”An ultra-high sensitivity quantification method of veterinary drugs residues in food using the SCIEX 7500 system
Dr. Jianru Stahl-Zeng, Global Technical, Leader for food and environmental, SCIEX & Daniel McMillan, Snr. Market Development Manager, Food, Environmental & Forensics (EMEAI), SCIEX

12.30-13.15 Vendor Seminar: **BRUCKER**
Using advanced technology for screening

Fifth session **Parallel session Future trends**

Chairman: G. Dervilly
Co-chairman: L. vanHaecke

13.30-13.50 **Oral lecture 17 (O17)**
Drs. M.H. Blokland
WFSR, Wageningen, Netherlands
True mobile mass spectrometry for on-site analysis

13.50-14.10 **Oral lecture 18 (O18**)
Dr. C.C. Cloteau
LABERCA, Nantes, France
Livestock and sport animals doping towards the practical implementation of metabolomics based strategies

14.10-14.30 **Oral lecture 19 (O19)**
Dr. R. Stella
Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro, Italy
LC-HRMS based metabolomics to identify liver markers of clenbuterol abuse in beef cattle

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15.20-15.40 **Oral lecture 20 (O23)**

Dr. P. Regal
University of Santiago de Compostela, Analytical Chemistry, Nutrition and Bromatology, Lugo, Spain
Fattyacidomics to trace rbST administration in dairy cattle: strengths and limitations

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Dr. B.J.A. Berendsen
WFSR, Wageningen, Netherlands
The potential and drawbacks of (smart-)sampling approaches for residue testing

Sixth session **Parallel session Environmental issues**

Chairman: G. Hamscher
Co-chairman: S. Croubels

13.30-13.50 **Oral lecture 22 (O20)**
Dr. T.B.G. Goessens
Ghent University, Department of Pathobiology, Pharmacology and Zoological Medicine, Ghent, Belgium
Veterinary drugs and feed additives in amphibian breeding ponds: occurrence and risk

13.50-14.10 **Oral lecture 23 (O21**)
Dr. J, Huygens
Flanders Research Institute for Agriculture, Fisheries and Food (ILVO), Melle, Belgium
Antibiotic residues in soil and leeks

14.10-14.30 **Oral lecture 24 (O22)**
Msc. E. Lange
WFSR, Wageningen, Netherlands
Antibiotic and anti-parasitic drugs in black soldier fly (Hermetia illucens) larvae production

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Dr. N, Gillard
CER Groupe, Marloie, Belgium
Development of an integrated approach for the diagnosis of water quality in the river meuse (diadem project)

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Dr. R.H. Hannisdal
Institute of Marine Research, Bergen, Norway
Environmental impact of anti-sea lice agents on non-target species

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2021/808/EU regulation (from 2002/657 to 2021/808) - EURLs, EU Commission, Third countries perception
Roundtable Leaders Joachim Polzer & Eric Verdon,

Wednesday 25th of May 2022

Seventh session **QA and Risk assesment**

Chairman: J. Mc Evoy
Co-chairman: A.Mauricio de Queiroz

9.00-9.20 **Oral lecture 27 (O27**)
Dr. F. Tadjine (O21)
BVL, Berlin, Germany
Pharmacokinetics of toltrazuril in laying hens and residues in egg

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Dr. J.C. Cornejo
University of Chile, Preventive Medicine Department, Santiagon, Chile
Validation of a microbiological screening method for the detection of residues of six families of antibiotics in animal manure.

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Dr. P.R. Reybroeck
ILVO, Technology and Food science Unit, Melle, Belgium
ISO Technical spcification 23758 regardingthe validation of qualitative screening methods for antimicrobials in milk

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Dr. E.D. van Asselt
WFSR, Wageningen, Netherlands
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Dr. E.D. Dreana
ANSES, Fougeres, France

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Dr. M. Groot
WFSR, Wageningen, Netherlands
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Dr. A. Borzekovski
BVL, Berlin, Germany
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Dr. L.A. van Ginkel
WFSR, Wageningen, Netherlands
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Dr. A.A. Bergwerff

13.15 **Closing of the EuroResidue conference**
Dr. A.A. Bergwerff

Poster Presentations

P1. New approaches for validation: inter-laboratory study for a confirmatory method for beta-agonists in liver on basis of a factorial design
A. Borzekowski, Federal Office of Consumer Protection and Food Safety, Germany

P2. LC-MS/MS method for the quantification of tiamulin, trimethoprim, tylosin, sulfadiazine and sulfamethazine in medicated feed
E. Patyra, National Veterinary Research Institute, Poland

P3. Antibacterial substances in natural and organic fertilizers as a potential problem of environmental contamination
E. Patyra, National Veterinary Research Institute, Poland

P4. Development and validation method for detection and determination of formaldehyde in feeds and silage by HPLC-DAD detector after precolumn derivatization
E. Patyra, National Veterinary Research Institute, Poland

P5. Stability of antibiotic residues in incurred meat samples during frozen storage
F.L. Fioroni, Istituto Zooprofilattico Sperimentale Umbria e Marche
Togo Rosati, Italy

P6. High sensitivity automated turboflow online UPLC-MS/MS methods for detrmination of chloramphenicol residues in whole eggs and dry eggs products
G. Quaglia, Life Analytics Floramo Corporation, Italy

P7. Simple and efficient UPLC-ESI-MS/MS method for multi-residue analysis of 14 coccidiostatic agents in poultry liver and muscle tissues
M. Rydchuk, SCIVP, Ukraine

P8. Development of a method for the determination of steroids esters in hair by liquid chromatography with tandem mass spectrometry
S. Termes, Laboratori Agroalimentari (Government of Catalonia), Spain

P9. Method extention of the noko multi-class veterinary drug residue analysis method by liquid chromatography mass spectrometry for egg and milk
A. Hütteroth, Landeslabor Berlin-Brandenburg, Germany

P10. Improved SPE for LC-MS Determination of Ractopamine in Porcine and Bovine Liver: The Oasis MCX Method Using Otto SPEcialist
S. Hird, Waters Corporation, United Kingdom

P11. Multi-residue screening approach for the detection of veterinary drugs in animal tissues using
LC-MS/MS
S. Hird, Waters Corporation, United Kingdom

P12. Antibiotic residues in surface and groundwater in flanders
G. Rasschaert, ILVO, Belgium

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S. Hird, Waters Corporation, United Kingdom

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E. George, Thermo Fisher Scientific, USA

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G. Dervilly, Oniris, France

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I. Becue, ILVO, Belgium

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G. Dervilly, Oniris, France

P18. Investigating nandrolone status in poultry liver
G. Dervilly, Oniris, France

P19. Comprehensive LC-HRMS metabolomics analysis to determine urinary metabolites of altrenogest in gilts
S. Liesenfeld, CVUA Karlsruhe, Germany

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L. Tuts, Flanders Research Institute for Agriculture, Fisheries and Food, Belgium

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E. Papapanagiotou, Aristotle University Of Thessaloniki, Greece

P22. Serious alert and border rejection notifications on residues of veterinary medicinal products in
food in the EU RASFF (2012-2020).
E. Papapanagiotou, Aristotle University Of Thessaloniki, Greece

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I.E.A. Bongers, Wageningen food safety research, The Netherlands

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A. Poirier, Oniris, Inrae, Laberca, France

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T. Chen, Ecole Nationale Vétérinaire, Agroalimentaire et de l'Alimentation, France

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M. Rejtharová, USKVBL, Czech Republic

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M. Rejtharová, USKVBL, Czech Republic

P28 A straightforward strategy for steroid esters analysis in bovine
A. Poirier, Oniris, Inrae, Laberca, France

P29. Determination of gestagens and stilbens residues in goat colostrum
M. Rejtharová, USKVBL, Czech Republic

P30. Determination of Estradiol esters derivatives in animal blood plasma by GC-MS/NCI
M. Rejtharová, USKVBL, Czech Republic

P31. Microwave assisted extraction for efficient analysis of progestagen esters in perirenal fat
A. Poirier, Oniris, Inrae, Laberca, France

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M. Rejtharová, USKVBL, Czech Republic

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L. Sibanda, Randox Food Diagnostics, United Kingdom

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F. Schoutsen, Thermo, The Netherlands

P35. Delvotest® Accelerator Smart Verification of Detection Capabilities of Delvotest ® T and Delvotest® SP-NT External Validation at Qlip
T. de Graaf, DSM, The Netherlands

P36. Development and validation of an LC-MS/MS method for the determination of total florfenicol residues as florfenicol amine in kidney
C.J. Bot, Wageningen Food Safety Research, The Netherlands

P37. A strategy to determine the fate of active chemical compounds in soil
B.J.A. Berendsen, Wageningen Food Safety Research, The Netherlands

P38. Doxycycline adsorption and toxicity in earthworms after manure application to soil
B.J.A. Berendsen, Wageningen Food Safety Research, The Netherlands

P39. Antimicrobial peptides - new emerging antimicrobial and growth promoting agents?
E. de Lange, Wageningen Food Safety Research, The Netherlands

P40. Ultra-high sensitivity quantification of veterinary drug residues in animal by-products
J. Steed, SCIEX, United Kingdom

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E. Nowacka-Kozak, National Veterinary Research Institute, Poland

P42. The quantification of prohibited veterinary drugs in milk
S. Vonsovic, WFSR, The Netherlands

P43. Confirmation of five nitrofuran metabolites by LC-MS/MS including the nifursol metabolite in food-producing muscle tissues according to Regulation (EU) 2021/808.
P. Guichard, Anses - Fougères Laboratory, France

P44. Veterinary drug analysis: The role of isomers and metabolites
C.J. Bot, Wageningen Food Safety Research, The Netherlands

P45. Workflow for the identification of ‘unknown' substances in animal feed
R.S. Wegh, Wageningen Food Safety Research, The Netherlands

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L.J.M. Jansen, Wageningen Food Safety Research, The Netherlands

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L.J.M. Jansen, Wageningen Food Safety Research, The Netherlands

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M. Arends, Wageningen Food Safety Research, The Netherlands

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H.W. Gerritsen, Wageningen Food Safety Research, The Netherlands

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M.I.K. Pedersen, National Food Institute, Denmark

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H. Daguer, Brazilian Ministry of Agriculture, Livestock, and Food Supply (MAPA), Brazil

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M.I.K. Pedersen, National Food Institute, Denmark

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H. Daguer, Brazilian Ministry of Agriculture, Livestock, and Food Supply (MAPA), Brazil

P54. Determination of 67 veterinary drugs in feedingstuffs using two-step energized dispersive guided extraction
H. Daguer, Brazilian Ministry of Agriculture, Livestock, and Food Supply (MAPA), Brazil

P55. Determination of progesterone in bovine serum.
I. Matraszek-Zuchowska, National Veterinary Research Institute, Poland

P56. The presence of antibiotic residues in different matrices of veal calves after medical treatment
B. Duijnhouwer, Triskelion, The Netherlands

P57. Oxytetracycline in treated chicken droppings and litter, its dissemination to untreated animals and effect on the selection of resistant bacteria.
E. Pokrant, University of Chile, Chile

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I.J.W. Elbers, Wageningen Food Safety Research, The Netherlands

P59. Risk of residues of toltrazuril and its metabolites in animals products after oral administration
K. Pietruk, National Veterinary Research Institute (PIWet), Poland

P60. The physiological values of sex steroids in calves and the reference histology of the target organs.
M. Pezzolato, Istituto Zooprofilattico Sperimentale del Piemonte Liguria e Valle d'Aosta, Italy

P61. Screening of selective androgen receptor modulators by hand-held laser diode thermal desorption-transportable mass spectrometry
Arrizabalaga-Larranaga, Wageningen Food Safety Research, The Netherlands

P62. Optimization of QuEChERS methodology to assess the contamination of antibiotics level of manure for agronomic use.
R. Avolio, istituto zooprofilattico sperimentale di liguria piemonte e valle d'aosta, Italy

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J. Jager, Wageningen Food Safety Research, The Netherlands

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A. Maddaleno, Universidad de Chile, Chile

P65. Natural hormones profiles in bovine blood serum by LC-MS/MS: multi-year survey in northern Italy
M. Gili, Istituto Zooprofilattico Sperimentale PLV, Italy

P66. Do you think that a 48 multi-residue method for antibiotics in a single run is possible?
M.C. Castelló, Agència de Salut Pública de Barcelona, Spain

P67. Bovine Teeth and Claws: A Potential new Forensic Matrix for the Detection of Illicit Administration of Regulated and Banned Substances
J.J.P. Lasaroms, Wageningen Food Safety Research, The Netherlands

P68. Controls in the food chain: multiclass methods to detect low residual concentrations of veterinary drugs in feed and food
G. Biancotto, Istituto Zooprofilattico Sperimentale delle Venezie, Italy

P69. Illicit administration of natural steroids in cattle: a case report
M. Gili, Istituto Zooprofilattico Sperimentale PLV, Italy

P70. A fast and selective LC-MS/MS method for avermectins and milbemycins in liver and milk
H.W. Gerritsen, Wageningen Food Safety Research, The Netherlands

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I.M. Barbu, Wageningen Food Safety Research, The Netherlands

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C. Douillet, Teagasc Food Research Centre, Ireland

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RESIDUES OF VETERINARY DRUGS IN FOOD

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Extended and short abstracts

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CBG-MEB, Netherlands
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WFSR, Wageningen, Netherlands
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ORAL PRESENTATIONS

ORAL O1

PROFICIENCY TESTS AS TREASURE TROVES OF INFORMATION - INFERENCES REGARDING THE APPLICATION OF MULTI-RESIDUE SCREENING METHODS THROUGHOUT THE EUROPEAN UNION

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Abstract
Modern screening techniques using minimum sample preparation procedures followed by chemical analysis with high resolution mass spectrometry (HRMS) greatly facilitate the time and cost efficient identification of non-compliant samples. These methods are therefore often superior to conventional confirmation methods in routine operations which is why their application is promoted by the European Union Reference Laboratory (EURL) Berlin. In order to evaluate laboratories' command of their methods as well as to assess the level of implementation of HRMS screening techniques and the coverage of relevant substance groups throughout the network of National Reference Laboratories (NRLs), a proficiency test focusing on the screening of veterinary drug residues of groups A6, B1, B2a, and B2e in bovine milk was carried out by the EURL Berlin for the first time. The review of the participants' method data revealed that the relevant substance groups are adequately covered by all members of the laboratory network but only a limited number of laboratories apply (HRMS) screening methods in routine analyses with more HRMS users in Western and Central Europe. These findings were confirmed by the results of a follow-up survey which also provided insight into the perceived challenges of HRMS-based screening techniques.

Keywords: HRMS screening, proficiency test

ORAL O2

NEW OPPORTUNITIES FOR SMART SAMPLING USING COATED BLADE SPRAY MASS SPECTROMETRY

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Abstract
Risked-based approaches are increasingly implemented for residue testing programs and go from sampling to the end of the analysis traject. Currently, sample-analysis is performed by transporting the sample to the laboratory. This only allows a small number of samples to be taken and analysed. Preferably a higher percentile of samples should be tested. Analytically this requires new approaches for sampling which are preferably directly compatible with analytical detection techniques. The direct ionization technique Coated Blade Spray Mass spectrometry (CBS-MS) demonstrates great potential for testing in food safety-related fields. The blades contain a biocompatible sorbent material coated on their surface, which binds the analytes of interest. In short: CBS-MS devices can sample directly by placing it in the sample (e.g. urine) for 30 seconds, washing matrix components off using water. Applying a small volume of desolvation/ionization solvent on the coated part leads to the extraction of the analytes while applying at the same time a high voltage generates electrospray ionization. CBS-MS contributes to smart sampling by simplifying the sampling, shipping, and increasing the number of samples tested in a similar timespan. Here, we present the results of this smart sampling approach for food safety-related applications and the potential it offers.

Keywords: Ambient mass spectrometry, Coated Blade Spray, Smart sampling

ORAL O3

IMPROVED HIGH THROUGHPUT ROUTINE ANALYSIS OF ANABOLIC STEROIDS AND THE COMPREHENSIVE ANALYSIS OF THEIR FREE AND CONJUGATED FORMS IN HORSERACING

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Abstract
The gold standard method for the screening of androgenic anabolic steroids (AAS) in equine urine, in the context of anti-doping control, is GC-MS/MS after de-conjugation, extraction and derivatization steps. Daily, more than 150 urine samples are screened in our laboratory, undergoing this analytical process. Although performant, the duration of the whole process for this number of samples is 72 hours. Aiming for a better high throughput performance, LC-MS seems to offer an alternative option for the screening of AAS under their free and conjugated forms.
An adapted analytical strategy was therefore settled to allow the extraction of free and conjugated AAS and their analysis by LC-MS. The developed analytical strategy was based, first, on a steroid extraction performed by means of a C_{18} SPE cartridge characterized by an optimized washing step. Then, the optimization of chromatographic and mass spectrometric parameters resulted in satisfying separation and detection performances. This strategy allowed free and conjugated AAS analysis in equine urine insuring a gain in time, avoiding potential artifacts and confirming their phase II metabolisation. For instance, such a strategy granted the daily analysis of 150 samples in less than 36 hours, doubling thus the actual productivity without the loss of analytical performances.

Keywords: androgenic anabolic steroids, Equine doping, LC-MS high throughput ,screening

ORAL O4

NEW APPROACH FOR THE DETECTION OF HYDROPHILIC DRUGS IN HORSE PLASMA FOR DOPING CONTROL BY IC-MS

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Abstract
Bisphosphonates are prohibited hydrophilic drugs divided in two groups: non-nitrogen-containing bisphosphonate (*e.g.* clodronic acid) and nitrogen-containing bisphosphonate (*e.g.* zoledronic acid). They are used for the treatment of navicular and related diseases in horses. Their hydrophilic properties and high affinity for the bone matrix make control of their use quite difficult. Today analysis of such compounds is based on a solid phase extraction using 96-well plates coated with Oasis weak anion exchange sorbent on a Biotage® Extrahera™ followed by detection using UhplC-MS/MS on a TSQ Quantiva™ mass spectrometer (Thermo Scientific™) after methylation with trimethyl orthoacetate in acetic acid.
To get rid of the derivatization step a new approach using ion chromatography linked to mass spectrometry is considered with the challenge to apply this method to doping control screening. Analyses are conducted on an ICS-6000 HPIC system linked to a TSQ Altis™ (Thermo Scientific™). Horse plasma results demonstrate good performances of the method for the detection of non-nitrogen-containing and nitrogen-containing bisphosphonates with lower limit of detection of 100 pg/mL. Validation and results on post-administration samples will be presented.

Keywords: Bisphosphonates, Horse doping control, Ion Chromatography

ORAL O5

IMPROVING THE QUECHERS LIQUID/LIQUID EXTRACTION OF ANALYTES WITH WIDELY VARYING PHYSICOCHEMICAL PROPERTIES: EXAMPLE OF 201 VETERINARY DRUGS IN MILK

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Abstract

A novel liquid/liquid extraction and clean-up process for the multiresidue analysis of veterinary drugs in milk is described. The paper addresses limitations of existing QuEChERS based protocols. This refers to poor and irreproducible recoveries of frequently used drugs (e.g. penicillin, tetracyclines and quinolones). The methodology produces quantitative data and has been fully validated according to the current EU validation guidelines by utilizing two different high resolution mass spectrometry instruments coupled to liquid chromatography (LC-Q-Orbitrap and LC-Q-TOF)

Introduction

The QuEChERS methodology is now the most frequently used concept for multiresidue analysis of pesticides in fruits and vegetables. As a consequence, it has been adopted and modified to suit the needs for the multiresidue analysis of veterinary drugs in animal based food. QuEChERS is attractive because it is simple and does not require expensive consumables. It produces a sufficient sample clean-up and good recoveries for most analytes. Yet, some highly relevant compounds belonging to the families of penicillines, tetracyclines and quinolones show unacceptable low analyte recoveries.

Materials and Methods

Extraction/clean-up

Milk samples are extracted with an acetonitrile complexing agent/water mixture. A phase separation is induced by the use of a mixture of di-potassium hydrogenphosphate and potassium dihydrogenphosphate. The supernatant phase is recovered, evaporated with a keeper and reconstituted.

LC-MS analysis

Extracts are separated by a reversed phase chromatography system hyphenated to a high resolution mass spectrometry system (HRMS). The validation, following the current EU guidelines was done by using two different HRMS detector systems (Q-Orbitrap and Q-Time of flight).

Results and Discussion

Conventional QuEChERS is based on a mixture of magnesium sulfate and sodium chloride. It is known from literature that tetracyclines form complexes with bivalent cations like magnesium. Such complexes are polar and are therefore only poorly partitioned into the acetonitrile phase. Yet, we observed a similar behaviour for several penicillines and cephalosporines in the presence of a high sodium concentration. Based on this observation and a careful study of the Hofmeister series, an alternative salt mixture consisting of di-potassium hydrogenphosphate and potassium dihydrogenphosphate was evaluated. This mixture significantly improved the recoveries of the critical penicillins and cephalosporins, while not negatively affecting the recoveries of all other analytes. This alternative salt mixture not only produces an easy phase separation but also leads to a supernatant acetonitrile layer containing comparable amounts of water as when using the conventional QuEChERS salt mixture. Most important, the amount of dissolved salt in the resulting supernatant organic phase is two to three times less than when using conventional QuEChERS salts. Hence, the extracts can be used for LC and probably also for GC injectons.

Conclusion

The proposed concept permits the high throughput of milk samples. It is neither labour nor consumables intensive. It detects and quantifies virtually all relevant veterinary drug families (not included were only the aminoglycosides, polypeptides and nitrofurans). The number of compounds can likely be expanded by adding additional compounds and by analysing the same extract in the negative ionization mode.

ORAL O6

DETECTION OF NATURAL STEROID HORMONE ABUSE IN CATTLE USING A COMBINATION OF IMMUNOAFFINITY CHROMATOGRAPHY AND GAS CHROMATOGRAPHY/COMBUSTION/ISOTOPE RATIO MASS SPECTROMETRY

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Abstract

The use of anabolic substances to promote growth in livestock is not allowed within European Union, and the use of GC-C-IRMS is recommended by the EURL to prove this abuse in case substances are used which can also be of endogenous origin. To avoid interferences during measurement, this highly specialized technique requires a compound specific sample cleanup protocol for each targeted analyte, usually based on preparative HPLC. However, immunoaffinity chromatography offers a valuable alternative extraction tool. Several immunoaffinity gels were prepared and characterized for detection of androgen abuse in bovine urine with etiocholanolone and boldenone as targeted metabolites, and 5-androstene-3β,17α-diol as endogenous reference compound (ERC). For the analysis of porcine urine, gels were developed for dehydroepiandrosterone as ERC and β-nortestosterone as metabolite.

The development of analytical strategies for the purification both bovine and porcine urine samples prior analysis with GC-C-IRMS for the detection of androgens is presented. The sample preparation including immunoaffinity purification for the detection of testosterone abuse in bovine urine samples was successfully validated. Although the method provides promising results with regard to boldenone in bovine urine and β-nortestosterone in porcine urine, the validation remains incomplete due to the lack of analyzable samples from a compliant control population.

Introduction

In the European Union, the use of hormonal substances as growth promoter in cattle breeding has been prohibited since 1988 (Directive 88/146/EC repealed by Directive 96/22/EC then amended by Directive 2003/74/EC and by Directive 2008/97/EC). EU legislation (Regulation 2021/808) imposes a two-step strategy in laboratories in charge of the control: an initial rapid and multiresidue screening step to sift large numbers of samples for potential steroids abuse, followed by a confirmatory step discarding any doubts on the compliance of the suspicious samples. These surveillance programs are adequate for the detection of xenobiotic residues. However, when synthetic forms of naturally occurring steroid hormones are administered to cattle, this abuse cannot be proven in urine samples by the classical confirmation methods which are based on LC-MS(/MS) or GC-MS(/MS) detection. Sterk *et al*., 2014 stipulates in the "EURL Reflection paper" that gas chromatography coupled to combustion/isotope ratio mass spectrometry (GC-C-IRMS) provides a valid confirmation method for natural hormone abuse detection in urine samples, and encourages member states to implement GC-C-IRMS techniques for this purpose.

Knowing that it is difficult to set threshold values which indicate the treatment, at least two analytes are measured with IRMS: i) an endogenous reference compound (ERC), of which the $\delta^{13}C_{VPDB}$ value is not affected by the steroid treatment; $\delta^{13}C_{VPDB}$ value corresponds to the carbon isotopic composition ($^{13}C/^{12}C$) expressed relative to Vienna Pee Dee Belemnite (VPDB). ii) a metabolite of the administered substance. The difference between the $\delta^{13}C_{VPDB}$ value of the ERC and the metabolite, expressed as $\Delta^{13}C$ (‰), provides proof of administration.

For hormone abuse detection, urine is the preferred matrix because of its higher concentrations of steroids and availability in large quantities. Nevertheless, besides highly purified extracts, an IRMS-analysis also requires sufficiently high quantities of the targeted analyte to enter the detector in order to provide reliable

measurements. This poses a critical challenge because of the relatively high content of potentially interfering compounds in urine. Classical sample preparation for steroid analysis in cattle using solid phase extraction (SPE), liquid-liquid extraction (LLE), high performance liquid chromatography (HPLC) have already been described in the past (Bichon *et al*., 2007; Janssens *et al*., 2013a, 2013b, 2015 and 2016). For human sport doping control, similar procedures are also followed (Piper *et al*., 2011). These protocols are complex and very time consuming. In this context, the use of immunoaffinity chromatography (IAC) is considered a valuable alternative extraction tool due to its superior specificity and substantial extraction yield. Indeed, the successful use of IAC for the preparation of samples for regular steroid UHPLC-MS/MS analysis has been well described (Delahaut *et al*., 2014). Also, two successful uses of IAC for the preparation of samples for 19-androsterone (Desroches *et al*., 2002) and endogenous steroids (Putz *et al*., 2019) before analysis by GC-C-IRMS have been published.

Steroid hormones can be categorized into five groups according to their receptor binding potential: androgens, glucocorticoids, mineralocorticoids, estogens and progestagens. In mammalian species, all steroid hormones are derived from cholesterol by a multi-step pathway; cholesterol can be taken up from the feed directly, or can be biosynthesized from acetyl co-enzyme A. Representation of biosynthetic pathways for androgens can be found in two main publications (Janssens *et al.,* 2013a and Scarth *et al*., 2009). For the detection of androgen abuse in bovine urine, etiocholanolone and boldenone are selected as targeted metabolites and 5-androstene-3β,17α-diol as endogenous reference compound (ERC). Whereas in porcine urine, the targeted metabolite is β-nortestosterone and dehydroepiandrosterone is selected as ERC.

Materials and Methods

Chemicals
Steroids were obtained from Steraloids (Wilton, NY, USA).
Phosphate Buffered Saline (PBS) 50mM, pH 7.4.

Apparatus

HPLC-UV
Two different HPLC setups were used during sample preparation. Reversed phase HPLC was performed on a Waters Alliance 2690 system, coupled to a UV-detector (diode array detector, DAD) measuring from 205 to 235 nm, and subsequently to an automated fraction collector. The system was equipped with a precolumn (Kinetex Security Guard Ultra C18 for 2.1 mm ID) and a C18 functionalized analytical column (Kinetex XB-C18; 250 mm x 4.6 mm; 5 µm), held at 50°C. An HPLC method was developed on the basis of a mobile phase composition (A:B; v/v) of 35:65, with H_2O/MeOH (95/5; v/v) as solvent A and MeOH as solvent B. After elution of the analytes, the mobile phase composition (A:B; v/v) was changed to 0:100 as a rinsing phase at the end of the run. A constant flow rate of 0.8 mL min-1 and an injection volume of 100 µL were used.
For normal phase HPLC, another Waters Alliance 2695 system, set up with UV detector (UV-VIS detector), fraction collector and two diol functionalized columns (LiChrospher Diol; 250 mm x 4 mm; 5 µm) in series, was used. A constant mobile phase composition of isooctane/isopropanol (85/15; v/v) was used, with a rinsing phase at the end of the run. A flow rate of 1 mL min-1, a column temperature of 40°C and an injected volume of 100 µL were selected.

GC-C-IRMS
A Thermo Trace GC Ultra gas chromatograph, equipped with a Thermo Scientific Triplus autosampler, was connected to both a Thermo DSQ II single quadrupole mass spectrometer and a Thermo MAT 253 isotope ratio mass spectrometer, via the Thermo Scientific GC Isolink. To achieve the parallel coupling of the two detectors, the signal was split after GC using two SilFlows (TM SGE Analytical Science). The system was equipped with an Optima 17MS column (Machery Nagel – 30 m; 0.25 mm i.d.; 0.25 µm df) and gas flows were controlled using the Thermo Scientific Conflo IV interface. Helium was used as carrier gas at a flow rate of 1.2 mL min^{-1}. After injection of 8 µL, at 20 µL s^{-1}, the injector temperature was held at 115°C for 0.10 min, with a vent flow operating at 20 mL min^{-1}. Then, the temperature was increased at 8°C min^{-1} up to 300°C and held 2 min. The GC oven was held at its initial temperature of 95°C for 1.5 min. Next, the temperature was raised to 220°C at 30°C min^{-1}, to 270°C at 6°C min^{-1}, to 300°C at 2°C min^{-1} and finally to 310°C at 50°C min^{-1}, which was held for 4 min. The transfer line temperature was set at 300°C. Approximately 5% of the GC eluate was transmitted to a quadrupole mass analyzer for characterization; the steroid signals were acquired in the MS full scan mode (m/z 50 to 400) after electron ionization (70 EV). The main split fraction was carried out to a combustion furnace, perfectly conditioned (1 h of oxidation before use) and maintained at 950°C. Next, by passing through a Nafion membrane, water was removed from the gaseous eluate. Electron ionization was used to convert carbon

dioxide (CO_2) into the corresponding ionized species characterized by an odd number of electrons ($CO_2^{+\bullet}$). Finally, isotopomer's species at m/z 44, 45 and 46 were simultaneously measured in separated Faraday cups. From these data, $\delta^{13}C_{VPDB}$ values of the analytes were calculated after calibration of the CO_2 reference gas, which was executed by injection of 17β-testosterone acetate and dehydroepiandrosterone (DHEA) acetate with known and certified $\delta^{13}C_{VPDB}$ values. When steroids were measured after acetylation, the shift of the $\delta^{13}C_{VPDB}$ value, caused by the acetate moieties, was corrected using the formula DOH = DOAc + 2m (DOAc − DAc)/n,
in which DOH is the $\delta^{13}C_{VPDB}$ value of the underivatized steroid, DOAc the $\delta^{13}C_{VPDB}$ value of the acetylated steroids, DAc the $\delta^{13}C_{VPDB}$ value of the acetic anhydride used for the derivatisation, n the number of carbon atoms in the molecule and m the number of hydroxyl groups which were acetylated.

IAC preparation: antibody selection and immobilisation

One criterion for inducing a reasonable immune response is a certain size (3000 – 5000 daltons) of the antigen that must be recognized as foreign particle by the infected host animal (rabbits). Steroids themselves are too small to stimulate B-cells to produce antibodies; they can be rendered immunogenic by coupling them in sufficient number to a protein carrier such as Bovine Serum Albumin (BSA), Ovalbumin (OVA), Keyhole Limpet Hemocyanin (KLH), Blue Carrier Protein (BCP). The steroid derivative occurred through the formation of hemisuccinate (HMS) or the carboxymethyloximes (CMO); the position is precised just before the abbreviation. The androstene-3β,17α-diol was coupled to BSA at position 17 via carboxylic acid group (cBSA), see Table 1.

The titer, the sensitivity and the cross-reactivity of each antibody were previously determined by an indirect competitive ELISA (Enzyme-Linked ImmunoSorbent Assay) using the corresponding conjugate; concretely, horseradish peroxidase (HRP) is attached instead of the carrier protein keeping the same protocol.

Antisera must be purified on protein A (HiTrap Protein A, GE Healthcare) prior to immobilization. By removing the bulk and the non-antibody proteins, a higher capacity immunosorbent with minimal non-specific adsorption can be obtained since only IgG fraction is collected. After isolation of IgG fraction, the individual antibodies were immobilised on cyanogen bromide-activated sepharose 4B gel (GE Healthcare 17-0430-01) according to the supplier's instructions at a level of 3mg IgG per ml of gel. The gels were stored in phosphate-buffered saline (PBS, 50mM pH7.5) containing 0.01% NaN_3 at 4°C for further use.

Sample preparation

A schematic overview of the analytical strategy is shown in Figure 1.

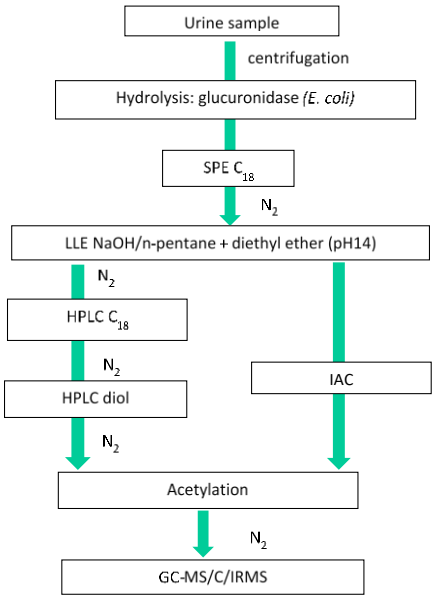

Figure 1: Overview of the analytical protocol for GC-C-IRMS analysis.

The urine samples were centrifuged (15 min, 3113g) prior to analysis to avoid obstruction of the solid phase extraction (SPE) cartridges later on. Ten milliliters of the sample were hydrolyzed at 37°C, after addition of 50 µL glucuronidase from *E. coli* and 3 mL phosphate buffer 0.8 M to maintain a pH of 6.8. After 15 hours, the

sample was loaded onto a C18 SPE column, which was previously conditioned by 6 mL of MeOH and 6 mL of H_2O. After stationary phase washing with 6 mL of H_2O and 5 mL of H_2O/acetonitrile (ACN) (80/20; v/v), target analytes were eluted with 8 mL of H_2O/ACN (5/95; v/v). Next, the eluted sample was evaporated to approximately 300 µL under a nitrogen stream at 45°C, before adding 0.5 mL of 1 M sodium hydroxide. Then, a liquid-liquid extraction (LLE) was performed using two times 4 mL of n-pentane/diethyl ether (92.5/7.5; v/v). Afterwards, 30 µL of a glycerol solution (10% in methanol) was added to the combined organic layers to serve as a keeper. From this stage, the sample is further purified by two successive HPLC or through an IAC column.

HPLC purification

The sample was evaporated at 37°C under a nitrogen stream and reconstituted into 80 µL of H_2O and 40 µL of fluoxymesterone in methanol (50 ng/µL), used as internal standard specifically during the reversed phase chromatography step. The collecting windows for targeted analytes on the reversed phase HPLC system were first determined through three successive injections of standard solutions. Next, the fractions were evaporated to dryness under a nitrogen stream at 37°C and before dissolving in 120 µL of isooctane/isopropanol (90:10; v/v) progesterone was added as internal standard before injection in the second HPLC system, after calculation of the fractionation windows by a threefold standard. The fractions, collected during the normal phase HPLC purification, were evaporated to dryness under a nitrogen stream at 37°C, and 25 µL of both acetic anhydride and pyridine were added before IAC purification.

IAC purification

Each column contains 2 ml of gel. This purification included the following steps: washing the column twice with 5 ml of PBS, equilibrating the gel with 10 ml of H_2O, applying the sample after LLE at the top of the gel, washing the gel with 5 ml of MeOH/H_2O (20:80, v:v), desorbing the bound fraction twice with 3 ml of MeOH/H_2O (80/20, v:v), rising the column with 10 ml of H_2O + 6 ml of MeOH/H_2O (80/20, v:v) and regenerating the column with 5 ml of H_2O + 10 ml of PBS. The gel may be stored at 4°C in PBS containing 0.1% NaN_3 as preservative.
In practice, the bovine extract follows the complete protocol through the IAC column immobilised either with antibodies against etiocholanolone or with antibodies against boldenone. Then, individual extracts pass through the IAC with antibodies for 5-androstene-3β,17α-diol (ERC). The porcine extract follows the complete protocol through the IAC column immobilised with antibodies against β-nortestosterone. Then, it passes through the IAC with antibodies for DHEA (ERC).

Acetylation and injection

After acetylation overnight at room temperature, the fractions were again evaporated to dryness under a nitrogen stream at 37°C. Finally, the fractions were dissolved in a calculated quantity of isooctane, depending the estimated concentration, containing 19-noretiocholanolone-3-acetate (4 ng/µL) as internal standard, before injection onto the GC-MS/C/IRMS.

Results and Discussion

Immunoaffinity chromatography is a form of affinity chromatography in which antigens or antibodies (this study) were immobilized on a solid phase support. The bio-specific antigen-antibody interaction makes it possible to obtain a high degree of purification.

Selection of antibodies for IAC

The polyclonal antibodies were derived by immunizing different haptens coupled to different carrier proteins and include the antibodies recovered from several animals. First, the characterisation of all potential antibodies is performed by competitive ELISA with the corresponding peroxidase-conjugate. The selection of the most promising antibody for a target analyte is based on the titre determination, the sensitivity and the cross-reactivity profile. The performance of all produced antibodies during this project were compared with each other; Table 1 gives an overview of studied antibodies and those selected for the preparation of IAC columns for further comparison.

Table 1. Overview of polyclonal antibodies studied then, some of them were selected for the preparation of IAC columns.

Target	Immunogen	Antibodies N°	Selected antibodies
Etiocholanolone	Etiocholanolone-3HMS-OVA	M13114 to M13116	/
	Etiocholanolone-3HMS-BSA	M13117 to M13119	M13119
	Etiocholanolone-17CMO-KLH	M150190 to M1501994	M150194
Boldenone	17β-boldenone-17-HMS-BSA	M160018 to M160022	M160020 + M160022
5-androstene-3β,17α-diol (ERC)	Epitestosterone-3CMO-BSA	M272 to M279	M273 + M277
	Epitestosterone-3CMO-BSA	M1800043 to M1800047	/
	Epitestosterone-3CMO-BCP	M1800048 to M1800052	/
	5-androstene-3β,17α-diol-17β-cBSA	M170027 to M170030	M170029
β-nortestosterone	17β-nortestosterone-3CMO-HSA	M200 to M204	M202
	17β-nortestosterone-17HMS-BSA	Ma9 to Ma16	Ma12 + Ma13
Dehydroepiandrosterone	DHEA-17-CMO-BSA	M150118 to M150122	M150118, M150120, M150121, M150122

Determination of IAC column capacity

The bio-specificity is often accompanied by extremely tight binding, making elution of active purified antigen very difficult. In order to improve the reproducibility of the extraction, the maximum binding capacity of the column was determined by adding a large amount of analyte diluted in water or solvent on the top of prepared columns. The different eluates were collected after deposition, washing and elution. After evaporation of each collected fraction, 1ml of ethanol was added and the extracts were analysed by UHPLC-MS/MS. The maximum capacity of each immunoaffinity column was determined for the steroid target, the amount of IgG immobilized per ml of gel and the volume of gel used for the preparation of the column. The results are presented in Table 2. The molecules recognized by the corresponding IAC are also cited in the same table.

Table 2. Determination of the IAC columns capacity.

Target	Antibody on IAC	Volume of gel	Amount IgG / ml gel	Maximum capacity[a]	Other recognitions
Etiocholanolone	M13119	1 ml	10 mg	610 ng	Androstenedione, androsterone,
	M150194	2 ml	10 mg	690 ng	Not determined
Boldenone	M160020	2 ml	3 mg	1500 ng	Epiboldenone, testosterone, epitestosterone, 5α-dihydrotestosterone, androstenedione, androstadienone, progesterone
	M160022	2 ml	3 mg	1450 ng	Epiboldenone, testosterone, epitestosterone, 5α-dihydrotestosterone, androstadienone, progesterone
5-androstene-3β,17α-diol (ERC)	M277	2 ml	10 mg	894 ng	Epitestosterone, epiboldenone, 5α-androstan-3β,17α-diol, testosterone
	M273	2 ml	10 mg	1500 ng	Epitestosterone, epiboldenone, 5α-androstan-3β,17α-diol, boldenone, testosterone, 5α-dihydrotestosterone
17β-nortestosterone	M202	2 ml	3 mg	1100 ng	17α-nortestosterone, 17β-testosterone, 17β-boldenone, 5α-dihydrotestosterone, 5-androstene-3β,17α-diol
	Ma12	2 ml	3 mg	1100 ng	17α-nortestosterone, 17β-testosterone, 17β-boldenone, 5α-dihydrotestosterone, 5-androstene-3β,17α-diol
	Ma13	2 ml	3 mg	1150 ng	17α-nortestosterone, 17β-testosterone, 17β-boldenone, 5α-dihydrotestosterone
Dehydroepiandrosterone (ERC)	M150118	2 ml	10 mg	1500 ng	5α-androstan-3β,17α-diol, pregnenolone, 17OH-pregnenolone, 5α-dihydrotestosterone, 5-androstene-3β,17α-diol
	M150120	2 ml	10 mg	1100 ng	5α-androstan-3β,17α-diol, pregnenolone, 17OH-pregnenolone, 5α-dihydrotestosterone, 5-androstene-3β,17α-diol
	M150121	2 ml	10 mg	305 ng	5α-androstan-3β,17α-diol, pregnenolone, 17OH-pregnenolone, 5α-dihydrotestosterone, 5-androstene-3β,17α-diol
	M150122	2 ml	10 mg	1500 ng	5α-androstan-3β,17α-diol, pregnenolone, 17OH-pregnenolone, 5α-dihydrotestosterone, 5-androstene-3β,17α-diol

[a]it concerns the target molecule.

The ability of an IAC to serve as a clean-up column relies on the specific retention of the analytes during the application and washing procedure and their recovery during the elution process. To isolate the different molecules of interest by IAC, several factors were optimised such as the wash solution, the volume applied, the number of washes, the elution solution, the volume applied as well as the preparation of IAC column corresponding to conditioning and the conditions for the regeneration. The optimisations of all these factors are important and influence the number of uses for the columns. The starting amount of IgG per ml of gel was usually 3mg; this amount was increased to 10mg in some cases to reach a better capacity.

GC-C-IRMS analysis of bovine urine

Different sample preparation strategies were compared such as the existing method including two HPLC-steps and the method including IAC steps. Figure 2 proved that the obtained fractions are sufficiently clean to provide accurate IRMS measurements.

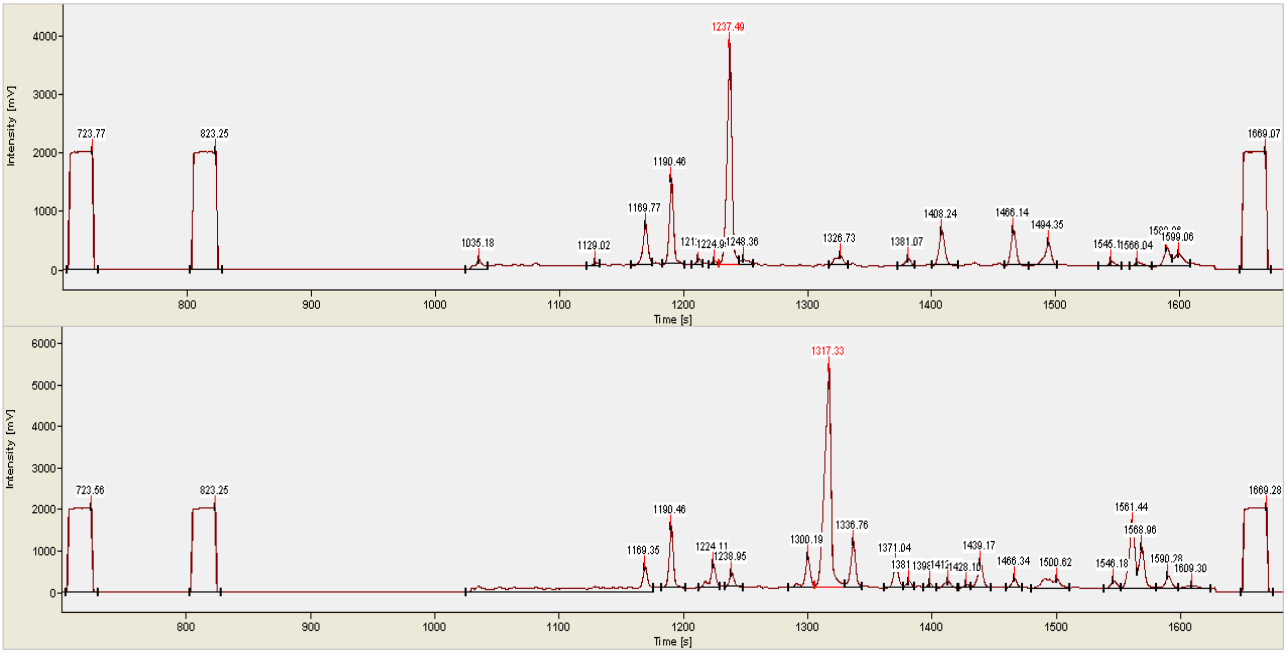

Figure 2. IRMS-chromatograms after IAC sample preparation of the etiocholanolone (above) and 5-androstene-3β,17α-diol (below) fractions.

$\Delta^{13}C_{VPDB}$ values of a control population were measured and a threshold value for compliance samples has been determined, which allows the distinction between samples from treated and non-treated bovines (Figure 3).

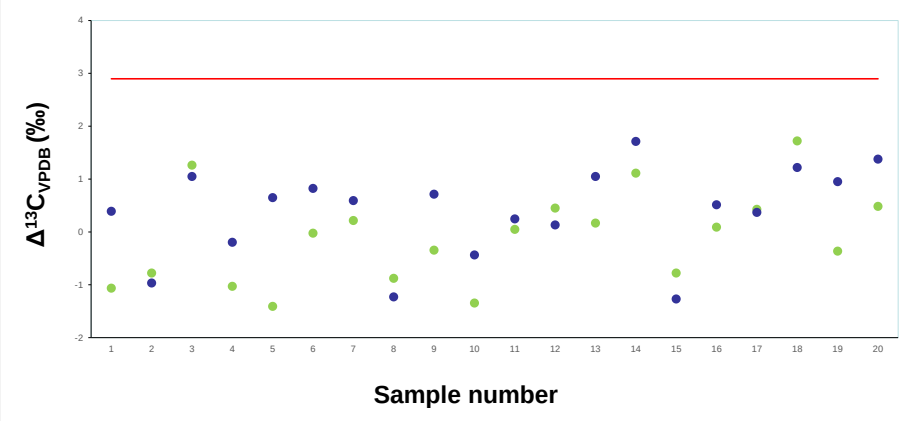

Figure 3. Results for a compliant control population (with etiocholanolone as metabolite, and 5-androstene-3β,17α-diol as ERC), analysed with IAC purification (blue) and HPLC purification (green). The red line indicates the compliance threshold value.

GC-CIRMS analysis of porcine urine

When purifying pig urine, the DHEA-IAC column must first be used in the configuration, and then the 17β-nortestosterone-IAC column, as the DHEA is carried away on 17β-nortestosterone-IAC column. For the purification of pork urine with gels 17β-nortestosterone/DHEA-IAC, a sample with a measured content of 114 ppb DHEA and 76 ppb 17β-nortestosterone was chosen. For 17β-nortestosterone, the 3 types of IAC gel for β-NT were of potential use. Based on minor differences in separation quality, M202 was selected. Wash step 2 and wash step 3 of the DHEA-IAC column were collected and applied to the Ma13 to minimize the loss of β-NT. The sample preparation procedures include IAC extraction of a single target analyte corresponding to a single antibody immobilized on the IAC gel. Preliminary experiments based on IAC for a group of target analytes

(multiple antibodies immobilized on IAC gel and/or antibodies with cross-reactivity) were conducted since it is considered as one of the most appropriate separation steps for mass spectrometry-based analysis (Tsikas, 2010). Unfortunately the results were disappointing because in urine with high concentrations of DHEA and β-NT, we see that the vast majority of DHEA and β-NT were not found in the expected elution fractions. Most of it was washed away. However, in porcine urine with low concentrations of DHEA and β-NT, the majority of both substances were found in the elution fractions.

Conclusion

IAC is a powerful tool to isolate substances from complex media such as urine by reducing the undesired matrix effects. IAC has a concentrating effect which enables convenient processing of large sample volumes. An IAC clean up by using two columns in series proved to be a good alternative for two steps HPLC of preparative HPLC to reduce impurities in urine samples before GC-C-IRMS injection.
Although there is the possibility of using gel mixtures for multi residue analysis, IAC purification offers a more appropriate sample preparation before GC-C-IRMS analysis of a limited number of compounds, whereas a HPLC offers a more flexible approach when larger numbers of targeted analytes are involved.

Acknowledgements

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ORAL O7

GC-HRMS FOR RISK-BASED FOOD MONITORING ON STEROIDS

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Abstract
National and international food and feed safety authorities have to shift from routine- to risk-based monitoring. Risk-based monitoring requires flexibility in the scope of analytes, matrices, and sampling. Also, risk-based monitoring implies a desire for retrospective analysis using a different scope(s) to follow trends, perform statistics with later insights, and identify new food safety threats. The current availability of sensitive and accurate high-resolution mass spectrometry (HRMS) enables this desired flexibility.

Our research investigated the applicability of gas chromatography (GC)-HRMS methods for risk-based monitoring food analysis on hormones. By using GC-HRMS Orbitrap technique, we explored the use of HRMS for application as a broad, flexible screening method for steroids in urine, validated according to (EU) 2021/808, and as a non-targeted follow-up for other effect-based screening methods. Besides, we investigated how retrospective analysis could contribute to risk-based monitoring and proved the concept by retrospectively mining using HRMS urine data.

By implementing HRMS for routine food and feed analysis, the competent authorities could extend lagging behind events by a predefined scope with opening the door for risk-based monitoring.

Keywords: GC-HRMS, retrospective analysis, risk-based monitoring

ORAL O8

DIRECT IONIZATION MASS SPECTROMETRY INSIGHTS AND ITS PERFORMANCE FOR THE SCREENING OF GROWTH PROMOTORS

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Abstract

Food and feed safety laboratories are demanding fast screening methods to identify/quantify different compounds at low concentration levels in complex food samples. However, the time-consuming sample clean-up and chromatographic separation hamper fast sample throughput. The recent developments on direct ionization mass spectrometry techniques may overcome some of these difficulties. They allow rapid, real-time, high-throughput, and in situ analysis of solids, liquids, and gases without the need for rigorous sample treatments.

In our research, different direct ionization techniques have been designed (optimizing geometrical parameters, subtract type, the addition of modifiers, etc.) and/or tested (check for false positive/negative results, library matches, etc.) for the fast screening of growth promoters. Our new homemade handheld laser diode thermal desorption electrospray ionization permitted to detect selective androgen receptor modulators in pills and powders, whereas the compact atmospheric solid analysis probe mass spectrometric system identified androgen steroid esters in oil preparations. Our initial results show the applicability of the developed simplified LDTD-ESI and ASAP-MS methods for future screening of organic compounds in complex matrices. These approaches can be very useful to support the control and enforcement authorities since they can fast filter suspect samples for further confirmatory identification improving the control laboratories throughput.

Introduction

In the analysis of drug residue in food, advanced and reliable screening and confirmatory analytical procedures are required to detect growth promotors in stock farming. The analytical methods are mainly based on liquid chromatography coupled with mass spectrometry methods. The strategy consists of a first screening method, and when a suspect result is obtained, these samples will be analysed with a confirmatory method. Although the screening methods showed great potential for identifying targeted compounds in supplements, analytical results are only available after a few days, and the running cost for sample storage and shipping can also be high. Therefore, there is a great interest in developing direct and fast analysis and easy-to-use methods for screening food samples, especially during food safety emergencies.

Direct and ambient ionization mass spectrometry (AIMS) has shown great potential in food and forensic control laboratories due to improved laboratory throughput with their implementation. This increase is because a high number of AIMS techniques are now available [1]. The main differences between AIMS techniques from other direct MS approaches rely on the fact that the sample processing is performed in real-time and proximal to the desorption and ionization of analytes. Hence, these steps take place almost simultaneously or sequentially in a few seconds, making these techniques very attractive for high-throughput laboratories in the field of food analysis, particularly for the detection of food contaminants or the forensic field for the rapid screening of banned compounds. Although today some techniques such as desorption electrospray ionization (DESI) [2], direct analysis in real-time (DART) [3], laser ablation electrospray ionization (LAESI) [4], and atmospheric solids analysis probe (ASAP) [5] are commercially available, still they are mostly used in specialized laboratories or for research purposes. In this context, their potential for future on-site testing is not well established yet, but the development of portable and miniaturized mass spectrometers will significantly support the progress of on-site chemical analysis.

So far, few studies have proposed using different AIMS techniques to analyze growth promotors such as selective androgen receptor modulators (SARMs) and anabolic steroid esters. For instance, Rijke *et al.* [6] proposed the use of DESI with a linear ion trap mass spectrometer in injection sites, whereas Doue *et al.* [7, 8] suggested the use of both DART and ASAP with an orbitrap and a triple quadrupole mass spectrometer for analyzing oily preparations. Nevertheless, although these AIMS approaches reduce the analysis time, the capability of AIMS

techniques to perform on-site analysis would be an added advantage for these controls. Thus, the use of robust transportable or miniaturized MS systems that provide acceptable analytical performance for the prescreening of samples and have a low risk of false-negative/positive results would permit this type of analysis, avoiding the costs of moving samples to the laboratories and further reducing the analysis time and follow-up decision of the product.

In this work, fast screening methods based on AIMS techniques are developed to detect SARMs and anabolic steroids used as growth promoters in food production. The main characteristics of these two new methods are discussed and compared, highlighting their interest in on-site inspections by official food controls [9, 10].

Materials and Methods

Standards

Growth promotor analytical standards of SARMs (ostarine, andarine, ligandrol, testolone, stenabolic and ibutamoren) were purchased from Selleck (Houston, TX, USA), whereas the seventeen androgenic steroid esters (Testosterone esters (8), Estradiol esters (3), Trenbolone esters (3), Boldenone undecylenate, Nortestosterone phenylpropionate, and drostalone enanthate were purchased from Steraloids Inc. Ltd. (London, England). Table 1 describes the growth promotors used in each of the developed AIMS technique in this work.

Table 1 . Chemical compounds and acronyms of the studied SARMs and androgen steroid esters by LDTD-ESI-Ms and ASAP-MS, respectively.

LDTD-ESI-MS	ASAP-MS
Ostarine (Ost)	Testosterone esters (Tx)
Andarine (And)	Estradiol esters (E2x)
Ligandrol (Lig)	Trenbolone esters (Trx)
Testolone (Tes)	Boldenone undecylenate (B Un)
Stenabolic (Sten)	Nortestosterone phenylpropionate (N PhPr)
Ibutamoren (Ib)	Drostalone enanthate (D En)

Instrumentation

Laser diode thermal desorption-electrospray-mass spectrometry (LDTD-ESI-MS) uses an infrared diode laser for the thermal desorption of sample and the generation of gas-phase molecules and an electrospray ionization source for their subsequent ionization through interactions with charged droplets (Figure 1A). On the other hand, the atmospheric solid analysis probe-mass spectrometry (ASAP-MS) technique is based on plasma ionization and combines the thermal desorption carried out by hot nitrogen gas stream flow with atmospheric pressure chemical ionization to produce gas-phase ions through a corona discharge (Figure 1B).

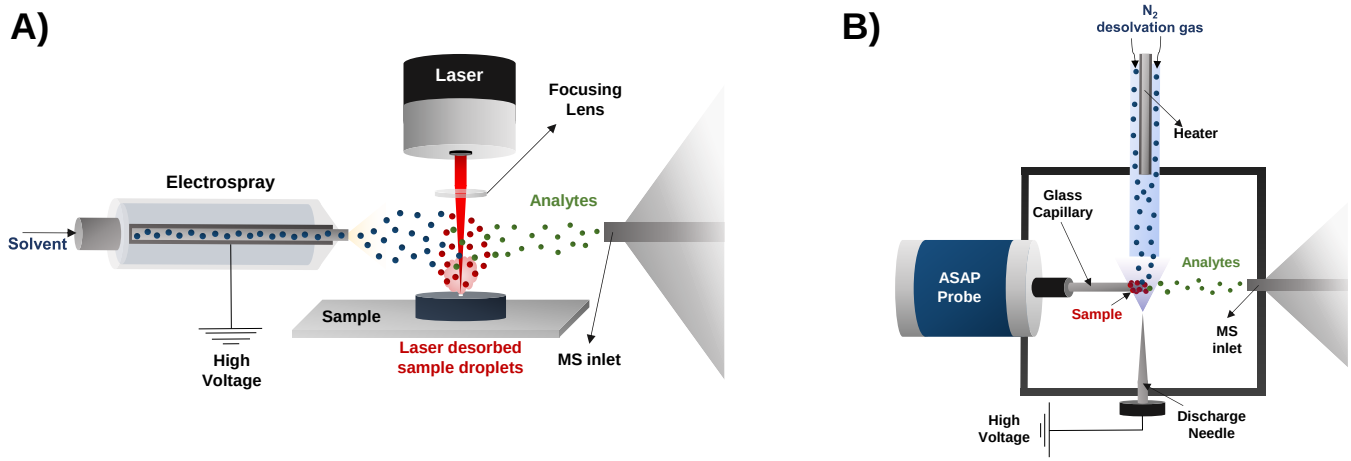


Figure 1. Scheme of (A) Laser diode thermal desorption electrospray (LDTD-ESI) and (B) atmospheric solid analysis probe (ASAP) AIMS techniques.

In our study, the LDTD-ESI-MS technique is equipped with a handheld diode laser (iLase, Biolase Tech, Irvine, CA, USA), an electrospray (ESI) emitter (Waters Corporation, Milford, Manchester, UK), and a transportable single quadrupole Acquity QDa detector MS system (Waters Corporation, Manchester, UK). The laser was operated at 940 nm while the optical peak power output was 3 W (average power density of 1 W), pulse length of 10ms. In positive ion mode, the ESI capillary voltage was 4000 and methanol:water (50:50, *v/v*) with 0.1% formic acid at

50 µL h^{-1} was used as ESI solvent whereas 3500 V and methanol:water (50:50, *v/v*) with 0.1% ammonia were used in negative ion mode. MS-data were acquired in full scan in positive ion mode. The handheld laser is positioned by hand at 90º from the nanoESI emitter. The distance between the mass spectrometer inlet and the spray tip was 5 mm at an angle of 180º, whereas the distance from the portable laser tip to the sample surface was set as 15 mm, the distance from the sample surface to the MS 10 mm. The laser tip distance to the sample cone was 5 mm.

The ASAP-MS method was performed on a RADIAN ASAP instrument based on the model QDa (single quadrupole) mass analyzer and equipped with a horizontal loading fixed geometry ASAP source (Waters Corporation, Manchester, UK). The sampling was carried out by spotting 2 µL onto a sealed glass capillary. Nitrogen gas flow was set at 3.0 L min^{-1}, the corona current at 3 µA and the thermal and probe temperature at 450 °C and 150 °C, respectively. The sampling cone voltages were established at 12, 20, 30, and 40 V and their acquisition were carried out in one run using different channels. Data were acquired in full scan positive ion mode (*m/z* 50–600) and selected ion recording (SIR) at 10 Hz scan speed. Instrument control and MS data analysis were carried out using Mass Lynx *v*4.2 software (Waters Corporation, Manchester, UK).

Samples

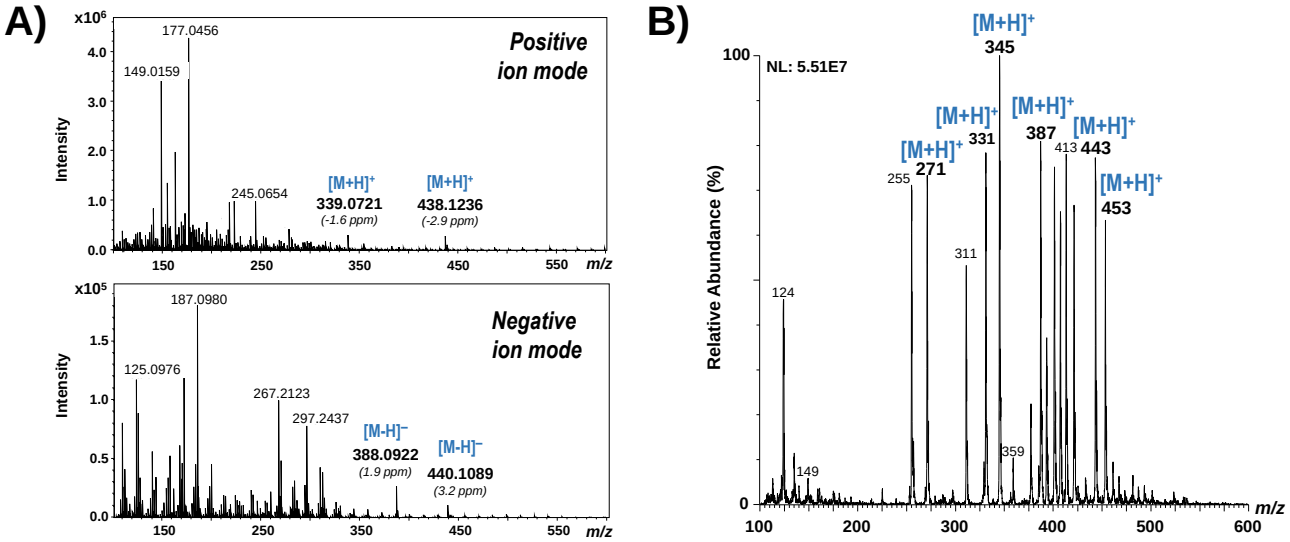
To evaluate the performance of the developed methods, samples suspected to have an illicit growth promoter were analysed. In the case of LDTD-ESI-MS, ten samples in which four were pills and six powders were placed onto the sample stage and they were directly analysed from both outside and inside parts. For the ASAP-MS study, seven oily injection preparations diluted 100-fold in acetonitrile were studied.

Results and Discussion

The use of a laser diode thermal desorption to screen SARMs and the atmospheric solids analysis probe to monitor anabolic steroids have been evaluated, aiming to propose rapid and easy-to-use methods to facilitate the work of official control inspectors and improve the throughput of control laboratories. In general, have commercially lasers a fixed and aligned setup, hindering their applicability for on-site analysis. However, the laser employed in this work is a handheld diode laser. This portable laser was developed for dental surgical applications as a source of infrared radiation. It is equipped with 1-hour lifetime rechargeable battery, making it capable of analyzing solid samples in the field and saving time and costs. On the other hand, the new ASAP mass spectrometry transportable system permits to obtain real-time results and includes a library matching tool.

Ionization behaviour using AIMS techniques

The ionization behaviour of target compounds using the corresponding ambient ionization technique was evaluated and as can be seen, protonated $[M+H]^+$ and deprotonated $[M-H]^-$ molecules were observed for SARMs by LDTD-ESI (Figure 2A), whereas androgen steroid esters present protonated molecule $[M+H]^+$ by ASAP (Figure 2B). Besides, as the mass analyser used in both studies is a single quadrupole and working at low resolution with ambient ionization techniques hinders the unequivocal identification of organic compounds in full scan mode, the use of in-source fragmentation by increasing the cone voltage was evaluated. Under this scenario, it has shown an improvement of the technique in terms of specificity. For this reason, four different cone voltages which allow the characterization of target compounds are usually employed for identification purposes. In this



way, in general, the lowest cone voltage showed the protonated/deprotonated molecule as the base peak of the mass spectrum, while as the voltage value increases, a greater number of characteristic fragments could be observed.

Figure 2. Full scan mass spectra of (A) SARMS by LDTD-ESI-MS and (B) anabolic steroid esters by ASAP-MS.

Method optimization

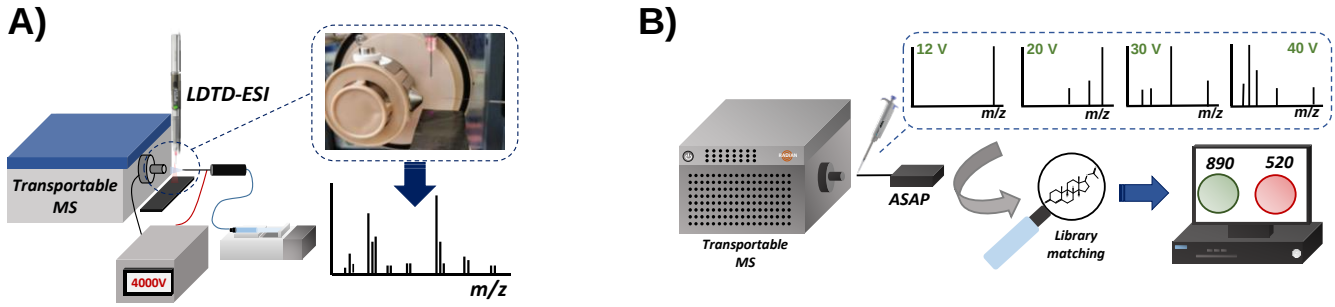
To develop these fast screening methods for the analysis of growth promoters, the nature of the substrate where the sample should be deposited as well as the main operational parameters, were optimized in each case. However, since the first approach is a homemade setup, some operational parameters should be optimized because they are critical for the efficient ionization of target compounds. In contrast, since the ASAP was a commercial device where most of the critical parameters were already pre-set, only a few needed to be optimized. Regarding LDTD-ESI different substrates were evaluated to obtain an intense and reproducible signal response. Among them, the glass and paper substrates were discarded since they led to a low irreproducible signal. In contrast, Teflon substrates look more promising in terms of signal intensity and stability. This fact may be due to its roughness and low porosity that reduce the area seized by the volume of liquid extract deposited, thus increasing the concentration of analyte per unit of area. Additionally, the signal intensity varies depending on the subtract color being 100-fold higher when using the black Teflon. This fact could be attributed to the absorption of all wavelengths by the black Teflon converting them into heat and consequently heating up the surface resulting in a better sublimation and transference to the gas phase of analytes. Regarding the main operational parameters of the developed homemade setup, the electrospray solvent composition has a crucial effect on both the ionization and transfer of the analytes to the mass spectrometer. Therefore, different solvent mixtures of methanol/water and acetonitrile/water and the addition of modifiers were evaluated. Among the organic solvent mixtures, the highest signal intensity in negative ion mode was achieved using 50% methanol/water mixture with ammonia, whereas in positive ion mode, the addition of formic acid provided the highest ionization efficiency. Moreover, the solvent flow rate was also tested from 30 to 500 µL h^{-1} showing the best performance at 50 ul h^{-1}. On the other hand, in the case of ASAP, the sample solvent could also influence the proton transfer or charge exchange reactions, but no differences were observed between methanol and acetonitrile-based solvents and when organic modifiers such as formic acid and acetone were added. Therefore, to simplify the method, 50% acetonitrile-water mixture was selected.

Regarding the laser-related parameters, a considerable increase in the signal intensity of the target compounds was observed when applying program 1, which was based on 3 W peak power and CP1 pulse mode consisting of 0.1 ms on followed by 2.0 ms off. Additionally, the laser diode thermal desorption geometrical parameters were optimized by varying the ESI emitter's distance to the inlet and the portable laser. In this way, the highest signal responses were observed to be at a distance of 5 mm from the mass spectrometer to the spray tip and laser tip and 10 mm and 15 mm from the sample surface to the mass spectrometer and laser tip. On the other hand, since complete vaporization of compounds without thermal degradation is the main objective to reach higher signal intensity with atmospheric solid analysis probe, the source temperature also has a crucial role in the ionization process. In this way, the total ion chronogram exhibits several peaks referring to the vaporization of different compounds due to the temperature increase over time. In fact, the esters with a short side chain showed the lowest desorption temperature, whereas those exhibiting a long side chain showed the highest temperature values. Thereby, since the side chain of target compounds influences the desorption temperature, to ensure the complete desorption of all target compounds from the glass probe, the source temperature was set at 450 ^{o}C.

ASAP-MS technique also enables the use of real-time library matching workflow, and for this purpose, a homemade library using the MSP librarian tool was created for each compound of interest with four acquisitions in full scan mode at different cone voltages. Several mixtures of target compounds in both solvent and oily-based blank matrix were prepared to evaluate the performance of the developed method and establish the library matching identification criteria. In this context, this software correctly recognizes the spiked standards in an oily solution resulting in high average match scores. Therefore, after these studies, an average threshold and individual match scores in each function were set to ensure the correct identification of the analytes. The

established thresholds were as follows: average match score ≥ 800, function 1 (12V) score ≥ 850 and function 2,3,4 (20,30,40 V) score ≥ 825.

In this way, on one hand, a homemade LDTD-ESI-MS setup consisting of a battery-powered surgical laser diode, a black teflon substrate, a nano ESI emitter with an external high voltage supply, and a syringe pump and a transportable MS system was developed for the screening of SARMs (Figure 3A). Additionally, within this project, the workflow using a commercially available instrument was developed, consisting of a first solution deposition followed by the acquisition of mass spectral data at four cone voltages and a library matching process to detect



suspect compounds (Figure 3B).

Figure 3. Scheme of (A) LDTD-ESI-MS system set up and (B) ASAP-MS workflow.

Sample analysis

To evaluate the applicability of the developed LDTD-ESI method, pills and other powders suspected to have a potential illicit application of SARM were analysed. In these studies, no signal was observed in the mass spectrum when analysing the outside or inside parts of the pills, although when the powders were placed on the Teflon substrate, SARMs could be identified. The powders were analysed in full scan mode applying 30 V and stenabolic was tentatively identified in one sample. Afterwards, the obtained mass spectrum was compared with its corresponding standard and they were almost identical, including the chlorine isotope signals and hence confirming the identification of the analyte Thereby, these initial results demonstrated the applicability of the developed method for future on-site analysis of organic compounds in solid samples.

On the other hand, the developed ASAP-MS method was applied to commercially available oily preparations. In many cases, the obtained spectra showed several mass to charge values that correspond to the suspicious target compounds. Additionally, the library matching tool identified several steroids, although few of them did not accomplish the previously established matching criteria and therefore, they were discarded as positive results. Additionally, samples were reanalysed using a selected ion monitoring method consisting of protonated molecules of all target compounds. In one of the samples, Drostanolone was not identified as a false positive in this case. Moreover, all positive samples were analysed by the official LC-MS method in Wageningen Food Safety Research to check the feasibility of the technique in real samples. Most compounds identified by LC-MS were also detected by the ASAP method. This fact makes the proposed methodology promising for the screening of suspect compounds since it identified the sample as non-compliant and therefore, the sample should be submitted to a confirmatory method avoiding a false negative result.

Conclusion

These *AIMS approaches can be very useful to support the control and enforcement authorities since they can fast filter suspect samples for further confirmatory identification improving the control laboratories' throughput. These developed methods permitted to perform screening of SARMs and anabolic steroid esters in food supplements on both benchtop and transportable MS instruments. However, when using the AIMS techniques with transportable low-resolution mass spectrometry instruments (e.g., single quadrupole), the general lack of selectivity and sensitivity to allow on-site screening of regulated substances at relevant levels should be improved in future applications. For this purpose, transportable MS systems could achieve more selectivity and detectability*

by implementing tandem mass spectrometry, either based on ion-trap or triple-quadrupole miniaturized mass analysers.

Acknowledgements
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ORAL O9

FULLY AUTOMATED BOVINE DRIED BLOOD SPOT ANALYSIS OF ON-CARD DERIVATISED STEROID-ESTERS BY SOLID-PHASE EXTRACTION LIQUID CHROMATOGRAPHY TRIPLE QUADRUPOLE MASS SPECTROMETRY

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Abstract
Anabolic steroids can be used to enhance growth. In animal production, the use of steroids for this purpose is forbidden within the European Union, and strategies and methods to detect abuse of exogenously administered steroids have been developed and are used in routine control programs. One particular part of the control involves the analysis and confirmation of intact steroid-esters in biological matrices, e.g., hair and blood. In this study, a new fast method was developed to detect steroid-esters based on dried blood spot (DBS) analysis. The use of dried blood spots for analysis of steroid-esters offers advantages in sample collection, transportation and storage. Dried blood spot cards, in combination with a dried blood spot autosampler (DBSA), were used for sample preparation. Subsequently, the DBSA was coupled on-line with a solid phase extraction (SPE) module for sample clean-up and concentration. The detection of steroid-esters was performed by LC-MS/MS analysis. To enhance the sensitivity, derivatisation of steroid-esters was performed on the dried blood spot card. Bovine blood and serum samples were spiked with testosterone-decanoate, testosterone-propionate, testosterone-phenylpropionate and testosterone-isocaproate and used to develop and validate the new method. Repeatability in blood and serum respectively ranged from 3-19 % and 2-22 %. The CCα values in blood and serum ranged from 0.22-0.33 picogram per microliter in blood to 0.17-0.44 picogram per microliter in serum. This applies for the compounds that can be determined quantitatively; testosterone-decanoate, testosterone-propionate, testosterone-phenylpropionate. The method was characterised as a qualitative screening method for testosterone-isocaproate. To demonstrate the applicability of the newly developed on-line DBSA-SPE-HPLC-MS/MS method, serum samples were obtained from an animal experiment in which bovines had been treated with a formulation containing these four testosterone-esters, were successfully analysed.

Introduction
The use of growth promoters in cattle breeding, including steroid hormones, β-agonists and growth hormones like myostatin, provides a higher growth rate of animals and thereby reduces the age at slaughter and, consequently, lowers the production costs (Heitzman 1976, Lone 1997, Scarth, Akre et al. 2009). However, the European Union Council Directives 96/22/EC states that substances having a hormonal action are prohibited for use in animals intended for meat production for human consumption. The directives also cover synthetically produced versions and analogues of endogenous steroids, like the male sex hormone testosterone and its analogues and derivatives (e.g., boldenone and testosterone-decanoate). These steroids can be used as illegal growth promoters in animal production and also by athletes or can be used for medical purposes. Testosterone and its analogues and derivatives can be applied via the skin (pour-on, cream, implants) (Stolker, Groot et al. 2009), orally (tablets, supplements) (Nielen, Lasaroms et al. 2006) or by intramuscular injection (Duffy, Rambaud et al. 2009).

A large number of esterified steroid preparations are available on the market, and the availability has increased via internet sales (Antonopoulos and Hall 2016). From an abuse perspective, the use of mixtures of testosterone-esters by intramuscular injection is beneficial. Once injected into muscle tissue, the steroid-esters are slowly released into the bloodstream, after which esterase enzymes hydrolyse them over time into their free active form. Generally, the longer the ester side chain, the lower the hydrophobicity of the compound, and the longer it will take to diffuse into the bloodstream (Gray, Teale et al. 2011, Forsdahl, Erceg et al. 2015). Therefore a mixture of esters can be used to provide a constant and sustained dose of the active parent steroid without the need for daily injections or oral administrations (Gray, Viljanto et al. 2013). Sustanon, a popular

injection cocktail, contains four esters, from which testosterone-propionate and testosterone-phenylpropionate are released fast, typically in the first days, while testosterone-decanoate and testosterone-isocaproate are released more slowly (Figure 1) (Gray, Teale et al. 2011, Forsdahl, Erceg et al. 2015).

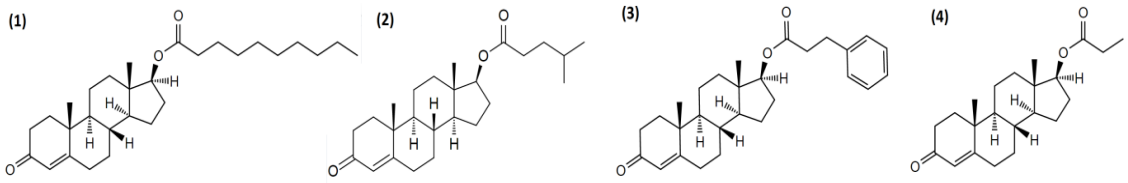

Figure 1: Structures of four testosterone-esters present in Sustanon®, (1) Testosterone-decanoate, (2) Testosterone-isocaproate, (3) Testosterone-phenylpropionate, and (4) Testosterone-propionate.

The use of Sustanon in the animal product is illegal, and enforcement of the European directives requires detection techniques able to analyse steroid hormones, including steroid-esters mixture as Sustanon. Most methods to detect steroid-esters are based on the analysis of urine samples where the ester chain is not present anymore. As a result, it is impossible or very difficult to distinguish between exogenous and endogenous natural steroids, i.e. not possible by LC-MS/MS analysis (Stolker, Groot et al. 2009) and difficult and laborious by use of GC-c-IRMS (Blokland, Van Tricht et al. 2012). From a regulatory point of view, the detection of an intact steroid-ester provides unambiguous evidence of an illegal administration. However, the detection of intact steroid-esters is not easy due to low concentrations of intact steroid-esters present in blood (Kim, Choi et al. 2000, Gray, Teale et al. 2011). The low concentrations of steroid-esters require derivatisation of the esters before analysis to increase the sensitivity of the detection method (Scarth, Akre et al. 2009, Stolker, Groot et al. 2009, Marcos and Pozo 2015). Matrices in which the intact ester form of the steroid can be detected are injection sites (Costain, Fesser et al. 2008, de Rijke, Hooijerink et al. 2013), blood (Tretzel, Thomas et al. 2014, Forsdahl, Erceg et al. 2015) and hair (Kintz, Cirimele et al. 1999, Nielen, Lasaroms et al. 2006, Stolker, Groot et al. 2009).

The simple concept of applying biological fluids, like blood, to a filter paper for drying and transport and subsequent chemical analysis in the laboratory is not new. With dried blood spot (DBS) sampling, a blood sample is typically collected on a filter paper card made of cellulose or polymer materials (Hannon and Therrell 2014). Guthrie first introduced DBS in 1963 for new-borns (Guthrie and Susi 1963). Next to new-born screening, DBS techniques are used in the field of toxicological and toxicokinetic studies, therapeutic drug monitoring and forensics. Commercial DBS kits consist of a filter paper card for the collection of the blood and contain a lancet for the blood punch, e.g., in the shape of a press button or a pen. A blood droplet will be collected on the DBS-card, and the card can be sent to the lab for analysis. In de lab, the blood spot can be punched out, followed by classical off-line chemical analysis (Tretzel, Thomas et al. 2014). In the study of Tretzel (Tretzel, Thomas et al. 2014), a method based on DBS for the off-line detection of steroid-esters was published. An alternative to off-line DBS analysis came with the introduction of (semi) automated DBS sample pre-treatment systems. These systems reduce the manual sample pre-treatment time and minimise the risks of human errors. The combination of the DBS sample pre-treatment systems with advances in mass spectrometric analysis has offered new possibilities for DBS analysis (Tretzel, Thomas et al. 2016, Verplaetse and Henion 2016, Lange, Walpurgis et al. 2019, Lange, Thomas et al. 2020, Solheim, Jessen et al. 2020, Thevis, Kuuranne et al. 2020). Furthermore, when using the DBS technique, sample storage space can be saved, but also sampling, sample transport and sample processing costs can be reduced.

The goal of the presented study was to develop and validate a method for the determination of steroid-esters (testosterone-propionate, testosterone-phenylpropionate, testosterone-isocaproate, and testosterone-decanoate) in bovine blood, making use of DBS cards. Due to the physicochemical properties of the steroid-esters and the low blood concentrations after treatment, on-card derivatisation of the steroid-esters was incorporated to increase the sensitivity of the method.

Materials and Methods

Reagents, chemicals and materials

Dried blood spot cards were obtained from Ahlstrom Munksjö (Bärenstein, Germany). The reference standards testosterone-propionate, testosterone-phenylpropionate, testosterone-isocaproate and testosterone-decanoate were obtained from Sigma-Aldrich (St. Louis, USA). Deuterated testosterone-propionate, testosterone-phenylpropionate and testosterone-decanoate were obtained from Steraloids (Newport, USA) and the pharmaceutical testosterone-ester mix Sustanon® was obtained from Organon N.V., Oss, The Netherlands (nowadays registered by Aspen Pharma Trading Limited, Dublin, Ireland).

Optimised card preparation and on-card derivatisation
Bovine blood or serum, 10 µL, was pipetted onto the designated part of the DBS card and the spot was dried at room temperature for at least one hour. Derivatisation was performed by pipetting 10 µL of 500 mM methoxyamine solution (in 50% methanol) onto the spot and the card was placed in a stove at 60°C for 20 min. After this derivatisation step, the DBS card was placed in the DBS autosampler for further preparation and analysis.

Optimised on-line sample preparation, clean-up and analysis
After preparation of the DBS-card, an automatic dried blood spot autosampler (Spark Holland, The Netherlands) was used to desorb the blood/serum sample from the card. The DBS-card was positioned in-between two 6 mm clamps which encloses the spot under pressure. Flow-through desorption takes place by passing 2 ml of 40 (v/v)% ACN (heated at 60 °C) through the clamped card. The DBSA is coupled with the on-line SPE unit, Automatic Cartridge Exchanger™ (Spark Holland, The Netherlands), where the compounds were trapped on a HySphere C_{18} HD SPE cartridge (10 x 2 mm internal diameter, 7 µm particle size, 100 Å pore diameter, Spark Holland). The C_{18} SPE cartridge was activated, conditioned and equilibrated with 1 mL ACN followed by 1 mL water, before the desorption output was loaded on the SPE cartridge. Matrix compounds were removed by washing the cartridge with 2 mL 1 (v/v)% TFA followed by 1 mL Milli-Q, 1 mL 30 (v/v)% ACN, and finally 1 mL Milli-Q. Following, the cartridge was placed into the HPLC flow and the compounds were eluted and transported to an the analytical HPLC column for separation. The HPLC system consisted of a Waters Acquity Ultra Performance LC (Waters, Milford, England) equipped with a Restek Raptor ARC C_{18} analytical column (100 x 2.1 mm, 2.7 µm). Column temperature was set at 60 °C and flow rate at 0.4 $mL \cdot min^{-1}$. Gradient elution was performed using mobile phases 5 mM formic acid in water (A) and 5 mM formic acid in ACN (B). The gradient started with the mobile phase held at 50 % A for 1 minutes followed by a linear increase of 2 minutes to 100 % B. After a 3-minute hold at 100 % B the flow was set to 50 % A again. The total run time of the complete instrumental preparation and analysis was 11 minutes. Detection was carried out using a Waters Xevo TQ-S mass spectrometer (Waters, Milford, England) in the positive electrospray ionisation (ESI) mode using selective reaction monitoring. Capillary voltage was set at 3.0 kV, source temperature at 150 °C, desolvation temperature at 600 °C, cone gas flow at 150 $l \cdot h^{-1}$ and desolvation gas flow at 600 $l \cdot h^{-1}$. Argon ($p=2.2 \cdot 10^{-3}$ mbar; purity > 99.998 %) was used as collision-induced dissociation (CID) gas.

Method validation
The developed DBSA-SPE-HPLC-MS/MS method for the analysis of steroid-esters in bovine blood samples was fully validated, according to Commission Decision 2002/657/EC. The following validation parameters were determined: linearity, trueness, repeatability, within-laboratory reproducibility, decision limit (CCα), detection capability (CCβ), selectivity and robustness. Additional validation was carried out for bovine serum samples. Trueness should be determined by use of certified reference material. However, for the compounds and materials, as used in this study, no certified reference material is available. In such case, trueness can be determined by using blank matrix, 100 µL blood or serum, to which known amounts of the compounds of interest are added (10 µL of different standard mixtures). Based on pre-validation studies, individual validation levels were set for the different testosterone-esters. These levels are 2 $pg \cdot \mu L^{-1}$ for testosterone-propionate and testosterone-phenylpropionate and 1 $pg \cdot \mu L^{-1}$ for testosterone-isocaproate and testosterone-decanoate. The validation was carried out on three different days with 21 different bovine blood samples and, additionally, one day with 7 different bovine serum samples.

Quantification of the testosterone-esters in the fortified blood and serum samples was performed using a matrix-matched standard (MMS). Using these MMS, a calibration curve was made by plotting the added concentration of a compound against the response of that compound corrected by the response of the corresponding deuterated standards. For testosterone-isocaproate, no internal standard was used. Linearity was calculated with linear regression on the MMS curve for a concentration range of 0.2 – 5 times the

validation level for validation, and the calculated correlation coefficient (R^2) should be > 0.990. Seven different blank blood samples were fortified with 0.5, 1.0 and 1.5 times the validation level for validation to determine the repeatability (including matrix-variation), within-laboratory reproducibility and accuracy of the method. The same procedure was repeated on 2 additional days with seven different fortified blank blood samples each day. For a quantitative method, the repeatability was accepted below 14.7 %, the within-laboratory reproducibility below 22 % and the accuracy of the method were accepted between 50-120 % for concentration ≤ 1 $pg \cdot \mu L^{-1}$ and 70-110 % for a concentration between 1-10 $pg \cdot \mu L^{-1}$. The CCα and CCβ were calculated from the fortified blank samples according to Commission Decision 2002/657/EC. The identity of a compound was confirmed when the deviation of the relative retention time of the chromatographic peak (relative to the internal standard) and the deviation of the ion ratio of the two product ions compared to the MMS was fulfil the criteria specified in Commission Decision 2002/657/EC. The specificity was checked by the analysis of the 21 blank samples without addition of the compounds, and the chromatograms were monitored for peaks interfering with those of the compounds of interest. When no blank peak interference on the relevant retention time is observed, the method is considered specific for this compound. In addition to the full validation of blood, also serum was validated, but according to a one-day-validation additional procedure. Therefore, only 7 bovine serum samples were used.

Animal experiment
For the proof-of-principle of the method serum of treated calves were used. An animal study was performed whereby to five calves of about 100 kg, Sustanon® (250 $mg \cdot mL^{-1}$) intramuscularly was injected in the neck. The animal experiment was approved by the animal experiment committee of Wageningen University and Research with code 2016007.c. Sustanon® was administered once a week for four weeks, based on intramuscular injection of 1 mL in the neck of the animals. Three days after each injection, blood was sampled (about 10 mL) from the tail or jugular vein. After the final injection, blood was sampled every day for two weeks. The blood collection tubes were centrifuged and the obtained serum was transferred to a test tube and stored in a freezer.

Results and Discussion
Method optimisation
The following parameters of the derivatisation step were studied; concentration, volume, and the way the derivatisation reagent was added in combination with the incubation temperature and time. The spot desorption was optimised, taking the influence on the SPE loading into account. The relevant parameters were the desorption solvent, volume, temperature and flow rate. The desorption volume flow directly to the SPE after the spot desorption. Various SPE sorbents were tested again in combination with different solvents and flow rates, and the number of wash steps. The chromatographic separation was optimal using the core-shell LC column technology, as the maximum pressure the SPE system can handle is only 300 bar. This analytical LC column has a similar performance as totally porous silica HPLC columns but at lower backpressure (Gritti, Cavazzini et al. 2007). All optimised parameters lead to the final method described in the Materials and Methods chapter.

As result of the optimisation of the DBSA-SPE-LC-MS/MS method, steroid-esters could be detected in bovine dried blood spots, as shown in Figure 2. Two chromatograms are presented, a standard containing the four steroid-esters and a fortified blood sample containing the four steroid-esters. In both cases, 10 µL sample was pipetted on the card. As can be depicted from the chromatogram for each compound, two peaks are observed and are due to the methoxime derivatisation reaction whereby an isomeric mixture is formed. The double peak will decrease the overall sensitivity but will enhance the specificity since, for each compound, two peaks have to be observed. For quantification, the sum of both peaks is used. In the fortified sample, all steroid-esters were detected, demonstrating that the method is applicable for the intended goal.

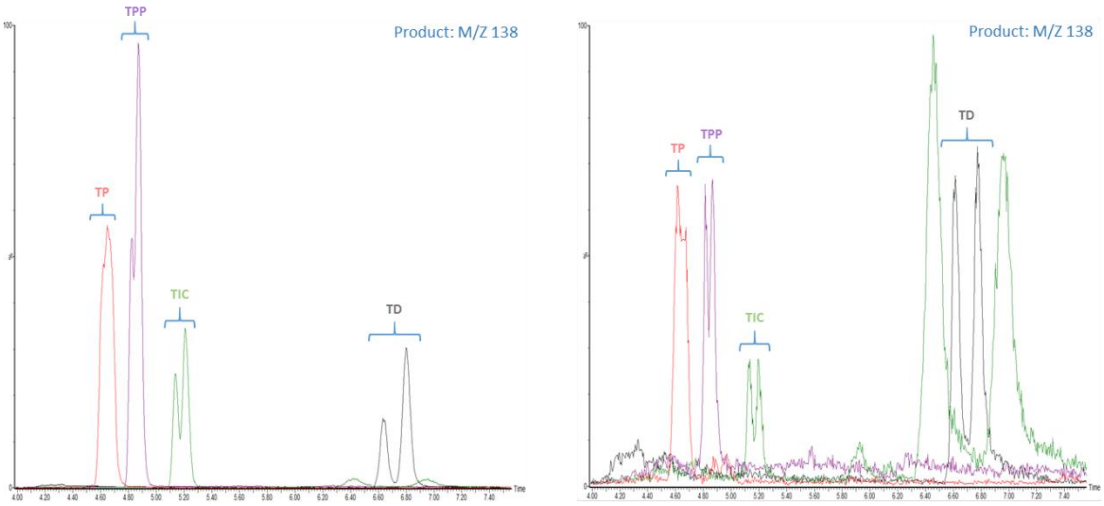

Figure 2: Chromatograms of a complete DBSA-SPE-HPLC-MSMS run, 10 µL standard solution, A) Standard solution of 10 pg·µL^{-1} testosterone-isocaproate and testosterone-decanoate and 20 pg·µL^{-1} testosterone-propionate and testosterone-phenyl-propionate, B) fortified blood with 1 pg·µL^{-1} testosterone-isocaproate and testosterone-decanoate and 2 pg·µL^{-1} testosterone-propionate and testosterone-phenyl-propionate.

Method validation

The newly developed DBSA-SPE-HPLC-MS/MS method was validated following Commission Decision 2002/657/EC. Validation results for bovine blood and serum are given in table 1.

Table 1. Validation results in bovine blood and serum. The underlined number did not comply the criteria.

Parameter	Testosterone-propionate		Testosterone-phenyl-propionate		Testosterone-isocaproate		Testosterone-decanoate	
	Blood	Serum	Blood	Serum	Blood	Serum	Blood	Serum
Validation Level (pg·µL^{-1})	2	2	2	2	1	1	1	1
Linearity (R^2)	>0.990	>0.990	>0.990	>0.990	>0.990	>0.990	>0.990	>0.990
Accuracy (%)	93	<u>117</u>	94	85	55	<u>191</u>	108	92
Repeatability (RSD%)	3	2	4	5	<u>19</u>	<u>22</u>	6	7
Reproducibility (RSD%)	8	4	5	7	19	<u>35</u>	9	12
CCα (pg·µL^{-1})	0.25	0.17	0.33	0.44	0.80	1.3	0.22	0.35
CCβ (pg·µL^{-1})	0.49	0.34	0.65	0.89	1.6	2.7	0.45	0.70
Confirmation*	Yes	Yes	Yes	Yes	<u>No</u>	<u>No</u>	Yes	Yes

*Comply with the criteria for confirmation according to 2002/657/EC

Following the Commission Decision 2002/657/EC, the method fulfills the criteria for a confirmatory method for testosterone-propionate, testosterone-phenylpropionate and testosterone-decanoate for blood but not for testosterone-isocaproate. An interfering chromatographic peak was present for testosterone-isocaproate for one of its fragment ions (m/z 126), and as a result confirmation of testosterone-isocaproate was not possible. For testosterone-isocaproate, also the accuracy, repeatability and reproducibility did not all fulfill the requirements for a quantitative method, even as testosterone-propionate did not fulfill the requirements for the accuracy in serum. A deuterated internal standard for testosterone-isocaproate could improve the quantitative performance of the method. Therefore, the new method is characterised as a qualitative screening method with a CCβ of 1.6 pg·µL^{-1} for testosterone-isocaproate and as a quantitative method for the other testosterone-esters with a CCα of 0.25, 0.33 and 0.22 pg·µL^{-1} for testosterone-propionate, testosterone-phenylpropionate and testosterone-decanoate, respectively, in blood. The method was proven to be selective, as in none of the blank blood and serum samples chromatographic peaks were observed at retention times that could interfere with the targeted compounds, except for testosterone-isocaproate, for which a peak was

observed that interfered with its fragment m/z 126. The robustness of the method was tested during method development.

Proof of principle
As a proof of principle serum samples from animals treated intramuscularly with a mix of 4 testosterone-esters were analysed to verify the feasibility of the newly developed method. Eleven serum samples were selected and analysed with the DBSA-HPLC-SPE-MS/MS method. These samples were also analysed according to our standard operating procedure, this SOP is based on the method published by (Gray, Teale et al. 2011). This off-line sample preparation and LC-MS/MS analysis was performed approximately one year before her-analysis of the serum samples with the newly developed DBSA-SPE-HPLC-MS/MS method. Figure 3 shows the quantitative results of both methods for the analysis of testosterone-decanoate. Both methods are able to confirm and quantify testosterone-decanoate in samples at relevant levels. The concentrations as determined by the DBSA-SPE-HPLC-MS/MS method are lower compared to the conventional method which can be explained by the time between analysis of both methods was over one year, whereby the serum samples were stored at -20 °C. It is known that steroid-esters in serum can degrade over time, even when stored in the freezer. The concentrations of the other steroid-esters were below CCα in serum in both methods (data not shown).

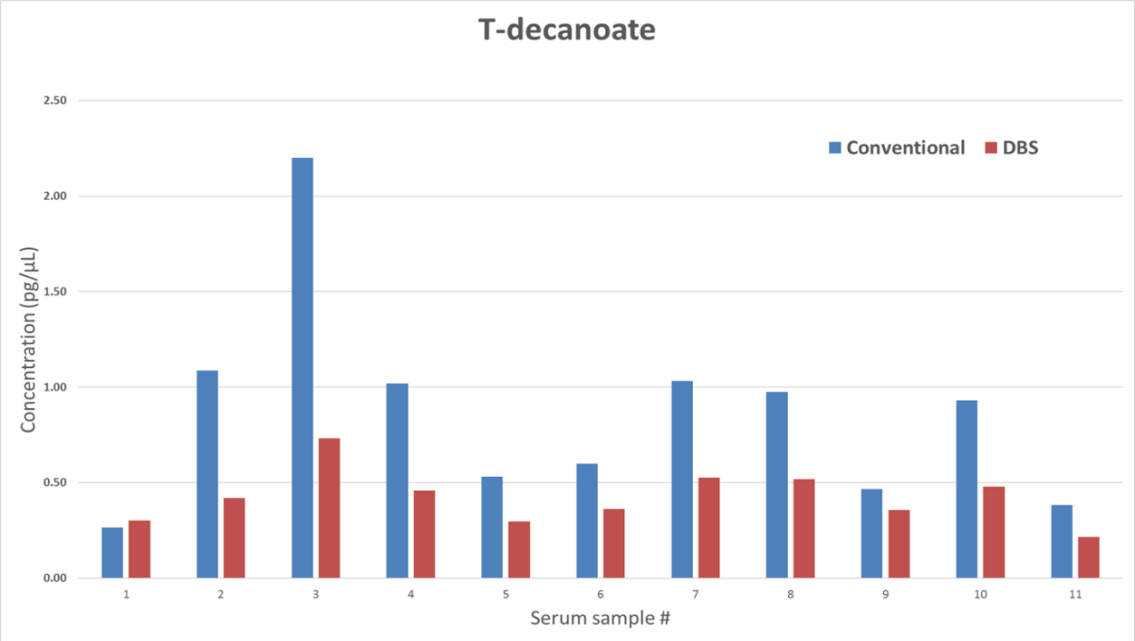

Figure 3. Concentration of testosterone-decanoate in serum determined with the conventional HPLC-MS/MS method (blue bars) and with the on-line DBSA-SPE-HPLC-MS/MS method (red bars).

Conclusion
A (semi) automated method was developed and validated based on dried blood spot analysis of derivatised testosterone-esters. Four commonly used testosterone-esters, testosterone-decanoate, testosterone-propionate, testosterone-phenylpropionate and testosterone-isocaproate were analysed in bovine blood and serum samples. The validation entirely fulfilled the criteria of the Commission Decision 2002/657/EC for a quantitative confirmatory method for testosterone-propionate, testosterone-phenylpropionate and testosterone-decanoate in bovine blood. The analysis of testosterone-isocaproate is characterised as a qualitative screening method. The developed DBS method was applied to samples from an animal study, and comparable results were found as with the reference method. These results show the possibilities and added value of the technique for the detection of illegal hormone use in cattle. This method can be applied in routine

control programs as samples can easily be taken at the farm on the card and transported, stored, derivatised and analysed at the laboratory.

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ORAL O10

DEVELOPMENT AND APPLICATION OF A MULTI-RESIDUE METHOD FOR NITROFURAN ANALYSIS, INCLUDING NEW BOUND MARKER METABOLITES

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Abstract

The aim of this work was to extend the scope of analysis and develop a faster method to include four additional nitrofuran compounds, detected as their markers dinitrosalicylic acid hydrazine (DNSAH), hydroxybenzhydrazide (HBH), oxamic acid hydrazide (OAH) and aminoguanidine (AGN). The analysis time was shortened from four to two days by developing a rapid sample preparation approach, using a microwave-assisted derivatisation step and a modified QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) -based extraction. This rapid method was fully validated in accordance with the new 2021/808 EU guidelines and decision limits (CCα) for all analytes ranged from 0.013 to 0.200 µg kg^{-1}. It is now an accredited testing method for nitrofuran analysis on the National Residue Control Plan in Ireland.

Introduction

Nitrofurans are a class of broad spectrum antibiotics widely used in the past as growth promotors, and for the treatment of a range of infections and diseases. However, these drugs are currently banned from use in food-producing animals in the EU due to concerns regarding their undesirable toxicological properties, and are listed under "prohibited substances" for which a MRL (Maximum Residue Limit) cannot be established. The analysis of nitrofurans has not changed significantly since the implementation of the FoodBRAND (Bound Residues and Nitrofuran Detection) methodology, a confirmatory LC-MS/MS method developed for the analysis of four nitrofurans, namely furaltadone, furazolidone, nitrofurantoin and nitrofurazone, as their respective marker residues (Cooper *et al.*, 2005). It was only recently that a fifth bound residue, namely DNSAH (marker residue for nifursol), was added to the monitoring list in the EU. A number of other nitrofuran drugs exist, namely, nitrovin, nifuroxazide and nifuraldezone that can form bound residues in animal tissue. A further recent change was the reduction of the EU Reference Point for Action (RPA) from 1.0 µg kg^{-1} to 0.5 µg kg^{-1}.

The analysis of nitrofuran residues in testing laboratories is challenging because of the need to maintain low sub-ppb detection levels for analytes and the desire to achieve short turnaround times. The original bound residue protocol is the most effective means of achieving improved sensitivity but is time consuming due to the extensive washing steps employed, the inclusion of a 16 h overnight derivatisation step, the double liquid-liquid extraction and solvent evaporation. Overall this process takes four days to complete. Alternative strategies have been proposed to provide more rapid analysis of nitrofuran residues, including analysis of total residues (no washing of samples), analysis of bound residues using simplified washing steps, and methods incorporating more rapid derivatisation of analytes at higher temperatures. The drawback of employing a total residue approach/simplified washing is that it generally results in less sensitive analysis, and can lead to shorter chromatographic column lifetimes and more mass spectrometry instrument downtime and source contamination problems.

The objectives of this work were to extend nitrofuran analysis to eight nitrofuran drugs and shorten turnaround times through improvements in the sample preparation.

Materials and Methods

Chemicals, materials and apparatus

3-amino-2-oxazolidinone (AOZ), 3-amino-5-morpholinomethyl-1,3-oxazolidin-2-one (AMOZ), 1-aminohydantoin (AHD), oxamic acid hydrazide (OAH), 5-dintrosalicylic hydrazide (DNSAH), 3-((2-nitrophenyl)methylene-amino-2-oxazolidinone (NPAOZ), 5-methylmorfolino-3-((2-nitrophenyl)methylene)-3-amino-2-oxazolidinone

(NPAMOZ), 1-((2-nitrophenyl)methylene)-amino-2-hydantoin (NPAHD), (2-nitrophenyl)methylene-semicarbazide (NPSEM), 3,5-(2-nitrophenyl)-dinitrosalicylic acid hydrazide (NPDNSAH), 4-(2-nitrophenyl)-hydrozybenzhydrazide (NPHBH) and (2-nitrophenyl)-aminoguanidine (NPAGN) were all purchased from Witega (Berlin, Germany). Semicarbazide hydrochloride (SEM) and aminoguanidine hydrochloride (AGN) were both supplied by Sigma-Aldrich (Dublin, Ireland). 4-hydroxybenzhydrazide (HBH) was obtained from Toronto Research Chemicals (Toronto, ON, Canada). 2-nitrophenyl-oxamic acid hydrazide (NPOAH) was obtained via laboratory synthesis at Teagasc Food Research Centre (Ashtown, Ireland). All internal standards were purchased from Witega (Berlin, Germany), namely, 3-amino-2-oxazolidinone-d4 (AOZ-d4), 3-amino-5-morpholinomethyl-1,3-oxazolidinon-2-one-d5 (AMOZ-d5), 1-aminohydantoin-$^{13}C_3$ (AHD-$^{13}C_3$), semicarbazide-^{13}C $^{15}N_2$ (SEM-^{13}C $^{15}N_2$), 3,5-dinitrosalicylic acid hydrazide-$^{13}C_6$ (DNSAH-$^{13}C_6$), 4-hydroxybenzhydrazide (HBH-$^{13}C_6$), oxamic acid hydrazide-$^{15}N_3$ (OAH-$^{15}N_3$) and aminoguanidine-$^{13}C^{15}N_4$ (AGN-$^{13}C^{15}N_4$). Individual primary stock solutions of nitrofuran standards and their isotopically labelled internal standards were prepared at a concentration of 50 µg mL^{-1} in MeOH. Two stock mixtures of the eight nitrofuran metabolites (MM1) and eight internal standards (IS1) was prepared in MeOH both at a concentration of 1 mg L^{-1}. A stock mixture (NP1) of the eight nitrophenyl derived standards was prepared in MeOH at a concentration of 1 mg L^{-1} (free metabolite equivalents). The NP1 solution was further diluted in MeOH to a concentration of 10 µg L^{-1} to make an intermediate working solution (NP2) of the nitrophenyl derived standards. Three intermediate standard mixtures were prepared daily by dilution of the MM1 solution in MeOH to give concentrations of 50 µg L^{-1} (MM2), 5 µg L^{-1} (MM3) and 0.5 µg L^{-1} (MM4). Two intermediate mixtures of internal standards were also prepared daily, by dilution of the IS1 solution in MeOH to give concentrations of 50 µg L^{-1} (IS2) and 5 µg L^{-1} (IS3).

Sample preparation

Muscle tissue (1.0 ± 0.01 g) aliquots were weighed into 50 mL polypropylene tubes, homogenised and washed sequentially with water and organic solvents, ice-cold MeOH, ice-cold EtOH and EtO_2 as described by Hoogenboom *et al.* (1992). The samples were placed in a fumehood, and the residual EtO_2 was left to evaporate overnight. Once fully evaporated, seven matrix calibrants were fortified across a range of concentrations, from 0.02 µg kg^{-1} to 5.00 µg kg^{-1}. A 100 µL volume of the internal standard solution (IS3) was added to all calibrants, controls and test samples. A 9 mL volume of HCl (0.1 M), a 100 µL volume of NBA (100 mM) and a magnetic stirrer bar was added to each sample. The samples were derivatised at the "high" stirring speed setting in a MARS 6 microwave system by ramping from room temperature to 60 °C over 4 min, followed by a 2 h hold at 60 °C. After removal from the microwave, samples were immediately neutralised, by adding 1 mL trisodium phosphate buffer (0.3 M) and approximately 570 µL NaOH (1 M). The pH of neutralised samples was checked using pH strips (satisfactory range of pH 6.5 – 7.5), and adjusted accordingly using 1 M HCl or 1M NaOH if necessary. Neutralised samples were then subjected to a QuEChERS-based extraction, which excluded the sorbent clean-up step due to the extensive pre-washing of the samples. 10 mL MeCN, a ceramic homogeniser, and 1 g NaCl were added to each tube, before being vortexed for 1 min. Approximately 4 g of $MgSO_4$ was added, and samples were shaken on a Minimix vibrational unit for 5 min. The shaken samples were then centrifuged at 2800 × *g* (4 °C, 12 min). The supernatant was transferred into a 15 mL polypropylene tube and evaporated to dryness under nitrogen stream on a Turbovap at 40 °C. After evaporation, the dried extracts were reconstituted in 500 µL of injection solvent (5 mM ammonium formate in H_2O: MeOH (90:10, *v/v*) and vortexed for 1 min prior to filtration through 0.2 µM PTFE syringe filters directly into autosampler vials. A 10 µL volume was subsequently injected into the UHPLC-MS/MS system.

UHPLC-MS/MS analysis

Samples were analysed using an Exion UHPLC system, coupled to an AB Sciex 5500+ QTRAP mass spectrometer (Warrington, UK), equipped with a TurboV Ion Source. Separation was performed on a stainless steel Agilent ZORBAX Eclipse Plus Phenyl-Hexyl RRHD analytical column (2.1 × 50 mm, particle size 1.8 µm), fitted with an in-line filter of 0.2 µm pore size, maintained at 40 °C. A binary gradient separation comprising of (A) 5 mM ammonium formate in H_2O: MeOH (90:10, *v/v*) and (B) 5 mM ammonium formate in H_2O:MeOH (10:90, *v/v*) pumped at 0.6 mL min^{-1}. The needle wash was MeOH:H_2O (90:10, *v/v*). Mass spectrometry conditions for analytes and internal standards are presented in Table 1.

Method validation

Method validation was carried out in accordance with 2021/808/EC guidelines (Commission Implementing Regulation, 2021) to evaluate the following parameters: identification, selectivity, linearity, matrix effects, trueness, within-laboratory repeatability (WLr), within-laboratory reproducibility (WLR) and decision limit (CCα). Identification was carried out through assessment of identification points, ion ratios and retention times. Selectivity was evaluated by injection of standard solutions of each individual analyte and internal standard, to

ensure that there were no observed interferences when monitoring all transitions. A matrix effects evaluation was carried out by analysing avian (*n* = 10), bovine (*n* = 10), ovine (*n* = 10), and porcine (*n* = 10) muscle samples, and comparing the signal obtained from standards spiked into solvent to those obtained from post-extraction spiked muscle samples. These samples were also analysed non-spiked to determine if any matrix interferences co-eluted with the analytes. Linearity of calibration curves was evaluated through the assessment of residues, R^2 values and visual inspection. Within-laboratory repeatability (WLr) studies were carried out using negative samples fortified at 0.5 (*n* = 8), 1.0 (*n* = 8) and 1.5 (*n* = 8) times the Reference Point for Analysis (RPA) of 0.5 µg kg^{-1}, which is set for only five nitrofurans but was applied to all eight nitrofurans in this work. WLr validations were carried out by the same analyst on three different days using repetitions of the same sample from each species (avian bovine, and porcine) on each day. WLR studies were carried out by three different analysts on separated days. Each WLR run included 32 different samples from avian (*n* = 8), eight bovine (*n* = 8), ovine (*n* = 7), porcine (*n* = 8), cervine (*n* = 1) and equine (*n* = 1). The WLR runs were carried out at 0.5 (*n* = 8), 1.0 (*n* = 8), 1.5 (*n* = 8) and 2.0 (*n* = 8) times the RPA. The CCα values were calculated as defined in 2021/808, by plotting the signal against the added concentration. The corresponding concentration at the y-intercept plus 2.33 times the standard deviation of the within-laboratory reproducibility of the intercept equals the decision limit (α = 1%). If the generated CCα values were lower than the levels achievable for certain analytes, they were set to higher concentrations. All CCα values were verified in an analytical run by fortification of 32 muscle samples at the selected CCα values.

Table 1. UHPLC-MS/MS conditions for nitrofuran bound residues

Analyte	Precursor (*m/z*)	Product (*m/z*)	RT (min)	DP	CE	CXP	MRM
NPAHD	249.0	134.1/104.0	3.87	95/91	18/31	7/13	20
NPAHD-13C3	252.1	134.1	4.08	89	18	7	20
NPAOZ	236.1	134.1/104.2	4.00	91/87	19/31	16/13	20
NPAOZ-d4	240.1	134.3	4.00	90	19	7	20
NPSEM	209.1	192.1/166.2	3.53	80/67	16/15	11/9	20
NPSEM-13C15N2	212.1	168.1	3.53	59	16	9	20
NPAMOZ	335.1	291.1/262.2	4.88	80/84	18/25	15/14	20
NPAMOZ-d5	340.1	296.1	4.88	64	17	17	20
NPDNSAH	374.0	226.0/182.1	5.67	93/87	34/30	7/5	30
NPDNSAH-13C6	380.1	188.0	5.67	75	30	10	30
NPHBH	286.0	121.1/93.0	4.43	66/67	27/54	7/11	25
NPHBH-$^{13}C_6$	292.0	127.2	4.43	97	31	7	25
NPOAH	237.1	192.1/135.2	3.37	66/59	20/30	10/7	20
NPOAH-$^{15}N_3$	240.1	194.1	3.37	69	19	10	20
NPAGN	208.1	191.0/119.2	2.84	87/86	20/28	11/6	60
NPAGN-$^{13}C^{15}N_4$	213.0	92.2	2.84	71	34	16	60

The mass spectrometer conditions were ion spray voltage set at +/- 1400 V, 30 psi for curtain gas, 650 °C for source temperature, 8 psi for CAD gas, and 70 psi for both gas 1 and gas 2.

Application of method to incurred tissues

The performance of the method when applied to incurred tissue was assessed by participating in a FAPAS proficiency test and through the analysis of incurred pig and turkey muscle samples incurred with AHD, DNSAH, SEM, AOZ or AMOZ supplied by ANSES Fougères,. The method was also applied in the analysis of 118 EU and non-EU poultry samples (chicken, quail, duck and turkey) purchased from local Irish supermarkets.

Results and discussion

UHPLC-MS/MS method development

The majority of methods developed for analysing bound nitrofuran residues report the separation of the nitrophenyl ester derivatives using alkyl-bonded silica stationary phases, with a predominant focus on only four or fewer compounds, namely NPAHD, NPAOZ, NPAMOZ and NPSEM. In this work, a BEH C_{18} column chemistry (2.1 × 100 mm; 1.7 µm) was initially evaluated but gave an unsatisfactory peak shape, particularly for NPAGN, NPAHD and NPDNSAH. In addition, matrix interfering peaks were observed in chromatographic traces that were

not fully resolved from the analytes. Consequently, seven different phenyl-hexyl column chemistries were evaluated because they can provide improved selectivity for compounds containing aromatic functionalities. An Agilent ZORBAX Phenyl-hexyl column (2.1 × 50 mm; 1.8 μm) performed best for the analysis of eight bound nitrofuran residues. MeOH was found to be the most suitable organic modifier because it gave increased retention and analyte separation, while the use of ammonium formate as an additive achieved the best peak shape. The LC gradient required careful optimisation to separate analytes and matrix interferences. A small interfering peak was observed in the quantifier (209.1 > 192.1 *m/z*) and qualifier (209.1 > 166.2 *m/z*) transitions for NPSEM, in all samples tested during this evaluation, the largest of which was measured at approximately 6% of the RPA. For approximately 50% of samples tested, this peak satisfied the ion ratio criteria and as such, the CCα for NPSEM was established at a level (0.20 μg kg^{-1}), whereby the interfering peak contributed to less than 10% of the CCα and did not impact on ion ratio tolerances. During chromatographic developmental work, matrix-interfering peaks were observed for other analytes, namely NPAHD, NPAOZ, NPHBH and NPDNSAH. However, these peaks were successfully resolved from the analyte peaks of interest, and did not affect quantification. The chromatographic separation of the eight nitrofuran analytes is shown in Figure 1.

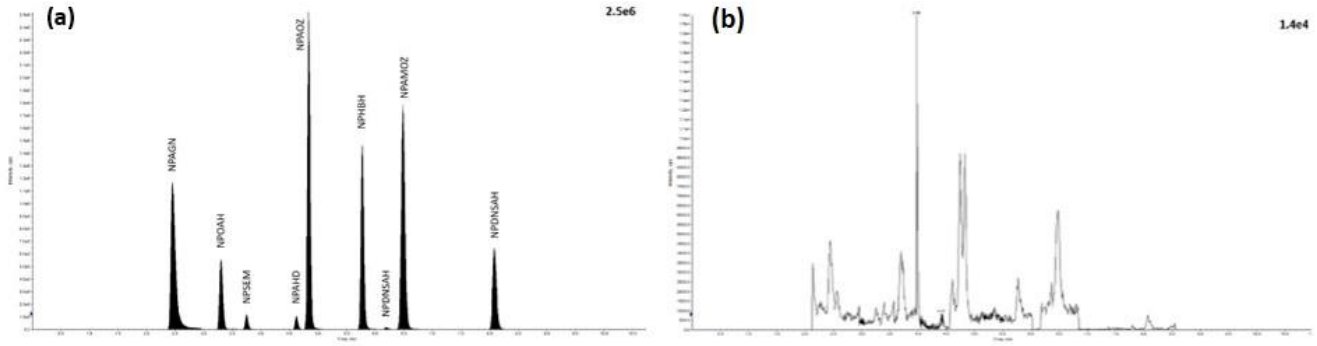

Figure 1. Chromatogram of quantifier transitions for (a) a bovine muscle sample spiked at 0.5 μg kg^{-1} and (b) a blank bovine muscle sample.

Optimisation of microwave-assisted derivatisation parameters

One of the main objectives of this work was to develop a microwave-assisted derivatisation reaction, which could replace the lengthy conventional approach of overnight incubation in a heated waterbath, with the aim of significantly reducing the 16 h derivatisation time. Preliminary testing indicated that the optimisation trials should be carried out in matrix rather than solvent, given that particularly poor derivatisation occurred in solvent for NPAMOZ and NPAGN when compared with matrix (93% and 97% differences, respectively). Optimisation of the microwave-assisted derivatisation reaction was carried out using porcine muscle incurred with AOZ. The impact of longer hold times and varying temperatures in the microwave reaction, as well as the performance of other published derivatisation methods, was assessed. Two such protocols were reported using a reaction at 55 °C for 4 h (Verdon *et al.*, 2007) and a temperature of 60 °C for 2 h (Chen *et al.*, 2020). An evaluation of these methods was carried out, showing significantly higher ($P < 0.05$) yields of bound AOZ residues using both of the EURL methods, when compared to a 16 h waterbath incubation at 37 °C. A range of different reaction times were also evaluated using the original run temperature of 65 °C. The results from the optimisation indicated a derivatisation temperature between 55 °C and 65 °C and a run time of > 90 min gave satisfactory yield of AOZ bound residues. However, it was not possible to optimise the method thoroughly for other analytes due to a lack of incurred tissues with the different drugs. Further studies were carried out to investigate the stability of nitrophenyl derivatives by spiking with NP derivatives just prior to derivatisation, and the findings showed that NPSEM was more stable at a reaction temperature of 37 °C than 55 – 65 °C, while NPDNSAH appeared to degrade more rapidly at 37 °C. As a compromise, a 2 h reaction at 60 °C selected as optimal.

Method Validation

Identification and confirmatory criteria, linearity, selectivity and matrix effects

In the revised EU method validation guidelines laid down in 2021/808, the retention time of the analyte in a test sample must correspond to that of the calibration standard with a tolerance of ± 0.1 min and the deviation

between relative retention times must be ≤ 1%. Secondly, ion ratios must be within the ± 40% tolerance for each analyte in the validation samples. These criteria were met during all validation runs. Linearity was determined with a $1/x^2$ weighting and linear fit, and was achieved across each analyte's calibration range, with regression coefficient values (R^2) ≥ 0.998 and residuals in the ± 20% range from the curves. The establishment of selectivity is a critical aspect of method development and was achieved by efficient chromatographic separation and was subsequently verified through selectivity studies. Matrix effects were evaluated at the RPA. Ion suppression was observed for all analytes, ranging from -7.4% (NPAHD) to -63.6% (NPOAH), with the exception of NPSEM for which +10.2% ion enhancement was seen. However, the seven analytes that showed ion suppression could be analysed to their lowest calibration level of 0.02 µg kg^{-1} (25 times lower than the RPA).

Trueness, precision and CCα

The mean trueness for all eight analytes was satisfactory under WLr and WLR conditions, ranging between 99% and 105%. WLr (RSD_r) and WLR (RSD_R) precisions across the three validation levels were acceptable, ranging from 0.6% to 3.9% and 0.9% – 10.7%, respectively. CCα values were calculated using the calibration curve procedure and ranged between 0.013 µg kg^{-1} (NPAMOZ) and 0.058 µg kg^{-1} (NPDNSAH). However, for two of the eight analytes, namely NPSEM and NPOAH, these calculations produced CCα values at a concentration lower than achievable. Based on the chromatography generated from the WLr and WLR studies, these values were not considered achievable from the perspective of satisfying both quantitative and qualitative criteria, such as acceptable ion ratios and satisfactory signal-to-noise. Hence, to ensure the CCα values for this method were reliable and represented an achievable decision limit, the CCα concentrations were set to a higher level, and verified in an analytical run comprising of bovine, avian, porcine and ovine muscle tissues. The validation results are summarised in Table 2.

Application of method to incurred tissues

The method presented in this study detected SEM and was assigned a *z*-score of 0.0 in the FAPAS proficiency study, showing satisfactory method performance. Additional incurred turkey and pork muscle tissues, of known concentrations, were analysed and the levels measured were compared to their respective assigned concentrations. Given that a *z*-score of $-2.0 \geq x \leq 2.0$ is deemed satisfactory in a proficiency study, the six muscle samples, incurred with AHD, AOZ, AMOZ, SEM or DNSAH, met the criteria for suitable method performance. The method was also applied to 118 poultry samples as part of a retail sampling study, which were all found to be negative. The poultry sampling survey presented in this extended abstract highlights the suitability and robustness of this confirmatory method, given that it was applied to a wide range of different processed and unprocessed samples. The method was fit to analyse all sample types, and no additional interferences were observed.

Table 2: Validation results for the analysis of eight bound nitrofuran metabolites in a range of bovine, avian, porcine and ovine muscle samples.

Analyte	Calibration Range (µg kg $^{-1}$)	R^2	WLR Trueness (%) (RSD_R) (%)				CCα (µg kg^{-1})
			L1	L2	L3	L4	
NPAHD	0.02 – 5.00	0.998	99 (2.4)	100 (2.0)	99 (3.9)	101 (4.0)	0.030
NPAOZ	0.02 – 5.00	0.999	100 (1.6)	100 (2.5)	99 (2.8)	99 (1.9)	0.019
NPAMOZ	0.20 – 5.00	0.998	101 (2.4)	100 (1.8)	100 (1.4)	101 (1.7)	0.013
NPSEM	0.02 – 5.00	0.998	101 (3.7)	100 (3.8)	100 (2.1)	100 (2.8)	0.200
NPHBH	0.02 – 5.00	0.999	100 (2.4)	99 (4.3)	100 (9.6)	98 (6.0)	0.070
NPAGN	0.02 – 5.00	0.999	101 (2.0)	101 (0.9)	101 (2.6)	101 (2.1)	0.017
NPOAH	0.02 – 5.00	0.999	101 (2.2)	100 (1.4)	100 (2.5)	100 (2.6)	0.200
NPDNSAH	0.02 – 5.00	0.999	99 (4.5)	101 (3.5)	105 (10.7)	100 (3.4)	0.058

Conclusions

A rapid and sensitive analytical method, incorporating a microwave-assisted derivatisation reaction and a modified QuEChERS extraction, has been developed for the confirmatory analysis of eight bound nitrofurans in animal tissue. The method has undergone extensive validation in accordance with the new 2021/808 legislation across a range of concentrations, in-line with the 0.5 µg kg^{-1} RPA for nitrofurans. The traditional approach to bound nitrofuran analysis is lengthy due to the overnight derivatisation step, followed by a double liquid-liquid extraction. This work proposes an alternative rapid approach using a 2 h microwave reaction and a modified QuEChERS extraction, shortening analysis time from four to two days. Based on currently available literature, this method is the first of its kind to comprehensively detect each of the eight nitrofurans as their respective marker residues. The findings during this study highlighted the importance of applying newly developed methods to incurred materials, particularly when analysing for bound residues, to ensure fitness for purpose. Overall, through rigorous validation studies and partaking in proficiency tests, the method presented in this paper has shown satisfactory performance in the analysis of eight bound nitrofurans in meat. This method can play a major role going forward in the surveillance for the illegal use of nitrofuran drugs.

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ORAL O11

HEADACHE WITH ANALYSIS OF NSAIDS IN FOODS OF ANIMAL ORIGIN

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Abstract
NSAIDs are an important group of veterinary medicines for which residues in food of animal origin have to be tested. The use of LC-MS/MS significantly facilitates the achievement of the required detectability of the methods used but does not solve several problems and challenges associated with both the methodology and the interpretation of the results.
To mention just a few, e.g. the occurrence of salicylic acid (a marker for acetylsalicylic acid) in nature, which can lead to falsely inconsistent results. Another challenge is the extremely wide range of MRLs in milk, starting at 0.1 µg/kg for diclofenac and ending at 50 µg/kg for metamizole. The whole picture of difficulties is completed by the instability of phenylbutazone during the analysis.
The lecture will present the authors' efforts to develop and apply successive versions of the method for determining NSAIDs, as well as the results of selected animal experiments.

Introduction
Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used in human and animal medicine. They can reduce pain, fever, and inflammation and prevent blood clots. NSAIDs are a relatively safe group of drugs. However, their usage can still cause side effects, including peptic ulcer disease, kidney and liver failure, or hypersensitivity (up to 2% of adults), from which the most dangerous is anaphylactic shock. Despite the similar mechanism of action (inhibition of the enzymes responsible for proteinoid synthesis – COX), NSAIDs are a diverse group of drugs. They became an essential class of medications for most mammalian animals in veterinary medicine.
Although the risk associated with the intake of drugs with food is low, it still must be assured that the levels of these substances will not exceed the limits introduced in regulatory documents. Although some NSAIDs are registered as veterinary drugs, according to the regulation, only a few have defined maximum residue limits (MRL) (Table 1.). They are allowed to use in food-producing animals. According to European Safety Food Authority (EFSA), in 2019, European laboratories involved in the official food control analysed 24,387 samples (muscle, milk), of which 46 (0.19%) were reported as non-compliant (EFSA, 2018). That is also the reason to monitor food of animal origin, paying attention to the type and quantity of the substances that can occur.

Many methods for determining NSAIDs in animal tissue and milk have been developed. However, some single-residue methods are based on HPLC-UV methods; LC-MS/MS has become the technique of choice due to European regulations and constant evaluation. Developing the multi-residue method for NSAIDs is still a challenge because of their chemical variety. Most of them have a chemical structure of weak acids. However, there are some alkaline compounds (metamizole and its' metabolites). That is why previous methods for determining NSAIDs residues in milk were using separate different sample preparation and analytical conditions for acidic and basic NSAIDs [Jedziniak et al., 2010; Jedziniak et al., 2013]. Also, analysis of muscle tissue requires good clean-up, including SPE and filtration, which make sample preparation time-consuming and labour-intensive.

In this publication, we would like to highlight the difficulties and analytical challenges associated with determining NSAIDs' residues in food of animal origin.

Salicylanes everywere!

While most NSAIDs are human formulated molecules, the use of salicylates was inspired by discovering the anti-inflammatory effect of salicylic acid isolated from white willow and named by German chemist Johann Andreas Buchner in 1828. This discovery gave rise to many compounds still in use today, most notably sodium salicylate, acetylsalicylic acid (aspirin), and others. Compounds from this group are also used to treat animals. It was considered unnecessary for most of these compounds to set an MRL. Still, in the case of, e.g. sodium salicylate, an MRL was set for tissues (turkey muscle, liver and kidney), and a note was made that it should not be used in animals, for animals producing eggs for human consumption. Interestingly, salicylic acid was

indicated as a marker residue in turkeys for aluminium salicylate and sodium salicylate. In addition, a milk MRL of 9 µg/kg was also entered for aluminium salicylate.

While the analysis of salicylic acid, mainly using the LC-MS/MS technique, is not challenging, the interpretation of the results can be problematic. Salicylic acid occurs naturally in many plants, e.g. maize, commonly used for animal feed (Protasiuk et al. 2018). Our study confirmed the transfer of salicylic acid into eggs and meat despite relatively low native salicylate levels in feed (Protasiuk et al., 2020). Also, a survey carried out by EURL Berlin to check salicylic acid levels in milk indicated that in some countries, the results were significantly above the MRL, which may be due to the specific feed composition. It also seems controversial to set low levels for salicylates in food of animal origin in a situation of 9-400 ug/kg when the average daily intake of these compounds consumed with plant products is several milligrams per day.

Limits and marker residues for advanced analysts

Different MRL values for tissues and food from other animal species have been a long-standing problem for laboratories controlling residues. This requires adapting the determination methodology and validating several matrix combinations over an extensive concentration range.

For NSAIDs, a particular challenge is the determination of residues of diclofenac (MRL = 0.1 µg/kg) alongside other drugs for which MRLs are in the range of 15 µg/kg (meloxicam) and 50 ug/kg (tolfenamic acid) (Table 1).

Analyte	Animal species	MRL/Level*	Matrix
Diclofenac	Bovine	5 µg/kg	Muscle
		0.1 µg/kg	Milk
	Porcine	5 µg/kg	Muscle
Firocoxib	Equidae	10 µg/kg	Muscle
Flunixin	Bovine	20 µg/kg	Muscle
	Porcine	50 µg/kg	Muscle
	Equidae	10 µg/kg	Muscle
5-Hydroxyflunixin	Bovine	40 µg/kg	Milk
Tolfenamic acid	Bovine, porcine	50 µg/kg	Muscle
	Bovine	50 µg/kg	Milk
Meloxicam	Bovine, caprine, porcine, rabbit, *Equidae*	20 µg/kg	Muscle
	Bovine, caprine	15 µg/kg	Milk
Metamizole (as 4-Methylaminoantipyrin)	Bovine, porcine, *Equidae*	100 µg/kg	Muscle
	Bovine	50 µg/kg	Milk
Carprofen (Sum of carprofen and carprofen glucuronide conjugate)	Bovine, *Equidae*	500 µg/kg	Muscle
Phenylbutazone Oxyphenbutazone Ibuprofen	-	5 µg/kg*	Muscle, milk
Naproxen Mefenamic acid Acetylsalicylic acid Acetylsalicylic acid DL-lysine	-	10 µg/kg*	Muscle, milk
Sodium acetylsalicylate Hydroxyethylsaalicylate Methyl salicylate Salicylic acid	All food-producing species except finfish	No MRL required	Not applicable
Aluminium salicylate, basic	All food-producing species except bovine, caprine, *Equidae*, rabbit, and finfish	No MRL required	Not applicable
Sodium salicylate	All food-producing species except finfish	No MRL required	Not applicable
	Turkey	400 µg/kg	Muscle
		200 µg/kg	Liver
		100 µg/kg	Kidney

Table 1. MRL levels were established by the EU in Commission Regulation No 37/2010, and levels recommended by the CRL guidance paper [Commision regulation, 2010].*

The determination of diclofenac in milk requires suitably sensitive mass spectrometers and appropriately selected sample preparation (Jedziniak et al., 2021).
The second major challenge is determining metabolites directly (5-hydroxyflunixin in milk and 4MAA in tissues and milk) or indirectly (carprofen: sum of carprofen and carprofen glucuronide conjugate in tissue). In the case of flunixin, determining the metabolite in milk is not a problem. However, our studies had shown that flunixin glucuronide is also present in milk, which was detected indirectly when milk samples were subjected to an enzymatic hydrolysis step (Jedziniak et al., 2013).
In the case of the metamizole residue (marker residue of 4MAA), the problem is even more significant because these compounds have a higher pKa value and are pretty tricky to determine next to "acidic" NSAIDs. In addition, 4MAA is further metabolised to 4-formylaminoantipyrine (4FAA) and 4-amino antipyrine (4AA), the latter being acetylated to 4-acetylaminoantipyrine (4AcAA), and determination of these metabolites is also helpful in interpreting results. Recent years have shown that determining these metabolites in a multi-component method is possible. However, the challenge is the proper shape of the peaks and selecting an appropriate mobile phase and column (Jedziniak et al., 2013).
Determination of carprofen in tissues is a particular case; most methods use enzymatic or chemical hydrolysis to convert glucuronide to the matrix compound. A significant problem is optimising the more commonly used enzymatic hydrolysis step using beta-glucuronidase. In practice, most authors of the available publications have not carried out this process. We used the available ibuprofen glucuronide standard (Jedziniak et al., 2016).

Phenylbutazone and oxyphenbutazone uncertainty
One of the most problematic issues in residue analysis of NSAIDs in food of animal origin is the determination of phenylbutazone (PBZ). This compound used extensively in veterinary medicine, especially in treating horses, is not permitted in food-producing animals. The analytical recommended limit for this compound in all tissues is 5 µg/kg. Phenylbutazone became more widely known to the public due to the so-called "horsemeat scandal" in some European countries where foods advertised as containing beef were undeclared or improperly declared horse meat. As a result of the EU measures, more than 4000 food samples were tested for phenylbutazone and regular monitoring.
Phenylbutazone and oxyphenbutazone present analytical difficulties due to their tendency to degrade during sample preparation. Some authors have used chemical reagents in their methodologies to reduce the degradation of PBZ and OPBZ (e.g., ascorbic acid) (Jedziniak et al. 2010) or to lessen the effect of the sample concentration step by adding dimethyl sulfoxide to the solution (Jedziniak et al. 2012). Experience in analysing incurred samples provided as part of proficiency testing indicates that using, e.g. enzymatic hydrolysis with the enzyme beta-glucuronidase in the sample preparation step makes the determination of PBZ and OPBZ impossible in practice, probably due to an increase in matrix effect or decomposition of the analytes. These observations conclude that the determination of PBZ and OPBZ simultaneously with other compounds may not be possible when using a confirmatory procedure.

Conclusion
These examples confirm that the residue analysis of NSAIDs is one of the most demanding. We deal with compounds with different chemical structures resulting in other properties among the analytes. From the perspective of chromatographic separation, the challenge is to simultaneously determine both acidic NSAIDs and alkaline compounds (metamizole metabolites) to obtain suitable peak shapes and appropriate retention times. A wide range of calculated MRLs and recommended limits causes the necessity of correct calibration of methods for different ranges of determined matrices. The very low MRL for diclofenac in milk requires the use of appropriate sample preparation as well as a sensitive mass spectrometer. The tendency of phenylbutazone and oxyphenbutazone to decompose during analysis completes the problem.
From the interpretation of the results, salicylic acid in the environment will undoubtedly cause problems in the performance of discordant results.
The problems described above are a significant challenge for any laboratory determining the residues of NSAIDs. Despite many years of experience analysing these compounds, new effective multi-component methods are still needed.

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ORAL O12

LC-MS METHODS FOR DETECTION OF INHIBITOR AND GROWTH PROMOTER PROTEINS

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Abstract
Proteins that have inhibitor- or growth-promoting properties are of interest for increasing of meat or milk production. Insulin-like growth factors (IGFs) are key mediators of growth hormone action and biomarkers for growth hormone abuse. Myostatin is an inhibitory growth factor for muscle development. Animals without myostatin or treated with compounds that block the activity of myostatin have significantly more muscle mass. These proteins are therefore of interest to increase meat production. Recombinant bovine somatotropin (rbST) is a protein used to enhance milk production, approved by several countries but prohibited in EU. Insulin-like growth factors, somatotropin, myostatin and myostatin inhibitors are prohibited compounds by the World Anti-Doping Agency (WADA) and for animal use according to Directive UV0 20221/808/EC, therefore methods for detection of these proteins are required.
To detect abuse, targeted LC-MS/MS methods combined with purification of enrichment methods such as precipitation with acetonitrile or immunoaffinity were set up to measure the concentration of myostatin, IGFs and rbST in bovine serum.

The results of these studies show that the confirmatory LC-MS/MS methods are specific, sensitive and combination of several biomarkers can be used in one method to obtain more accurate information in clinical and doping research.

Introduction
Proteins and peptides with growth-promoting properties are a class of compounds that can be illegally used by athletes and farmers looking for prohibited enhancement muscle growth. Here we discuss three cases: the case of insulin-like growth factors and somatotropin which are part of a specific class (S2), 'peptide hormones, growth factors, and related substances and myostatin and myostatin inhibitors which are part of a specific class (S4), hormone and metabolic modulators'. All three are prohibited compounds by the World Anti-Doping Agency (WADA, www.wada-ama.org) and also prohibited for animal use according to Directive UV0 20221/808/EC.

IGFs are bioactive hormones that are endogenously produced single-chain peptides with high sequence similarity to insulin. They are part of a complex system that cells use to communicate with their physiologic environment. Although IGF-1 and IGF-2 are structurally related and bind to the same receptor and IGFBPs (IGF binding proteins), they have different functions. For example, IGF-1 plays an important role in childhood and adult growth and IGF-2 has primarily effect during gestation. Both IGF-1 and IGF-2 are biomarkers proposed for detection of growth hormone (GH) administration and for diseased state (e.g. breast cancer, GH-related disease) [1-3], consequently specific methods of analysis of these compounds are in high demand. In particularly, IGF-1 and its synthetic variants are important biomarkers for doping control due to their effects on growth hormone therefore their analysis is important for the detection of growth hormone and IGF-1 misuses [3]. The synthetic IGF-1 analogues (des1-3-IGF-1, R3-IGF-1 and longR3-IGF-1) were developed in order to hinder their association with IGFBP after administration. However, they are not approved for use and are distributed illegally on the black market or via internet [4-6].

Somatotropin (ST), also known as growth hormone, is a single-chain polypeptide hormone of 191 aminoacids long and approximately 22 kDa. It controls differentiation, growth and metabolism of many cell types of vertebrate species [7]. Therefore, it plays an important role in the control of growth and reproduction. It is a hormone produced by the anterior pituitary gland and belongs to the same hormonal family as prolactin and placental lactogen [8]. One of its characteristics is stimulation of mammary gland growth and regulation of milk production [9]. As ST stimulates the mammary gland growth and milk production, exogenous administered ST will also stimulate this production. Monsanto Co. and Genetech Inc. researched first the possibility to produce recombinant bovine ST (rbST) with biotechnological techniques [10]. This biotechnology offered the opportunity to produce rbST in large amounts which led to commercialization of the rbST protein. Since then

various forms have been produced over the years. Nowadays two forms are commercially available Posilac® and BoostinS®. Posilac®, developed by Monsanto, is chemically almost identical to the endogenous form, with only a distinct N-terminal end. Therefore methods for detection of these proteins are required to prevent abuse.

Myostatin is an inhibiting growth factor for muscle development. It is also known as growth differentiation factor 8, in short GDF-8 [11]. It is a protein that is coded in humans on the MSTN (myostatin) gene and is primarily produced in skeleton muscle cells. In blood it is bonded to the activin type II B- receptor (ActRIIB). Agents which modulate the action of myostatin are of interest for medical use in degenerative muscle diseases and muscle wasting in conditions like cancer cachexia and other debilitating situations. For athletes myostatin blockers are of interest to increase muscle mass and strength. Animals without myostatin or treated with compounds that block the activity of myostatin have significantly more muscle mass. This protein is therefore of interest in the meat industry to increase meat production therefore development of methods for myostatin detection is needed.

Three inhibition mechanisms are possible: 1) injecting antibodies that capture myostatin, this is an expensive and unlikely application 2) adding an additive, for example follistatin, which binds to myostatin and therefore inhibits the action of myostatin, this is a likely form of administration 3) injecting antibodies that bind to the receptor, causing myostatin to stop functioning, this is also an expensive and unlikely application.

Materials and Methods

Samle preparation

IGF-1 and IGF-2 proteins circulate in serum as a complex with binding proteins. Therefore prior to extraction, the protein needs to be released from the binding protein. For this reason 1 % acetic acid is used. High abundant proteins are removed from the serum by protein precipitation with acetonitrile and the supernatant is reduced with dithiothreitol, alkylated with iodoacetamide and digested overnight with trypsin. Each protein is quantified by a single unique tryptic peptide. Synthetic stable isotope labelled peptides homologues (RAPQTGIVDECCFR for IGF-1 and GIVEECCFR for IGF-2) are used to assist in identification and for ion signal correction. Validation and quantification is performed using recombinant IGF-1 and IGF-2, through the construction of a calibration curve.

Immunoenrichment was performed for both rbST and myostatin (GDF-8) before sample digestion with trypsin. Polyclonal anti-rbST and anti-GDF-8 respectively antibodies were immobilized to monolithic micro-columns loaded with protein A by affinity binding. The pipet tips containing the protein A monolithic micro-columns were placed on the Finnpipette® Novus i multichannel, which is an automated device having a repetitive cycling function. First the monolithic micro-columns were washed with 20 mM phosphate buffer pH 7 before the anti-rbST or anti-GDF-8 antibodies were bound. The same buffer was used to remove the unbound antibodies. For immuno-enrichment, 1 mL serum sample or spiked serum was diluted with 1 mL 20 mM phosphate buffer pH 7; for extraction of rbST from milk 2 ml were used (see protocol [12-13]). The captured rbST was then eluted from the monolith micro-column by 50 µL 200 mM NaOH. The eluate was collected, and 50 µL 50 mM Tris pH 7.9 was added before further use (final pH>10). Eluates were further digested with trypsin after the solution was adjusted to 8–8.5 with 1 M HCl. The proteins were reduced with dithiothreitol, alkylated with iodoacetamide and digested 1 hour with trypsin. Synthetic stable isotope labelled peptide homologues for AFPAMSLSGLFANAVLR is used to assist in identification and for ion signal correction for rbST. The digest was than concentrated on an Agilent Bond Elut Plexa SPE column and eluted in water/acetonitrile/formic acid (25:70:5, v/v/v).

LC-MS/MS analysis

Analysis was performed using an I-Class UPLC system connected to a Xevo TQS mass spectrometer Waters (Manchester,
UK). The final extracts were analysed by UHPLC-MS/MS in multiple reaction monitoring (MRM) mode in positive electrospray ionization mode. Chromatographic separation was achieved on different type of C18 columns and gradients. Gradient elution was performed using water/formic acid (100:0.1, v/v) and acetonitrile/formic acid (100:0.1, v/v).

Results and Discussion

IGFs

Here we developed a method for the identification and quantification of bovine or human insulin-like growth factor -1 and -2 (IGF-1 and IGF-2) in bovine or human serum (the chosen peptides are identical in bovine and human serum) by selected reaction monitoring LC-MS/MS at a level of 50 - 1000 µg/l [2, 4, 14,15]. Ion transitions chosen for the quantifier peptide RAPQTGIVDECCFR from IGF-1 are m/z 570.3 > 771.3, 886.3, 985.4 and ion transitions for the quantifier peptide GIVEECCFR from IGF-2 are m/z 585.3 > 642.2, 771.3, 900.3. Different biochemical (e.g. extractions, dissociation and purification) and chromatographic parameters were optimizes and consecutively the method was validated conform 2021/808 and the performance characteristics of the analysis were evaluated. In order to create a calibration curve that covers these levels of IGF-1, 2 in plasma, human recombinant IGF-1, and IGF-2 were spiked into bovine plasma at different concentration. Since both components occur naturally in serum, the observed increase in concentration due to the 100, 200 and 300 µg/l spikes in all measured samples was calculated. Confirmation of the identity of IGFs is based on EU criteria (2021/808) and those requirements were meat. From our validation study we found that the due to the broad variation in the endogenous concentration of IGFs in serum, the RSDr < 20 % RSDrl < 30% and could not meet the requirements from 2021/808 for all levels (see Table 1).

Component	Validation level (µg/l)	RSDr* (%)	RSDrl (%)
IGF-2	100	39.6	40.9
	200	27.2	27.8
	300	21.0	21.1
IGF-1	100	27.9	28.2
	200	17.5	18.7
	300	12.5	14.2

Table 1. Performance characteristics in bovine serum

When an identical sample is processed in 6-fold (see Table 2), the repeatability does meet the specified criterion. As already mentioned, the cause probably lies in the large variation in the endogenous concentration of the serum samples. It is therefore not possible to accurately determine the concentration of a sample via an external calibration line. Standard addition is therefore needed for accurate quantification of IGFs in serum.

Sample	Concentration (µg/l)	
	IGF-2	IGF-1
Serum E-1	463.9	345.9
Serum E-2	401.3	362.9
Serum E-3	451.9	385.0
Serum E-4	454.7	361.5
Serum E-5	398.2	379.3
AVG:	434.0	366.9
STDEV:	31.6	15.6
RSD (%):	7.3	4.2

Sample	Concentration (µg/l)	
	IGF-2	IGF-1
Serum A-1 + 200 µg/l	734.4	240.7
Serum A-2 + 200 µg/l	755.1	237.3
Serum A-3 + 200 µg/l	763.0	248.2
Serum A-4 + 200 µg/l	758.2	219.2
Serum A-5 + 200 µg/l	710.9	238.8
AVG:	744.3	236.8
STDEV:	21.6	10.7
RSD (%):	2.9	4.5

Table 2. Within day repeatability of identical samples

We concluded that this method is sensitive enough to obtain quantitative information for both IGF-1 and IGF-2 in a single analysis and can provide important information for detection of growth hormone abuse. It also has the advantage that it can be further combined with detection of other biomarkers in plasma.

rbST

A novel approach to pinpoint rbST abuse has been developed based on rbST enrichment by immunoaffinity on monolith micro-columns combined with LC-MS/MS. High sensitivity is reached with a CCα of 0.8 ng/ml in serum and CCα of 2.3 ng/ml

in milk. Applicability of the confirmatory method was demonstrated by analysis of serum and milk samples from a treated animal for which positive screening results were obtained [12-13].

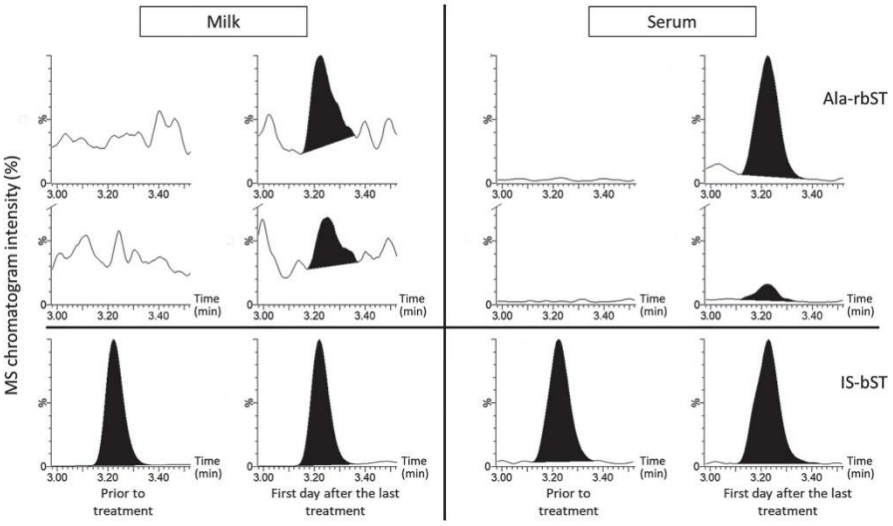

Figure 1. MRM transitions (from top to bottom) m/z 883.1 > m/z 774.1 and m/z 883.1 > m/z 960.6 for the alanine-recombinant bovine somatotropin (Ala-rbST) marker peptide and m/z 888.1 > m/z 779.1 for the methionine-bovine somatotropin (bST) internal standard (IS-bST) of, on the left side, a milk sample taken before treatment and a milk sample taken 1 d after the last Ala-rbST treatment and, on the right side, the corresponding serum samples. All samples were obtained from the same cow.

Ion transitions m/z 883.1 > 774.1, 1047.6, 960.6 were measured to detect the bST- and Ala-rbST–specific N-terminal peptide with amino acid sequence AFPAMSLSGLFANAVLR, and ion transitions m/z 913.1 > 774.1, 1,047.6, 960.6 were measured to detect the Met-rbST specific N-terminal peptide with amino acid sequence MFPAMSLSGLFANAVLR, all formed after tryptic digestion. To check the retention time of the N-terminal rbST peptide of interest, a synthetic analog of the rbST peptide was injected at the beginning and end of each series. For the 13C6-bST internal standard, the transition m/z 888.1 > 779.1 was monitored.

Smits *et al.* [12] demonstrated that the blood-milk barrier for rbST is very specific. Both rbST formulations are detectable in serum, but Met-rbST was barely transferred to milk (see Figure 1). As expected, Ala-rbST, which is similar to endogenous bST, was transferred to the milk, and the levels were about 5 times lower than those in the serum samples. Thus, a difference of 1 amino acid (methionine) makes an important difference for elimination into milk.

Myostatin (GDF-8)

To detect abuse, concentration of myostatin in blood can be measured by LC-MS/MS [16, 17]. The growth inhibitory factor GDF-11 has 90% homology in the amino acid sequence with GDF-8, therefore a targeted LC-MS/MS method was set up to measure and ultimately quantify these two proteins to prevent overestimation of serum myostatin levels. A distinction which, for example, cannot be made with immunoassay methods, which results in a structural overestimation of myostatin. The following peptides have been found to be specific and sensitive: IPAMVVDR for GDF-8 and IPGMVVDR for GDF-11. A targeted LC-MS/MS method has been set up to measure and ultimately quantify these two peptides.

The expected endogenous level for GDF-8 is a few ng/ml and for GDF-11 less than 1 ng/ml. For method development, GDF-8 and GDF-11 (50:50) has been added to bovine plasma at different levels (50, 100, 200, 400 ng/ml). For isolation of GDF-8 and -11, Mass Spectrometric Immunoassay (MSIA) microcolumns filled with polyclonal antibody directed against GDF-8 (and indirectly against GDF-11) were used. The binding of polyclonal antibody to GDF-8 was first tested by ELISA with a positive outcome. After isolation, both GDF-8 and GDF-11 have been detected.

Protein A and Protein G MSIA microcolumns were both tested. Better results were achieved for both GDF-8 and GDF-11 peptides when Protein A MSIA microcolumns were used. Using Protein A MSIA microcolumns,

both peptides could be detected at 100 ng/ml, but only just above the detection limit. The response must therefore increase significantly as expected levels will be around 1 ng/ml. LC-MS/MS chromatograms of GDF-11 and GDF-8 added in PBS buffer (top) and plasma (bottom) at 200 ng/ml are shown in Figure 2.

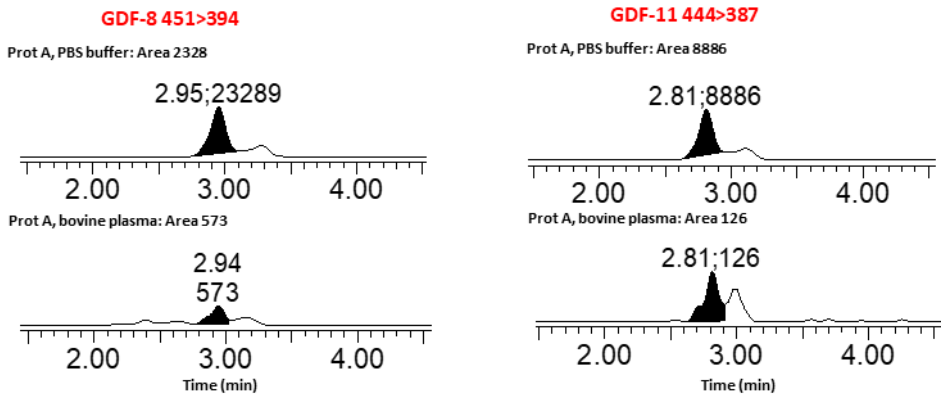

Figure 2. LC-MS/MS chromatograms of GDF-11 and GDF-8 added in PBS buffer (top) and plasma (bottom) at 200 ng/ml.

The effect of the matrix and MSIA microcolumns were evaluated. Samples spiked with GDF-8 and GDF-11 in PBS buffer (no plasma) were enriched using the MSIA columns to evaluate the effect of the matrix (plasma). The signal is 40 times higher for GDF-8 and 70 times higher for GFD-11 when no plasma is used as a matrix. The effect of the MSIA microcolumns was also investigated by spiking GDF-8 and GDF-11 (50:50) in the digestion buffer (200 mM ammonium bicarbonate). Samples were digested without use from MSIA microcolumns. The signal is 300 times higher for GDF-8 and 600 times higher for GFD-11 when no plasma is used as a matrix and the samples are not enriched via the MSIA microcolumns. The lowest spiked level (2 ng/ml) could be detected and the linearity in the range of 0-50 ng/ml (the endogenous level of GDF-8 and -11) is R2 = 0.9994 for GDF-11 and 0.9987 for GDF-8.

Conclusion

IGFs: This approach is sensitive enough to obtain quantitative information for both IGF-1 and IGF-2 in a single analysis and can provide important information for detection of growth hormone abuse. It also has the advantage that it can be further combined with detection of other biomarkers in plasma. For quantification of IGFs in serum standard addition method is recommended.
rbST: From our studies we concluded that LC-MS/MS analyses of rbST in serum samples is possible at low levels of detection. The detection of rbST in serum is needed to confirm the type of rbST used, as Met-rbST is not able to cross the blood-milk barrier.
GDF-8 (myostatin): A specific immunoaffinity LC-MS/MS method has been set up to determine GDF-8 and GDF-11 in plasma. To improve the detection sensitivity, so that measurements can be made at relevant levels, the method will have to be further improved.

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ORAL O13

BENEFITS OF INTEGRATING TRAVELING WAVE ION MOBILITY SPECTROMETRY INTO LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY WORKFLOWS FOR STEROID ANALYSIS

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The advantages provided by the implementation of ion mobility spectrometry (IMS) in traditional liquid chromatography (LC)-mass spectrometry (MS) are discussed. IMS provides new analytical information on the structure of ions, the so-called collision cross section (CCS), which in combination with retention indexes and mass spectra increases confidence in compound identification in both targeted and non-targeted analysis. Among recent CCS databases available, a $^{TW}CCSN_2$ database for 300 steroids, including endogenous and exogenous steroids, has recently been reported. This database has been experimentally cross-validated by three external laboratories, showing measurement errors within the ±2% range. Computational studies and the application of machine learning approaches have also been evaluated as plausible tools for generating CCS values. Furthermore, it is shown that CCS measurements in the presence of matrix, specifically urine, are not influenced by the matrix. From the point of view of compound separation, the integration of IMS as a third separation dimension in LC-MS workflows increases the resolving power of the method, allowing the separation of isobaric and isomeric steroids. Furthermore, it also allows the separation of the steroids from the chemical background related to the urine sample, improving the limits of detection (LODs) between 2 and 7 times.

Keywords: Collision Cross Section, Ion Mobility, Steroids

ORAL O14

INNOVATIVE LOW-FLOW LC-HRMS APPROACH OPENS NEW PERSPECTIVES FOR HIGH THROUGHPUT DOPING CONTROL ANALYSES: APPLICATION TO SOMATOTROPIN DETECTION

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Abstract
Doping control analytical workflows often involve an extraction procedure followed by a narrow bore liquid chromatography coupled to tandem mass spectrometry. Over the past decade, this approach underwent several breakthrough improvements in terms of throughput and accuracy thank to UHPLC and high-resolution mass spectrometry (HRMS). First generation nanoLC significantly improved sensitivity at the expanse of robustness and analysis throughput. Indeed, the use of such technology was often associated with the risk of retention time fluctuation and time-consuming sample loading. These constraints limited the use of nanoLC for equine drug testing purposes to confirmatory analysis of few polypeptides.
The recent advances in low-flow separation through innovative sample trapping combined to controlled elution and on-column refocusing is now available by means of the “Evosep One” system which fills the gap between conventional high-throughput ultra-high-performance liquid chromatography (UHPLC) and high-sensitivity nanoLC. Novel methods on the Evosep One reveal that the former proteomics-dedicated technology provides enhanced performances for small-molecules analysis such as androgenic anabolic steroids (AAS) in highly complex matrices such as equine urine and hair. Furthermore, the adaptation of our LC-MS/MS Somatotropin/Growth Hormone (GH) method demonstrate significant improvements in terms of robustness and throughput, without compromising the analytical performances.

Keywords: Growth hormone, Horse doping control, Low-flow LC-MS/MS

ORAL 15

OCCURRENCE OF ANTIBIOTIC RESIDUES IN RAW BOVINE MILK AND THEIR RELEVANCE TOWARD FOOD SAFETY AND TECHNOLOGICAL IMPLICATIONS

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Abstract

Antimicrobial drugs are used to control, prevent, treat infections, and to enhance animal growth. The occurrence of these residues can represent a problem of food safety, increase resistance of pathogenic bacteria but can also interfere with cheese making techniques, causing improper ripening of cheese. In this regard, we developed and validated a confirmatory multiclass method for the analysis of 66 antibiotics in 150 bovine raw milk samples by HPLC–HRMS Orbitrap. All samples showed residues lower than the maximum residue limits: lincomycin was the most frequently found antiobiotic, with an average concentration of 15.35 ng g^{-1}, with sporadic traces of spiramycin, sulfapyridine and oxytetracycline. Our confirmation analyses also demonstrated the ineffectiveness of the screening kits previously used to test the raw milks, showing false positives due to the presence of interferents. Moreover, considering the range concentration of the most detected antibiotic, lincomycin, we spiked some milk samples at 10, 20 and 40 ng g^{-1} in order to investigate how lincomycin can interfere on lactic acid bacteria acitivity. Finally, we found the same spike concentration in the final product, with a direct relation and impact leading a pH increasing and reduction of lactic bacteria counts that are essential for the caseification.

Introduction

Antibiotics are widely administered in animal husbandry for prevention, for disease treatment, and/or as growth promoters (Rama *et al.*, 2017). These practises may leave antimicrobial residues in food intended for the consumer.

Milk is a staple food and consumed in large-scale throughout the world also in the form of derivatives. For this reason, milk safety plays a crucial role and a critical issue. Usually, chemicals represent the main source of residues since they are directly used during dairy management and milking process. In particular, tetracyclines, quinolones, β-lactams, streptomycin, chloramphenicol and sulfonamides, are the most frequently administered in dairy cattle and their presence as residues in milk could increase the risk of allergies in susceptible people and promote the phenomenon of antibiotic resistance (Jank *et al.*, 2015). The most popular antibiotic resistant strains are antibiotic resistant salmonellae, fluoroquinolone or macrolide resistant campylobacters, glycopeptide or streptogramin resistant enterococci and multiple antibiotic resistant E. coli (Tempini *et al.*, 2018). The European Food Safety Authority (EFSA) collects and analyses data on antibiotic resistance from EU States by official reports. Quinolones, cephalosporins, macrolides and polymyxins are in the list of the most critical antimicrobials, encouraging the incidence monitoring of these compounds (EFSA, 2016). For consumer health safety, maximum residue limits (MRLs) are fixed for the different residues of antimicrobials (European Commission, 2010).

Another critical point is the technological impact since antimicrobials can interfere with the dairy production, decreasing acid formation, reducing the milk curdling and causing an improper cheese ripening (Quintanilla *et al.*, 2018).This could be a serious issue for some geographical areas where milk is primarily used for PDO or IGP cheese-making, as for example for Parmigiano Reggiano and Grana Padano. In this case a residue level even below the MRL can cause economic losses for this negative impact (Pogurschi *et al.*, 2015).

Some studies deal with the role of temperature treatments in the possible inactivation of antimicrobials even if the topic is a little bit controversial (László *et al.*, 2018). Some Authors reported a partial degradation accentuated by long heating time for β-lactams, sulfonamides, quinolones, macrolides, aminoglycosides and tetracyclines (Ianni *et al.*, 2018).

With regard to the determination of antibiotics, there are some rapid screening tests that although they allow a large number of samples to be screened even in livestock, they suffer from aspecificity. On the other side there are the confirmatory analises, typically based on LC-MS/MS or HRMS detection. In this case the challenge is to develop multiclass confirmatory methods that enable sensitive and accurate detection of a large number of antimicrobials.

In the light of these considerations the aims of this study were to develop an LC-HRMS method based on multiclass antimicrobial analysis in raw bovine milk used for cheese production to evaluate the incidence of antimicrobial residues in comparison with results obtained by screening tests and finally, to investigate their impact during caseification by simulalting a cheese-making process of a PDO cheese in small scale.

Materials and Methods

Chemicals and reagents

All solvents, reagents and the 66 selected antimicrobials were from Merck (KGaA, Darmstadt, Germany). The SPE cartridges (Oasis HLB 3 mL, 60 mg), were from Waters (Milford, MA, USA). Screening tests were: Delvotest® SP NT plates from DSM (Heerlen, the Netherlands), Milk Antibiotic Testing 3 in 1 Macrolides Erythromycin – Lincomycin – Tylosin – Tilmicosin 96 Tests from Shenzhen Bioeasy Biotechnology Co., Ltd. (Shenzhen, China) and ROSA Charm QUAD1 Test from Charm Sciences Inc (Lawrence, MA, USA).

Standard solutions

Stock solutions at concentration of 1 mg mL^{-1} and working solutions at 10 and 100 ng $mL^{-1}$of all compounds were prepared in methanol and kept at -20 °C.

Milk samples

150 anonymous raw bovine milk samples were collected from local farms of Piedmont Region, in North Italy, a geographical area where most of the milk is used for Grana Padano PDO cheese production. The samples were previously processed by 3 screening tests and then by a LC-HRMS confirmation methods in order to assess their conformity in compliance with MRLs and food safety regulations.

Screening methods analyses

The 3 different screening tests were used following the supplier instructions. In brief, Delvotest® SP NT consist of 96 wells plates with *Bacillus stearothermophilus var. calidolactis* where 100 µLof milk sample were inserted and incubated for 3 hours, considering a water bath preheated to 64°C . Charm QUAD1 Test is a rapid immunoreceptor assay based on lateral flow technology in which 300 µL of milk sample were inserted and incubated at 56°C for 5 minutes. Bioeasy Milk Antibiotic Testing 3in1 (Erytromycin+Lyncomycin+Tylosin & Tilmicosin) is also a rapid immunoreceptor assay with lateral flow technology, where 200 µL of sample, placed in microwell were incubated at 40°C for 3 minutes. For all tests the results were obtained immediately after incubation.

HPLC-HRMS analyses

Raw bovine milk sample extraction was performed following the procedure of Chiesa *et al.* (2018a). Briefly, 2ml of sample were spiked with Enrofloxacin-d5, as internal standard, at 2 ng g^{-1}, added of 5 mL of McIlvaine buffer (pH 4.0) and 100 µL, 20% w/v of Trichloroacetic acid for protein precipitation. After vortex, sonication for 10 min and centrifugation for 10 min (2500×g, 4°C), the extract was defatted with 2×3 mL of n-hexane. The purification was done by an SPE cartridge preconditioned with 3mL of methanol and 3 mL of Milli-Q water. After sample loading, the cartridge was washed with 2×3 mL methanol:water (5:95 v/v) and finally eluted with 5 mL of methanol. After evaporation the extract was reconstituted in 200 µL of methanol: 0,1% formic acid (10:90 v/v).

The analyses were carried out by an HPLC system (Thermo Fisher Scientific, San Jose, CA, USA) coupled to a Thermo Q-Exactive Orbitrap (Thermo Fisher Scientific). The analyses were performed through a full-scan (FS) acquisition combined with a data-independent acquisition (DIA), for the confirmatory response. All HPLC and HRMS parameters were reported in previous work (Chiesa *et al.*, 2018b).

HPLC-HRMS method validation
Method validation was carried out according to the Commission Decision 2002/657/EC guidelines (European Union, 2002) and described in Chiesa *et al.* (2018b). In particular, the recovery was calculated as the percentage of the true concentration of the analyte recovered after the analytical protocol; the decision limit (CCα) and detection capability (CCβ) were calculated to define our method sensitivity; the precision, in terms of intra- and inter-day repeatability, was evaluated through the calculation of coefficient of variation (CV%) obtained from six replicates at 3 different concentration levels analysed during the single batch of the daily validation protocol and among 3 different days.

Antimicrobial trials in cheesemaking simulation
A laboratory-scale process simulated the original cheese-making process focusing in particular on the first step of milk acidification and subsequent addition of whey starter in order to assess the impact of antimicrobial residues at 3 different concentration levels. This monitoring plan was performed by choosing the compound most frequently detected and quantified during the screening of the samples.

In particular, 3 different lincomicyn concentrations in triplicate were selected to fortify blank milk previously selected for the absence of antimicrobials: blank (B), C1 (fortified with 10 ppb), C2 (fortified with 20 ppb) and C3 (fortified with 40 ppb). Briefly, 500 mL of milk respectively of B, C1, C2 and C3 were added of 1 % (weight/weight) of natural whey starter to simulate cheese production, followed by a thermostatic incubation at 45°C for 14 hours. The natural whey was obtained from the processing with semi-fat hard cheese of the previous day, so it is characterised by autochthonous microflora. The acidification milk kinetic was periodically investigated by a pH-meter (model Basic 20, Crison, Barcelona, Spain) with a penetration probe (model 5232, Crison, Barcelona, Spain).

At the end of the acidification process during which whey starter was initially added to the milk, lactic acid bacteria and total microbial count were investigated to understand their possible reduction or inactivation due to the possible negative impact of lincomycin added during the caseification process.

pH and microbiological determination
Possible negative impact on reduction or inactivation on caseification process was investigated by checking lactic acid bacteria and total microbial count combined with pH measurement during 12 h acidification and at the end of the process.

The pH and microbiological parameters were evaluated in triplicate in 10 mL of milk samples added with whey starter and fortified at the 3 levels of lincomycin (C1, C2 and C3), homogenized in a stomacher for 1min at room temperature after addition of 90mL of sterile diluent solution (0.85% NaCl and 0.1% peptone). Mesophilic aerobic bacteria were determined using Plate Count Agar (Oxoid CM0325) after incubation at 30°C for 72 hours, instead lactic streptococci were determined using M17 agar after incubation at 37° for 2 days. Total Lactobacillus species were counted by MRS agar incubated at 30 °C for 72 h under microaerobic atmosphere. The reading was performed by an Interscience - Scan® 4000 and the result were expressed as colony-forming unit (CFU) mL^{-1}.

Results and Discussion

HPLC-HRMS method validation parameters
All validation parameters are presented in Table 1. Good method sensitivity was showed by CCα and CCβ much lower than MRLs set for the different antimicrobials (European Union, 2010). Good selectivity and specificity were achieved by the extraction and purification of the analytical protocol. Effective recoveries were obtained from 88 to 99% and the CV% were all ≤ 20% (European Union, 2002). Comparing with literature there are few confirmatory methods with a wide number of antibiotics of different classes in milk and dairy products, most of all are of a screening type or low resolution MS confirmatory methods.

Antimicrobial residue in real samples
All 150 samples showed residues lower than the maximum residue limits. All the quantified residue concentrantions were reported in Table 2. In particular, lincomycin was the most frequently found antiobiotic, with an average concentration of 15.35 ng g^{-1} (0.77 minimum - 51.68 maximum) and a detection frequency of 10%. Sporadic traces of spiramycin, sulfapyridine and oxytetracycline were detected. Scarce data are available in literature about lincomycin residues in bovine milk. The most common antibiotics used in cow breeding are β-lactams and tetracyclines administered for prevention and treatment of mastitis (Landers *et al.*, 2012). The presence of antimicrobial residues in milk derives by direct administration but can also occur during machine washings after milking a treated cow before anuntreated one (Kebed *et al.*, 2014).

Even if below the MRLs, the detected residue can spread the antibiotic resistant bacteria. Comparing the confirmatory results with those obtained by screening tests, only false positive results were found. In particular, Delvo and Charm reported 3 and 8 % false positive results respectively toward sulfonamide and β-lactam residues. These frequencies are similar to those reported in other studies on antibiotic detection in milk (Bion *et al.,* 2015). For the 3easy test also 8 % frequency of false positives (for lincomycin) were calculated.

Considering that the screening tests are frequently used at the farm due to higher costs for confirmation techniques, it is crucial to assess and validate multiclass antimicrobial screening tests in order to set up reliable monitoring plans both for food safety and technological issues.

Effect of antimicrobial residues on caseification process

As described before, 3 batches of the same blank milk involved in PDO cheese production were fortified at 3 different lincomycin concentration (C1=10, C2=20 and C3=40 ng g^{-1}).

Data obtained from pH measurements showed that pH value during the 12 h of acidification decays over time both in all blank and fortified samples, but with different trends (Figure 1). In particular at the end of the acidification process, the most acid pH value (3.76) was found in the blank unfortified samples (Figure 1). According to Loftin *et al.* (2008) lincomycin is stable to pH variations and the 3 fortified amounts were detected and quantified at the end of the process.

The lowering of pH value is very important in the cheese-making process because it increases the activity of enzymes and the speed of coagulation (Lawrence *et al.*, 2000), so an insufficient lowering of pH can cause early fermentation, supported by clostridia or by yeasts, with formation of large spongy cavities in the cheese (Pecorari *et al.*, 2003).

On the microbiological point of view, the total bacterial count should never exceed a maximum value to avoid reduction in terms of cheese-making capacity (Hill, 2000). At the end of 2 h acidification process, microbiological analyses were performed. As expected, mesophilic aerobic bacteria number decreased from 8,26 log CFU/ml^{-1} of the unfortified samples to 8.11, 7.44 and 6.86 log CFU/ml^{-1} in the fortified C1, C2 and C3 samples, respectively. The number of *Lactobacillus spp*. decreased from 10,32 log CFU/ml^{-1} in the unfortified samples to 10.20, 9.50 and 8.92 log CFU/ml^{-1} in the fortified C1, C2 and C3 samples, respectively. Also for the lactic streptococci there was a slight decrease from 7.12 log CFU/ml^{-1} to 7.20, 6.67 and 6.79 log CFU/ml^{-1} in the fortified C1, C2 and C3 samples, respectively.

On the light of these evidences, even if the concentration of the antimicrobial was lower than the MRL, the presence of residues have a negative effect on milk starter cultures and in general on cheese-making.

Table 1. Validation parameters for antimicrobial detection in milk samples

Chemical Class	Compound	Decision limit CCα (ng mL^{-1})	Detection capability CCβ (ng mL^{-1})	Recovery %	CV intra-day %	CV inter-day %
Quinolon	Enrofloxacin	0.10	0.23	98	9	11
Quinolon	Ciprofloxacin	0.11	0.25	96	10	13
Quinolon	Difloxacin	0.11	0.24	93	12	16
Quinolon	Danofloxacin	0.13	0.27	95	11	13
Quinolon	Levofloxacin	0.12	0.22	94	10	12
Quinolon	Lomefloxacin	0.11	0.2	93	11	13
Quinolon	Marbofloxacin	0.13	0.25	96	10	15
Quinolon	Norfloxacin	0.12	0.24	95	13	18
Quinolon	Enoxacin	0.13	0.26	94	12	18
Quinolon	Flumequine	0.11	0.24	97	12	20
Quinolon	Nadifloxacin	0.11	0.25	95	14	17
Quinolon	Oxolinic acid	0.22	0.31	96	12	15
Quinolon	Nalidixicacid	0.24	0.33	94	11	15
β-Lactam	Amoxicillin	0.50	0.62	89	14	17

β-Lactam	Ampicillin	0.53	0.63	90	14	18
β-Lactam	Phenoxymethylpenicillin	0.54	0.64	93	13	16
β-Lactam	Benzylpenicillin	0.54	0.66	94	12	16
β-Lactam	Cefadroxil	4.37	5.10	89	11	16
β-Lactam	Cefalexin	0.49	0.60	92	14	20
β-Lactam	Cefalonium	4.28	5.08	91	14	20
β-Lactam	Cefalothin	4.25	5.06	91	13	18
β-Lactam	Cefazolin	4.30	5.11	88	12	15
β-Lactam	Cefoperazone	4.12	4.99	92	13	15
β-Lactam	Cefquinome	0.47	0.59	93	10	14
β-Lactam	Cefapirin	4.06	5.09	91	11	13
β-Lactam	Ceftiofur	0.18	0.30	93	11	15
β-Lactam	Desfuroylceftiofur	0.21	0.31	94	14	19
β-Lactam	Cloxacillin	0.57	0.72	92	13	19
β-Lactam	Dicloxacillin	0.59	0.75	92	14	17
β-Lactam	Desacetylcefapirin	4.01	5.04	94	12	18
β-Lactam	Nafcillin	0.10	0.22	96	12	15
β-Lactam	Oxacillin	0.11	0.24	89	11	14
β-Lactam	Piperacillin	0.11	0.23	95	10	12
Macrolide	Tylosin	0.13	0.25	93	10	14
Macrolide	Tilmicosin	0.12	0.25	94	8	12
Macrolide	Oleandomycin	0.89	1.04	92	11	16
Macrolide	Spiramycin	0.39	0.50	87	14	17
Macrolide	Neospiramycin	0.85	1.02	91	13	17
Macrolide	Kitasamycin	0.90	1.04	93	12	15
Macrolide	Josamycin	0.87	1.05	96	12	16
Macrolide	Tulathromycin	0.38	0.51	95	12	18
Macrolide	Erythromicyn A	0.40	0.49	95	11	14
Sulfonamide	Sulfadiazine	0.10	0.22	97	8	13
Sulfonamide	Sulfadimethoxine	0.12	0.23	98	9	12
Sulfonamide	Sulfadimidine	0.12	0.25	97	9	11
Sulfonamide	Sulfamerazine	0.11	0.24	97	10	12
Sulfonamide	Sulfamethoxazole	0.11	0.24	96	11	13
Sulfonamide	Sulfamonomethoxine	0.11	0.22	98	10	13
Sulfonamide	Sulfapirydine	0.13	0.25	98	9	11
Sulfonamide	Sulfatiazole	0.13	0.24	99	9	12
Sulfonamide	Trimethoprim	0.10	0.22	99	10	13
Tetracycline	Chlorotetracycline	0.15	0.24	96	10	12
Tetracycline	Oxytetracycline	0.10	0.22	97	10	13
Tetracycline	Tetracycline	0.12	0.24	95	12	15
Tetracycline	Doxyicline	0.12	0.23	96	11	14
Lyncosamide	Lincomycin	0.09	0.20	98	7	11
Amphenicol	Chloramphenicol	0.06	0.12	91	13	14
Amphenicol	Tiamphenicol	0.11	0.26	93	11	14
Amphenicol	Florfenicol	0.21	0.3	92	14	18

Amphenicol	Florfenicol amine	0.20	0.30	90	13	20
Pleuromutilin	Tiamulin	0.18	0.25	94	13	20
Pleuromutilin	Valnemulin	0.88	1.02	93	12	16
Nitroimidazole	Dimetridazole	0.10	0.22	93	12	14
Nitroimidazole	Ronidazole	0.10	0.21	94	14	15
Nitroimidazole	Tinidazole	0.12	0.24	96	11	16
Nitroimidazole	Metronidazole	0.10	0.21	95	12	15

Table 2. Range, average concentrations and detection frequency of the detected antimicrobials in the 150 raw milk samples by LC-HRMS analysis.

Compound	Mean ± SD n=150 (ng mL^{-1})	Min-Max (ng mL^{-1})	Detection frequency (%)
Cefapirin	<CCβ	-	0.7
Spiramycin	<CCβ	-	0.7
Oxytetracycline	3.47 ± 6.66	0.82-10.13	1.3
Lincomycin	15.35 ± 22.92	0.77-51.68	10

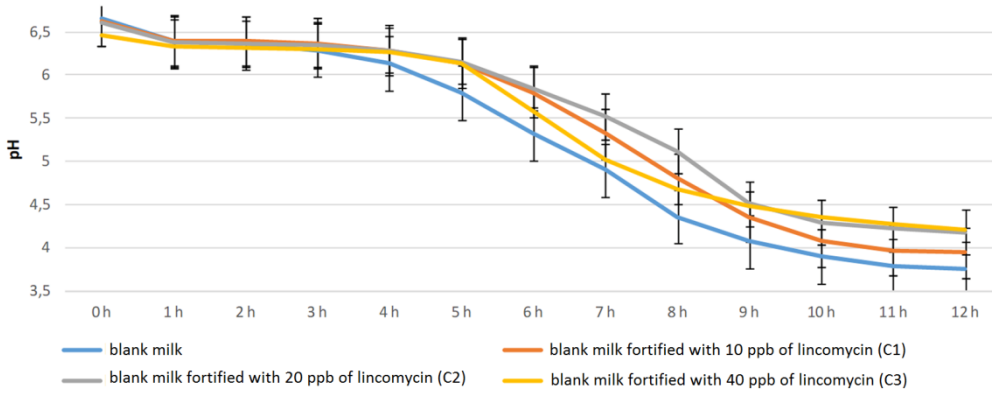

Figure 1. pH trend during the acidification process of both blank milk and fortified samples at 3 different lincomycin concentrations

Acknowledgements

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ORAL O16

HORMONES RESIDUES IN BOVINE MATRICES: SAMPLING, ANALYSIS, AND HEALTH RISK ASSESSMENT.

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Abstract
Hormonal substances have been widely used in food-producing animals for the growth promotion and to fulfill the high increase in consumption demand. However, the misuse of these compounds has led to the accumulation of their residues in animal tissues which may pose serious human health risks. Therefore, the use of hormones in cattle husbandry was banned since 1981. So far, each country should monitor the use of anabolic hormones to protect the consumer's health. Thus, in Lebanon, it was necessary to establish a monitoring to control the misuse of these substances in livestock practices. This study presents the development and validation of new liquid chromatography coupled to mass spectrometry (LC-MS/MS) method for the analysis of fourteen hormones in bovine matrices (muscle, liver, kidney, bile, and hair). Results showed good linearity as well as acceptable accuracy with coefficients of variation for repeatability and reproducibility lower than 23%. Moreover, the values of decision limit (CCα) and detection capability (CCβ) w

ere calculated indicating satisfied values. Indeed, this method has been successfully applied to detect anabolic hormones in two-hundred forty-seven samples collected from different slaughterhouses. As a result, four hormones were found. Finally, the performance of a preliminary risk assessment was carried out.

Keywords: bovine matrices, Hormones, Liquid chromatography mass spectrometry

ORAL O17

TRUE MOBILE MASS SPECTROMETRY FOR ONSITE ANALYSIS

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Abstract

Nowadays, we use optical sensors in our smartphones and smartwatches to measure all kinds of mostly health-related parameters. Unfortunately, their application is limited. Imagine the possibilities if you could take high-end laboratory equipment to the field. Current mass spectrometers are, at best transportable, not truly portable. What if you could fit an MS in a small backpack or carry it in your hand?

In recent years, we have invested in research, leading to effective onsite mass spectrometry (MS). After evaluating the commercially available portable MS instruments, one was purchased by Wageningen Food Safety Research (WFSR). This MS is fully self-sustaining in the field and consists of a battery, small gas cylinder, computer, GPS, WiFi, and gas chromatograph, and it is connected to WFSR via the cloud. Different sample inlet options are available, e.g. membrane inlet MS (MIMS), thermal desorption, and split/splitless injection. This truly portable MS is currently evaluated for various food applications. For example, the detection of illegal drug waste dumping (volatiles) in crop fields and manure pits or other environmental-related questions. Also, confirmatory analysis of formulations is possible. The results of the evaluation of this MS system and related onsite sample preparation techniques for food safety-related applications will be presented.

Introduction

At the moment, there are many trends and external factors that will change the way we look and probably how we will work in the food safety systems. For instance, we want to reduce food waste, close the food cycle, and decrease our carbon footprint. For this purpose, a reduction in meat consumption or a change in its production is needed. Also, the food price is increasing, and thus, food fraud is more likely. All these factors will increase the number of samples for food safety control laboratories. However, as a food safety laboratory, this fact will be a bottleneck in the future since we cannot do all these measurements, and therefore existing methodologies need to be changed to decrease our workload. Consequently, we believe that the demand for measuring onsite will grow. Basically, this means that we have to bring the laboratory to the sample. So, partly sampling, administration, and transport to a central laboratory is therefore expected to be a thing of the past, just like waiting, a day, a week, or even a month, for a result. This development applies not only to food control but also to environmental and forensics measurements. In this context, lab tests will be increasingly in the hands of operators, inspectors, drivers, farmers, and ultimately even in those of the consumer. Many experts say 'this will never work', but many citizens are already walking around with wearables and smartwatches in the meantime.

Due to recent scientific and technological breakthroughs, it is expected that in the near future, non-experts users will be able to measure in the food chain on location. This will be supported due to the fact that classic laboratory equipment is increasingly miniaturized and made more robust, transportable, and even portable. Due to legal requirements and flexibility, mass spectrometers are widely used in control laboratories but not yet onsite. However, these advanced instruments are available in a portable variant. We evaluated several of these portable mass spectrometers and eventually purchased one to gain experience with onsite mass spectrometric measurements. The purchased instrument is equipped with a battery, a simplified user interface, and Wifi for communication with stakeholders.

In this work, the potential of a true mobile mass spectrometry for onsite analysis has been evaluated. For this purpose, several cases related to food safety have been studied. Within our studies, the portable MS has been used to analyze chemical incidents such as illegal drugs waste dumping in the fields or the manure pits of farms.

Materials and Methods

Standards

Several steroid ester analytical standards such as Testosterone acetate, Testosterone propionate, Testosterone propionate and Trenbolone acetate were purchased from Steraloids Inc. Ltd. (London, England). SPME fibers were purchased from Supelco (Sigma, the Netherlands).

Instrumentation

A Person-Portable gas chromatography-mass spectrometer, Griffin 510 instrument (Flir, Wilsonville, OR, USA), has been used in this study. This Portable GC-MS is fully battery-operated, consisting of a split-splitless injector, a 15 m polysiloxane DB-5 column, an electron ionization (EI) source, and a single quadrupole mass analyzer. The high vacuum is obtained using a small turbopump. Besides a traditional liquid injection, a heated sample probe can be used, which sucks in vapors. These vapors can be analyzed directly via membrane introduction mass spectrometry (MIMS) or concentrated via an internal dual-bed pre-concentrator. The portable MS can run continuously for 4 hours without external power or helium supply using small helium bottles and a swappable battery. In figure 1, a schematic overview of the portable MS is given.

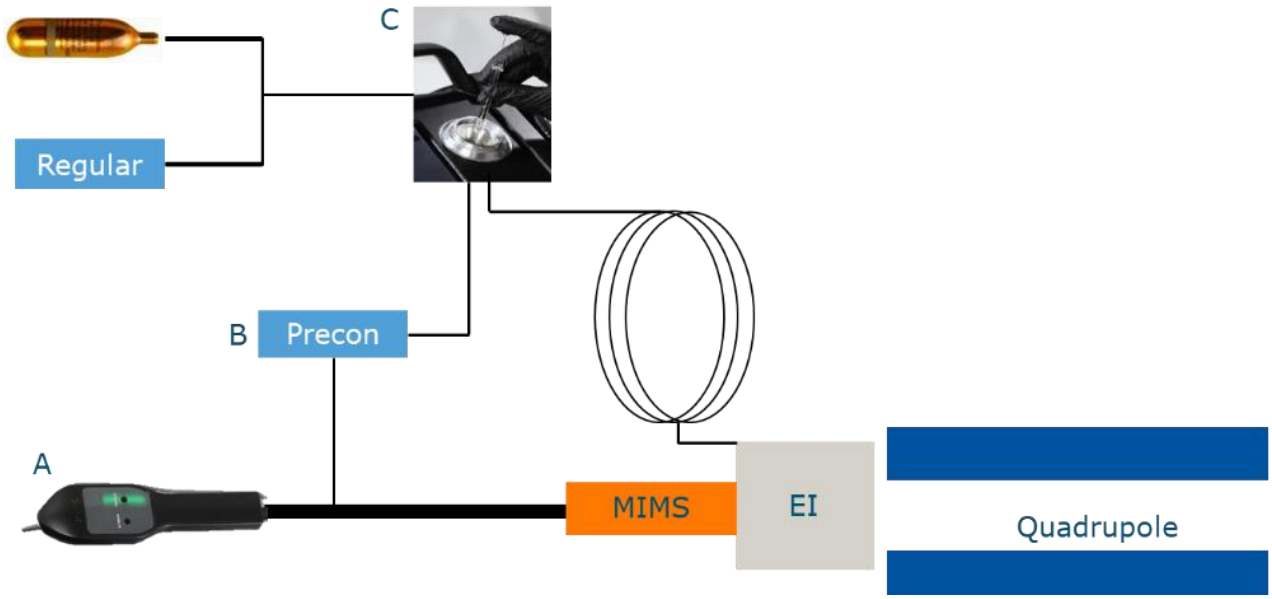


Figure 1. Schematic overview of the portable GC-MS. A: heated sample probe, B: internal dual-bed pre-concentrator, C: split-spitless injector.

Additionally, in case 1, a handheld diode laser (iLase, Biolase Tech, Irvine, CA, USA) operated at 940 nm, and with an optical peak power output of 3 W (average power density of 1 W), pulse length of 10 ms was used.

Results and Discussion

Spectral quality

The MS produces spectra using an electron ionization source at 70 eV in combination with a quadrupole. So, in theory, it should be capable of producing spectra comparable to spectra collected on benchtop mass spectrometers. To check the spectral quality, different steroid-esters were analyzed, and the full scan spectra were compared with library spectra. In figure 2, an example of a spectrum of testosterone-acetate and the corresponding library spectrum from NIST is given. There is an excellent match between the two. This is important since this MS is mainly used in full-scan in the field and will rely heavily on library matching to identify compounds.

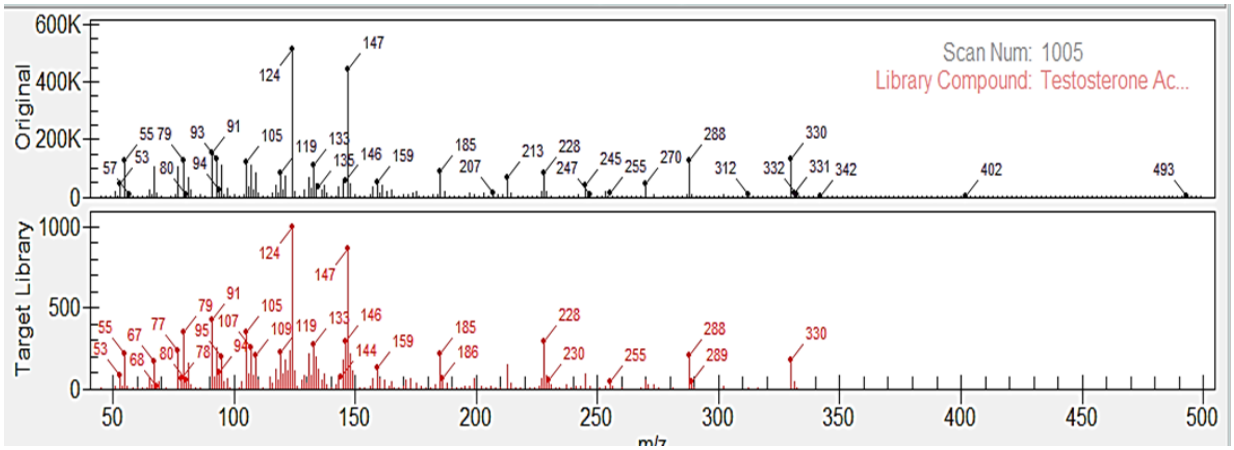
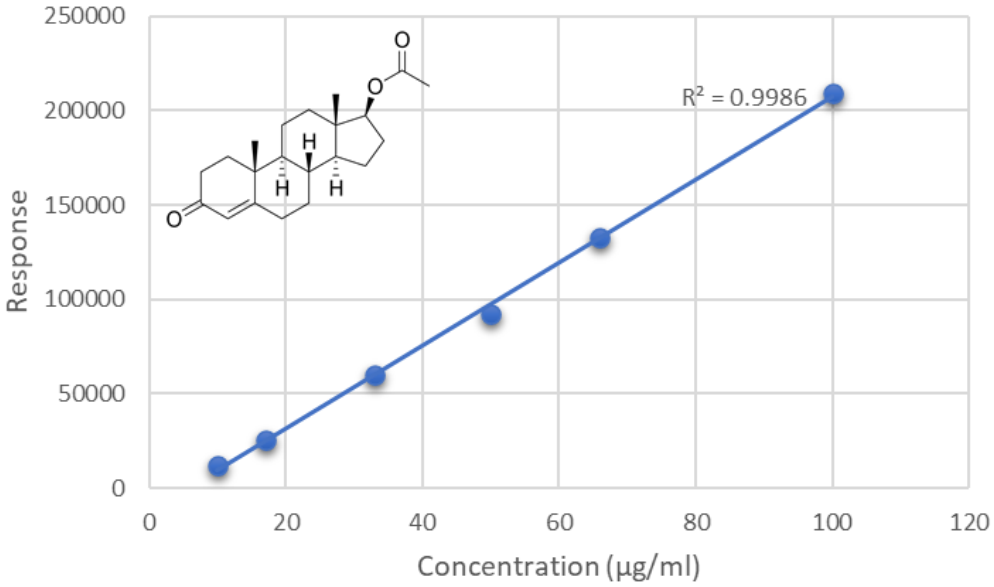


Figure 2. Comparison of the measured spectrum (upper panel) and a corresponding spectrum from the NIST library (lower panel).

The linearity was checked by injecting different concentrations of steroid-esters. The injection was performed by means of the solid-phase microextraction (SPME) technique. So, in this case, if a sample has to be taken onsite, no extraction has to be performed other than sticking the needle into the sample and, afterward, placing the SPME needle into the injector. In figure 3, an example of a calibration curve of testosterone-acetate prepared in water is given. The correlation coefficient is >0.99 without using an internal standard, which is considered



adequate for quantitative analysis.

Figure 3. Example of a calibration curve of testosterone-acetate obtained with SPME extraction (quantifier ion m/z 288).

Evaluated case's

To evaluate the potential of the acquired portable MS, several cases were studied. Within these cases studies, the portable MS has been used in real life to analyze chemical incidents such as illegal drugs waste dumping in the fields or the manure pits of farms.

- *Case 1: Identification of an unknown white powder*

The first case evaluated in this study was an unknown white powder found next to an analytical balance in the laboratory. In this context, our work aimed to identify the origin of this powder using the mobile MS. The sample could be taken, diluted, and afterward analyzed in one of the available high-end mass spectrometers. However, we wanted to examine if we could identify this powder with minimal sample clean-up using the portable MS. So first, the sniffer of the mobile MS was used to determine if there was any odor we could identify. Our results showed that in this white powder sample, there was not any odor. Next, the odor was concentrated using the internal trapping device, and still, no relevant hits appeared apart from acetonitrile which belonged to the laboratory environment. Therefore, an intelligent way to introduce the powder without any chemicals was tested. A handheld-diode portable laser was used in order to evaporate the sample. Afterward, the vapor was trapped using the heated sampling probe, and the portable MS started identifying the vapor by heating the trapping device and performing GC-MS analysis. Figure 4 shows the performed experiment with the portable laser and the heated sampling probe.

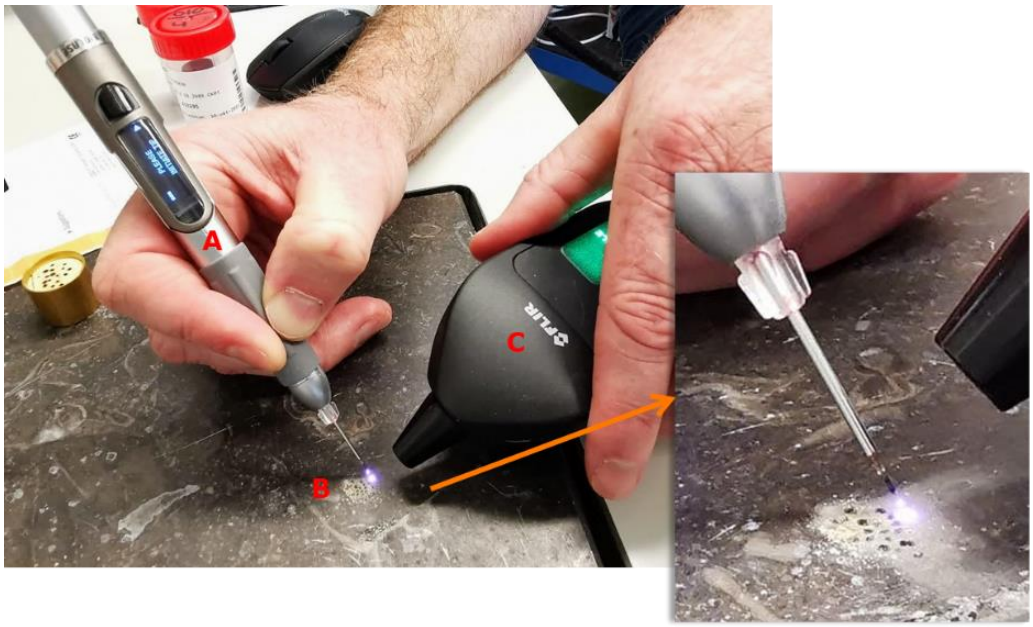


Figure 4. Overview of the experiment to identify a white powder left next to an analytical balance, A: Portable laser, B: white powder, C: Heated sampling probe.

In figure 5, the obtained chromatogram and spectra from this experiment are shown. As can be observed, most of the observed peaks could be identified. In this way, after some puzzling, we concluded that the white powder was diethylstilbestrol. Of course, this is an extreme example, but this case demonstrates the possibility of how to sample a solid substance in a relatively easy way.

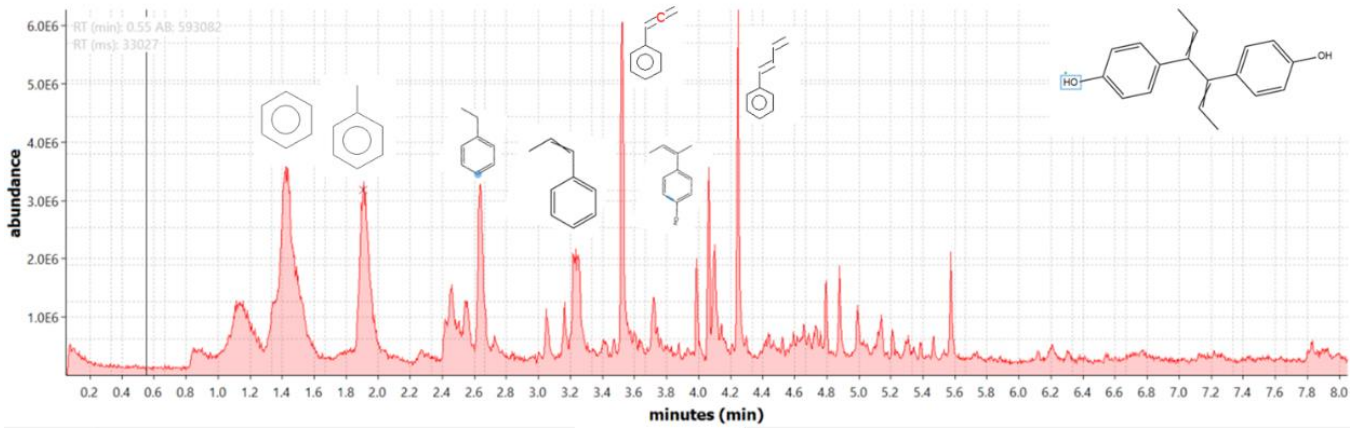


Figure 5. Chromatogram obtained after evaporation of the sample with the portable laser. The peaks were identified based on their spectrum, and by combining the information, the structure was elucidated.

- *Case 2: Detection of drugs waste dumping*

As it is well known, unfortunately, many hard drugs are produced in the Netherlands. During drugs production, there is also chemical waste produced. However, drug labs do not take proper care of these waste streams, and often these waste streams are dumped inside farms and the surrounding farmlands. Previously, it has been investigated how MDMA ended up in the corn. In this particular case, manure had been applied from a manure pit into which chemical waste from an ecstasy lab had also been discharged. To gain experience, we performed measurements above an old manure pit on the farm where drugs waste was dumped. To determine if drugs or any precursors are present, an air sample was first taken using the heated sampling probe, and the sucked air was concentrated for a few minutes on the internal trapping device. After three minutes of trapping, a GC-MS run was performed, and the peaks present were automatically annotated using the NIST library. In the samples taken, clearly a precursor of drugs production was present, phenylacetone. As shown in figure 6, the obtained chromatogram, spectra, and library search in this study are shown. This finding also demonstrated that using a simple direct measurement by a portable MS can help onsite inspectors or law enforcers determine if illegal practices at a farm occur.

Conclusion

After evaluating commercially available portable MS instruments, one was purchased by WFSR. This MS is fully self-sustaining in the field and consists of a battery, small gas cylinder, computer, GPS, WiFi, and gas chromatograph, and it is connected to WFSR via the cloud. This study demonstrates the different sample inlet options, e.g. membrane inlet MS (MIMS), thermal desorption, and split/splitless injection. Two cases were studied: 1) the analysis of an unknown white substance and 2) detection of illegal drug waste dumping in manure pits. These two cases demonstrated that portable MS could, and will, have a future in food-safety analysis.

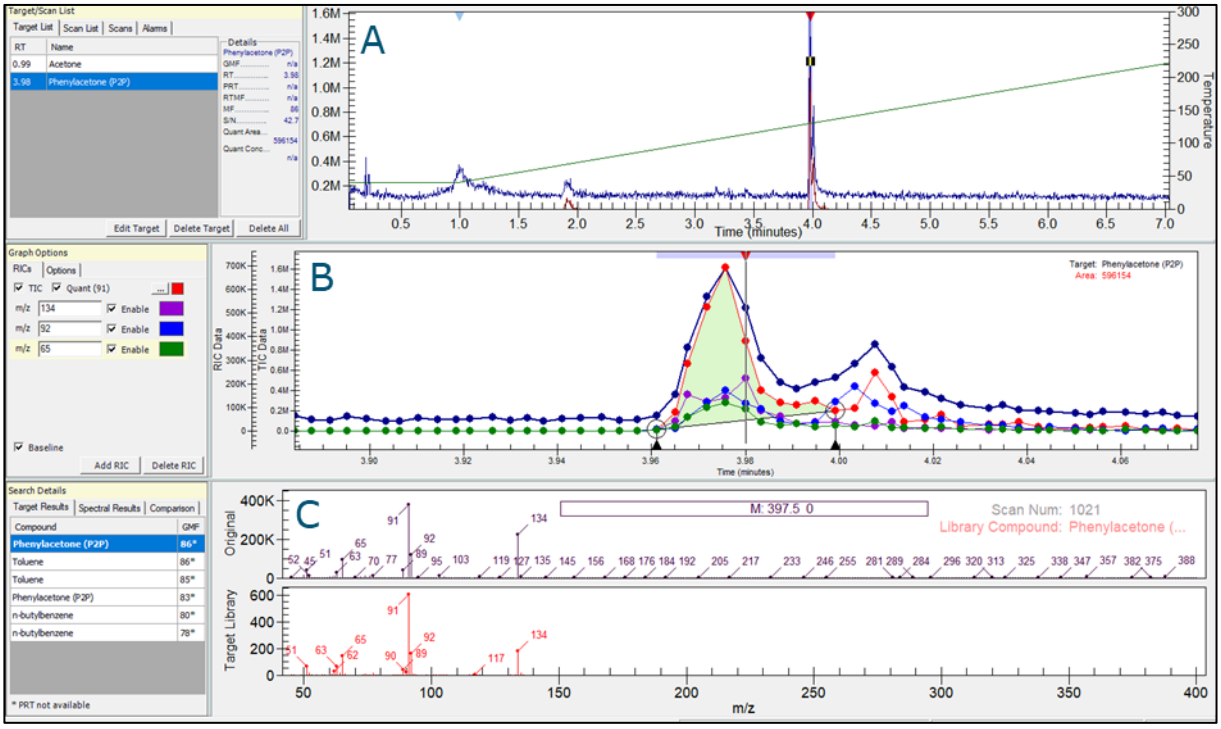


Figure 6. Overview of the results obtained from an air sample taken above a manure pit on a farm where in the past drugs were dumped, A: Chromatogram, B: Extracted traces of phenylacetone, C: Library search window

Acknowledgements

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ORAL O18

LIVESTOCK AND SPORT ANIMALS DOPING: TOWARD THE PRACTICAL IMPLEMENTATION OF METABOLOMICS BASED STRATEGIES

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Abstract
Over the last past 15 years, non-targeted strategies have emerged as relevant screening tools to detect the misuse of growth promoters in livestock and sport animals, allowing characterization of physiological disruption upon substances administration Among other omics approaches, metabolomics turned out to be the most promising to highlight biomarkers of these practices. While numerous studies have demonstrated the proof of concept of metabolomics-based workflow [1], it is now important to extend their scope and address validation steps.
A LC-HRMS metabolomics workflow was applied to investigate markers associated to boldenone administration in cattle and testosterone in equine. Resulting statistical models enabled efficient classification of the sample status. To validate the models and define their respective scopes, samples from additional *in vivo* studies involving animals' variability (e.g., gender, age) together with the administration of different substances, doses, administration routes etc., were characterized. Finally, with the aim of implementation in field laboratories, methodological transfer towards low resolution (LC-LRMS) devices was performed. The samples from other *in vivo* studies were predicted and classified on both models demonstrating the robustness of the approach towards other anabolics. Moreover, the performances were preserved during inter-instrument transfers.

[1] Cloteau *et al.* 2022

Keywords: Anabolics, Metabolomics, Screening

ORAL O19

LC-HRMS BASED METABOLOMICS TO IDENTIFY LIVER MARKERS OF CLENBUTEROL ABUSE IN BEEF CATTLE

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Abstract

Metabolomics analysis was applied to liver samples to overcome limitations of classical analytical methods adopted in surveillance of illegal use of growth promoters in food producing animals. Liver extracts were collected from an *in vivo* study in which 8 adult charolaise bulls were experimentally treated with clenbuterol combined with dexamethasone to mimic a potential anabolic practice, whereas other 8 bulls did not received any pharmacological treatment and were kept as control animals. LC-HRMS analysis of liver extracts using either RPLC or HILIC in both negative and positive ionization polarity produced 4 data sets. Statistical analysis enabled us to reveal two compounds as potential markers of treatment. Such compounds and other two metabolites of the same metabolic pathway were exploited to develop a predictive model to classify animals according to their corresponding group (treated or control).

The predictive model was validated on external sample sets collected from additional *in vivo* studies involving 86 animals subjected or not to experimental administration of corticosteroids, sexual steroids and clenbuterol. Data collected indicate that the indirect screening method based on the targeted quantification of 4 metabolites altered by β_2-agonists administration possesses 100% accuracy to detect clenbuterol abuse.

Introduction

The use of pharmacologically active compounds aimed at improving the animal growth is banned in the European Union (Council directive 96/22/EU, 1996). Currently, surveillance is based on analytical methods combining mass spectrometry and chromatographic separation (Mainero Rocca *et al*., 2017). These conventional methods have been extensively used, but are now being challenged by unknown drug analogues illicitly used as growth promoters in animal production that will not be monitored by existing targeted methods (Li *et al*., 2014). Administration of pharmacologically active compounds can influence the physiology of an organism, thus detection strategies based on the recognition of metabolic changes induced by anabolic practices can be used to strengthen existing targeted monitoring plans. In this context, metabolomics based on mass spectrometry has emerged as a promising technology for quantitative and qualitative analysis of small metabolites in complex biological samples to track physiological actions of anabolic practices. Among active compounds illegally administered to food producing animals, β_2-agonists have been reported to improve carcass composition by increasing growth rate, and reducing fat content. Several reports have described the use of non-targeted metabolomics approaches to screen for the abuse of β_2-agonists in food animals and athletes (Wang *et al*., 2016; Tang *et al*., 2016, Wu *et al*., 2015, Kiss *et al*., 2013, Dervilly-Pinel *et al*., 2018), indicating the feasibility of indirect detection methods.

In this study, we applied liquid chromatography coupled with high-resolution mass spectrometry (LC-HRMS) to study the metabolome of bovine liver after a putative growth-promoting schedule combining an anabolic dosing of clenbuterol with dexamethasone. The overall objective was to assess the ability of metabolomics to detect significant differences between a control group and a treated group of animals in terms of metabolic profile and to build a predictive model able to detect administration of clenbuterol.

Materials and Methods

Animal treatment and sample collection
In the present study, liver samples coming from adult bovines collected in the frame of several controlled experimental trials have been included. Sixteen clinically healthy Charolaise bulls at an average age of 20 months were divided in two groups of 8 animals each, and used as training set for metabolite markers discovery. The first group (n = 8) was used as control, while the second group (n = 8) received a cocktail containing a fixed amount of dexamethasone and an increasing dose of clenbuterol. The trial mimicking a putative anabolic practice lasted for 28 days. After 7 days of drug withdrawal time, animals were slaughtered. The remaining liver samples included in the study as validation set (i.e. 86 animals) came from field conditions or from previous controlled trials in which animals were either non-treated or administered with clenbuerol, or dexamethasone, or trenbolone acetate combined with β-estradiol.
Small portions of the liver were collected from slaughtered animals, immediately frozen in dry ice and stored at -80°C. Eyeballs were collected and kept at -20°C for the majority of animals.
The facilities used in the study, animal welfare, and all the experimental procedures executed agreed with the European regulations regarding the protection of animals used for experimental or other scientific purposes.

Extraction of metabolites and LC-HRMS analysis
Small portions of liver tissue were pulverized in presence of liquid nitrogen and then were freeze-dried. Extraction of metabolites was carried out adding 1.5 mL of methanol/water solution (50/50, v/v) to 50 mg of liver powder. Liver extracts were centrifuged at 12,000×g for 5 min at 4 °C and the supernatant was analysed by LC-HRMS. Profiling of the liver metabolites was performed using a UHPLC system (Ultimate 3000, Thermo Scientific) coupled with a quadrupole-orbitrap mass spectrometer (Q-Exactive, Thermo Scientific), using a reversed phase C_{18} column (Hypersil gold, 100 mm × 2.1 mm, 1.9 µm, Thermo Scientific) or a HILIC column (Zic-HILIC, 100 mm × 2.1 mm, 3.5 µm, Merck). The mass spectrometer was operated in full scan mode at a mass spectral resolution of 70,000 full width at half maximum (FWHM) at 200 *m/z* and the acquired mass range was between 70 and 1000 *m/z*. Quality control (QC) samples prepared by pooling equal amounts of each liver extract were injected at the beginning, the end and every five samples along the analytical run to check LC and HRMS performance during the analytical sessions and to normalize instrumental response.

Statistical analysis and bioinformatics
Acquired LC–HRMS data were analyzed using Compound Discoverer software (version 2.1, Thermo Scientific) to perform alignment of chromatographic peaks, integration of compound peaks and normalization of area values. Only signals exhibiting a CV% values below 10% in QC samples analyzed throughout each analytical sequence were accepted.
Principal component analysis (PCA) was performed using the SIMCA-P software (version 13.0, Umetrics, Sweden) to visualize sample distribution. Data were mean centered and Pareto scaled before analysis. Normal distribution of data was assessed and differences in abundance of liver metabolites were identified using the two-tailed Student's T-test, calculating the ratio T/C by dividing the average normalized area of a given metabolite in treated samples to that of the same metabolite in control samples. Potential markers of treatment with clenbuterol were selected as follows: ratio T/C value < 0.25 or > 4.0 and Student's T-test *p-value* < 0.005.

Quantification of metabolite markers
After identification of markers of treatment and related metabolites, their quantification was achieved using a full scan method combined with parallel reaction monitoring (PRM). A six-point calibration curve was constructed with reference standards dissolved in solvent to quantify markers of treatment by integrating the peak area corresponding to the most intense precursor-to-product ion transition recorded in PRM mode and chosen according to the fragmentation patterns of reference standards.

Classification model development
Concentration values of two liver metabolite markers and two additional related molecules were used to devise a classification model based on partial least square discriminant analysis (PLS-DA) using SIMCA-P software (version 13.0). The resulting statistical model allowed to set up a mathematical equation to classify samples as a function of ratio values of 4 endogenous liver metabolites. We defined a suspicion threshold for this classification model on the basis of 16 animals of the training set. The model was tested on a validation set consisting of 86 animals of different sex and age.

Results and Discussion

Metabolomics analysis

The aim of this study was to reveal significant alteration of metabolic profiles between bulls subjected to an anabolic dosing of clenbuterol or not, by non-targeted metabolomics. Two different animal sets comprising 102 animals were considered in this study: one set of 16 bulls was used for markers discovery, and another set consisting of 86 animals was used for validation purposes.

A non-targeted metabolomics approach based on LC-HRMS was used to compare the liver metabolome of bulls administered with clenbuterol at anabolic dosage to that of control bulls. PCA was applied to highlight sample distribution and grouping. A clear separation between the β_2-agonist treated group and control group was observable in the training set in both negative (Figure 1A) and positive ion modes (Figure 1B) in RPLC, while in HILIC no clear separation between treated and non-treated samples was achieved (data not shown). Such clear separation of non-treated animals from treated bulls indicated that clenbuterol administration was responsible for alterations of liver metabolism.

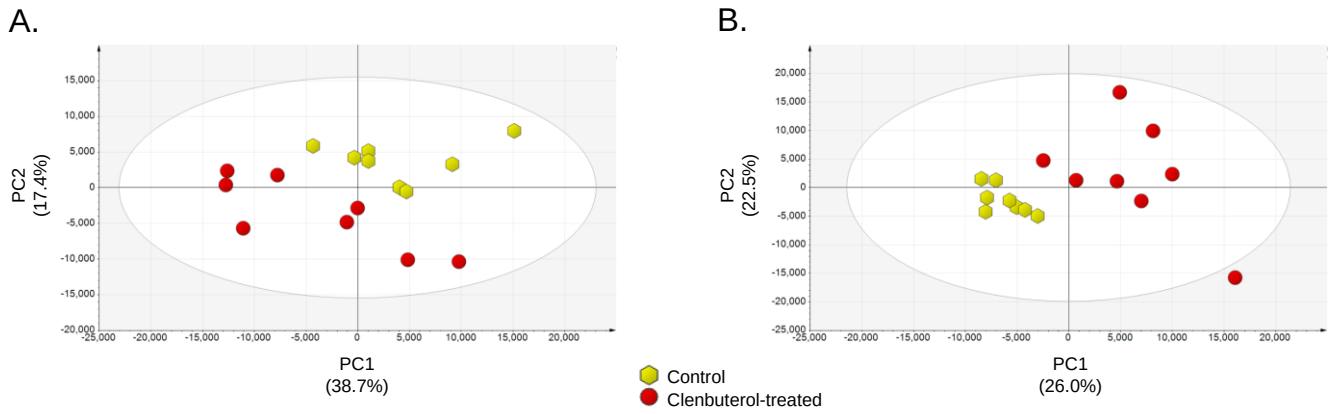

Figure 1. Principal component analysis (PCA) score plots. The unsupervised PCA analysis performed on metabolomics data acquired in negative ionization mode (A.) and in positive ionization mode (B.) show that control animals (blue hexagon) and treated animals (orange circle) are clearly separated attesting for an alteration of the metabolomics profile induced by the anabolic treatment (figure adapted from Stella et al., 2021).

Potential markers of treatment driving the separation between control and treated animals, were selected on the basis of their fold-change and statistical significance resulting from the Student's T-test *p-value*. By applying these two criteria, 15 metabolites were selected, 11 of which were detected in positive ionization mode while the remaining 4 were detected in negative ionization mode. We tried to identify such 15 potential metabolite markers by LC-HRMS/MS. Retention time, deviation from nominal mass (Δ ppm) and fragmentation spectra were used for the annotation. We identified of 2 out of 15 metabolites as nicotinic acid and 5'-deoxy-5'-methylthioadenosine (Figure 2A) by analyzing commercially available standard compound under the same LC-HRMS/MS conditions. The differential abundance of such markers between the two groups of the training set is depicted in box plots to highlight the effect of clenbuterol administration on the hepatic profile (Figure 2B).

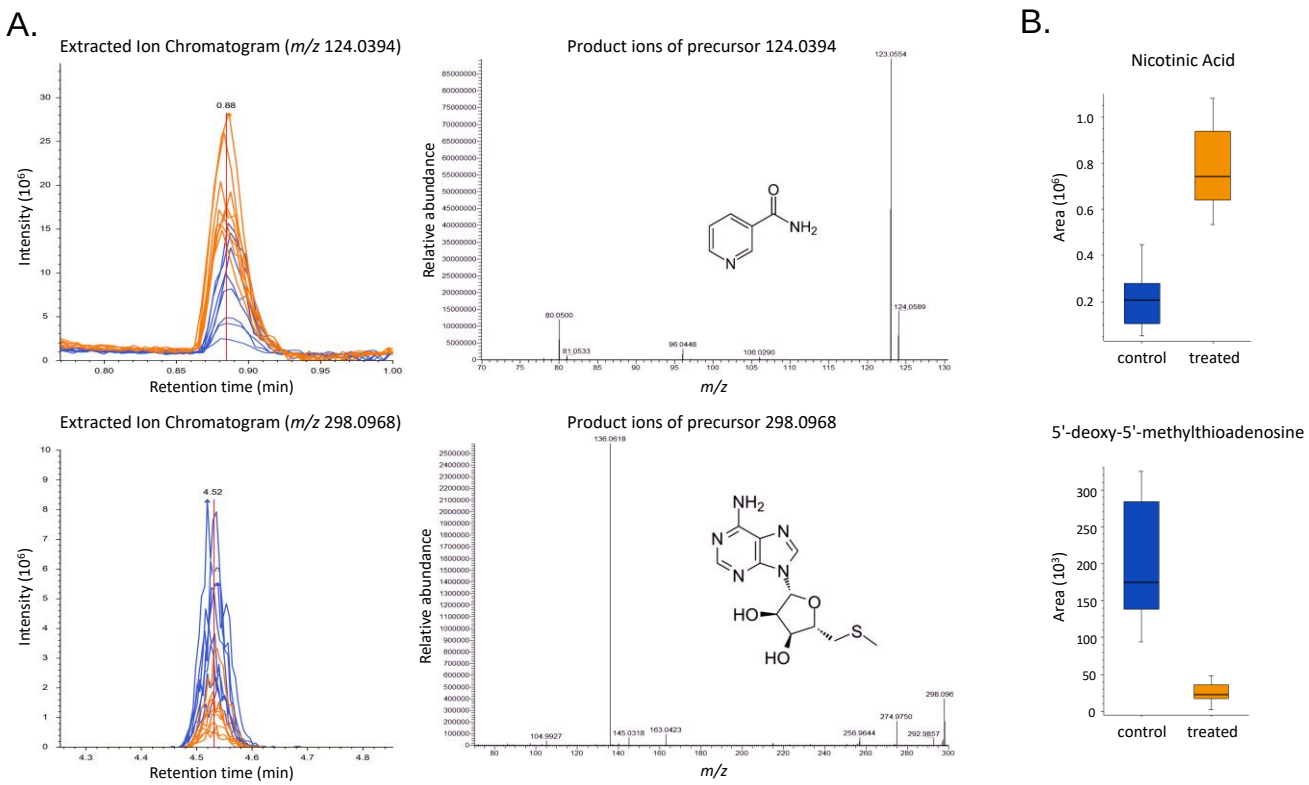

Figure 2. Extracted Ion Chromatograms and fragmentation spectra of identified metabolites. (A) The upper panel shows the extracted ion chromatogram corresponding to nicotinic acid ionized in positive electrospray (theoretical m/z value of 124.03931, experimental m/z value 124.03940, Δ ppm = 0.73) and the corresponding fragmentation spectrum. The lower panel shows the extracted ion chromatogram corresponding to 5'-deoxy-5'-methylthioadenosine in positive electrospray (theoretical m/z value of 298.09684, experimental m/z value 298.09680, Δ ppm = -0.13) and the corresponding fragmentation spectrum. Control samples are depicted in blue, while treated samples are depicted in orange. (B) Box plots summarizing the relative intensity values measured for identified markers of treatment with clenbuterol (figure adapted from Stella et al., 2021).

Such metabolites belong to the nicotinate and nicotinamide metabolism and/or to the cysteine and methionine metabolism. Interestingly, previous studies conducted in food-producing animals dosed with β_2-agonists, reported that nicotinate and nicotinamide metabolism and cysteine and methionine metabolism, were altered in plasma and urine of pigs subjected to an experimental treatment with salbutamol (Wu *et al*., 2015; Li *et al*., 2016). Similar results were achieved in urine from athletes undergoing an anabolic treatment with β_2-agonists (Kiss *et al*., 2013).

Other research groups selected potential urinary markers for diagnosis of β_2-agonist administration in pigs, indicating their practical applicability (Wu *et al*., 2015), or applied statistical analysis to devise a mathematical equation that has been validated and accredited as screening tool for β_2-agonist treatment in bovines on the basis of 3 unknown metabolites (Dervilly-Pinel *et al*., 2015).

In the present study, we found that nicotinic acid and 5'-deoxy-5'-methylthioadenosine were potential markers of clenbuterol administration. However, concentration values of these two metabolites showed a great animal to animal variation. For this reason, we normalized their concentration values to other metabolites of the same altered metabolic pathways (i.e. nicotinamide and methionine) to reduce the biological variation. We then applied a multivariate regression analysis based on partial least square discriminant analysis (PLS-DA) using SIMCA-P software, taking into account ratio values of endogenous metabolites (i.e. nicotinamide/nicotinic acid and methionine/5'-deoxy-5'-methylthioadenosine). Ratio values were used to derive the regression equation and to determine the probability of classifying an animal as treated with β-agonists. Additional details about the classification model can be found in the published version of this research study (Stella *et al*., 2021).

The result of the equation corresponds to the probability (prediction value) for an unknown sample to be regarded as potentially treated or non-treated with β-agonists. The probability (P) values obtained from control animals of the training set were used to calculate a threshold value (i.e. $P < 0.487$) defined as average of such predicted values plus 1.64 times their standard deviation, as established by the guidelines for the validation of screening methods for residues of veterinary medicines. By applying such threshold value to 16

liver samples of the training set, we obtained 100% sensitivity and 100% specificity with no misclassified samples. External validation of the classification model was obtained by calculating probability values for 86 liver samples of the validation set. Calculated probability values for training and validation sets are depicted in a trend chart (Figure 3).

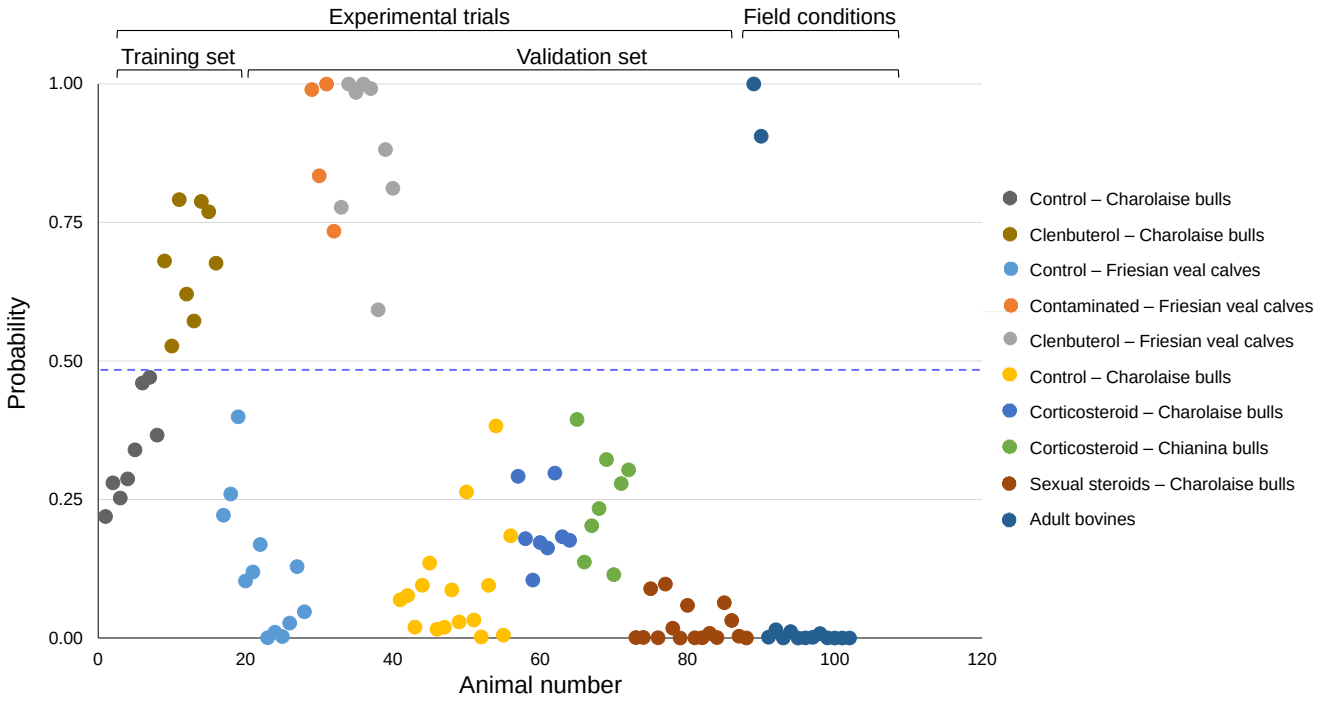

Figure 3. Probability distribution and classification of liver samples of the validation set. Estimated probability of being classified as treated animal, based on the proposed mathematical equation. The blue dashed line corresponds to the calculated cut-off level (figure adapted from Stella et al., 2021).

The classification model correctly assigned all experimentally treated veal calves and two additional incurred samples that were found to be treated with clenbuterol or salbutamol, suggesting that the proposed biomarkers may be specific to different β-agonist drugs. Additionally, 12 untreated veal calves, 16 untreated bulls and 12 additional bovines (2 bulls and 10 cows) certified free of pharmacological treatments and sampled from field conditions were assigned as non-treated. The specificity of the classification model was attested by analyzing samples treated with trenbolone acetate and β-estradiol or dexamethasone that were all correctly classified as non-treated with β-agonists. Such findings proved that selectivity of the method is good enough given that nor corticosteroid (i.e. dexamethasone) or sexual steroids (i.e. trenbolone and β-estradiol) administration affected the profiles of the proposed biomarkers.
However, four untreated veal calves were misclassified as potentially treated with β-agonists. To verify such findings, a thorough investigation was carried out on liver and retina samples from these four animals revealing an unexpected presence of clenbuterol in the retina, which is a tissue known to accumulate β-agonists. On the other hand, liver samples of these four animals showed no clenbuterol residues, making these animals virtually compliant. Taken together, these findings indicate either that the administration of clenbuterol took place before the experimental trial begun, or that a low-dose cross-contamination of control samples occurred during the experimental trial, as reported in similar experiments (Groot, *et al*., 2013). Nonetheless, our findings indicate that the altered metabolomics profile of the proposed liver biomarkers is still evident even when very low amount of β-agonists, well below the CCα of classical detection method, are present in the liver.

Conclusions

In summary, this study demonstrated that the application of LC–HRMS based metabolomics analysis on liver tissue was successful in discovering indirect markers of clenbuterol administration. Metabolomics data were thus used to develop a classification model that proved to be promising to overcome current limitations of classical detection method. This new proposed diagnostic tool based on the targeted detection of four metabolites was validated on a large animal cohort. To ensure sample integrity, liver tissue portions were

immediately frozen after collection and stored at –80 ◦C until analysis. Under these conditions, our results indicate that global performance of the classification model in terms of specificity (100%) and sensitivity (100%) fully falls within required performance for screening method where a false compliant rate below 5% is required, proving to be fit-for-purpose.

Acknowledgements
Authors would like to thank Macelleria De Gaspari, Campodarsego (Padova, Italy) for providing liver samples collected under field conditions. This work was supported by the Italian Ministry of Health as part of project n. RC IZS VE 03/2016 (CUP B22F17000340001) and project n. IZS VE 2008 RF 1157188.
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ORAL O20

Veterinary drugs and feed additives in amphibian breeding ponds: occurrence and risk

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Abstract
Increasing anthropogenic pressure is affecting freshwater ecosystems. Most compounds find their way into the aquatic environment by application in agriculture and mechanisms of run-off. The overall objective of this study was to develop multi-residue methods to assess the contamination of ponds over time, as well as the ecotoxicological risk and correlation to surrounding agriculture. Methods were developed for five groups of contaminants (n=178) and grab samples from 26 ponds situated across Belgium were obtained monthly between March and June 2019. Non-halogenated pesticides, mycotoxins and antiparasitic agents were analyzed using solid phase extraction (SPE) followed by liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). Gas chromatography with electron capture detection was used to detect halogenated pesticides. Antimicrobial drugs were analyzed using SPE followed by UHPLC-high-resolution mass spectrometry. Inductively coupled plasma-optical emission spectrometry was used for measuring heavy metals. Highest concentrations were found for heavy metals and pesticides. Based on reported ecotoxicological endpoints, detected concentrations of cadmium, copper, mercury, cypermethrin, terbuthylazine and zinc posed a risk to *Daphnia magna*. No significant correlation was demonstrated between the detected compounds per pond, and the percentage of surrounding arable land (p-values > 0.05). This research identifies hazardous substances which may be added to the European watch list.

Keywords: agricultural contaminants, freshwater ponds, spatiotemporal screening

ORAL O21

ANTIBIOTIC RESIDUES IN SOIL AND LEEK SAMPLES

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Abstract
In Belgium, especially in Flanders, a high livestock density is coupled to a high antibiotic use in the animal production. Antibiotic residues can enter the environment when using the raw manure of those animals as fertilizer. In this study, 288 soil samples were collected in a longitudinal study and a method was optimized in order to analyze 56 antibiotic residues by UHPLC-MS/MS in the soil samples.
Between March and June 2018, soil samples were taken at 8 different farms; 2 calve farms and 6 pig farms. Each field was sampled 3 times: before fertilization, immediately after fertilization and 2 till 3 weeks after fertilization. The extraction was consecutively performed with acetonitrile containing 0.125 % TFA and a McIlvaine-EDTA buffer at pH 4. A clean-up procedure with tandem SPE (HLB/MCX) was performed. The extracts were injected into the UHPLC-MS/MS system and data processing was performed using Targetlynx XS.
Twenty-four different antibiotic residues from 8 different classes were detected in soil and slurry. Flumequine was detected in each soil sample in concentrations below 100 µg/kg despite being detected in only half of the corresponding slurry samples. Doxycycline, oxytetracycline, lincomycin and sulfadiazine were also frequently detected in the soil and slurry.

Keywords: antibiotic residues, fertilization, oil

ORAL O22

ANTIBIOTIC AND ANTI-PARASITIC DRUGS IN BLACK SOLDIER FLY (*HERMATIA ILLUCENS*) LARVAE PRODUCTION

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Abstract
The full text can be found in Hoek- van den Hil *et al.* (2022).

Insect proteins are expected to be increasingly used for food and feed. Black soldier fly larvae (BSF, *Hermetia illucens*) can convert low quality organic substrates, such as manure, into protein-rich ingredients for food and feed. However, pig and chicken manure can contain residues from antibiotics and anti-parasitic drugs, resulting from treatments of the animals for diseases. This study aimed to evaluate the possible effects of veterinary drugs on black soldier fly larvae rearing, including growth and survival, as well as on the presence of residues in the larvae. The study focused on regularly found veterinary drugs in manure. Five-days old larvae were exposed to either 0.05 and 0.5 mg/kg flubendazole (FLUB), 0.05 and 0.5 mg/kg ivermectin (IVM), 0.5 and 5 mg/kg doxycycline (DOX), 0.5 and 5 mg/kg flumequine (FLUM) or 0.5 and 5 mg/kg sulfadiazine (SULF) for one week. The growth of larvae reared on substrate with IVM (0.5 mg/kg) was significantly lower than the control, while the survival of the larvae was not affected. The growth and survival of the larvae was not affected by the other treatments. Chemical analyses showed that concentrations of the veterinary drugs in the larvae, after exposure, were generally low. Only DOX concentrations in the larvae were high; these levels would exceed the European Commission maximum limit for DOX in meat products. Mass-balance calculations showed possible degradation or metabolism of veterinary drugs by the larvae, except for SULF. In conclusion, when using manure as substrate for BSF rearing, the possible presence of veterinary drugs in manure should be carefully controlled to ensure optimal insect growth and safety of the insect products.

Introduction
Edible insects are gaining more and more interest as they are expected to become an important alternative ingredient for food and feed with minimal use of natural resources (Foley et al., 2011; Godfray et al., 2010; Van Huis et al., 2013; Veldkamp et al., 2012). Edible insect larvae have the capacity to convert low quality organic resources into protein-rich ingredients for food and feed (Fasolin et al., 2019). In particular, the black soldier fly (BSF, *Hermetia illucens*) is capable to grow efficient on low organic waste streams, like plant based side streams or manure (Bosch et al., 2019; Miranda et al., 2019, 2020; Oonincx et al., 2015). BSF have high protein content and a good amino acid composition for feed (Bosch et al., 2014). The environmental impact of BSF reared on residual streams is lower than BSF reared on conventional feed resources (Bosch et al., 2019). Rearing insects on manure will make insect production more economic and competitive towards other animal proteins, and will increase the resource use efficiency and circularity of our food system. However, before bringing BSF to the market for feed and food use, their safety should be investigated. To date, very limited data related to the safety of insects reared on residual materials are available and, therefore, such data should be collected, among which data related to veterinary drugs (Charlton et al., 2015; Fels-Klerx et al., 2018; Meyer et al., 2021). Manure may contain antibiotic and/or anti-parasitic drug residues as a result of treatment of the production animals, like fattening pigs, broilers and laying hens, with these veterinary drugs against animal diseases (Massé, 2014).

In previous research it was shown that 70 to 88% of the manure samples collected in the Netherlands contained antibiotics and/or anti-parasitic drugs (Berendsen et al., 2015; Jansen et al., 2019). These veterinary drug residues present in the manure could be taken up by the insect larvae hereby affecting the safety of the insect products. Also, antibiotic and anti-parasitic drugs may affect insect viability reducing insect growth and survival and thus insect rearing productivity (Gao et al., 2019; Roeder et al., 2010). This study aimed to investigate effects of veterinary drugs on the survival and growth of black soldier flies (*H. illucens*) as well as on the possible transfer of veterinary drug residues from substrates into BSF larvae. To this end, experiments were performed in which

larvae of the black soldier fly were grown on substrate spiked with different antibiotic and anti-parasitic drugs. Insect survival and growth, as well as the presence of the veterinary drugs in the harvested larvae were determined.

Materials and Methods

Materials

Acetonitrile (ACN), ammonium (25%), ammonium acetate, citric acid monohydrate, di-sodium hydrogen phosphate, ethanol (EtOH), ethylenediaminetetraacetic acid (EDTA), formic acid (FA), and methanol (MeOH), and were purchased at Merck (Kenilworth, NJ, USA). Ammonium formate (97%) and dimethyl sulfoxide (DMSO), lead acetate trihydrate, trifluoric acetic acid (TFA), were purchased at Sigma-Aldrich. Bondesil-PSA (40 µM) was purchased at Agilent (Santa Clara, CA, USA).

Doxycycline (DOX, 97%), flubendazole (FLUB, 98%), flumequine (FLUM, 99%), ivermectin (IVM, 97%), selamectin, and sulfadiazine (SULF, 100%), were purchased at Sigma-Aldrich (St. Louis, MO, USA). Aminoflubendazole (FLUB-A, 100%), hydroxyflubendazole (FLUB-H, 100%) and flubendazole-d3 were purchased at Witega (Darmstadt, Germany). Natamycin (98%), sulfadiazine N4 acetyl (97%) and the internal standards doxycycline-d3, sulfadiazine-d4, flumequine-13C3 and ivermectin-d2 were purchased at Toronto Research Chemicals (Toronto, ON, Canada).

Reagents

McIlvain-EDTA buffer was prepared by adding 500 mL 0.1 M citric acid, 280 mL 0.2 M di-sodium hydrogen phosphate and 74.4 g sodium-EDTA to 1 L water into a 2 L volumetric flask. The pH was adjusted to 4.0 using citric acid solution or di-sodium hydrogen solution and the solution was diluted with water up to the mark.

Stock solutions of the reference standards and internal standards were prepared at a concentration at 100 mg/L for flumequine-$13C_3$, FLUB-A, FLUB-H, IVM, flubendazole-d3, ivermectin-d2 and selamectin. The other compounds were prepared at 1000 mg/L and dissolved in MeOH. FLUM and flumequine-$13C3_3$ were dissolved in a solution of 2% 2M ammonium hydroxide in MeOH, FLB was dissolved in DMSO, and FLUB-A and FLUB-H were dissolved in a solution of 5% FA, 45% water and 50% EtOH.

For the addition of the compounds to the insect substrate, solutions of DOX, SULF, and FLUM were prepared at a concentration of 1000 mg/L and solutions of IVM and FLUB were made at a concentration of 100 mg/L. A 2,000 mg/L natamycin solution was made to prevent mould formation in the stability test. For the analysis, a mixed solution of reference standards was prepared at a concentration of 10 mg/L in MeOH. The internal standard solution prepared at 5 mg/L and at 2.5 mg/L for the antibiotic and antiparasitic compounds, respectively.

Insect experiment

Five-day old black soldier fly (*H. illucens*) larvae were reared on the substrate spiked with veterinary drugs for seven days. One hundred larvae (manually counted) were reared per insect breeding dish (100×40 mm with ventilation cap of 40 mm, Novolab NV, Geraardsbergen, Belgium), with three dishes (biological replicates) per treatment. The BSF larvae were exposed to either 0.05 and 0.5 mg/kg FLUB, 0.05 and 0.5 mg/kg IVM, 0.5 and 5 mg/kg DOX, 0.5 and 5 mg/kg flumequine or 0.5 and 5 mg/kg SULF. In addition, breeding dishes containing only the substrate spiked with veterinary drugs were added to the experiment in duplicate to check the stability of the veterinary drugs during the experiment. Natamycin with a final concentration of 40 mg/kg in substrate was added to these additional breeding dishes to prevent fungal growth. The insect breeding dishes were randomly placed in a climate chamber at 28 ˚C, humidity of 70% and a light regime of 12:12 h. After seven days, the insects were removed from the substrate and weighted. The weights of the substrate was also measured at the start and the end of the experiment. All samples, including the harvested insects, substrate prior to the treatment and the residual materials were stored at -20 ˚C for further analyses.

Antibiotic analysis

Antibiotics were analysed with a validated and optimized LC-MS/MS method for antibiotic analyses. Sample preparation was performed according to Jansen *et al.* (2019) with slight modifications. Of each sample, 2 grams was weighted into 50 mL polypropylene tubes (Greiner Bio-One, Alphen aan de Rijn, the Netherlands). Internal

standard solutions were added to each sample. The aliquots were shaken on a vortex mixer and left at room temperature for 20 minutes. Next, 4 mL of a freshly prepared 0.125% TFA in ACN solution was added, shaken thoroughly by hand and 4 mL of McIlvain-EDTA buffer was added. Subsequently, the samples were then shaken head-over-head for 15 minutes followed by the addition of 2 mL lead acetate solution (200 g/L) and centrifuged for 10 minutes at 3500 g. The supernatant was decanted entirely into a 12 mL glass tube, the ACN was evaporated at 40 ˚C under a gentle nitrogen flow. The extracts were diluted by adding 13 mL of 0.2 M EDTA solution before SPE. A reversed-phase polymeric SPE cartridge 200 mg, 6 ml (Strata-X, Phenomenex, Torrance, CA, USA) was subsequently conditioned with 5 ml of MeOH and 5 ml of McIlvain-EDTA buffer. The entire extract was transferred onto the cartridge, which was washed with 5 ml of water and dried by applying vacuum for 1 min. The residues were eluted into a 12 ml glass tube using 5 ml of MeOH which was then evaporated until dry (40 °C, N2). Residues were reconstituted in 100 µl MeOH by vortex mixing and diluted with 400 µl of water. Due to the high concentration of veterinary drugs in the substrate and residual material, evaporation of the eluate was not necessary. The eluate was immediately diluted with water. The final extracts were centrifuged for 10 min at 2,500×g and transferred into UHPLC vials. The samples were analysed immediately or stored at -20 °C and analysed at a later point.

Chemical analysis of the substrate, larvae and residual material samples was performed according to Jansen et al. (2019) with slight modifications. In short, LC-MS/MS analysis was carried out using an AcquityUPLC system, coupled to an AB Sciex Q-trap 6500 mass spectrometer (Sciex Framingham, MA, USA). Chromatographic separation was done using a Kinetex C18 2.1×100 mm 1.7 µm analytical column (Phenomenex, Torrance, CA, USA), placed in a column oven operating at 40 °C. Both liquid chromatography and mass spectrometry settings, including ion transitions, were used as described by Berendsen et al. (2015). The mobile phases used were 2 mM ammonium formate and 0.016% FA in water (Solvent A) and 2 mM ammonium formate and 0.016% FA in MeOH (Solvent B). Operating at a flow rate of 0.3 ml/min, the used gradient was: 0-0.5 min, 1% B, 0.5-5.0 min, a linear increase to 100% B with a final hold of 1.0 min and an equilibration time of 3.5 min. The injection volume was 10 µl or 3 µl for high concentrations. The limit of detection varied from 0.5 µg/kg up to 10 µg/kg depending on the matrix.

Anti-parasitic analysis

Antiparasitic compounds were also analysed with an validated and optimised LC-MS/MS method for antiparasitics. Details about this analysis will be described below. Of each sample 2 g sample was weighed into 50 ml polypropylene tubes (Greiner Bio-One). Internal standard solutions were added. The aliquots were shaken for 5 s on a vortex mixer and then left at room temperature during 20 min. Hereafter 5 ml of ACN was added and samples were shaken thoroughly by hand. For the analyses of the larvae an ultra-torex was used (1 min, 3,300×g) to optimise the extraction recovery. This was not needed for the substrate and residual material samples. The samples were shaken head-over-head (Heidolph REAX-2) during 15 min. Samples were centrifuged (Biofuge Stratos centrifuge) for 10 min at 3,500×g. The supernatant was entirely decanted into a 12 ml tube containing 200 mg ‘primary secondary amine’. The aliquots were shaken head-over-head for 5 minutes and centrifuged (10 min, 3,500×g). The supernatant was entirely decanted into a 12 ml glass tube which was then evaporated until dry (55 °C, N2). Residues were reconstituted in 200 µl 50% MeOH by vortex mixing. The final extracts were transferred to UHPLC vials and analysed immediately or stored at -20 °C and analysed at a later point.

LC-MS/MS analysis was carried out using an Acquity UPLC system, coupled to an AB Sciex Q-trap 6500 mass spectrometer. Chromatographic separation was done using a Acquity UPLC HSS T3 2.1×100 mm 1.8 µm analytical column (Waters, Milford, MA, USA), placed in a column oven operating at 40 °C. The mobile phases used were 2 mM ammonium formate and 0.016% FA in water (Solvent A) and 2 mM ammonium formate and 0.016% FA in MeOH (Solvent B). Operating at a flow rate of 0.4 ml/min, the used gradient was: 0-1.0 min, 5% B, 1.0-6.0 min, a linear increase to 100% B with a final hold of 4.5 min and an equilibration time of 1.5 min. The injection volume was 5 or 2 µl for high concentrations. The limit of detection varied from 0.1 up to 5 µg/kg depending on the matrix.

Results and Discussion

Potential substrates for insect rearing could be contaminated with veterinary drugs. Therefore, the effects on growth and survival of BSF larvae and possible accumulation and uptake of veterinary drugs in the larvae have been studied. The results of this study showed that both the growth and survival rate of the larvae was not affected by the presence of DOX, FLUM, SULF, FLUB, and IVM-0.05 in the substrates. However, the growth rate of the larvae reared on substrate spiked with IVM-0.5 was significantly lower than the control, though the total survival of the larvae was not affected. It was found that the minimum survival rate was 92% for all treatments.

The concentrations of the individual veterinary drugs were analysed in insects and residual material to study the accumulation of veterinary drugs in larvae. For DOX-0.5 and DOX-5 treatments, the mean DOX concentrations in larvae were 203±25 and 1800±58 µg/kg, respectively. Concentrations of these compounds in the larvae were 46 and 37% of the concentration in the substrate. The mean concentration of FLUM found in the larvae reared on FLUM-0.5 and FLUM-5 were 7.2±1.7 and 66±13 µg/kg, respectively (Figure 2B). Concentrations in the larvae were, respectively, 1.5 and 1.3% of the concentrations found in the substrate. SULF concentrations found in larvae reared on SULF-0.5 and SULF-0.05 were 9.4±3.2 and 61±19 µg/kg, respectively (Figure 2C). Concentrations in the larvae were 2.9 and 1.8% of the concentrations found in the substrate, respectively. The metabolite N-acetyl sulfadiazine was not found in the larvae. Mean IVM concentrations found in the larvae after 7 days were approximately 5 µg/kg for rearing on IVM-0.05 and IVM-0.5, but for one treatment related to larvae reared on IVM-0.05 the concentration of the larvae sample was <LOD of 5 µg/kg. The IVM concentrations in the larvae were 18 and 1.3% of the measured concentrations in the substrates at the start of the experiment (Figure 2D). The mean concentrations found in larvae reared on FLUB-0.5 were 2.0±0.34, 0.45±0.03 and 3.8±0.9 µg/kg, respectively, for FLUB, FLUB-OH and FLUB-NH2. Concentrations of FLUB and the FLUB metabolites in larvae reared on FLUB-0.05 were at or below LOD. The concentrations in the larvae reared on FLUB- 0.5 were 0.07, 0.1 and 0.8% of the FLUB concentrations found in the substrate at the start of the experiment, respectively for FLUB, FLUB-OH and FLUB-NH2, the total FLUB concentration (FLUB + FLUB metabolites) in the larvae, reared on FLUB-0.5, is in total 1% of the concentrations in the start substrate. Overall, the concentrations found in the larvae for all tested veterinary drugs were lower than the concentration in the substrate, percentages of concentrations in the larvae relative to the measured start substrates were between 1 and 46%. thus, results showed that there was no accumulation of the tested veterinary drugs in the larvae.

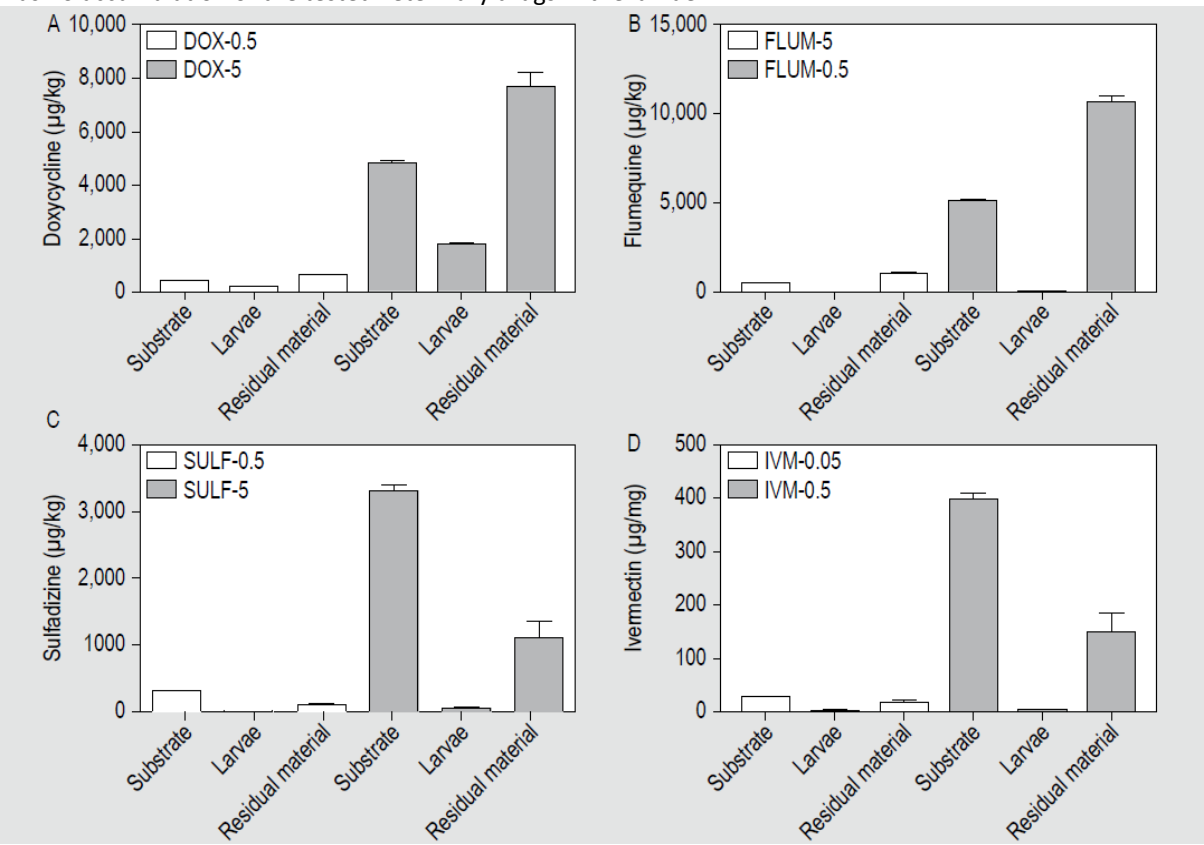

Figure 1: (A) doxycycline (DOX), (B) flumequine (FLUM), (C) sulfadiazine (SULF), and (D) ivermectin (IVM) concentrations in substrate, larvae and residual material during rearing on different substrates with 0.05, 0.5 or 5 mg/kg of the individual

veterinary drugs for 7 days. LOD: 5 µg/kg for DOX, 0.5 µg/kg for FLUM, 2 µg/kg for SULF, and 5 µg/kg for IVM. Results are presented as mean ± SEM.

As no maximum residue limits (MRLs) are available for the presence of veterinary drugs used as feed or food ingredients, the detected compounds were compared with the most relevant MRL for food producing animals (Regulation (EU) 37/2010). The concentration of DOX in the larvae were high (200-1800 µg/kg) and these concentrations exceed the MRL for bovine or poultry muscle of 100 µg/kg. This is concerning since the concentrations spiked to the substrate were in the range of DOX levels regularly found in chicken or pig manure (ref). The most relevant MRLs for comparison were not exceeded for the other studied veterinary drugs. these included the MRL of FLUB of 50 ug/kg for muscle of poultry and porcine, the MRL of FLUM of 200 ug/kg for muscle of bovine, ovine, caprine and porcine, the MRL of IVM of 100 ug/kg for liver and fat of mammalian food producing species, the MRL of the sum of all substances belonging to the sulfonamides of 100 ug/kg for all food producing species (Regulation (EU) No 37/2010).

A mass balance was calculated to investigate how much of the original absolute amount of veterinary drugs spiked into the substrate was found back in the insects and residual material, which was expressed as percentage of the original (measured) amount in the substrate. At the start of the experiment, the amount of substrate was 58 g per treatment while the residual material was between 8.5-11 g for all treatments, except for IVM-0.5 for which it was 23 g. The mass balance showed that – for all veterinary drugs – <40% of the initial amount of added veterinary drugs was found back, as shown in the grey columns in figure 2. Part of the missing amounts could be due to natural breakdown of the compounds in this experimental set-up, which was also observed in the stability experiment without larvae. The percentage of natural breakdown of the compounds is shown in the white columns in figure 2. However, the mass balance of sulfadiazine is almost 100% when taking the natural breakdown of this compound into account, which implies that BSF larvae did not metabolize or degrade sulfadiazine in this study. This is confirmed by the fact that the metabolite N4-acetyl sulfadiazine was not found in neither the larvae or the substrate. FLUB was partially metabolized to FLUB-OH and FLUB-NH_2 by the larvae. However, only up to 50% was found when taking into account the formed metabolites and their stability in the mass balance calculations. Therefore, it is recommended to study possible formed breakdown products and to investigate if these breakdown products are still biologically active.

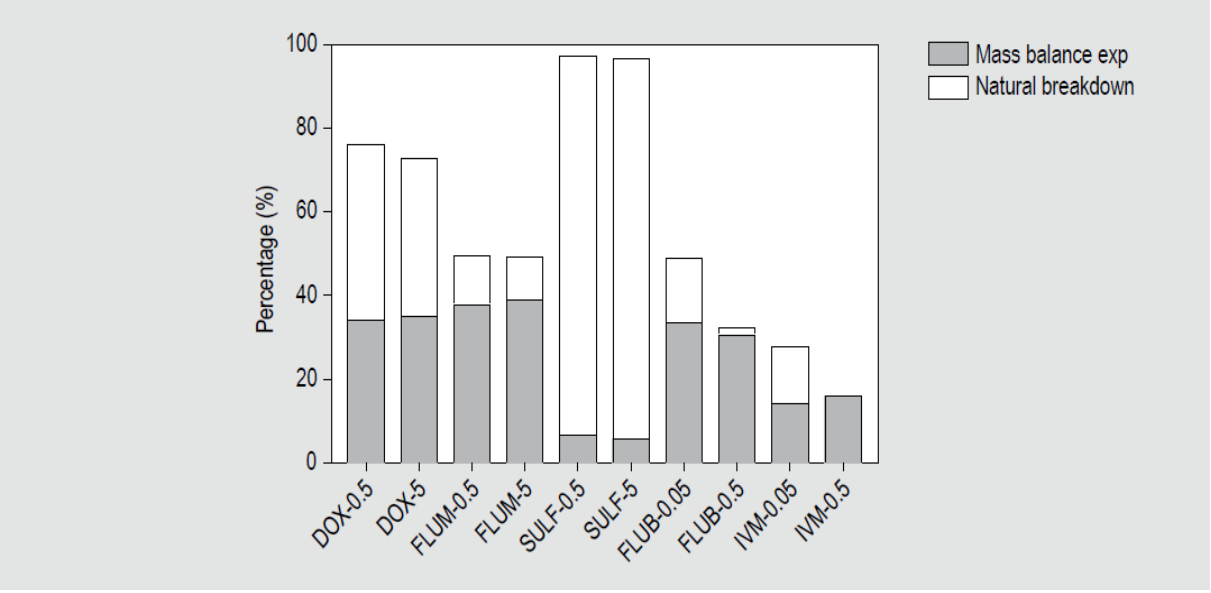

Figure 2: Mean mass balance of the amount of veterinary drugs found back in the insects and residual materials compared to the original amount of veterinary drugs measured in the substrate in percentages (grey columns). Mean percentage of natural breakdown products during the experimental set-up of veterinary drugs in the substrates is shown in the white columns.

Conclusion

In conclusion, this study found high DOX concentrations in the larvae, while concentrations of other investigated veterinary drugs in the larvae were relatively low. It is possible that breakdown products are formed which could

end up in the larvae, these breakdown products could be bioactive. Therefore, possible breakdown of veterinary drugs by insect larvae should be further studied. Results of this study are promising since veterinary drugs residues that may be present in manure did not affect the survival of BSF larvae reared on the spiked substrates. Growth of the larvae was only decreased for the IVM-0.5 treatment, however, this concentration is about 10× the concentration regularly found in manure pits. Other veterinary drugs studied did not show a decrease in larval growth. Overall, this study showed that the presence of veterinary drugs in manure should be determined prior to selecting manure types, which could possibly be used as substrate for insect rearing, so as to ensure optimal insect growth and safety of insect products.

Acknowledgements
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ORAL O23

FATTYACIDOMICS TO TRACE RBST ADMINISTRATION IN DAIRY CATTLE: STRENGTHS AND LIMITATIONS

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Abstract
Somatotropin is a species-specific polypeptide hormone produced in the pituitary gland of vertebrates. When administered exogenously to dairy cattle it has galactopoietic effects and is capable of increasing the milk yield. The commercial production of recombinant bovine somatotropin (rbST) had a major economic impact in the dairy industry. The trade and administration of rbST to farm animals is banned in the European Union (EU) since 1999, invoking animal welfare reasons, but also the impact on the European milk policy and consumer fears. Confirmation of rbST (ab)use is not always easy, since it requires tedious sample preparation and very low detection limits. Also, the use of natural-like variants, complicate the scenario. Alternative methods are always welcome, in particular to screen large number of samples. Hence, a targeted omics profiling was assessed as a potential screening strategy for rbST (ab)use in cattle. A wide profile of fatty acids (*fattyacidome*) was determined by GC-FID in red blood cell and white blood cell membranes, collected from control and rbST-treated lactating cows at different time points after treatment. Milk collected from the same animals was also profiled. Appropriate multivariate and univariate statistical analyses were used to confirm the existence of lipid disturbance upon treatment.

Keyword 1: fatty acids, milk, rbST

ORAL O24

THE POTENTIAL AND DRAWBACKS OF (SMART-)SAMPLING APPROACHES FOR RESIDUE TESTING

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Abstract

Competent authorities mainly focus on monitoring products of animal origin for prohibited substances and MRL violations. They are aiming for efficient yet effective approaches for monitoring and enforcement. To achieve this, the whole chain of the sample taking process and the laboratory procedures are taken into account. Technical developments offer new opportunities for innovative and efficient control. Another driver for new approaches is the implementation of regulation (EU) 2017/625, stating that part of the official control should be performed risk-based. Furthermore, enforcement of specific (national) policies requires new approaches, like ante-mortem antibiotic testing based on pig saliva and the use of swab samples and feathers/hair to study the correct registration of antibiotic use.

Traditionally, for the detection of prohibited substances, mainly urine and hair are analysed. Food products of animal origin are used for MRL enforcement. Recently, smart sampling approaches and 'new' matrices are being developed, used and/or evaluated, including but not limited to saliva, feathers, manure, blood (or dried blood spots), various types of swab samples and coated blade spray. In this presentation, the benefits and drawbacks of these (smart-)sampling approaches and their applicability in residue testing will be discussed from a physico-chemical (e.g. persistence and contamination), analytical (applicability in the laboratory) and practical (sample taking) perspective.

Introduction

With current measurement methods, analysis can be performed on a wide variety of samples taken from animals during the primary or slaughter phase. In the slaughter phase, besides the time pressure, sample taking is relatively easy and a variety of samples can be taken ante- or post-mortem. In the primary production phase, sample taking options are more limited as they cannot be invasive and animals are not static objects.

Traditionally, urine samples are taken to analyze prohibited substances in the primary production phase, and for two decades, also, hair sampling and analysis are routinely applied for enforcement purposes. Even though, monitoring and enforcement based on such samples are effective, the analysis strategy, including the sampling, is not very efficient. For urine, an inspector needs to wait for an animal to urinate. For hair analysis, an animal needs to be fixed (two inspectors are required to do so) and hairs have to be shaven with an instrument which introduces the risk of contamination between samples.

For allowed veterinary drugs, the traditional approaches require a quantitative result as, for enforcement, the result is compared to a maximum residue limit. As these maximum limits are merely established for edible products, the sampling was limited to the slaughter phase except for milk and eggs. However, nowadays, new research questions allow for qualitative or semi-quantitative results. These research questions include: what antibiotics have been administered by a farmer and are these correctly registered in the administration? Are Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) administered to mask symptoms of infections or the animal's unfitness for transportation? To answer these questions, the traditional matrices might not be the most effective nor efficient.

Here, we present an assessment of animal matrices for residue analysis in the perspective of the whole analysis strategy, focusing on the primary production phase. This strategy does include not only laboratory practices but also sampling, transport and storage. 'New' matrices were evaluated, including but not limited to saliva, feathers, manure, blood and various types of swab samples. Furthermore, innovation and miniaturization of analytical methods, has significantly reduced the amount of sample material required and have opened the door to the implementation of new sampling and analysis strategies. An example is dried blood spot sampling,

in which a small drop of blood is placed on a card. Another is the use of coated blade spray. In both approaches, the sample can be sent to the laboratory (sometimes unconditioned) where the analysis takes place automatically. This approach makes the entire chain of sampling, transport, analysis and storage more efficient. We call this smart sampling. The benefits and drawbacks of these (smart-)sampling approaches and their applicability in residue testing are discussed from a physico-chemical (e.g. persistence and contamination), analytical (applicability in the laboratory) and practical (sample taking) perspective.

Smart-sampling

Currently, sample taking for veterinary drug residue analysis during the primary production phase is labour intensive and time consuming. For urine, an inspector needs to wait for an animal to urinate. For hair analysis, an animal needs to be fixed (two inspectors are required to do so) and hairs have to be shaven with an instrument which introduces the risk of contamination between samples. Also, the analysis of such samples is not easily automated in the laboratory. There is a clear need for next-generation samples that are easy to obtain and that can be efficiently yet effectively analysed in the laboratory. We call such samples: smart samples. Among the smart samples are: swab samples, blood sampling devices, liquid spot analysis, and coated blade spray.

In controlled animal studies, the value of swab samples has been demonstrated. A moist disposable cloth is wiped over the back (or any other part of the animal) and put into a plastic container. The sample is completely extracted for analysis in the lab. The samples are very easy to take in practice and they are relatively simple to analyse. This approach was proven successful for both instrumental analysis, as well as for biosensors including lateral flow devices and multi-plex binding-assays.

Blood sampling is of high interest as blood is a matrix that is not directly in contact with the animal's surrounding. Blood sampling is traditionally labour intensive and not animal-friendly. A capillary is used to prick a vain, e.g. in the ear of the animal, and some blood can be collected in a tube subsequently. Devices, developed for blood pricking in humans (e.g. for diabetes testing) have been developed. The TAP Blood Collection Device (Seventh Sense Biosystems Inc, Medford, MA, USA) was tested in controlled animal studies. The device is positioned on the back of the animal and it is supposed to remain in place by a glue layer that is attached to the device's bottom. By pushing a button, microneedles penetrate the skin and in five minutes approximately 200 µL of blood is collected inside the device. The device is uniquely labelled with a code and barcode and the sample can be shipped, unconditioned to the lab. There, the device can be opened and a drop of blood can be transferred to a dried blood spot card to allow fully automatic analysis. We successfully applied this device on calves and horses, but it is applicable only after shaving to ensure the device sticks to the skin. Currently, we conclude that this device is not applicable for routine sampling in the primary production phase, but further development of this technique could be promising. Automated dried blood spot analysis was developed in our lab and is currently being implemented for routine analysis of blood samples taken at the slaughterhouse.

A coated blade is a sharp metal device of approximately 5 cm long to which a coating has been applied. The blade is kept in a liquid sample or pressed into a solid sample (e.g. tissue or specifically an injection site). Substances are directly extracted from the sample by adsorption to the blade. The metal device can then be sent to the laboratory for further analysis. The analysis of a blade requires little further pre-treatment. Substances can first be eluted from the coating by an organic solvent, but the substances can also be analysed without any further sample preparation. The coated blade can be placed in an interface, directly in front of the mass spectrometer. Adsorbed substances on the blade can be released by putting a solvent and current on the blade and directly be transferred to the mass spectrometric source for detection.

Controlled animal studies

The application of saliva, blood and swab samples was evaluated is several animal studies.

Hair and feces in bovine

Four calves were treated with oxytetracycline (20 mg/kg body weight) two times a day through milk, during 5 days at an age of 5 weeks and another four at the age of 13 weeks. As an infection occurred in one animal, a treatment with sulphadoxin was carried out at 17 weeks of age. Samples taken from this calf after its treatment were additionally analysed for sulphadoxin. Of the group treated at 5 weeks of age, rectal feces samples were taken daily during treatment, five consecutive days after the last treatment and then at a lower frequency until 20 weeks after start of the treatment. A similar sampling plan was adopted for the older animals, but it only continued until 13 weeks after start of the treatment. Hair of the younger group was

sampled after 2, 6, 9, 13, 17, 21 and 26 weeks after start of treatment. Of the older group, this was at 2, 6, 10 and 14 weeks after start of the treatment. Samples were stored in the freezer until analysis. Feces was homogenized by stirring and hair was grinded before analysis.

Saliva in porcine
Pigs of 26 days old were treated with oxytetracycline or sulphamethoxazole, both by oral administration (2 x n=4) or intramuscular injection (2 x n=4). The pigs were treated for 5 consecutive days. Saliva samples were taken daily during treatment and during the five days after the last day of treatment using a chewing rope. After that, samples were taken weekly.

Urine, feces and swab samples in porcine
Four pigs were treated with sulphadimidine and enrofloxacin, both by oral administration in which an exact amount of the drug was mixed with propylene glycol and administered directly in the pigs' mouth. The pigs were treated for 5 consecutive days. Urine, feces and swipe samples were taken before treatment and 1, 4, 7, 10, 14 and 17 day after start of treatment. Rectal feces and urine samples were taken. Swab samples were taken by wiping a moist ethanolic disposable cloth over the pigs' back.

Feces, urine, hair, swab samples, blood and saliva in bovine
Two calves were orally treated with oxytetracycline during 5 days (2 doses per day). Samples were taken 1, 4, 8, 10, 14, 21, 28, 35 and 42 days after start of treatment. At each time point, urine, feces (rectal samples), saliva, hair, blood and hair were sampled. Also swab samples of the animals were taken by swapping a moist disposable cloth over the animal's back.

Results

Hair and feces in bovine
The determined concentration of oxytetracycline in the feces and hair samples is graphically presented in figure 1. In the oxytetracycline treated calves, oxytetracycline was detectable in feces until 80 – 100 days after the last day of treatment. In the end of the excretion phase the oxytetracycline concentration seems to drop very slow. This could be a result of re-absorption of oxytetracycline that is adsorbed to bones. In hair a similar detection window was observed: residues were still detectable 60 - 80 days after end of treatment. Therefore, for detection of oxytetracycline, both hair and feces could be of interest from a physiological perspective. For sulphadoxin, even though only limited data points are available, a clear difference is observed between detectability in feces and hair. Sulphadoxin is not detectable in feces, approximately 4 weeks after the end of treatment. In hair, the excretion is much slower. After 9 weeks, the sulphadoxin concentration in hair has only dropped by 65% from the highest concentration measured. Extrapolation indicates that it is still detectable 200 days after end of treatment. Here, hair is clearly favourable over feces.

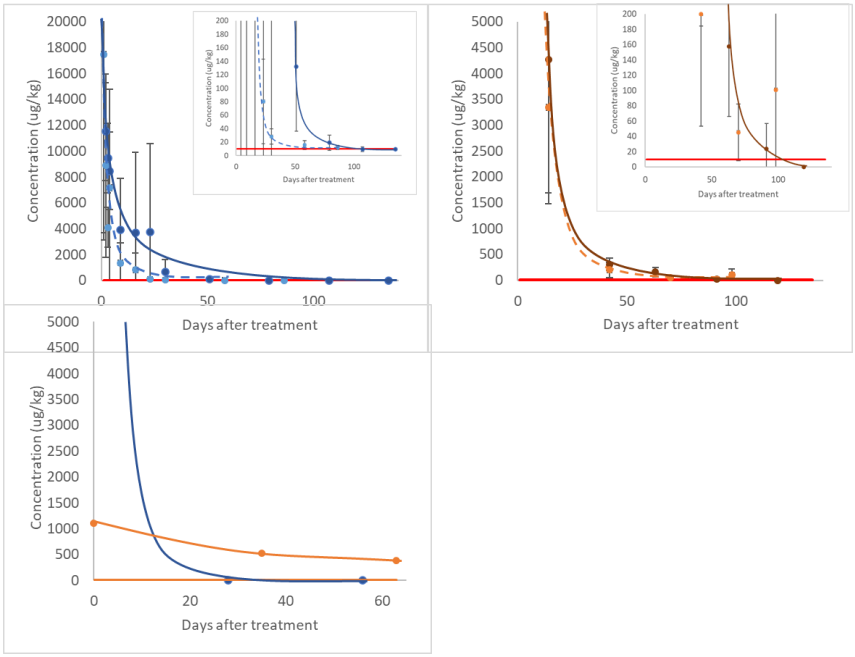

Figure 1. Concentration (µg/kg) of oxytetracycline (left and middle panels) and sulphadoxin (right panel) in feces (blue) and hair (orange) after the end of the treatment. The calves were treated (bold lines) at 5 and (dashed) 17 weeks of age for oxytetracycline. Sulphadoxin treatment occurred at 17 weeks age.

Saliva in porcine

In all orally treated pigs, oxytetracycline was detectable until approximately 2 weeks after end of the treatment. For intramuscular treatment, this was about 5 days after end of treatment. Trimethoprim was detectable in all pigs until 3 weeks after end of oral administration and 2 weeks after the latest injection. Sulphamethoxazole showed a longer detection window: it was detectable in all animals until 6 weeks for oral and 4 weeks for intramuscular administration. Surprisingly, residue concentrations in saliva after oral and intramuscular treatment were in the same range during treatment and shortly thereafter. A representative example is presented in figure 2 for sulphamethoxazole. Clearly, the antibiotics are quickly distributed throughout the body after intramuscular injection. After approximately 3 weeks, the residue concentration in saliva of the injected pigs clearly decreases, whereas the residue concentration in the orally treated pigs remains fluctuating.

An important observation and a clear difference compared to excretion via feces or hair is that the concentration in saliva strongly fluctuates in time; both during and after treatment. The logical explanation is that the residue concentration in saliva depends on the time of eating and drinking of the animal. Also large differences were observed among animals.

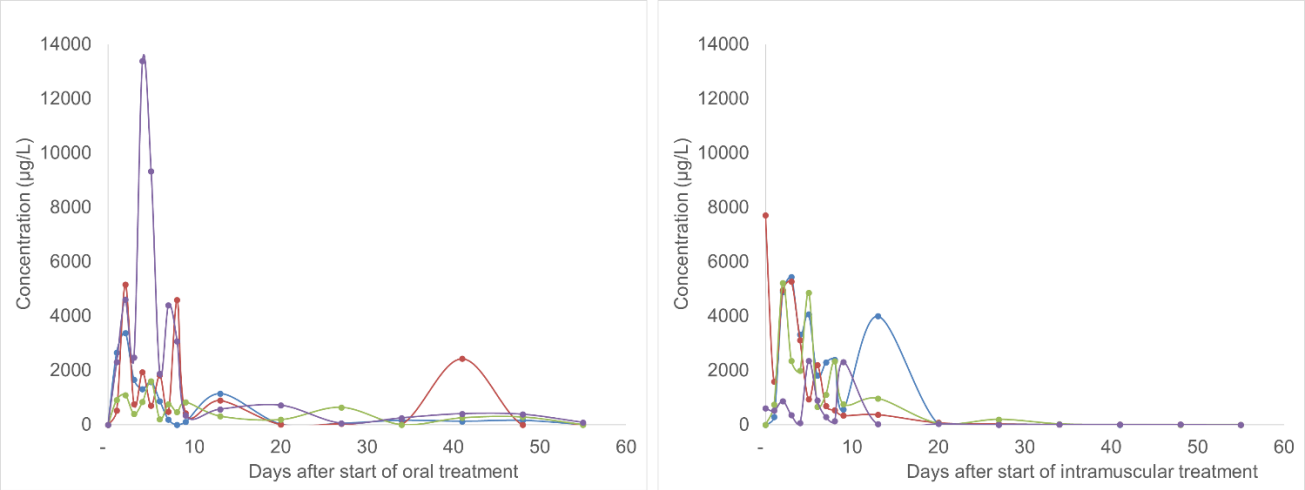

Figure 2. Concentration (µg/kg) of sulphamethoxazole in saliva of four individual pigs after (left) oral treatment and (right) intramuscular injection during treatment and several weeks thereafter.

Urine, feces and swabs of porcine

The determined concentration of sulphadimidine and enrofloxacin in feces, urine and swab samples is graphically presented in figure 3. Sulphadimidine was detectable in all matrices until the latest sampling time point. Concentrations in urine and feces were in the same order. At the end of the study, residues were best detected in the swab samples, also considering that the limit of detection for swab sample analysis is lower compared to urine and feces. This is a direct result of urine and feces being very complex sample materials.

Enrofloxacin shows a different excretion pattern (figure 3). Enrofloxacin is mainly excreted through feces and to much less extent in urine. A few days after the end of treatment, enrofloxacin is not anymore detectable in urine. In feces it is detected until approximately 9 days after end of treatment. In the swab samples, enrofloxacin is detectable the longest: until the last sampling time point at 12 days after end of treatment.

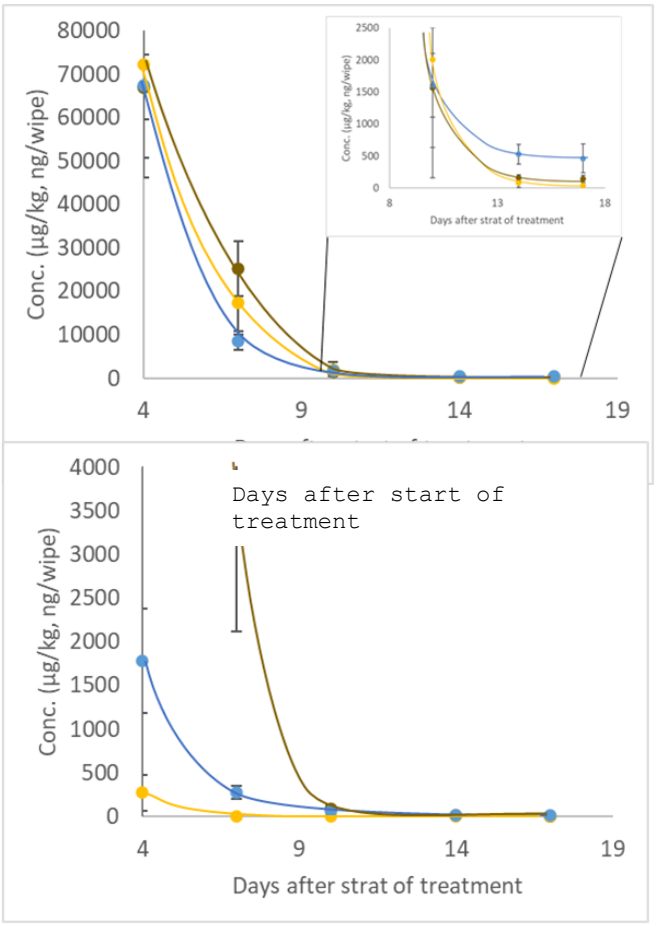

Figure 3. Concentration (µg/kg, urine and feces; ng/wipe, swab samples)) of sulphadimidine (left panel) and enrofloxacin (right panel) in urine (yellow), feces (brown) and swab samples (blue) of treated pigs (n=4) during the excretion phase. Error bars indicate the standard deviation.

Feces, urine, hair, swab samples, blood and saliva in bovine

The determined concentration of oxytetracycline in the samples is graphically presented in figure 4. Oxytetracycline was detected in all samples up until the last sampling time point, 42 days after start of treatment, except for in the saliva and blood samples. In saliva, oxytetracycline was latest detected in the samples taken on day 14, 9 days after end of treatment. The same is true for the blood sample of one of the animals. It seems like the concentration of oxytetracycline in most matrices does not go towards 0. This might be explained by long term re-absorption of oxytetracycline from the animals' body.

Manure contained the highest concentration of oxytetracycline (160 mg/kg during treatment) up until approximately 1 week after end of treatment. This is related to the relatively poor absorption of the drug in the animals gut. From approximately a week after end of treatment, the highest concentrations are found in hair samples even though after 1 month, the difference is not anymore significant. Also, urine has relatively high concentrations (7 mg/kg during treatment), but the concentration rapidly decreases over time. The concentrations in blood (250 µg/kg during treatment) and saliva (40 µg/kg during treatment) are much lower.

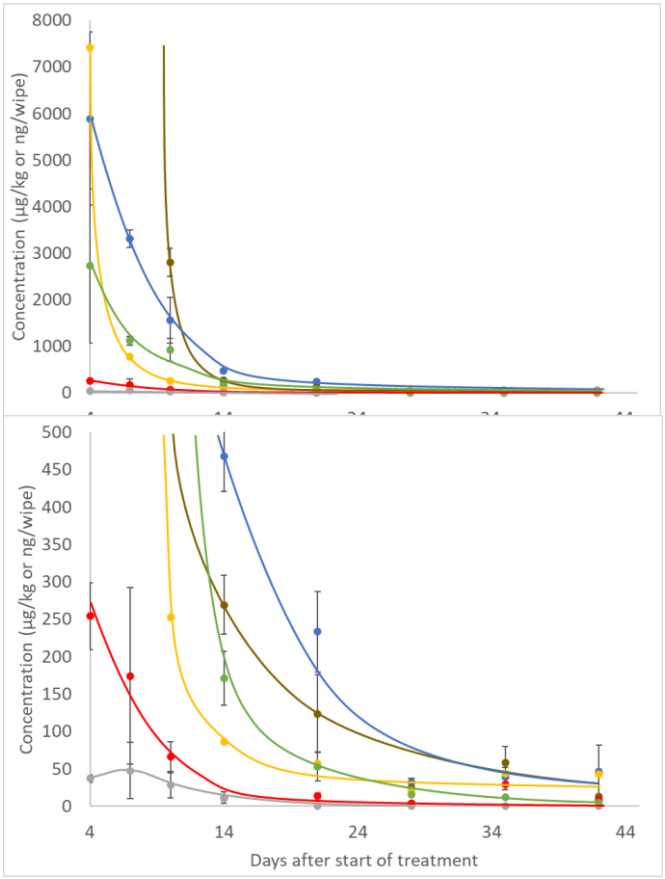

Figure 4. Concentration (µg/kg, urine, feces, hair, blood, saliva); ng/wipe, swab samples)) of oxytetracycline in urine (yellow), feces (brown), hair (blue), saliva (grey), blood (red) and swab samples (green) of treated calves (n=2) during the excretion phase. Error bars indicate the standard deviation. Both panels present the same data, but y-axis is different.

Discussion

The data obtained from controlled animal studies demonstrates that the excretion and translocation can differ from one veterinary drug to the other. Only a limited number of veterinary drugs have been included in the assessment of the use of alternative matrices. In general, some observations are done:

- During and shortly after treatment, concentrations in feces are highest. Concentrations depend on many factors, including the drug administered, the dose, the treatment, the animal's age, and to what extent the drug is adsorbed in the animal's gut.
- Excretion through urine and feces is generally quick. Residues are, in some cases, detectable for a long time due to the high initial concentration or through re-absorption from the body. In general, hair concentrations are higher than other matrices long after treatment. Therefore, hair is a very effective matrix for detecting veterinary drug applications. Unfortunately, the sample collection of hair is laborious and the analysis (especially if a quantitative result is required) is an extensive process.
- Residues are detectable in saliva, both after oral and intra-muscular administration. Sulphamethoxazole was detectable up until 6 weeks after the end of treatment. Concentrations in saliva vary depending on the time of sampling. For oxytetracycline, concentrations in saliva were much lower and were not detectable after approximately a week after the end of treatment. Collection of saliva seems simple in pigs and young bovines. For older bovines, the sample collection is more complicated. Also, in some cases, the sample volume obtained is limited.
- The results of swab samples cannot be directly compared to residue concentrations in other matrices, as the swab analysis result is expressed as an absolute amount of the drug per wipe. Nonetheless, residues are detectable in swab samples and the detection window is promising. Here it should be taken into account that swab sample analysis usually shows a relatively low limit of detection compared to analysis of more complex matrices. Sample collection is very easy and analysis is relatively simple as samples are cleaner and more uniform compared to e.g. urine and feces.

Conclusion

Little has changed in how samples were collected in the past decades. In the primary production phase, monitoring and inspection of veterinary drugs heavily relied on urine and hair samples. Sample taking is a labour-intensive process that hampers the number of samples collected over a specific time. Efficient monitoring and inspection not only require efficient yet effective sample collection, but can also benefit from innovative laboratory procedures including full automation. Innovations and scientific knowledge open up new possibilities for sampling and analysis. These include the use of swab samples, liquid spots and coated blades. Evidence is available that swab samples are a great addition or replacement for urine and hair samples: sample collection is easy and quick and analysis is straightforward. The use of dried blood spots and coated blade spray in the primary production stage is currently in its infancy. Good results have been obtained from samples obtained at the slaughterhouse. Further development, testing, and optimisation of these strategies are foreseen for the upcoming years. However, smart-sampling techniques will change how we currently perform monitoring and inspection and aid the transition towards a more risk-based approach.

ORAL O25

DEVELOPMENT OF AN INTEGRATED APPROACH FOR THE DIAGNOSIS OF WATER QUALITY IN THE RIVER MEUSE (DIADEM PROJECT)

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Abstract

The DIADeM (Development of an Integrated Approach for the Diagnosis of Water Quality in the Meuse) project developed an interdisciplinary, cross-border approach in order to improve the diagnosis and monitoring of the chemical quality of the water in the Meuse river and two affluents. The project proposed bringing together chemical and biological analyses (biomarkers) carried out on enclosed organisms of species representative of cross-border hydrosystems and mathematical models to predict the effect at a population level.

Wihin the project, CER Groupe had assessed contamination level of water based on bioaccumulation analysis in the tissues of the target organisms on the basis of a well-controlled UHPLC-MS /MS methodology.

These analyzes were carried out, on one hand, on the organisms exposed in aquarium, in artificial rivers and, on the other hand, on organisms caged on the various study sites.

An important objective was to compare the 5 species, to confirm the accumulation capacity of these molecules within the biota and to identify, the species or the most appropriate species to assess the contamination of water.

The progress made by the DIADeM project will help protect the environment through the integrated management of cross-border resources.

Introduction

In the last decades, exposure to pharmaceutical contaminants in the environment have been recognized as an emerging health risk due to their intrinsic biological effects in non-target species and also contributes to the development of antimicrobial resistance.

Therefore, it is important to assess the exposition of aquatic organism to these contaminants.

As part of the Interreg DIADeM program ("Development of an integrated approach for the diagnosis of the water quality of the River Meuse"), a global study was conducted to establish the proof of concept that the use of a multispecies active biomonitoring approach improves diagnostic of aquatic systems. The complementarity of the biomarker responses was tested in five model species including : the bryophyte *Fontinalis antipyretica* (moss), the bivalve *Dreissena polymorpha* (freshwater mussel), the amphipod *Gammarus fossarum* (freshwater shrimp) and the fish *Gasterosteus aculeatus* and *Oncorhynchus mykiss* (rainbow trout). The study was carried out at 3 scales for certains species : in laboratory conditions in aquarium, in artificial river mesocosms and in the Meuse river upstream and downstream from five wastewater treatment plants.

Among contaminants, five molecules being acetaminophen, carbamazepine, diclofenac, irbesartan and naproxen were selected amongst the most abundant contaminants present in surface waters along the Meuse River in Belgium between February 2015 and July 2016 (Imhotep 2013–2017) (Nott et al., 2018). The target

organisms were then artificially exposed to three different concentrations, including 1-time, 10-times and 100-times the environmental concentration observed in the Meuse river.
After the exposure, concentrations of each analyte including their main metabolites were determined.
One of the aim of this study was to check the ability of several aquatic species to accumulate 5 selected contaminants. These data were then used to corroborate sensitivity of the species to the exposure of pharmaceutical contaminants trough the analysis of several biomarkers.

Materials and Methods

Rearing experiment
Selected organisms were exposed to the 5 contaminants at 0 (Control), 1, 10 or 100-times environmental concentration in aquarium or in mesocosm. More information on the aquarium experiments can be found in Schmitz et al., 2018 and mesocosm experiments were depicted in detail in David et al., 2020 and Alaoui et al., 2021.
For both experiments, concentrations of paracetamol, irbesartan, carbamazepine, naproxen and diclofenac in water were set at level described in table 1. *Fontinalis antipyretica*, *Dreissena polymorpha* and *Gasterosteus aculeatus* and *Oncorhynchus mykiss* (rainbow trout) were assayed in aquarium during up to 6 weeks and *Fontinalis antipyretica*, *Dreissena polymorpha*, *Gammarus fossarum* and *Gasterosteus aculeatus* were also exposed to the xenobiotics in mesocosms for 23 weeks.

Table 1 : Concentration (ng l^{-1}) of paracetamol, irbesartan, carbamazepine, naproxen and diclofenac in water for aquarium and mesocosm studies

		Concentration (ng l^{-1})				
		Carbamazepine	Diclofenac	Irbesartan	Naproxen	Paracetamol
Control	0	0	0	0	0	0
Condition A	1x	25	25	50	25	100
Condition B	10x	250	250	500	250	1000
Condition C	100x	2500	2500	5000	2500	10000

All species have been also caged upstream and downstream from five wastewater treatment plants in the Meuse watershed as described by Hani et al. (2021) and Catteau et al. (2022). Experiments were conducted at least for 3 weeks and concentration of 45 pharmaceuticals were monitored – including the five target contaminants (Catteau et al., 2022).

Standards
4-hydroxy diclofenac, 5-hydroxy diclofenac, irbesartan, irbesartan-d4 (internal standard, IS), desmethyl naproxen, and naproxen were purchased from LGC Standards (Molsheim, Germany). Carbamazepine, carbamazepine-d8 (IS) and naproxen-d3(IS) were supplied by Sigma-Aldrich (Bornem, Belgium). carbamazepine 10,11-epoxide, diclofenac-d4 (IS), naproxen Acyl-B-D-glucuronide, diclofenac Acyl-B-D-glucuronide, and irbesartan-glucuronide were from TRC (Toronto, Canada). Individual standard stock solutions of compounds were prepared at 1 mg ml^{-1} in methanol. Mixed working standard solutions were prepared by diluting the stock solutions with methanol and contained all the analytes at 1 µg ml^{-1}. All stock and working solutions were stored for one year at -18°C.

Sample preparation
Organisms were first rinse well with distilled water and dry with paper towel. Then, a homogenised sample (5 g - 2 g for moss and mussel) was spiked with 50 µl of the internal solution and the mixture was allowed to stand for 15 min. Twenty ml of acetonitrile was used to extract drug residues and precipitate proteins. The sample was then thoroughly shaken at room temperature for 15 min and centrifuged at 4650 *g* for 5 min. The extract was collected, evapored under a stream of N_2 at 50°C. After reconstitution with 1 ml of 2 ml of a mixture of methanol/water (20/80 ; v/v), the extract was loaded on an Oasis HLB cartridge (preconditioned with 3 ml of methanol and 3 ml of water) under vacuum to obtain a flow rate of about 1 ml min^{-1}). The cartridges were then rinsed with 3 ml ultra-pure water and

Liquid chromatography/mass spectrometry analysis

Analyses were performed using an Acquity UHPLC system (Waters, Milford, MA, USA) and chromatographic separations were done by injecting 20 µl of sample on an Acquity UPLC HSS T3 column (150 × 2.1 mm, 1.7 µm particle size, Waters). The mobile phases consisted of water with 0.05% formic acid (A) and ACN with 0.05% formic acid in (B). The linear gradient used was as follows: 0-1.0 min: 0% B, 1.0-6.0 min: increase to 100% B, 6.0-7.0 min: 100% B; 7.1-8.0 min: 0% B) at a flow rate of 0.5 ml min^{-1}. The column and autosampler were set, respectively, at 50°C and 15°C. Detection was carried out with a Waters Acquity TQ mass spectrometer (Waters, Manchester, UK) equipped with an electrospray ionisation source operating in the positive (ESI+) ionisation mode and using the following parameters: capillary voltage 2.50 kV, source temperature 150°C, desolvation temperature 600°C, cone gas (nitrogen) flow 50 l h^{-1}, desolvation gas (also nitrogen) flow 1200 l h^{-1}. Collision-induced dissociation was performed with argon as the collision gas at 4×10^{-3} mbar pressure in the collision cell. The cone voltages, the collision energies, the precursor and product ions were optimised by direct infusion of the pure individual standard solution at a concentration of 10 µg ml^{-1} and a flow-rate of 5 µl min^{-1}. The dwell times were optimised for each drug to achieve at least 12 points per peak. Data acquisition was done with the MassLynx 4.1 software and the TargetLynx 4.1 software (Waters). Matrix matched calibration curves ranging from 0.5 µg kg^{-1} to 100 µg kg^{-1} were prepared in each blank tissues. For quantification of analytes, their corresponding deuterated analogues were used as internal standards.

Results

Method evaluation

The specificity and selectivity of the method were demonstrated by comparing chromatograms of 5 matrix blanks with blank matrix spiked at the LOQ. Representative chromatograms ate shown in figure 1 for rainbow trout. The absence of any peaks in the blank sample at the retention times of the target contaminants indicated that there was no matrix interference that could lead to false positive signals.

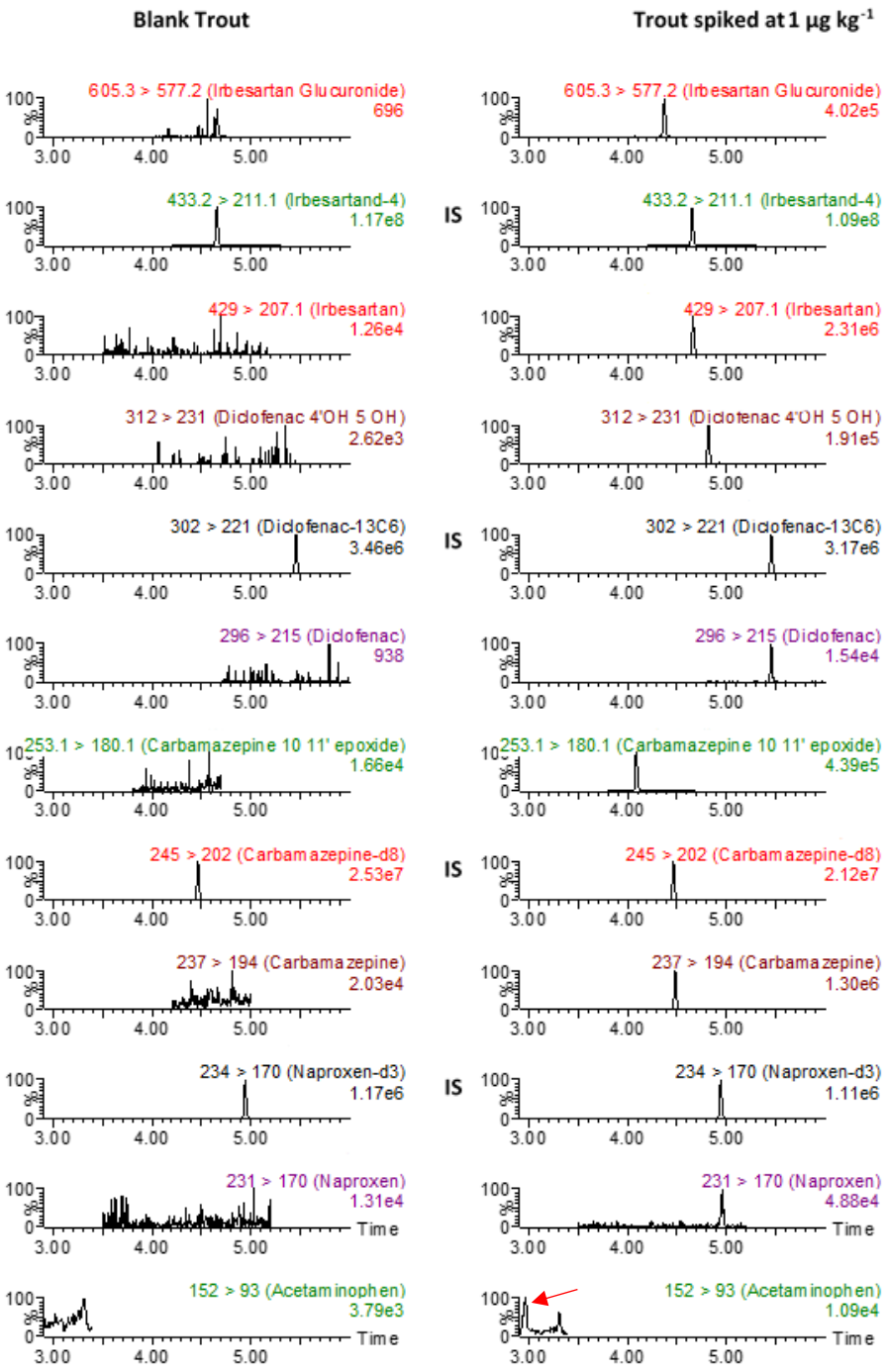

Figure 3 : Comparison of chromatogram of a blank sample (left) and a QC sample of trout spiked at 1 µg kg^{-1} (right)

The linearity of calibration curves was evaluated by using a seven-level matrix matched calibration curve for all analytes. Blank samples were spiked with working standards to obtain 0.5, 1, 2,5,10 20, 50 and 100 µg kg^{-1} for each analyte. Relative peak areas were used as the analytical response versus concentrations for all target compounds. As the results, acceptable linear regression values (r^2 > 0.99) were obtained for all target analytes over the concentration range and for the matrix matched calibration curves.

To limit matrix effects, calibration curves were all established with matrix-matched and the use of isotope labeled internal standards of the target compounds added at the at the beginning of the extraction. The limit of quantification (LOQ) of each compound in each matrix was determined based on the criterion that the analyte response peak heightat LOQ is ten times of the baseline noise. LOQ were found to be around 1 µg kg^{-1} expect for diclofenac and naproxen for which much higher value were found (50 µg kg^{-1}) in moss species.

Chemical analysis results in all tissues and the 3 experiments are reported in table 1.

Table 2 : Maximal concentration of the target compounds/metabolites found in the selected species during the different experiments

Species	Trageted compounds	LOQ µg kg^{-1}	Detected concentration µg kg^{-1}		
			Aquarium	Mesocosm	Natural rivers
Gammarus fossarum	Carbamazepine	1	nt	7.9	0.78
	Naproxen	1		2	<LOQ
	Irbesartan	1		5.6	0.57
	Diclofenac	1		4.5	3.9
	Paracetamol	1		<LOQ	<LOQ
Fontinalis antipyretica	Carbamazepine	1	50	35.5	<LOQ
	Naproxen	50	<LOQ	<LOQ	<LOQ
	Irbesartan	1	<LOQ	37	<LOQ
	Diclofenac	50	<LOQ	<LOQ	<LOQ
	Paracetamol	1		<LOQ	<LOQ
Dreissena polymorpha	Carbamazepine	0.5	12	5.7	<LOQ
	Naproxen	1	2.7	4.3	<LOQ
	Irbesartan	0.5	1.4	1.6	<LOQ
	Diclofenac	0.5	13	2.7	<LOQ
	Paracetamol	1		<LOQ	<LOQ
Gasterosteus aculeatus	Carbamazepine	0.5	nt	1.75	nt
	Carbamazepine 10 11’ epoxide	0.5		6.3	
	Naproxen	1		<LOQ	
	Irbesartan	0.5		4	
	Diclofenac	1		11	
	Paracetamol	1		<LOQ	
Oncorhynchus mykiss	Carbamazepine	0.5	<LOQ	nt	<LOQ
	Naproxen	0.5	<LOQ		<LOQ
	Irbesartan	0.5	<LOQ		0.8
	Diclofenac	0.5	<LOQ		1.3
	Paracetamol	1	<LOQ		<LOQ

nt : non tested

The highest levels of contaminants were found during artificial exposure studies but only for exposure to the highest concentrations (condition 100x). Same analytes and concentrations in the same range were detected for both aquarium and mesocosm experiments for the moss species and the zebra mussel.
Paracetamol was never found in all experiments.
None of the analytes were present at quantifiable concentrations in the different trout samples artificially exposed to the contaminants whatever the tested concentration and the exposure time.
The carbamazepine metabolite (carbamazepine-10-11'-epoxide) was the only metabolite detected and was found in the three-spined stickleback for exposure to the high concentrations (condition 10- and 100 times the environmental concentration).

The evolution of the concentration of contaminants in dreissene after 8, 16 or 23 weeks of exposure at 100-times the environmental concentration the in mesocosm studies in illustrated by Figure 2. Zebra mussels show their ability
to accumulate pollutants notably because their high filtration capacities.

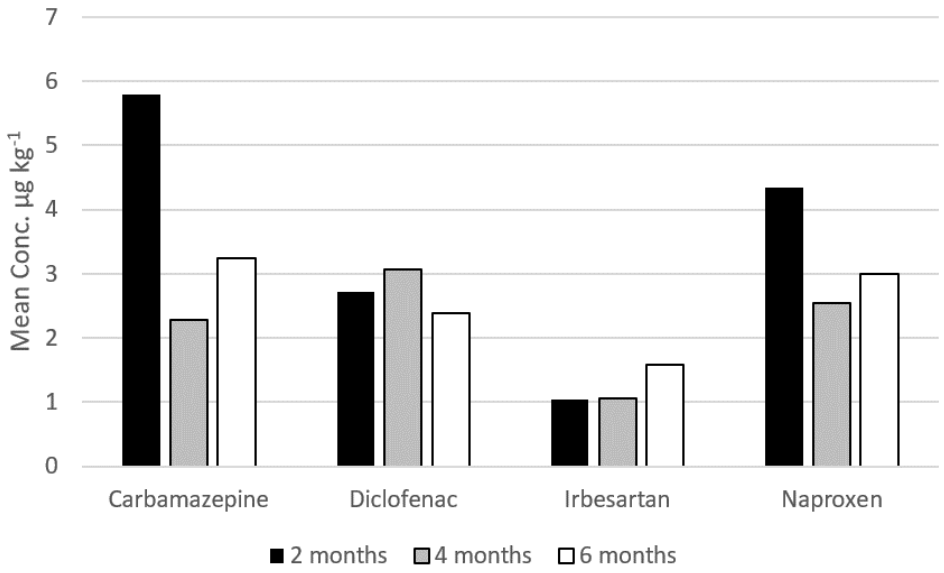

Figure 4 : Evolution of the concentration of carbamazepine, diclofenac, irbesartan and naproxene in the soft tissues of the dreissene after 8, 16 or 23 weeks of exposure at 100-times the environmental concentration the in mesocosm studies

In natural rivers, drug concentrations measured in caged organisms are lower than those detected under controlled laboratory conditions (aquaria and mesocosms) except for rainbow trout for which low level of diclofenac and irbesartan were detected. The highest mean concentration of 45 pharmaceuticals was 4400 ng l-1 detected downstream from the wastewater treatment plants located in Namur (Catteau et al., 2022).
The shorter duration of caging, the lower concentration of contaminants in the natural aquatic environment and the presence of others contaminants are the probable reasons for these lower levels.
All these results highlight the ability of contaminants to accumulate in aquatic organisms.

Conclusions

Following this project, we confirmed that certain organisms are able to accumulate contaminants. Among them, it was demonstrated that *G. fossarum* could be perfectly suitable for monitoring contamination in the natural environment as they were the sole species for which analytes were detected. Il was also attest that the during of exposure and the concentration of contaminant affect its accumulation by the organism.

Acknowledgements

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ORAL O26

ENVIRONMENTAL IMPACT OF ANTI-SEA LICE AGENTS ON NON-TARGET SPECIES

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Abstract
Sea lice are a major challenge for the Norwegian aquaculture. The use of veterinary drugs is one of the methods used against sea lice. The anti-sea lice agents are used either as in-feed agents or through bath treatment, and the mode of action differ between the drugs used.
We have used Bayesian networks as a qualitative tool for visualising the risk, the individual risk factors, and the strength of knowledge for the individual risk factors, of anti-sea lice agents impact on non-target species.
In this risk assessment we have used the presence of anti-sea lice agents in the environment, overlap between the presence of non-target species and the use of anti-sea lice agents during the season, as well as the toxicity of the anti-sea lice agents, to assess the impact anti sea lice agents have on non-target species.
For the anti-sea lice agents used in Norway in the last years, azamethiphos has been consider having a low risk to non-target species, while deltamethrin, emamectin, hydrogen peroxide, and flubenzurons have a moderate risk.

Keywords: aquaculture, non-target species, sea lice

ORAL O27

PHARMACOKINETICS OF TOLTRAZURIL IN LAYING HENS AND RESIDUES IN EGG

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Toltrazuril is effective against all coccidial parasites in most animal species. Pharmacological studies showed that toltrazuril was well absorbed in the gastrointestinal tract and rapidly metabolised to toltrazuril sulfoxide and toltrazuril sulfone (ponazuril), which is considered as marker substance of toltrazuril residues.
The MRLs for toltrazuril (as toltrazuril sulfone) in edible poultry tissues range from 100 to 600 µg/kg. However, toltrazuril is not authorised for use in poultry producing eggs. Nevertheless, veterinary medicinal products containing the active substance toltrazuril are approved for pullets with withdrawal periods for edible tissues between 16 and 21 days. Since egg laying can start between the 17th and 20th week of age in laying hens, residues in eggs might be detectable after the end of a legal treatment.
The objective of this study was to evaluate the pharmacokinetic profiles of toltrazuril and its major metabolites. The treatment of the pullets was carried out on two consecutive days during the 15th week of age in line with legal conditions. Egg samples were collected after the beginning of the laying activity and were analysed for toltrazuril and its metabolites. In the egg samples residues were detected, even though the specified restricted periods had been respected.

Extended Abstract

Started pullets can be legally treated with coccidiostats which are not allowed for use in laying hens. It was reported by official control laboratories (OCLs) that – depending on the time span between the treatment of the started pullets and the first production of eggs – elevated residue levels in egg could occur after the end of the legal treatment of the hens. For example, positive results for toltrazuril sulfone in eggs from 20-week-old laying hens were reported. At the inspection of the concerned farm, no evidence of an illegal use of toltrazuril and no indication of misuse were found. For pullets all treatments with authorised toltrazuril veterinary products are authorised either up to 4 weeks before the start of laying or not beyond the 15th week of age. Since egg laying can start between the 17th and 20th week of age in laying hens, residues in egg might be detectable after the end of a legal treatment. For treatments with toltrazuril, the following five preparations, each containing 25 mg/ml of toltrazuril, are authorised: Zorabel, Toltra-k, Zuritol, Baycox and Dozuril. The following instructions apply: for TOLTRA-K, Zorabel and Zuritol, the withdrawal periods after treatment are 4 weeks before the start of laying, and for Baycox and Dozuril the treatment must not be conducted on pullets after the 15th week of age. Additionally, none of the products are authorised for or applicable to animals from which eggs are produced for human consumption.
A study was carried out with the objective of evaluating the pharmacokinetic profiles of toltrazuril and its major metabolites. For this purpose, 12-week-old brown laying hens were kept in one group in floor husbandry on wood shavings. Once a week the litter was exchanged completely. Pelleted laying-hen compound-feed and water were accessible ad libitum. After a two-week familiarisation phase, the animals were treated with TOLTRA-K (25 mg/ml) on two consecutive days during their 15th week of age via drinking water containing the active substance. Toltrazuril was administered continuously at a dosage of 7 mg of toltrazuril per kg of body weight (bw) per day over 24 hours as oral suspension.
After the start of the laying activity, all egg samples were collected as single samples. The collected egg samples were individually broken, homogenised roughly with an electric mixer, filled into Sarstedt

tubes and stored at -25 °C. A total of approximately 190 eggs were collected. The samples were analysed by LC-MS/MS and the residue depletions of toltrazuril and its metabolites were identified and determined.

The obtained results showed that residues of toltrazuril sulfone of up to 99.9 µg/kg could be detected after the legal withdrawal period of four weeks. Seven weeks after treatment a majority of the eggs still contained residues above CCα, and only at the end of week 8 (55.5 days) after treatment, the residues were below CCα. The pharmacokinetics of toltrazuril sulfone were investigated by statistically evaluating the obtained results. The statistical analysis considers the CCα value to determine and calculate the restricted periods necessary before egg samples become marketable. The restricted period to be imposed so that the residues are no longer detectable in the eggs, is calculated by tracking the variation of the concentration of toltrazuril sulfone in egg after treatment. Depletion results indicate that the established restricted period after a legal treatment with toltrazuril preparations should be reviewed.

ORAL O28

SIX-PLATE SCREENING METHOD FOR THE DETECTION OF ANTIMICROBIAL RESIDUES IN ANIMAL WASTE.

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Abstract
Antibiotic residues are of great importance in food production for their consequences on human health, such as antimicrobial resistance. It has been described that a large percentage of these drugs are excreted and persist in animal waste, so this could be used as a non-invasive matrix for antibiotic analysis, and therefore a tool to control the use of antibiotics in animal production. A new microbiological screening method for the detection of antibiotics for six families in manure was implemented, validated, and verified in vivo. Validation was performed by assessing the specificity, CCβ, robustness and applicability according to 2002/657/EC. For in vivo verification, broilers were treated with a representative drug formulation of each antibiotic family. Manure samples were analysed by screening method and HPLC-MS/MS, as confirmation. The screening method was specific to detect all antibiotics tested, and a CCβ of 203, 104, 103, 103, 402, 203, 203, 203 μg kg^{-1} was determined for tetracyclines, β-lactams, macrolides, quinolones, sulphonamides and lincosamides, respectively. The concentrations quantified in the incurred samples were higher than the CCβ of the microbiological screening method. In conclusion, the microbiological screening method presented performance characteristics that demonstrate utility for the intended analytical applications, ensuring traceability of the results.

Introduction
Poultry droppings are the main component of poultry litter and raw/treated manure, by-products of the industry used mainly for fertilization of agricultural soils or for the formulation of diets for feeding other productive species (Ghirardini et al., 2020; Richa et al., 2020; Rojas G & Catrileo S, 2006; Zhang et al., 2015).

It has been described that poultry treated with different antimicrobials have excreted residues of these drugs from less than 10% to above than 90% of what was administered (Ghirardini et al., 2020; Peng et al., 2016). According to reports, after the end of treatment with chlortetracycline at therapeutic doses in broilers, it is possible to detect this antimicrobial in faeces, observing in a study that concentrations of this antimicrobial were gradually decreasing during sampling, which covered from day 5 to day 25 post-treatment (Cornejo et al., 2018).

This aspect becomes relevant considering that by-products are not intended for direct human consumption, and therefore are not controlled or monitored for the presence of antimicrobial residues. Hence, the waste of productive animals can be an uncontrolled route of antimicrobial transfer through the food chain and the environment (Bacanlı & Başaran, 2019; Berendsen et al., 2015; Kwon et al., 2011).

Nowadays, different methods based on liquid chromatography coupled to mass spectrometry provide the necessary degree of confidence for the analysis of veterinary drug residues in biological samples, due to their selectivity. Despite the fact that different antimicrobials have been detected in animal waste from different species such as cattle, poultry, and swine (Berendsen et al., 2015; Jansen et al., 2019; Patyra et al., 2020;

Pokrant et al., 2021; Yévenes et al., 2018), the drawback with these techniques is that they require high cost installed analytical capacity as well as a high level of technical training (Majdinasab et al., 2020).

For this reason, microbiological screening methods, which are mainly based on microbiological inhibition, were the first techniques used to detect antibiotic residues and are still a tool used for preliminary detection of antibiotic residues in edible animal tissues such as milk, egg, muscle, liver and kidney (Cháfer-Pericás et al., 2010; Gaudin et al., 2010; Wu et al., 2020). However, we are not aware of microbiological screening methods for the analysis of antimicrobials in animal waste.

Therefore, the development of a methodology for the detection of antimicrobial residues in faeces of productive animals would allow an early determination of these drugs in a rapid, economical, and non-invasive way, with respect to the currently existing methodologies. Bearing this in mind, the main objective of the present study was to implement and validate a microbiological screening method in the broiler droppings matrix. In addition, the methodology was verified in vivo samples by analysing broiler droppings after administration of antimicrobials representing the families of macrolides, β-lactams, quinolones, sulphonamides, lincosamidas and tetracyclines.

Materials and Methods

Solvents, reagents and standards.
For the fortification of droppings samples, standards of certified purity were used, corresponding to Erythromycin (ERI) (CAS: 114-07-8), Ampicillin trihydrate (AMP) (CAS: 7177-48-2), Enrofloxacin (ENR) (CAS: 93106-60-6), Ciprofloxacin hydrochloride (CIP) (CAS: 93107-08-5), Sulfachloropyridazine (SCP) (CAS: 80-32-0), Lincomycin hydrochloride monohydrate (LIN) (CAS: 7179-49-9), Oxytetracycline hydrochloride (OTC) (CAS: 2058-46-0), and 4-Epioxytetracycline (EPI) (CAS: 14206-58-7). Manufacturer by Dr. Ehrenstorfer Gmbh (Augsburg, Germany). Stock solutions of each analyte were prepared at a concentration of 1000 ng mL^{-1} in methanol and working solutions at a concentration of 5000 ng mL^{-1}, in the same solvent.

Water, methanol (MET), acetonitrile (ACN) and acetone (ACT) of the LiChrosolv® line, HPLC grade (Merck KgaA, Darmstadt, Germany) were used as extraction solvents for both the confirmatory analysis by LC-MS/MS and the extraction method for analysis by microbiological screening method. EDTA-McIlvaine buffer was prepared with citric acid monohydrate, disodium hydrogen phosphate dihydrate and ethylenediaminetetraacetic acid (EDTA), manufactured by Merck KgaA (Darmstadt, Germany). Phosphate Buffer, 0.1M pH 6 was prepared with 11.2 g of KH_2PO_4 and 2.8 g of K_2HPO_4, and the Phosphate Buffer, 0.1M pH 8 was prepared with 0.523 of KH_2PO_4 and 16.73 g of K_2HPO_4, both buffers diluted in 1000 mL of distilled water.

Culture Media
A specific culture medium was used for plaque preparation: Difco™ Antibiotic Medium 5, Difco™ Antibiotic Medium 8, and Difco™ Mueller Hinton Agar. These media were sterilized and subsequently inoculated with a collection strain, sensitive to the analyte each analyte. Subsequently, 10 mL of inoculated medium was poured into a sterile 90 x 15 mm Petri dish. Six specific plates were prepared for the detection of antimicrobials belonging to the different families of interest, which corresponded to macrolides, quinolones, tetracyclines, lincosamidas, sulphonamides and β-lactams. The plaques were determined according to the sensitivity assay.
I. *Kocuria rhizophila* ATCC 9341 was inoculated at a final concentration of 1.5 x 10^6 bacteria/mL in: Medium N° 8 at pH 8.0 for the detection of macrolides, in Medium N° 5 pH 6.0 for the detection of β-lactams, and Mueller Hinton (MH) Medium pH 8.0 for the detection of lincosamidas.
II. *Bacillus subtilis* ATCC 6633 was inoculated at a final concentration of 3.0 x 10^6 spores/mL in Medium N° 5 pH 8.0 for the detection of quinolones.
III. *Geobacillus stearothermophilus* ATCC 10149 was inoculated at a final concentration of 4.5 x 10^6 spores/mL in MH medium pH 7.3 for the detection of sulphonamides.
IV. *Bacillus cereus* ATCC 11778 was inoculated at a final concentration of 3.0 x 10^6 spores/mL in Medium N° 8 pH 6.0 for the detection of Oxytetracycline.

Extraction procedure of residues for microbiological screening detection.
For the analysis by microbiological screening method, a shorter procedure for the extraction of antibiotic residues from the manure samples was implemented and validated. In resume, 2.0 ± 0.01 g of sample was weighed into a 50 mL polypropylene tube. Samples were fortified at concentrations of 25, 50, 100, 200, 400 and 600 µg kg^{-1} with certified standards of erythromycin, ampicillin, enrofloxacin and ciprofloxacin, sulfachloropyridazine, lincomycin, oxytetracycline and 4-epioxytetracycline. Subsequently, 3 mL of an organic solvent was added, according to the analytes of interest and specific plate, where acetonitrile was used for the extraction of macrolides, acetonitrile + Buffer phosphate, 0. 1M pH 6 for the analysis of sulphonamides, and

acetonitrile + Buffer phosphate, 0.1M pH 8 for the analysis of lincosamides, tetracyclines, quinolones and β-lactams. The samples were shaken in a Multi Reax® (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) for 10 min at maximum speed, and stored at -20 °C for 10 min. Subsequently, they were centrifuged at room temperature in an Eppendorf® Centrifuge 5804 (Merck KGaA, Darmstadt, Germany) for 10 minutes at 4000 rpm, the supernatant was aspirated and filtered using a CLEAR PTFE-22/25 syringe filter (Macherey Nagel) with 0.2 µm pore size and 25 mm diameter.

The filtered extract was transferred to a sterile 5 mL microcentrifuge tube (Jet-Biofil®), and 200 µL of this filtrate was immediately inoculated into each cylinder arranged on each plate. The plates were incubated for 18 to 24 hours at the optimal growth temperature of the microorganism. *Kocuria rhizophila* plates were incubated at 35°C; *Geobacillus stearothermophilus* plates were incubated at 55°C; and *Bacillus subtilis* and *Bacillus cereus* plates were incubated at 30°C. The inhibition halos radii were measured with a calliper and reported in millimetres. The presence of an inhibition halo ≥ 2 mm, with respect to the blank sample, indicated a positive detection of the analyte.

Validation of microbiological screening method

The validation of the microbiological screening method was carried out in accordance with Decision 2002/657/EC (European Commission, 2002). For this purpose, an internal validation protocol was elaborated, which contemplated the evaluation of several parameters to demonstrate that the analytical method complies with the applicable criteria for the detection of antibiotic residues from chicken droppings. The following analytical parameters were determined.

Detection capability (CCβ): 20 samples of faeces fortified at concentrations similar to the MRL in muscle, plus a blank sample, were analysed. Tested concentrations were 100 µg kg^{-1} for erythromycin, ampicillin and oxytetracycline with 4-epioxytetracycline, 200 µg kg^{-1} for sulfachloropyridazine and lincomycin, and 400 µg kg^{-1} for enrofloxacin with ciprofloxacin. Finally, the CCβ of the DML with a 5% error (β = 5 %) was equal to the concentration value plus 1.54 times the corresponding standard deviation. The acceptance criterion was a maximum of 1 false result every 20 replicates.

Specificity: 40 samples of chicken faeces fortified with the chosen fortification concentration and 40 blank samples were analysed in triplicate for each antibiotic, in two independent experiments and with two different analysts. The radio of halos and the standard deviations of both fortified and blank samples were compared.

Robustness: The robustness study was based on a Youden factorial design for the analysis of the effects and interactions of three independent analytical variables, which can influence the results of the measurements. These variables correspond to amount of bacterial inoculum on the plate ± 30%, pH of the culture medium ± 0.5, and the operator (two different operators participated in this determination, to estimate the variability between them).

Stability: The persistence of the different compounds was analysed after four weeks of storage at 4°C and -20 °C in fortified matrix and working solution. The percentage of analyte remaining in solution was calculated, taking as 100 % the measured halo of the freshly prepared solution at the time of analysis (t=0), to define the maximum time and optimal storage conditions.

Verification in vivo of the microbiological screening method: Animals, experimental groups, and drug administration

Forty-eight male Broiler chicks, Ross 308 genetics, were used. The birds were kept from day 1 of life in rearing batteries under controlled environmental conditions (a temperature of 25 ± 5°C, adapting according to the days of life of the birds, and 50-60% relative humidity), ad libitum access to water and non-medicated feed. The feed was previously analysed to rule out the presence of residues of the antimicrobials of interest and formulated according to the requirements of the breed. The cages had a raised wire floor in order to obtain faecal samples. The birds were raised and monitored throughout the experiment in the Animal Management Unit of the Department of Animal Preventive Medicine of the Faculty of Veterinary and Livestock Sciences of the University of Chile. For the maintenance of the experimental animals, the animal welfare conditions approved by the Institutional Animal Care and Use Committee (CICUA) of the University of Chile (Nº: 19331-VET-UCH) were respected, and the biosafety measures for working with experimental animals and working in laboratories approved by the FAVET Biosafety Committee (Certificate N° 153) were followed.

Six experimental groups were used for the study of each of the selected antimicrobial families. To define the sizes of the experimental groups, the criteria established by the European Medicines Agency, Guideline: Approach towards harmonization of safeguard periods EMEA/CVMP/SWP/735325 (2016) were considered. In this way, we worked with 6 animals per sampling point to ensure obtaining the necessary results to provide validity to the study.

On day 21 of life, the birds were weighed, and 7 experimental groups were randomly formed, which were maintained under the same conditions. The drug was administered orally, through an esophageal tube, 2 consecutive days. In the case of ampicillin, since there was no authorized oral pharmaceutical formulation, a certified standard was used, which was administered intravenously in a single dose.

The experimental groups and their doses were administered as follows:
Group 1: sulfachloropyridazine 10% with trimethoprim 2% was administered at a dose of 30 mg/kg.
Group 2: erythromycin thiocyanate 80% was administered at a dose of 90 mg/kg.
Group 3: enrofloxacin 20% was administered at a dose of 10 mg/kg.
Group 4: ampicillin trihydrate was administered at a dose of 10 mg/kg.
Group 5: oxytetracycline hydrochloride 80% was administered at a dose of 59 mg/kg.
Group 6: lincomycin hydrochloride 4.4% was administered at a dose of 0.4 mg/kg.
Group 7: Control group, corresponding to untreated animals.

Samples were collected at 4- and 8-hours each day post-administration. In the case of ampicillin, being a single intravenous dose, samples were taken at 1, 2, 4, 6 hours post-administration. All samples were stored at -20°C individually until analysis for residue detection by microbiological screening and HPLC-MS/MS.

Microbiological analysis of experimental samples
Samples from each experimental group, were tested against the respective plates for the detection of the presence of enrofloxacin + ciprofloxacin, erythromycin, lincomycin, oxytetracycline + 4-epi-oxytetracycline and sulfachloropyridazine residues. Also, blank samples of droppings from untreated birds (animals free of antibiotic residues) were tested in six-plates.

Confirmation of the presence of antimicrobial residues using HPLC-MS/MS in experimental samples

Screening-positive samples were confirmed by a multi-residual analytical method previously implemented and validated at the Veterinary Pharmacology Laboratory (FARMAVET) of the Faculty of Veterinary and Livestock Sciences of the University of Chile. The extraction protocol and chromatographic conditions of the confirmatory method were based on a previously published analytical methodology (Pokrant et al., 2021). Sample analysis was performed in a liquid chromatograph, consisting of a quaternary pump, an autosampler and a column oven, (Agilent 1290 infinity series) coupled to a triple quadrupole mass spectrometer (API 5500, ABSCIEX). The analytical column corresponded to a Synergi 4u fusion RP 30a 50 x 2.0 mm. The software used to operate the equipment was Analyst 1.6.3 and the Multiquant 3.0 program was used for sample integration.

The concentrations of the detected residues were determined by the equation of the line, from a linear regression analysis of fortified matrix calibration curves. A coefficient of determination greater than or equal to 0.99 was considered for quantification purposes.

Finally, it was verified if the quantified concentration, determined by HPLC-MS/MS, were greater than or equal to the detection capacity of the microbiological screening method. The results were considered concordant when the concentration obtained by HPLC-MS/MS was greater than or equal to the CCβ of the screening method.

Results and Discussion
The parameters of detection capacity, specificity, robustness, and stability indicated that the method is robust and detect all the antibiotics under study in droppings matrix (Table 1).

The results of the stability tests suggest that all analytes are stable in solution at the storage temperatures tested (4°C and -20 °C), with, on average, 95-99% of analyte remaining in each solution after the first week (t=1), and approximately 90% after 4 weeks of analysis (t=4), compared to time zero (t=0). On the other hand, when the analytes are in the matrix, for most of them a decrease in the size of the inhibition halos is observed during the first week of storage, and then it remains constant over time, both at 4 °C and at -20 °C. For erythromycin, enrofloxacin + ciprofloxacin, sulphonamides and lincomycin, this decrease was similar at both storage temperatures. For ampicillin and oxytetracycline + 4-epi-oxytetracycline the decrease was greater when the samples were stored at 4 °C.

Table 1. Legend.

Antimicrobial class	Representative antimicrobial	CCβ (μg kg^{-1})	Specificity		Robustness	
			Concentration** (μg kg^{-1})	Average ZI***(mm) ± SD	Mean ZI (mm) ± SD: CCβ	Average ZI (mm) ± SD: Robustness
Quinolones	Enrofloxacin + Ciprofloxacin	402	400	11 ± 1	12 ± 1	11 ± 1
Tetracyclines	Oxytetracycline + 4-epi-oxytetracycline	203	100	16 ± 3	21 ± 2	14 ± 1
Sulphonamides	Sulfachloropyridazine	203	200	21 ± 2	22 ± 1	21 ± 2
Macrolides	Erythromycin	103	100	16 ± 2	15 ± 2	14 ± 1
β-lactams	Ampicillin	104	100	26 ± 3	31 ± 2	23 ± 11
Lincosamidas	Lincomycin	203	200	13 ± 2	14 ± 2	14 ± 2

[a] note.

During the in vivo verification of the validated method, all the samples from treated animals presented inhibition halos and the difference in radius was greater than 2 mm with respect to the blank samples, the latter coming from the group that did not receive any antimicrobial. Specifically, it was observed that the droppings obtained from the birds that received the antimicrobial enrofloxacin presented an inhibition halo between 13.4 ± 0.7 and 15.8 ± 0.3 mm in radius. While the residue-free samples presented inhibition halos of 0 to 1.2 mm radius.

Samples from the birds to which oxytetracycline was administered presented inhibition halos between 14 and 17.7 mm with respect to the blank samples that presented a halo between 1.3 and 2.2 mm. In the analysis of sulfachloropyridazine residues, it was observed that the specific plates for detection of this antimicrobial showed inhibition halos between 9.3- and 22.5-mm radius for the samples obtained from the birds to which the antimicrobial was administered, and a radius between 4.3 and 4.5 for the blank samples.

For erythromycin, the samples analysed from the four sampling points were positive for the presence of this antimicrobial by microbiological screening analysis, where the inhibition halos had a radius between 15.3 and 17.4 mm. On the other hand, the blank samples showed a radius between 6.8 and 9.2 mm, although these radii were the highest values with respect to the other plates, the difference between the positive and residue-free samples complied with a difference of more than 2 mm in diameter to accept the sample as positive. Samples obtained from the group of birds to which lincomycin was administered presented a radius between 15.3 and 17.9 mm, and the blank samples did not present inhibition halos when analysed on the specific plate for the detection of this family. Regarding the analysis of ampicillin residues, it was observed that the average of the inhibition halos fluctuated between 20.5- and 28.8-mm radius, with respect to the blank samples that presented a radius between 0.9- and 3-mm.

For the detection of all the antimicrobials, clearly delimited inhibition halos were observed in direct view on each of the plates, except in the case of sulfachloropyridazine. For this antimicrobial, partial inhibition was observed as no "tapestry-like" bacterial growth was visible for a clear reading, something that has been described in the validation of other microbiological screening methods (Gaudin et al., 2004). Figure 1 shows the inhibition halos for each specific plate, with respect to the blank samples analysed, and the table shows the detail of the values obtained per family of antimicrobials analysed. Bacterial growth is visible but incomplete, i.e., most, but not all, of the bacteria are inhibited in the presence of the antimicrobial. In addition, the plates were incubated at 55°C, so they tended to dehydrate faster than the other plates, so the reading was taken after 18 hours of incubation.

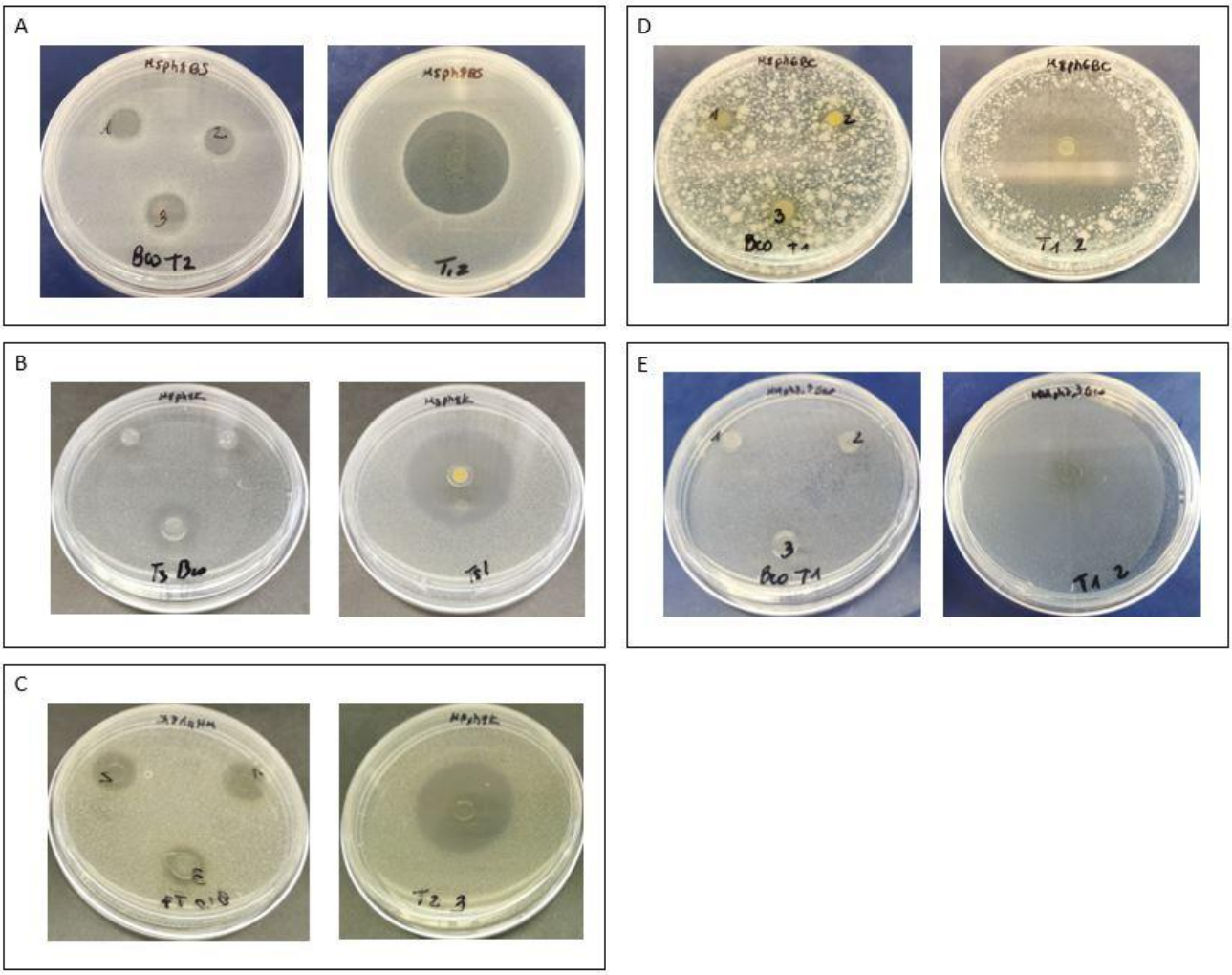

Figure 1. Six-plate method plate on the left of each image corresponds to the analysis of blank sample (free of residues), plate on the right of each image corresponds to the analysis of experimental sample. (A) Enrofloxacin + ciprofloxacin, (B) Erythromycin, (C) Lincomycin, (D) oxytetracycline + 4-epi-oxytetracycline, (E) Sulfachloropyridazine.

Both the positive samples and those from the control group were verified by HPLC-MS/MS, and residues of these antimicrobials were quantified. Concentrations were above the CCβ of the microbiological screening methodology and exist concordance between microbiological screening and HPLC-MS/MS analysis. No presence of enrofloxacin + ciprofloxacin, erythromycin, lincomycin, oxytetracycline + 4-epi-oxytetracycline or sulfachloropyridazine were detected from the waste samples of control group. The table 2 shows in details the results for each experimental group comparing the results obtained from the microbiological screening and the confirmatory method.

Table 2. Average halo radius (mm) and quantified concentrations for Enrofloxacin + Ciprofloxacin, Oxytetracycline + 4-epi-oxytetracycline, Sulfachloropyridazine, Erythromycin, Ampicillin and Lincomycin from experimental samples.

Antimicrobial	Sampling[a]	CCβ (µg kg^{-1})	Average halo radius (mm)	Quantified concentration (ng gr^{-1})
Enrofloxacin + Ciprofloxacin	1	402	15.8 ± 0.3	46,882 ± 4568
	2		13.4 ± 0.7	45,759 ± 4766
	3		14.5 ± 0.5	22,725 ± 3787
	4		13.8 ± 0.3	15,111 ± 9107
Oxytetracycline + 4-epi-oxytetracycline	1	103	16.4 ± 3.7	10,769 ± 7544
	2		14 ± 0.7	12,916 ± 16,455
	3		17.7 ± 0.7	4442 ± 3998
	4		16.6 ± 0.4	11,613 ± 11,844
Sulfachloropyridazine	1	203	22.5 ± 0	895 ± 32
	2		19.2 ± 1.7	982 ± 297
	3		14.3 ± 3.8	4693 ± 1032
	4		9.3 ± 0.8	2578 ± 136
Erythromycin	1	103	14.7 ± 3	46,193 ± 28,545
	2		16.8 ± 0.4	13,076 ± 1772
	3		15.3 ± 0.4	31,919 ± 26,833
	4		17.2 ± 1.6	15,368 ± 7738
Ampicillin[b]	1	104	28.8 ± 2.9	57,751 ± 7007
	2		24.8 ± 2.8	27,934 ± 26,541
	3		21.5 ± 3.8	24,429 ± 4717
	4		20.5 ± 0	14,491 ± 16,133
Lincomycin	1	203	16.9 ± 1.8	752,118 ± 431,729
	2		15.7 ± 0.4	731,288 ± 4628
	3		15.3 ± 5	404,562 ± 313,206
	4		17.9 ± 0.2	1,578,003 ± 1,453,197

[a] sampling 1: 4 hours post-administration, first day; sampling 2: 8 hours post-administration, first day; sampling 3: 4 hours post-administration, second day; sampling 4: 8 hours post-administration, second day.
[b] sampling 1: 1-hour post-administration; sampling 2: 2 hours post-administration; sampling 3: 4 hours post-administration; sampling 4: 6 hours post-administration.

Conclusion

The process of verification in vivo of the methodology allowed demonstrating that the six-plate microbiological screening method is capable of detect antimicrobials of tetracyclines, β-lactams, macrolides, quinolones, sulphonamides and lincosamidas classes in the broiler droppings matrix after the metabolization process.

This method constitutes a competent tool for monitoring antimicrobials in animal production, since it allows the simultaneous detection of six families of antimicrobials, using a non-invasive matrix, with the purpose of avoiding or reducing the presence of residues in the final product, which will allow the application of policies on the use of antimicrobials in animal production throughout the production chain. It was possible to develop the adaptation of the six-plate test to the purpose for which it was designed. Also, this methodology is easy to apply and cost-effective for the detection of antimicrobial residues.

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ISO TECHNICAL SPECIFICATION 23758 REGARDING THE VALIDATION OF QUALITATIVE SCREENING METHODS FOR ANTIMICROBIALS IN MILK

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Abstract
In 2021 ISO published the Technical Specification ISO/TS 23758 | IDF RM 251 'Guidelines for the validation of qualitative screening methods for the detection of residues of veterinary drugs in milk and milk products'.
In addition to the CRL Guidelines for the validation of screening tests for the detection of residues of veterinary medicines (January 2010), this document is providing more detailed information how to perform a validation (initial and transfer validation). The technical specification is dealing with several aspects like standard blank matrix, preparation of stock & working solutions and spiked milk samples. The technical specification describes in detail how to perform and to determine the detection capability, test specificity/selectivity, test robustness (influence of test protocol, milk quality & composition, type of milk, animal species, batch & age of reagents, and stability of readers & reagents) and reader and test repeatability.
The Technical Specification could help to harmonize the set-up of initial and transfer validations of qualitative screening tests for antimicrobials in milk.

Introduction
Screening tests for the detection of antimicrobial residues are used worldwide. Before use, people need to be sure that the test is fit for purpose. By means of validation of the method, it shall be demonstrated that the screening test complies with the criteria applicable for the relevant performance characteristics. Commission Implementing Regulation (EU) No 2021/808, repealing Commission Decision (EC) No 2002/657, is specifying the performance characteristics which need to be determined for analytical methods. For qualitative screening tests it concerns following parameters: detection capability CCβ, selectivity/ specificity, stability and ruggedness. Regarding stability, if stability data for analytes in a matrix are available from scientific literature or from another laboratory, these data do not need to be determined again on condition that identical conditions are applied.
The European legislation provides limited explanation how validation and verification of the performance characteristics for screening tests should be performed. In 2010 the European Union Reference Laboratories (EU-RL) distributed a guidance document and launched the idea of the initial validation of screening methods in the originating laboratory and the shortened or 'abridged' validation of these methods in the receiving laboratory following their transfer to that laboratory (Community Reference Laboratories, 2010). This guidance is currently being revised to adjust to the requirements of the new regulation (EU) No 2021/808. Nevertheless there was still need to more precise instructions and this gap is now filled up by the publication of a technical specification by ISO|IDF (International Organization for Standardization|International Dairy Federation, 2021). This international guideline describes general workflows and protocols for the validation and the verification of biological and qualitative screening tests for the detection of residues of veterinary drugs in raw or liquid milk (pasteurized, UHT and reconstituted milk powders and whey protein extracts). This guideline is intended to be useful for manufacturers of screening test kits, laboratories validating screening methods or tests, competent authorities and dairies or end users of reagents or tests for the detection of veterinary drug residues in milk products. The goals of this guideline are a harmonization in validation of tests kits or methods in order that all stakeholders have full trust on the result of a residue screening and to limit overlap and multiplication of validation work in different laboratories by sharing the validation results generated by an independent expert laboratory.

General requirements

The developer or the kit manufacturer should provide information regarding methodology, test kit reagents, additional chemicals not necessarily included in the kit, operating requirements (information about the reading system, cut-off value), scope of the test (matrices suitable to be tested), spectrum of the test (list of veterinary drugs and expected detection capabilities (so far known)), test specifications and documentation.

Key requirement

The key requirement for a screening method is its ability to reliably detect the analyte in question at the chosen screening target concentration (STC). The screening target concentration should be chosen to avoid false-negative results, i.e. low enough to ensure that if the analyte in question is present in the sample at the Regulatory Limit, the sample will be classified as 'Screened Positive'. Both validation and verification should provide the objective evidence that this key requirement is met.

Reagents

Standard blank matrix

The raw milk used is commingled milk coming from at least 4 animals not treated with veterinary drugs within the last 2 months, in mid lactation, and delivering milk with a low to moderate number of somatic cells. The raw milk is collected in sterile containers and kept below 4°C. The use of thawed or reconstituted lyophilized milk could also be authorized on condition the equivalence of results for negative and positive raw milk samples was demonstrated.

Antibiotics

Only analytical grade or certified reference material for validation or verification purposes could be used.

Standard stock solution

Standard stock solutions of the antibiotic at 100 mg L^{-1} are made in water or a suitable solvent and kept below 4°C. The shelflife is depending on the stability of the molecule. In the preparation of the stock solution, correction for impurity and water content is performed.

Working stock solutions

Dilutions of 10 to 0.1 mg L^{-1} are freshly prepared on a daily basis.

Spiked sample

For the preparation of end concentration, the final spiking is performed in the standard blank matrix. The blank milk will be spiked with each analyte.

Test kit

Reagents of at least two and by preference three different production lots are used.

Original validation

The validation is a procedure applied to characterize the performances of a test, in the originator laboratory. The validation demonstrates that the method is fit for purpose. The originator laboratory could be the laboratory which developed the new analytical method or the first laboratory performing a full validation study. The originator laboratory performing the validation should be an independent expert laboratory with experience in the field and a quality management system (e.g. ISO17025) in place and accredited for analogue methods for the same matrix.

Detection capability (CCβ)

The detection capability (CCβ) should be determined in the main matrix which in most cases will be raw bovine milk. However two approaches are possible: the CCβ is determined in the main matrix as such (for example in raw bovine milk) then other matrices are studied as part of the applicability and/or robustness testing or the detection capabilities are determined directly within a mix of matrices in the same study.

All substances relevant for end users in their routine application should be validated. For example: the marker residues of all pharmacologically active substances of the involved group(s) of veterinary drugs (e.g. Council Regulation (EU) N° 37/2010) or the marker residues of all active substances occurring in brands/trade names registered for use in dairy cattle in the country of interest and belonging to the involved group(s) of veterinary drugs.

For each compound at least two concentrations around the initial test concentration will be tested. The aim is to find the lowest concentration of compound that returns a positive test in 95% of the cases = detection capability. The increment between the different concentrations is dependent on the concentration level, as indicated in Table 1. In case of testing of concentrations below 0.5×RL (Regulatory Limit) or above RL, the increment could be doubled.

For broad-spectrum tests with a lot of substances involved larger increments could be applied e.g. based on factors of the regulatory limit like RL, 3⁄4 RL, 1⁄2 RL, 1⁄4 RL, and 1⁄10 RL.

Table 1. Increment between the concentrations tested.

Concentration (in µg/kg)	Increment (in µg/kg)	Concentration (in µg/kg)	Increment (in µg/kg)
1-10	1	101-250	25
11-20	2	251-500	50
21-50	5	501-1,000	100
51-100	10	1,001-5,000	500

The number of replicates tested at each concentration is based on the closeness of the predicted detection capability (CCβ) to the regulatory limit. Each concentration will be tested 20 (concentration ≤ 0.5 RL or >RL), 40 (0.5 RL< concentration <0.9 RL), or 60 (0.9 RL ≤ concentration ≤ RL) times, in a time period of at least three days, with at least two operators and by using at least two and by preference three test kit lots.

Test selectivity/specificity

This includes testing of all other substances belonging to the same antibiotic group as mentioned in Commission Regulation (EC) N° 37/2010 and the testing of substances (minimum one per family) of all other antibiotic families, spiked at a high concentration (100 × RL) in raw milk. Tested in duplicate. If any cross-reactivity is noticed, the substance(s) should be added to list of substances for detection capability testing.

Rate of positive results not caused by residues of veterinary drugs

This is required to show that the test will perform as expected on a range of samples that will be encountered as part of routine use of the test. This can be done in parallel with routine testing in a laboratory. It concerns the testing of 300 individual farm milk and 300 tanker milk samples.

Robustness testing

Robustness testing is designed to check the impact of small changes in the test protocol and to identify the range of samples in which the test will operate. These samples are typically designed to be at the extreme ends of the normal range of the sample received into a laboratory for routine testing. Robustness testing is further designed to measure the influence of production and age of reagents.

Parameters include:

- Influence of test protocol (incubation temperature, incubation time, delay in reading, setup time, volume of test portion, temperature of test portion).

.- Influence of milk quality and composition (somatic cell count, bacterial count, fat content, protein content, pH, lactation stage).

- Influence of type of milk (pasteurized, sterilized, UHT, thawed, reconstituted) or animal species (goat, ewe, mare,…) (optional).
- Influence of production batch and age of reagents.
- Stability of readers and reagents.

For the determination of a base line for the variations covered by the test, 4 to 10 different blank raw milk samples with a normal content, quality and pH; 4 to 10 different blank raw milk samples with a normal content, quality and pH and spiked with a substance (A) or a substance (B) at or just above detection capability (maximum +20 %) are tested following the protocol as given in the kit insert.

Then for each parameter described in the following sections test, 4 to 10 different blank raw milk samples, 4 to 10 different raw milk samples spiked with a substance (A) or a substance (B) at or around 20 % above detection capability are tested with a slightly adapted test protocol (influence of test protocol) or with the correct test protocol but using milk with a specific composition/quality or milk of a different type or milk from another animal species.

Regarding the testing of the influence of production of reagents, in the case that three different lots of reagents were used during the testing of the detection capability, there is no more need for extra testing of batch differences. The impact of the age of reagents is checked by testing with reagents used shortly after production versus the use of reagents just before expiry date. Some of the reagents are stored till shortly before expiry date and then tested, using samples of 'normal' milk.

For stability testing frozen raw milk, lyophilized samples or reconstituted skimmed milk powder may be used if necessary.

Reader and test repeatability

The repeatability of the reader is determined based on the in duplicate reading of minimum 20 blank milk samples, minimum 20 positive samples (low positive), and minimum 20 positive samples (high positive).

The repeatability of the test is calculated on the results of in duplicate testing of minimum 10 blank milk samples, minimum 10 positive samples (low positive), and minimum 10 positive samples (high positive).

Participation in a(n) (inter)national ring trial

Participation with the test under validation in a ring trial for the detection of antibiotics in milk with microbiological and rapid tests is strongly recommended. The samples used in the ring trial should by preference contain residues in a concentration at or around the detection capability for the compound concerned.

Verification testing of a transferred screening method

Certain laboratories require a validation report but do not have the possibility to complete a full validation study. In such a case these laboratories can take over the results of the initial validation study and perform verification testing. A verification procedure is undertaken when transferring a method from the validation laboratory to another laboratory (receptor laboratory). The receptor laboratory needs to demonstrate that they are able to achieve the same CCβ for the test on the scope of matrix/analytes relevant for their daily routine operations as in the validation report.

The receptor laboratory should have access to the complete test procedure and the complete validation report. The laboratory should perform the test in the same conditions as for the validation using the same type of equipment (reader type, incubator, etc.), using the same screening target concentrations and, if applicable the same cut-off level(s).

Detection capability

As the detection capability of the test has already been determined by the original validation, this testing is to show that the detection capability of the test is the same in the receptor laboratory.

For each involved family of veterinary drugs, at least one representative pharmacologically active substance (by preference the most difficult compound to be determined or the most relevant compound) should be

tested. The screening target concentration (STC) for verification should be the CCβ (or higher up to +20% on condition the regulatory limit is not surpassed).

Each concentration is tested 20 times (replicates), with at least two operators. The detection capability is defined as the lowest concentration tested giving at least 19 positive results out of the 20 tests. The preparation of the standards and the spiking of milk samples may be performed by another laboratory, rather than by the receptor laboratory.

Test selectivity/specificity and robustness testing

Since the same reagents and the same test procedure are used, this testing does not need to be repeated.

Reader and test repeatability

Reader and test repeatability should be checked in the verification study in the same way as in the original study.

Participation in a(n) (inter)national ring trial

Ongoing participation with the test under verification in a ring trial for the detection of antibiotics in milk with microbiological and rapid tests is strongly recommended. The samples used in the ring trial should by preference contain residues in a concentration at or around the detection capability for the compound concerned.

Conclusions

The ISO Technical Specification is a serious help in the harmonization of the set-up of initial and transfer validations of qualitative screening tests for antimicrobials in milk. The verification study with a significant reduced workload compared to the original validation should be feasible for most end-user laboratories of antimicrobial screening tests.

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PRIORITIZATION OF SUBSTANCES FOR A RISK BASED NATIONAL RESIDUE CONTROL PLAN

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Abstract
National monitoring plans on veterinary drug residues should be risk-based. For this purpose, we developed decision trees that can be used to prioritize substances into low, medium and high priority to include in the monitoring. The methodology was applied to antibiotics, antiparasitics and NSAIDs in 8 different animal products. In some cases, data were limited hampering a definite prioritization. Overall, the developed methodology worked well to prioritise VMP residues in animal products to be included in the national monitoring plans.

Introduction
According to Regulation (EU) 2017/625, European Union Member States need to establish a risk-based multi-annual national monitoring program that is regularly updated. This regulation, however, does not prescribe how to establish such a risk-based monitoring program. Various methods are available that allow for prioritizing hazard-food combinations (Van der Fels-Klerx et al., 2018). Depending on time, available data and budget, a quantitative, semi-quantitative or qualitative method can be selected. Risk assessments are examples of quantitative methods, whereas risk matrices are seen as semi-quantitative methods (van der Fels-Klerx et al., 2015). The National Residue Control Plan (NRCP) contains a wide range of substances and a number of animal products that need to be monitored according to the Implementing Regulation (SANTE 10216-2022) for the control on residues of relevant substances as indicated in article 19 section 3a and 3b of Regulation (EU) 2017/625. As a result, a quantitative method for prioritizing hazard-food combinations is not feasible for establishing a NRCP.
Therefore, previously, decision trees were derived that allow the prioritization of both authorised and unauthorised substances (van Asselt et al., 2018).
The aim of the current research was to prioritize residues of Veterinary Medicinal Products (VMPs) in animal products based on the previously derived decision trees. As a first start, the substance groups antibiotics, antiparasitics and NSAIDs were prioritized for the following species: bovine, porcine, poultry, horse, goat and sheep, as well as eggs and cow milk.

Materials and Methods

Approach
A list of substances to be prioritised was established as indicated below. For each of the substance groups of VMPs, the selected substances were run through the decision trees per animal species in order to classify their individual priority for monitoring resulting in a low, medium or high priority to include the substance in the NRCP. These decision trees include both questions related to the probability of residues of the substance in the animal product and data on the effect of the substance on human health (the severity). For this purpose, the results of Dutch and EU monitoring programs were used as well as information on the use of the substances (e.g. antibiotics use as registered by the Netherlands Veterinary Medicines Institute (SDa), and sales data for other substances from the Dutch branch organisation for VMPs (FIDIN)). Potential human health effects were assessed based on EFSA

or JECFA reports.

List of substances

For the authorised substances, Table 1 of Regulation (EU) 37/2010 was used. For unauthorised substances, in principle an endless list of substances can be established. The only category that is strictly defined is the group A2 substances, which comprises the substances in Table 2 of Regulation (EU) 37/2010. For the other unauthorised substances, in essence, we included those substances currently monitored in the Plan (NRCP), supplemented with the ones mentioned in the EURL guidance document (EURL, 2020). In case non-compliances were found in the EU monitoring data for a particular substance group that was not represented yet, this substance was also added to the list. Additionally, substances retrieved from non-EU regulatory information concerning legal limits with respect to feed additives and veterinary drug residues were included, as well as substances available for treatment of non-food producing animals (www.diergeneesmiddeleninformatiebank.nl).

Decision tree for authorised substances

The established list of substances was evaluated using the decision tree for authorised substances (Figure 1). Each question was answered using the following information:

1. Is this an essential antimicrobial for humans?
   For this question, the 2017 WHO report was used to identify the highest priority critically important (HPCI) antimicrobials for human medicine. These antimicrobials included quinolones, 3rd and higher generation cephalosporins, macrolides and ketolides, glycopeptides and polymyxins (WHO, 2017).
2. Have MRLs been set for this substance in this animal species?
   This question was answered using table 1 in the Annex of Regulation (EU) 37/2010. The extrapolation of MRLs (except for milk and egg) in species with MRLs to species without MRLs as outlined in Regulation (EU) 2017/880 was not taken into account.
3. Were any non-compliant residue data of the substance found in the last five years?
   In order to answer this question, monitoring data on residues of the substances was used.
   EFSA reports on the results from the monitoring of veterinary medicinal product residues and other substances in live animals and animal products were used for the years 2012-2016 to identify non-compliances in EU MS (EFSA, 2014, 2015, 2016, 2017, 2018) as well as RASFF notifications (2012 – 2016, https://webgate.ec.europa.eu/rasff-window/portal). Furthermore, national monitoring data was extracted from the Dutch Quality Program for Agricultural Products (KAP), which is an extensive cooperation between the Dutch government and Dutch agribusiness (www.chemkap.rivm.nl). Data originated from RIKILT- Wageningen UR and the NVWA and were available for the years 2012, 2013 and 2017.
4. Is the substance regularly used in this animal species?
   This question was answered by using data on use of antibiotics as registered by the Netherlands Veterinary Medicines Institute (SDa, https://www.autoriteitdiergeneesmiddelen.nl) for 2017. As a cut-off value, a DDDA ("Defined Daily Dose Animal", the defined average dose of a specified medicine per kg of a specified animal per day, applied for its main indication (EMA, 2015; Postma et al., 2014)) of 50,000 was set. This threshold was set such that at least 95% of the total antibiotics use in each of the animal species would be included in the analyses. Since the SDa data only indicated antibiotics use in poultry, no distinction could be made between use for broilers and use for laying hens. As a result, it was assumed that antibiotics use for poultry meat and eggs was the same (worst case assumption).
5. Do drugs with this active substance have a long withdrawal period?
   Withdrawal periods were obtained from the product specifications retrieved from the CBG-MEB veterinary medicines database. In case the longest withdrawal time was longer than 10 days for beef, pork and poultry meat and longer than 5 days for milk and eggs (Danaher et al., 2016), this question was answered with a 'yes'.

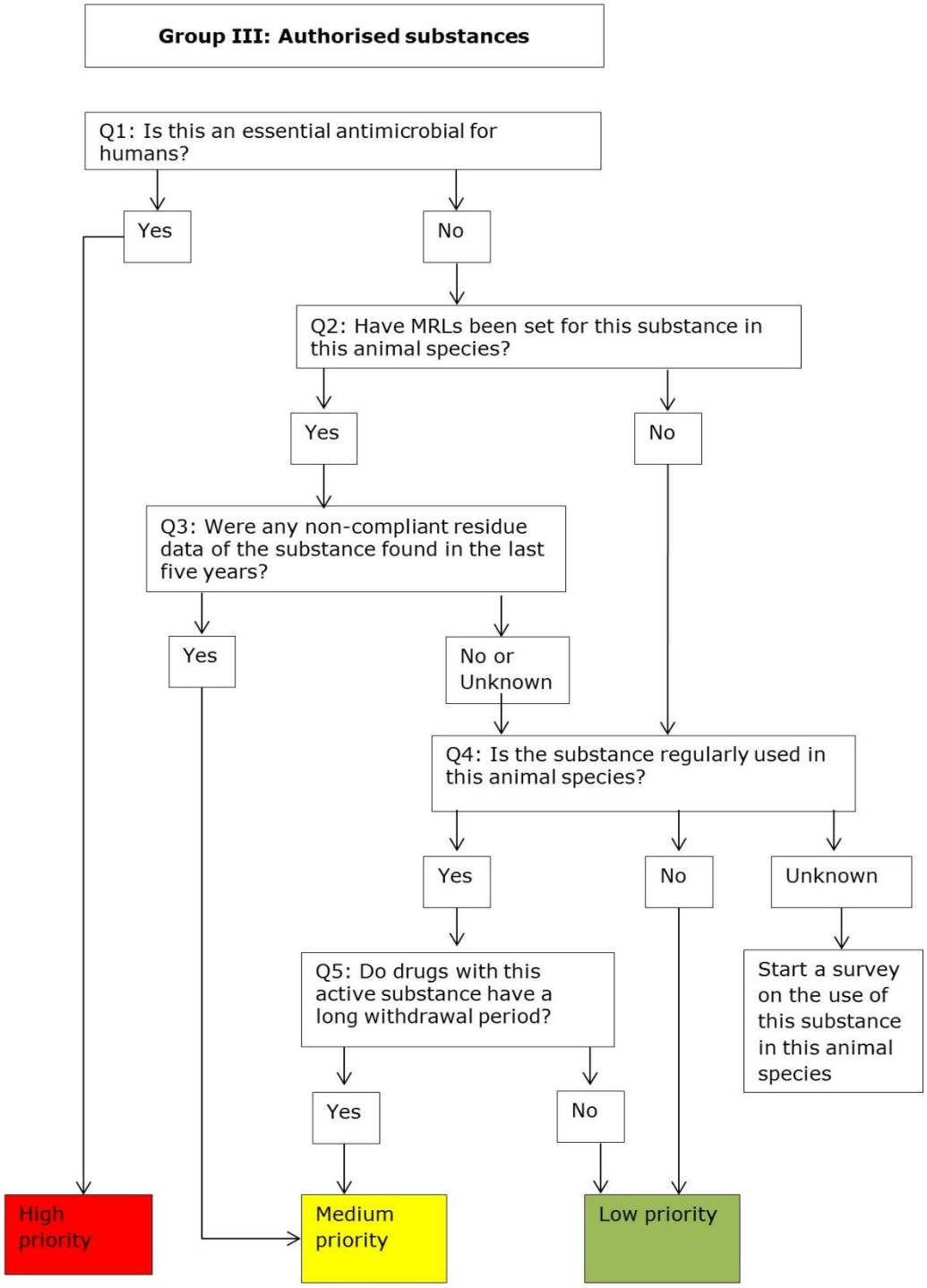

Figure 1. Decision tree for authorised substances (updated from van Asselt et al. (2018)).

Decision tree for unauthorised substances

The established list of substances was evaluated using the decision tree for unauthorised substances (Figure 2). Each question was answered using the following information*:*

1. Were any non-compliant residue data of the substance found in the last five years?
   In order to answer this question, monitoring data on residues of the substances was used.
   EFSA reports on the results from the monitoring of VMP residues and other substances in live animals and animal products were used for the years 2012-2016 to identify non-compliances in EU MS (EFSA, 2014, 2015, 2016, 2017, 2018) as well as RASFF notifications (2012 – 2016, https://webgate.ec.europa.eu/rasff-window/portal). Furthermore, national monitoring data was extracted from the Dutch Quality Program for Agricultural Products (KAP), which is an extensive cooperation between the Dutch government and Dutch agribusiness (www.chemkap.rivm.nl). Data originated from RIKILT- Wageningen UR and the NVWA and was available for the years 2012, 2013 and 2017.
2. Is a human health risk due to residues of the substance scientifically proven to be absent or negligible?
   Reports from EFSA, JECFA and scientific papers were checked for the effect of the substance on human health. In case no severe and/or irreversible adverse effects were reported, this question was answered positively.
3. Are there indications for use of this substance in production systems for food producing animals?
   Use in livestock outside EU, availability of products for companion animals and use as a pesticide were evaluated to answer this question. In case national monitoring results or data from other EU MS showed that residues were found in other animals (mammals for bovine and porcine, poultry meat for eggs), this question was answered positively. For substances that are currently not monitored, the possible availability on the market was checked. For this purpose, the CBG-MEB database was checked to determine whether products are authorised for other animal species, such as companion animals as well as the internet (ebay.com and alibaba.com).

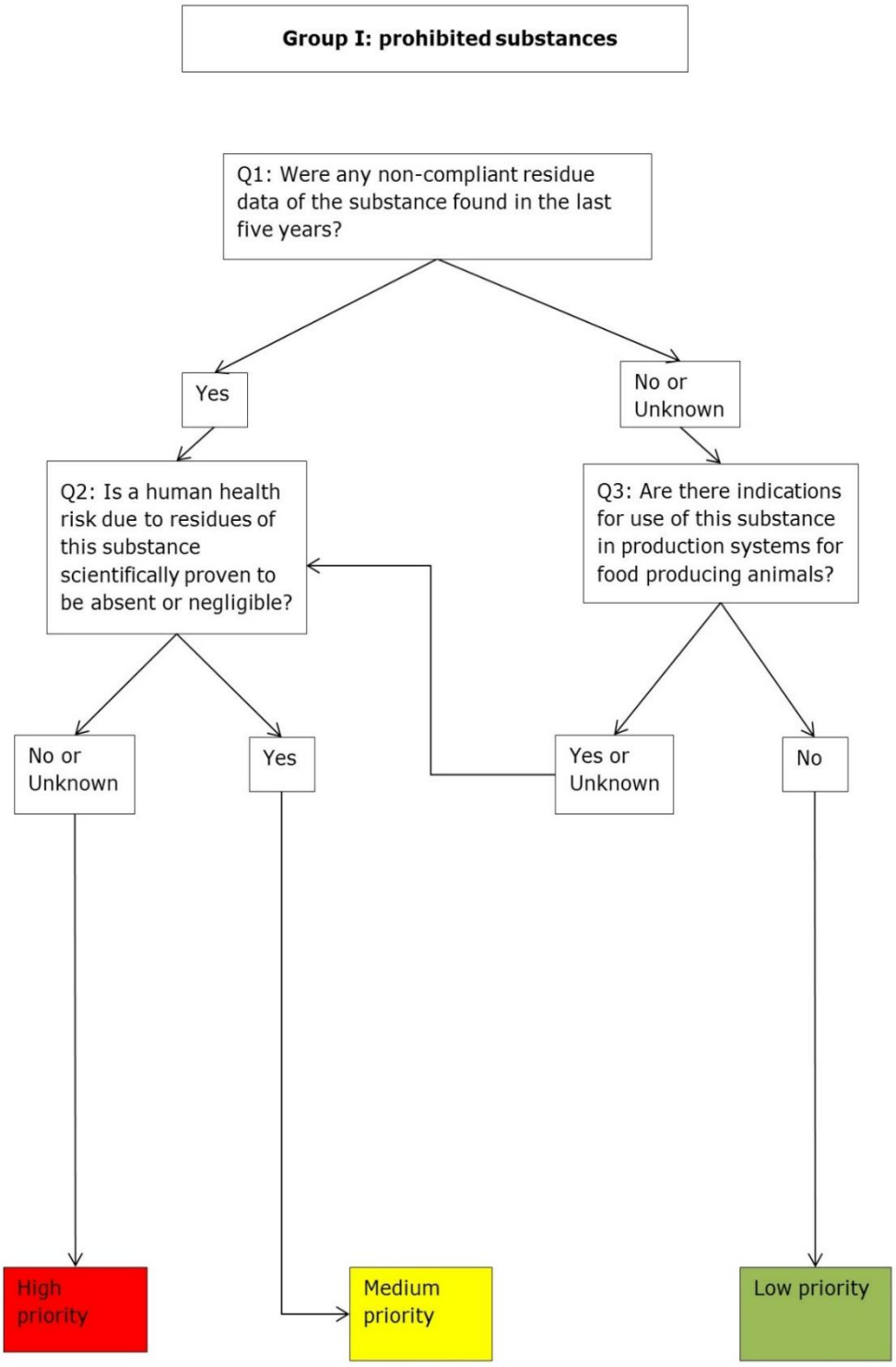

Figure 2. *Decision tree for unauthorised substances (updated from van Asselt et al. (2018)).*

Results and Discussion

In total, 68 antibiotics were evaluated, 51 antiparasitics and 29 NSAIDs in 8 different animal products. The results of the prioritization for authorised substances are indicated in Figure 3 and the results for unauthorised antiparasitics and NSAIDS are indicated in Figure 4.

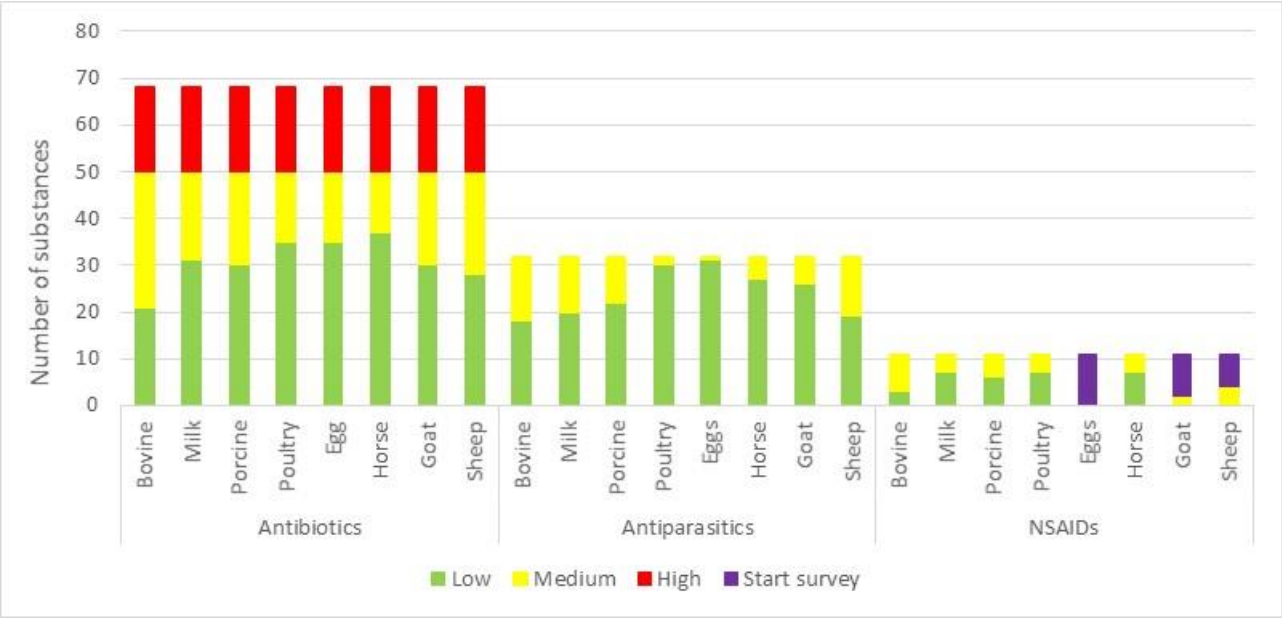

Figure 3. overview of prioritization for authorised antibiotics, antiparasitics and NSAIDS in various animal products

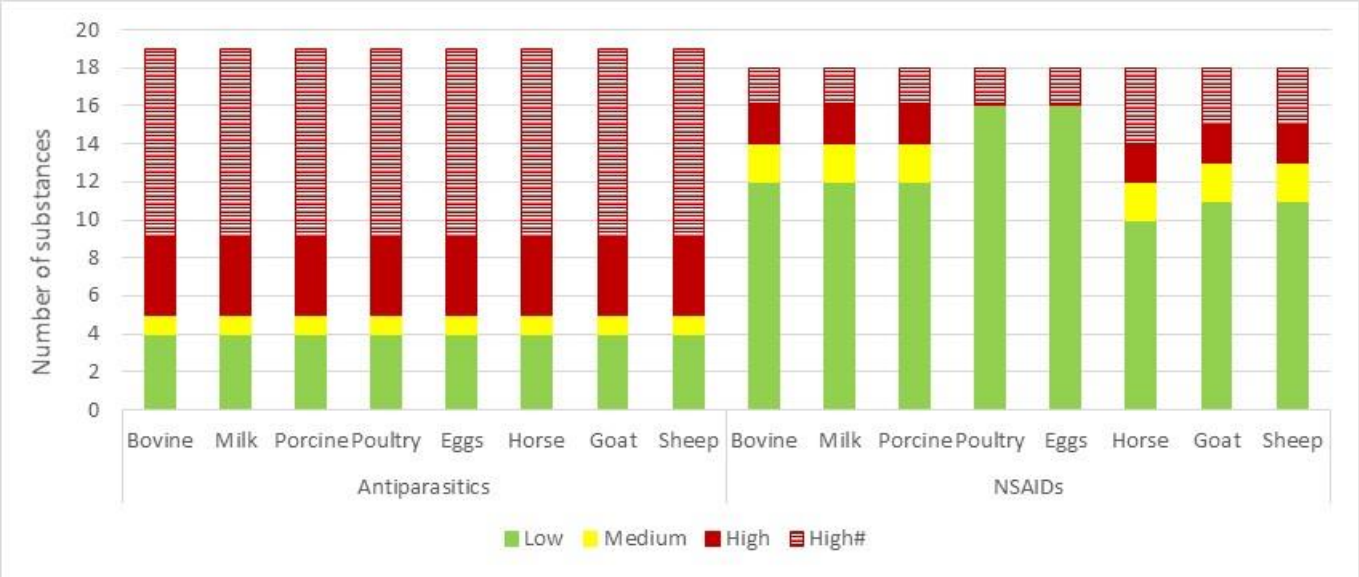

Figure 4. overview of prioritization for authorised antibiotics, antiparasitics and NSAIDS in various animal products

Figure 3 shows that in total 16% of the authorised substances obtained a high priority, 28% a medium priority and 53% a low priority. For 3% of the substances, data was lacking hampering a prioritization into low, medium or high. For those substances, it is recommended to start a survey. Figure 4 indicates that 15% of the unauthorised antiparasitics and NSAIDs obtained a high priority, 7% a medium priority and 45% a low priority.

As a worst case approach, some substances obtained a high priority due to a lack of data. These were indicated with a proviso #.

The decision trees were drafted based on the definition of risk as a combination of probability and severity. For the authorised substances, MRLs have been established indicated that their human health risks have been assessed. As a result, severity is seen as low and these substances are prioritized primarily based on potential occurrence into medium or low priority depending on non-compliances found or high use of VMPs. Only the antibiotics classified by WHO as HPCI antimicrobials will end up as high priority substances according to the currently applied methodology since these can cause human health risks due to potential antibiotic resistance. For the unauthorised substances, human health risks in most cases could not scientifically be proven to be absent or negligible. Depending on non-compliances found or indications of use, most substances thus were prioritized either as high or as low priority.

Since not all substances are currently included in the NRCP or in EU monitoring, data were lacking resulting in either a recommendation to start a survey (for authorised substances) or a worst case approach for unauthorised substances was used resulting in a high priority that was marked with an asterisk to indicate a lack of data. This was especially the case for authorised antiparasitics. Furthermore, specific data for goat and sheep were limited. Also, only a limited number of VMPs is available to treat these minor species, resulting in relatively frequent cascade application. Moreover, in many cases, a distinction between goat and sheep data was impossible, as these are often considered as a single category in reports on monitoring data or on VMP use. Therefore, given the lack of data, the prioritisation of substances for these animal species should be seen as a worst-case classification.

The research performed in this study showed that the decision trees work well to prioritise residues of veterinary drugs in animal products and could be used to prioritize all VMP residues for the NRCP.

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ORAL O31

ANTIMICROBIAL RESIDUES ALONG THE BROILER FEATHERS: A NON-INVASIVE SAMPLE MATRIX FOR MONITORING AND SURVEILLANCE OF VETERINARY TREATMENTS USED IN POULTRY

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Abstract

In poultry industry, feathers are a by-product, rich in nitrogenous matter, which are generally valorised and transformed into feather meal for feed (pets, aquaculture or pigs) or can be incorporated into fertilizers. Through its introduction into the food chain, the presence of antimicrobial residues in feathers represents a one-health issue as it may contribute to the selection of resistant bacteria. Within the framework of the French national plan Ecoantibio 2 plan, the behaviour of oxytetracycline, sulfadiazine and trimethoprim in feathers was investigated on treated broiler chickens (n = 6) by analysing the transfer and the distribution over time of these antibiotics residues in feathers after treatment. Feathers were sampled and segmented into four segments and then analysed using a previously validated LC-MS/MS method. The results confirmed that residues persist for a long time in the feather, up to 50 days after the end of the treatment, especially in the barbs in the upper part of the feather. For the first time, this study shows the distribution of antibiotic residues both in different parts of the feathers and at different times. In the future, feather analysis could be applied as a non-invasive monitoring of antibiotic residues in poultry.

Introduction

With the objective of a one-health approach, new non-invasive methods are being implemented to monitor the presence of antibiotic residues in the environment, and in human and animal health (San Martín et al. 2007; Cornejo et al. 2010; Heinrich et al. 2013; Berendsen et al. 2013; Jansen, Bolck, and Berendsen 2016; Cornejo et al. 2016; Haag, Marin, and Errecalde 2016; Cornejo, Pokrant, Krogh, et al. 2017; Cornejo, Pokrant, Carvallo, et al. 2017; Gajda et al. 2019). The feather could be a non-invasive means of monitoring the use of antibiotics in poultry production. Moreover, feathers, rich in nitrogenous matter, are valorised and transformed into feather meal. Feather meal is then used in animal feed (aquaculture, swine or pets) or incorporated in fertilizers. However, following a veterinary treatment in poultry farming, studies have shown the presence of antibiotic residues in the feathers while no residues were detected in the tissues (San Martín et al. 2007; Cornejo et al. 2010; Cornejo, Pokrant, Krogh, et al. 2017; Cornejo, Pokrant, Carvallo, et al. 2017; Pokrant et al. 2018; Maddaleno et al. 2019). In addition, an American study was conducted on feather meal marketed in Asia and the United States (Love et al. 2012). 2 to 12 antimicrobials were detected in all samples (Love et al. 2012). Feathers and feather meal are therefore potential reservoirs of antimicrobial residues and may contribute to the emergence of antibiotic resistance.

A few studies have focused their interest on feather segmentation (Berendsen et al. 2013; Jansen, Bolck, and Berendsen 2016; Gajda et al. 2019) in order to study the transfer of these residues into the feathers and to differentiate between the treatments administered. One of these studies focused on the feathers segmentation to analyse the barbs and rachis separately 20 days after the end of oxytetracycline treatment (Berendsen et al. 2013). Their results shown that the majority of oxytetracycline residues were found in the barbs (Berendsen et al. 2013).

The objective of the study presented in this paper is to better understand the behaviour and distribution of antibiotic residues in the feather. Our study was carried out on antibiotics commonly used in French poultry farms (oxytetracycline: OTC, sulfadiazine: SDZ and trimethoprim: TMP). Feathers from chicken were collected, segmented and analysed at different times during the 55-day study.

Materials and Methods

Animal treatment

With the approval of the Ethics Committee according to French and European legislation (Directive 2010/63/EU), an experimental study was conducted on eighteen Ross 308 broilers. For the duration of the study, the chickens were had *ad libitum* access to drinking water and antibiotic-free feed the absence of which had been previously checked using an internal validated method (Gaugain et al. 2020). After a seven-day acclimation period, the four weeks old animals were randomly divided into three groups (I, II and III), observing male-female parity. All groups, consisting of six broilers, were placed in cages with an elevated wire floor and faeces collection trays to avoid any external contamination of the feathers via the faeces. Group I was treated with a veterinary speciality composed of a mixture of SDZ/TMP at a therapeutic dose of 25/5 $mg.kg^{-1}$ body weight, respectively. Group II was treated with a veterinary speciality of OTC at the therapeutic dose of 20 $mg.kg^{-1}$ body weight. Group III was a negative control group *i.e.* the animals in this group were untreated and not exposed to antibiotics for the fifty-five day study. All animal were treated with antibiotic once a day for five consecutive days by oral gavage except control animals (group III) which were given water without antibiotics. At the end of the study, all animals were slaughtered after electronarcosis.

Samples collection and storage.

One sample per group (a few wing feathers from each animal in each group) was collected in a plastic bag at different times: D0 (start of antibiotic treatments), D1, D4 (end of antibiotic treatments), D5, D6, D7, D11, D14, D21, D29, D46 and D55 (day of slaughter). Wing feathers, selected for their size and to have sufficient matrix to study the distribution of antibiotic residues in the feather, were segmented. All samples were stored at -80°C until analysis.

Chemicals and reagents

The oxytetracycline, sulfadiazine and trimethoprim standard was obtained from Sigma Aldrich (Saint-Quentin-Fallavier, France) with a purity higher than 90%. The 4-epi-oxytetracycline standard was from Fisher Scientific (Illkirch-Graffenstaden, France), with a purity greater than 80%. The internal standard tetracycline-d6 was provided from C.I.L. Cluzeau (Saint-Foy-La-Grande, France), sulfadiazine-13C6 was obtained from Witega (Berlin, Germany) and trimethoprim-d9 was from Sigma Aldrich. Acetonitrile (ACN), methanol (MEOH), disodium ethylenediaminetetraacetic dihydrate (Na_2EDTA), sodium hydroxide (NaOH) and sodium disulphate (Na_2SO_4) were from Fisher Scientific. Trisodium citrate dihydrate was provided by Sigma Aldrich. Formic acid, isooctane, ammonium acetate and sodium chloride (NaCl) were obtained from Merck (Darmstadt, Germany). Dimethyl sulfoxide (DMSO) was supplied by VWR (Fontenay-sous-Bois, France). Milli-Q water was prepared using a Milli-Q Gradient purification system (Millipore, Yvelines, France). All organic solvents used were of HPLC grade. Filters were Millex – HV PVDF 0.22 µm from Millipore.

Stock standards solutions of sulfadiazine, oxytetracycline and 4-epi-oxytetracycline were individually prepared at 0.5 $mg.mL^{-1}$ in MeOH. Stock standard solution of trimethoprim was dissolved at 0.5 $mg.mL^{-1}$ in MeOH and water (50/50, v/v). The stock internal standard of tetracycline-d6 was prepared in MeOH at 0.1 $mg.mL^{-1}$ and stocks internal standards solutions of sulfadiazine-13C6 and trimethoprim-d9 were prepared in MeOH at 0.5 $mg.mL^{-1}$. Stock standard solutions were all stored at -18°C and were stable for six months (Gaugain, Chotard, and Verdon 2013). An intermediate solution was prepared at 100 $\mu g.mL^{-1}$ by diluting the stock standard solution of sulfadiazine, trimethoprim, oxytetracycline and 4-epi-oxytetracycline in MeOH. Working solutions were prepared at 0.1 $\mu g.mL^{-1}$ and 10 $\mu g.mL^{-1}$ the day of analysis by diluting the intermediate solution to 100 $\mu g.mL^{-1}$ in purified water. An internal standard solution of sulfadiazine-13C6, triméthoprime-d9 and tetracycline-d6 in a mixture was daily prepared in purified water.

Feathers segmentation

All feather samples were segmented into nine segments. Firstly, the calamus was separated from the rest of the feather to be analysed separately. Next, the feather was segmented into four segments of equal length (Figure 1). For each segments, the rachis was separated from the barbs using scissors. At each sample time, all feather segments of the animals in a given group were pooled in order to have enough matrix to perform the analysis. The rachis A an B were systematically pooled due to their very low weight.

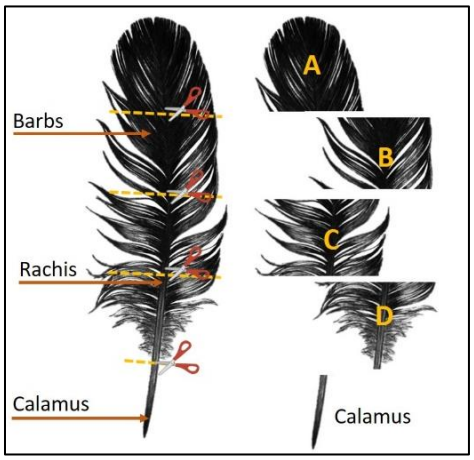

Figure 5. Nomenclature and overview of a feather.

All samples were then previously ground with a ball mill MM400 from Retsch (Haan, Germany) prior to the extraction of SDZ, TMP, OTC and its epimer.

Extraction and LC-MS/MS analysis

The analysis of feather segments was based on a method previously developed and validated for a dosage range in feathers between 0.05 and 0.2 $mg.kg^{-1}$ (Dréano et al. 2021). This method is based on a solid/liquid extraction followed by the salting out technique. This method was validated again for a dosage range between 0.02 and 4.0 $mg.kg^{-1}$ for the analysis of our samples.

Residues of SDZ, TMP, OTC and 4-epi-OTC detected in the different feather segments were quantified using a calibration curve performed with calibration standards (CS) covering the validated dosing range. For each day of analysis, quality control (QC) and CS were performed on spiked blank feather samples. QCs were performed to validate the analysis and quantification of the day.

Results and Discussion

Results of the sulfadiazine and trimethoprim segmentation

The measured concentrations of SDZ and TMP in the calamus, rachis and barbs of group I are presented in Table 1. The different segments of the rachis and barbs were summed respectively at each time point.

The results obtained show a persistence of SDZ and TMP residues in the wing feathers until 51 days after the end of the treatment and in particular in the barbs where, on average, more than 80% of the residues of SDZ and TMP were found (Table 1). The observation made in the study by Berendsen and his team for OTC (Berendsen et al. 2013) was also found in the present study for SDZ and TMP. The substances are predominantly present in the barbs after treatment of the animals.

It should be noticed that the withdrawal period for edible foodstuff is 12 days after treatment before the animal is slaughtered. In the event of a disease in a poultry farm, the farmer may have to treat his animals and then slaughter them after this regulatory withdrawal period. The collected feathers are then processed into feather meal. However, according to our results, high concentrations of SDZ and TMP were found after 17 days after the end treatment in the barbs, close to 270 $mg.kg^{-1}$ and 17 $mg.kg^{-1}$, respectively. This potentially increases the risk of transmission of antibiotic residues in animal feed.

Table 1. SDZ and TMP distribution along the feather in the barbs, rachis and calamus of group I.

	Time (days)	Calamus (mg.kg^{-1})	Rachis (mg.kg^{-1})	Barbs (mg.kg^{-1})
SDZ	1	3.46	1.79	75.83
	4	7.57	16.07	242.06
	5	10.11	17.74	249.83
	6	8.70	16.79	219.69
	7	3.89	34.06	417.94
	11	0.97	18.38	187.91
	14	0.52	22.18	243.93
	21	0.33	13.85	274.54
	29	0.04	15.62	62.07
	46	0.01	2.91	5.89
	55	<LOQ	0.06	1.28
TMP	1	1.60	0.38	15.15
	4	3.94	3.96	42.30
	5	6.84	4.81	50.39
	6	3.84	3.86	28.37
	7	1.52	8.28	42.35
	11	1.08	7.21	21.92
	14	0.70	8.86	29.59
	21	0.59	8.51	17.29
	29	<LOQ	1.61	22.16
	46	<LOQ	<LOQ	2.84
	55	<LOQ	<LOQ	0.23

Subsequently, we focused on the different barb segments (A, B, C and D) to better understand the migration of SDZ and TMP residues along the feather. The relative distribution of each segment was calculated in relation to the sum in all barb segments (Figure 2). Indeed, at D1, more than half of the SDZ and TMP residues are located in segments C and D, nearest the calamus. From D7 to D21, more than 60% of the SDZ and TMP residues are located in the middle of the feather and more precisely in the B and C segments. From D29 to D55, SDZ and TMP residues are mainly located at the apex of the feather, *i.e.* in the A segment. According to the literature, other substances like enrofloxacine were also located at the apex of the feather on day 34 (Jansen, Bolck, and Berendsen 2016). SDZ and TMP residues appear to migrate along the feather as it grows. However, unlike hair, feathers do not grow in a linear progression, which makes it difficult to date the antibiotic treatment (Vargas et al. 2020).

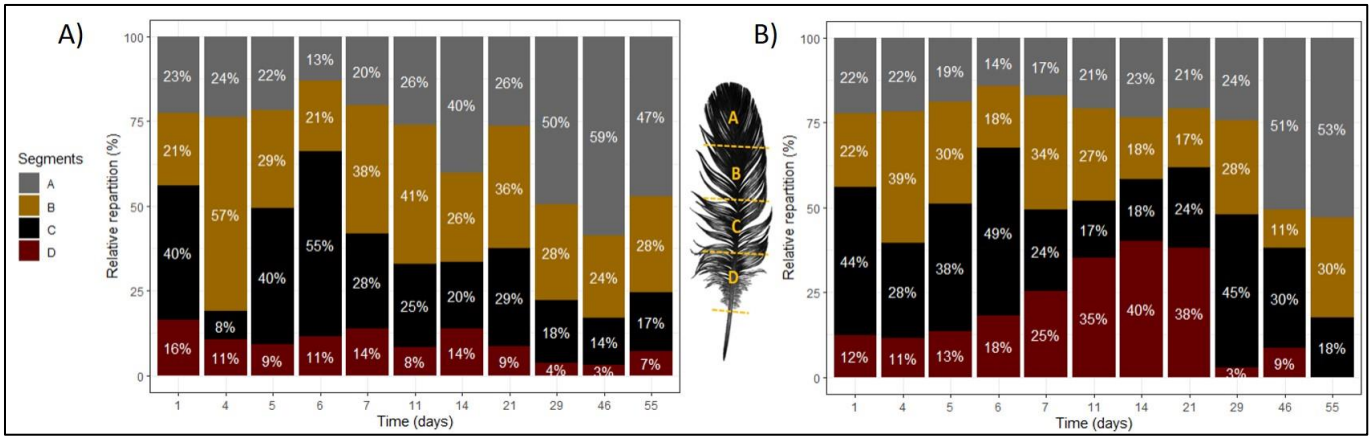

Figure 6. Relative distribution of SDZ (A) and TMP (B) along the feather as a function of time in the barbs of group I.

Results of the oxytetracycline segmentation

The different measured concentrations of the group II of the rachis segments and the barb segments (A, B, C and D) were summed respectively for each sample time to observe the part of the feather with the highest concentration of OTC (Table 2). The results show that the OTC residues are mainly in the barbs, just as for SDZ and TMP. However, OTC persisted in the wing feathers for a shorter time period than SDZ and TMP, *i.e.* 24 days after the end of the treatment versus 50 days. For information, the epimer of OTC, 4-epi-OTC, was measured below the limit of quantification throughout the study.

The withdrawal period after treatment with OTC before slaughter is 7 days. OTC is known to be eliminated faster in the body than SDZ and TMP. However, at 14 days, 4.16 $mg.kg^{-1}$ of OTC in the barbs were measured.

Table 2. OTC distribution along the feather in the barbs, rachis and calamus of the group II

Time (days)	Calamus ($mg.kg^{-1}$)	Rachis ($mg.kg^{-1}$)	Barbs ($mg.kg^{-1}$)
1	0.04	0.06	4.25
4	0.10	1.12	16.13
5	0.10	0.96	13.64
6	0.06	0.95	9.12
7	0.09	0.49	6.98
11	0.03	0.19	4.40
14	<LOQ	0.20	4.16
21	<LOQ	0.08	1.47
29	<LOQ	0.06	1.08
46	<LOQ	<LOQ	<LOQ
55	<LOQ	<LOQ	<LOQ

As with SDZ and TMP, the focus was on the different barb segments (A, B, C and D) of the feather in this study. The relative distribution of each segment was calculated in relation to the sum in all barb segments (Figure 3). Less than 20% of OTC residues were located in the lower part of the feather, *i.e.* the D segment, which has also been observed by a team from the Netherlands(Berendsen et al. 2013). Throughout the study, it was observed that the OTC was equally distributed between the A, B and C segments (30% each). The OTC therefore does not appear to migrate along the feather during its growth. In a contrast, a rapid migration of doxycycline, another tetracycline, along the feather has been observed in another study (Gajda et al. 2019). Indeed, the molecule was mainly found at the apex of the feather at days 8, 15, 18 and 22 (Gajda et al. 2019).

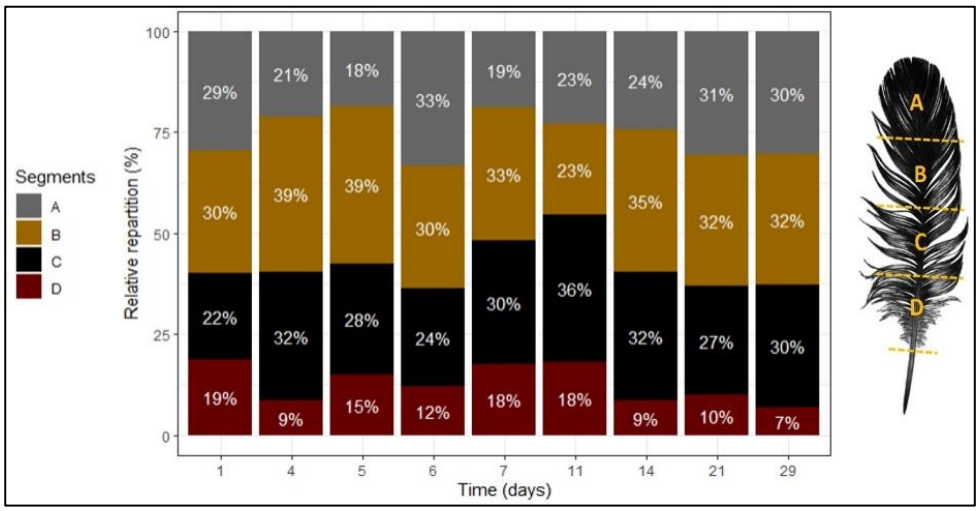

Figure 7. Relative distribution of OTC along the feather as a function of time in the barbs of group II.

Conclusion

The feather could be used for the control and monitoring of antibiotic residues during veterinary treatments in poultry farms. In addition, the feather has the advantage of being a non-invasive means to perform these controls and monitoring. The study presented in this paper shows that there is a migration of the majority of the target residues into the barbs. However, as the growth of the feathers is not linear and the distribution of antibiotics is not the same within the feather depending on the antibiotics administered, it seems to be more relevant to consider the whole feather for control monitoring. This study also shown that high concentrations may be found several days after the regulatory withdrawal period established for edible foodstuff. The high levels of SDZ, TMP and OTC found in the feathers indicate that antibiotic residues may persist for a long time in the feather compared to edible foodstuffs, potentially increasing the risk of the development of resistant bacteria.

Funding

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ORAL O32

PARACETAMOL LEVELS IN TREATED AND UNTREATED VEAL CALVES, AN UPDATE

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Abstract
Recently, residues of paracetamol (4-acetaminophen) have been found in urine of slaughter animals. To investigate its possible origin, a 7 week animal experiment was performed with 5 groups of 4 calves fed different amounts of roughage, and treated or not with paracetamol. Group 1 (control): new-born calves were fed, after the first days of colostrum, solely dairy based calf milk replacer (CMR). The animals from the other groups were 2-3 weeks of age at the start of the experiment and were fed the same CMR. Group 2 was administered oral Pracetam® 30 mg acetaminophen/kg Body Weight (BW) in the CMR, 5 days a week at alternate weeks, with maximum roughage. Group 3 was fed legal minimum roughage. Group 4 received the maximum roughage. Group 5 was administered human paracetamol pills 30 mg/kg BW once a week at weeks 2-6 with maximum roughage. The animals were housed in separate pens on slatted rubber floors without bedding. The controls were housed in a different room than the other animals. Urine was sampled once in the acclimatization period and for 5 days after treatment. At week 2, 4 and 6 at respectively 1, 2 and 3 days after treatment, one animal from groups 2 and 5 was sacrificed. An additional animal from group 4 was sacrificed at week 4, the rest of the animals at day 43. Urine, muscle, liver and kidney were analysed for residues of paracetamol and its metabolites. It appeared that controls and the low and high roughage animals had almost no 4-acetaminophen (<3 µg/L) in the urine and low levels of metabolites, whereas high roughage had at slaughter higher levels of metabolites than low roughage. Pracetam® treated animals showed high levels 4-acetaminophen (up to 130.000 µg/L) and metabolites during treatment, and lower levels (2800 µg/L) at slaughter. In the paracetamol treated animals 4-acetaminophen and metabolites were detectable during treatment (up to 130.000 µg/L) and until 7 days after treatment (110 µg/L). 4-Acetaminophen and metabolites were only present in tissues of treated animals, during treatment (11-14.800 µg/L) and a few days after treatment (7-55 µg/L). A striking effect was the decrease in concentration of 4-acetaminophen and metabolites in time during treatment in the tissues. Liver enzyme induction may provide a possible explanation for this.

Conclusions

We aimed to investigate the possible source of paracetamol in veal calf urine, kidneys and meat and to evaluate the possibility to distinguish therapeutic treatment from “background” levels. The results show that acetaminophen is present in a dairy based calf milk replacer and roughage in low levels and may contribute to the presence of 2 and 4-acetaminophen in urine in calves, which was detected in some untreated calves. Aniline levels in feed were very low or not detected and are unlikely to contribute to formation of 4-acetaminophen in urine. In treated calves predominantly 4-acetaminophen was found in high levels, whereas no or low levels of 2-acetaminohen were found. In the tissues liver, kidney and muscle only residues were found in treated animals. Further research is needed in older animals to ascertain if this is also true for animals older than 7 weeks.

Urine:

- Controls and low roughage: almost no 4-acetaminophen and low levels of metabolites
- High roughage: low levels of 4-acetaminophen. At slaughter: levels of metabolites higher than low roughage

- Pracetam®: 4-acetaminophen + metabolites during treatment, at slaughter. 3 days after last treatment low levels
- Paracetamol: 4-acetaminophen + metabolites during treatment and until 7 days after treatment

Tissues

- Only 4-acetaminophen and metabolites present in tissues of treated animals, during treatment and a few days after stopping treatment
- Levels of 4-acetaminophen and metabolites in tissues reduce in time during treatment, liver enzyme induction possible explanation

Enzyme induction

- (UGT) activity increased in 4-acetaminophen treated animals and was higher in high roughage animals as compared with low roughage animals

Since all the 4-acetaminophen treated animals also had high roughage diets the two parameters could not be separated.

ORAL O33

SURVEY ON SALICYLIC ACID RESIDUES IN MILK

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Abstract
Salicylates are non-steroidal anti-inflammatory drugs (NSAIDs) and authorised for animal treatment. Salicylic acid (SA) is the basic substance of the salicylates. In Regulation (EU) No 37/2010, maximum residue limits (MRL) are laid down for SA in different matrix/species combinations.
In plants, SA is a secondary metabolite known to participate in the defence against physical, chemical and biological damage. Animal consumption of those plants may lead to relevant SA amounts in tissues and liquids. SA residues are often present in milk samples at low concentrations. These residues can exceed the MRL of 9 µg/kg, even though a treatment with this drug was unlikely or not indicated. In order to get an overview of possible residue levels in milk of untreated animals, 141 milk samples from different EU countries and origins were analysed.
The results of the survey show that different parameters influence the determined SA residues, and will be presented and discussed in the poster.

Keywords: Salicylic acid, MRL, milk

POSTER PRESENTATIONS

POSTER P1

NEW APPROACHES FOR VALIDATION: INTER-LABORATORY STUDY FOR A CONFIRMATORY METHOD FOR BETA-AGONISTS, IN LIVER ON BASIS OF A FACTORIAL DESIGN

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Abstract
Regulation (EU) 2017/625, Article 34, defines requirements for methods used in official residue control. It belongs to the tasks of EURLs to coordinate the application of these methods by National Reference Laboratories.
Within the framework of the EURL work programme, a new approach was tested, which aimed at promoting the harmonisation of residue control by supporting NRLs in implementation of new methods through a series of measures: (1) by training; (2) by providing standards and reference materials; (3) by organising a multi-laboratory validation study based on experimental plans; (4) by providing individual validation protocols and a validation protocol for the EURL reference method.
The results of this study are a) an in-house validation according to Regulation (EU) 2002/657 for each participating laboratory on the basis of the criteria approach; b) robust overall method performance data and identification of main contributions to measurement uncertainty; c) fulfilment of requirements of Regulation (EU) 2017/625 with regard to coordination of the application of control methods.
Details and advantages of this approach will be discussed at the example of an inter-laboratory study with 12 participating laboratories (8 NRLs, 4 German RFLs) of a method for 24 betaagonists in liver, based on an international factorial design.

Keywords beta-agonists , inter-laboratory study , validation

POSTER P2

LC-MS/MS METHOD FOR THE QUANTIFICATION OF TIAMULIN, TRIMETHOPRIM, TYLOSIN, SULFADIAZINE AND SULFAMETHAZINE IN MEDICATED FEED

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Abstract
Since 2006, when the European Commission banned the use of antibiotic growth promoters, increased use of medicated feedingstuffs has been observed in animal production. This paper reports the development of a selective and sensitive new method for simultaneously analysing five antimicrobial agents (tiamulin, trimethoprim, tylosin, sulfadiazine and sulfamethazine) in one single extraction protocol in medicated feed for different animal species (poultry, swine and cattle) using only liquid extraction and liquid chromatography with tandem mass spectrometry. Antibacterial substances were extracted with the use 0.1% formic acid in acetonitrile, next centrifuged, diluted in Milli-Q water, filtered and analysed by high performance liquid chromatography coupled to tandem mass spectrometry. The separation of the analytes was performed on a biphenyl column with a gradient of 0.1% formic acid in acetonitrile and 0.1% formic acid in Milli-Q water. The validated method was successfully applied to the medicated feeds obtained from the interlaboratory studies and feed manufactures from Spain. In these samples tiamulin, tylosin and sulfamethazine were detected at the concentration levels declared by the manufacturers. The developed method can therefore be successfully used to routinely control the content and homogeneity of these antibacterial substances in medicated feed.

Keywords: antibacterial substances, LC-MS/MS, medicated feed

POSTER P3

ANTIBACTERIAL SUBSTANCES IN NATURAL AND ORGANIC FERTILIZERS AS A POTENTIAL PROBLEM OF ENVIRONMENTAL CONTAMINATION

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Abstract
Veterinary antibiotics are used globally to treat diseases and to protect the health of animals. Most pharmaceutical active ingredients can be excreted by the medicated animals, and depending on the structure up to 90% of the initial compound can be found in the urine and/or faeces afterwards. However if animals are treated, residues of the antibiotic substances can frequently be detected in manure due to poor absorption of the respective substance in the animal gut or unmetabolized extraction. In this paper, a simple and robust method is proposed for the simultaneous analysis of oxytetracycline, tetracycline, chlortetracycline, doxycycline, enrofloxacin, ciprofloxacin, trimethoprim, tiamulin and tylosin from faeces, liquid manure and digestate. The sample preparation was done by using ultrasonic extraction with McIlvaine-Na_2EDTA buffer solution and purified by SPE (Strata-X-CW cartridges) and analysed by HPLC-MS. The analysis of 38 samples revealed that 13 samples (34.2%) were positive for the presence of doxycycline, enrofloxacin, oxytetracycline and tiamulin. The obtained results in the presented study demonstrated that veterinary antibacterial substances can lead to the contamination of agricultural soils via fertilization with animal manure.

Keywords: antibacterial substances, HPLC-MS analysis, natural and organic fertilizers

POSTER P4

DEVELOPMENT AND VALIDATION METHOD FOR DETECTION AND DETERMINATION OF FORMALDEHYDE IN FEEDS AND SILAGE BY HPLC-DAD DETECTOR AFTER PRECOLUMN DERIVATIZATION

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Abstract
Formaldehyde had been used for many years as a feed additive as an additive from the group of technological additives “and the functional group” preservatives.On 7th February 2018, Commission Implementing Regulation (EU) 2018/183 was issued refusing to authorize the use of formaldehyde as a feed additive belonging to the functional groups ‘preservatives’ and ‘hygiene improving substances’. Due to the ban on the use of formaldehyde, an analytical method based on the HPLC-DAD technique was developed for the purposes of official control. The method is based on the determination of the product of chemical reaction between formaldehyde and 2,4-dinitrophenylhydrazine. In 2018-2021 a total of 194 samples were analysed for detection and determination formaldehyde by HPLC-DAD technique. Our study showed that only 1 (0.51%) tested feed sample for milk cows was contaminating formaldehyde. A concentration of formaldehyde in this sample was 59.8 mg/kg. Our study showed that feed and silage produced in Poland was free from formaldehyd using. Good manufacturing practice and good hygiene practice allows for a low level of contamination of feed and silage of Salmonella, so that formaldehyde is not used as a preservative for feed and silage in Poland.

Keywords: feed and silage, formaldehyde, HPLC-DAD

POSTER P5

STABILITY OF ANTIBIOTIC RESIDUES IN INCURRED MEAT SAMPLES DURING FROZEN STORAGE

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Abstract
Official control laboratories routinely check the presence of antibiotic residues in food of animal origin to verify that their levels are below the Maximum Residue Limits (MRLs) set by the European Union. If residues exceed their MRLs, a legal proceeding is initiated and it may be required to reanalyze samples after several months, according to the Italian legal system. To allow this, samples are normally stored under frozen conditions, though little is known about the stability of residues over time. Here, we have tested the stability of two β-lactams residues, Amoxicillin and Cefquinome, and a sulfonamide residue, Sulfadimethoxine, in incurred meat samples upon storage at -24±6°C or -80±15°C for 6 months (Amoxicillin), 12 months (Cefquinome) and 24 months (Sulfadimethoxine). We show that, while the concentration of Cefquinome remained constant at both storage temperatures, the concentration of Sulfadimethoxine decreased over time, with decay levels reaching 28% at -24°C and 15% at -80°C, respectively, after 2 years.
The samples incurred with Amoxicillin showed a rapid and constant degradation when stored at -24°C, resulting in the residue concentration being more than halved after six months. Conversely, storage at -80°C allowed to maintain the stability of this analyte. Our results show that the storage temperature of frozen samples may become a critical factor for the detection of antibiotic residues.

Introduction
Antibiotics and chemotherapy drugs are widely used in veterinary medicine for treating bacterial infections.

Within the European Union, member states are required to implement a National Residue Control Plan to organize and monitor the testing of meat and food of animal origin for the presence of several groups of residues of veterinary drugs and contaminants (Directives 96/22/EC and 96/23/EC). As set by the national plan, official control laboratories routinely analyse the concentration of residues in meat and animal-derived food to verify that the residue levels are below the Maximum Residue Limits (MRLs) set by the European Union (EU Regulation 37/2010).

In Italy, when residues are found to exceed their respective MRLs, a legal proceeding is initiated, and this may require samples to be analyzed again to confirm the results, even after several months. Therefore, samples are normally stored under frozen conditions for long time and, consequently, it becomes essential to ensure that the residue levels remain stable.

However, most reports on the stability of veterinary drugs have tested drugs in tablets or in solution (Y.Liang *et al*., 1998; M.Cerezo *et al.* 2001; B.J.A. Berendsen *et al.* 2011; S. Pellicciotti *et al.* 2014), and not much is known about the stability of drug residues in incurred matrices over time.

β-lactams are a group of antibiotics known to be chemically unstable, but so far only the stability of the β-lactam Ampicillin has been tested in naturally contaminated (incurred) meat samples frozen at different temperatures (E.Verdon *et al*., 2000). Other studies have investigated the stability of penicillins and others antibiotics in animal tissues but the experiments were based on spiked samples and not incurred samples (G.K.Thomas *et al.* 2001; B.J.A. Berendsen *et al.* 2011).

In this study, we have tested the stability of two β-lactams residues, Amoxicillin and Cefquinome, and a sulfonamide residue, Sulfadimethoxine, in incurred meat samples upon storage at -24±6 °C and -80±15°C, respectively. We show that, while normal storage at -24±6°C may be sufficient for Cefquinome and Sulfadimethoxine, storage at -80±15°C is required for Amoxicillin.

Materials and Methods

Incurred Tissues

A male calf was dosed by intra-muscular injection of Cefquinome on four successive days at a dose of 2 mg Kg^{-1} bodyweight, to mimic an antibiotic treatment that may be performed as part of routine veterinary therapy. Another male calf was dosed by intra-muscular injection of Amoxicillin on three successive days at a dose of 15 mg Kg^{-1} bodyweight. A single male swine was orally dosed at a rate of 100 mg Kg^{-1} bodyweight of Sulfadimethoxine for four days in the drinking water. One day after last administration (DALA), the animals were euthanised and muscle tissues were harvested.

Sample collection and storage

The muscle tissues from each thigh were immediately homogenized and divided into multiple aliquots weighed in polypropylene tubes.

Triple aliquots for Amoxicillin and Cefquinome incurred samples, respectively, were immediately analysed (DALA, t=0). Half of the remaining aliquots were stored in the freezer at -24±6°C and the other half was stored at -80±15°C. Samples were analysed monthly for up to 6 months. For Cefquinome, incubation was prolonged for 6 further months, with samples analysed bimonthly.

Sulfadimethoxine incurred samples were aliquoted and frozen at -80±15°C for 3 days. Then, 3 aliquots were thawed and analysed (t=0). Remaining aliquots were either kept -80±15°C or transferred to -24±6°C and incubation was continued for up to 24 months. Aliquots were analysed monthly.

Sample extraction

All analytical work was carried out using protocols for the determination of veterinary drug residues that are routinely used in our official control laboratories.

Briefly, samples for the analysis of Amoxicillin and Cefquinome were prepared as follows: 5 g of tissue were spiked with internal standards (Amoxicillin-d4 and Ampicillin-d5, respectively) and extracted with 10 mL of mixture acetonitrile-water (80:20 v/v). Samples were then homogenized with Ultraturrax, shaken on a horizontal shaker for 10 minutes, placed in an ultrasonic bath for 10 minutes and centrifuged. The extraction was repeated with 5 mL of acetonitrile. The two aliquots of extracts were combined and the volume was raised up to 20 mL with acetonitrile. 2 mL of extract were evaporated to dryness and redissolved in Ammonium acetate solution. Samples were subsequently ultra-centrifuged and transferred in a vial for LC-HRMS analysis.

Samples for the analysis of Sulfadimethoxine were prepared using the following procedure: 10 g of homogenized tissue were spiked with internal standard (Sulfadoxin) and extracted with 30 mL of ethyl acetate and 50 µL Acetic acid and dried with sodium sulphate. Samples were shaken with a vortex and a horizontal shaker and then centrifuged. The extraction was repeated with 30 mL of ethyl Acetate. Extracts were combined and evaporated to dryness by rotavapor. The residue was solubilized with 30 mL of HCl 0.5 N and degreased with n-hexane. Then, a Solid Phase Extraction (SPE), using Strata X-C 200 mg/6 mL cartridges, was carried out. After reconstitution in 1 mL of acetate buffer, the extract was transferred in a vial for HPLC-DAD analysis.

Sample analysis

Samples for Amoxicillin and Cefquinome were analysed by LC-HRMS using a Thermo Scientific ACCELA 1250 LC system connected to a Thermo Scientific LTQ Orbitrap XL. Separation was carried out on a Poroshell 120 EC-C18 2.7µm Column (100 x 3.0 mm, using 0.1% formic acid – methanol gradient). The extract was analysed in positive mode.

Samples for Sulfadimethoxine were analysed by HPLC-DAD using an Agilent 1200 system connected to an Agilent SL diode-array detector (DAD). Separation was carried out on a Luna C-8 Column (250 x 3.0 mm, using acetate buffer pH 4.5 – acetonitrile gradient).

Data analysis

For Amoxicillin and Cefquinome, the residue concentration was obtained from the ratio between the area peak of the analyte and the area peak of the internal standard (Amoxicillin-d4 and Ampicillin-d5, respectively) added to each analysis. Values shown are normalized to the recovery at t=0 (100%). The DALA samples were used for the determination of the initial drug concentration in meat tissue (t=0), average of three replicates. Values obtained for the subsequent time points were normalized to t=0 point (100%). For Sulfadimethoxine, the concentration was calculated by a single level point prepared in solvent and corrected by the recovery of the standard process. The averaged value of the three t=0 aliquots was used to normalize other samples.

Results and Discussion

Bovine meat samples incurred with Cefquinome were analysed monthly or bimonthly upon frozen storage for up to 12 months at -24±6°C or -80±15°C, respectively (Table 1). The residue levels did not appear to be affected by the storage temperature or time (Figure 1).

Swine meat samples incurred with Sulfadimethoxine were analysed monthly for up to 24 months of frozen storage (Table 1). In this case, the observed levels of residue were stable for the first two months of incubation both at -24±6°C and -80±15°C (Figure 2). After the first couple months the residue levels of Sulfadimethoxine decreased constantly and dropped down to 28% at -24°C and 15% at -80°C, respectively, after 2 years (Figure 2).

Bovine meat samples incurred with Amoxicillin were analysed monthly for up to 6 months of frozen storage (Table 1). Samples stored at -24±6°C showed a rapid and constant degradation of the analyte, with the levels of recovered residue decreasing to 40% of the initial value in six months (Figure 3). On the contrary, samples stored at -80±15°C did not show any degradation (Figure 3).

Table 1. Levels of Cefquinome, Sulfadimethoxine and Amoxicillin residues in incurred meat samples upon storage of the samples at -24±6°C and -80±15°C, respectively. Aliquots were analysed monthly (or bi-monthly) for up to 24 months. Values shown are the average of three replicates and are normalized to the recovery at t=0 (100%).

Residue	Temp.	Time (months)																		
		0	1	2	3	4	5	6	7	8	9	10	11	12	14	16	18	20	22	24
Cefquinome	-24°C	100	114	111	86	111	106	106	-	92	-	106	-	131						
	-80°C	100	109	111	97	118	117	110	-	103	-	113	-	126						
Sulfadimethoxine	-24°C	100	100	98	89	92	102	83	85	77	85	89	83	82	80	79	84	87	79	72
	-80°C	100	101	105	90	96	100	95	91	93	97	88	94	94	93	89	86	96	83	85
Amoxicillin	-24°C	100	84	68	60	47	42	41	-	-	-	-	-	-						
	-80°C	100	97	92	92	102	92	110	-	-	-	-	-	-						

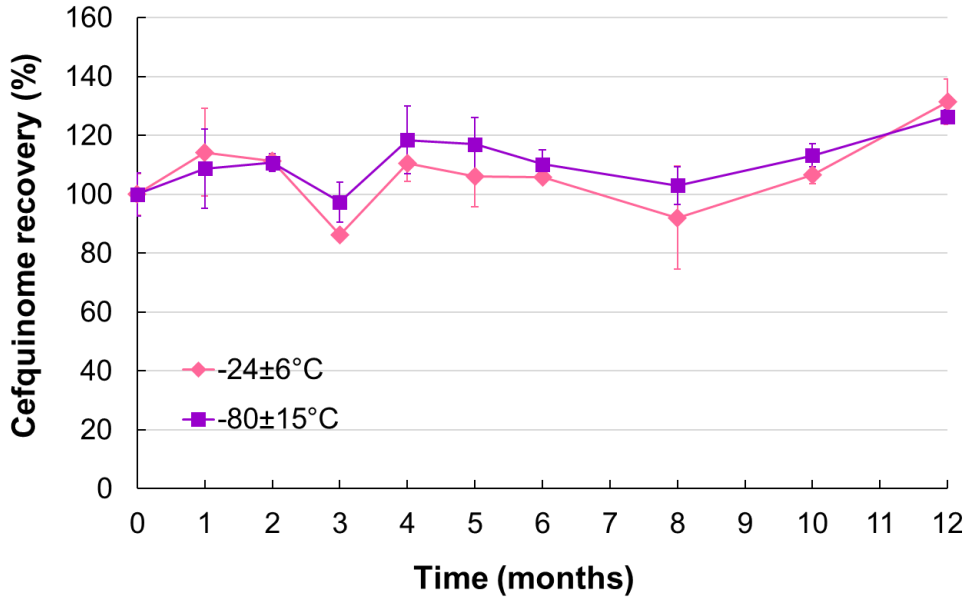

Figure 1. Recovery of Cefquinome over time in meat samples frozen at -24±6°C and -80±15°C, respectively. Recovery was calculated as the ratio between Cefquinome and Ampicillin-d5, as detailed in Materials and Methods. Values shown are normalized to the recovery at t=0 (100%) and are the average of three replicates (error bars indicate the standard deviation).

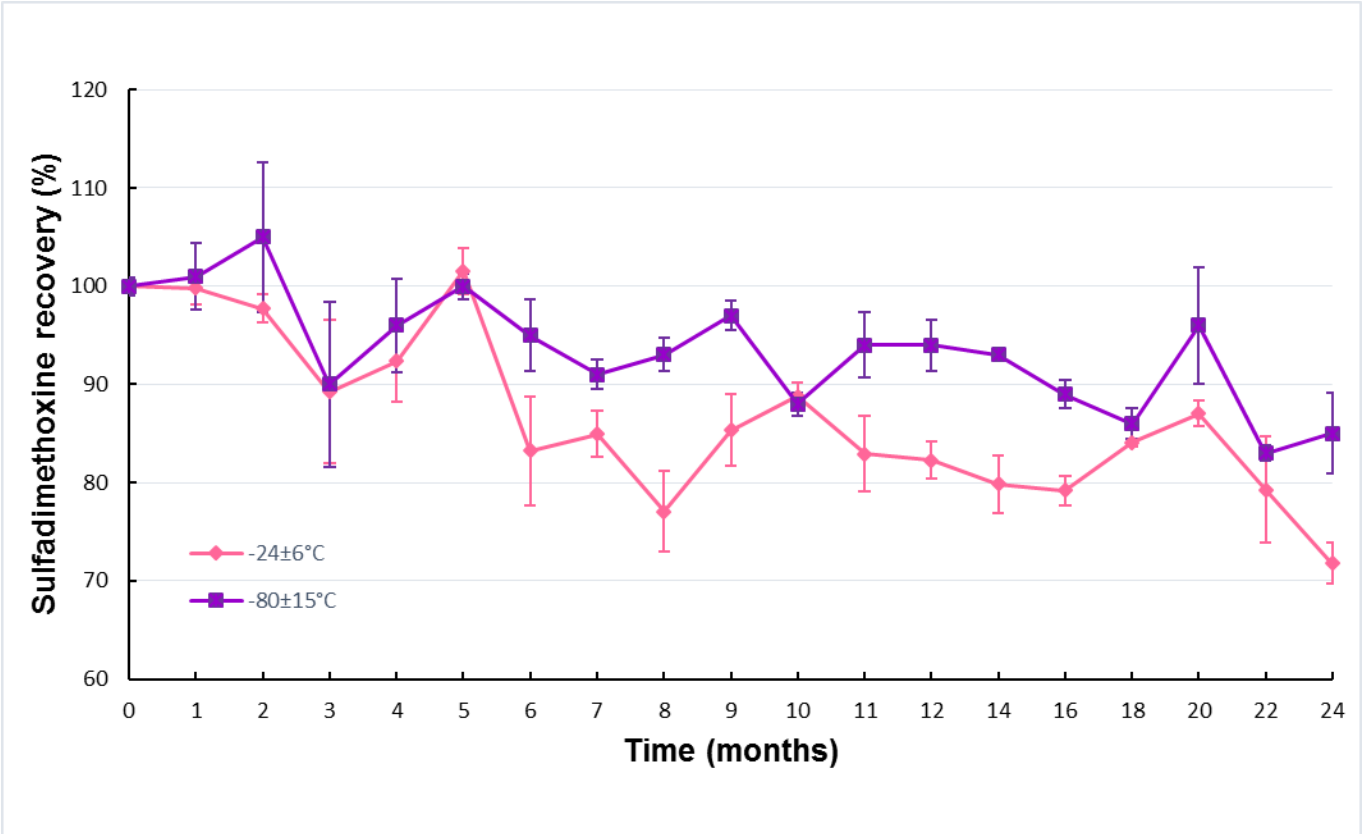

Figure 2. Recovery of Sulfadimethoxine over time in meat samples frozen at -24±6°C and -80±15°C respectively. Recovery was calculated by a calibration curve prepared in solvent and corrected by the recovery of the standard process. Values shown are normalized to the recovery at t=0 (100%) and are the average of three replicates (error bars indicate the standard deviation).

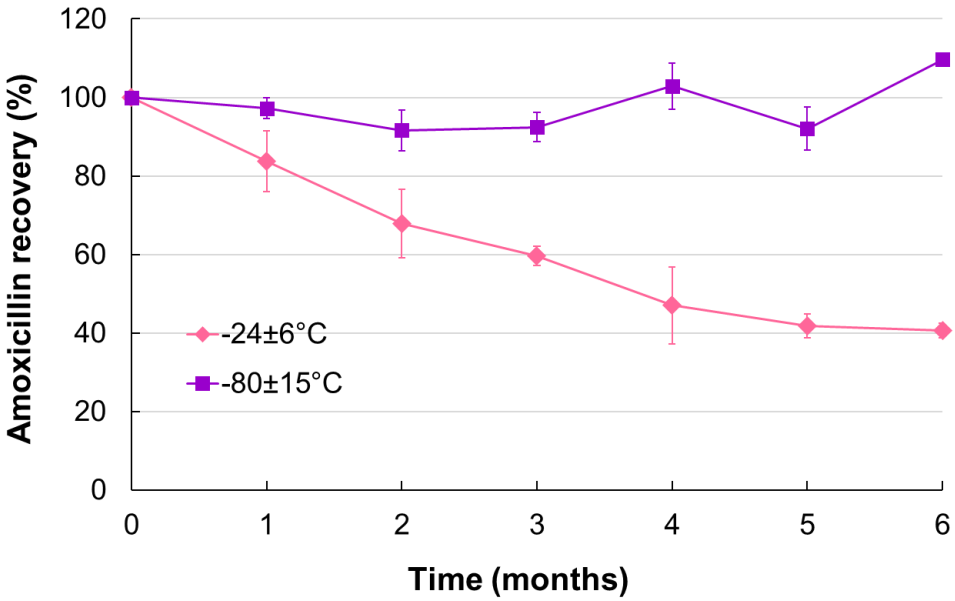

Figure 3. Recovery of Amoxicillin over time in meat samples frozen at -24±6°C and -80±15°C respectively. Recovery was calculated as the ratio between Amoxicillin and Amoxicillin-d4, as detailed in Materials and Methods. Values shown are normalized to the recovery at t=0 (100%) and are the average of three replicates (error bars indicate the standard deviation).

Conclusions

We have shown that frozen storage at -24±6°C is not sufficient to ensure the stability of Amoxicillin in incurred meat samples, and therefore storage at -80±15°C is required. On the contrary, similar levels of Cefquinome residues were recovered up for up to twelve months regardless of the storage temperature. The concentration of Sulfadimethoxine decreased over time, with decay levels reaching 28% at -24°C and 15% at -80°C, respectively, after 2 years.

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POSTER P6

HIGH SENSITIVITY AUTOMATED TURBOFLOW ONLINE UPLC-MS/MS METHODS FOR DETRMINATION OF CHLORAMPHENICOL RESIDUES IN WHOLE EGGS AND DRY EGGS PRODUCTS

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Abstract
A very fast, reliable and high throughput method has been developed and validated for the identification and quantification of Chloramphenicol and Amphenicols antibiotics in whole eggs and dry eggs products. A fast and simple step preparation sample prior to Turbulent flow on-line UPLC-MSMS is involved.
All matrix are spiked with isotopically labeled internal standard Chloramphenicol D5, then dissolved in acetonitrile by ultrasonic aid, followed by centrifugation and filtration on 0,45µm PTFE filter.
The filtered extract was injected into the Turboflow-UPLC-MS/MS Thermo Fisher Scientific Trascend II system coupled with Thermo Fisher Scientific TSQ-Vantage triple stage quadrupole, programmed in H-MRM mode.
Method validation was carried out according to European Union Directive 2002/657/CE at concentration level of 0.1-0.3-1.0 µg/kg for all matrix type.
Limit of detection and limit of quantification have been calculated on the basis of report limit fixed at 0,3 µg/kg; the limits of detection (LODs) were lover than 0.1 µg/kg for all matrix.
Method high throughput by Turbulent flow purification mode has been very useful in order to better estimate many samples/day with screening/confirmatory analysis simultaneously.

Keywords: Eggs / dry egg products, LC-MS/MS, Turboflow

POSTER P7

SIMPLE AND EFFICIENT UPLC-ESI-MS/MS METHOD FOR MULTI-RESIDUE ANALYSIS OF 14 COCCIDIOSTATIC AGENTS IN POULTRY LIVER AND MUSCLE TISSUES

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Abstract

Coccidiosis is one of most common fatal parasitic diseases of poultry. Ionophore coccidiostats are the most effective veterinary preparations traditionally used in industrial poultry farming, but EU legislation classifies them as feed additives and makes obligatory the systematic control of their residues in food of animal origin. Therefore, we have developed, validated and approved in our laboratory practice the simple and rapid UPLC-MS/MS protocol for simultaneous determination of salinomycin, monensine, lasalocide, halofuginone, robenidine, nicarbazine, narasin, maduramycin, decoquinate, diclazuril, toltrazuril, semduramycin, clopidol and amprolium in chicken and turkey meat and liver in efficient and cost-effective way. Main steps of sample preparation are as follows: homogenization, ultrasonication with buffer solution, centrifugation, extraction by organic solvents mixture, evaporation, defatting and reconstitution in mobile phase. The technique is capable for multi-analyte determination of coccidiostats with LODs of 1-5 ppb depending on analyte using matrix matched calibration. Procedure's adequacy was evaluated according to EU Commission Decision 2002/657/EC. Proposed method's efficiency is provided by extractants rational selection and ultracentrifugation of cooled samples at last stage of sample preparation. Thus, usual long and costly procedures with ion suppressive desalting agents or SPE purification are not involved, which is our method's benefit comparing with known multi-residue LC-MS/MS techniques.

Introduction

Coccidiostats are veterinary drugs used in industrial poultry farming to treat and prevent coccidiosis, the most common severe poultry infection, caused by parasitic species of genus *Eimeria*. Among them, narasin, lasalocid, monensin, salinomycin, maduramycin, semduramycin, robenidine, diclazuril, decoquinate, halofuginon and nicarbazine are approved as feed additives within the EU (Peek *et al.*, 2011; Regulation (EC) No 1831/2003). The legislation of EU and Ukraine makes obligatory a systematic control of their residues in food of animal origin, in particular in eggs, meat, liver etc., owing to the potential harmfulness of these drugs residual quantities for the health of consumers (Commission Regulation (EU) No 37/2010; Moloney *et al.*, 2012).

Coccidiostat residues have been routinely analysed in food of animal origin and other animal tissues mainly using ELISA as screening and LC-MS/MS as confirmatory methods (Clarke *et al.*, 2014; Moloney et al., 2012). Main disadvantages of known multi-analyte LC-MS/MS methods are high cost, time consuming and complicated sample preparation (necessary use of solid phase extraction clean-up, utilization of considerable amounts of organic solvents etc.) and not satisfactory sensitivity relatively to the established MRLs or set permitted levels of target coccidiostats, taking into account their various chemical properties (Chang *et al.*, 2019; Klimek-Turek *et al.*, 2019; Piatkowska *et al.*, 2016; Wang *et al.*, 2020). The availability of reliable testing methods suitable for the simultaneous determination of coccidiostats residues in food of animal origin is very important for analytical laboratories, especially in those, involved in official food safety control, state monitoring and in the registration of veterinary drugs, when establishing withdrawal periods of veterinary drugs based on single or few coccidiostats. Therefore, the aim of our research was to develop, validate and approve universal simple and rapid UPLC-MS/MS confirmatory technique for simultaneous and individual determination of salinomycin, monensine, lasalocide, halofuginone, robenidine, nicarbazine, narasin, maduramycin, decoquinate, diclazuril, toltrazuril, semduramycin, clopidol and amprolium in chicken and turkey meat and liver in efficient and cost-effective way.

Materials and Methods

Chemicals and Reagents

Ultrapure water at a resistivity of at least 18.4 MΩ·cm^{-1} was prepared using a Millipore Direct-Q 3 system (France). Methanol, acetonitrile, ethyl acetate and hexane of HPLC grade were obtained from Sigma-Aldrich (Germany). Formic acid and phosphoric acid were obtained from Riedel-de-Haën (Germany), and sodium hydroxide and potassium hydrogen phosphate – from Sigma-Aldrich (Germany).

Standards

Standards of coccidiostats (salinomycin, monensine, lasalocide, halofuginone, robenidine, nicarbazine, narasin, maduramycin, decoquinate, diclazuril, toltrazuril, semduramycin, clopidol and amprolium) of VETRANAL® analytical standard grade were purchased from Sigma-Aldrich (Germany). Individual stock solutions of coccidiostats at the concentration of 1.0 mg mL^{-1} were prepared in methanol or acetonitrile, and working solutions were prepared by diluting stock solutions in methanol to obtain concentrations from 100 to 10 µg mL^{-1}, and standards mixtures were prepared by mixing corresponding volume of each working solution and diluting by methanol up to the final concentration between 10 and 0.01 µ g mL^{-1}.

Instrumentation

UPLC-ESI-MS/MS analysis was carried out using Waters ACQUITY UPLC H-Class chromatographic system equipped with tandem triple quadrupole mass spectrometric detector Waters Xevo TQ-S Micro, equipped with Waters AQUITY UPLC BEH C18 analytical column (50×2.1 mm; 1.7 µm) connected with Waters AQUITY UPLC BEH C18 VanGuard guard column (5×2.1 mm; 1.7 µm) (USA). LC eluents were as follows: 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in methanol (mobile phase B). The system was operated at the flow rate of 0.5 mL·min^{-1}. Gradient initial composition was 50% A / 50% B, maintained for 0.25 min, and continued with linear increase of phase B to 100% to 1.50 min and kept till 2.50 min, followed by linear ramp to 50% A to 3.50 min and equilibrated to initial conditions till 4.0 min. Total separation time was 4.5 min. The column temperature was 40 °C, and the sample temperature was kept at 10 °C. The injection volume was 10 µL.

Detection was carried out in Multiple Reaction Monitoring (MRM) scan mode using the mass spectrometer in positive and negative electrospray ionization mode (ES+/ES-). The operating parameters were: capillary voltage 0.5 kV; source temperature 150 °C; desolvation temperature 600 °C; cone gas (nitrogen) flow 50 L·h^{-1}; desolvation gas (nitrogen) flow 1000 L·h^{-1}; collision gas (argon) pressure 3.8·10^{-3} mbar; dwell time was 0.25 sec. Data were acquired and processed using MassLynx 4.1 software (Waters). Coccidiostats retention times (RT), MS/MS transitions and optimal operational conditions used for the analysis are summarized in Table 1.

Table 1. Retention Times and MRM parameters for UPLC-ESI-MS/MS determination of target coccidiostats in poultry muscles and liver

Analyte	RT, min	Ionization Mode	Precursor Ion, m/z	Product Ion, m/z	Cone Voltage, V	Collision Energy, V
Salinomycin	1.83	ES+	773.8	265.3; 431.4; 531.5	40	56; 50; 42
Monensin	1.73	ES+	693.6	461.4; 479.5; 501.5	40	48; 50; 50
Lasalocid	1.81	ES+	613.3	359.2; 377.3; 577.3	26	34; 34; 30
Halofuginone	1.21	ES+	416.0	100.0; 120.1; 138.0	26	24; 18; 18
Robenidine	1.34	ES+	334.1	110.9; 137.9; 155.0	26	40; 24; 18
Nicarbazine (DNC)	1.43	ES-	301.1	106.9; 136.0	30	34; 12
Narasin	1.90	ES+	787.8	431.4; 531.5	22	48; 42
Maduramycin	1.79	ES+	939.6	719.5; 877.7	25	64; 28
Decoquinate	1.70	ES+	418.3	147.9; 203.9; 232.0; 372.3	18	56; 40; 32; 20
Diclazuril	1.48	ES-	405.0; 407.0	334.0; 301.0; 336.0	40	15; 26; 15
Toltrazuril	1.49	ES-	424.0	41.8; 424.0	25	14; 5
Semduramycin	1.66	ES+	895.5	833.6; 851.5	20	30; 35
Clopidol	0.53	ES+	192.0	87.0; 100.9	10	28; 24
Amprolium	0.37	ES+	243.2	93.9; 122.0; 150.0	22	8; 24;10

Auxiliary equipment

Laboratory balances AXIS AD 200 and AXIS AD 200C (AXIS, Poland); a centrifuge MULTIFUGE 1S-R (Thermo Scientific, USA); a centrifuge Sigma 1-16K (Sigma, Germany); an orbital shaker Multi Bio RS-24 (Biosan, Latvia); a

vortex MAXI MIX II (Barnstead Thermolyne, USA); a homogenizer IKA T-25 ULTRA-TURRAX (IKA, Germany); a centrifugal vacuum evaporator EZ-2 Envi (Genevac, England); ultrasonic bath Elmasonic S 120 H (Elma, Germany).

Sample Preparation

2.0 g of grinded poultry tissue (liver, muscle) was weighed into 50 mL plastic centrifuge test tube, and 5 ml of acetonitrile and 5 mL of 0.2 M phosphate buffer solution (pH 9.7) was added. Then the sample was vortexed for 30 sec, shaken on an orbital shaker at 60 rpm for 10 min and placed into ultrasonic water bath for 15 min at room temperature. Next step was the neutralization of extraction medium by adding ~ 0.5 ml 0.25 M H_3PO_4. After centrifuging at 4700 rpm and 10 °C for 10 min the upper liquid layer was poured into another 50 mL plastic centrifuge test tube, 9 ml of ethyl acetate was added, and the sample was shaken on an orbital shaker at 60 rpm for 10 min. 10 ml aliquot of upper organic layer was transferred into a centrifugal glass vial and evaporated to dryness on a vacuum centrifugal evaporator at 40 °C. Then the residue was reconstituted in 1.5 ml of 30 % methanol and degreased by 0.75 ml of hexane by vortexing for 30 sec. Thereafter, the phases were separated by centrifuging for 4 min at 1500 rpm at room temperature, and 1 ml aliquote of lower phase was transferred from a vial into a microcentrifuge tube to be additionally cleaned up in a microcentrifuge at 21000×*g* and 10 °C for 7 min. 0.3 ml aliquote of purified sample extract was transferred into a chromatographic vial and analysed by LC-MS/MS.

Results and Discussion

First stage of our research was to optimize MRM parameters of analytes by means of direct infusion of individual standard solutions of each target coccididiostat in that valve position, when analyte flow was combined with a mobile phase. As a result of tuning using IntelliStart™ Software (Waters), three of examined compounds were most effectively ionised in ESI negative ion mode and the rest of analytes (11 coccidiostatic agents) – in ES+ mode (see details in Table 1). In order to obtain sharp and symmetrical chromatographic peaks of target compounds and to enhance the yields of protonated precursor ions $[M+H]^+$, which are characteristic for the majority of analytes, we used 0.1 % formic acid as an additive to LC eluents (water and methanol). It should be mentioned, that due to the high sensitivity of MS/MS detector, the presence of formic acid in such concentration appeared not to be an obstacle for the obtaining of deprotonated precursor ions $[M-H]^-$ of nicarbazine, diclazuril and toltrazuril, which are ionised in ES- mode. Also we have optimized LC separation parameters, which in combination with advanced MS detector operational parameters made it possible to distinguish clearly all 14 coccidiostats simultaneously from one standard mix solution at the concentration level of 50 ng kg^{-1}.

It is a known fact, that sample preparation is critical and very important point of multi-analyte LC-MS/MS methods. As for coccidiostats, the assay of their residues in animal tissues is complicated by different chemical properties of analytes, e.g., their pK_a. So the extraction conditions were studied very carefully, taking into consideration high protein content in muscle tissues and enzymes and lipids presence in liver samples, as well. Thus, we have developed a simple and effective sample preparation method for poultry muscle tissue and liver assay. The first step was coccidiostats extraction from homogenized tissues in the presence of basic phosphate buffer solution as an extraction medium and acetonitrile as an extragent in ultrasonic bath at ambient temperature to intensify the process. Extraction of analytes with ethyl acetate as auxiliary and less polar solvent than acetonitrile, was carried out after neutralization of the extraction medium to pH 7.5 with 0.25 M phosphoric acid and separation of tissues by centrifugation. That separation step allowed us to clean-up the extract additionally from matrix proteins. The obtained extract was concentrated by drying on a rotary evaporator and the residue was defatted with hexane and reconstituted in mobile phase. The use of hexane as a degreasing agent has some disadvantages, because after vortexing and centrifugation it forms an upper phase and should be discarded, whereas the extract of target compounds in 30 % methanol is a lower phase, which is inconvenient when transferring sample into a chromatographic vial. We have studied the option to substitute hexane with non-polar solvents with higher density, e.g. dichloromethane or chloroform, or to use definite hexane mixtures with these solvents as degreasing agents. It appeared not to be successful, since considerable amounts of few assayed coccidiostats (e.g. halofuginone, nicarbazine, diclazuril, semduramycin et al.) are distributed both in mobile phase and in non-polar solvent, which reduced analytes' recoveries, and thus the sensitivity parameters of the developed multi-residue technique significantly decreased.

Examples of mass chromatograms of the prepared extracts of blank chicken muscle and liver, fortified with coccidiostats mix standard at the level of 10 μg kg^{-1}, are presented on Figure 1 and Figure 2, correspondingly.

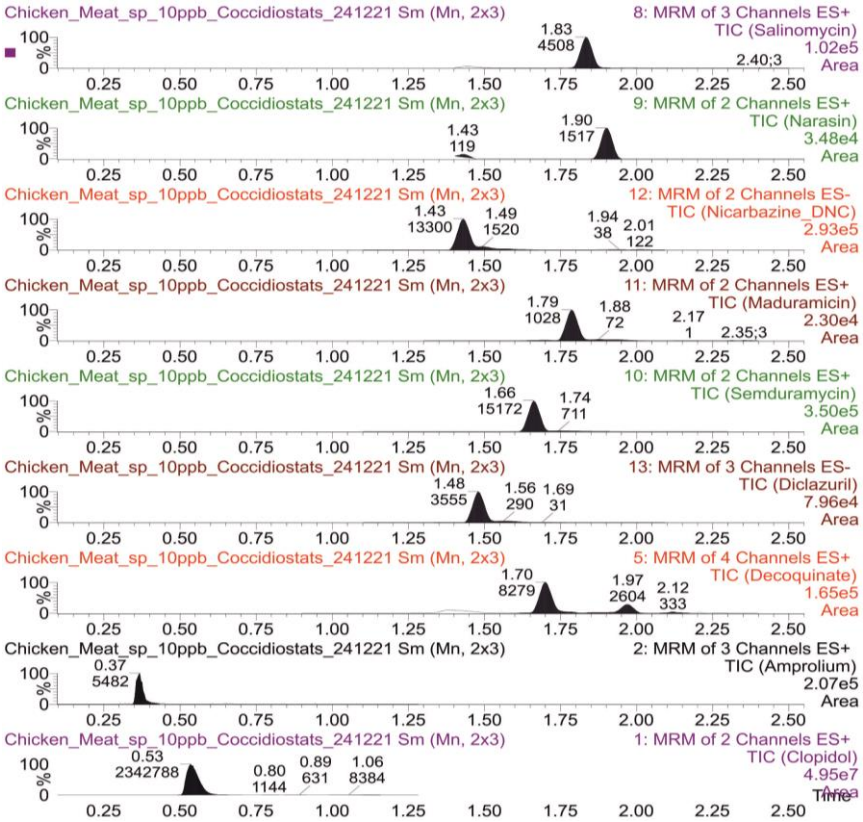

Figure 1. Examples of LC-MS/MS chromatograms of matrix matched standards of target analytes at the concentration levels of 10 µg kg^{-1}, prepared using blank chicken muscle.

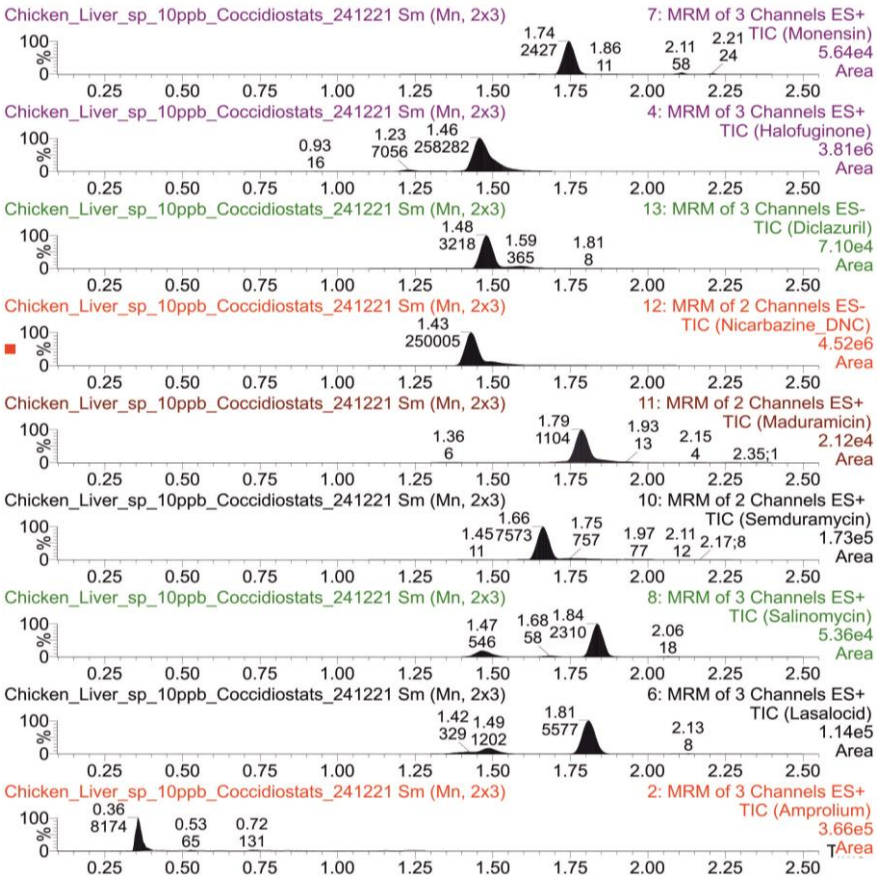

Figure 2. Examples of LC-MS/MS chromatograms of matrix matched standards of target analytes at the concentration levels

of 10 µg kg^{-1}, prepared using blank chicken liver.

UPLC-ESI-MS/MS multi-analyte determination of coccidiostats residues in chicken muscle and liver tissues was carried out using matrix matched calibration, which is the most common approach to compensate the suppression or enhancement of the analytical signal. To build the calibration plot, grinded blank muscle or liver sample was fortified with coccidiostats mix standard solution at eight calibration levels: 0; 0.5; 1.0; 2.0; 5.0; 10.0; 20.0 and 50.0 µg kg^{-1} of each analyte. Good linearity ($R^2 \geq 0.99$) for matrix matched calibration plots was achieved for all drugs in the set concentration ranges. Target analytes' content in samples was quantified using total ion chromatograms (TIC) of selected MRMs with respect to relative ion intensities and according to the calibration plot by means of MassLynx 4.1 software (Waters).

The proposed analytical technique is fast, accurate and selective. It is capable for simultaneous multi-analyte determination of coccidiostatics residues in poultry tissues and liver with LOD values from 1 to 5 ppb depending on an analyte (see Table 2) using matrix matched calibration, which corresponds or even exceeds the requirements of MRLs or provided regulatory limits with the sufficient level of precision and percentage of analyte's absolute recoveries.

Table 2. Sensitivity parameters of the developed UPLC-ESI-MS/MS multi-analyte confirmatory method of coccidiostats residues assay in chicken muscles and liver (Clarke et al., 2014; Update of Worldwide Regulatory Limits. Eurofins Technologies; Order of the Ministry of Health of Ukraine № 2235, 2021)

Analyte	Muscle tissue			Liver			MRL EC (UA)[a], µg kg^{-1}	
	LOD, µg/kg	LOQ, µg/kg	Abs. Rec., %	LOD, µg/kg	LOQ, µg/kg	Abs. Rec., %	Muscle	Liver
Salinomycin	2	5	25	2	5	20	5 (15)	5 (150)
Monensin	2	5	22	1	2	33	8 (2)	8
Lasalocid	1	2	40	2	5	33	20 (5)	100
Halofuginone	2	5	45	2	5	25	3[b] (3)	30[b]
Robenidine	5	10	8	2	5	5	200 (5)	800
Nicarbazine (DNC)	2	5	50	2	5	10	4000 (50)	15000
Narasin	1	2	22	2	5	16	50 (5)	50
Maduramycin	2	5	25	2	5	35	30 (2)	150
Decoquinate	2	5	5	2	5	6	20[b] (500)	1000
Diclazuril	1	2	150	1	2	100	500 (5)	1500
Toltrazuril	1	2	120	1	2	100	100 (100)	600
Semduramycin	1	2	100	1	2	100	2[b]	2[b]
Clopidol	2	5	60	2	5	50	5000[c]	15000[c]
Amprolium	1	2	1	2	5	0.5	500[c]	1000[c]

[a] MRLs set by Ministry of Health of Ukraine; [b] non-target species; [c] MRLs set by FDA

Thus, this technique is universal for muscle tissues and parenchymal organs assay, it requires less time consuming sample preparation and relatively small amount of organic reagents, being suitable for the control of coccidiostats residues in food of animal origin, and for clinical trials.

Conclusions

The elaborated method is relatively fast, robust, reliable and cost-effective. It has sufficient advantages comparing with known multi-residue LC-MS/MS methods for coccidiostats determination in animal tissues and parenchymal organs, since it does not demand the use of expensive and time consuming solid phase extraction without a loss of sensitivity and selectivity, and the analysis is performed in one sample preparation procedure. This technique is routinely used in our laboratory to test the safety parameters of poultry meat, to establish the withdrawal periods of veterinary drugs based on these coccidiostats, to assay turkey tissues after mortality cases on the farm, as well as during taking part in international proficiency testing rounds.

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POSTER P8

DEVELOPMENT OF A METHOD FOR THE DETERMINATION OF STEROIDS ESTERS IN HAIR BY LIQUID CHROMATOGRAPHY WITH TANDEM MASS SPECTROMETRY

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Abstract

The European Union, in the Council Directive 96/22/EC, bans the use of substances with hormonal or thyrostatic effect in stock farming. The Regulation (EU) 2017/625 controls the use of these unauthorized substances and regulates how the member states have to elaborate the monitoring plans for their detection. In this scope, the "EURL Guidance on minimum method performance requirements (MMPRs) for specific pharmacologically active substances in specific animal matrices" is a technical guidance to improve and harmonize the performance of analytical methods used for the analysis of residues of unauthorized or prohibited pharmacologically active substances and it's used as a guidance in the elaboration of the national residue control plans.

One of this unauthorized substances due to its anabolic effect, are the steroids esters. The development of methods for their control represents an important advance because their presence may indicate a steroids exogenous administration.

The Laboratori Agroalimentari has developed a method for the determination of steroids esters in hair. Twelve steroids esters have been optimized obtaining good results. The Laboratory has participated with it in the proficiency test esters in bovine hair 2020-09 of Wageningen Food Safety Research (WFSR). The next step is the validation of the method.

Introduction

The European Union, in its Farm to Fork strategy, aims to guaranty a safety alimentation without health risks for its citizens. One of the objectives is the control of pharmacologically active substances administrated to the food-producing animals, as they may leave residues that can enter the human food production chain and be harmful to the consumer. For this reason, the Council Directive 96/22/EC bans the use of several substances that have hormonal or thyrostatic effect in stock farming in the European Union. In order to control the application of this prohibition, the Regulation (EU) 2017/625 sets the common grounds of how each country has to prepare and execute the national control plans that cover all the areas governed by Union agri-food chain legislation, including the Council Directive 96/22/EC.

Also, for harmonise the performance of the analytical methods used in the control plans for the analysis of unauthorized or prohibited substances, the European Union Reference Laboratories, published in September 2020 the "EURL Guidance on minimum method performance requirements (MMPRs) for specific pharmacologically active substances in specific animal matrices". In this guidance minimum method performance requirements are defined and it's indicated that laboratories should ensure that the CCalfa for confirmatory methods are lower than the MMPR indicated in the guidance.

One group of the substances that are banned for its use in the European Union and that are controlled by this national control plans, are the anabolic steroids due to its health risks if heavily consumed and because they can fraudulently be used as growth promoters. Some of this steroids (like testosterone, nortestosterone – nandrolone-, boldenone) are natural occurring, so a detection of their presence in animal sample (like urine), may not mean that an illegal use has been done. As explained by Gray et alt., 2013, the natural hormones can be found in all matrices, and their concentration depends on the species, sex, age and individual characteristics of each animal.

As several papers indicate (Gray et alt., 2013; Matraszek-Żuchowska et alt., 2019; Stolker et alt., 2009), the common way of administrating steroids to animals is by injection of its synthetic ester derivatives, as this form is more easily absorbed and transported to the target organs. And, because steroids esters are not naturally metabolized, their detection in an animal matrix is indication that a non-natural steroid administration has been done. Hair has been described as a good matrix for the analysis of steroids esters (Matraszek-Żuchowska et alt., 2019; Stolker et alt., 2009) due to its easy, stress-free, non-invasive collection, but most importantly

because these substances can be detected long after its administration. Therefore, the analysis of steroids esters in hair can be a good complement to inconclusive analysis of steroids in urine.
The "EURL Guidance on minimum method performance requirements (MMPRs) for specific pharmacologically active substances in specific animal matrices" has defined a MMPR of 10 µg kg^{-1} for testosterone esters and of 20 µg kg^{-1} for estradiol esters in hair.
The Laboratori Agroalimentari, as part of the laboratories that execute the control plans for the analysis of prohibited or not-authorized substances in the region of Catalonia (Spain), analyses, among others, steroids in urine. In order to better resolve if the positives of steroids identified, came from natural occurring or for illegal administration, the need of developing a method for the analysis of steroids esters in hair was perceived.

For the extraction of intact steroids esters in hair, the procedure described by Matraszek-Żuchowska et alt., 2019 was followed. In this paper, five steroids esters with three internal standards were studied. The main objective of the present work was to increase the number of steroids esters analysed (up to sixteen steroids esters) within the range of concentrations indicated in the EURL MMPR Guidance.

Materials and Methods

Chemicals and standards
Seventeen steroids esters standards were employed: ten standards were purchased from Merck Life Science (testosterone benzoate, testosterone isocaproate, testosterone propionate, testosterone decanoate, testosterone cypionate, testosterone enanthate, testosterone phenylpropionate, estradiol valerate, estradiol benzoate, nandrolone decanoate) and seven where purchased from Cymit Quimica (testosterone cypionate-d3, testosterone acetate, testosterone undecanoate, boldenone undecylenate, 17b-estradiol-16,16,17-d3 3-benzoate, estradiol dipropionate, estradiol enanthate). Stock solutions were prepared in a concentration of 1 mg L^{-1} and stored at -20ºC for 6 months.
All chemicals used in this work were of analytical, HPLC or LC-MS grade. Ultra-pure water was obtained from a Milli-Q system (Millypore, Bedford, USA). Methanol, acetonitrile and ethyl acetate where purchased from Honeywell. Formic acid was purchased from Fisher Scientific, and Tris(2-carboxyethyl)phosphine hydrochloride solution 0.5 M, pH 7.0 (TCEP) from Merk Life Science. For the sample clean-up, Strata-X 33u 200mg from Phenomenex was used.

Sample treatment
The hair samples were previously cut at about 5mm. 200 ± 2 mg of hair were weight in propylene tubes and spiked with 200 µl of the solution of the internal standards (0.1 mg L^{-1}). Then, 2 ml of TCEP were added to each sample and the tubs were shaken horizontally one hour at 250 rpm. After that, 4 ml of methanol were added ant the tubs were shaken with a vortex mixer and centrifuged for 5 minutes at 5000 rpm.
The extract was then transferred to a tub and added 4 ml of water Milli-Q, and shaken again via vortex mixer. A SPE clean-up was then performed. All the extract obtained was applied on Strata-X 33u 200mg cartridges who were previously conditioned with 6 ml of methanol and 6 ml of waters. Subsequently, the cartridges were washed with a methanol/water mixture (60:40) and then, the steroids esters were eluted with 3 mL of acetonitrile and 3 mL of ethyl acetate.
The eluted extract was then evaporated to dryness at 40ºC and reconstituted with 200 µL of a mixture of acetonitrile:water:0.1% formic acid (70:30:2 v/v/v). Finally, the extracts were filtered with 0.22 µm membrane filters and injected to the HPLC system.

Instrumentation
The chromatographic separation of the steroids esters was carried out using a high-performance liquid chromatography (UHPLC) system Acquity (Waters) equipped with a reversed-phase Kinetex C18 (150 mm x2.1mm, 2.6µm) from Phenomenex. Separation under gradient elution based on 0.1% Formic acid in water (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B) was as follows: 0 – 8 min, isocratic conditions at 15% A, flow rate 250 µL min^{-1}; 8 – 11 min, linear gradient from 15% to 2% A, flow rate 400 µL min^{-1}; 11 – 18 min, isocratic conditions at 2% A , flow rate 400 µL min^{-1}; 18 – 20 min, linear gradient from 2% to 15% A, flow rate 400 µL min^{-1}; 20.0 – 20.1 min, isocratic conditions at 15% A, flow rate 250 µL min^{-1}; 20.1 – 22 min, back to initial conditions, isocratic conditions at 15% A, flow rate at 250 µL min^{-1}. The injection volume employed was 10 µL.
The UHPLC System was connected to a triple quadrupole mass spectrometer (Xevo TQ-S micro) equipped with a ESI ionization source operated in positive ionization mode. A desolvation temperature at 650 ºC, source temperature 150 ºC, capillary voltage at 3.00 kV, cone gas flow at 75 L hr^{-1} were applied. Gas desolvation was carried out using nitrogen at flow rate of 950 L hr^{-1}. In the collision cell, argon was used as collision gas at a

pressure of 3-3.5*10^{-3} mbar. The signal acquisition was performed by multiple reaction monitoring (MRM). Parameters such as chromatographic retention times, MS/MS transitions, collision energy and cone voltage are summarized in Table 1.

Table 1. MS/MS conditions.

Analyte	RT (min)	Quantification transition (m/z)	Cone Voltage (V)	Collision energy (eV)	Confirmation transition (m/z)	Cone Voltage (V)	Collision energy (eV)
Testosterone cypionate-D3 (I.S. for testosterone's esters)	5.15	416.6 > 97.0	25	25	416.6 > 79.0	25	35
Testosterone acetate	2.02	331.5 > 96.9	40	25	331.5 > 109.0	40	25
Testosterone propionate	2.37	345.0 > 97.0	35	25	345.0 > 109.0	35	25
Testosterone benzoate	2.96	393.5 > 105.0	50	20	393.5 > 97.0	50	25
Testosterone phenylpropionate	2.97	421.6 > 105.0	35	30	421.6 > 97.0	35	25
Testosterone isocaproate	3.79	387.6 > 81.0	55	25	387.6 > 97.0	55	25
Testosterone cypionate	5.18	413.6 > 97.0	35	25	413.6 > 107.0	35	20
Testosterone decanoate	9.61	443.7 > 97.0	50	25	443.7 > 155.1	50	20
Testosterone undecanoate	10.72	457.0 > 97.0	35	25	457.0 > 109.0	35	25
Estradiol-D3 benzoate (I.S. for the rest)	2.30	380.5 > 77.0	40	55	380.5 > 105.0	40	20
Estradiol benzoate	2.31	377.5 > 77.0	40	40	377.5 > 105.0	40	20
Boldenone undecylenate	6.62	453.7 > 121.0	40	30	453.7 > 269.3	40	15
Nandrolone decanoate	8.53	429.7 > 155.0	60	20	429.7 > 109.0	60	30

Results and Discussion

During the development, satisfactory results were obtained for eleven steroids esters (of the initial sixteen): testosterone acetate, testosterone propionate, testosterone benzoate, testosterone phenylpropionate, testosterone isocaproate, testosterone cypionate, testosterone decanoate, testosterone undecanoate, estradiol benzoate, boldenone undecylenate and nandrolone decanoate and for the internal standards testosterone cypionate-D3 and estradiol-D3 benzoate. Good linearity results were obtained in the studied range (5 µg kg^{-1} to 50 µg kg^{-1}). An example of the chromatograms obtained from a blank bovine hair sample spiked at 10 µg kg^{-1} can be seen in Figures 1 and 2.

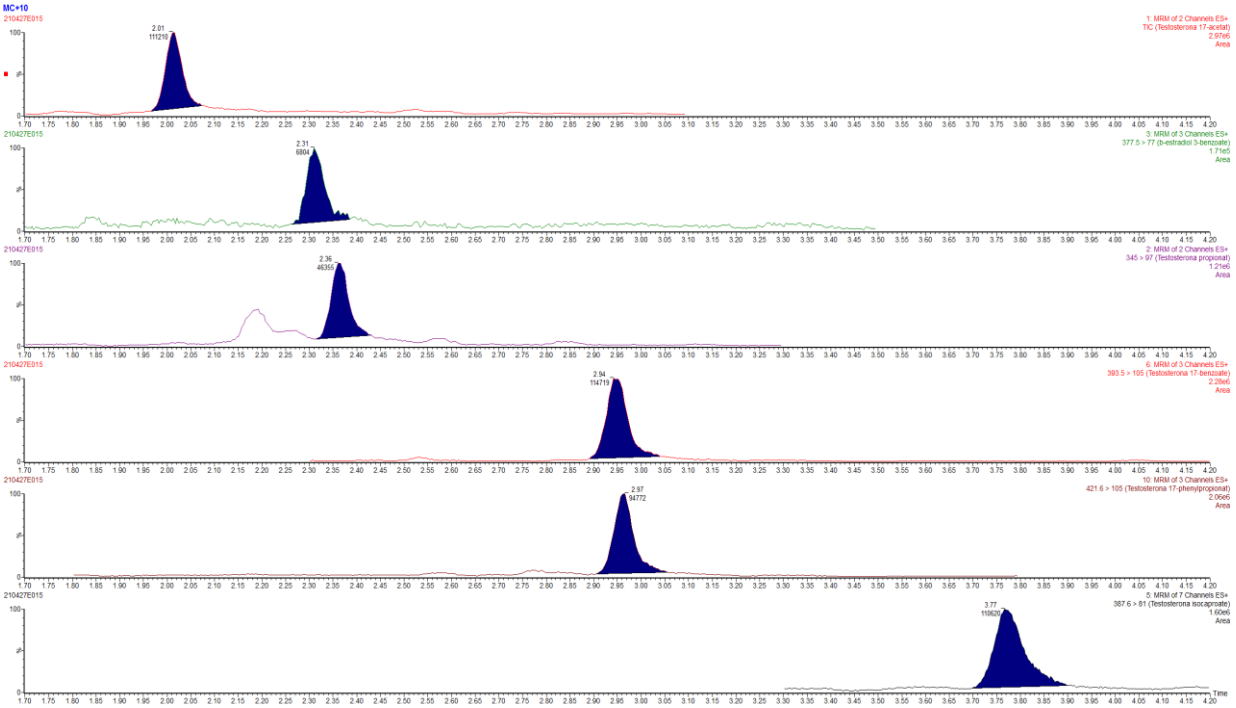

Figure 1. Quantification transitions for testosterone acetate, estradiol benzoate, testosterone propionate, testosterone benzoate, testosterone phenylpropionate and testosterone isocaproate at 10 µg kg^{-1} in hair.

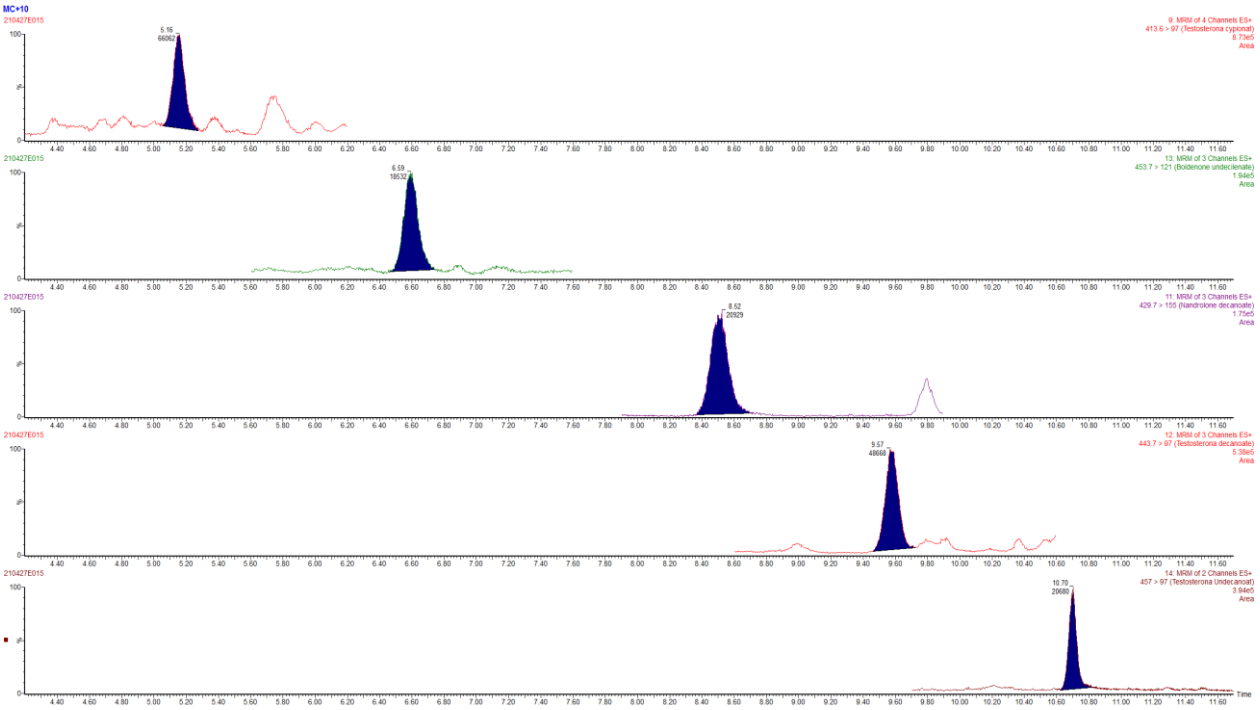

Figure 2. Quantification transitions for testosterone cypionate, boldenone undecylenate, nandrolone decanoate, testosterone decanoate and testosterone undecanoate at 10 µg kg^{-1}.

Conclusion

The development of the method shows that is ready for being validated according to the Commission Implementing Regulation (EU) 2021/808.

Acknowledgements
The authors would like to specially thank the Laboratori Agroalimentari veterinary drug residue technical staff Anna Boquet, Albert Abril and Carles Bruguera for their technical support.

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POSTER P9

EXTENSION OF THE NOKO MULTI-CLASS VETERINARY DRUG RESIDUE ANALYSIS METHOD BY LIQUID CHROMATOGRAPHY MASS SPECTROMETRY FOR EGG AND MILK

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Abstract

The Multi-class veterinary drug residue analysis method developed in 2017 by the North German Cooperation of State Laboratories (NOKO) [1] is extended for the matrices egg and milk. The goal is an enlarged method for the analysis of antibiotics and other veterinary drugs of relevance in all matrices of the national control plan. With the matrices egg and milk, the next step is been taken to achieve this goal.

Introduction

The use of antibiotics and other pharmacologically active substances (PAS) is a common application to maintain a high health standard in livestock farming. There are many authorised substances for the permitted use in livestock farming. Some drugs are restricted for the use in diary farming or for the application on laying hens. On the other hand, there are PAS which are prohibited or unauthorized for the use in animal production throughout the EU und for import into the EU, e.g. dapson, chloramphenicol or the compound group nitroimidazoles. These substances have to be detected as low as reasonably achievable but at least lower then the reference point of action (Regulation (EU) 2019/1871). To accomplish a comprehensive screening for PAS in milk and egg, a detailed validation of a wide range of relevant compounds has been performed.

Methods

Sample preparation and Measurement

2 g of sample were extracted with acetonitrile and evaporated to a small volume. Before clean-up EMI-buffer[2] was added to stabilize to pH 4. For the solid phase extraction with Oasis-HLB 200 mg / 6 mL (Waters®), the cartridge was conditioned with methanol and water, the sample extract was slowly passed through the cartridge, washed with water/methanol (95/5 v/v) and finally the analytes were eluted with methanol. The SPE eluate was evaporated to dryness and reconstituted in water. Subsequently residues of pharmacologically active substances were analysed by liquid chromatography and tandem mass spectrometry (LC-MSMS) on an Infinity 1290 LC-system and 6495 B triple quadrupole from Agilent Technologies®. For LC separation a BEH C18 column (1,7 µm; 2,1 x 100 mm) from Waters® was used with water/acetonitrile gradient and 0,1 % formic acid.

Validation

Due to the large amount of analytes (129) from many different classes (16) and existing single-class confirmatory methods, this validation study was primarily intended for screening purposes. Therefore, the validation was conducted according to Method 2 of CIR (EU) 2021/808 EU (2.7.1(b) and 2.7.2(b)). Fortified blank material with levels at the screening target concentration (STC = 0,25 x regulation level) was analysed. The regulation level depended on the nature of the analyte: for unauthorised or prohibited pharmacologically active substances, it is the reference point of action (RPA) [3] or minimum method performance requirement (MMPR) [4] and for authorized substances, it is the maximum residue level (MRL) [5]. For each concentration level, 20 fortified blanks were analysed and compared to unfortified blanks. If the minimum of the 20 fortified blanks is higher than the maximum of the 20 unfortified blanks, the STC equals to the detection capability (CC-beta), which means that no false compliant result is generated. With a needed false compliant rate below 5% (beta error), it is possible to take one false compliant result of the 20 blanks out of the calculation and still meet the regulation necessities. According to CIR (EU) 2021/808 (1.1.2) the detection capability (CCβ) needs to be below the regulation level.

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[2] EMI-buffer: combine 0.1 molar citric acid solution and 0.2 molar disodium-hydrogenphoshat-buffer 60/40 v/v, add 0.1 molar disodium-ethylenediaminetetraacetic acid (EDTA) and adjust to pH 4.

Results and Discussion

Table 1 summarizes all the results for egg and milk and gives an overview of the successful validated compounds. In total, the study started with 129 compounds of which only 96% and 95% met the requirements of CIR (EU) 2021/808. For 5 compounds in egg and 6 compounds in milk the validation did not succeed. Most of the unsuccessful compounds are NSAIDs. This compound group needs a different sample pre-treatment und also an adapted LC method. Some failures can be due to the fact that only one mix of fortification solution was prepared and used for both matrices. The concentrations for different regulation levels are compromises and that is why some corticosteroids were successful for egg and not for milk. Another reason are difficulties detecting the compounds in such low concentrations and the different matrix effect of milk and egg, e.g. the corticosteroid Triamcinolon in egg or the NSAID Diclofenac in milk.

Conclusion

The validation results show the suitability of the method to screen 124 and 122 different veterinary drugs in egg and milk, respectively. The method is robust for routine analysis according to CIR (EU) 2021/808. By screening egg and milk with the multi-screening-method 5 times more parameters are covered in comparison to the formerly used method [6]. New unauthorized compound groups are added, e.g. nitroimidazoles and Chloramphenicol. The spectrum of authorized compounds, such as amphenicols, sulphonamides or quinolones is widened. All of which will results in a much higher probability to detect wrong applications (e.g. shortened withdrawal period) or misuse.

Acknoledgment

Many thanks to the lab team for tedious validation studies and loads of data evaluation and processing.

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- [2] COMMISSION IMPLEMENTING REGULATION (EU) 2021/808 of 22 March 2021 on the performance of analytical methods for residues of pharmacologically active substances used in food-producing animals and on the interpretation of results as well as on the methods to be used for sampling and repealing Decisions 2002/657/EC and 98/179/EC.
- [3] COMMISSION REGULATION (EU) 2019/1871 of 7 November 2019 on reference points for action for non-allowed pharmacologically active substances present in food of animal origin and repealing Decision 2005/34/EC.
- [4] EURL Guidance 09/2020: EURL Guidance on minimum method performance requirements (MMPRs) for specific pharmacologically active substances in specific animal matrices.
- [5] COMMISSION REGULATION (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin.
- [6] L 01.00-85, Januar 2011: Untersuchung von Lebensmitteln: Bestimmung von Rückständen der Antibiotikagruppen Macrolide, Lincosamide, Chinolone, Tetracycline, Sufonamide und Trimethoprom in Milch – HPLC-MS/MS-Verfahren. Amtliche Sammlung von Untersuchungsverfahren nach § 64 LFGB.

		Egg		Milk	
analyt	group	CC-beta µg/kg	validaded CC-beta < RL	CC-beta µg/kg	validaded CC-beta < RL
Chloramphenicol	Amphenicol	0,1	yes	0,1	yes
Florfenicol	Amphenicol	25	yes	25	yes
Florfenicol-amine	Amphenicol	25	yes	25	yes
Thiamphenicol	Amphenicol	25	yes	25	yes
Rifaximin	Ansamycin	40	yes	40	yes
Cefalexin	Cefalosporin	100	yes	100	yes
Cefalonium	Cefalosporin	10	yes	10	yes
Cefapirin	Cefalosporin	30	yes	30	yes
Cefazolin	Cefalosporin	25	yes	25	yes
Cefoperazone	Cefalosporin	25	yes	25	yes
Cefquinome	Cefalosporin	10	yes	10	yes
Ceftiofur	Cefalosporin	50	yes	50	yes
Cefuroxim	Cefalosporin	25	yes	25	yes
Desacetyl Dephapirin (DACP)	Cefalosporin	30	yes	30	yes
Desfuoryl Ceftiofur (DFCF)	Cefalosporin	50	yes	50	yes
Cinoxacin	Quinolone	25	yes	25	yes
Ciprofloxacin	Quinolone	25	yes	25	yes
Danofloxacin	Quinolone	15	yes	15	yes
Difloxacin	Quinolone	25	yes	25	yes
Enoxacin	Quinolone	25	yes	25	yes
Enrofloxacin	Quinolone	25	yes	25	yes
Flumequin	Quinolone	12,5	yes	12,5	yes
Lomefloxacin	Quinolone	25	yes	25	yes
Marbofloxacin	Quinolone	37,5	yes	37,5	yes
Moxifloxacin	Quinolone	25	yes	25	yes
Nalidixic acid	Quinolone	25	yes	25	yes
Norfloxacin	Quinolone	25	yes	25	yes
Ofloxacin	Quinolone	25	yes	25	yes
Orbifloxacin	Quinolone	25	yes	25	yes
Oxolinic acid	Quinolone	25	yes	25	yes
Sarafloxacin	Quinolone	25	yes	25	yes
Sparfloxacin	Quinolone	25	yes	25	yes
Betamethasone	Corticosteroide	0,8	yes	0,8	no
Dexamethasone	Corticosteroide	0,8	yes	0,8	no
Flumethasone	Corticosteroide	5	yes	5	yes
Methylprednisolone	Corticosteroide	5	yes	5	no
Prednisolone	Corticosteroide	2	yes	2	yes
Triamcinolone	Corticosteroide	x	no	5	yes
Baquiloprim	Diaminopyrimidine	15	yes	15	yes
Ormethoprim	Diaminopyrimidine	12,5	yes	12,5	yes
Trimethoprim	Diaminopyrimidine	12,5	yes	12,5	yes
Clindamycin	Lincosamide	10	yes	10	yes
Lincomycin	Lincosamide	50	yes	50	yes
Pirlimycin	Lincosamide	50	yes	50	yes
3-O-acetyltylosin	Makrolide	50	yes	50	yes
Azithromycin	Makrolide	10	yes	10	yes
Clarithromycin	Makrolide	10	yes	10	yes
Erythromycin	Makrolide	37,5	yes	37,5	yes
Gamithromycin	Makrolide	20	yes	20	yes

		Egg		Milk	
analyt	group	CC-beta µg/kg	validaded CC-beta < RL	CC-beta µg/kg	validaded CC-beta < RL
Josamycin	Makrolide	20	yes	20	yes
Neospiramycin	Makrolide	50	yes	50	yes
Oleandomycin	Makrolide	10	yes	10	yes
Roxythromycin	Makrolide	10	yes	10	yes
Spiramycin	Makrolide	50	yes	50	yes
Tildipirosin	Makrolide	10	yes	10	yes
Tilmicosin	Makrolide	12,5	yes	12,5	yes
Tulathromycin	Makrolide	12,5	yes	12,5	yes
Tulathromycin-marker	Makrolide	12,5	yes	12,5	yes
Tylosin A	Makrolide	50	yes	50	yes
Tylosin B = Desmycosin	Makrolide	50	yes	50	yes
Tylvalosin	Makrolide	37,5	yes	37,5	yes
Carnidazole	Nitroimidazole	0,75	yes	0,75	yes
Dimetronidazole	Nitroimidazole	0,75	yes	0,75	yes
HMNNI=Hydroxy-dimetridazole	Nitroimidazole	0,75	yes	0,75	yes
Hydroxy-ipronidazole	Nitroimidazole	1,75	yes	1,75	yes
Hydroxy-metronidazole	Nitroimidazole	0,75	yes	0,75	yes
Ipronidazole	Nitroimidazole	1,75	yes	1,75	yes
Metronidazole	Nitroimidazole	0,75	yes	0,75	yes
Nimorazole	Nitroimidazole	0,75	yes	0,75	yes
Ornidazole	Nitroimidazole	0,75	yes	0,75	yes
Ronidazole	Nitroimidazole	0,75	yes	0,75	yes
Secnidazole	Nitroimidazole	0,75	yes	0,75	yes
Ternidazole	Nitroimidazole	0,75	yes	0,75	yes
Tinidazole	Nitroimidazole	0,75	yes	0,75	yes
5-Hydroxy-flunixin	NSAIDs	20	yes	20	yes
Carprofen	NSAIDs	25	yes	x	no
Diclofenac	NSAIDs	0,1	yes	x	no
Flufenamic acid	NSAIDs	x	no	x	no
Flunixin	NSAIDs	20	yes	20	yes
Ibuprofen	NSAIDs	x	no	x	yes
Ketoprofen	NSAIDs	5	yes	5	yes
Meclofenamic acid	NSAIDs	50	yes	50	yes
Mefenamic acid	NSAIDs	8	yes	x	yes
Meloxicam	NSAIDs	7,5	yes	7,5	yes
Naproxen	NSAIDs	x	no	x	yes
Niflumic acid	NSAIDs	8	yes	8	yes
Oxyphenbutazone	NSAIDs	3,5	yes	3,5	yes
Phenylbutazone	NSAIDs	3,5	yes	3,5	yes
Tolfenamic acid	NSAIDs	25	yes	25	yes
Vedaprofen	NSAIDs	x	no	x	no
Virginiamycin M1	other	10	yes	10	yes
Amoxicillin	Penicilline	2	yes	2	yes
Ampicillin	Penicilline	2	yes	2	yes
Benzylpenicillin = Pen G	Penicilline	2	yes	2	yes
Cloxacillin	Penicilline	15	yes	15	yes
Dicloxacillin	Penicilline	15	yes	15	yes
Methicillin	Penicilline	5	yes	5	yes
Nafcillin	Penicilline	15	yes	15	yes

		Egg		Milk	
analyt	group	CC-beta µg/kg	validaded CC-beta < RL	CC-beta µg/kg	validaded CC-beta < RL
Oxacillin	Penicilline	15	yes	15	yes
Phenoxymethylpenicillin = PenV	Penicilline	12,5	yes	12,5	yes
Dapsone	Phenyl sulphone	4	yes	4	yes
8-alpha-Hycdroxy-mutilin	Pleuromutilin	50	yes	50	yes
Tiamulin	Pleuromutilin	50	yes	50	yes
Valnemulin	Pleuromutilin	10	yes	10	yes
Bacitracin A	Polypeptid	50	yes	50	yes
Bacitracin B	Polypeptid	50	yes	50	yes
Rifampicin	Polypeptid	40	yes	40	yes
Dichlorphenamide	Sulfonamide	25	yes	25	yes
Phthalylsulfathiazole	Sulfonamide	25	yes	25	yes
Sulfachlorpyridazine	Sulfonamide	25	yes	25	yes
Sulfaclozine=Sulfachlorpyrazine	Sulfonamide	25	yes	25	yes
Sulfadiazine=S.pyrimidine	Sulfonamide	25	yes	25	yes
Sulfadimethoxine	Sulfonamide	25	yes	25	yes
Sulfadimidine=S.methazine	Sulfonamide	25	yes	25	yes
Sulfadoxine	Sulfonamide	25	yes	25	yes
Sulfalene=S.methoxypyrazine	Sulfonamide	25	yes	25	yes
Sulfamerazine	Sulfonamide	25	yes	25	yes
Sulfamethoxazole	Sulfonamide	25	yes	25	yes
Sulfamethoxypyridazine	Sulfonamide	25	yes	25	yes
Sulfaquinoxaline	Sulfonamide	25	yes	25	yes
Sulfathiazole	Sulfonamide	25	yes	25	yes
Chlortetracycline	Tetracycline	50	yes	50	yes
Chlortetracycline-epi	Tetracycline	50	yes	50	yes
Doxycycline	Tetracycline	10	yes	10	yes
Minocycline	Tetracycline	10	yes	10	yes
Oxytetracycline	Tetracycline	50	yes	50	yes
Oxytetracycline-epi	Tetracycline	50	yes	50	yes
Tetracycline	Tetracycline	50	yes	50	yes
Tetracycline-epi	Tetracycline	50	yes	50	yes

Table 1: Validation results for egg and milk (CC-beta = detection capability for screening according to CIR (EU) 2021/808; RL = regulation level)

POSTER P10

IMPROVED SPE FOR LC-MS DETERMINATION OF RACTOPAMINE IN PORCINE AND BOVINE LIVER: THE OASIS MCX METHOD USING OTTO SPECIALIST

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This poster describes a simple, rapid, and effective cleanup strategy to remove co-extractives from porcine and bovine liver extracts. This is executed via Oasis MCX 96-well SPE plate, processed using Otto SPEcialist, a semi-automated positive pressure manifold, prior to UPLC-MS/MS quantification of total ractopamine, a beta-agonist veterinary drug, with a limit of detection of 0.1 ng/g. This method quantifies ractopamine and ractopamine-glucuronide metabolites to accurately measure ractopamine in animal tissue. The use of Otto SPEcialist to process samples in 96-well plate format not only increases sample throughput and reproducibility, but also eliminates the risk of cross contamination when using manual vacuum manifold.

Keywords: Automation, Ractopamine, Solid-phase extraction

POSTER P11

MULTI-RESIDUE SCREENING APPROACH FOR THE DETECTION OF VETERINARY DRUGS IN ANIMAL TISSUES USING LC-MS/MS

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Abstract
LC-MS/MS is a versatile technology, broadly accepted and recognized as the 'gold standard' for determination of contaminants in food. Whilst originally utilized for confirmation of vet drug residues, we can also use validated analytical techniques based upon LC-MS/MS for screening. This ensures we have great compound coverage, avoid false negatives results, have few false positives, and can use the methodology for a variety of different sample types. In this poster, we describe development and validation of a comprehensive screening method based on LC–MS/MS for over 140 veterinary drugs, from different classes, in representative animal tissues. Bovine muscle tissue was extracted with acetonitrile containing 1% oxalic acid to improve recovery of tetracyclines. The effectiveness of quick and easy to use clean-up options was evaluated. Validation was carried out in accordance with the guidelines laid down by Commission Decision 2002/657/EC for qualitative screening methods. In all cases, values for CCβ were established at concentrations below MRLs and in most cases at 0.1 or 1.0 µg/kg.

Keywords: LC-MS/MS, Multi-residue, Screening

POSTER P12

ANTIBIOTIC RESIDUES IN SURFACE AND GROUNDWATER IN FLANDERS

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Abstract
Due to antibiotic use in animal production, manure may contain antibiotic residues which enter the environment after soil fertilization. Due to run-off or infiltration, antibiotic residues may reach surface and ground water. In this study, 58 antibiotic residues were quantified by UHPLC-MS/MS in 85 Flemish surface and 73 ground water samples.
No antibiotic residues were detected in 9 out of the 85 surface water samples. In the remaining samples, between 1 and 10 residues were detected. A total of 23 different components were encountered in the surface water samples. The most frequently detected antibiotic residues were lincomycin (66%), flumequine (58%), sulfadiazine (29%), trimethoprim (28%), sulfamethazine (27%) and sulfamethoxazole (25%). With a few exceptions, the measured concentrations were lower than 1 µg/L.
Of the 73 groundwater samples, no antibiotic residues were detected in 31 samples. Between 1 and 4 residues were detected in the remaining samples. A total of 13 different antibiotic residues were detected in the groundwater samples. The most frequently detected components were sulfamethazine (30%) and sulfamethoxazole (19%). The measured concentrations were lower than 0.1 µg/L.
This research indicates that water samples, especially surface water, may often contain antibiotic residues but at rather low concentrations

POSTER P13

THE ANALYSES OF MACROLIDES, TETRACYCLINE AND SULFONAMIDE ANTIBIOTICS IN ANIMAL TISSUES USING LC-MS/MS

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Abstract
LC-MS/MS can be used to determine residues of the vet drugs of interest in a variety of matrices. Employing selective extraction and clean up options offers opportunities for the greatest sensitivity and robustness without relying on the highest specification instrument. This poster describes two methods for the determination of important classes of vet drug residues in some representative matrices. One deals with the determination of macrolides and another for the determination of a series of different antibiotics, namely tetracyclines, sulfonamides, trimethoprim, ormetoprim, and dapsone antibiotics. Both use solid-phase extraction (SPE) followed by LC-MS/MS using an ACQUITY UPLC I-Class PLUS System coupled to a Xevo TQ-S micro MS/MS system. The methods both allow for a fast and reliable quantitation down to concentrations well below typical MRLs and have been successfully validated according the European Commission Decision 2002/657. These cost-effective methods can be easily implemented in routine testing laboratories, has been demonstrated as suitable for checking compliance with MRLs, and has the potential for screening at much lower concentrations, such as for food business operators' due diligence testing.

Keywords: Class-specific, LC-MS/MS, validated

POSTER P14

Multi-class Veterinary Drugs Analyses of QuEChERS Extracts using an Automated Online µSPE Clean-up Coupled to LC-MS/MS

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Abstract

This study describes a fully automated approach to online cleanup and injection of QuEChERS extracts of animal tissues for LC-amenable veterinary drugs, based on the use of µSPE cartridges. A total of 103 veterinary drug residues spiked into bovine muscle, liver, and kidney tissues were evaluated using automated µSPE-LC-MS/MS methods. Data on recovery, precision, robustness, matrix effects, and linearity will be presented using the automated technique.

Introduction

Veterinary drugs are administered to animals to ensure animal welfare. It is necessary to screen food products for veterinary drug residues at the maximum residue limits (MRL) set by global regulatory agencies. This screening typically involves both identification and quantification of veterinary drugs using LC-MS/MS.

A sample preparation process commonly used for veterinary drug screening in animal tissues is a modified QuEChERS (quick, easy, cheap, effective, rugged, and safe) extraction. This process involves a liquid-solid extraction of the sample with acetonitrile followed by a simple clean-up. One common clean-up approach is dispersive solid phase extraction (dSPE), which involves adding a fixed amount of a powdered reagent (such as C18 or PSA) to the sample, vortexing, then centrifugation and transfer into an autosampler vial. These techniques require laboratory technicians to deal with weighing or dispensing reagents into labelled tubes and/or time-consuming SPE conditioning and extraction with a manual vacuum manifold. An automated, on-line workflow to address QuEChERS extract clean-up will reduce potential errors, save time, and allow use of lower volumes of solvents and materials.

Materials and Methods

Samples and extraction

Bovine muscle samples were purchased at a local grocery store. Bovine liver and kidney samples were obtained through the Veterinary Diagnostic Laboratory at Iowa State University (ISU VDL). All tissues were homogenized using a laboratory blender. Five grams of tissue was added to a 50 mL Falcon tube. Next, 0.5 mL of 0.2 M ammonium oxalate/EDTA solution was added to the tube followed by acetonitrile to bring the total volume to 15 mL. The tubes were shaken at 2500 rpm on a Fisherbrand™ Digital MultiTube Vortexer for 10 minutes. Matrix extracted standards (MES) were prepared by spiking a mix of 103 veterinary drug residues prior to QuEChERS extraction into bovine muscle, kidney, and liver at different concentration levels. Matrix matched standards (MMS) were prepared by spiking the same mixture of veterinary drugs into extracts after the clean-up step. The concentration levels investigated were from 1 to 100 ng/g. All standards were cleaned up using either a manual dSPE and/or two different automated µSPE workflows. Samples were transferred to LC-MS/MS autosampler vials prior to analysis.

Dispersive SPE (dSPE) and Micro SPE (µSPE) Clean-up.

Extract clean-up approaches included manual dispersive solid phase extraction (dSPE), as well as two fully automated online µSPE cartridge clean-ups utilizing the robotic TriPlus RSH µSPE autosampler. For the manual dSPE experiments, 500 mg CEC18 was added to the supernatant and shaken on a vortexer for 15 minutes, and then centrifuged at 3000 rpm for 10 minutes. These were then placed into the autosampler for injection. Automated µSPE clean-up was performed using the robotic TriPlus RSH µSPE autosampler system (P/N C0950-01-00611), based on a PAL3-RTC autosampler from CTC Analytics (CTC Analytics AG, Zwingen, Switzerland).

Thermo Scientific™ TraceFinder™ software was used for system control. Two miniaturized SPE cartridges, each with a different sorbent, were developed and optimized based upon experiments performed earlier at Iowa State University or the PAL System North America Regional Office laboratory located in Lake Elmo, MN. The result was two µSPE cartridges for further evaluation as described here: a) µSPE, QuEChERS blend for LC, P/N 60101-15VDC18 containing 15 mg CEC18 and b) µSPE, QuEChERS blend for LC, P/N 60101-10VDHPR containing 10 mg Thermo Scientific™ HyperSep™ Retain-PEP material (HRP). A leak-proof seal is obtained when the needle is compressed by the PAL into the cartridge, which allows the syringe to push sample extracts or solvents through the sorbent bed with a constant force. Essentially, the syringe replaces the vacuum system of the classical SPE methodology working at defined flow rates. Procedurally, uncleaned QuEChERS extracts were transferred into 2 mL autosampler vials and placed into a 54-position tray (sample tray). The corresponding number of collection vials [500 µL, fused insert snap-top (P/N C4011-LV1) sealed with star-slit snap-it caps (P/N C4011-59)] were placed into a second 54-position tray (eluate tray). Uncleaned extracts were loaded onto the cartridges and the extracted samples were injected directly into the LC-MS/MS system.

LC-MS/MS Analysis

The LC-MS/MS system comprised a Vanquish Flex UHPLC binary system interfaced with a TSQ Altis triple quadrupole mass spectrometer equipped with a H-ESI ionization probe. Chromatographic separation was carried out on a Thermo Scientific™ Accucore™ VDX column, 100 mm × 2.1 mm × 2.6 µm (P/N VDX-102130) at a temperature of 40 ˚C, using a gradient elution of 0.05 % formic acid in water (mobile phase A) and 1:1 methanol : acetonitrile with 0.05% formic acid and 5% water (mobile phase B). The mass spectrometer was operated in both positive and negative ionization modes.

Results and Discussion

The veterinary diagnostic lab at Iowa State University routinely screens for a wide variety of veterinary drugs with QuEChERS extraction followed by manual dSPE or SPE clean-ups. Extracts of three tissue types, (bovine muscle, liver, and kidney) were selected to evaluate the automated online µSPE-LC-MS/MS clean-up methods described herein. Method performance criteria, including linearity, absolute recovery, precision, reproducibility, and robustness were evaluated. Absolute recovery was verified at 50 ng/g and reproducibility of the method was verified at 5 and 50 ng/g. Method effectiveness was checked by comparing the conventional manual dSPE with CEC18 QuEChERS clean-up procedure. Also, the effectiveness of the automated µSPE CEC18 clean-up was compared to that of the automated µSPE procedure using the Hypersep Retain-PEP (HRP). The selected 103 veterinary drugs represent a wide variety of compound classes and are listed in the AOAC Standard Method Performance Requirements document (SMPR 2018.010).

Calibration linearity

For screening and quantitation of the veterinary drugs, calibration levels using matrix extracted standards (MES) and/or matrix matched standards (MMS) were prepared in all three tissue types. Calibration standards ranging from 1 to 100 ng/g were analyzed and excellent linearity was achieved, with coefficient of determination R2>0.99 for most compounds in the three different extracts. For screening workflow, the Veterinary Diagnostic Lab at Iowa State University created calibration curves using an automatic calibration script on the TriPlus RSH µSPE system. In this case, the analyst prepared a stock standard in solvent and used the blank QuEChERS matrix as a diluent. The system proceeds to prepare all specified standard levels and mixes them before passing them through the µSPE cartridge and subsequent injection into the LC-MS/MS system. This saves time and reduces potential handling and labelling errors in the lab.

µSPE method performance

The absolute recoveries of 103 veterinary drugs using the cleanups described above (manual dSPE, CEC18-µSPE, and HRP-µSPE) were evaluated in bovine muscle, liver, and kidney tissues. For each experiment, five biological replicates were prepared as matrix-extracted spikes (MES) containing all the target residues at 50 ng/g. The MES were compared to standards spiked into the cleaned samples (MMS) for each of the different clean-ups at the same concentrations. Recovery was calculated as the ratio of the average peak area response of the MES to the average peak area response of the MMS. Absolute recoveries within 30–140% with corresponding %RSDs less than or equal to 20% are required for satisfactory method validation according to the EU SANTE 12682/2019 document for pesticides.

Recoveries within this range were achieved for over 95% of the target veterinary drugs in the three tissue extract types for both the manual CEC18 dSPE procedure and the automated online clean-up using the miniaturized CEC18 µSPE cartridges. Using the HRP material, it was noted that the recoveries of rafoxanide, closantel, and lasalocid are very poor (typically less than 5%). This is likely due to retention of these analytes on the sorbent during the clean-up, and we are undergoing further study to determine the root cause. Some of the compounds also demonstrated low sporadic recoveries with high %RSD in some matrices. However, these are easily detected at less than 5 ng/g in the bovine extracts.

A precision study was also carried out using the CEC18 µSPE miniaturized cartridge cleanup in all three tissue types. MES calibration curves were prepared at calibration levels of 0.5, 1, 5, 10, 25, 50, 70, and 100 ng/g, along with five biological replicates of each matrix prepared at 5 and 50 ng/g. Excellent precision of less than 20% RSD and recovery between 70 and 120% were obtained for over 90% of the veterinary drugs in each matrix at both concentration levels.

Overall time savings and µSPE method robustness

The online automated approach to clean-up QuEChERS extracts using the TriPlus RSH µSPE autosampler saves a tremendous amount of labor, especially for large batches of samples. At the Iowa State Veterinary Diagnostic lab, measurements were made to determine the operator time saved during a batch preparation of 50 samples. For the µSPE-CEC18 workflow, a time savings of up to 4 hours was achieved as compared to the manual dSPE method. For the HRP method, which normally is an SPE procedure requiring a vacuum manifold, over 5 hours is saved. The operators noticed that labelling and moving vials around the lab costs time and is prone to errors. The time savings noted here can even be improved by the autocalibration function mentioned earlier, as this is clearly another key advantage of the system. Finally, this automation provides an easy way to evaluate different clean-up sorbents packed into the miniaturized cartridges, since control of flow rates is crucial, and these settings are easily adjusted in an automated experiment.

The TSQ Altis triple quadrupole mass spectrometer has been operating routinely in the Iowa State Veterinary Diagnostic laboratory for over three years analyzing veterinary drugs in animal tissues and biological fluids. Method robustness enabling increased uptime is the main reason extract clean-up is so important. Two compounds, levamisole and albendazole- 2-aminosulfone in bovine muscle extract, cleaned up using the µSPE CEC18 cartridges. The data shows that the response was well within the expected ±20% range for at least 100 consecutive injections without maintenance.

Conclusion

The fully automated and online µSPE clean-up workflow with LC-MS/MS described here is a reliable, accurate, reproducible, and robust solution for multi-class veterinary drug screening and quantitation in a variety of animal tissues (muscle, liver, and kidney).

References

The full application note with complete method details is available and can be downloaded here:
https://assets.thermofisher.com/TFS-Assets/CMD/Application-Notes/an-66000-lc-ms-vet-drugs-quechers-uspe-an66000-en.pdf

POSTER P15

OMICS APPROACHES TO DETECT GROWTH HORMONE ADMINISTRATION IN ATHLETES

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Abstract
A cohort of well-trained volunteers was administered with micro-doses of EPO or EPO/GH every second day for two weeks. Urine and plasma samples have been collected before, during and after the treatment. The metabolomics results [1] show that, applying a direct discriminant analysis, it is possible to distinguish the different metabolic profile of the two treatments, and to obtain a high classification accuracy for each subject. A time 0 centering of the data enabled performing a longitudinally tracking of each subject leading to the observation of higher difference between the groups. A perfect classification of the samples before and after the treatments could thus be obtained. The study further demonstrated [2] that individuals supplemented with micro-doses of rhGH exhibited different leukopoietic and steroidal profiles compared to the control population, suggesting a role of the rhGH in both pathways. The use of leukopoietic and steroidal biomarkers together with endocrine biomarkers (IGF-1 and P-III-NP) allowed to correctly classify over 98% of samples with no false positives, misclassifying only one single sample (false negative) over a total of 56; a promising result, if compared to the current rhGH detection strategies.
[1] Narduzzi et al. DrugTestAnal. 2020
[2] Narduzzi et al. Front.Mol.Biosci. 2021

Keywords: Growth Hormone, Metabolomics, Steroidomics
Abstract nr: 165

POSTER P16

DETERMINATION OF COCCIODIOSTATS, NITRO-IMIDAZOLES AND FIPRONIL IN MILK BY UHPLC-MS/MS

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Abstract
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Coccidiostats are widely used for prevention and treatment of coccidiosis. A screening and confirmatory ultra-high performance liquid chromatographic-tandem mass spectrometric (UHPLC-MS/MS) multiresidue method was validated for the detection and quantitation of 22 coccidiostats, 10 nitro-imidazoles + metabolites and fipronil +metabolites in milk.

Validation was performed according to the guidelines stipulated in Commission Implementing Regulation (EU) 2021/808 taking into account the maximum levels in milk (Commission Regulation 37/2010, 124/2009 and 610/2012). In addition to the veterinary drugs considered in this study, the insecticide fipronil, which is not approved for use in food producing animals, was included as well.
The validation parameters linearity, precision, recovery, specificity, relative matrix-effect, decision limit (CCα) and detection capability (CCβ) were determined. It can be concluded that the validated method is easy to perform, fast, and suitable for both screening and confirmation purpose. Final results will be presented during the symposium.

Keywords: Coccidiostats, milk, nitro-imidazoles

POSTER P17

ANDROGENS AND ESTROGENS BIOMARKERS IDENTIFICATION IN BOVINE URINE APPLYING A METABOLOMICS APPROACH

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Abstract
Despite steroids have been banned in the EU for growth promoting purposes, their illegal use in livestock is regularly suspected in Europe and in edible tissues imported from third countries. To tackle associated practices (natural hormones, designer drugs, low dose cocktails), innovative screening strategies based on biomarkers monitoring have already proven their efficiency, e.g. in highlighting a range of β-agonists practices [1]. Further, such approach has proved its applicability for control purposes under ISO17025 accreditation [2].
To extend the work to other anabolic practices, markers of effects related to steroids application in livestock have been investigated. Specific urinary patterns associated with three steroid treatments (17β-nandrolone, 17β-estradiol, 17β-nandrolone+17β-estradiol) in bovine have been investigated through RPLC-ESI(+/-)-HRMS. While specific fingerprints with strong differences could be highlighted between urinary metabolite profiles within urine samples collected on control and treated animals, it appeared further that significant discriminations could also be observed between steroid treatments, evidencing thus specific patterns and candidate biomarkers associated to each treatment. An MS^2 structural elucidation step enabled level-1 identification of two biomarkers mainly involved in energy pathways, in relation to skeletal muscle functioning [3].
[1] Dervilly-Pinel Metabolomics 2015
[2] Dervilly-Pinel Food Additives & Contaminants 2018
[3] Ouzia Drug Test Anal. 2021

Keywords: Metabolomics, Screening, Steroids

POSTER P18

INVESTIGATING NANDROLONE STATUS IN POULTRY LIVER

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Abstract
Among the matrices analysed in the framework of official national control plans, poultry liver is one of those for which a particular steroid, nandrolone, is frequently detected. The frequency of detection of this compound does not, however, appear to be compatible with widespread and fraudulent use of this compound. Therefore, the hypothesis of its endogenous status might explain these observations. This work has made it possible to study nandrolone metabolism in greater detail in order to define whether a particular metabolite profile is associated with the presence of hepatic nandrolone. The combination of a preparative liquid chromatography with specific detection by GC-MS/MS allowed to propose an efficient method for the detection of estranediol isomers in the liver. The application of this method to poultry livers exhibiting high nandrolone levels allowed the identification of a specific metabolite profile associated with these atypical cases. These innovative results will need both to be confirmed on a larger panel of samples to establish the occurrence distribution and to be confronted with the analysis of samples from animals actually treated with nandrolone in order to determine the scope of this work and the possibility of defining a new criterion of suspicion.

Keywords: Estranediols, Nandrolone, Natural Hormone

POSTER P19

COMPREHENSIVE LC-HRMS METABOLOMICS ANALYSIS TO DETERMINE URINARY METABOLITES OF ALTRENOGEST IN GILTS

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Abstract
Altrenogest (ALT), a synthetic progestogen, is used in pig farming for estrus synchronisation in gilts. Residues of ALT and its metabolites may reach the aquatic environment via the spread of liquid manure and may present a risk for fish and other higher aquatic organisms due to its endocrine disrupting potential. Thus, we conducted a pilot study to estimate the environmental input of ALT and its metabolites (Liesenfeld et al., 2022). LC-HRMS was applied to perform targeted analysis of ALT and known metabolites as well as non-targeted metabolomics analyses in order to find previously unknown metabolites. The targeted investigation showed that glucuronide conjugates of ALT and its photo-isomerisation product are main urinary metabolites of ALT in gilts. The chemometric analysis of non-targeted data revealed a clear difference between ALT-treated gilts and control animals. A hydroxylated ALT glucuronide was identified as highly significant in the ALT-treated group. Additional biomarker annotation and pathway mapping uncovered changes in the metabolism of ALT-treated animals which can be explained by ALT's hormonal action. This study illustrates the exceptional potential of LC-HRMS and metabolomics for the detection of potentially new environmental contaminants with high biological activity.

Introduction
Altrenogest (ALT), a synthetic progestogen, is one of a few hormonally active substances permitted for zootechnical use in livestock farming in Europe (96/22/EC, 1996). It is administered to gilts at 20 mg/d for 15–18 days to induce a blockage of estrous cycle enabling estrus synchronisation and thus batch farrowing. The main pharmacodynamic actions of ALT are based on progestomimetic and anti-gonadotrophic effects (EMA, 2012). An extrapolation from the sales numbers in Lower Saxony resulted in an estimated consumption of about 250 kg ALT per year in Germany (Petri, 2019). Published data on urinary excretion of ALT in gilts is limited, fragmented and contradictory. Elimination via bile as the major excretion pathway with only 20 % renal excretion as well as primarily renal excretion (60 %) is reported in gilts (EMA, 2012). Urinary metabolites of ALT are only described in horses (Lampinen-Salomonsson et al., 2006).

Synthetic substances with hormonal actions are subject to public discussion since they are very critical environmental contaminants and belong to the group of endocrine disrupting chemicals (UNEP/WHO, 2013; Hamscher and Bachour, 2018). Veterinary medicinal products containing ALT are also discussed to pose a risk to fish and other aquatic organisms (EMA, 2016). ALT (< 0.4 ng/L) showed relevant effects on the fertilisation and survival rate in fish as well as a shift in sex ratio toward males caused by its androgenic effect (EMA, 2016). Considering the predicted environmental concentration a risk for fish and other aquatic organism associated with the zootechnical use of ALT is likely for certain geographical areas (Di Nica et al., 2015; EMA, 2016). Environmental residue monitoring programs detected ALT in 4 % of 50 tested lakes in Minnesota, U.S. and in influent and effluent of a wastewater treatment plant in the Czech Republic in environmentally relevant concentrations (Ferrey et al., 2015; Golovko et al., 2018). Furthermore, sorption studies at a laboratory scale showed further that an accumulation of ALT in different soils may occur and biotic as well as abiotic elimination needs to be considered (Petri, 2019; Yang et al., 2020). We therefore conducted a pilot study with spot urine samples from gilts taken under real world, routine breeding conditions (Liesenfeld et al., 2022).

Materials and Methods

Samples

Spot urine was sampled in the course of a pilot study with conventionally housed gilts treated with ALT as part of routine, real world breeding procedures without any impairment to the animals at the "Landesanstalt für Schweinezucht" in Boxberg (Baden-Wuerttemberg, Germany) in December 2019. Spontaneous urine samples were collected from 5 non-medicated gilts (Baden-Wuerttemberg hybrid, female, age 6 month) and 3 ALT-treated gilts (Baden-Wuerttemberg hybrid, female, age 6 month) at different sampling times, resulting in 5 control samples and 7 positive samples (multiple samples per animal), respectively. All animals were clinically healthy and weighed appr. 100 kg. In the treatment group, 20 mg ALT were administered daily at 08:00 a.m. over the course of 18 day as part of standard breeding procedures. The samples from medicated gilts were taken at day 17 and 18 of treatment, i.e., during the steady-state phase (EMA, 2012). Both groups were given the same feed stuff. Please note that permission from the relevant ethics committee is not required for these studies.

Sample preparation and LC-HRMS analysis

Samples were pH-adjusted using ammonium formate buffer and used for a solid phase extraction procedure (SPE; OASIS HLB®, 200 mg, 6 cc). The extracts were evaporated, reconstituted with the eluent starting conditions and analysed via LC-HRMS in a targeted and non-targeted setting (Liesenfeld et al., 2022). Sample preparation was performed without a hydrolysis step in order to check for the presence of known metabolites such as ALT glucuronide, sulfate conjugate, isomers and the parent drug (ALT) as well as with a hydrolysis step in order to quantify ALT glucuronides after deconjugation to ALT.

For details regarding data processing, statistical analysis and biomarker annotation for non-targeted analysis see Liesenfeld et al. (2022). In brief, after data processing in MZmine 2 (Pluskal et al., 2010), the data was subjected to multivariate statistical analysis, i.e., principal components analysis (PCA) using an evaluation procedure that was implemented in MATLAB R2019 (The MathWorks Inc., Natick, Massachusetts). The calculation of a 0.9-prediction ellipse for the PCA data was performed using EXCEL 2010 (Steliopoulos, 2013). For metabolite identification, the data was submitted to MetaboAnalyst 4.0 (Chong et al., 2019) and Compound Discoverer 3.1 (Thermo Scientific, Idstein Germany). Biomarker annotation was performed for the top 25 significant features as described in Liesenfeld et al. (2020). Additionally, pathway mapping was performed to complement manual biomarker identification.

Results and Discussion

The targeted investigation showed that glucuronide conjugates of ALT and its photo-isomerisation product (ALT-CAP) are main urinary metabolites of ALT in gilts. The concentrations in urine ranged from 1.90 mg/L to 4.14 mg/L, which is comparable to a worst case estimation of 5 mg/kg ALT in manure used for laboratory scale sorption studies (Petri, 2019). Due
to the division of labor between pig breeding and pig fattening, this worst case scenario is reasonable, and regional hot spots are likely. Hence, it cannot be excluded that even considering a dilution factor of 1.0×10^6 by water, sorption processes in run-off scenarios or manure from untreated animals concentrations of ALT reaching surface water exceed the predicted no effect concentration of ALT for surface water (0.04 ng/L). Consequently, a risk for fish and other aquatic organisms associated with the zootechnical use of ALT cannot be ruled out completely.

Sulfate conjugates as well as ALT and its photo-isomerisation product were not observed. However, an unknown isomer of ALT was detected at trace level. Our results are in line with the Committee for Medicinal Products for Veterinary Use (CVMP) opinion stating that glucuronide conjugation is a major metabolic pathway for ALT (EMA, 2012). Furthermore, our data clearly shows that ALT conjugates need to be considered in the environmental risk assessment. ALT conjugates may exhibit a hormonal effect after microbial deconjugation (EMA, 2012) and its photo-isomerisation product ALT-CAP has also shown hormonal activity (Pflug et al., 2019).

Table 1. Results of targeted LC-HRMS analysis of urinary ALT gluc. (sum of ALT glucuronide and ALT-CAP glucuronide) (n = 2; mean ± measurement uncertainty).

Sample	Hours afer last ALT treatment	ALT[a] (ng/mL)	ALT isomer[b] (ng/mL)	ALT sulfate (qual.)	ALT gluc[c]. (ng/mL)
Gilt A					
3:30 p.m. (day 17)	7.5	n.d. (<CL)	< LOQ	n.d.	3900 ± 250
11:15 a.m. (day 18)	3.25	n.d. (<CL)	< LOQ	n.d.	3950 ± 250
Gilt B					
4:00 p.m. (day 17)	8	n.d. (<CL)	< LOQ	n.d.	1900 ± 250
11:15 a.m. (day 18)	3.25	n.d. (<CL)	< LOQ	n.d.	4140 ± 250
Gilt C					
3:00 p.m. (day 17)	7	n.d. (<CL)	< LOQ	n.d.	3780 ± 250
7:00 a.m. (day 18)	23	n.d. (<CL)	< LOQ	n.d.	3320 ± 250
10:15 a.m. (day 18)	2.25	n.d. (<CL)	< LOQ	n.d.	2780 ± 250

n.d.: not detected, decision limit CL: 1.6 ng/mL; determination limit LOQ: 3.9 ng/mL; qual: qualitative analysis; [a]ALT analyzed by method without enzymatic hydrolysis; [b]Unknown ALT isomer analyzed by method without enzymatic hydrolysis; [c]ALT gluc. (sum of ALT glucuronide and ALT-CAP glucuronide) quantified as ALT after enzymatic hydrolysis.

The multivariate statistical analysis of non-targeted data revealed a clear difference between ALT-treated gilts and control animals. As can be seen in Fig. 1 the prediction ellipses only overlap merely slightly.

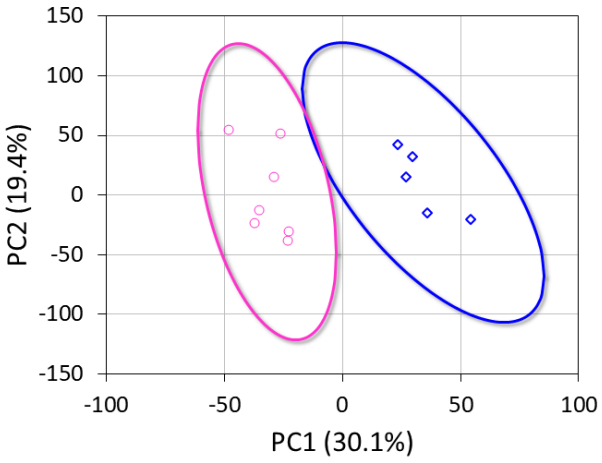
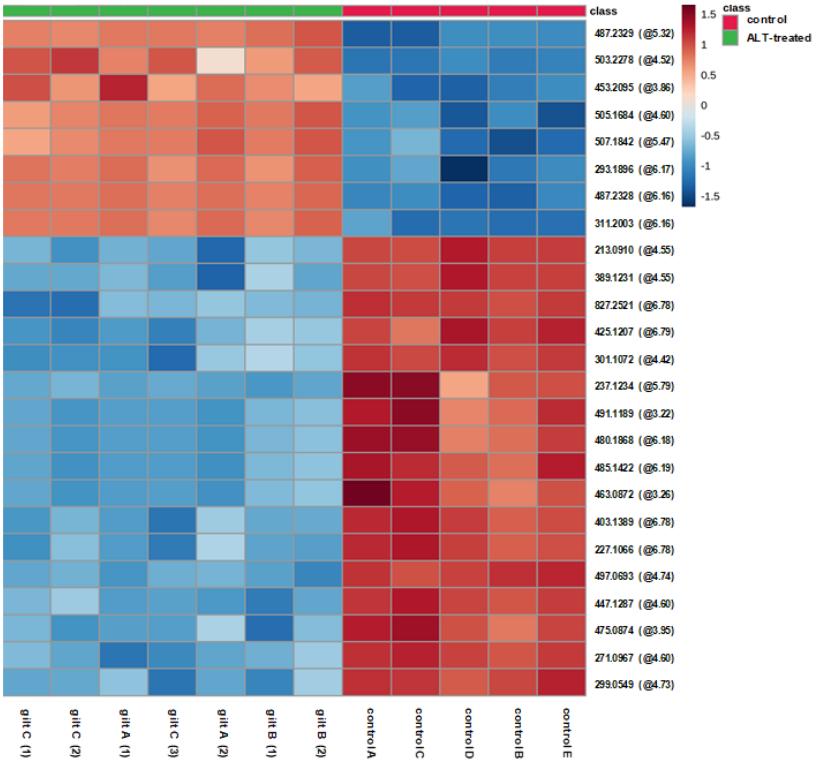


Figure 1. 2D scores plot of PCA of LC-HRMS data of urine samples; blue rhombi: control samples (non-medicated gilts) with 0.9-prediction ellipse, magenta circles: positive samples (ALT-treated gilts) with 0.9-prediction ellipse; PC: principal component with explained variance shown in brackets.

Univariate statistical tests were used for biomarker identification. ALT glucuronide, ALT-CAP glucuronide and their in-source fragmentation products were in the top 25 significant variables distinguishing the two test groups. All adjusted p-values of the top 25 features were highly significant (p-adj. < 3.1×10^{-9}, for details see Liesenfeld et al., 2022). In order to visualize the results and aid pattern recognition a 2D heatmap was constructed (see Fig. 2). Eight of the top 25 significant features were increased in the medicated group and 5 of these 8 increased features were identified as metabolites and in-source fragments of ALT metabolites. One metabolite was tentatively identified as a hydroxylated ALT glucuronide. Interestingly, 17 of the 25 top significant features showed higher intensities in the control group. Six features correspond to glucuronide conjugates given the characteristic neutral loss of 176.0309. Database hits and mass spectral library hits suggest a similarity to isoflavonoids and stilbene derivatives. This may lead to the assumption that the



metabolism of nutrients, i.e., phytoestrogens, is altered under ALT treatment. However, further studies are recommended to support this hypothesis.

Figure 2. 2D heatmap in order to visualize the top 25 significant features of Student's t-test and to aid pattern recognition; 5 control gilts (A–E) and 3 ALT-treated gilts (A–C) at different sampling times (1–3, see Table 1) were clustered with dendograms at sample and analyte level.

Furthermore, pathway mapping based on database hits indicated differences in the tryptophan degradation as well as C21-steroid bio-synthesis pathways in the ALT-treated group compared to the control group. These changes can be explained by the mechanism of action of the hormonally active substance ALT.

Conclusion

The targeted investigation of real world urine samples from ALT-treated gilts showed that glucuronide conjugates of ALT and its photo-isomerisation product (ALT-CAP) are main urinary metabolites of ALT in gilts. The concentration of ALT glucuronides (1.90–4.14 mg/L) is comparable to worst case calculations used for laboratory scale sorption studies. These findings confirm that even considering a dilution factor of 1.0×10^6 environmentally relevant concentrations in surface water cannot be excluded. Furthermore, another isomerisation product of ALT was observed at trace level, which to the best of our knowledge has not yet been described in the literature. These results underline the need to perform field studies to determine the environmental input of ALT considering regional hotspots, i.e., pig breeding facilities. The non-targeted analysis of real world urine samples showed a clear separation of ALT-treated gilts and control animals. ALT glucuronides and their in-source fragmentation products were in the top 25 significant hits in order to distinguish the two test groups. Furthermore, a hydroxylated ALT glucuronide could be identified at substance

class level. Besides, metabolic changes in the treatment group were highlighted that can be explained by the mechanism of action of the hormonally active substance ALT. These results illustrate the exceptional potential of non-targeted LCHRMS analyses, since they render valuable information for metabolite and biomarker discovery. Further advantages of the method described are the sampling during routine breeding conditions, a relatively small number of animals required and no particular stress for the animals.

Acknowledgements
The authors thank the Landesanstalt für Schweinezucht in Boxberg for giving them the opportunity to collect spot urine samples from gilts.

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POSTER P20

ANTIBIOTIC RESIDUES IN AQUATIC ENVIRONMENTS: VALIDATION OF A UHPLC-MS/MS METHOD

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Abstract
The occurrence of antibiotic residues in the environment has received considerable attention due to their potential to select for bacterial resistance, which may render some infections untreatable. Overuse of antibiotics in human medicine and animal production leads to antibiotic residues reaching aquatic environments, both surface, ground and marine water, but currently it is not yet well known at which concentrations.
A large number of water samples in regions with both low and high load of antibiotic usage will be collected. These include freshwater samples from areas with high livestock concentrations and hospital effluents and furthermore marine samples taken from aquaculture, harbors (marine and brackish) and seas. For this purpose, a method for detection of antibiotic residues in water had to be developed.
This study presents the validation of a multi-residue method for simultaneous quantitative analysis (screening + confirmation) of 80 different antibiotics from different classes: beta-lactam, sulfonamides, tetracyclines, lincosamides, phenicols, quinolones, macrolides, pleuromutilins and diamonopyrimidine derivates. Solid-phase extraction with OASIS-HLB cartridges (6 cc, 500 mg) was followed by analysis with UHPLC-MS/MS (BEH C_{18} column). The parameters limit of detection, limit of quantification, linearity, precision, recovery, specificity and relative matrix effect have been addressed. Results will be published at the conference.

Keyword: Antibiotics

POSTER P21

THE EU RASFF AND FOODBORNE ANTIMICROBIAL RESISTANCE. COULD A COLLABORATION WITH THE WHO GLASS BE ENVISAGED?

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Abstract

For the first time, a search (from 1979 until 2020) in the EU RASFF Portal (https://webgate.ec.europa.eu/rasff-window/portal/?event=SearchForm&cleanSearch=1#) was undertaken with the only search subject being the term "resistant" to effectively come up with 118 notifications that pertain to antimicrobial resistance (AMR), in food and feed submitted from 2002 until 2015. All 118 (except for three) of these notifications were on multiresistant *Salmonella* (mostly *Salmonella typhimurium*). The most frequently found combinations of *Salmonella* and specific food product categories, were the following: i) *Salmonella typhimurium* + "meat and meat products (other than poultry)", and ii) *Salmonella typhimurium* + "poultry meat and poultry meat products". Denmark was the EU RASFF notifying country that had most frequently submitted notifications with the term "resistant" included in their subject and Germany was the country of origin that was most frequently notified against. A proposal is thus put forward to use the EU RASFF again as a risk communication tool for AMR in food and feed and in essence to continue from 2015 when this reporting was discontinued.

All this information on AMR could certainly help the EU and give support to the WHO GLASS initiative in offering valuable data on resistant microorganisms in the EU RASFF Member States but in Third Countries (that export products to the EU). Thus, a successful collaboration could indeed be envisaged between the two supranational organizations for the benefit of the protection of Public Health against AMR, in the context of One Health.

Introduction

Antimicrobial resistance (AMR) poses an important, complex, and priority global public health challenge (CXC 61-2005) with

significant global economic and security implications as acknowledged by the World Health Organisation (WHO). In 2015, WHO Member States unanimously approved a **Global Action Plan to tackle AMR**. **On 22 October 2015, WHO launched the Global Antimicrobial Resistance and Use Surveillance System (GLASS)**, the first global collaborative effort to standardize AMR surveillance. Endorsed by the Sixty-eighth World Health Assembly in resolution WHA68.7, GLASS was created to support the second objective of the GAP-AMR initiative to "strengthen knowledge through surveillance and research", and to continue filling knowledge gaps, with the aim to inform strategies at all levels (https://www.who.int/initiatives/glass). GLASS has been conceived to progressively incorporate data from surveillance of AMR in humans, such as monitoring of resistance and the use of antimicrobial medicines, including AMR in the food chain and in the environment. GLASS is supported by the **WHO AMR Surveillance and Quality Assessment Collaborating Centres Network**. It enjoys strong commitment from participating countries and close collaboration with **AMR regional networks** such as the Central Asian and European Surveillance of Antimicrobial Resistance (CAESAR), the European Antimicrobial Resistance Surveillance Network (EARS-Net), the Latin American Network for Antimicrobial Resistance Surveillance (Rede Latinoamericana de Vigilancia de la Resistencia a los Antimicrobianos [ReLAVRA]), and the Western Pacific Regional Antimicrobial Consumption Surveillance System WPRACSS (https://www.who.int/initiatives/glass).

The EU Rapid Alert System for Food and Feed (RASFF) has its legal basis in Regulation 178/2002 and is an important risk communication tool for EU Member States (MS) in exchanging information concerning risks found in food or feed. The members of this network are the EU MS, the Commission Services, the European Food Safety Authority, the countries of the European Economic Area (EEA), and Switzerland. The notifications contained in the EU RASFF database pertain to either food, or feed or food contact materials and refer to hazards (biological, chemical, physical) found in the three aforementioned categories. Antimicrobial resistance (AMR) has to this day not been investigated in the RASFF database and would be identified as a reason for a RASFF notification after the inclusion of the term "resistant" in the search subject.

Apart from the RASFF, other important EU Institutions such as the Health and Food Audits and Analysis (HFAA) of the European Commission have contributed greatly in identifying good practices *vis a vis* AMR through fact-finding missions and auditing of the EU MS on that subject.
Examples of DG Health and Food Safety fact-finding missions to gather information on the prudent use of antimicrobials in animals, considered for the latest Overview Report (2019-6788) were the following: i) France (2017-6200), ii) Latvia (2017-6202), iii) Norway (2017-6199), iv) Sweden (2017-6201) and v) Italy (2018-6371). Also, more audits have been performed in order to gather information on the prudent use of antimicrobials in animals, in the following EU MS: Romania (2016-8888), Spain (2016-8887), Cyprus (2016-8884), Netherlands (2016-8889), Finland (2016-8886), Germany (2016-8890), Denmark (2016-8882), Slovenia (2016-8883). Additionally, a large number of audits have been performed in order to evaluate the monitoring and reporting of antimicrobial resistance in zoonotic and commensal bacteria in certain food-producing animal populations and food in the following MS: Poland (2018-6308), France (2018-6307), Lithuania (2017-6197), United Kingdom (2017-6194), Italy (2017-6191), Bulgaria (2017-6192), Romania (2016-8677), Netherlands (2016-8919), Hungary (2016-8676), Spain (2016-8678), Slovakia (2016-8917), (Germany 2015-7404), Denmark (2015-7383) that can be accessed at the HFAA website of the European Commission (https://ec.europa.eu/food/audits-analysis/audit_reports/index.cfm).
The aim of this study was firstly to search for notifications on AMR in the EU RASFF database from 1979 until 2020 and reveal data on "resistant" foodborne microorganisms in food and feed, but also to raise awareness on the possible collaboration of the EU RASFF and the WHO GLASS for the risk communication of foodborne AMR.

Materials and Methods
A search in the EU Rapid Alert System for Food and Feed database (https://webgate.ec.europa.eu/rasff-window/portal/?event=searchForm&cleanSearch=1) retrieved the data used for this paper (accessed on 21/01/2021). The sole criterion used in the search undertaken for all types of notifications (from 1979 till 31/12/2020) was the term: "resistant". All data were extracted in Excel files.

Results
The first ever notification which was an alert notification was submitted on 12/03/2002 by Finland (Ref. 2002.089) its subject being: "*Salmonella enteritidis* (2 out of 6 samples) and *Salmonella Hadar* multiresistant (4 out of 6 samples (2 multiresistant)) in Chicken breasts fillets" the product category being "poultry meat and poultry meat products" in food, with a risk decision "undecided".
The last notification to appear in the EU RASFF was an information for attention notification was submitted on 24/08/2015 by the United Kingdom (Ref. 2015.1096) its subject being:" *Salmonella typhimurium* DT 193 - multiresistant (presence /25g) in fresh vine leaves (Piper lolot / Piper saigonensis) from Cambodia, via Vietnam" the product category being "fruits and vegetables" in food, with a risk decision being "serious".
Of the 118 notifications in total that were retrieved from the EU RASFF database, the types of notifications that contained the term "resistant" in their subject in the RASFF database were 80 information notifications, 34 alert notifications, 1 information for attention notification, 3 information for follow-up notifications. These 118 notifications were submitted from 2002 until 2015 and from that year on, none such notification containing the term "resistant" was found in the RASFF database.
All the 118 notifications were on food, except for only two that were on feed and these were the following: i) one alert notification submitted on 03/10/2014 (Ref. 2014.1352) by Austria its subject being: "*Salmonella typhimurium* DT 193 - multiresistant (presence /25g) and high count of *Enterobacteriaceae* (8800 CFU/g) in dog chews from Poland" in pet food on feed, with a risk decision designated as "serious", and ii) one alert notification submitted on 05/10/2007 (Ref. 2007.0714) by Iceland its subject being: "*Salmonella typhimurium* (multiresistant) in dog chew from the Netherlands" in pet food on feed with a risk decision designated as "undecided".
The food product categories involved in all 118 notifications were the following (in descending order of frequency of appearance in the RASFF database): i) poultry meat and poultry meat products (64/118 or 54.23%), ii) meat and meat products (other than poultry) (49/118 or 41.52%), iii) pet food (2/118 or 1.69%), iv) cephalopods and products thereof (1/118 or 0.84%), v) eggs and egg products(1/118 or 0.84%) and vi) fruits and vegetables (1/118 or 0.84%).
Only two of these 118 were "serious" (all the rest were designated as "undecided" in terms of "risk decision") and these two were the following: an information for attention notification submitted on 24/08/2015 (Ref. 2015.1096) by the United Kingdom, its subject being: "*Salmonella typhimurium* DT 193 - multiresistant (presence /25g) in fresh vine leaves (Piper lolot / Piper saigonensis) from Cambodia, via Vietnam" in fruits and

vegetables on food, and an alert notification submitted on 03/10/2014 (Ref. 2014.1352) by Austria, its subject being: "*Salmonella typhimurium* DT 193 - multiresistant (presence /25g) and high count of *Enterobacteriaceae* (8800 CFU/g) in dog chews from Poland" in pet food on feed.
In the subject of each notification the hazard identified was *Salmonella* (n=142) in many of which there were more than one *Salmonella* present in the food item examined, such as the following examples of: i) an alert notification submitted on 10/07/2007 by Denmark (Ref. 2007.0456), its subject being: " *Salmonella Agona* (multiresistant, presence in 5/12 samples) and *Salmonella typhimurium* (multiresistant, presence in 5/12 samples) in turkey breast cuttings from France" and ii) an alert notification submitted on 09/04/2003 by Finland (Ref. 2003.092), its subject being: "*Salmonella enteritidis* (positive in 1/5 samples /25g), *Salmonella Hadar* multiresistant (positive in 2/5 samples /25g) and Salmonella Newport (positive in 1/5 samples /25g) in chicken breast fillets" and iii) an information notification submitted on 29/05/2009 by Denmark (Ref. 2009.0681), its subject being: "*Salmonella Bredeney* (1 out of 12), *Salmonella typhimurium* U302 multiresistant (4 out of 12) and Salmonella typhimurium U311 (1 out of 12) in pork loin from Spain".
Only one notification which was an information notification did not include *Salmonella* in its subject but *Campylobacter* and this was submitted on 14/08/2007 by Italy (Ref. 2007.BYC), its subject being: "*Campylobacter* (multiresistant, in 1 out of 5 samples present /25g) in fresh whole chicken from France" on poultry meat and poultry meat products, in food with a risk decision designated as "undecided".
In all 118 notifications the word 'multiresistant' was consistently included in the subject, except for only three notifications, namely: i) an alert notification submitted on 11/07/2007 by Denmark (Ref. 2007.0462), its subject being: "*Salmonella enterica ser. Enteritidis* (in 3 out of 12 samples, quinolon-resistant) in frozen salted boneless skinless chicken breast from Brazil, via the United Kingdom" on poultry meat and poultry meat products, in food with an "undecided" risk decision, ii) an information notification submitted on 15/10/2004 by the United Kingdom (Ref. 2004.CFT), its subject being: "*Salmonella enterica ser. Enteritidis* (ampicillin resistant) in raw chicken eggs from Spain" on eggs and egg products in food with an "undecided" risk decision, iii) an information notification submitted on 17/12/2007 by Italy (Ref. 2007.CYI), its subject being: "*Salmonella Weltevreden* (sulfonamide resistant) in cuttlefish (*Sepia aculeata*) from India" on cephalopods and products in food with an "undecided" risk decision.
In fact, the word 'multiresistant was mentioned 120 times in total in all 118 notifications. The most frequently mentioned *Salmonella* was *Salmonella typhimurium* (73 times or 61.86%).
The EU RASFF MS that have notified in their capacity as notifying countries, the greatest numbers of notifications to the RASFF were (in descending order) the following: Denmark (94), Italy (11), Finland (9), the United Kingdom (2), Austria and Iceland (1 of each).
In the subject of the notifications, the country of origin of the transgression that was mentioned most frequently was Germany, and was thus the most frequently notified against country of origin (51 times) followed by Poland (17 times) and France (12 times).
The yearly breakdown of the 118 notifications was the following; 2015 (1), 2014 (1), 2013 (0), 2012 (0), 2011 (3), 2010 (11), 2009 (16), 2008 (34), 2007 (30), 2006 (11), 2005 (3), 2004 (10), 2003 (4), 2002 (3). No other notifications were found before 2002 or after 2015 containing the term "resistant" in their subject in the EU RASFF database.
It is worth-mentioning that after 2015, the HFAA has been actively engaged in the issue of AMR, whereas the respective involvement of RASFF in terms of notifications submitted on AMR seems to have been discontinued from 2015 onwards.

Discussion

The vast majority of the 118 notifications on AMR were on food, and more specifically in food product categories of animal origin e.g. "poultry meat and poultry meat products" and "meat and meat products (other than poultry)".
Interestingly, in the vast majority of the 118 notifications the word 'multiresistant' was consistently included in the subject, which points to a rather significant challenge with regards to the microorganism identified *vis a vis* public health.
Denmark has exhibited the greatest notification activity concerning the notifications on AMR throughout the years that such notifications were found to heve been submitted in the EU RASFF. On the other hand, the country of origin of the transgression that was mentioned most frequently was Germany. The year that presented the maximum number of notifications on AMR in the EU RASFF was 2008 to be followed by 2007.
The fact that notifications on AMR have ceased to appear after 2015 in the EU RASFF database possibly indicates a shift in the decision-making process at a central level in the EU. It is hereby advocated that a re-introduction of such valuable information in the EU RASFF database would be of great value to all stakeholders

(competent authorities, industry and consumers) both within the EU but also to Third Countries since many such countries export foods of animal origin to the EU. The excellent work that has been performed by the HFAA of the European Commission, through fact-finding missions to gather information on the prudent use of antimicrobials in animals but also through audits in order to evaluate the monitoring and reporting of antimicrobial resistance in zoonotic and commensal bacteria in certain food-producing animal populations and food, especially after 2015 until today could work hand-in-hand with the aforementioned proposal put forward herein for the re-introduction of AMR-related notifications in the EU RASFF to holistically address the challenge of AMR.

This reinstatement of EU RASFF notifications on AMR could also play a significant role as a complementary database for the WHO GLASS which for now encompasses AMR data on humans. These two highly esteemed institutions could enjoy a fruitful collaboration in the years to come and strengthen the information basis on AMR on a global scale. Thus, the interaction between the EU RASFF and the WHO GLASS could indeed be envisaged for the benefit of consumer health against foodborne AMR, in the context of One Health.

Conclusion

The notification of (multi)resistant strains of *Salmonella* in food and feed in the EU RASFF database from 2002 until 2015 was an excellent paradigm of risk communication on AMR on the part of the EU, which has since then ceased to appear in the EU RASFF database. The re-introduction of submitting notifications on AMR on food and feed in the EU RASFF would offer a wealth of valuable information that could assist in risk management within the EU (but also outside the EU as well). All this information on AMR is additionally hereby proposed to be incorporated into a "RASFF MS country profile" as already proposed for serious alert and serious border rejection notifications on food by Papapanagiotou (2021) within the EU RASFF context, and in accordance with the Food and Veterinary Office (now HFAA of the European Commission) "country profile". Furthermore, this information could be effectively utilised in close collaboration by the WHO GLASS (for Europe) since such information on food is not yet offered by the WHO GLASS, which focuses its efforts on humans.

This collaboration of RASFF and the WHO GLASS would effectively establish the former as a key supra-national system in the global context on AMR incidents' notifications and offer its long-standing experience and esteemed operational expertise to the WHO. In this way, the EU RASFF would be the first risk communication network on AMR on food to collaborate with the WHO GLASS with others to follow from other parts of the world.

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POSTER 22

SERIOUS ALERT AND BORDER REJECTION NOTIFICATIONS ON RESIDUES OF VETERINARY MEDICINAL PRODUCTS IN FOOD IN THE EU RASFF (2012-2020)

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Abstract

For the first time, serious alert (sA) and serious border rejection (sBR) notifications on RVMP from the RASFF Portal (https://webgate.ec.europa.eu/rasff-window/portal/?event=SearchForm&cleanSearch=1#) were used to generate lag phases (from sampling to notification days), expressed as a percent (%) share of all such notifications, in order to highlight the timely reporting of serious hazards, on RVMP in food. On average (from 2012 until 2020), 9.89 ± 6.39% and 13.11 ± 9.18 % of all A and all BR notifications on RVMP were serious, respectively. The most challenging lag phase, namely, the 51-≥101 days, as an overall value (of all RASFF Member States collectively examined), in sA and sBR notifications was 17.98% and 4.23%,respectively, whereas the 0-30 days lag phase, in sA and sBR notifications was 35.95% and 79.66%,respectively. A lack of harmonization was observed between the top-4 EU RASFF MS (having contributed the highest numbers of sA and sBR notifications). In sA notifications on RVMP, the combinations of the highest prevalence of specific hazards in the most frequently notified food product categories were: i) meat and meat products (other than poultry) + (oxy)phenylbutazone, and ii) fish and fish products + (leuco)malachite green, whereas in the sBR were i) crustaceans and products thereof + nitrofuran (metabolite) and ii) fish and fish products + nitrofuran (metabolite). A more rapid notification of sA is hereby proposed to effectively attain harmonisation with the revealed timely notification of sBR on RVMP in food by the EU RASFF MS. This could be realized with focused audits on the part of the European Commission's Health and Food Audits and Analysis, aiming at the enhanced protection of Public Health.

Introduction

The use of veterinary medicines in food-producing animals **in case of abuse** may **leave residues in the food from treated animals.** Residues of veterinary medicinal products (RVMP) are chemical hazards that can be found in food of animal origin and are thus the target of official controls. A number of legal texts such as Regulation (EC) No 470/2009 that lays down the procedure for setting Maximum Residue Limits (MRLs) for residues of pharmacologically active substances (in veterinary medicinal products) in products of animal origin is one example of a legal text pertaining to RVMP. Another example is Commission Regulation (EU) No 37/2010 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin.

A recent EFSA report summarises the monitoring data collected in 2018 on the presence of residues of veterinary medicinal products and certain substances in live animals and animal

products in the European Union. The presence of unauthorised substances, residues of veterinary medicinal products or chemical contaminants in food may pose a risk factor for public health. The EU legislative framework defines maximum limits permitted in food and monitoring programmes for the control of the presence of these substances in the food chain. Overall, the percentage of non-compliant samples in 2018 (0.30%) was comparable to the previous 10 years (0.25%-0.37%), although slightly lower compared to 2017 (0.35%) (EFSA, 2020).

The EU Rapid Alert System for Food and Feed (RASFF) has its legal basis in Regulation 178/2002 and is an important tool for all EU Member States (MS) in exchanging information concerning risks found in food or feed. The members of this network are the EU MS, the Commission Services, the European Food Safety Authority, the countries of the European Economic Area (EEA), and Switzerland. A RASFF notification by a member of the network is submitted when a serious direct or indirect risk to public health in food or in feed is identified. Of all the types of notifications only the serious alert and serious border rejection notifications will be examined herein with a view to offer an insight into the transgressions regarding RVMP in the EU RASFF and attempting to assess the performance effectiveness of the EU RASFF MS in their timely reporting.

The aim of this study was threefold. Firstly, to determine lag phases (from sampling to notification dates) of only the serious alert and serious border rejection notifications on RVMP submitted to the EU RASFF from 2012 till 2020 in food, as percent (%) shares, for all RASFF EU MS as an overall value and individually for the top-4 EU RASFF MS (in numbers of each type of notification). Secondly, to define the time from sampling to notification in serious alert and serious border rejection notifications of the most important hazards namely, nitrofuran (metabolite) and chloramphenicol and to define the most frequently found combinations of hazard/food product category from the RASFF database. Finally, to offer proposals regarding targeted actions aiming at a higher harmonization of a timely notification performance effectiveness in reporting of RVMP, by harmonising the lag phases percent (%) shares of sA and sBR notifications.

Materials and Methods

A search in the EU Rapid Alert System for Food and Feed database (https://webgate.ec.europa.eu/rasff-window/portal/?event=searchForm&cleanSearch=1) retrieved the data used for this paper (accessed on 30/01/2021).

The following criteria were used in the search undertaken for alert (A) and border rejection (BR) rejection notifications with a “serious” risk decision (from 01/01/2012 till 31/12/2020): RVMP AND food, notifying country, country of origin, hazard category, year of notification, type of notification, subject, risk decision, distribution status, classification basis and dates of sampling and dates of notification from the details of every single notification. The subtraction (notification dates minus sampling dates) from each serious alert and serious border rejection notification enabled the calculation of the lag phases (in days). The lag phases have been grouped in specific time periods 0-10 days, 11-20 days, 21-30 days etc., so as to include groups of days for a more comprehensive presentation of the results and as percent (%) shares of all sA or sBR notifications. All data were extracted in Excel files, in the various combinations chosen and descriptive statistical methods were applied.

Additionally, the notification basis [Border Control-Consignment Released (BCCR), Company’s own check (COC), Consumer Complaint (CC), Food poisoning (FP), Monitoring of media (MoM) which mainly points to monitoring of products sold online, Official control on

the market (OCTM)] was also retrieved for all serious alert notifications, as well as Border Control-Consignment Detained (BCCD) and Border Control-Consignment Under Customs (BCCUC) for all serious border rejection notifications.
The Performance Effectiveness Reporting-50/30 (PER-50/30) as a proposed indicator of effectiveness (Papapanagiotou, 2021) for the timely reporting of sA and sBR in food in the RASFF has additionally been applied herein for RVMP. This indicator describes a cut-off threshold of at least 50% of sA or sBR being notified to RASFF within the lag phase of 0-30 days, for the EU RASFF MS.

Results

Serious alert (sA) and serious border rejection (sBR) notifications on RVMP in food in the EU RASFF

The sA (n=89) and the sBR (n=118) notifications on RVMP in food in the EU RASFF database were submitted for a rather small variety of food product categories of animal origin. More specifically, in the sA notifications the following foods were implicated (in descending order of frequency of notifications): i) meat and meat products (other than poultry) (48/89 or 53.93%), ii) fish and fish products (21/89 or 23.59%), iii) poultry meat and poultry meat products (11/89 or 12.35%), iv) crustaceans and products thereof (5/89 or 5.61%), v) honey and royal jelly (3/89 or 3.37%), vi) milk and milk products (1/89 or 1.12%).
On the other hand, in the sBR notifications the following food product categories were implicated (in descending order of frequency of notifications): i) crustaceans and products thereof (66/118 or 55.93%), ii) fish and fish products (28/118 or 23.72%), iii) meat and meat products (other than poultry) (18/118 or 15.25%), iv) other food product/mixed (3/118 or 2.54%), v) cephalopods and products thereof (2/118 or 1.69%), vi) poultry meat and poultry meat products (1/89 or 0.84%).
The overall percent (%) share of serious alert and border rejection notifications on RVMP in food over the examined period (2012-2020) was 18.58% (89/479), and 34.10% (118/346), respectively.

Serious alert (sA) notifications on RVMP in food

The first ever sA notification on RVMP was notified by Belgium on **27/07/2012 (with the date of sampling being 13/06/2012, Reference** 2012.1078**) and the subject was** "unauthorised substances clenbuterol (0.0023 mg/kg - ppm) and phenylbutazone (0.0013; 0.0015; 0.0010 mg/kg - ppm) in chilled deboned horse meat and frozen deboned horse meat from Canada in meat and meat products (other than poultry)".
The eighteen (of a total of 32, excluding Commission Services) RASFF Member States (MS) that have submitted sA notifications on RVMP were the following (in descending order of number of notifications submitted): Belgium (n=17), Italy (n=13), Germany (n=11), the United Kingdom (n=9), the Netherlands (n=7), the Czech Republic (n=6), Poland, France (n=5 each), Greece (n=3), Portugal, Sweden, Lithuania, Bulgaria (n=2 each), Ireland, Denmark, Latvia, Slovenia, Luxemburg (n=1 each). Collectively, the contribution of sA notifications, of the top-4 greatest contributors (in number of notifications submitted to RASFF on RVMP in food) namely, Belgium, Italy, Germany and the United Kingdom, collectively accounted for 56.17% of all such notifications.
The greatest part (67/89 or 75.28%) of all sA notifications were submitted on the basis of an official control on the market (OCTM) which signifies that the Competent Authorities (CA) of the respective MSs were responsible for sampling and analysis of just over three quarters of

all RVMP-related transgressions in food. The remaining notifications were made on the basis of: i) border control-consignment released, BCCR (14/89 or 15.73%), ii) company's own check, COC (n=5/89 or 5.61%), which reflects the industry's commitment to self-control and ii) official control in non-member country, (3/89 or 3.37%).
A point worth highlighting is the percent (%) share of sA notifications found to have been made on the basis of "border control - consignment released" or BCCR on RVMP in food. In essence, in such an incident the consignment was released without awaiting the analytical result, a fact that could point to the possible requirement to retrace it if the result was unfavourable and the product would need to be withdrawn from the market. Thus, these incidents could lead to alert or information notifications.
The WHO INFOSAN was included in 14.60% (13/89) of all sA notifications on RVMP in their "countries/organisations concerned" as shown in their notifications details in the RASFF database. This finding concerns a number of countries (both within and outside the EU) which could potentially have been affected by a health-threatening incident involving RVMP. Furthermore, a 26.96% (24/89) of all sA notifications involved Commission Services (CS) in their "countries/organisations concerned" as shown in their notification details in the RASFF database. Only 7/89 (7.86%) sA notifications had both INFOSAN and CS mentioned in their "countries/organisations concerned" as shown in their notifications details in the RASFF database.
Another finding of interest was the actual number of countries involved in the distribution of faulty consignments especially when it was ≥15 countries per notification. Five (5) such notifications were identified and this means that that the "risk dispersion" was quite widespread in these notifications. One such example, having 27 countries of distribution (D), implying widespread distribution (and hence potentially extensive "risk dispersion") to numerous other countries was the following, having been notified by Denmark on 30/6/2014, Reference 2014.0890, in meat and meat products (other than poultry), the subject being "prohibited substance diethylstilbestrol (1.3 µg/l) in pork from Denmark", which was an OCTM, with 27 distribution countries (excluding CS), namely Australia (D), Belgium (D), Bulgaria (D), Commission Services, Croatia (D), Czech Republic (D), Denmark (D/O), Germany (D), Greece (D), Hong Kong (D), Hungary (D), Ireland (D), Italy (D), Japan (D), Lithuania (D), Netherlands (D), Norway (D), Poland (D), Portugal (D), Romania (D), Singapore (D), Slovakia (D), Slovenia (D), Spain (D), Sweden (D), Switzerland (D), United Kingdom (D), United States (D). The action taken was withdrawal from the market and distribution to other member countries was reported. The significance of "risk dispersion" in sA notifications reflects on both the actual high number of countries potentially involved in the distribution of the faulty consignment but also to the geographical dissemination which was global by and large.

Lag phases (from sampling dates to notification dates) in sA notifications on RVMP in food, of the EU RASFF MS as notifying countries (NC)

Lag phases, represent a novel approach to decipher critical information from sA notifications used herein for the first time for RVMP in food and was calculated by the subtraction of the sampling dates from the notification dates (given in days). Ideally, this should be as short as possible, denoting that the submission of a sA notification to the RASFF should be as close as can be practically feasible to the sampling date for the protection of consumer health. Since, just over half of the sA notifications were submitted as an OCTM, the lag phases revealed herein have portrayed the Competent Authority's (CA's) timely effectiveness in successfully managing each RVMP sample from beginning to end.

The lag phases have been reported as groups of days e.g. 0-10 days, 11-20 days, 21-30 days etc. for a more comprehensive presentation of the results. A few shortcomings with regards to the reporting of the sA notifications were identified and these referred to: i) notifications with no sampling date (n=25 or 10%) where lag phases could not be calculated.
The percent (%) share of lag phases (from sampling to notification dates) in sA for all EU RASFF MS (collectively as an overall value) and individually for the top-4 EU RASFF MS that have notified the highest numbers of sA, is shown in Table 1.

Table 1. *Percent (%) share of lag phases (from sampling to notification dates) in serious alert notifications (sA) on RVMP in food from the EU RASFF database (from 2012 until 2020) for all EU RASFF MS as an overall value, and individually for the top-4 notifying countries.*

Lag phases (days)	**Percent (%) share of sA notifications on RVMP in food**				
	All EU RASFF MS (overall)	**BE**	**IT**	**DE**	**GB**
0-10	8.99	0.00	0.00	0.00	22.22
11-20	10.11	5.88	0.00	0.00	11.11
21-30	16.85	29.41	23.08	0.00	11.11
31-40	22.47	29.41	23.08	45.45	0.00
41-50	12.36	5.88	15.38	36.36	11.11
51-100	14.61	17.65	15.38	18.18	11.11
≥101	3.37	0.00	0.00	0.00	22.22
No sampling date	10.11	5.88	23.08	0.00	11.11
1/1/1970	1.12	5.88	0.00	0.00	0.00

The overall percent (%) share of sA notifications for all EU RASFF MS collectively calculated, that have a lag phase of: i) 0-30 days was 35.96%, ii) 31-50 days was 34.83%, iii) 51-≥101 days was 17.98%. No lag phases could be calculated for the notifications that presented shortcomings and this represented 11.23% of all sA notifications on RVMP.
The top-4 EU RASFF MSs (that have notified the majority of sA notifications) namely Belgium, Italy, Germany, and the United Kingdom have a percent (%) share of sA notifications in the lag phase of 0-30 days of 35.29%, 23.08%, zero % and 44.44%, respectively. According to Papapanagiotou (2021), if the indicator Performance Effectiveness Reporting 50/30 (PER-50/30) were to be applied to the lag phases of all sA notifications on RVMP submitted by all EU RASFF MS (overall) and to those submitted individually by the top-4 EU RASFF MS, the compliance to the aforementioned indicator would not be secured for any of these.
In the lag phase of 0-10 days (which is the most important in order to secure a timely response to RVMP transgression incidents) the highest percent (%) share was attained by the United Kingdom and this is indeed a most promising finding. Examples of such notifications are the following, having been notified by: i) The Czech Republic on 17/04/2019 (Reference 2019.1463), ii) France on 14/04/2017 (Reference 2017.0487), iii) Poland on 08/08/2014 (Reference 2014.1118), iii) Netherlands on 14/04/2017 (Reference 2014.0883), iv) The Czech Republic on 08/08/2014 (Reference 2013.0985), v) The Czech Republic on 26/06/2014 (Reference 2013.0988), vi) The United Kingdom on 12/07/2013 (Reference 2013.0187), vii) The United Kingdom on 14/02/2013 (Reference 2012.1251).
The top-4 EU RASFF MSs namely Belgium, Italy, Germany, and the United Kingdom have a

percent (%) share of sA notifications in the lag phase of 31-50 days of 35.29%, 38.46%, 81.81% and 11.11%, respectively, whereas their respective values in the lag phase of 51-≥101 days were 17.65%, 15.38%, 18.18% and 33.33%, respectively. The most challenging lag phase, namely, the ≥101 days when expressed as an overall value (of all RASFF MS as a whole) was 3.37% which was exceeded only by the United Kingdom.

Hazards identified in sA notifications on RVMP in food and their respective lag phases (from sampling dates to notification dates)

The sA notifications on nitrofuran (metabolite) be it furazolidone (AOZ), or nitrofurazone (SEM), or furaltadone (AMOZ), in various food product categories were 14/89, with an average of 40.64±23.54 days from sampling to notification in the RASFF database. The sA notifications on chloramphenicol (only) in various food product categories were 5/89, with an average of 65.40±48.73 days from sampling to notification in the EU RASFF database.
In the sA notifications on RVMP in food, the highest prevalence of a specific food product category (53.93%) identified was "meat and meat products (other than poultry)", of which the highest prevalence of a specific hazard was identified to be (oxy)phenylbutazone in a percent share of 31.25%. Thus, the combination of (oxy)phenylbutazone in meat and meat products (other than poultry) was the one of the highest importance in sA notifications.
In the sA notifications on RVMP, the second highest prevalence of a specific food category (23.59%) identified was "fish and fish products", of which the highest prevalence of a specific hazard was identified to be (leuco)malachite green in a percent share of 47.61%. Therefore, the combination of (leuco)malachite green in fish and fish products was the one of the second highest importance in sBR notifications.

Serious border rejection (sBR) notifications on RVMP in food

The first ever sBR notification was notified by the United Kingdom on 30/08/2012 **(with no date of sampling reported, Reference** 2012.BSY**) and the subject being** "unauthorised substance leucocrystal violet in frozen whole catfish (*Clarias* spp.) from Indonesia in fish and fish products"**.**
The twelve EU RASFF MS (of a total of 32, excluding Commission Services) that have submitted sBR notifications on RVMP in food, are the following (in descending order of numbers of notifications): Belgium (n=26), Spain, the Netherlands, the United Kingdom (n=14 each), Denmark (n=11), Poland (n=10), Germany, Italy (n=9 each), France (n=6), Portugal (n=3), Bulgaria, Slovenia (n=1 each). Interestingly, the top-4 EU RASFF MS namely, Belgium, the United Kingdom, Spain and the Netherlands together have contributed 57.63% of all sBR notifications to the EU RASFF.
The vast majority of all sBR notifications (96.63%) were submitted on the basis of border control-consignment detained (BCCD) which means that the CA were responsible for sampling and analysis for the greatest part of RVMP transgressions that were refused entry into the EU market. The remaining one notification was made on the basis of border control-consignment under customs (BCCUC).
In contrast to the respective finding in sA notifications, the WHO/INFOSAN was not included in any of the sBR notifications as shown in their "countries/organisations concerned" in their notification details in the RASFF database, whereas the Commission Services (CS) were included in their notification details, on 36 occasions (30.50%).
With regards to the countries of origin (CO), three Third Countries (TC), namely India,

Vietnam and China, were most frequently notified against, in sBR notifications on RVMP in food. The collective percent (%) share of these top-3 TC in their capacity as CO amounted to 88.98% (105/118). The rest of the transgression Third Countries designated as CO were as follows: Brazil (n=3), Bangladesh, Turkey (n=2 each), Armenia, Indonesia, Egypt, Iran, Thailand (n=1 each).
In more detail, India was notified against 54 times for RVMP in food (mostly for nitrofuran (metabolite)). The notifying countries that submitted the most sA notifications against India were Belgium and the Netherlands. Vietnam was notified against 29 times for RVMP in food (mostly for nitrofuran (metabolite)). The notifying countries that submitted the most sA notifications against Vietnam were Spain and Italy . China was notified against 23 times for RVMP in food (mostly for chloramphenicol, but also for nitrofuran (metabolite)). The notifying countries that submitted the most sA notifications against China were Poland and Denmark.

Lag phases (from sampling dates to notification dates) in sBR notifications on RVMP in food, of the EU RASFF MS as notifying countries (NC)

Like in sA notifications the same procedure was followed for sBR notifications in order to display the percent (%) shares of the lag phases (from sampling to notification dates). The lag phases have been reported again as 0-10 days, 11-20 days etc. so as to encompass groups of days for a more comprehensive demonstration of the results. A few shortcomings in the reporting of the sBR notifications were also recorded and these pertained to notifications with no sampling date (n=2). The percent (%) share of lag phases in sBR for all RASFF MS as a whole and for the top-4 MS that have notified the highest numbers of sBR, is shown in Table 2.

Table 2. *Percent (%) share of lag phases (from sampling to notification dates) in serious border rejection notifications (sBR) on RVMP in food from the EU RASFF database (from 2012 until 2020) for all EU RASFF MS as an overall value, and for the top-4 notifying countries.*

Lag phases (days)	Percent (%) share of sBR notifications on RVMP in food				
	All EU RASFF MS (overall)	BE	NL	ES	GB
0-10	15.25	7.69	0.00	0.00	7.14
11-20	29.66	38.46	50.00	14.29	42.86
21-30	34.75	30.77	42.86	50.00	21.43
31-40	8.47	15.38	7.14	14.29	7.14
41-50	3.39	0.00	0.00	14.29	7.14
51-100	2.54	3.85	0.00	0.00	7.14
≥101	1.69	0.00	0.00	0.00	0.00
No sampling date	2.54	3.85	0.00	0.00	7.14
1/1/1970	0.85	0.00	0.00	7.14	0.00
inverse	0.85	0.00	0.00	0.00	0.00

The overall percent (%) share of sBR notifications for all EU RASFF MS collectively calculated, that have a lag phase of: i) 0-30 days was 79.66%, ii) 31-50 days was 11.86%, iii) 51-≥101 days was 4.23%. No lag phases could be calculated for the notifications that presented shortcomings (no sampling date, date reported as “1/1/1970” and “inverse date”).

The top-4 EU RASFF MSs (that have notified the majority of sBR notifications) namely Belgium, the Netherlands, Spain, and the United Kingdom have a percent (%) share of their sBR notifications in the lag phase of 0-30 days of 76.92%, 92.86%, 64.29 % and 71.42%, respectively. According to Papapanagiotou (2021), if the indicator Performance Effectiveness Reporting 50/30 (PER-50/30) were to be applied to the lag phases of all sBR RVMP notifications submitted by all EU RASFF MS (overall) and to those submitted by the top-4 EU RASFF MS, the compliance would be secured for all of the above.
In the lag phase of 0-10 days (which is the most important in order to secure a timely response to RVMP transgression incidents) the highest percent (%) share was attained by Belgium and this is indeed a most promising finding.
The top-4 EU RASFF MSs namely Belgium, the Netherlands, Spain, and the United Kingdom have a percent (%) share of sBR notifications in the lag phase of 31-50 days of 15.38%, 7.14%, 28.58% and 14.28%, respectively, whereas their percent (%) shares in the lag phase of 51-≥101 days were 3.85%, zero %, zero% and 7.14%, respectively.
The most challenging lag phase, namely, the ≥101 days when expressed as an overall value (of all RASFF MS as a whole) was 1.69% which was not exceeded by any of the top-4 EU RASFF MS examined here.
In the lag phase of 0-10 days (which is the most important in order to secure a timely response to RVMP transgression incidents) in sBR notifications the highest percent (%) share was attained by Belgium and this is indeed a most promising finding. Examples of such notifications are the following, having been notified by: i) Belgium on 20/01/2017 (Reference 2017.ACV), ii) Denmark on 30/05/2016 (Reference 2016.ATT), iii) Italy on 10/03/2016 (Reference 2016.AKH), iv) Italy on 22/02/2016 (Reference 2016.AIH), v) Italy on 01/02/2016(Reference 2016.AFB), vi) Slovenia on 29/10/2014 (Reference 2014.BRY), vii) Belgium on 25/06/2014 (Reference 2014.BBA), viii) United Kingdom on 24/01/2014 (Reference 2014.AED), viii) Denmark on 15/01/2014 (Reference 2014.ACD), viii) Denmark on 10/12/2013(Reference 2012. 2013.CBX), viii) Germany on 16/09/2013 (Reference 2013.BPY), viii) Denmark on 16/10/2012(Reference 2012.CAR), viii) Denmark on 21/09/2012 (Reference 2012.BXL), viii) Italy on 10/09/2012 (Reference 2012.BUN), and viii) Denmark on 06/09/2012 (Reference 2012.BTT).

Hazards identified in sBR notifications on RVMP in food and their respective lag phases (from sampling dates to notification dates)

The sBR notifications on nitrofuran (metabolite) be it furazolidone (AOZ), or nitrofurazone (SEM), or furaltadone (AMOZ), in various food product categories were 85/118, with an average of 24.64±15.54 days from sampling to notification in the RASFF database. Fifty-one of these, originated from India and nineteen from Vietnam (as countries of origin).
The sBR notifications on chloramphenicol (only) in various food product categories were 17/118, with an average of 15.53±7.41 days from sampling to notification in the RASFF database. Twelve of these, originated from China (as a country of origin).
In the sBR notifications on RVMP, the highest prevalence of a specific food category (55.93%) identified was “crustaceans and products thereof”, of which the highest prevalence of a specific hazard was identified to be nitrofuran (metabolite) in a percent share of 83.33%. Thus, the combination of nitrofuran (metabolite) found in “crustaceans and products thereof” was the one of the highest importance in sBR notifications.
In the sBR notifications on RVMP, the second highest prevalence of specific food category

(23.72%) identified was “fish and fish products”, of which the highest prevalence of a specific hazard was identified to be nitrofuran (metabolite) in a percent share of 75.00%. Consequently, the combination of nitrofuran (metabolite) found in fish and fish products was the one of the second highest importance in sBR notifications.

Lag phases (from sampling dates to notification dates) in both sA and sBR notifications on RVMP in food, in one of the top-4 EU MS that appear in both rankings, namely Belgium

Additionally, another comparison could easily be envisaged between the one EU MS that has been found to be present in the top-4 positions as notifying country in both sA and sBR notifications, in such a way so as to reveal its lag phases percent share (%) attainment in both types of serious notifications (Table 3).

Table 3. *Percent (%) share of lag phases (from sampling to notification dates) in serious alert (sA) and serious border rejection (sBR) notifications on RVMP in food from the EU RASFF database (from 2012 until 2020) for Belgium, the EU RASFF MS that was among the top-4 notifying countries in both types of serious notifications.*

Lag phases (days)	Percent (%) share of sA and sBR notifications on RVMP in food	
	Belgium (BE)	
	sA	sBR
0-10	28.57	17.65
11-20	10.71	41.18
21-30	28.57	17.65
31-40	17.86	0.00
41-50	3.57	23.53
51-100	7.14	0.00
≥101	0.00	0.00
No sampling date	3.57	0.00
Inverse date	0.00	0.00

Belgium for example, has achieved 59.85% and 76.48%, in the 0-30 days lag phase in sA and sBR respectively, whereas in the 51-≥101 days lag phase the respective percent (%) shares were 7.14% and zero%, respectively. Clearly, the Performance Effectiveness Reporting 50/30 (PER 50/30) indicator would be complied with for Belgium in both types of serious notifications and this is an example of a EU RASFF MS that deserves attention and could be used as a paradigm for other MS.

Discussion

An attempt was made in this paper to analyse exclusively the sA and sBR notifications on RVMP in food, in the context of the EU RASFF database, for the first time in bibliography. Serious notifications on RVMP in the EU RASFF undoubtedly entail a food safety risk and this should be assessed regarding their timely notification. The present analysis of the sA and sBR notifications on RVMP has revealed some quite interesting differences between the two, such as the number of MS involved in submitting sA (n=18) in comparison to those submitting sBR (n=12) from the total of 32 EU RASFF MS.

With regards to the sA notifications, the top-4 notifying countries (in numbers of sA notifications submitted to the EU RASFF) collectively accounted for just over 56% of all sA notifications on RVMP, and these were Belgium, Italy, Germany and the United Kingdom. The top-4 notifying countries (in numbers of sBR notifications submitted to the EU RASFF) collectively accounted for just under 57% of all sBR notifications. It seems that the RVMP

transgression consignments enter the EU market through a rather small portion of all the EU RASFF MS, and hence their control could be rather easily facilitated by the enhanced vigilance by the top-4 EU RASFF MS, namely the Belgium, Spain, the Netherlands and the United Kingdom (in descending order of sBR notifications submitted). The top-3 countries of origin involved in the RVMP transgressions (which collectively accounted for 89% of all sBR notifications), were India, Vietnam and China. In more detail, only eleven (11) TC have been notified against as countries of origin (CO) throughout the examined period, a fact that shows that only a small number of TC have been implicated in transgressions regarding RVMP in food, and this could be dealt with a focused set of audits by the Health and Food Audits and Analysis of the European Commission.

Lag phases as a novel criterion to be used for specific individual hazards identified in the sA and sBR notifications have been estimated. The examination of the lag phases has revealed that a lack of harmonisation exists in the percent (%) share of the lag phases calculated for both sA and sBR notifications on RVMP in food, when each MS was observed alongside the respective set of values of the overall lag phases (all EU RASFF MS collectively assessed), but also when observed alongside other MS (as exemplified in the top-4 MS examined here), or itself for that matter (e.g. Belgium).

The 0-30 days lag phase of all EU RASFF MS examined collectively was 35.96% and 79.66% for sA and sBR notifications, respectively, denoting a 2.2 times faster response time in submitting the latter type of notification. The PER-50/30 indicator would be complied with in the case of sBR but unfortunately not in the case of sA notifications. The 31-50 days lag phase of all EU RASFF MS examined collectively was 34.83% and 11.86% for sA and sBR notifications, respectively, signifying a 2.9 times difference. On the other hand, the 51-≥101 days lag phase of all EU RASFF MS examined collectively was 17.98% and 4.23% for sA and sBR notifications, respectively, portraying a 4.25 times difference. This finding undoubtedly points out to the fact that the reaction time in sBR notifications was much shorter than that of sA notifications. This is quite important since the sBR notifications originate from transgressions coming from Third Countries (TC), but at the same time highlights that more could be done towards the timely notification of the sA associated with the RVMP transgressions.

Lag phases heterogeneity was evidenced between the top-4 notifying MS for both types of notifications, for example, in sA notifications a rather large range of percent (%) share of lag phases was evidenced, such as in the 0-10 days (0.00-22.22%), or the 21-30 days (0.00-29.41%), etc. Similarly, in sBR notifications in the top-4 notifying MS an equally large range of percent share of lag phases was evidenced, such as in the 11-20 days (14.29-50.00%), the 31-40 days (7.14-15.38%), etc. It is not surprising that EU RASFF MS differ with regards to their percent share of lag phases in their sA and sBR notifications and this highlights a lack of harmonization. This heterogeneity could be ameliorated by fact-finding missions by the European Commission's HFAA/ex-FVO in view of attaining some degree of harmonization between MSs by and large, with regards to RVMP notifications' timely reporting.

It has become apparent that for both types of notifications examined herein, examples of EU RASFF MS that have attained excellent lag phases (less than 10 days between sampling and notification) do actually exist and their experience perhaps would be most valuable to other MSs. Eight (8) such examples were found in sA and 14 in sBR notifications in the examined period in the EU RASFF database. Additionally, a comparison of lag phases attained by each EU RASFF MS against the overall average of all EU RASFF MSs collectively, could perhaps represent a starting point for a novel *modus operandi* aiming at the harmonization in timely

reporting of sA and sBR notifications on RVMP in food.
The application of the PER-50/30 indicator in all EU RASFF MS (overall), individual MSs, individual hazards of RVMP, individual food product categories, individual hazards within individual food product categories, can easily be performed.
Thus, it has been shown that the PER-50/30 indicator was better in the sBR notifications of RVMP in food (where all top-4 EU RASFF MS complied) in comparison to sA (where none of the top-4 EU RASFF MS complied with it). Moving on to a more in-depth analysis of the reporting time of the individual hazards identified in the sA and sBR notifications on RVMP in food, it was found that in sA notifications with nitrofuran (metabolite) as the hazard involved, an average of 40.64 days was required (from sampling to notification date) whereas in sBR only 24.64 days. The respective times for chloramphenicol in sA and sBR notifications were 65.40 and 15.53 days, respectively, pointing to a much faster response time for sBR in comparison to sA for both chemical hazards. In this respect, the revealed situation is by far better in the sBR than in the sA notifications on RVMP.
Nevertheless, the findings revealed from the analysis of the RASFF database should always be carefully interpreted since the RASFF system provides information only on non-compliance data, so the percent (%) of compliance is not known and also no volumes of consignments are given in the details of each notification. These limitations should always be taken into consideration, and a rather thorough analysis of the RASFF data should in principle be performed.
A number of new findings provided herein could potentially present the stimuli to instigate additional action on the part of the EU Institutions, namely the European Commission's, Health and Food Audits and Analysis (ex-FVO), in order to further harmonise the current situation concerning serious notifications' timely reporting of RVMP transgressions in the RASFF. A lack of harmonization in the allocation of sA and sBR into the different lag phases represented as a relative percent (%) share, was revealed among the top-4 EU RASFF MS for RVMP examined. This raises concerns about the uniformity of a truly integrated EU approach to the timely reporting of serious notifications (be it sA or sBR) on RVMP in food. This lack of harmonization would undoubtedly require an in-depth examination by the European Institutions, taking into consideration the specific individual situations existing in each MS (e.g. funding, culture, priorities).

Conclusion

The effective incorporation of the novel "lag phases" criterion into the already proposed PER-50/30 by Papapanagiotou (2021) in the sA and sBR notifications on RVMP in food has revealed the existing non-harmonised situation in terms of percent (%) shares of sA and sBR notifications on individual EU RASFF MS. All this information on RVMP could potentially be incorporated into a "RASFF MS country profile" as already proposed for sA and sBR notifications in food by Papapanagiotou (2021). Furthermore, another proposed option would be to assess the lag phases of each MS individually concerning specific hazards related to RVMP on a yearly basis to evaluate long-term timely notification consistency in both sA and sBR. Data gathered could be analysed to identify, at EU level, the possible challenges faced by the authorities in facilitating the implementation of a harmonization in lag phases of each RVMP hazard in each food product category, in each EU RASFF MS, identified in sA and sBR notifications and ultimately effectively increasing the percent (%) share of shorter lag phases rather than longer ones for each MS individually.

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POSTER P23

RISK OF SALICYLIC ACID FORMATION IN MILK DURING SAMPLE STORAGE

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Abstract
For a correct qualification and quantification of residues in matrices, proper handling of samples is required. Improper storage conditions could be a potential risk for the stability of analytes in samples. Reanalysis of Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) in milk samples showed an increase of the extractable salicylic acid concentration by storage outside the freezer. Within a day of storage at room temperature, concentrations severely exceeded the MRL. Thus, improper storage of milk samples can lead to false non-compliant results for salicylic acid. The increased salicylic acid concentration partly originates from the hydrolysis of salicyluric acid, a metabolite of salicylic acid, which is naturally present in milk. Further investigation proved that the processes responsible for the salicylic acid increase in milk are biologically. Therefore, an in-house stabilisation procedure was developed in which the biological activity is deactivated by adding 0.5 (v/v) % acetic acid, therefore stabilising the salicylic acid content in the milk samples. This study clearly shows the importance of proper stabilisation and storage of samples for residue analysis.

Introduction
Salicylic acid is a phenolic plant hormone that is found in plants with roles in plant growth and development, photosynthesis, transpiration, ion uptake and transport. It is known to occur in animal feed ingredients like alfalfa, rapeseed scrap, citrus pulp and wheat. Salicylic acid is the basic substance of the salicylates, which are non-steroidal anti-inflammatory drugs (NSAIDs). Salicylic acid and its sodium, aluminium salt and methyl salicylate are used as NSAIDS in cattle, horses, sheep, goats and poultry. In 2016 the Maximum Residue Limit (MRL) for salicylic acid has been lowered from 25 µg/kg to 9 µg/kg in milk in the European legislation (EU/2010/37).

For enforcement of MRLs, stable samples are required in order to measure pharmaceutical active substances exactly as their presence in the sample during sample collection. That means that the concentration of the pharmaceutical active substance that has to be detected in the matrix does not decrease during the time period from sample collection until analysis. On the other hand, an increasement is also not allowed. In this study, the (in)stability of the raw milk for the salicylic acid level has been studied in a structured way. Firstly, the problem was identified, then the cause was established, followed by the origin/source and finally this led to the solution (Figure 8).

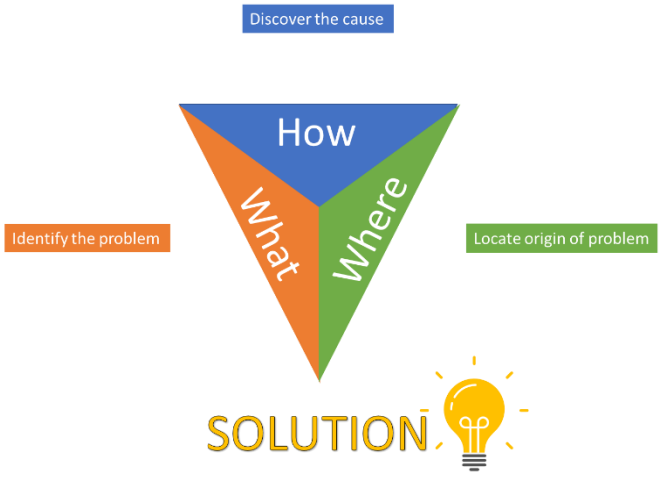

Figure 8: The problem approach.

Materials and Methods

Reagents, chemicals and materials
The reference standards of salicylic acid, 3-hydroxybenzoic acid and 4-hydroxybenzoic acid were obtained from Sigma Aldrich (St. Louis, MO, USA) and salicyluric acid from Santa Cruz Biotechnology (Dallas, TX, USA). The isotopic labelled standard of salicylic acid was obtained from Toronto Research Chemicals (Toronto, ON, Canada) and of salicyluric acid from Santa Cruz Biotechnology.

Sample preparation
Weigh 2 g of milk (possible after incubation) into a 14 mL polypropylene centrifuge tube. Add internal standard solution and 5 mL of acetonitrile. Shake for 15 minutes using a rotary tumbler and centrifuge for 10 min at 3500 g. Transfer the supernatant info a new polypropylene tube containing 200 mg bondesil-primary-secondary Amine (PSA). Shake for 5 minutes using a rotary tumbler and centrifuge for 10 min at 3500 g. Transfer the supernatant into a glass tube and evaporate the acetonitrile layer and redissolve it in 800 µL methanol.

Analysis
The general UHPLC-MS/MS triple quadrupole analyses were performed as follows; The UHPLC system consists of an Acquity model (Waters, Milford, MA, USA) with an Acquity HSS-T3 C_{18} column of 2.1 * 100 mm, 1.7 µm (Waters), placed in a column oven at 40 °C. The mobile phase consists of 2 mM ammonium formate and 0.016% formic acid in water (Solvent A) and 2 mM ammonium formate and 0.016% formic acid in MeOH (Solvent B). The gradient: 0–1.0 min, 0% mobile phase B, 1.0–2.5 min, linear increase to 45% B, 2.5–8.5 linear increase to 100% B with a final hold of 2.0 min. The gradient is returned to its initial conditions within 0.1 min and the column is allowed to equilibrate for 1.4 min before the next injection is initiated, resulting in a total run of 12 min. The flow rate is 0.4 mL min^{-1} and the injection volume is 5 µL. Detection is carried out by MS/MS using Q-trap (Sciex, Framingham, MA, USA) in negative electrospray ionisation (ESI) mode. The operating parameters are curtain gas 35, ion source gas 1 60, ion source gas 2 60, source temperature 550 °C and ion spray voltage -5500 V. The substances were fragmented using collision induced dissociation with nitrogen as collision gas. Selected reaction monitoring (SRM) transitions are presented in Table 2.

The UHPLC-HRMS/MS analyses were performed using an Ultimate 3000 UHPLC system, which was coupled to a Q-Exactive OrbitrapTM system with a HESI-II electrospray source (Thermo Scientific, San Jose, CA, USA). Chromatographic separation was performed using an Atlantis T3 (100 x 3 mm, 3 µm particles) analytical column (Waters) at a column temperature of 40°C. The mobile phases used were 2 mM ammonium formate and 0.016% formic acid prepared in water (solvent A) and in MeOH (solvent B). Operating at a flow rate of 0.3 mL min−1, the used gradient was 0–0.1 min, 0% B, 0.1–2.0 min, a linear increase to 45% B followed by a linear increase to 100% B from 2.0–8.0 min with a final hold of 9.5 min and an equilibration time of 5 min. The total runtime was 23 min and the injection volume was 5 µL. Detection is carried out in the negative scan mode with the following general MS settings: capillary voltage −2.5 kV, sheath gas 47.5 AU, auxiliary gas 11.25 AU, cone gas 2.25 AU, capillary temperature 250°C and heater temperature 400°C. A total of six scan events were combined: one full-scan event (mass range *m/z* 135–1000) with a resolving power of 70 000 (defined at *m/z* 200, FWHM) and five MS2 vDia events. In the MS2 scan events the precursor ion ranges *m/z* 95–205, 195–305, 295–405, 395–505 and 495–1005 were selected consecutively, fragmented in the collision cell and the resulting ions measured in five separate Orbitrap scans with a resolving power of 35 000.

Table 2: The SRM transitions of salicylic acid and salicyluric acid.

ID	Q1 (*m/z*)	Q3 (*m/z*)	DP	CE	CXP
Salicyluric acid	193.8	120.8	-35	-28	-15
Salicyluric acid	193.8	147.7	-35	-24	-17
Salicyluric acid	193.8	149.8	-35	-18	-17
Salicyluric acid	193.8	64.9	-35	-56	-9
Salicyluric acid	193.8	92.9	-35	-30	-11
Salicylic acid	136.8	93.0	-40	-18	-11
Salicylic acid	136.8	64.9	-40	-40	-7
Salicyluric acid-IS	196.8	151.9	-25	-18	-13
Salicyluric acid-IS	196.8	92.7	-25	-30	-11
Salicyluric acid-IS	196.8	64.8	-25	-58	-7
Salicylic acid-IS	142.9	99.0	-40	-18	-11

Results and Discussion

Identification of the problem: (In)stability of milk
A raw milk sample which has been screened negative (< 0.5*MRL) for the presence of salicylic acid (a blank milk sample), can show an increase of the salicylic acid levels after storage at room temperature from a few hours to a few days, as shown in Figure 9 (left, blue line). An addition of salicylic acid to the same sample before incubation resulted in a total higher level of salicylic acid but did not affect the rising trend (Figure 9 left, green line).

From these results, the following question arises; Is the detected signal really salicylic acid? The identification of the formed salicylic acid was confirmed following the European legislation about confirmation of the identity (EU/2002/657) based on relative retention time and relative abundance of two product ions. Additionally, a high-resolution mass spectrometry (HRMS/MS) measurement was performed and the detect mass was within the margin of a 5 ppm error. Finally, another chromatographic method (Ayaz 2005) was used with an extended separation of salicylic acid (2-Hydroxybenzoic acid) from several other phenolic acids, including the meta- and para-isomers 3-hydroxybenzoic acid and 4-hydroxybenzoic acid, respectively. Taking all these results together, it cannot be proven that the increasing signal is not salicylic acid and therefore we have strong indications to accept this identity.

We identified the problem as an increase of the salicylic acid level in milk samples after storage from a few hours to a few days at room temperature. An instable salicylic acid levels in milk complicates enforcement. In order to solve the problem, the origin of the problem must be investigated.

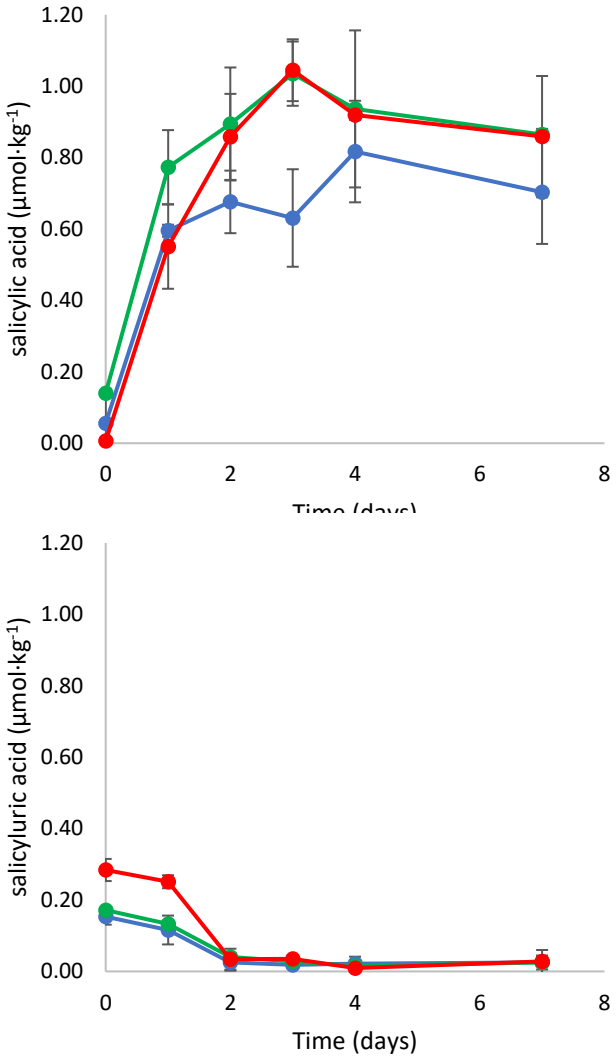

Figure 9: the average (n=2) increases of salicylic acid (left) or decrease of salicyluric acid (right) of a blank milk sample stored at room temperature for in total 7 days. The blue line indicates the blank milk sample without any addition, the green line indicates the same sample spiked with 25 µg·kg^{-1} (0.18 µmol·kg^{-1}) salicylic acid and the red line indicates the same sample spiked with 25 µg·kg^{-1} (0.13 µmol·kg^{-1}) salicyluric acid. The MRL is 9 µg·kg^{-1} which correspond with 0.065 µmol·kg^{-1}.

Locate the origin of the problem: Salicyluric acid

The increase of one component, in this case salicylic acid, could mean a decrease of another component or several components. The increase of salicylic acid may be due to the formation of salicylic acid from two (or more) other molecules or, more likely, the degradation of a molecule with a high molecular mass. This last hypothesis was tested using a parent scan on a triple quadruple mass spectrometer (MS) in combination with a full scan on the LC-HRMS/MS. For these experiments, a blank milk sample was stored 4 days at room temperature and aliquots were collected at t=0 days and t=4 days.

A parent scan provides information of all present precursor ions in a sample extract that has a certain product ion. The assumption was that a molecule that can potentially degrade to salicylic acid will partly have an overarching structure with salicylic acid and therefore probably the same product ion *m/z* 93, which is a high abundance product ion of salicylic acid after fragmentation in the MS. The sample extract of t=0 was used for the parent scan.

Both sample extracts (t=0 and t=4) were measured on the LC-HRMS/MS. The full scan data of t=4 days was subtracted from the full scan of t=0 days. This results in several masses that are present in a higher intensity in t=0 compared to t=4. Therefore, these masses are possible candidates to degradation over time, and perhaps into salicylic acid.

The information of the parent scan and the subtracted full HR scans were combined and prioritized based on literature. The *m/z* 94 was prioritised highest. As shown in Figure 10, the extracted *m/z* value of 194 from the full scan data shows a decrease from t=0 till t=4, while the *m/z* value of 173, salicylic acid, shows an increase. In the extracted ion chromatograms (EIC) of salicylic acid, two peaks are visible. Not only the salicylic acid level increases, also the 4-hydroxybenzoic acid signal increases over time. The peak with *m/z* 194 was identified as salicyluric acid, a known metabolite of salicylic acid (Lui, 1996). The identity was confirmed based on a reference standard.

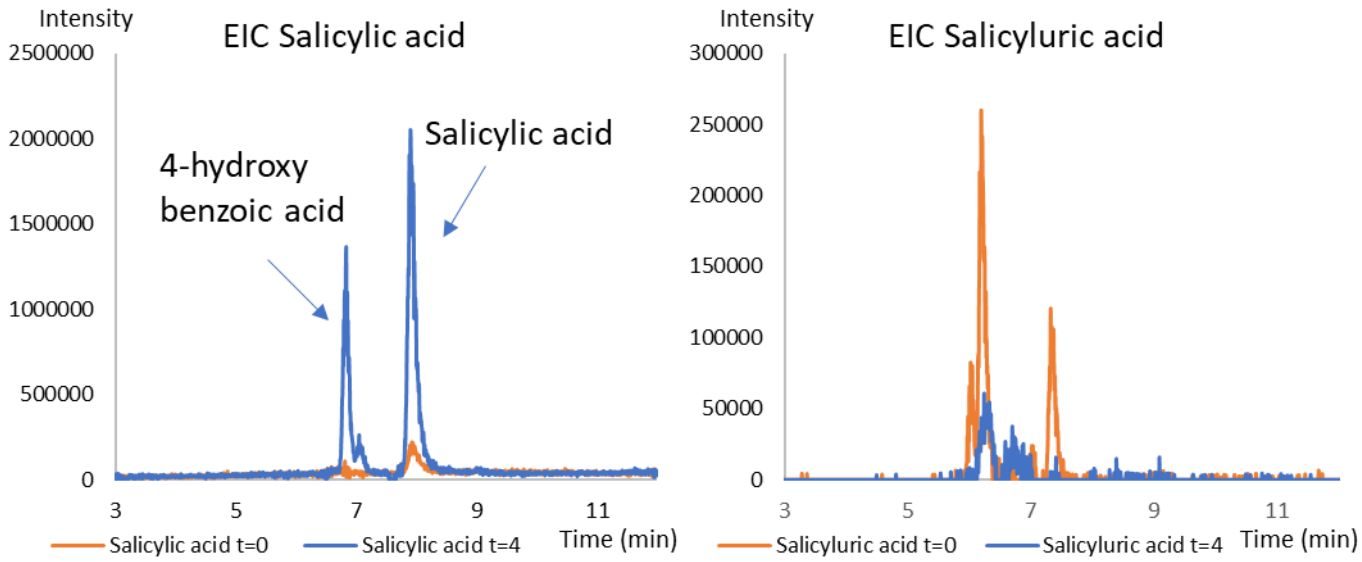

Figure 10: The extracted ion chromatograms (EIC) of the m/z values of 137.02440-137.02578 for salicylic acid (left) and the m/z values of 194.04491-194.04685 for salicyluric acid (right) of a 'blank' milk sample before storage (t=0) and after storage for four days (t=4) at room temperature.

The degradation of salicyluric acid into salicylic acid was tested in the proof-of-principle. The same blank milk samples as used in the first experiment (Figure 9), were not only fortified with salicylic acid, but also with additional salicyluric acid (red line). As can be seen at the y-intercept of the graphic, salicyluric acid is already present in the milk sample. After incubation at room temperature for several hours until several days, a decrease of salicyluric acid (Figure 9, right) was observed in the milk samples without an addition (blue line). The red line, which represent the milk spiked with additional salicyluric acid showed a higher level at t=0 because of the addition, but after 2-3 days most of the salicyluric acid is degraded including the additional added standard. On the other hand, the total salicylic acid concentration (Figure 9, left) is higher after incubation in the samples with additional salicyluric acid (red line) compared to the samples without (blue line).

Comparing the left and right picture of Figure 9 shows that de decrease of salicyluric acid leads to the increase of salicylic acid. However, a decrease of one mol salicyluric acid should lead to an increase of one mol salicylic acid. Th results show that this is not the case, which probably means that salicyluric acid is not the only source of the salicylic acid formation. The origin is partly located, however the process responsible for the problem is still unknown.

Discover the cause of the problem: Microbiological or enzymatic activity?

The process responsible for the instability of the milk was investigated using the following hypothesis: degradation of substances can be caused by physical instability or microbiological/enzymatic activity.

To determine if the formation of salicylic acid from salicyluric acid is caused by physical instability the effect of time, temperature and pH was investigated by comparing water fortified with salicyluric acid with milk fortified

with salicyluric acid. Results show that in the fortified water no salicylic acid was formed. Consequently, physical instability of salicyluric acid is less likely.

The other option, microbiological/enzymatic activity was tested by sterilisation of the milk. The sterilisation process (121 °C, 15 min) could not completely prevent the formation of salicylic acid. There could be two reasons for this (1) the sterilisation process was not sufficient or (2) thermostable enzymes are responsible for the process. Alternative options to inactive microbials and enzymes are adding organic solvent to the samples or decrease the pH. Both options were tested and results show that the addition of methanol (10 (v/v)% in the sample), formic acid (0.1 (v/v)% in the sample) or acetic acid (0.5 (v/v)% in the sample) seems to be sufficient to stabilise the milk samples. Acetic acid is preferred above the other options, because the stabilization process is easy to check afterwards due to the smell.

Thus, the cause of the problem could not be proven, but a solution was found. Addition of, among other things acetic acid (0.5 (v/v)% in the sample) prevents the possible formation of salicylic acid in milk samples during storage. The efficiency of the stabilization was tested during storage at room temperature over the weekend using several blank milk samples (screened as negative) and positive milk samples (exceeding the MRL concentration) originated form an animal experiment where cows were treated with sodium salicylate. The results are presented in **Error! Reference source not found.**. After the stabilization of milk samples with acetic acid, the salicylic acid concentrations in the positive milk samples (n=3) remained stable during storage at all conditions (freezer, refrigerator and room temperature), while in the non-stabilised samples the concentrations increased at room temperature and even in the refrigerator for one sample. In the blank milk samples, no salicylic acid was formed in the stabilised samples during storage but was formed in the non-stabilised samples during storage at room temperature. The salicyluric acid concentration was also monitored (data not shown) and showed a decrease in the non-stabilised samples, but not in the stabilised samples.

The detection of salicylic acid is part of a multiresidue method for 29 different NSAIDs. In the final experiment, the influence of the stabilisation with acetic acid on the other substances was tested in combination with a stability test of the other NSAIDs. The absolute recovery of all NSAIDs in stabilised milk was comparable with the absolute recovery of all NSAIDs in non-stabilised milk. No other NSAID than salicylic acid showed instability over a time period of a weekend in both stabilised milk and non-stabilised milk.

POSTER P24

IS FAECES A RELEVANT MATRIX TO CONTROL B-AGONISTS MISUSE IN LIVESTOCK ?

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Abstract
Faeces can be considered as an alternative interesting biological matrix to detect residues or their metabolites in livestock especially for substances preferentially eliminated by the biliary route. The relevance of such matrix has already been demonstrated for detecting a range of steroids[1] , glucocorticoids[2] or SARMs[3,4]. While the excretion of b-agonists in faeces is reported since the early 90s [5, 6], the use of such matrix for monitoring their misuse is not widespread within EU national monitoring plans. Considering the main advantage of faeces over urine and blood being the collection ease in breeding conditions, the French risk manager has strongly encouraged analytical developments toward faeces. In the present work, an analytical workflow dedicated to the analysis of at least 19 b-agonists in faeces, including Clenbuterol, Salbutamol and Ractopamine at concentrations between 0.2 and 10 µg/kg has been optimised. It involves an SPE Strata X-C step and UHPLC-MS/MS Waters Xevo TQS1 analysis.
[1] Nielen et al JCB (2004) [2] De Clercq et al. JCA (2014) [3] Cesbron et al. FAC (2017) [4] Rojas et al. DTA (2017) [5] Courtheyn 1991 [6] Batjoens 1996

Keywords: B-AGONISTS, FAECES, LIVESTOCK

POSTER P25

DEEP INVESTIGATION ON STEROIDS METABOLISM AND BIOMARKERS IN LIVESTOCK USING INNOVATIVE HIGH-RESOLUTION GC AND LC-MS APPROACHES AND MOLECULAR NETWORKS

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Abstract
Studying the metabolism of steroids in detail was an early and successful strategy to identify metabolites of interest in the control of steroid misuse. The present work is part of this continuity in that it aims to reinvestigate the metabolism of certain steroids of interest using innovative analytical strategies and recent chemoinformatics advances. Molecular networking (MN) has proved to be efficient to identify new natural products. Based on the assumption that structurally related molecules produce similar fragmentation patterns, this emerging computational approach allows to visualize and organize tandem MS/MS data sets and to propagate annotation to unknown but related molecules. In this context, first step consisted in the implementation of a steroid database to study the clustering power of MN on more than 100 reference compounds. MN analysis was subsequently applied to detect novel nandrolone derived metabolites in urine samples from treated bovines. Urine samples were characterized with non-targeted approaches involving high-resolution mass spectrometry (HRMS/MS) coupled with liquid and gas chromatography (LC-Q-Exactive / GC-Q-Exactive) to generate global spectrometric fingerprints. Data analysis using statistical methods such as Galaxy and Global Natural Products Social Molecular Networking enabled annotating data with databases and creating molecular networks to investigate metabolism products and pathways.

Keywords: biomarkers, molecular network, teroids

POSTER P26

MULTISCREENING METHOD FOR THE DETERMINATION OF FIVE NITROFURAN METABOLITES IN VARIOUS BIOLOGICAL MATRIXES.

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Abstract
Improvement of nitrofuran metabolites analyses is currently very up-to-date due to several legislative changes. Not only nifursol metabolite (5-dinitrosalicylhydrazide - DNSH) has been recently included in the compulsory monitored substances, but also RPA limits were reduced from 1 to 0.5 ppb for all nitrofuran metabolites in all biological matrixes. This change has required adjustments of previous analytical methods associated primarily with a modification of measurement conditions for analytical instruments. This proceeding presents an analytical multimethod that has an universal application for a determination of five basic nitrofuran metabolites in the most common biological matrixes - muscle, milk, eggs and honey. It was proved that this analytical method can be also used for a determination of nitrofuran metabolites in a much broader spectrum of proteinaceous matrixes such as intestines, cheeses, dried egg white, etc. The proceeding describes laboratory sample preparation, HPLC-MS/MS measurement conditions and method validation results achieved according to Regulation (EU) 2021/808.

Introduction
Nitrofurans are synthetic chemotherapeutic agents with a broad antimicrobial spectrum; they are active against both gram-positive and gram-negative bacteria, including Salmonella and Giardia spp, trichomonads, amebae, and some coccidial species. Due to their potential carcinogenity, nitrofurans belong among the drugs prohibited in food animals in the European Union and other countries (e.g. the USA). Currently, there is a requirement for National Residue Control Plans to monitor the illegal use of 5 selected synthetic broad-spectrum antimicrobial agents belonging to a group of nitrofurans, that are, furazolidone, furaltadone, nitrofurantoin, nitrofurazone and nifursol. In vivo these compounds are rapidly absorbed and transformed, giving rise to protein-bound residues 3-amino-2-oxazolidinone (AOZ) from furazolidone, 3-amino-5-methylmorpholino-2-oxazolidinone (AMOZ) from furaltadone, 1-aminohydantoin (ADH) from nitrofurantoin, semicarbazide (SEM) form nitrofurazone and 3,5-dinitrosalicyclic acid hydrazide (DNSH) from nifursol. These metabolites are routinely used as markers for control of the prohibited treatment with nitrofurans of food-producing animals.
To correctly evaluate the situation, it is then also necessary to determine the origin of SEM and to distinguishing between SEM from nitrofurazone and SEM from other sources in food products (e.g. from the shells of crustaceans after heat treatment; SEM as dough improver flour treatment agent etc.) Therefore, it is necessary to perform a tissue washing step prior to the analysis of nitrofurans to distinguish between tissue-bound SEM as a drug metabolite and unbound SEM from another source.

Materials and Methods

Sample preparation

2.0 g of homogenized matrix (muscle, honey, egg, milk, cheese) or 1.0 g of homogenized matrix (intestines, dried egg white, dried milk powder) are taken into the analyse. The sample is spiked with internal standards (AOZ-d4, AMOZ-d5, AHD-13C3, SEM-13C,15N2, DNSH-15N2) at the concentration of 1.0 µg/kg. 15 mL 0.1 M hydrochloric acid and 100 µL 2-nitrobenzaldehyde (NBA) solution (15 mg/mL in methanol) is added and derivatization takes place overnight at the temperature of 37 °C. After this the sample is centrifuged for 10 minutes at 15 °C, 3000 rpm (1180 g) and the upper layer is separated and transferred into the 20 mL vial. Then 1 mL of 1M phosphate buffer pH 7 is added and pH of sample is adjusted in a range of 6.5 - 7.5 using sodium hydroxide. Extractions with 2 x 5 mL of ethyl acetate with centrifugation for 10 minutes at 3000 rpm (1180 g) at 10 °C between extraction steps are performed. Combined extracts are evaporated under a nitrogen stream at the temperature of 40 °C to

dryness. The sample is re-dissolved in 250 µL of a mixture methanol+ water (40+60) and 1 mL of hexane is added. The sample is shaken vigorously and then centrifuged for 10 minutes at 3000 rpm (1180 g) at 10 °C. The bottom layer is used for the analysis.

Note: To distinguish between tissue-bound SEM as a drug metabolite and unbound SEM from another source a washing step is applies in a case of muscle analysis. An unbound SEM is gradually washed out from a muscle matrix using 50%, 75% and 100% methanol before performing derivatization with the NBA.

LC-MS/MS method

HPLC chromatograph 1260 (Agilent) connected with a mass spectrometer LC/MSD Triple Quadrupole G6495A (Agilent) and HPLC Accela (Thermo) connected with a mass spectrometer TSQ Vantage (Thermo) were used. Chromatographic separation was achieved using Symmetry C8, 2.1 x 100 mm, 3.5 µm, Waters column. The mobile phase consisted of 10 mM ammonium formate (A) and methanol (B). The column temperature was set at 25 °C and the flow rate was set at 0.2 mL/min. The injection volume was 20 µL, chromatographic conditions are reported in table 1 with an overall run time of 18 min.

MS/MS measurement was proceeded in ESI+ for AOZ, AMOZ, AHD and SEM and in ESI- for DNSH mode using capilary voltage

2900 V in ESI+ or 3700 V in ESI-, gas temperature 160 °C, gas flow 14 L/min, nebulizer 30 psi; sheath gas temperature 310 °C; sheath gas flow 12 L/min; nozzle voltage 700 V in ESI+ or 300 V in ESI- (conditions for LC/MSD Triple Quadrupole G6495A) or Spray voltage: 4000 V in ESI + or 3000 V in ESI-, drying gas flow 50 units, auxiliary gas flow 45 units, capillary temperature

230 °C, drying gas temperature 370 °C (conditions for TSQ Vantage).

The multiple reaction monitoring (MRM) conditions for the analytes are given in table 2.

Table 1. LC conditions

Time (min)	[A] (%)	[B] (%)	Flow rate (µL/min)	Analytical column
0,00	65	35	200	Symmetry C8, 2.1 x 100 mm, 3.5 µm, Waters
8.00	10	90	200	
13.00	10	90	200	
13.01	65	35	200	
18.00	65	35	200	

Table 2. MRM transitions, retention time, collision energies and internal standards

Component	Retention time (min)	MRM transition 1	MRM transition 2	Internal standard
AOZ	5.4	236 > 134 (10 eV)	236 > 104 (23 eV)	AOZ-d4
AMOZ	6.4	335 > 291 (9 eV)	335 > 262 (15 eV)	AMOZ-d5
AHD	5.1	249 > 134 (9 eV)	249 > 104 (22eV)	AHD-13C3
SEM	5.9	209 > 166 (7 eV)	209 > 192 (8 eV)	SEM-13C,15N2
DNSH	8.8	374 > 183 (31 eV)	374 > 226 (25 eV)	DNSH-15N2
AOZ-d4	5.4	240 > 134 (10 eV)		
AMOZ-d5	6.4	340 > 296 (9 eV)		
AHD-13C3	5.1	252 > 134 (9 eV)		
SEM-13C,15N2	5.9	212 > 168 (7 eV)		
DNSH-15N2	8.8	376 > 227 (25 eV)		

Results and Discussion

Validation study

The validation study was performed according to the Regulation (EU) 2021/808 requirements. Each matrix samples were spiked with standards of investigated nitrofuranes at the concentration range of 0 - 3.0 µg/kg (µg/L respectively) and the concentration of internal standards was 1.0 µg/kg (µg/L respectively). Six six-point calibration curves were prepared and measured within 4 days. Concentration levels 0.0; 0.5; 1.0; 1.5; 2.0 and 3.0 µg/kg (µg/L respectively) were used for all investigated analytes. Each sample was twice injected i.e. 72 values were statistically calculated. The CCα limits were calculated from this dataset. The laboratory reproducibility was calculated using results of 6 spiked samples at the level of 0.5 µg/kg (µg/L respectively) that were measured within 4 days. Validation results are shown in table 3 and table 4.

Table 3. Validation study - validation results for frequently analyzed matrixes

Component	Matrix	CCα limit [µg/kg or µg/L]	Reproducibility [RSD (%)]	Linearity Intercept	Linearity Slope	Correlation coefficient R^2
AOZ	muscle	0.3	4.29	-0.0104	1.0559	0.999
AMOZ	muscle	0.2	9.09	0.0084	1.2307	0.998
AHD	muscle	0.4	8.52	0.0034	0.1992	0.999
SEM	muscle	0.4	8.83	0.0128	0,5838	0.999
DNSH	muscle	0.3	12.71	0.0538	0.4949	0.996
AOZ	eggs	0.2	2.87	0.0069	0.9472	0.999
AMOZ	eggs	0.3	2.91	0.0495	1.2077	0.999
AHD	eggs	0.4	7.68	0.0036	0.1800	0.999
SEM	eggs	0.4	2.85	0.0022	0.6201	0.999
DNSH	eggs	0.3	3.33	0.0103	0.4038	0.999
AOZ	milk	0.2	5.73	-0.0015	1.1146	0.999
AMOZ	milk	0.3	3.00	0.0005	1.3467	0.999
AHD	milk	0.4	6.19	-0.0084	0.2566	0.998
SEM	milk	0.4	8.26	0.0035	0.6948	0.999
DNSH	milk	0.3	8.81	-0.0226	0,4297	0.998
AOZ	honey	0.3	5.12	0.0183	0.9222	0.999
AMOZ	honey	0.2	9.43	0.0067	1.1580	0.997
AHD	honey	0.4	5.56	-0.0013	0.1934	0.995
SEM	honey	0.4	7.53	0.0126	0.5283	0.994
DNSH	honey	0.3	4.75	0.0406	0.4887	0.999

Table 4. Validation study - validation results for not common matrixes

Component	Matrix	CCα limit [µg/kg or µg/L]	Reproducibility [RSD (%)]	Linearity Intercept	Linearity Slope	Correlation coefficient R^2
AOZ	cheese	0.2	4.38	0.0253	1.0025	0.999
AMOZ	cheese	0.2	8.25	0.0514	1.1658	0.998
AHD	cheese	0.4	9.33	0.0125	0.2033	0.998
SEM	cheese	0.4	10.02	0.0625	0.6563	0.998
DNSH	cheese	0.3	13.08	0.0426	0.3686	0.999
AOZ	intestines	0.2	5.68	0.0054	0.9354	0.999
AMOZ	intestines	0.3	4.87	0.0842	1.2365	0.999
AHD	intestines	0.4	6.98	0.0019	0.1953	0.998
SEM	intestines	0.4	5.74	0.0145	0.7032	0.998

DNSH	intestines	0.3	6.03	0.0536	0.3395	0.999
AOZ	dried egg white	0.3	6.24	-0.0152	1.0369	0.998
AMOZ	dried egg white	0.3	4.35	0.0125	1.2006	0.998
AHD	dried egg white	0.5	7.12	-0.0056	0.2351	0.996
SEM	dried egg white	0.4	7.95	0.0188	0.5456	0.997
DNSH	dried egg white	0.3	9.51	-0.0186	0.4035	0.998
AOZ	dried milk powder	0.3	5.01	0.0258	0.9985	0.995
AMOZ	dried milk powder	0.3	6.24	-0.0176	1.1362	0.994
AHD	dried milk powder	0.5	6.38	-0.0246	0.2197	0.992
SEM	dried milk powder	0.4	9.45	0.0253	0.6328	0.993
DNSH	dried milk powder	0.3	9.11	0.0046	0.3958	0.999

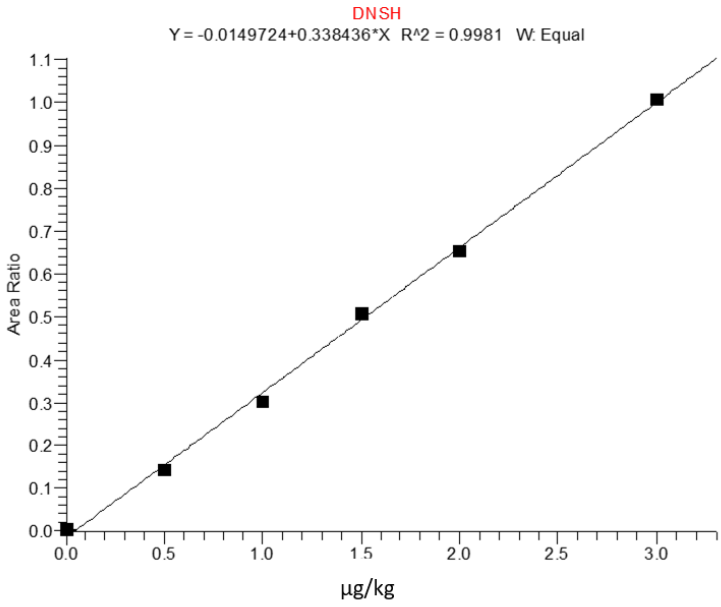

Figure 1. Calibration curve of DNSH in intestines

Conclusions

The analytical method for the determination of five nitrofuranes in various biological matrixes was described. As it was shown, this analytical method is sufficiently sensitive, provides reproducible and linear responses for all five determined analytes and it is very robust in terms of use. Due to this, it can be used for the analysis of less common samples such as intestines, various types of cheese (hard cheeses, soft cheeses) dried protein samples (dried milk powder, dried eggs, dried egg white, etc.) As a result, the method can help laboratories expand the portfolio of samples analyzed beyond the usual ones.

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POSTER P27

MONITORING OF RESIDUES OF SELECTED BETA-AGONISTS IN BROILER CHICKENS DURING TREATMENT AT THERAPEUTIC DOSES IN VARIOUS MATRICES (MUSCLE, LIVER, LUNGS, FEATHERS, CLAWS)

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Abstract

Beta-agonists are a group of drugs whose use is banned in food-producing animals within EU. Residues of beta-agonists are officially monitored in food raw materials of animal origin within the framework of the National Monitoring Plan for Residues of Exogenous Substances. The aim of this study was to evaluate the persistence of residues in selected tissues in individual poultry animals (muscle, liver, lungs, feathers and claws). The obtained data can also help to understand the process of distribution of beta-agonists residues in a living organism which is important for the detection of possible illegal use of these substances. The study was conducted on a statistically representative number of chicken broilers (32 pcs) that were individually orally treated with the selected beta-agonists (clenbuterol, terbutaline or zilpaterol) in an appropriate amount close to the theoretical therapeutic dose. Clenbuterol was chosen as the most common substance of beta-agonist group, zilpaterol was chosen as an authorized substance for cattle outside EU and terbutaline was chosen due to its accumulation in broiler tissues, which has already been confirmed by other studies. Validated analytical LC-MS/MS method using SPE cartridges Clean Screen Dau for sample preparation was used for the determination of beta-agonists residues in all selected matrixes.

Introduction

Beta-agonists are phenyl-ethanolamines derivative compounds related to catecholamines, having different substituents on the aromatic ring and on the terminal amino group, with a pharmacological activity. Beta-agonists are synthetic compounds that mimic some of the effects of naturally-occurring compounds by binding to beta-receptors on the surface of cells within the muscle, fat, and other tissues of animals. They belong to the most common veterinary drugs using for specific effects on smooth muscle. They are used to treat acute heart failure, bradyarrhythmia, asthma and chronic obstructive pulmonary disease for livestock as well as to treat diseases and infections for humans. At higher doses, they can also act as growth promoters by stimulating the increase of the muscular mass and reducing the adipose tissue in livestock production. However, using of beta- agonists is banned in food-producing animals within the European Union since 1996 by Directive 96/22/EC except for well-defined therapeutic purposes and under strict veterinary control. The consumption of food contaminated by beta-agonists may cause serious adverse effects, such as food intoxication and cardiovascular and nervous system disorders.

Control of the illegal use of beta-agonists as well as other veterinary drugs and growth promoters in intensive animal production is regularly performed within the EU by the application of Residue Monitoring Programs. The overall strategy of control includes the surveillance of live animals with the collection of urine, feed, water and edible tissues like muscle, liver, kidney and more at slaughterhouses. Most countries have established strict surveillance programs for official control purposes to check compliance with regulatory limits, for both domestic and imported production. However, non-compliant samples are often identified in Member States. Every year, the European Food Safety Authority (EFSA) publishes an overview of findings of non-compliant samples from national authorities and food safety agencies in EU Member States. In 2018, 33,165 targeted samples were analysed for beta-agonists within EU, with 4 non-compliant samples reported in total. The non-compliant samples were found for bovines (three non-compliant samples: for salbutamol – Italy, sotalol hydrochloride - Ireland, terbutaline and tulobuterol – Germany) and poultry (1 sample for clenbuterol - Spain). In 2019, 36,698 targeted samples were analysed with no non-compliant samples reported. And in 2020, 34,624 targeted samples were analysed with one non-compliant sample reported for salbutamol in bovines.

In our study, we focused on 3 selected analytes - clenbuterol, terbutaline, and zilpaterol. They were chosen as the target beta-agonists because their residues have been detected in various matrixes within the Framework

of national European residue monitoring. Clenbuterol is the most preferred beta-agonist used as a growth promoter in livestock outside EU. It is also used for expanding the bronchi in horses and calves and slowing down uterine contractions in cows. Treatment dose is very often added to the feed of cattle, sheep and horses for economic gain. Previously it has been reported that the amount of the drug in the muscle of bovine animals given an anabolic dose of clenbuterol decreases below maximum residue limits - MRLs (0.1 µg kg^{-1} according to Commission Decision 37/2010) after more than seven days. Terbutaline is illegally used as growth promoter to increase skeletal muscle mass, protein deposition and to decrease carcass fat of broiler chickens. Zilpaterol is administrated as a feed additive for cattle outside EU. It decreases the rate of fat/protein of a carcass, increases growth rates, and decreases feed consumption in ruminants. It is widely used in Central America and Mexico. Zilpaterol has been licensed for zootechnical use in South Africa and Mexico.

Worth mentioning is the beta-agonist - ractopamine, which is used as a growth promoter for ruminants, pigs and turkeys. It has potential risk to consumers for adverse cardiovascular and central nervous system effects. Despite that fact, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) has established Maximum Residue Limits (MRLs) for ractopamine in cattle and pig muscle, kidney, liver and fat (10, 90, 40 and 10 µg kg^{-1}, respectively), which have been adopted by the Codex Alimentarius Commission and implemented in some countries (e.g. Canada). However, the using of ractopamine in cattle and pigs is still disputable. Residue depletion study of ractopamine in swine tissues has been reported. According to the results of the study, ractopamine residues were still detected after 11 days of medication in all tissues examined (muscle, liver, kidney, heart, lung, stomach-pylorus, large intestine-colon, small intestine-ileum) with the exception of muscle. The highest ractopamine level was found in lung tissue.

Approximately 25 countries, including the United States, Australia, Canada, Japan, Mexico and Brazil, allow the use of clenbuterol, ractopamine, and zilpaterol within the Codex Alimentarius Commission guidelines. A residual concentration in a tissue must be below a given maximum residue limit (MRL) when the animals are slaughtered and sent on the market. But many other countries (including EU) have banned their use as a growth promoter and have implemented traceability programs. They established an action level for some beta-agonists in imported consignments lower than Codex MRL. If they apply a zero tolerance policy, when an official laboratory founds any confirmed residues at lower concentrations, the consignment would be rejected.

Several analytical techniques for determination of beta-agonists in biological matrixes have been employed, such as liquid chromatography, gas chromatography with mass spectrometry and liquid chromatography coupled to mass spectrometry with high mass accuracy or tandem mass spectrometry. LC-MS/MS is an important analytical technique for the qualitative and quantitative analysis of beta-agonists due to their high selectivity and sensitivity. For some beta-agonists Minimum Method Performance Requirements (MMPRs – for specific pharmacologically active substances in specific animal matrices) were laid down in the EURL guidance (September 2020) and these are binding on official laboratories. Urine, liver, lung, retina, kidney, muscle, faeces, plasma, hair, drinking water and feed are the recommended matrixes for routine beta-agonists residues analyses. Unfortunately, the short persistence of beta-agonists in biological matrixes is one of the most limitative factors for successful detection of their illegal use. Due to this, our study was targeted to some other matrixes where the persistence of those compounds seems to be much better.

Therefore, the aim of this study was to observe residues of beta-agonists in chicken muscle, liver, lungs, feathers and claws after oral administration of selected beta-agonists and to compare the time needed for their depletion, when residue concentrations decrease under detection limit.

Experimental

Animal treatment

Clenbuterol hydrochloride (Sigma-Aldrich), terbutaline hemisulfate salt (Sigma-Aldrich) and zilpaterol hydrochloride (Lonza) used for chicken treatment were supplied by Merck in a purity higher than 99%. For the application to animals, aqueous solutions of individual substances were used. Due to the limited solubility of beta-agonists in water, solutions for application were prepared at a concentration of 1 mg mL^{-1} (for each substance separately).

21-days-old broiler chickens, vaccinated against coccidiosis, were randomly divided into four experimental groups (non-treated group - 9 pcs., clenbuterol group - 9 pcs., terbutaline group - 9 pcs. and zilpaterol group - 9 pcs.). 3 broilers were slaughtered and their matrixes were analysed at the beginning of the experiment to verify that the delivered animals did not contain the test substances in their tissues. All experimental groups of animals were put into separated boxes and left for 7 days to adapt to the environment under controlled conditions of 23 ± 5°C and 50–70% relative humidity. The boxes were furnished with a raised mesh floor in

order to avoid contamination of the feathers with chicken droppings. The design of the experiment was approved by the Ministry of Agriculture of the Czech Republic (permission No. MZE-30908/2021-18134). Ethical and welfare guidelines according to Directive 2010/63/EU were followed throughout the whole animal breeding process. Throughout the entire experiment, all birds had ad libitum access to fresh water and non-medicated feed (complete feed mixture - grower for broilers fattening).

After being reared for 5 days, chickens were weighed and these weights were used to calculate the dose of drugs. Individual weights of animals before medication are shown in Table 1. Broilers were treated with 1 mg of clenbuterol, terbutaline or zilpaterol per kg body weight. Aqueous solutions of selected beta-agonists (of which the real concentrations were analytically confirmed) were administered for 4 consecutive days to 4weeks old chickens once daily (the same day time of administration on the respective day, i.e. the same inter-dose intervals followed). Administrations were performed by gavage to ensure exact dose. Non-treated chickens were deemed the control group and they were excluded from the treatment. In established time intervals the representative numbers of randomly selected chickens in each group were slaughtered. The first batch of treated birds was sacrificed 1 day after the last treatment when the highest residual concentrations were expected. The second and third batches of animals were sacrificed days 4 and 8, respectively, after the last treatment. Individual weights of animals are shown in Table 2.

Table 1. Weight of chickens before medication (in grams)

Animal	Control group (without medication)	Clenbuterol group	Terbutaline group	Zilpaterol group
1	890	940	1130	1030
2	840	1170	930	880
3	1110	1120	1050	1030
4	1060	1130	870	840
5	930	1110	930	1070
6	920	990	1030	1090
7	960	930	970	930
8	940	1030	980	1070
9	1040	1030	930	970
Mean	**966**	**1050**	**980**	**990**

Table 2. Weight of chickens before slaughter (in grams)

Days after the last treatment	Animal	Control group (without medication)	Clenbuterol group	Terbutaline group	Zilpaterol group
1	1	1550	1450	1760	1640
	2	1610	1840	1880	1740
	3	1560	1620	1540	1330
	mean	**1573**	**1637**	**1727**	**1570**
4	4	1910	2030	1810	1660
	5	1810	2280	1810	1460
	6	2020	2260	1890	2080
	mean	**1913**	**2190**	**1837**	**1733**
8	7	2250	2200	2420	2400
	8	2530	2900	2500	2420
	9	2810	2360	2440	2680
	mean	**2530**	**2283**	**2453**	**2500**

Materials and Methods

Sample preparation

Muscle and liver samples: 5 g of homogenized liver or muscle were subjected to analysis. Then 5 mL of dissolving solution (90 mL 5 mM ammonium formate + 10 mL MeOH + 0.1 mL formic acid) were added. The sample was spiked with internal standards at a level of 0.1 µg mL^{-1}, mixed and it was allowed to stand for 15 minutes. Then 10 mL of phosphate buffer pH 5 were added. The mixture was allowed to incubate for 20 minutes at 40°C, it was shaken occasionally. Then the mixture was cooled to room temperature and it was centrifuged for 10 minutes at 4°C, 1530 *g*. The upper layer was transferred to clean centrifugation tube. Then 5 mL of phosphate buffer pH 5 were added and mixed for 30 seconds. The mixture was centrifuged for 10 minutes at 4°C, 1530 *g* and both of supernatants were combined. Hydrolysis: 50 µL of β-Glucuronidase were added, mixed and the mixture was heated 1 hour for 40°C. The sample was allowed to cool and pH was adjusted to 6 by 5M NaOH. The supernatant was completed by 200 µL of MeOH and it was centrifuged for 10 minutes at 4°C, 1530 *g*. This extract was transferred onto SPE column.

Lungs samples: 2 g of sliced lungs were subjected to analysis. 10 mL of phosphate buffer pH 5 were added a mixed by homogenizer. The sample was spiked with internal standards at a level of 0.1 µg mL^{-1} and mixed again. If it necessary, pH was adjusted to 5 by 1 M NaOH or 25% HCl. The mixture was allowed to incubate for 20 minutes at 40°C and shaken occasionally. Then the mixture was cooled to room temperature and it was centrifuged for 10 minutes at 4°C, 1530 *g*. The upper layer was transferred to clean centrifugation tube. Then 5 mL of phosphate buffer pH 5 were added and mixed for 30 seconds. The mixture was centrifuged for 10 minutes at 4°C, 1530 *g* and both of supernatants were combined. Hydrolysis: 50 µL of β-Glucuronidase were added, mixed and the mixture was heated 1 hour for 40°C. The sample was allowed to cool and pH was adjusted to 6 by 5M NaOH. The supernatant was completed by 200 µL of MeOH and it was centrifuged for 10 minutes at 4°C, 1530 *g*. This extract was transferred onto SPE column.

Feather samples: The feathers from the whole chicken wing were homogenised in toto to eliminate the inhomogeneous distribution of beta-agonists in them. Feathers were visually inspected, mechanical impurities were removed and feathers were ground up. Close attention was paid to the optimization of the milling conditions. Insufficient milling led to a poor sample state; on the other hand, excessive milling led to burning of the matrix and the degradation of the sample. A time period of 1 minute and frequency of 30 vibrations per second was determined as optimal when a ball mill MM 400 Retsch (Haan, Germany), a stainless ball (weight 63 g, Retsch part. No. 05.368.0105) and a stainless grinding jar (Retsch part. No. 01.462.0216) were used for the milling.

0.2 g of homogenized feathers were subjected to analysis. The sample was spiked with internal standards at a level of 0.1 µg mL^{-1}, then 8 mL of tris buffer pH 8 and 100 µL of protease was added. The sample was then shaken and left overnight at 55°C. The sample was allowed to cool and 2 mL of 0.1 M phosphate buffer were then added. The sample was inserted into ultrasonic baths and then it was centrifuged for 15 minutes at 4°C, 1530 *g*. pH of the sample was checked and the upper layer was separated and transferred onto SPE column.

Claws samples: 2.5 g of homogenized claws were subjected to analysis. Then 5 mL of dissolving solution (90 mL 5 mM ammonium formate + 10 mL MeOH + 0.1 mL formic acid) were added. The sample was spiked with internal standards at a level of 0.1 µg mL-1, mixed and it was allowed to stand for 15 minutes. Then 10 mL of phosphate buffer pH 5 were added. The sample was spiked with internal standards at a level of 0.1 µg mL^{-1} and mixed again. The mixture was allowed to incubate for 20 minutes at 40°C, it was shaken occasionally. Then the mixture was cooled to room temperature and it was centrifuged for 10 minutes at 4°C, 1530 *g*. The upper layer was transferred to clean centrifugation tube. Then 5 mL of phosphate buffer pH 5 were added and mixed for 30 seconds. The mixture was centrifuged for 10 minutes at 4°C, 1530 *g* and both of supernatants were combined. The supernatant was completed by 200 µL of MeOH and it was centrifuged for 10 minutes at 4°C, 1530 *g*. This extract was transferred onto SPE column.

SPE clean up

Clean Screen 500 mg/6 mL (CSDAU506, UCT Bristol) were used for SPE extraction of beta-agonists from matrix. The cartridges were conditioned with 2 mL of methanol, 2 mL of deionized water and 2 mL of phosphate buffer (pH 6.0). After sample application, the cartridges were washed with 1 mL of 1M acetic acid and dried at high vacuum for 20 minutes. 2 mL of methanol were then loaded and the cartridges were dried under a strong vacuum for 10 minutes. The analytes were eluted with 3x2 mL of ammonia elution solution (a mixture of ethyl acetate and ammonia (32 % in water) in a ratio 97:3) and the samples were evaporated at 40°C almost to dryness. The samples were re-dissolved in 300 µL of dissolving solution (90 mL 5 mM ammonium formate + 10

mL MeOH + 0.1 mL formic acid) or 600 µL of dissolving solution (feathers and claws) and used for the chromatographic analysis.

LC-MS/MS method

All samples were analysed by LC-MS/MS method using chromatography column X-Terra C18, 2.1 x 150 mm, 3.5 µm, Waters and Agilent Technologies chromatograph 1260 Infinity connected with Triple Quadrupole G6495A. The mobile phase consisted of 10mM solution of ammonium acetate (A) and methanol gradient grade (B). The column temperature was set at 40°C and the flow rate was set at 0.2 mL min^{-1}. The injection volume was 25 µL.

The mobile phase gradient started at A/B (93/7) and was programmed to A/B (20/80) over 16 minutes and allowed to stand for 2 minutes. The mobile phase ratio was then changed back to A/B (93/7) and left to the end of the analysis (28 min). MS/MS measurement was carried out in ESI+ mode using a gas temperature of 100°C, gas flow 14 L min^{-1}, nebulizer 50 psi, sheath gas temperature 370°C, sheath gas flow 12 L min^{-1}, capillary voltage 2600 V, nozzle voltage 400 V, ion funnel high pressure (positive) 200 V, low pressure (positive) 100 V, dwell time 50 ms and scan width 0.07 min for all measured transitions. The multiple reaction monitoring (MRM) conditions for all analytes are given in Table 3.

Table 3. MRM transitions, retention times, collision energies and internal standards

Component	Retention time (min)	MRM transition 1	MRM transition 2	Internal standard
Clenbuterol	15.1	277 > 203 (16 eV)	277 > 132 (33 eV)	Clenbuterol-d6
Terbutaline	10.1	226 > 170 (11 eV)	226 > 152 (17 eV)	Salbutamol-d6
Zilpaterol	11.2	262 > 244 (12 eV)	262 > 202 (21 eV)	Zilpaterol-13C3
Clenbuterol-d6	15.0	283 > 204 (17 eV)		
Salbutamol-d6	10.4	246 > 148 (20 eV)		
Zilpaterol-13C3	11.2	265 > 247 (12 eV)		

Results and Discussion

The aim of the study was to determine how the residues were distributed throughout the chicken body. The contents of beta-agonists residues obtained from analyses of chicken samples taken at the appropriate time intervals following the beta-agonists administration are shown in Tables 4-11 and Figures 1-3 (N/D means not detected). The concentrations of beta-agonists residues in feathers, then in the claws and lungs decline more slowly than in liver and muscle. Regarding the muscle samples, breast and thigh (leg) muscles were considered separately. Differences in measured residues for individual tissues within one batch of matrix (samples from animals slaughtered in one time interval) are not significant: residue levels vary within one concentration order. The highest concentrations were detected at feathers, as was the case with a similar depletion study of nitroimidazoles. The amount of residue of beta-agonists in liver were higher than at the muscle (which is in accordance with findings of EU-RL BVL Berlin). The residues of beta-agonists found in the lungs were higher than in the liver and muscle, thus confirming the conclusion of the study from Dong et al., 2011. More specifically, the corresponding average concentrations of beta-agonists residues found in descending order were feathers > claws > lungs > liver > muscle.

Clenbuterol was detected in almost all chicken matrixes, and its maximum concentration (at feathers) was higher than that of the other selected analytes. Almost no residues were detected in tissues obtained from chicken animals at the last slaughter. The absence of detectable residues in most of muscle, liver and lung samples shows very quick depletion of beta-agonists amount intake.

Table 4. Clenbuterol residue mean concentrations (µg/kg) in chicks' tissues

Matrix	1	4	8
		(day of slaughter)	
feathers	444	389	321

claws	11.9	8.3	4.3
lungs	11.5	0.3	0.1
liver	9.2	0.5	0.3
muscle	1.1	0.2	N/D

Table 5. Terbutaline residue mean concentrations (µg/kg) in chicks' tissues

Matrix	1	4	8
		(day of slaughter)	
feathers	95	68	60
claws	5.1	2.2	1.8
lungs	0.8	N/D	N/D
liver	0.9	N/D	N/D
muscle	0.4	0.2	N/D

Table 6. Zilpaterol residue mean concentrations (µg/kg) in chicks' tissues

Matrix	1	4	8
		(day of slaughter)	
feathers	86	89	83
claws	3.3	2	2
lungs	0.8	N/D	N/D
liver	0.3	N/D	N/D
muscle	0.3	N/D	N/D

Table 7. Results for muscle (µg/kg)

Day of slaughter	Clenbuterol breast / leg	Terbutaline breast / leg	Zilpaterol breast / leg
1	1.24 /1.27	0.25 / 0.39	0.16 / 0.43
	0.70 / 0.86	0.42 / 0.39	0.39 / 0.35
	1.28 / 1.41	0.40 / 0.57	0.35 / 0.45
4	0.31 / 0.30	N/D / 0.13	0.13 / N/D
	0.22 / 0.22	0.18 / 0.19	N/D
	0.19 / 0.24	N/D / 0.15	N/D
8	N/D	N/D	N/D
	N/D	N/D	N/D
	N/D	N/D	N/D

CCα: Clenbuterol – 0.1 µg/kg; Terbutaline – 0.5 µg/kg; Zilpaterol – 0.5 µg/kg

Table 8. Results for liver (µg/kg)

Day of slaughter	Clenbuterol	Terbutaline	Zilpaterol
1	11.07	1.32	0.31
	5.18	0.52	N/D
	11.50	0.82	0.32
4	0.57	N/D	N/D
	0.40	N/D	N/D

	0.43	N/D	N/D
8	0.31	N/D	N/D
	0.33	N/D	N/D
	0.39	N/D	N/D

CCα: Clenbuterol – 0.08 µg/kg; Terbutaline – 0.3 µg/kg; Zilpaterol – 0.3 µg/kg

Table 9. Results for lungs (µg/kg)

Day of slaughter	Clenbuterol	Terbutaline	Zilpaterol
1	15.07	0.93	1.13
	5.87	0.61	0.33
	13.51	0.72	0.85
4	0.31	N/D	N/D
	0.19	N/D	N/D
	0.35	N/D	N/D
8	0.14	N/D	N/D
	N/D	N/D	N/D
	0.10	N/D	N/D

CCα: Clenbuterol – 0.1 µg/kg; Terbutaline – 0.5 µg/kg; Zilpaterol – 0.5 µg/kg

Table 10. Results for feathers (µg/kg)

Day of slaughter	Clenbuterol	Terbutaline	Zilpaterol
1	468.79	70.30	109.45
	445.17	84.07	61.99
	417.33	131.06	87.24
4	448.27	60.16	86.63
	395.28	75.16	101.32
	323.91	69.81	78.58
8	389.62	46.87	75.38
	356.83	80.18	79.90
	217.16	54.28	94.70

Table 11. Results for claws (µg/kg)

Day of slaughter	Clenbuterol	Terbutaline	Zilpaterol
1	13.98	5.54	3.05
	10.14	5.22	3.65
	11.69	4.47	3.36
4	9.97	1.40	2.24

	5.89	1.66	1.73
	9.11	3.59	2.09
8	6.03	2.10	2.28
	3.23	1.61	2.02
	3.69	1.62	1.80

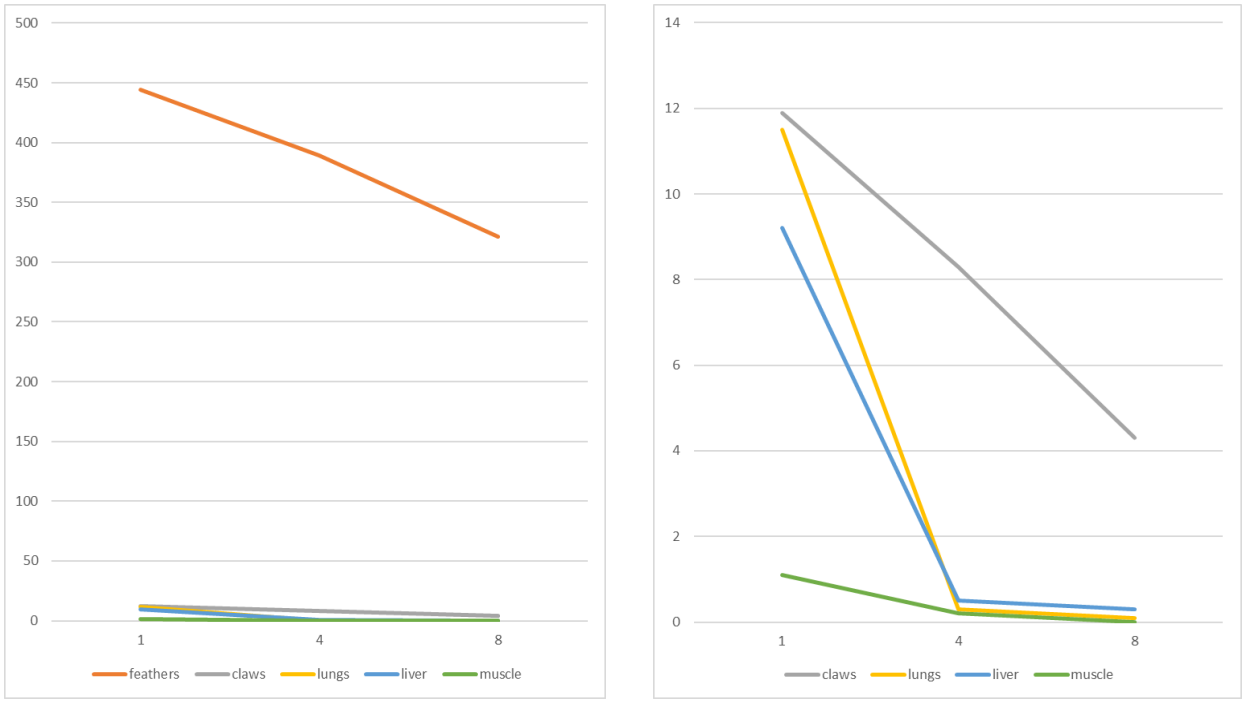

Figure 1. Concentration of Clenbuterol in individual matrixes (and extended scale) in µg/kg at respective days

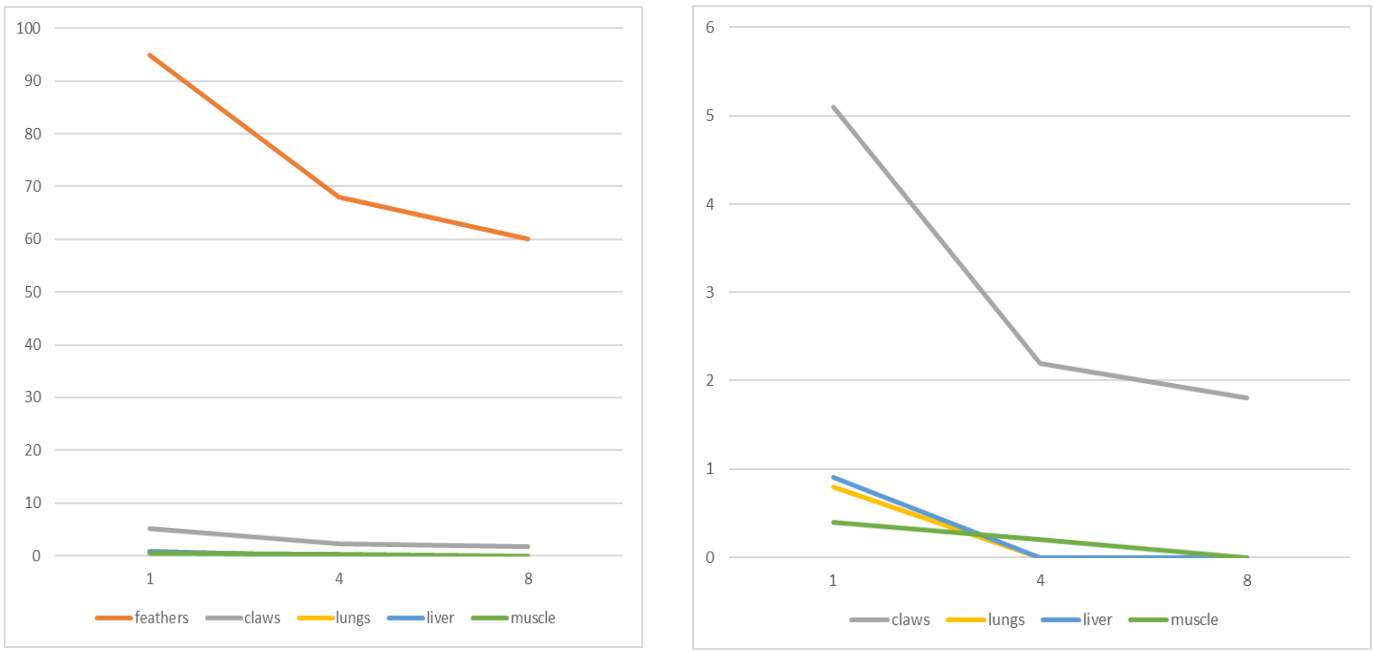

Figure 2. Concentration of Terbutaline in individual matrixes (and extended scale) in µg/kg at respective days

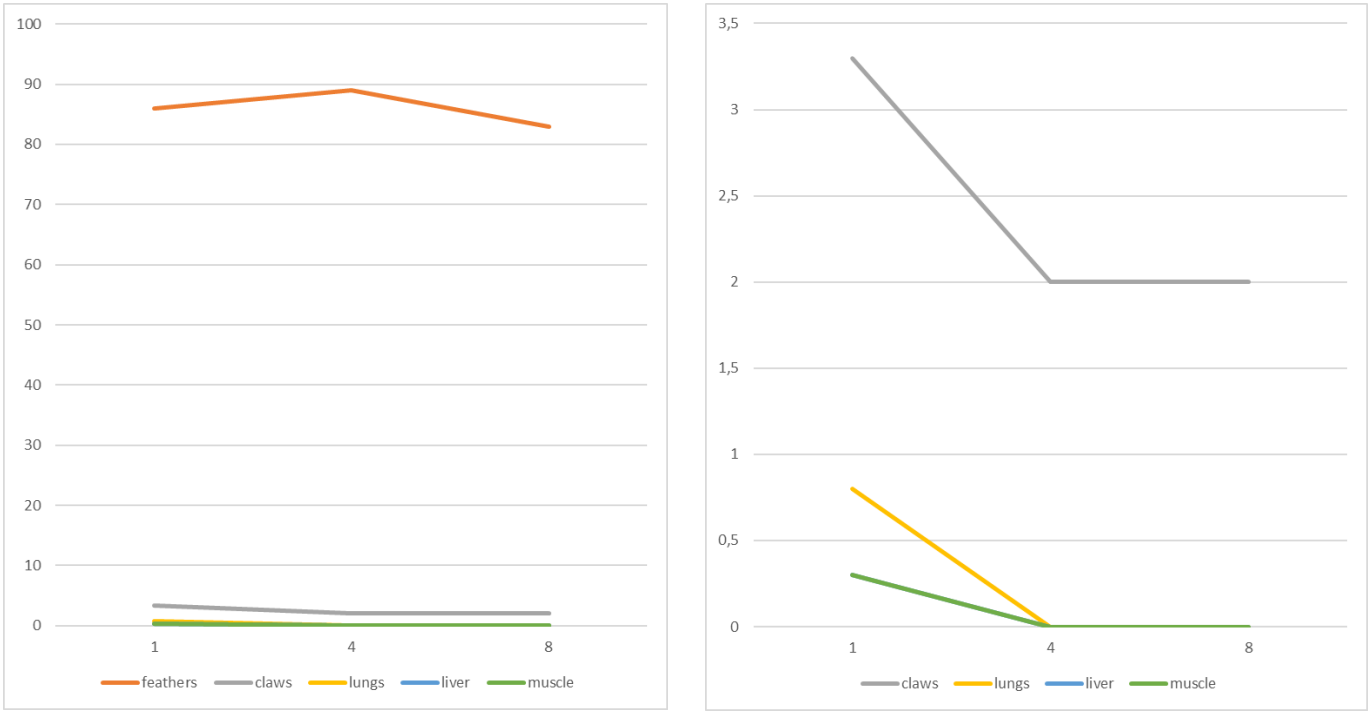

Figure 3. Concentration of Zilpaterol in individual matrixes (and extended scale) in µg/kg at respective days

Conclusions

Beta-agonists are substances forbidden for use on food-producing animals. Except for clenbuterol (MRL was established for bovine muscle, liver, kidney and milk), there is zero tolerance concerning the concentration of their residuals.

The objectives of this study were to measure the residues of beta-agonists in broiler chickens incurred samples after treatment with clenbuterol, terbutaline and zilpaterol and to compare depletion time in relation to the matrix. An efficient and sensitive analytical method was used for the detection of beta-agonists in individual chicken matrixes by LC-MS/MS based on SPE residues extraction. The method validation was undertaken

according to the Regulation (EU) 2021/808 requirements. Beta-agonists were individually administered to chickens at expected therapeutic doses with an oral probe in order to minimize any external contamination of feathers which could occur when drug administration via drinking water or medicated feed had been used.

In addition to the sensitivity of the analytical method, also the persistence of residues and choice of suitable matrix for official control affects whether illegal use of prohibited substances is discovered or not. As the results show, depletion of beta-agonists especially in feathers and claws runs much slower than muscle or liver. This conclusion was confirmed by the evaluation of depletion characteristics of all monitored substances. It has also been found that the concentrations of all analytes are higher in feathers, subsequently in claws and lungs than in other studied matrixes.

These findings lead to the conclusion that beta-agonists residues in feathers are detectable throughout the whole life of broiler chickens and this enables the detection of the illegal use of beta-agonists after a longer period than in the case of analysis of other matrixes. The results of study might certainly be taken into account when suitable matrixes for monitoring beta-agonists residues in birds are selected and when National Residue Control Plans are compiled. The analytical method used is also very robust and versatile and it can be used for beta-agonists residues analyses in a wide range of biological matrixes.

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POSTER P28

A STRAIGHTFORWARD STRATEGY FOR STEROID ESTERS ANALYSIS IN BOVINE

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Abstract
Hair represents a matrix of choice for detecting steroids residues under intact esterified form, signing xenobiotic administration even in the case where natural steroids are involved. Moreover, hair can be considered has a reliable matrix for the long term measurements of such esters, even several months after administration.
In 2012, Bichon and al.[1], reported a method relying on LC-MS/MS for steroid esters analysis in hair, using dansylation of estrogen esters. However, such derivatization is time-consuming and leads to unselective fragment ions related to the derivative reagent. Therefore, the screening and the confirmatory steps remain an issue due to a large number of interfering compounds.
The purpose of this work was the development and optimization of an efficient analytical strategy dedicated to the determination of steroid esters in hair samples, without any derivatization step, based on the article published by Kwok et al. [2].
The proposed analytical method allows monitoring 36 steroid esters related to testosterone, estradiol, boldenone, nortestosterone with calculated decision limits below 100 µg/kg, and with a majority of targeted compounds below than 10 µg/kg, fulfilling the European requirements.
[1] Bichon and Al. *Rapid Commun. Mass Spectrom.* (2012)
[2] Kwok et al. *Journal of Chromatography A*. 1493 (2017) 76-86

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POSTER 29

DETERMINATION OF GESTAGENS AND STILBENS RESIDUES IN GOAT COLOSTRUM POWDER

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Abstract

Methods for the determination of residues of two gestagens and four stilbens in goat colostrum powder were developed. The procedure for analysis of two selected steroids with gestagenic action – progesterone and medroxyprogesterone acetate - consists of colostrum homogenization with water, methanol extraction, cleaning on an alumina column and LC MS/MS measurement. The other method includes testing of four stilbens – diethylstilbestrol, hexestrol, dienestrol and benzestrol. It comprises homogenization of colostrum samples with water, liquid/liquid extraction and cleaning on C-18 columns. For a chromatographic separation and detection of samples GC-MSD was used.

Introduction

Colostrum is produced by all mammals in the first few days after giving birth. It is known as concentrated source of proteins, growth factors and antibodies that are essential for early development of newborns. Both hormones and non-hormonal biologically active compounds can be synthesized in the mammary gland and are moving to the circulation of mother. Bioactive compounds, which are transported in the blood and can be detected in the milk, due to mammary secretion consist of components or precursors from blood plasma.

Our laboratory was asked to investigate samples of goat colostrum as a potential source of gestagens and stilbenes residues. In our laboratory the group of gestagens is routinely analysed in kidney fat and stilbenes are commonly determined in urine, muscle and liver. The modified methods were developed for colostrum samples.

These methods have been validated according to Commission Decision 2002/657/EC. Decision limits were observed below 1.0 $\mu g.kg^{-1}$ for gestagens and below 2.0 $\mu g.kg^{-1}$ for stilbens. Our method developed can be applied both for screening and confirmatory analyses in official residue monitoring.

Materials and Methods

The reference standards of analytes were supplied by Sigma-Aldrich (progesteron, medroxyprogesterone acetate, diethylstilbestrol, hexestrol, dienestrol) and by Santa Cruz Laboratories (benzestrol). Dienestrol-d_4 was used as the internal standard, supplied by Sigma Aldrich, too. The other internal standards medroxyprogesterone acetate-d_3, hexestrol-d_4 and diethylstilbestrol-d_8 were provided by Bank of Reference standards WFSR. Substances were dissolved in methanol and diluted to low concentration working solution. All solvents used in both methods were obtained from Merck and they were of SupraSolv quality. The alumina sorbent with activity grade 1, neutral, was purchased also from Merck as a bulk pack. Glass columns for alumina had an internal diameter cca 1 cm, and they were 10 cm long with glass frit and stopcock. The Discovery DSC-18 SPE tubes were purchased from Supelco, a member of the Merck Group. Heptafluorobutyric anhydride (HFBA), as a derivatization reagent, was purchased from Sigma Aldrich.

Sample preparation

Primary sample preparation is the same for both methods. 5 g of dry colostrum with 45 mL of deionized water were thoroughly homogenized using vortex mixer and afterwards centrifuged at 3000 x *g*, 10 minutes at the room temperature. By taking these steps, there are differences with following extraction and cleaning on columns between two our methods.

Primary extraction of gestagens

4 g of clear fat-extracted supernatant (0.5 g of dry colostrum) was instilled to a centrifugation tube. The sample was spiked with internal standards working solution (at the final concentration in colostrum 10.0 $ng.g^{-1}$) and eventually fortified with gestagens standards working solution. 10 mL of methanol was added and the sample was properly homogenised by using a mechanical dispergator and afterwards centrifuged at 4000 x *g* 10

minutes at the room temperature. The clear supernatant was transferred to an evaporation flask. The extraction was repeated with another 10 mL of methanol. The combined methanol extract was evaporated. The rest of the sample in the flask was dissolved in 3 mL of solution toluene/hexane (5/9 v/v), and it was applied to the alumina columns.

Gestagens extract clean-up on alumina column

Sorbent for cleaning-up on alumina column was prepared. Activity grade 1 alumina (20 g) was weighed into a glass flask, and 1.2 mL of deionized water was added. The flask was tightly closed, and water was uniformly diffused by rotation of the flask. The tightly closed flask was left at room temperature overnight. Deactivated alumina (2 g) was weighed into a small beaker, and the sorbent was immediately covered with toluene. The alumina and toluene were transferred into the glass column with a frit, and the sorbent was washed with 10 mL of a solution toluene/hexane (1/9 v/v). The dissolved extract from the primary extraction was applied on the column. The evaporation flask was washed with an additional 2 mL of toluene/hexane (5/9 v/v), which were also applied to the column. The column was subsequently washed with 5 mL of toluene/hexane (5/9 v/v), 10 mL of toluene and 5 mL of toluene/ethanol (99/1 v/v). The analytes were eluted with 15 mL of toluene/ethanol (99/1 v/v) to a clean evaporation flask, and all solvents were evaporated to dryness at 40 °C. The residues in the flask were re/dissolved in 0.5 mL of methanol/water solution (6/4 v/v) by using ultrasonic bath and filtered through a syringe filter (regenerated cellulose , 0.2 µm) into a chromatographic vial.

LC MS/MS Measurement of gestagens

The chromatographic analysis was performed by using Agilent Technologies equipment LC 1260 Infinity II coupled with Triple Quad 6495 detector. The chromatographic column was a ZORBAX SB-C18 (2.1 x 50 mm, 1.8 µm) and pre-column ZORBAX SB-C18 (2.1 x 50 mm, 1.8 µm) was used, the column temperature was 45 °C and the column flow was 0.3 $mL.min^{-1}$. The injection volume was 20 µL. The mobile phase consisted of methanol (A) and deionized water (B), and the gradient used is described in Table 1. The detection conditions were as follows: ion source AJS ESI+, drying gas temperature 350 °C , drying gas flow 12 $L.min^{-1}$, capillary voltage 4000 V. The ion transition and retention times of both gestagens are stated in the Table 2.

Table 1. Mobile phase gradient

Time	(A) %	(B) %
0 minutes	60	50
10 minutes	100	0

Table 2. MRM transitions and retention times for gestagens

Analyte	Precursor ion m/z	Product ion m/z	Fragmentor (V)	Collision energy (eV)	Retention time (min.)
progesterone[a]	315.2	297.2	166	15	5.9
medroxyprogesterone acetate	387.2	327.2	166	12	5.6
medroxyprogesterone acetate – d3	390.2	327.2	166	12	5.6

[1] Medroxyprogesterone acetate – d3 was used as the internal standard for the progesterone

Primary extraction of stilbens

4 g of clear fat-extracted supernatant (0.5 g of dry colostrum) was instilled to a centrifugation tube. The sample was spiked with internal standards working solution (at the final concentration in colostrum 10.0 $ng.g^{-1}$) and eventually fortified with stilbens standards working solution. 15 mL of butyl methyl ether was added and the sample was homogenized using vortex mixer and afterwards centrifugated 10 minutes. The clear supernatant was transferred to an evaporation flask. The extraction was repeated with another 10 mL of butyl methyl ether. The combined butyl methyl ether extract was evaporated. The rest of the sample in the flask was dissolved in 3 mL of methanol/water solution (3/7 v/v). The sample was newly treated with 2 x 3 mL of hexane (discarded) and the rest of methanol in sample was evaporated to volume 0.5 mL at 60 °C.

Stilbens extract clean-up on SPE columns

2 mL of deionized water was added to the tube with 0.5 mL of evaporated extract and briefly treated in a ultrasonic bath. An SPE-C18 cartridge (1 g, 6 mL tube Supelco Discovery DSC-18) was conditioned with 5 mL of

methanol and 5 mL of deionized water. Then the sample extract was put onto the cartridge. The column was subsequently washed with 5 mL of deionized water, 5 mL of methanol/water solution (4/6 v/v) and the analytes was eluted with 5 mL of acetonitrile. The sample was evaporated to dryness at 40 °C and transferred to a derivatisation vial with 2 x 0.5 mL of butyl methyl ether. The butyl methyl ether was evaporated with a gentle stream of nitrogen and 40 µL of dehydrated acetone and 10 µL of HFBA were added to the dry residues. The sample was incubated for 60 minutes and 60 °C, and afterwards evaporated with a stream of nitrogen. The sample was dissolved in 50 µL of dehydrated toluene.

GC-MSD Measurement of stilbens

The chromatographic analysis was perfomed on an Agilent Technologies GC 6890M chromatograph with Single Quad MS detector 5973 Inert. An Agilent Technologies HP-1MS (length 30 m, I.D. 0.25 mm, film 0.1 µm) capillary column was used. The injection was performed by on-column method; carrier gas was He 6.0 with constant flow 1.2 $mL.min^{-1}$ and interface temperature was 280 °C. The temperature program was T_{init}. 80 °C, 0 min, β1 30 $°C.min^{-1}$ to 200 °C, β2 4 $°C.min^{-1}$ to 240 °C, β3 10$°C.min^{-1}$ to 280 °C, 5 minutes at 280 °C. Detector conditions were as follows: temperature of ion source 230 °C and temperature of quadrupole 150 °C. The m/z ion values and retention times (RT) of all stilbens are stated in Table 3.

Table 3. Ions m/z (EI - SIM) and retention times (RT)

Analyte	Ion m/z (quantifier)	Retention time (min.)
benzestrol[a]	303.1	11.8
hexestrol	331.0	9.1
hexestrol – d_4	333.0	9.0
dienestrol	658.0	9.0
dienestrol – d_4	662.2	8.9
diethylstilbestrol	660.0	9.2
diethylstilbestrol – d_8	668.0	9.1

[1] hexestrol – d4 was used as the internal standard for the benzestrol

Results

The both methods were validated according to Commission Decision 2002/657/EC.

Validation of gestagens method

The linearity was observed by measuring a blank colostrum samples and samples fortified at different concentration levels (0, 0.5, 1.0, 2.0, 5.0, 10.0 $ng.g^{-1}$). The relative response was plotted against the entered concentration, the curve was constructed and the linear regression was applied. The correlation and regression coefficients are described in the Table 4. The reproducibility was obtained by the measurement of the set of 6 samples, prepared at the different days, fortified at the same concentration level (2.0 $ng.g^{-1}$). The relative standard deviations for all analytes are also stated in the Table 4.

Table 4. Linearity and reproducibility

Analyte	Slope	Intercept	Regression coefficient	RSD %
progesterone	0.093	0.009	0.986	10.4
medroxyprogesterone acetate	0.121	0.035	0.999	6.4

Blank samples and samples fortified at 6 different concentration levels were prepared repeatedly (6 times) on different days from colostrum samples. From the Student's t value at the 99 % confidence level (2.5), the standard deviation of the intercept and the regression coefficients, the decision limit and the detection capabilities were established. Regression coefficient, standard deviations of the intercepts, decision limits (CCα) and detection capabilities (CCβ) obtained are stated in the Table 5.

Table 5. Results of validation study

Analyte	Slope	Intercept	Standard deviation of the intercept	CCα (ng.g^{-1})	CCβ (ng.g^{-1})
progesterone	0.085	0.026	0.0309	0.9	1.5
medroxyprogesterone acetate	0.103	0.035	0.0224	0.6	0.9

Validation of stilbens method

The linearity was observed by measuring a blank colostrum samples and samples fortified at different concentration levels (0, 2, 4, 10, 14, 20 ng.g^{-1}). The relative response was plotted against the entered concentration, the curve was constructed and the linear regression was applied. The correlation and regression coefficients are described in the Table 6. The reproducibility was obtained by the measurement of the set of 6 samples, prepared at the different days, fortified at the same concentration level (10.0 ng.g^{-1}). The relative standard deviations for all analytes are also stated in the Table 6.

Table 6. Linearity and reproducibility

Analyte	Slope	Intercept	Regression coefficient	RSD %
benzestrol	0.120	0.048	0.992	9.6
hexestrol	0.245	0.134	0.998	12.9
dienestrol	0.172	0.031	0.998	13.6
diethylstilbestrol	0.558	0.067	0.999	13.9

Blank samples and samples fortified at 6 different concentration levels were prepared repeatedly (6 times) on different days from colostrum samples. From the Student's t value at the 99 % confidence level (2.5), the standard deviation of the intercept and the regression coefficients, the decision limit and the detection capabilities were established. Regression coefficient, standard deviations of the intercepts, decision limits (CCα) and detection capabilities (CCβ) obtained are stated in the Table 7.

Table 7. Results of validation study

Analyte	Slope	Intercept	Standard deviation of the intercept	CCα (ng.g^{-1})	CCβ (ng.g^{-1})
benzestrol	0.082	0.027	0.0474	1.4	2.4
hexestrol	0.138	0.129	0.0392	0.7	1.2
dienestrol	0.080	0.009	0.0474	1.5	2.5
diethylstilbestrol	0.279	0.020	0.1002	0.9	1.5

Conclusions

The validation according to Commission Decision 2002/657/EC verified the suitability of the both method for the screening and confirmatory purposes for monitoring of gestagens and stilbens residues. The method described provides sufficient sensitivity and robustness for routine laboratory control of goat colostrum samples. The sample preparation procedure allows a high throughput of handled samples at low laboratory material cost.

References

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POSTER P30

DETERMINATION OF ESTRADIOL ESTERS DERIVATIVES IN ANIMAL BLOOD PLASMA BY GC-MS/NCI

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Abstract

In 1996, the EU prohibited the use of substances with anabolic action for food-producing animals (EU Directive 96/22/EC). In cases of illegal use of steroid hormones, these substances are usually applied to the animals in the form of esters. The reliable determination of intact steroid esters in animal tissues or body fluids is an unequivocal proof of illegal treatment of animals with EU prohibited anabolic substances. Previously our laboratory developed a sensitive method for determination of oestradiol benzoate and other steroid esters in blood plasma using LC-MS/MS, validated according to Commission Decision 2002/657/EC. This study describes a GC-MS method which has been developed for five oestradiol esters in blood plasma. The sample preparation procedure consisted of protein precipitation, phospholipids removal and cleaning on an alumina column. Oestradiol esters were derivatised with 2, 3, 4, 5, 6-pentafluorobenzoyl chloride (PFBCl) and pyridine in dichloromethane. The measurement of oestradiol esters was carried out by GC-MS/NCI with Cool On-Column injection. Methane was used as a negative chemical ionisation reagent gas. The method for determination of oestradiol esters in blood plasma has been validated according to Commission Decision 2002/657/EC. Decision limits for all analytes were observed below 0.05 ng mL^{-1}. The method is robust for bovine and porcine plasma analyses and can be applied both for screening and confirmatory determination in routine residue monitoring.

Introduction

The European Union (EU) has banned the use of hormonal substances for fattening purposes in livestock since 1988. In cases of illegal use of steroid hormones, these substances are usually administered intramuscularly and in the form of pour-on hormone cocktails in places from the neck to the tail under their ester forms (synthetically produced) in lipophilic excipients providing longer biological availability and lasting effects. Depending on the length of the side chain, steroid esters show different release times and different properties, e.g. volatility. After administration (especially by injection) they are hydrolysed or epimerised and transported with body fluids to peripheral blood (Doué *et al.*, 2014; Matraszek-Żuchowska *et al.,* 2019).

The detection of the abuse of synthetic steroid esters using urine testing of animals is practically impossible, because within a few hours of administration hormone esters are quickly hydrolysed to parental synthetic steroid hormones. The detection of intact steroid esters in bovine hair has shown to be an attractive sample matrix for prolonged detectability of residues of anabolic steroids. In the case of detection in animal hair, contamination issues need to be carefully considered in order to eliminate environmental contamination as a possible cause of false non-compliant results (Stolker *et al.*, 2009; Scarth *et al.*, 2012). Plasma is the matrix that is most commonly used for measurement of steroid hormones in clinical laboratories. Advantages of this matrix are that it reflects the concentrations of circulating steroid hormones in the body. Disadvantages are that collection is invasive, difficult and has to be carried out by a medical professional (French, 2016). The steroid esters are present in plasma in small concentrations since there is extensive enzymatic cleavage activity of the esters in blood. Historically, the detection of intact steroid esters in plasma was hampered by their low concentrations in blood relative to analytical limits of detection. However more recently, screening and confirmatory approaches based on the detection of intact steroid esters in blood plasma have been developed, e.g. Scarth *et al.* (2012); Rejtharová *et al.* (2017); Rocha *et al.* (2020) or Van Renterghem *et al.* (2020).

In order to individually quantify these compounds, they have to be separated by either GC or LC before they enter the mass spectrometer. In order to be measured by MS methods, compounds have to be ionized. Since the steroid hormones are neutral compounds, they do not ionize efficiently as they lack acidic or basic functional groups making it more challenging to measure them by LC-MS/MS (French 2016). GC-MS gives us

the opportunity to choose several ionization techniques for steroid analysis. The use of GS-MS as a detection method has spread both in the human and veterinary fields. Hansen *et al.* (2011) presented an analytical procedure, which allows for the simultaneous determination of nine native key steroid hormones expressed in the steroidogenesis, relying on chemical analysis by solid phase extraction (SPE) and clean-up followed by derivatization and gas chromatography tandem mass spectrometry (GC-MS/MS). The method is applicable for blood from several animal species with high variation in plasma lipids (e.g. rat, frog and polar bear). Van Renterghem *et al.* (2020) used chemical ionization and tandem mass spectrometry (GC-CI-MS/MS) to improve detection sensitivity compared to earlier GC-MS/MS methods, e.g. De la Torre *et al.* (1995) or Peng *et al.* (2002). The Van Renterghem research group developed a direct detection method for a broad range of testosterone and nortestosterone esters that allows detection of trace amounts (less than 0.2 ng mL^{-1}) of these compounds in an athlete's blood.

One of the main issues regarding sample preparation for GC-MS is derivatization. One of the classical approaches used for derivatization is oxime-trimethylsilylation. The most common oximation and trimethylsilyl derivatization reagents are methoxyamine (MOX) and *N, O*-bis-(trimethylsilyl) trifluoroacetamide (BSTFA). Unfortunately, multiple derivatives are formed due to syn- and anti- formation. This approach was then replaced by simultaneous derivatization of keto and hydroxyl groups using mixture reagents such as silylation reagent, catalyst and reductant *N*-methyl-*N*-alkylsilyltrifluoroacetamide (MSTFA), trimethyliodosilane (TMIS) and ammonium iodide (NH_4I), dithioerythreitol (DTE), ethanethiol, 1-propanethiol, and 2-meraptoethanol (Marques *et al.*, 2007; Fang *et al.*, 2010). Summarized derivatization reaction, enolization of the keto group occurs and then simultaneous silylation of the enol and a nucleophilic substitution by an MSTFA were observed. Commonly, the derivatization reagent is used in a thousand-fold excess over the analyte and the derivatization can be carried out at room temperature (Noppe *et al.*, 2008) or at 65°C for 5 min (Haber *et al.*, 2001; Parkinson 2014). Other derivatization reagents that can be used for steroid hormones and their esters are *O*-(2,3,4,5,6-Pentafluorobenzyl)hydroxylamine (PFBHA) and 2,3,4,5,6-Pentafluorobenzoyl chloride (PFBCl). Polyfluoro adducts of PFBHA have been extensively used to investigate carbonyl groups in aldehydes and ketones (Tsai and Chang 2003; Jakober *et al.*, 2006). Derivatization reaction using PFBHA can be performed under mild conditions and this reaction is very fast (Parkinson 2014). Chemical reaction of carbonyls with PFBHA forms their corresponding oxime isomers (*E*- and *Z*- isomers) (De Boer *et al.*, 1995) and then two GC peaks can be observed, which are characteristic of these two isomers. PFBCl derivatization reagent can be used for esterification of phenolic and alcoholic groups of steroid hormones and their derivatives.

The first objective of this work was to investigate and develop derivatization technique, using PFBCl/pyridine/dichloromethane as the derivatization reagent. The effects of reaction time, temperature and derivatization reagent ratios were systematically investigated. The stability of estradiol esters derivatives, linearity, precision and decision limits in matrix samples were also studied. Methods based on GC-MS detection have evolved significantly in sensitivity using chemical ionization and tandem mass spectrometry. This work presents a novel analytical procedure, which allows for the simultaneous determination of five estradiol esters analysis by solid phase extraction (SPE) and clean-up followed by derivatization, gas chromatography with negative chemical ionization and (tandem) mass spectrometry(GC-MS or GC-MS/MS). The method is applicable for blood from several animal species. The estradiol esters can be determined in low concentrations (tens of picograms per millilitre) with high selectivity.

Materials and methods

The reference standards of the analytes were supplied by Sigma-Aldrich (β-estradiol 17-acetate, β-estradiol 3-benzoate, β-estradiol 17-cypionate), and by Toronto Research Chemicals (β-estradiol 17-valerate and β-estradiol 17-enanthate). Internal standard β-estradiol 3-benzoate-d_3 was supplied by CDN ISOTOPES and internal standard β-estradiol 17-valerate-d_9 by Toronto Research Chemicals. The substances were individually dissolved in methanol and then diluted to low concentration working solutions. All solvents (methanol, acetonitrile, toluene, ethanol, n-hexane, dichloromethane, pyridine, tert-butylmethyl ether, isopropyl ether) were obtained from Merck and were of SupraSolv® quality. Formic acid 98 – 100 % and hydrochloric acid 30 % were purchased from Merck and they were of Suprapur® quality. *2, 3, 4, 5, 6* - Pentafluorobenzoyl chloride (PFBCl), as a derivatization reagent, was purchased from Merck. Helium 6.0, nitrogen 5.0 and methane 4.5 were supplied by Linde. Derivatization glass vials used for derivatization reactions were acquired from Agilent Technologies. Bovine and porcine blood plasma samples for method validation were procured by official veterinary inspectors from various fattening animals, which had not been treated with any steroids or steroid esters.

Cleaning procedure
The sample cleaning procedure follows our previously published method (Rejtharová *et al.*, 2017).

Derivatization
The sample was transferred to a derivatization vial with 2 x 0.5 mL terc-butylmethylether (BME). The BME was evaporated under a gentle stream of nitrogen and 20 µL of a solution of pyridine in dichloromethane (1 mg mL^{-1}) and 50 µL of a solution of PFBCl in dichloromethane (1 mg mL^{-1}) were added to the dry residue. The sample was incubated for 5 min at room temperature. The excessive reagent was then evaporated with a stream of nitrogen and the sample was dissolved in 50 µL of dehydrated toluene.

Measurement
The chromatographic analysis was performed on an Agilent Technologies (Santa Clara,CA, USA) GC 6890 chromatograph with mass detector 5973N (MSD) or on an Agilent Technologies GC chromatograph 7890A with mass detector 7000C (QQQ), both in NCI mode with on-column injection. An Agilent Technologies VF-35ms (length 30 m, I.D. 0.25 mm, film 0.1 µm) capillary column was used. The injection was performed by cool on-column method; carrier gas was He 6.0 with constant flow 1.8 mL min^{-1} and interface temperature 280°C. The temperature programme was T_{inic}. 80°C, 0 min., β_1 30°C min^{-1} to 280°C, β_2 1°C min^{-1} to 290°C, 26 min. at 290°C. Detector conditions were as follows: methane flow 40 %, temperature of ion source 150°C, temperature of quadrupoles 150°C. The m/z ion values and retention times (RT) of all esters measured in NCI-SIM mode are stated in Table 1. Dwell times for MRM transitions were set to 200 ms.

Table 1. Ions m/z (NCI −SIM) and retention times (RT).

Analyte	*Ion m/z (quantifier)*	*Internal standard m/z*	*Approximate RT*
Estradiol acetate	508.2		12.9 min
Estradiol valerate	550.4	559.4	17.5 min
Estradiol enanthate	578.5	(Estradiol valerate-d_9)	22.6 min
Estradiol cypionate	590.6		33.5 min
Estradiol benzoate	570.5	573.5 (Estradiol benzoate-d_3)	40.6 min

Results and Discussion

Cleaning procedure on alumina column
The last sample cleaning step is the elution of analytes from the alumina sorbent. In the previous washing procedure samples were applied on the column (2 mL solution of toluene/hexane (5/9, v/v)). The evaporation tube was washed with an additional 5 mL of this solution, which was also applied on the column. The column was subsequently washed with 5 mL of toluene. The analytes were eluted from the alumina column with 10 mL of toluene/ethanol (99/1, v/v) and were evaporated to dryness at 40°C.

Cleaning procedure on Supel™-Select HLB column
To improve the cleaning procedure we had tried to employ HLB columns. In this case, alumina sorbent in glass columns was replaced by the hydrophilic modified styrene polymer Supel™-Select HLB columns to clean-up the extract. Cleaning procedure is the same as in our previously published article (Rejtharová *et al.*, 2016). In terms of cleaning ability, the HLB procedure did not differ from the alumina cleaning method.

Derivatization
Development of the derivatization procedure was the main objective of this work. We wanted to develop an universal derivatization procedure for both groups of estradiol esters (3- and 17-), which can be easily used in laboratory practice. We used PFBCl as a derivatization reagent and optimized the derivatization conditions. It was necessary to optimize the derivatization temperature, reaction time, solvents, type of base and molar ratios of each reagent. Derivatization of estradiol esters via PFBCl is, when simplified, the benzoylation of phenol. This reaction is carried out in the presence of some base (usually aqueous NaOH or pyridine solution). Temperature conditions from 0°C to 60°C and reaction times from 0 min to 30 min were tested. We found that neither temperature nor reaction time affected the derivatization efficacy. The derivatization reaction proceeds immediately at room temperature after mixing of the derivatization reagent with analytes in an

appropriate solvent in the presence of a base. The solvent in which the PFBCl is dissolved has to be without any water traces (i.e. dry). The presence of water can partly hydrolyse the derivatization reagent and the derivatization reaction may not be successful. Dry pyridine works perfectly in acetylation reaction. The base is necessary to encourage an equilibrium shift towards the formation of a reaction product. The base catalyst has absorbed the acidic proton and this HCl is neutralized by the base catalyst as well and pyridinium chloride is formed. The pyridinium salt thus formed can be removed by extraction into water or isopropyl ether. However, removal of these salts leads to a reduction of reaction yield and we did not use this type of extraction at our low concentration levels.

We tested different reaction molar ratios of estradiol ester: PFBCl: pyridine varying from 1: 20: 20 to 1: 250 000: 250 000. The estradiol ester amount tested was 0.01ng. The derivatization reaction was not successful with reaction ratios from 1: 20: 20 to 1: 500: 500. Derivatization product can be observed at ratio 1: 1000: 1000 for only four esters with small peak areas, for estradiol benzoate the derivatization was unsuccessful. Reaction ratios from 1: 5000: 5000 to 1: 250 000: 250 000 show a rising response. The best reaction ratios after optimization turned out to be the following: 1: 145 000: 125 000 (estradiol ester: PFBCl: pyridine). Due to a strong covalent bond between the ester and the derivatizing agent, the resulting derivatives are highly stable.

Chromatographic conditions

In addition to the optimization of chromatographic conditions as a temperature program or carrier gas flow, we had to choose an optimal stable phase of the chromatographic column for estradiol esters separation. Finally, we used column Agilent Technologies VF-35ms (length 30 m, I.D. 0.25 mm, film 0.1 µm). Agilent J&W VF-35ms is a medium polarity column and contains stabilized arylene-modified equivalent of a 35 % phenylmethyl phase. This column is sufficiently polar for efficient separation of estradiol esters from blood plasma impurities. All retention times of analytes for this column are mentioned in Table 1.

Method validation

Validation according to Commission Decision 2002/657/EC verified the suitability of the optimized derivatization reaction for estradiol esters (ester/PFBCl/pyridine) after appropriate sample preparation for screening and confirmatory purposes for monitoring of ester residues. The linearity was observed by measuring blank blood plasma samples and samples fortified at different concentration levels (0, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5 and 1.0 ng mL^{-1}). The relative response was plotted against the concentration, the curve was constructed and the linear regression was determined. The correlation and regression coefficients obtained for five estradiol esters are described in Table 2. The results for all analytes present good linearity and low intercept values which reflect negligible measurement interferences. To determine reproducibility, a series of thirteen samples (ten for estradiol valerate) were prepared under various conditions with an added concentration of individual analytes of 0.025 ng mL^{-1}. The relative standard deviations (RSD) for all analytes at this level are also listed in Table 2.

Table 2. Linearity and reproducibility.

Analyte		Intercept	Slope	Correlation coefficient R^2	RSD %
Estradiol	acetate	-0.0025	1.8304	0.9816	19.7
	benzoate	0.0182	1.7845	0.9913	32.3
	cypionate	0.0068	0.8623	0.9826	25.7
	enanthate	0.0061	1.0154	0.9898	24.3
	valerate	0.0076	1.4850	0.9924	23.6

Stability of estradiol esters derivatives was investigated both on pure standards solutions and on matrix spiked standards. For matrix spiked standards, eight plasma samples were spiked at 0.05 ng mL^{-1} and treated according to protocol including the derivatization step. Samples were measured immediately after preparation and then stored at 4°C in a fridge. They were measured repeatedly up to ten days after derivatization. Differences in response obtained for all estradiol esters were observed within established reproducibility, no response reduction was noted during this time.

Samples fortified at eight different concentration levels (0, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5 and 1.0 ng mL^{-1}) were prepared repeatedly (six times) on different days from blood plasma samples obtained from various

animals for determination of within-laboratory reproducibility according to Commission Decision 2002/657/EC, which requires that the validation procedure shall cover all expectable scenarios during real sample analyses. Porcine and bovine blood plasma were used for the robustness examination and the applicability of this method was verified for both animal species. There was no evidence of any difference between results obtained from bovine and porcine samples. From the Student's *t* value at the 99 % confidence level (2.5), the standard deviation of the intercept obtained from all within-laboratory reproducibility measurements and the regression coefficients, the decision limit and the detection capabilities were established. The regression coefficients, standard deviation of the intercepts, decision limits (CCα) and detection capabilities (CCβ) obtained are stated in Table 3.

Table 3. Results of within-laboratory reproducibility, decision limits, detection capabilities.

Analyte		Intercept	Slope	Standard deviation of the intercept	CCα [ng mL^{-1}]	CCβ [ng mL^{-1}]
Estradiol	acetate	-0.0025	1.8304	0.01784	0.024	0.040
	benzoate	0.0182	1.7845	0.01320	0.018	0.031
	cypionate	0.0068	0.8623	0.00969	0.028	0.047
	enanthate	0.0061	1.0154	0.00822	0.020	0.033
	valerate	0.0076	1.4850	0.01346	0.023	0.038

The method described provides sufficient sensitivity and robustness for routine laboratory control of bovine and porcine blood plasma samples by GC-MS/NCI; all decision limits and detection capabilities were below 0.05 ng mL^{-1}. Such sensitivity allows this method to be used for revealing possible illegal application of estradiol esters for a reasonable period after the last treatment. The application of GC-MS in estradiol esters residue analysis confirmed this separation and measurement technique as a useful alternative to the widely used LC-MS/MS, where sensitivity for estradiol – type compounds suffers from weak ionization in LC-MS/MS ESI ion source. The derivatization with PFBCl under the optimal conditions prior to GC-MS enhances the scope of reasonable analytical methods applicable for the official determination of banned hormonally active substances (Lišková *et al.*, 2021).

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POSTER P31

MICROWAVE ASSISTED EXTRACTION FOR EFFICIENT ANALYSIS OF PROGESTAGEN ESTERS IN PERIRENAL FAT

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Abstract
Among the matrices of interest for the detection of residues of banned substances in livestock, perirenal fat is considered a reference matrix for its ability to store compounds such as progestagen esters. An analytical strategy for the detection and identification of such esters has been developed in bovine, porcine, equine, ovine and goat fat samples. While the previous analytical workflow used to rely on Pressurized Liquid Extraction System (PLE), an innovative extraction step involving microwave assisted extraction (Anton Paar MAE system) was optimised. Further, the chromatographic and spectrometric methods were developed on a Waters Xevo TQS UHPLC-(ESI+)-MS/MS system. The efficiency of the newly developed workflow allowed reducing the sample size by a factor of 4 (from 4 g to 1 g fat). Performances of the validated method enables monitoring flugestone acetate, delmadinone acetate, chlormadinone acetate, melengestrol acetate, norgestomet, altrenogest and in particular medroxyprogesterone acetate at a level below 1 µg/kg (Minimum Required Performance Limit for MPA in pig kidney fat).

Keyword 1: microwave assisted extraction, perirenal fat, progestagen esters

POSTER 32

EFFICIENCY COMPARISON OF FOUR B-GLUCURONIDASE TYPES UNDER DIFFERENT CONDITIONS

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Abstract

Deconjugation of steroid glucuronides is crucial step for reliable determination of steroid residues in urine samples. Standards of α- and β-19-Nortestosterone Glucuronides were treated under different conditions with four types of enzyme β-Glucuronidase of different origin (Helix Pomatia juice, Bovine liver, Red Abalone and Escherichia Coli). An efficiency of enzymes was evaluated based on GC-MS/MS measurement of liberated α- and β-19-Nortestosterone. Deconjugated samples were extracted with tertbutylmethylether, purified on HPLC with fraction collection and derivatized with BSTFA prior to GC-MS/MS measurement. β-19-Nortestosterone-d_3 was used as an internal standard. The deconjugation procedure was verified on bovine and porcine urine samples containing endogenous β-19-Nortestosterone metabolites.

Introduction

Steroids in animal body are partially metabolised in the liver and kidney to phase 2 metabolites i.e. glucuronic acid or sulphate conjugates. For reliable determination of steroids in urine and other biological samples, where residues may appear as conjugates, either determination of intact steroid-glucuronides is needed or complete deconjugation to free steroids is required. Analyses of intact glucuronide metabolites are partially limited by narrow accessibility of reference standards and also by availability of LC-MS instrumentation which is fundamental technic for their confirmatory determination. In majority, the conjugated metabolites are primarily deconjugated enzymatically with aryl sulphatase and glucuronidase resulting in free steroids in the extract for the purpose of residue analysis (Scarth et al., 2009). Historically, plenty of methods was published and used for determination of free steroids using both GC-MS and LC-MS technics. However, these procedures require a comprehensive deliberation of steroids from their glucuronide metabolites.

Each routine method implemented in laboratories for determination of free steroid residues in urine includes a deconjugation step by deconjugation enzyme (glucuronidase, sulphatase). Enzyme manufacturers recommend an optimal amount and conditions for successful deconjugation. Laboratories on their own, however, have very limited possibilities to verify whether their deconjugation procedure applied is sufficiently effective and robust. This can be proved either on incurred (urine) samples obtained from an animal treated with respective steroid (where phase 2 metabolites were created in an animal body) or on steroid-glucuronide standards which are subjected to deconjugation step. Moreover, the enzyme solutions may introduce additional impurities into sample which obstruct purification procedure.

Our laboratory investigated four types of enzyme β-Glucuronidase (Helix Pomatia juice, Bovine liver, Red Abalone and Escherichia Coli) and compared their efficacy under two different conditions by deconjugation of α- and β-19-Nortestosterone Glucuronides reference standards. The liberated α- and β-19-Nortestosterone were determined by validated procedure comprising extraction, extract purification, BSTFA derivatization and GC-MS/MS measurement.

Materials and Methods

The reference standards of α- and β-19-Nortestosterone Glucuronides were supplied by Steraloids Inc., the internal standard β-19-Nortestosterone-d_3 was provided by Bank of Reference standards WFSR. Substances were dissolved in methanol and diluted to low concentration working solution. We tested the following types of β-Glucuronidase enzymes: Helix Pomatia juice (HP), Merck (1.04114), Bovine liver B-1 (B1), Sigma (G 0251, aqueous solution activity 100 000 units mL^{-1}), Escherichia coli VII-A (EC), Merck (G 7646) and Red Abalone - Abalonase+ (RA), UCT (ASF-BETA-GLUC). All solvents used were obtained from Merck and they were of SupraSolv quality. BSTFA - bis(trimethylsilyl)trifluoroacetamide with 1% TMCS, as a derivatization reagent, was purchased from Sigma Aldrich (T-6381).

Deconjugation conditions
20 ng of α- and β-19-Nortestosterone Glucuronides (separately) in water sample (3 mL) were subjected to β-Glucuronidase treatment under two controlled conditions – laboratory temperature (22°C) overnight (18 hours) or 50°C for 1 hour. Sample pH was set according to enzyme manufacture recommendations. For HP it was pH 5.2, for B1 pH 5, for EC pH 7 and for RA provided buffer was used. Samples after deconjugation and internal standard addition (10 ng of β-19-Nortestosterone-d_3) were extracted and purified.

Sample preparation
3 mL of sample were extracted with 2 + 8 mL of tertbutylmethylether (BME) using Extrelut NT3 extraction column (Merck). The extract was evaporated and subjected to HPLC purification on C18 column (Zorbax Eclipse XDB-C18, 4.6 x 150 mm, 5-Micron Agilent Technologies 7995118-595), mobile phase water/methanol, 1.3 $mLmin^{-1}$ with gradient from 50% methanol to 100% methanol in 12 min. Respective fraction (9.8 – 11.2 min.) was collected by fraction collector and evaporated. Dry sample was transferred to a derivatisation vial with 2 x 0.5 mL of BME. BME was evaporated with a gentle stream of nitrogen and 40 µL BSTFA were added to the dry residue. The sample was incubated for 30 min. at 75°C and afterwards evaporated with a stream of nitrogen. The sample was dissolved in 50 µL of dehydrated toluene.

GC-MS/MS measurement
The chromatographic analysis was performed on an Agilent Technologies GC chromatograph 7890A with mass detector 7000C (QQQ) with on-column injector. An Agilent Technologies HP-1MS (length 30 m, I.D. 0.25 mm, film 0.25 µm) capillary column was used. The injection was performed by cool-on-column method; carrier gas was He 6.0 with constant flow 1.2 $mL.min^{-1}$ and interface temperature 280°C. The temperature programme was ramped from 80°C to 280°C. Detector conditions were as follows: temperature of ion source 230°C, temperature of quadrupoles 150°C, CE 13V, dwell 100ms. The MRM transitions were 346-215 and 346-256 for 19-Nortestosterone and 349-215 for internal standard.

Urine samples
After evaluation of α- and β-19-Nortestosterone Glucuronide standard results, two urine samples (bovine and porcine) with endogenous contain of β-19-Nortestosterone metabolites were investigated. Six aliquots of each sample were treated with HP, EC and RA enzymes under the same conditions as standards, i.e. pH set, laboratory temperature (22°C) overnight (18 hours) or 50°C for 1 hour. Urine samples were subjected to the same sample preparation procedure and measurement as described above.

Results and Discussion
Relative responses of liberated α- and β-19-Nortestosterone from α- and β-19-Nortestosterone Glucuronide standards are stated in Tables 1 and 2 and graphically represented in Figures 1 and 2. Relative responses were obtained as a rate of α- or β-19-Nortestosterone peak area and internal standard peak area.

Table 1. Relative responses of α-19-Nortestosterone.

Glucuronidase	22°C, overnight	50°C, 1 hour
HP	0.792	1.041
B1	0.209	0.018
EC	1.439	1.611
RA	1.321	1.470

Figure 1. Relative responses of α-19-Nortestosterone.

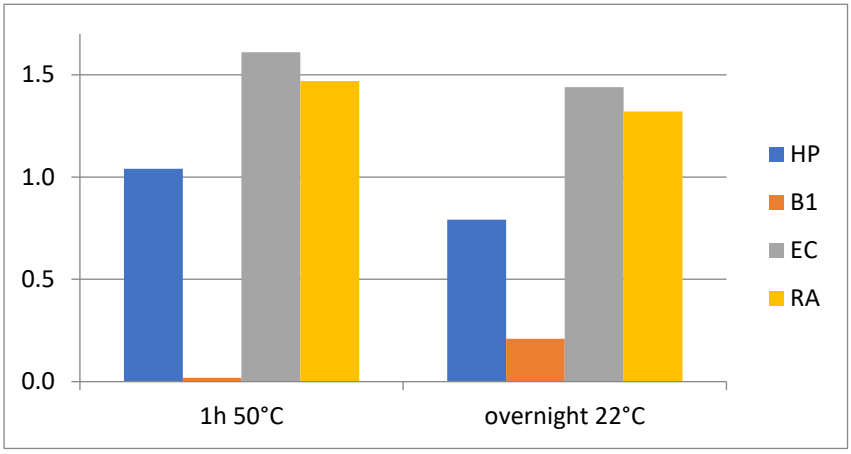

Table 2. Relative responses of β-19-Nortestosterone.

Glucuronidase	22°C, overnight	50°C, 1 hour
HP	1.045	1.135
B1	0.373	0.015
EC	1.051	1.207
RA	1.136	1.162

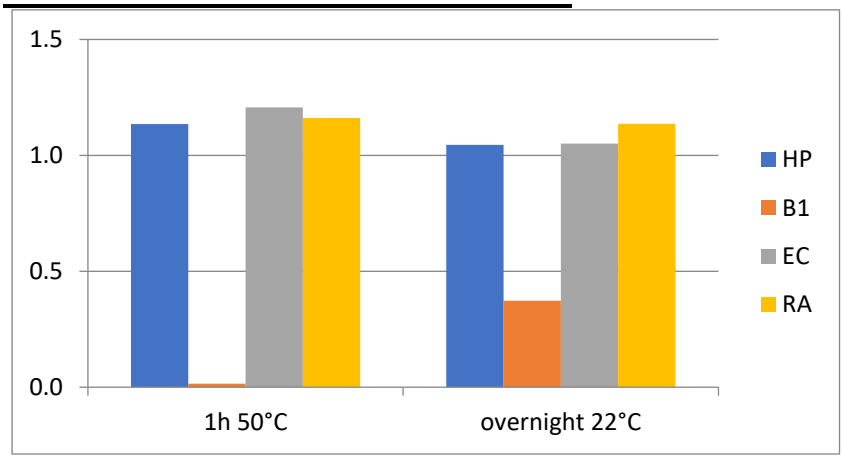

Figure 2. Relative responses of β-19-Nortestosterone.

The presented results show surprisingly poor efficiency of β-Glucuronidase B1. Three remaining enzymes demonstrated relatively satisfactory activity, both for α- and β-19-Nortestosterone Glucuronides. Slightly weaker efficiency was observed only for HP on α-19-Nortestosterone Glucuronides. No significant differences were observed between two tested conditions, i.e. laboratory temperature (22°C) overnight (18 hours) and 50°C for 1 hour. Similar results were obtained on bovine and porcine urine samples, too. β-19-Nortestosterone concentrations measured in urine aliquots did not differ more than 20% which is about uncertainty of the method.

It could be concluded that three β-Glucuronidase enzymes tested, HP, EC and RA, can be successfully used for an official determination of steroids in urine samples. Despite the fact, that no significant differences were measured between these three enzymes in terms of liberated β-19-Nortestosterone concentrations, it was realised by empiric observation that HP enzyme introduce significant visual impurities into samples which are not fully removed by purification procedure. When EC and RA enzymes were used the extracts look much cleaner.

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POSTER P33

FULLY AUTOMATED SCREENING OF VETERINARY DRUG RESIDUES AND MYCOTOXINS SIMULTANEOUSLY IN MILK UTILISING THE EVIDENCE MULTISTAT BIOCHIP ARRAY BENCHTOP ANALYSER

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Abstract
Veterinary drug residues (VDR) may exist in raw milk due to their misuse or violation of withdrawal periods while mycotoxins in milk result from contaminated feed. Consequently, these contaminants can persist in animal-derived foods and present potential food safety risks. For consumer safety, the levels of VDR and mycotoxins in milk are regulated. The Infiniplex for Milk (IPM) assay, utilising biochip array technology on the Evidence MultiSTAT analyser, screens for mycotoxins and multiple VDR, including antibiotics, anti-inflammatories, corticosteroids, growth promoters and anti-parasitics simultaneously.
Utilising as little as 250 µl of raw bovine milk, IPM is a rapid, competitive, qualitative immunoassay array, covering 117 contaminants across 44 individual reportable assays. Evidence MultiSTAT employs a self-contained cartridge comprising all the necessary components for the IPM immunoassay, providing results in under 30 mins. Depending on the level of VDR/mycotoxin present in the sample being above or below the Decision Level, results are automatically reported as ''Positive'' or ''not detected''.
The use of the fully automated benchtop Evidence MultiSTAT allows the rapid screening of VDR and mycotoxins from a sample of raw bovine milk employing IPM biochip array. The application of IPM increases the screening capacity on-site ensuring compliance with maximum residue limits.

Keywords: MultiSTAT**,** mycotoxins, Veterinary drug residues

POSTER P34

HIGH RESOLUTION ACCURATE MASS (HRAM) SCREENING AND QUANTITATION OF VETERINARY DRUGS

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Abstract
The use of veterinary drugs can have adverse effects on animals, the environment, and human health. As a result, the determination and efficient analysis of veterinary drugs is an important part of routine food quality control. The European Union (EU) have developed specific regulations to address these growing concerns. The requirements of low limits of quantification on different chemical classes and properties, along with a wide variety of matrices of veterinary drugs create analytical challenges. A comprehensive and rigorous monitoring program for residues in animal feed and tissue helps ensure the well-being of the animals and safe consumption. Routine target analysis is typically carried out by using liquid chromatography mass spectrometry (LC-MS/MS) and a rapid sample preparation procedure like QuEChERS. A target approach is limited to the target list, and to the analytical properties of the technique. When applying a full scan approach in High Resolution Accurate Mass mode there is a benefit to detect all peaks in a sample extract rather than a limited target list.In this presentation a screening solution will be shown on a large number of veterinary drugs and option to confirm and quantitate to the low detection limits that are required by governing authorities.

Keywords: High Resolution, MS, Veterinary Drugs

POSTER P35

VERIFICATION OF DETECTION CAPABILITY OF DELVOTEST® T AND DELVOTEST® SP-NT WITH APPLICATION OF DELVOTEST® ACCELERATOR SMART

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Abstract
On request of DSM Food Specialties, Qlip has conducted a verification study on the detection capabilities of the Delvotest® T and Delvotest® SP-NT in a microtiter plate format using Delvotest® Accelerator Smart (DAS) equipment. DAS is a fully automated incubation and microtiter plate reading device. It automatically determines the control time and generates test result readings accordingly. The study focused on verifying indicated detection limits for 11 antibiotics spiked in two different herd bulk cow milks.

Introduction
Royal DSM, a global science-based company active in Nutrition, Health and Sustainable Living, recently launched the Delvotest® Accelerator Smart, a fully automated system designed to be used in combination with DSM's internationally validated Delvotest® plates to optimize antibiotic residue detection in milk. Delvotest® Accelerator Smart is intended as a tool to help milk testing laboratories and dairies reduce costs, bolster accuracy, and achieve faster, more reliable results, while also preventing milk loss and ensuring complete traceability.

Delvotest® T and Delvotest® SP-NT are microbial inhibition assays specifically designed to detect inhibiting concentrations of antibiotic residues in raw milk. Their sensitivity towards certain antibiotics in raw milk was determined internally at DSM Food Specialties. The sensitivities and detection capabilities of both tests using the Delvotest® Accelerator Smart were carefully established. The inhibiting concentrations of several different antibiotics are mentioned in the technical documentation of Delvotest® T and Delvotest® SP-NT tests. On request of DSM Food Specialties, Qlip has conducted a verification study on the detection capabilities of the Delvotest® T and Delvotest® SP-NT of 11 different antibiotics using Delvotest® Accelerator Smart equipment.

POSTER P36

DEVELOPMENT AND VALIDATION OF AN LC-MS/MS METHOD FOR THE DETERMINATION OF TOTAL FLORFENICOL RESIDUES AS FLORFENICOL AMINE IN KIDNEY

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Abstract

Florfenicol is a broad-spectrum antibiotic for which the EU has established MRL values to ensure food safety. For these MRLs florfenicol residues are defined as the sum of florfenicol and its metabolites measured as florfenicol amine (2010/37/EU). In treated animals florfenicol is metabolised to florfenicol amine via several other metabolites. Both the parent compound and its metabolites can be present as residues in different tissues. Therefore, an analytical method using acidic hydrolysis to convert florfenicol and metabolites to florfenicol amine was developed to allow for the determination of total florfenicol as florfenicol amine in accordance with MRL requirements. Kidney samples were hydrolysed using hydrochloric acid to yield free florfenicol amine. The hydrolysed samples were purified using reversed phase solid phase extraction. After sample preparation, the extracts were diluted with water and analysed using LC-MS/MS. For quantification matrix fortified samples were used that were spiked with florfenicol amine. The completeness of the hydrolysis was checked with each analysis series based on a blank sample which was pre-spiked with florfenicol. The method is fully validated in accordance with 2002/657/EC at the MRL concentrations for porcine and bovine kidney.

Introduction

Florfenicol is a broad-spectrum antibiotic for which a Maximum Residue Limit (MRL) is defined in Commission Regulation (EU) No. 37/2010. According to this MRL definition, the marker residue of florfenicol is defined as the "s*um of florfenicol and its metabolites measured as florfenicol amine*". In treated animals florfenicol (FF) is metabolised to florfenicol amine (FFA) via several other metabolites. This metabolic pathway is shown in figure 1. Both the parent compound and its metabolites can be present as residues in different tissues. Therefore, an analytical method that is used for MRL-enforcement of florfenicol should use a hydrolysis step to convert florfenicol and its metabolites to florfenicol amine during sample preparation. The aim of this study was to develop and validate a method for the determination and quantification of total florfenicol residues as free FFA in porcine and bovine kidney at the MRL concentrations as established in Regulation 37/2010. The MRL for porcine kidney is 500 µg kg^{-1} and for bovine kidney it is 300 µg kg^{-1}.

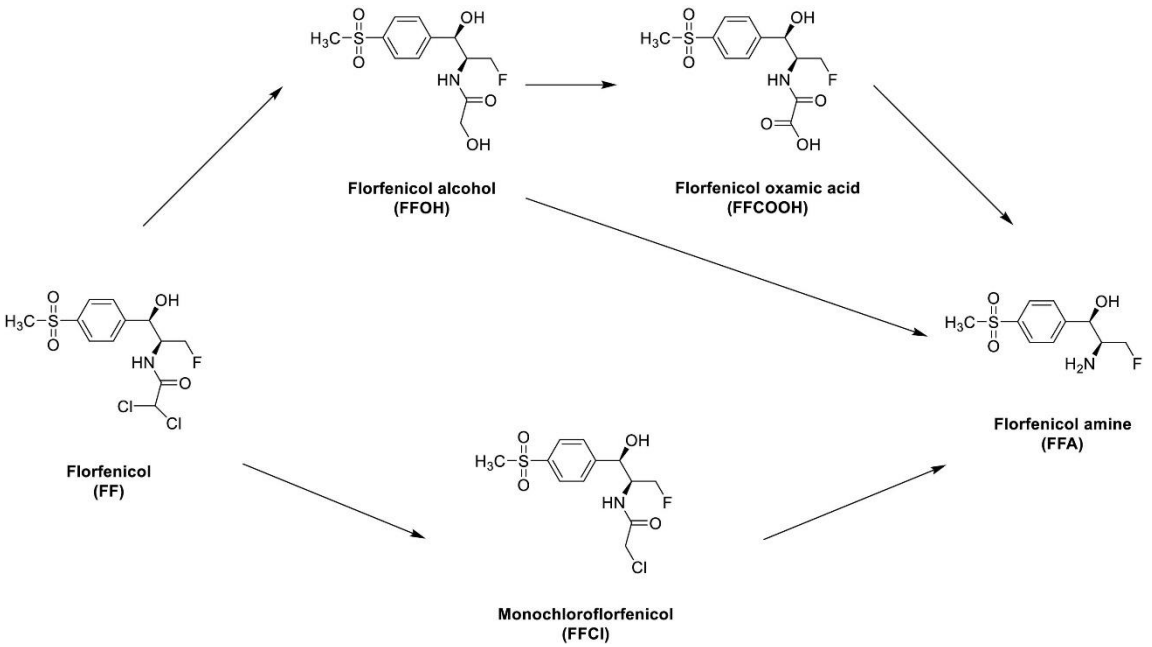

Figure 1. Metabolic pathway of florfenicol (FF). FF is metabolised to FFA via several other metabolites (Saito-Shida et al. 2019).

Materials and Methods

Chemicals, standards and solutions

Milli-Q water was prepared using a Milli-Q system at a resistance of at least 18.2 MΩ cm (Millipore, Billerica, MA, USA). Ultra LC-MS grade methanol and acetonitrile were obtained from Actu-All Chemicals (Oss, The Netherlands). Sodium hydroxide pellets were obtained from Merck (Darmstadt, Germany). Formic acid, hydrochloric acid (37%) and ammonia solution (25%) were obtained from VWR International (Radnor, PA, USA). The reference standards of florfenicol and florfenicol amine were obtained from Sigma Aldrich (St. Louis, MO, USA). FFA-d_3 was obtained from Toronto Researsch Chemicals (Toronto, ON, Canada). Strata-X reversed phase SPE cartridges (200 mg; 6 mL) were from Phenomenex (Torrance, CA, USA).

Extraction procedure and clean-up

2.0 ± 0.05 g of homogenised kidney sample was weighed into a 50 mL polypropylene tube and 50 µL of internal standard solution (FFA-d_3 20 mg L^{-1}) was added. Subsequently, 5 mL 6M hydrochloric acid solution was added. After manual shaking of the tubes the samples were hydrolysed for 2 hours at 90 °C. Then, samples were removed from the water bath and allowed to cool down to ambient temperature. 5 mL sodium hydroxide solution 30% (w/v) was added to the samples to adjust the pH to ≥12.5. Next, 10 mL of Milli-Q water was added after which samples were shaken head-over-head for 5 minutes and centrifuged for 15 minutes at 3600 g.

The Strata-X cartridges were conditioned by first adding 5 mL methanol followed by 5 mL Milli-Q water. Then, 5 mL of the sample extract was added to the cartridge. The cartridge was washed by adding 6 mL basic methanol/water (15:85; v/v). After drying the cartridges the samples were eluted with 3 mL methanol/water (80:20; v/v) from the cartridges into 12 mL polypropylene tubes. 100 µL of the eluted extract was diluted with 900 µL water before LC-MS/MS analysis.

Instrumentation

After sample preparation, the UPLC-MS/MS analysis was carried out on an Acquity UPLC (Waters, Milford, MA, USA). An Acquity UPLC BEH C18 analytical column (Waters, Milford, MA, USA) of 100 × 2.1 mm with a particle size of 1.7 µm, was placed in a column oven at 30°C. Into the LC column, 10 µL of the sample extract was injected. The substances were chromatographically separated by gradient elution using a flow rate of 0.4 mL min−1. The gradient started at 100% mobile phase A during the first minute after which it was linearly increased to 25% mobile phase B in 3 minutes. The percentage of mobile phase B was then increased to 70% in 2 minutes, followed by an increase to 100% mobile phase B in 0.1 minute. The next 1.3 minutes were isocratic, followed by an requilibration time of 1.5 minutes at 100% A. After the chromatographic separation, the

substances were introduced directly into a Q-Trap 6500+ or Q-Trap 6500 mass spectrometer (Sciex, Framingham, MA, USA). The mass spectrometer was operated in positive electrospray ionisation mode. The operating parameters were set for curtain gas (N_2) at 35, nebulising gas (N_2) at 60, heater gas (N_2) at 60 psi , source temperature at 500°C and ion spray voltage at 5500 V. The precursor ions were fragmented to product ions using collision induced dissociation with nitrogen gas (N_2). The scheduled Multiple Reaction Monitoring (MRM) transitions are presented in Table 1. The response factors were calculated by dividing the area of the most abundant product ion of the compound by the area of the internal standard.

Table 1. Mass spectrometry conditions for FF and FFA. Quantifier ions are underlined.

Q1 m/z precursor ion	Q3 m/z product ion	Time (ms)	ID	DP (V)	EP (V)	CE (V)	CXP (V)
248.0	130.1	100	FFA_130.1	71	10	39	10
248.0	91.0	100	FFA_91	71	10	65	22
251.0	132.0	100	FFA-d3_132	56	10	31	20
358.0	240.9	50	FF_240.9	101	10	23	16
358.0	130.1	50	FF_130.1	101	10	67	10

Validation procedure
The developed method was validated in accordance with 2002/657/EC at the MRL concentrations for porcine and bovine kidney. The following performance characteristics were determined: trueness, precision (repeatability and within-laboratory reproducibility), selectivity/specificity, CC_α, CC_β, robustness, stability and linearity. A three-day validation was performed for porcine kidney with concentration levels of 250, 500 and 750 µg kg^{-1}. An additional one-day validation was performed for bovine kidney with concentration levels of 150, 300 and 450 µg kg^{-1}.

Results and Discussion

Extraction and hydrolysis
For the extraction and hydrolysis of FF and its metabolites to FFA it is necessary to use an acidic hydrolysis in the extraction procedure. In literature the hydrolysis is often described using high concentrations of HCl at high temperatures during several hours. Because of safety reasons we did an experiment to determine if it was also possible to use lower acid concentrations. An experiment was performed with different HCl concentrations (0.2M, 1M, 2M and 6M), temperatures (37°C, 60°C and 90°C) and different incubation times (1, 2, 4 and 16 hours). The results of this experiment demonstrated that the hydrolysis was complete when a HCl concentration of 6M at a temperature of 90°C and incubation time of 2 hours was used. Unfortunately, using a lower acid concentration or lower temperature did not result in complete hydrolysis.

Sample clean-up
The acidic hydrolysis of kidney samples results in a dark brown dirty emulsion. For sample clean-up solid phase extraction (SPE) was used. During method development different SPE approaches were evaluated. An experiment was performed to test reversed phase SPE at acidic, neutral and basic pH sample conditions. Additionally, a strong mixed-mode cation exchange SPE was tested. The results of this experiment showed that using reversed phase SPE in combination with basic pH sample conditions yielded the best results. To make sure that FFA was retained on the reversed phase SPE columns the samples were adjusted to basic pH ≥12.5. After diluting the samples with 10 mL Milli-Q water, 5 mL of the sample extract was added to the SPE cartridges for sample clean-up.

Quantification
For quantification matrix fortified samples were used that were fortified with florfenicol amine. The completeness of the hydrolysis was checked with each analysis series based on a blank sample which was fortified with florfenicol.

Validation
To determine selectivity/specificity blank porcine (n=21) and bovine (n=7) kidney samples were analysed. All samples were free of interference signals. The linearity of the method was evaluated based on matrix fortified

calibration curves. The concentration range was 125-2500 µg kg^{-1} for porcine kidney and 75-1500 µg kg^{-1} for bovine kidney. Regression coefficients of R^2 >0.99 were obtained.

The validation results for trueness, precision (repeatability and within-laboratory reproducibility), CC_α and CC_β are shown in table 2. The developed method provided good performance characteristics in accordance with EU criteria (2002/657/EC).

Table 2. Trueness, repeatability, within-laboratory reproducibility, CC_α and CC_β for FFA in porcine and bovine kidney as determined during method validation.

Species	Concentration (µg kg^{-1})	Trueness (%)	Repeatability (CV%)	Within-laboratory reproducibility (CV%)	CC_α (µg kg^{-1})	CC_β (µg kg^{-1})
Porcine (n=21)	250	98	2.4	4.1		
	500	100	3.3	3.9	532	563
	750	102	2.5	2.7		
Bovine (n=7)	150	103	1.3	2.1		
	300	102	0.9	1.4	307	314
	450	102	0.7	1.2		

Conclusion

In this study an LC-MS/MS method for the determination and quantification of free florfenicol as florfenicol amine was developed. The method uses acidic hydrolysis to convert florfenicol and its metabolites to florfenicol amine. The hydrolysed samples were purified using reversed phase solid phase extraction. The method was fully validated in accordance with 2002/657/EC at the MRL concentrations for porcine and bovine kidney.

Acknowledgements

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POSTER P37

A STRATEGY TO DETERMINE THE FATE OF ACTIVE CHEMICAL COMPOUNDS IN SOIL

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Abstract

This work was described in more detail in Berendsen 2021. Data on the fate of chemical substances in the environment after e.g. manure application is mandatory input for risk assessment in perspective of a more circular biobased economy. Such fate studies include a persistence study to determine a half-life value and a mobility study. It is recognized that not only the native substance should be considered, but that also degradation products should be included that might exert a similar effect as the native substance. We report a tiered fate study strategy that starts with a persistence study. For non-persistent substances a study is performed to determine if degradation products have a similar effect as the native compound. If so, a procedure using high resolution mass spectrometry is suggested to identify the potentially active degradation products. Based on the outcomes, substances are divided into three categories: (I) persistent, (II) degradable to inactive products or (III) degradable to active products. Even though the priority is with category I and III, for all substances and possible degradation products a mobility study is proposed. The fate strategy is successfully applied to ten antimicrobially active substances originating from the tetracyclines, sulfonamides, diaminopyrimidines, fluoroquinolones, macrolides and lincosamides. The fluoroquinolones, tetracyclines and trimethoprim were relatively persistent. The sulfonamides, macrolides and lincomycin (the latter also depending on soil type) degraded relatively quickly. Tylosin A proved to degrade to antimicrobially active degradation products which were tentatively identified as tylosin C, tylosin A acid, tylosin B acid and tylosin C acid.

Introduction

This work was described in more detail in Berendsen 2021. Chemical contaminants end up in agricultural soils after manure application as a result of disease treatment in animal husbandry (e.g. veterinary drugs), crop production (e.g. biocides) or through irrigation using contaminated surface water (e.g. biotoxins). Manure is known to be one of the major pathways through which antibiotic residues are introduced into the environment (Halling-Sørensen 1998;Chee-Sanford 2009). When further implementing the concepts of a circular biobased economy, other waste streams might also contribute to soil exposure. As the number of chemicals that is (unwillingly) applied to agricultural soil is high, so is the number of organisms that are potentially affected. It is, therefore, impossible to carry out an extensive risk assessment for all chemicals and organisms in all relevant reservoirs. Consequently, it is important to understand the fate of these chemicals in a circular system and use this knowledge to predict the fate of chemicals by translocation modelling. As a result risk assessment studies can be prioritized for the most hazardous chemicals in the reservoirs they occur in most.

The fate of chemical substances in soil depends on their persistence and their mobility. The persistence relates to the kinetic degradation of the native contaminant and the mobility determines the transport of the chemical to other reservoirs. Immobile contaminants are retained in the soil and, if persistent, can accumulate there. Mobile contaminants leach to ground water or run off to surface water (mobile) and are potentially available for plant uptake.

The persistence of a chemical substance depends on the physico-chemical properties of the substance itself and on the soil composition and its biotic and abiotic properties, including the fraction of organic matter, pH and the active microbiome. Many studies focus on the persistence of the native substance. However, it is recognized that degradation products can still exert a negative impact on the environment. Therefore, degradation studies followed by a studies to annotate potential degradation products should be included in strategies to assess the fate of chemicals in the agricultural environment.

The mobility is related to the soil-water partitioning coefficient (K_d), which expresses the potential of a chemical contaminant to be adsorbed by soil. Since adsorption occurs predominantly by partition into the soil organic matter, it is more useful to normalize K_d to the organic carbon content of the soil and express the distribution coefficient as a organic carbon-water partition coefficient (KOC) (Wegst-Uhrich 2014). Additionally, estimation of K_{OC} by K_{OW} (octanol water partition coefficient) leads to significant underestimation of the KOC. Mechanisms other than hydrophobic partitioning, e.g. ionic interactions, complex formation and hydrogen bonding are not accurately accounted for using this approach which results in underestimation (Tolls 2001).

We developed a tiered fate study strategy including the determination of (1) persistence, (2) active degradation products and (3) mobility in soil. The fate strategy was applied to antimicrobial active compounds that are most frequently detected in animal manure.

Materials and Methods

Soil samples

The fate of ten model antibiotics in two different soil types was studied. One soil type was a sandy, the other was a clay soil. These were selected due to being the two major soil types used for agricultural practices. in The Netherlands. The soil type was pH 5.5, with soil organic matter (SOM) of 6.4 % and the 16S RNA count was $9.3*10^9$. The clay soil had a pH of 6.8, a SOM of 5.9 % and a less abundant microbiome: S9 RNA of $1.8*10^9$.

Fate study strategy

An overview of the developed tiered fate study strategy is presented in figure 1. The starting point is a laboratory scale degradation study (1). Only if significant degradation of the native compound is observed, a study into the potential formation of active degradation products is carried out (2a). If this test indicates the presence of active degradation products, these are identified (2b). On the basis of these tests, three categories are distinguished: in soil the chemical substance is (I) persistent, (II) degrades to inactive products or (III) degrades to active products. Substances belonging to category I and III are subjected to a mobility study and the final fate is used as input for the translocation model (outside the scope of this paper).

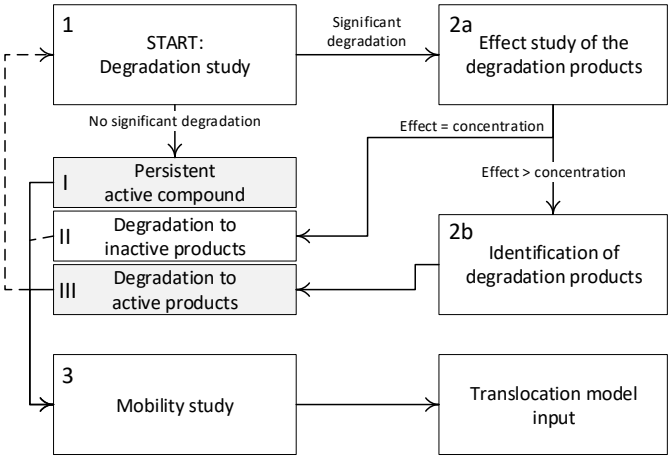

Figure 1. Overview of the tiered fate study strategy consisting of a degradation study, study for active degradation products and a mobility study.

Details about the degradation study, activity analysis of degradation products, identification of potential degradation products and the mobility study can be found in Berendsen 2021.

Results and Discussion

Degradation study

In the degradation experiment, the antibiotic amended soils were placed at room temperature for a maximum of 37 days. The remaining fraction of the antibiotics during the long term experiment is graphically presented in figure 2 for both sand and clay. Note that for enrofloxacin and tilmicosin in clay no data are presented as the

analysis yielded unreliable results due to high variability. The calculated DT50 and DT90 are presented in table 1.

From the persistence study it is concluded that the sulfonamides, lincomycin and tylosin A are very non-persistent with DT50 values of below 5 days in at least one of the soil types. These are prioritized to be subjected to tier 2, a study for potential antimicrobially active degradation products. Tier 2 is also relevant for the tetracyclines and trimethoprim. The other antibiotics are more persistent and additional effect studies have lower priority: in this study these are considered as category I compounds, (relatively) persistent, and are directly subjected to tier 3, the mobility study.

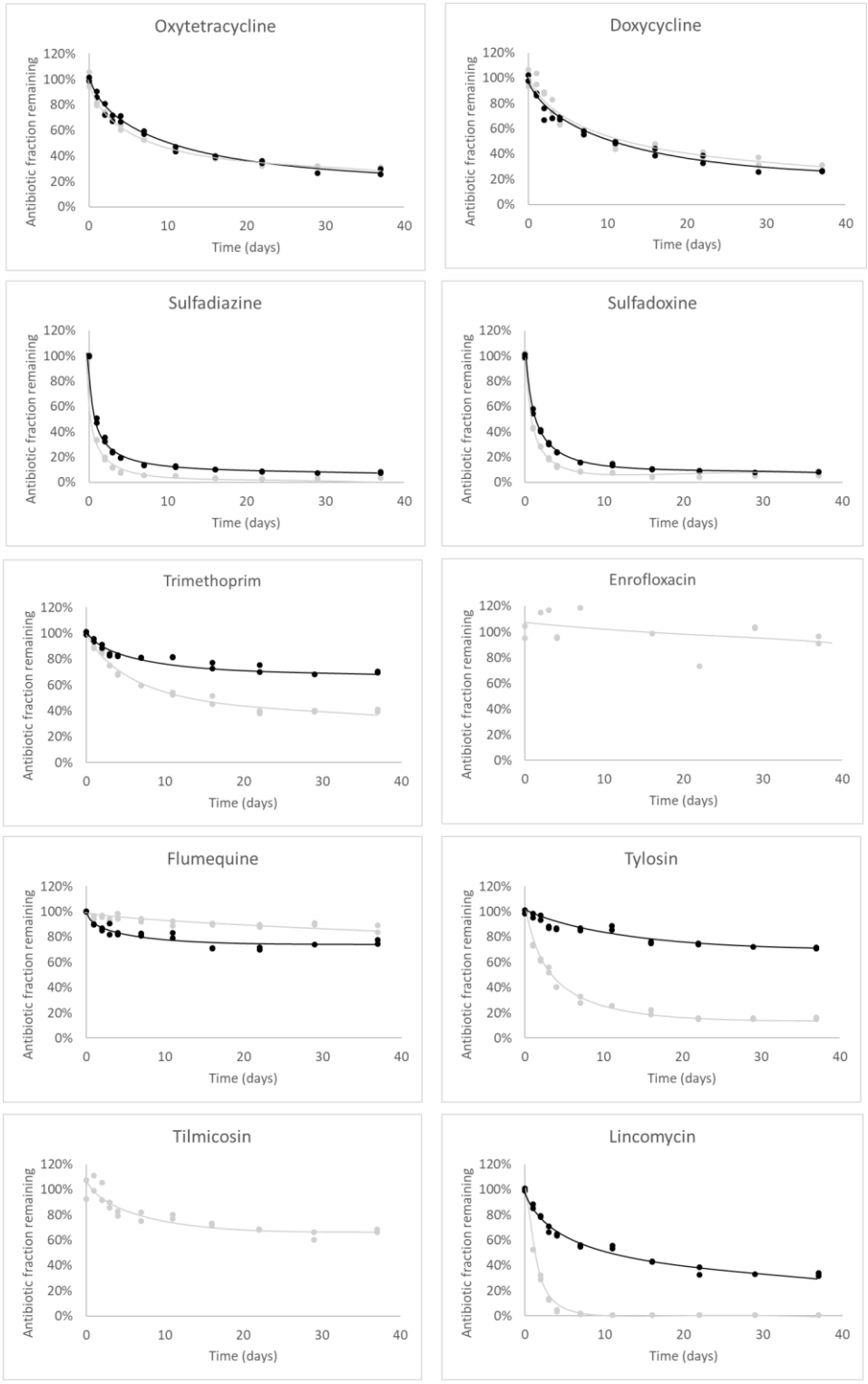

Figure 2. Degradation of the antibiotics in (grey) sandy soil and (black) clay soil in the long term degradation study. For enrofloxacin and tilmicosin in clay, no data was obtained.

Table 1. Determined DT50 and log K_{OC} for the antibiotics in sand and clay.

Antibiotic	Soil type	DT50 (days)	DT90 (days)	Log K_{OC} (L kg^{-1})
Oxytetracycline	Sand	8	110	≥ 3.0*
	Clay	9	85	3.3
Doxycycline	Sand	9	100	3.5
	Clay	11	66	3.0
Sulfadiazine	Sand	0.6	3	0.5
	Clay	1.0	20	0.7
Sulfadoxine	Sand	0.9	7	0.5
	Clay	1.4	23	0.5
Trimethoprim	Sand	12	270	≥ 4.2*
	Clay	75	250	≥ 4.3*
Enrofloxacin	Sand	137	460	≥ 2.9*
	Clay	NR	NR	≥ 3.3*
Flumequine	Sand	226	750	≥ 4.2*
	Clay	97	320	≥ 4.3*
Tylosin	Sand	3	55	≥ 3.2*
	Clay	73	240	2.0
Tilmicosin	Sand	108	620	≥ 3.2*
	Clay	NR	NR	≥ 3.4*
Lincomycin	Sand	1.1	4	2.1
	Clay	11	93	1.6

Effect study of the degradation products

Sulfadiazine, lincomycin and tylosin A were subjected to a study to determine if during degradation, products are formed that still have antimicrobial properties. In case of sulfadiazine and lincomycin, the inhibition zone of the incubated soil sample was in agreement with the expected inhibition zone at the concentration of the native antibiotic still present in these extracts. Both compounds were classified as category II (Figure 1): substances that degrade to inactive products. For tylosin the extract of the incubated soil sample showed an inhibition zone equal to a tylosin A concentration of approximately 0.95 µg mL^{-1} whereas the actual concentration of tylosin A was 0.50 µg mL^{-1}. From this it is concluded that the extract exerts approximately 60% more antimicrobial activity than expected if only the native compound would be antimicrobially active. It is concluded that a significant antimicrobial effect is caused by degradation products. This requires that for tylosin tier 2b is carried out aiming for identification of the antimicrobial active degradation products.

Identification of antimicrobially active degradation products

By applying different high-resolution mass spectrometry approaches like suspect screening and fragment ion flagging, several degradation products of tylosin were tentitatively identified. These are presented in table 2.

Table 2. Tylosin A and degradation products thereof, including molecular formula and abundace in the soil sample extract at t=0 and t=7.

Substance	Molecular formula	Abundance t=0	Abundance t=7	Reference
Tylosin A	***$C_{46}H_{77}NO_{17}$***	***$4.1*10^6$***	***$1.4*10^6$***	
Tylosin C (macrosin)	$C_{45}H_{75}NO_{17}$	$3.8*10^4$	$6.0*10^5$	(Hu 2007)
Tylosin A acid	$C_{46}H_{77}NO_{18}$	$6.1*10^4$	$8.1*10^5$	(Sassman 2007)
Tylosin B acid	$C_{39}H_{65}NO_{15}$	-	$9.5*10^4$	(Sassman 2007)
Tylosin C acid	$C_{45}H_{75}NO_{18}$	$3.9*10^3$	$5.3*10^5$	

Mobility study

The mobility of the antibiotics was determined by a column leaching method in which 5 g of antibiotic amended soil was flushed through with 10 mL water. This miniaturized set-up proved to be quick and reproducible, allowing the cost-efficient study of many different substances in many different soil samples. The determined KOC values of the antibiotics is presented in table 1. The cases in which the analytical limit of detection was the limiting factor in the determination of Kd, this is indicated. Determined KOC values were compared with previously reported values. For most antibiotics, the KOC determined in the current study is in agreement with values previously reported (Rabølle 2000;Thiele-Bruhn 2003;Boxall 2006;Park 2008;Sukul 2008;Cycoń 2019;University of Hertfordshire 2019). In perspective of the fate analysis strategy, it is concluded that the sulfonamides are the most mobile antibiotics tested and it is expected that, if they are introduced into the environment, they will leach to groundwater or run off to surface water. The tetracyclines, trimethoprim and flumequine demonstrated to be immobile: they will hardly be translocated after introduction into the soil.

Input translocation study

By applying the fate strategy reported, data is obtained to be used as input for translocation models. Based on these model it can be determined to what extend agricultural soils, ground water or surrounding surface waters are exposed to antibiotics. Based on the outcomes of the fate study the sulfonamides degrade quickly in soil. However they are quickly translocated to other reservoirs (e.g. surface water). Trimethoprim, enrofloxacin, flumequin, tilmicosin and, in clay also tylosin, are very persistent: for the quinolones and tilmicosin, the estimated DT90 is over one year in sand. Especially trimethoprim, the quinolones and tilmicosin have a relatively low mobility; these will remain in agricultural soils for a long time after introduction and have a high risk of contaminating agricultural soils. Tylosin in clay is slightly more mobile and is therefore expected to be partly translocated to the aqueous environment. Based on these findings specific substances (and their degradation products) can be prioritised for risk assessment in specified reservoirs.

Conclusion

The reported fate study strategy consists of several tests in a tiered approach and includes a degradation and a mobility study. The risk of the formation of active degradation products during soil incubation should not be overlooked as was demonstrated by the results for tylosin. Therefore, an assay to determine if produced degradation products show biological activity is mandatory. The presented approach was successfully applied to ten model antibiotics of which, for some, no fate data has been reported previously. The data can be used as input for translocation models and together with input on the antibiotic usage data, excretion models, persistence data in manure (Berendsen 2018) and manure distribution data, the spatial distribution of antibiotics over agricultural soils can be predicted. Additionally, the applied strategy can easily be applied to other substances. In that case an appropriate effect assay has to be applied in tier 2a.

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POSTER P38

DOXYCYCLINE ADSORPTION AND TOXICITY IN EARTHWORMS AFTER MANURE APPLICATION TO SOIL

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Abstract
In modern society, we unintentionally release pharmaceutical active substances into the environment. For instance, with every application of animal manure the soil is potentially contaminated with hormones, antibiotics and antiparasitics used in conventional farming practices. The number of potentially harmful substances is large and therefore prioritization of risk assessment is urgent. We investigated effects of doxycycline, the most frequently used antibiotic in animal production in The Netherlands, on earthworms, as model soil species, after soil application. Doxycycline was added to soil at environmentally relevant to high concentrations (5 µg/kg – 500 mg/kg). Effects of doxycycline on earthworm growth and mortality were quantified. The uptake of doxycycline in earthworms was measured as function of the degradation of doxycycline in soil over 28 days. The calculated environmental halftime of doxycycline was approximately 20 days. The doxycycline concentration in earthworms initially increased to 5 – 10% of the soil concentration and then followed similar decreasing trends as the soil concentration. We conclude that doxycycline was taken up by the earthworms fast upon initial application, but also rapidly excreted. No effects on the earthworm growth nor mortality were observed.

Introduction
Chemical contaminants end up in agricultural soils after manure application as a result of disease treatment in animal husbandry (e.g. veterinary drugs), crop production (e.g. biocides) or through irrigation using contaminated surface water (e.g. biotoxins). When further implementing the concepts of a circular biobased economy, other waste streams might also contribute to soil exposure. In a sustainable food production system, the use of persistent chemicals is unwanted as they are a hazard for soil health, biodiversity, environmental sustainability and food safety, especially in a circular system. For traditional chemicals and chemicals that are unintendedly introduced onto agricultural soils, the ecotoxicological effects are largely unknown.

To understand the ecotoxicological effects of chemical residues, we aim to develop an *in silico* tool to assess the potential accumulation and effects of chemicals for bioindicator species, in our case earthworm. More specifically, this will consist of (1) the development of the conceptual dynamic energy budget toxicology (DEBtox) earthworm model and (2) the completion of experimental studies for model training and verification. Here we present a first pilot experiment using one of the most frequently applied antibiotic in animal husbandry: doxycycline.

Materials and Methods

Earthworm experiment
Doxycycline was added to soil in concentrations ranging from 0 to 500 mg/kg. The experimental unit consisted of a plastic jar with a vented lid filled with 420 g of standard LUFA 2.2 soil moistened with additional water (22.5% w/w, ~45% water holding capacity). Soil was spiked with doxycycline to reach seven nominal concentrations ranging from 0 to 500 mg/kg (n=3). For concentration 0.5 and 5 mg/kg, additional jars were prepared in order to allow sampling at five different time point. In each jar, 7 adult earthworms (*Eisenia fetida)* were added after allowing them to empty their guts. The jars were kept in an incubator at 20 °C. At day 3, 7, 14, 21 and 28 soil aliquots and earthworms were sampled from individual jars at exposure concentrations of

0.5 and 5 mg/kg doxycycline in soil. Earthworms were washed and allowed to purge their gut in a Petri dish before analysis. At day 28, mortality and weight change in earthworms was recorded in all the jars at all tested concentrations. Concentrations of doxycycline in soil and earthworms were determined by liquid chromatography coupled to tandem-mass spectrometry.

Calculation of uptake and elimination kinetic rate constants
The internal concentrations in *E. fetida* were fitted with a one compartment toxicokinetic model (Ardestani et al., 2014; Van Den Brink et al., 2019) by the following equation:

$$C_{int} = k_1/k_2 * C_{exp} * (1 - e^{-k_2 * t})$$

where C_{int} is the internal concentration of doxycycline in the earthworms (µg kg^{-1} wet body weight), k_1 is the uptake rate constant (µg dox kg dry soil µg dox^{-1} kg^{-1} wet body weight day^{-1}), k_2 is the elimination rate constant (day^{-1}), C_{exp} is the measured concentration in the soil (µg kg^{-1}wet weight soil), t is the time of exposure (day^{-1}).

Results and Discussion

Ecotoxicological effects
No mortality was observed at any tested concentrations. Growth was not affected by the presence of doxycycline since it did not show any statistically significant difference with the control.

Doxycycline in soil
For the exposure concentrations of 0.5 and 5 mg/kg in soil, soil samples were taken at five time points during the experiment. Measured doxycycline soil concentrations are presented in Figure 1. A similar degradation constant rate was calculated for both exposure concentrations: 0.035 day^{-1} and 0.039 day^{-1} for 0.5 and 5 mg/kg, respectively. Doxycycline showed a half-life (DT50) of 20 days at an exposure concentrations of 0.5 mg/kg and similarly 18 days at 5 mg/kg. This is in accordance to expectations. Taking into account the measurement uncertainty, no significant difference was observed in the degradation rate for both soil concentrations.

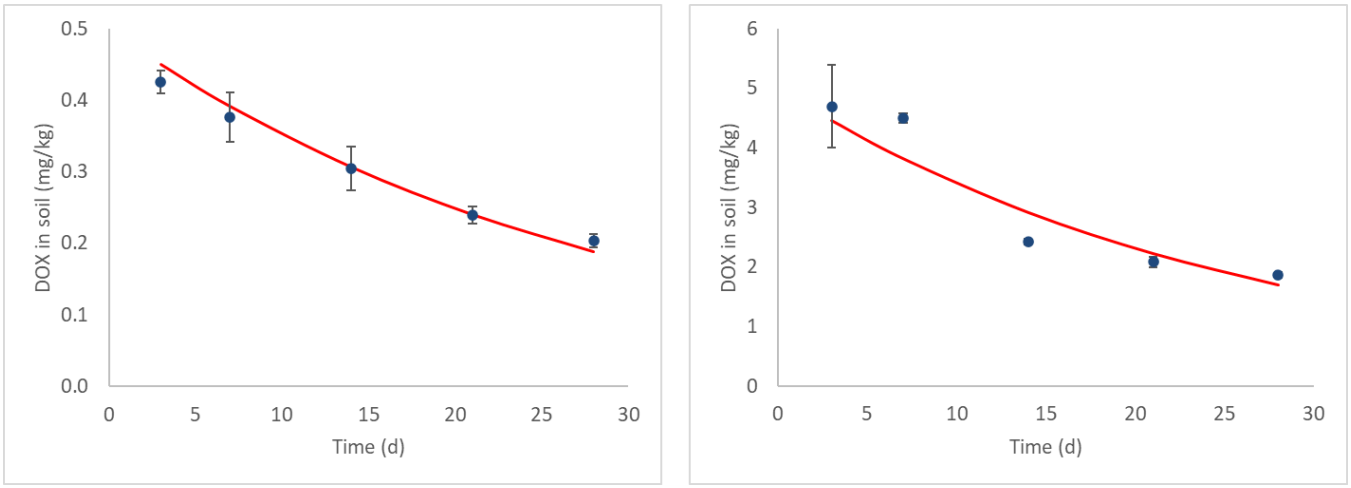

Figure 1. Degradation of doxycycline in soil over time. The exposure concentration is 0.5 mg/kg (left panel) and 5 mg/kg (right panel). Blue circles are the measured concentration (average and standard deviation (n=3)). The red line is the predicted concentration.

Doxycycline in earthworms
The doxycycline concentration determined in the earthworms is presented in figure 2. Earthworms accumulated higher concentrations of doxycycline when exposed to 5 mg/kg compared to the 0.5 mg/kg treatment which were 94 ± 18 and 11 ± 6 µg/kg after 28 days, respectively. The internal doxycycline concentrations in the earthworm reflected the doxycycline in the soil. Indeed, the concentrations in the earthworms decreased along with the degradation of the compound in the soil. This showed that the earthworms successfully eliminated doxycycline either by excretion or biotransformation. The estimated uptake and elimination kinetic rate constants are reported in table 1.

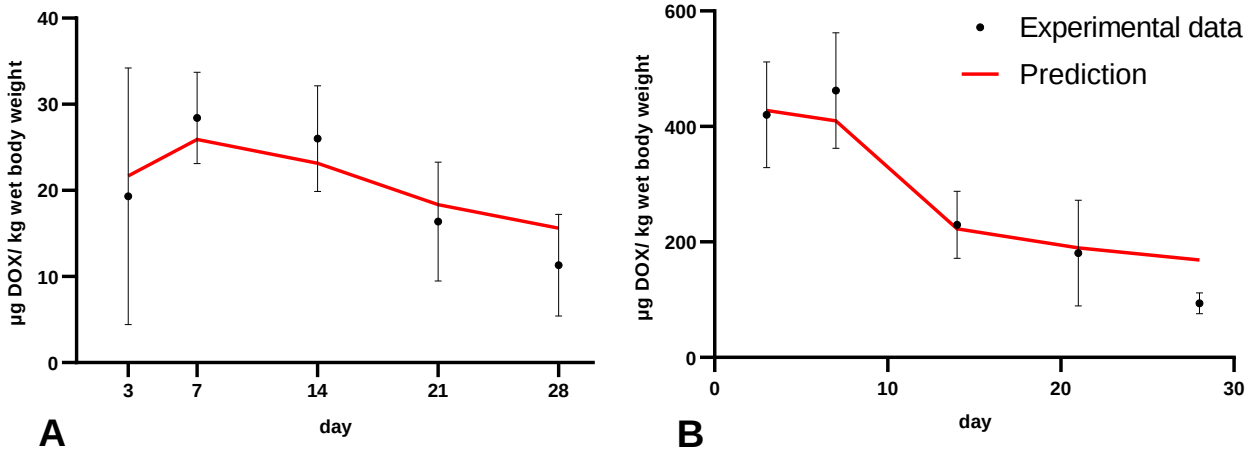

Figure 2. Kinetic of doxycycline accumulation in earthworms exposed to 0.5 mg doxycycline/kg soil (A) and 5 mg/kg (B). Circles represent the measured concentrations (average and standard deviation, n=3). The red line represents the predicted concentrations.

Table 1. Uptake and elimination kinetic constant rates estimated for each exposure concentration separately and for the joined concentrations.

	k1	k2
0.5 µg DOX kg^{-1} soil	0.025	0.333
5 µg DOX kg^{-1} soil	1.724	18.927
0.5 µg DOX kg^{-1} soil + 5 µg DOX kg^{-1} soil	0.088	0.955

Conclusion

Degradation of doxycycline in the soil was determined. The DT50 was estimates at 18 and 20 days for 0.5 and 5 mg/kg soil concentrations respectively. Mortality and growth of earthworm are not affected at the tested concentrations. Earthworms take up and excrete doxycycline effectively. The kinetic constants are given in Table 1.

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POSTER P39

ANTIMICROBIAL PEPTIDES: NEW EMERGING ANTIMICROBIALS AND GROWTH PROMOTING AGENTS?

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Abstract

Antimicrobial peptides (AMPs) are compounds with broad-spectrum activity against microorganisms. In the last decades, AMPs have been identified as a potential alternative to conventional antibiotics in both human and veterinary medicine because of their unique membrane disruption mechanism. Next to their antimicrobial activity, AMPs might also show regulatory activity for gut microbes and have been considered as inductors of growth performance. Therefore, a literature study was conducted to answer the question: "Are AMPs new emerging antimicrobials and growth-promoting agents that should be addressed?". It is concluded that research on AMPs has significantly increased in the last 10 years, but their application in food production is currently limited. However, an increase in the application of AMPs in food production is expected because of the growing economic peptide drug market, the increasing number of AMPs in clinical trials, and their growth-promoting properties. It is therefore important to continue to monitor the development and applications of AMPs in animal production.

Introduction

Over the last decade, the rapid emergence of (multi)drug-resistant bacteria has become a global concern. In the EU alone, resistant bacterial infections are the cause of an estimated 33,000 deaths annually and cause significant production losses in livestock farming (Cassini et al., 2019). In addition, the number of approvals of new antibiotics has decreased significantly in the past three decades (Alanis, 2005). The development of alternatives for antibiotics is urgent to overcome these problems, but might introduce new food safety risks. A promising alternative group of antimicrobials are the antimicrobial peptides (AMPs), which are small positively charged molecules that have been described as evolutionary ancient weapons against microbial infections (Erdem Büyükkiraz & Kesmen, 2022). They are produced by all organisms, from prokaryotes to human beings, and serve a fundamental role in innate immunity (Zhang & Gallo, 2016). Currently, more than 4000 natural AMPs have been discovered and included in various databases including DRAMP, DBAASP, APD, and CAMP (Kang et al., 2019). AMPs have been reported to have broad-spectrum activity against bacteria, fungi, protozoa, and viruses (Figure 1). Moreover, AMPs have been reported to be effective against microorganisms with resistance to conventional antibiotics and have shown growth-promoting properties in pigs and broilers (Shuai Wang, Zeng, Yang, & Qiao, 2016). These advantages make AMPs a suitable candidate for pharmaceutical and veterinary applications, but also make these compounds a potential candidate for misuse. Therefore, a literature study was conducted to answer the question: "Are AMPs new emerging antimicrobials and growth-promoting agents that should be addressed?".

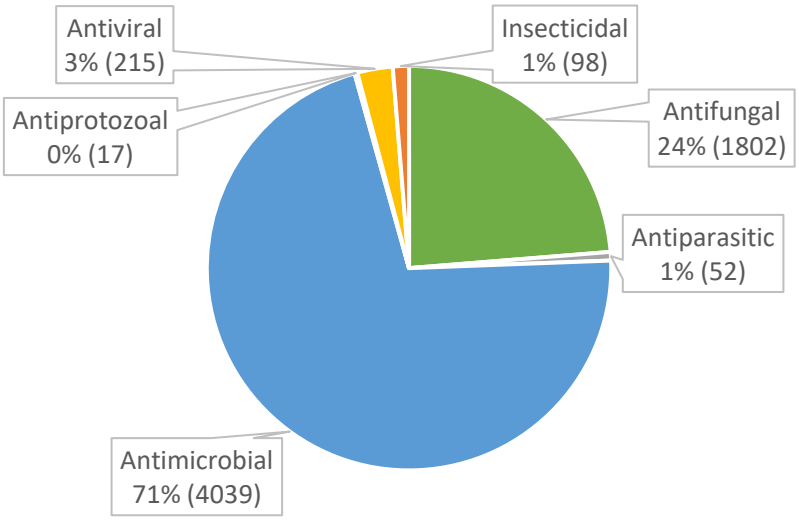

Figure 11: Activity of AMPs that are included in the DRAMP database. Data of the DRAMP database was obtained from Kang et al. (2019) and Fan et al. (2016) (Fan et al., 2016; Kang et al., 2019).

Structural classification of AMPs

AMPs have been classified according to a variety of properties including their biological origin (insects, mammals, marine, etc.) and their activity spectrum. However, the classification based on their secondary structure is the most common (Koehbach & Craik, 2019). The AMP secondary structures are generally classified into α-helical, β-sheet, mixed (α-helical/β-sheet), and extended structures (Figure 2). The first and largest group, α-helical AMPs, are the most studied group and include the extensively studied human LL37 (Figure 2a). These peptides exhibit little secondary structure in aqueous solution but adopt their amphipathic α-helical structure when they enter non-polar environments such as the bacterial membrane. The second group of AMP is characterized by their β-sheet conformation which consists of at least two β-sheets, which are stabilized by disulfide bonds. The bovine lactoferrin B, shown in figure 2b, is an example of an AMP in this class. The third group consists of AMPs that exhibit both α-helical and β-sheet conformations in their structure that are stabilized by three or four disulfide bridges. Figure 2c shows an example of such a peptide, which is the human β-defensin 1 peptide. Lastly, the extended peptides do not contain any α-helical or β-sheet motifs but are rather defined by a high content of specific residues such as histidine, arginine, glycine, or tryptophan (figure 2d).

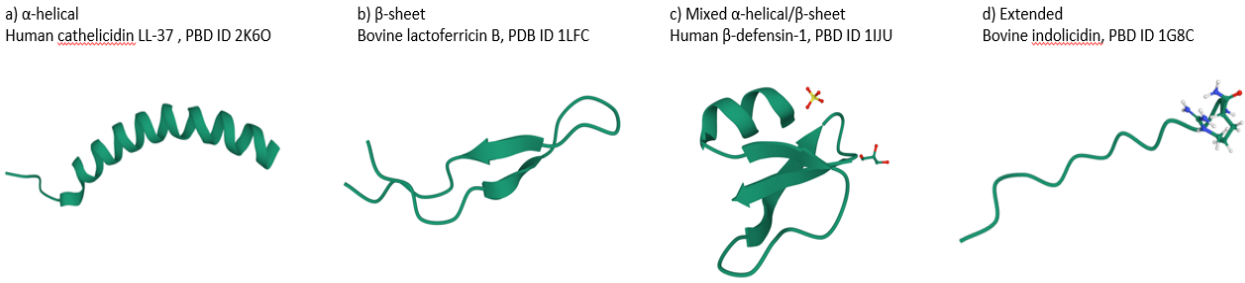

Figure 12: Protein models representing the four secondary structures of antimicrobial peptides; a) α-helical human cathelicidin LL-37 (G. Wang, 2008), b) β-sheet bovine lactoferricin B (Hwang, Zhou, Shan, Arrowsmith, & Vogel, 1998), c) mixed α-helical/β-sheet human β-Defensin-1 (Hoover, Chertov, & Lubkowski, 2001) and d) extended bovine indolicidin (Rozek, Friedrich, & Hancock, 2000). All structures where obtained freely from the RCSB Protein Data Bank (PDB) (Berman et al., 2000).

Activity of AMPs

The antimicrobial activity of AMPs is exerted through two different mechanisms: 1) membrane-targeting AMPs impair the structural integrity of the bacterial cytoplasmic membrane resulting in lyses of the bacterial cell and 2) non-membrane targeting AMPs inhibit DNA and protein synthesis, protein folding, enzymatic activity, and cell wall synthesis (Matsuzaki, 2019). AMPs have also been reported to exert multiple mechanisms against microorganisms. Membrane-targeting AMPs interact with bacterial cells because of their cationic and

amphipathic properties. The initial binding between the positively charged AMPs and the negatively charged bacterial membrane is formed through electrostatic attraction. Next, the AMPs penetrate the hydrophobic core of the bilayer, where they interact with the fatty acid chains of the membrane lipids. This leads to the formation of transmembrane pores in the bacterial membrane that eventually results in the lysis of the bacterial cells. Non-membrane targeting AMPs translocate into the cell membrane without perturbing it and then inhibit critical cellular processes by interacting with intracellular targets (Koehbach & Craik, 2019; Nawrocki, Crispell, & McBride, 2014; Nguyen, Haney, & Vogel, 2011).

Advantages and limitations

The major advantage of AMPs over conventional antibiotics is that bacteria may have less chance to develop drug resistance due to its unique membrane disruption mechanism and their ability to exert multiple mechanisms against microorganisms, although bacterial resistance against AMPs has been reported in recent literature. These resistance mechanisms include proteolytic degradation, export by efflux pumps, and modifications of the bacterial outer membrane (Joo, Fu, & Otto, 2016; Spohn et al., 2019). However, Spohn et al. suggested that the frequency of mutations conferring against AMPs is low and the fact that the applied dose of AMPs is below the concentration under which resistance can evolve (Spohn et al., 2019). A second advantage of AMPs is that their exertion of multiple mechanisms against microorganisms results in broad-spectrum activity against microorganisms and even activity against multi-drug resistant pathogens (Shuai Wang et al., 2016). Lastly, AMPs are effective against both Gram-positive and Gram-negative bacteria (Koo & Seo, 2019).

Unfortunately, AMPs also have some major limitations that hamper the applications of AMPs in human and veterinary medicine. A major limitation is the interactions of some AMPs with mammalian cells through hydrophobic interactions, resulting in the lysis of mammalian cells. In addition, AMPs are prone to degradation by pH or protease, loss of activity in serum differences between *in vivo* and *in vitro* experiments are shown, and their complicated structure leads to high research and development costs (Li, Hu, Yang, Lu, & Wang, 2018; Mahlapuu, Hakansson, Ringstad, & Bjorn, 2016; McIntosh, Donia, & Schmidt, 2009; Xiao et al., 2015). Currently, the production costs of an AMP is estimated to be $50-400 per gram of AMP produced (Marr, Gooderham, & Hancock, 2006). Research has therefore partially moved towards small molecule-based peptidomimetic AMPs to mimic structural features and biological functions of AMPs without the limitations described above (Rezende, Oshiro, Júnior, Franco, & Cardoso, 2021). A short description of the different classes of synthetic AMPs is presented below (see Brogden *et al.*. (Brogden & Brogden, 2011) for a comprehensive review on this topic):

- Antimicrobial mimetics are small, synthetic compounds that mimic the properties and bioactivity of naturally occurring AMPs. These compounds are constructed from peptoids, β-peptides, arylamides, oligomers, or phenylene ethynylenes.
- Hybrid AMPs are constructed of two or three naturally occurring peptides to capture and combine the potential benefits of each fragment while reducing the limitations of naturally occurring AMPs.
- AMP congeners are compounds that are closely related to their parent AMP in composition, exerting either similar or antagonistic effects.
- Cyclotides and stabilized AMPs are prepared by cyclization of AMPs making them more active and less prone to degradation as a result of changes in the pH or the presence of proteases.
- AMP conjugates are prepared by coupling the AMP to a specific outer surface antibody or ligand for a receptor on a specific pathogen. These types of compounds could be used at lower concentrations with 'targeted' antimicrobial activities while inducing fewer side effects.
- Immobilized AMPs are AMPs that are adsorbed to a variety of surfaces without losing their activity against pathogens.

Applications of AMPs

AMPs for food preservation

To date, the most prevalent use of antimicrobial peptides has been in the preservation of foods, although the number of AMPs approved as a preservative is limited (Thacker, 2013). Nisin is currently the most commonly applied AMP and has been approved in the EU as a food additive under Annex II of Regulation (EC) 1333/2008. The application of nisin as a food preservative is approved in over 50 countries and can be used in processed

cheese, pasteurized dairy products, and canned vegetables. Recently, the use of nisin has been reported in high moisture products, hot baked flour products, and pasteurized liquid eggs (Hwanhlem, Ivanova, Haertlé, Jaffrès, & Dousset, 2017).

ε-Polylysine can be used for food preservation. It is an AMP produced by the *Streptomyces albulus* and consists of 25-35 L-lysine residues that are linked through their carboxylic and ε-amino groups. ε-Polysyine has broad-spectrum activity against Gram-positive and Gram-negative bacteria, yeasts, and mold. Furthermore, it has an antibacterial effect against the Gram-negative bacteria *E. coli* and *Salmonellae*, which are difficult to control with other natural preservatives. The use of ε-polylysine is not permitted as a food additive in most countries, but the substance is generally used as a food preservative in Japan and Korea. In the United States, ε-polylysine is recognized as GRAS material (Luz et al., 2018).

The AMP pediocin PA-1 is a bacteriocin composed of 44 amino acid residues and is produced by *Pediococcus acifilactici*. This bacteriocin is on the market under the name ALTA^{tm} 2431. Pediocin has been used to improve shelf-life, mainly on ready-to-eat meat products, and has shown activity against *L. monocytogenes* growth (Santos et al., 2018). Pediocin PA-1 is currently not approved as a food additive in the EU.

AMPs in veterinary medicine

The number of AMPs that are approved in veterinary medicine is currently limited to colistin and in some countries nisin. Cao *et al.*. (2007) reported that a nisin-based formulation was effective in the treatment of clinical mastitis in lactating dairy cows (Cao, Wu, Xie, Hu, & Mo, 2007; Shin et al., 2016). Nisin is currently on the market under the name of RE-TAIN^{TM} for the treatment of mastitis in lactating cows. However, the increasing microbial resistance to conventional antibiotics has resulted in the study of the effects of AMPs on swine and poultry. In these studies, it was reported that a few AMPs, such as antimicrobial peptide-A3, P5, and cecropin AD had beneficial effects on growth performance, nutrient digestibility, intestinal morphology, and gut microbiota in weanling piglets (Shuai Wang et al., 2016; Wu et al., 2012; Xiao et al., 2015; J. H. Yoon et al., 2012; J. H. Yoon et al., 2014; Jung Ho Yoon et al., 2013). In addition, antimicrobial peptides-P5 and P3 showed to reduce coliforms in both weanling piglets and broilers (Choi et al., 2013a, 2013b; J. H. Yoon et al., 2012; Jung Ho Yoon et al., 2013). Treatment with antimicrobial peptides A3 and P3 resulted in growth performance similar to antibiotic treatment, which suggests that AMPs have potential as novel alternatives to antibiotic growth promotors. This growth-promoting effect may be related to the improvement in nutrient digestibility (Shuai Wang et al., 2016). A selection of the studied AMPs and their application effects are shown in table 2.

Table 3: Selection of AMPs studied in production animals

AMP	Animal	Application effects	References
Cecropin AD	Weanling pigs	Enhances pig performance through increasing immune status and nitrogen and energy retention as well as reducing intestinal pathogens	(Wu et al., 2012)
Antimicrobial peptide-P5 (AMP-P5); 40 or 60 mg/kg	Weanling pigs	Improvement of growth performance and apparent total tract digestibility of nutrients. Reduces coliforms.	(Jung Ho Yoon et al., 2013)
Antimicrobial peptide-P3 (AMP-A3); 0, 60 or 90 mg/kg	Weanling pigs	Has beneficial effects on performance, coefficient of total tract apparent digestibility of nutrients, intestinal morphology and intestinal and fecal microflora.	(J. H. Yoon et al., 2012)
AMP-A3 or AMP-P5	Weanling pigs	Improves the performance, nutrient digestibility, intestinal morphology and reduces pathogenic bacteria	(J. H. Yoon et al., 2014)
Lactoferrin	Weanling pigs	Reduced total viable counts of *E. coli* and *Salmonella* in the small intestine. Enriched the colonic *Lactobaccillus* and *bifidobacterium* in the small intestine.	(Y.-Z. Wang, Shan, Xu, Feng, & Wang, 2007)
Bovine lactoferrin	Weanling pigs	Decreased the counts of *E. coli* in the ileum, caecum and colon. Increased the counts of *Lactobacilli* and *Bifidobacteria* in the ileum, caecum and colon.	(Tang et al., 2008)
Cecropin A-D-Asn	Broilers	Decreased aerobic bacteria counts in both jejunal and ceacal digesta	(Wen & He, 2012)
Antimicrobial peptide-A3	Broilers	Improved growth performance, nutrient retention,	(Choi et al.,

		intestinal morphology and reduced harmful microorganisms in broilers.	2013a)
Antimicrobial peptide-P5	Broilers	Improved growth performance, nutrient retention, intestinal morphology, and reduces intestinal and excreta coliforms.	(Choi et al., 2013b)
Sublancin	Broilers	Reduced *Clostridium perfringens* in the cecum	(S. Wang et al., 2015)

AMPs on the market and used for humans

Currently, a limited number of AMPs are currently approved by the FDA, although many AMPs are in clinical trials (Figure 3. According to the DRAMP database, there are currently 33 AMPs in clinical trials and 33 more AMPs in preclinical stages (Figure 3) with the most AMPs being in phase 2. The majority of AMPs in clinical trials have antibacterial activity, although antiviral, anticancer, and antifungal AMPs are also in current clinical trials (Fan et al., 2016; Kang et al., 2019). It is expected that the number of AMPs clinical trials increases in the coming years because it is forecast that the global peptide drug market will double between 2018 and 2026(Henninot, Collins, & Nuss, 2018; Koo & Seo, 2019).

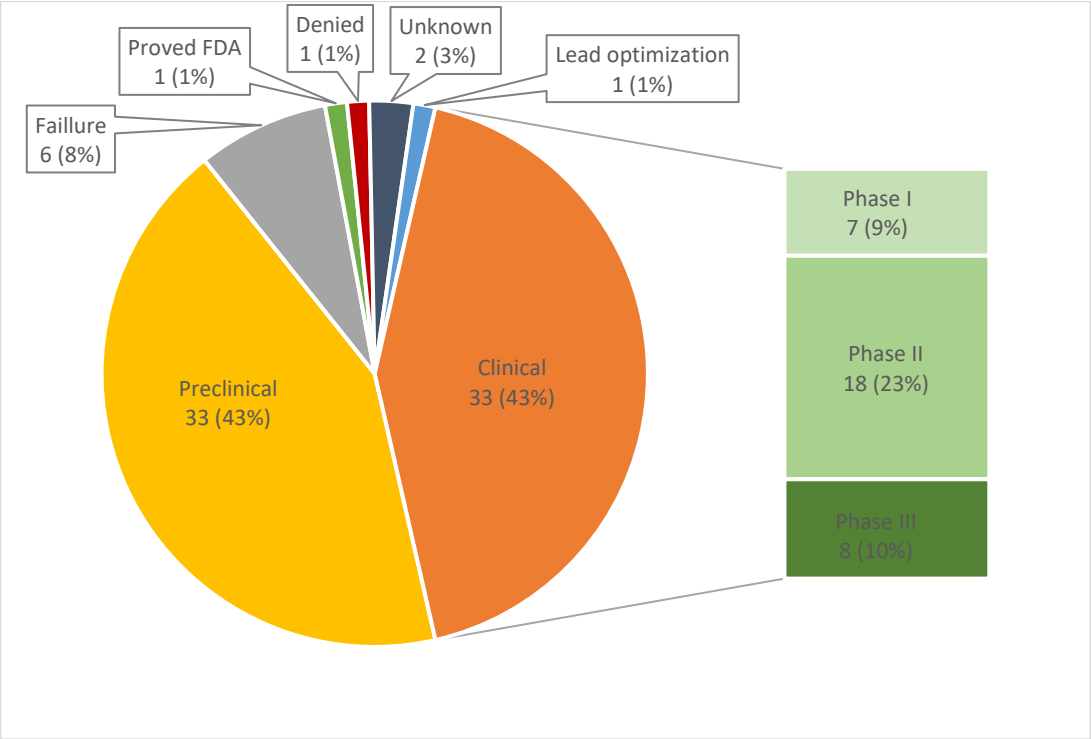

Figure 13: Overview of the number of AMPs that are currently, or have been, in clinical trials. The data was obtained from the DRAMP database from Kang et al. (2019) and Fan et al. (2016) (Fan et al., 2016; Kang et al., 2019).

Conclusion

Research on AMPs has increased significantly in the last 10 years, but their application in animal production is currently limited to AMPs such as colistin and nisin. However, because of the increasing number of AMPs in clinical trials, the growing economic value of the peptide drug market, and their growth-promoting properties, an increase in AMP application in food production is expected. It is therefore important to continue to monitor the development and applications of AMPs in food production.

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POSTER P40

ULTRA-HIGH SENSITIVITY QUANTIFICATION OF VETERINARY DRUG RESIDUES IN ANIMAL BY-PRODUCTS

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Abstract

As regulations continue to tighten around food testing, it has become increasingly challenging to achieve the necessary levels of sensitivity during analysis while maintaining a high level of accuracy and precision. Within this method, LOQ values down to 0.005 ng/mL have been achieved while keeping high levels of accuracy and precision in standard solutions. When spiked into matrices, LOQs of 0.01 µg/kg in pork and chicken and 0.005 µg/kg in milk were observed. This high level of sensitivity allows routine laboratories to further dilute their samples to minimize any matrix effects observed. The linear range of each compound analyzed has been assessed, with ranges spanning up to 4 orders of magnitude and r values >0.99. sMRM acquisition helps ensure that both quantifier and qualifier transitions can be measured to increase the specificity of the analysis without the need to compromise on data quality by reducing the number of data points across each peak. In addition, chromatographic separation is important to minimize the number of compounds analyzed at any one time. This allows for a balance between the cycle time of the mass spectrometer and the dwell time for each analyte so that accurate quantification can be performed for each compound.

In addition to MRLs (maximum residue limits), minimum method performance requirements (MMPRs) are recommended by the EU for some prohibited compounds, which are summarized here. In these instances, the sensitivity of the analysis is paramount to ensure the MMPR is achieved or exceeded. In this method the MMPR has been met or improved upon, showing that even for the most difficult to analyze compounds, this method can achieve or improve on the recommended levels of sensitivity.

Introduction

The use of pharmacologically active substances in veterinary settings has been scrutinized for several years due to their sometimes inappropriate or intensive use. This is particularly worrying to authorities due to possible allergic reactions and an increase in antibiotic resistance which is becoming increasingly problematic both in livestock and human populations.[1] Therefore, the use of these substances needs to be controlled and limited to mitigate these known issues. One such way these controls are implemented is by analytical testing of products of animal origin, with a number of compounds receiving a MRL to reduce the levels of particular compounds. In addition to MRL values, some compounds have subsequently been prohibited due to their inherent toxicity. Therefore, in this case it is important to achieve LOQ values as low as is reasonably possible to limit these compounds in case of illegal use in the food industry.[2] Outside of this, minimum method

performance requirement (MMPR) values have also been set for certain compounds to ensure than an acceptable level of sensitivity is reached.[3]

Here, a method for analyzing over 180 relevant compounds used in the veterinary industry has been developed, with LOQ values down to 0.005 ng/mL.

See Figure 1 which highlights the sensitivity of the SCIEX 7500 System for the analysis of chlorpromazine, which is one of the compounds listed to be prohibited by the European Union.[4]

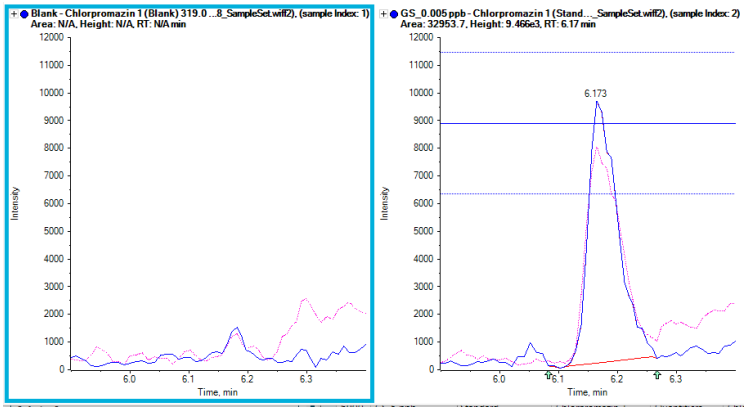

Figure 1. Overlaid XICs for chlorpromazine in solvent. Both the quantifier (blue) and qualifier (pink) MRM transitions of a chlorpromazine are shown in the solvent blank (left) and the LOQ of 0.005 ng/mL in solvent (right). The LLOQ image shows two MRM transitions for chlorpromazine overlaid along with ion ratio lines which outline the acceptable height (± 30%) of the qualifier peak.

Materials and Methods

Standard preparation: A mixed standard solution was provided by the official food control authority and veterinary affairs of Geneva at a concentration of 1 µg/mL. A series of dilutions were prepared to cover a range of 0.005 - 100 ng/mL.

Sample preparation: Each sample was homogenized prior to weighing. 5 g of each sample (pork, milk and chicken) was then weighed before the addition of 0.1 M EDTA-McIlvaine buffer (4 mL for pork and chicken, 3 mL for milk). All samples were then homogenized (pork and chicken for 1 min 15 secs using a FASTH21, milk for 10 minutes using a mechanical shaker). To each solution acetonitrile and ammonium sulfate was added (pork and chicken – 16 mL and 5 g, milk – 8 mL and 5 g) before being further homogenized and centrifuged for 5 min at 4700 rpm (4°C). 4 mL of the upper layer was removed and transferred into an evaporative vial, with 400 µL of DMSO added to each. Evaporation of the solution was performed using nitrogen at 40 °C until a final weight of ~ 0.5 g was achieved. 1 g of H2O was added to each solution before vortexing for ~15 sec, centrifuging for 5 mins at 13,000 rpm and finally filtering through a 0.45 µm nylon filter prior to analysis.

Spiked sample preparation: 90 µL of prepared matrix sample (pork, chicken, milk) was added to 10 µL of a relevant standard solution. Three spike concentrations were prepared at 0.01, 0.1 and 1 ng/mL with standard solutions at 0.1, 1 and 10 ng/mL being used as spiking solutions.

Chromatography: An ExionLC AD system was used to perform the chromatographic separation along with a Phemomenex Kinetex Polar C18 (2.6 µm, 100 Å, 100 x 2.1 mm) column.

Mass spectrometry: The analysis was performed using the SCIEX 7500 system. The system was operated using time-scheduled multiple reaction monitoring (sMRM) mode (see Figure 2) using positive and negative

electrospray ionization (ESI) switching. Q0D optimization was performed to enhance some of the compound's signal.[5] Data was acquired using SCIEX OS software.

Data Processing: Data was processed using SCIEX OS software, with the AutoPeak algorithm for peak integration.[6-7]

Results and Discussion

Sensitivity and specificity

Sensitivity and specificity are two of the main drivers of a successful analysis, with triple quadrupole mass spectrometers typically being used to achieve high levels of these metrics. See Figure 2 which shows three analytes at their respective LLOQ of 0.005 ng/mL in solution, highlighting the levels of sensitivity which can be achieved when using the SCIEX 7500 system. A blank injection has also been included in the figure to show that the blank is free of interferences and the peak observed at the LLOQ is genuine. In addition to sensitivity, the use of two MRM transitions (quantifier and qualifier) is shown within the figure and is used to provide an extra level of specificity to the analysis by implementing the use of ion ratios which can better confirm the identity of the peak when compared to a single MRM transition, especially in matrix. The ion ratio lines shown represent where the qualifier peak needs be within to be ±30% of the expected value.

Sensitivity in solvent is an important factor for any analysis however, what is more important is to carry this performance over to analysis in matrix. See Figure 3 which shows the same three compounds highlighted in Figure 2 when spiked into a pork matrix. The XICs shown are at the lowest spike level analyzed (0.01 ng/mL) which equates to 0.01 µg/kg in the pork matrix, again showing the ultra-levels of sensitivity which can be achieved.

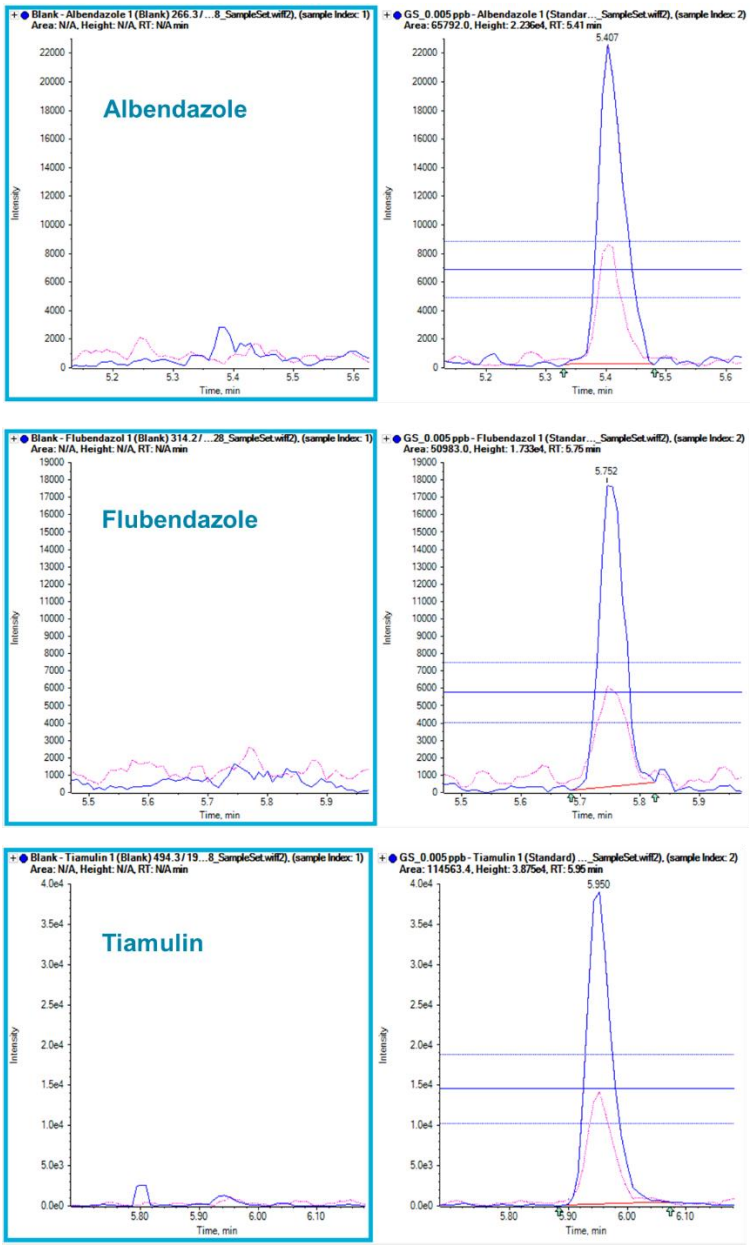

Figure 2. Overlaid XICs of the quantifier (blue) and qualifier (pink) MRM transitions of albendazole (top), flubendazole (middle) and tiamulin (bottom). The above images show the respective solvent blank (left) for each compound alongside the compounds' LLOQ (right). Each compound achieved an LLOQ of 0.005 ng/mL.

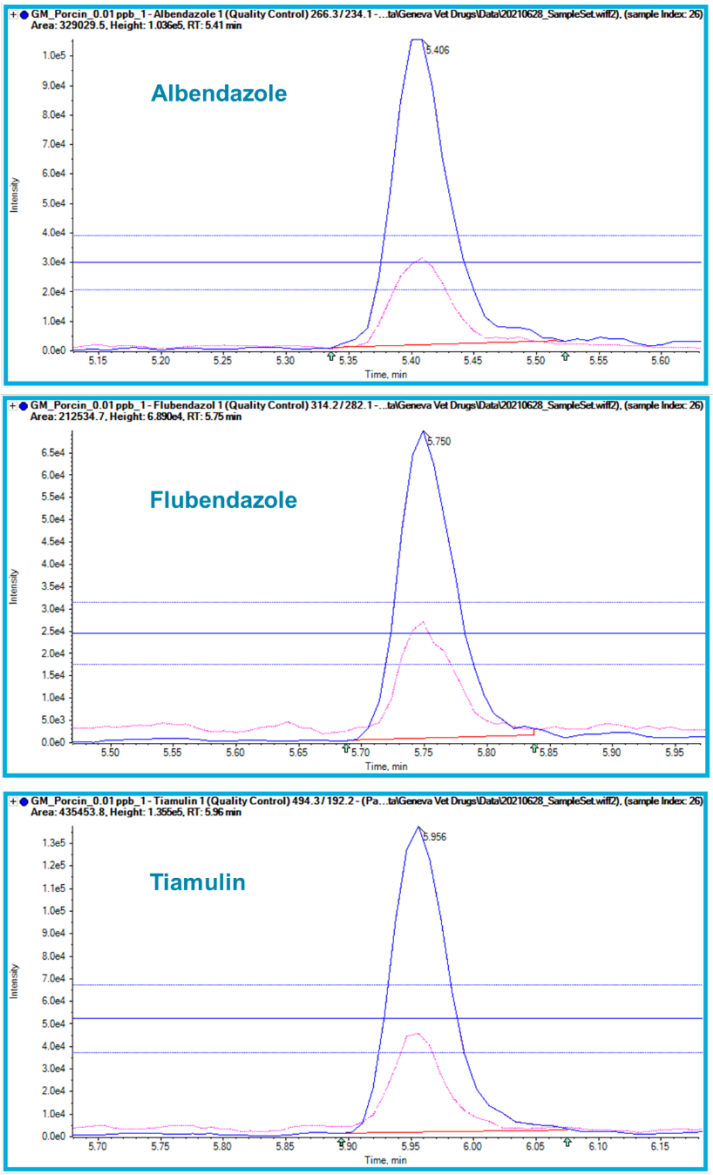

Figure 3. XIC overlays of both quantifier (blue) and qualifier (pink) with ion ratio lines for three analytes spiked into a pork matrix at the lowest spike concentration analyzed (0.01 ng/mL). This concentration equates to 0.01 µg/kg in sample.

Linear dynamic range

The linear range of an analyte is the span at which quantification is possible, while achieving acceptable levels of precision and accuracy. See Figure 4 which shows the linear dynamic range of three compounds across 4 orders of magnitude while still achieving accuracy levels between 70 – 130% and an r value >0.99.

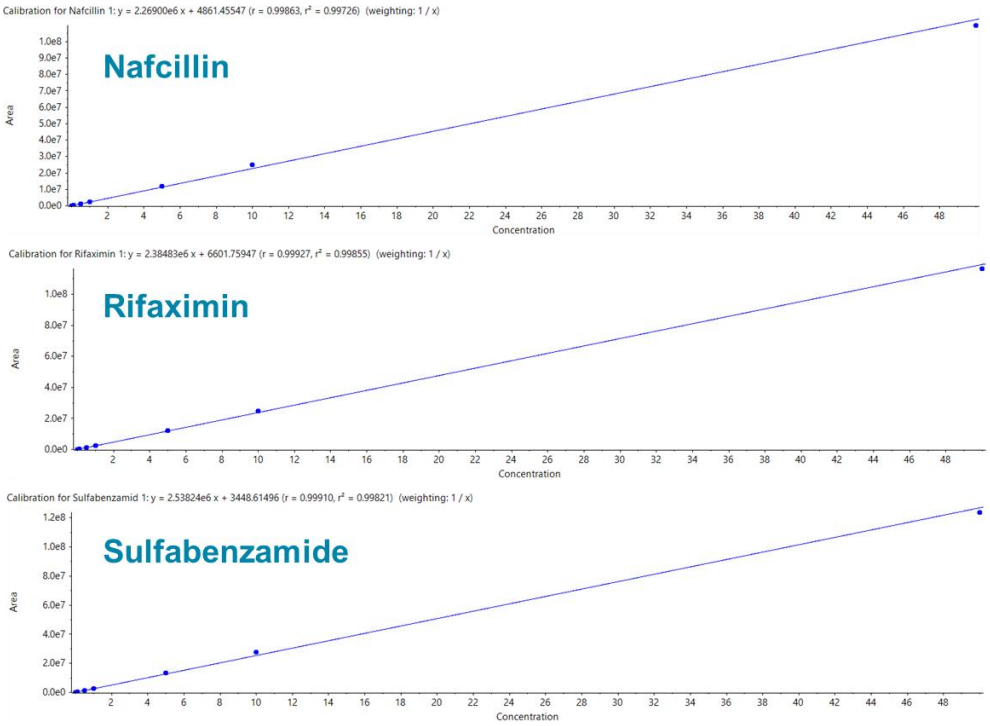

Figure 4. The linear ranges for three of the compounds analyzed. Each compound provides a linear range from 0.005 to 50 ng/mL, therefore highlighting a linear range over 4 orders of magnitude. The r value for each linearity was >0.99.

Accurate and precise quantification

To ensure accurate and precise quantification, it is important that each individual peak has enough data points. See Figure 5 which shows the data points of chlorpromazine for both the quantifier and qualifier ion MRM transitions at a concentration of 10 ng/mL in solution. Each peak shows >10 data points which is consistent across the compounds analyzed.

Accuracy was also accessed at each point of the calibration curve, with all analytes providing accuracy values between ± 30 % at each level when back calculated against the curve.

In addition, precision is paramount to show the consistency of the instrument both in solution and in sample. See Table 1 below which shows the %CV values achieved for the three compounds in Figure 7 at the lowest concentration which was assessed for precision (0.01 ng/mL in solution and 0.01 µg/kg in pork and chicken, 0.005 µg/kg in milk) in solution and when spiked into matrix.

Table 1. The %CV (calculated concentration) values of chlorpromazine, metronidazole and triclabendazole sulfoxide at the lowest concentration assessed for precision (0.01 ng/mL in solution. 0.01 µg/kg in pork and chicken, 0.005 µg/kg in milk) (N=5).

Compound name	Solvent	Pork	Milk	Chicken
Chlorpromazine	*4.00*	*4.15*	*5.67*	*6.60*
Metronidazole	*5.33*	*5.24*	*5.84*	*6.99*
Triclabendazole sulfoxide	*12.11*	*9.89*	*11.36*	*5.99*

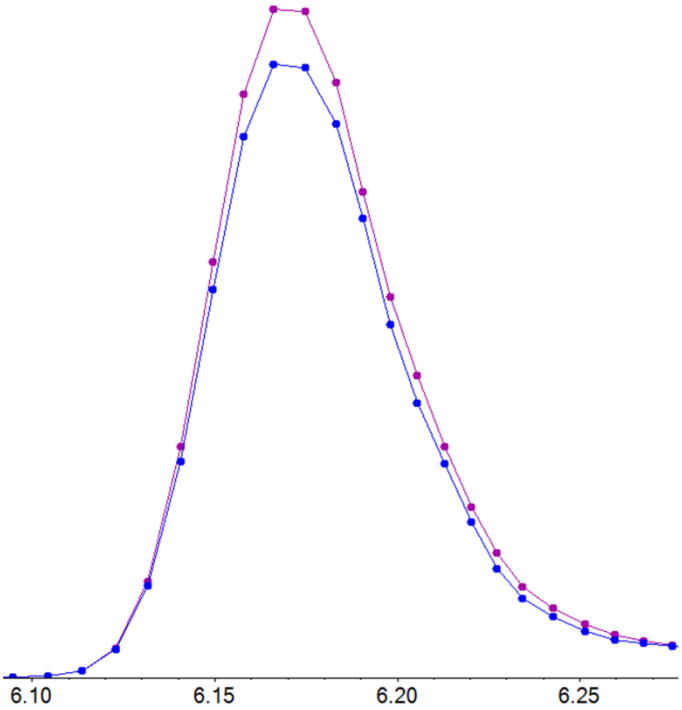

Figure 5. Overlaid XICs of quantifier (purple) and qualifier (blue) MRM transitions, highlighting the number of data points acquired for chlorpromazine. Over 10 data points have been achieved for both the quantifier and qualifier transition across the peak, indicating that accurate and reproducible quantification can be performed.

Minimum method performance requirements (MMPRs)

Many compounds which have been analyzed in this method are prohibited to be used in veterinary settings. For these compounds it is paramount to achieve high levels of sensitivity to ensure illegal use is not carried out. Therefore, these prohibited compounds do not have MRL values as any level is not permitted. However, some of these compounds have been detailed in a specific EURL guidance that defines MMPR values, which correspond to the minimum concentrations that should be achieved by official laboratories.3 See Table 2 where a compiled list of prohibited compounds has been shown which have an MMPR value. As can be seen in the table, the assigned MMPR value has been achieved for all compounds analyzed.

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Table 2. A comparison of the MMPR value and the lowest quantifiable concentration in a chicken matrix. For all compounds listed, sensitivity is important due to being banned substances in animal by-products. Therefore, the LOQ needs to be as low as is feasibility possible. Note: chicken was spiked at 0.01, 0.1 and 1 µg/kg.

Compound Name	Lowest quantifiable concentration in chicken (µg/kg)	MMPR value (µg/kg)
Brombuterol	*0.1*	*0.1*
Carbadox	*0.1*	*5*
Cimbuterol	*0.01*	*0.1*
Clenbuterol	*0.1**	*0.1*
Clenproperol	*0.1**	*0.1*
Dapsone	*0.1*	*5*
Dimetridazol-hydroxy	*0.1*	*1*
Flufenamic acid	*0.1*	*10*
Metronidazole-hydroxy	*0.1*	*1*
Mefenamic acid	*0.1*	*10*
Ronidazole	*0.1*	*1*
Tulobuterol	*0.1**	*0.1*
Zeranol	*1*	*1*
Phenylbutazone	*0.1*	*5*
Salbutamol	*0.1*	*0.5*

**= Peak observed in the blank matrix injection therefore, it was not possible to assess at lower levels.*

Conclusions

- An ultra-sensitive and fast method for the quantification of over 180 pharmacologically active compounds has been showcased
- Analysis has been performed in three relevant food matrices down to 0.01 µg/kg for pork and chicken, 0.005 µg/kg for milk
- Linear dynamic ranges which span up to 4 orders of magnitude without the use of internal standard
- Accurate and precise quantification including the use of ion ratios to increase the specificity of the analysis
- The assigned MRL or MMPR values can be achieved for all compounds analyzed.

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9. Commission implementing regulation (EU) No 2021/808, 22nd March 2021.

POSTER P41

UHPLC-MS/MS ANALYSIS OF ANTIMICROBIAL RESIDUES IN MEAT AND WATER AS A PART OF SURVEILLANCE SYSTEM ON PIG AND POULTRY FARMS

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Abstract
Antibiotic use plays a major role in the emerging public health crisis of antibiotic resistance. The aim of this study was to analyse an antibacterial residues in meat and water samples as a part of surveillance system on pig and poultry farms within the project Healthy Livestock. The *Healthy Livestock* is a research programme to study the contributions of enhanced animal health and welfare on reducing the need to use antimicrobials in pigs and poultry. Within the workpackages 5, subtask 5.1.1 (Analysis of antimicrobial residues in meat and water on pig and poultry farms), samples of muscles and drinking water were collected from farms in EU countries: Greece and Cyprus (22 farms), France (19 farms), Italy (30 farms) and The Netherlands (14 farms), in two steps as pre and post intervention. Two UHPLC-MS/MS methods for the determination of 61 substances including penicillins, cephalosporins, macrolides, tetracyclines, quinolones, sulfonamides, pleuromutilins, diaminopirymidynes, lincosamides, in muscle and water were developed. Veterinary drugs were analysed in 880 muscles and 190 water samples. Doxycycline was detected in muscle samples from 15 farms. Detected concentrations of doxycycline in post intervention (15.9- 70.8 µg/kg) were lower than concentration in pre intervention step (20.6-100 µg/kg). In water samples, doxycycline in an average concentration of 119 µg/L in pre and 23.1 µg/L in post intervention, as well as enrofloxacin in an average concentration 170 µg/L in pre and 1.72 µg/L in post intervention were quantified. The obtained results confirm the effectiveness of the intervention actions.

Introduction
Intensive animal breeding for food production has led during last decades to a significant increase in the use of veterinary medicines. To protect the health and welfare of livestock and to improve their performance, antimicrobial agents, especially antibacterial compounds, are used throughout the world, in a variety of extensive and intensive livestock production systems. The overuse and excessive administration of antibacterials, as well as failures to follow the label directions of antibiotics and withdrawal inadequacy, can lead to residues occurrence in products of animal origin. Drug residues in foods derived from animals, may pose many adverse health effects for the consumer [1,2]. The residues may result in many biological adverse effects as allergic reactions, increase immunological responses in susceptible individuals and intestinal microbiota disturbance in consumers [3]. The spread of drug-resistant bacterias, as well as bacterial resistance acquisition led to the recognition by the WHO of antimicrobial resistance (AMR) as one of the major threats to public and animal health [4,5]. The EU legislative framework defines maximum limits permitted in food for a number of antimicrobial residues and monitoring programmes for the control of the presence of these substances in the food chain. Regulation (EU) No 37/2010 establishes maximum residue limits (MRL) for residues of veterinary medicinal products in food-producing animals and animal products [6]. In the EU countries, the monitoring of veterinary medicinal product residues and other substances in live animals and animal products is carried out every year and non-complaint results are presented in annual reports. The latest report summarises the

monitoring data generated in 2020 in the frame of official control activities. For group B1 (antibacterials), in 2020 0.14% of the samples analysed under the Directive 96/23/EC monitoring were non-compliant and the same non-conformity rate was reported in 2019 [7,8].

One of the most practical and economical routes of veterinary drugs administration mainly for chickens, less frequently in pigs, is drinking water [9,10]. It provides rapid administration of medicines to all animals in the early stages of disease, low cost of solution preparation, easy distribution and drug storage, as well as facile quick changes of dosage [11]. However, in such way of drugs administration an important factor is to guarantee adequate water quality and water hygiene [12]. The physicochemical properties of drugs should be consider, like solubility in water and adsorption in the solid phase, because some substances can form complexes with ions present in drinking water [13,14]. This method of treatment has a higher probability of improper dosing of the drugs and contributes to the (over)exposure of healthy or non-infected animals to medicines [15]. Inadequate hygiene in water supply systems may produce many undesirable effects such as antibiotic contamination of dispensers and application containers [16]. Contaminated water supply systems can cause the spread of medicines to the farm environment. Therefore, a regular cleaning and a system of regular sanitation procedures in water supply systems with special cleaners should be implemented in each farms, where food producing animals are housed.

The aim of this study was to analyse an antibacterial residues in meat and water samples as a part of surveillance system on pig and poultry farms within the project Healthy Livestock. The Healthy Livestock is a research programme to study the contributions of enhanced animal health and welfare on reducing the need to use antimicrobials in pigs and poultry. Within the workpackages 5, subtask 5.1.1 (Analysis of antimicrobial residues in meat and water on pig and poultry farms), samples of muscles and drinking water were collected from farms in EU countries. For the determination of antimicrobials in meat and water, two UHPL-MS/MS methods were developed. The simple and efficient methods enable the analysis 61 compounds in muscle and 45 substances in water including penicillins, cephalosporins, macrolides, tetracyclines, quinolones, sulfonamides, pleuromutilins, diaminopirymidynes, lincosamides.

Materials and methods

Sample collection

Meat and water samples were collected from poultry and pig farms. Seven, fifteen and thirteen poultry houses were recruited to participate in this study in Cyprus, Greece and the Netherlands, respectively. Nineteen pig houses in France and 30 pig farms in Italy were involved as well. All pig and poultry farms were identified and documented. The objective was to identify the biosecurity and health standards on the site and what was considered to be the key areas needing improvement with the aim of decreasing the need for antimicrobial use while maintaining biological and economic performance. Such health plans included changes which could mitigate risks, could be implemented and could reduce the use of antimicrobials. Biological and economic data, as well as antimicrobial use was recorded for each farm in pre intervention cycles, and post intervention cycles. Samples for detecting any residues in meat, water and manure were also collected pre and post intervention.

UHPL-MS/MS analysis of muscle

Chicken or pig meat samples were minced, homogenized and stored at -20°C until analysis. These samples were analysed for the presence of 61 antimicrobial drugs by UHPLC-MS/MS method using two extraction methods [17].

Briefly, the first extraction methods by acetonitrile is suitable to detect and quantify 45 antibiotic belonging to the following classes: β-lactams, sulfonamides, macrolides, fluoroquinolones, dapsone, pleuromutilins and diaminopyrimidines. The protocol of the first method was as follows. To a muscle subsample (2 g), 8 ml of acetonitrile was added, mixed thoroughly and centrifuged. Then 6 ml of supernatant were taken and evaporated to dryness at 45°C. The dry residue was dissolved in 0.6 ml of 0.025% heptafluorobutyric acid (HFBA) and filtered through a 0.22 μm PVDF filter into a LC vial.

The second extraction method by aqueous solution of 5% trichloroacetic acid (TCA) allows isolation of 16 antibiotics (aminoglycosides, tetracyclines, lincosamides). Extraction with this method involved adding 6 ml of 5% TCA to 2 g of muscle subsample. The sample was vortex mixed and centrifuged. Finally, the TCA extract (1ml) was taken and filtered by 0.22 um PVDF filter to vial for UHPLC-MS/MS analysis.

UHPLC-MS/MS analysis was performed by the NEXERA X2 UHPLC system (Shimadzu, Japan) connected to the QTRAP 4500 triple quadrupole mass spectrometer (AB Sciex, Canada). Separation of target compounds was performed on the Poroshell C18 column (150 x 2.1 mm, 2.7 μm) using acetonitrile (A) and 0.025% HFBA (B)

as mobile phases in gradient mode, different for TCA and ACN extractions. The flow rate of mobile phase was 0.3 mL/min., the injection volume - 5µL. For quantification, two product ions were monitored to ensure specific and accurate quantification. The information on ion transitions and optimal conditions for fragmentation of monitored antibiotics are provided in Table 1.

UHPL-MS/MS analysis of water

Water from breeding animal watering supply was analysed by UHPLC-MS/MS for 45 veterinary compounds belonging to 9 different antibiotic groups, including aminoglycosides (4), β-lactams (13), diaminopyrimidines (1), fluoroquinolones (10), lincosamides (1), macrolides (5), pleuromutilins (1), sulfonamides (6) and tetracyclines (4). The tested antibiotics are marked with an asterisk in Table 1 and Table 2.

Isolation of antimicrobial substances from water sample was based on extraction with sodium acetate and addition of ionic pairs followed by solid phase extraction (SPE) [16]. Concisely, to 250 ml of water 6 ml of 0.5 M sodium acetate pH = 5.6 and 30 µL of HFBA were added and shaken briefly for 5 min. Next, the sample was transferred on conditioned Strata-X SPE column. The analytes were eluted from the SPE with 3 mL of a mixture of acetonitrile : 0.05 M HFBA (9:1, v/v) and the eluate was evaporated to dryness. The dry residue was dissolved in 500 ul of 0.025% HFBA and filtered through 0.22 um PVDF syringe filters into LC vials.

UHPLC-MS/MS analysis of water was performed on the same instrument as the meat sample. However, chromatographic separation of analytes was performed on the Zorbax SB column (50 x 2.1 mm, 1.8 µm) using the same mobile phases but in different gradient mode. The flow rate of mobile phase was 0.7 mL/min., the injection volume - 5µL.

Table 1. List of analytes and mass spectrometry parameters for detection of antibacterial compounds

Class	Analyte	Precursor (m/z)	Products 1/2 (m/z)	DP (V)	CE 1/2 (V)
β-lactams	Amoxycillin*	366	349/208	45	14/18
	Ampicillin*	350	106/160	58	27/19
	Penicillin G*	335	160/176	60	17/19
	Penicillin V	351	160/114	54	17/48
	Oxacillin*	402	160/243	50	18/18
	Cloxacillin	436	160/277	50	20/20
	Nafcillin*	415	199/171	50	20/50
	Dicloxacillin*	470	160/311	50	20/20
	Cephapirin*	424	154/124	50	35/70
	Cefoperazone*	646	530/143	60	17/50
	Cephalexin*	348	158/106	50	10/23
	Cefquinome*	529	134/125	50	25/75
	Cefazolin*	455	323/156	50	15/23
	Cefalonium*	459	337/152	46	16/28
	Ceftiofur*	524	241/125	50	25/70
Sulfonamides	Sulfaguanidine	215	156/108	20	20/30
	Sulfadiazine	251	156/108	53	22/30
	Sulfathiazole*	256	156/108	53	20/34
	Sulfamerazine*	265	156/108	45	25/37
	Sulfamethazine*	279	156/108	50	25/36

	Sulfamethoxazole*	254	156/108	50	23/35
	Sulfamethoxypyridazine	281	156/108	60	25/35
	Sulfamonomethoxine*	281	156/108	50	23/37
	Sulfadoxine	311	156/108	60	25/40
	Sulfadimethoxine*	311	156/108	50	23/37
	Sulfaquinoxaline	301	156/108	50	23/40
Diaminopyrimidine	Trimethoprim*	292	231/262	52	33/36
Macrolides	Tylosin*	916	174/772	110	52/42
	Erythromycin*	734	158/576	75	42/27
	Spiramycin*	843	174/540	120	52/44
	Tilmicosin*	869	174/696	135	61/56
	Josamycin*	828	174/229	80	46/44
	Tulathromycin	806	577/158	95	37/59
Fluoroquinolones	Danofloxacin*	358	340/255	60	33/50
	Difloxacin*	400	382/356	50	30/28
	Enrofloxacin*	360	342/286	72	30/50
	Ciprofloxacin*	332	314/231	61	30/47
	Flumequine*	262	244/202	44	25/45
	Sarafloxacin*	386	368/348	50	31/46
	Marbofloxacin*	363	345/320	70	30/22
	Norfloxacin*	320	302/231	60	33/50
	Oxolinic acid*	262	244/216	53	25/40
	Nalidixic acid*	233	215/187	42	30/35
Pleuromutilines	Tiamulin*	494	192/118	128	30/56
	Valnemulin	565	263/164	45	20/40
4	Dapsone	249	156/108	67	21/31
Tetracyclines + epimers	Chlortetracycline*	479	444/462	56	31/25
	Tetracycline*	445	410/427	36	27/19
	Doxycycline*	445	428/154	55	25/42
	Oxytetracycline*	461	426/443	41	27/19
Aminoglycosides	Streptomycin*	582	263/246	166	45/52
	Dihydrostreptomycin*	584	263/246	150	42/53
	Gentamycin	478	322/157	44	22/31
	Paromomycin	616	163/293	112	49/33
	Spectinomycin*	351	333/207	67	27/32
	Kanamycin	485	163/205	70	35/36
	Neomycin*	615	161/163	109	46/33
Lincosamides	Lincomycin*	407	126/359	74	36/28

**- analytes tested in water, DP – declustering potential, CE – collision energy.*

Results and Discussion.

LC-MS/MS conditions and optimization

For all compounds (61 in muscle and 45 in water), the two most abundant product ions produced from each precursor ion were chosen as the ion transition in order to comply with the criteria needed for qualitative and

quantitative methods. According to the EU criteria, two MRM transitions were monitored. The characteristic MS/MS parameters: collision energy (CE), declustering potential (DP) and dwell time have been optimised separately for each analyte. The analyses were performed in the positive ionisation mode. Mobile phase compositions of methanol and acetonitrile coupled with formic acid or water including one of two ion-pair agents such as heplafluorobutyric acid and pentafluoropionic acid were tested. The use of the ion-pair chromatography was necessary because of the polar character of aminoglycosides The best results such as better aminoglycosides elution from chromatography column, satisfactory peak shapes and reproducible retention time were obtained with 0.025% HFBA combined with acetonitrile. Furthermore, the mobile phase gradient was also optimised in order to achieve the best chromatographic result with the minimum analysis time.

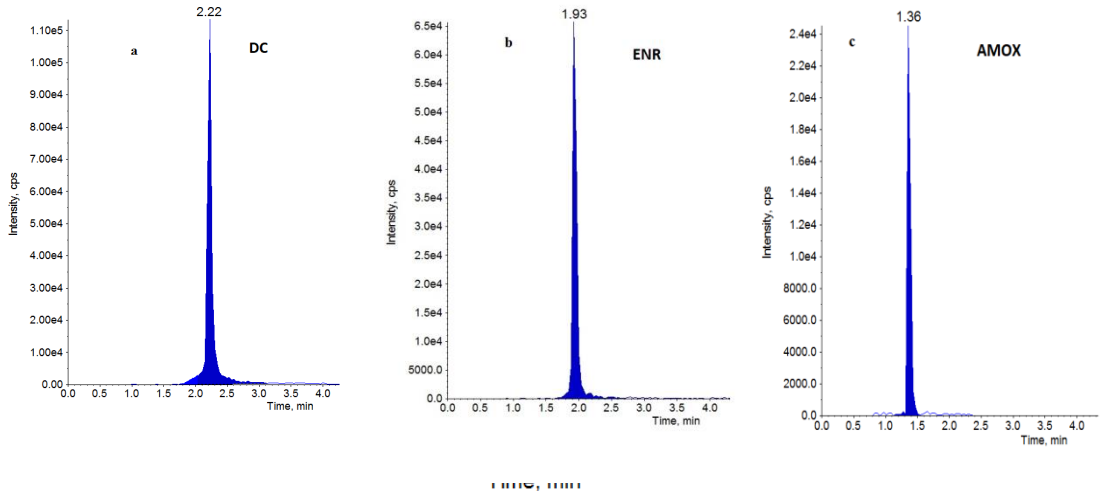

Fig. 1. Chromatograms of water samples with: a- doxycycline, b- enrofloxacin, c- amoxicillin at concentration 5 µg/L.

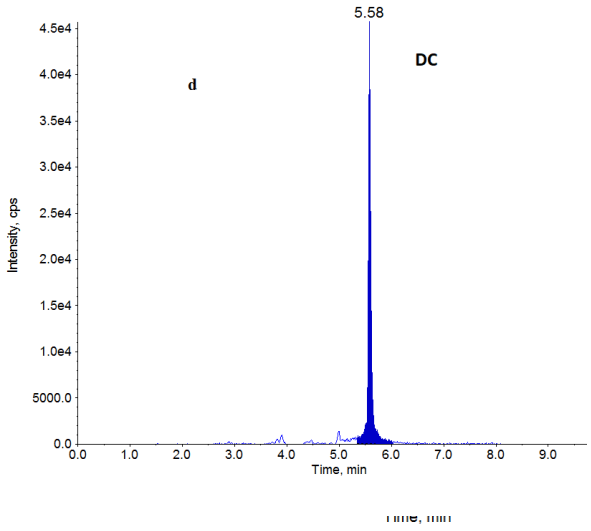

Fig. 2. Chromatograms of muscle samples with doxycycline at concentration 5 µg/kg.

Validation results

The method was validated according to the criteria of Commission Decision 2002/657/EC. Matrix matched calibration were used for quantification in order to reach a high accuracy. The method is linear in a wide range as confirmed by the correlation coefficient r > 0.99, where the lowest concentration on the calibration curve refers to the limit of quantification (LOQ). The recoveries ranged from 88% to 105% and within-laboratory reproducibility was lower than 15% (Table 2).

The validation results of the method are reported in Table 2. The matrix matched calibration curves achieved good linearity (r > 0.99). The recoveries are in the range between 84% and 109%, within-laboratory reproducibilities are below 14% and the LOQ values are in the range 0.02 – 10 µg/L, depending of analyte.

Table 2. Recoveries, reproducibilities and LOQs achieved during validation study on spiked muscle and water

Analyte	Muscle			Water		
	Recovery (%)	Reproducibility (%)	LOQ (µg/kg)	Recovery (%)	Reproducibility (%)	LOQ (µg/L)
Amoxycillin*	98	14.4	2	98	4.0	10
Ampicillin*	100	11.5	2	106	12.2	0.05
Penicillin G*	102	14.3	2	97	12.7	10
Penicillin V	99	13.1	2	-	-	-
Oxacillin*	98	7.2	2	90	9.3	0.05
Cloxacillin	100	7.0	2	-	-	-
Nafcillin*	101	6.7	2	100	9.2	0.05
Dicloxacillin*	100	7.0	2	105	8.7	0.05
Cephapirin*	92	9.3	25	92	13.6	0.05
Cefoperazone*	98	7.3	25	91	9.9	0.02
Cephalexin*	100	8.6	50	93	13.9	0.05
Cefquinome*	91	13.9	10	103	9.7	0.02
Cefazolin*	100	10.0	25	99	7.9	0.02
Cefalonium*	99	11.5	10	96	11.2	0.02
Ceftiofur*	98	4.6	50	103	8.8	0.05
Sulfaguanidine	88	9.3	5	-	-	-
Sulfadiazine	92	5.9	5	-	-	-
Sulfathiazole*	93	7.3	5	105	7.9	0.02
Sulfamerazine*	97	6.3	5	104	11.4	0.02
Sulfamethazine*	97	6.6	5	107	10.1	0.02
Sulfamethoxazole*	98	5.8	5	102	7.0	0.02
Sulfamethoxypyridazine	97	6.6	5	-	-	-
Sulfamonomethoxine*	99	7.3	5	91	9.1	0.02
Sulfadoxine	99	7.6	5	-	-	-
Sulfadimethoxine*	100	7.7	5	99	8.1	0.02
Sulfaquinoxaline	99	10.0	5	-	-	-
Trimethoprim*	99	9.6	5	95	12.5	0.05
Tylosin*	95	10.4	5	91	10.5	0.02
Erythromycin*	97	11.8	5	96	9.4	5
Spiramycin*	99	12.1	5	96	8.0	0.05

Tilmicosin*	99	13.1	5	103	12.2	0.05
Josamycin*	100	10.6	5	104	11.3	0.05
Tulathromycin	100	5.5	10	-	-	-
Danofloxacin*	95	11.6	5	96	8.3	0.02
Difloxacin*	98	12.0	5	95	6.1	0.02
Enrofloxacin*	98	10.3	5	89	7.1	0.02
Ciprofloxacin*	93	13.6	5	88	10.5	0.02
Flumequine*	105	11.9	5	102	10.2	0.02
Sarafloxacin*	100	13.9	5	84	9.1	0.02
Marbofloxacin*	93	9.3	5	86	10.9	0.02
Norfloxacin*	92	11.2	5	85	11.6	0.02
Oxolinic acid*	102	12.1	5	105	9.1	0.02
Nalidixic acid*	100	11.5	5	109	11.1	0.02
Tiamulin*	98	7.6	1	97	7.3	0.02
Valnemulin	100	11.8	5	-	-	-
Dapsone	94	8.7	0.5	-	-	-
Chlortetracycline*	97	13.1	5	99	10.5	0.05
Tetracycline*	100	13.6	5	96	8.0	0.05
Doxycycline*	100	13.2	5	96	13.3	0.05
Oxytetracycline*	96	14.0	5	99	12.9	0.02
Streptomycin*	96	9.7	25	96	7.1	1
Dihydrostreptomycin *	95	10.6	25	91	7.6	2
Gentamycin	99	13.2	25	-	-	-
Paromomycin	97	9.7	250	-	-	-
Spectinomycin*	96	11.4	100	94	7.6	1
Kanamycin	95	11.2	50	-	-	-
Neomycin*	102	11.3	250	97	6.8	10
Lincomycin*	95	10.2	5	99	8.5	0.02

**- analytes tested in water.*

Analysis of meat and water samples

Veterinary drugs were analysed in 880 muscles and 190 water samples. 585 muscle and 116 water samples from poultry farms were analysed. 295 and 74 pigs muscle and water samples were tested. Doxycycline was detected in muscle samples from 15 poultry farms, were antibiotics usage were documented and confirmed. Detected concentrations of doxycycline in post intervention (15.9- 70.8 µg/kg) were lower than concentration in pre intervention step (20.6- 100 µg/kg). In water samples, doxycycline in an average concentration of 119 µg/L in pre and 23.1 µg/L in post intervention, as well as enrofloxacin in an average concentration 170 µg/L in pre and 1.72 µg/L in post intervention were quantified. All water samples with antibiotics were from poultry. The obtained results confirm the effectiveness of the intervention actions. The concentrations of antibiotics in muscles were slightly lower after implementation health plan on farms. The differences in the levels of confirmed drugs are more evident in water samples. Seven water samples contained 3 antibiotics (doxycycline, enrofloxacin and amoxicillin) before intervention, while no antibiotics in muscles were found. After heath plan application, only enrofloxacin was detected with significantly lower levels. The obtained results were presented in Table 3.

Table 3. Average concentrations of antibiotics detected in muscle and water samples.

Farm	Muscle		Water					
	Doxycycline (μg/kg) average concertation		Doxycycline (μg/L) average concertation		Enrofloxacin (μg/L) average concertation		Amoxicillin (μg/L) average concertation	
	pre intervention	post intervention	pre intervention	post intervention	pre intervention	post intervention	pre intervention	post intervention
1.	80	70.8	13.1	6.3	6.3	ND	ND	ND
2.	30.7	28.2	315	1.8	20.4	ND	ND	ND
3.	42.2	30.1	42	ND	2.5	ND	ND	ND
4.	39.9	35.6	18.2	5.8	0.4	0.7	ND	ND
5.	23.6	49	14.1	ND	0.2	ND	ND	ND
6.	20.6	20	21.4	2.0	4.3	0.1	ND	ND
7.	24.4	20.8	40.5	2.1	7.7	ND	ND	ND
8.	20.8	40	405	216	3.8	9.3	ND	ND
9.	64.7	64.8	66	0.8	3.4	0.8	ND	ND
10.	67.6	39	15.6	0.5	0.3	0.05	ND	ND
11.	100	34.4	28.3	3.5	ND	ND	ND	ND
12.	49.2	23.6	64.9	1.3	1.0	ND	ND	ND
13.	83.4	48.6	42.6	0.9	0.3	ND	ND	ND
14.	36.5	25.1	774	12.6	20	0.6	ND	ND
15.	29.3	15.9	7.3	0.3	ND	0.2	ND	ND
16.	ND	ND	ND	ND	1.2	1.2	ND	ND
17.	ND	ND	12.5	ND	1880	ND	ND	ND
18.	ND	ND	ND	ND	0.7	ND	2475	ND
19.	ND	ND	ND	ND	49.2	0.08	ND	ND
20.	ND	ND	ND	ND	35.5	ND	163	ND
21.	ND	ND	ND	ND	13.3	ND	2926	ND
22.	ND	ND	ND	ND	5.1	0.2	ND	ND

ND – not detected

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H2020-SFS-46-2017 Healthy Livestock- Alternative production system to address anti-microbial drug usage, animal welfare and the impact on health, WP5-Validation of potential solutions - Analyses of anti-microbial residues in meat and water on pig and poultry farms)

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POSTER P42

METHOD DEVELOPMENT OF PROHIBITED VETERINARY DRUGS IN MILK

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Abstract
Chloramphenicol, nitrofuran metabolites (AOZ, AMOZ, SEM, AHD and DNSH), three nitroimidazoles (dimetridazole, metronidazole and ronidazole), chlorpromazine, dapsone and colchicine are prohibited veterinary drugs according to Commission Regulation (EU) 37/2010 (table 2). Previously six different methods were applied to analyse these compounds in milk. Therefore, an UPLC-MS/MS method was developed for the analysis of the compounds and five additional nitroimidazoles in bovine milk. The method consists of an acid hydrolysis combined with a derivatization procedure, followed by a supported liquid extraction (SLE) using an ethyl acetate elution. For the analysis of all analytes in a single run, the chromatographic separation was optimized and combined with a polarity switching mode. The method was developed for bovine milk with target values of 0.1 µg kg-1 for chloramphenicol, 0.5 µg kg-1 for the nitrofurans, 1 µg kg-1 for the nitroimidazoles, 2 µg kg-1 for chlorpromazine, 5 µg kg-1 for dapsone and 6 µg kg-1 for colchicine. This new method demonstrated that six different single compound(group) methods were successfully combined into a single multi compound method at low levels.

Introduction
The European Union established regulations to control and enforce veterinary drug use in food-producing animals (e.g. milk-producing bovine and caprine) (European Commission 2017) because of potential public health risks (Baynes et al. 2016). These risks include direct exposure to toxic veterinary drug residues and related metabolites and the chance of developing antimicrobial resistance in bacteria. Strict legislation regarding the application of veterinary drugs and their enforcement are established in the European Union: Regulation (EU) 2017/625 (European Commission 2017) and Commission Regulation (EU) No. 2010/37 (European Commission 2010). Commission Regulation (EU) No. 2010/37 (European Commission 2010) includes, in addition to maximum residue limits for registered veterinary drugs, a table with prohibited veterinary drugs, classified as A6 group in Council Directive 96/ 23/EC Annex 1 (European Commission 1996). In this abstract these substances are referred to as prohibited pharmacologically active substances (PPAS). The PPAS group evaluated in this study includes nitroimidazoles, nitrofurans, chloramphenicol, dapsone, chlorpromazine and colchicine.
Nitroimidazoles (NIZs) are prohibited because concerns have been raised about toxicity, mutagenicity and genotoxicity of this substance class (WHO 1987; Boechat et al. 2015). However, NIZs are important antiparasitic agents with a high biological activity (Boechat et al. 2015). The NIZs mentioned in Table 2 of Commission Regulation (EU) No. 2010/37 (European Commission 2010) are metronidazole (MNZ), ronidazole (RNZ) and dimetridazole (DMZ). In addition, the corresponding metabolites hydroxymetronidazole (MNZ-OH) and 2-hydroxymethyl-1-methyl-5-nitroimidazole (DMZ-OH) are included in this study. DMZ-OH is the hydroxyl metabolite of both RNZ and DMZ. Finally, ipronidazole (IPZ), hydroxy-ipronidazole (IPZ-OH) and ternidazole (TNZ) are also included in this study.
The second veterinary drug class of interest are the nitrofurans (NFs), including furazolidone, furaltadone, nitrofurantoin, nitrofurazone and nifursol. These substances are broad spectrum antimicrobial agents, with genotoxic and carcinogenic properties (EFSA 2015) and are therefore prohibited. The NFs rapidly metabolise to 3-amino-2-oxazolidinone (AOZ), 3-amino-5-methylmorpholino-2-oxazolidinone (AMOZ), 1-aminohydantoin (AHD), semicarbazide (SEM) and 3,5-dinitrosalicylic acid hydrazide (DNSH), respectively (Mologni et al. 2021). For that reason, instead of the native substances, the metabolites are included in the analyses of NFs in food products as mentioned in the Commission Regulation (EU) 2019/1871.
Four other relevant PPAS are chloramphenicol (CAP), dapsone (DAP), chlorpromazine (CPZ) and colchicine (COL). CAP is a broad-spectrum antibiotic and has been prohibited as results of concerns about its genotoxicity, embryotoxicity and fetotoxicity, carcinogenicity and the possible contribution to aplastic anaemia (JECFA 2004; Baynes et al. 2016). DAP is under debate because of its carcinogenic and genotoxic properties (EFSA 2005; EMA

2012). The lack of information about reproductive toxicity and teratogenicity has made DAP a PPAS since 1996 (EMA 1996b). CPZ belongs to the class of tranquillizers. Prohibition of CPZ is based on the advice of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) because of, among other things, the lack of relevant toxicological data (JECFA 1991; EMA 1996a). COL is an alkaloid isolated from Colchicum Autumnale. So it is plant based. COL is used in veterinary drug to treat papillomas and warts in cattle and horses. COL has a narrow therapeutic index, so overdosing is a significant risk that makes COL genotoxic even at low concentrations.
The EURLs responsible for the analysis of PPAS have created guidelines for the analysis of most of these substances in food, including minimum required method performance requirements (MMPRs) in all kinds of food products, including milk (EURL Guidance on MMPRs) (EURL 2020). The MMPR for the three prohibited NIZs including the corresponding metabolites is 1 µg kg−1 and for DAP 5 µg kg−1. The RPA for NFs are 0.5 µg kg−1 and for CAP 0.15 µg kg−1 as described in Commission Regulation (EU) 2019/1871 (European Commission 2019). For CPZ and COL in milk, no MMPR or RPA is established yet.
The analysis of PPAS requires highly sensitive, selective and accurate methods. In 2021 a paper has been published where five different single compound(group) methods in milk were successfully combined into a single multi compound method (Bongers et al.2021). The method described by Bongers et al.2021 was an ultra-high-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) method consists of an acid hydrolysis combined with a derivatization procedure, followed by a dispersive SPE-kit clean-up and double liquid-liquid extraction with ethyl acetate. For NFs analysis, a hydrolysis is required to include protein-bound residues and a derivatisation is applied to stabilise the metabolites and to enhance the mass spectrometric signal (Mologni et al. 2021).
This method development describes a new multi-class method where six (COL added) different single compound (group) methods in milk are combined into a single one. It is still a UPLC-MS/MS method that consists of an acid hydrolysis combined with a derivatization procedure, but this is now followed by a supported liquid extraction (SLE) using an ethyl acetate elution. For the analysis of all compounds in a single run, the chromatographic separation was optimized and combined with a polarity switching mode. This new approach is less time-consuming and therefore more-effective surveillance of the PPAS in milk. In addition, less sample material is used with the new method.

Materials and Methods

Reagents, chemicals and materials
Milli-Q water was prepared using a Milli-Q system at a resistance of at least 18.2 MΩ cm (Millipore, Billerica, MA, USA). Ultra LC-MS grade methanol, acetonitrile and ethyl acetate were obtained from Actu-All Chemicals (Oss, The Netherlands). Hydro chloric acid (37%), trisodium phosphate dodecahydrate, sodium hydroxide, acetic acid (100%) and formic acid (100%) were obtained from Merck (Darmstadt, Germany). 2-Nitrobenzaldehyde and ammonium formate were obtained from Sigma Aldrich (St. Louis, MO, USA). Supported liquid extraction (SLE) ISOLUTE SLE+ 1mL sample volume columns were obtained from Biotage Sweden AB. Hydrochloric acid solution (0.5 mol L−1) was prepared by diluting 4.3 mL hydrochloric acid (37%) in 100 mL Milli-Q.
2-Nitrobenzaldehyde solution (100 mmol L−1) was prepared by dissolving 1.51 g 2-nitrobenzaldehyde in 100 mL methanol. Trisodium phosphate solution (0.3 mol L−1) was prepared by dissolving 11.4 g trisodium phosphate dodecahydrate in 100 mL Milli-Q. Sodium hydroxide solution (2 mol L−1) was prepared by dissolving 8.0 g sodium hydroxide in 100 mL Milli-Q. Ammonium formate solution (1 mol L−1) was prepared by dissolving 6.3 g ammonium formate (1 mol L−1) in 100 mL Milli-Q. Mobile phases A and B were prepared by diluting 2 mL ammonium formate solutions and 160 µL formic acid to 1 L with Milli-Q and methanol, respectively.
The reference standards of MNZ, RNZ, DMZ, MNZ-OH, CAP, DAP, CPZ and COL were obtained from Sigma Aldrich. DMZ-OH, IPZ, IPZ-OH, TNZ HCl, AMOZ, AHD HCl and SEM HCl were obtained from Witega (Berlin, Germany). AOZ was obtained from Santa Cruz Biotechnology (Dallas, TX, USA). DNSH was obtained from Toronto Research Chemicals (Toronto, ON, Canada). The isotopic labelled standards MNZ-d4, DAP-d8, and DNSH-15N2 were obtained from Toronto Research Chemicals (Toronto, ON, Canada). RN Z-d3 and AHD-13C3 were obtained from Sigma– Aldrich (St. Louis, MO, USA). DMZ-d3, HMM NI-d3, IPZ-d3, IPZ-OH-d3, AOZ-d4, AMOZ-d5, SEM-13C15N2, CAP-d5, and CPZ-d6 HCl were obtained from Witega (Berlin, Germany)

Sample preparation
The procedure for the sample preparation is as follows: 500 ± 5 mg of homogenised raw milk was weighed into a 12 mL polypropylene tube and 20 µL of internal standard solution (15–250 µg L−1) was added. Subsequently,

500 µL 0.5M hydrochloric acid solution and 12.5 µL 100 mM 2-nitrobenzaldehyde solution were added. The sample solution was shaken head-over-head (Heidolph REAX-2, Schwabach, Germany), overnight at 37°C to hydrolyse protein-bound NF metabolites and to derivatise the metabolites into their nitrophenyl (NP)-derivatives. After cooling of the sample solution to room temperature, 125 µL of 0.3M trisodium phosphate solution and 75 µL of 2M sodium hydroxide solution were added to adjust the pH to 7.0 ± 1.0. Next, the sample solution was centrifuged for 10 minutes at 3500 g (MSE Falcon 6/300, Heathfield, UK). After centrifugation, the sample solution was applied to a 1 mL supported liquid extraction (SLE) column. The sample was let to distribute evenly over the column for at least 5 minutes. Then, the sample was eluted into a 12 mL glass tube with 2 times 2.5 mL ethyl acetate. The ethyl acetate was evaporated under nitrogen at 40°C (TurboVap LV Evaporator Zymark, Hopkinton, MA, USA), and the remaining part was reconstituted in 100 µL Methanol. Finally, the sample extract was transferred into a vial to facilitate analysis by LC-MS/MS.

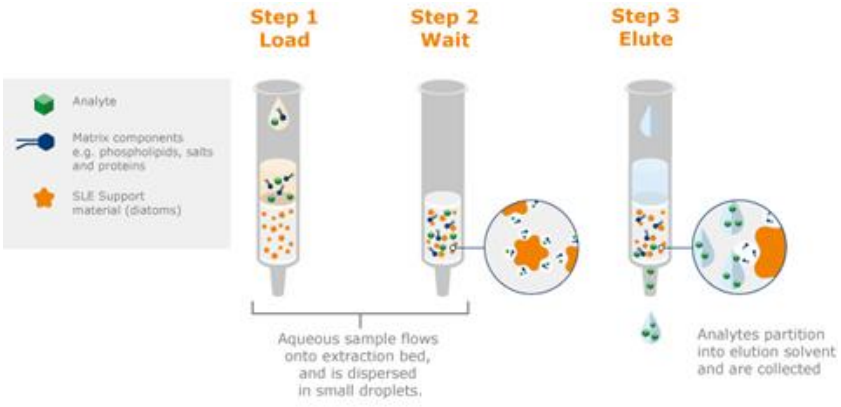

Figure 1: Principle of SLE extraction (Biotage, Stephanie J. Marin, Ph.D. et al.2020)

UPLC-MS/MS analysis

After sample preparation, the UPLC-MS/MS analysis was carried out on an Acquity UPLC (Waters, Milford, MA, USA) or a Nexera UHPLC (Shimadzu, Kyoto, Japan). An Acquity UPLC BEH C18 analytical column (Waters, Milford, MA, USA) of 100 × 2.1 mm with a particle size of 1.7 µm, was placed in a column oven at 35°C. Into the LC column, 2 µL of the sample extract was injected. The compounds were chromatographically separated by gradient elution using a flow rate of 0.4 mL min−1. The gradient started at 100% mobile phase A and linearly increased to 30% mobile phase B in 4 minutes. The next 2 minutes were isocratic, followed by an increase of the percentage mobile phase B to 70% in 1.5 min and to 100% in the next 1.5 min, with a final hold of 1.0 min and a requilibration time of 2 min at 100% A. After the chromatographic separation, the substances were introduced directly into a Q-Trap 6500+ or Q-Trap 6500 mass spectrometer (Sciex, Framingham, MA, USA). The mass spectrometer was operated in polarity switching mode (i.e., alternately in positive and negative electrospray ionisation mode). The operating parameters were curtain gas flow 40 psi (N2), nebulising gas flow 50 psi (N2), heater gas flow 50 psi (N2), source temperature 400°C and ion spray voltage (-) 4500 V. The precursor ions were fragmented to product ions using collision induced dissociation (N2). The scheduled Multiple Reaction Monitoring (MRM) transitions (60 s window in positive and 120 s window in negative mode) are presented in Table 1. Since no labelled internal standards were available for MNZ-OH and TNZ, DMZ-OH-d3 was used for these two substances. The response factors were calculated by dividing the area of the most abundant product ion of the substance by the area of the internal standard.

Table 4: MRM transitions of the PPAS and their internal standards, including the declustering potential (DP), collision energy (CE) and collision cell exit potential (CXP).

Substance	Ionisation mode	Precursor ion (m/z)	Product ion (m/z)	DP (V)	CE (eV)	CXP (V)
MNZ	+	172.0	128.0	26	19	16
			82.0	26	33	12
MNZ-d4	+	176.0	128.0	26	19	16
RNZ	+	201.0	140.0	36	15	14
			55.0	36	29	8
RNZ-d4	+	204.0	143.0	36	15	14
DMZ	+	142.0	81.0	36	35	10
			96.0	36	21	10
DMZ-d4	+	145.0	99.0	36	21	10
MNZ-OH	+	188.1	123.0	36	19	8
			125.9	36	23	6
DMZ-OH	+	158.0	140.0	31	17	14
			55.0	31	25	8
DMZ-OH-d3	+	161.0	143.0	31	17	14
IPZ	+	170.0	109.0	56	33	12
			124.1	56	23	8
IPZ-d3	+	173.0	127.1	56	23	8
IPZ-OH	+	186.1	122.0	41	27	12
			168.0	41	17	16
IPZ-OH-d3	+	189.1	171.0	41	17	14
TNZ	+	186.0	128.0	40	30	10
			82.0	40	30	13
NPAOZ	+	236.2	104.1	41	17	20
			134.1	41	17	20
NPAOZ-d4	+	240.2	134.1	41	17	12
NPAMOZ	+	335.3	262.3	60	15	10
			291.3	60	15	13
NPAMOZ-d5	+	340.4	296.3	60	15	13
NPAHD	+	249.0	104.0	60	27	4
			134.0	60	17	4
NPAHD-$^{13}C_3$	+	252.2	134.1	60	12	12
NPSEM	+	209.2	166.2	60	13	12
			192.2	60	15	12
NPSEM-$^{13}C^{15}N_2$	+	212.2	168.2	60	10	12
NPDNSH	-	374.0	183.0	-60	-30	-13
			226.0	-60	-30	-13
NPDNSH-$^{15}N_2$	-	376.0	181.9	-60	-30	-13
CAP	-	321.0	152.0	-35	-25	-12
			194.0	-35	-15	-12
CAP-d5	-	325.9	157.0	-35	-12	-12
DAP	+	249.1	107.8	46	29	12
			155.9	46	19	10
DAP-d8	+	257.0	160.0	46	20	10
CPZ	+	319.0	58.0	60	67	8
			86.0	60	25	12
CPZ-d6	+	325.0	92.0	60	25	12
COL	+	339.4	357.9	51	31	10
			309.9	51	35	10

Metronidazole (MNZ), ronidazole (RNZ), dimetronidazole (DMZ), hydroxymetronidazole (MNZ-OH), 2-hydroxymethyl-1-methyl-5-nitroimidazole (DMZ-OH), ipronidazole (IPZ), hydroxy-ipronidazole (IPZ-OH), ternidazole (TNZ), nitrophenyl derivative of 3-amino-2-oxazolidinone (NPAOZ), nitrophenyl derivative of 3-amino-5-methylmorpholino-2-oxazolidinone (NPAMOZ), nitrophenyl derivative of 1-aminohydantoin (NPAHD), nitrophenyl derivative of semicarbazide (NPSEM), nitrophenyl derivative of 3,5-dinitrosalicylic acid hydrazide (NPDNSH), chloramphenicol (CAP), dapsone (DAP), chlorpromazine (CPZ) and colchicine (COL).

Results and Discussion

Method development
Three different experiments were performed to develop a method for the analysis of PPAS in milk. The first experiment was carried out to see if it was possible to reach the new target values for the nitroimidazoles of 1 µg kg-1 and to add COL to the method. The single multi compound method (Bongers et al.2021) was used as the starting method. COL could be added to the method without any problems and the target value of 1 µg kg-1 for nitroimidazoles was easily achievable for all nitroimidazoles.

The next experiment was performed to see if reprocessing could be scaled down and simplified using supported liquid extraction (SLE). The experiment is performed by using the original method (Bongers et al.2021) but the sample volume is downscaled twice as a starting point. In the current experiment, the original method is also downscaled 40 times. Also downscaled 2 times in combination with 10 mL SLE column, downscaled 40 times in combination with 1 mL SLE column in a 48 well plate and 40 times downscaled in combination with 1 mL SLE column in a 48 well plate but without AOAC SPE kit (used in the original method). In all the reprocessing's where it has been downscaled 40 times, the results were poor. Which means it was downscaled too far. In addition, reprocessing was difficult due to the starting sample volume being too low. Reprocessing using a 10 ml SLE column gives promising results. In addition, processing with SLE is much faster and easier. The extracts also look cleaner.

The next step was therefore the optimization of the SLE reprocessing. For the optimization of the SLE workup the same method as the starting point was used again as in the previous experiment. The experiment was varied with 2 times and 4 times downscaling, with 1ml and 2 mL SLE column and with or without AOAC kit/Ethyl acetate extraction. Of course, the maximum volume of the SLE column had to be taken into account. This means that the amounts and concentrations of chemicals used during the reprocessing have been adjusted accordingly.
While reviewing the results, it was clear quite quickly that the processing hoped for also gave the best results. That was the one with 4 times downscaling with 1 ml SLE column without AOAC kit and without ethyl acetate extraction.

Conclusion
In this method development an UPLC-MS/MS method was developed for the analysis of the prohibited pharmacologically active substances (PPAS) in bovine milk. The PPAS group evaluated in this study includes nitroimidazoles, nitrofurans, chloramphenicol, dapsone, chlorpromazine and colchicine. The method was developed for bovine milk with target values of 0.1 µg kg-1 for chloramphenicol, 0.5 µg kg-1 for the nitrofurans, 1 µg kg-1 for the nitroimidazoles, 2 µg kg-1 for chlorpromazine, 5 µg kg-1 for dapsone and 6 µg kg-1 for colchicine. This new method demonstrated that six different single compound(group) methods were successfully combined into a single multi compound method at low levels.

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POSTER P43

CONFIRMATION OF FIVE NITROFURAN METABOLITES BY LC-MS/MS INCLUDING THE NIFURSOL METABOLITE IN FOOD-PRODUCING MUSCLE TISSUES ACCORDING TO REGULATION (EU) 2021/808

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Abstract

A liquid chromatography–tandem mass spectrometry method for the simultaneous confirmation of five nitrofuran metabolites in food-producing muscle tissues, and including the nifursol metabolite (DNSH), was developed and fully revalidated in accordance with the new Regulation (EU) 2021/808. The method is taking into consideration the enforcement to enter into force in Nov 2022 and lowering the reference point for action (RPA) to 0.50 µg.kg-1 under Regulation (EU) 1871/2019. The five nitrofuran metabolites investigated were AOZ, AMOZ, AHD, SEM, and DNSH respectively. The impact of the regulatory change has been assessed and determined for both qualitative and quantitative parameters, as regard to these prohibited substances. The validated data proved that the method is suitable for the confirmation of the five nitrofuran metabolites when implemented for official control in meat muscle tissues and in aquaculture products with CCα estimated below 0.50 µg.kg-1, corresponding to the enforcing RPA recently adopted by the European Commission.

Introduction

Nitrofurans (NFs) are synthetic, broad-spectrum antibiotics frequently used in food producing animals. They also have been largely used as growth promoters in the poultry and pig industries (Vass, Hruska & Franek, 2008). Because these compounds are persistent in the tissues of treated animals in the form of their protein-bound metabolite, and since toxicological studies has shown carcinogenic, teratogenic and mutagenic effects of both the parents compounds and their metabolites (Bogialli & Di Corcia, 2009; McCalla, 1983), the European Union (EU) has banned the veterinary use of these compounds in human food-producing animals because no acceptable daily intakes nor maximum residue levels (MRLs) have ever been established (European Commission, 1993, 1995, 2002b, 2009).

In order to ensure that all the EU Member States (EU-MS) have harmonised analytical methods for the control of prohibited substances, the European Commission (EC) finally adopted the minimum required performance limit (MRPL) concept in 2003, set at 1 $\mu g.kg^{-1}$ for NF metabolites, in accordance with the Decision (EC) No 2003/181 (European Commission, 2002a, 2003). More recently, EFSA delivered a scientific opinion on nitrofurans and their metabolites in food (EFSA Panel on Contaminants in the Food Chain (CONTAM), 2015). This report led the EC to strengthen the control of these substances by enforcing lower reference points for action (RPAs) set at 0.5 $\mu g.kg^{-1}$ for NF metabolites in accordance with the Commission Regulation (EU) 2019/1871 (European Commission, 2019). This Regulation impelled the national reference laboratories (NRLs) of the EU-MS, during the 2019-2022 period, to verify the performance of their methods in order to ensure that these substances are properly controlled at a level below or equivalent to the new adopted RPA right after Nov 2022 for the 2023 annual national residue control plans.

As EU reference laboratory (EU-RL), the Anses Fougeres Laboratory had recently developed and fully validated according to European Decision 2002/657/EC (European Commission, 2002a) a new confirmatory method for the control of the five nitrofuran metabolites by LC-MS/MS in muscle tissues (Guichard et al., 2020). The method is taking into account the new regulatory limit and includes the metabolite of the nifursol banned in the EU since 2005 (European Commission, 2002b). However, European Decision 2002/657/EC has been replaced by the Commission Implementing Regulation (EU) 2021/808 of 22 March 2021 dedicated to the performance of analytical methods for residues of pharmacologically active substances used in food-producing animals and to the interpretation of results (European Commission, 2021a). Methods validated before 10 June 2021 under 2002/657/EC have a transitional period of 5 years to comply with the CIR (EU) 2021/808 (European Commission 2021b). Due to the implementation of this new regulation, the performance of the methods

validated according to 2002/657/EC are still applicable but, must be re-validated under the new Regulation during the transitional period. In this context, the Anses Fougeres Laboratory updated and rechecked the performance of its confirmatory method for nitrofuran metabolites in meat (Guichard et al., 2020), according to the new Regulation (EU) 2021/808.

Materials and Methods

Raw data

As the method was fully validated under European Decision 2002/657/EC in 2019 (Guichard et al., 2020), the raw data from this validation was reused for applying the criteria defined in the new Regulation (EU) 2021/808. The validation plan was built on five different concentration levels (0.10 – 0.25 – 0.50 – 1.0 and 2.0 µg.Kg^{-1}) for each of the compounds, in muscle tissues from food-producing species and in flesh from aquaculture products.

Identification parameters

The signal-to-noise of all calibrating levels has been firstly checked to ensure that the ratio is greater than or equal to 3:1. The case of concentration level at 0.1 µg.kg-1 does not meet this criterion, therefore this level is not taken into account for the method validation.

The relative retention times and ion ratio tolerances have been changed under the new Regulation (EU) 2021/808. So, these two identification criteria have been rechecked taking into account the new tolerances of ± 1% for the relative retention time (all compounds have an analogous isotopically labelled internal standard) and ± 40% for relative ionic intensities, respectively.

Quantitative parameters

The quantitative parameters were checked using the Enoval 4.1® software (Pharmalex). Calibration curves are obtained from the five levels of spiked standard calibration samples (SC) or using only four levels (0.25 to 2.0 µg.kg^{-1}) when the first level at 0.10 µg.kg^{-1} does not respect identification criteria. Three first ranges of data from three first days of validation using pork muscle, poultry muscle and red meat to collect data from different muscle tissues from slaughter animals and poultry categories; and three additional ranges of data from three other days of validation using thin fish, oily fish and crustaceans to collect data from different fleshes from aquaculture product categories. A linear model was chosen for each of the analyte/matrix pairs. The coefficient of determination r^2 is verified as greater than or equal to 0.99.

Then, standard validation samples (SV) were analysed to determine the quantitative performances by means of back calculation of the SV concentrations at the same SC levels. Accuracy of measurements in terms of trueness and precision were estimated from the mean of six replicates for each concentration level, and compared to the acceptability limits defined in Table 1 and 2 of the Regulation (EU) 2021/808. Trueness is expressed in terms of relative bias according to the following formula: Relative bias (%) = ((estimated mean concentration – theoretical concentration) / theoretical concentration) x 100. The required performance limits for trueness are set from -50 % to 20% when the concentration level studied is below 1 µg.kg^{-1} and from -30 % to 20 % when the concentration level studied is between 1 to 10 µg.kg^{-1}. Precision is assessed by calculating the intra-day and inter-day coefficient of variation according to the Standard ISO 5725 (AFNOR., 1994). Precision includes repeatability and intra-laboratory reproducibility. The acceptable coefficient of variation presented in the new Regulation is set at 30 % but should be set as low as reasonably possible.

Limits of decision CCα are calculated according to the method 3 of parag 2.6.1.c of the Regulation (EU) 2021/808 concerning prohibited substances: CCα = LCL + k(one-sided, 99 %) x (combined) standard measurement uncertainty at LCL ; using a k factor of 2.33. Capability of detection CCβ for screening is defined as the first validated level, corresponding to method 2 of parag 2.7.1.b of the Regulation (EU) 2021/808 concerning prohibited substances.

Other performance characteristics such as stability and matrix effects were determined during the primary validation process and are in line with the new regulation. Only the specificity requires further data, because twelve different batches of blank matrices devoid of nitrofuran residues were tested during the first validation process, when twenty are now required.

Results and Discussion

Identification parameters

Signal-to-noise ratio (S/N)

All S/N were satisfactory except for AHD and SEM in aquaculture products and SEM in muscle tissues at 0.10 $\mu g.kg^{-1}$. SEM is the less sensitive substance to analyse due to its low molecular weight, even after derivatization and fragmentation the signal remains less selective than expected as for the other substances: the transition of SEM used for quantification is even the least intense; however, it was selected as it shows less noise and matrix interferences.

Retention time

All compounds successfully pass the new criteria for the relative retention time lower than 1% except for AHD and SEM at concentration level 1 in aquaculture products, were relative retention times vary from -3.38% to 2.93%, due to low peak intensities. Despite the regulatory changes, the validation levels for this criterion are kept identical for each of the compounds.

Ion ratio

All compounds successfully pass the new criteria, except at concentration level 1 for AHD in aquaculture products and SEM in aquaculture products and muscle tissues, again due to low peak intensities. On the other hand, new Regulation is less restrictive with regard to ion ratio tolerances, set at 40% regardless of the intensity of the base peak. Thus, some validation levels that did not meet this identification criterion are now valid under the new Regulation, such as concentration level 2 for AHD and SEM and concentration level 1 for AOZ, all in aquaculture products.

Globally, the identification criteria are fully respected for all compounds at each concentration levels except for three analyte/matrix pairs at 0.10 $\mu g.kg^{-1}$: AHD and SEM in aquaculture products and SEM in muscle tissues. LCL are thus established according to Table 1.

Table 1. Lowest Calibrated Levels (LCL) fully validated based on analyte/matrix pairs.

Compound	Muscle tissues	Aquaculture products
AOZ	0.10	0.10
AMOZ	0.10	0.10
AHD	0.10	0.25
SEM	0.25	0.25
DNSH	0.10	0.10

Quantitative parameters

Calibration curves

The calibration curves have been established according to the Table 1 from the LCL to the 2.0 $\mu g.kg^{-1}$ level. All linear regressions were satisfactory with coefficient of determination r^2 greater than 0.99 whatever the analyte/matrix pair studied.

In our previous validation process, linear regressions were built using only four of the concentration levels studied. Here, the calibration range was extended to the fifth concentration level at 2.0 $\mu g.kg^{-1}$ when LCL is set to 0.10 $\mu g.kg^{-1}$. These differences are reflected in different accuracies of measurements and in different limits of decision CCα.

Accuracy of measurements in terms of trueness and precision

Trueness

All compounds reached the trueness criteria whatever the concentration level starting from the LCL and the meat/flesh category considered. Values are ranged from -16.9 % to 7.7 %, lower and higher values corresponding to SEM in muscle tissues and AOZ in aquaculture products, respectively. Results, presented in Table 2, are slightly higher than those from our previous validation process where they ranged from -13.5% to 8.2% for SEM in aquaculture products and AMOZ in muscle tissues, respectively. Regarding the specific cases of SEM and AHD, it is important to stress that these compounds could be validated at 0.25 $\mu g.kg^{-1}$ instead of the previous 0.50 $\mu g.kg^{-1}$ level in aquaculture products and thanks to the new tolerance of the ion ratios which was extended to 40%. It is therefore logic for these compounds to reach a greater bias.

Precision

In terms of intra-laboratory reproducibility, the precision remains fully compliant for all compounds and in all meat/flesh categories from the LCL to the 2.0 $\mu g.kg^{-1}$ levels. Values, presented in Table 2, ranged from 3.10 %

to 28.22 % from AMOZ in muscle tissue to SEM in aquaculture products, respectively. As well as for the trueness of the method, values are similar to those of our previous validation process, except for AHD and SEM validated at 0.25 µg.kg^{-1} instead of the previous 0.50 µg.kg^{-1} level in aquaculture products; and except for AOZ for which the LCL could be lowered to 0.10 µg.kg^{-1} in aquaculture products.

Limits of decision for confirmation (CCα) and capability of detection for screening (CCβ)

The capability of the detection for screening, CCβ, is estimated at 0.10 µg.kg^{-1} for most of the analyte/matrix pairs, except for SEM in both meat/flesh categories and for AHD in the aquaculture products flesh were values are set to 0.25 µg.kg^{-1}.

The limits of decision for confirmation, CCα, are calculated according to the LCL defined in Table 1. Results are presented in Table 2. Most of the estimated values are below 0.15 µg.kg^{-1} with the best result for AHD in muscle calculated to 0.117 µg.kg^{-1}. Other values range from 0.318 to 0.434 µg.kg^{-1} for SEM in muscle tissues and in aquaculture products, respectively. All results are satisfactory with values strictly below 0.50 µg.kg^{-1}, corresponding to the new RPA set for nitrofurans by the Commission Regulation (EU) No 2019/1871. These results are slightly different from those determined in our previous method validation process where was used a different calculation based on the Standard ISO 11843-2 (AFNOR., 2000) method; corresponding to the method 1 of paragraph 2.6.1.a under the new Regulation. The new estimated CCα values raised to 1.7 to 4.0 times higher than previous ones. Thus, it has to be stressed that if the method 3 of paragraph 2.6.1.c of CIR 2021/808 is used for calculation of CCα, the choice of the LCL for prohibited substances as far as possibly analytically low is of utmost importance and must be chosen, in all cases, enough below the regulatory level (RPA or MMPR) set or recommended for these prohibited substances.

Table 2. Results of the validation process concerning quantitative parameters.

Meat/flesh category		Muscle tissues					Aquaculture products				
Analyte		DNSH	AMOZ	AOZ	AHD	SEM	DNSH	AMOZ	AOZ	AHD	SEM
Trueness : Bias (%) from 0.10 to 2.00 µg.kg^{-1}	0.10	-0.77	7.50	4.70	-2.48	ND[a]	-2.36	-0.67	7.70	ND[a]	ND[a]
	0.25	-0.19	3.24	0.94	-3.02	-16.93	-4.94	-4.59	-3.37	-7.74	1.21
	0.50	2.25	6.04	3.01	-0.66	-7.41	-5.26	-7.55	-4.80	-4.11	-5.90
	1.00	-5.33	0.58	-5.00	-5.65	-7.09	-8.59	-10.54	-7.36	-6.49	-9.40
	2.00	-1.99	3.46	0.68	-0.18	-1.51	-5.27	-7.68	-6.07	-5.63	-5.55
Precision : CV_R (%) from 0.10 to 2.00 µg.kg^{-1}	0.10	16.61	10.44	13.51	6.82	ND[a]	9.89	12.61	14.9	ND[a]	ND[a]
	0.25	5.45	3.10	6.28	5.99	10.70	4.78	6.21	7.19	11.55	28.22
	0.50	6.05	4.12	7.76	6.28	4.04	7.78	6.92	5.77	3.18	4.17
	1.00	7.12	6.83	8.54	8.97	8.56	9.22	8.49	7.89	6.20	7.73
	2.00	5.87	4.42	6.87	6.80	4.40	9.79	8.93	8.17	9.23	8.80
Uncertainty of measurement (%) from 0.10 to 2.00 µg.kg^{-1}	0.10	18.88	11.70	15.42	7.30	ND[a]	11.15	14.28	15.99	ND[a]	ND[a]
	0.25	6.15	3.25	7.06	6.82	11.74	10.06	6.69	7.88	12.88	31.62
	0.50	6.79	4.33	8.88	7.17	8.49	17.75	7.89	6.45	3.52	4.28
	1.00	7.80	7.33	9.65	10.24	19.49	20.84	9.51	8.82	6.87	8.39
	2.00	6.60	4.93	7.72	7.57	9.67	22.42	10.18	9.30	10.49	10.03
CCα (µg.kg^{-1})		0.144	0.127	0.136	0.117	0.318	0.126	0.133	0.137	0.325	0.434
CCβ = LCL (µg.kg^{-1})		0.10	0.10	0.10	0.10	0.25	0.10	0.10	0.10	0.25	0.25

[a] ND : Not Determined

Conclusion

The method previously fully developed end validated under European Commission 2002/657/EC was fully re-validated according to the new Regulation (EU) 2021/808. The identification parameters were not problematic and successfully passed the new criteria. Important to stress that each analyte is thankfully supported with its own isotopically labelled internal standard. The 1% new criterion on the retention time is therefore perfectly respected. Moreover, the tolerance of the relative ion ratios having been extended to 40%, it was possible to extend the validation range of the method for AOZ in aquaculture products down to 0.10 µg.kg^{-1} and for AHD and SEM in aquaculture products down to 0.25 µg.kg^{-1}.

Regarding the quantitative parameters, all criteria are met. The extension of the validation range, lowering the LCL from 0.50 to 0.25 µg.kg^{-1} for SEM and AHD in aquaculture products was crucial in order to obtain CCα

values below the current RPA of 0.50 µg.kg^{-1}. Finally, the use of the new CCα calculation method (method 3 of 2.6.1.c of CIR 2021/808) with estimation against the LCL and taking into account the combined standard uncertainty demonstrates that, the choice of the lowest LCL is crucial in order to obtain a satisfactory regulatory CCα.

Acknowledgements
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POSTER P44

VETERINARY DRUG ANALYSIS: THE ROLE OF ISOMERS AND METABOLITES

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Abstract

Recent issues have shown that isomers and/or metabolites can influence the (in)correct identification or quantification of veterinary drugs. One of the most well-known examples are the isomers chloramphenicol versus dextramycin. An inventory showed that for almost all veterinary drug groups isomers/metabolites occur, in particular in the groups of the macrolides, coccidiostats, avermectins, and aminoglycosides. Even though validation protocols demand a thorough selectivity study, the analytical consequences of the presence of metabolites and/or isomers on the enforcement of Maximum Residue Limits (MRLs) remain largely unknown. To assess this a literature review was performed per veterinary drug group. The main goal of this review was to determine the analytical consequences of possibly present metabolites and/or isomers on the analysis of the marker molecules. For each individual compound, an assessment was carried out focussing, amongst others, on the production, the similarities and differences, the metabolism and the possible degradation products of all metabolites and isomers. The collected information was used to assess whether these compounds could interfere in the official analysis, e.g. by yielding false-positive findings. Recommendations to prevent such interferences are given. Findings will be presented.

Introduction

In recent years, it is shown that the presence of isomers and/or metabolites of veterinary drugs can affect the identification of compounds present in a sample. If not handled with care, results of a substance may be reported incorrectly and subsequent enforcement may be incorrect. An example that has occurred internationally is the reporting of the banned antibiotic chloramphenicol, while an unregistered isomer of chloramphenicol (dextramycin) was found to be present. Similar false positive results can result in false accusations and wrongful consequences, which should be avoided. This example illustrates the importance of thorough research into isomers, metabolites, and other potentially interfering substances and the possible risks they pose for MRL enforcement.

WFSR has conducted a study to investigate the analytical consequences of possibly present metabolites and/or isomers on the analysis of the marker molecules. For each individual compound, an assessment was carried out focussing on the production, the similarities and differences, the metabolism and the possible degradation products of all metabolites and isomers. The collected information was used to assess whether these compounds could interfere in the official analysis, e.g. by yielding false-positive findings. This study showed that isomers and/or metabolites occur for almost all groups of veterinary drugs.

Here, two cases are presented that illustrate the analytical challenges when it comes to isomers and/or metabolites in official analyses. Each of these cases poses different challenges and were chosen to demonstrate the importance of being aware of the possible issues that can arise when (un)knowingly dealing with isomers and/or metabolites. First, the possible degradation of D-ampicillin to L-ampicillin during sample handling will be highlighted. Second, the importance of continuous quality control on purchased analytical standards is illustrated based on the case of 3-O-acetyltylosine.

Results and Discussion

D-ampicillin and L-ampicillin

An interesting case of stereoisomers was identified in D-ampicillin and L-ampicillin (see figure 1). Findings resulted in the suspicion that D-ampicillin is converted to L-ampicillin in bovine urine. In literature it is described that this conversion can occur at pH ±7. Therefore, it is important to understand what is happening during sample preparation. If indeed this conversion takes place in the matrix or during sample preparation or extraction, it is important to be aware of this. The absence of D-ampicillin in a sample, without also screening for L-ampicillin, may result in the reporting of false-negative results.

To rule out that this conversion is taking place during routine analyses performed by WFSR, blank matrices (cheese, milk, kidney and muscle) were spiked with D-ampicillin. The samples were then analysed both with and without derivatisation to see whether conversion had taken place. This experiment showed that during the applied method no conversion of D-ampicillin to L-ampicillin is taking place. However, it is still important to keep the possibility of the conversion reaction in mind. Especially if changes will be made to a method or if the method is applied to other matrices.

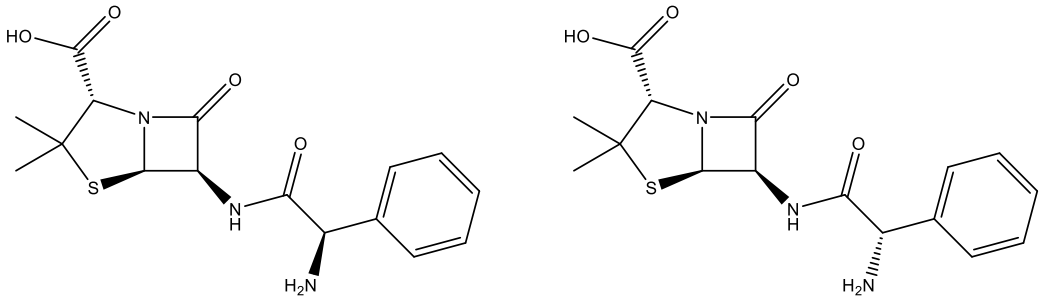

Figure 1. Structural formulas of D-ampicillin (left) and L-ampicillin (right).

3-O-acetyltylosine

After analysing two analytical standards of 3-O-acetyltylosine that were purchased from different suppliers (P1 and P2), it was noted that the standards produced similar product ions but with a difference in ion ratio. Moreover, a slight difference in retention time was observed of 0.01 min between the two standards. Following this, both standards were analysed for identification using liquid chromatography coupled with high resolution mass spectrometry. Identification of the compounds was achieved based on retention times, isotope patterns and fragments ions.

The chromatography shows a similar retention time between the two standards, with a small difference in retention time of 0.04 min. However, a difference was observed in their fragmentation spectra. The obtained fragmentation spectra were studied and the proposed fragmentation pattern is shown in figure 2. In short, the fragmentation starts with the cleavage of the glycosidic bond between the mycaminose and mucarose at the latter's side resulting in a loss of 144 Da and 186 Da for P1 and P2, respectively. Consequently, the product ions at *m/z* 814 was formed in P1 and at *m/z* 772 for P2. Next, the product ions of P1 and P2 lost the mycaminose sugar (174 Da) because of the cleavage of the glycosidic bond between mycinose and the tylonolide ring at the sugar's side to yield product ions at *m/z* 640 and *m/z* 598, respectively. This fragmentation is followed by the cleavage of the glycosidic bond between the mycaminose sugar and the C-5 hydroxyl group of the tylonolide ring, resulting in the formation of aglycones with *m/z* 449 for P1 and *m/z* 407 for P2.

The fragmentation described above shows that the fragments formed in P1 are consistently 42 Da higher compared to the fragments formed in P2, which could be explained by a difference in position of the acetyl group between P1 and P2. Therefore, it was concluded that the acetyl group is positioned in the mycarose sugar in P2, whereas in P1 the acetyl group is attached to the tylonolide ring. This means that P1 is 3-O-acetyltylosine. This finding highlights the importance of a thorough quality control of newly obtained analytical standards. In addition to the retention time and product ions, attention should also be paid to a correct ion ratio compared to a reference standard.

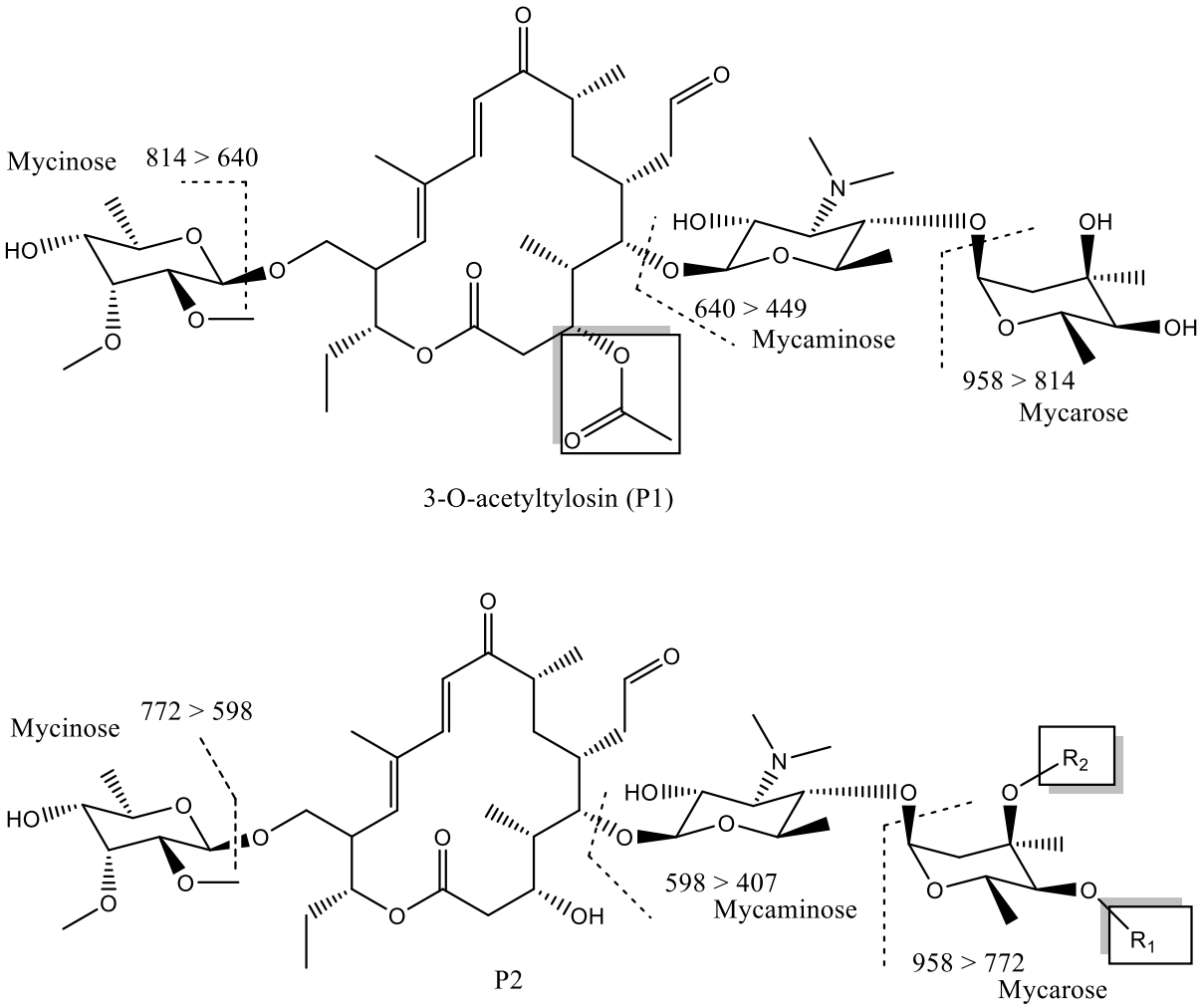

Figure 14. Structural formulas of 3-O-acetyltylosine (top) and P2 (bottom). The proposed fragmentation route is indicated with the dotted line. The difference in functional groups is shown using a black outline. The position of the acetyl group is positioned in the mycarose sugar in P2, positioned at either R1 or R2.

Conclusion

WFSR has conducted a study to investigate the analytical consequences of possibly present metabolites and/or isomers on the analysis of the marker molecules of veterinary drugs. Here, two cases are presented that illustrate the analytical challenges when it comes to isomers and/or metabolites in official analyses. These examples illustrate the importance of thorough research into isomers, metabolites, and other potentially interfering substances and the possible risks they pose for MRL enforcement.

Acknowledgements

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POSTER P45

WORKFLOW FOR THE IDENTIFICATION OF 'UNKNOWN' SUBSTANCES IN FEED AND FOOD

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Abstract
Regularly biological effects or instrumental signals are observed in food or feed samples that cannot be explained by the presence of a chemical substance that is included in target mass spectrometric methods. It is desirable to further identify those signals or effects, thus facilitating risk-based monitoring. This extended abstract presents a workflow for identification of unknown compounds responsible for observed effects or signals. The workflow is based on multi-disciplinary approaches. These include analytical chemistry (mass spectrometry, NMR), biochemistry (biosensors, DNA analysis), physical chemistry (microscopy) and knowledge of bioactive compounds.
The starting point of the search for 'unknowns' in the workflow can be threefold: (1) a case from the field, for example negative health effects, (2) an observed effect in a bioassay and (3) a suspect signal in a chemical analysis, e.g. high resolution mass spectrometry. The developed workflow was first evaluated using two test cases. In both test cases, the identity of the 'unknown' was successfully confirmed, demonstrating applicability for food and feed samples and supplements. Subsequently, the workflow was successfully applied to real sample, demonstrating an until-then unidentified fraud case. We experienced that the success rate of identification of an unknown compound increases when multidisciplinary research is applied in which biochemistry, analytical chemistry and physical chemistry are combined.

Introduction
A frequently used strategy for food and feed safety is the targeted monitoring of known substances. Consequently, unexpected or new substances that might pose a risk for food and feed safety will not be detected. [1] Within WFSR, a workflow was developed for identifying 'unknown' issues. These issues may be suspect food or feed samples for which an unexpected observation (activity, effect, signal) was done, that could not be explained within the targeted analysis scope. For example a positive result from a screening analysis, that could not be confirmed with the corresponding confirmatory analysis.

A central role in the workflow is reserved for the technique of high-resolution mass spectrometry (HRMS) (letters p and q in figure 1). HRMS is important in the identification of unknown masses. The dataset consisting of full-scan, and sometimes fragment mass spectra, is mined for unknowns using systematic workflows. Spectra are matched to broad on-line databases, such as NIST and PubChem. Systematic workflows for unknown analysis based on HRMS data have been published [2,3]. Hits may be tentatively identified by comparison with databases [4] and/or in-silico simulation of retention time and fragmentation [5, 6]. HRMS-based identification of unknowns has been combined with other techniques, such as NMR or bioassays. Literature shows some examples of unexpected veterinary drug, hormone and/or pesticide residues in food matrices that were successfully identified using HRMS untargeted approaches [7,8,9]. For evaluating the overall efficacy of a workflow for identification of unknowns a test set of compounds spiked to the matrix might be used. We have used such workflows, combining untargeted HRMS with fractionation and anti-microbial activity test. Using such workflow, we identified the presence of didecyldimethylammonium chloride, a substance showing antimicrobial activity in animal feed. Next, successfully detecting and identifying spiked substances in two similarly designed test cases [10]. Similarly, Fu *et al* detected 17 model compounds from different classes in fish, and identified an unexpected residue (difloxacin) by an untargeted approach [3]. The HRMS data play an essential role in order to prioritize hits and decide on the subsequent steps. Jansen *et al* worked on acquisition and data-processing parameters in the suspect screening of veterinary drugs in archive matrices using LC-HRMS. The optimised suspect-screening method proved to be fit for the purpose of finding veterinary drugs in feather meal. These veterinary drugs are not in the scope of the current monitoring methods. The suspect screening method gives added value in the perspective of a risk-based monitoring [11].

Key to solving unknown issues are multidisciplinary approaches. Important for estimating the relevance of hits is the coupling between HRMS spectra and biological properties of the molecules, such as anti-microbial or hormonal activity, by e.g. screening and fractionation approaches. Proposals have been made for risk assessment based on 'unknown' analysis-workflows. Here such a workflow based on multi-disciplinary approaches is presented. This includes microscopy, biochemistry, analytical chemistry and physical chemistry.

Workflow

The developed workflow is presented in figure 1. The starting point of the search for 'unknown' substances may vary. We assume 3 starting points: (a) an observed effect in a bioassay of other screening test and (b) a case from the field, for example an observed negative health effect (c) a suspect signal in a chemical analysis, e.g. high resolution mass spectrometry.

Every case starts with a case manager assembling a multidisciplinary team to solve the case. First, the case manager collects information about the specific case. Next, meetings are planned in which the case is discussed with colleagues from different disciplines. After these meetings and information collection, a multidisciplinary team is assembled.

An interactive scheme, of which figure 1 shows a 2D impression, helps the team to make decisions and provides information. Every button (marked with a letter) provides practical information. The information consists for instance of operational procedures, articles, expert names etc.. Every step (click) made in the scheme is documented in a standard report format. When closing a case, a report is created showing the applied approach, expert discussion, results and evaluation of the approach. A structured report will facilitate to solve cases in the future more efficient.

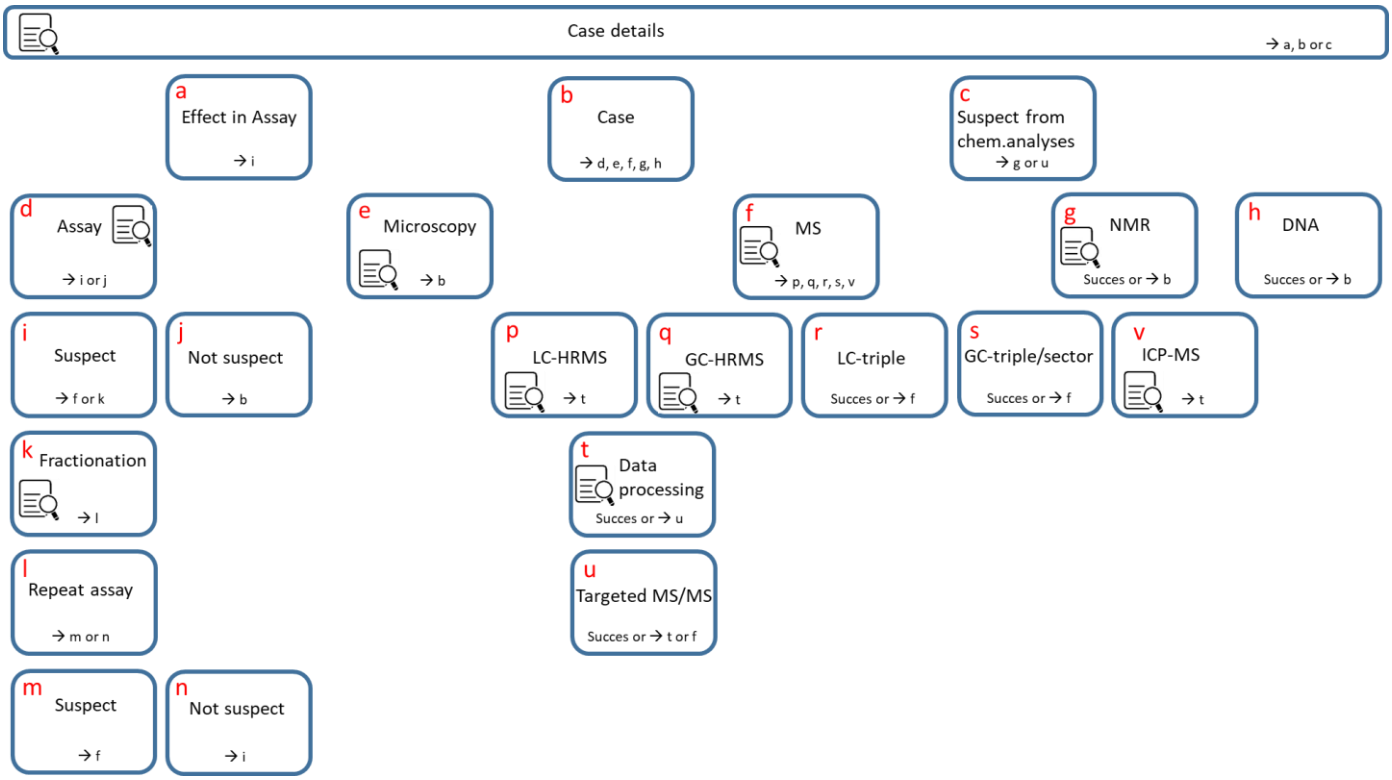

Figure 1. 2D impression of the interactive workflow

Results

The developed workflow was applied for solving of 'unknown' issues in food and feed samples. First, the workflow was evaluated using two artificial example cases. In both example cases, the identity of the

'unknown' was successfully confirmed, demonstrating applicability for real food and feed samples and supplements.
Figure 2 shows the workflow path taken to solve one of the artificial cases.

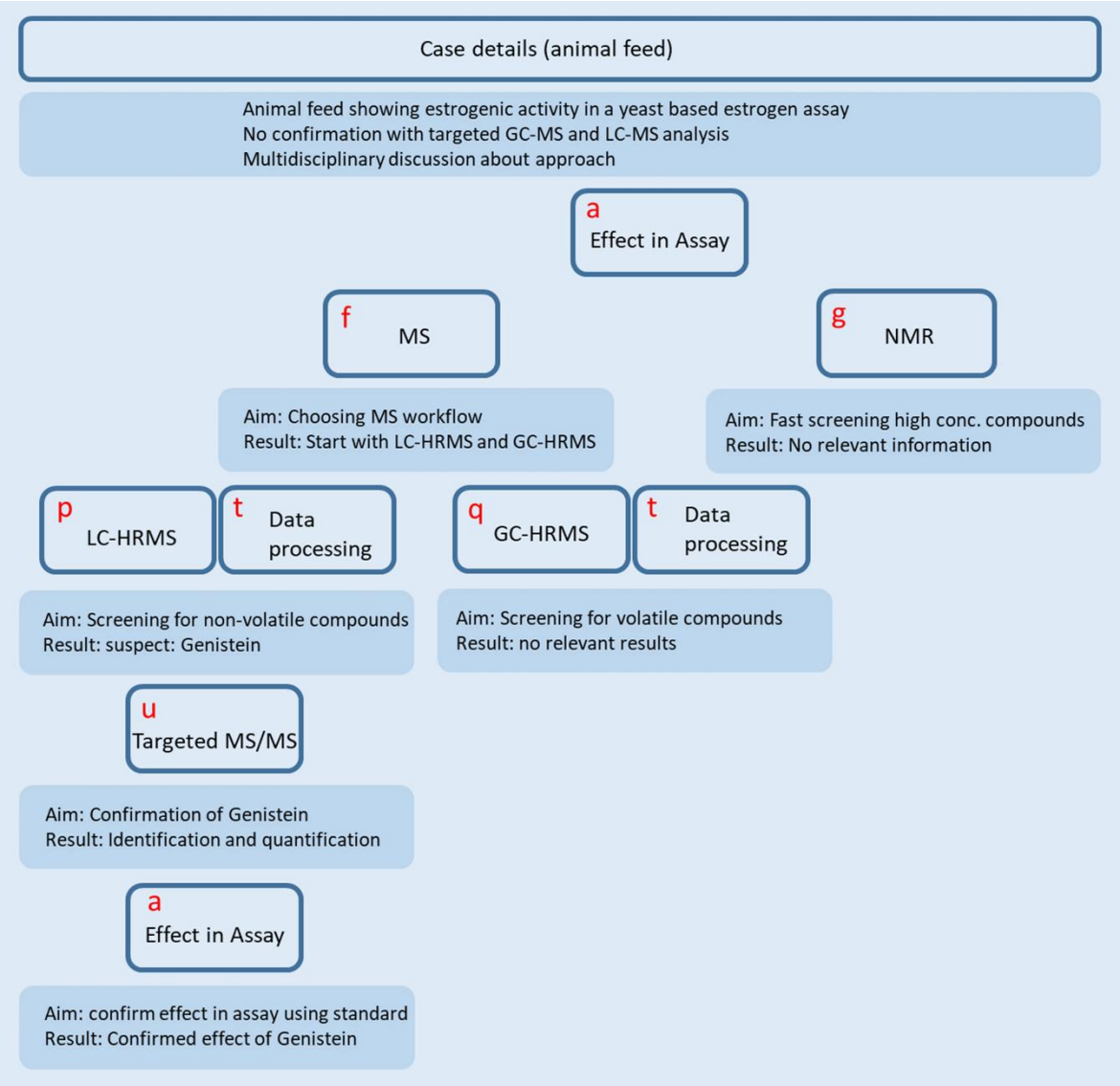

Figure 2. workflow path, solved artificial case

Subsequently, the workflow was successfully used to real samples. Here an example is given for the analysis of a fishmeal sample showing antimicrobial effects. This case yielded the detection of an until-then unknown fraud case. Figure 3 shows the workflow path of the real sample.

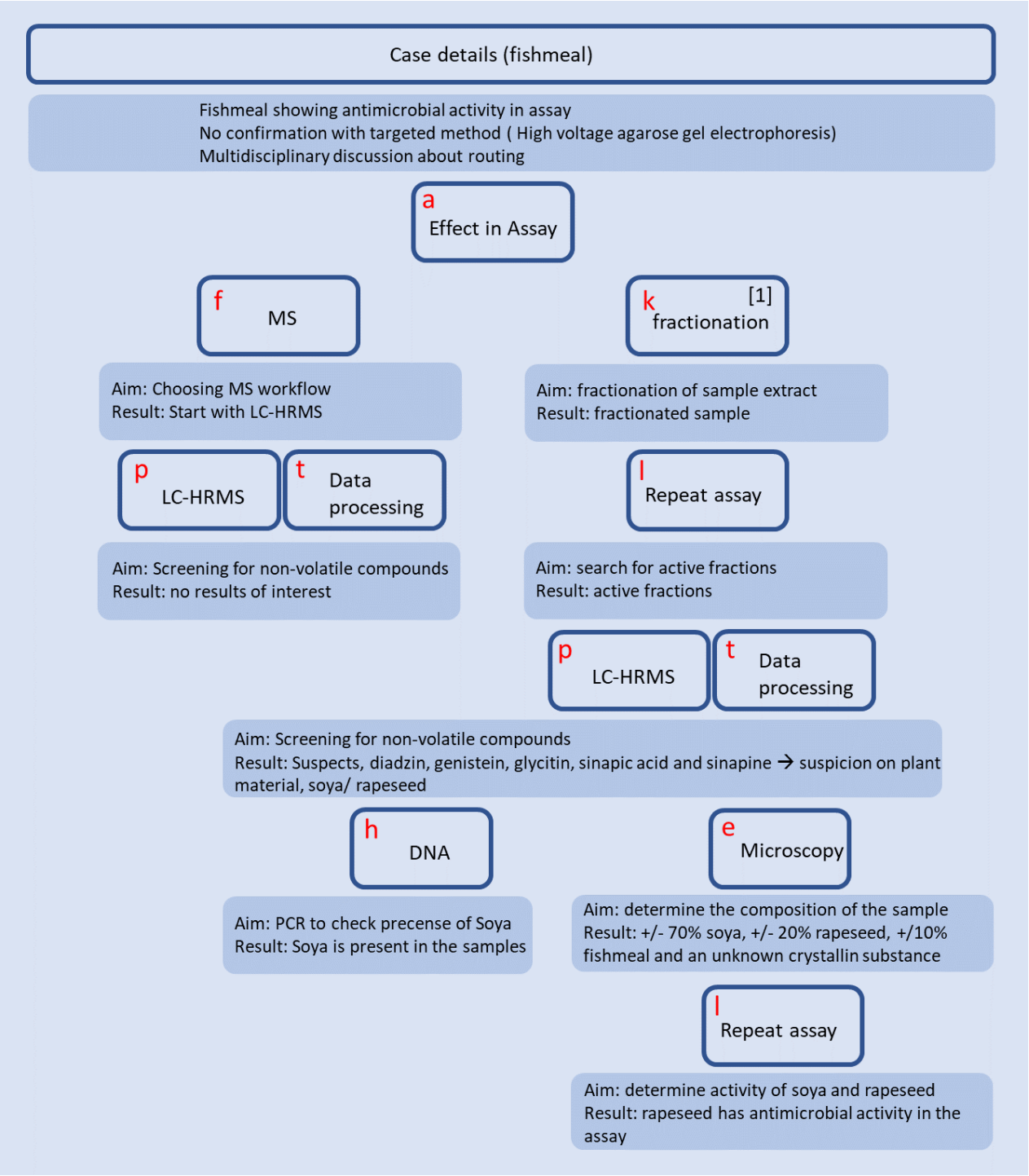

Figure 3. workflow path, real case

Conclusion

The identity of the 'unknown' substance(s) in the artificial case was successfully confirmed. It was confirmed that a large fraction of the "fishmeal" sample consists of soya and rapeseed (fraud) instead of fish. It was confirmed that the antimicrobial activity shown in the assay can be explained by the presence of rapeseed. We

experienced that the success rate of identification of the unknown substance increases when multidisciplinary research is applied in which biochemistry, analytical chemistry and physical chemistry are combined.

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POSTER P46

SYSTEMATIC ASSESSMENT OF ACQUISITION AND DATA-PROCESSING PARAMETERS IN THE SUSPECT SCREENING OF VETERINARY DRUGS IN ARCHIEVE MATRICES USING LC-HRMS

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Abstract

Effective application of screening strategies is hampered by complex data interpretation and therefore targeted data analysis is commonly applied. In this study, the performance of a suspect screening approach using a dedicated suspect list and online spectral database mzCloud™ was explored to facilitate detection of antibiotics in archive matrices.

Different data evaluation parameters were studied and results show that application of a narrow mass tolerance of 1.5 ppm had a negative effect on the number of false positives, as does omission of retention times in the suspect list. Using mzCloud™, the quality of the fragmentation data proved to be a critical factor and thus data dependent acquisition is preferred. The advantage of mzCloud™ is that no retention time data is required, eliminating the need for access to a reference standard for screening.

A proof of principle was carried out on feather meal samples and fourteen different antibiotics were detected and confirmed of which three were not included in the suspect list. The suspect screening method proved to be fit for the purpose of finding veterinary drugs which are not in the scope of the current monitoring methods and therefore is of added value in the perspective of a risk-based monitoring.

Introduction

For years, targeted analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS) has been used to monitor specific veterinary drugs in products of animal origin. Now, monitoring strategies start shifting towards a non-targeted, more risk-based approach, as is mandatory according to the official controls regulation (EU) 2017/625 (European Commission, 2017). Part of such a risk-based approach is not only targeting known substances, but also searching for unexpected substances. This shift has pushed the utilization of high resolution mass spectrometry (HRMS) and suspect screening, an approach for processing untargeted data in which reference standards are not a necessity (Caballero-Casero *et al*., 2021; Pourchet *et al*., 2020).We advocate that applying suspect screening to archive matrices, in which residues can be detected longer, could greatly advance the early detection of the application of unexpected veterinary drugs. Studies that investigate the performance of suspect screening workflow strategies for the detection of veterinary drugs in complex matrices are however still scarce.

In the current research, two suspect screening data processing workflows were designed to facilitate detection of potential unexpected veterinary drugs in the complex matrices animal feed and feather meal; a workflow based on an in-house created suspect list and a workflow using the online spectral database mzCloud™. Several parameters that potentially influence the data processing with respect to the detected suspects, in terms of false positives and false negatives, were assessed and optimized. The optimized method was then applied to feather meal samples as a proof of principle.

This study is described in detail in a scientific journal (Jansen *et al*., 2022).

Materials and Methods

Data acquisition

A fortified animal feed and feather meal sample containing 114 veterinary drugs was prepared, with concentrations ranging from 20 – 4000 µg L^{-1}. These matrices and the standard mix solution itself (with equal concentrations) were analysed using Liquid Chromatography High Resolution Mass Spectrometry (LC-HRMS). The analysis was performed using an Ultimate 3000 UHPLC system which was coupled to a Q-Exactive Orbitrap™ system with HESI-II electrospray source (Thermo Scientific, San Jose, CA, USA). Chromatographic

separation was done using an Atlantis T3 (100 x 3 mm, 3 µm particles) analytical column (Waters, Milford, MA, USA) at a column temperature of 40˚C. The mobile phases used were 2 mM ammonium formate and 0.16% FA prepared in water (solvent A) and in MeOH (solvent B), operating at a flow rate of 0.3 mL min^{-1}.

The performance and possibilities of data processing are influenced by settings applied for data acquisition (Wu *et al*., 2020). In order to explore the effects of these settings, different data acquisition settings were evaluated. The different acquisition methods (AM) combine full scan (FS) with different types of fragmentation (MS2), such as data-independent acquisition (vDIA) or all ion fragmentation (AIF) in combination with data dependent acquisition (DDA). The different AM are presented in table 1.

Table 1. The tested acquisition methods (AM) using different resolution settings, based on combinations of FS analysis with vDIA, where the number of mass ranges (DIA's) used are shown in brackets, or with AIF in combination with DDA.

AM	FS	AIF	vDIA	DDA
1	70,000	-	35,000 (5)	-
2	140,000	-	35,000 (5)	-
3	140,000	-	35,000 (3)	-
4	140,000	-	17,500 (5)	-
5	70,000	70,000	-	17,500
6	140,000	35,000	-	17,500

The vDIA methods were based on the method described by Zomer and Mol (Zomer *et al*., 2015) and include several consecutive scan events (shown in brackets in table 1). During this research, no 'inclusion list', a list of masses to select for MS2, were used in the analysis. Also no 'exclusion lists' were used in any of the DDA methods. We chose to firstly observe the results without use of an exclusion list as this requires the least a priori input and is independent of the system used or matrices investigated. Therefore also, in all cases, data were not subjected to retention time correction or mass correction preliminary to data processing, by means of for example QC samples or 'lock masses'.

Data processing

Data processing was done using Compound Discoverer 3.1. (Thermo Scientific). Compound Discoverer is a commercially available small molecule structure identification software, using accurate mass data, isotope pattern matching, and mass spectral database searches. Compound Discoverer has the option to search the mzCloud™ database, a mass spectral database for the identification of small molecules using tandem mass spectrometry. The database includes a freely accessible, growing collection of over 18,500 compounds and over 7 million high resolution/accurate mass spectra (LLC H., accessed in October 2020).

In Compound Discoverer, data is processed by means of a 'workflow'. The workflow consists of different sections in which different parameters can be set. Two different suspect screening workflows were created; a workflow where matching is based on an in-house created suspect list and a workflow using the online spectral data base mzCloud™. Data evaluation parameters specifically investigated for application of a suspect list were mass tolerance (1.5, 3 or 5 ppm) and the addition or omission of retention times from the suspect list. The effect of different AM and resolution were investigated for both the suspect list workflow and the mzCloud™ database workflow.

Acquired data were processed using both workflows and the number of true positives, false negatives and false positives were evaluated. Using the mzCloud™ database more compounds were detected than only the compounds in standard mix solution of this research, which could originate from the fact that they are originally present in the sample. These compounds could not be regarded as false positives, and were therefore called 'other mzCloud™ hits'.

Results and Discussion

The effect of data acquisition parameters

Data processing using variations in mass tolerance in the suspect list pointed out that lower mass tolerance, especially 1.5 ppm, has a negative effect on data evaluation, as it results in more false positives. The application of a retention time in the suspect list leads to more correct identification and therefore aids in lowering the false positive rate. Omission of retention times clearly resulted in more false positives. These

observations were made for both the standard mix solution as the fortified matrices.

The results for comparison of the different AM using the suspect list and mzCloud™ database workflows are presented for the standard mix solution, fortified animal feed and feather meal in figure 1. For the suspect list, there are only minor differences observed among different AMs applied, confirming that the AM is not a highly critical parameter when using a suspect list, since feature assignments are done based on FS data. A reduction of the number of DIA windows (AM 3) or a lower MS2 resolution (AM 4-6) does seem to increase the number of true positives. The improvement is most likely the result of a shorter scan cycle time, which gives an increase in the number of data points for each peak.

The use of the mzCloud™ database, independent of the AM applied, did not result in a large increase of false positives compared to using a dedicated suspect list including retention times, whereas this was the case when retention times were omitted in the suspect list. The largest increase was with 2 to 4 false positives for AM 3. Clearly, the addition of MS2 data significantly decreases the number of false positives. This means that, in terms of false positives, addition of MS2 data matching compensates for the lack of a retention times in a suspect list.

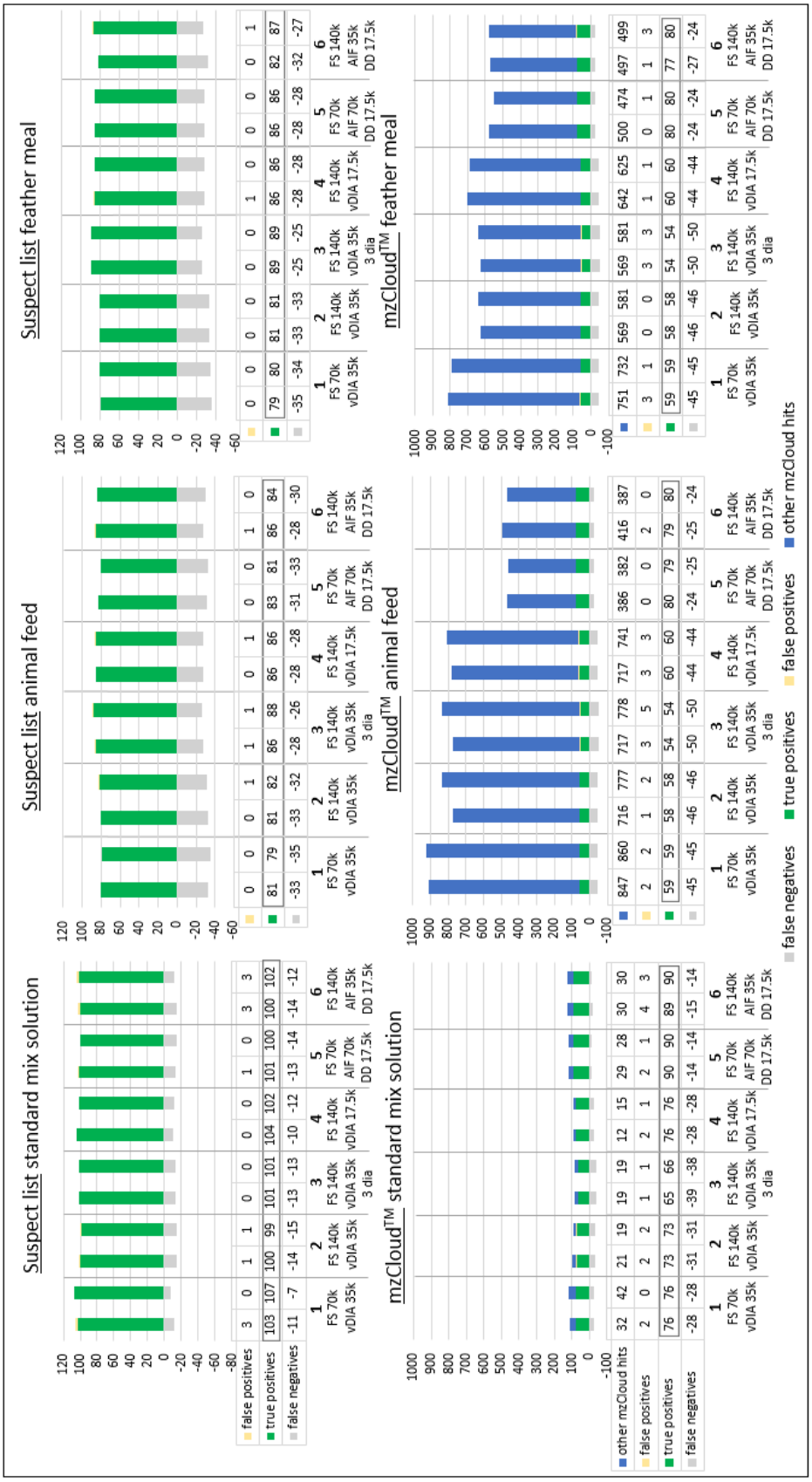

Figure 1. The number of true positives, false negatives and false positives in standard mix solution, fortified animal feed and feather meal matrix based on 6 different acquisition methods using either a suspect list workflow (including retention times

(±0.1 min), 5 ppm mass tolerance) or the mzCloud™ database. Using the latter, the number of other mzCloud™ hits were also evaluated.

Another observation when considering the mzCloud™ data output, is the difference in the number of 'other mzCloud™ hits' for the animal feed and feather meal. It is observed that, especially for animal feed, the number of other mzCloud™ hits is lower when using DDA compared to vDIA. This is also attributed to the higher MS2 data quality that is obtained by DDA compared to vDIA. In order to prevent the loss of valuable signals, when for example using data for retrospective analysis, the combination of AIF and DDA is essential. By combining these two, the masses not selected for DDA top 5 analysis will still be recorded in the AIF scan event, although the spectra will be less specific.

Taking into account the results of the different workflow variations, it would be most optimal to combine both into a single workflow. The following is required in order to effectively combine these two workflows: (1) high quality FS (high resolution) data, (2) high quality MS2 data as well as (3) an adequate number of scans per peak. Therefore, AM 6 was selected and a combined workflow was prepared using both a suspect list, including retention times and a mass tolerance of 5 ppm, and the mzCloud™ database search.

Proof of principle on feather meal samples

Feather meal samples that had a high risk of containing antibiotics were used to conduct a proof of principle for the optimized suspect screening method. In this proof of principle the focus was on the antibiotic compounds. A total of 7 feather meal samples were prepared by a method described for feathers (Jansen *et al*., 2017). The samples were then analysed using AM 6, and the data were processed using the optimized suspect screening workflow. The samples were also analysed using LC-MS/MS to check for tetracyclines, quinolones, macrolides, lincosamides, pleuromutilins and sulfonamides, according to the settings described by Jansen et al (Jansen *et al*., 2017). In table 2 the suspect screening results in combination with the LC-MS/MS data are presented.

Table 2. Antibiotics found in imported feather meal samples, with determined concentrations (µg kg^{-1}) of confirmed compounds by LC-MS/MS and indications.

Sample	1	2	3	4	5	6	7
Azithromycin						x[b]	
Ciprofloxacin	29[a]		7[a]		18[a]		
Doxycycline		89		>500[a,b]		20	500[a,b]
Enrofloxacin	>1000[a,b]		200[a,b]		380[a,b]		
Gatifloxacin						x[b]	
Levofloxacin						x[b]	
Sulfadiazine						5	
Sulfadimethoxine	9				30		
Sulfadimidine						61[a,b]	
Sulfamethoxazole						8[a]	
Sulfaquinoxaline						150[a,b]	
Tiamulin	3		180[a,b]			>500[a,b]	
Tilmicosin		>500[a,b]	41				
Trimethoprim	30[a,b]		3		30[a,b]	250[a,b]	

[a] Compound had a suspect list hit in suspect screening

[b] Compound had a mzCloud™ hit in suspect screening

x The compound was not in the scope of the LC-MS/MS analysis but was confirmed according to 2002/657/EC

In some cases, a compound was detected using the LC-MS/MS method but not in the suspect screening. This is to be expected, as lower detection limits can be achieved using LC-MS/MS compared to LC-HRMS. This also indicates the boundaries when using the suspect screening method for these antibiotics in terms of concentration.

Fourteen different antibiotics were detected and confirmed. Three antibiotics were found using the suspect screening method based on only an mzCloud™ hit; azithromycin, a macrolide antibiotic and gatifloxacin and levofloxacin, both fluoroquinolones. These compounds were not included in the suspect list nor in the scope of

the routine LC-MS/MS monitoring method. Standards were bought and the identity of all three compounds could be confirmed according to 2002/657/EC (European Commission, 2002).

Based on this proof of principle, the developed suspect screening method was found to be fit for the purpose of finding unexpected antibiotics in feather meal and the developed strategy can thus be applied for risk-based monitoring.

Acknowledgements
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POSTER 47

THE VERTICAL TRANSMISSION OF ANTIBIOTIC RESIDUES FROM PARENT HENS TO BROILERS

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Abstract
Imprudent and superfluous use of antibiotics contributes to the selection of resistant bacteria, which is a large treat to human health. Therefore, methods have been developed to determine whether animals have been exposed to antibiotics, using matrices in which antibiotic residues persist for a relatively long time, like feathers. To be able to state that antibiotics were administered, other routes besides direct administration, through which animal matrices could contain antibiotic residues, should also be taken into account.
In this research the vertical transmission from parent hen to broiler was investigated for the antibiotics enrofloxacin, doxycycline and sulfachloropyridazine in combination with trimethoprim through a controlled animal study. Vertical transmission was observed for all antibiotics in egg, egg shell and the broiler's manure, but no vertical transmission was observed to the muscle and kidney of the broiler at an age of 4 weeks. Vertical transmission can result in the detection of low concentrations of antibiotic residues in the broilers' feathers at a higher age. In all cases <50 μg kg^{-1} of freely extractable residues and <10 μg kg^{-1} of non-freely extractable residues where found. This research provides relevant information regarding the possible concentration of residues originating from vertical transmission.

Introduction
Antibiotics are of great importance to cure disease in humans and animals by killing or inhibiting the growth of bacteria. Therefore antibiotics are used worldwide to treat infections in both humans and animals. However, forbidden use for growth-promoting purposes, treating animals at sub-therapeutic levels over a long period of time, or off-label and superfluous use cannot be excluded. Products of animal origin in the EU and The Netherlands are monitored for antibiotic residues by enforcing maximum residue limits (MRL) (European Commission, 1990, 2010). However, these monitoring methods proved not to be sufficient in enforcing correct registration of antibiotics administered, as residues are usually relatively quickly excreted from these matrices (San Martín *et al*., 2007; Cornejo *et al*., 2011; Berendsen *et al*., 2013). Consequently, there was a need for matrices in which antibiotics could be detected over a longer time span. For the monitoring of antibiotic use in poultry, the analysis of feathers provided a means to detect antibiotic residues with a long detection window, even after they were excreted fully from muscle and liver (San Martín. *et al*., 2007; Cornejo *et al*., 2011; Berendsen *et al*., 2013; Cornejo *et al.*, 2017; Pokrant *et al*., 2018; Církva *et al*., 2019; Maddaleno *et al*., 2019). Because of the potential of these matrices to detect antibiotic use over a long time span, other pathways (besides direct treatment) through which antibiotics might enter the animal should be taken into account. For parent hens the antibiotics transfer to eggs is expected to occur as well. There is a chance that these residues subsequently transfer to the new-born chick and are detectable in the next generation broiler. However, to what extent vertical transmission of antibiotic residues from parent hens to broilers occurs has not been studied before.

In this research the vertical transmission from parent hen to broiler was investigated for the antibiotics enrofloxacin (ERF), doxycycline (DC) and sulfachloropyridazine (SCP) by analysing the broiler chick's manure, breast muscle, kidney, tail feathers and wing feathers. Even though the transmission of antibiotic residues to eggs has frequently been reported, we included this step in our study and additionally analysed the egg shell, as this is a waste stream of the process under investigation.

This study is described in detail in a scientific journal (Jansen *et al*., 2020).

Materials and Methods

Animal study
Following ethical approval, a parallel animal study was conducted with sulfachloropyridazine, doxycycline and

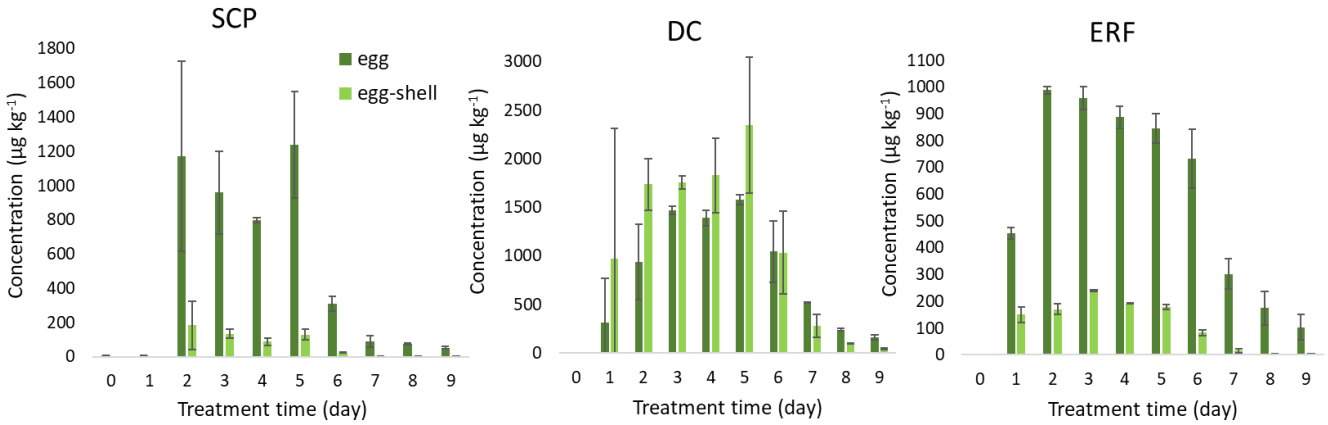
enrofloxacin. These antibiotics are all critically important antibiotics for animal health, represent different antibiotic groups and cover the spectrum from very persistent to mobile (Berendsen et al. 2018). During this study, only the parent hens were treated. The experiment consisted of two phases. In phase 1, eggs were collected from the treated parent hens on the day before commencing the treatment (day 0), daily during the treatment (day 1 – 5) and at the 4 days after the end of the treatment (day 6 – 9). Every day, two eggs per treatment group were taken for analysis. In phase 2 the eggs were hatched and matrices of the broilers were investigated. Manure samples were taken from the hatching trays, one day after placing the chicks in their cages and then weekly until slaughter. For feathers, at 4, 5 and 6 weeks of age, three broilers were sampled for wing and tail feathers, with exception of DC at week 5, where only one broiler could be sampled. The feathers were first analysed for freely extractable residues, which are the residues that can be extracted when extracting whole or cut feathers with extraction solvent. After analysing for freely-extractable residues, for a selection of the feather samples, non-freely extractable residues were determined as well. Non-freely extractable residues are the residues which are extracted after washing/extracting with extraction solvent and grinding of the feathers.

Analysis of matrices

All matrices were analysed using LC-MS/MS, where egg and egg shell were analysed using a Micromass Quattro Ultima Pt with ESI interface (Waters, Manchester, UK) and other matrices using a Q-trap 6500 (Sciex, Framingham, MA, USA). The concentration at which SCP and ERF could still be confirmed in breast muscle, kidney, egg and manure was 1 µg kg^{-1} and for DC this was 5 µg kg^{-1}. In feathers, this limit was determined to be 2 µg kg^{-1} for all compounds. In egg shell, 10 µg kg^{-1} could be determined for DC and 1 µg kg^{-1} for SCP and ERF.

Results and Discussion

The results for transfer from parent hen's to egg yolk and egg shell are presented in figure 1. After commencing the treatment, residues in egg increase rapidly. For all treatments, residues are detectable from at least 1 day after the start of the treatment (day 2 in figure 1). For SCP, residues in egg shell are about 10 times lower compared to egg. For ERF, transmission to egg shell seems to be slightly higher, where



concentrations in egg shell are a factor 5 lower compared to egg. Interestingly, DC concentrations found in egg shell were higher than in egg. ERF is known to be persistent and DC is known to bind to divalent metal ions, such as calcium, which is one of the main constituents of egg shell. Note that in some poultry production systems, the animals are hatched in the animal housing and the egg shells are consumed by the chicks as a calcium source. This could result in an additional transfer of antibiotics. Also these results indicate that egg shell is a useful non-invasive matrix which could be used to detect the application of antibiotics in parent hens and probably also in laying hens.Figure 1. Detected residues of SCP, DC and ERF in egg (dark green) and egg shells (light green) of animals treated during day 1 – day 5. Vertical lines indicate the standard deviation of the concentrations found in the two samples taken (biologic variation).

Although eggs contained significant concentrations of antibiotics, none of the muscle and kidney samples contained any detectable residues of SCP, ERF or DC. Clearly vertical transmission cannot result in MRL violations in the broiler at slaughter.

For manure, the highest concentration was found on the first sample day, which was 2.0 mg kg^{-1} for SCP, 2.4 mg kg^{-1} for DC and 3.0 mg kg^{-1} for ERF. All residues decrease exponentially in time, of which SCP showed the most rapid excretion. After 8 days SCP was not detectable anymore. Both DC and ERF were detected up to 30

days after hatching. The slow degradation of DC is a common observation, which is likely to be the result of adsorption and desorption to for example bone material (Körner *et al*., 2001; Odore *et al*., 2015). For ERF no data is available of these kind of processes.

For feathers, the detected freely extractable residues are presented in figure 2. For SCP, freely extractable residues are especially found on the tail feathers compared to the wing feathers. For DC and ERF no difference between tail feathers and wing feathers is observed. After treatment of the parent hens, the broilers' tail feathers contain on average < 50 µg kg^{-1} and the wing feathers < 10 µg kg^{-1} freely extractable antibiotic residues.

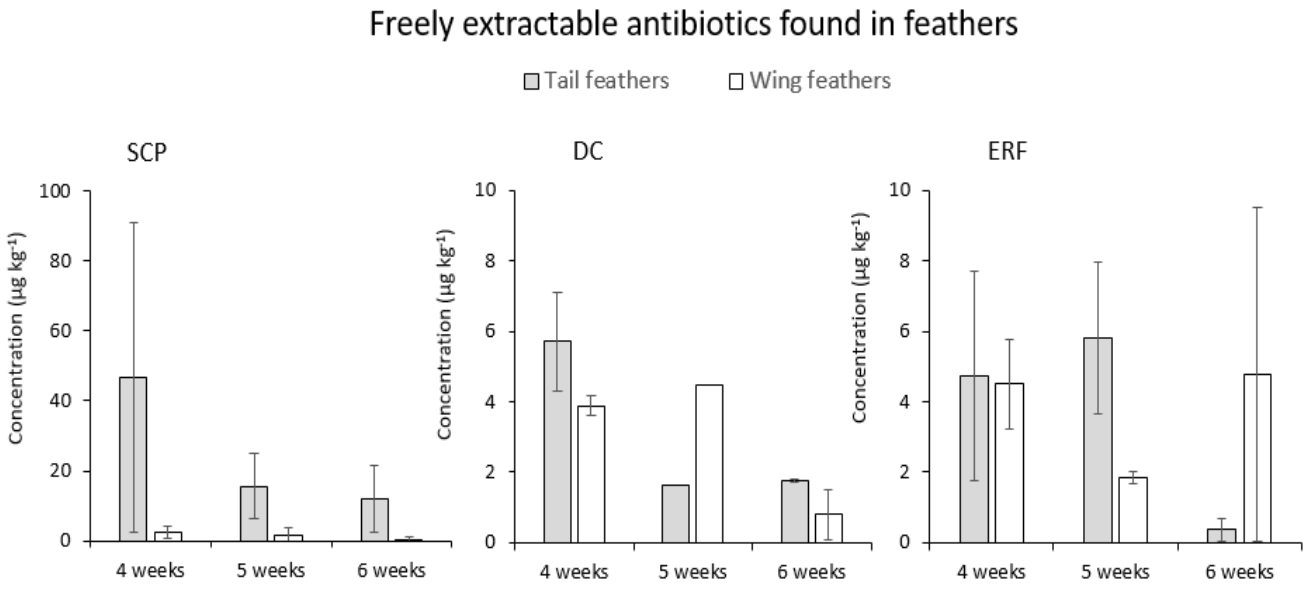


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Figure 2. Detected (freely extractable) concentration of SCP, DC and ERF in tail and wing feathers of broilers of the 3 respective antibiotic treatments at 4, 5 and 6 weeks of age. Vertical bars represent the standard deviation, n=3 (except for DC at 5 weeks: n=1).

Freely extractable residues can originate from direct vertical transmission during egg incubation or through indirect exposure after transmission via the chicks' manure. To discriminate external exposure from actual vertical transmission (direct or through manure picking) the amount of non-freely extractable residues was determined in selected antibiotic containing feather samples. Only in a few samples very low levels of non-freely extractable residues (< 10 µg kg^{-1}) were detected. If vertical transmission from parent hen to broiler chicken feathers occurs, it is clearly only at very low levels.

Conclusion

The results in this research provide relevant information regarding the possible concentration of residues originating from vertical transmission. Vertical transmission cannot result in MRL violations in the broiler kidney or meat at slaughter but does take place to other sampled matrices. Residues are present in high concentrations in broiler manure in the first days after hatching and remain detectable until 8 days (SCP) or even 30 days after hatching (DC and ERF). In untreated broilers from 4 weeks of age, low levels of residues can be detected in the feathers. These residues can be the result of direct vertical transmission, oral exposure of the broilers by picking their own contaminated droppings or external contact with the contaminated manure or environment. These routes of exposure should be considered when low levels of antibiotic residues (freely or non-freely extractable) are detected in feathers.

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POSTER P48

ACARICIDES AND RELATED COMPOUNDS IN BOVINE HAIR AND POULTRY FEATHER: DEVELOPMENT AND VALIDATION OF A LC-HRMS SCREENING METHOD

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Abstract

Acaricides could be used as veterinary drugs or pesticides to eliminate mites and ticks. Several acaricides are allowed to be used in animal husbandry and are registered as veterinary drugs. However, most acaricides are not registered as veterinary drugs. In 2017 unregistered (for poultry) Fipronil was used in many poultry farms in the Netherlands and the compound was detected in eggs. This raised the question: which (unregistered) acaricides and related compounds, with similar properties, are used in animal husbandry. To answer this question, a qualitative UHPLC-high resolution MS method was developed for the screening of a high number of acaricides and related compounds in feathers and hair. The procedure consisted of a full scan data acquisition followed by an all ion fragmentation (AIF) scan. A database was designed containing compound specific information such as retention time, parent and fragment ion with 5 ppm mass accuracy. This database was used for the identification of 198 acaricides and related compounds in hair and feather samples. The qualitative screening method was fully validated according to CD 2002/657/EC. Approximately 60% of the 177 compounds were validated at a level of 10 µg/kg, 30% at 100 µg/kg and 10% at 1000 µg/kg.

Introduction

In animal husbandry, animals can suffer from mites and ticks. Acaricides are used to eliminate these ticks and mites. Some acaricides are allowed to be used as a pesticide in animal husbandry and are registered as a veterinary drug, for example deltamethrin, ivermectin and permethrin. Others, however, are not registered and are forbidden for use as veterinary drug. Acaricides are primarily used as a pesticide to protect crops.

In 2017 the Netherlands was struggling with the egg-crisis, also called fipronil-crisis. Halfway through 2017 the Dutch food and consumer product safety authority (NVWA) blocked approximately 350 poultry farms because the prohibited/unregistered (for poultry) acaricide Fipronil was found in their eggs. Because of this, large numbers of chicken eggs were destroyed and millions of chickens were killed. This incident raised the question as to which (unregistered) acaricides and related compounds, with similar properties, were used in animal husbandry. To answer this question a survey study was carried out to monitor the use of (registered and unregistered) acaricides and related compounds in animal husbandry. Subsequently, a qualitative UHPLC-high resolution MS method was developed for the screening of 198 acaricides and related compounds in bovine hair and poultry feathers. These compounds fall under different groups, including organophosphate pesticides, pyrethroid pesticides, carbamate pesticides and anthelmintics.

The compounds were analyzed in an untargeted way and processed in an targeted way. The mass and retention time were known based on infusion and standard injection experiments. The orbitrap mass spectrometric detection procedure consists of a full scan data acquisition followed by an all ion fragmentation (AIF) scan. An advantage of this procedure is the possibility to perform a retrospective search on already recorded data for other acaricides or related compounds of interest. The procedure is used for qualitative screening and for that reason confirmation is not required. However, for many compounds one fragment ion was included into the acquisition method to enhance the results based on accurate mass. A database was designed containing compound specific information such as retention time, parent and fragment ion with the recommended requirements of Zomer *et al.* (2015). According to Zomer *et al.* (2015), the compounds should be detected within an accurate mass error of 5 ppm and detected within 30 seconds of the expected retention time.

The method was validated as a qualitative screenings method. The analyzed compounds belong to different compound groups (pesticides and veterinary drugs) and therefore require different validation regulations. For that reason a choice had to be made for a validation procedure. The validation was performed according to Commission Decision 2002/657/EC and the following performance characteristics have been established: detection capability (CCβ), selectivity, robustness and stability.

Materials and Methods

Hair and feather samples
For the validation of the method bovine hair, turkey feather and chicken feather samples were collected by the Dutch food and consumer product safety authority (NVWA) at the farm. For the survey, a total of 63 bovine hair samples, 32 turkey feather samples were collected by NVWA at the farm. 16 chicken feather samples were collected by a poultry farm which was contaminated with fipronil.

Sample preparation
Bovine hair and cut poultry feather samples were ground with a ball grinder (Retsch MM301) and aliquots of 100 mg were weighed into 14 ml tubes. After adding the internal standard solution the samples were allowed to stand for about 5 minutes. Then, 2 ml acetonitrile was added for extraction and tubes were shaken on a vortex. After a minimum of 12 hours ultrasonicating the samples were centrifuged at 3600 g during 10 minutes. From the supernatant 500 µl was evaporated under a gentle flow of nitrogen at 40°C and directly resolved in 250 µl methanol and 250 µl water. The sample extracts were transferred into a filter vial.

U-HLPC-Orbitrap-MS method
Analyses were carried out on Ultimate 3000 Vanquish (Thermo Scientific Inc., San Jose, USA) ultra-high performance liquid chromatography (U-HPLC) system. Chromatographic separation was achieved with a flow rate of 0.4 ml/min at 40 °C using an Atlantis T3 C18 column (3 µm, 100 x 3 mm, Waters, Milford, USA). The mobile phase was composed of two solutions: [A] 1 M ammonium formate/formic acid/water (2/0.02/1000 (v/v/v)) and [B] 1 M ammonium formate/formic acid/water/methanol (2/0.02/48/950 (v/v/v)). The following gradient was used: [0-0.1 min] 100% A, [2-8 min] 55% A, [8-14.5 min] 0% A and [15-20 min] 100% A. The injection volume was 5 µl and the samples were kept in the autosampler at a temperature of 10 °C.
The mass-spectrometry measurement was performed on an Q Exactive Focus Hybrid Quadrupole- Orbitrap MS system (Thermo Scientific Inc., San Jose, USA) equipped with an electrospray ion source (ESI) in positive or negative mode using a mass range of 80 – 1000 m/z with a resolution of 70 000. The procedure was consisted of full scan data acquisition followed by an all ion fragmentation (AIF) scan. The ion source parameters used are 3500 V (positive) and 2500 V (negative) spray voltage, capillary temperature of 250 °C, sheath gas flow of 47.5 arbitrary units, auxiliary gas flow of 11.25 arbitrary units, cone gas flow of 2.25 arbitrary units, and a probe heater temperature of 412 °C.

Method validation
The method was validated as a qualitative screening method according to Commission Decision 2002/657/EC. The detection capability (CCβ) was determined at a concentration level for which 95% of the cases a peak is detected that do not originate from the blank signal. Therefore, the CCβ was determined at the level at which the component is detected in 19 of the 20 hair/feather samples over three days with an addition of analytes at the levels of 10, 100 and 1000 µg/kg prior to sample preparation.
For the selectivity, every day 10 samples without the addition of analytes were prepared and analyzed.
The robustness of the method was determined by slightly changing two parameters in duplicate as shown in Table 1.

Table 1. Parameters used for robustness testing of the acaricides multi-method.

Day	Level (duplicate)	Method (A)	Variable (B)	Criteria
3	1000 µg/kg	Centrifugation at 3600 g	Centrifugation at 3600 rpm	The analytes detected with method A = The analytes detected with method B
3	1000 µg/kg	Immediately resolving after evaporation.	Evaporate the sample for half an hour after dry	

The stability of the analytes in extract was determined by reanalyzing six samples (three feather and three hair samples) of validation day 1 with an addition of 1000 µg/kg that were stores in the freezer for one month.

Results and Discussion

Development
Within the organization Wageningen Food and Safety Research, two methods are available for the analysis of hair: [1] veterinary drugs in bovine hair and [2] pesticides in human hair. The method for veterinary drugs in bovine hair consists of an extraction with methanol using a beadruptor and an incubation time of 16 hours. The

method for pesticides is almost similar as the final method described in section “Sample preparation”, but with a concentration step and resolving in 50% acetonitrile. Both methods were tested for a group of acaricides in bovine hair and poultry feathers without the concentration step. During sample preparation with method [1] the methanol is boiling during beadrupting. There is also leakage. Because of this method [2] was selected. Results show that concentrating does not result in higher signals for all compounds due to matrix concentration. In addition, the final extract must consist of 50% organic solvent to prevent peak broadening. As organic solvent, methanol is chosen, because of the composition of the mobile phase.

Validation

The validation was performed for 198 acaricides and related compounds according to Commission Decision 2002/657/EC. The CCβ was determined for hair and feather. For most of the compounds the CCβ in hair and feathers are the same, except for abamectin, chlorpyrifos-methyl, cinerin II, oxantel, dichlorphos and fluralaner. For these compounds an overall CCβ was determined, which corresponds to the highest CCβ of the two matrices. Therefore only one mixed standard solution had to be made. The results of the determination of the total CCβ is shown in figure 1. Of the 198 compounds 21 are not detectable at a level of 1000 µg/kg. About 60% of the 177 compounds are detectable at a level of 10 µg/kg, 30% at 100 µg/kg and 10% at 1000 µg/kg. Cinerin, pyrethrin and fenvalerate have a different CCβ in comparison with the other compounds, because the reference substance was a mixture of multiple compounds or a mixture of compounds with its isomers.

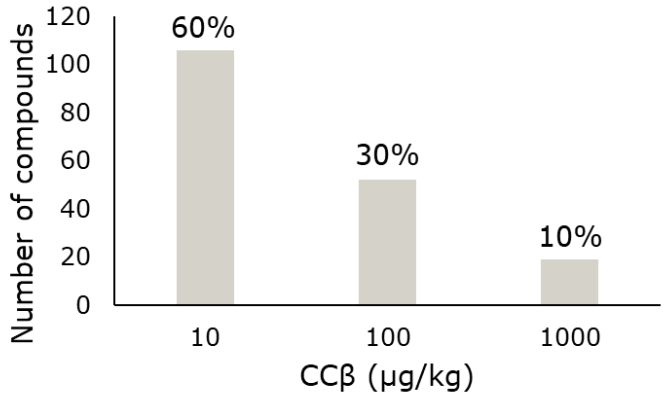

Figure 1: Overall CCβ of 177 acaricides and related compounds in bovine hair and poultry feather.

The method is selective for all compounds with the exception of pyrethrin I (without measuring pyrethrin II). Almost all samples (especially feathers) were suspect for pyrethrin I, but the same samples were negative for pyrethrin II. The suspected pyrethrin I samples were measured with an LC-MS/MS confirmatory method and with this method an interfering peak close to pyrethrin I was detected.

Pyrethrin I and II naturally occur together, because they originate from a natural plant extract (Chrysanthemum cinerariifolium) where there is always a mixture of pyrethrin I and pyrethrin II (and also cinerin I, cinerin II, jasmolin I and jasmolin II). So if a sample which is analyzed with this LC-Q-Orbitrap-MS-method appears to be suspected for pyrethrin I, it should also be suspected for pyrethrin II. If not, the pyrethrin I peak originates from an interference and the sample is probably negative for pyrethrin I. Because of this, no CCβ was set for pyrethrin I.

The method is robust, except for dichlorphos and formothion. Evaporating the sample extract for half an hour after dryness has a negative effect on these compounds. It must be ensured that the sample extracts are not in the evaporator too long after dryness. Moreover, the residue must be dissolved immediately after drying.

The stability of the compounds in extracts is determined for all compounds at 1000 µg/kg while some compounds appear to have a higher CCβ. For these compounds the stability is not determined. From the results of the other compounds it can be concluded that the compounds are stable in extract for 1 month in the freezer, except for acequinocyl, bifenazate, bifenthrin, cinerin I, formothion and pyridalyl. For these components the extracts should be analyzed immediately after sample preparation.

Analysis of hair and feather samples

For the survey 63 bovine hair samples and 48 feather samples were screened with this method. Results are presented in table 2. A number of registered compounds for use as anti-coccidiosis (ponazuril/toltrazuril) or insecticide (thiamethoxam) were found in turkey feathers. A number of registered compounds were also found in the chicken feathers (ponazuril/toltrazuril, flubendazole, pyriproxyfen and spinosyn A & D), next to two unregistered compounds, i.e. bendiocarb (insecticide) and fipronil . The presence of fipronil is not surprising because the feathers came from a location infected with fipronil.

Two unregistered compounds were found in the bovine hair samples, i.e. allethrin (insecticide) and diazinon (insecticide/acaricide). Also registered compounds were found in several hair samples . These results were confirmed with a targeted LC-MSMS method according to CD 2002/657/EC. This survey demonstrates the usefulness of the developed acaricide screening method.

Table 2: Confirmed compounds in bovine hair and poultry feathers based on initial screening results

Feathers		**Hair**
Chicken	Turkey	Bovine
Unregistered compounds		Unregistered compounds
• Fipronil (+metabolite) • Bendiocarb		• Allethrin • Diazinon
Registered compounds		Registered compounds
• Ponazuril/Toltrazuril • Flubendazole • Pyriproxyfen • Spinosyn A & D	• Ponazuril/Toltrazuril • Thiamethoxam • Levamisole	• Thiamethoxam • Cyromazine • Permethrin • Pyrethrin/Cinerin • Imidacloprid • Ivermectine • Triclabendazole (+metabolite)

Conclusion

The method of analysis developed in this study is suitable for the screening of 177 acaricides and related compounds in bovine hair and poultry feather. The qualitative screening method was fully validated according to CD 2002/657/EC. Approximately 60% of the 177 compounds were validated at a level of 10 µg/kg, 30% at 100 µg/kg and 10% at 1000 µg/kg. For 99% of the compounds, the method is selective and robust for the parameters tested. Six compounds are not stable in extracts for one month in the freezer.

Several registered and unregistered acaricides and related compounds were found in the survey in bovine hair and poultry feather. The survey demonstrates the usefulness of the developed screening method. The method could be used for monitoring the use of acaricides and related compounds in animal husbandry.

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POSTER P49

POINTS OF ATTENTION WHEN USING ISOTOPE LABELLED STANDARDS IN LC-MS/MS

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Abstract
Isotope-labelled internal standards are commonly used in LC-MS/MS analysis of veterinary drugs. The benefits are great like correction for extraction loss and correction for matrix effects during the LC-MS/MS analysis. However, there are some points of attention when using isotope labeled internal standards. If the number of labeled atoms is too low or when the number of C-atoms of the analyte is high it's is possible that the unlabeled analyte of interest will contribute on the signal of the internal standard. For that reason it's advisable to consider if the concentration of internal standard is appropriate in relation to the concentration of the analyzed compound. For the analysis of the macrolide Spiramycin, Spiramycin-d3 as internal standard can be used. Due to the high number of C-atoms in Spiramycin, the third isotope peak of Spiramycin will contribute 3.2% to Spiramycin-d3. For the avermectin Ivermectin, Ivermectin-d2 can be used as internal standard, in this case the second isotope peak of Ivermectin will contribute 16.8% to Ivermectin-d2. It is recommended that the right concentrations for the internal standards are chosen to minimize these effects of contribution. If not, the calibration curve will be influenced and the directional coefficient and the correlation coefficient might be too low and that may result in overestimated results.

Introduction
The use of internal standards is generally applied in LC-MS/MS analyses of veterinary drugs. An isotope labeled standard of the test substance is considered the most ideal internal standard because its physical and chemical properties are similar to the substance being analyzed. When these internal standards are added to samples before extraction, they will correct for extraction loss. During the LC-MS/MS analysis they also correct for matrix influences that can occur during the measurement because the retention time is the same as the native compound. However, there are also risks associated with using such labeled internal standards which are not of common awareness. During the method development and implementation of an analysis method for avermectins in liver, it was found that an isotope labeled internal standard for Ivermectin, Ivermectin-d2 was not useful. The response for ivermectin-d2 was influenced during the LC-MS/MS analysis by Ivermectin itself. It turned out that the second isotope peak of Ivermectin contributed to the signal of the Ivermectin-d2 peak. This was a significant contribution of about 16.8% that effected the linearity. Another example was the results of an proficiency test for macrolides in bovine muscle. The result for Spiramycin was too high, Spiramycin-d3 was used as internal standard and it appeared that Spiramycin contributed 3.2% to the signal of Spiramycin-d3. When the concentration of the internal standard is too low in relation to the concentration of the native compound it will result in an overestimated result in the test sample.

Examples and inventory strategy

Spiramycin in bovine muscle

After participation in a proficiency test for macrolides in bovine muscle the results were evaluated by the organiser.The reported content of spiramycin of 182 µg/kg was too high, the assigned value was 120 µg/kg and the z-score was 2.3. The result was based on a standard addition analysis. As a result of the deviating value, a follow-up action was started to see what the cause of the deviation was. It was noted that the correlationcoefficient of the curve from 0.9924 was sufficient, but that the peak area of the internal standard Spiramycin-d3 increased when increasing the concentration of Spiramycin (see table 1).

Table 1. Increasing peak area of spiramycin-d3 at increasing addition of Spiramycin

addition spiramycin µg/kg	Spiramycin	Spiramycin-d3 (100 µg/kg)	respons factor
	m/z 422.4 > 174.0	*m/z* 423.8 > 174.1	*m/z* 423.8 > 174.1
0	229032	240696	0.95
0	237780	247393	0.96

100	443466	262754	1.69
200	615716	279039	2.21
300	777020	284168	2.73
400	977138	307407	3.18

It turns out that the increasing peak area of spiramycin-d3 was caused by the third isotope peak of spiramycin. Due to the large number of carbon atoms, this contribution is significant. Spiramycin, $C_{43}H_{74}N_2O_{14}$ with a molecular mass of 843.1 Da is analyzed during the LC-MS/MS analysis as a double charged precursor ion $[M+2H]^{2}+$ with *m/z* 422.3. For the internal standard Spiramycin-d3, $C_{43}H_{71}D_3N_2O_{14}$ with molecular mass 845.5 Da is analyzed during LC-MS/MS as a double charged precursor ion $[M+2H]^{2}+$ is measured with *m/z* 423.8. Figure 1 shows that the contribution of Spiramycin to spiramycin-d3 is 3.2%. This can result in a significant contribution if the concentration of the internal standard is chosen too low.

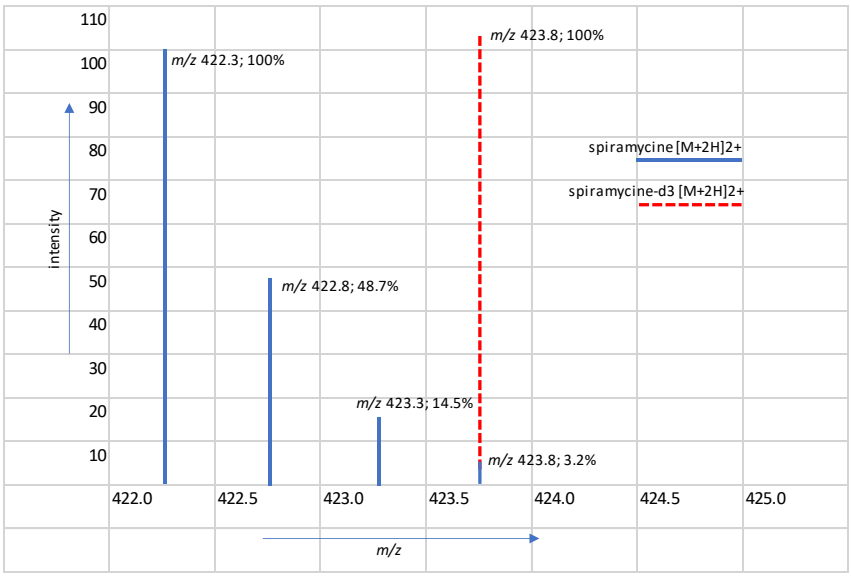

Figure 1. Isotope spectrum from Spiramycin and the addition on Spiramycin-d3

It is necessary to correct for this contribution from the isotope peak if the contribution is significant. Table 2 shows the expected contribution from Spiramycin to Spiramycin-d3. Responsfactors were corrected and the content of Spiramycin in the test sample after this correction is 151 µg/kg and the z-score of 1.2 met the criteria (between -2 and 2) . The correlationcoeffiecient of the curve of 0.9975 was sightly increased by the corrected response factors.

Table 2. Correction on Spiramycin-d3 concentration and respons factor

addition spiramycin µg/kg	spiramycin-d3 concentration in µg/kg after correction	responsfactor	responsfactor corrected
0	100.0	0.95	0.95
0	100.0	0.96	0.96
100	103.2	1.69	1.74
200	106.4	2.21	2.35
300	109.6	2.73	3.00
400	112.8	3.18	3.59
600	119.2	3.76	4.48

Additional, the effect of increasing the concentration of spiramycin-d3 was investigated, by comparing a calibration line with a concentration of Spiramycin-d3 of 100 µg/kg with a calibration line with a concentration of Spiramycin-d3 of 300 µg/kg. When spiramycin-d3 is added at higher concentrations the relative contribution from Spiramycin on Spiramycin-d3 is lower and the calibration is positively influenced which results in a higher directional coefficient and a sufficient correlation coefficient, see Figure 2.

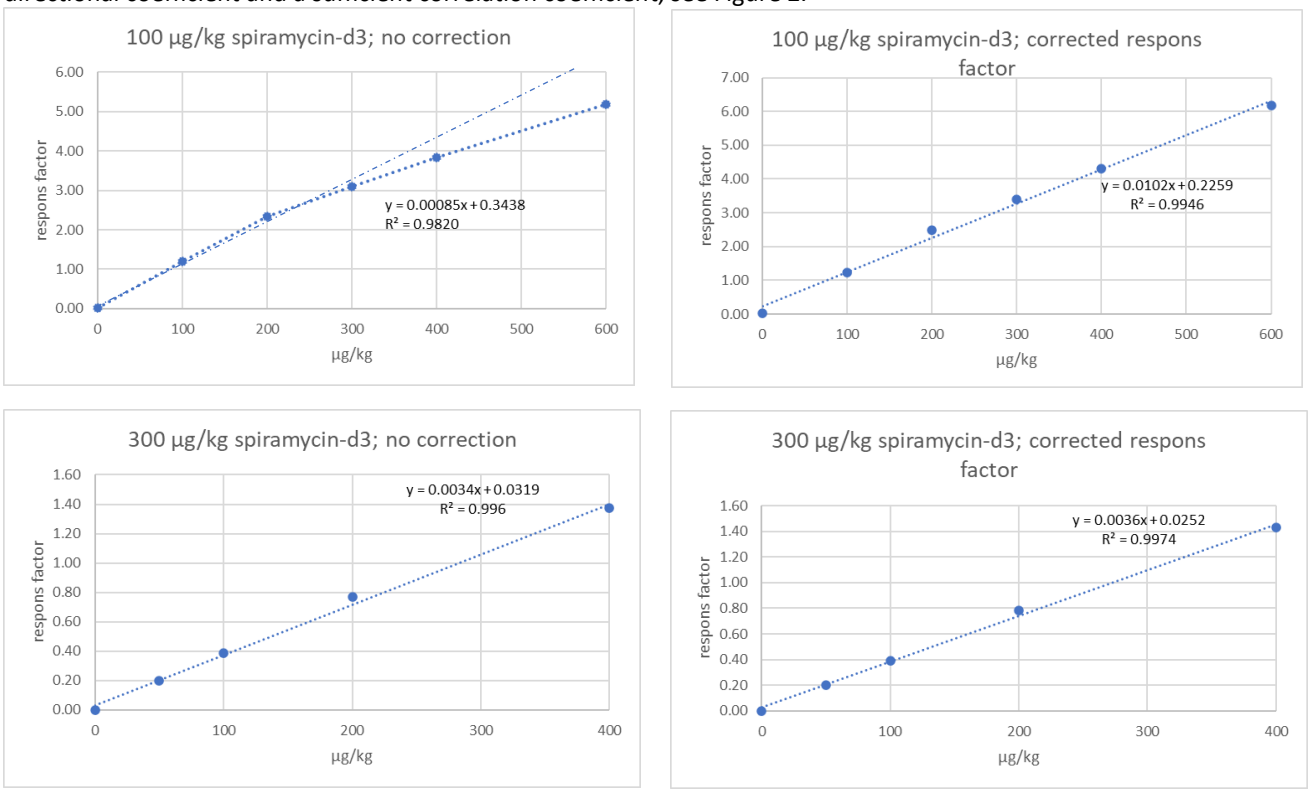

Figure 2. Calibration curves with 100 µg/kg and 300 µg/kg Spiramycin-d3

Ivermectin in porcine kidney

For ivermectin in porcine kidney a maximum residue level of 30 µg/kg is established in Regulation EU/37/2010. During method development Iveremectin-d2 was used as internal standard. First experiments shows flattening calibration curves due to the contribution from Ivermectin on Ivermectin-d2. Due to the large amount of carbon atoms in ivermectin ($C_{48}H_{74}O_{14}$), isotope peaks will contribute on Ivermectin-d2. During LC-MS/MS for Ivermectin precursor ion [M+Na]+ with *m/z* 897.5 is detected and for Ivermectine-d2 ($C_{48}H_{72}D_2O_{14}$) precursor ion [M+Na]+ with *m/z* 899.5 is detected. Figure 3 shows the contribution of 16.8% from the second isotope peak from Ivermectin to Ivermectin-d2, In figure 4 the calibration curves are shown with and without correction for this contribution.

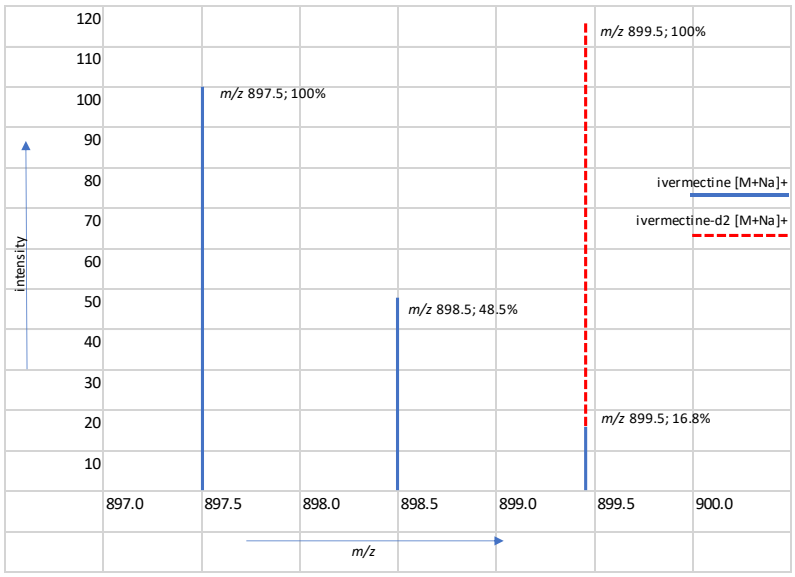

Figure 3. Isotope spectrum from Ivermectin and the addition on Ivermectin-d2

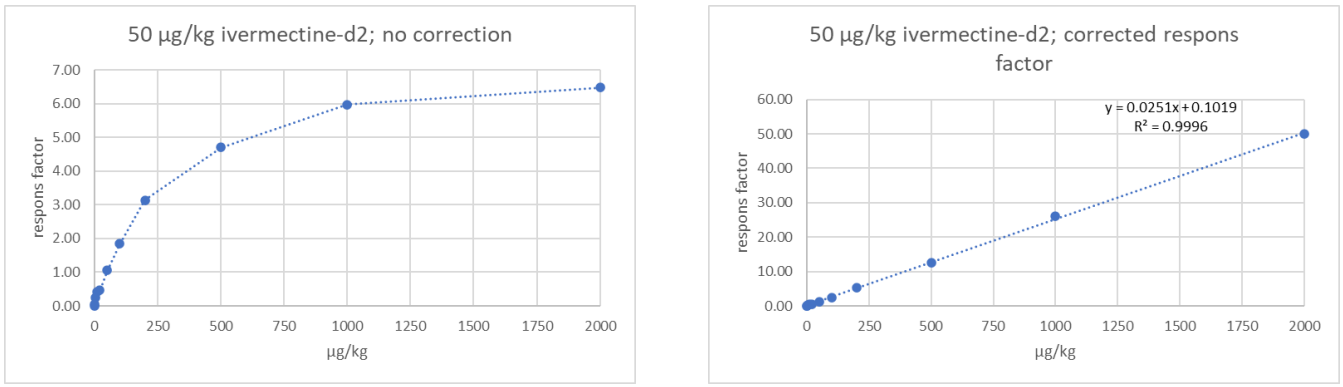

Figure 4. Calibration curves Ivermectin in porcine kidney without and with correction for isotope contribution from Ivermectin

Recommendations for the use of isotope labeled standards

When using isotope labeled internal standards it is recommended to calculate the predicted contribution from the isotope peaks from the native compound on the signal of isotope labelled internal standard. A predicted contribution <1% is considered to be sufficient. As an example the appropriate concentration for Spiramycin is calculated. The maximum residue limit for Spiramycin in bovine muscle is according to Regulation EU/37/2010 established at 200 µg/kg. When a sample contains 250 µg/kg, the expected contribution on the internal standard is 0.032* 250 = 8 µg/kg. The recommended minimum concentration of Spiramycin-d3 is 800 µg/kg, however it is advisable to increase the concentration in accordance to the highest calibration sample level, for example at 400 µg/kg (2*MRL). In this case the recommended concentration is (0.032*400)*100 = 1280 µg/kg Spiramycin-d3. For the analysis of Ivermectin in porcine kidney with a MRL of 30 µg/kg a calibration range from 15-60 µg/kg is usual. The recommend concentration for Ivermectin-d3 is (0.168*60)*100=1008 µg/kg.

Acknowledgements
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References

- COMMISSION REGULATION (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin

POSTER 50

VETERINARY DRUG- AND PESTICIDE RESIDUES IN PIG MUSCLE BY LC-QTOF-MS

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Abstract
A liquid chromatography-high resolution mass spectrometry (LC-HRMS) method for screening and identification of veterinary drugs (antibiotics, NSAID, anthelmentics, coccidiostatics, tranquilizers) and pesticides was validated with pig muscle.
Tissue was extracted with acidic methanol/water with EDTA, followed by dispersive solid phase extraction to remove unpolar interferences. Before analysis the extract was centrifuged at high speed (12.000g).
A MaXis Q-TOF mass spectrometer equipped with an electrospray ion source was operated in positive mode. In the same analysis full scan acquisition with a mass range of 100 to 1100 amu and broadband collision induced dissociation (bbCID) acquisition was used for screening and identification.
Identification criteria's for screening were established for response (intensity and area), mass accuracy, isotopic pattern, retentiontime and qualifier ions were established. Samples tested positive from routine control (using LC-MS/MS) were analysed with the new LC-HRMS method for testing the method. The method is validated for 65 veterinary drug residues and 114 pesticides and part of the validation included testing stability of selected veterinary drugs in pig muscle incubated at -20, +4 and +20 ^{o}C during a 5 month period.

Introduction
The analytical methods used for official control for residues in animal and animal products according to Council Directive 96/23 legislation are mainly based on liquid-chromatography and gas-chromatography with UV-, fluorescence or mass spectrometry detection. With improved sensitivity and selectivity for methods based on mass spectrometry and with instruments that have become fast enough, the methods can analyse several groups of analytes in one analytical run (Granelli K. *et al.* 2009). For antibiotics it is has for many years been normal procedure to use different LC methods for different classes of compounds. Methods has since more than 15 years been published making it possible to analyse several groups of antibiotics and other veterinary drug residues (anthelmentics, coccidiostatics, NSAIDs, tranquilisers) in one analytical procedure (ANSES, 209; Miossec *et al*, 2020; Stubbings *et al*, 2009; Casey *et al*, 2020; Kaufmann *et al*, 2011; Stolker *et al*, 2008). With the continuously increased sensitivity of instruments based on high-resolution mass spectrometry, methods based on this technique has been published during the recent years (ANSES, 2019; Gago-Ferrero *et al*, 2020; Kaufmann *et al*, 2011).

The extraction of tissue should be capable of removing as many interfering substances as possible without removing the compounds of interest. With use of analytical methods for analysis of a wide range of compounds the extraction should be as delight as possible for keeping the compounds of interest in the extract, but at the same time it should be possible to remove components like lipids, phospholipids, carnitines, proteins and peptides before introduction in the instrument. Despite of having a very high mass accuracy with high-resolution instruments, thereby making it possible to remove the interferences by mass accuracy, these interferences will contaminate the instrument and reduce or block the ionisation of the compounds of interest.

Proteins and peptides are often removed by precipitation with an organic solvent with intermediate polarity and lipids can be reduced by use of heptane, freezing out or solid-phase extraction, SPE. Phospholipids are shown to reduce ionisation efficiency and they can be difficult to remove from the analytical column. SPE is, like liquid-liquid extraction, used for reducing interferences by use of a sorbent and a liquid phase where affinity to the sorbent decides whether separation can occur. SPE further makes it possible to concentrate the sample without an evaporation step, and for analysis of large volume of samples (e.g. wastewater analysis) this has been very important for reaching low detection limits. For a more quickly and cheap extraction, but at the same time use of a sorbent for reducing interferences, the sorbent, as a powder, can be added to the sample and interferences that have a higher affinity to the sorbent material will be reduced. This has been used for a long time for analysis of pesticides, where different sorbents are used for reducing interferences.
Phospholipids and lipids can be reduced by this step with use of a non-polar sorbent. According to Commission regulation (EU) 2021/808 when confirming a finding a certain number of identification points has to be earned

for the result, where use of chromatography is obligatory and criteria for use of mass spectrometry are described.

According to the CRL guideline on validation of screening methods a method used for screening should have a β-error < 5%, giving a risk of a false compliant (false negative) results below 5% and when confirming a finding the α-error should be < 5% or 1% (for group A, forbidden, substances), giving a risk of a false non-compliant (false positive) result below 5%/1%. When setting up a method for screening it is therefore important to be able to detect the compounds at a certain concentration (CCβ) and at the same time not have to many false positives (high α-error). When using HR-MS threshold values for key-parameters are important for reducing the number of hits that are given. Gago-Ferrero and co-workers described a workflow for target screening of emerging contaminants in wastewater where criteria for these factors were established for reducing the rate of false negative and false positive results (Gago-Ferrero *et al*, 2020). In this study a method, based on high-resolution mass spectrometry, is validated and threshold values are suggested when processing data. Furthermore samples, with findings of veterinary drug residues, previously analysed with LC-MS/MS, will be analysed by the new method for testing the treshold values.

It is important to know how long time an analyte is stable in the matrix before analysis. After a sample has been sampled several days can pass before analysis has been made and the sample might be stored at a temperature above 0 °C for a period. The European Reference Laboratories, EURL, for residues are continuously testing stability of analytes within their scope (Maris and Hurtaud-Pessel, 2000; BVL, 2019) and the group with Berendsen and coworkers published data for stability of several antibiotics in matrix (Berendsen *et al*., 2011).

In Denmark muscle from porcine is tested for coccidiostatics, anthelmentics and NSAID and with this study stability data is established for selected compounds from these groups in pig muscle.

Materials and Methods

Materials and reagents

All reagents were of analytical-, HPLC- or LC-MS-grade and supplied by Merck (Darmstadt, Germany) and Rathburn Chemicals (Walkerburn, Scotland). Water was ultra-purified using a Maxima purification system from USF Elga (Bucks, UK). C18 and Z-Sep were supplied by Sigma-Aldrich (Brøndby, Denmark).

The compounds included in the study were veterinary drug residues and pesticides. 14 NSAIDs, nine anthelmentics, four tranquilizers, three coccidiostatics, 27 antibiotics, nine dyes and 140 pesticides were included. Sulfadimethoxine-d6, chlorpromazine-d3 and flunixin-d3 were used as internal standards. Analytical standards were purchased from Sigma-Aldrich.

Stock solutions of each compound were prepared separately at concentrations of 1 mg mL^{-1}. Methanol or ethanol was used as solvent for most, and for some 50% acetonitrile with or without sodiumhydroxide, acetone, acetonitrile and dimetylformamide was used. Seven working solutions containing a group of compounds were prepared with methanol at a concentration of 10 µg mL^{-1} for many compounds and 2 µg mL^{-1} for the solution with pesticides. For some compounds the concentration depends on the MRL-value or the sensitivity of the method for the specific compound. All solutions were stored at –18 °C until use.

Porcine muscle were obtained from local supermarkets. As soon as the samples were received, they were homogenized in a blender and stored at –18 °C until analysis.

Sample preparation

Muscle tissue was homogenised, and 2 g was transferred to a centrifuge tube. 20 µL internal standard working solution was added and after 10 min 1.5 mL of EDTA-solution (0.1 M) was added and the sample was vortexed. 6 mL Methanol with 0.1% formic acid and a ceramic grinding cylinder was added and the sample was shaken on a Geno/GrinderR homogenisator for 2 min. with 750 rpm. After centrifugation (4 °C, 10 min, 4000 g), 1.5 mL was transferred to an Eppendorf tube with 100 mg C18 sorbent and another 1.5 mL transferred to an Eppendorf tube with 100 mg Z-Sep sorbent. The tubes were shaken (1 min.) and centrifugated at high speed (4 °C, 10 min, 12.000g). To the supernatant 10 µL DMSO was added and the sample was evaporated until dryness (40 °C with nitrogen). 500 µL 15% methanol in water was added and 5 µL of the sample was injected on the instrument.

LC-Q-TOF analysis

LC separation was performed on a Dionex Ultimate 3000 RS (Thermo Scientific, CA) equipped with a high-pressure binary pump, degasser, auto sampler and a column heater. The analytes were separated on a

Poroshell SB C-18 (100×2.1 mm, 2.7 µm particle size) with the LC-column held at 25 °C (Agilent technologies, Walbron, Germany). A gradient was used for separating the analytes using 0.1% formic acid and acetonitrile as mobile phases, and the flow rate was set at 0.3 mL min^{-1}. The sample volume injected was 2 µL.

The LC system was connected to a Bruker Daltonics, MaXis Q-TOF MS equipped with an electrospray ion source operated in positive ion mode (Bruker Daltonics, Bremen, Germany). Sodium formate dissolved in 50% 2-propanol was introduced in the ion source in a 0.2-0.4 min time segment and used for internal calibration of the data files. Hexakisperflouroetoxyphophazene was used as lock mass calibrant to compensate for drift in the mass axis during analysis.

The ion source settings were: dry gas temperature 200 °C, nebulizer pressure 2 bar, capillary voltage 4500 V, drying gas flow 10 l min^{-1}. The scan range was from 80 to 1100 m/z with an acquisition rate of 2 Hz.

Spiked standards were used to quantify the compounds. Six concentration levels for the calibration curve was used with concentration depending on the MRL level for the compound. For the lowest level the concentration was 5-250 µg kg^{-1} and for the highest level the concentration was 50-2500 µg kg^{-1}, and a fixed amount of internal standard (100, 500 or 1000 µg kg^{-1} depending on the compound) was added to all the samples. Calibration curves were obtained relating relative responses of the compound (chromatographic peak area of the compound at different concentration levels in relation to the concentration of the internal standard divided by the peak area of the internal standard) versus nominal concentrations (µg kg^{-1}) added of the compounds. Data files were processed using TASQ 2.1 (Bruker Daltonics, Bremen, Germany).

Results and Discussion

Method development

Several methods has been described elsewhere for analysis of antibiotics in tissue (Anses, 2000; Miossec et al. 2020; Yamagushi et al., 2015) and we wanted to implement a fast method for screening of known veterinary drug residues with minimum sample treatment, e.g. sample preparation, sample extraction and LC-QTOF-MS analysis within one day. When implementing the method we used a standardsolution with 55 veterinary drug residues covering the different groups (antibiotics, NSAID, anthelmentics, tranquilizers and coccidiostatics).

The starting point was precipitation with organic solvent, where acetonitrile, methanol and acetone were tested. The sensitivity was improved for some compounds when adding 0.1% formic acid to the extraction solution, which is explained by the sensitivity to pH for polar compounds and furthermore compounds bearing a carboxylic group will have a higher recovery in an acidic solution. By adding EDTA the recovery of especially tetracyclins and quinolones were improved, by chelating with divalent cations, like Mg^{2+} and Ca^{2+}, occurring in the sample (Aguilera-Luiz et al. 2008). After high-speed centrifugation (12.000g) the extract was clear and it was decided to try not to include a filtering step thereby reducing the risk of loss of compounds, especially if the extraction should be used for many different compounds. Moissec et al described e.g. loss of some NSAIDs with use of a nylon filter and loss of antibiotics with use of PVDF filter. By looking at the chromatogram it was clear that many unwanted compounds were introduced in the instrument. With use of methanol as organic solvent instead of acetonitrile and acetone the best recovery was overall achieved, but still with many interfering peaks in the chromatogram.

For removing interferences we wanted to use a sorbent either as SPE or dSPE. Since the prioritisation was on having a quick screening method without loss of too many compounds we used dSPE thereby loosing the possibility of washing the SPE-sorbent before eluting the compounds from the sorbent. Using 2 g of muscle we tested two different sorbent types: C18 and zSep. The latter proven to be especially effective for removing pigments and lipids via interactions between hydroxyl groups and Zirconium. 50, 100 and 200 mg of added sorbent was tested and using 100 or 200 mg comparable results were acchieved. For the coccidiostatics a significant loss of compounds was seen when using C18 sorbent compared to zSEP and a loss of appr. 50% was seen for the NSAIDs and anthelmentics tested, but for the tested tetracyclins and quinolones the loss was appr. 50% when using the zSEP sorbent. For the other groups of analytes and the tested pesticides the results for the two sorbents we comparable. The method was validated with use of both sorbents, by splitting the sample before dSPE but the results presented in this study is with use of 100 mg zSEP which showed best results overall for all tested compounds.

For processing the data we used TAQ 2.1 where a database, as a csv file, was made with 355 of the veterinary drug residues listed in the SANTE database (Directorate F, 2022). The formular and name was registered and a test-solution with 152 compounds was analysed with full scan and bbCID acquisition. The database was updated with the results, giving retention time and fragment ions (qualifiers). For some compounds the adduct (sodium or ammonium) was the preferred precursorion and for six compounds (bacitracin, gamithromycin,

spiramycin, tildipirosin, tulathromycin and tilmicosin) the divalent ion was used. In table 1 the values are listed for some anthelmentics.

Method validation

For calculating precision and recovery the compounds were added at three concentration levels to three different samples of pig muscle. A low level (½×MRL), medium level (MRL) and high level (2×MRL) was used and for the calibration curve six samples of pig muscle was used with the levels 0, ¼×MRL, ½×MRL, MRL, 2×MRL and 5×MRL. For some compounds, with MRL, lower spike levels were used for practical reasons. The analysis was repeated on three different days. The dyes were also included in the validation study and in two sequences the method was tested with 114 pesticides, known to be analysed by LC-MS. The precision and recovery for some anthelmentics are listed in table 1 and for most compounds the recovery is between 70-130%. For most antibiotics the recovery is 80 – 120% fulfilling the criteria in Commission implementing regulation (EU) 2021/808 but for some compounds in the other groups the recovery is outside the interval 80-120%. For improving the recovery use of a dedicated internal standard might improve the recovery, since we only included two antibiotics and one tranquilizer as internal standard.

Table 1. Database values and results from method validation for selected anthelmentics

Cmpd	Formular	Rt (min.)	Qual1 *(m/z)*	Qual2 *(m/z)*	MRL (µg kg^{-1})	Range (µg kg^{-1})	Recovery (%)	RSDr (%)
Albendazol	C12H15N3O2S	8.0	234.070		100	5 - 100	95	18
Albendazolsulfon	C12H15N3O4S	6.4	266.060	159.043	100	5 - 100	101	11
Albendazolsulfoxide	C12H15N3O3S	5.5	240.044	208.018	100	25 - 500	98	11
Fenbendazole	C15H13N3O2S	8.9	268.054		50	25 - 500	85	22
Fenoterol	C17H21NO4	4.5				25 - 500	115	19
Flubendazole	C16H12FN3O3	7.9	282.068		50	25 - 500	90	12
Mebendazole	C16H13N3O3	7.6	264.077	318.085	60	5 - 100	93	11
Oxfendazole	C15H13N3O3S	6.4	284.049	191.069	50	25 - 500	92	12
Oxibendazole	C12H15N3O3	6.7	218.093	176.046	100	25 - 500	89	19

At the medium and high concentration level the precision is below 25% for most compounds except some dyes and robenidine (coccidiostatic). According to EU-regulation 2021/808 the repeatability standard deviation must be below 25% if the concentration is 10–120 µg kg^{-1}. The precision could be improved by using a faster scan rate (4 Hz instead of 2 Hz) due to more datapoints for the chromatographic peak, but since the sensitivity was reduced 2-fold with faster scan rate we used 2 Hz for better sensitivity. It is well known that the dyes are highly influenced by pH when extracting the samples and during the LC-analysis, and for some dyes a stabilising agent is needed during extraction.

For processing data several parameters has to be optimised for extracting correct results. Criterias for retention time (when it is known), isotopic fit (given by a mSigma-value in the software), mass accuracy, number of qualifier ions for the compound, area and intensity. They should be set in order to reduce the β-error and α-error (thereby getting fewer hits) for the method. The parameters were first set to prevent any false negative results. The threshold for Area, Intensity, mSigma, Mass accuracy and dRT were in the beginning fixed at 800, 500, 1000, 10 ppm and 0.4 min. In every sequence the retentiontime was stable with a deviation less than 0.05 min. except azaperone, azaperol and crystalviolet where the deviation was less than 0.3 min, due to the broadness of the peaks.

Mass accuracy is less than 5 ppm for all compounds except ibuprofen where the C13/C12 ratio is not acceptable. 98.7% of the results are below 3 ppm mass accuracy and if a threshold for mass accuracy is fixed at 5 ppm for ibuprofen and 3 ppm for others 99.6% of the results are below the threshold-value. The mSigma-value is less than 200 for 98.2% of the results if ibuprofen is removed from the data.

The rate for false positive results were tested by analysing blank samples and processing with the established database and processing data using different settings for mSigma and mass accuracy. A tolerance of 0.4 min for the retention was fixed but for 198 compounds without an established retentiontime this threshold is not

used. Treshold for Area and Intensity was fixed and threshold values for mSigma and mass accuracy was varied as shown in table 2.

Table 2. Factors tested for dataprocessing.

Factor	Treshold
dRT	0.4 min
Area	800
Intensity	500
Mass accuracy	3 ppm; 5 ppm
Isotopic fit (mSigma)	100; 200; 300; 800

26 blank samples were analysed and using 5 ppm and 800 mSigma 9-17 hits were found in every sample and using 3 ppm and 200 mSigma 4-9 hits were still found in every sample. A list, with compoundname and retentiontime, was then made with the compounds that were found in three or more samples and they were afterwards substracted from the hits generated in every sample. Looking at the hits generated using this exclusion list only one hit was found in five samples using 5 ppm and 200-800 mSigma tolerances (four different compounds found in the five samples). One hit was neomycin (retention time 11.04 min.) and since aminoglycosides are probably eluted in the beginning of the time program this hit is excluded and included in the exclusion list. By looking at chromatograms for all blank samples two of the hits, chlorprothixene and levafloxacin, were also found, but with an area below the threshold, these were also included in the exclusion list. Only one hit was then found in a sample. The mSigma value was 199 and the mass accuracy was 4.5 ppm and after reinjecting the sample the mSigma value was 208 and the mass accuracy was 6.3 ppm, and it was included in the exclusion list. Injecting the sample several times for reducing number of hits and false positive results is recommended when doing non-targeted screening (Kunzelmann et al., 2017) and for this study when retention time is unknown this is relevant to do.

When screening with the database the setting 5 ppm and 200 mSigma will be used and the hits found in several blank samples will be continously registered in the exclusion list for reducing false positive hits.

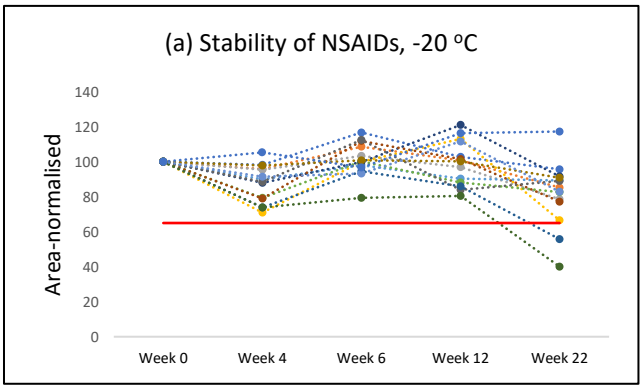
The method was tested for a group of pesticides as described above. 114 pesticides were analysed and the concentration levels were 25, 50 and 100 µg kg-1 and the calibration curve further included the levels 12.5 and 200 µg kg^{-1}.

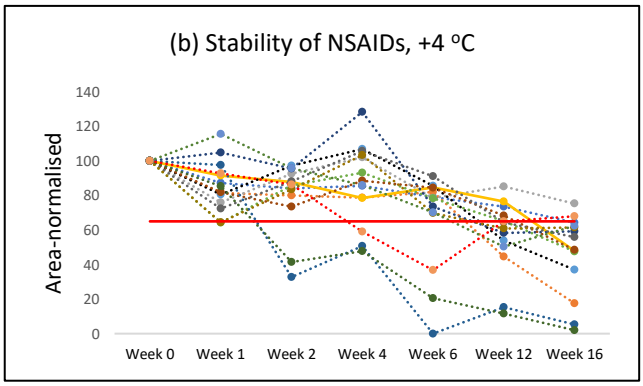
100 of the tested pesticides were detected with the screening approach where one non-detected pesticide (triflumuron) was due to a bad mass accuracy (> 5 ppm) and for cyhalothrin the mSigma-value was higher than 200. 14 pesticides had a response below the noise level. For all 100 detected pesticides the mean recovery is between 60-140 which is the criteria given in the guidance, SANTE/12682/2019, and for 85 of the 100 pesticides the precision was below 25%.

Stability testing

Stability of selected compounds were tested. The groups that were tested were as follows: NSAID's, coccidiostatics, tranquilizers and anthelmentics. A spike solution was prepared by mixing 400 µL of stocksolutions (1 mg mL^{-1}) for 30 compounds and 3 mL acetone.

500 g of porcine beef was homogenised and the spike solution was added to 400 g, giving a concentration of 1 mg kg-1 of all compounds. The sample was again homogenised and 6 samples were analysed for testing homogeneity. The homogenised spiked sample was transferred to sarstedt tubes with 5-6 g in each. The tubes





were stored at -80 °C, -20 °C, +4 °C and +20 °C. Sample were tested at week 1, 2, 4, 6, 12, 16 and 22 by analysing two samples from every storage condition. Samples stored at +20 °C was not tested after 6 weeks. When evaluating the stability a threshold value of 65% is used corresponding to an uncertainty of 25%, coverage factor 2 and duplicate analysis. All compounds were stable more than three months at -20 °C. After 22 weeks only oxyphenbutazone and phenylbutazone are below the threshold value at -20 °C. For the tested compounds all anthelmentics and coccidiostatics are above the treshold after 22 weeks. For the four tested tranquilizers all, except chlorpromazine, are above the threshold. The NSAIDs are stable at least four weeks at + 4 °C except phenylbutazone,

Figure 1. Stability of NSAIDs at -20 °C (a) and +4 °C (b)

oxyphenbutazone and vedaprofen (fig. 1). For the other groups of compounds they are stable at least 2 weeks except fenbendazole, fenoterol and robenidine. At +20 °C the compounds are stable less than one week except most NSAIDs that are stable for at least one week except phenylbutazone and oxyphenbutazone, they are degraded rapidly.

Real sample analysis

Samples analysed by another laboratory with LC-MS/MS with finding of sulfadoxin, tiamulin and oxytetracyclin in three samples, were analysed using the screening approach described here. In two samples hits were found, with sulfadoxin (124 µg kg^{-1}) in one sample and tiamulin in another sample. These finding were in line with the original findings in the other laboratory. For the finding of sulfadoxin criteria for mass accuracy and retentiontime are acceptable and the two qualifier ions are detected with acceptable ion ratio (fig 2). In a third sample oxytetracyclin was found (7 µg kg^{-1}) with LC-MS/MS but this finding was not found with our screening method, probably due to the low concentration.

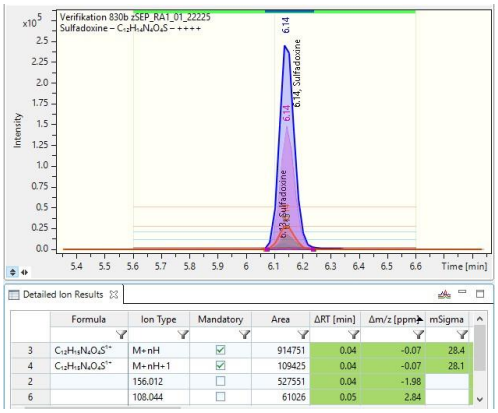


Figure 2. Pig muscle with finding of sulfadoxin (124 µg kg^{-1})

The last three years the screening approach has been tested by analysing 100 extracted samples on the LC-HR instrument. The samples were extracted at another laboratory with their in-house extraction method and analysed for a few groups of veterinary drug residues as part of the residue control. This year 46 of the extracts were samples of muscle from pig and bovine and by using the screening approach described with the exclusion list, in only four samples of bovine, thiabendazol were found. The mSigma-value and mass accuracy was within the tolerance but since the expected qualifierions were not seen the extracts were confirmed by analysing them with LC-MS/MS with the same LC-conditions as presented here.

Conclusion

A method for simultaneous analysis of several groups of veterinary drug residues and pesticides has been documented using liquid chromatography coupled to high-resolution mass spectrometry. For processing of data it is important to optimise the setting for reducing the rate of false compliant results and at the same time not end up with too many hits and false non-compliant results and when retentiontime is unknown this can especially be a problem. For this work an in-house database has been used where retentiontime has been established for 152 veterinary drug residues and 114 pesticides. The settings has been tested by analysing

blank samples and samples previously tested positive for some antibiotics. In the blank samples no suspect samples occurred and for the “positive” samples, the findings were confirmed.

As part of the validation a stability study was done on selected compounds from several groups of veterinary drug residues. For most compounds they were stable at three month stored at -20 °C and two weeks stored at + 4 °C.

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POSTER P51

USE OF PLACKETT-BURMANN DESIGN TO OPTIMIZE ANALYTICAL METHODS OF VETERINARY DRUGS AND FOOD CONTAMINANTS

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Abstract

Experimental design is a way to optimize analytical process minimizing time and the number of experiments to be performed. However, when a high number of critical factors is involved, it is necessary a previous study to select the main factors that impact method efficiency. The Plackett –Burman (PB) designs are a useful tool to this purpose.

Herein, we describe the assembly of a simple PB spreadsheet to be used for factors selection before the use of complete factorial designs. In the two described cases, PB was followed by Box-Behnken design, once the main impacting factors were set. The workflow was used for the analysis of polycyclic aromatic hydrocarbons in fish and pharmaceuticals in mussels, using matrix solid phase dispersion followed by pressurized liquid extraction (PLE) as sample preparation techniques. In both cases, the critical factors were extraction solvent volume, amount of organic solvent in the extraction solvent, sample mass, holding time in PLE, temperature (PLE), amount of solid phase (MSPD) and ratio of extraction solvent in the PLE system. The PB data analysis facilitated the observation of factors with major influence under the analytes response before method optimization by Box-Behknen design.

Introduction

Different statistical tools are available in the literature to assess the influence of factors on an expected response. The traditional univariate method allows understanding the impact of a single variable on a data set. However, when a set of critical factors need to be optimized to provide an efficient analytical process, the interaction between these factors may require multiple experiments and disregard the interaction between them. In these cases, where it is necessary to evaluate several critical factors, multivariate chemometric approaches are applied to optimize the process. These approaches offer greater economy, speed and efficiency when compared to univariate ones.

Experimental designs (DoE) are efficient multivariate approaches for identifying and optimizing critical factors in an analytical method. DoE designs provide multiple responses with a minimal number of experiments, in addition to enabling analysis of the interaction between critical factors (Narenderan et al., 2019).

Analytical process optimization is usually carried out in two-step projects: screening (I) and optimization (II). Screening designs are used to select the significantly important factors for subsequent optimization. A popular, efficient, and cost-effective screening design was developed by RL Plackett and JP Burman (PB) (Plackett; Burman, 1946). BP is a two-level fractional factorial design, of at most N-1 factors with N experiments (N being a multiple of 4). Although BP does not consider the interaction between factors, it allows the evaluation, with few experiments, of a large number of variables, and therefore, allows the identification of critical factors for optimization.

The critical factors identified in the screening project (BP) are submitted to optimization projects. The Box-Behnken (BBD) optimization design is a three-level design and can be applied to three or more factor optimization. The number of experiments is defined as 2 k (k − 1) + Cp , where k is the factor numbers and Cp is the number of center points. The BBD allows designing a second-order response surface, enabling the identification of optimal conditions and the interaction between each of the factors (Box-Behnken, 1960).

The development and optimization of analytical methods are steps prior to validation and which, in general, are the steps that most require analysts' time, resources and expertise. The use of chemometric tools allows obtaining data of great importance, allowing the reduction of costs and time. In addition, well-designed experiments can statistically guarantee the maximum verification or approximation of the optimal points of an analytical method, however, the routine use of such tools in the area of drug residues and contaminants in food is still incipient.

In our study, we describe the assembly of a simple BP spreadsheet to be used in the selection of critical factors preceded by full factorial designs (BBD). The spreadsheet was applied in the evaluation of critical factors in the processes of extraction of polycyclic aromatic hydrocarbons (PAHs) in fish and medicines in mussels. In both cases, the impacts of the following critical factors were evaluated: volume of extraction solvent, amount of organic solvent in the extraction solvent, sample mass, residence time in the pressurized liquid extractor (PLE), temperature, amount of solid phase (MSPD) and extraction solvent ratio in the PLE system.

Materials and Methods

Chemicals and reagents

All reagents used were analytical grade and LC-MS grade solvents. Ultrapure water (minimum resistivity of 18.2 MΩ cm at 25 °C) obtained by Milli-Q® Integral Millipore (Molsheim, France). The sorbent materials used were diatomaceous earth supplied by Honeywell, Fluka (Charlotte, USA) and Q-Matrix Hydra supplied by CEM Corporation (Charlotte, USA).

All analytical standards of pharmaceuticals and PAHs were of the highest available purity (≥ 95% of purity) and were supplied by Dr. Ehrenstorfer GmbH (Augsburg, Germany) or Sigma-Aldrich (St. Louis, USA). Stock and work solutions were prepared as described in previous reports (de Melo et al. 2022; Hoff et al., 2020). All stock solutions were separately prepared with 1000 µg mL^{-1} and stored at -18 °C.

Samples

Samples of fish, crustaceans and bivalve mollusks were used in the study. Samples were obtained from commercial capture or cultivation areas through the Brazilian Federal Inspection Service (SIF). All samples were cleaned with ultrapure water to remove dirt. Only portions of muscle tissue were used and homogenized in a food processor. The samples were stored in aluminum packages with laminated paper lids at a temperature of –30 to –10 ºC until analysis.

Apparatus and analytical instrumentation

Liquid chromatography was performed in the 1290 Infinity system, manufactured by Agilent Technologies (Waldbronn, Germany) coupled to a 5500 QTrap hybrid triple quadrupole-linear ion trap mass spectrometer, manufactured by Sciex (Framingham, USA). The analysis of pharmaceuticals was achieved using the conditions previously describe by our research group (Hoff et al., 2020). Determination of PAHs were performed according to our previous report (de Melo et al., 2022).

Pressurized liquid extraction apparatus

The PLE system was an automated pressurized fluid extraction system EDGE® (CEM Corporation, Matthews, USA), with twelve positions. The general procedure for EDGE extraction starts with the preparation of an extraction cell (Q-cup®), where one or more filtration membranes were assembled. Thus, the sample (with or without previous dispersion on solid phase) was transferred to the tubes and submitted to the extraction process.

Sample preparation

MSPD-PLE method for pharmaceuticals was performed with 2.0 g of sample dispersed in diatomaceous earth (2.0 g). After that, the mixture of sample and dispersing agent was transferred to an aluminium tube (model Q-Cup®, CEM Corporation, Matthews, USA), which was assembled with three filtration discs in a sandwich of C9 + G1 + C9 (Q-Discs®, CEM Corporation). Extraction solvent was H_2O: ACN, 80:20, with 0.1% FA. Samples were extracted in just one cycle, programmed as follows: 10 mL of extraction solvent added on top of the extraction cell; 10 mL of solvent added on the bottom of the extraction cell; temperature 80 °C, held by one minute. After

that, the extract was automatically transferred to a clean tube. The tubes were kept at -30 ± 5 °C for 30 minutes. Subsequently, tubes were centrifuged at 3488-g for 10 min. An aliquot of 1 mL of the supernatant was directly transferred to a glass vial to be submitted to analysis.

For PAHs analysis, the procedure was the same previously reported (de Melo et al., 2022), but with the transfer of PLE process from a hard cap coffee machine to the EDGE PLE system, maintaining the extraction conditions as similar as possible to the initial validated parameters (data in preparation for submission).

Plackett-Burman design

In the process of optimizing the preparation of samples for the determination of PAHs and pharmaceuticals, considering the large number of variables involved, we started the optimization by chemometric tools using a Plackett-Burman (BP) design, a screening method in which it is possible to prioritize those parameters with the greatest influence on the response.

In order to find out which factors were the most impactful in extracting samples using the EDGE system through the PB design, the following parameters were chosen for PAHs analysis: (1) solvent extraction volume (in mL); (2) percentage of ACN in the extraction solvent; (3) sample mass; (4) residence time of the sample in the extraction chamber; (5) extraction temperature; (6) amount of diatomaceous earth and (7) proportion of solvent dispensed at the top and/or bottom of the extraction cell (Table 1).

Table 1. Plackett-Burman design to evaluate seven variables in the extraction procedure of polycyclic aromatic hydrocarbons (PAHs) in fish by pressurized liquid extraction.

Code	Independent variables	Levels		Center point
		1	-1	0
1	Extraction solvent volume (mL)	30	10	20
2	ACN amount in extraction solvent (%)	60	20	40
3	Sample mass (g)	1.0	3.0	2
4	Holding time (min)	15	5	10
5	Temperature (°C)	100	50	75
6	Diatomaceous earth (dosage)	4	2	3
7	Ration of extraction solvent delivery (top: bottom)	1:2	2:1	1:1

ACN = acetonitrile.

In the case of pharmaceuticals determination in mussels and oysters, the following factors were chosen: extraction solvent volume (A), percentage of ACN in extraction solvent (B), percentage of formic acid in extraction solvent (C), holding time (D), temperature (E), mass of adsorbent (F) and ratio of solvent dispensed in top and/or bottom of extraction cell (G).

According to the standard procedure for PB design (Analytical Methods Committee, 2013), each value was evaluated in two levels (+1 and -1) in 8 trials (Table 2). The dependent response was peak area (cps). The data were evaluated using the software Minitab version 17 (Minitab, State College, PA, USA).

Table 2. Codified trials for Plackett-Burman design.

Trials	X1	X2	X3	X4	X5	X6	X7
1	1	-1	-1	1	-1	1	1
2	1	1	-1	-1	1	-1	1
3	1	1	1	-1	-1	1	-1
4	-1	1	1	1	-1	-1	1
5	1	-1	1	1	1	-1	-1
6	-1	1	-1	1	1	1	-1
7	-1	-1	1	-1	1	1	1
8	-1	-1	-1	-1	-1	-1	-1

Box-Behnken Design

The key factors determined by the BP approach were optimized using a 2-factor Box-Behken design. Control variables were extraction solvent volume (mL) and percentage of ACN in extraction solvent (%). The response variable was the analyte peak area (cps). The regular experimental design has included 3 levels for each factor and 3 replicates for the center point. The center point conditions were the best conditions observed in the PB experiment. Data analysis and mathematical modelling were performed using Design-Expert version 12 (Stat-Ease, Minneapolis, MN, USA). Prior to the elaboration of the contour plot, the mathematical models were validated using ANOVA (95% confidence interval).

Results and Discussion

Variables selection for PAHs analysis using PB

The BP results demonstrate that the extraction solvent volume is the main overall factor, with a positive effect, except for BbF (Table 3). The second most influential factor on the analytical response is the percentage of acetonitrile, with a positive effect. The third factor is predominantly the sample residence time under the EDGE extraction conditions, with a negative effect. The other variables analyzed are less impacting (sample mass, amount of diatomaceous earth and temperature) and the proportion of solvent introduction into the extraction cell is not significant.

Table 3. Main significant factors obtained by Plackett-Burman design screening for PAH analysis.

Analyte	1st factor	2nd factor	3rd factor
BaP	Solvent volume (+)	Acetonitrile (+)	Holding time (-)
BaA	Solvent volume (+)	Acetonitrile (+)	Diatomaceous earth (+)
CR	Solvent volume (+)	Diatomaceous earth (+)	Temperature (-)
BbF	Acetonitrile (+)	Solvent volume (+)	Holding time (-)
BkF	Solvent volume (+)	Acetonitrile (+)	Holding time (-)
DbA	Solvent volume (+)	Acetonitrile (+)	Holding time (-)
BgP	Solvent volume (+)	Sample mass (-)	Acetonitrile (+)
IcP	Solvent volume (+)	Acetonitrile (+)	Sample mass (-)

BaP = benzo[a]pyrene; BaA = benzo[a]anthracene; CR = chrysene; BbF = benzo[b]fluoranthene; BkF = benzo[k]fluoranthene; DbA = dibenzo[a,h]anthracene; BgP = benzo[ghi]perylene; IcP = indeno[1,2,3-cd]pyrene.

Variables optimization for PAH analysis using Box-Behnken design

Considering that BP is a variable prioritization approach, the results showed that carrying out a factorial design 2^2 (volume of solvent + acetonitrile) or 2^3 (volume of solvent + acetonitrile + residence time) with axial points should produce the ideal conditions. This experiment was then designed using a Box-Behnken type experimental design with two factors (volume of extraction solvent and percentage of acetonitrile in the extraction solvent).

The results demonstrate that the preponderant factors are the percentage of ACN in the mixture (BbF, BkF, DbA) and the interaction of the two factors (BaP, IcP and BgP). For CR and BaA, low analytes signals were detected in the blank sample, which interfered with the interpretation of the results and did not allow the elaboration of response surface graphs for these two analytes. There are two distinct profiles of response surfaces (Figure 1A and 1B). For those analytes whose most influential factor was the percentage of ACN, the optimal response point seems to be located at higher values of ACN, around 85%.

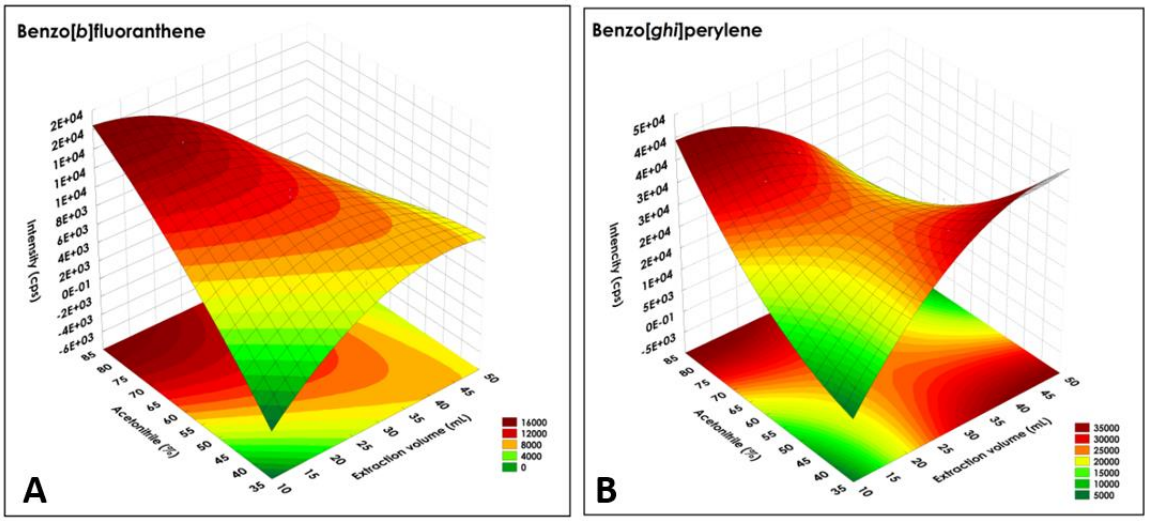
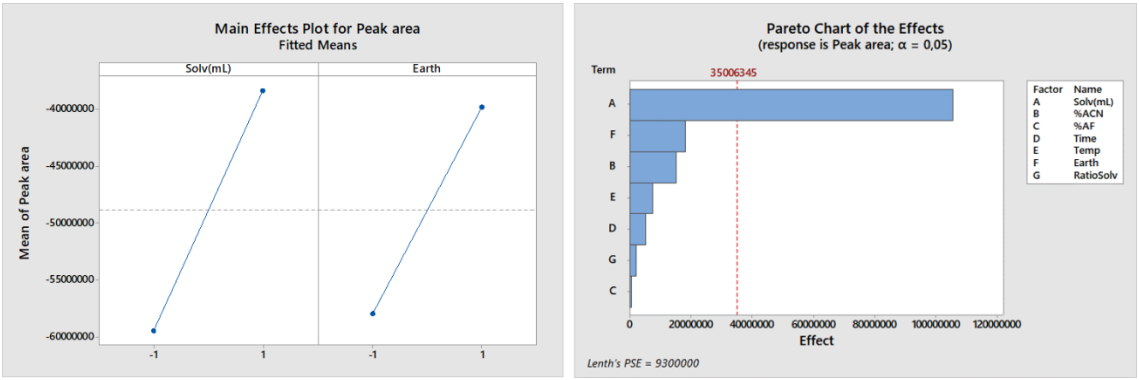


Figure 1. Surface responses for benzo[b]fluoranthene (A) and benzo[ghi]perylene (B).

Variables selection for pharmaceuticals determination using PB

Considering that PLE using the automated system EDGE is susceptible to several parameters, a PB design was applied to differentiate between those factors with influence under the response and the noise (factors with negligible impact). The results clearly show the extraction solvent volume as an impacting factor for representative analytes from the main pharmacological class, as well as diatomaceous earth amount and ACN percentage. Extraction time, extraction temperature, and the ratio of solvent delivery inside the Q-cup® were non-significant for all analytes. As an example, the Figure 2 shows the main effects plot and the Pareto chart of



the effects for quetiapine.

Figure 2. Main effect plot and Pareto chart of the effects for quetiapine, obtained using Plackett-Burman design.

All main factors showed positive responses: all +1 factors increased the response. The screening results show that an optimization experiment including the main factors (solvent volume + diatomaceous earth + %ACN) should produce the optimum conditions. Thus, the second chemometric study was designed as a Box-Behnken model to evaluate these three parameters and its interactions.

Variables optimization for PAH analysis using Box-Behnken design

The results obtained from the Box-Behnken design show that the extraction solvent volume plays the major role in the peak area response. The example of ractopamine illustrates the main behavior observed for all evaluated analytes, the same used for PB study (Figure 3).

Figure 3. Surface plot for main effects and interactions between extraction solvent (ES) volume (A), acetonitrile percentage (B), and diatomaceous earth mass (C) under ractopamine analytical signal (peak area in cps). From left to right: interactions A:B, A:C, and B:C.

Conclusions

In general, it appears that for matrices with significant amounts of potentially interfering substances, the use of larger volumes of solvent produces an increase in the analytical response, probably due to a decrease in matrix effects caused by the dilution of the interferents. However, even though the optimization results indicate that a larger volume of extraction solvent would increase the responses, there is a volume limitation due to the characteristics of the EDGE system. In addition, the use of solvent volumes above 40 mL directly affects the costs and environmental impact of the method, given that the objective is to develop a routine method. Thus, the observed effect of increasing solvent can probably be replicated by a post-extraction dilution.

The overall conclusion is that both PB and BB are very useful chemometric tools that can be easily inserted into the laboratory routine. The application of these tools in the development and optimization of analytical methods can generate objective information about how closer to the optimal sample preparation conditions our methods are really are. Moreover, the data obtained using this approaches can clear indicates to what direction we can go to achieve the best responses.

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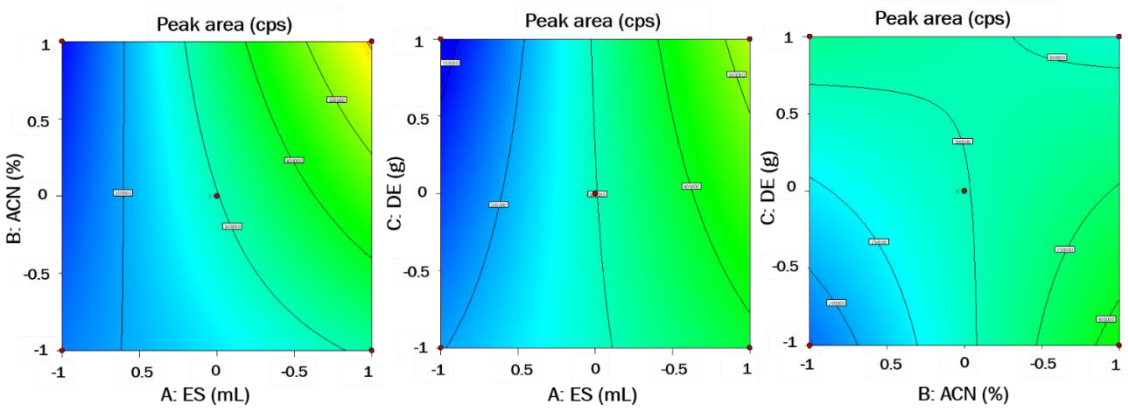

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POSTER P52

QUANTIFICATION OF STEROIDS IN IN-VITRO AND IN-VIVO ASSAYS WITH ON-LINE SPE-LC-MSMS

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Abstract
As part of testing drugs for their endocrine disrupting effect and their effects on steroidogenesis the H295R cell line assay is an OECD approved in-vitro test. For measuring the steroids in the final cell extracts a LC-MS/MS is developed using on-line solid phase extraction for detection of androgens, corticosteroids, estrogens and gestagens in cell extracts.
The cell extract is centrifugated at high speed (12.000 g) and 100 µl is injected on the LC-MS/MS (EVOQ Elite Triple Quadropole mass spectrometer from Bruker and an Ultimate 3000 UPLC system with DGP-3600RS dual-gradient pump from Thermo Scientific). The steroids are retained on a short HLB column (20 *2,1 mm, 15 µm) and eluted on an analytical column with a gradient with ammoniumfluoride (0,2 mM) and methanol (estrogens) or formic acid (0,1%) and acetonitrile (androgens, gestagens and corticosteroids). The method is also developed for detection of the steroids in plasma and gonad tissue.
The method is validated for 15 steroids (covering androgens, corticosteroids, estrogens and gestagens). The limit of detection is below 100 ppt for most steroids and 10 ppt for the estrogens.

Keywords: LC-MS/MS, Online SPE, Steroid analysis

POSTER P53

DETERMINATION OF RACTOPAMINE IN FEEDINGSTUFFS AT PPT LEVEL USING MIP-SPE

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Abstract

Ractopamine (RAC) is a β -agonist agent which has been banned from animal production in several countries. Other countries like Brazil still allows its use, but pig production units dedicated to meat export must adopt zero tolerance environment for RAC. Thus, the analytical methods employed in RAC monitoring need to achieve the lower detection limits as reasonable. In order to assure that there is no cross-contamination even at trace levels (ng kg^{-1}), we developed a sample preparation protocol based on solid-liquid extraction followed by molecularly-imprinted polymer/solid phase microextraction (MIP-SPE) and LC-MS/MS analysis. Briefly, 10 g of sample were extracted with 30 mL of 90:10 acetonitrile:methanol with 0.1% formic acid and 1 g of Na_2SO_4. The extraction was achieved by ultrasound-assisted extraction and low-temperature clean-up. After that, the supernatant was applied to MIP-SPE cartridges (SupelMIP SPE-Beta Agonists) and the final eluate was concentrated to dryness, reconstituted in ACN and injected in LC-MS/MS system. Using internal standard calibration (with RAC-d3) and matrix-matched analytical curves, it was possible to correctly determine real samples fortified with 100 ng kg^{-1} of RAC in pig feed, allowing the monitoring of feed cross-contamination in very low concentrations.

Introduction

Ractopamine (RAC) is a β -agonist drug used as an additive to increase meat deposition and reduce fat accumulation in pigs (Aroeira et al., 2019). However, RAC is banned in many important import markets such as the European Community, Taiwan, Russia and several others. Brazil is the largest meat exporter in the world, and to meet the requirements of international trade in the area of food safety, the Ministry of Agriculture, Livestock and Food Supply (MAPA) started to issue special certificates, such as the RAC exemption, after proving that the companies comply with good practices (GMP) and that they do not use this ingredient in their production process (Aroeira et al., 2019). This verification takes place through an on-site audit involving a delegation of MAPA inspectors, belonging to different sectors of the Ministry. Therefore, MAPA regulates industries and carries out a special certification process for companies that focus on production for foreign markets with this restriction.

In Brazil, the use of RAC is still authorized and was approved by MAPA to be used in dosages from 5 to 20 mg kg^{-1} in pig feed during 28 days prior to slaughter, without a withdrawal period. In the EU, additives belonging to the β-agonist class were banned through Directive 96/23/EC. Although Brazil allows RAC in pig production, MAPA developed the proposal of a Pig Production Program without RAC (Split System), in cooperation with the production sector, to ensure a safe product in order to meet international requirements.

Consequently, this split system requires a strict monitoring system, and the challenge task for the official laboratories is to associate the analysis of tissue samples, animal feed, and feedingstuffs ingredients collected in both production systems: RAC-free and authorized for RAC use. In the cases of RAC-free production systems, international requirements are increasingly restricted and analytical methods with high sensibility and low detections limits are required.

Meat and bone meal is considered an important co-product from the animal industry once it is composed by a wide variety of animal organs, which have high nutritional value. Thus, these ingredients can be in pig feeding as a protein source, decreasing feed costs and increasing competitiveness. This fact generates a concern about the potential presence of RAC residues in this ingredients, leading to RAC occurrence in pig feed and, consequently, affecting the RAC-free status of the production plants (Feddern et al., 2018).

MAPA official method for RAC determination in feedingstuffs (along with other 61 veterinary drugs and contaminants) has a limit of quantification of 75 µg kg^{-1} (Hoff et al., 2020). In the case of analysis from RAC special investigations, alternative methods are applied, with lower detection limits. In the present study, we have evaluated alternative procedures to achieve the lower detection limits as possible for RAC determination in pig feed samples.

Materials and Methods

Chemicals and reagents

Analytical standard of ractopamine hydrochloride (RAC) with 95 ± 0.71% of purity and were supplied by Dr. Ehrenstorfer GmbH (Augsburg, Germany). Analytical standard od deuterated ractopamine (RAC-d3) with 98.4% of purity were obtained from TLC Pharmachem (Canada). All stock solutions were separately prepared at 1000 mg L^{-1} and stored in freezer. Working solutions were prepared diluting aliquots of stock solutions in ACN. All solvents were in chromatographic grade. All reagents were in analytical grade. Acetonitrile (ACN), anhydrous magnesium sulfate and methanol (MeOH) were supplied by J. T. Baker Chemical Co. (Phillipsburg, NJ, USA)., acetic acid (AA) and formic acid (FA) were supplied by Tedia Co. (Fairfield, OH, USA). Sodium chloride (NaCl) was from LS Chemicals (India). Ammonium acetate and anhydrous sodium sulphate (Na_2SO_4) were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Molecularly-imprinted polymers SPE cartridges SupelMIP® SPE - Beta-agonists (bed weight 25 mg, volume 10 mL) were from Supelco Merck KGaA, Darmstadt, Germany).

Samples

Blank samples of pig feed were selected from official samples previously analyzed in our laboratory.

Apparatus and analytical instrumentation

Liquid chromatography was performed in the 1290 Infinity system, manufactured by Agilent Technologies (Waldbronn, Germany) coupled to a 5500 QTrap hybrid triple quadrupole-linear ion trap mass spectrometer, manufactured by Sciex (Framingham, USA). Chromatographic separation was performed with a C_{18} Simmetry column (50 × 2.1 mm; 3.5 µm, 100 Å, from Waters) with a C_{18} guard column (4.0 mm × 3.0 mm) from Phenomenex (Torrance, CA, USA). Water and MeOH were used as mobile phases A and B, respectively, with ammonium acetate (5 mmol L^{-1}) and AA (0.1%) in each phase. The elution gradient was set to 95% A (1–4 min), 10% A (4–12 min), and 95% A (12–13 min), plus 5 min to system auto-equilibrium at the same initial conditions. The injection volume was set to 5 µL and the flow was 0.3 mL min^{-1}. The column was kept at 40 °C.

Mass spectrometry analysis were carried out using electrospray ionization source (ESI) in positive mode, with the following parameters: ion spray (IS) voltage: 5500 V; curtain gas: 25 psi; nebulizer gas (GS1): 55 psi; auxiliary gas (GS2): 55 psi; source temperature: 400 °C. Nitrogen was used for nebulization and collision. The Analyst 1.6.2 and the MultQuant softwares (Sciex, Foster City, CA) performed data acquisition and processing. Analyte-specific mass spectrometry parameters were optimized in previous reports and were confirmed for the application in the present method (Hoff et al., 2020; Jank et al., 2018; Molognoni et al., 2018; Valese et al., 2017) (Table 1).

Table 1. Parameters of multiple reaction monitoring (MRM) conditions for the residue analysis of RAC and RAC-d3 in animal feed by LC-MS/MS.

Analytes	Internal standard	Precursor Ion (ESI+)	Quantification transition (CE, in V)	Confirmation transition (CE, in V)	DP (V) Q/C	CXP(V) Q/C
Ractopamine (RAC)	RAC-d3	302	164.2 (21)	121.2 (29)	161/161	8/8
Ractopamine-d3 (RAC-d3)	---	305	167 (23)	124.2 (29)	86/161	10/8

CE = collision energy; CXP = exit cell potential; DP = declustering potential; Q/C = quantification/confirmation.

Information-dependent acquisition and enhanced product ion experiments

In order to increase the method confidence for the identification of RAC, additional experiments of information-dependent acquisition (IDA) and enhanced product ion (EPI) were associated to the MRM experiment. The IDA criteria was optimized to select the peaks from the MRM experiment which exceeds at least 5000 cps, after dynamic background subtraction of survey scan, and with mass tolerance of 250 mDa.

Peaks were selected after the first occurrence during at least 2 seconds. The EPI experiment was designed to trap the selected peak in the ion trap in order to increase the concentration of the selected peak and produce a MS^2 spectrum which is compared with the library constructed with analytical standard. The EPI parameters were the following: mass range from 120 to 350 Da, the time period was 13 minutes with 2226 cycles. The scan rate was 10000 Da/s. Other parameters were declustering potential (DP) of 100 V, entrance potential (EP) of 10 V, collision energy (CE) de 35 V, and collision energy spread (CES) of 15 V; scan mode profile with step size of 0.12 Da, dynamic fill time selected, settling time of 50 ms, and 8 V of Q3 entry barrier.

Optimized sample preparation method for 1-5 ppb level determination
Samples of 2.0 g or blank pig feed were exactly weighted. After that the samples were fortified with 1.0 µg kg^{-1} of RAC and 5.0 µg kg^{-1} of RAC-d3 (10 µL of RAC working solution + 50 µL of RAC-d3 working solution). Samples were extracted using the matrix solid phase dispersion (MSPD) followed by pressurized liquid extraction (PLE) using a hard cap coffee machine as previously reported by our research group (Hoff et al., 2020). Briefly, after fortification all samples were mixed using a mechanical vortex and approximately 2.0 g of diatomaceous earth was added. The tubes were vigorously mixed and the resulting mixture sample + solid phase were transferred to a capsule and extracted using H_2O:ACN (80:20) with 0.1% FA. The extract produced by the PLE apparatus was received in a clean polypropylene tube containing 2.0 g of NaCl and 2.0 g of anhydrous magnesium sulphate. The extract volume was up to 20 mL with the extraction solution. The extract was mixed with the salts using mechanical vortex (10-15 s), followed by an orbital shaker (180 rpm by 20 minutes). After that, the tubes were centrifuged (3488 G-Force for 10 min) to promote the salting-out. The organic supernatant phase was transferred to a 15-mL polypropylene tube. An aliquot of 800 µL was evaporated to dryness in a water bath (45 °C) with the aid of a gently of nitrogen, and reconstituted with 200 µL of MeOH:H_2O (80:20) into a HPLC vial and submitted to LC-MS/MS analysis.

Optimized sample preparation method for ppt level determination
Samples of 10 g of blank pig feed were weighed in 50-mL polypropylene tubes and were fortified with 1 ng of RAC and 1 ng of RAc-d3, producing 0.1 µg kg^{-1} (100 ppt). Then, 30 mL of the extraction solvent (ACN:MeOH, (90:10) with 0.1% FA was added. After that, 1.0 g of Na2SO4 were added and the tubes were using mechanical vortex (10-15 s), followed by an orbital shaker (180 rpm by 20 minutes). Following, the tubes were placed in a freezer by 1 hour to promote low temperature cleanup pf the extracts. Then, the tubes were centrifuged (3488 G-Force for 10 min) and the supernatant were applied to the SPE cartridge.
SPE-MIP extraction was preceded by cartridge condition/equilibrate with 1 mL of MeOH, 1 mL of H_2O, and 1 mL of 25 mM ammonium acetate pH 6.7. Then, the sample was loaded at a flow rate of approximately 0.5 mL min^{-1}. The wash was performed using 1 mL of H2O followed by vacuum application (2 minutes). After that, the SPE cartridge was washed sequentially with 1 mL of 1% Aa in ACN, 1 mL of 50 mM ammonium acetate pH 6.7, and 1 mL of ACN:H2O (60:40). After the washing steps, the cartridge was dried again applying vacuum by 2 minutes. Finally, the analyte was eluted with 2 × 1mL 10% AA in MeOH. The eluate was concentrated to dryness in a water bath (45 °C) with the aid of a gently of nitrogen, and reconstituted with 200 µL of ACN into a HPLC vial and submitted to LC-MS/MS analysis.

Quality control parameters
Each experiment was composed by at least 5 replicates and was accompanied by blank and double blank samples.

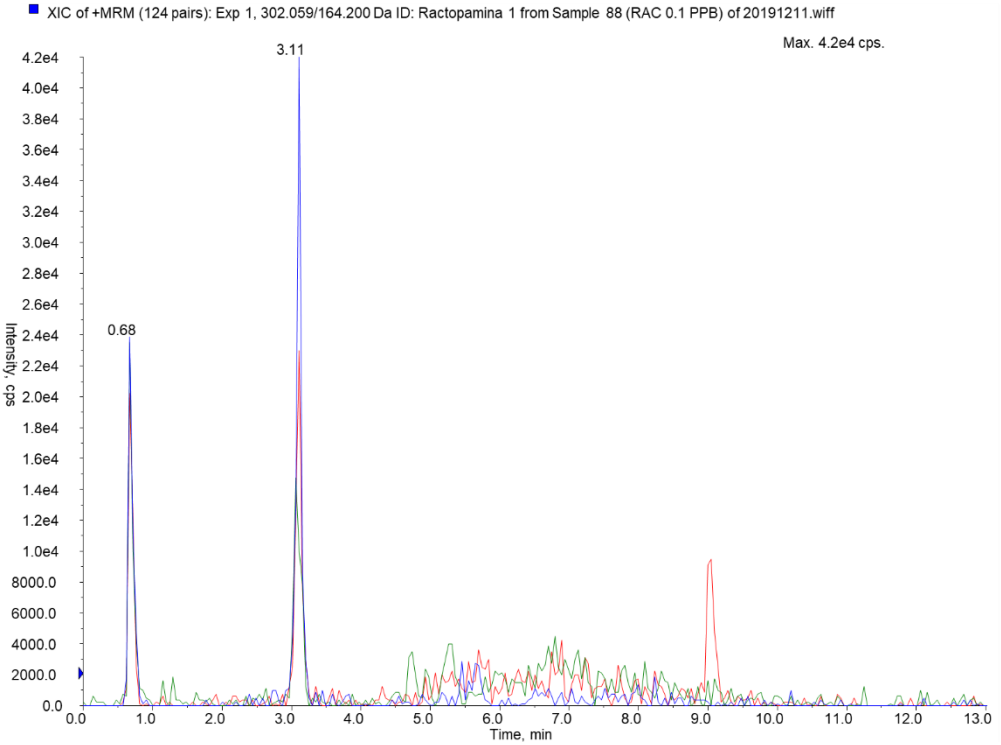
Results and Discussion

Sample extraction for 1-5 ppb level
The extraction of RAC using the salting-out process proved to be quite efficient to obtain detection limits much lower than those previously obtained with the hard cap coffee machine method (Hoff et al., 2020). The physicochemical properties of RAC foment the concentration of this analyte in the organic phase, which, after concentration by evaporation, allowed obtaining very relevant concentration factors. However, the concentration process can also cause intensification of matrix effects, leading to results contrary to those initially desired, i.e., ionic suppression instead of analytical signal enhancement. To verify the most appropriate procedure and identify the balance point between concentration and matrix effect, the organic phase obtained after the salting-out process was subjected to different treatments: i) analysed directly; ii) diluted with MeOH:H_2O (1:1); iii) concentration by evaporation of aliquots of 400, 600 and 800 µL. The result of all these tests demonstrates that the procedure of concentrating 800 µL of the extract and then reconstituting the dry extract with 200 µL MeOH:H_2O (1:1) was the approach that obtained the most intense analytical signals, with

matrix effects similar to the other tests. The concentration factor obtained with this procedure was 4 times in relation to the organic phase obtained after the salting-out. The procedure proved to be very valid to obtain detection and quantification limits in the range of 1 to 5 ppb in matrix without the need to use SPE or other more complex preparation procedures. Future studies can be carried out in order to fully validate this protocol not only for RAC but also for other banned drugs that are soluble in the organic phase.

Sample extraction for ppt level

Regarding the procedure using SPE-MIP, the challenge becomes greater because animal feed matrices are very



complex, composed of fat, protein, carbohydrates, mineral salts and a series of additives, such as vitamins, amino acids, trace elements and others. In order to obtain an analytical signal for concentration levels as low as 100 ng kg^{-1}, it is necessary to use sample preparation processes with very high selectivity and that allow obtaining a high concentration factor for the analytes. The SPE cartridge used was originally developed for animal tissues and biological fluids such as urine. The development of a protocol using this cartridge for different matrices as feedingstuffs required a certain level of adaptation, mainly with regard to the initial sample mass. Different initial masses of samples were evaluated, starting with 2.0 g, which was the amount of sample used in the official method. As no analytical signs of RAC were verified using this initial mass, we evaluated larger amounts of samples. The aliquots tested were 5.0, 7.5 and 10.0 g of sample. Only when 10 g of sample were used, an analytical signal distinguishable from noise was obtained and capable of being confirmed by the MRM-IDA-EPI experiments. Thus, the protocol used was 10 g of sample, which is extracted with 30 mL of a mixture of acetonitrile and MeOH. The use of a dehydrating agent such as sodium sulphate proved to be essential to obtain the result. The SPE-MIP conditioning, washing and elution protocol did not require any changes, being performed exactly as proposed by the manufacturer for biological fluids. The concentration of the final eluate by evaporation in a water bath at 40°C with the aid of a gentle flow of nitrogen provided an additional concentration factor to the SPE. Following this protocol, chromatograms with adequate signal (signal-to-noise ratio >3) were obtained and blank samples were obtained without the presence of RAC signal or interference in the same retention time window (around 3.10 min) of the monitored m/z transitions. However, a potential interferent peak was observed eluting together with the column dead volume (Figure 1). The concentration factor obtained considering 10 g of sample and final extract reconstituted in 200 µL was 50 times.

Figure 1. Extracted ion chromatogram (XIC) for RAC in pig feed sample fortified with 0.1 µg kg^{-1} and extracted suing SPE-MIP protocol.

Conclusions

Although the determination of RAC at levels as low as 0.1 µg kg^{-1} has not yet been required by any regulatory body, it is interesting that the official laboratories working with the determination of residues of veterinary drugs in food anticipate the demands and have tools capable of providing analytical answers in the face of increasingly strict requirements. In the present work, we demonstrate a proof of concept that it is possible to determine RAC levels in swine feed in parts per trillion using highly selective and specific sample preparation protocols. Moreover, the addition of a salting-out step in our official method for veterinary drugs determination in animal feed allows to obtain detection limits much lower than those originally validated. Such methods can be applied in case of audits in production plants that wish to obtain or maintain RAC-free certification. Future studies may be carried out to fully validate the procedures and provide analytical tools that can be applied in routine laboratories for the analysis of a large number of samples.

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POSTER P54

DETERMINATION OF 67 VETERINARY DRUGS IN FEEDINGSTUFFS USING TWO-STEP ENERGIZED DISPERSIVE GUIDED EXTRACTION

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Abstract

The monitoring of veterinary drugs in feedingstuffs is critical to prevent the occurrence of residues above the MRLs in edible tissues. Using the energized dispersive guided extraction, a new system of pressurized liquid extraction (PLE), 67 veterinary drugs can be extracted in just two steps. Briefly, samples (2.0 g) were mixed with diatomaceous earth and submitted to PLE extraction using ACN: H_2O (60:40) as extraction solvent. The resulting extract was transferred to a freezer to promote low-temperature clean-up. This procedure spontaneously promoted salting-out.

After 30 min at -20°C, samples were centrifuged, and an aliquot of the upper layer was directly injected in a LCMS/MS system. The same extract was injected in two chromatographic columns (C_{18} and ciano), allowing the separation and determination of a wide range of analytes, from high hydrophilic analytes (e.g. amoxicillin) to very lipophilic substances such as avermectins. The method was validated according to 2021/808/CE, covering intra and interday precision, CCα, trueness, and matrix effects. The method was applied to several kinds of feedingstuffs intended for the main animal species and ingredients and made with both animal and vegetal ingredients.

Introduction

In Brazil, residues of veterinary drugs in feedingstuffs is one of the risk management actions for monitoring the production conditions and are a complementary effort to the controls carried out by the National Residues Control and Plan (NRCP), which aims to provide adequate residues control in foods (Mauricio et al., 2009). In feed factories, the production of both medicated and non-medicated feed is a common scenario. The carry-over of compounds between successive batches is considered unavoidable and traces of the first produced feed may remain in the production line and contaminate the next feed batch. As a consequence, the non-compliant animal feed production can lead to veterinary drugs residues in edible animal tissues. Feedingstuffs analysis to determine levels of veterinary drugs are a challenging task, due to the distinct scenarios regarding the use of those substances as additives or therapeutics. Moreover, an ideal method should be able to analyze since feed ingredients (e.g. oat bran) to concentrated products (premixes) with a very high concentration level (g.kg-1 or even %). Thus, the present method was developed with the aim of: a) identifying drug residues banned in the manufacture of animal feed; b) identify pharmaceuticals in animal feed produced by establishments that do not have authorization to manufacture such products; c) verify the level of a drug which was declared by the manufacturer in the product label; e) evaluate the presence or absence of carry-over contamination in production lines and f) investigations of the food production chain in the case of non-compliance samples of edible animal tissues with drug residues above the maximum residue limit (MRL).

Since 2012 our laboratory has been applying analysis methods for veterinary drugs in animal feed. The current method was developed in order to expand the scope initially applied to this analysis, initially developed for 12 forbidden antibiotics. Later, the scope was increased to include more 12 analytes, covering 24 antibiotics and antibacterial not allowed to be used as feed additives (Jank et al., 2018). Recently, the method was full changed to include a wide scope, comprehending 62 veterinary drugs and contaminants (Hoff et al., 2020).

Our official method was based on matrix solid phase dispersion (MSPD) followed by pressurized liquid extraction (PLE) using a hard cap espresso machine. This method was applied to the analysis of hundreds of samples in the last years. However, to increase the method capability regarding number of samples and also to achieve a higher degree of automation, the hard cap espresso machine has been replaced by a new PLE system, nominated as energized dispersive guided extraction (EDGE). The change in the extraction method, associated with significant variations in the separation method, has resulted in the need of a validation study to assure the fitness to purpose of the scope extension.

Thus, in the present study, we have performed a scope extension validation study to assure the analytical quality of a new method for veterinary drugs and contaminants determination in feedingstuffs.

Materials and Methods

Chemicals and reagents

All analytical standards were of the highest available purity and were supplied by Dr. Ehrenstorfer GmbH (Augsburg, Germany) or Sigma-Aldrich (St. Louis, MO, USA), with the exception of zilpaterol (supplied by Toronto Research Chemicals Inc., North York, Canada). The analytes included in the present study was described in our previous report (Hoff et al., 2020), with the exception of the added compounds virginiamycin S1, narasin, salinomycin, salbutamol and diaveridine. All stock solutions were separately prepared at 1000 mg L^{-1} and stored in freezer. The exception was amoxicillin, which stock solution was fractioned in aliquots of 0.5 mL and stored in an ultrafreezer (-80°C). Working solutions were prepared diluting aliquots of stock solutions in ACN.

All solvents were in chromatographic grade. All reagents were in analytical grade. Acetonitrile (ACN) and methanol (MeOH) were supplied by J. T. Baker Chemical Co. (Phillipsburg, NJ, USA). Acetic acid (AA) and formic acid (FA) were supplied by Tedia Co. (Fairfield, OH, USA). Ammonium acetate and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Diatomaceous earth (Hydromatrix®) was supplied by Agilent Technologies. Optimized extraction solution was prepared by mixing ultrapure water and ACN (40:60, v/v) and adding 0.1% of FA.

Samples

Blank samples of animal feed were selected from official samples previously analyzed in our laboratory. The validation was performed using a blank sample composed by a feed formulation for laying hens. For premix analysis, a previously analyzed premix for turkey feed without any of the analytes was used as blank sample.

Apparatus and analytical instrumentation

Liquid chromatography was performed in the 1290 Infinity system, manufactured by Agilent Technologies (Waldbronn, Germany) coupled to a 5500 QTrap hybrid triple quadrupole-linear ion trap mass spectrometer, manufactured by Sciex (Framingham, USA). Chromatographic separation was evaluated in three independent methods. Hydrophilic interaction liquid chromatography (HILIC) was performed with the Hypersil Gold HILIC column (150 × 3.0 mm, 5 µm) from Thermo Fisher Inc. (Rockford, USA). Reversed-phase chromatographic separation was achieved in a C_{18} Simmetry column (50 × 2.1 mm; 3.5 µm, 100 Å, from Waters) and in a di-isopropyl-3-aminopropyl silane bound to hydroxylated silica (CN) stationary phase (Zorbax 300 Stand Boat-CN, 150 × 4.6 mm, 5 µm, 300 Å, from Agilent Technologies). The details about mobile phases and gradient mode are previously described (Hoff et al., 2020) for HILIC and C_{18}. In the present study, the same conditions applied for C_{18} separation were used to evaluate the CN column.

Mass spectrometry analysis were carried out using electrospray ionization source (ESI) working in positive and negative mode, with the following parameters: ion spray (IS) voltage: 5500 V; curtain gas: 25 psi; nebulizer gas (GS1): 55 psi; auxiliary gas (GS2): 55 psi; source temperature: 400 °C. When working in negative mode, the same parameters were settled, except the IS voltage, set in −4500 V. Nitrogen was used for nebulization and collision. The Analyst 1.6.2 and the MultQuant softwares (Sciex, Foster City, CA) performed data acquisition and processing. Analyte-specific mass spectrometry parameters were optimized in previous reports and were confirmed for the application in the present method (Hoff et al., 2020; Jank et al., 2018; Molognoni et al., 2018; Valese et al., 2017).

Pressurized liquid extraction apparatus
The PLE system was an automated pressurized fluid extraction system EDGE® (CEM Corporation, Matthews, USA), with twelve positions. The general procedure for EDGE extraction starts with the preparation of an extraction cell (Q-cup®), where one or more filtration membranes were assembled. Thus, the sample (with or without previous dispersion on solid phase) was transferred to the tubes and submitted to the extraction process.

Optimized sample preparation method
In few words, the sample (2 g) was dispersed using diatomaceous earth (approximately 2 g) in a polypropylene tube of 50 mL using a mechanical vortex. The resulting mixture was transferred to an EDGE extraction cell with a sandwich of membrane filters assembled in the bottom of the tube (C9+G1+C9) which was extracted by PLE using water and acetonitrile (H2O/ACN 40:60) as extraction solvent. PLE parameters was: residence time – 2 min; top volume – 10 mL; bottom volume – 10 mL; temperature – 85°C; rinse solvent - H2O/ACN 40:60; wash volume – 30 mL; wash solvent – H2O at 100°C. After PLE, the extract (approximately 25 mL) was submitted to cleanup under low temperature and, after centrifugation, 1 mL of the supernatant was directly analyzed by LC-MS/MS.

Method validation
Method validation was carried out in accordance with 2021/808/EC guidelines, to evaluate the following parameters: selectivity, linearity, matrix effects, trueness, within-laboratory repeatability (WLr), within-laboratory reproducibility (WLR) and decision limit (CCα).

Selectivity was evaluated based on standard solutions of each analyte and internal standards, to ensure that there were no interferences peaks, plus the analysis of 20 blank samples. Linearity and matrix effects were evaluated through the construction of 3 calibration curves, with 9 levels of concentrations (including zero), namely: 0, 15, 30, 75, 150, 300, 450, 600 and 900 μg kg^{-1}. The only substance that was evaluated in a different concentration range was amoxicillin, whose curve was prepared with the following concentrations: 0, 75, 150, 300, 450, 600, 750, 900 and 1200 μg kg^{-1}. Matrix effects evaluation was carried out comparing the response of calibration curve prepared in pure solvent, in samples fortified before the extraction, and from post-extraction spiked samples.

Trueness and precision were assessed by carrying out within-laboratory repeatability and within-laboratory reproducibility studies using fortified blank samples. The evaluated concentration levels were 0.5, 1.0 and 1.5 times at the reference point for action (RPA) of 300 μg kg^{-1}. WLr studies were performed on three different days by the same analyst, with batches composed by 7 replicates of each fortification levels plus blanks samples and matrix matched calibration curve. WLR studies were performed on three different days by three different analysts, with batches composed as described to WLr study. CCα values were calculated from the within-laboratory reproducibility data, as defined in the Method 1 as described in the 2021/808/EC.

Results and Discussion

Sample extraction
Sample preparation was done using MSPD followed by PLE. In our previous report, a hard cap coffee machine was used to perform the PLE step after the dispersion of samples with diatomaceous earth. To transfer the PLE method from the coffee machine to an automated PLE system, the initial evaluation was done using the same parameters previously validated, assuming a similar performance between both PLE methods. However, one of the disadvantages of the coffee machine was the limitation to use organic solvents as an extraction solution. As the new system does not show this limitation, extraction solutions with more organic solvent in its composition were tested (H_2O/ACN 20:80, H_2O/ACN 40:60 and H_2O/ACN 60:40, all of them with 0.1% FA). Table 1 shows the results for these three different sample treatments according to the pharmacological/chemical groups, in terms of increase or decrease of the response, assuming the previous solvent extraction composition (H_2O/ACN 80:20) as 100%. Some groups have shown very significant increase in the response such as avermectins, benzimidazoles, nitrofurans and several coccidiostats.

Table 1. Percentage decrease or increase of response (peak area) according to the extraction solvent composition used in PLE.

	Extraction solvent composition		
Pharmacological/chemical group	H_2O/ACN 20:80	H_2O/ACN 40:60	H_2O/ACN 60:40
Aminopirimidines [a]	-95.6 [b]	-46.2	17.0
Amphenicols	157.2	252.7	4.4
Avermectins	1032.0	1873.3	146.8
Benzimidazoles	390.8	752.6	75.9
β-agonists	92.1	172.2	-23.4
Coccidiostats	51.3	219.9	-24.6
Fluorquinolones	83.7	17.4	108.4
Lincosamides	37.1	-14.0	-36.4
Macrolides	249.8	75.1	-17.8
Nitrofurans	272.5	370.9	24.8
Quinoxalines	-46.6	43.1	-48.9
Sulfonamides	399.3	277.6	24.4
Tetracyclines	-49.0	4.1	4.0
Internal standards	204.5	444.0	26.9
β-lactams	31.6	4.7	41.5

[a] The results are the average of all analytes of each group; [b] n= 3 for each analyte.

An unexpected advantage was the occurrence of a spontaneous salting-out process in the low temperature cleanup procedure for those samples extracted with higher levels of acetonitrile were used (up to 60%). In this case, both aqueous (lower) and organic (upper) lawyers were injected. The results for the organic phase are those presented in Table 1. For the aqueous phase, just amoxicillin showed a better response than the organic phase. Considering the results, the mixture chosen to perform the PLE was H_20/ACN, 40:60.

Chromatographic separation

The wide range of physico-chemical characteristics of the analyse included in this study results in a considerable obstacle to achieve a unique chromatographic separation. In our previous report (Hoff et al., 2020), we described the use of two columns to resolve this question. The most hydrophilic analytes (amoxicillin, oxibendazole, melamine, halquinol, among others) were separated using a HILIC column while all other analytes were resolved using a C_{18} column. In the present study, we evaluated the use of a CN column with the aim to separate all compounds using just one column. The CN column was first evaluated using the mobile phase composition and gradient mode equal to the C_{18} column. After that, a second test was made using the same conditions used before for the HILIC separation. The best responses were obtained when the CN column was used using the same C_{18} conditions. The results were very similar to the C_{18}, with a slightly decrease in tetracyclines resolution. Moreover, the same analytes unable to be separated adequately using C_{18} were poorly separated using CN. Thus, to achieve the best separation for all compounds, the array C_{18} + HILIC were maintained.

Validation

The method selectivity/specificity was evaluated by analyzing 20 feed samples from different producers to assess the presence of possible interferences in the retention times and transitions of each analyte. No peaks that could contribute to errors in the analyte identification and quantification procedures were identified.

The unweighted linear model was used for calibration curves by adding a matrix standard and internal standardization, verifying its fit to the linear model. For some analytes, the model that best fitted was the weighted linear model (1/y). Based on the matrix matched calibration curve, the majority of the analytes showed linearity through all the evaluated range. To aid the routine analysis performance, a balanced range from 75 to 600 µg kg^{-1} were chosen as work range. Likewise, although not contemplate by 2021/808/EC - the method limit of quantification (LOQ) was established as equivalent to the least point on the curve in which the

coefficient of variation between the triplicate peak areas was equal to or less than 30% (CV ≤ 30%). Similarly, the limit of detection (LOD) was established as being equal to the point on the curve immediately below the LOQ at which analyte peaks with a signal-to-noise ratio greater than point 0 can be identified. In the working range used, all curves showed linearity with regression coefficient greater than 0.95.

Matrix effects were present for almost all analytes, which means that the use of matrix matched calibration curves is mandatory. The matrix effects were negligible just for benzimidazoles (4% of ion suppression). The higher effects were observed for fluorquinolones (50% of ion suppression). Recoveries were also determined using the same equations, with the most analytes with a recovery around 60%. Values of CCα were calculated considering the level of 300 µg kg^{-1} as the centre level. An example of the curves used for the calculation of CCα is given in Figure 1. In average, the CCα values were very dispersed, but is notably that those compounds analysed suing HILIC chromatographic separation have produced the highest values, which can be associated not to the separation method but to the hydrophilic characteristic of those substances and a heterogeneous separation in the salting-out step of the sample preparation protocol.

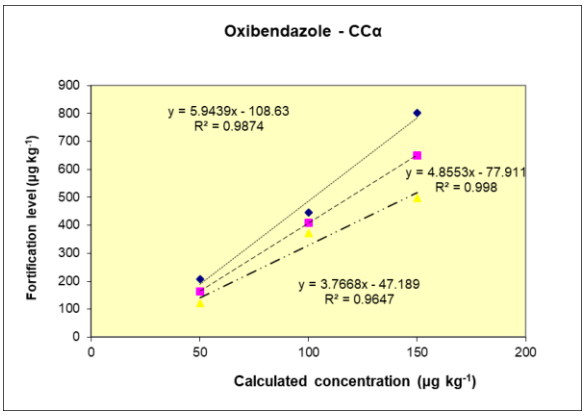

Figure 1. Example of calibration curve approach used for CCα determination, with a plot of all results determined for 21 replicates for each concentration level and the derived calibration curves produced by the sum of 1.64 x the standard deviation of within-laboratory reproducibility for each one of the 3 levels.

Trueness was also satisfactory with global values from 80 to 123%, considering the average for the 3 evaluated concentration levels, corresponding to 150, 300 and 450 µg kg^{-1}. WLR and WLr were evaluated considering the calculated concentration for all batches analysed by the same analyst (WLr) and the combination of all batches (WLR). Most of the analytes have showed satisfactory result, with CV < 30%, with the exceptions of melamine, salbutamol, diaveridine, and halquinol. Enramicin was the only analyte with totally unacceptable results and consequently was removed from the method scope.

Conclusions

The official method for determination of veterinary drugs and contaminants in feedingstuffs were improved using a new PLE system. In comparison with our previous report, the new sample preparation method was fast, able to be automatized, and have better recoveries results for the majority of the analytes, especially for avermectins and benzimidazoles. Validation parameters were within the internal laboratory criteria and the method have been applied to determination of real animal feed samples.

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POSTER P55

DETERMINATION OF PROGESTERONE IN BOVINE SERUM

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Abstract

Progesterone- a steroid female sex hormone can be used for anabolic purposes in cattle. Endogenous levels of progesterone are known to be highly variable and systematic studies on progesterone levels in different matrices are limited. The aim of the study was to develop a method for determining progesterone in bovine serum samples and to use it in testing of samples obtained under the National Residue Control Program. The progesterone was analysed and quantified as heptafluorobutyric anhydride derivatives by gas chromatography–mass spectrometry after extraction from serum with tert-butyl methyl ether/petroleum ether. The method is characterized by good repeatability, reproducibility and the recovery (89%). A total of 341 bovine serum samples were examined, including 175 taken from females and 166 from males. The natural presence of progesterone was detected in 105 female bovine serum samples (60%) above CCα limit (0.10 μg L^{-1}- 79.4 μg L^{-1}). Of the serum samples taken from male bovine 53 had progesterone concentration in the range from 0.10 μg L^{-1} to 3.9 μg L^{-1}. The mean progesterone concentration in female serum was 2.8 μg L^{-1}, and in male serum only 0.20 μg L^{-1}, while the median was 0.22 μg L^{-1} and 0.04 μg L^{-1} respectively.

Introduction

The use of anabolic steroids such as oestrogens, androgens and gestagens in food production of animal origin have been forbidden in the European Union, due to possible toxicological effects on human health (Council Directive 96/22/EC, 1996). Progesterone (formerly called lutein) is one of the most important natural female sex hormones (EURL reflection paper, 2014). It is mainly produced by the cells of the ovarian corpus luteum during the luteal phase of the menstrual cycle, in early pregnancy, and by the placenta during the later stages of pregnancy (Bomba-Opon *et al*., 2015). Progesterone belongs to the group of progestogens, hormones with a steroid structure. The precursor of progesterone, analogous to many other numerous and important steroid hormones, is cholesterol, which is converted to pregnenolone, its direct precursor. The main function of progesterone is to prepare the uterus for implantation of the embryo in its mucosa and to protect it throughout pregnancy. The effects of progesterone on the reproductive organs are the facilitation of ovulation, the development or inhibition of excessive endometrial hyperplasia, and cyclical changes in the appearance of the epithelium of the reproductive organs (Cicinelli *et al.*, 2000; Posaci *et al*., 2000). It also acts as a neurotransmitter, transporting information between nerve cells in the brain, especially in areas responsible for the regulation of sexual behaviour and sex drive, which is under constant research. Progesterone works synergistically with oestrogens to stimulate lactation. In human medicine, this hormone is used preventively in women at the risk of miscarriage, corpus luteum failure, pregnancy poisoning, infertility treatment, menstrual cycle disorders resulting from endogenous hormone deficiency and in postmenopausal hormone replacement therapy for protective purposes (Czajkowski *et al*., 2007; Miles *et al.*, 1994). Progesterone, although a female sex hormone, plays an important role in the male body. It inhibits the process of conversion of testosterone into dihydrotestosterone, the excessive concentration of which can even lead to such a serious disease as prostate cancer. In men, progesterone is produced by the testes and adrenal glands in small amounts compared to the amount of progesterone in the female body.

The metabolism of progesterone takes place mainly in the liver (90%), the kidneys and in trace amounts in the muscles, it is mainly excreted in the urine. In veterinary medicine, progesterone also plays an important role in the reproductive process and embryogenesis. Individually or in combination with oestradiol, it can be administered to animals in the form of an implant in order to improve the rate of muscle mass gain and better use of feed, i.e. for anabolic purposes. Extending the duration of action and improving the effectiveness of progesterone can be obtained by converting it into synthetic derivatives such as medroxyprogesterone acetate (MPA), megestrol acetate (MGA), chlormadinone acetate (CMA) or melengestrol acetate (MLGA). The residues of the above substances are monitored in animals and in animal products in tests carried out under National Residue Monitoring Plans, obligatory for all EU Member States (Regulation EU 2017/325, 2017). Their residues

are tested in muscle and kidney fat samples. Progesterone has not been included in the minimum list of compounds to be tested in monitoring programs. Since we determine progesterone in muscle samples in our laboratory, a project was created to check the levels of this natural hormone in serum samples from cattle, taken for tests for the determination of oestradiol and testosterone. Progesterone, on the other hand, is measured in various matrices in human and veterinary medicine. Control of the misuse of anabolic hormones as well as quantitative determination of their concentration is effective only by sensitive and specific analytical techniques (Posthuma-Trumpie *et. al*., 2009). Actually chromatographic techniques play the most important role in residue analysis of anabolic substances like natural, pseudo-endogenous or synthetic hormones, both for screening and for confirmatory analysis (Commission Implementing Regulation EU 2021/808, 2021; Stolker *et al.*, 2005; Wozniak *et al.*, 2011).

Materials and Methods

Reagents and chemicals

Standards of Progesterone (98%) and the deuterated internal standard of 17β testosterone-d2 (>95%) were obtained from Sigma Aldrich (Germany) and WFSR (Wageningen Food Safety Research, EURL, the Netherlands). All standards were stored in accordance with the recommendations of the certificates. Primary standard stock solutions were prepared in methanol at a concentration of 10 µg L^{-1} and were stored below –18°C. Working solutions with a concentration of 1.0 µg L^{-1}, 0.1 µg L^{-1}, and 0.01 µg L^{-1} (depending on the required situation) were prepared by appropriate dilution of the stock solutions with methanol. All solutions were stored at about 4°C (range 2–8°C) for no longer than six months. All chemicals were of high purity quality. Solvents: petroleum ether, tert-butyl methyl ether and methanol (residue grade) were purchased from Mall Baker (the Netherlands), isooctane (GC grade) and heptafluorobutyric anhydride (HFBA) were obtained from Sigma-Aldrich (Germany).

Samples.

The bovine serum samples were obtained within the frame of Polish Residue Monitoring Programme. The samples were collected from both animals living on farm and from animals intended for slaughter. Serum samples were obtained from 341 animals, including 175 cows of 1 – 168 months of age and from 166 bulls of 1 – 48 months of age. The information about cattle breed, physiological condition, and feeding system of the animals was not available. At the laboratory, the serum samples were stored below –18°C and thawed before analysis.

Sample preparation.

Serum samples were analysed by gas chromatography-mass spectrometry technique (GC-MS) as described before (Wozniak *et al., 2011)*. Briefly, hormones were extracted from serum samples with a mixture of tert-butyl methyl ether/petroleum ether (30:70, v/v). After phase separation, the tube contents were centrifuged and frozen at < –18°C for 1.5–2 h. Then, the organic phase was decanted and evaporated to dryness under a stream of nitrogen. The residue was subjected to derivatisation process with HFBA reagent, and analysed by GC-MS. For each analytical batch calibration curve was prepared over analyte concentration range. The Progesterone determined by GC-MS was quantified by using internal standard technique. In each series of analysis, spiked samples were tested to verify the quality of the results. The method is accredited for determination of natural sex hormones: testosterone and oestradiol in serum and positively verified in proficiency testing and interlaboratory comparisons. It is planned to extend the method to progesterone after the validation.

GC-MS measurement.

An Agilent 6890N GC chromatograph with an Agilent 5973 Quadrupole MSD mass spectrometer and Chemstation Software was used for the analysis. Chromatographic separation of progesterone and testosterone-d2 was achieved on a non-polar HP-5MS capillary column (30 m, i.d. 0.25 m, 0.25 µm film thickness) using 0.9 mL min^{-1} constant flow of helium. The samples (2 µL of injection volume) were injected in pulsed splitless mode in 250°C. The oven temperature was kept constant at 120°C for 2 min and was then increased by 14°C per min to 270°C and kept on this level for 7 min, a further rise in temperature was 15°C to 280°C and kept for 2 min also. The injection port, MS source and Quadrupole temperatures were set as 250, 230 and 150°C respectively. The GC-MS apparatus was operated in positive electron impact (EI) ionization

mode at 70 eV with selected ion monitoring (SIM).

Validation study.
The method actually is under validation in accordance with Commission Implementing Regulation (EU) 2021/808. All required validation parameters will be estimated. Of course before measuring the concentration levels of progesterone initial validation was performed. For this purpose blank serum samples spiked with the progesterone to the concentration levels of a 0.5 μg L^{-1}, 1.0 μg L^{-1} and 2 μg L^{-1} were analysed. Based on that results, apparent recovery, precision (repeatability) and linearity of standard and also matrix matched calibration curves were evaluated. The linearity was estimated on the basis of calibration curves of standard of progesterone with seven calibration levels, prepared to each series of tested samples.
For the evaluation of the test samples the CCα determined for 17β-testosterone was adopted. The performance of the method for progesterone detection in bovine serum samples is presented in Table 1.

Results and Discussion

The study was carried out using a single ion monitoring mode which causes increasing of sensitivity of analytes and minimises background noise. For progesterone mass m/z 510 and for the internal standard of testosterone-d2 mass m/z 682 were observed. For the applied technical conditions a satisfactory chromatographic separation was obtained. Typical chromatograms of serum sample spiked with progesterone and also example of serum sample tested with the estimated concentration of this compound are presented in Figure 1.

The linear regression parameters of standard and matrix-matched calibration curves were correct in the whole range of concentrations (corresponding to the concentration of progesterone in samples 0-6 μg L^{-1}) as proved by the correlation coefficient exceeding 0.98 value.
The apparent recovery values were within the reference range defined in the legislation. The method was characterised by good precision less than 25%.

Table 1. Method parameters for progesterone detection in bovine serum samples

Parameter		
Calibration curve (standard)	Correlation coefficient	0.9864
	Slope	3.0284
	Intercept	0.9103
Calibration curve (matrix-matched)	Correlation coefficient	0.9887
	Slope	2.9058
	Intercept	-0.3410
Apparent recovery [%]	0.5 μg L^{-1}	86.5
	1.0 μg L^{-1}	89.3
	2.0 μg L^{-1}	88.9
Repeatability C.V. [%]	0.5 μg L^{-1}	15.2
	1.0 μg L^{-1}	21.6
	2.0 μg L^{-1}	20.6

Natural progesterone was found in 60% of the female samples tested. Of these samples, in 39% the concentration was within the range of 0.10-0.50 μg L^{-1}; in 20% of samples the concentration was in the range from 0.50-1.00 μg L^{-1}; in 32% of samples the concentration was in the range from 1.00-10.00 μg L^{-1} and in 8.6% it exceeded 10 μg L^{-1}. The highest concentration of progesterone equal to 79.43 μg L^{-1} was found in the serum taken from 3.5-year old female bovine animal.

Natural progesterone was also found in male animals. Of course the concentrations were much lower. Of these samples, in 60% the concentration was within the range of 0.10-0.50 μg L^{-1}; in over 18% the concentration was in the range from 0.50-1.00 μg L^{-1} and above 1.00 μg L^{-1}.

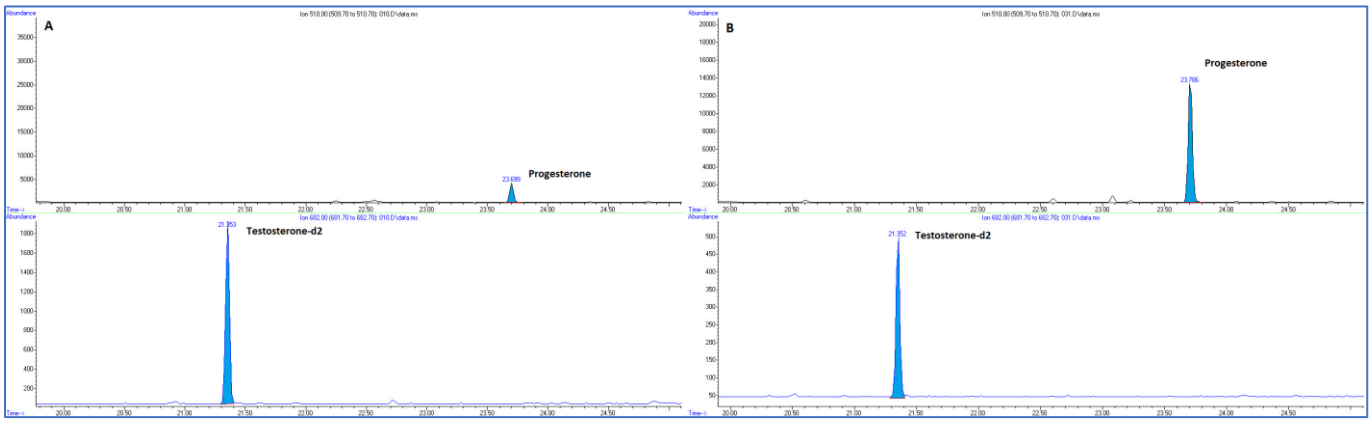

Figure 1. GC-MS chromatogram of (A) a serum sample fortified with Progesterone at 1 µg L^{-1} concentration level; (B) a tested serum sample from 7 years old female animal with the concentration of Progesterone 14.280 µg L^{-1}.

Conclusion

The GC/MS method developed for the determination of progesterone in serum is very simple and can be used only in screening after full validation. In the near future a confirmatory method based on LC-MS/MS or GC-MS/MS will be developed and the correlation between the content of progesterone and other natural sex hormones will be investigated.

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POSTER P56

THE PRESENCE OF ANTIBIOTIC RESIDUES IN DIFFERENT MATRICES OF VEAL CALVES AFTER MEDICAL TREATMENT

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Abstract
The presence of antibiotic residues has been determined in 10 veal calves in hair and saliva at the age of 5 months and additionally in hair, muscle, kidney, liver and teeth after slaughter, using an UPLC-MS/MS multimethod. The findings have been linked with the veterinary treatments.

Gentamycin traces have been detected in the kidney of all parenterally treated veal calves 6½ months after treatment.
Florfenicol, given as individual treatment parenterally, has been found in saliva and hair of the treated animals but also in non-treated veal calves in the same group housing system. Oxytetracycline residue traces have been found in hair and in teeth at slaughter in all veal calves although only 1 animal had been treated (6½ months before slaughter).
The presence of antibiotic residues in different matrices during fattening or after slaughter could not always be linked to the treatment of the specific animals. It is suspected that cross contamination between group-mates caused by veterinary treatment is a possibility.

Introduction
The use of antibiotics in livestock is a topic with a lot of attention in our society. Several livestock sectors set goals to minimize the use of antibiotics and therefore decrease antibiotic resistance. To reach these goals it is important to monitor the use and mis-use of antibiotics during the life of livestock. Since every antibiotic is absorbed differently in the body it is important that the correct matrices are analysed to detect the compound of interest. In this study antibiotic treatments will be followed in 10 veal calves to determine the migration of administrated medication in the matrices hair, saliva, kidney, muscle, liver and teeth.

Materials and Methods

Sample collecting
10 veal calves from one farm with a known medical (farm contains 1200 veal calves) history are sampled on the farm and during slaughter. All veal calves are from different groups of calves and in different pens. On the farm the saliva and hair are collected. During slaughter the matrices hair, teeth, kidney, liver and muscle are collected.

Analytical method
Kidney, liver and muscle samples are extracted with 70% methanol (fraction 1) and with 5% TCA (fraction 2) while being milled. The fractions are centrifuged. Hair and teeth samples are milled with a ball mill to a homogeneous sample. The milled sample is extracted with 2.5% TFA in methanol and McIlvain-EDTA buffer (fraction 1) and with 5% TCA (fraction 2). SPE extraction with HLB columns takes place on the fractions. Saliva samples (absorbed in salivates) are extracted with 2% FA in acetonitrile (fraction 1). The extractions are centrifuged.
The obtained extractions are measured with UHPLC-MS/MS Sciex 6500 (Fraction 1: 0,1% FA in H_2O & methanol; acquity UPLC BEH C18. Fraction 2: 0,1% in H_2O & acetonitrile HSS T3).

Results and Discussion
In total 11 different antibiotics have been administrated to different veal calves and have been followed in several matrices during the lifecycle of these veal calves. The results are demonstrated in table 1 and have been reported qualitatively. The results show the detectability of the antibiotic in the specific matrix.

Table 1. Summary of the results.

Antibiotics	Administration	Farm		Slaughter				
		Hair	Saliva	Hair	Liver	Kidney	Muscle	Tooth
Ampicillin	Oral	x	x	x	x	x	x	x
Amoxicillin	Oral	x	x	x	x	x	x	x
Doxycycline	Oral	+++	+	++	x	+	x	+++
Florfenicol	Parental	++	+++	+	x	x	x	x
Oxytetracycline [1]	Oral	x	x	x	x	x	x	x
Sulfadiazine	Oral	+	x	+	x	x	x	x
Tilmicosin	Oral	++	+	+	+	x	x	x
Trimethoprim	Oral	+	x	x	x	x	x	x
Paromomycin	Oral	x	x	x	x	x	x	+++
Gentamycin [2]	Parental	x	x	x	+	+++	x	+
Neomycin	Oral	x	x	x	x	x	x	x

[1] Sum of OTC and metabolites
[2] Sum of gentamycin C1+C1a+C2+C2a

x = Unusable
+ = Indicative
++ = Useful matrix
+++ = Recommended matrix

Saliva could not be extracted sufficiently from the sample containers. Therefore, it is difficult to conclude if all antibiotics are extracted from the salivates. However, it can be said that florfenicol, doxycycline and tilmicosin can be extracted and detected in the saliva. Hair is also a good matrix to detect the use of florfenicol, doxycycline and tilmicosin. Also trimethoprim/sulfadiazine can be detected in the hair when a veal was administrated with the drug. These compounds where still detectable in the hair during slaughter of the veal calves.

For gentamycin analysis in animal tissue kidney is clearly the preferred matrix. Gentamycin is detected in high concentration in the kidney, even after the withdrawal period described in the drug label prescription.

Teeth is an interesting matrix to detect doxycycline where it could not be detected in matrices like kidney, liver and muscle. Paromomycin has also been detected in teeth.

Ampicillin, amoxicillin and neomycin have not been detected in any matrix.

Oxytetracycline has been detected in five veal calves of different groups and in all veal calves during slaughter. However, only one group received oxytetracycline as medical treatment. The same was observed with trimethoprim/sulfadiazine which had been detected in nine veal calves of different groups where only three groups received treatment.

Conclusion

The detection of antibiotics is matrix dependent due to different administration routes and pharmacokinetics. Not all compounds can be detected in the same matrices. Obtained information about the most useful matrices can be crucial for an efficient antibiotic monitoring program.

Cross contamination of antibiotic residues between veal calves in group housing seems plausible whit the obtained data. Detection of antibiotic residues in different matrices in a veal calf during fattening or slaughter seems not always conclusive of the use of antibiotics in this specific animal. It could also be caused by the veterinary treatment of group-mates.

References

- Regulation EU 37/2010 of 22 December 2009; on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin.
- Regulation EU 2021/808 of 22 March; on the performance of analytical methods for residues of pharmacologically active substances used in food-producing animals and on the interpretation of results as well as on the methods to be used for sampling and repealing Decisions 2002/657/EC and 98/179/EC.

POSTER P57

OXYTETRACYCLINE IN TREATED CHICKEN DROPPINGS AND LITTER, ITS DISSEMINATION TO UNTREATED ANIMALS AND EFFECT ON THE SELECTION OF RESISTANT BACTERIA.

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Abstract

Oxytetracycline (OTC) is used in the poultry industry through feed and drinking water. To determine the excretion and dissemination of OTC to litter and other animals, and its effect on the selection of resistant Escherichia coli strains during post-treatment, OTC+4-epi-OTC were quantified in droppings and litter from treated and untreated birds (sentinel), by a validated LC-MS/MS method. Escherichia coli was isolated from all samples and its susceptibility to tetracyclines was determined by the Kirby-Bauer test. Fifty resistant isolates were for the presence of *tet* genes by PCR. Average OTC+4-epi-OTC concentrations between 347.63 and 2244.66 µg/kg were detected in the treatment group. On the other hand, in the sentinel groups OTC were below the detection limit (12.2 µg/kg). The highest proportion of tetracycline-resistant *Escherichia coli* strains were isolated from the treated birds and the susceptibility of the strains was group-dependent. A high clonal diversity of the isolates was observed and 80% presented the *tet*(A) gene. It is concluded that OTC there is a low probability of transfer to other animals since traces of this antimicrobial were detected.

Introduction

One of the most important effects generated by the use of antimicrobials in productive animals is the selection of resistant bacteria. It is in this sense that important efforts have been made to control the improper or excessive use of these drugs, highlighting the global action plan on antimicrobial resistance adopted at the World Health Assembly in May 2015, where different objectives related to this issue were established (Sumpradit et al., 2021).

Specifically, tetracyclines have led to the emergence of resistant bacterial variants, in particular those containing *tet* genes, which are generally associated with mobile genetic elements or conjugative transposons (Chopra & Roberts, 2001; Roberts, 2005; Thaker et al., 2010), which encode for different resistance mechanisms against these antimicrobials, such as efflux pumps, ribosomal protection, enzymatic inactivation and different mutations, such as the one described in the 30S ribosomal subunit (Bryskier, 2005).

Genes are currently argued to be emerging contaminants because they have been detected in different environmental compartments, including soils, river sediments, watercourses, and wastewater (Pruden et al., 2006; Zalewska et al., 2021). Their presence in different environments is a risk because they can be acquired by other pathogenic bacteria affecting humans and animals (Xiong et al., 2018; Zhao et al., 2017).

Escherichia coli is a Gram-negative bacillus that is part of the normal intestinal microbiota of animals and humans. However, there are some pathogenic strains that can cause fatal diseases in humans, mammals, and birds (Torres, 2016). These bacteria are used as indicator bacteria for antimicrobial resistance levels in different productive species, as they have been described to be a reservoir of resistance genes, which could be transmitted to pathogenic and zoonotic bacteria (Agyare et al., 2019; Moreno, 2000; Nguyen et al., 2009; Torres, 2016). Antimicrobial stewardship has been observed to increase the risk of antimicrobial resistance in E. coli in pigs. However, there is a lack in the study of doses or concentrations, and effects over time (Burow et al., 2014).

Regarding the relationship between the presence of antibiotic residues and prevalence of resistance in veterinary medicine, Chantziaras et al. (2014) evaluated the correlation between antibiotic use and the prevalence of *E. coli* strains isolated from pigs, poultry, and cattle. Their results indicated that there is a correlation between the use of specific antimicrobials and the level of resistance of these microorganisms. However, there are many data restrictions in this study, so they conclude that more detailed data collection and harmonization is needed.

Currently, there are no known controlled studies that determine the effect of OTC administration, at therapeutic doses, in broilers on the selection of post-treatment resistant strains. For this reason, the objective of the present study was to determine the presence of *E. coli* isolates not susceptible to this antibiotic in broiler droppings and litter from treated and untreated birds, to define if there is a relationship between treatment and the selection of resistant bacteria in these study matrices.

Materials and Methods

Culture media, certified standards, solvents and reagents

For chemical analysis, were uses certified standards of OTC, 4-epi-OTC and Tetracycline-d6, which was used as an internal standard, manufactured by Dr. Ehrenstorfer™ and Toronto Research Chemicals (Toronto, Canada). The working solution was prepared in methanol at a concentration of 1000 ng mL^{-1}.

Solvents and reagents used for chemical analysis correspond to acetonitrile, methanol, water, and formic acid from LiChrosolv® (MERCK KGaA, Darmstadt, Germany) line and LC-MS grade. Citric acid, disodic phosphate and Na2EDTA (Titriplex® III ACS, ISO, Reag. Ph Eur (MERCK KGaA, Darmstadt, Germany) were required to prepare Na2EDTA-McIlvaine buffer.
For the isolation and identification of E. coli from the experimental samples the following culture media and reagents were used: MacConkey agar, buffered peptone water (APT), mobility-indole-ornithine agar (MIO), prepared with yeast extract (3 g), peptone (10 g), tryptone (10 g), L-ornithine (5 g), dextrose (1 g), bromocresol purpura (0.02 g), agar (2 g) in 1 L of distilled water. Voges-Proskauer medium (prepared with peptone (7 g), glucose (5 g), K2HPO4 (5 g), in 1 L of distilled water), Simmons citrate, Methyl red, 95% ethanol analytical grade, potassium hydroxide and α-naphthol.

Trypticase soy broth (TSB), sodium chloride (NaCl), Mueller Hinton agar (M-H), nutrient agar and antimicrobial disc with tetracycline 30 µg (OXOID®) were required for sensitivity testing. PCR analysis was performed with ultrapure water, TAE buffer 1x, agarose, nuclease-free water, master mix (containing DNA Polymerase, dNTPs, MgCl2 and reaction buffers) (GoTaq® G2 green master mix, Promega, WI, USA), *tet* gene primers.

Experimental animals
For the in vivo study, male broiler chickens of the Ross 308 genetic line (Ross®, Aviagen Inc., Huntsville, AL, USA) were used. The birds were reared from the first day of life in an experimental unit specially designed to carry out this study. In this experiment, 1.5 m2 pens were conditioned with clean and previously analysed shavings (chemical and microbiological analysis), since these shavings later formed part of the birds' litter. Environmental conditions were controlled, such as temperature, according to age requirements, humidity (50-60%) and ventilation.

During the experiment, the birds had ad libitum access to water and unmedicated feed. Diets were formulated according to the nutritional requirements of the breed as recommended in the Aviagen™ manual (Aviagen Ross, 2019).
The protocol for handling and monitoring the experimental birds was based on Law 20.380 "On the protection of animals" and Directive 2010/63/EU on the protection of animals used for scientific purposes (EU, 2010). Regulation (EC) No. 1099/2009 on the protection of animals at the time of slaughter was respected (Ministry of Health, 2009). The experiment was approved by the Institutional Animal Care and Use Committee (CICUA) of the University of Chile, certificate N°: 18187-VET-UCH-E1.

Experimental groups and treatment of birds
From the first day of life, the birds were randomly grouped into three experimental groups. Group A included six birds treated with a pharmaceutical formulation containing OTC at 100 mg mL^{-1} (10%), which is authorized for use in broilers. All birds in group A were treated orally using an orogastric tube (Levin No. 6) and a sterile syringe, with a therapeutic dose of 80 mg kg^{-1} for 10 consecutive days, according to label directions. The drug was administered directly into the crop, according to individual weight ensuring delivery of the calculated doses.

The second and third groups (Groups B and C), also consisting of six birds each, were not treated; group B was kept in a pen adjacent to group A, while group C was kept 30 cm away from group A. In the latter, an effective separation of 30 cm was determined to avoid total direct contact between birds. All pens had an area of 1.5 m^2, divided by a solid wall 1.5 m high. All measures were taken to avoid contamination by handling, such as the use of shoe covers and gloves for handling each experimental group.

Collection of faecal and broiler litter samples
Samples of droppings were collected from each bird by cloacal swabbing and placed in sterile glass tubes. Broiler litter samples were obtained from each pen and stored in sterile plastic bags. Six 10 g samples were obtained from each pen and stored in sterile plastic bags.

six 10 g samples from each pen according to the soil sampling protocol of the Servicio Agrícola y Ganadero (SAG), which establishes homogeneity requirements according to article 28 of DS: nº 4/09 (SAG, 2019). Accordingly, samples were collected equidistantly within each pen, covering the entire area, using an a-systematic sampling method.

For the isolation of *E. coli*, the samples were processed immediately, and the sampling points corresponded to days 1, 7, 14 and 21 after the end of the treatment. Litter samples were also analysed one and two weeks after the birds were slaughtered. Chemical analyses were also performed on the samples obtained at the same sampling points.

LC-MS/MS sample analysis and OTC quantification
Sample extraction procedure and chromatographic analysis was performed according to the published study (cita). Samples were analysed by high-performance liquid chromatography system, where an API 5500 (AB Sciex, Darmstadt, Germany) mass spectrometer was used in multiple reaction monitoring mode (MRM) through an electrospray interface. OTC and their epimer concentration in experimental samples were calculated using the line equation derived from the regression analysis of spiking sample calibration curves, which was done concurrently with each sampling.

Isolation and confirmation of E. coli from broiler faecal samples and broiler litter.
All samples were analysed immediately after sampling. A 1 g litter sample was weighed and 9 mL of APT was added. In the case of cloacal swabs, 4.5 mL of APT was added. Subsequently, the samples were homogenized and seeded by streak depletion on MacConkey agar. The pink, round and medium-sized colonies were selected as suspected *E. coli* colonies and were subjected to IMVic tests, which consists of four tests: indole production test, Voges-Proskauer test, methyl red and Simmons citrate agar test (Lupindu, 2017; Nkogwe, et al, 2011).

Susceptibility testing of strains Kirby-Bauer disk diffusion method
For analysis, 4 to 5 colonies of pure cultures were taken and incubated at 35°C for 18 hours in TSB (Hudzicki, 2009). Subsequently, the turbidity of the bacterial suspension was checked in a spectrophotometer (Halo RB-10, Dynamica Ltd., Kuala Lumpur, Malaysia) and the concentration of the suspension was adjusted by dilutions in 0.85% saline solution until an OD between 0.08 and 0.1 at 600 nm was reached, which is equivalent to 0.5 McFarland.

After adjusting the turbidity, the agar was inoculated using a sterile swab. The plate was rotated by 60° and the step was repeated twice to obtain a uniform distribution of the inoculum. An antimicrobial disk of tetracycline (30 µg) was placed on the plate 15 minutes after inoculation and the disk was lightly pressed down to ensure contact with the agar surface. The plates were incubated inverted at 35°C for 16-18 hours. The zone of inhibition was observed after incubation and the diameter of the inhibition halos was measured with a meter foot. The diameter of the inhibition zone of the test isolates was compared with the interpretation criteria of the Clinical and Laboratory Standards Institute (CLSI, 2015) document.

Determination of tet genes by conventional PCR
To determine the resistance genes, present in the isolates, a conventional PCR was performed using the extracted DNA as a template. DNA extraction, resistant strains were seeded and incubated on MacConkey agar at 37°C for 18-24 hours. First a wash was performed by inoculating a batch in 1 ml of nuclease-free water and then centrifuging and discarding the supernatant. Subsequently, 1 ml of nuclease-free water was added and then boiled for 10 min at 100°C, centrifuged at 10,000 rpm for 10 min and 500 µl of supernatant were recovered (Noll *et al.,* 2015). The extracted DNA was quantified with a spectrophotometer (NANO-400 microspectrophotometer, Hangzhou Allsheng instruments Co.). Samples that exhibited an absorbance ratio of 260/280 nm close to the optimal range (1.8-2.0) were analysed by PCR.

The genes to be analysed corresponded to *tet* genes, specifically *tet*(A), *tet*(B), *tet*(C), *tet*(D), t*et*(E) and *tet*(M) (Ng et al., 2001); the 16S rRNA gene was included for confirmation of DNA presence (Mamun et al., 2016) and the *uspA* gene for confirmation of *E. coli* identity (Toro et al., 2018). *E. coli* strain ATCC® 25922 was used as a negative control, and DNA from strains that were previously sequenced and present the target genes was used as a positive control.

DNA amplification was carried out on a LifeECO thermal cycler (Hangzhou Bioer Technology Co. Ltd. Zhejiang, China) using the following conditions: an initial denaturation for 5 min at 94°C, followed by 35 cycles of 1 min at 94°C for denaturation, annealing at 54°C for 1 min for *tet* genes, and 58°C for 1 min for *UspA* and *16S rRNA* genes, and 72°C for 1-5 min for elongation. Finally, 5 µL of the PCR product was visualized on electrophoresis gels (2% (w/v) agarose in 1×TAE buffer), previously stained with Safeview®. The bands were visualized by ultraviolet transillumination, and the sizes of the PCR products were determined by comparing with the 100-base pair (bp) size scale (Maestrogen Hsinchu, Taiwan).

Statistical analysis

Descriptive statistical analysis was performed for qualitative variables by calculating absolute and relative frequencies. In the case of quantitative variables (concentration in µg kg^{-1} of OTC + 4-epi-OTC), position and dispersion parameter analysis were performed using the mean of the data and the standard deviations obtained. These data were previously published (Pokrant et al., 2021). To determine the independence between groups, the non-parametric Chi-square test of independence was performed, where the classification criteria were susceptibility and condition (treated/untreated), and the frequencies corresponded to the percentages of non-susceptible and susceptible isolates. The analysis was carried out using InfoStat software version 2020I. A statistically significant difference was considered when the p value < 0.05, rejecting the null hypothesis of independence.

Results and Discussion

OTC and their epimer were detected in the droppings of treated birds after treatment period had ended. On day 14 following the end of treatment, the highest concentrations of OTC+4-epi-OTC were measured (2244.66 µg kg^{-1}). In treated birds, OTC+4-epi-OTC concentrations were never less than 300 µg kg^{-1}. (Table 1 and 2). Trace concentrations of OTC were detected in sentinels' groups (Pokrant et al., 2021)

Table 1. Percentage of resistant isolates/intermediate susceptibility (not susceptible), and OTC + 4-epi-OTC concentrations detected in bird droppings, according to experimental group throughout the post-treatment days.

Days	Treatment Group		Sentinel Group Adjacent to treatment		Sentinel Group 30 cm from treatment	
	No susceptible (%) / Total no[a]	Concentration (µg Kg^{-1})	No susceptible (%)/ Total no.	Concentration (µg Kg^{-1})	No susceptible (%)/ Total no.	Concentration (µg Kg^{-1})
1	100 (R[b]) / 20	2.087.4	4,6 (R) / 22	<LOD[d]	76.9 (R) / 13	<LOD
7	84.2 (R) / 19	347.6	22.2 (R) / 9	<LOD	41.2 (R) / 17	<LOD
14	54,5 (R); 9,1 (I[c]) / 22	2.244.7	23.8 (R) / 21	<LOD	16.7 (R) / 12	N/D[e]
21	17.4 (R); 17,4 (I) / 23	733.0	22.2 (R); 11.1 (I) / 9	<LOD	28.6 (R) / 14	N/D

[a] Total number of isolates; [b] R: resistant; [c]I: intermediate susceptibility; [d]<LOD: below the detection limit of the analytical technique (12.5 µg kg-1); [e]ND: not detected.

Table 2. Percentage of resistant/intermediate susceptibility (non-susceptible) bacterial isolates and OTC+ 4-epi-OTC concentrations detected in bedding, according to experimental group throughout the post-treatment days.

Days	Treatment Group		Sentinel Group Adjacent to treatment		Sentinel Group 30 cm from treatment	
	No susceptible (%) / Total no[a]	Concentration (µg Kg^{-1})	No susceptible (%) / Total no	Concentration (µg Kg^{-1})	No susceptible (%) / Total no	Concentration (µg Kg^{-1})
1	82.8 (R[c]) / 29	22,741.7	16.7 (R) / 30	<LOD[e]	6.7 (R) / 30	<LOD
7	66.7 (R); 8,3 (I[d]) / 12	15,594.1	10.5 (R); 5.3 (I) / 19	<LOD	N/D[f] / 15	<LOD
14	27.8 (R); 44,4 (I) / 18	12,236.7	5.0 (R) / 20	<LOD	N/D / 15	<LOD
21	11.8 (R); 5,9 (I) / 17	12,946.1	9.1 (R) / 11	<LOD	20.0 (R); 10.0 (I) / 10	<LOD
29[b]	45.5 (R); 36,4 (I) / 22	15,557.6	55.0 (R); 15.0 (I) / 20	<LOD	30.0 (R) / 10	<LOD
36[b]	65.0 (R); 28,0 (I) / 25	11,429.1	25.0 (R); 25.0 (I) / 16	<LOD	8.0 (R) / 25	<LOD

[a] Total number of isolates; [b]Days post slaughter, corresponding to days 7 and 14 after the slaughter of the experimental birds; [c]R: resistant; [d]I: intermediate susceptibility; [e]<LOD: below the detection limit of the analytical technique (12.5 µg kg^{-1}); [f]N/D: not detected.

Treatment group present high concentrations of OTC+4-epi-OTC in litter samples that exceeded concentrations in droppings by more than 10 times, at the first sampling point. A representative OTC (461.0/426.0) chromatogram of a dropping and litter sample fortified at 50 µg kg^{-1} and from experimental samples are shown in Figure 1.

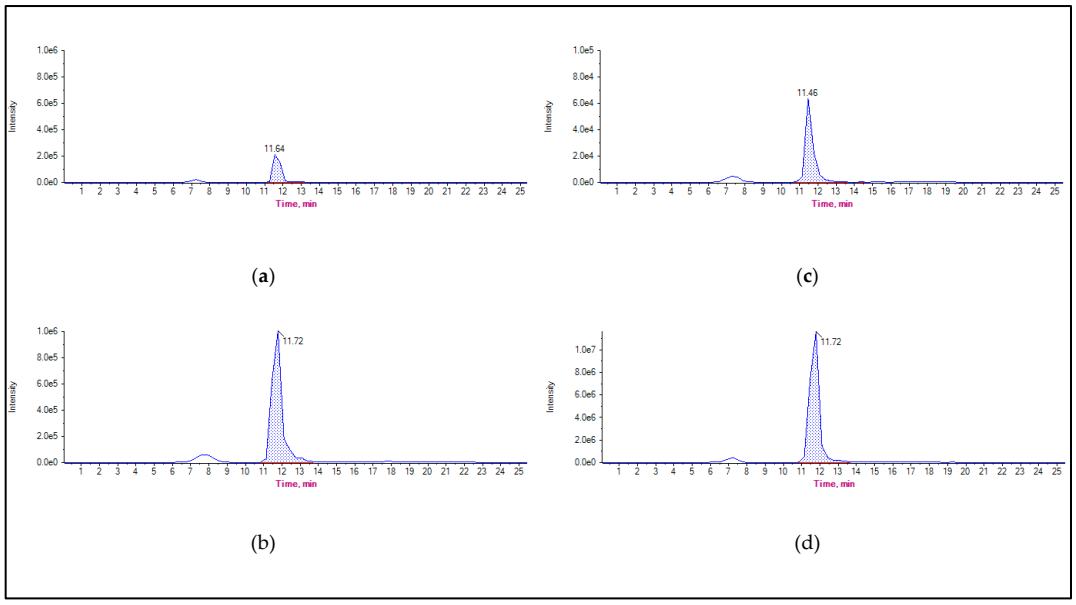

*Figure 1. Chromatograms of OTC (461.0/261.0) from (**a**) droppings fortified at 50 µg kg^{-1}, (**b**) experimental droppings sample from group A day 1 post the end of treatment, (**c**) litter sample fortified at 50 µg kg^{-1} and (**d**) experimental litter sample from group A day 1 post the end of treatment.*

The manure and litter samples were analysed prior to the experiment and *E. coli* was isolated to determine the resistance profile prior to treatment. *E. coli* was isolated only from the manure samples, and of the 90 colonies isolated, 80 were confirmed by biochemical tests. On the other hand, in the shaving's samples, which corresponded to the substrate of the poultry litter, no microorganism growth was observed after sowing in the selective agars for Enterobacteriaceae.

On the other hand, E. coli colonies were isolated from the different samples analysed from the post-treatment samples, which were also confirmed by biochemical tests. Six manure samples and six litter samples were analysed for each experimental group at days 1, 7, 14 and 21 post-treatments. Additionally, post-faecal litter

samples were sampled at 7 and 14 days after the pens remained unoccupied (equivalent to days 29 and 36 post-treatment). From typical colonies on MacConkey agar, five colonies were obtained from each sample and confirmed by the IMViC test (Figure 1). Strains that tested positive for Ornithine, motility, and methyl red, and negative for Voges-Proskauer and citrate were confirmed as *E. coli*. Positive and negative results were considered for the Indole test (with Kovacs reagent) since biotype I presented a positive reaction and biotype II a negative reaction to this test.

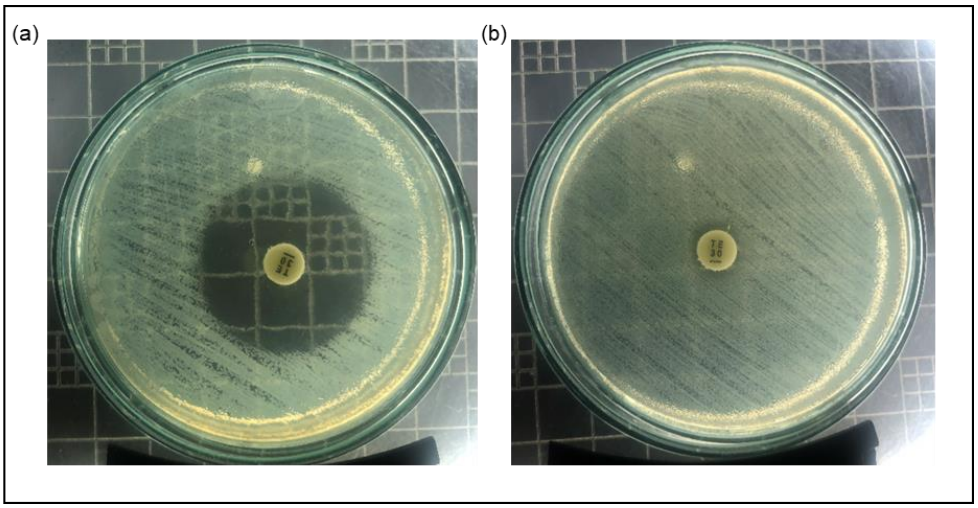

Figure 1. Representative image of the analysis by the disk diffusion method (Kirby-Bauer) of E. coli strains isolated from broiler litter and droppings samples. Sensitivity was determined according to the diameter of bacterial inhibition halos, according to CLSI, 2019 criteria for Enterobacteriaceae and tetracycline discs at 30 µg. (Resistant: ≤11 mm; Intermediate: 12-14 mm; Sensitive: ≥ 15 mm). Left image: sensitive strain, with a diameter of 30 mm; Right image: resistant strain without inhibition halo.

Sensitive and resistant strains were detected at all sampling points for the two study matrices. Also, strains with intermediate sensitivity were detected, mainly in the post-feeding litter samples, where a higher proportion of these isolates was determined (Table 1 and 2). Post-slaugter samples were taken on days 7 and 14 after the birds were slaughtered, i.e., the pens were sampled when they were uninhabited for a period of 1 and 2 weeks, so there was no contribution of faecal material, and the temperature and humidity conditions were ambient. The OTC residues and their epimer in each of the samples were previously quantified (Pokrant et al., 2021) and compared with the percentage of non-susceptible strains isolated from the same samples and the different experimental groups. Table 1 and 2 show that the highest percentage of non-susceptible strains was detected in the samples of the treatment group, both in droppings and litter.

According to the non-parametric Chi-square statistical analysis of independence, p-values of < 0.0001 were obtained for the results obtained from the matrix analysis (droppings and broiler litter). As the value was lower than the significance level of 0.05 selected, the null hypothesis in favours of the dependence between susceptibility and treatment condition of the groups studied was rejected. So, it was observed that there is a dependence between the two variables. The highest proportion of resistant isolates was determined in the treated group, where the concentrations of OTC and its epimer were higher than 22,000 g kg^{-1}. These results are expected, as it has been described that high concentrations of antimicrobials produce a selection pressure on resistant bacteria (Martínez, 2017). However, it has been described that low residue concentrations, even below the minimum inhibitory concentration (MIC) may also be related to the presence of resistant microorganisms (Andersson & Hughes, 2014; Gullberg et al., 2014; Wistrand-Yuen et al., 2018), which is consistent with the detection of some resistant strains in the untreated groups where, OTC residues were below the detection limits, i.e. below 12.5 µg kg^{-1}.

This aspect is of worldwide concern since currently, OTC is not only used for the treatment of productive animals but is also administered in the poultry industry through feed to promote growth of birds in subtherapeutic doses. The use for this purpose is still allowed in some countries, such as Brazil and China (Roth et al., 2019).

In the present study, it was observed that most of the resistant isolates came from droppings and litter from birds treated with an OTC pharmaceutical formulation. Fifty non-susceptible *E. coli* strains from manure and litter samples from this sampling point (day 1 post-treatment) and 10 *E. coli* strains from pre-treatment

sampling were analysed by conventional PCR. These isolates were previously confirmed as *E. coli* by detection of the *uspA* gene (Toro et al., 2018). From molecular analysis it was determined that the phenotype for tetracycline resistance matched the genotypic resistance, which was determined by *tet* gene positivity in almost all of the *E. coli* isolates. Eighty percent of the isolates had *tet*(A) genes, 12% had *tet*(B) genes, 10% had *tet*(C) genes and 14% had *tet*(M) genes. Only four resistant isolates were observed to have none of the resistance genes analysed in the present study; therefore, the resistance of these strains could be mediated by a different gene. The pre-treatment resistant E. coli strains were found to have only *tet*(A) genes. Figure 2 shows the PCR products.

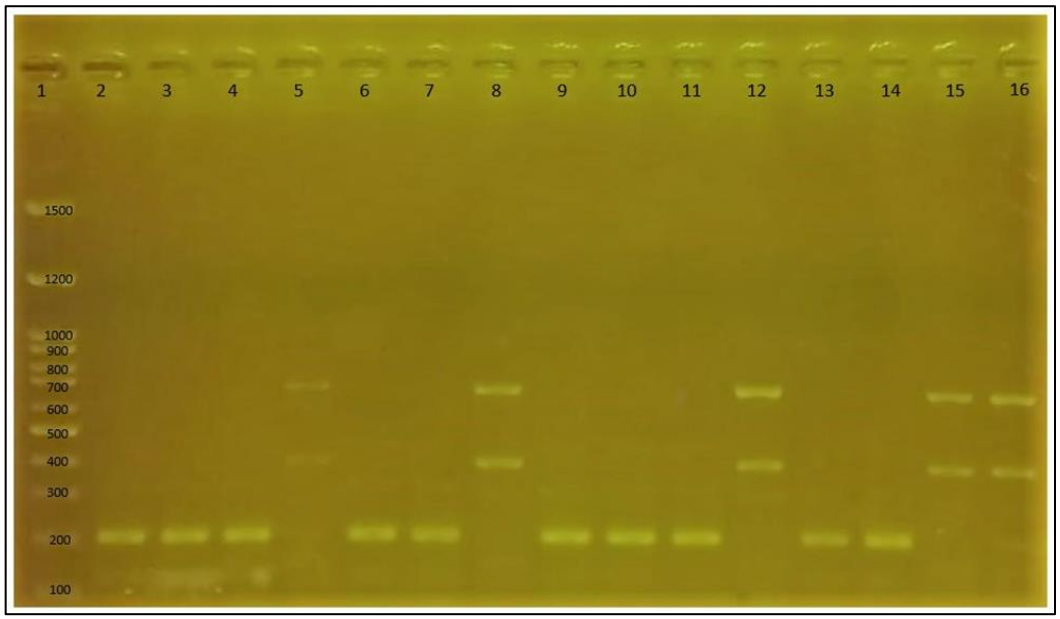

Figure 2. PCR products in agarose gel electrophoresis. Lines; (1) molecular mass marker; (2), (3), (4), (6), (7), (9), (10), (11), (13), (14) tet(A)*; (5), (8), (12), (15), (16) tet*(B) *and tet*(C)*.*

Conclusion

Broiler chickens excrete high concentrations of OTC and their epimer even up to 21 days post-treatment. In the present study concentrations of these analytes were detected in dropping that ranging from 347.63 g kg1 to 2244.66 g kg^{1}. These residues were detected in higher concentrations in their litter, ranged from 10,360.60 to 22,741.68 g kg^{-1}. Sentinel bird droppings and litter contained just trace amounts of OTC. These findings provide the first evidence that OTC residues from treated birds are unlikely to be transferred to the environment or to untreated birds in nearby or separate pens.

The highest proportion of non-susceptible E. coli isolates (resistant and of intermediate sensitivity) to tetracyclines was detected in the stool and bedding samples of the group treated with OTC, with respect to the untreated groups. Therefore, it is concluded that, even at therapeutic doses, there is a selection pressure on *E. coli* strains resistant to antimicrobials of this family. The *tet(*A) genes were the most frequent, therefore, it is concluded that the main mechanism of resistance of *E. coli* isolates to tetracyclines is mediated by active efflux pumps.

Acknowledgements

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POSTER P58

PROFICIENCY TEST FOR CORTICOSTEROIDS IN BOVINE AND PORCINE URINE

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Abstract

The proficiency test for corticosteroids in bovine and porcine urine was organised by the European Union Reference Laboratory (EURL) for hormonal growth promoting compounds, sedatives and mycotoxins. Twenty-five participants received three bovine and two porcine urine samples. Eight National Reference Laboratories passed this test by correct quantification of the corticosteroids, the absence of false positive and false negative results and reporting in time.

Introduction

Proficiency testing is conducted to provide laboratories a powerful tool to evaluate and demonstrate the reliability of the data that are produced. Next to validation and accreditation, proficiency testing is an important requirement and is demanded by ISO 17025:2017 [1].

The aim of this proficiency test (PT) was to give laboratories the opportunity to evaluate or demonstrate their competence for the analysis of corticosteroids in bovine and porcine urine.

This proficiency test was conducted in accordance with guidelines ISO 17043 [3] (R013 at www.rva.nl) and ISO 13528 [2]. The preparation of the materials, including the suitability testing of the materials and the evaluation of the quantitative results were carried out by Wageningen Food Safety Research (WFSR).

Materials and methods

Preparation of the materials

Three bovine (ABC) and two porcine (DE) urine samples were prepared. The samples did not originate from incurred material, since the incurred concentrations were too low to prepare PT-samples. The materials were prepared by adding a methanol solution of triamcinolone acetonide to A, B and E, dexamethasone to B and E, betamethasone to material C and clobetasol to material D. Each of the materials was homogenized according to in-house standard operating procedures [4] and lyophilized. Samples were stored in the freezer.

Homogeneity

The homogeneity of the materials was tested according to The International Harmonized Protocol for Proficiency Testing of Analytical Laboratories [5] and ISO 13528 [2], taking into account the insights discussed by Thompson [6] regarding the Horwitz equation. With this procedure the between-sample standard deviation (s_s) and the within-sample standard deviation (s_w) are compared with the target standard deviation for proficiency assessment derived from the Horwitz equation (σ_P). The method applied for homogeneity testing is considered suitable if $s_w < 0.5*\sigma_P$ and a material is considered adequately homogeneous if $s_s < 0.3* \sigma_P$.
Ten containers of all materials were analysed in duplicate for all compounds to determine the homogeneity of the materials [9].

Table 1. Concentrations of corticosteroids in bovine and porcine urine based on homogeneity test

µg/l	Material A	Material B	Material C	Material D	Material E
Triamcinolone acetonide (TCA)	2.4	5.2			4.9
Dexamethasone (DEX)		0.86	0.74		0.77
Betamethasone (BET)			1.0		
Clobetasol (CLO)				0.94	

All materials demonstrated to be sufficiently homogeneous for use in the proficiency test. The results are included in Table 1.

Stability
The stability of the corticosteroids in bovine and porcine urine was tested by storing samples in the ultrafreezer (assumption that analytes are stable) and in the freezer and at room temperature for one day. After the deadline the samples of the different storage conditions were analyses under repeatability conditions. Instabilities for TCA and BET were taken into account for the calculation of z-scores.

Evaluation of the performance
The statistical evaluation was carried out according to the International Harmonized Protocol for the Proficiency Testing of Analytical Laboratories [5], elaborated by ISO, IUPAC and AOAC and ISO 13528 [2] in combination with the insights published by the Analytical Methods Committee [7,8] regarding robust statistics. For the evaluation of the quantitative results, the assigned value, the uncertainty of the assigned value, a target standard deviation and z_a-scores were calculated according to ISO 13528. If the calculated uncertainty of the assigned value was significant ($u>0.3\sigma_P$) it influenced the evaluation. Therefore the uncertainty was taken into account when calculating the z_a-scores.
In addition, the Common EURL protocol for proficiency testing in the field of veterinary drug residues [9] was used to evaluate the participants' results. In this protocol a scoring system for the evaluation of the participants is used. In short, points are assigned for a z-score <2 (1.5 point), z-score >2 (1 point) and one or two false positive results (-1 point). The maximum score was 10.5 points and a participant passed the test by gaining more than 6.5 points. Participants with a limited scope (not all analytes were tested) could pass in the following assessment; however, they would be advised to extend the scope of the method.

Applied methods
All participants applied (UP)LC-MS/MS except two, which applied ELISA-detection. Enzymatic hydrolysis was applied by using a mixture of glucuronidase/arylsulfatase or only glucuronidase. Extraction was performed with tertbutylmlethylether, hexane, ethylacetate and acetonitrile. The applied SPE purification was based on polymers, C18, NH2, dispersive SPE, IAC and anion exchange. Several different internal standards were used.

Results and discussion
Twenty-five participants, of which 22 European National Reference Laboratories (NRLs) subscribed for the proficiency test and 24 reported results. Seventeen labs included BET, DEX and TCA in their method and seven labs included only DEX in their method. CLO was not taken into account since only two participants reported quantitative results.

The results of this test are presented in Table 2 and Figures 1 and 2.

Table 2. Results of RALs in materials B-E

material	analyte	# results	# quantitative results	# false negative results	consensus value µg/l	correct quantitative results
A	TCA	17	15	1	2.4	12
B	DEX	24	20		1.2	17
	TCA	17	16	1	5.2	15
C	BET	23	18	2	1.5	17
	DEX	24	20		1.2	17
D	CLO	2	2		-	-
E	DEX	24	21		1.3	18
	TCA	18	17		5.6	15

Two false positive results were reported for the presence of isoflupredone in materials D and E. Four false negative results were reporting by failing to detect the presence of TCA in materials A and B and BET in material C (twice).

Figure 1 presents the results per analyte in terms of the absolute z-scores; either exceeding 2 or between 0 and 2. Additional, the percentage false negative and qualitative results are shown.

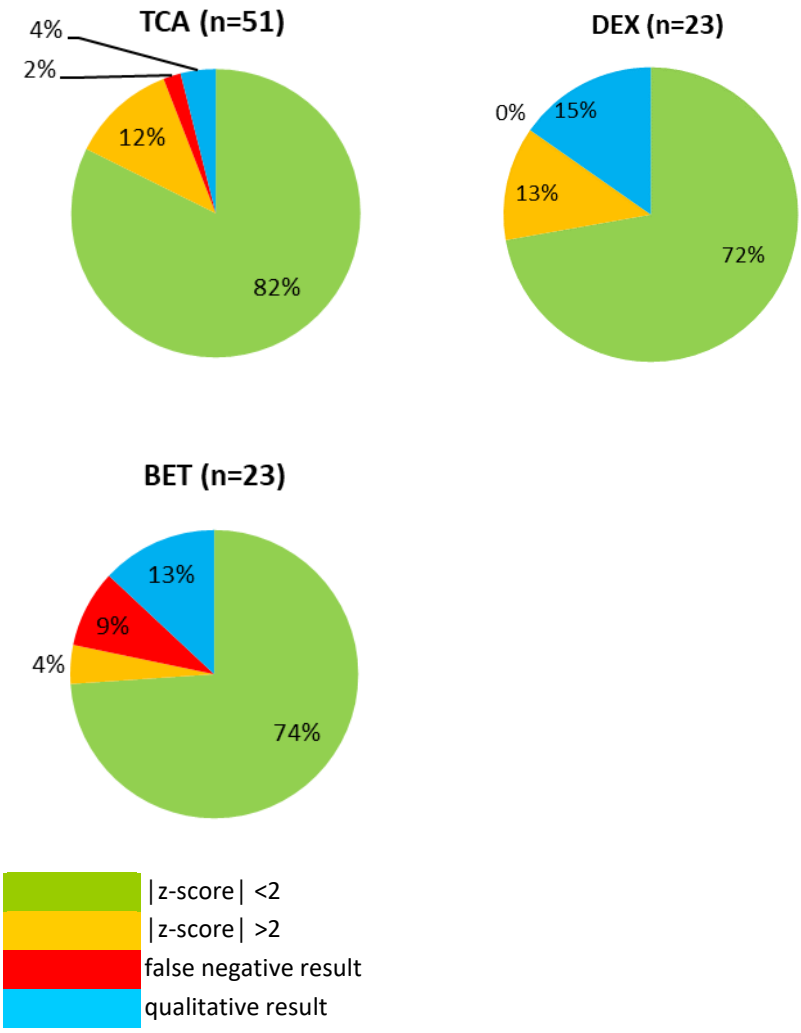

Figure 1. Overview of |z|-scores <2 and >2, false negative results and qualitative results

The correct quantitative results vary from 72% for DEX to 82% for TCA. Recently the Minimum Method Performance Requirement (MMPR) [10] for DEX was lowered from 2µg/l to 0.5 µg/l. Taken into account the consensus values of DEX in this PT, ranging from 1.2 to 1.3 µg/l, quite some labs should improve the quantification at a lower level, since at a level of more than two times higher than the MMPR only 72% correct quantitative results were reported.

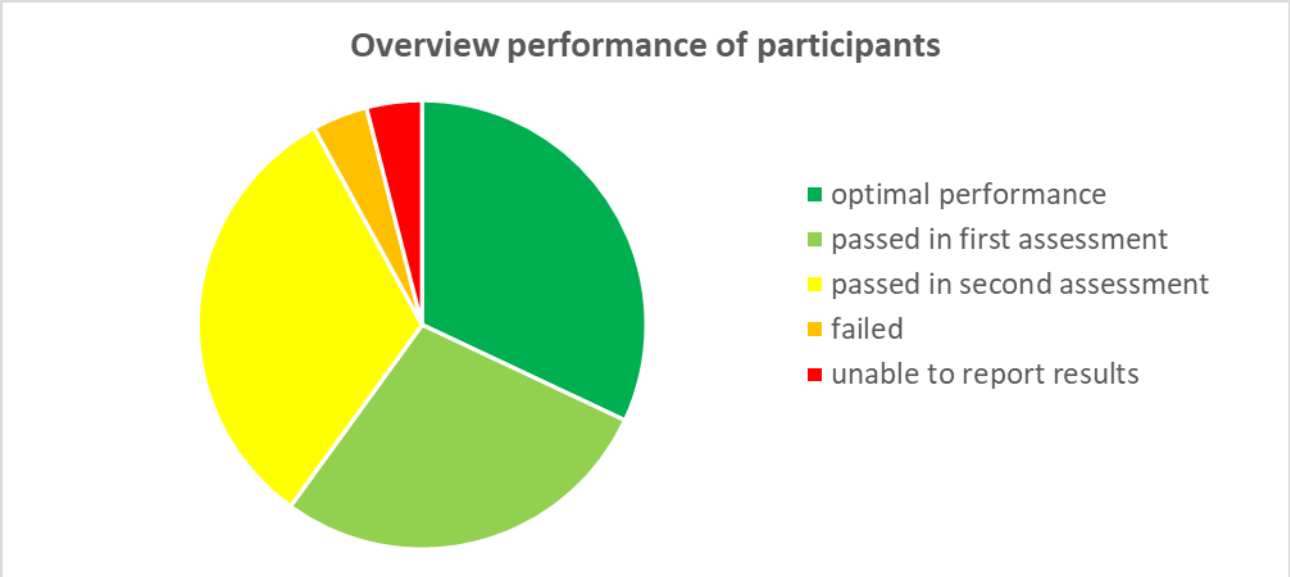

Figure 2. Overview of performance of participants according to uniform EURL point score system.

In total 16 questionable/unsatisfactory z-scores, 4 false negative and 2 false positive results were reported. Eight NRLs passed the PT achieving a maximum score by correct quantification of all compounds, the absence of false positive (FP) and false negative (FN) results and reporting in time. Seven labs (five NRLs) passed the PT in the first assessment by scoring more than 65% and less than 100% of achievable points. Another eight (seven NRLs) passed the PT in the second assessment by scoring more than 65% of achievable points taking into account the participant's scope. Overall, 60% of the participants passed the test in the first round and 32% in the second round. One NRL failed the PT and one NRL was unable to report results.

Acknowledgements [optional]
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Colleagues at WFSR: Bert Brouwer, Eric van Bennekom, Melissa Broeren and Paula Rutgers
Questions/remarks? E-mail to pt.wfsr@wur.nl

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POSTER P59

RISK OF RESIDUES OF TOLTRAZURIL AND ITS METABOLITES IN ANIMALS PRODUCTS AFTER ORAL ADMINISTRATION

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Abstract
Toltrazuril is a pharmacologically active substance used to treat coccidiosis. Due to the high efficacy, toltrazuril was authorized as a veterinary drug in many animal species, including chicken and turkey broilers. Although, toltrazuril is not authorized for animals producing eggs, due to long depletion time there is a potential risk of residues of toltrazuril metabolites in eggs.
Results obtained in this work confirm the slow elimination of toltrazuril and its metabolites in eggs. Residues of toltrazuril sulfone were still found 42 days after administration in whole eggs (22.5 µg/kg) and yolks (92.5 µg/kg).Almost 70 days was required to residues of toltrazuril sulfone reach zero in whole eggs samples. An exceptionally slow elimination process can cause residues of toltrazuril sulfone in eggs even if the medication was performed before the laying period.

Keywords: depletion study, residues, toltrazuril

POSTER 60

THE PHYSIOLOGICAL VALUES OF SEX STEROIDS IN CALVES AND THE REFERENCE HISTOLOGY OF THE TARGET ORGANS

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Abstract

Among forbidden substances included in the EU legislation, endogenous steroids represent a challenge in the framework of veterinary Official Monitoring Plans. To date natural hormonal profiles were less investigated because results are difficult to interpret. The goal of this study was to verify, in animals housed under controlled conditions, the level of natural steroids hormones during housing period from 3 to 8 months of age vs. values reported in the national and European regulation, in order to eventually update the legislation in force. At the same time sexual glands, prostate and bulbo urethral glands for male and ovaries, uterus and mammary glands for female were collected at the slaughterhouse for histological characterization.

The absence of residues of oestradiol and progesterone and the value of testosterone within the legal limits in our animals confirms that the values currently reported in the national legislation are consistent with the objective of ensuring the safety of commercial food. The histological pictures of target organs showed absence of lesions in agreement with control parameters adopted in the ongoing Italian histological monitoring plan.

Introduction

The use of anabolic agents in food animal production is prohibited in Eu. Notwithstanding sex hormones are still used as anabolic agents. They can be divided into steroids natural to the body (endogenous steroids), steroids foreign to the body and other compounds foreign to the body (Grooth et al.,2007).

In Italy monitoring plans are based on physiological hormonal levels reported in a Ministerial Decree of 14 November 1996. The levels reported are different for categories of production. 17β-estradiol limit is 0,04 ng/ml for all categories (males <6 months and > 6 months and for females <6 months and > 6 months); progesterone limits are: 1ng/ml for males and females <6 month. 1,5ng/ml for males > 6 months and 14 ng/ml for females > 6 months); testosterone limits are 10 ng/ml for males <6 months; 0,5 ng/ml for ffemales <6 month and > 6 months and 30 ng/ml for males.

To date research has mainly focused on setting up methods to identify illicit treatments, while the natural hormonal profiles was less investigated, nerveless it is fundamental to know physiological level of natural sex hormone to identify their use for illicit administration.

This study aims to verify, in animals housed under controlled conditions, if the level of natural steroids hormones reported in the national regulation are still actual in order to update the legislation in force. Moreover, the second aim is to update the normal histology of target tissues of the veal calf

Materials and Methods

The animals included in the experimental trial were housed under controlled conditions faithfully reproducing the zootechnical practices of veal industry farm. The experiment was approved by the Italian Ministry of Health (242/2020-PR)

Blood samples were collected every month and at slaughter and stored at −20 °C. LC analysis was carried out

through an HPLC system Exion (Sciex, Framingham, MA, USA). The analytes were detected in ESI positive (testosterone and progesterone) and negative (estradiol) MRM mode. At the slaughterhouse prostate and bulbourethral gland were sampled from the male animals and mammary gland, Bartholin's gland and ovaries from the female animals. All samples were fixed in 4% buffered formaldehyde at room temperature for about three days, routinely processed, embedded in a paraffin wax, sectioned in 3–5 µm slices and stained with haematoxylin and eosin.

Results and Discussion

Serum samples collected during the study (n =184) were all analyzed by multiresidue screening method. Only 87 samples were subjected to confirmatory method in order to quantify results of testosterone. Estradiol and progesterone were not detected in serum samples. Results of the confirmatory method confirm that the value in calves were below limits indicated in the national legislation (DM 1996).

The bulbourethral glands showed peripheral mucinous epithelium and central more immature epithelium and the prostate of all animals showed normal histology. Both organs did not showed the characteristic lesions present in animals treated with illicit substances, such as mild to severe metaplasia of glandular tissue or ducts. In females, mammary gland, ovaries and Bartholin glands were evaluated as target organs. The mammary gland tissue of all the females consisted of an immature duct system and a stromal portion that is proportionately larger and immature form. The ovaries showed growing follicles (primary and secondary) and atretic follicles. Bartholin's glands did not showed metaplasia in analogy with the target organs of males.

Conclusion

The absence of residues of oestradiol and progesterone and the normal level of testosterone in our animals confirms the values currently reported in the national legislation (DM 1996), The limits ensure, at the same time, the safety of commercial food and the protection of farming systems.

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POSTER P61

SCREENING OF SELECTIVE ANDROGEN RECEPTOR MODULATORS BY HANDHELD LASER DIODE THERMAL DESORPTION-TRANSPORTABLE MASS SPECTROMETRY

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Abstract

Rapid and easy-to-perform (on-site) analysis methods have a bright future in forensic, environmental, and food analysis. Implementing on-site pre-screening of target compounds in samples would reduce the number of suspicious samples to be transported to the control laboratories for their analysis. Therefore, it would improve the efficiency of control and monitoring programs. However, today, it is still challenging to carry out an on-site analysis because most of the available methods are based on spectroscopic techniques. They do not permit the differentiation of molecules with similar physicochemical properties. This fact is essential to avoid false-positive results. In this context, on-site mass spectrometry (MS) could solve these drawbacks because of its specificity on the mass spectra, leading to the direct identification of specific compounds. Before analysis, the ambient ionization mass spectrometry (AIMS) techniques offer simplified sample preparation and sample introduction protocols [1]. Today, only a few studies reported ambient ionization of food contaminants and drug analysis with a (trans)portable MS system [2-4].

This work [5] developed a handheld laser diode thermal desorption electrospray ionization (LDTD-ESI) mass spectrometry (MS) method to rapidly screen illegal substances in solid samples. A surgical laser diode which is simple to operate, cheap and battery-powered at 940 nm was employed to ablate the solid samples. After ablation, the ionization occurs through nano-ESI. The flow rate (50 µL h^{-1}) and solvent (methanol/water, 50:50, *v/v*) were critical to obtain the best sensitivity among the optimized ESI parameters. In addition, a black polytetrafluoroethylene substrate was investigated and demonstrated to enhance the desorption of the analyte to the gas phase. The applicability was demonstrated to rapidly identify selective androgen receptor modulators (SARMs) in powders and pills using accurate mass measurements by time-of-flight MS. Also, the handheld LDTD-ESI was combined with a transportable single quadrupole MS. The same SARMs samples were analysed, and identifications were based on observed in-source cone voltage fragmentation patterns. For future on-site testing of organic compounds in solid samples, the initial results demonstrated the applicability of the developed simplified LDTD-ESI MS method.

Conclusion

The developed handheld diode laser thermal desorption electrospray ionization mass spectrometry method successfully screened selective androgen receptor modulators in solid samples such as powders. Unlike previous expensive and complex laser setups, this development consists of a simple, inexpensive, battery-powered surgical laser diode combined with a nano ESI emitter that can be coupled to either a lab-based HRMS or a simple transportable MS system. Identification of samples can be performed based on in-source fragmentation patterns observed. These initial results demonstrate the applicability of the simplified LDTD-ESI–MS method for future on-site analysis of organic compounds in solid samples.

Acknowledgements

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POSTER P62

OPTIMIZATION OF QUECHERS METHODOLOGY TO ASSESS THE CONTAMINATION OF ANTIBIOTICS LEVEL OF MANURE FOR AGRONOMIC USE

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Abstract

This research aims to develop and validate an *in-house* multi-residue method for screening of antibiotics residues in manure, providing a tool for the evaluation of the related risk of their agronomic use.

Development of this proposed modified QuEChERS approach required several experiments, to settle the right weight of sample, polarity and pH of extraction solvent, different salting out agent, and different clean-up absorbents. Antibiotics were detected by LC-MS/MS.

Validation process was carried out according to the Regulation (EU) 2021/808 and specificity, linearity (range 0.25-2.0 mg/kg), detection capability (CCβ <0.500 mg/kg) and ruggedness were evaluated. This method demonstrated to be effective to detect the sulphonamides, quinolones and tetracyclines residues in manure.

This project will broaden the spectrum of knowledge on the degree of chemical contamination of swine manure, and findings may be useful to help to define targeted surveillance approaches along the supply chain, and eventually reschedule process control actions.

1. Introduction

Based on EFSA expert knowledge, fertilisers of faecal origin (i.e. manure), irrigation and surface water are major sources and transmission routes of contamination for plant-based food, and reducing the chances of introduction and persistence of faecal contamination represents a priority. To treat bacterial infections in zootechnical animals, several classes of antibiotics are commonly used in breeding. The absorption of antimicrobials takes place during digestion and a significant fraction of these molecules and their metabolites can be excreted with faeces and urines. Therefore, the accumulation of antibiotics or metabolites residues could occur in livestock manure. Antibiotics residues could encourage the development of antimicrobial resistance (AMR), because of their ability to be persistent active substances.

This research aims to develop and validate an *in-house* multi-residue method to screen antibiotics residues in manure and provide data useful to assess the potential risk associated with their use as fertilizers in regenerative agriculture.

In recent years, the QuEChERS (quick, easy, cheap, effective, rugged, and safe) methodology allowed to carry out accurate qualitative and quantitative analyses of various compounds, especially in multi-residue determination field and for some complicated matrices. Thanks to low environmental impact and reduced use of organic solvents, QuEChERS methodology is simpler, more effective, and even more oriented to green chemistry than traditional preparation protocols. Development of the proposed modified QuEChERS methodology requested a series of experiment performed with different weight of sample, polarity and pH of extraction solvent, different salting out agent, and different clean-up absorbent.

Validation process was carried out according to the Regulation (EU) 2021/808 and specificity, linearity (range 0.25-2.0 mg/kg), detection capability for screening (CCβ <0.500 mg/kg) and ruggedness were evaluated. This method demonstrated to be effective to detect the sulphonamides, quinolones and tetracyclines residues in swine manure.

The project will broaden the spectrum of knowledge on the degree of chemical contamination of manure and gather useful information regarding their reintroduction into the agronomic production cycle. These findings will be used to define targeted surveillance approaches along the supply chain and to reschedule new process control actions.

2. Materials and Methods

2.1 Chemicals and materials

All references and internal standards for quinolones (QUI), sulphonamides (SUL) and tetracyclines (TCL) with a minimum purity of 95% were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol (MeOH) and acetonitrile (ACN) LC-MS grade were purchased by VWR (Milan, Italy). The stock standard solutions of each compound (200 ng µL-1) were mixed in MeOH to prepare a working solution (40 ng μL^{-1}). Their stability was evaluated at -20°C for 12 and 6 months, respectively.
Disodium hydrogen phosphate dihydrate anhydrous salt (Na_2HPO_4) and citric acid ($C_6H_8O_7$) were obtained from VWR (Milan, Italy). Formic acid (CH_2O_2) and ethylenediaminetetraacetic acid disodium salt dihydrate ($Na_2EDTA \bullet {}_2H_2O$) were bought from Carlo Erba Reagents (Cornaredo, MI, Italy). Succinic acid ($C_4H_6O_4$) and phosphoric acid (H_3PO_4) were purchased by Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water was prepared by a Milli-Q purification system (Millipore, Bedford, MA, USA). 15g of disodium dihydrogen phosphate and 13g of citric acid monohydrate were weighted and dilute to the mark in the 1L flask to prepare McIlvaine's buffer (pH=4). 0.1 M EDTA-McIlvane buffer was prepared by dissolving 36.62 g of disodium EDTA per liter of McIlvaine's buffer. The extraction mixture consisting of 0.1M EDTA-McIlvane \ MeOH \ ACN 50: 12.5: 37.5. The pH was adjusted to 4.0 adding concentrated H_3PO_4. Copure® QuEChERS Premixed Extraction Salts and QuEChERS clean-up kits used for the extraction and clean-up of manure samples were purchased from Biocomma (Shenzhen, China).

2.2 LC–MS/MS analysis

Mass spectrometer parameters were optimised for each compound and acquisition was performed in Multiple Reaction Monitoring (MRM) to increase sensitivity. The precursor and product ions, declustering potential (DP), collision
energy (CE) and collision cell exit potential (CXP) were established for each compound. Electrospray ionization (ESI) operated in a positive mode and two transitions were selected to identify analytes unequivocally and to reach five identification points. Experimental UHPLC-MS/MS parameters and investigated compounds are reported in table 1 and 2, respectively.

Table 1. Experimental UHPLC-MS/MS parameters.

MASS SPECTROMETER:	QTRAP 5500- AB SCIEX				
UHPLC-SYSTEM	EXION LC-AB SCIEX				
Column	Luna Omega Polar- C18 100 Å (150 x 3.0 mm, 3 µm, Luna Omega) - Phenomenex				
Flow	0,25 mL/min	Injection volume	10 µL	Temperature	30°C
Autosampler temperature:	8°C				
Mobile phase:	0.1 % formic acid in ACN/ 0.1% formic acid aqueous				

Table 2. Detailed list of investigated antibiotics.

QUINOLONES	SULFONAMIDES	TETRACYCLINES

NALIDIXIC ACID	SULPHACHINOXALINE	CHLORTETRACYCLINE
OXOLINIC ACID	SULFADIAZINE	DOXYCYCLINE
CIPROFLOXACIN	SULPHADIMETHOXIN	OXYTETRACYCLINE
DANOFLOXACIN	SULFAMERAZINA	TETRACYCLINE
DIFLOXACIN	SULPHAMETZINE	
ENROFLOXACIN	SULFAMETHOXAZOLE	
FLUMECHIN	SULFAMETHOXYPYRIDAZINE	
LEVOFLOXACIN	SULFAMONOMETHOXINE	
MARBOFLOXACIN	SULFAPYRIDINE	
NORFLOXACIN	SULFATHIAZOLE	
SARAFLOXACIN		

2.3 Sample extraction and clean up procedure

The extraction procedure was adapted by previous works with some modifications. Briefly, 4g of swine manure were weighted in a 50 ml polypropylene centrifuge tube. Then 40 mL of extraction mixture was added to each sample. 4g $MgSO_4$+1g NaCl salts were added before each sample was vortexed for 1 min and then centrifuged for 10 min at 8°C at 4000 rpm. 5mL organic phase + 40 mg PSA+20mg C18, centrifuge for 15 min, at 8°C at 4000 rpm. Evaporation of 1 ml of surnatant was carried out under nitrogen at 60°C. Resisue was dissolved in 125 µL of ACN HCOOH 0.1% /HCCOH0.1% aq (20/80 v/v), filtered through a nylon membrane with 0.22 µm pore size (Millipore, Bedford, USA) and transferred in vials for direct injection into LC-MS/MS system.

2.4 Method validation

Requirements of validation process were established according to Commission Decision (EC) No 2021/808 and specificity, linearity (concentration range 0.25-2.0 mg/kg), detection capability for screening (CCβ <0.500 mg/kg) and ruggedness were evaluated. Specificity was performed analysing twenty representative blank swine manure samples. Admissible criteria were S/N≥3 and the absence of interfering signal in the range ±1% of expected t_R of monitored molecules. Instrumental linearity was tested for each analyte as follows. Three replicates of standard calibration curve were prepared in a mixture of 0.1% formic acid in ultrapure water/0.1% formic acid in ACN (80/20 v/v) and analysed by LC-MS/MS. Linearity range was evaluated by relative area peak at five different levels. Concentrations of each analyte in standard calibration curves were 0.25, 0.50, 0.75, 1.00, 2.00 mg kg^{-1}, corresponding to 0.8, 1.0, 2.0, 3.0, 4.0 ng μL^{-1} injected. The IS was added at each level in concentration of 0.50 mg kg^{-1} (1 ng μL^{-1}). Instrumental linearity was estimated by coefficient of determination R^2 and response factor distribution yx^{-1}. Acceptability criteria were R^2>0.99 and yx^{-1} mean ±10%.
Ruggedness test was conducted according to Youden approach by the introduction of slight deliberate changes (±10%) to 7 potentially critical analytical variables in experimental conditions. The selected parameters were shaking time (extraction step), sonication time, centrifugation time (1st extraction step), shaking time (purification step), centrifugation time (2nd extraction step), evaporation temperature, spinning speed. The resulting changes in terms of response on blank swine manure samples spiked at CCβ level were observed. F-test and t-test were carried out to evaluate ruggedness. Acceptability criteria were F observed lower than F scheduled and the t-test for each variable lower than t critic at 95%.
The detection capability CCβ was estimate twenty blank manure samples were spiked with all antibiotics and internal standards at 0.50 mg kg^{-1}. Acceptable criteria were a maximum false compliant result of 5 % (β error ≤ 5 %).

Results and Discussion

Validation results showed that this method fit for purpose. No peaks with S/N ≥ 3 in the retention time window (Rt±1%) of each analyte were detected. Hence, the developed method proved to be specific for all antibiotics tested. Coefficient of determination (R^2) and response factor distribution (yx^{-1}) showed a linear correlation of

calibration curves (R^2 ≥0.99 and mean yx^{-1} values ranged from 0.42 to 1.8). β error was estimated minor than 5 % for 0.50 mg kg^{-1} level that was fixed as CCβ of screening method. Results on eight samples tested with slight deliberated variations of selected parameters did not exhibit significant changes, compared to those obtained under standard conditions. F test was carried out and it was verified that F observed < F scheduled proving that the developed analytical method is rugged. The proposed method has proven to be suitable for the identification of sulphonamides, quinolones and tetracyclines in swine manure. It will provide useful data to evaluate chemical risks associated with swine manure used as agricultural fertilizer, and to support the manure-based fertilizer production.

Acknowledgements

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POSTER P63

FIRST EXPERIENCES WITH LIQUID CHROMATOGRAPHY-ISOTOPE RATIO MASS SPECTROMETRY IN FOOD RESIDUE CONTROL

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Abstract
A confirmatory analysis is the most important step between suspected results and reporting non-compliant findings and is therefore essential in food quality and safety control. In most cases, confirmatory testing of residues is covered by routinely operated tandem-MS analyses. However, it gets more complicated if residues also have a presumable endogenous origin. A well-known example is synthetic testosterone and estradiol. In these cases, a difference in carbon isotope ratio could confirm the identity of the substance, as already demonstrated by gas chromatography-combustion-isotope ratio mass spectrometry (GC-c-IRMS). However, non-volatile's are often not applicable or require derivatization before GC separation. To overcome this limitation for non-volatile's, liquid chromatography-isotope ratio mass spectrometry (LC-IRMS) can be used. Nevertheless, the interface design between LC and IRMS requires the need for carbon-free eluents, therefore restricting usable LC separation techniques. Our research investigated the use of LC-IRMS as a confirmatory technique for steroids, antipyretics and thyreostats found in animal urine and of presumable natural and/or exogenous origin. Here, we demonstrate our findings and encountered challenges in developing LC-IRMS methods for bovine urine and its applicability for confirmatory testing within residue food analysis.

Introduction
Food quality and safety control methods can roughly be divided into two categories, 1) methods for identifying substances, commonly known as screening methods, and 2) methods for confirming the suspected results from the screening methods, known as confirmatory methods[1]. Both are essential for guaranteeing safe and reliable food for consumers. When one focusses on the available confirmatory methods, the most used analytical techniques consist of a chromatography system either liquid- or gas chromatography (LC or GC) coupled to tandem-mass spectrometry (MS/MS). These combinations of separation and detection systems are excellent because of their proven robustness, sensitivity and selectivity. However, it gets more complicated when targeted substances in these confirmatory methods also have a presumable endogenous origin. Well known examples are α-testosterone, α-estradiol, 2-thiouracil and acetaminophen (paracetamol) [2-5] . While the LC- or GC-MS/MS techniques can reliably detect residues of these substances in matrices, residues will, in most cases, be present in blank samples. Thus, different methods are required to confirm if the detected residues are of synthetic or natural origin.

For the steroids testosterone and estradiol the technique GC-combustion-Isotope Ratio Mass Spectrometry (GC-c-IRMS) has been proven to be effective in confirming the identity of the detected residues [6, 7]. Differences in carbon isotopic ratio can be detected by the GC-c-IRMS, between endogenous and synthetic steroids. These differences are caused by the starting product and/or the synthesis used. However, in order to be compatible with GC separation, substances require to be volatile or in the cases of steroids, require derivatization prior to analysis. This limits the use of IRMS based techniques in food safety control, as most substances are polar, non-volatile and not all easily modified using derivatization reactions. Additionally, the use of derivatization reactions often alters the isotopic signature, even further limiting IRMS analysis. Overcoming these limitations of polarity and volatility was found possible by using LC separation prior to IRMS detection.

Here, we present our research focused on using LC-IRMS as a confirmatory technique for multiple relevant examples that occur presumable natural and/or exogenous in urine, in the field of food quality and safety control.

Materials and Methods

Chemicals

Analytical standards of 17α-testosterone and 17α-estradiol were purchased from Steraloids (Newport, Rhode Island, USA) dehydroepiandrosterone (DHEA), salicylic acid and paracetamol were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands).

Methanol, acetonitrile, formic acid (99%), n-pentane and water (analytical or ULC/MS grade) were purchased from Actu-All (Oss, The Netherlands), disodium hydrogen phosphate dihydrate, potassium dihydrogen phosphate, acetic acid, sodium acetate, ortho-phosphoric acid (85%) and potassium dihydrogen phosphate were purchased from Merck (Darmstadt, Germany), orthophosphoric acid (99%) was from Fluka (Buchs, Switzerland), and isooctane was purchased from Biosolve (Valkenswaard, the Netherlands). Sodium peroxodisulfate (99%) and methanolic HCl (3N) were from Sigma-Aldrich (Zwijdrecht, the Netherlands). β-glucuronidase from *Escherichia coli* K12 was from Roche Diagnostic (Mannheim, Germany).

Stock solutions, each containing one standard in methanol at a concentration of 1000 mg L^{-1} were prepared. Test standards were prepared by evaporating the methanol and redissolving in the required amount of water.

Urine samples

Routine samples in which no or low concentrations of 17α-estradiol and DHEA in a concentration above 25 µg L^{-1}, were pooled and used as blank urine for the preparation of spiked samples. Urine samples of healthy pregnant cows were collected to be used as reference population samples. Urine samples of estradiol treated animals, were available at WFSR from controlled animal studies. All samples were distributed in volumes of 5 mL and kept frozen (-20°C) until analysis.

LC-IRMS optimization

The LC-IRMS system consisted of a 1290 Infinity II LC-setup, equipped with a 1290 Infinity II Diode Array Detector (DAD) (Agilent, Santa Clara, USA), connected to an IRMS system existing out an IsoLink LC-interface and a MAT253 IRMS (Thermo Fisher Scientific, Germany). For the optimization studies of chromatographic conditions, the LC-setup consisted of an Agilent Infinity II system equipped with a 1290 Infinity II Diode Array Detector (DAD) (Agilent, Santa Clara, USA). Temperature gradient separation was performed using a Polaratherm Series 9000 Total Temperature Controller. Tested analytical columns were Zorbax Bonus-RP RRHD (100x2,1 mm, 1,8 µm), Zorbax 300 SB-C_3 (50x2,1 mm, 1,8 µm), Acquity UPLC HSS T3 (100x2,1 mm, 1,8 µm), Xbridge Protein BEH C_4 (50x2,1, 3,5 µm), and Synergi Polar-RP (150x3 mm, 4 µm). Under optimized conditions, temperature gradient chromatography was performed using a Xbridge Protein BEH C4 (50x2,1, 3,5 µm) column, with an initial temperature of 80°C held for 1 minute and increasing to 140 °C (15 °C/min), and held for 20 minutes before going back to initial conditions. The total run time was set to 35 minutes. Under all conditions LC flow remained constant at 0.5 ml/min, unless mentioned otherwise.

Procedure urine samples

Bovine urine samples were homogenized, and 5 ml was taken into preparation. Samples were extracted using C_{18} SPE (xxxxx). During SPE clean-up the SPE tubes were activated with 4 ml of MeOH, followed by 4 ml of MilliQ water. Extracts were applied and washed with 5 ml of water, followed by 3 ml of MeOH/water (10/90 v/v). Columns were eluted with 5 ml of MeOH.

Eluted samples were evaporated until dry, and 0.15 ml of methanolic HCl (3N) was added for methanolysis of conjugates and placed at 60 °C for 2 hours. After methanolysis, samples were evaporated and 3 ml of phosphate buffer pH 7.4, and 0.05 ml of glucuronidase were added. Deconjugation was performed at 37 °C over night.

For further purification purposes, 7 ml of n-pentane was added to the hydrolysed samples. Samples were shaken and centrifuged at 2500 rpm for 5 minutes. The organic upper layer was transferred into a new glass tube and the extraction was repeated. The combined n-pentane layers were evaporated at 45 °C and redissolved into 0.1 ml MeOH. To all samples 5 ml of MilliQ water was added for SPE purposes. The second SPE clean-up was performed using xxxxx SPE tubes. SPE tubes were activated using 4 ml of MeOH, followed by 4 ml of MilliQ water. Extracts were applied and washed with 4 ml of MilliQ water, followed by 4 ml of MeOH/water (40/60 v/v). Columns were eluted using 4 ml of MeOH/water (80/20 v/v), and evaporated at 55 °C until dry.

Eluted samples were transferred to LC vials, for preparative UPLC separation equipped with a fraction collector. Fractions of α-testosterone, α-estradiol and DHEA were collected, evaporated until dry and redissolved into MilliQ water for LC-IRMS analysis.

Results and Discussion

Chromatography

The IRMS system is a specialised mass spectrometer dedicated for detecting relative stable isotopes abundances of multiple elements, including carbon ($^{13}C/^{12}C$) determined by the carbon isotope abundance of CO_2. The isotope ratio is expressed in delta notation (e.g. $\delta^{13}C$), as the values are small numbers in the range of *per mil* (‰) [8]. In GC operation mode, CO_2 is formed from organic molecules by combustion, hence the name combustion-IRMS. In LC operation mode the CO_2 is formed out of organic molecules by wet-chemical oxidation, converting all organic material towards CO_2.

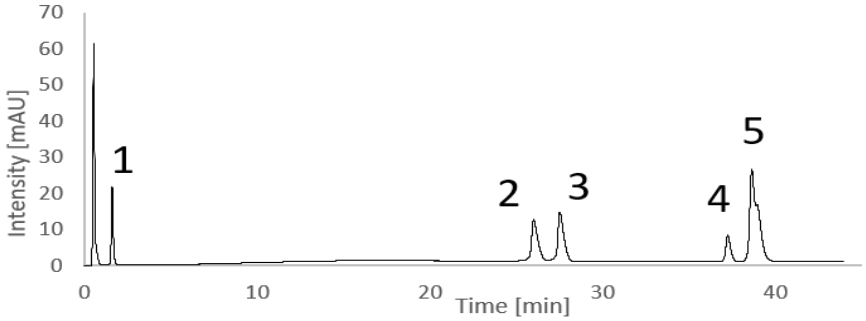
Consequently, the operation of LC-IRMS thus requires the absence of any organic modifiers (e.g. MeOH or ACN) in the mobile phases. Separation of target substances has to be performed using aqueous solutions. Different from the routinely operated LC-MS systems, the IRMS can be operated using non-volatile buffers or modifiers like phosphate salts. As the formed CO_2 gas is separated using semi-permeable membranes from the eluent flow.

Different methods of separation for relevant substances were evaluated. These can roughly be divided into two categories, a) high-temperature liquid chromatography, and b) water-only reversed phase liquid chromatography [9]. High-temperature liquid chromatography was tested for the steroids 17α-testosterone (α-T), 17α-estradiol (α-E2) and dehydroepiandrosterone (DHEA), resorcylic acid lactones and paracetamol. As these substances have to strong interactions with reversed phase stationary phases compared to the aqueous mobile phase. To achieve sufficient elution strength in the absence of organic modifiers, elevated temperature can be used [10]. In isothermal mode, high temperatures of 60, 80 and 100 °C on the XBridge BEH C_4 column were tested for zeranol and compared to chromatography using the same column at 25°C. improvements in peak shape were observed, with optimal peak shape at 80 °C. However, the interaction with the stationary phase was limited, as retention times were limited to only 3.5 min.

Isothermal mode was also tested for paracetamol using a HSS T3 column. Using optimized settings and a column temperature of 80 °C, the observed signal from a paracetamol standard was sufficiently separated from the dead volume (data not shown). Over time the signal remained at a stable retention time at 6.5 min, during multiple injections.

Temperature gradient mode

In temperature gradient mode, the column oven is programmed to run a temperature gradient to achieve retention for the a selection of growth promoters. The analytes have strong interactions with the XBridge BEH C_4 column at lower temperatures. Only when increasing the temperature the elution strength increase to the point of elution. This process enables sufficient retention from other matrix artifacts in the sample prior to converting all organic material to CO_2. Injecting a mixture of different growth promoting substances using temperature gradient mode, effectively separated 1) thiouracil, 2) prednisolone, 3) prednisone, 4) α-zearanol,



and 5) zearalenone, as shown in figure 1.

Figure 1. Chromatogram obtained by high-temperature LC photodiode array detection of 1) 2-thiouracil, 2) prednisolone, 3) prednisone, 4) α-zearanol, and 5) zearalenone.

The application of temperature gradient LC was also tested for the steroids α-E2, DHEA and α-T using the Xbridge BEH C_4 column. This chromatography method was able to separate the 3 steroids, achieving retention times of 12.6; 16.9 and 21.0 min, respectively. In matrix, tests were performed using bovine urine samples. Bovine urine samples were spiked to relevant levels of α-E2, DHEA and α-T and sample preparation was performed similar to bovine urine samples dedicated for GC-c-IRMS analyses skipping the derivatization step often used. The final extracts were analysed using the proposed LC chromatography method. Good separation

from other matrix artefacts were obtained for α-E2 and α-T, and $\delta^{13}C$ values could be determined. DHEA, which is used to determine the endogenous $\delta^{13}C$, achieved unsatisfying separation from other substances. Further optimisation did not result in improved retention from other, presumable matrix artefacts. Potentially additional selectivity for DHEA could be achieved in additional sample preparation steps.

It was observed that in high-temperature gradient mode, the background steadily increased after multiple chromatographic runs. This is potentially caused by bleeding and degradation of the column packing, as their advised operation temperatures are often exceeded. This is also observed in shifting retention times, as each chromatographic run a shorter retention time was observed for mainly α-E2. Additionally, temperature gradient mode is affected by the equilibration step between chromatographic runs. Sufficient time is required to cool and equilibrate the column back to the initial temperature.

Water-only reversed phase liquid chromatography

Water-only liquid chromatography uses reversed-phase columns that are suitable at 100% aqueous conditions. The packing of these columns are in most cases end capped, to overcome chain collapse. Compared to high-temperature LC, this separation mechanism relies on "normal" LC temperature conditions. With IRMS detection, this can directly be observed as reduced background signal is present. Furthermore, columns were found not to degrade after multiple runs resulting in more stable retention times. In order to be compatible with water-only RP-LC, the analytes should be (highly) polar since the mobile phase strength is limited. Therefore, this mode of chromatography was tested mainly for 2-thiouracil using Acquity HSS T3, Synergi Polar-RP and Zorbax Bonus-RP columns. Of the three tested columns the Synergy Polar-RP demonstrated sharp peaks and a retention time of 4.5 min, compared to only 2.3 min for the Zorbax Bonus-RP column. Optimal conditions were found to be 25 °C using 100% LC-grade MiliQ water (figure 2), higher temperatures resulted into decreasing retention times for 2-thiouracil. It was hypothesized that the additional separation acquired by the Synergi Polar-RP column, was due to π-π interactions between 2-thiouracil and the ether-linked phenyl groups of the column packing.

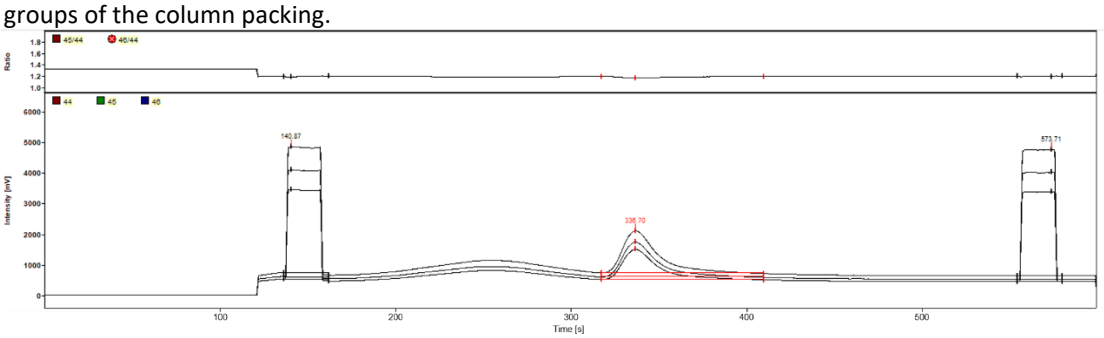


Figure 2. LC-IRMS chromatogram of an aqueous 2-thiouracil standard, using water-only reversed phase liquid chromatography. Square signals at 150 and 570 s are CO_2 pulses for carbon isotope ratio calibration purposes.

Detection limits of LC-IRMS

Multiple options for chromatography were found to be compatible with IRMS measurements, in LC-IRMS mode. To be applicable for confirmatory testing for residue analysis in food-related topics, detection limits should be at relevant and realistic. While in other confirmatory methods, this can be determined based calculations on areas of the integrated signals in chromatograms, the detection limits in IRMS detection are depended on repeatable and accurate carbon isotope ratio's. To determine the detection limits for confirmatory testing of LC-IRMS, the dispersion of the carbon isotope ratio of one standard at different concentrations was determined. Individual standards of α-T, α-E2, paracetamol and salicylic acid were analysed in 5-fold and the dispersion at each concentration was determined. Dispersion of < ±0.5‰ was accepted for reliable detection. The concentration where this criteria was met, was established as the detection limit. This limit is displayed as the absolute amount of nanograms (ng) on column, as in practise this can be established by concentrating the sample during sample preparation. For paracetamol and α-E2, the 5-fold measurements at 100 ng on-column had sufficient precision, staying within the margin of ±0.5‰, resulting in detection limits of 100 ng on-column. Salicylic acid and α-T higher SD than ±0.5‰ at 100, 200 and 300 ng on-column, resulting in detection limits of 500 ng on-column. The results including the linear regression of the peak area and the dispersion of the $\delta^{13}C$ are shown in figure 3.

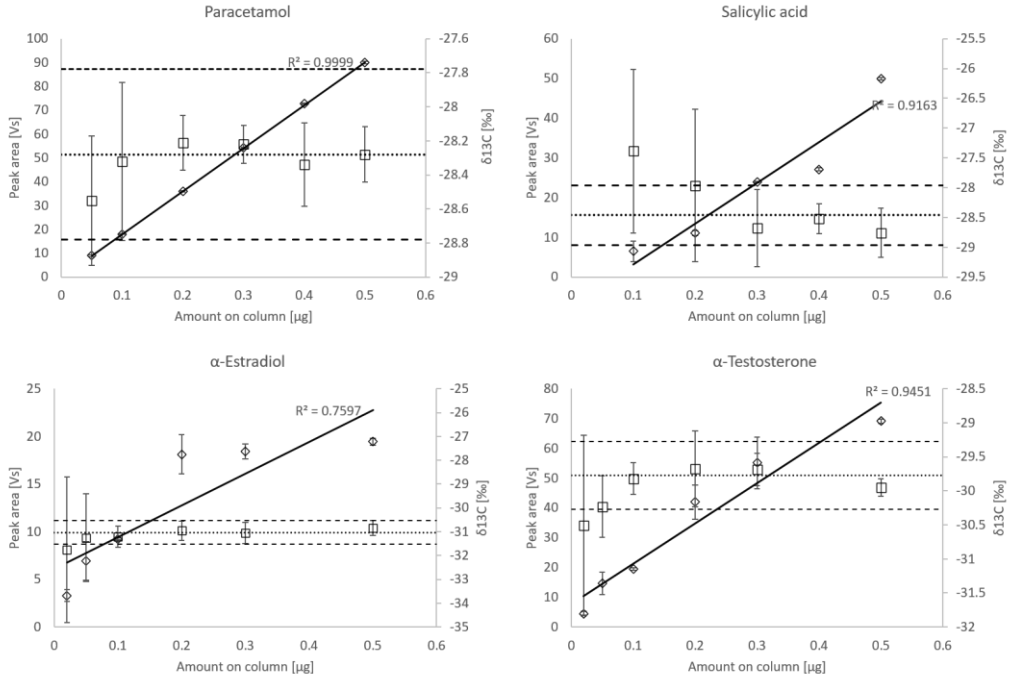
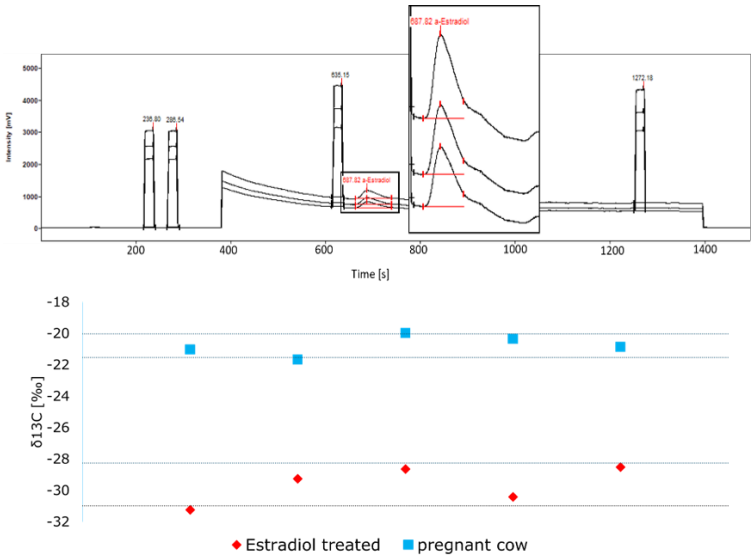


Figure 3. Determination of the method detection limit for paracetamol, salicylic acid, α-estradiol and α-testosterone. The squares represent the $\delta^{13}C$. The linear curve fit of the peak area of $^{44}CO_2$ and the correlation coefficient for plotting peak area vs. absolute amount are shown. Error bars indicate the SD of 5-fold measurements. The dotted line indicates the calculated mean value of $\delta^{13}C$. The horizontal dashed lines represent the interval of mean value ±0.5‰.

LC-IRMS in practise for food-related control

The developed chromatographic LC-IRMS method was tested using small sets of bovine urine samples. Two sets of bovine urine samples, a) from a pregnant animal and b) from an estradiol treated animal were prepared according the GC-c-IRMS protocol, apart from the derivatization step which was omitted. Urine from a pregnant bovine animal was used as an endogenous control, since pregnancy increases the amount of endogenous α-E2 excretion. After sample preparation, the final extracts were injected on LC-IRMS, using the temperature gradient separation. Good separation from other matrix artefacts that were still present in the final extracts was achieved (figure 4). After data handling, the data demonstrated clear differences in the $\delta^{13}C$ between the two groups, as shown in figure 5. Clearly showing the method allows for the detection of estrogen abuse, with α-E2 as the urinary metabolite of β-E2. However, since the current method was unable to sufficiently separate the endogenous reference compound DHEA, further testing has to be performed using



multiple control groups exposed to different types of feed and environments.

Figure 4. LC-IRMS chromatogram of α-estradiol and the δ13C difference between an urine batch from a pregnant animal and an urine batch from an estradiol treated animal. The dashed lines represents the 99% confidence intervals..

Conclusion

With the installation of the LC-IRMS system at WFSR, multiple chromatographic conditions have been evaluated for multiple substances with presumed endogenous and/or exogenous origin. Chromatography based on higher than normal column temperatures demonstrated promising results for the urinary steroid metabolites α-estradiol and α-testosterone in bovine urine. Besides sufficient separation from other matrix artefacts still present in the final extracts, practical detection limits were achieved as further confirmed by urine samples from pregnant and estradiol treated animals.

Besides the evaluation of LC-IRMS for detecting steroid abuse, aqueous chromatographic methods for 2-thiouracil, paracetamol, salicylic acid and zeranol were explored. Further research will be conducted on these polar substances to develop a reliable LC-IRMS confirmatory method.

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POSTER P64

EVALUATION OF BIOEQUIVALENCES AND DEGREE OF AGREEMENT WITH MANUFACTURERS LABELLING OF DIFFERENT OXYTETRACICLINE PREMIXES FOR USE IN THE POULTRY INDUSTRY

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Abstract
The objectives of the present study sought the determination of concentrations differences of active ingredient of three oxytetracycline premixes commercially authorized by the Agricultural Livestock Service (SAG-CHILE), as declared on their labels, and through bioavailability studies in broiler Ross 308 chickens, the therapeutic bioequivalence between them. OTC is one of the most used antibiotics in the national poultry industry, there are different commercial alternatives available for use, but they are not classified as pharmaceutical bioequivalents. This does not ensure its therapeutic effectiveness when applied in poultry plants according to their label registration. Differences in the profiles and pharmacokinetic parameters obtained with non-bioequivalent products can cause therapeutic failures, antimicrobial resistance and poisoning. The premixes and chicken´s plasma treated with OTC, were quantified by standardized HPLC- MS/MS methods complying with the requirements of European Union Commission Decision 2002/657/EC. In the case of the determination of bioequivalence, ANOVA ($p>0.05$) and Schuirman's "two one sided tests" were used. The results indicated that two OTC premixes presented differences greater than 20% in the percentage composition of the active ingredient with respect to its label, exceeding the limit required by the European Medicine Agency (EMA). For the premixes evaluated in this study, therapeutic bioequivalence was determined.

Introduction
Antimicrobial use in premixes has improved the health and welfare of broiler chickens and laying hens in the poultry industry by lowering the incidence and mortality of bacterial infectious illnesses while also making antibiotic delivery simple and effective (Venkitanarayanan et al., 2019). According to several research in broiler chickens, oxytetracycline is poorly absorbed from the gastrointestinal tract.
The absorption half-life time of a pharmaceutical formula is the time it takes for one half of the substance to be absorbed from its absorption site. For oxytetracycline (OTC), it has been described an absolute bioavailability of 12.13 ± 4.56% following its oral administration (Ziółkowski et al., 2016). This aspect is especially important for the poultry sector, as antimicrobials are administered by feed and drinking water (Agyare et al., 2019; European Commission, 2015).

Several of these formulations have been approved for usage in broiler chickens in Chile and are now accessible. However, the bioequivalence of these many on-the-market alternatives has not been determined, therefore their therapeutic equivalence against a bacterial infectious disease is unknown. In the United States, the Food and Drug Administration (FDA) oversees bioequivalence studies for veterinary medicines, but in the European Union, the Committee for Veterinary Medicinal Products (CVMP) of the European Medicines Agency is in charge (USDA, 2014)

In terms of active compound concentrations in the formulations, Fernandez-Gonzalez et al (2002) studied at over-the-counter medicated feeds and premixes and found that after the extrusion procedure, these medications were 30 percent less concentrated (Fernandez-González et al., 2002). Moreover, various investigations using high performance liquid chromatography (HPLC) for the characterization and identification of veterinary pharmaceuticals in premixes and medicated feeds (Han et al., 2020; Krasucka et al., 2010; Song et al., 2018).

Any deviation from the active ingredient's labelled concentration, or if the formulation loses its homogeneity, might result in unfavourable outcomes, such as improper dosage administration, intoxication induction, therapeutic failures, alterations, and varied therapeutic outcomes (Pauli et al., 2014). Several international organisations have documented these inadequacies, which have been dubbed "current administration gaps" (Patel et al., 2020). As a result, determining and validating the concentration of active components as stated on the premix label is critical. Determining if the chemicals are bioequivalent is also important.

In order to interpret bioequivalence, numerous parameters must be determined, each of which must meet distinct confidence intervals. The area under the curve (AUC) and maximum concentration (Cmax) must both have ranges between 80 and 125 percent, with a 90 percent confidence interval. Cmax is a metric that is strongly reliant on the sampling process, therefore similarity ranges between 70% and 143% are acceptable. Because a variation of 20% for a maximum Time (Tmax) of 10 minutes is not the same as a variation of 20% for a Tmax of 120 minutes, the range of time-dependent parameters such as Maximum Time (Tmax) must be carefully defined and justified, even if it is not decisive in determining bioequivalence between formulations (CVMP, 2018).

Antimicrobial bioequivalence has yet to be established in Chile between several pharmaceutical formulations designed for veterinary use that are already on the market. Bearing this in mind, the objectives of the present study sought the determination of concentrations differences of active ingredient of three oxytetracycline premixes commercially authorized by the Agricultural Livestock Service (SAG-CHILE), as declared on their labels, and through bioavailability studies in broiler Ross 308 chickens, the therapeutic bioequivalence between them.

Materials and Methods

Reagents and solvents

Methanol, formic acid and ammonium formate were used for the analysis of our experimental samples. All of these solvents were of HPLC-grade purity and were manufactured by MERCK® (Merck KGaA, Darmstadt, Germany). Also, oxytetracycline (CAS: 6153-64-6) and florfenicol (CAS: 73231-34-2) standards of certified purity were used for the implementation, validation, and analysis of plasma samples collected from broiler chickens.

Additionally, chloramphenicol D5 is a deuterated form of an amphenicol molecule and was used as an internal standard due to its similar chemical characteristics with florfenicol. Likewise, oxytetracycline C13 is an isotopically marked molecule that was used as an internal standard for oxytetracycline.

From these antimicrobials, we then prepared stock and working solutions at a concentration of 1000 µg/g. All of these solutions were prepared in a mobile phase, at a ratio of 85:15 (V/V) for the mobile phases A and B, respectively. In the case of the oxytetracycline analysis, mobile phase A was a solution of 2 mM ammonium formate plus 0.16% formic acid in water, whereas mobile phase B was a solution of 2 mM ammonium formate plus 0.16% formic acid in methanol. Meanwhile, the florfenicol analysis required preparing a mobile phase A solution using 0.1% acetic acid in water (pH 3.7±0.2), and a mobile phase B solution containing 0.05% acetic acid in an acetonitrile/water mix (50:50 (V/V), with pH 3.7±0.2).

Commercial Premixes of OTC

Two commercial OTC formulations for broiler chickens were chosen from the list of those approved by Chile's National Agriculture and Livestock Service (SAG, by its Spanish acronym). Product A contained 10 g of Oxytetracycline (10%), and Product B contained 80 g of Oxytetracycline hydrochloride (equivalent to 74.2 g of Oxytetracycline base) (80%).

Experimental animals

The experimental animals were male broiler chickens of the genetic line Ross® 308 (Ross®, Aviagen Inc., Huntsville, AL, USA). These birds were cared for following the husbandry guidelines provided by the genetic company regarding the requirements of the particular breed, and they were housed in the Animal Management Unit (AMU) in the Department of Animal Preventive Medicine from the Faculty of Veterinary and Animal Sciences of the University. of Chile (FAVET, by its Spanish acronym). This facility provided the required isolation, as well as allowing controlled conditions suitable for the age of the birds, such as temperature, humidity (50-60%), ventilation, and ad libitum access to both water and non-medicated food. The animal handling and welfare conditions of these birds were approved by the Institutional Committee for the Care and Use of Animals (CICUA, by its Spanish acronym) from the University of Chile, as it is detailed on the Certificate N° 21492 – VET – UCH.

The number of samples required—from the minimum number of individuals—that would allow performing a bioequivalence study was calculated following the recommendations described in the "Guideline on the

conduct of bioequivalence studies for Veterinary Medicinal Products" (CVMP, 2018). Consequently, six birds were used on each experimental group to properly assess the bioequivalence of these four drugs.

All these birds were identified by plastic bracelets placed on each of their legs and then assigned to one experimental group. The experimental groups were separated and housed independently to avoid any chance of interaction between groups. Groups 1, 2, 3, and 4 were the experimental groups where the antimicrobial drug was administered orally, using an orogastric tube to ensure that the calculated dose was ingested entirely. The recommended dose on the label of each product was 35 mg/kg for the OTC formulations and 30 mg/kg for FF formulations. As for Group 5, this was assigned as the negative control group, hence it received no treatment, but it was kept under the same experimental conditions than the other groups throughout the entire experiment.

Sample Treatment

OTC premixes were dissolved in mobile phase and shaking for 15 minutes in a Multi Reax® agitator (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany). After, samples were sonicated for 15 minutes, and the solution was diluted twice in the mobile phase to check that the levels remained within the calibration curve values. To reduce the analytical variability of calculations and thus increase the certainty of detecting significant findings, these dilutions were standardized at 50 g/L and examined in batches of 20 samples per product (a total of 120 samples). All analyses from the same production batch were completed in a single assessment time.

The concentrations in each OTC premix were compared to the concentrations listed on the labels, and the difference between the two numbers was calculated and reported as a percentage.

Plasma Analysis was performed following the procedures described by Anadón et al. (2012). Briefly, samples were weighed in 1±0.01 g of blood plasma and adding 1.5 mL McIlvaine buffer (pH 4). These samples were shaken for 30 seconds in a Heidolph® orbital shaker and then centrifuged at 1500 rpm for 10 minutes, collecting the supernatant. This was transferred to a 1 g Varian Bond Elut C18® extraction column, which was previously conditioned using 5 mL of methanol and 5 mL of McIlvaine buffer. Then, the column content was eluted using 1 mL of a solution made of acetonitrile and McIlvaine buffer (50:50 (V/V)) before injecting it into a mass spectrometer.

Instrumental Analysis

Samples were analyzed by a liquid chromatography (LC) coupled to mass spectrometry technique. In the case of OTC, it was detected using a Xevo® TQs micro, which comprised an Acquity® pump, an Acquity® FTN oven, and an Acquity® FTN autosampler. All of these modules were manufactured by Waters® Technologies. Meanwhile, FF residues in plasma samples were analyzed using an ABSciex® API4000 triple quadrupole mass spectrometer. The LC system comprised an Infinity® 4000 series (Agilent Technologies®) pump, an oven, and an autosampler.

Quantification of OTC concentrations in plasma

The concentration of OTC residues (in µg/L) detected in plasma samples after extraction was determined by using the linear equation, which was calculated from the regression analysis of the matrix calibration curves, using fortification concentrations of 25, 50, 100, 200, 300, 500, 700, 800, 1000, 5000, 8000, 11,000, and 14,000 µg/L.

To ensure statistical validity when quantifying these analytes, the curves, and each set of analyzed samples required a coefficient of determination (R^2) ≥ 0.95.

The analytes were then quantified by replacing the value of the area ratio, for each sample, in the following equation:

Concentration mg/Kg = ((Area ratio - Intercept of the calibration curve)) /Slope of the calibration curve

Determination of the area under the curve and Cmax

The area under the curve (AUC) was calculated by using the trapezoidal area method for determination of the AUC_{0-24} and $AUC_{0-\infty}$, adding to the latter the Elimination Constant (K_{el}) of each product.

As for the maximum concentration (C_{max}), this was determined by visualizing the pharmacokinetic profile of each pharmaceutical formulation. The range of acceptance of areas and pharmacokinetic parameters of the premixes was then determined by using confidence intervals.

Determination of bioavailability and bioequivalence

The normality of the distribution of the pharmacokinetic parameters was assessed using the Shapiro−Wilk test. Then, any possible differences between the pharmacokinetic parameters C_{max} and AUC was evaluated by using the multivariate analysis of variance (one-way ANOVA). Values transformed to the natural logarithm of $p<0.05$ were deemed statistically significant. Also, the p values for variability factors, such as sequence, treatment, and period were determined by analyzing the sum of the squared errors and adjusted means of the ANOVA model. These factors explain different sources of variation that can be defined as follows. The sequence effect quantifies the variation of the model according to the sequence followed when entering data, whereas the treatment effect quantifies the variation of the data that is not explained by the predictors used in the model. Also, the period effect calculated by the analysis of the adjusted mean squares determines the variations of the model that arise independently of the order followed when entering data.

The significance level was set at 0.05, hence no significant differences were assumed when a p value exceeded that mark. The relative bioavailability for each set of formulations was determined by comparing the $AUC_{0-\infty}$ of each formulation, and including the administered dose of each premix as a common factor.

A confidence interval of 90% was set for the ratio between the average pharmacokinetic parameters that were calculated between the formulations of the same active ingredient. This involved transforming them to the natural logarithm (ln) of the concentration detected.

For the final calculation of bioequivalence, the Schuirmann TOST analysis (two one-sided test) was run using the statistical software RStudio® V 0.99.903. This statistical test allows for assessing the bioequivalence of both formulations by means of analyzing the means calculated for each parameter, along with the analysis of confidence intervals set at 90%. These results are then adjusted using two t−tests to find the maximum limits (lower and upper) that are allowed for each variable. The formulations were considered as bioequivalent whenever their p values were lesser than 0.05 for each tested parameter.

Results and Discussion

The concentration of oxytetracycline detected for all formulations exceeded the concentration stated on the label, averaging an additional 29.68% and 21.54 % of the active ingredient for products A and B, respectively. The differences were statistically significant (p value < 0.0001) for all formulations according to the Wilcoxon Test, for a confidence level of 95% and a p value ≤ 0.05 (Maddaleno et al., 2021). The amounts measured (mg/kg) in each premix sample (n = 20) were corrected by the recovery of the analytical method, and the comparison with the information indicated on each product label is documented in Table 1.

Table 1. Bioequivalence tests for pharmaceutical formulations, including confidence intervals and Schuirman test.

Premix Active	Premix Active	Premix Active	Premix Active	Premix Active	Premix Active
Product A	OTC 10%	100	129,678.38 ± 5987.27	29,678.38	29.68
Product B	OTC HCL 80%	742	901,804.57 ± 24,297.15	159,804.57	21.54

The trapezoidal area of each sampling point was calculated to assess the bioequivalence of these formulations and their relative bioavailability. The K_{el} was calculated on the basis of the depletion slope of their pharmacokinetic model, the AUC_{0-24} and the $AUC_{0-\infty}$, and the C_{max} of every formulation. Figures 5 and 6 show the pharmacokinetic profiles at ln scale.

No statistically significant differences ($p>0.05$) were found in any of the pharmacokinetic parameters tested in either the oxytetracycline groups or the florfenicol groups. The average pharmacokinetic parameters calculated between the formulations of the same active ingredient were transformed to the natural logarithm (ln) of their values, and then used to calculate 90% confidence intervals. The results from all parameters met the confidence interval, thus signalling that no statistically significant differences could be assumed for the parameters means between formulations (see Table 10).

Table 10. Confidence intervals calculated for ln C_{max}, ln AUC_{0-24}, ln $AUC_{0-\infty}$, and ln T_{max} for each formulation.

Parameter	Product A[a]	Product B[b]	90% Confidence interval
ln Cmax	6.82	7.13	92.7% - 102.7%
ln AUC 0-24	13.14	13.32	96.4% - 101.4%
ln AUC 0- INF	13.19	13.36	93.9% - 103.6%
ln Tmax	4.69	4.79	99.1% - 101.3%

[a] Product A contained 10 g of Oxytetracycline (10%); [b]Product B contained 80 g of Oxytetracycline hydrochloride (equivalent to 74.2 g of Oxytetracycline base) (80%).

A multivariate analysis of variance (ANOVA) was used to test for the possibility of statistically significant differences between the pharmacokinetic parameters C_{max} and AUC. This test showed a statistically significant difference for these parameters when their values were transformed to a natural logarithm scale (see Tables 11).

Table 11. ANOVA for parameters Cmax, AUC_{0-24} and $AUC_{0-\infty}$ of formulations.

Variation Source	*ln* C_{max}	*ln* AUC_{0-24}	*ln* $AUC_{0-\infty}$
Interindividual			
*Sequence effect	0.0912	0.6541	0.6811
Intraindividual			
*Treatment effect	0.1238	0.4541	0.6122
*Period Effect	0.0997	0.9112	0.8812

The calculated p-values were greater than 0.05 in both formulations, thus no statistically significant differences could be assumed for the parameter variances of all the formulations, either for interindividual or intraindividual effects on each group (p>0.05). This meant that no residual effects that impact on the model came from sources other than the premix used in this study or the plasma concentrations found in it.

The Schuirmann TOST "two one side test" analysis was used for the final calculation of bioequivalence, using the statistical software RStudio® V 0.99.903. The analysis tested the parameters for ln C_{max}, ln AUC_{0-24}, and ln $AUC_{0-\infty}$ for each group of formulations (see Tables 13).

Table 13. Bioequivalence tests for phormulations, including confidence intervals and Schuirman test.

	IC_{90} test limits		Two one side test (TOST)	
Parameter	Lower limit	Upper limit	Lower limit	Upper limit
ln C_{max} (µg/mL)	92.71%	102.12	0.032	0.044
ln AUC_{0-24} (µg*h/mL)	97.12%	101.54	0.003	0.008
ln $AUC_{0-\infty}$ (µg*h/mL)	98.99%	102.63	0.012	0.013

All parameters, and in both upper and lower limits, showed calculated p-values lower than the significance level of 0.05. Therefore, the null hypothesis proposed by Schuirmann was rejected, which signalled that the formulations were bioequivalent.

Conclusion

The results of this study show that, although the premixes analysed were uniform in composition, the concentrations observed did not coincide with the amounts specified on the packaging labelled by the manufacturer. This implies that a strict control of the manufacturing process of formulations is essential.

The pharmacokinetic parameters that were tested for formulations did not show significant differences on the indicators ln C_{max}, ln AUC_{0-24}, and ln $AUC_{0-\infty}$, thus showing similar values in plasma for each group of OTC formulations. Different environmental factors can alter the pharmacokinetic parameters of an active ingredient, thus causing differences in their values as well as in the kinetics of the plasmatic curves of each antimicrobial analysed. That is the reason why the environmental variables of temperature and humidity were controlled during the entire experiment: to avoid alterations in the pharmacokinetic profiles and parameters

identified in this work. These alterations could arise as a consequence of a modification in the rate of water and/or food consumption by animals, and it should be considered not only for future studies but also in animal production facilities. This is especially relevant now, in light of the climate change scenario already affecting the world. Additionally, the results of the pharmacokinetic parameters indicated above for OTC would allow performing PK/PD studies for the purpose of establishing specific treatment doses for each antimicrobial. These doses could be calculated by running the Monte Carlo simulation on pathogens that are susceptible to the drug of interest. Therefore, readjusting and optimizing the total doses of these antimicrobials that are currently administered in the poultry industry could improve production quality, and also reduce costs associated with loosing animals because of diseases in the farm.

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POSTER 65

NATURAL HORMONES PROFILES IN BOVINE BLOOD SERUM BY LC-MS/MS: MULTI-YEAR SURVEY IN NORTHERN ITALY

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Abstract

Among forbidden substances included in the EU legislation, endogenous sex steroids constitute a challenge in the framework of veterinary official monitoring plans. Indeed, these molecules are naturally present at low and variable concentrations depending on the animal race, sex and age.

17β-estradiol physiological concentrations in blood serum are much lower than that of testosterone and progesterone, so two different sample preparation protocols are generally applied to analyze the three steroids.

To overcome this issue and improve the selectivity, we developed a multiresidue, rapid and effective analytical procedure for the quantitative determination of progesterone, testosterone and 17β-estradiol in bovine blood serum, based on the use of molecularly imprinting polymers (MIPs) cartridges for cleanup step (Lucci *et al*, 2011).

The analytes were detected by LC-MS/MS in ESI positive and negative mode. The method was successfully validated both for screening and quantification purposes in the concentration range: 0.020-0.080 μg/L for 17β-estradiol, 0.25-10 μg/L for testosterone and 0.5-20 μg/L for progesterone.

Finally, the results of three-years of controls carried out analyzing 203 bovine serum samples were discussed.

Introduction

Natural and synthetic steroid hormones have been widely illegally used in animal husbandry for several decades as growth promoters especially in bovine farming. Their use in animal fattening has been prohibited as legislated in the Directive 96/23/EC (Dir. 1996) because of their toxic effects on public health. In Italy the Ministerial Decree 14/11/1996 (D.M. 1996) has fixed Maximum Physiological Limits (LM) for natural hormones in bovine blood serum. Limit levels are different in production categories of animals according to sex and age: progesterone limits are: 1ng/ml for males and females <6 month; 1,5 ng/ml for males > 6 months and 14 ng/ml for females > 6 months); testosterone limits are 10 ng/ml for males <6 months; 0,5 ng/ml for females <6 month and > 6 months and 30 ng/ml for males, 17β-estradiol limit is 0,040 ng/ml for all categories.

These levels allowed to discriminate between treated or untreated animals. In order to monitor possible illicit use of these three steroids in bovine, very sensitive analytical methods are required since these molecules are naturally present at low and variable serum concentrations depending on the animal race, sex and age. Currently, due to the strong legal impact involved in such a kind of investigation for the official controls in Food Safety, chromatographic techniques associated with mass spectrometric detection play a major role in this kind of analysis, due to their high sensitivity and selectivity.

However, it should be noted that the rearing methods have changed, especially for calves, as well as the killing categories have been defined on the basis of a weight of about 240 kg and the slaughtering age increased from six to eight months. (Rutherford et al., 2021). Therefore, it becomes a priority to better study the physiological profile of natural sex hormones in order to identify their use for illicit administration but also to understand if the actual maximum physiological limits are still valid.

This study presents a rapid and effective analytical procedure for the simultaneous quantitative determination of progesterone, testosterone and estradiol in bovine blood serum. Moreover, the results of two-years of controls carried out analyzing bovine serum samples were discussed.

Materials and Methods

Chemicals and Materials
Acetonitrile, methanol, ammonium fluoride were of analytical or HPLC grade quality and were supplied by Sigma-Aldrich (St. Louis, MO, USA). Molecularly imprinting polymers (MIPs) SPE cartridges Affinmip Estrogens (100 mg, 3 mL) were purchased from Affinsep (Petit-Couronne, France). 17β-estradiol, 17β-estradiol-d4, 17β-testosterone, 17β-testosterone-d3, progesterone and progesterone-d9 were supplied by Sigma-Aldrich (St. Louis, MO, USA). The stock standard solutions of each analyte and internal standard (ISTD) were prepared in methanol at the concentration of 2 µg/mL and stored at -20 °C in the dark; solutions were stable for 2 years. Suitable working standard solutions in methanol were obtained by appropriate dilution of the corresponding stock solutions and stored at -20 °C.

Blood serum sample preparation
Blood serum (2 ml) was spiked with IS and diluted with 7 ml water. The sample was loaded on AFFINIMIP® SPE (0,5 drop/s), previously conditioned with 3 ml acetonitrile and 3 ml water; after washing with 2x3 ml water and 3 ml water/acetonitrile (6:4), the cartridge was dried; analytes were eluted with 3 ml methanol. After evaporation to dryness in nitrogen stream at 50 °C, the residue was dissolved in 0.1 ml methanol/water (1:1) mixture.

LC-MS/MS Analysis
LC analysis was carried out through an HPLC system Exion (Sciex, Framingham, MA, USA). The analytes were detected in ESI positive (testosterone and progesterone) and negative (estradiol) using Multiple Reaction Monitoring (MRM) mode. Chromatographic separation was performed on a Waters XSelect HSS T3 XP (3 x 100 mm; 2,5 µm) column, kept in a column oven at 40 °C, using a gradient elution with NH4F 0.2 mM in water (A) and NH4F 0.2 mM in methanol (B). Estradiol-d4, testosterone-d3 and progesterone-d9 were used as internal standards. The mass spectrometer was a QTRAP 6500+ (Sciex, Framingham, MA, USA). Injection volume was 25 µL and flow rate was 0.3 ml/min, with an overall run time of 20 min. The gradient profile was as follows: 0–1 min 50% (B); 1-6 min linear increase up to 95% (B); 6-14 min 95% (B); 14-18 min ramp linearly to 50% (B); 18-20 min 50% (B). At least 3 product ions were monitored for each analyte. The MRM conditions are given in Table 1.

The method was validated both for screening and quantification purposes according to Commission Decision 2002/657/EC (Com. Dec. 2002) at the following concentration range: 0.020-0.080 µg/L for 17β-estradiol, 0.25-10 µg/L for testosterone and 0.5-20 µg/L for progesterone.

Table 1. MRM conditions.

Analita	DP	EP	Parent ion	Product ion	CE	CXP
Estradiol	-100	-10	271.2	145.0	-50	-12
				183.1	-54	
				143.0	-67	
Estradiol-d4	-130	- 102	275.2	147.0	-53	-12
				187.0	-54	
				145.0	-65	
Testosterone	74	10	289.1	97.2	29	12
				109.0	33	
				79.1	70	
Testosterone-d3	90	10	292.2	256.4	25	12
				109	32	
				97.2	29	
Progesterone	103	10	315.2	97.1	26	12
				109.1	30	
				297.4	22	
Progesterone-d9	80	10	324.3	100.1	29	12
				113.1	33	
				306.4	25	

Results and Discussion

The developed method allowed to achieve the simultaneous extraction of the three natural sex hormones from blood serum, avoiding two different sample treatment procedures.
Matrix effect, variation and losses during sample preparation are minimized by using of the stable isotope-labelled internal standard (SIDA approach). The use of the AFFINIMIP® cartridges allows a very good clean up. Furthermore, ammonium fluoride as a mobile phase provides an enhancement in the estradiol signal, the analyte with the lowest limits.

In the period 2019-2021, in Piedmont Region 203 different bovine blood serum samples were collected during official controls. They were examined in order to evaluate the physiological levels of steroid hormones based on animal age and sex and to verify compliance with the legal limits.

As shown in Table 2, in all the samples analysed steroid levels were lower than the Maximum Physiological Limits. It can be observed that for males, testosterone levels were compliant. For females > six months of age, progesterone levels were compliant, instead for the age < 6 months the progesterone is not detected. For both sexes estradiol is always undetected.

Table 2. Comparison between results of two years of controls and Maximum Physiological Limits

	Average concentration found (μg/L)				Maximum Physiological Limits (D.M. 14/11/1996)			
	M < 6 months	M > 6 months	F < 6 months	F > 6 months	M < 6 months	M > 6 months	F < 6 months	F > 6 months
Testosterone	0.98	3.4	n.d.	n.d.	10	30	0.5	0.5
Progesterone	n.d.	n.d.	0.2	1.8	1	1.5	1	14
17β-estradiol	n.d.	n.d.	n.d.	n.d.	0.04	0.04	0.04	0.04

n.d.=not detected

Conclusion

The absence of residues of estradiol and the normal level of testosterone and progesterone in all the animals confirms the values currently reported in the national legislation (DM 1996), The limits ensure, at the same time, the safety of commercial food and the protection of farming systems.

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POSTER P66

MAKING POSSIBLE A 48 MULTI-RESIDUE METHOD FOR ANTIBIOTICS IN A SINGLE RUN

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Abstract
A simple and rapid method has been developed for the analysis of 48 antibiotics (tetracyclines, quinolones, penicillins, sulfonamides, macrolides, cephalosporins and trimethoprim) in animal origin foodstuffs. A multi-residue method has been validated, not only in muscle/fish, but liver, kidney, milk, eggs, honey and cheese, according to European Union Decision 2002/657/EC and, subsequently, considering the Regulation (EU) 2021/808.
The method combines an effective extraction with occasionally specific clean-ups. The chromatographic separation is performed with BEH C18 column and mobile phases consisting of water and acetonitrile containing formic acid and oxalic acid. MS/MS detection is performed with a triple quadrupole (QqQ) mass spectrometer. UHPLC-MS/MS allows both confirmation and quantification in a single chromatographic run. A total of 48 analytes and 7 internal standards elute within 13 minutes.
This method is already accredited under the ISO/IEC17025 and is used daily within official controls at the Agència de Salut Pública de Barcelona (ASPB). About 2400 samples have been analysed along 2021, of which 250 were suspicious, immobilized or contradictory analysis. Thus, it is necessary to have a rapid and robust confirmatory analysis.

Introduction

Antibiotics are substances that kill and inhibit the growth of microorganisms, being prevented the spread of infections caused by them. Antimicrobial agents could be of natural origin, in other words, that are produced by microorganisms to protect themselves from others microorganisms, or synthetic origin, obtained by chemical synthesis.

The use of antimicrobial agents from the veterinary view is closely linked with the practice of intensive stock-breeding. It is used for two basic purposes: therapeutic (for diagnosed diseases treatments as well as a prophylactic measure) and for diseases prevention.

In the European Union (EU) since 2006 the use of antibiotics for growth promotion is forbidden. The inappropriate use of antimicrobial agents could mean the appearance of waste of them in animal origin food, creating different types of risk for human health. To ensure food safety and protect the consumers, maximum residue limit (MRL) of antibiotics in the food of animal origin was established by the European Commission in the Commission Regulation 37/2010. There are also some antibiotics that are forbidden in certain matrices or species. Table 1 shows the analytes, matrix and LQ validated in the laboratory.

Initially, antibiotic residue analysis methods on food were based on microbiological and immunoassay techniques. Microbiological trials are based on inhibiting the growth of microorganisms (usually bacteria) by the presence of the antimicrobial in the sample. The advantages presented by microbiological methods are the possibility of detecting a large number of compounds simply and at low cost. This method, however, has many drawbacks; it takes a lot of time, is unspecific and not confirmative. Immunoassays are based on the specific reaction between antigen and antibody; the most widely used technique within this group is ELISA (from non-linked immunoassay). ELISA methods are very specific and selective. But the fact that they are so specific means that there are no assays for many of the compounds, and in general, they are quite expensive.

Over the years, the great evolution of analytic instrumentation has resulted in microbiological methods being gradually replaced by chromatographic methods with different types of detectors. Today, in order to comply with the points system present in Decision 657/2002/EC and, subsequently, Regulation (EU) 2021/808, liquid chromatography coupled with mass spectrometry (LC-MS/MS) clearly predominates in mostly laboratories that control veterinary drugs.

In an official laboratory, the demand for antibiotic analysis is very high, multi-residue methods are used including as many compounds as possible of different families. In multi-residue methods, the extraction of a large number of compounds with different properties is prioritized, and therefore sample treatment procedures are very generic. In these methods, clean-up is often removed, in order to avoid heavy losses from some analytes. This causes relatively complex extracts. Therefore, it is necessary to use high selectivity detection systems, being the MS/MS the most appropriate and, in fact, being mandatory in the case of prohibited compounds.

Table 1. Analytes, matrixes and LQ validated in the laboratory.

Family	Antibiotic	Muscle/Fish (µg/kg)	Liver (µg/kg)	Kidney (µg/kg)	Milk (µg/kg)	Egg (µg/kg)	Honey (µg/kg)	Cheese (µg/kg)
Sulfonamides	Sulfapyridine	10	10	10	1	2	1	20
	Sulfaquinoxaline	10	10	10	1	4	1	20
	Sulfadiazine	10	10	10	1	2	1	20
	Sulfamethoxazole	10	10	10	1	2	1	20
	Sulfamethazine	10	10	10	1	2	1	20
	Sulfamonomethoxine	10	10	10	1	2	1	20
	Sulfachloropyridazine	10	10	10	1	2	1	20
	Sulfamethizole	10	10	10	1	2	1	20
	Sulfadoxin	10	10	10	1	2	1	20
	Sulfamethoxypyridazine	10	10	10	1	2	1	20
	Sulfathiazole	10	10	10	1	2	1	20
	Sulfamerazine	10	10	10	1	2	1	20
	Sulfadimethoxine	10	10	10	1	2	1	20
	Sulfisoxazole	10	10	10	1	2	1	20
	Trimetroprim	10	10	10	1	2	1	20
Cephalosporins	Cefalexin	10	10	10	-	-	-	-
	Cefapirin	20	20	20	-	-	-	-
	Cefquinome	10	10	10	-	-	-	-
Quinolones	Marbofloxacin	10	10	10	1	4	1	20
	Ciprofloxacin	20	20	20	1	4	1	20
	Danofloxacin	20	20	20	1	4	1	20
	Enrofloxacin	20	20	20	1	4	1	20
	Difloxacin	10	10	10	1	4	1	20
	Flumequine	10	10	10	1	4	1	20
	Oxolinic Acid	10	10	10	1	4	1	20
	Sarafloxacin	20	20	20	1	4	1	20
	Norfloxacin	10	10	10	1	4	1	20
Macrolides	Tylosin	20	20	20	1	4	1	20
	Espiramicin	10	10	10	1	4	1	20
	Eritromicin	10	10	10	1	4	1	20
	Josamicin	10	10	10	1	4	1	20
	Tilmicosin	-	-	-	1	4	1	20
	Lincomicin	10	10	10	1	4	1	20
Tetracyclines	Oxytetracycline + 4-epioxytetracycline	10	10	10	1	4	1	20
		10	10	10	-	-	-	20
	Tetracycline + 4-epioxitetracycline	10	10	10	1	4	1	20
		10	10	10	-	-	-	20
	Clortetracycline + 4-epiclortetracycline	10	10	10	1	8	1	20
		10	10	10	-	-	-	20
	Doxycycline	10	10	10	1	4	1	20
Penicillins	Amoxicillin	20	20	20	2	4	2	20
	Ampicillin	20	20	20	2	4	1	20
	Penicillin G	10	10	10	1	4	1	20
	Penicillin V	10	10	10	2	4	1	20
	Oxacillin	10	10	10	1	4	1	20
	Cloxacilin	10	10	10	1	4	1	20
	Dicloxacilin	10	10	10	1	4	1	20
	Nafcillin	10	10	10	-	-	-	20

– means that the analyte is not validated in the matrix currently.

Materials and Methods

Standards

1000 mg L-1 stock standard solutions of each analyte are prepared by dissolving the antibiotics or their internal standards (IS) in an appropriate solvent (ACN, MeOH, CH3Cl, H2O or 0.1 M NaOH). The antibiotics included in the study are listed in Table 1. The internal standards included in the study are: sulfamethazine-C13 and Sulfadiazine-C13 (sulfonamides and trimethoprim), norfloxacin-d5 (quinolones), roxitromycin (macrolides), piperacillin (penicillins), demeclocycline (tetracyclines) and Cefoxitin (cephalosporins). Working solutions were prepared by aqueous dilution. All solutions were stored at -20 ºC.

Reagents

Methanol HPLC (Fisher Chemical), Acetonitrile HPLC Hypergrade type and P.A. type (Fisher Chemical), Formic acid P.A. (Fisher Chemical), EDTA Disodium Salt 2-hydrate (Reag. Ph Eur.) P.A. (Panreac), Oxalic Acid 2-hydrate P.A. (Sigma Aldich), Sodium hydroxide P.A. (Honeywell), Chloroform (Panreac), N,N-Dimethylformamid P.A. (Merck), type I Water (Milli Q, Millipore, Molsheim, France), Ethyl acetate HPLC (Honeywell), 2-Propanolol (Honeywell), Sodium phosphate dibasic 99+% (Acros Organics), Citric acid monohydrate (Merck), n-Hexane 95% for pesticides analysis (Panreac), Strata XL 100µm Polymeric Reversed Phase 200mg/60mL (Phenomenex).

LC-MS/MS conditions

A Waters Acquity Ultra Performance Liquid Chromatography (UPLC) system coupled to Xevo TQ-S micro triple-quadrupole mass spectrometer equipped with an electrospray ionisation (ESI) source is used.

BEH C18 column (2.1 x 100 mm, 1.7 µm, Waters) is used. Separation is performed at 40ºC in gradient elution mode at a flow rate of 0.3 mL min-1; (t,%A): (0,95);(1,95);(5,75);(8.5,10); (9.5,10); (10,95); (13,95). Mobile phase A: 0.2% formic acid and 0.1 mM oxalic acid in water and mobile phase B: 0.1% formic acid in acetonitrile is used. 5 µl of sample is injected. The electrospray ionisation source is operated in positive mode with following conditions: capillary voltage of 0.75 kv; source block and desolvation temperatures of 150°C and 550°C, respectively; desolvation and nebulizer gas (nitrogen) flow rates of 1000 lh-1 and 50 lh-1, respectively. The Multiple Reaction Monitoring (MRM) method is used. Two transitions are followed for each analyte (quantification and confirmation) and one for each IS. Identification was carried out by retention time and confirmation was performed using ion ratio criteria (2002/657/EC).

Results and Discussion

The same instrumental method is used for all the analytes of the families: tetracyclines, quinolones, penicillins, sulfonamides, macrolides, cephalosporins and trimethoprim. The analysed matrices are: muscle/fish, liver, kidney, milk, honey, egg and cheese.

For solid matrices, a liquid-liquid extraction without cleaning step is used. Initially the muscle was validated and later fish, liver and kidney of different species were validated completely with their different MRLs.

For liquid matrices and cheese, a solid-liquid extraction is used using specific cartridges. The initial validation was in egg matrix and later in honey, milk and cheese. MRM chromatograms from milk spiked at LQ level of an analyte of each family is shown in Figure 1.

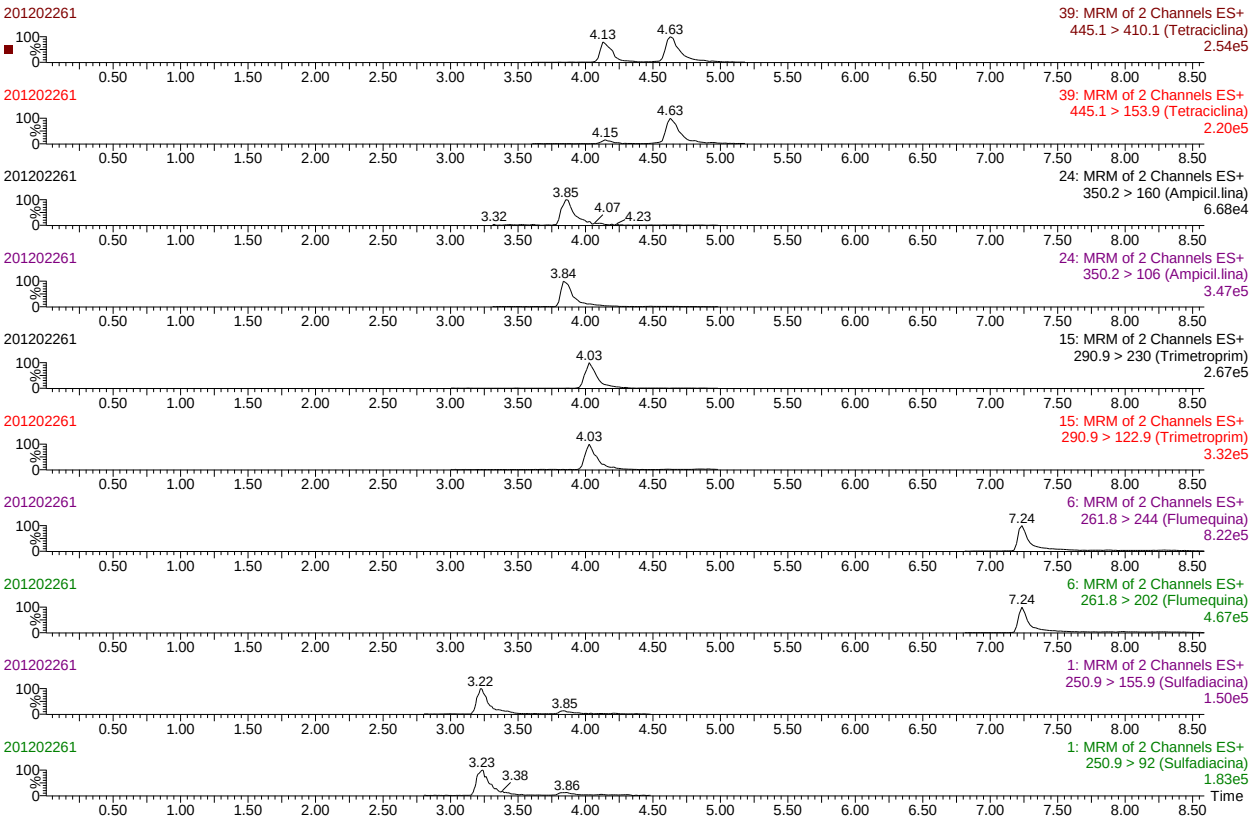

Figure 1. MRM chromatograms from milk spiked at LQ level (Tetracycline, Ampicillin, Trimetroprim, Flumequine, Sufadiazine).

Owing to the optimized gradient program, all the analytes and IS's eluted within only 13 min. Moreover, monitoring of the two transitions per compound was possible with just one chromatographic run.

Once the whole analytical method was optimized, the method was validated against EU Decision 2002/657/EC. The following parameters were tested: linearity, precision, trueness, specificity, limit of quantification (LQ), CCα and CCβ.

Calibration curves were obtained from spiked blank samples that were processed the same way as the samples. Quantification was performed using the internal standard method, by adding the ISs to all samples (including calibration samples) before extraction. Thus, no correction for extraction recovery was required.

Linearity was tested in the range from LQ to 3/2 MRL except for that analytes which have no MRL (from LQ to 75 µg/kg) with five points curves. The value of r was higher than 0.99 in all cases, while residuals were lower than 20% for the first calibration point, and lower than 15% for the others.

Trueness and precision (repeatability and within-laboratory reproducibility) were determined at LQ, 1/2 MRL, MRL and 3/2 MRL, except that analytes which have no MRL. These compounds were instead checked at levels of LQ and two equidistant levels. For each concentration level, 18 samples of each matrix were processed in three different days. The relative standard deviation (RSD) values obtained were below those calculated by the Horwitz equation. Trueness were always between the ranges following Decision 657/2002/CE.

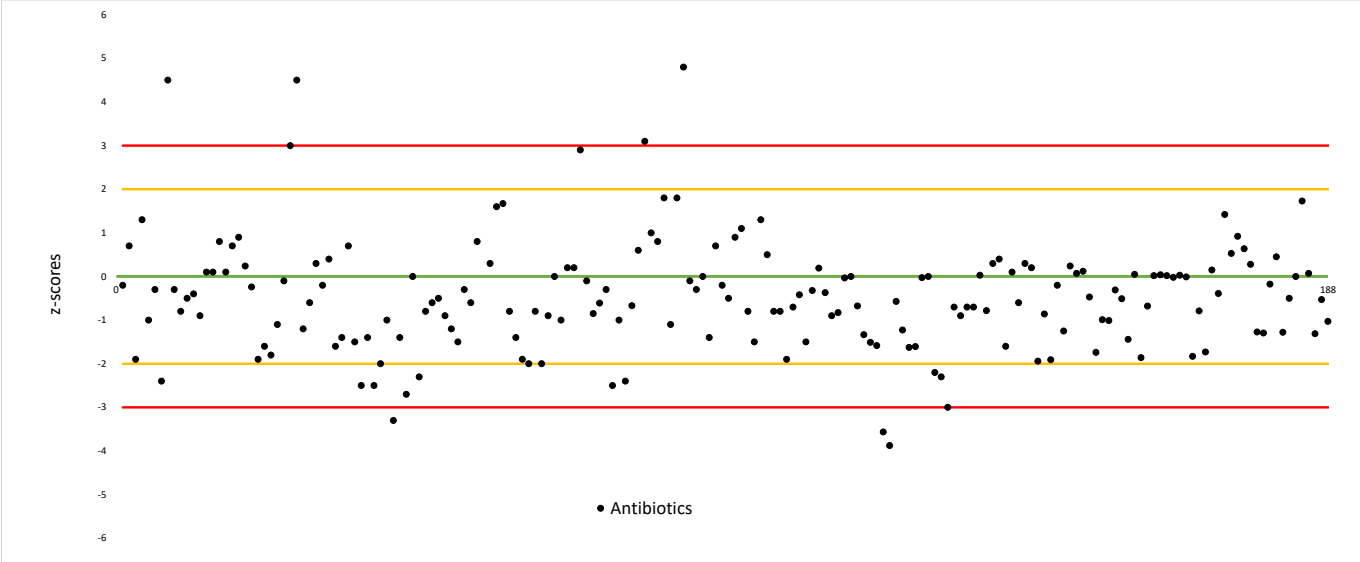

Figure 2. Results of z-score obtained in the analysis of 37 proficiency test and 32 reference material in different antibiotics and matrices.

For compounds having an MRL, CCα was calculated according to the following equation: CCα = MRL + 1.64(SD); whereby SD = the standard deviation from 18 samples spiked at MRL level. For compounds that not have MRL CCα was calculated as the concentration at the y-intercept plus 2.33(SD).

The specificity was assessed by analysing blank tissue samples. The absence of background peaks with a signal-to-noise ratio greater than 3 at the retention times of the target compounds indicated that the method is free of any endogenous interference.

The applicability of the method in different matrices was explored using reference materials and proficiency test exercises represented in figure 2. The x-axis represents the compounds and the y-axis the z-score obtained. It is also represented with a yellow line the margin of ±2 and with red the ±3.

In the last 5 years, a total of 37 proficiency test and 32 reference material have been performed, evaluating a total of 188 compound in different samples: muscle, liver, kidney, crustaceans, fish, honey, milk and eggs.

Figure 3 shows the percentage of satisfactory, questionable and unsatisfactory results. 90% of successful results, 6% questionable results and 4% unsatisfactory results have been obtained.

In unsatisfactory cases, the cause has always been studied.

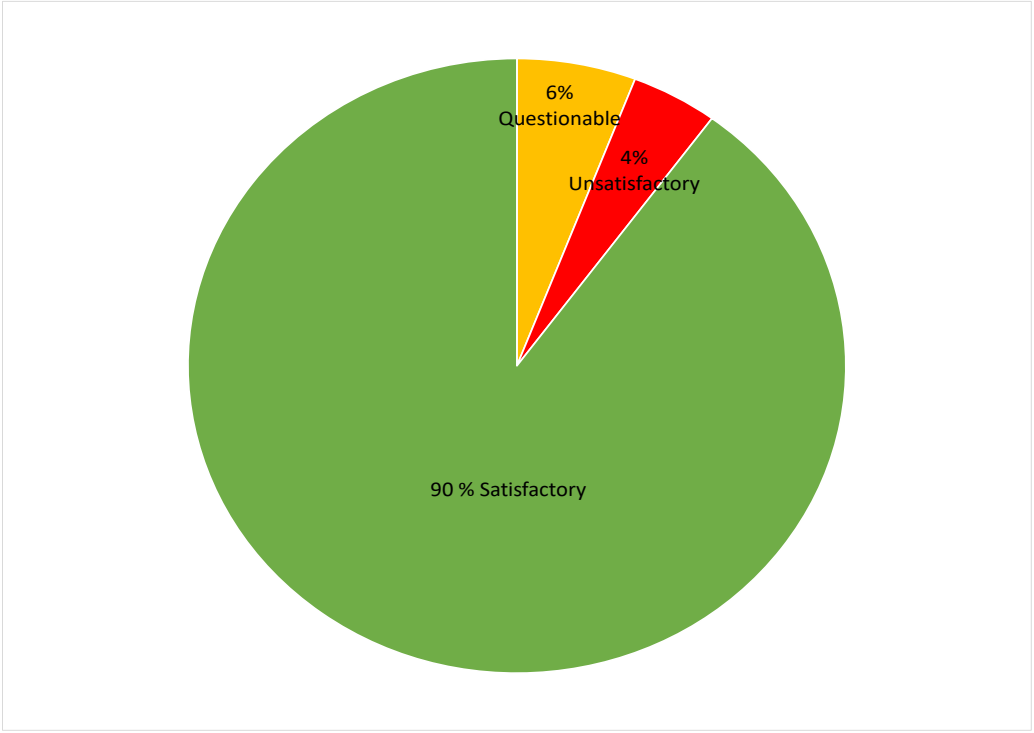

Figure 3. Percentage of satisfactory, questionable and unsatisfactory z-score of proficiency test and reference material in the last 5 years.

The results obtained in the external and internal quality control in the last years demonstrates that the method is suitable as a confirmatory method for regulatory purpose. The method is being used for routine analysis at the *Agència de Salut Pública de Barcelona.*

Conclusion

Due to the need to report results of different types of samples received in an official laboratory and its open scope, it is necessary to have a method in which a wide range of matrices can be analysed in a rapid and robust way.

The proposed method has been shown to be useful both as a screening and for confirmation and quantification of antibiotic residues. It is also a robust method as it can be applied to different matrices and meets the requirements of Directive 2002/657/EC. In addition, it is a relatively fast method, as the samples require very little manipulation.

The method has been successfully validated and is suitable as a confirmatory method for regulatory purpose. About 2400 samples in different matrix are analised each year at the *Agència de Salut Pública de Barcelona*. The method is included in its list of accredited methods under ISO/IEC 17025.

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POSTER P67

BOVINE TEETH AND CLAWS: A POTENTIAL NEW FORENSIC MATRIX FOR THE DETECTION OF ILLICIT ADMINISTRATION OF REGULATED AND BANNED SUBSTANCES

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Abstract
To investigate illicit administration of regulated and banned substances used for growth-promoting purposes in cattle breeding, it is common to analyse the usual matrices like urine, feed, animal tissue, and hair samples nowadays. Each type of material has its advantages but also its disadvantages. For example, urine samples are easy to collect, but the compounds of interest could only be detected a few hours or days after administration, hair samples are not that simple to collect but do have a longer detection time window. We wondered if there is a different type of sample material available that could overcome these disadvantages? A requirement is that the samples should be tracebale to the animals and be easy to collect, for example, at slaughterhouses. Possible 'offal'? Possibly bovine teeth and claws could be used to detect regulated and banned substances. Methods for these matrices were developed, and samples from a 'real case', where an illegal regulated compound was administrated, and also samples from animal studies, were analysed with these methods. The findings of the administered compounds in the bovine teeth samples proved that the developed method is fit for the purpose.

Keywords: Forensic, New Matrices

POSTER P68

CONTROLS IN THE FOOD CHAIN: MULTICLASS METHODS TO DETECTLOW RESIDUAL CONCENTRATIONS OF VETERINARY DRUGS IN FEED AND FOOD

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Abstract
Last generation UPLC-MS systems enable to monitor the presence of a wide range of different veterinary drugs by means of a simplified sample preparation protocols, with no or minimum purification steps, reducing the time and costs of the analyses. Consequently, an increasing trend in developing these analytical strategies is taking place in routine laboratories.
In this study, two similar multiclass-multiresidue methods, for semi-quantitative screening purpose are presented.
One method for feed, with a CCß ≤ 0.5 mg/kg to monitor possible cross contamination effects or undeclared inclusion of veterinary drugs in feed; the other for muscle, with a CCß ≤ 10-25 µg/kg to monitor residual concentrations of antibiotics << MRLs.
76 and 56 active substances were monitored respectively in muscle and in feed.
These methods were applied over a total of 195 targeted samples (50 feeds and 145 muscles of bovine, swine, poultry, rabbit and fish) collected for the implementation of the National Residue Monitoring Plans (NRMPs) 2019-2021.
In both matrices the percentage of samples containing detectable concentrations of residues was lower than 10%; residual levels of antibiotics were higher than MRLs in 5 muscles and lower than MRLs in 8 muscles, and in the range 0.5-696 mg/kg in feed.

Introduction
In recent years, the implementation of ultra-highly performing Liquid Chromatography (UPLC) coupled to very sensitive triple quad Mass Spectrometry (MS), enabled to develop "multi-residue and multiclass" methods, i.e. one single analytical protocol to analyse different classes of chemical compounds in one shot.
This new approach could represent an evolution in the strategy of the implementation of NRMPs, especially in consideration of the new Regulation (EU) 2017/625 on official controls for food and feed, inforce since December 2019, which places a strong emphasis on the need to ensure consistency and coordination among official controls in the food chain.
The availability of multiclass methods would permit to move to a new sampling strategy, where each sample would be tested not just for one single compound or class of compounds but for a wide range of substances, enabling to achieve a broader information on possible –even unexpected– source of food/feed contamination.
Therefore, the principal goal of this project was to develop and validate a multiclass screening methods based on LC-MS for the determination of different classes of veterinary drugs (mainly but not exclusively antibiotics) in feed and in muscle samples.
Each method was internally developed and validated according to CRL Guideline for the Validation of Screening Methods for Residues and in agreement with Regulation (EU) 2021/808.
Their effectiveness has been tested through participation in two ring tests for antibiotics organized by the Wageningen University, obtaining, for both, favourable outcomes.
Both methods have been accredited according to EN ISO /IEC 17025, and are now applied routinely on samples of the NRMPs to collect data and highlight any critical issues.

Materials and Methods

Sample preparation
Feed samples (2.5 g) were spiked with 62.5 ng of amoxicillin-D4, ampicillin-D5, chloramphenicol-D5, ipronidazole-D3, robenidine-D8, nicarbazin-D8, virginiamycin-S1, sulfadoxin-D3, sulfadimetoxin-D6, trimethoprim-D9, sarafloxacin-D8, difloxacin-D3, enrofloxacin-D5, tetracycline-D6, demeclocycline, roxithromycin, and pipemidic acid as internal standards and/or process standards. The extraction of analytes was performed by adding 15 ml of 0.1M succinic acid (pH=4): samples were vortexed at high speed for 2 minutes on a vertical shaker (Genogrinder), and sonicated in an ultrasound bath for 10 minutes, centrifuged (6500 × g, 10 min, 4°C) and transferred in a new tube. The extraction was repeated two more times, the first

with acetonitrile (5ml), the second with methanol (12.5). Supernatants were collected with the previous one (about 27.5 ml). The extract was splitted and treated in 2 different ways:

- For coccidiostats and avermectins: 0.5 ml of extract were diluted to to a final volume of 5 ml with methanol.
- For ß-lactams, quinolones, macrolides, tetracyclines, sulfonamides lincosamides, pleuromutilins, furans, nitrofurans, trimethoprim, virginiamycin-M1: 0.5 ml of extract were diluted to a final volume of 5 ml with acetonitrile/deionised water (90/10).

An appropriate aliquot of each of them were transferred in HPLC vials for subsequent analysis.

Muscle samples (5 g) were spiked with 50 ng of deuterated β-lactams (ampicillin-D5, cafapirin-D4, cefalexin-D5, ceftiofur-D3, cloxacillin-^{13}C4, penicillinG-D7, penicillinV-D5), sulfadimetoxin-D6, demeclocycline, roxithromycin and pipemidic acid as internal standards. The extraction was performed by adding 10 ml of refrigerated extraction mixture (acetonitrile/deionised water 80/20) and 0.5 ml of 250 mM EDTA (pH, 8.0). Samples were vortexed for 10 minutes on a shaker, then centrifuged (4000 × g, 5 min, 4°C). The supernatant was transferred in a new tube and the extraction was repeated with acetonitrile (5ml). After centrifugation (4000 × g, 5 min, 4°C), the supernatant was combined to the previous one (about 15 ml) and diluted to a final volume (20 ml) with acetonitrile. The extract was splitted and treated in 2 different ways:

- For ß-lactams, dapson, quinolones, phenicols, tetracyclines, sulphonamides and trimethoprim: 2 ml of extract, defatted with n-heptane (2 ml), were evaporated until dryness under a liquid nitrogen stream at 40°C and reconstituted with 0.5 ml of Ammonium Acetate 0.2 M.
- For macrolides lincosamides, pleuromutilins, rifamycin and novobiocin: 2 ml of extract, defatted with n-heptane (2 ml), were evaporated until dryness under a liquid nitrogen stream at 40°C and reconstituted with 0.8 ml of methanol and 0.2 ml of Ammonium Acetate 0.2 M.

Appropriate portions of each of them were transferred in HPLC vials for subsequent analysis.

Liquid Chromatography and Mass Spectrometry

A UFLC (Nexera X2, Shimadzu) interfaced with an API 4000 triple quadrupole (AB Sciex) was used for LC-MS/MS analyses of feed samples. Two transitions per molecule were monitored using a scheduled MRM method. The separation was achieved using 0.1% formic acid (A) and acetonitrile (B) as mobile phase and a Poroshell 120 EC-C18 2.7 µm (2.1 x 100) mm column (Agilent Tecnologies). Two gradient were generated by mixing A, B as follows:

- Gradient named “SLOW” for the separation of ß-lactams, quinolones, macrolides, tetracyclines, sulfonamides lincosamides, pleuromutilins, furans, nitrofurans, trimethoprim, virginiamycinS1: initial conditions using 90:10 (A:B), kept unchanged until 0.5 min, then 50:50 at 11 min, 5:95 from 11.5 to 14.5 min to wash the system, and finally 90:10 from 15 to 18 min to re-equilibrate the column. Flow 0.25 ml/min, injection volume 5 µl, column temperature 30°C, sample temperature 10°C
- Gradient named “RAPID” for the separation of coccidiostats and avermectins: initial conditions using 90:10 (A:B), kept unchanged until 0.5 min, then 40:60 at 1.5 min, 5:95 from 3.5 to 7 min to wash the system, and finally 90:10 from 7.5 to 10 min to re-equilibrate the column. Flow 0.25 ml/min, injection volume 5 µl, column temperature 30°C, sample temperature 10°C.

A UPLC Acquity I class plus (Waters) coupled with a Xevo TQ-XS triple quadrupole (Waters) was used for LC-MS/MS analyses of muscle samples. Two transitions per molecule were monitored using MRM method in ESI positive mode. The separation was achieved using 0.1% formic acid (A) and acetonitrile (B) as mobile phase and a Poroshell 120 EC-C18 2.7 µm (2.1 x 100) mm column (Agilent Tecnologies). Two gradient were generated by mixing A, B as follows:

- Gradient named “NORMAL” for the separation of ß-lactams, quinolones, phenicols, sulfonamides, tetracyclines, dapson and trimethoprim: initial conditions using 90:10, kept unchanged until 0.5 min, then 85:15 at 6 min, 50:50 at 11 min, 5:95 from 11.5 to 14.5 min to wash the system, and finally 90:10 from 15 to 18 min to re-equilibrate the column. Flow 0.25 ml/min, injection volume 5 µl, column temperature 30°C, sample temperature 10°C.

Gradient named “FAST” for the separation of macrolides, lincosamides, pleuromutilins, rifamycin and novobiocin in muscle samples: initial conditions using 90:10, kept unchanged until 1min, 100 (B) from 5 to 6 min, and finally 90:10 from 6.1 to 10 min to re-equilibrate the column. Flow 0.40 ml/min, injection volume 5 µl, column temperature 30°C, sample temperature 10°C.

Results and Discussion

Method validation
The methods were fully validated as qualitative screening method as described in Commission Decision 2002/657/EC and in CRL Guidelines for the Validation of Screening Methods for Residues: specificity, ruggedness, and detection capability (CCβ) were evaluated. The validation fulfils also the requirements of Regulation (EU) 2021/808.
The screening Target Concentration (STC) for each analyte was chosen considering MRLs, CCßs recommended in the Italian NRCP and preliminary results acquired during method development.

- STC for feed at 0.5 mg kg^{-1} for all veterinary drugs selected (amoxicillin, ampicillin, spiramycin, tilmicosin, tylosin, erythromycin, chlortetracycline, doxycycline, oxytetracycline, tetracycline, enrofloxacin, ciprofloxacin, difloxacin, danofloxacin, flumequine, marbofloxacin, nalidixic acid, norfloxacin, oxolinic acid, sarafloxacin, sulfachloropyridazine, sulfadiazine, sulfadimethoxine, sulfadoxine, sulfamerazine, sulfamethazine, sulfamethoxazole, sulfamethoxypyridazine, sulfamonomethoxine, sulfaquinoxaline, sulfathiazole, sulfisoxazole, nitrofurazone, furazolidone, nitrofurantoin, furaltadone, nifursol, dimetridazole, ronidazole, metronidatole, ipronidazole, carbadox, olaquindox, florfenicol, thiamphenicol, ivermectin, emamectin, abamectin, tiamulin, valnemulin, nicarbazin, robenidine, metilclorpindol, lincomycin, trimethoprim, virginiamycin M1. When appropriate internal standard correction was adopted.

- STC for muscle: at 5 µg kg^{-1} for dapsone, at 10 µg kg^{-1} for amoxicillin, ampicillin, cloxacillin, dicloxacillin, oxacillin, nafcillin, penicillin G, penicillin V, cefalexin, cefalonium, cefapirin, desacetyl cefapirin, cefazolin, cefquinome, cefoperazone, cefacetril, cefuroxime, trimethoprim, erythromycin, gamithromycin, josamycin, lincomycin, pirlimycin, spiramycin, neospiramycin, tildipirosin, tilmicosin, tylosin, tylvalosin, 3-O-acetyltylosin, tulathromycin, florfenicol, florfenicol amina, thiamphenicol, tiamulin, valnemulin, rifaximin, novobiocin, enrofloxacin, ciprofloxacin, difloxacin, danofloxacin, flumequine, marbofloxacin, nalidixic acid, norfloxacin, oxolinic acid, sarafloxacin, chlortetracycline, epi-chlortetracycline, doxycycline, oxytetracycline, epi-oxytetracycline, tetracycline, epi-tetracycline, phthalylsulfathiazole, sulfachloropyridazine, sulfaclozine, sulfadiazine, sulfadimethoxine, sulfadoxine, sulfaguanidine, sulfalene, sulfamerazine, sulfamethazine, sulfamethizole, sulfamethoxazole, sulfamethoxypyridazine, sulfamonomethoxine, sulfapyridine, sulfaquinoxaline, sulfathiazole, sulfisoxazole and at 25 µg kg^{-1} for ceftiofur and desfuroylceftiofur.
  When appropriate internal standard correction was adopted.

A qualitative approach was used to determine the performance parameter CCß as described in the CRL Guidelines: a minimum set of twenty blank feed intended for different animal species and muscle samples from different species (poultry, bovine, swine and trout) were analysed for specificity test; the same samples spiked at the STC were analysed for ß error verification.
Ruggedness (minor change) was evaluated by Youden fractional factorial design: seven variables were chosen and deliberately altered. 8 experiments were carried out on samples fortified at STC, according to the Youden scheme.

The reliability and actual performance of the methods were tested by participating to proficiency tests (PTs) organized by the University of Wageningen for the identification of antibiotics belonging to different families in pig feed and porcine muscle samples.
The feed PT involved the analysis of 3 feed samples to search for one or more analytes from a total of 35 potential compounds belonging to the families of tetracyclines, sulfonamides, macrolides, pleuromutilins, lincosamides, quinoxalines, phenicols, coccidiostats.
The muscle PT involved the analysis of 3 samples of porcine muscle to search for one or more analytes from a total of 60 compounds belonging to the main categories of antibiotics (ßlactams, quinolones, tetracyclines, macrolides, lincosamides, sulfonamides included trimethoprim and phenicols).
In both cases, the samples were analyzed using the validated procedures.
The results of PTs were summarized in Table 1.

Table 1. Proficiency Test Results:

Feed

Sample	Analyte	Result (mg kg^{-1})	Assigned value (mg kg^{-1})	z-score
AB/2019/feed/772	/	/	/	
AB/2019/Feed/753	carbadox	2.7	3.1	0.46
	nicarbazin	0.8	0.9	0.43
	tiamulin	1.5	1.7	0.42
AB/2019/feed/204	sulfadiazine	3.1	3.0	0.20
	trimethoprim	<CCß*	0.6	Not applicable
	salinomycin	1.0	1.0	0.13
Muscle				
Analyte	Analyte	Result (mg kg^{-1})	Assigned value (mg kg^{-1})	z-score
AB/2020/muscle/557	/	/	/	/
AB/2020/muscle/637	florfenicol	252	297	-0.8
AB/2020/muscle/320	cefalexin	202	189	0.3
	sulfadoxin	84	97	-0.6

**: The applied method correctly revealed the presence of trimethoprim but in a concentration lower than the cut-off: for this reason, no concentration value was reported. Considering the measurement uncertainty applicable to the assigned value, the sample could be lower than the CCß equal to 0.5 mg / kg. The data was acceptable for the lab.*

Method application

Once the reliability of the method was verified, 50 feeds and 145 muscle samples were collected and analysed within the 2019-2020-2021 NRMPs.

Feed: All selected feed samples derived from sampling activity for the NMCPs. 4 out of 50 samples resulted suspect after multiclass screening analysis for the presence of one or more analytes: 2 suspect samples were feed for rabbit, one was for poultry and one for swine.

Muscle: 13 out of 145 samples resulted suspect on multiclass screening analysis for the presence of one or more analytes. 11 out of 13 were bovine muscles samples collected from emergency slaughtering, the other 2 suspect samples were broiler and fish muscles collected from NMCPs.

Sample types are summarized in table 2

Table 2: Sample types

	Feed	**Muscle**			
Target Species	Sampling Strategy				
	NMCPs	NMCPs	Import Controls	Emergency Slaughter	Total
Aquaculture fish	5	22 (1 S)			22
Cattle	10	49	4	14 (11 S)	67
Equine	1	1	2		3
Farmed game	/	4			4
Poultry	10 (1S)	32 (1 S)			32
Rabbit	9 (3S)	6			6
Swine	15 (1S)	7	4		11
Total	50	121	10	14	145

All antibiotics identified by the proposed multiclass screening methods were confirmed by subsequent confirmatory analysis.

Even if the screening method was not intended for quantitative purposes, a good correlation was usually observed between the results of the screening and the confirmatory tests. Some differences may derive by the different scope of the methods: the screening is targeted for the detection of low concentrations and is based on a single level calibration curve, whereas the confirmatory analysis is applied for a larger concentration

range, and consequently include also a multilevel calibration curve. The results of the qualitative and quantitative analyses are reported in table 3.

Table 3: Comparison Screening and Confirmatory results

Feed				
Species	Suspect Samples			
	Analytes detected	Cut off Screening Level mg kg^{-1}	Semi-quantitative Screening value (mg kg^{-1})	Confirmatory value (mg kg^{-1})
swine	tiamphenicol	0.42	1.79	1.190
	florfenicol	0.23	0.48	0.136
rabbit	osisitetracycline	0.31	0.45	0.33
rabbit	tetracycline	0.34	7.2	> 2 (about 3.4)
	osisitetracycline	0.31	456	< 2 (about 696)
poultry	clortetracycline	0.37	4.18	> 2 (about 3.9)
	ossitetracycline	0.31	1.2	1.18
Muscle				
Species	Suspect Samples			
	Analytes detected	Cut off Screening level	Semi-quantitative Screening value (µg kg^{-1})	Confirmatory value (µgkg-1)
Culled cow	ciprofloxacin	5.5	15	11.8
	enrofloxacin	7.0	14	15.5
Culled cow	amoxycillin	5.4	120	336
Beef veal	ciprofloxacin	5.5	24	26.3
	enrofloxacin	7.0	24	27.5
Beef cattle	marbofloxacin	6.3	47	49.2
Beef veal	ampicillin	9.1	283	339
Beef cattle	penicillin-G	9.2	39	48.2
Beef cattle	ampicillin	9.1	200	232
	sulfadiazine	8.1	189	273
	trimetoprim	6.4	59	34
Beef veal	ampicillin	9.1	1164	1361
Turkey	amoxycillin	5.4	9	43.8
Trout	trimetoprim	6.4	16	11.1
Beef cattle	ciprofloxacin	5.5	15	25.6
	enrofloxacin	7	58	80.1
Beef cattle	ossitetrcycline	7.4	100	96.7
Culled cow	ossitetracycline	7.4	566.0	499.6
	epi-ossitetracycline	7.1	25	35.2

Conclusions

The principal aim of this research was the development and validation of qualitative screening methods for the determination of multiple classes of antibiotics (multiclass) in muscle tissue and feed.

To achieve this goal, highly sensitive UPLC coupled to the latest generation triple quadrupole MS were adopted.

Two simple, quick and sensitive method based on UPLC-MS/MS analysis were validated for semi-quantitative screening methods of 76 active substances in muscle and 56 in feed, in agreement with the CRL Guideline for the Validation of Screening Methods for Residues and Regulation (EU) 2021/808. These methods were accredited in accordance with EN ISO/IEC 17025 and are now in routine use in our laboratory as multiclass screening method.

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POSTER P69

ILLICIT ADMINISTRATION OF NATURAL STEROIDS IN CATTLE: A CASE REPORT.

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Abstract

Natural and synthetic hormones have been widely illegally used in animal husbandry for several decades as growth promoters especially in bovine farming. Their use in animal fattening has been prohibited because of their toxic effects on public health. Controls are regulated under Directive 96/23/EC (Dir 1996).

In Italy, maximum physiological levels have been established in 1996 for 17β-estradiol, progesterone and testosterone in bovine serum (D.M.1996): these levels allowed to discriminate between treated or untreated animals.

During a police investigation performed in 2016 in Italy, an undeclared animal treatment was observed by hidden cameras. Next day, anonymous liquids were impounded and serum of ten animals was collected and transferred to our laboratory.

A rapid LC-MS/MS procedure was developed for simultaneous determination of progesterone, testosterone, 1-testosterone, 17β-estradiol, residues in bovine serum.

In 9 serum samples 17β-estradiol was found at concentration higher than Maximum limit. In all anonymous liquids the prodrug 17β-estradiol benzoate was found at 14 mg/ml.

Just one serum was found compliant. The practice of keeping one untreated animal in the same box with treated calves is typical in cattle breeding: it suggests the corruptive intent of the breeder to focus unfair veterinary controls on the untreated animal only.

Introduction

Among prohibited substances included in the EU legislation, steroid compounds and, in particular, endogenous steroids, i.e., androgens, estrogens, progestins constitute a challenge in the framework of veterinary official monitoring plans. Indeed, these molecules can be naturally present at variable levels in tissues, organs and body fluids depending on the species, sex and age of the individual animal (Duffy 2009 JCA) and their physiological levels make difficult for possible identification of illicit administration as growth promoters.

Estrogens represent hormones involved in several biochemical processes such as the oestrous cycle. Besides their function in the reproductive system, these steroid compounds play an important role in a number of other physiological processes including mineral, fat, sugar and protein metabolism as well as sodium and water retention. Owing to their wide systemic effects, estrogens are also illegally administered to stimulate growth in calves and increase meat production.

In relation to its physiological presence, an upper limit of 40 ng/l for 17β-estradiol was set for bovine serum and plasma by Italian legislation in order to distinguish natural level of the unconjugated 17β-estradiol from illicit exogenous administration of the compound.

The aim of this work was to describe a case of illicit administration of 17β-estradiol to cattle in order to highlight a national control process that involved different competent authorities, that is, police and official laboratories. Detailing, in February 2014 a police investigation was conducted for a possible illicit cattle treatment, within a livestock in Piedmont, Northern Italy. An animal treatment by an intramuscular administration was observed by hidden video-cameras and the police found in the stable four unlabelled bottles containing liquids and then these anonymous liquids were impounded. Serum samples from 10 animals were immediately collected after police investigation and then transferred to laboratories for the analysis.

Materials and Methods

Chemicals and Materials

Acetonitrile, methanol, ammonium fluoride were of analytical or HPLC grade quality and were supplied by Sigma-Aldrich (St. Louis, MO, USA). Molecularly imprinting polymers (MIPs) SPE cartridges Affinmip Estrogens

(100 mg, 3 mL) were purchased from Affinsep (Petit-Couronne, France). 17β-estradiol, 17β-estradiol-d4 were supplied by Sigma-Aldrich (St. Louis, MO, USA). The stock standard solutions of each analyte and internal standard (ISTD) were prepared in methanol at the concentration of 2 µg/mL and stored at -20 °C in the dark; solutions were stable for 2 years. Suitable working standard solutions in methanol were obtained by appropriate dilution of the corresponding stock solutions and stored at -20 °C.

Blood serum sample and anonymous liquids preparation
Blood serum (2 ml) was spiked with IS and diluted with 7 ml water. The sample was loaded on AFFINIMIP® SPE (0,5 drop/s), previously conditioned with 3 ml acetonitrile and 3 ml water; after washing with 2x3 ml water and 3 ml water/acetonitrile (6:4), the cartridge was dried; analytes were eluted with 3 ml methanol. After evaporation to dryness in nitrogen stream at 50 °C, the residue was dissolved in 0.1 ml methanol/water (1:1) mixture. Anonymous liquids samples were diluted 1:100 with 0.1% formic acid in acetonitrile solution and infused in the mass spectrometer.

LC-MS/MS Analysis
LC analysis was carried out through an HPLC system Exion (Sciex, Framingham, MA, USA). The analytes were detected in ESI negative using Multiple Reaction Monitoring (MRM) mode. Chromatographic separation was performed on a Waters XSelect HSS T3 XP (3 x 100 mm; 2,5 µm) column, kept in a column oven at 40 °C, using a gradient elution with NH4F 0.2 mM in water (A) and NH4F 0.2 mM in methanol (B). Estradiol-d4 was used as internal standard. The mass spectrometer was a QTRAP 6500+ (Sciex, Framingham, MA, USA). Injection volume was 25 µL and flow rate was 0.3 ml/min, with an overall run time of 20 min. The gradient profile was as follows: 0–1 min 50% (B); 1-6 min linear increase up to 95% (B); 6-14 min 95% (B); 14-18 min ramp linearly to 50% (B); 18-20 min 50% (B). At least 3 product ions were monitored for each analyte.

The method was validated for quantification purposes according to Commission Decision 2002/657/EC at the concentration range 0.020-0.100 µg/L.

Concerning anonymous liquids samples, mass spectrum was registered in scan mode. The isolated pick was fragmented, and its product ion spectrum was collected.

Results and Discussion
As result of the analysis performed on serum, nine out of ten samples resulted positive for 17β-estradiol in the range of 0.059-0.208 µg/L (Table 1). The collected values were higher than the Maximum Physiological Limits established in the Ministerial Decree. Only one serum sample resulted compliant to the limit of action, that is, 0.040 µg/L.

Table 1. 17β-estradiol concentration in blood serum samples

Animal	17β-estradiol concentration (µg/L)
1	n.d. [a]
2	0.094
3	0.208
4	0.154
5	0.116
6	0.103
7	0.150
8	0.059
9	0.082
10	0.089

[a] n.d.= not detected

For the four anonymous liquids samples, the mass spectrum registered in scan mode showed an interesting pick at m/z 377, while the product ion spectrum reveals a loss of 105 m/z, typical of the benzoate moiety. Esters are the typical dosage form of many drugs, in particular hormones. It was not difficulty interpreting the MS/MS spectrum, which results typical of 17β-estradiol-benzoate. In order to proof our hypothesis, the analytical standard of this analyte was purchased, and the match verified. 17β-estradiol benzoate was detected

in each four liquids investigated and the concentrations of the compound ranged from 12.0 to 14.4 mg/mL (Table 2).

Table 2. 17β-estradiol-benzoate concentration in anonymous liquids

Anonymous liquids	17β-estradiol-benzoate concentration (mg/mL)
1	13.7
2	14.4
3	12.0
10	14.4

The illicit drug treatment was found thanks to a collaboration between Police and the laboratory staff. The direct observation that an animal treatment had been carried out permitted to collect the animal serums immediately after, making it possible to find strong evidence of the exogenous treatment. It is also interesting the finding of one compliant animal out of ten controlled. This practice of keeping one untreated animal in the same box with treated veals suggests the corruptive intent of the breeder, addressed to obtain dishonest veterinary controls focused only on the untreated animal.

Conclusion

The illicit drug treatment was found thanks to a very good collaboration between Police and the laboratory staff. The requisition of anonymous liquids permitted to identify the analyte to look for. The direct observation that an animal treatment had been carried out permitted to collect the animal serums immediately, thereafter, making it possible to find strong evidence of the exogenous treatment. It is also interesting the finding of one compliant animal out of ten controlled. This practice of keeping one untreated animal in the same box with treated veals is quite typical in cattle breeding: it is suggestive of the corruptive intent of the breeder, addressed to obtain dishonest veterinary controls focused only on the untreated animal. Serum matrix requires a sampling very close to the drug administration in order to find a non-compliant result.

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POSTER P70

A FAST AND SELECTIVE LC-MS/MS METHOD FOR AVERMECTINS AND XVIDEMYCINS IN LIVER AND MILK

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Abstract
Avermectins and milbemycins are deworming agents that can be administered in livestock. The use of some avermectins and milbemycins is permitted within the European Union (EU) with an established maximum residue limit, all others are unauthorized. Traditional methods based on liquid chromatography (LC) coupled to ultraviolet (UV) or fluorescent (FLD) detection are relatively time-consuming and don't yield the high selectivity that is obtained by mass spectrometric (MS) detection. Avermectins and milbemycins are difficult to include in a wide-scope veterinary drug multimethod due to their relatively nonpolar properties and relatively low sensitivity in MS detection. Therefore, a specialized analytical method was developed based on a simple extraction applying a QuEChERS method and detection with triple quadrupole LC-MS/MS. The method was fully validated in accordance with EU Commission Decision (EU) 2021/808 for all avermectins and milbemycins at relevant concentration levels in bovine and porcine liver and bovine milk. The identity of all avermectins and milbemycins could be confirmed in the liver and milk samples at the required levels with sufficient selectivity, robustness, reproducibility and repeatability. This work has resulted in a rapid and reliable method for the screening, quantification and confirmation of avermectins in liver under the Netherlands Residue Control Programme.

Keywords: avermectins, LC-MS/MS, validation
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POSTER P71

MULTIRESIDUE METHOD FOR ANALYSIS OF VETERINARY MEDICINAL PRODUCT RESIDUES IN MEAT AND MILK USING LC-HRMS: VALIDATION.

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Abstract

Liquid chromatography coupled to high-resolution mass spectrometry is a powerful tool for multi-analyte residue determination, as it can detect all ionized compounds without prior selection at their low ppb levels. It is therefore particularly suitable for screening methods in food safety area.

As part of our reference laboratory activities for regulatory control of veterinary drug residues in food from animal origin, a method was developed using LC-HRMS for the screening in meat and milk of about 200 compounds belonging to four main therapeutic classes of veterinary drugs: i.e. antibacterials, antiparasitics, non-steroidal anti-inflammatory drugs, and sedatives. A Q-Exactive system was operated in variable data-independent acquisition (v-DIA) mode. Target screening of compounds of interest was achieved from a compound database, based on retention time, and accurate mass of parent and fragment ions. A comprehensive validation process was undertaken to assess the performance characteristics of the method in terms of trueness, precision, detection capability and specificity. The results showed that the method allows detecting about 75 % of the compounds at the lowest spiking level of 5 -10 µg/kg (milk-muscle) and highlighted the few problematic compounds. The combined examination of quantitative performance characteristics, such as trueness and precision, allowed each compound to be classified into one of the following three categories : qualitative, semi-quantitative or quantitative screening method as described in Regulation (EU) 2021/808.

Introduction

Within the European Union, residue control of pharmacologically active compounds in foodstuffs of animal origin is based on the Regulation (EU) 2017/625. In this context, new control plans to monitor both authorised, prohibited and unauthorised pharmacological substances will have to be implemented starting in 2023. To meet the request of a new specific surveillance plan dedicated to food exposure of residues, multi-residue methods built to look for a wide range of substances are well suited once their performance characteristics in terms of capability of detection has been assessed. High-resolution mass spectrometry (Tof and Orbitrap systems) is a technology of choice for residue analysis collecting information on targeted and non-targeted substances. The objective of the project was the development of a multi-residue target screening method for wide range of veterinary medicinal product residue in meat and in milk using HRMS detection with a Q-Exactive system. This paper describes the process employed to validate the method for the screening purpose, and according to the EU Regulation 2021/808.

Materials and Methods

Chemical and reagents

Analytical standards were purchased from Sigma-Aldrich (Saint-Louis, USA), Witega (Berlin, Germany), Cluzeau (Ste Foy-La-Grande, France) and Acros (Geel, Belgium). A few analytical standards were specifically obtained from the pharmaceutical companies such as gamithromycin (Sanofi, Germany), hydroxymutiline (Sandoz, Kundl Austria), O-acetyltylosin (Eco animal health, Southgate, London), Semduramicin (Philbro, Brazil), Tulathromycin marker (Pfizer-zoetis, Spain).

Stock solutions of each analytical standard were prepared individually in appropriate solvents . Then eleven mixed intermediate solutions were prepared at 10 $\mu g.mL^{-1}$ preferentially grouping standards per chemical family (beta-lactams, quinolones, NSAIDs, coccidiostats, …). Finally, two working standard solutions were prepared fresh daily at 1 $\mu g.mL^{-1}$ in acetonitrile, one containing 154 compounds and the second one containing 42 compounds. Additionally, two solutions of selected internal standards were prepared, one containing sulfadiazine $^{13}C_6$, albendazole sulfone D_3, erythromycin D_6, oxyclozanide $^{13}C_6$, flunixin D_3 and the second one containing alone the ronidazole D_3.

Liquid chromatography-high resolution mass spectrometry

Analyses were performed using a Vanquish Flex UHPLC binary pump (Thermo scientific) linked to a Q-Exactive plus mass spectrometer (Thermo scientific). The software used was Trace Finder 4.1. The chromatographic separation was operated in gradient mode using a Phenyl-Hexyl Accucore column (100 x 2.1 mm, 2,6 µm particle size) with methanol and water containing 0.1 % formic acid as mobile phase. Flow rate was 0.3 $ml.min^{-1}$, column oven temperature was set at 30°C and the injection volume was 1 µL. The mass spectrometer was operated in variable data-independent acquisition mode (FS-vDIA), consisting of a combination of full scan followed by fragmentation in HCD cell of wide-isolation ranges of precursor ions. The full-scan MS acquisition was made with resolution set at 70,000 FWHM (Full Width at Half Maximum) in the m/z range of 120-1200. The automatic gain control (AGC) target was set at $1e^6$ ions and the maximum injection time (IT) was set at 240 msec. For the v-DIA events, 6 m/z isolation windows of precursor ions were applied as followed : m/z isolation windows of 145-255; 245-355 ; 345-455; 445-555 @resolution 35,000 FWHM, m/z isolation windows of 550-880 and 875-1205 @resolution 17,500 FWHM; AGC target was set at 5.10^5 ions, IT was set at 110 msec for the first 4 isolation window ranges and at 50 msec for the 2 others. Stepped normalized collision energy (NCE) was carried out at 10, 30 and 60. The instrument was operated using heated electrospray either in positive or in negative mode in independent runs. No switch pos/neg was applied.

Sample preparation

Two grams of ground muscle or two grams of milk were weighed in a Falcon tube. A volume of 130 µL of 0.1 $mol.L^{-1}$ Na_2EDTA was added and sample was vortex-mixed before adding 4 ml of acetonitrile/water (80:20; V/V) and a ceramic rod. The sample was then placed on a mechanical rotary shaker (Heildoph) for 10 minutes at 100 rpm and centrifuged for 5 minutes at 3000 g under refrigerated conditions set at +4°C. A first supernatant was obtained. This supernatant was transferred to a clean 15 ml glass tube. A second extraction was then performed on the remaining pellet by adding 4 ml of acetonitrile, then by shaking and centrifuging in the very same conditions as described before The second supernatant was added to the first one in the same tube and 200 µl of DMSO was added. The acetonitrile part was evaporated under a light flow of nitrogen set at 50°C using a Turbovap (LV System). After evaporation, 800 µl of 0.2 $mol.L^{-1}$ ammonium acetate/acetonitrile (80:20; V/V) was added to the remaining 200 µL volume of non-evaporated DMSO. Reconstituted extract was briefly vortexed, transferred to an Eppendorf tube for centrifugation for 5 minutes at 20 000 g under refrigerated conditions set at 0 °C. The final extract was filtered through a 0.22 µm PVDF filter into a (glass or plastic) vial. A volume of 1 µL of the extract was injected into the LC-HRMS instrument.

Data processing

Data processing was performed using TraceFinder® applying a quantitative workflow which allows identification and quantification of a targeted compound belonging to a compound database. This compound database includes dedicated information (formula, fragment ions, retention time, polarity) for the 196 compounds and 6 internal standards. The criteria for identification applied for screening were as follows : presence of the molecular ion with accuracy mass tolerance of 3 ppm within a retention time window of 30 sec. Additional criteria are applied to confirm identification and avoid false positive identification : presence of at least one fragment ion with an accuracy mass tolerance of 5 ppm and an isotope pattern match was set at 70%.

The quantification was then performed based on the area of the molecular ion of the analyte obtained in full MS scan mode.

Method validation

The validation of the method was performed according to a conventional approach applying the validation scheme described in Table 1. Each day 8 different batches were used: 1 batch for the calibration samples and 7 batches for the validation samples. For milk validation, an additional lower level of fortification has been tested at 5 µg/kg for 154 analytes. Microsoft Excel® and Enoval software® were used to perform calculations. The quantitative performance characteristics in terms of trueness and precision and the qualitative performance characteristics in terms of detection capability and specificity were assessed according to the Regulation (EU) 2021/808.

Table 1. Validation experiments for muscle.

Day	Matrix		Calibration samples (Matrix-fortified)					Validation samples (Matrix-fortified)				Total Nb samples
		Fortification level (µg/kg)	0	10	50	100	250	0	10	50	100	
Day 1	porcine	Nb extractions	1	1	1	1	1	7	7	7	7	33
Day 2	poultry	Nb extractions	1	1	1	1	1	7	7	7	7	33
Day 3	bovine	Nb extractions	1	1	1	1	1	7	7	7	7	33
		Nb samples						21	21	21	21	99

Results and Discussion

The validation of the multi-residue screening method was performed taking into account several technical guidance and regulatory documents, such as the Commission Decision 2002/657/EC, the EU-RL Guidance on screening methods , and the EU Regulation 2021/808 .

The specificity of the method was assessed by calculating the false positive rate (FP%) evaluated against the 21 blank samples collected from different batches. For some of the analytes, it appeared necessary to take into account of at least one fragment ion in addition to the parent ion at the expected RT in order to sufficiently reduce the FP rate. Forty out of the 196 substances can generate false positive results applying criteria of identification based on the presence of the molecular ion with accuracy mass tolerance of 3 ppm within a retention time window of 30 sec and a fragment ion with accuracy mass tolerance of 5 ppm. However, it should be noted that for a strictly screening method, the full identification of the compounds in accordance with point 1.2 of EU Regulation 2021/808 is not requested. This requirement is set only for the attention of confirmatory methods.

Detection capability (CCβ) for screening was assessed by investigation of the 21 fortified samples at each fortification level. The concentration level, where only 5% false compliant results remain, equals to the detection capability of the method. The CCβ are displayed in table 2.

Trueness and precision were calculated for each compound in the milk and the muscle matrices. It was considered that the level of interest was validated when the requirements for trueness and precision laid down in EU Regulation 2021/808 (point 1.2.2.1 and 1.2.2.2) were met, i.e. the trueness in the range -20% to +20% when concentration raises ≥ 10 µg/kg and in the range -30% to +20% for concentration at 5 µg/kg ; the reproducibility CV lower than 25 % for concentration between 10-120 µg/kg. In this case the analysis is qualified as a quantitative screening method. In case the variability is too high, so that precision and/or trueness are out of regulatory limits, the method is qualified as semi-quantitative. When no acceptable response function could be obtained from Enoval® software, the method is defined as a qualitative screening method only.

Consequently, the combined examination of quantitative performance characteristics, such as trueness and precision, allowed each compound to be classified into one of the following three categories : qualitative, semi-quantitative of quantitative screening method as described in EU Regulation 2021/808. The results are displayed in table 2.

Table 2. : Classification of the method for each compound according to the performance characteristics.

	SCREENING in MUSCLE					SCREENING in MILK				
Compound	**CCβ Muscle (µg/kg)**	QUAL	SEMI-QUANT	QUANT	lowest validated level (µg/kg)	**CCβ Milk (µg/kg)**	QUAL	SEMI-QUANT	QUANT	lowest validated level (µg/kg)
AMZ-amitraz	> 100	V				50	V			
4-Acetylaminoantipyrin	10			V	10	10			V	10
4-Aminoantipyrin	10			V	10	10			V	10
4-Dimethylaminoantipyrin	10			V	10	10			V	10
4-Formylaminoantipyrin	10			V	10	10			V	10
4-Methylaminoantipyrin	10			V	10	10		V		
Abamectin B1a	100	V				> 100	V			
Acepromazine	10		V			5			V	5
Acid fusidic	10			V	10	10			V	10
Acid mefenamic	10			V	10	5		V		
Acid nalidixic	10		V			5		V		
Acid oxolinic	10		V			5			V	5
Acid tolfenamic	10			V	10	5		V		
Albendazol	10		V			50			V	50
Albendazol amino sulfon	10			V	10	5			V	5
Albendazol Sulfon	10			V	10	5			V	5
Albendazol Sulfoxid	10			V	10	5			V	5
Amino flubendazole	10		V			50		V		
Amino Mebendazole	10		V			5			V	5
Amoxycillin	50		V			100	V			
Ampicillin	10		V		50	5			V	5
Antipyrine -phenazone	10			V	10	10			V	10
Avilamycin	10		V			5		V		50
Azaperol	10		V			5		V		
Azaperone	10		V			5			V	5
Bacitracie A	50	V				50	V			
Baquiloprim	> 100	V				> 100	V			
Carazolol	10		V			5			V	5
Carprofen	10			V	10	5			V	5
Cefacetrile	50	V				50	V			
Cefalexin	10		V			5			V	5
Cefalonium	50		V			10		V		
Cefapirin	50			V	50	5			V	5

Cefazolin	50	V				10			V	50
Cefoperazon	50			V	50	50		V		
Cefquinom	10		V			5		V		10
Ceftiofur	50			V	50	10		V		
Ceftriaxone	> 100	V				> 100	V			
Cefuroxim	100	V				50			V	50
Cephradin		nd				5			V	5
Chlorpromazine	10		V			5		V		10
Chlorprotixene	10		V			5		V		50
Chlortetracyclin	10		V			5			V	5
Cinoxacin	10		V			10	V			
Ciprofloxacin	10			V	10	5			V	5
Clarithromycin	10			V	10	10		V		
Clindamycin	10			V	10	10			V	10
Clorsulon	10			V	10	5			V	5
Closantel	100	V				5		V		
Cloxacillin	50				50	> 100		V		
Colistin	100	V				10			V	50
Danofloxacin	10			V	10	5			V	5
Dapsone	10		V		50	5			V	5
Demeclocyclin	50		V			10		V		
Desacetylcephapirin DAC	10			V	10	5			V	5
Desfuroylceftiofur DCCD	100	V				50		V		
Diclazuril	10			V	10	5			V	5
Diclofenac	10			V	10	5			V	5
Dicloxacillin	50				50	100		V		
Difloxacin	10		V			5			V	5
Dimetridazole	10			V	10	10			V	10
DMA-Dimethylanilin	10		V			50			V	50
DMF-Methyl formamidin	10	V				5			V	5
DMPF-Dimethyl formamidin	10		V			5			V	5
Doramectin	100	V				> 100	V			
Doxycyclin	10		V			5			V	5
Emamectin B1a	10		V			50	V			
Enrofloxacin	10		V			5			V	5
Epi CTC	50		V			5		V		10
Epi OTC	50	V				5		V		10
Epi tetra	10		V			5		V		10
Eprinomectin B-1a	50		V			> 100	V			
Erythromycin	10		V		50	10		V		
Fenbendazole	10		V			5		V		50
Fenbendazole sulfoxide	10	V				5			V	5
Fleroxacin	10			V	10	10			V	10
Florfenicol	10	V				5			V	5
Florfenicol amine	50			V	50	5			V	5
Flubendazole	10			V	10	5		V		
Flumequine	10		V			5		V		50
Flunixin	10			V	10	5			V	5
Fluralaner	10		V			10		V		
Gamithromycin	10		V		50	5		V		
Halofuginone	10		V			5			V	5
Haloperidol	10		V			5			V	5
HMMNI	10			V	10	10			V	10
Hydroxy flubendazole	10			V	10	5			V	5
Hydroxy flunixin	10			V	10	5		V		50
Hydroxy mebendazole	10		V		50	5			V	5
Hydroxy-Ipronidazole	10			V	10	10			V	10
Hydroxy-Metronidazole	50			V	50	10			V	10
hydroxymutilin	10		V			5			V	5
Imidocarb	10			V	10	10			V	10
Ipronidazole	10			V	10	10			V	10
Ivermetin B-1a	50	V				> 100	V			

Josamycin	10		V			50	V			
Ketoprofen	10			V	10	50			V	
Ketotriclabendazole	50		V		50	10		V		
Kitasamycin (Leucomycin hydrat	> 100	V				> 100	V			
Lasalocid A	50	V				50	V			
Levamisole	10			V	10	5			V	5
Lincomycin	10		V			5			V	5
Lomefloxacin	10		V			10		V		
Maduramicin	50	V				> 100	V			
Magnamycin A	50	V				> 100	V			
Marbofloxacin	10		V			5			V	5
Mebendazole	10			V	10	5			V	5
Meloxicam	10			V	10	5		V		
Metronidazole	10			V	10	10			V	10
Monensin	50	V				10	V			
Monepantel	10	V				10		V		
Monepantel-sulfon	10	V				5		V		
Morantel	10		V			5			V	5
Moxidectin	100	V				> 100	V			
Nafcillin	10		V		50	10			V	50
Naproxen	10			V	10	10		V		
Narasin	50	V				100	V			
Neospiramycin	10		V			5			V	5
Nicarbazin	10			V	10	10			V	10
Nigericin	100	V				> 100	V			
Nitroxinil	10			V	10	5			V	5
Norfloxacin	10		V			5			V	5
Novobiocin	10		V		50	10		V		
O-acetyltylosin	50		V			> 100	V			
Ofloxacin	10		V			10			V	10
Oleandomycin	10			V	10	10			V	10
Orbifloxacin	10			V	10	10			V	10
Ormetoprim	10			V	10	10			V	10
Oxacillin	10			V	50	10		V		
Oxfendazole sulfon	10			V	10	5		V		10
Oxibendazole	10			V	10	5			V	5
Oxycloxanide	10			V	10	5			V	5
Oxyphenbutazone	10		V			5		V		50
Oxytetracyclin	10		V			5			V	5
Penicillin-G	10		V		50	50		V		
Penicilllin-V	10		V		50	> 100	V			
Phenylbutazone	10		V			5		V		50
Phthalylsulfathiazole	10			V	10	10		V		
Pirlymycin	10			V	10	5			V	5
Propionylpromazine	10		V			5			V	5
Pyrantel	10			V	10	5			V	5
Quinine	10			V	10	10		V		
Rafoxanide	100	V				5		V		
Rifampicin	10			V	10	10			V	10
Rifamycin	10		V			10			V	10
Rifaximin	10		V			> 100	V			
Robenidine	100	V				100	V			
Ronidazole	10			V	10	10			V	10
Roxithromycin	10			V	10	50		V		
Salinomycin	10	V				> 100	V			
Sarafloxacin	10		V		50	5			V	5
Semduramicin	10		V			5		V		
Spiramycin	10		V		50	5			V	5
Sulbactam	10			V	10	10			V	10
Sulfacetamide	10		V			5			V	5
Sulfachloropyridazine	10		V			5			V	5
Sulfaclozide	10		V		50	5			V	5

Sulfadiazine	10			V	10	5			V	5
Sulfadimethoxine	10			V	10	5			V	5
Sulfadoxin	10			V	10	5			V	5
Sulfaguanidine	10		V		50	5			V	5
Sulfamerazine	10			V	10	5			V	5
Sulfamethazin	10			V	10	5			V	5
Sulfamethizol	10		V		50	5			V	5
Sulfamethoxazole	10			V	10	5			V	5
Sulfamethoxypyridazine	10			V	10	5			V	5
Sulfamonomethoxine	10		V		50	5			V	5
Sulfanilamide	50		V			5		V		10
Sulfaphenazol	100	V				5			V	5
Sulfaquinoxaline	10			V	10	5			V	5
Sulfathiazole	10			V	10	5			V	5
Sulfisoxazol	10			V	10	10			V	10
Telithromycin	10		V			10			V	10
Tetracyclin	10		V			5		V		
Thiabendazole	10			V	10	5			V	5
thiabendazole-OH	10		V			5			V	5
Thiamphenicol	10		V			5			V	5
Tiamulin	10		V			5		V		
Ticarcillin	> 100	V				> 100	V			
Tildipirosin	10		V			5		V		
Tilmicosin	10		V			5			V	5
Toltrazuril	10			V	10	10			V	10
Toltrazuril sulfon	10			V	10	10			V	10
Triclabendazole	10		V			10		V		50
Triclabendazole sulfon	10		V		50	10		V		
Triclabendazole sulfoxyde	10		V		50	5		V		10
Trimethoprim	10		V			5			V	5
Trovafloxacin	10		V			10		V		
Tulathromycin	10		V			5			V	5
Tulathromycin marker	10		V			5			V	5
Tylosine	50	V				50	V			
Tylvalosin	50		V			> 100	V			
Valnemulin	10			V	10	5		V		
Vedaprofen	50	V				50	V			
Virginiamycin M1	50			V	50	50	V			
Xylazine	10		V			5			V	5

Conclusion

Qualitative and quantitative performance characteristics have been determined through a complete validation process and this made it possible to define precisely the use of the screening method according to table 5 of Reg EU 2021/808. The method can be used as a semi-quantitative screening method for 67 compounds in muscle and for 50 compounds in milk, and as a quantitative screening method for 94 compounds in muscle and for 115 compounds in milk. For the few other compounds, qualitative screening results can be obtained but a particular attention should be paid regarding the detection capability CCβ for the less sensitive or problematic compounds, notably the avermectins (abamectin, doramectin, eprinomectin, ivermectin, moxidectin), some polyether ionophores (maduramicin, nigericin, salinomycin), some β-lactams (cloxacillin, ceftriaxone, penicillin V, ticarcillin), some macrolides (kitasamysin, magnamycin, tylvalosin, o-acetyltylosin), and few others like rifaximine, baquiloprim, amitraz.

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POSTER P72

VALIDATION OF ANTICFAST® BTS RAPID TEST KIT: FAST TESTING FOR BETA-LACTAM, TETRACYCLINE AND SULFONAMIDE RESIDUES IN MILK

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Abstract

AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit (Meizheng Bio-Tech, CN) is a qualitative two-step (2+5 minutes) rapid lateral flow assay to detect β-lactam (both penicillins and cephalophorins), tetracycline and sulfonamide residues in raw commingled cows' milk. In 2021, a validation study was performed at ILVO according to Commission Decision 2002/657/EC, the CRL guidelines for the validation of screening methods for residues of veterinary medicines (2010) and ISO TS 23758 | IDF RM 251 (2021). AnticFast® BTS detects all β-lactam, tetracycline (including 4-epimers) and sulfonamide (marker) residues at their MRL at least in 95% of the replicates except for desfuroylceftiofur and cefalexin. The kit is very specific, with no interference by any compound different except for clavulanic acid from 2,000 µg/kg onwards. The kit shows to be robust to variations in milk quality and test protocol. Upon 600 blank milk samples, no false positive results were obtained on the β-lactam and sulfonamide channel and one false positive result (=0.002%) was observed on the tetracycline channel. Another 98 blank milk samples were analysed with 2 additional lots with increased sensitivity for ceftiofur, gave no false positive results (=0%). Validation results show that next to raw cows' milk, the AnticFast® BTS could also be used to screen UHT milk and reconstituted milk powder.

Introduction

AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit (Meizheng Bio-Tech, CN) is a qualitative two-step (2 min + 5min) rapid lateral flow assay to detect β-lactam (penicillins and cephalophorins), tetracycline and sulfonamide residues in raw commingled cows' milk.

A validation study was performed at ILVO-T&V (Technology & Food Science Unit of the Flanders research institute for agriculture, fisheries and food) according to Commission Decision 2002/657/EC and to the CRL guidelines for the validation of screening methods for residues of veterinary medicines (*Anonymous*, 2010). The following analytical parameters were checked: test specificity, detection capability, and test robustness (impact of deviation of the test protocol, impact of the milk composition or milk type) and the stability of reagents. The test was also included in an interlaboratory study organised by ILVO in spring 2021 (Ooghe and Reybroeck, 2021).

Materials and Methods

Test preparation and procedure

All reagents and kit components should be at room temperature (20-25°C) before use. Ensure that the milk is homogenous (no precipitation no clotting). In this validation study, raw milk temperature was standard 1-4°C.

Remove the cover of the tube and take out the appropriate number of test strips and microwell. Immediately cover the tube and restore the remaining components at 2-8°C.

- Step 1: Turn on the incubator and wait until it is stable at 40°C. Place the empty microwells into the incubator.
- Step 2: Transfer 200 µL of raw milk to each microwell placed in the incubator. Dissolve the coating conjugate in the microwell by pipetting the content up and down for 5 to 6 times.
- Step 3: Incubate the sample for 2 minutes at 40°C, then insert the test strip into the microwell.
- Step 4: Let the test strip develop color for 5 minutes at 40°C.
- Step 5: Take out the strip and remove the absorbent pad. Interpret the results within 1 minute visually or by using a BMZ6000 Portable Strip Reader and software.

For the test lines (T1 to T3) following counts: Negative: If the test line is stronger than or equal to the control line, the milk sample contains no antibiotics or contains antibiotics at lower level than the detection limits. Positive: test line is weaker (less intense) than the control line, the milk sample contains antibiotics above or equal to the detection limits.

For instrumental reading: The BMZ6000 Portable Strip Reader is comparing the intensity of each test line with the intensity of the control (reference) line and calculates for each channel a ratio = intensity test line / intensity control line. This ratio for each test line is compared to a fixed cut-off value (ratio = 1.00). In order to perform an instrumental interpretation of a strip, the QR code, included for each channel in the test kit, needs to be scanned by the reader.

Spiking of antibiotic-free (blank) raw milk with β-lactams (penicillins and cephalosporins), tetracyclines and sulfonamides

Blank milk was collected from 4 individual cows in mid-lactation which had not been treated with any veterinary drug for the last 2 months and which had a low to moderate number of somatic cells in the milk. The milk was kept in sterile containers and below 4°C to limit the bacterial count. The maximum period for the cold storage of the fresh raw milk was 56 hours which is shorter than the local milk collection interval (3 days in Belgium).

Determination of the detection capability of the test kit

For all different compounds belonging to the β-lactam and tetracycline family, mentioned as marker residue in Table 1 of the annex of Commission Regulation (EU) No 37/2010, and for 19 different sulfonamides, the detection capability was determined. The spiking was performed as described in the ISO Draft Technical Specification 23758 (ISO|IDF, 2020). Each compound was individually spiked in blank raw milk at fixed concentrations. For each compound a minimum of 2 concentrations around the test sensitivity (test detection capability) were tested. The increment between the concentrations tested for each compound was dependent on the level of spiking and the closeness to the respective MRL (Table 1). Each concentration was tested 20, 40 or 60 times in a time period of at least three days.

Table 1. Increment between the concentrations tested for each compound was dependent on the level of spiking.

Concentration (in µg/kg)	Increment (in µg/kg)
1-10	1
11-20	2
21-50	5
51-100	10
101-250	25
251-500	50
501-1,000	100
1,001-5,000	500

The detection capability is defined as the lowest concentration tested where at least 19 out of 20 tests, 38 out of 40 tests or 57 out of 60 tests were positive, respectively.

Detection capability tests were performed with 4 different lot numbers of reagents: lot 1 20200918G (expiration date 18/09/2021) and lot 2 20200821G (expiration date 21/08/2021), and for the detection capability of ceftiofur, two extra (more sensitive) lots were used: lot 3 20210909G (expiration date 09/09/2022) and lot 4 20211009G (expiration date 09/10/22). All were used following the manufacturer's instructions. The intensity of color formation of each test line was compared to the intensity of the control line and was interpreted by means of a BMZ6000 Portable Strip Reader and software. The cut-off value is 1.00 (≥1.00: negative; <1.00: positive). All results (reader values) were collected in a data base.

Certified reference material from different reagent suppliers was used: Sigma-Aldrich N.V. (Overijse, BE), Toronto Research Chemicals (TRC) (Ontario, CA); Dr Ehrenstorfer (Augsburg, DE), LGC Standards (Molsheim, FR) and Acros Organics (Geel, BE).

Selectivity/specificity and false positive rate

The selectivity of the different test lines of the AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit was tested by analysing milk spiked with β-lactam, tetracycline and sulfonamide compounds and by analysing milk spiked with compounds belonging to different antibiotic or chemotherapeutic families (1 per

family). Raw milk was spiked at a high concentration (100×MRL or 100×MRPL in milk) in raw milk. All testing was completed in duplicate. In case of a positive result also lower concentrations were tested. Following compounds were used: benzylpenicillin (penicillins) and cefalonium (cephalosporins), oxytetracycline (tetracyclines), sulfadiazine (sulfonamides), neomycin B (aminoglycosides), erythromycin A (macrolides), enrofloxacin (quinolones), chloramphenicol (amphenicols), colistin (polymyxins), lincomycin (lincosamides), clavulanic acid (β-lactamase inhibitors), trimethoprim (diamino pyrimidine derivatives) and dapsone (other chemotherapeutics).

To determine the false positive rate, 300 farm and 301 tanker load milk samples were tested with AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit (lot 1 20200918G (expiration date 18/09/2021), lot 2 20200821G (expiration date 21/08/2021)) and other microbiological and receptor screening tests. With the improved reagents of lot 3 20210909G (expiration date 09/09/2022) and lot 4 20211009G (expiration date 09/10/22), 48 additional blank farm and 49 tanker load milk samples were tested, as it was noticed that the ratios obtained for blank samples were lower than with lot 1 and 2.

Reader and test repeatability.

Samples of 10 blank, 10 low positive samples and 10 high positive samples for each channel were measured twice. For the spiked samples, any compound found positive could be used for the testing of the reader repeatability.

For the test repeatability, twin samples of 10 blank, 10 low positive samples and 10 high positive samples for each channel were analysed. For the spiked samples, any compound found positive could be used for the testing of channel.

Robustness study.

All robustness analyses were performed in general in triplicate with blank and spiked milk samples containing benzylpenicillin at 1.5 µg/kg and oxytetracycline at 7 µg/kg or spiked milk containing sulfadoxine at 3 µg/kg.

Influence of changes in the test protocol (length of incubation steps, delay of reading and milk volume) on the test results were determined as followed. While the normal incubation takes 2+5 minutes, following combinations were tested: 1’45 + 4’30; 1’45 + 5’; 1’45 + 5’30; 2’ + 4’30; 2’ +5’30; 2’15 + 4’30; 2’15 +5’ and 2’15 + 5’30. Next to the incubation times, a delay of 5 and 10 minutes was tested and the results compared with no delay in reading (= reference). The kit manufacturers advises to read the result within 1 minute. To test the influence of the milk volume on the test results, a volume of 180, 200 (protocol = reference), and 220 µl of milk was tested.

Also the impact of the milk temperature was tested by analysing milk of 20°C and of 1-4°C (= reference).

To test the impact of the milk quality and composition, results obtained with normal milk samples (as specified by ISO|IDF, 2020) were compared to milk samples with a high total bacterial count (TBC >1.4 $\times 10^6$ CFU per ml), milk samples with a low (<1.40 g per 100 g) fat content and milk samples with a low (<2.99 g per 100 ml) protein content. For each 10 replicates per situation were tested except for SCC blank milk: 20 replicates.

Raw milk, UHT milk and reconstituted milk powder were analysed in order to determine if the AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit is a suitable test for these types of milk cow (10 replicates, except for 20 replicates of blank goats’ and ewes’ milk).

Stability of reagents

The following control samples were analysed daily in duplicate with the AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit to check the stability of the reagents and consistency of results:

- Blank milk (antibiotic-free raw milk)
- Raw milk spiked with 1.5 µg/kg of benzylpenicillin and oxytetracycline at 7 µg/kg
- Raw milk spiked with 3 µg/kg of sulfadoxine

Each day, also a negative and positive control (lot numbers and expiry dates are equal to those of the kit reagents (lot 1 20200918G (expiration date 18/09/2021) and lot 2 20200821G (expiration date 21/08/2021)) as provided in the kit were analysed. These controls were dissolved in 2 ml of HPLC water. The positive control contained 3 µg/kg of benzylpenicillin, 25 µg/kg of oxytetracycline and 25 µg/kg of sulfamethazine.

Interlaboratory testing - National ring trial

ILVO organizes twice a year a national ring trial for the (Belgian) dairy industry regarding the detection of residues of antibiotics in milk by microbiological and rapid tests. In April 2021, AnticFast® Beta-lactams &

Tetracyclines & Sulfonamides Rapid Test Kit was integrated as rapid test. Following compounds were included in the ring trail each at their respective MRL level in raw milk: sulfadoxine, cefquinome, cloxacillin, benzylpenicillin, chlortetracycline, cephalexin, ampicillin and one blank.

Results and Discussion

Detection capability

The AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit is capable to detect the residues of β-lactams (penicillins and cephalosporins), tetracyclines and sulfonamides (19 different compounds) present on the EU-MRL list in milk (Commission Regulation (EU) No 37/2010) as included in Table 2. With the improved reagents, all β-lactams can be detected at least in 95% of the replicates at their respective MRL except for cefalexin and desfuorylceftiofur. The 95% detection capability of these two compounds was not determined as these could not be detected at MRL and it was therefore not requested by the kit manufacturer. It's worth noting that ceftiofur and cefazolin could not be detected at MRL with the first two lots of reagents. Based on this information the kit manufacturer decided to adjust the reagents (lots 3 and 4). With these new improved reagents a CCβ of 60 µg/kg was determined for ceftiofur and of 30 for cefazolin. The impact of the improvement of the reagents on the CCβ for the other β-lactams and other families was not determined.

All tetracyclines, including their 4-epimers can be detected at least in 95% of the replicates at their respective MRL. Doxycycline, not for use in animals from which milk is produced for human consumption, can be detected at least in 95% of the replicates from 2 µg/kg on.

All tested sulfonamides were detected at least in 95% of the replicates at their respective MRL.

Table 2. Detection capability (in µg/kg) of AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit (Meizheng Bio-Tech, CN) in raw bovine milk with instrumental reading (BMZ6000 Portable Strip Reader) with cut-off ratio = 1.00. Detection capability defined as the lowest concentration tested giving minimum 19, 38 or 57 positive results out of 20, 40 or 60 replicates, respectively.

Antibiotic Group/antibiotic	EU MRL (µg/kg)	Detection capability (µg/kg)	Antibiotic Group/antibiotic	EU MRL (µg/kg)	Detection capability (µg/kg)
Penicillins & Cephalosporins			chlortetracycline	100[c]	20
benzylpenicillin	4	2	4-epimer of chlortetracycline	100[c]	50
ampicillin	4	3	doxycycline	--[d]	2
amoxicillin	4	4	**Sulfonamides**		
oxacillin	30	7	sulfamethazine	100[e]	20
cloxacillin	30	7	sulfamerazine	100[e]	5
dicloxacillin	30	5	sulfadiazine	100[e]	3
nafcillin	30	25	sulfamonomethoxine	100[e]	2
ceftiofur	100[a]	60[lot3-4]	sulfadimethoxine	100[e]	3
desfuroylceftiofur	100[a]	-- (**>MRL**)*	sulfadoxine	100[e]	3
cefquinome	20	20	sulfametoxydiazine	100[e]	3
cefazolin	50	30[lot3-4]	sulfisomidine	100[e]	4
cephapirin	60[b]	16	sulfachlorpyridazine	100[e]	5
desacetylcephapirin	60[b]	50	sulfamethoxypyridazine	100[e]	25
cefacetrile	125	60	sulfabenzamide	100[e]	10
cefoperazone	50	3	sulphaquinoxaline	100[e]	25
cefalexin	100	--(**>MRL**)*	sulfamethoxazole	100[e]	60
cefalonium	20	2	sulfamethoxypyrazine	100[e]	4
Tetracyclines			sulfamethoxydazine	100[e]	18
tetracycline	100[c]	6	sulfaclozine	100[e]	6
4-epimer of tetracycline	100[c]	12	sulfisoxazole	100[e]	45
oxytetracycline	100[c]	6	sulfamethizole	100[e]	4
4-epimer of oxytetracycline	100[c]	10	sulfaethoxypyridazine	100[e]	45

Note: lot 1 20200918G (expiration date 18/09/2021), lot 2 20200821G (expiration date 21/08/2021), lot 3 20210909G (expiration date 09/09/2022) and lot 4 20211009G (expiration date 09/10/22). *: detection capability >MRL, exact detection capability not tested. Bold and red font detection capabilities are above the drug MRL. MRL: Maximum Residue

Limit, Regulation (EC) No 470/2009 and Commission Regulation (EU) No 37/2010 and amendments (situation on 01/02/2021). Detection capability defined as the lowest concentration tested giving a minimum of 19 positive results out of 20, 38 positive results out of 40 or 57 positive results out of 60, respectively. [a]The MRL of 100 µg/kg is applied on the sum of all residues retaining the β-lactam structure expressed as desfuroylceftiofur, [b]The MRL of 60 µg/kg in milk is applied on the sum of cephapirin and desacetylcephapirin, [c]The MRL of 100 µg/kg in milk is applied on the sum of parent drug and its 4-epimer, [d]: No MRL in milk, not for use in animals from which milk is produced for human consumption, [e]: The combined total residues of all substances within the sulfonamide group should not exceed 100 µg/kg.

Selectivity/specificity and false positive rate

AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit is a highly specific test for detection of β-lactams, tetracycline and sulfonamides in milk and does not detect compounds from the aminoglycosides, macrolides, quinolones, amphenicols, polymyxins, lincosamides and diamino pyrimidine derivatives, nor dapsone. Clavulanic acid, a β-lactamase inhibitor, gave an interference at the beta-lactam channel. This interference is expected since this molecule contains a β-lactam structure resembling that of the penicillin, except that the fused thiazolidine ring of the penicillins is replaced by an oxazolidine ring (*Anonymous*, 2005). Interference by clavulanic acid with a 95% or higher detection was from 2,000 µg/kg on.

The false positive rate was determined on the 2 sets of lots:

For the original lot 1 and 2: Of the 300 farm milk samples and 301 tanker milk samples, all tested negative for β-lactams and sulfonamides on AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit, except for one farm milk samples which tested positive on the tetracycline channel (ratio: 0.3385). It was however noticed that the ratios of all channels were significantly lower than otherwise. Repetition of the sample gave twice negative results. Also one tanker load milk samples tested positive on the tetracycline channel (ratio: 0.5074). Repetition of the sample gave positive results (ratios: 0.4073 and 0.4164). Testing with other screening tests showed that this sample was a real positive. Giving these results, it is concluded that in total no false positive results were obtained upon 600 samples on the β-lactam and sulfonamide channel. And one false positive result (=0.002%) is obtained on 600 samples on the tetracycline channel. For the improved reagents of lot 3 and 4: Of the 48 farm milk and 49 tanker load milk samples, all tested negative for β-lactams, tetracyclines and sulfonamides on AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit. Giving these results, it is concluded that in total no false positive results (=0%) were obtained upon 97 samples on all test channels.

Reader and test repeatability.

Both the repeatability of the reader and of the test was good. Very low relative standard deviations were obtained (highest value 3.55%) for the reader repeatability. The highest variance value for the test repeatability is 7.40%.

Robustness testing

Most variations in the length of the incubation steps did not impact results significantly; all negative results remained negative and all positive results stayed positive. Deviating the length of the incubation from the standard 2’ + 5’, is giving less variation in ratios for spiked milk and in general slightly more positive results on all channels. When delaying the reading, no significant impact of the interpretation of test results was obtained: all negative results remained negative and all positive results stayed positive. A small increase of ratio (less positive) is observed for spiked milk on the β-lactam and tetracycline channel with increasing delay of reading. Also a volume of milk differing of 20 µl (10%) from the prescribed volume of 200 µl did not impact the interpretation of test results; the negative results remained negative and positive results stayed positive. For the spiked milk, a milk volume of 220 µl gave slightly decreased ratios (become more positive).
The milk temperature (20°C) did not significantly impact the AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit results: blank milk was always tested as negative while the spiked milk samples gave a clear positive result on their respective channel. For milk at 20°C, slightly lower (more positive) ratios were obtained for all channels and a smaller range of variation for spiked milk was noticed on the sulfonamide channel.

Concerning the impact of the milk quality and composition: in general, a high total bacterial count or a low fat or protein content had mostly no significant influence on the performance of the results. One blank milk sample with low protein content was found positive on the sulfonamide channel (ratio: 0.5879). The sample also tested positive on another screening test and LC-MS/MS analysis (semi-quantitative) showed the presence of sulfadoxine at 3.0 µg/l and trimethoprim at 0.44 µg/l (result not included in Table 13 or Figure 4). So no false

positives were obtained with the blank milk. All positive samples were positive except for one milk sample with a low protein content spiked with benzylpenicillin at 1.5 µg/kg and oxytetracycline at 7 µg/kg, with one borderline negative result (ratio 1.0220) on the tetracycline channel. This indicates a small hampering of detection for low protein milk at these low concentrations. However, since the samples were spiked at concentrations far below the MRL (MRL of oxytetracycline = 100 µg/kg), it is expected not to have any detection problems at MRL.

There could also be interest to use the AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit, although developed for the testing of raw cows' milk, to test UHT milk or reconstituted milk powder. For blank milk, all types of milk gave negative results. For spiked milk types always positive results were obtained. It was however noticed that for UHT milk, the control line was much lighter than usual. The AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit can be used to analyse UHT milk and reconstituted milk powder.

Stability of reagents

Very stable ratio values were obtained for daily control samples with the AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit reagents over the test period. Correct values were obtained for the different daily standards: all blank milk standards gave a negative result on all channels. The milk samples spiked with a concentration of 1 µg/kg of benzylpenicillin and 4 µg/kg of tetracycline and the milk samples spiked with a concentration of 4 µg/kg of cefalexin always resulted in positive results, except for one borderline negative result (ratio 1.0322) on the tetracycline channel. For both blank milk as well as spiked milk samples with the improved reagents (lot 3: 20210909G (expiration date 09/09/2022) and lot 4: 20211009G (expiration date 09/10/22)), the ratios are lower, but always correct results were obtained. The negative and positive controls inserted in the kit always gave correct results.

Interlaboratory testing - National ring trial

Good results were obtained with AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit.

Except for the sample spiked with cefalexin at 100 µg/kg, all other milk samples fortified with ß-lactam antibiotics were screened positive with AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit. This is in line with the detection capability reported by Meizheng Group for cefalexin (> MRL). The milk sample spiked with chlortetracycline at 100 µg/kg was screened positive on the tetracycline test line and the milk sample fortified with sulfadoxine at 100 µg/kg was screened positive on the sulfonamide test line. Hence, in this ring test sulfadoxine, cefquinome, cloxacillin, benzylpenicillin, chlortetracycline and ampicillin are detected at MRL with AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit. Negative results were obtained for the blank milk sample on all channels and for the milk samples spiked with antibiotics that are supposed to give a negative result on the respective test lines. So, there were no false positive results with AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit.

Conclusion

Results of this validation show that the AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit is an easy, reliable, robust and highly specific test for screening of raw cows' milk for residues of β-lactam antibiotics (penicillins and cephalosporins), tetracyclines and sulfonamides. With the improved reagents (lot 20210909G (expiration date 09/09/2022) and lot 20211009G (expiration date 09/10/22)), all β-lactams can be detected at least in 95% of the replicates at their respective MRL except for cefalexin and desfuroylceftiofur. The exact detection capability (above MRL) of cefalexin and desfuorylceftiofur was not determined. All tetracyclines, including their 4-epimers can be detected at least in 95% of the replicates at their respective MRL. Doxycycline, not for use in animals from which milk is produced for human consumption, can be detected at least in 95% of the replicates from 2 µg/kg on. All tested sulfonamides are detected at least in 95% of the replicated at their respective MRL. Next to raw cows' milk, AnticFast® Beta-lactams & Tetracyclines & Sulfonamides Rapid Test Kit is suitable to be used to screen UHT milk and reconstituted milk powder.

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POSTER P73

DEVELOPMENT OF AN ANALYTICAL METHOD FOR THE DETECTION OF SELECTIVE ANDROGEN RECEPTOR MODULATORS (SARMS) IN URINE AND HAIR

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Abstract

In this work, impounded products containing ibutamoren were analysed including capsules where approximately 20 times the expected concentration was found. Following this, two liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) methods were developed and validated to determine Selective Androgen Receptor Modulators (SARMs) in bovine, calf, and porcine urine and in bovine hair. First, urine samples were analysed using deconjugation and liquid-liquid extraction. Using this method, screening analysis of SARMs was performed in 1200 urine samples from the Dutch National Residue Plan. Ostarine incurred urine samples from an old animal study were also analysed using this method, and concentrations up to 28 $\mu g \cdot L^{-1}$ were found.
Additionally, with only limited data on pharmacokinetics and metabolism, the second method was developed in the archive matrix hair because it is a matrix in which drug residues can be found long after administration. A methanol extraction of hair was applied, carried out simultaneously with the milling step using a BeadRuptor homogenizer. Sample clean-up is performed using Strata-X 96-wells SPE, resulting in a high throughput method which will go through validation in early 2022.

Introduction

Selective Androgen Receptor Modulators (SARMs) are non-endogenous compounds that have similar anabolic properties to anabolic steroids. All SARMs are to this date investigational drugs, no SARMs have passed clinical trials or are approved as human/veterinary drugs. Because of their androgenic effect, SARMs are popular agents in sports doping (Thevis & Schänzer, 2018). Their mode of action is proposed by activating the androgen receptors in muscles and bones (Gheddar, Raul, & Kintz, 2021).
The World Anti-Doping Agency (WADA) prohibited SARMs in 2008, both in and out of competition, because of their potential abuse in sports (Gheddar et al., 2021). During a police raid back in 2020, an illegal production laboratory actively producing SARMs was discovered, which led to a gained interest in these compounds regarding food safety. Due to the affordability, availability, and steroid-like effects, the use of SARMs in animal husbandry cannot be excluded. However, currently many of these SARMs are not actively monitored and are often not yet included in routine methods. Therefore, we set to develop a liquid chromatography tandem-mass spectrometry (LC-MS/MS) method to detect SARMs in relevant animal matrices. As only limited information is available regarding metabolism and pharmacokinetics of these SARMs, two methods were developed for 1) bovine and porcine urine and 2) bovine hair. The urine method was successfully applied to incurred bovine urine samples.

Materials and Methods

Materials

Ethanol (EtOH), methanol (MeOH), dimethyl sulfoxide (DMSO), acetonitrile (ACN) and tert-butyl methyl ether (TBME) were obtained from Biosolve (Valkenswaard, The Netherlands). Formic acid (FA), 32% ammonia, and β-glucuronidase (E. Coli) were obtained from Merck (Darmstadt, Germany). Milli-Q water (H_2O) was prepared using a Milli-Q system at a resistivity of at least 18.2 $M\Omega \cdot cm^{-1}$ (Millipore, Billerica, MA, USA). The analytical standards and internal standards of the SARMs were obtained from various suppliers. Using these standards, stock solutions of 1000 $mg \cdot L^{-1}$ were prepared in EtOH, MeOH, or DMSO depending on the solubility of the compound. All mixed standard solutions (MSS) and internal standard solutions (ISS) were made using MeOH.

Analytical procedure

LC and MS analysis

LC-MS/MS analyses were performed using a Waters Acquity LC coupled to a Waters Xevo TQ-XS (Waters, Milford, MA, USA) for urine analyses or a Q-trap 6500+ (Sciex, Darmstadt, Germany) for bovine hair analysis. The compounds were separated using a Luna Omega Polar C_{18} analytical column (1.6 µm, 100x2.1 mm) (Phenomenex, Torrance, CA, USA) at 40 °C. The eluents used for separation consisted of (A) 0.1% FA in water and (B) 0.1% FA in acetonitrile with a flow rate of 0.5 $mL \cdot min^{-1}$, and a gradient as shown in Table 5 using an

injection volume of 10 µL. The MS conditions of the Waters Xevo TQ-XS were: 3.0 kV capillary voltage, a source temperature of 130 °C, desolvation gas flow of 550 $L \cdot h^{-1}$, cone gas flow of 150 $L \cdot h^{-1}$, nebuliser gas flow of 7 bar, Argon as CID gas with a flow of 0.18 $mL \cdot min^{-1}$, and the MS conditions of the Q-trap 6500+ were: Curtain gas of 40 $L \cdot h^{-1}$, collision gas at medium, Ion Spray voltage of 4500 V, temperature of 400 °C, ion source gas 1 and 2 of 50 $L \cdot h^{-1}$. The negatively and positively ionised compounds were analysed using the MRM transitions as mentioned in Tables 2 and 3.

Table 5. LC gradient

t (min)	Mobile phase A (% v/v)	Mobile phase B (% v/v)
0	90	10
0.5	90	10
9.0	1	99
10.0	1	99
10.1	90	10
12.0	90	10

Table 6. MRM transitions of the negatively ionised compounds and target levels for the urine and hair analyses

Component	$[M-H]^-$ Mw (CV Waters & Sciex)	Product ion 1 (CE Waters & Sciex)	Product ion 2 (CE Waters & Sciex)	TL urine ($\mu g \cdot L^{-1}$)	TL hair ($\mu g \cdot kg^{-1}$)
Andarine	440.0 (30 & 45)	150.2 (20 & 30)	261.1 (18 & 27)	1	1
Bicalutamide	428.9 (30 & 45)	255.1 (35 & 53)	185.1 (15 & 23)	1	1
Ostarine	388.1 (30 & 45)	118.2 (20 & 30)	269.1 (15 & 23)	1	1
S23	414.9 (30 & 45)	144.9 (20 & 30)	185.0 (30 & 45)	1	5
SK33	499.2 (45 & 68)	228.9 (20 & 30)	268.9 (20 & 30)	1	5
Bicalutamide-d4 (IS)	433.1 (30 & 45)	255.0 (15 & 23)	-	-	-

Table 7. MRM transitions of the positively ionised compounds and target levels for the urine and hair analyses

Component	$[M+H]^+$ Mw (CV Waters & Sciex)	Product ion 1 (CE Waters & Sciex)	Product ion 2 (CE Waters & Sciex)	TL urine ($\mu g \cdot L^{-1}$)	TL hair ($\mu g \cdot kg^{-1}$)
AC-262536	279.0 (50)	168.9 (25 & 38)	195.0 (25 & 38)	1	10
ACP-105	291.1 (30)	167.0 (30 & 45)	193.0 (30 & 45)	1	5
BMS-564929	305.95 (50)	86 (20 & 30)	192.9 (20 & 30)	20	40
Cardarine	454.0 (30)	257.0 (45 & 68)	188.0 (30 & 45)	1	1
Cl-4AS-1	441 (30)	154 (40 & 60)	373 (30 & 45)	1	10
GLPG0492	390.1 (50)	118.0 (35 & 53)	132 (25 & 38)	20	40
Ibutamoren	529.2 (30)	91.0 (50 & 75)	267.0 (20 & 30)	1	5
LGD-2226	393.1 (60)	241.0 (50 & 75)	223.0 (35 & 53)	1	5
Ligandrol	339.1 (30)	220.0 (30 & 45)	199.0 (30 & 45)	1	10
Ly2452473	375.1 (50)	272.0 (40 & 60)	180.0 (20 & 30)	1	2
MK-0773	480 (30)	149 (30 & 45)	480 (30 & 45)	1	10
PF-06260414	303.17 (60)	168 (30 & 45)	210.0 (30 & 45)	1	10
Stenabolic	438.0 (30)	125.0 (30 & 45)	142.1 (20 & 30)	1	5
Testolone	394.0 (30)	223.0 (30 & 45)	170.0 (10 & 15)	1	10
Yk-11	399.0 (30)	307.0 (30 & 45)	325.0 (30 & 45)	20	400
Methyltestosterone-d3	306.1 (35)	97.1 (25 & 38)	-	-	-

Impounded products

Based on the stated concentrations on the packaging, and/or physical appearance of the samples the expected concentrations were estimated. Capsules were emptied of its contents and homogenised. Of the homogenised sample 50 mg powder was dissolved in 50 mL ethanol. For the liquid sample, 100 mg was diluted with 100 mL ethanol and crystalline powders were homogenised and 6.5 mg was dissolved in 10 mL ethanol. All extracts were diluted 100-fold (A), 1000-fold (B), 10,000-fold (C), and 50,000-fold (D) with ACN:H_2O (20:80, *v/v*). Using these diluted extracts, the samples were analysed using Multi Level Standard Addition (MLSA).

Urine analysis

Bovine or porcine urine samples were homogenised, and 1 mL of sample was taken into preparation. After additions of internal standards at 1 µg·L^{-1}, 1.5 mL 0.2 M phosphate buffer (pH 6.8-7.0) was added, and the samples were adjusted to pH 6.5-7.5 using HAc or NaOH. Matrix fortified standards (MFS) were prepared at 0.25-5*TL with each sample series. To all samples 20 µL β-glucuronidase was added, and samples were deconjugated at 50 °C for 1 hour. For extraction purposes, 400 µL 3% NH_4OH was added to all hydrolysed samples to bring the pH above 10. All samples were extracted using TBME. The organic upper layer was transferred into a new glass tube and the extraction was repeated. The combined organic layers were evaporated to dryness at 50 °C under a flow of nitrogen and reconstituted in 100 µL ACN:H_2O (20:80, *v/v*). Samples were analysed using LC-MS/MS as described previously.

Hair analysis

For bovine hair analyses, 100 mg of sample was taken into preparation. All samples were fortified with internal standard solution at 1 and 10 µg·kg^{-1} for bicalutamide-d4 and methyltestosterone-d3 respectively. To the MFS, standards were added in a range of 0.25-2.5 TL. To all samples 1 vial cap of 2.3 mm zirconium/silica beads and 2 mL MeOH was added. The hair samples were milled and extracted by means of a BeadRuptor homogeniser [s=6; c=3; t=2; d=0.3], whilst cooling with liquid nitrogen. The milled and extracted samples were centrifuged for 10 minutes at 3500 rcf, after which the MeOH layer was transferred to an empty 96-wells plate, and evaporated to dryness at 60°C under a gentle flow of nitrogen. The samples were reconstituted in 1 mL ACN:H_2O (20:80, *v/v*), and transferred to an activated and conditioned Strata-X 96-wells SPE plate. During SPE clean-up the Strata-X 96-well plate was activated with 1 mL MeOH. Extracts were applied and washed with 1 mL MeOH:H_2O (60:40, *v/v*), dried for 1 minute using positive pressure and eluted using MeOH:ACN:FA (50:50:2, *v/v*). The elutes were evaporated until dryness at 60°C under a gentle flow of nitrogen and reconstituted in 100 µL ACN:H_2O (20:80, *v/v*). Samples were analysed using LC-MS/MS as described previously.

Results and Discussion

Impounded products

The samples that were confiscated were initially diluted as described earlier and analysed using LC-MS/MS separately in negative and positive ionisation mode. The samples were tested specifically for the substances mentioned on the label of the sample. For ibutamoren, the negative ionisation resulted in poor peak shape, improved peak shape and sensitivity was achieved in positive ionisation mode as shown in figure 1. The substances in the confiscated samples were confirmed to be SARMs, by the use of an analytical reference standard. Additionally, the samples were tested using H-NMR for further structure identification (data not shown).

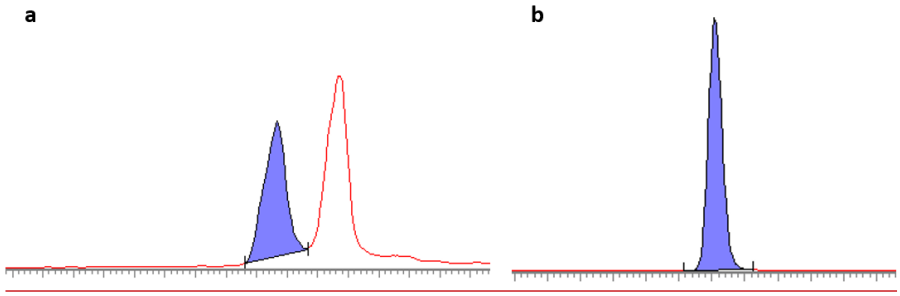


*Figure 15, peak shapes of ibutamoren measured using (**a**) negative ionisation and (**b**) positive ionisation. (peak heights are not set to the same scale in this image)*

For quantification purposes, the samples were analysed using MLSA. Concentrations of 2.27% and 103% were found for the liquid and the crystalline samples and 70.8% for the capsule powder, approximately 20 times higher than mentioned on the label. The above case sparked a gained interest into SARMs regarding food safety. While these products were most likely targeted for human use, their illegal use in animal husbandry cannot be overlooked. Consequently, a LC-MS/MS method that includes a variety of SARMs was required to detect these substances in matrices of animal origin.

Urine is the often the matrix-of-choice for detecting the use forbidden substances. Mainly, because urine often contains high concentrations of forbidden substances (or their metabolites) and because it can be sampled non-invasively. Therefore, an analysis method for SARMs in bovine and porcine urine was developed to detect the potential use of SARMs in animal husbandry.

Optimization

The optimized method included in total 20 SARMs, which are analysed both in positive and negative ionisation mode. The latter was required since ostarine, andarine, bicalutamide, S23, and SK33 are mostly detected as deprotonated $[M-H]^-$ molecules. The mass spectrometer settings, including electrospray voltage, gas flow rates and temperatures were optimized to get maximum response. Dwell times, inter-scan and inter-channel delays were selected to be compatible with polarity switching, while maintaining sufficient data points across a peak.

Different chromatographic settings, including different mobile phase compositions were tested and optimised. Optimal LC conditions were found using mobile phases 0.1% FA in H_2O (A) and 0.1% FA in ACN (B), employing a Luna Omega Polar C_{18} analytical column (1.6 µm, 100x2.1 mm). As the method includes a variety of SARMs, with different physico-chemical properties concessions in terms of sensitivity and/or peak shape were required for BMS-564929, GLPG0492 and YK-11.

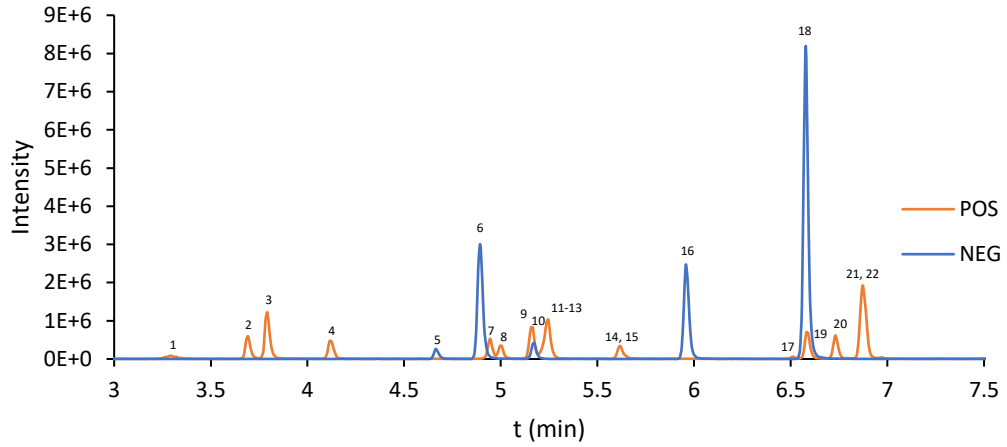


Figure 16. Overlay of the SARMs in a working standard (Hair method). With: BMS-564929 (1), ibutamoren (2), MK-0773 (3), PF-06260414 (4), andarine (5), bicalutamide & bicalutamide-d4 (6), testolone (7), GLPG0492 (8), LY2452473 (9), ostarine (10), AC-262536 (11), methyltestosteron-d3 (12), LGD-2226 (13), ligandrol (14), ACP-105 (15), S23 (16), Cl-4AS-1 (17), SK33 (18), cardarine (19), GW0742 (20), stenabolic (21), YK-11 (22). * GW0742 was added during the method development of the hair method, and has not been incorporated into the urine method as of yet.Sample preparation bovine and porcine urine*

Sample preparation was optimized to be suitable for the wide variety of SARMs included in the method. From literature it was readily suggested that liquid-liquid extraction (LLE) procedures were successful for the extraction of SARMs (Ventura et al., 2019). Therefore, both LLE and supported liquid extraction (SLE) were investigated with a variety of organic solvents. As figure 3 shows, the LLE (using TBME) results in higher signal intensities. This experiment was only carried out on a few of the SARMs because the remaining standards were not available at this time, but the LLE method also worked well for the remaining compounds. The pH at which the extraction is carried out was also optimised, and the optimal pH was determined to be >10.

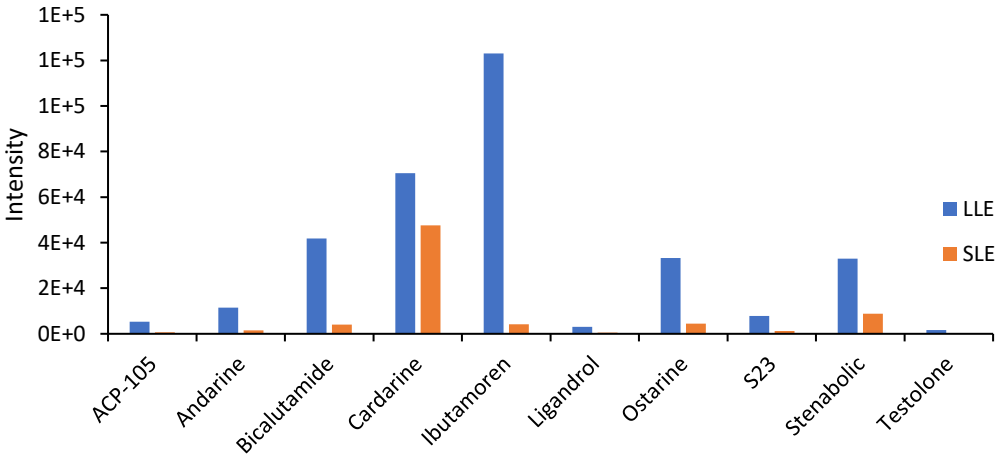


Figure 17. Comparison of the LLE and SLE methods for the determination of SARMs in bovine urine.

With this method, SARM concentrations of 1 µg·L^{-1} could be analysed for most of the 20 SARMs. The only 3 analytes which are harder to analyse are BMS-564929, GLPG0492 and YK-11 which can be analysed at 20 µg·L^{-1}.

Sample preparation bovine hair

With the limited amount of literature available to the metabolism and pharmacokinetics of SARMs, an additional method was developed for the analysis of SARMs residues in bovine hair. Hair is regarded as an archive matrix, displaying use for a longer period of time (Stolker et al., 2009). During optimization studies it was found that solely LLE using MeOH was insufficient in cleaning-up the extracts, resulting in increased matrix-effects during ionisation. Therefore, further clean-up by means of 96-wells solid phase extraction was investigated. Ultimately, Strata-X polymeric reversed phase was deemed to be the best option. The SARMs remained on the SPE when rinsing with a MeOH percentage of up to 60% and elution was achieved using MeOH:ACN:FA (50:50:2, *v/v*). This method will go through validation in early 2022 according to 2021/808/EC with lowest calibrated levels (LCL) of 0.5 - 5 µg·kg^{-1} for most of the SARMs, and 20 µg·kg^{-1} for BMS-564929 and GLPG0492 and 200 µg·kg^{-1} for YK-11.

Validation

The method for bovine and porcine urine was validated as a quantitative confirmatory method according to 2007/657/EC. The specificity of the method was investigated by monitoring interfering signals in the LC-MS/MS traces. Additionally, trueness, repeatability (RSD_r*), within-laboratory reproducibility (RSD_{rl}), CCα, and CCβ were calculated.

Table 8. Overview of the validation results of the analysis of SARMs in bovine urine. Samples which are quantitative are underlined, and screening compounds are asterisked.

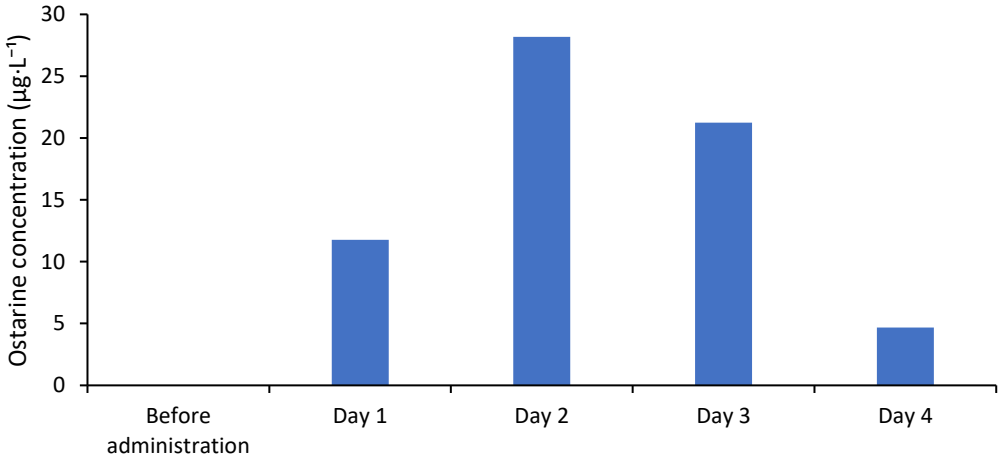
Compound	Level (µg•L^{-1})	Trueness (%)	RSD_r* (%)	RSD_{rl} (%)	CCα (µg•L^{-1})	CCβ (µg•L^{-1})
AC-262536	0.5	96.4	13	14%	0.52	-
	1.0	91.6	11	11%		
	1.5	90.6	13	14%		
ACP-105	0.5	95	12	13%	0.52	-
	1.0	93	13	13%		
	1.5	89	12	12%		
Andarine*	0.5	109	18	21%	-	0.50[a]
	1.0	105	18	19%		
	1.5	102	18	19%		
Bicalutamide	10	100	13	14%	0.48	-
	20	102	17	20%		
	30	99	7	9%		
BMS-564929	10	311	46	117%	≤20	-
	20	346	38	100%		
	30	405	37	103%		
Cardarine	0.5	99	28	28%	≤1.0	-
	1.0	96	34	35%		
	1.5	87	29	31%		
GLPG0492*	10	109	47	39%	-	30[a]
	20	114	73	47%		
	30	116	40	35%		
Ibutamoren	0.5	96	27	38%	≤1.0	-
	1.0	89	27	39%		
	1.5	87	28	37%		
LGD-2226	0.5	69	22	25%	≤1.0	-
	1.0	73	33	35%		
	1.5	85	32	37%		
Ligandrol	0.5	103	23	24%	≤1.0	-
	1.0	97	23	23%		

Compound	Level (µg·L^{-1})	Trueness (%)	RSD$_r$* (%)	RSD$_{rl}$ (%)	CCα (µg·L^{-1})	CCβ (µg·L^{-1})
	1.5	92	27	27%		
Ly2452473	0.5	104	16	22%	≤1.0	-
	1.0	96	15	46%		
	1.5	90	23	29%		
Ostarine	0.5	92	41	50%	≤1.0	-
	1.0	89	39	54%		
	1.5	90	39	49%		
PF-06260414	0.5	106	28	28%	≤1.0	-
	1.0	103	35	36%		
	1.5	100	37	37%		
S23	0.5	81	41	49%	≤1.0	-
	1.0	83	40	48%		
	1.5	85	41	45%		
Stenabolic	0.5	40	72	75%	≤1.0	-
	1.0	73	55	55%		
	1.5	95	68	71%		
Testolone	0.5	94	23	33%	≤1.0	-
	1.0	89	19	28%		
	1.5	94	20	29%		
Yk-11*	10	377	79	127%	-	30 [a]
	20	279	104	167%		
	30	436	54	87%		
Cl-4AS-1*	0.5	97	59	95%	-	1.5 [a]
	1.0	87	58	92%		
	1.5	128	30	48%		
MK-0773	0.5	77	108	173%	≤1.0	-
	1.0	66	66	105%		
	1.5	86	59	95%		
SK33*	0.5	483	88	141%	-	1.5 [a]
	1.0	346	83	133%		
	1.5	592	69	111%		

[a] This is a CCβ which is set on the lowest fortification at which signals were present in 20/21 samples.

Application on incurred samples

Using the analysis method in urine, old urine samples from an animal study where ostarine was administered were analysed. The results of this analysis (figure 4) show that after administration of ostarine, high concentrations are found in the urine samples. For ostarine a maximum of 28 µg·L^{-1} was found. On the fourth day (i.e. the final sample), 5 µg·L^{-1}. The ostarine concentration quickly diminishes, so it is reasonable to assume it will not be detectable after a week or so. It should be noted however, that these results are from urine samples which have been stored in a freezer since 2011 so degradation cannot be excluded. There is limited pharmacokinetic information regarding SARMs. Some literature describes the metabolism of some SARMs in humans or equines. But overall, the information is quite limited. In equine urine, three SARMs were reported at



maximum concentrations of approximately 4 to 500 µg·L^{-1} depending on the compound (Cutler et al., 2020, 2021, 2022).

Figure 18. Ostarine concentration before and after administration.

Survey study

The analysis method was also applied to screen about 1200 urine samples (approximately evenly distributed between bovine, porcine and calf). In these analyses, no SARMs were confirmed to be present.

Conclusion

After optimisation, a method for the analysis of SARMs in bovine, porcine, and calf urine has been developed validated, and validated. The method has also been applied on incurred samples, in which ostarine has been confirmed in concentrations > CCβ. Using this method, about 1200 urine samples were analysed in which no SARMs were confirmed to be present. A method to analyse SARMs in hair has also been developed, and this method will go through validation in early 2022.

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POSTER P74

VETERINARY DRUG ANALYSIS IN ROUTINE FOOD CONTROL USING LC-HRMS WITH AUTOMATED SAMPLE PREPARATION

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Abstract

A wide range of veterinary drugs are used for animal breeding to prevent or treat diseases or to promote growth. The misuse of veterinary drugs and their residues in food are becoming an increasing problem. Therefore, the use of veterinary drugs in the EU is regulated by Commission Regulation (EU) 37/2010, which sets Maximum Residue Limits (MRL) and lists prohibited substances.

Routine monitoring requires robust multi-class methods that incorporate sensitive measurements and rapid, reproducible sample preparation to accurately analyze both as many samples as possible and as many substances as possible in a short time. Therefore, a Thermo Scientific QExactive LC-HRMS system was used to set up a 15-minute method for the simultaneous analysis of ~120 veterinary drug residues in food (amphenicoles, benzimidazoles, quinolones, beta-lactams, macrolides, nitroimidazoles, sulfonamides, tetracyclines and triphenylmethane dyes). With the help of a Gerstel MPS sample preparation robot, spiking of 30 samples per day, as well as their QuEChERS-like extraction with phase separation, evaporation step and filtration, could be fully automated. Only the weighing step still has to be done manually.

The method is validated and successfully applied in the routine analysis of eggs, meat, fish and milk, showing good agreement with specific LC-MS/MS single-class methods.

Introduction

Veterinary drugs are widely used for animal breeding to prevent or treat diseases or to promote growth (Stolker and Brinkman 2008). Abuse of veterinary drugs and their residues in food have become an increasing problem due to the risk to human health. Therefore, for routine control of veterinary drug residues in food sensitive and robust analytical methods are needed.

To cover the whole spectrum of relevant analytes there is a need for screening and quantification method for the simultaneous determination of about 120 veterinary drug residues (amphenicoles, benzimidazoles, quinolones, beta-lactams, macrolides, nitroimidazoles, sulfonamides, tetracyclines and triphenylmethan dyes) in various matrices. In the past, such approaches have been realized on LC-MS/MS systems. (Biselli et al. 2013) However, more specific LC-HRMS systems systems allow to screen for even more compounds, are more reliable in compound identification, and enable also fast quantitative evaluation. Hence, a new method was to be set up using a Thermo Scientific QExactive LC-HRMS system.

In addition, due to the increasing demand for robust methods with a rapid, reproducible sample preparation a fully automated sample preparation was to be developed. Therefore, a Gerstel MPS sample preparation robot was implemented.

Goal was the, except weighing, which still has to be done manually, the entire sample preparation is carried out by the sampler.

The results of the automated sample preparation were to be compared with the manual sample preparation of the multi-class method and with specific LC-MS/MS single-class methods. The method has been validated and can be used for various matrices.

Materials and Methods

Chemicals

All chemicals were at least analytical grade unless stated otherwise. Acetonitrile was of HPLC grade and the ultrapure water was made by Milli-Q® IQ 7000 (Merck, Darmstadt Berlin, Germany). Formic acid and sodium chloride were also in analytical grade. The analytical standards were purchased from Merck (Hamburg, Germany), LGC Standards (Wesel, Germany) or Witega (Berlin, Germany). The stock solutions were prepared in methanol or acetonitrile. From these solutions the measurement standards and the spiked solutions and internal standard solutions were prepared.

Sample preparation
The homogenous sample was weighed twice into glass tubes. One was spiked with the internal standards (IS), the other with a standard solution containing all analytes and the IS. Since experience has shown that each matrix has a different influence on the intensity of the MS signals, the recovery rate of each sample is determined individually. For quantification, an external calibration curve is used. Concentrations are corrected with an internal standard for each analyte group and the recovery rate.
After weighing, the tubes were placed on the Gerstel MPS sample preparation robot (figure 1). The addition of buffer followed by acetonitrile, shaking, phase separation, evaporation and filtration were performed by the sampler. At the end, the vial was taken from the robot ready for measurement. For comparison all steps were performed manually using the existing validated routine procedure.

Figure 1. Gerstel MPS sample preparation robot.

LC-HRMS
A LC-HRMS system (table 1) was used for the simultaneous analysis of the ~120 veterinary drug components (amphenicoles, benzimidazoles, quinolones, beta-lactams, macrolides, nitroimidazoles, sulfonamides, tetracyclines and triphenylmethane dyes) within 15 minutes.

Table 1. LC-HRMS conditions.

HPLC	Thermo Scientific Ultimate 3000
HPLC column	Thermo Scientific Accucore XL C8, 150 x 4.6 mm, 4 µm; 30°C
Mobile phase A	Water (0.1 % formic acid)
Mobile phase B	Acetonitrile (0.1 % formic acid)
HRMS	Thermo Scientific Q Exactive™
Mode	ESI positive / negative

For comparison, measurements were carried out using LC-MS/MS single class methods or multiclass methods that were fully validated according to 2002/657/EG. (Biselli et al. 2013)

Results and Discussion
An automated sample preparation with the help of a Gerstel MPS sample preparation robot for the simultaneous determination of about 120 veterinary drug residues of 9 analytical groups (amphenicoles, benzimidazoles, quinolones, beta-lactams, macrolides, nitroimidazoles, sulfonamides, tetracyclines and

triphenylmethane dyes) was developed, validated and successfully applied in the routine analysis of eggs, meat, fish and milk. For the automation, the method had to be miniaturized. Due to technical reasons such as the robust withdrawing of aliquots from the upper phase of the QuEChERS style extract the sample weight to final volume ratio was slightly smaller than in the manual version. Nevertheless, similar LODs were achieved, when the samples were analysed by LC-HRMS with a Thermo Scientific Q Exactive/Ultimate 3000 system in full-scan/data-dependent fragmentation mode with positive/negative switch.
Table 2 shows all target analytical groups with the calculated limit of detection (LOD) for the automated LC-HRMS screening method.

Table 2. Validation data.

Analytical group	LOD (µg/kg)
Amphenicoles	0.25 – 1.5
Benzimidazoles	1.3 – 3.2
Quinolones	2.5 – 8.6
Beta-Lactames	6.3 – 10
Makrolides	2.0 – 6.5
Nitroimidazoles	0.8 – 2.0
Sulfonamides	1.6 – 6.3
Tertracyclines	3.7 – 10
Triphenylmethan dyes	0.6 – 2.7

Since 2013, about 800 positive samples of several matrices have been measured and compared by LC-HRMS and LC-MS/MS in our laboratory. Comparison show good accordance between both techniques.
Positive routine samples were prepared using the automated method and measured using LC-HRMS. The automated method provides comparable results (table 3) to the routine methods (LC-HRMS multi-class method or LC-MS/MS single-class methods).

Table 3. Comparison of automated LC-HRMS method and manual routine methods.

Analyte	Analytical group	Sample matrix	LOD method (µg/kg)	Automated LC-HRMS (µg/kg)	Routine (µg/kg
Flubendazole	Benzimidazoles	Liquid whole egg	5	16	12
Oxfendazole	Benzimidazoles	Liquid whole egg	5	27	25
Doxycycline	Tetracyclines	Liquid whole egg	10	29	26
Florfenicol	Amphenicoles	Egg powder	1	0.8	1.5
Enrofloxacin	Quinolones	Egg powder	10	50	56
Chloramphenicol	Amphenicoles	Condensed milk	0.3	2.9	2.5
Oxytetracycline	Tetracyclines	shrimp	10	20	22
Tylosin	Makrolides	beef	10	72	73

Conclusion
The sample preparation of the multi method could be automated with the Gerstel MPS sample preparation robot. For the conversion to automated reprocessing, a few obstacles had to be solved (e.g. reduction of volumes, phase separation), but eventually it was successfully implemented and validated. The required limit of detection was achieved.
The comparison of the results of the automated method with the results of the routine multi-class method or with specific LC-MS/MS single-class methods provided comparable results.

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POSTER P75

FORMATION OF SEMICARBAZIDE IN POWDER PORCINE RED BLOOD CELLS

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Abstract

Semicarbazide (SEM) is a metabolic marker of the antibiotic nitrofurazone. The use of nitrofurazone in food-producing animals has been banned in the EU since 1993 because of its carcinogenicity. Therefore, a minimum required performance limit (MRPL) of 1µg/kg has been set in EU legislation for SEM (Commission of the European Communities 2002).

SEM is not only a metabolite of nitrofurazone, but can also be formed in various foodstuffs such as milk or egg powder and carrageenan – as a reaction product during storage and processing.

Aim of the experiments was to study the occurrence of SEM in powder porcine red blood cells dependent on the drying process (lyophylised and spray-dried) and after the addition of different amounts of urea. The amount of SEM in liquid and dried samples was determined after acidic hydrolysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

The experiments show a correlation between the drying process and the increasing levels of SEM. The SEM content in dried samples cannot only result from the concentration effect of drying. Presence of urea and heat leads to an increase in SEM. The addition of urease reduces the formation of SEM during the drying process, but does not prevent it completely.

Introduction

SEM is not only a metabolite of nitrofurazone, but can also occur naturally in products (e.g. carrageenan). In addition, it is detected more and more frequently in products for which there is evidence that no nitrofurazone has previously been applied. Several studies indicate that processing, drying and subsequent warm storage are critical steps for the formation of SEM (Gatermann et al. 2004; Pearson et al. 2016; Stadler et al. 2015)

SEM was found in chicken from Brazil and traced back to the flour coating. The flour had been treated with azodicarbonamide a bleaching agent that reacts with thiols to form biurea. Biurea is then converted into SEM (figure 1). (Pereira et al. 2004, Stadler et al. 2015).

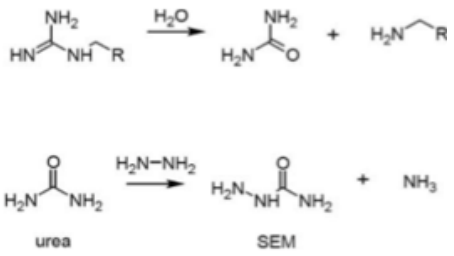

Figure 1. Formation of SEM from guanidinum moieties via urea and hydrazine (Stadler et al. 2015)

Low levels of SEM that were not associated with a nitrofurazone treatment were detected in milk powder (Hoenicke et al. 2004; Pearson et al. 2016), egg powder and whey powder (Gatermann et al. 2004), as well as fish and meat powders (Stadler et al. 2015) or carrageenan derived from dried and bleached algae extracts (Hoenicke et al. 2004). In egg, whey, and milk powder SEM was formed during warm storage (Gatermann et al. 2004; Pearson et al. 2016).

All of these examples show that SEM can be a by-product during the manufacturing process and not merely a marker to identify the abuse of nitrofurazone (Gatermann et al. 2004; Hoenicke et al. 2004; Pearson et al. 2016; Stadler et al. 2015;
Yu et al. 2019).
Powdered porcine red blood cells (RBC) are a high-quality source of protein. Formulated into diets for aquaculture, swine, poultry and adult ruminants, they can be used as a partial or complete replacement for other high-quality protein ingredients. During spray drying of RBC SEM can be formed, similar to milk and egg powder. Furthermore, the matrix is rich in amino acids and free urea can be present in blood plasma, which can have an effect on the formation of SEM during processing. The free urea content in blood depends on three factors: the protein content and quality of the diet, as well as time of sample collection with respect to feeding. (Eggum 1970; Whang and Easter 2000). Within 3-4 h after feeding, the urea content in the blood rises. Moreover, it rises with the quality and the quantity of protein supplied in the diet. An imbalance of amino acids can lead to an increase of urea in blood. The urea content in porcine blood is between
160-450 mg/L, depending on when the sample is taken. The highest amount is reached 5 h after feeding (Eggum 1970; Whang and Easter 2000).
Aim of the work was to study the occurrence of SEM in powder porcine RBC after the addition of different levels of urea and dependent on the drying process (spray-dried or lyophilized). Spray drying is used to dry liquids whose properties make it almost impossible to heat them without significantly changing the properties of the liquids. (Patel et al. 2009) During lyophilisation the product is frozen and then dried in a vacuum. The vacuum sublimates the frozen moisture. (Smith et al. 2013)

Materials and Methods

Chemicals
All chemicals were at least analytical grade unless stated otherwise. Acetonitrile was of HPLC grade and the ultrapure water was made by Milli-Q® IQ 7000 (Merck, Darmstadt Berlin, Germany). Formic acid and sodium chloride were also in analytical grade. The solution for derivatisation was prepared fresh daily from 2-nitrobenzaldehyde (2-NBA, Merck, Darmstadt Berlin, Germany) and dimethyl sulfoxide solution. The analytical standard SEM-$^{13}C_3$ was purchased from Witega (Berlin, Germany). The stock solutions were prepared in methanol. From these solutions the measurement standards and the internal standard solution were prepared. Urea (Panreac 131754) and Urease (Sigma 94281) were provided by APC-Europe.

Sample preparation
The homogenous sample was weighed into plastic tubes. According to their dry mass, 0.5 g of liquid red blood cells (RBC) and 0.2 g of dried RBC were weighed in. The samples were spiked with internal standard (SEM-13C3) and dispensed in hydrochlorid acid. Afterwards, 2-nitrobenzaldehyde was added to the extract followed by derivatisation for 2 h at 50°C. After derivatisation, K2HPO4-buffer was added to the samples and the pH was adjusted to 6.5-7.0. Ethyl acetate, NaCl and MgSO4 were added for liquid-liquid extraction. After shaking and centrifugation the ethyl acetate phase was taken and evaporated to dryness. The dried residue was reconstituted in methanol:water (40:60, V/V) and measured with LC-MS/MS.
The method is used to determine the total SEM content of both bound and unbound forms. Therefore, acid hydrolysis is carried out at the beginning of the sample preparation to release e.g. protein bound nitrofuran residues. The limit of quantification (LOQ) of the method is 0.5 µg/kg

From single batch of liquid RBC collected in one of the centrifuges of the production plant of APC-Europe (Granollers, Spain) several samples and products were produced for the determination of SEM:

1. Liquid samples with added urea (0, 1 and 5 g/kg of liquid RBC) without water addition
2. Spray-dried samples produced at laboratory scale (Buchi Mini Spray Dryer B-290, Buchi Labortechnik, Switzerland) from the liquid samples describes above (1.)
3. Lyophilised samples produced (VirTis BenchTop lyophilizer, VirTis, Inc., NY, USA) from the liquid samples described above (1.)
4. Spray-dried samples produced at laboratory scale (Buchi Mini Spray Dryer B-290, Buchi Labortechnik, Switzerland) from liquid samples described above (1.) which were additionally treated with urease (8 %) to eliminate the added urea in the reaction conditions indicated in the technical sheet of the enzyme (25° and pH 8.00)

These experiments were repeated three times (Experiment 1 – 3).

Additional experiments carried out in the laboratory:

5. Samples of pure urea and urease were sent for analysis. Urea was diluted at 15% water to be analysed.
6. Urea (5g/kg) was added to RBC (Control RBC untreated) prior to analysis and incubated at 25°C and pH 8.00 over 5, 10, and 15 minutes.

Liquid and spray-dried samples were produced by APC-Europe from porcine blood certified for animal consumption from authorized slaughterhouses and were analysed at Eurofins WEJ Contaminants GmbH.

LC-MS/MS
Samples were measured with a HPLC 1200 series (Agilent, Waldbronn, Germany) and an AB Sciex API 4000 Triple Quad mass spectrometer (Concord, Ontario, Canada). 0.2 mM ammonium acetate + 0.01 % formic acid and methanol were used as eluents. Separation was achieved on a Raptor Biphenyl column, 100 x 3 mm, 2.7 µm (Restek, Bad Homburg, Germany). The flow rate was 0.3 mL/min and the column temperature was 30°C. The total runtime was 12 min.

Results and Discussion
Results for the SEM analysis in RBC samples are shown in table 1.

Table 1. Overview of the SEM results.

Description	Experiment 1		Experiment 2		Experiment 3	
	SEM (µg/kg) solids)	Increase (%)	SEM (µg/kg) solids)	Increase (%)	SEM (µg/kg) solids)	Increase (%)
Liquid Samples						
Control RBC	<0.5		<0.5		<0.5	
RBC + 1 g/kg Urea	<0.5		<0.5		<0.5	
RBC + 5 g/kg Urea	<0.5		<0.5		<0.5	
Spray-dried Samples						
Control RBC	7.4		7.8		6.4	
RBC + 1 g/kg Urea	33	346	22	182	23	259
RBC + 5 g/kg Urea	77	941	64	721	53	728
Lyophylised Samples						
Control RBC	1.6		1.6		2.5	
RBC + 1 g/kg Urea	4.0	150	31	1838	11	340
RBC + 5 g/kg Urea	98	6025	124	7650	31	1140
Spray-dried Samples treated with urease (8 %)						
Control RBC	1.1		2.3		2.7	
RBC + 1 g/kg Urea	7.7	600	4.9	113	6.2	130
RBC + 5 g/kg Urea	8.7	691	13	465	9.6	256

In liquid samples no SEM was detected (Limit of Detection <0.2 µg/kg) irrespective of addition of urea. Hence, a contamination of the raw material resulting from nitrofuran treatment of the pigs is unlikely, particularly as the method determines both the free and the bound forms of nitrofurans. In contrast, SEM was detected in the dried RBC from both spray-drying and lyophilisation. Due to a low limit of detection and the higher weight of liquid samples, it can be excluded that SEM became detectable merely based on a concentration factor of approx. 3 between liquid and dried samples.
After spray-drying, pure RBC without added urea contained more SEM (ø 7.2 µg/kg) then lyophilised RBC (ø 1.9 µg/kg). This was in agreement with literature which suggests that heat promotes SEM formation in protein-rich powdered produce such as egg or milk powders (Gatermann et al. 2004; Pearson et al. 2016; Stadler et al. 2015). Addition of urea before drying lead to an increase in the content of SEM with both types of drying. The

increase of SEM depended on the amount of urea that was added. The content of SEM in lyophilised samples increased significantly from 15 µg/kg (1 mg/kg urea) to 84 µg/kg (5 µg/kg urea) on average. When urease was added in addition to urea before drying the RBC, the content of SEM also increased, but to a lower extent. Even in the Control RBC samples, the amount of SEM was lower after urease treatment (ø 2.0 µg/kg) than without (ø 7.2 µg/kg). Overall, the effect of spray-drying with urease treatment was comparable to lyophilisation. In the urea treated samples, the content of SEM was up to 10 times lower after the addition of urease. The three replicates of the experiment show similar results. Only in the third experiment, the increase of SEM in the lyophilised samples after addition of 5 g/kg urea is lower than in the other two. However, as stated for other food products (Bendall 2009; Gatermann et al. 2004; Hoenicke et al. 2004; Pearson et al. 2016; Stadler et al. 2015), urea presumably has a significant influence on SEM formation during RBC drying.

SEM was not detected in pure urea nor urease (table 2). The additives can thus be excluded as a source of SEM in the samples. Furthermore, in the incubation test, when urea was added to liquid RBC samples in the lab prior to sample preparation no SEM was detected. Formation of SEM in presence of urea or free amino acids under acidic conditions during analysis was suggested in previous studies (Abernethy 2015; Hoenicke et al. 2004). However, as it was detected in none of the three samples irrespective of incubation time of 5, 10 and 15 minutes, the sample preparation as source of SEM is also unlikely.

Table 2. Overview of the SEM results of the additional experiments.

Description	SEM (µg/kg)
Urea	<0.5
Urea, 15 % in water	<0.5
Urease	<0.5
Control RBC + 5 g/kg urea (5 min incubation)	<0.5
Control RBC + 5 g/kg urea (10 min incubation)	<0.5
Control RBC + 5 g/kg urea (15 min incubation)	<0.5

Conclusion

The experiments show a connection between the drying process and increasing levels of SEM in RBC. The SEM content in the dried samples cannot arise from the concentration effect of the drying process as only an enhancement of solids by a factor of three has taken place, and not even low levels of SEM were detectable in the liquid samples. Thus, SEM most likely forms during drying of liquid RBC samples. Heat during the drying process promotes the SEM formation especially at low urea levels. The presence of urea leads to an increase of the content of SEM in RBC. The addition of urease reduces the formation of SEM during the drying process, but does not completely prevent its formation.

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POSTER P76

BOVINE TUBERCULOSIS IN SPAIN: EMERGING RESIDUE PERSPECTIVES

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Abstract
The incidence rate of bovine tuberculosis in Spain is among the highest values in the European Union (EU). It remains a disease surrounded by controversy due to the so-called “false positives”. The tuberculin skin tests performed in official veterinary controls are considered as unspecific test, and their results depend very much on the correct execution and interpretation. The recent increase in the prevalence of bovine tuberculosis worries Spanish farmers, giving rise to a tense and distrustful environment between stockbreeders and government. Animals that test positive are compulsory slaughtered, leading to serious economic losses for the farmer. In this context, topical formulations containing corticosteroids have emerged as attractive alternatives to avoid positive results, since they prevent skin reaction or inflammation after intradermal tuberculin test. Conversely, some farmers deliberately inject turpentine (and similar solvents) into the animal’s skin right after the test has been carried out, provoking false positive results, in order to benefit from insurance policies and public subsidies. In both situations, animal products contining residues may end up entering the food chain. Hence, analytical methods to screen for corticosteroids and turpentine residues in bovine skin samples have been developed, using chromatography coupled to mass spectrometry.

Keywords: corticosteroids, tuberculosis, turpentine

POSTER P77

A NEW CONTROL STRATEGY TO DETECT THIOURACIL ABUSE

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Abstract

Thiouracil (TU) is a thyreostat that can be used as an illegal growth promoter. However, naturally low levels of TU can occur in urine. There is an ongoing debate on which concentrations are 'natural' and which are caused by abuse of thyreostatics and how to distinguish between the two. In this study, an animal experiment was carried out on bovine and porcine animals with rapeseed and/or TU (low or high dose) mixed in the feed. By using the urine samples obtained before, during, and after the study TU concentrations levels were determined. Also, a biomarker discovery study was conducted to determine if there are biomarkers that can discriminate between natural and endogenous TU. Conclusions: administration of TU leads to high concentration levels (range from 0.1-5 mg/l) of TU and metabolites in urine, much higher than the current threshold of 10 µg/l. Several metabolites of TU were identified after the administration of TU. These metabolites were not detected in the control- and/or rapeseed treated groups. The most promising marker metabolites were purchased and samples from the animal experiment and routine monitoring programs were reanalyzed using the developed confirmatory method for the proposed biomarkers. Results of this study are presented on a poster during Euroresidue. All of the presented work is published (Blokland *et al*, 2021)

Acknowledgements

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POSTER P78

SCREENING FOR BETA-LACTAM, SULFONAMIDE, TETRACYCLINE & QUINOLONE RESIDUES IN MILK BY MILKSAFE™ 4BTSQ: VALIDATION RESULTS

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Abstract

MilkSafe™ 4BTSQ Beta-lactam + Sulfonamide + Tetracycline + Quinolones Residues Test in Milk (Chr. Hansen S/A, Hørsholm, Denmark) is a qualitative one-step 5-min rapid lateral flow assay to detect the respective antibiotic families in commingled raw cows' milk.

The validation of this test kit was performed at ILVO, according to Commission Decision 2002/657/EC, the CRL guidelines for the validation of screening methods (2010) and ISO TS 23758 | IDF RM 251 (2021).

The MilkSafe™ 4BTSQ detects all β-lactams, sulfonamides, tetracyclines and quinolones with an MRL in MRL (EU Regulation 37/2010) at or below MRL except for nafcillin and the cephalosporins (desfuroyl)ceftiofur, desacetylcephapirin, cefquinome, cefazolin, and cefalexin. The kit is very specific, with no interference by any compound except for clavulanic acid in concentrations above 2,000 µg/kg. Testing of 600 blank milk samples showed no false positive results. The kit shows to be robust to variations in milk quality and test protocol. This validation study shows that next to raw cows' milk, MilkSafe™ 4BTSQ is also suitable to be used as a fast, simple and reliable specific test to screen heat-treated milk (UHT, sterilized milk, reconstituted milk powder) and milk from other animal species (goats', ewes' and buffalo milk).

Introduction

Milksafe™ 4BTSQ 96 Beta-lactam + Sulfonamide + Tetracycline + Quinolones Residues Test in Milk (Chr. Hansen S/A, Hørsholm, Denmark) is a qualitative one-step 5-min rapid lateral flow assay to detect β-lactam, sulfonamides, tetracycline and quinolones antibiotic residues in raw commingled cows' milk.

This test was validated at ILVO-T&V (Technology & Food Science Unit of the Flanders research institute for agriculture, fisheries and food) according to Commission Decision 2002/657/EC, Commission Implementing Regulation 2021/808 and to the CRL guidelines for the validation of screening methods for residues of veterinary medicines (*Anonymous*, 2010). The following analytical parameters were checked: test specificity, detection capability and test robustness (impact of deviation of the test protocol, impact of milk composition and impact of reagents). Further the suitability of the Milksafe™ 4BTSQ Test for Milk to screen different milk types (UHT milk, sterilized milk, reconstituted milk powder and thawed milk) or milk from animal species (goat, ewe and buffalo) other than the cow was also tested. The test was also included in the interlaboratory study organised by ILVO on 22 April 2021.

Materials and Methods

Test preparation and procedure

Ensure that the milk has no precipitation or is clotted.

- 1st step: Turn on the MilkSafe™ Mini Incubator and wait until it is stable at 40°C.
- 2nd step: Take out the strips and micro-wells needed for each sample, add 200 µl milk to a well and mix well by pipetting up and down 6-10 times.
- 3rd step: Insert the test strip into the well and incubate for 5 minutes at 40°C.
- 4th step: Take out the strip from the well and remove the sample pad at the lower end. Read the results visually or by using a CHR. HANSEN MilkSafe™ Desktop Reader.

Interpret the test strip within 5 minutes. For each of the test lines (T1-T4) following counts: Negative: the test line is stronger than the control line, the milk sample contains no antibiotics or contains antibiotics at lower level than the detection limits. Positive: test line is weaker than the control line, the milk sample contains antibiotics above the detection limits. Weak positive: test line has the same colour intensity as the control line, the sample contains antibiotics close to the detection limits. For instrumental reading, the CHR. HANSEN

MilkSafe™ Desktop Reader is used. The reader is comparing the intensity of each test line with the intensity of the control (reference) line and calculates for each channel a ratio = intensity test line / intensity control line. This ratio for each test line is compared to a fixed cut-off value (ratio = 1.1). In this validation study we consider ratio values ≤1.10 as positive without making a discrimination between 'weak positive' and 'strong positive'.

Spiking of antibiotic-free (blank) raw milk with β-lactams (penicillins and cephalosporins), tetracyclines, sulfonamides and quinolones

Blank milk was collected from 4 individual cows in mid-lactation which had not been treated with any veterinary drug for the last 2 months and which had a low to moderate number of somatic cells in the milk. The milk was kept in sterile containers and below 4°C to limit the bacterial count. The maximum period for the cold storage of the fresh raw milk was 56 hours which is shorter than the local milk collection interval (3 days in Belgium).

Determination of the detection capability of the test kit

The detection capability of the Milksafe™ 4BTSQ was determined for all different compounds belonging to the β-lactam, tetracycline, sulfonamide and quinolone family mentioned as marker residue in Table 1 of the annex of Commission Regulation (EU) No 37/2010. The spiking was performed as described in the ISO TS 23758 | IDF RM 251 (ISO/IDF, 2021). Each compound was individually spiked in blank raw milk at fixed concentrations. For each compound a minimum of 2 concentrations around the test sensitivity (test detection capability) were tested. The increment between the concentrations tested for each compound was dependent on the level of spiking and the closeness to the respective MR(P)L (Table 1). Each concentration was tested 20, 40 or 60 times in a time period of at least three days.

Table 1. Increment between the concentrations tested for each compound was dependent on the level of spiking.

Concentration (in µg/kg)	Increment (in µg/kg)
1-10	1
11-20	2
21-50	5
51-100	10
101-250	25
251-500	50
501-1,000	100
1,001-5,000	500

The detection capability is defined as the lowest concentration tested where at least 19 out of 20 tests, 38 out of 40 tests or 57 out of 60 tests were positive, respectively.

Detection capability tests were performed with at least 3 different lot numbers of reagents (lot 2103298002, 2103308003, 2103318004, with as expiration date 28/09/2022, 29/09/2022 and 30/09/2022, respectively) following the manufacturer's instructions. The intensity of colour formation of each test line was compared to the intensity of the control line and was interpreted by means of a CHR. HANSEN MilkSafe™ Desktop Reader. The cut-off value is 1.1.

Certified reference material from following different reagent suppliers was used: Sigma-Aldrich N.V.(Overijse, BE), Toronto Research Chemicals (Ontario, CA); LGC Standards (Molsheim, France); HPC Standards GmbH (Borsdorf, Germany) and Acros Organics B.V.B.A. (Geel, Belgium).

Selectivity/specificity and false positive rate

The selectivity of the different test lines of the Milksafe™ 4BTSQ was tested by analysing milk doped with β-lactam, tetracycline, sulfonamide and quinolone compounds and by analysing milk doped with compounds belonging to different antibiotic or chemotherapeutic families (1 per family) to check the selectivity of the β-lactam, tetracycline, sulfonamide and quinolone test line. Raw milk was doped at a high concentration (100×MRL or 100×MRPL in milk) in raw milk. All testing was completed in duplicate. In case of a positive result also lower concentrations were tested and even some compounds were added to the list of compounds for detection capability testing. Following compounds were used: benzylpenicillin (penicillins) and cefalonium (cephalosporins), oxytetracycline (tetracyclines), sulfadiazine (sulfonamides), neomycin B (aminoglycosides), enrofloxacin (quinolones), colistin (polymyxins), chloramphenicol (amphenicols), erythromycin A (macrolides), lincomycin (lincosamides), clavulanic acid (β-lactamase inhibitors), trimethoprim (diamino pyrimidine

derivatives) and dapsone (other chemotherapeutics). Standard material from Sigma-Aldrich was used, except for chloramphenicol and clavulanic acid from LGC Standards (Molsheim, France).

To determine the false positive rate, 300 blank farm and 300 tanker load milk samples were tested with Milksafe™ 4BTSQ and other microbiological and receptor screening tests.

Reader and test repeatability.

Samples of 10 blank, 10 low positive samples and 10 high positive samples for each channel were measured twice. For the spiked samples, any compound found positive could be used for the testing of the reader repeatability.

For the test repeatability, twin samples of 10 blank,10 low positive samples and 10 high positive samples for each channel were analysed. For the spiked samples, any compound found positive could be used for the testing of channel.

Robustness study.

All robustness analyses were performed in triplicate (except where mentioned different) with blank and spiked milk samples containing benzylpenicillin at 2 µg/kg, oxytetracycline at 14 µg/kg, sulfadimethoxine at 2 µg/kg and enrofloxacin at 10 µg/kg.

Influence of changes in the test protocol (length of incubation steps, temperature of incubation, delay of reading, milk volume and removal of the sample pad) on the test results were determined as followed. While the normal incubation takes 5 minutes, incubating 30 seconds shorter and longer were tested. Also, the temperature of incubation was deviated with 10% from the advised temperature of 40°C. A delay of 5 and 10 minutes was tested and the results compared with no delay in reading (= reference). The kit manufacturer advises to read the result within 5 minutes. To test the influence of the milk volume on the test results, a volume of 180, 200 (protocol = reference) and 220 µl of milk was tested. Also the results when removing the sample pad (=reference) were compared to not removing the sample pad after incubation and before reading.

The impact of the milk temperature was tested by analysing milk of 20°C and of 1-4°C(= reference).

To test the impact of the milk quality and composition on the results, results obtained with normal milk samples (as specified by ISO|IDF, 2020) were compared to milk samples with a high somatic cell count (SCC $>10^6$ per ml), with a high total bacterial count (TBC $>5\times10^5$ CFU per ml), milk samples with a low (<0.89 g per 100 ml) or a high (>6.01 g per 100 ml) fat content, milk samples with a low (<3.00 g per 100 ml) or a high (>4.14 g per 100 ml) protein content and milk samples with a low pH (6.0) or a high pH (7.5). Each time 10 replicates per situation except for high SCC blank milk: 20 replicates.

Next to raw milk, also UHT milk, sterilized milk, reconstituted milk powder and thawed milk were analysed in order to determine if the MilkSafe™ 4BTSQ is a suitable test for these types of milk. Raw goats' milk, raw ewes' milk and raw buffalo milk samples were analysed to determine if the MilkSafe™ 4BTSQ is a suitable test for these types of milk coming from an animal species other than the cow. For each 10 replicates were tested, except for 20 replicates of blank goats' and ewes' milk

Stability of reagents

The following control samples were analysed daily in duplicate with the MilkSafe™ 4BTSQ to check the stability of the reagents and consistency of results:

- Blank milk (antibiotic-free raw milk)
- Raw milk doped with benzylpenicillin at 2 µg/kg, oxytetracyline at 14 µg/kg, sulfadimethoxine at 2 µg/kg and enrofloxacin at 10 µg/kg

Each day, also a negative and positive control (lot numbers and expiry dates are equal to those of the kit reagents) as provided in the kit were analysed.

Interlaboratory testing - National ring trial

Twice a year, ILVO-T&V organizes a national ring trial for the Belgian dairy industry regarding the detection of antibiotic residues in milk by microbiological and rapid tests. In April 2021, MILKSAFE™ 4BTSQ was integrated as rapid test, with lot 2103318004 (exp. date: 30/09/2022). The drugs and concentrations of blind-coded doped raw milk samples were as follows: sulfadoxine at 100 µg/kg, cefquinome at 20 µg/kg; cloxacillin at 30 µg/kg, benzylpenicillin at 4 µg/kg; chlortetracycline at 100 µg/kg; cefalexin at 100 µg/kg; a blank sample and ampicillin at 4 µg/kg.

Results and Discussion

Detection capability

The Milksafe™ 4BTSQ is capable to detect residues of all β-lactams, sulfonamides, tetracyclines and quinolones with a MRL in milk (EU-Regulation 37/2010 and amendments). All β-lactams, tetracyclines (parent drugs and their 4-epimers), sulfonamides and quinolones can be detected at least in 95% of the replicates at their respective MRL except for nafcillin (CCβ=50 µg/kg, MRL=30 µg/kg), ceftiofur (CCβ=350 µg/kg, MRL=100 µg/kg), desfuroylceftiofur (CCβ=3,000 µg/kg, MRL=100 µg/kg), cefquinome (CCβ=25 µg/kg, MRL=20 µg/kg), cefazolin (CCβ=150 µg/kg, MRL=50 µg/kg), desacetylcephapirine (CCβ=100 µg/kg, MRL=50 µg/kg) and cefalexin (CCβ=3,000 µg/kg, MRL=100 µg/kg).

Doxycycline (no MRL in milk) was detected at least in 95% of the replicates at 14 µg/kg. Also difloxacin and oxolinic acid (both no MRL in milk) were detection at least in 95% of the replicates at 7 µg/kg and 25 µg/kg respectively. These drugs are not for use in animals from which milk is produced for human consumption. A summary of the ILVO Milksafe™ 4BTSQ detection capabilities is given in Table 2.

Table 2 Detection capability (in µg/kg) of the Milksafe™ 4BTSQ Test in Milk (CHR. Hansen S/A, Hørsholm, Denmark) in raw bovine milk with instrumental reading (CHR. Hansen MilkSafe Desktop reader and software) with cut-off ratio = 1.1. Detection capability defined as the lowest concentration tested giving minimum 19, 38 or 57 positive results out of 20, 40 or 60 replicates, respectively.

Antibiotic Group/ antibiotic	EU MRL (µg/kg)	Detection capability (µg/kg)	Antibiotic Group/ antibiotic	EU MRL (µg/kg)	Detection capability (µg/kg)
Penicillins			**Sulfonamides**		
benzylpenicillin	4	2	sulfadiazine	100[d]	2
ampicillin	4	3	sulfapyridine	100[d]	12
amoxicillin	4	3	sulfathiazole	100[d]	2
oxacillin	30	12	sulfamethazine	100[d]	12
cloxacillin	30	12	sulfadimethoxine	100[d]	2
dicloxacillin	30	10	sulfadoxine	100[d]	70
nafcillin	30	**50**	**Tetracyclines**		
phenoxymethylpenicillin	(25[a])	3	tetracycline	100[e]	20
Cephalosporins			4-epimer of tetracycline	100[e]	30
ceftiofur	100[b]	**350**	oxytetracycline	100[e]	12
desfuroylceftiofur	100[b]	**3,000**	4-epimer of oxytetracycline	100[e]	30
cefquinome	20	**25**	chlortetracycline	100[e]	25
cefazolin	50	**150**	4-epimer of chlortetracycline	100[e]	30
cephapirin	60[c]	35	doxycycline	--[f]	14
desacetylcephapirin	60[c]	**100**	**(Fluoro)Quinolones**		
cefacetrile	125	100	enrofloxacin	100[g]	9
cefoperazone	50	3	danofloxacin	30	18
cefalexin	100	**3,000**	difloxacin	--[f]	7
cefalonium	20	2	marbofloxacin	75	8
			flumequine	50	16
			oxolinic acid	--[f]	25

Note: Bold and red font detection capabilities are above the drug MRL. MRL: Maximum Residue Limit, Regulation (EC) No 470/2009 and Commission Regulation (EU) No 37/2010 and amendments (actual situation). Detection capability defined as the lowest concentration tested giving a minimum of 19 positive results out of 20, 38 positive results out of 40 or 57 positive results out of 60, respectively. [a]: No MRL in milk, MRL based on Commission Implementing Regulation (EU) 2018/470; [b]: The MRL of 100 µg/kg is applied on the sum of all residues retaining the β-lactam structure expressed as desfuroylceftiofur; [c]: The MRL of 60 µg/kg in milk is applied on the sum of cephapirin and desacetylcephapirin; [d]: The combined total residues of all substances within the sulfonamide group should not exceed 100 µg/kg; [e]: The MRL of 100

µg/kg in milk is applied on the sum of parent drug and its 4-epimer; [f]: Not for use in animals from which milk is produced for human consumption; [g]: The MRL of 100 µg/kg in milk is applied on the sum of enrofloxacin and ciprofloxacin.

Selectivity/specificity and false positive rate

Milksafe™ 4BTSQ is a highly specific test for detection of β-lactams, tetracyclines, sulfonamides and quinolones in milk and does not detect compounds from the aminoglycosides, polymyxins, amphenicols, macrolides, lincosamides and diamino pyrimidine derivatives, nor dapsone. Clavulanic acid, a β-lactamase inhibitor, gave an interference at the β-lactam channel. This interference is expected since this molecule contains a β-lactam structure resembling that of the penicillin, except that the fused thiazolidine ring of the penicillins is replaced by an oxazolidine ring (*Anonymous*, 2005). Interference by clavulanic acid is possible from ≥2,000 µg/kg on (95% detection capability was not determined). .

All 300 farm and 300 tanker load milk samples tested negative for β-lactams, tetracyclines, sulfonamides and quinolones on Milksafe™ 4BTSQ. So in total no false positive results are obtained upon 600 samples on all channels (= 0 %).

Reader and test repeatability.

The repeatability of the reader was good; very low standard deviations of repeatability were obtained. The highest variance value of 7.75 % is very acceptable. Also the repeatability of the test was very good, low standard deviation values were obtained. The highest variance value of 14.88 % is still acceptable since this variance is noted for low ratio values far away from the cut-off value.

Robustness testing

Variations in the length of the incubation step, a deviation of 10% of the recommended incubation temperature or a delay in reading the devices did not impact results; all negative results remained negative and all positive results stayed positive. Also a volume of milk differing some 20 µl (10%) from the prescribed volume of 200 µl did not impact the interpretation of test results; the negative results remained negative and positive results stayed positive. But a decrease in volume of doped milk generates a small increase in ratio values (less positive) on all channels. An increase in volume of doped milk generates a very small decrease in ratio values (more positive) on the tetracycline, β-lactam and sulfonamide channel.

Not removing the sample pad before reading had no significant impact on the results for blank milk samples (all negative results obtained). Also positive results were obtained for all doped milk samples, however, for doped milk, 0.00 ratio values were obtained on the tetracycline channel when not removing the sample pad before reading.

Also the milk temperature (20°C) did not significantly impact the MilkSafe™ 4BTSQ results: blank milk was always tested as negative while the doped milk samples gave a clear positive result on their respective channel.

Concerning the impact of the milk quality and composition; in general, there was little influence on the performance of the MilkSafe™ 4BTSQ. No false positives were obtained with the blank milk, except for milk samples with a very low pH, where 2 out of 10 results were false positive on the sulfonamides channel with ratio values of 0.46 and 1.03. Before adjusting the pH, all samples were analysed negative. Also in milk with a very high protein content one false positive result was obtained out of 10 results (ratio 0.91). LC-MS/MS analysis of this sample showed no presence of any antibiotic residue. For doped samples, always positive results were obtained. No false negative results were obtained.

There could be interest to use the MilkSafe™ 4BTSQ, although developed for the testing of raw cows' milk, to test UHT milk, sterilized milk, reconstituted milk powder or thawed milk (monitoring samples are often kept frozen during transport and storage). People could also have interest to test milk from an animal species different from the cow (goat, ewe, buffalo).

For all milk types, goats', ewes' and buffalo milk, all blank samples tested clearly negative and all doped samples tested positive. This test is therefore also very suitable to use in UHT milk, sterilized milk, reconstituted milk powder, or thawed milk and for types of milk coming from animal species other than the cow (goats', ewes' and buffalo).

For ewes' milk, one blank sample was found positive on the sulfonamide channel (ratio 0.73); by LC-MS/MS analysis it was shown to be a real positive containing 0.54 µg/kg sulfadiazine and 0.16 µg/kg trimethoprim. This result shows that the test is capable of detecting a very low level of sulfadiazine in an incurred sample.

Stability of reagents

In general stable ratio values were obtained for daily control samples with the MilkSafe™ 4BTSQ reagents over the test period on all four tests lines. Always correct values were obtained for the different daily standards. All blank milk standards gave a negative result on all channels. All doped milk samples gave positive results. The negative and positive controls inserted in the kit always gave correct results.

Interlaboratory testing - National ring trial

In the proficiency testing of April 22, 2021 (Ooghe & Reybroeck, 2021) no false positive results were obtained with Milksafe™ 4BTSQ. Except for the sample spiked with 20 µg/kg of cefquinome and the sample spiked with 100 µg/kg of cefalexin, the other milk samples fortified with β-lactam antibiotics were screened positive on the β-lactam test line of Milksafe™ 4BTSQ. This is in line with the detection capabilities obtained with Milksafe™ 4BTSQ at ILVO for cefquinome (25 µg/kg) and cefalexin (3,000 µg/kg). The milk sample spiked with 100 µg/kg of chlortetracycline screened positive on the tetracycline test line of Milksafe™ 4BTSQ and the milk sample doped with 100 µg/kg of sulfadoxine screened positive on the sulfonamide test line of Milksafe™ 4BTSQ. Hence, in this ring test sulfadoxine, cloxacillin, benzylpenicillin, chlortetracycline and ampicillin are detected at MRL with Milksafe™ 4BTSQ. So good results in line with the detection capabilities study were obtained in this proficiency testing.

Conclusion

Results of this validation show that the Milksafe™ 4BTSQ Test in Milk is a very fast, simple and reliable highly specific test for screening of raw cows' milk for residues of β-lactam antibiotics (penicillins and cephalosporins), tetracyclines, sulfonamides and quinolones in raw commingled cows' milk (EU-Regulation 37/2010 and amendments) with at least a 95% detection. All β-lactams can be detected at least in 95% of the replicates at their respective MRL except for nafcillin (CCβ=50 µg/kg, MRL=30 µg/kg), ceftiofur (CCβ=350 µg/kg, MRL=100 µg/kg), desfuorylceftiofur (CCβ=3,000 µg/kg, MRL=100 µg/kg), cefquinome (CCβ=25 µg/kg, MRL=20 µg/kg), cefazolin (CCβ=150 µg/kg, MRL=50 µg/kg), desacetylcephapirin (CCβ=100 µg/kg, MRL=60 µg/kg) and cefalexin (CCβ=3,000 µg/kg, MRL=100 µg/kg).

The test could also be used to screen UHT or sterilized milk, reconstituted milk powder, thawed milk, goats', ewes' and buffalo milk on the presence of residues of β-lactams, tetracyclines, sulfonamides and quinolones.

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POSTER P79

VALIDATION OF BIOEASY 3IN1 BST TEST KIT FOR FAST DETECTION OF RESIDUES OF BETA-LACTAMS, TETRACYCLINES AND SULFONAMIDES IN MILK

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Abstract

3IN1 BST Beta-lactams + Sulfonamides + Tetracyclines Rapid Test for Milk (Bioeasy Biotechnology Co. Ltd., Shenzhen, CN), a two-step 9-minute lateral flow test for the detection of β-lactam, tetracycline and sulfonamide residues in raw cows' milk, was validated at ILVO according to Commission Decision 2002/657/EC and the CRL Guidelines for the validation of screening methods (2010) and ISO TS 23758|IDF RM 251 (2021). From all three antibiotic target groups, all compounds having a MRL in milk could be detected at MRL, except for desfuroylceftiofur, cefazolin and cefalexin. The test showed to be very specific, with only an interference by clavulanic acid on the β-lactam channel for concentrations of 2,500 µg/kg and above. Testing of 630 residue-free milk samples gave no false positive results. The kit was able to detect an incurred sample with very low levels of sulfadiazine (2.3 µg/kg). Variations in test protocol, milk quality or composition had no significant influence on the performance, except for high protein milk: a false positive result on the sulfonamide channel can occur.

3IN1 BST is also suitable to screen UHT milk, sterilized milk, reconstituted milk powder and thawed milk, or goats' and ewes' milk on the presence of antimicrobial residues.

Introduction

The Bioeasy 3IN1 Beta-lactams, Sulfonamides and Tetracyclines (BST) in Milk (Shenzhen Bioeasy Biotechniology Co., Ltd. , Shenzhen, CN) is a competitive receptor test for the rapid detection and discrimination of β-lactams, sulfonamides and tetracyclines in milk based on colloidal gold immunochromatography technology. It takes about 9 minutes for one test. This test was validated at ILVO-T&V (Technology & Food Science Unit of the Flanders research institute for agriculture, fisheries and food of the Flemish Community) according to Commission Decision 2002/657/EC and to the CRL guidelines for the validation of screening methods for residues of veterinary medicines (*Anonymous*, 2010). In this validation study, the following analytical parameters were checked: test specificity, detection capability, and test robustness (impact of deviation of the test protocol, impact of the milk composition, and stability of reagents). Further the suitability of the 3IN1 BST Test for Milk to screen different milk types (UHT milk, sterilized milk, reconstituted milk powder, thawed milk) or milk from animal species (goat and ewe) other than the cow was also tested. Finally, the test was integrated in the monitoring of dairy samples to check the occurrence of false negative or false positive results, and the test was also included in a national ring trial (Ooghe and Reybroeck, 2019).

Materials and Methods

Test preparation and procedure

Bring the milk temperature to 20-25°C. No other sample pre-treatment is necessary.

- 1st step: Put microwell in incubator (40±2°C), add 200 µl of milk (ideal temperature 20-25°C) and mix well by pipetting the mixture 5 to 10 times up and down
- 2nd step: Incubate 3 minutes
- 3rd step: Insert the teststrip into the microwell after first incubation. Incubate another 6 minutes at 40±2°C.
- 4th step: Take out the dipstick from the microwell and remove the absorbent sponge pad at the lower end. Read the results visually or by using a TSR-20 (Test Strip Reader) and software. Interpret the test strip within 5 minutes.

For each of the test lines (T1-T3) following counts: Negative: the test line is darker than the control line, the milk sample contains no antibiotics or contains antibiotics at lower level than the detection limits. Positive: test

line is lighter than the control line, the milk sample contains antibiotics above the detection limits. Weak positive: test line has the same color intensity as the control line, the sample contains antibiotics close to the detection limits.

For instrumental reading, a TSR-20 (Test Strip Reader) (Shenzhen Bioeasy Biotechnology Co., Ltd) and Bioeasy Data Export software version AS174A180827_1 or AS174B180501_1 was used. The reader is comparing the intensity of each test line with the intensity of the control (reference) line and calculates for each channel a ratio = intensity test line / intensity control line. This ratio for each test line is compared to a fixed cut-off value (ratio = 1.1).

Spiking of antibiotic-free (blank) raw milk with β-lactams (penicillins and cephalosporins), tetracyclines and sulfonamides

Blank milk was collected from 4 individual cows in mid-lactation which had not been treated with any veterinary drug for the last 2 months and which had a low to moderate number of somatic cells in the milk. The milk was kept in sterile containers and below 4°C to limit the bacterial count. The maximum period for the cold storage of the fresh raw milk was 56 hours which is shorter than the local milk collection interval (3 days in Belgium).

Determination of the detection capability of the test kit

For all different compounds belonging to the β-lactam and tetracycline family mentioned as marker residue in Table 1 of the annex of Commission Regulation (EU) No 37/2010, and for 4 different sulfonamides the detection capability was determined. The spiking was performed as described in the ISO TS 23758 | IDF RM 251 (ISO|IDF, 2021): Each compound was individually spiked in blank raw milk at fixed concentrations. For each compound a minimum of 2 concentrations around the test sensitivity (test detection capability) were tested. The increment between the concentrations tested for each compound was dependent on the level of spiking and the closeness to the respective MRL (Table 1).

Each concentration was tested 20, 40 or 60 times in a time period of at least three days.

Table 1. Increment between the concentrations tested for each compound was dependent on the level of spiking.

Concentration (in µg/kg)	**Increment (in µg/kg)**
1-10	1
11-20	2
21-50	5
51-100	10
101-250	25
251-500	50
501-1,000	100
1,001-5,000	500

The detection capability is defined as the lowest concentration tested where at least 19 out of 20 tests, 38 out of 40 tests or 57 out of 60 tests were positive, respectively.

Detection capability tests were performed with at least 3 different lot numbers of reagents (lot 19M012600001C, 19M012600002C, 19M012600003C, with all 3 as expiration date: 10/01/2020) following the manufacturer's instructions. All data was interpreted by means of TSR-20. The cut-off value is 1.1 (>1.1: negative; 0.9 ≤R≤1.1: low positive; <0.9: positive).

Additional testing was performed on the detection capability of a few compounds (compounds with a CCβ above or close to MRL) with reagents (lot 19M072600016C (exp. 28/01/2021), lot 19M112600022C (exp. 15/05/2021) and lot 19M112600023C (exp. 16/05/2020)) with altered sensitivity for ceftiofur.

Standard material from Sigma-Aldrich was used except for cefacetrile, desacetylcephapirin and desfuroylceftiofur from Toronto Research Chemicals (Ontario, CA) and 4-epimer of chlortetracycline, 4-epimer of oxytetracycline and 4-epimer of tetracycline from Acros Organics (Geel, BE).

Selectivity/specificity and false positive rate

The selectivity of the different test lines of the 3IN1 BST was tested by analysing milk doped with compounds belonging to different antibiotic or chemotherapeutic families (1 per family) to check the selectivity of the β-lactam, sulfonamides and tetracycline test line. Raw milk was doped at a high concentration (100×MRL or 100×MRPL in milk) in raw milk. All testing was completed in duplicate. In case of a positive result also lower

concentrations were tested and even some compounds were added to the list of compounds for detection capability testing. Following compounds were used: benzylpenicillin (penicillins), cefalonium (cephalosporins), sulfadiazine (sulfonamides), oxytetracycline (tetracyclines), erythromycin A (macrolides), enrofloxacin (quinolones), chloramphenicol (amphenicols), neomycin B (aminoglycosides), colistin (polymyxins), lincomycin (lincosamides), clavulanic acid (β-lactamase inhibitors), trimethoprim (diamino pyrimidine derivatives) and dapsone (other chemotherapeutics). So far as available, standard material from Sigma-Aldrich was used except for erythromycin A, enrofloxacin, and clavulanic acid from Fluka (Bornem, Belgium) and lincomycin from Pfizer (Kalamazoo, MI).

To determine the false positive rate, 315 blank farm and 315 tanker load milk samples were tested with 3IN1 BST and other microbiological and receptor screening tests.

Robustness study.

All robustness analyses were performed in triplicate (except where mentioned different) with blank and spiked milk samples with benzylpenicillin at 2 µg/kg, sulfadiazine at 2 µg/kg, and oxytetracycline at 14 µg/kg.

Influence of changes in the test protocol (length of incubation steps, delay of reading and milk volume) on the test results were determined as followed. While the normal incubation takes 3+6 minutes, Different combinations were tested: 2'30 + 6'; 3'30 + 6'; 3' + 5'30; 3' + 6'30; 2'30 + 5'30; 2'30 + 6'30; 3'30 +5'30 and 3'30 + 6'30. Next to the incubation times, a delay in reading of 1, 5 and 10 minutes was tested and the results were compared with no delay in reading (= reference). The kit manufacturer advises to read the result within 5 minutes. To test the influence of the milk volume on the test results, a volume of 180, 200 (protocol = reference), and 220 µl of milk was tested.

Also the impact of the milk temperature was tested by analysing milk of 20°C (= reference) and of 0-4°C.

To test the impact of the milk quality and composition, results obtained with normal milk samples (as specified by ISO|IDF, 2020) were compared to milk samples with a high somatic cell count (SCC $>10^6$ per ml), with a high total bacterial count (TBC $>5\times10^5$ CFU per ml), milk samples with a low (<2 g per 100 ml) or a high (>6 g per 100 ml) fat content and milk samples with a low (<3 g per 100 ml) or a high (>4 g per 100 ml) protein content. For each 10 replicates per situation were tested, except for SCC and TBC blank milk: 20 replicates.

Next to raw milk, also UHT milk, sterilized milk, reconstituted milk powder, and thawed milk were analysed in order to determine if the 3IN1 BST is a suitable test for these types of milk. Raw goats' milk and raw ewes' milk samples were analysed to determine if the 3in1 BST is a suitable test for these types of milk coming from an animal species other than the cow. For each 10 replicates were tested, except for 20 replicates of blank goats' milk.

Stability of reagents

The following control samples were analysed daily in duplicate with the 3IN1 BST to check the stability of the reagents and consistency of results:

- Blank milk (antibiotic-free raw milk)
- Raw milk doped with 1 or 2 µg/kg of benzylpenicillin
- Raw milk doped with 2 µg/kg of sulfadiazine
- Raw milk doped with 14 µg/kg of oxytetracycline

Also the negative and positive control standards (Bioeasy) were analysed (lotn° 19M012600001C, 19M012600002C and 19M012600003C – all expiration date 10/01/2020).

Interlaboratory testing - National ring trial

ILVO organizes twice a year a national ring trial for the (Belgian) dairy industry regarding the detection of residues of antibiotics in milk by microbiological and rapid tests. In the ring trial of Spring 2019 the 3IN1 BST was integrated as a rapid test with lot number: Lot 19M012600001C (Exp. 10/01/2020). The drugs and concentrations of blind-coded doped raw milk samples were as follows: cloxacillin at 30 µg/kg, benzylpenicillin at 4 µg/kg; cefquinome at 20 µg/kg; tetracycline at 100 µg/kg; gentamycine/sulfadoxine at 50/100 µg/kg; benzylpenicillin at 3 µg/kg; amoxicillin at 4 µg/kg and cloxacillin at 10 µg/kg. Results were determined visually and using a TSR-20 reader and Bioeasy Data Export software.

Results and Discussion

Detection capability

The BIOEASY 3IN1 BST is capable to detect the marker residues of all β-lactams (penicillins and cephalosporins) and tetracyclines present on the EU-MRL list in milk (Commission Regulation (EU) No 37/2010) and the 4 tested sulfonamides. With the adapted kit version, all β-lactams, all tetracyclines (parent drugs and their 4-epimers) and the 4 tested sulfonamides can be detected at least in 95% of the replicates at their respective MRL except for desfuroylceftiofur (CCβ=800 µg/kg, MRL=100 µg/kg), cefazolin (CCβ=60 µg/kg, MRL=50 µg/kg) and cefalexin (CCβ= >1,500 µg/kg, MRL=100 µg/kg).

Doxycycline (no MRL in milk) was detected at least in 95% of the replicates at 20 µg/kg. This drug is not for use in animals from which milk is produced for human consumption.

Selectivity/specificity and false positive rate

No interference by any compound was noted on any test line except for clavulanic acid, a β-lactamase inhibitor. This interference is expected since this molecule contains a β-lactam structure resembling that of the penicillin, except that the fused thiazolidine ring of the penicillins is replaced by an oxazolidine ring (*Anonymous*, 2005). Interference by clavulanic acid with a 95% or higher detection was from 2,500 µg/kg on. 3IN1 BST is a highly specific test for detection of β-lactams, sulfonamides and tetracyclines in milk and does not detect compounds from the macrolides, quinolones, amphenicols (other than chloramphenicol), aminoglycosides, polymyxins, lincosamides and diamino pyrimidine derivatives, nor dapsone.

314 farm milk samples and all 315 tanker load milk samples tested negative for β-lactams, tetracyclines and cefalexin on 3IN1 BST. One real positive farm was detected on the sulfa channel (ratio = 0.108) with the presence of 2.3 µg/kg sulfadiazine confirmed by LC-MS/MS. The positive result on the sulfa test line is in line with the detection capabilities and proofs that also incurred sulfadiazine residues (2.3 µg/kg) are detected at that low level. So no false positive result was obtained upon a total of 630 samples (= 0 %).

Table 2. Detection capability (in µg/kg) of the BIOEASY 3IN1 BST (Shenzhen Bioeasy Biotechnology Co., Ltd, Shenzhen, P.R. China) for β-lactams, sulfonamides and tetracyclines in raw bovine milk with instrumental reading (TSR-20) with cut-off

ratio = 1.1. Detection capability defined as the lowest concentration tested giving minimum 19, 38 or 57 positive results out of 20, 40 or 60 replicates, respectively.

Antibiotic Group/ antibiotic	EU MRL (µg/kg)	Detection capability (µg/kg)	Detection capability (µg/kg) Adapted kit version	Antibiotic Group/ antibiotic	EU MRL (µg/kg)	Detection capability (µg/kg)	Detection capability (µg/kg) Adapted kit version
Penicillins & Cephalosporins				**Tetracyclines**			
benzylpenicillin	4	2		tetracycline	100[c]	35	
ampicillin	4	3	≤3*	4-epimer of tetracycline	100[c]	30	
amoxicillin	4	3	≤3*	oxytetracycline	100[c]	12	
oxacillin	30	20		4-epimer of oxytetracycline	100[c]	25	
cloxacillin	30	5		chlortetracycline	100[c]	20	
dicloxacillin	30	4		4-epimer of chlortetracycline	100[c]	30	
nafcillin	30	20		doxycycline	--[d]	20	
ceftiofur	100[a]	**150**	100*	**Sulfonamides**			
desfuroylceftiofur	100[a]	**1,000**	**800***	sulfadiazine	100[e]	2	
cefquinome	20	9		sulfadimethoxine	100[e]	2	
cefazolin	50	**100**	**60***	sulfadoxine	100[e]	70	
cephapirin	60[b]	18		sulfamethazine	100[e]	16	
desacetylcephapirin	60[b]	60	≤60*				
cefacetrile	125	60					
cefoperazone	50	3					
cefalexin	100	**>1,500**					
cefalonium	20	2					

Note: indicated in red and bold: ccβ > MRL. EU MRL: Maximum Residue Limit, Regulation (EC) No 470/2009 and Commission Regulation (EU) No 37/2010 and amendments (situation on 03/03/2019). Detection capability defined as the lowest concentration tested giving a minimum of 19 positive results out of 20, 38 positive results out of 40 or 57 positive results out of 60, respectively. [a]: The MRL of 100 µg/kg is applied on the sum of all residues retaining the β-lactam structure expressed as desfuroylceftiofur; [b]: The MRL of 60 µg/kg in milk is applied on the sum of cephapirin and desacetylcephapirin; [c]: The MRL of 100 µg/kg in milk is applied on the sum of parent drug and its 4-epimer; [d]: No MRL for doxycycline fixed in milk; [e]: The combined total residues of all substances within the sulfonamide group should not exceed 100 µg/kg; *Additional testing performed in 2020 on new lots (lot 19M072600016C (exp. 28/01/2021), lot 19M112600022C (exp. 15/05/2021) and lot 19M112600023C (exp. 16/05/2020) with altered sensitivity for ceftiofur.

Robustness testing

Variations in the length of the incubation steps did not impact results; all negative results remained negative and all positive results stayed positive with no big shift in ratio values. Longer delay in reading the devices also did not impact the interpretation of test results; all negative results remained negative and all positive results stayed positive. But there were limited changes in the ratio values for negative samples; for positive samples the ratio values on the β-lactam and tetracycline channel increased from 5 minutes of waiting before reading the strips on. Therefore the kit manufacturer advises to read the strips within 5 minutes. A volume of milk differing some 20 µl (10%) from the prescribed volume of 200 µl did not impact the interpretation of test results; the negative results remained negative and positive results stayed positive. With a volume of 180 µl of milk we remark that the ratio values for doped milk slightly increase on the sulfa and tetra channel.

The milk temperature (0-4°C) did not impact the 3IN1 BST results: blank milk was always tested as negative while the doped milk samples gave a clear positive result on their respective channel. There were no significant changes in ratio values noticed.

The milk quality and composition had little if any influence on the performance of the 3IN1 BST. Two false positive values (ratio: 0.664 & 1.094) were obtained for blank milk on the sulfonamide channel for milk with a high protein content. Lowered ratio values were noticed for blank milk with a high number of somatic cells, with a ratio of 1.261 as lowest value. But all doped milk samples tested positive. Summarizing could be

concluded that the somatic cell count, total bacterial count, fat and protein content of the milk do not significantly influence the results of the 3IN1 BST test. False positive results on the sulfonamide channel for milk with a high protein content cannot be excluded.

3IN1 BST, although a test developed for the testing of raw cows' milk on the presence of residues of β-lactam, sulfonamides and tetracycline antibiotics for the dairy industry, can be used by other laboratories to test UHT milk, sterilized milk, reconstituted milk powder and thawed milk (monitoring samples are often kept frozen during transport and storage). For blank milk no false positive results were obtained for any milk type or for any milk of different animal species. But for goats' and ewes' milk mainly on the sulfonamide channel lower ratio values were noticed. For the ewes' milk samples no 100% guarantee could be given that the blank samples were fully blank and not containing a small background quantity of sulfonamide residues since some milk samples from that farm contained sulfonamide drug residues confirmed by LC-MS/MS. The results also indicated that 3IN1 BST is a suitable test to screen milk of species other than the cow (goat, ewe).

Stability of reagents

In general very stable ratio values were obtained for daily control samples with the 3IN1 BST reagents over the test period on all three tests lines. Always correct values were obtained for the different daily blank and doped milk standards: all blank milk standards gave a negative result on all channels; the pooled milk sample doped with 1 or 2 µg/kg of benzylpenicillin, 2 µg/kg of sulfadiazine and 14 µg/kg of oxytetracycline always tested positive on all three test channels. For the positive and negative controls of the kit correct results were obtained.

Interlaboratory testing - National ring trial

In the proficiency testing of April 16, 2019: no false positive results were obtained with 3IN1 BST. All milk samples doped with β-lactam antibiotics, tetracyclines or sulfonamides were screened positive with 3IN1 BST. Hence, in this proficiency testing, cloxacillin, benzylpenicillin, cefquinome, amoxicillin, tetracycline and sulfadoxine are detected at (below) MRL with 3IN1 BST.

So good results in line with the detection capabilities study were obtained in this proficiency testing.

Conclusion

The test was very specific; no interaction was noticed by compounds of antibiotic families or chemotherapeutics other than β-lactams except for the interference by clavulanic acid. With the latest version all β-lactams and tetracyclines with a Maximum Residue Limit (MRL) in milk were detected at their respective MRL except for desfuroylceftiofur (CCβ=800 µg/kg, MRL=100 µg/kg), cefazolin (CCβ=60 µg/kg, MRL=50 µg/kg) and cefalexin (CCβ=>1,500 µg/kg, MRL=100 µg/kg). The CCβ for the four tested sulfonamides was below MRL.

The test result remained reliable even when performing the BIOEASY 3IN1 BST with the timing changes of different steps in the testing protocol including delay of reading. Also, the impact of milk parameters (somatic cells, total bacterial count, fat and protein content, pH) was tested as part of the robustness testing. No interference by these milk parameters on blank milk was noticed except for some false positives in milk with high somatic cell counts. No significant differences were noticed in testing different milk types (raw milk, UHT-milk, sterilized milk, reconstituted milk powder or thawed milk) and other species animal milk (goats' or ewes' milk). Therefore, BIAEASY 3IN1 BST offers the flexibility of testing several different milks. No significant differences in testing capability were found between three batches of reagents BIOEASY 3IN1 BST. Finally, excellent results were obtained in a national proficiency testing. Out of the data of this validation study it can be concluded that the BIOEASY 3IN1 BST is a very fast, simple, and reliable test for the monitoring of milk on residues of β-lactam, sulfonamides and tetracyclines. The test is also giving reliable test results for different milk types and milk of animal species different from the cow (goat, ewe).

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POSTER P80

DETECTION OF BETA-LACTAM AND TETRACYCLINE RESIDUES IN MILK: VALIDATION OF TWINSENSOR PLUS (KIT106)

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Abstract

TwinSensor Plus (KIT106) (UNISENSOR s.a., Seraing, BE) is a qualitative two-step 6-min rapid lateral flow assay to detect β-lactam (penicillins and cephalosporins, including cefalexin) and tetracycline antibiotic residues in raw commingled cows' milk. The test detects all β-lactams with a MRL in milk (EU regulation 37/2010) at MRL, except for nafcillin which is detected below 5×MRL. Therefore KIT106 is fulfilling the current and the future more strict acceptance criteria of the Belgian Federal Agency for the Safety of the Food Chain (FASFC) for tests used by the Belgian dairy companies to check incoming milk on the presence of β-lactam residues (FASFC, 2021). The new criteria shall enter into force on July 1st 2023. Also all tetracyclines are detected at MRL, except for the 4-epimers, but these do not occur in milk. The TwinSensor Plus also shows to be specific and robust for test protocol variations. At the tetracycline channel, using spiked milk with oxytetracycline below 0.5xMRL, it is shown that milk quality and composition can have an influence, but at MRL no problems are expected.

The test is also suitable for residue detection in heat-treated milk (UHT milk, sterilized milk, reconstituted milk powder) and milk from goats' and mares.

Introduction

TwinSensor Plus (KIT106) (UNISENSOR s.a., Seraing, BE) is a qualitative two-step 6-min rapid lateral flow assay to detect β-lactam (penicillins and cephalosporins), including cefalexin and tetracycline antibiotic residues in raw commingled cows' milk.

This new test was validated at ILVO-T&V (Technology & Food Science Unit of the Flanders research institute for agriculture, fisheries and food) according to Commission Decision 2002/657/EC and to the CRL guidelines for the validation of screening methods for residues of veterinary medicines (*Anonymous*, 2010). The following analytical parameters were checked: test specificity, detection capability and test robustness (impact of deviation of the test protocol, impact of the milk composition and milk type and batch differences of reagents). The test was also be included in the interlaboratory study organized by ILVO on April 22th, 2021 (Ooghe and Reybroeck, 2021).

Materials and Methods

Test preparation and procedure

Read the instructions before starting the experiment. Before opening the reagents, take the kit out of the fridge and wait until the temperature of the reagents reaches room temperature. Milk samples must be liquid and homogeneous without any agglomeration and sedimentation. The ideal temperature of the milk sample is between 4°C and 10°C. Switch on the "HeatSensor" and select the program set at the right temperature (40°C) and timing (3 + 3 minutes). Wait until the temperature has stabilized at 40°C. Switch on the "ReadSensor" if instrumental reading is chosen for result interpretation. Make sure that the check / calibration was performed according to the Users' Manual. Take out the required number of microwells and strips, and make proper marks. Store the rest of the unused kit.

- 1st step: Place the microwells in the heating block which shows 40±3°C.
- 2nd step: Immediately transfer 200 µl of the milk sample into the microwell, then mix gently to homogenize by pipetting up and down 5 to 10 times. Start the Heatsensor or use a timer. The initial incubation of 3 minutes begins. Place the dipsticks into the "HeatSensor" holder if using "HeatSensor" DUO(OCTO). After the initial incubation, the dipstick will be dipped automatically into the microwell if using the "HeatSensor" insert manually the test strip into the well with the "Immersed" end fully dipped in to the mixture of reagent and sample.

- 3rd step: Incubate for 3 min at 40±3°C. Take out the strip, and gently remove the sample pad. Determine the result by visual interpretation or with the ReadSensor 2 within 2 minutes.

For each of the test lines (T1-T3) following applies: Negative: the test line is stronger than the control line, the milk sample contains no antibiotics or contains antibiotics at lower level than the detection limits. Positive: test line is weaker than or equal to the control line, the milk sample contains antibiotics above the detection limits. For instrumental reading, the ReadSensor 2 is comparing the intensity of each test line with the intensity of the control (reference) line and the software calculates for each channel a ratio = intensity test line / intensity control line. Calculation is obtained based on the measurement of the area of the color formation. Ratio values ≤1.10 are considered as 'positive' for the antibiotic family/compound detected by the test line concerned.

Spiking of antibiotic-free (blank) raw milk with β-lactams (penicillins and cephalosporins) and tetracyclines

Blank milk was collected from 4 individual cows in mid-lactation which had not been treated with any veterinary drug for the last 2 months and which had a low to moderate number of somatic cells in the milk. The milk was kept in sterile containers and below 4°C to limit the bacterial count. The maximum period for the cold storage of the fresh raw milk was 56 hours which is shorter than the local milk collection interval (3 days in Belgium).

Determination of the detection capability of the test kit

The detection capability of the TwinSensor Plus (KIT106) was determined for all different compounds belonging to the β-lactam and tetracycline family mentioned as marker residue in Table 1 of the annex of Commission Regulation (EU) No 37/2010. The spiking was performed as described in the ISO Draft Technical Specification 23758 (ISO|IDF, 2020). Each compound was individually spiked in blank raw milk at fixed concentrations. For each compound a minimum of 2 concentrations around the test sensitivity (test detection capability) were tested. The increment between the concentrations tested for each compound was dependent on the level of spiking and the closeness to the respective MRL (Table 1). Each concentration was tested 20, 40 or 60 times in a time period of at least three days.

Table 1. Increment between the concentrations tested for each compound was dependent on the level of spiking.

Concentration (in µg/kg)	Increment (in µg/kg)
1-10	1
11-20	2
21-50	5
51-100	10
101-250	25
251-500	50
501-1,000	100
1,001-5,000	500

The detection capability is defined as the lowest concentration tested where at least 19 out of 20 tests, 38 out of 40 tests or 57 out of 60 tests were positive, respectively.

Detection capability tests were performed with at least 3 different lot numbers of reagents (lot 20240H (with exp. date 27 August 2021), 20328M (with exp. date 24 November 2021) and 20350R (with exp. date 16 December 2021) following the manufacturer's instructions. The intensity of color formation of each test line was compared to the intensity of the control line and was interpreted by means of a ReadSensor 2 reader (model APP088) and software u1.12a 200728 1.00b 180501. The cut-off value is 1.10 (>1.10: negative; ≤1.10: positive).

So far as available, standard material from Sigma-Aldrich was used except for ticarcillin, 4-epimer of tetracycline, 4-epimer of oxytetracycline, 4-epimer of chlortetracycline from Acros Organics (Geel, Belgium); ceftiofur, desacetylcephapirin, desfuroylceftiofur and ceftizoxime from Toronto Research Chemicals (Ontario, CA); aspoxicillin, cefquinome, cefacetrile, and doxycycline from LGC Standards (Molsheim, France); cefalotine from British Pharmacopoeia (London, UK) and ceftriaxone from Alfa Aesar (Kandel, Germany).

Selectivity/specificity and false positive rate

The selectivity of the different test lines of the TwinSensor Plus (KIT106) was tested by analysing milk doped with β-lactam and tetracycline compounds and by analysing compounds belonging to different antibiotic or chemotherapeutic families (1 per family) to check the selectivity of the β-lactam, cefalexin and tetracycline test

line. Raw milk was doped at a high concentration (100×MRL or 100×MRPL in milk) in raw milk. All testing was completed in duplicate. In case of a positive result also lower concentrations were tested. Following compounds were used: benzylpenicillin (penicillins), cefalexin and cefalonium (cephalosporins), oxytetracycline (tetracyclines), sulfadiazine (sulfonamides), neomycin B (aminoglycosides), erythromycin A (macrolides), enrofloxacin (quinolones), chloramphenicol (amphenicols), colistin (polymyxins), lincomycin (lincosamides), clavulanic acid (β-lactamase inhibitors), trimethoprim (diamino pyrimidine derivatives) and dapsone (others chemotherapeutics). All standard material from Sigma-Aldrich was used.

To determine the false positive rate, 300 blank farm and 300 tanker load milk samples were tested with TwinSensor Plus (KIT 106) and other microbiological and receptor screening tests.

Test repeatability.

For the test repeatability, twin samples of 10 blank,10 low positive samples and 10 high positive samples for each channel were analysed. For the spiked samples, any compound found positive could be used for the testing of channel.

Robustness study.

All robustness analyses were performed in triplicate (except where mentioned different) with blank and spiked milk samples containing benzylpenicillin at 3 µg/kg and oxytetracycline at 40 µg/kg or spiked milk containing cefalexin at 80 µg/kg.

Influence of changes in the test protocol (length of incubation steps, incubation temperature, delay of reading and milk volume) on the test results were determined as followed. Normal incubation of 3+3 minutes was compared to different combinations: 3’ + 2’45; 2’45 + 3’15; 2’45 + 3’; 2’45 + 2’45; 3’ + 3’15; 3’15 + 2’45; 3’15 +3’ and 3’15 + 3’15. Also the incubation temperature was varied at 37, 40 (=reference) and 43°C. (The kit manufacturer advises to incubate at 40±3°C). Differences in results for a delay of reading of 5 and 10 minutes was compared with no delay in reading (= reference). (The kit manufacturer advises to read the result within 2 minutes.) The influence of the milk volume on the test results, was tested with adding a milk volume of 180, 200 (protocol = reference), and 220 µl. Also the removal (= reference) or no removal of the sample pad after incubation and before reading, was tested.

Further, the impact of the milk temperature was tested by analysing milk of 20°C and of 1-4°C (= reference).

To test the impact of the milk quality and composition, results obtained with normal milk samples (as specified by ISO/IDF, 2020) were compared to milk samples with a high somatic cell count (SCC >10^6 per ml), with a high total bacterial count (TBC >2.9 ×10^6 CFU per ml), milk samples with a low (<0.91 g per 100 g) or a high (>6.01 g per 100 g) fat content, milk samples with a low (<3.04 g per 100 ml) or a high (>4.00 g per 100 ml) protein content, milk samples with a low pH (6.0) or a high pH (7.5) and milk samples from an early lactation (<30 days after calving but no colostrum milk) or late lactation stage (>270 days after calving). For each 10 replicates per situation were tested except for SCC blank milk: 20 replicates.

Next to raw milk, also UHT milk, sterilized milk, reconstituted milk powder, and thawed milk were analysed in order to determine if TwinSensor Plus (KIT106) is a suitable test for these types of milk. Also milk from other origin than cows’ milk (goats’, ewes’, mares, buffalo and camel milk) were analysed to determine if the TwinSensor Plus (KIT106) is a suitable test for these types of milk coming from an animal species other than the cow (10 replicates, except for 20 replicates of blank goats’ and ewes’ milk).

Stability of reagents

The following control samples were analysed daily in duplicate with the 3IN1 BST to check the stability of the reagents and consistency of results:

- Blank milk (antibiotic-free raw milk)
- Raw milk doped with benzylpenicillin at 3 µg/kg, oxytetracycline at 35 µg/kg and cefalexin at 70 µg/kg

Each day, also a negative (lot 20198, exp. date: 17 July 2022; lot 20254, exp. date: 11 September 2022 or lot 20324, exp. date: 20 November 2022) and a positive control (lot 20252, exp. date: 9 September 2022 or lot 20338, exp. date: 3 December 2022) as provided in the kit were analysed. These controls were dissolved in 1ml of HPLC water.

Interlaboratory testing - National ring trial

ILVO organizes twice a year a national ring trial for the (Belgian) dairy industry regarding the detection of residues of antibiotics in milk by microbiological and rapid tests. In the ring trial of April 22, 2021, TwinSensor

Plus (KIT 106) was integrated as rapid test with lot number: Lot 20240H (Exp. 27/08/2021). Following compounds were included in the ring trail each at their respective MRL level: sulfadoxine, cefquinome, cloxacillin, benzylpenicillin, chlortetracycline, cefalexin, ampicillin and one blank sample. Results were determined visually and using a ReadSensor2.

Results and Discussion

Detection capability

The TwinSensor Plus (KIT106) is capable to detect all residues of β-lactams (penicillins and cephalosporins) and tetracyclines present on the EU-MRL list in milk (Commission Regulation (EU) No 37/2010). All β-lactams, including cefalexin and tetracyclines (parent drugs) can be detected at least in 95% of the replicates at their respective MRL except for nafcillin (CCβ=125 µg/kg, MRL=30 µg/kg). The 4-epimers of tetracyclines are also not detected at their respective MRL (4-epimer of tetracycline (CCβ >1,000 µg/kg, MRL=100 µg/kg); 4-epimer of oxytetracycline (CCβ=1,000 µg/kg, MRL=100 µg/kg) and 4-epimer of chlortetracycline (CCβ=350 µg/kg, MRL=100 µg/kg). But in general these 4-epimers are not found in milk (ILVO, internal information). Doxycycline, not for use in animals from which milk is produced for human consumption, can be detected at least in 95% of the replicates from 7 µg/kg on.

The test is fulfilling the current and the future more strict acceptance criteria of the Belgian Federal Agency for the Safety of the Food Chain (FASFC) for tests used by the Belgian dairy companies to check incoming milk on the presence of β-lactam residues (FASFC, 2021). The new criteria shall enter into force on July 1st 2023.

Table 2. Detection capability (in µg/kg) of the BIOEASY 3IN1 BST (Shenzhen Bioeasy Biotechnology Co., Ltd, Shenzhen, P.R. China) for β-lactams, sulfonamides and tetracyclines in raw bovine milk with instrumental reading (TSR-20) with cut-off ratio = 1.1. Detection capability defined as the lowest concentration tested giving minimum 19, 38 or 57 positive results out of 20, 40 or 60 replicates, respectively.

Antibiotic Group/ antibiotic	EU MRL (µg/kg)	Detection capability (µg/kg)			Antibiotic Group/ antibiotic	EU MRL (µg/kg)	Detection capability (µg/kg)		
		T	B	C			T	B	C
Penicillins					desacetylcephapirin	60[b]		25	
benzylpenicillin	4		3		cefacetrile	125		16	
ampicillin	4		4		cefoperazone	50		4	
amoxicillin	4		4		cefalexin	100			70
oxacillin	30		9		cefalonium	20		4	
cloxacillin	30		9		cefuroxime	--[b]		125	
dicloxacillin	30		6		cefalotine	--[b]		6	
nafcillin	30		**125**		ceftriaxone	--[b]		10	
phenoxymethylpenicillin	(25[a])		4		ceftizoxime	--[b]		450	
piperacillin	--[b]		4		cefotaxime	--[b]		40	
ticarcillin	--[b]		20		**Tetracyclines**				
aspoxicillin	--[b]		5		tetracycline	100[c]	90		
Cephalosporins					4-epimer of tetracycline	100[c]	**>1,000**		
ceftiofur	100[a]		14		oxytetracycline	100[c]	30		
desfuroylceftiofur	100[a]		70		4-epimer of oxytetracycline	100[c]	**1,000**		
cefquinome	20		20		chlortetracycline	100[c]	20		
cefazolin	50		14		4-epimer of chlortetracycline	100[c]	**350**		
cephapirin	60[b]		6		doxycycline	--[d]	7		

Note: Bold and red font detection capabilities are above the drug MRL. MRL: Maximum Residue Limit, Regulation (EC) No 470/2009 and Commission Regulation (EU) No 37/2010 and amendments (situation on 01/02/2021). Detection capability defined as the lowest concentration tested giving a minimum of 19 positive results out of 20, 38 positive results out of 40 or 57 positive results out of 60, respectively. T: tetracycline channel; B: β-lactam channel; C: cefalexin channel. [a]: No MRL in milk, MRL based on Commission Implementing Regulation (EU) 2018/470; [b]: Not in MRL list; [c]: The MRL of 100 µg/kg is applied on the sum of all residues retaining the β-lactam structure expressed as desfuroylceftiofur; [d]: The MRL of 60 µg/kg in milk is applied on the sum of cephapirin and desacetylcephapirin; [e]: The MRL of 100 µg/kg in milk is applied on the sum

of parent drug and its 4-epimer; [f]: No MRL in milk, not for use in animals from which milk is produced for human consumption.

Selectivity/specificity and false positive rate

TwinSensor Plus (KIT106) is a highly specific test for detection of β-lactams and tetracyclines in milk and does not detect compounds from the macrolides, quinolones, amphenicols, aminoglycosides, polymyxins, lincosamides and diamino pyrimidine derivatives, nor dapsone. On the β-lactam channel, as expected, cefalexin (beta-lactam) gave also positive results in high concentrations (100x and 10x MRL). Clavulanic acid, a β-lactamase inhibitor, gave an interference at the beta-lactam channel at high concentrations (100x MRL, 20x MRL, 10x MRL and 1,500 µg/kg). This interference is expected since this molecule contains a β-lactam structure resembling that of the penicillin, except that the fused thiazolidine ring of the penicillins is replaced by an oxazolidine ring (*Anonymous*, 2005). The specificity of the cefalexin channel was not tested. So the possibility to get a positive result on the cefalexin channel caused by high concentrations of a β-lactam compound different from cefalexin could not be excluded.

All 300 farm milk samples and 300 tanker load milk samples tested negative for β-lactams and tetracyclines on TwinSensor Plus (KIT106). So in total no false positive result was obtained upon 600 samples (= 0%).

Test repeatability.

The repeatability of the test was also very good, low standard deviation values were obtained. The highest variance value of 7.35 % is very acceptable.

Robustness testing

Variations in the length of the incubation step did not impact results significantly; all negative results remained negative and all positive results stayed positive. But benzylpenicillin was the best detected by respecting the incubation periods as given in the test protocol. Also, a deviation of 10% of the recommended incubation temperature, delay in reading or not removing the sample pad does not significantly impact the interpretation of test results; all negative remained negative and all positive results stayed positive. A volume of milk differing some 20 µl (10%) from the prescribed volume of 200 µl did not impact the interpretation of test results; the negative results remained negative and positive results stayed positive. For the doped milk a much larger range of ratios on all channels was obtained when 10% deviation is applied, and higher ratios (less positive) especially for the β-lactam channel. An excess of 10% of volume of milk is also slightly diminishing the detection of oxytetracycline (higher ratio values).

Testing milk with a temperature of 20°C resulted in correct TwinSensor Plus (KIT106) results: blank milk was always tested as negative while the doped milk samples gave a clear positive result on their respective channel but resulted in higher ratio values compared to testing milk of 1-4°C.

The milk quality and composition had little if any influence on the performance of the TwinSensor Plus (KIT106). No false positives were obtained with the blank milk. Four samples with a high SCC tested positive on the β-lactam channel but addition of β-lactamase ES and LC-MS/MS showed real presence of β-lactam residues. So these samples were excluded from this validation study. For the doped samples, milk with a high total bacterial count, high fat or low protein content, low pH or from an early lactation stage all tested positive. For the other doped milk samples some hampering of detection was observed, mainly on the tetracycline channel. On the tetracycline channel, negative results were obtained for milk with a high somatic cell count (1 out of 10 samples – ratio: 1.140), low fat milk (1 out of 10 samples – ratio: 1.247), high protein milk (2 out of 10 samples – ratio: 1.111 and 1.460), high pH (4 out of 10 samples – ratios between 1.141 and 1.353) and milk from a late lactation stage (6 out of 10 samples – ratios between 1.154 and 1.507) negative results were obtained on the tetracycline channel. For doped milk with high somatic cell count also one negative result was obtained on the beta-lactam channel (ratio: 1.137). It is worth noting that all tested concentrations were 25% (benzylpenicillin) to 60% (oxytetracycline) below their respective MRL. So one could expect positive results for all milk conditions when testing concentrations at MRL.

There could be interest to use the TwinSensor Plus (KIT106), although it is a test developed for the testing of raw cows' milk, to test other milk types such as UHT milk, sterilized milk, reconstituted milk powder or thawed milk or milk from another origin (goats, ewes', mares', buffalo or camel milk). For all the blank milk samples negative results were found. So no false positives were noticed. One blank ewes' milk sample suffered from a bad flow. The reader indicated "invalid" but due to the color of the sample there was uncertainty whether it might contain colostrum, so the sample was left out. For all doped samples, all positive results were found except for ewes' milk (9 out of 10 false negative results; ratios between 1.420 and 2.391), buffalo milk (7 out of

10 false negative results; ratios between 1.144 and 1.668) and camel milk (7 out of 10 false negative results; ratios between 1.298 and 1.469). The results indicate that TwinSensor Plus (KIT106) is suitable to test besides raw cows' milk also goats' and mares' milk. When testing ewes', buffalo and camel milk users should be aware that the detection capabilities as given in Table 4 for raw cows' milk do not apply. Extra testing is needed to indicate which compounds are detectable at MRL in these special milk types.

Stability of reagents

In general, stable ratio values were obtained for daily control samples with the TwinSensor Plus (KIT 106) reagents over the test period on all three tests lines. Mainly correct values were obtained for the different daily standards: all blank milk standards gave a negative result on all channels. The pooled milk sample doped with a concentration of 3 µg/kg of benzylpenicillin, 35 µg/kg of oxytetracycline and 70 µg/kg of cefalexin, gave some false negative results on the tetracycline channel over time. It is worth noting that these daily positive control samples are frozen and kept for a longer period. The samples for ccβ analysis in fresh milk and the lyophilized positive controls from the kit did not show any lack of detection of tetracyclines over time.

The negative and positive controls inserted in the kit always gave correct results, except for one value of the positive control (ratio: 1.633).

Interlaboratory testing - National ring trial

All milk samples fortified with β-lactam antibiotics were screened positive on the β-lactam test line of TwinSensor Plus (KIT 106), except for sample containing cefalexin, which was screened positive on the cefalexin test line. The milk sample spiked with 100 µg/kg of chlortetracycline was screened positive on the tetracycline test line of TwinSensor Plus (KIT 106). Hence, in this ring test, cefquinome, cloxacillin, benzylpenicillin, chlortetracycline, cefalexin and ampicillin are detected at MRL with TwinSensor Plus (KIT 106).

Negative results were obtained for the blank milk on all channels and for the milk samples doped with antibiotics that are supposed to give a negative result (on the respective test lines). So, there were no false positive results with TwinSensor Plus (KIT 106).

Conclusion

Results of this validation show that the TwinSensor Plus (KIT 106) is an easy, reliable and highly specific test for screening of raw cows' milk for residues of β-lactam antibiotics (penicillins and cephalosporins), including cefalexin and tetracyclines. All β-lactams, including cefalexin and tetracyclines (parent drugs) can be detected at least in 95% of the replicates at their respective MRL except for nafcillin (CCβ=125 µg/kg, MRL=30 µg/kg) and cefuroxime (CCβ=125 µg/kg, MRL=50 µg/kg). The 4-epimers of tetracyclines are also not detected at their respective MRL (4-epimer of tetracycline (CCβ>1,000 µg/kg, MRL=100 µg/kg); 4-epimer of oxytetracycline (CCβ=1,000 µg/kg, MRL=100 µg/kg) and 4-epimer of chlortetracycline (CCβ=350 µg/kg, MRL=100 µg/kg). But in general these 4-epimers are not found in milk (ILVO, internal information). Doxycycline, not for use in animals from which milk is produced for human consumption, can be detected at least in 95% of the replicates from 7 µg/kg on.

The test could also be used to screen UHT, sterilized milk, reconstituted milk powder and thawed milk on the presence of residues of β-lactams and tetracyclines. Also goats' and mares' milk can be screened, but the test is less suited to be used to test ewes', buffalo or camel milk, as higher detection capabilities will apply than those given in Table 2.

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POSTER P81

VERIFICATION OF BOUND AMINOGUANIDINE AS A SUITABLE MARKER RESIDUE FOR THE DETECTION OF NITROVIN IN PIG TISSUES

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Abstract

Nitrovin (NTV) is a veterinary drug that belongs to a class of antibiotics called nitrofurans, which are prohibited from use in food-producing animals. Recent methods have included aminoguanidine (AGN) as the marker residue for NTV but to date, no animal studies have been reported to confirm the suitability of AGN as the marker metabolite. In this work, pigs were fed with NTV-medicated feed (50 mg kg^{-1}) to assess the metabolism and depletion of the drug *in vivo*. Tissues (kidney, muscle and liver) and plasma were collected from the pigs on different withdrawal days, and the samples were analysed for bound AGN, total AGN and for the parent drug, NTV. At Day 0, the highest concentrations of both bound and total AGN were detected in liver at 8.4 µg kg^{-1} and 14.7 µg kg^{-1}, respectively, whilst the lowest levels were measured in muscle at 0.12 µg kg^{-1} and 0.28 µg kg^{-1}, respectively. AGN residues were detected in each matrix on all days of the study, whereas the parent drug was only detected in kidney tissue at low levels on Day 0. The research supports the use of AGN as the marker residue for monitoring illegal use of NTV in animal-derived products.

Introduction

Nitrovin (NTV) is a nitrofuran drug has been used as a growth promotor in animal feed for chickens, turkeys, pigs and calves at levels between 10 and 25 mg kg^{-1} feed, and also to prevent a wide array of diseases, such as salmonellosis in poultry, due to its bactericidal and bacteriostatic action (Analytical Methods Committee, 1991; Joner *et al.*, 1977). Due to concerns regarding the mutagenicity and carcinogenicity of nitrofuran residues and their potential adverse effects on human health, nitrofurans were completely banned from use in food-producing animals in the European Union (EU) in 1995 (Commission Regulation, 1995). Despite being a non-allowed pharmacologically active substance, NTV is currently not included on the EU monitoring list.

Nitrofuran drugs metabolise rapidly *in vivo* and have short half-lives, making the parent forms unsuitable for effective monitoring of their illegal use, while the metabolites bind to tissue proteins and can persist for much longer periods of time. Various methods have been published in literature for the detection of NTV as both the parent and as its metabolite AGN (Yan *et al.*, 2011; Chen *et al.*, 2020), and their chemical structures are shown in Figure 1. However, to date, no animal studies have been reported to confirm the suitability of AGN as the protein-bound marker metabolite of NTV. Therefore, the objectives of the study presented in this paper were to confirm AGN as a suitable marker residue of NTV for the first time using incurred material and to gain an understanding of the metabolism and depletion of NTV in pigs.

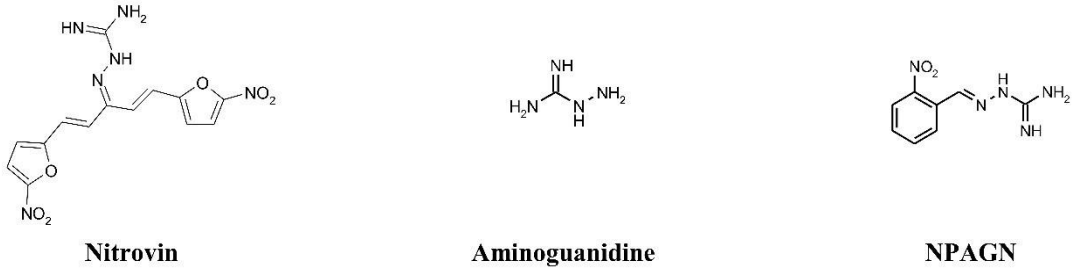

Figure 1. Chemical structures of the parent drug nitrovin (NTV), its metabolite aminoguanidine (AGN) and the nitrophenyl derivative marker residue, NPAGN.

Materials and Methods

Animal experiments

The animal experiments in this study were carried out in the Agri-Food and Biosciences Institute (AFBI) in Belfast, UK, in accordance with the Animals (Scientific Procedures) Act 1986 (HMSO, 1986). Eight pigs (Large White-Landrace cross), weighing approximately 30 kg each, were fed nitrovin-medicated feed *ad libitum* for seven consecutive days. The medicated feed contained a standard ration, premixed with nitrovin at a concentration of 50 mg kg^{-1}. Two control pigs were fed non-medicated feed. Normal drinking water was provided *ad libitum* throughout the study to all animals. After the seven days of medication, the treated pigs were moved to fresh pens. Two pigs were slaughtered with no withdrawal period (Day 0), and subsequently, another two pigs were slaughtered after three days (Day 3), five days (Day 5) and seven days (Day 7) withdrawal. The two control pigs were also slaughtered on Day 7. Muscle, liver, kidney, and blood (plasma) samples were collected on each withdrawal day, bagged separately and stored at -20 °C prior to analysis.

Analysis of bound and total residues of aminoguanidine

Samples were analysed for bound aminoguanidine residues using the method described by Regan *et al.* (2021), across a calibration range of 0.02 - 16.25 µg kg^{-1}. For total aminoguanidine analysis, samples were not washed prior to analysis to ensure that both the bound and free (total) residues were extracted. Alternatively, sample aliquots (1.0 ± 0.01 g) were weighed into 50 mL polypropylene tubes, a 4.5 mL volume of HCl (0.1 M) was added, and the samples were homogenised using an ultra-turrax® probe homogeniser. A further 4.5 mL HCl (0.1 M), 100 µL NBA (100 mM), and a magnetic cross stirrer were added to each tube, prior to the derivatisation and hydrolysis reaction in the MARS6 microwave system. From this point onwards, the extraction protocol was the same as the above for bound residue analysis. For plasma samples, aliquots were weighed (1.0 ± 0.01 g), and 9 mL HCl (0.1 M), 100 µL NBA (100 mM) and a magnetic cross stirrer were directly added to each tube prior to the derivatisation and extraction protocols. The UHPLC-MS/MS analysis for bound and total AGN was carried out as described by Regan *et al.* (2021).

Analysis of parent NTV residues

For parent nitrovin residue analysis, muscle, kidney and liver samples (2.0 ± 0.02 g) were weighed into 50 mL polypropylene tubes. Six matrix calibrants were fortified across a range of concentrations from 0.1 µg kg^{-1} to 5.0 µg kg^{-1}. Samples were shaken and homogenised with a 10 mL volume of MeCN:H_2O (1:1; *v/v*) and two ceramic homogeniser cutters. $MgSO_4$ (2 g) and NaCl (0.5 g) were added prior to shaking on a vibrational homogeniser for 7 min, followed by centrifugation at 4,800 × *g* (4 °C, 10 min). The supernatant was transferred into a 50 mL polypropylene tube containing 250 mg end-capped C_{18} and 5 mL *n*-hexane pre-saturated with MeCN was added. Samples were multi-vortexed for 60 s and centrifuged at 4,800 × *g* (4 °C, 10 min). The *n*-hexane layer was aspirated to waste and a 1.5 mL aliquot was transferred to 1.5 mL polypropylene centrifuge tubes. Samples were centrifuged at 21,000 × *g* (4 °C, 10 min). The supernatant was transferred to an autosampler vial, and a 2 µL volume was injected for the UHPLC-MS/MS analysis.

For parent nitrovin residue analysis in blood (plasma), 400 µL samples were weighed into 2 mL polypropylene centrifuge tubes. A volume of 1.6 mL MeCN (stored overnight at -20 °C) was added, and the samples were multi-vortexed for 60 s, followed by storage at -20 °C for 20 minutes. Following the protein precipitation, NaCl (200 mg) was added and the samples were centrifuged at 21,000 × *g* (4 °C, 10 min). A 1.4 mL aliquot then underwent further defatting with 600 µL *n*-hexane pre-saturated with acetonitrile. The top *n*-hexane layer was aspirated to waste, and 1 mL of the remaining extract was transferred to autosampler vials. A 2 µL volume was finally injected onto the UHPLC-MS/MS platform.

UHPLC-MS/MS analysis for NTV residues was carried out using a Waters Acquity UPLC® I-Class coupled to a Waters Xevo® TQ-XS triple quadrupole mass spectrometer (Manchester, UK), operating in a positive electrospray ionisation mode (ESI+) with an Acquity UPLC® HSS T3 analytical column (100 × 2.1 mm, 1.8 µm) equipped with a stainless steel in-line filter unit containing a stainless steel replacement filter (0.2 µm, 2.1 mm). A binary separation gradient comprising of 0.01% (*v/v*) acetic acid in H_2O:MeCN (9:1, *v/v*) (MPA) and 0.5 mM ammonium formate in MeOH:MeCN (3:1, *v/v*) (MPB) was used at a flow rate of 0.6 mL min^{-1}. UHPLC-MS/MS conditions are shown in Table 1.

Table 1. UHPLC-MS/MS conditions for NTV analysis.

Analyte	Measured Ion	Precursor (m/z)	Product (m/z)	Cone Voltage	CE	RT (min)
NTV 1	$[M+H]^+$	361.31	222.04*	50	16	4.52
NTV 2	$[M+H]^+$	361.31	180.04	50	18	4.52
NTV 3	$[M+H]^+$	361.31	153.91	50	34	4.52
NTV 4	$[M+H]^+$	361.31	301.99	50	18	4.52
NTV 5	$[M+H]^+$	361.31	197.07	50	26	4.52

* Quantitative ion. MS conditions were as follows: capillary voltage 1 kV; desolvation temperature 650 °C; source temperature 150 °C; desolvation 1200 L h^{-1}; cone 150 L h^{-1}; nebuliser 7 Bar; collision gas 0.15 mL min^{-1}.Results and Discussion

Suitability of aminoguanidine as a bound marker residue for nitrovin

In this work, samples collected from pigs fed with NTV-medicated feed were analysed using the bound residues method, and all matrices (muscle, liver and kidney) were found to contain bound AGN residues at concentrations >0.02 µg kg^{-1} (measured as its NPAGN derivative). A solvent standard of NPAGN was injected and the analyte was shown to elute at the same retention time of 2.47 min, with an ion ratio of 0.26. The tissues collected from the control pigs did not contain AGN, which provided additional confirmation that the residues present in the test samples were a result of the NTV-medicated feed. Thus, this study verified that AGN is a suitable bound residue marker of NTV in pigs.

Depletion of bound and total aminoguanidine residues in pig tissues

The highest concentrations of bound AGN residues were detected at day 0 with levels averaging 5.7 µg kg^{-1} (n = 6; RSD = 4.9%) in kidney, 8.4 µg kg^{-1} (n = 6; RSD = 12.3%) in liver and 0.12 µg kg^{-1} (n = 6; RSD = 13.0%) in the muscle of each pig. These levels depleted over the course of withdrawal period to measure 0.86 µg kg^{-1} (n = 6; RSD = 18.3%) in kidney, 2.7 µg kg^{-1} (n = 6; RSD = 12.8%) in liver and 0.05 µg kg^{-1} (n = 6; RSD = 9.3%) on Day 7 in muscle. For bound AGN, the fastest rate of depletion was observed in kidney, followed by liver and then muscle.

On Day 0 of withdrawal, the concentrations of total (bound + free) AGN residues were 2.6 times higher for kidney, 1.7 times higher for liver, and 2.3 times higher for muscle, relative to bound residues only. A concentration of 1.5 µg kg^{-1} (n = 6; RSD = 6.6%) was detected in the plasma samples. As depicted by the depletion curves shown in Figure 2, the concentrations of total residues depleted in a manner such that the majority of AGN present on Day 7 of withdrawal was in the bound form in all matrices.

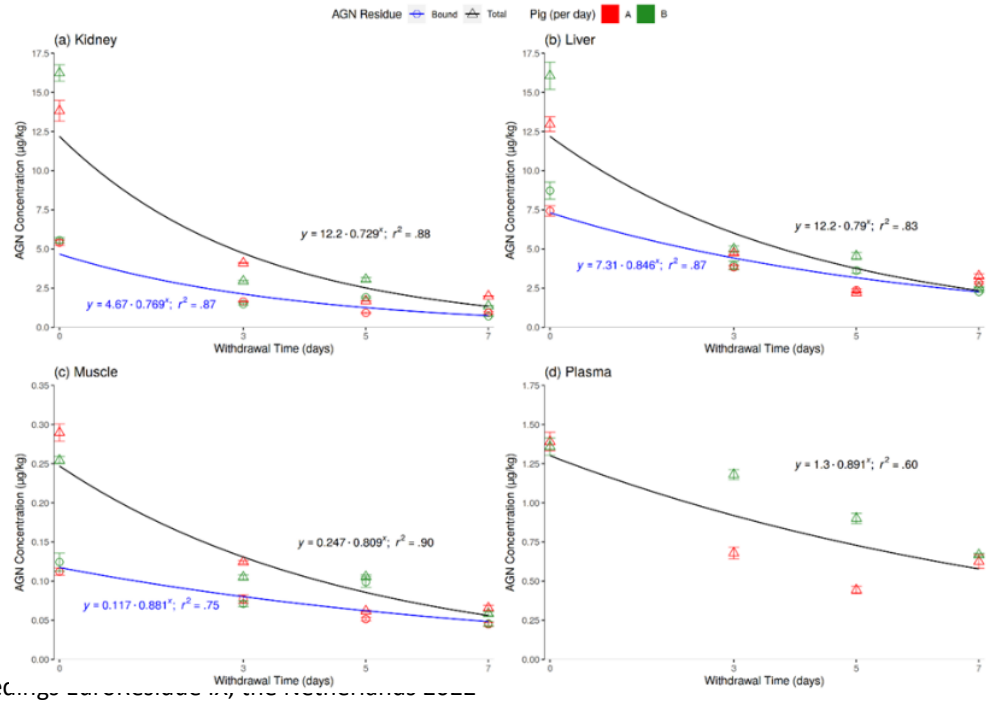

Figure 2. Depletion curves across a seven day period for bound and total residues of aminoguanidine hydrochloride (AGN) detected in (a) kidney, (b) liver, (c) muscle, and (d) plasma samples, collected from pigs fed with nitrovin-medicated feed. Triplicate measurements from each pig are depicted by a mean value ± standard error of the mean.

Detection on NTV parent residues

In this work, the concentration of NTV parent residues were measured in the porcine tissue and blood (plasma) samples at each withdrawal time point (Table 2). The parent drug was only detected in the kidney samples of pigs at Day 0 of the study (0.126 μg kg^{-1}). In the liver, muscle and plasma samples, no nitrovin was measured. It has been well established in previous studies that parent nitrofuran compounds have short *in vivo* half-lives, becoming undetectable very quickly after cessation of treatment.

The findings reported in this paper are in agreement with reports that the parent form of nitrofuran antibiotics are not suitable markers for monitoring their illegal use. However, the results are in contrast with the levels measured in NTV-treated chickens reported by Yan *et al.* (2011) and may indicate a difference in the metabolism and persistence of NTV in different species. The study presented here suggests that the parent drug is unsuitable for detection purposes, and that AGN should be considered as the appropriate marker residue for monitoring NTV in food-producing animals.

Table 2. Concentrations of NTV residues measured from parent analysis in porcine kidney, liver, muscle and plasma across the withdrawal periods.

Withdrawal Day	Concentration measured (μg kg^{-1})			
	Kidney	Liver	Muscle	Plasma
0	0.126	< 0.1	N.D.	N.D.
3	N.D.	N.D.	N.D.	N.D.
5	N.D.	N.D.	N.D.	N.D.
7	N.D.	N.D.	N.D.	N.D.

N.D. = not detected.

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POSTER P82

OXAMIC ACID HYDRAZIDE AS A SUITABLE MARKER RESIDUE FOR MONITORING THE ILLEGAL USE OF NIFURALDEZONE IN POULTRY

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Abstract
Nifuraldezone (NDZ) belongs to a class of antibiotic drugs called nitrofurans, which were previously licensed for use in veterinary medicine for the prevention and control of disease. However, nitrofurans are now prohibited from use in food-producing animals, due to the potential threat they pose to consumer health. As a result, strict legislation is in place which mandates monitoring for the illegal presence of their residues in animal-derived products. Detection of nitrofurans is by their protein-bound metabolites, as the parent drugs are rapidly metabolised upon administration and cannot be directly measured. Oxamic acid hydrazide (OAH) has been proposed as a metabolite of nifuraldezone, but to date, it has not been confirmed using animal studies. In this work, NDZ-medicated feed (50 mg kg^{-1}) was administered to broilers to determine the drug's metabolism *in vivo*, and on different withdrawal days, breast muscle, thigh muscle and liver samples were collected. These samples were analysed for both bound and total OAH in order to assess the depletion profile of NDZ. This study confirmed OAH as a metabolite and its suitability for use as a marker residue for monitoring the abuse of NDZ in food-producing animals.

Introduction
Nifuraldezone (NDZ) is a broad-spectrum antimicrobial that belongs to a class of drugs called nitrofurans. Also known as furamazone or framazone, NDZ can be used in veterinary medicine in the treatment of enteritis in calves and winter dysentery. It has been reported NDZ's effectiveness against enteric infections is to its low degree of absorption from the gut (Fulford, 1961). As is characteristic of nitrofuran drugs, NDZ contains a furan ring in its chemical structure (*Figure 1*).

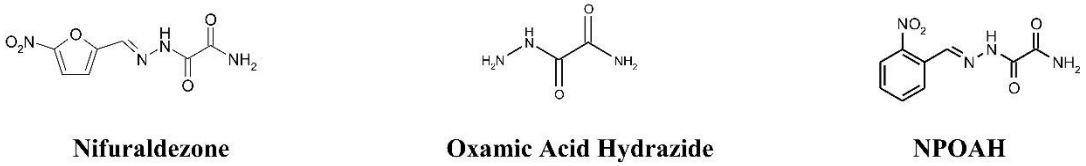


Figure 1. Chemical structures of the parent drug nifuraldezone (NDZ), its metabolite oxamic acid hydrazide (OAH) and the nitrophenyl derivative marker residue, NPOAH.

Nitrofurans are considered as zero tolerance substances, meaning that they are completely banned from use in food-producing animals due to their undesirable toxicological properties. Nitrofurans are administered in their parent forms but are rapidly metabolised *in vivo* to form highly stable, protein-bound metabolites. Given their short half-lives *in vivo*, the parent forms are unsuitable for monitoring the illegal presence of nitrofurans in food, so instead, their respective persistent metabolites are used as marker residues. The bound metabolites are formed by the cleavage of carbon-nitrogen double bond present in side chain of the nitrofuran ring. NDZ is not currently on the monitoring list in EU legislation, but its marker residue can be predicted by examining its chemical structure. It has been proposed that oxamic acid hydrazide (OAH) is a metabolite formed during NDZ metabolism *in vivo*, but to date, this has not been verified using animal studies. The study presented in this extended abstract aimed to confirm OAH as a suitable marker residue of NDZ for the first time using incurred material and to gain an understanding of the metabolism and depletion of NDZ in different avian tissues.

Materials and Methods

Chemicals and apparatus

Oxamic acid hydrazide (OAH) and oxamic acid hydrazide-$^{15}N_3$ (OAH-$^{15}N_3$) were purchased from Witega (Berlin, Germany). 2-nitrophenyl-oxamic acid hydrazide (NPOAH) was obtained via laboratory synthesis at Teagasc Food Research Centre (Dublin, Ireland). "SpS"-grade (super purity solvent) acetonitrile (MeCN) 200 far UV and methanol (MeOH) 215 were supplied by Romil Ltd. (Cambridge, UK). Diethyl ether (Et_2O) was sourced from Honeywell (Riedel-de-Haen, Seelze, Germany). Ethanol (EtOH) absolute and trisodium phosphate dodecahydrate (Na_3PO_4) were obtained from Merck kGaA (Darmstadt, Germany). Concentrated hydrochloric acid (HCl) (37%), 2-nitrobenzaldehyde (NBA) and ammonium formate puriss p.a. were purchased from Sigma Aldrich (Dublin, Ireland). Sodium hydroxide (NaOH) pellets were sourced from Lennox Laboratory Supplies Ltd. (Dublin, Ireland). Sodium chloride (NaCl) was obtained from Applichem (Darmstedt, Germany) and enviro-clean magnesium sulphate anhydrous ($MgSO_4$) was supplied by United Chemical Technologies Ireland Ltd. (Dublin, Ireland). Ultra-pure water (UPW) (18.2 MΩ cm^{-1}) was generated in-house using a Millipore purification system (Cork, Ireland).

An ME365 microbalance, sourced from Sartorius (Dublin, Ireland) was used for weighing during standard preparation. The following apparatus was used during sample preparation: an ultra-turrax® probe homogeniser from IKA (Staufen, Germany), a Mars 6 240/50 model microwave from CEM Microwave Technologies Ireland Ltd. (Dublin, Ireland), a minimix vibrational unit (Merris Engineering Ltd., Milltown, Co. Galway, Ireland) and a TurboVap® LV evaporator from Biotage (Sweden). Ceramic homogenisers were purchased from Agilent Technologies Ltd. (Cork, Ireland) and PTFE magnetic cross stirrers (8 × 20 mm) were sourced from VWR International Ltd. Ireland (Wexford, Ireland). Polypropylene tubes (15 mL and 50 mL) were obtained from Sarstedt Ltd. (Wexford, Ireland). Whatman Mini-Uniprep® syringeless filter devices (pore size 0.2 μm) were purchased from Cytiva (United States).

Animal experiments

The animal experiments in this study were carried out in the Agri-Food and Biosciences Institutes (AFBI) in Belfast, UK, in accordance with the Animals (Scientific Procedures) Act 1986 (HMSO, 1986). 32 chicks were split into two groups (Group 1 = NDZ × 24 chicks and Group 2 = controls × 8 chicks) and were allowed to acclimatise for 14 days. All birds received non-medicated starter meal from Day 1 - 10, followed by non-medicated grower meals from Day 11 - 16 of the study. Group 1 birds were then fed for seven consecutive days with feed that had been medicated with NDZ at a concentration of 50 mg kg^{-1}. The Group 2 control birds remained on non-medicated feed. At the end of the treatment period, Group 1 birds were moved to clean pens to ensure that on further medicated feed was ingested. After one day of withdrawal (Day 1), four birds from Group 1 and one control bird from Group 2 were randomly selected for sacrifice and sampling. Subsequently, another four medicated birds and one control bird were sacrificed after four days (Day 4), six days (Day 6) and eight days (day 8) of withdrawal. From this point in the study onwards, no control birds were sacrificed. Four medicated birds from Group 1 were selected and sacrificed after 11 days of withdrawal (Day 11) and after 14 days of withdrawal (Day 14). All birds were killed by exposure to isofluorane gas, followed by cervical dislocation. On each sacrifice day, breast muscle, thigh muscle and liver samples were collected. All samples were bagged separately and stored at -20 °C prior to analysis.

Analysis of bound and total residues of OAH

Samples were analysed for bound OAH residues using the method described by Regan *et al*. (2021). Test portions (1.000 ± 0.010 g) were weighed by taking a minimum of three aliquots from different parts of the tissue sample, combining by chopping and mixing, then weighing from this combined portion to ensure representative sampling. Tissues were homogenised and washed with ice-cold MeOH, ice-cold EtOH, and Et_2O prior to analysis, to isolate the bound residues only. The solvent was allowed to evaporate overnight and once the washed samples were fully dried, 11 matrix calibrants were fortified across a range of concentrations from 0.02 μg kg^{-1} to 50 μg kg^{-1}. All samples were spiked with a 100 μL volume of IS. 9 mL HCl (0.1 M), 100 μL NBA (100 mM) and a magnetic cross stirrer were added to each sample prior to a 2 h microwave-assisted hydrolysis and derivatisation of the samples. Following this reaction, all samples were neutralised and extracted using a modified QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) approach. The solvent extracts were evaporated to dryness under nitrogen gas, using an LV Turbovap®, and were reconstituted in a 500 μL volume of injection solvent (5 mM ammonium formate in H_2O:MeOH (9:1, v/v)). Extracts were filtered using Whatman Mini-UniPrep vials of 0.2 μm pore size and a 10 μL volume was injected for LC-MS/MS analysis.

For total OAH analysis, samples were not washed prior to analysis to ensure that both the bound and free (total) residues were extracted. Instead, sample aliquots (1.000 ± 0.010 g) were weighed into 50 mL polypropylene tubes, a 6 mL volume of HCl (0.1 M) and a ceramic homogeniser were added, and the contents of each tube were

homogenised by shaking for 1 min on a Minimix® vibrational unit. A further 3 mL HCl (0.1 M) and 100 µL NBA (100 mM) were added to each tube to wash any tissue adhering to the upper wall of the tube, prior to derivatisation and hydrolysis. From this point onwards, the extraction protocol was the same as the above for bound residue analysis.

UHPLC-MS/MS analysis was carried out as described by Regan *et al.* (2021) and conditions are shown in Table 1.

Table 1. UHPLC-MS/MS conditions for NPOAH analysis.

Analyte	Measured Ion	Precursor (*m/z*)	Product (*m/z*)	RT (min)	DP	CE	CXP	MRM
NPOAH 1	$[M+H]^+$	237.1	192.1	3.25	66	20	10	20
NPOAH 2	$[M+H]^+$	237.1	135.2	3.25	59	30	7	20
NPOAH-$^{15}N_3$	$[M+H]^+$	240.1	194.1	3.25	69	19	10	20

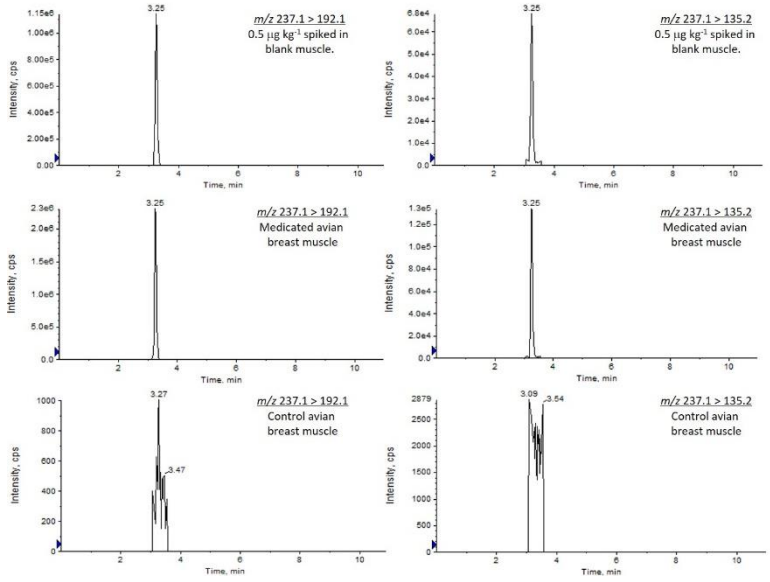
MS Condition	Value
Ion spray voltage	+1400 V
Source temperature	650 °C
CAD gas	8
Entrance potential (EP)	10
Curtain gas pressure	30 psi
Ion source gas 1 (GS1)	70 psi
Ion source gas 2 (GS2)	70 psi

Results and Discussion

Oxamic acid hydrazide as a marker residue for nifuraldezone

Nifuraldezone is not commonly included in methods for nitrofuran analysis and to date, only one analytical method has been published with the inclusion OAH as its marker residue (Regan *et al.*, 2021). OAH has been proposed as a product of NDZ metabolism *in vivo*, on the basis that it would form through the cleavage of the carbon-nitrogen double bond in NDZ's chemical structure. This cleavage is observed in other nitrofuran parent drugs when forming their respective metabolites, such as, AOZ for furazolidone. In this work, animal studies were carried out to investigate NDZ metabolism *in vivo* and to subsequently assess the suitability of using OAH as a marker residue for monitoring the abuse of NDZ in food-producing animals.

All breast muscle, thigh muscle and liver samples collected from the medicated birds were analysed for NPOAH residues using the bound method, and all matrices were found to contain NPOAH residues at concentrations ranging between 0.32 – 110.6 µg kg^{-1}. All NPOAH residues detected in the test samples were confirmed by injection of fortified matrix and solvent standards of NPOAH, which were observed to elute at 3.25 min, with ion ratios of 0.06. Additionally, all samples collected from the control birds (fed with non-medicated feed) were analysed and no NPOAH residues were detected. *Figure* 2 shows the chromatography from a blank breast muscle

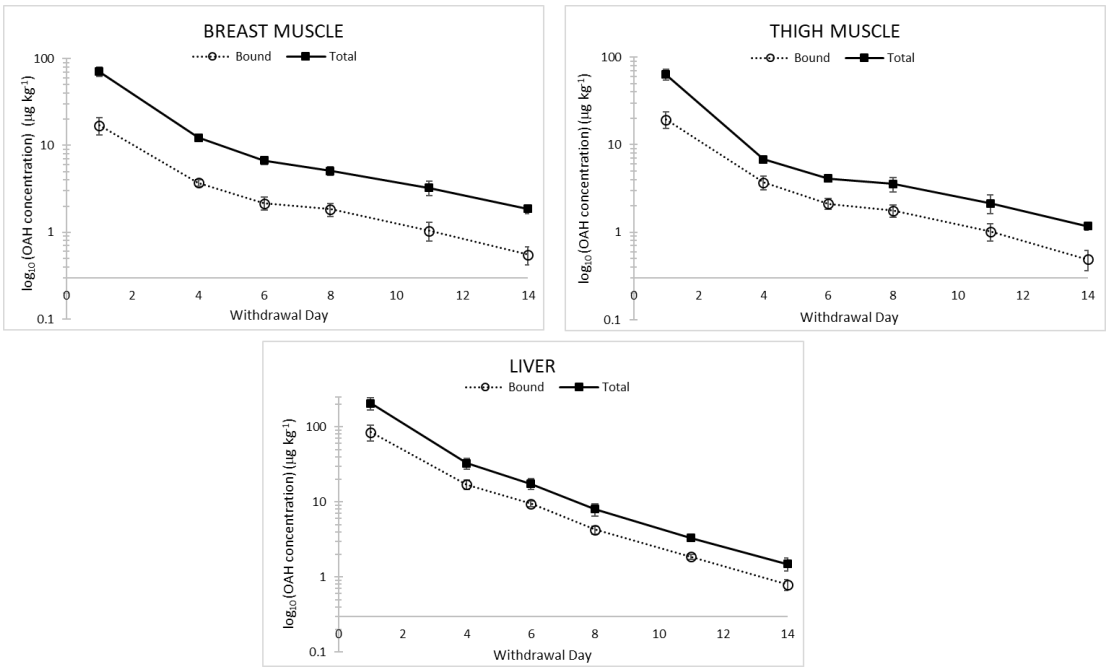


sample spiked with OAH, a breast muscle sample collected from a medicated bird and a breast muscle sample collected from a non-medicated control bird.

Figure 2. OAH chromatography for quantifier and qualifier transitions for a spiked breast muscle sample, a breast muscle sample (10.5 μg kg^{-1}) collected from a medicated bird and a breast muscle sample collected from a control bird.

Metabolism and depletion of OAH residues

The breast muscle, thigh muscle and liver samples collected from the NDZ-medicated birds were analysed for both bound and total residues of OAH. "Bound" residues refer to the protein-bound OAH only, which is isolated by washing the samples with organic solvents prior to analysis. "Total" residues refer to both the bound and free residues, which are measured by analysing unwashed samples. The depletion of OAH residues over time is depicted in the charts shown in *Figure 3.* As expected, the highest concentrations of both bound and total OAH were detected on Day 1, which was the shortest withdrawal period in the study, and the levels gradually decreased until the lowest concentrations were measured on Day 14. On the last day of the study, all matrices contained OAH bound and total residues at or above 0.5 μg kg^{-1}, which is the new reference point for action

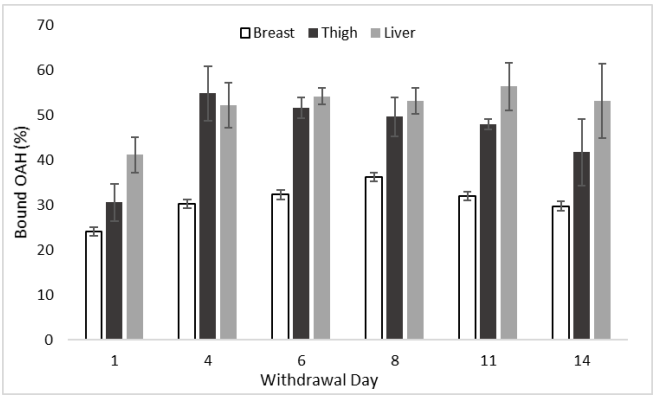


(RPA) for nitrofurans that will come into effect in November 2022 (Commission Regulation, 2019). The levels measured on Day 14 indicate the persistence of OAH residues in poultry tissues, even after treatment with NDZ has ceased. The liver samples contained the highest levels of both bound and total residues, at all time-points of the study, while the breast and thigh muscle samples contained similar concentrations of OAH.

Figure 3. Depletion of oxamic acid hydrazide (OAH) bound and total residues across a 14 day withdrawal period detected in breast muscle, thigh muscle and liver samples collected from birds fed with nifuraldezone-medicated feed.

The ratio of bound to total residues is displayed in *Figure 4*, and gives an indication of how the OAH residues persist in the various avian tissues. After one day of withdrawal, the bound residues accounted for approximately 24% in breast muscle, 30% in thigh muscle, and 41% in liver, of the total residues detected. Over the course of the study, the percentages varied depending on the matrix and the withdrawal day. On Day 14, the bound residues constituted approximately 30% in breast muscle, 41% in thigh muscle and 54% in liver, which suggests that the protein-bound OAH residues may persist for longer periods of time.

Figure 4. Percentage of protein-bound oxamic acid hydrazide (OAH) residues in breast muscle, thigh muscle and liver of birds



fed with nifuraldezone-medicated feed, across a withdrawal period of 14 days.

Conclusion

An *in vivo* metabolism and depletion study was carried out in chicken to determine the suitability of OAH as a marker residue for monitoring illegal NDZ use in poultry. All samples collected on all withdrawal days (Day 1 – 14) were analysed in triplicate and were found to contain levels of OAH ranging between 0.50 – 85 μg kg^{-1} for bound residues and between 1.2 – 206 μg kg^{-1} for total residues. This research is the first instance where OAH has been confirmed as a major bound metabolite of NDZ using animal studies and the findings clearly indicate that OAH is suitable for use as a marker residue for detecting NDZ misuse in poultry. In both thigh muscle and liver samples, the bound form of OAH accounted for more than 50% of the total residues from Day 4 of withdrawal. The greatest levels of both bound and total OAH residues were detected in liver samples, and the concentrations measured in the breast and thigh samples were still at or above the new RPA of 0.5 μg kg^{-1} on the final day of the study. This work has highlighted that both liver and muscle are suitable matrices for detecting OAH residues, but given that muscle tissue is more widely consumed than liver, perhaps muscle would be the most appropriate matrix.

Acknowledgements

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POSTER P83

DEVELOPMENT OF A LC-MS/MS METHOD FOR ANALYSIS OF COVID-19 AND HIV DRUGS IN MUSCLE TISSUE

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Abstract

The objective of this work was to develop a LC-MS/MS method for the analysis of HIV and COVID-19 antiviral drugs that have potential for illegal used in animal production. The compounds of interest were selected based on their previous reported use in pig farming or their use (or suspected use) for the treatment of COVID-19. These analytes included tenofovir, favipiravir, nevirapine, ritonavir, lopinavir and its main metabolite, efavirenz, hydroxychloroquine, azithromycin, remdesivir, darunavir and arbidol and two of its metabolites. Reversed-phase chromatography was found to give best retention and peak shape using a Poroshell 120 Sb-Aq column. A range of sample extraction procedures were evaluated to extract the analytes showing that acetonitrile-based extraction procedures such as simple solvent extraction or QuEChERS were not found to be suitable for more polar analytes such as tenofovir. Methanol was found to be the most efficient solvent achieving recoveries ranging from 78% to 107%. The extracts were evaporated and reconstituted in a methanol:water mixture to improve peak shape and solubility

Introduction

In the last two decades, concerns have grown over the illegal use of antiviral drug against influenza in poultry farming (Cyranoski 2005; Wan *et al.* 2013). However, it was recently reported that other antiviral drugs, namely HIV drugs including tenofovir, nevirapine and efavirenz, have been used in pig farming in Eastern Africa (Nakato *et al.* 2020). This illegal use could cause the emergence of drug-resistant HIV strains and these reports called for the need of a wider investigation (Ndoboli *et al.* 2021). In 2020, following the covid-19 pandemic, researchers have repurposed various drugs including HIV and Ebola drugs in order to find an efficient treatment for covid-19 (Riva *et al.* 2020). Apart from HIV drugs, other drugs have been suspected to be use in animal farming (Xiang *et al.* 2020). Therefore, there is a need to develop sensitive quantitative method for analysing residues of these drugs in meat.

Currently, no LC-MS/MS methods analyse for many of these drugs in animal meat, but HPLC work and extraction from other biological matrix have been researched by several studies (Mwando *et al.* 2017; Abafe *et al.* 2018).

The goal of this study is to develop a straightforward confirmatory LC-MS/MS method for the quantification of 15 HIV and covid-19 drug residues in pig muscle tissue. The development and optimisation work aim to achieve a fast method with a satisfactory sensitivity. The method is to be subsequently validated using the EU guidelines 2021/808 (EU 2021).

Materials and Methods

Chemicals and standard

Nevirapine, lopinavir, darunavir, hydroxichloroquine sulfate, efavirenz tenofovir hydrate, arbidol sulfoxide and arbidol were purchased from Sigma-Aldrich (Arklow, Ireland). Arbidol and favipiravir were sourced from Carbosynth (St Gallen, Switzerland). Remdesivir was supplied by Tebu-bio (Le Perray-en-Yvelines, France) while molnupiravir was supplied by Witega (Berlin, Germany). Nevirapine-d_3, arbidol-d_6 sulfate, azithromycin dehydrate, azithromycin -$^{13}Cd_3$, lopinavir-d_8, lopinavir-M1 and hydroxychloroquine-d_4 were purchased from Toronto Research Chemicals (North York, Canada). Darunavir-d_9 was supplied by Cayman Chemical (Ann Arbor, Michigan, United States).

Acetonitrile (MeCN) and Methanol (MeOH) were sourced from Romil (Cambridge, UK). Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich (Dublin, Ireland). Ultra-pure water was prepared in-house with a water purification system supplied by Merck-Millipore (Cork, Ireland). Formic acid was purchased from Honeywell Fluka (Seelze, Germany).

Standard preparation

Individual stock standard solutions at concentration of 1mg mL^{-1} were prepared. Darunavir, nevirapine, ritonavir, efavirenz, molnupiravir, lopinavir, lopinavir-M1, azithromycin, nevirapine-d_3, lopinavir-d_8, darunavir-d_9 and azithromycin-$^{13}Cd_3$ were dissolved in methanol. Remdesivir was dissolved in DMSO while tenofovir and hydroxychloroquine were dissolved in water. Mix solutions and were prepared in MeCN:H_2O (50/50, *v/v*). All solutions were stored at -30°C.

Sample preparation

Pork muscle tissue (2 ± 0.02g) aliquots were weighed into 50 mL polypropylene tubes. A volume of 50 µL of internal standard was added to each tube while calibrant samples were spiked with 100 µL of working calibrant standard solutions. Sample were let to rest 15 minutes after spiking, a ceramic homogeniser was then added into each tube. Samples were extracted by adding 10 mL of MeOH into each tube. Homogenisation was carried out using a minimix vibrational shaker for 5 minutes. The tubes were then centrifuged at 2725 x*g* at -4°C for 10 min. Subsequently, 3 mL of the supernatant was transferred into a 15mL propylene tube. The extract was evaporated until dryness in a turbovap at 50°C. The extracts were reconstituted in 1 mL of a mixture of MeOH:H_2O (50/50, v/v). The tubes were vortexed for 30s using a multivortexer. Extracts were filtered using 0.2 µm PTFE syringe filters prior to injection in LC-MS/MS system.

LC-MS/MS analysis

The liquid chromatography system was a Waters Acquity I Class UPLC system with an Agilent InfinityLab Poroshell 120 SB-AQ column (50 x 2.1 mm, 2.7 µm) fitted with a Waters in-line filter unit (0.2 µm filter). The chromatographic separation was operated at a temperature of 40°C, using a binary gradient system comprising mobile phase (A) 0.2% formic acid in H_2O and (B) 0.2% formic acid in MeCN. The flow rate was at 0.3 $mL.min^{-1}$ and the gradient profile was: (1) 0-1 min 99% A; (2) 1-5 min 10% A; (3) 5-6.5 min 10% A; (4) 6.5-7.5 min 99% A; (5) 7.5-10 min hold at 99% A. The injection volume was 1 µL and vials were kept at a temperature of 8°C.

The detection was achieved using a Waters Xevo TQ-XS. The MS method was run in electrospray (ESI+) and in positive mode. Concerning source parameters, the capillary voltage was 2.5 kV, the source temperature was 150°C and the desolvation temperature was 600°C. MS tuning was carried out by infusing an individual solution of 1 µg mL^{-1} of each compound while running mobile phases (A) 0.2% formic acid in H_2O and (B) 0.2% formic acid in MeCN at 0.3 mL min^{-1} and a composition of 50/50 A/B (*v/v*). The infusion was done at 10 µL min^{-1}. The optimal cone voltage was 20 V for all transitions. For each analyte, a parent ion and two product ions was selected depending on the sensitivity and the selectivity, while for internal standards one parent ion and one product ion were chosen. These parameters were summarised in Table 1.

Table 1: MS parameters.

Analyte	RT (min)	Precursor Ion (m/z)	Product Ions (m/z)	Dwell time (s)	Collision (eV)
Tenofovir	1.04	288.2	176.2/159.2	0.163	23/29
Favipiravir	1.51	157.9	140.9/84.9	0.163	12/21
Hydroxichloroquine	2.38	336.3	247.2/179.1	0.108	21/36
Azithromycin	2.86	749.6	591.6/158.2	0.015	30/40
Nevirapine	3.07	267.2	226.2/80.1	0.015	24/29
Arbidol Sulfoxide	3.18	495.3	370.3/325.2	0.015	14/25
Arbidol Sulfone	3.31	511.3	466.2/296.1	0.015	20/33
Arbidol	3.66	479.4	434.2/281.2	0.015	19/33
Remdesivir	3.68	603.4	200.2/402.3	0.015	38/15
Darunavir	3.93	548.3	392.3/69.1	0.015	12/26
Lopinavir-M1	4.26	643.5	412.4/429.4	0.018	22/23
Efavirenz	4.28	316.2	168.2/232.2	0.018	20/18
Ritonavir	4.34	721.4	268.3/197.2	0.018	28/41
Lopinavir	4.36	629.5	155.2/447.4	0.018	44/20
Molnupiravir	2.61	330.1	128.1/203.1	0.019	15/9
Hydroxichloroquine-d4	2.38	340.3	251.3	0.108	21

Azithromycin-$^{13}CD_3$	2.86	753.6	595.6	0.015	29
Nevirapine-d_3	3.06	270.3	229.2	0.015	24
Arbidol-d_6	3.66	485.2	434.1	0.015	20
Darunavir-d_9	3.93	557.4	401.4	0.015	12
Lopinavir-d_8	4.35	637.5	163.3	0.018	42

RT: Retention time.

Results and Discussion

Method development

Mass Spectrometry

All of the antiviral drugs used in this work were found to produce protonated ions in agreement with previously published papers. An evaluation of Electrospray and UniSpray ionization was subsequently carried out showing that the latter gave up to a 10-fold increase the signal. However, it was also observed that noise also increased when using Unispray and that lowest achievable calibrant levels were equivalent with both sources.

Liquid Chromatography

The analytes used in this analysis did not include any highly polar molecules and chromatographic challenges were mainly focussed on peak shape or sensitivity. Thus the natural choice to start stationary phase screening was using reversed-phase columns. Various reversed-phase columns were tested, including Poroshell EC C_{18}, Kinetex PFP, Speedcore Diphenyl, Fusion RP, YMC Triart C_{18}, Zorbax Eclipse Plus C_{18}, Luna Polar C_{18}, Cortecs C_{18+} and Poroshell 120 SB-AQ. This screening was performed at 0.3 $mL.min^{-1}$ and using mobile phases (A) 0.1% of formic acid in water and (B) 0.1% of formic acid in MeCN. It was observed that reversed-phase columns provide sufficient retention for this group of compounds because the retention factor for the first eluter (tenofovir) was always superior to 1. It was found that hydroxichloroquine, azithromycin and tenofovir were the most problematic compounds showing broader or tailing peak shapes using several columns. The Poroshell 120 SB-AQ column led to sharper peaks and less tailing for these analytes. This column type was successfully applied in other published methods for hydroxichloroquine analysis and is advantageous because it can be used in 100% aqueous conditions.

A number of additional experiments were carried out to evaluate the impact of organic modifiers and additives on chromatography and MS sensitivity. MeCN provided more efficient elution and sharper peaks than MeOH. While, the inclusion of formic acid in both mobile phases led to sharp peaks compared with the broad peaks produced with acetic acid for compounds with cationic characteristics (i.e. azithromycin, hydroxychloroquine and arbidols). The use of ammonium acetate in small quantity (0.1 to 5 mM) did not impact significantly on peak shapes but a high signal suppression of up to 10-fold was observed for efavirenz. For these reasons, formic acid in both mobile phases was chosen and 0.2% was found to be the optimal concentration.

Sample preparation

The objective of the sample preparation step was to perform an exhaustive extraction while keeping a clean extract. To extract analytes from the pork muscle matrix, various liquid-solid extraction procedures were tested. The standard QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) for this kind of application was found to be completely inefficient at extracting polar compounds including tenofovir. Solvent-based options were found to be more appropriate, use of pure MeCN was again too hydrophobic but the use of MeOH or MeCN:H_2O (80:20, *v/v*) gave satisfactory extraction efficiency results as seen on Figure 1. These two options were also found to be equivalent in terms of matrix effects, the methanol extraction was finally selected because it led to a slightly better signal for the least sensitive compounds (e.g. tenofovir, efavirenz and favipiravir). Use of formic acid in the methanol extraction led to very dirty extracts large matrix suppression for most analytes.

A clean-up step investigation was judged to be worth considering especially because the extract is concentrated by 3-fold. An ultrafiltration step using a cellulose based 3k Da filter was found to retain compounds with high molecular mass including azithromycin (749 g mol^{-1}) and ritonavir (721 g mol^{-1}). A screening of dispersive solid phase extraction sorbents including C_{18}, C_8, PSA, Zsep and NH_2 was carried out but low absolute recoveries were observed for some compounds (i.e. tenofovir when using PSA, Zsep and NH_2).

However, C_{18} and C_8 gave good results visually but were finally not selected due to linearity issues when injecting a curve.

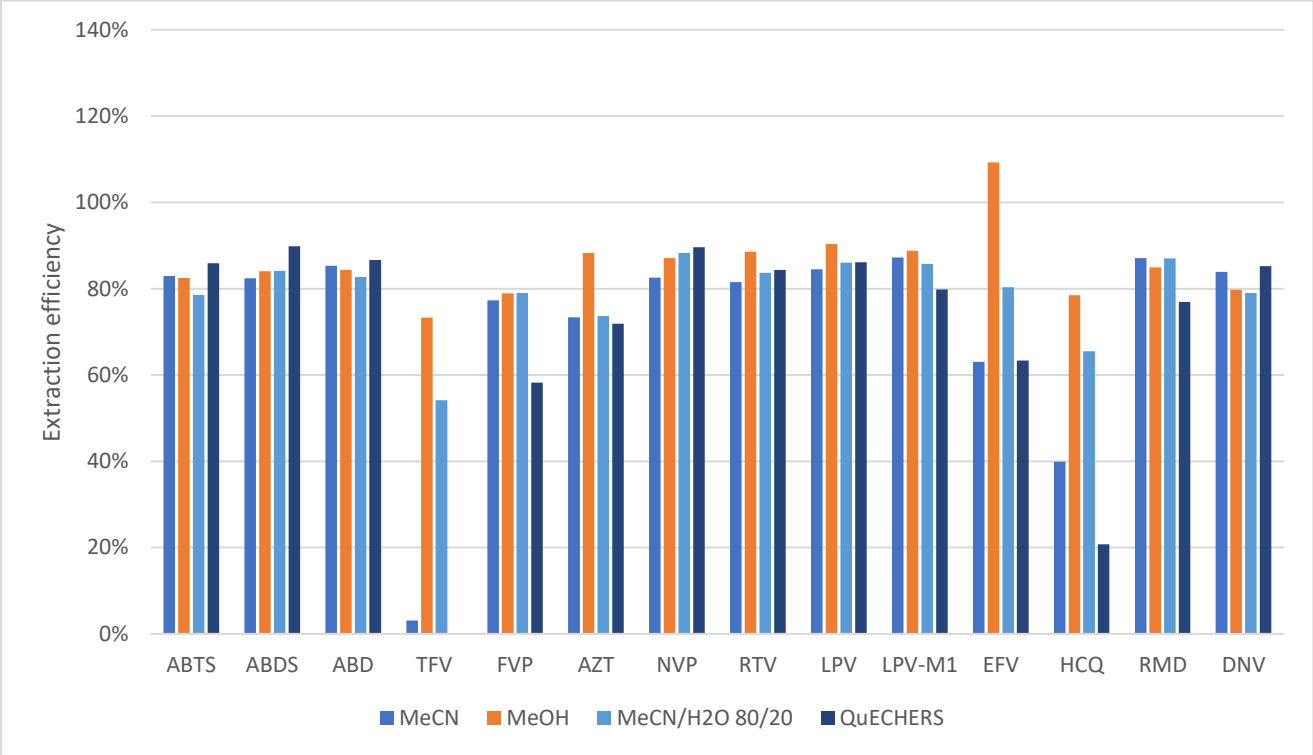

Figure 1. Extraction efficiency when extracting antiviral drugs from pig muscle tissue using various solvent.

Method performance

Prior to validation, performance test were run by injecting matrix-matched calibration curves. The lowest calibrant levels (LCL) were determined as they were equivalent to the limit of quantitation (LOQ). The LCL ranged from 0.2 µg kg^{-1} for arbidol to 5 µg kg^{-1} for efavirenz. Matrix effects ranged from the most suppressed at 41% (favipiravir) to the most enhanced at 146% (hydroxychloroquine) (Table 2).

Table 2. Calibration ranges in pork meat and matrix effects data.

Analyte	Range (µg/kg)	Matrix effects (RSD) (%)
Arbidol	0.2 - 1.6	76 (2.0)
Arbidol Sulfoxide	0.2 - 1.6	81 (0.6)
Lopinavir	0.2 - 1.6	88 (3.8)
Arbidol Sulfone	0.5 – 4.0	82 (3.3)
Nevirapine	0.5 – 4.0	68 (5.7)
Ritonavir	1.0 – 8.0	98 (1.0)
Hydroxichloroquine	1.0 – 8.0	146 (1.1)
Remdesivir	1.0 – 8.0	84 (2.3)
Darunavir	1.0 – 8.0	88 (4.2)
Tenofovir	2.0 – 16.0	54 (2.9)
Favipiravir	2.0 – 16.0	41 (3.2)
Azithromycin	2.0 – 16.0	85 (2.9)
Lopinavir-M1	2.0 – 16.0	86 (4.6)
Efavirenz	5.0 – 40.0	60 (2.9)

RSD: Relative Standard Deviation

Conclusions

A LC-MS/MS method was developed for the quantitative analysis of 15 antiviral drug residues in pig muscle tissue. The proposed sample preparation was a methanol-based extraction followed by evaporation and reconstitution in a MeOH:H_2O mixture. Various reversed phase columns were evaluated and it was found that a Poroshell 120 SB-AQ column led to satisfactory retention time and the overall best peak shapes. When comparing two sources of ionization, it was found that Unispray and electrospray were equivalent in terms of results. The sensitivity of the final method was found to be satisfactory as the LCL ranged from 0.2 µg kg^{-1} to 5 µg kg^{-1}. This method will be subsequently validated following EU guidelines 2021/808.

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POSTER P84

DETERMINATION OF CEFTIOFUR-RELATED RESIDUES IN BOVINE MUSCLE USING VIBRATIONAL SHAKING, MICROWAVE-ASSISTED EXTRACTION AND LC-MS/MS DETERMINATION

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Abstract
In this work, the applicability of vibrational shaking and microwave-assisted extraction (MAE) was evaluated for the determination of ceftiofur-related residues in bovine muscle using derivatisation and stabilisation to desfuroylceftiofur acetamide (DCA), followed by ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) determination. The performance of vibrational shaking was assessed, and compared to horizontal shaking and probe homogenisation. Microwave conditions were optimised to achieve the controlled derivatisation of all residues. DCA was separated from matrix interferences on a BEH C_{18} analytical column, and detected in positive electrospray ionisation mode (ESI+). The method validation showed trueness in the range 105-133%, while precision ranged from 3.5% to 11.9%. Limits of detection (LOD) and quantitation (LOQ) were 25 and 75 µg kg^{-1}, respectively. To the best of our knowledge, this was the first time that vibrational shaking and MAE were evaluated for the analysis of ceftiofur metabolites in food.

Introduction
Ceftiofur is a third-generation broad-spectrum cephalosporin approved for the treatment of respiratory infections and mastitis in swine, beef and dairy cattle (Anon. 1999). While this drug remains primarily as the parent compound in milk after intramammary administration, it is rapidly converted (half-life < 10 min) to a number of metabolites and conjugates following intramuscular and subcutaneous injections, including desfuroylceftiofur (DFC), desfuroylceftiofur cysteine disulfide (DCCD), 3,3-desfuroylceftiofur disulphide (DCD), desfuroylceftiofur thiolacton, desfuroylceftiofur-glutathione-disulphide, and protein-bound DFC (Berendsen *et al.*, 2012; Olson *et al.*, 1998). These metabolites retain the β-lactam ring and, as a consequence, the activity of the parent compound.

The monitoring of the MRL (Maximum Residue Limit) compliance of food-commodities is particularly important for ceftiofur because this drug is also used in human medicine as critically important antimicrobial (Anon. 2007). For this reason, a MRL has been set by Commission Regulation (EU) 37/2010 in milk and tissues from all mammalian food-producing species for the sum (expressed as DFC) of all residues retaining the β-lactam structure (Anon. 2010). Therefore, sensitive and accurate analytical methods are essential for the confirmatory analysis of these residues.

Two main approaches for the LC-MS monitoring of ceftiofur-related compounds in animal tissues have been reported in the literature. The first approach involves the direct analysis of parent ceftiofur and its metabolites, which however lacks the ability of detecting the protein-bound metabolite fraction, leading to an underestimation of the total content of ceftiofur (Feng *et al.*, 2012; Mastovska and Lightfield, 2008). The second approach allows a more accurate quantitation of all protein-bound metabolites and DFC-conjugates, and is based on their extraction and deconjugation using dithioerythriol (DTE), followed by the stabilisation of the resulting free DFC to desfuroylceftiofur acetamide (DCA) using iodoacetamide, which results in its quantitative conversion to DCA (Berendsen *et al.*, 2012; Jiang *et al.*, 2008). In these methods, the deconjugation of the DFC-conjugates to free DFC is achieved by shaking the samples on a mechanical shaker for approximately 15 min, and subsequently incubating them in a water bath at 50°C for 15 min. However, a more controlled extraction temperature can be achieved with alternative techniques, such as microwave-assisted extraction (MAE). The main advantages of MAE are the automated standalone extraction of analytes, the most efficient and rapid heating of samples, the control of the extraction temperature, and the possibility of simultaneously extracting and derivatising multiple samples with minimal labour (Llompart *et al.*, 2019). Therefore, the main objective of this work was to assess the applicability of a vibrational shaker and the

performance of MAE for the extraction of ceftiofur-related residues in bovine muscle tissue using derivatisation and stabilisation to DCA. To the best of our knowledge, this is the first time that these techniques are evaluated for the analysis of ceftiofur metabolites in food.

Materials and Methods

Chemicals, Materials and Apparatus

Ultra-pure water (18.2 MΩ cm^{-1}) was generated in-house using a Millipore water purification system (Cork, Ireland). Methanol (MeOH) and acetonitrile (MeCN) were HPLC grade and purchased from Romil Ltd. (Cambridge, UK). Dimethyl sulfoxide (DMSO), ammonia (NH_3) 32%, 99% acetic acid (CH_3COOH) and ammonium acetate ($C_2H_7NO_2$) were obtained from Sigma-Aldrich (Dublin, Ireland). Dithioerythritol (DTE) and iodoacetamide (IAA) were purchased from Acros Organics (Dublin, Ireland). Ceftiofur and its metabolites (namely desfuroylceftiofur (DFC), desfuroylceftiofur cysteine disulfide (DCCD) and desfuroylceftiofur dimer (DCD)), and desfuroylceftiofur S-acetamide (DCA) were sourced from Toronto Research Chemicals (Toronto, ON, Canada).

Polypropylene tubes (15 mL and 50 mL) with screw caps were obtained from Sarstedt Ltd (Wexford, Ireland). Ceramic homogeniser pellets (15 mL tubes, part number 5982-9312) were purchased from Agilent Technologies Ltd. (Cork, Ireland). Magnetic stirring bars (10 × 3 mm) were obtained from Fisher Scientific (Dublin, Ireland). The solid-phase extraction (SPE) cartridges used for sample preparation were Strata C_{18}-E 500 mg/3 mL and Strata-X-C 33µm Polymeric Strong Cation 300 mg/6 mL, and purchased from Phenomenex Ltd. (Macclesfield, UK). Membrane filters (Captiva Econo Filter PTFE 13m 0.2 µm) were sourced from Agilent Technologies Ltd.

A ME36S microbalance and an A200S digital electronic analytical balance (both from Sartorius, Dublin, Ireland) were used for standard preparation. A Rotanta 460R refrigerated centrifuge (Hettich, Kirchlengern, Germany), a Merris Minimix vibrational shaker (Merris Engineering Ltd., Galway, Ireland), a CEM MARS 6 microwave digestion system (Analytix, Boldon, UK) and a Gerhardt Analytical System shaker (Königswinter, Germany) were employed for sample preparation. An Ultra-Turrax probe blender from IKA (Staufen, Germany) was used for preliminary studies on sample preparation optimisation.

Preparation of Standards and Working Solutions

An individual stock solution of ceftiofur was prepared at a concentration of 0.5 mg mL^{-1} in DMSO. DCCD was dissolved in H_2O:MeCN (50:50, *v/v*) at a concentration of 0.5 mg mL^{-1}, while DCD stock solution was prepared in DMSO at a concentration of 2 mg mL^{-1}. Stock solutions of DFC and DCA were prepared in MeOH at 0.5 mg mL^{-1}. All standard solutions were stored at -80°C in 2.5 mL aliquots in 15 mL polypropylene tubes.

A 1 M ammonium acetate solution, a 0.1 M ammonium acetate solution, a 2% CH_3COOH solution, a 20 mg mL^{-1} DTE solution and a 0.2 M IAA solution were prepared as described by Berendsen *et al.* (2012). A 2% CH_3COOH in H_2O:MeCN (80:20, *v/v*) solution was prepared by diluting 2 mL of CH_3COOH to 100 mL with H_2O:MeCN (80:20, *v/v*). A 1 M ammonium acetate in H_2O:MeCN (85:15, *v/v*) solution was prepared by dissolving 7.71 g of ammonium acetate in 100 mL of H_2O:MeCN (85:15, *v/v*). A 1 M ammonium acetate in H_2O:MeCN (50:50, *v/v*) solution was prepared by dissolving 7.71 g of ammonium acetate in 100 mL of H_2O:MeCN (50:50, *v/v*).

Sample Preparation

Sample (1 ± 0.01 g) aliquots were weighed into 50 mL polypropylene centrifuge tubes. In order to promote the deconjugation of the DFC-conjugates to free DFC, a ceramic homogeniser and a 5 mL aliquot of a DTE solution were added to each tube, and samples subsequently homogenised on a Minimix vibrational extraction system for 1 min. A magnetic stirring road was then added to all tubes, and samples placed in a microwave digestion system. The temperature was set at 30°C, with a ramp time and a hold time of 6 min each. The deconjugated DFC was subsequently stabilised to DCA by adding a 5 mL aliquot of a IAA solution to each tube, and by shaking the samples for 15 min, following by incubation at room temperature for 30 min. Samples were then centrifuged at 3,735 *×g* for 30 min (4°C), and a 5 mL aliquot of the supernatant applied onto the SPE cartridges.

The Strata C_{18}-E cartridges were initially conditioned with 5 mL of MeOH, 5 mL of a 2% CH_3COOH in H_2O:MeCN (80:20 *v/v*) solution, and 5 mL of a 0.1 M ammonium acetate solution. A 5 mL aliquot of the sample extracts was applied onto the cartridges, which were then washed with 5 mL of the 0.1 M ammonium acetate solution, followed by 5 mL of a 2% CH_3COOH solution. DCA was eluted from the cartridges using 10 mL of a 2% CH_3COOH in H_2O:MeCN (80:20 *v/v*) solution, and collected into 15 mL polypropylene tubes. Strata-X-C Polymeric Strong Cation cartridges were subsequently conditioned with 5 mL of MeOH, followed by 5 mL of a 2% CH_3COOH solution. The samples obtained from the previous SPE step were applied onto the cartridges, and washed with

2 mL of MeOH. The cartridges were dried for 5 min using a vacuum pump, and DCA subsequently eluted using 5 mL of a 1 M ammonium acetate in H_2O:MeCN (50:50, *v/v*) solution. A volume of 300 µL of the final extracts was transferred into autosampler vials, and analysed by UHPLC-MS/MS.

UHPLC-MS/MS Conditions

Analysis was performed using a Waters Acquity UHPLC system coupled to a Waters Quattro Premier XE triple quadrupole mass spectrometer equipped with ESI ionisation probe (Milford MA, USA). The UHPLC-MS/MS system was controlled by MassLynx software (V4.1), and the results were processed by TargetLynx software (V4.1). Separation was achieved using a stainless steel Acquity BEH C_{18} analytical column (2.1 x 100 mm, particle size 1.7 µm) fitted with an in-line filter with a 0.2 µm pore size, which was kept in the column compartment at 50°C. Chromatography was performed using a binary gradient of 0.2% NH_3 in H_2O adjusted to pH 8 with 99% CH_3COOH (mobile phase A) and 0.2% NH_3 in MeOH adjusted to pH 8 (mobile phase B). The injection volume was 5 µL in full loop mode (overfill factor = 4). The gradient profile was as follows: 0 – 1.0 min (100% A); 1.0 – 5.0 min (linear decrease to 50% A); 5.0 – 6.0 min (linear decrease to 0% A); 6 – 6.5 min (hold at 0%A); 6.5 – 6.6 (linear increase to 100% A), with a final hold of 2.6 min at 100% A to allow for column re-equilibration. The flow rate was set at 4.0 mL min^{-1}. Samples were kept at 10°C in the autosampler, which was rinsed after each injection using a strong wash (H_2O:MeCN, 20:80 *v/v*, 750 µL) and a weak wash (H_2O:MeCN, 80:20 *v/v*, 1,000 µL).

MS detection was carried out in positive electrospray ionisation mode (ESI(+)). The operating parameters were as follows: capillary voltage 2.7 kV; source temperature 120°C; desolvation temperature 450°C. Nitrogen was used as desolvation and cone gases, with flow rates of 1000 L h^{-1} and 200 L h^{-1}, respectively. The optimised conditions for the ceftiofur metabolites previously described by Di Rocco *et al.* (2017) were added to the acquisition method for sample preparation development, and to verify their quantitative conversion to DCA. The MS tuning for ceftiofur and DCA was performed by injecting 1 µg mL^{-1} aqueous standard solutions with mobile phase A:B (50:50, *v/v*). Acquisition time segment was 0.5-9 min, with DCA eluted at 3.78 min. Inter-scan delay and inter-channel delay were set at 0.005 s. The optimised transitions and parameters are shown in Table 1.

Table 1. MS conditions for ceftiofur and DCA.

Analyte	Measured ion	Precursor ion (*m/z*)	Product ion (*m/z*)	CV (V)	CE (eV)
Ceftiofur	$[M+H]^+$	524	125[a]/241	28	40/17
DCA	$[M+H]^+$	487	167[a]/241	27	24/24

[a] Quantifier ion.

Method Validation

The following parameters were evaluated according to Commission Decision 2002/657/EC (Anon. 2002): identification, linearity, trueness, intra-day repeatability, matrix effect, limit of detection (LOD) and limit of quantitation (LOQ).

Identification was assessed by examining retention times, ion ratios and identification points. The linearity of the calibration curve was considered satisfactory if $R^2 \geq 0.98$ and if individual residuals did not deviate by more than ± 20% from the calibration curve. The trueness and precision of the analytical method were evaluated by using six blank samples fortified at 0.5, 1 and 1.5 times the MRL (1,000 µg kg^{-1}) established by current legislation (Anon. 2010). The matrix effect was investigated by spiking six different muscle samples post-extraction with DCA at the MRL level, and by comparing the signal obtained from those samples to the signal obtained from a standard solution at the same concentration (Trufelli *et al.*, 2011). The LOD and LOQ were estimated from three blank samples fortified at the lowest calibration level, and established at those concentration levels for which the measured signal-to-noise (S/N) ratios would be at least 3 and 10 for the quantifier and the qualifier ions, respectively.

Results and Discussion

Method Optimisation

Optimisation of Sample Homogenisation

The homogenisation and the consequent disruption of tissue samples is essential for the efficient extraction of the drug residues. Although validation studies in most published methods are performed by employing fortified samples, the drug residues are intracellular in the test samples, and could be more difficult to extract (Stolker and Danaher, 2012). In this work, three different approaches (A, B and C) were evaluated for homogenisation by employing three different bovine muscle samples fortified with ceftiofur at 1,000 µg kg^{-1}. Following the addition of 5 mL of the DTE solution, the three methods were applied. With method A, samples were shaken at 200 oscillations min^{-1} on a horizontal shaker for 15 min. With method B, a ceramic homogeniser pellet was added to each tube, and the samples vigorously shaken using a Minimix vibrational shaker. The duration of the homogenisation on the Minimix was also investigated (1, 5 and 8 min). Method C employed an Ultra-Turrax probe blender, with probe homogenisation of individual samples for 20 s. After homogenisation, the samples obtained from all methods were shaken in a water bath at 50°C for 15 min. DFC was subsequently stabilised to DCA by the addition of the IAA solution. The samples were then shaken for 15 min, incubated at room temperature for 30 min, and centrifuged at 3,735 ×*g* for 30 min (4°C). An aliquot of the extracts was subsequently filtered into autosampler vials, and directly injected into the UHPLC-MS/MS system.

The samples obtained by employing method B for 5 and 8 min could not be successfully centrifuged due to the excessive disruption of the matrix, therefore these approaches were immediately discarded. The highest peak area for DCA was obtained when using method B for 1 min, which also led to more reproducible results and the possibility of unattended extraction of 36 tubes at one time, while method C was found to provide the lowest DCA peak area, with also the limitation of having to homogenise one sample at a time. Quantitative conversion of ceftiofur to DFC, and subsequently to DCA, was observed for all the tested methods (Figure 1), which suggested a number of hypothesis explaining the difference in the DCA peak area. In particular, this could be due to the insufficient extraction of the metabolites from the matrix when using the shaker approach or the effect of ion suppression when employing the probe homogenisation, rather than the incomplete conversion of DFC to DCA. Another possible explanation could be the degradation of the analytes, which should be verified by using probe homogenisation over ice, or the degradation of DTE, which could be assessed by post-homogenisation addition. In view of the results obtained, the Minimix vibrational shaker for 1 min was chosen for sample preparation.

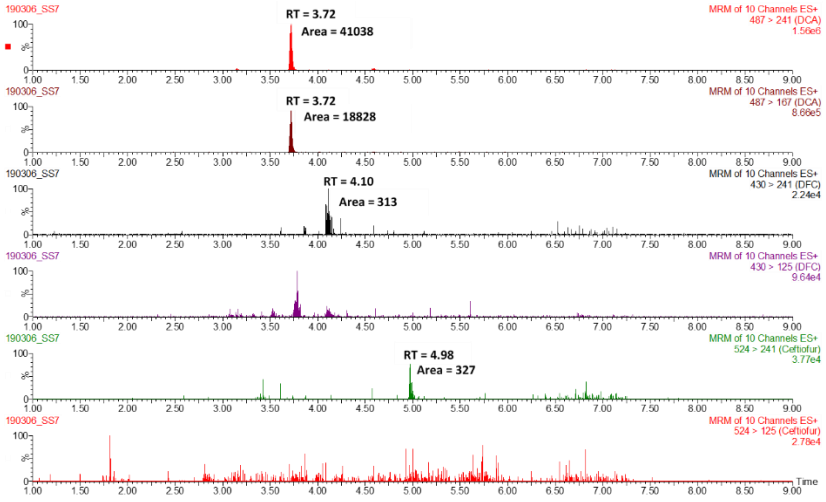

Figure 1. Chromatograms obtained from a muscle sample fortified with ceftiofur at 1,000 µg kg^{-1} and homogenised using the Minimix for 1 min. Ceftiofur and DFC were quantitatively converted to DCA.

Optimisation of Microwave-assisted Extraction

The initial deconjugation of DFC-conjugates to free DFC is particularly important for the subsequent derivatisation to DCA, a more stable marker compound that allows the measuring of the total amount of ceftiofur-related metabolites. As reported in the literature, this step of the sample preparation is normally achieved by shaking the samples and incubating them in a water bath at 50°C for 15 min (Berendsen *et al.*, 2012; Makeswaran *et al.*, 2005). However, due to the heat instability previously reported for β-lactam

compounds (Di Rocco *et al.*, 2017), a temperature of 50°C could cause degradation and result in an underestimation of the total content of residues. In addition, the water bath could lead to an uneven heating, as samples can actually affect the final temperature, and requires a certain heat up time. Furthermore, the oscillation speed can be affected by the weight of the sample tubes. For this reasons, the performance of MAE was evaluated in this work on four different muscle samples, and compared to the incubation of the same samples in a water bath at 50°C for 15 min.

An experimental design generated by Minitab 17 Statistical Software version 17.1.0 (MiniTab Inc., PA, USA) was used to optimise three factors: (1) temperature, (2) ramp time (time to reach the chosen temperature) and (3) hold time (time at the chosen temperature). As the deconjugation of ceftiofur-related compounds is normally performed at 50°C in all published methods (Berendsen *et al.*, 2012; Makeswaran *et al.*, 2005), the temperature was investigated across a range of values between a minimum of 30°C and a maximum of 90°C. The ramp time was assessed in the range 5-10 min to allow the desired temperature to be reached, while the hold time was evaluated between 0 and 20 min, considering that the time reported in the literature for a complete deconjugation is approximately 15 min. A quadratic model was selected to create 20 experimental combinations, which included six centre points to provide a measure of experimental error.

Three muscle sample aliquots fortified with individual compounds (ceftiofur, DCD and DCCD) at 1,000 µg kg^{-1} were analysed by using all the different experimental combinations. The input of the peak areas obtained for DCA from the fortified samples into the MiniTab generates a number of Response Surface Methodology (RSM) plots showing the trend and the optimal values within the established limits for the three parameters (Figure 2). The overall optimum temperature for the deconjugation of the ceftiofur-related compounds to DFC and their extraction from the matrix was determined to be 30°C, with a ramp time of 6 min followed by a hold time of 6 min. However, the lowest temperature was found to provide the optimal response, therefore further investigation would be needed to verify if a temperature < 30°C could result in a higher response.

Four different muscle samples were subsequently fortified with ceftiofur at 1,000 µg kg^{-1}, and the results obtained from the optimised microwave conditions compared to the results obtained on the same samples when using a water bath at 50°C for 15 min. As shown in Figure 3, the CEM microwave provided higher peak areas for DCA, and allowed better reproducibility between samples.

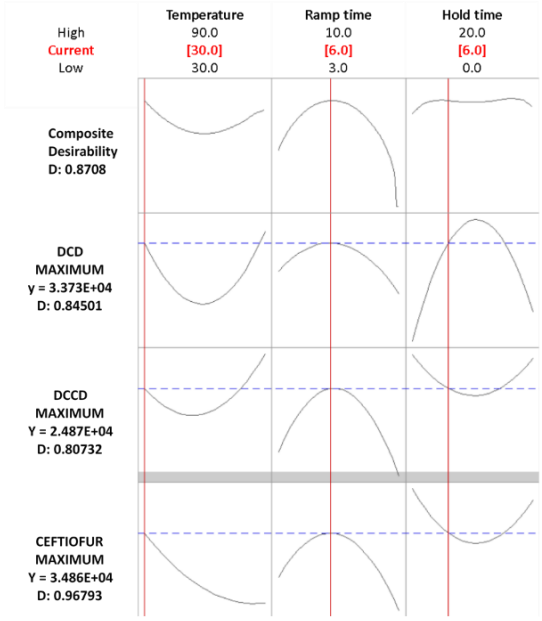

Figure 2. RSM plots obtained from the microwave experimental conditions.

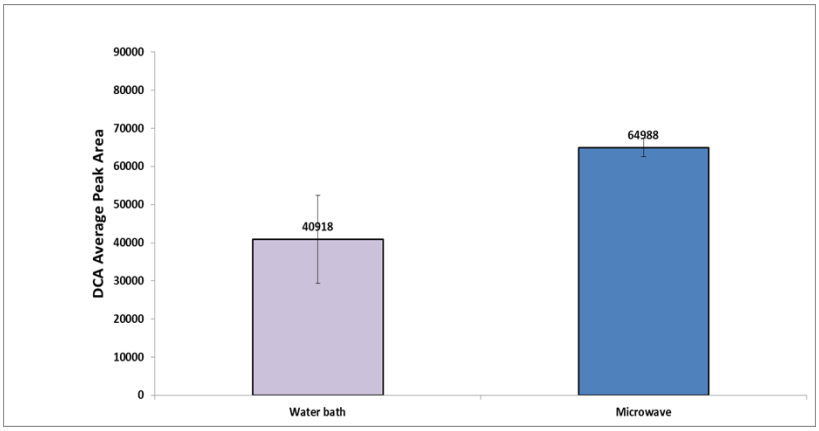

Figure 3. Results obtained from the analysis of samples (n=4) employing water bath and microwave derivatisation. Precision, expressed as standard deviation (± SD), is shown by error bars.

The peak area RSD% (Relative Standard Deviation) was found to be, indeed, 32.7% when using the water bath, while RSD% decreased to 4.6% with the CEM microwave. It could be concluded that the MAE achieved a more uniform heating of the samples, and a more efficient extraction of the residues. The magnetic stirring roads added to the sample tubes allowed for agitation of the tube content to promote deconjugation, while the temperature could be monitored by a probe and maintained at the required value to achieve optimal heating efficiency.

Method Validation

Analyte Confirmation

Analyte confirmation was accomplished according to 2002/657/EC guidelines (Anon. 2002), which require a minimum of three identification points for confirmation of group B substances. In this work, four identification points were obtained for DCA by monitoring two product ions from the 487 *m/z* precursor mass. Retention times and ion ratios were also all within the maximum permitted tolerances (Anon. 2002).

Linearity, LOD, LOQ and Matrix Effect

A matrix-matched calibration curve in the range 100-2,000 µg kg^{-1} was constructed on three different occasions by fortifying seven aliquots of three different negative control muscle samples with ceftiofur standard. The samples subsequently underwent the sample preparation protocol to obtain the marker compound DCA. Satisfactory linearity was achieved for DCA over the calibration range of the method, as $R^2 \geq 0.99$ were obtained on all occasions, with individual residuals in the ± 20% range of deviation tolerance from the calibration curve.

The LOD and LOQ values were estimated based on the signal-to-noise (S/N) ratios observed for the first calibration point on three different occasions, and set at 25 µg kg^{-1} and 75 µg kg^{-1}, respectively.

The matrix effect study showed 25% matrix suppression, with 9.3% variability (RSD%) between the six different muscle samples. This variability could be potentially reduced by implementing an internal standard into the method, ideally a deuterated internal standard.

Trueness and Precision

The trueness of the analytical method at 0.5, 1 and 1.5 times the MRL established for ceftiofur (1,000 µg kg^{-1}) was found to be 105, 117 and 133%, respectively. The precision, expressed as the intra-day repeatability between six samples fortified at the three validation levels, was found to be 3.5, 8.8 and 11.9%, respectively. Although precision satisfied the 2002/657/EC guidelines at all validation levels, the trueness of the method was above the recommended values (range 80-110%) at 1 and 1.5 times the MRL, and could be further improved with the addition of an internal standard into the method.

Conclusions

A derivatisation procedure was optimised for the simultaneous analysis of all ceftiofur-related metabolites in bovine muscle using vibrational shaking and MAE, followed by UHPLC-MS/MS determination. The conversion of the DFC-conjugates to DCA provided a more stable marker compound, and allowed the accurate measuring

of the total amount of ceftiofur-related residues. The application of the vibrational shaker combined to MAE was found to promote sample homogenisation and the deconjugation of the DFC-conjugates. Validation studies showed that trueness was in the range 105-133%, while precision ranged between 3.5% and 11.9%, demonstrating that the method represents a valid approach for the determination of ceftiofur residues in bovine muscle samples. To the best of our knowledge, this was the first time that the vibrational shaker and MAE were applied to a derivatisation procedure for the analysis of ceftiofur and its metabolites.

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POSTER P85

DETECTION AND QUANTIFICATION OF IGF-1 AND IGF-2 IN BOVINE PLASMA BY LC-MS/MS

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Abstract
Proteins and peptides with growth-promoting properties are a class of compounds that can be illegally used by athletes and farmers looking for prohibited enhancement muscle growth. IGF-1 and IGF-2 proteins are insulin-like growth factors with high similarity to insulin are key mediators of growth hormone action and biomarkers for growth hormone abuse.
Here, we developed a method for quantification of endogenous hormones IGF-1 and IGF-2 in bovine serum samples by selected reaction monitoring LC-MS/MS.
IGF proteins circulate in serum as a complex with IGF-binding proteins, therefore prior to extraction, the proteins are released from the complex by using 1% acetic acid. High abundant proteins are removed by protein precipitation with acetonitrile and the supernatant is reduced with dithiothreitol, alkylated with iodacetamide and digested with trypsin. Single unique tryptic peptides are used for protein quantification. Synthetic stable isotope labelled peptides homologues are assisting in identification and for ion signal correction. Validation and quantification is performed using recombinant IGF-1 and IGF-2, via the construction of a calibration curve.
This high-throughput method proved to be sufficiently sensitive to obtain quantitative information for both IGF-1 and IGF-2 in a single analysis and provides important information for detection of growth hormone abuse in bovine.

Keywords: IGF, LC-MS, proteins

POSTER P86

DETERMINATION OF QUINOLONES, MACROLIDES, SULFONAMIDES AND TETRACYCLINES IN HONEY- QUECHERS SAMPLE PREPARATION AND UHPLC-MS/MS ANALYSIS

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Abstract

Monitoring the health of the beehive and timely prevention of any possible infection with various bacteria, mould, viruses or parasites is exceptionally important in beekeeping. Antibiotics may be found in honey originating from the environment and resulting from improper beekeeping practices. Enabling the detection of antibiotic residues in honey and suppressing the development of antibiotic resistance, require the development of a sensitive multi-class method for the determination of antibiotics. For the purpose of analysing honey, a screening and confirmatory method for the determination of 36 antibiotics was developed. The QUECHERS procedure and ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) was selected to achieve high sensitivity and selectivity. Validation according to the new Commission Implementing Regulation (EU) 2021/808 included following performance characteristics: selectivity, trueness, precision, decision limit (CCα), detection capability (CCβ) and relative matrix effect. The method was validated in the measurement range from 1 to 20 µg/kg, where maximum trueness and acceptable coefficients of variation were achieved. For unauthorized pharmacologically active substances, the CCα was determined, ranging from 0.16 µg/kg for sulfaquinoxaline to 3.67 µg/kg for difloxacin. The high matrix influence of floral and chestnut honey indicated the need for quantitative analysis by using the matrix calibration curve.

Introduction

Honey bees are considered to be the biological indicators of environmental contamination. As a consequence of industrialization and intensive agriculture, honey can be contaminated by antibiotics, pesticides, heavy metals, bacteria and radioactive metals (Reybroeck et al., 2012).

The digestive system of bees is potentially the largest source of microbial contamination of honey since it contains 1% yeast, 27% Gram-positive bacteria (*Bacillus, Bacteridium, Streptococcus, Clostridium spp*) and 70% Gram-negative bacteria (*Achromobacter, Citrobacter, Enterobacter, Erwinia, Escherichia coli, Flavobacterium, Klebsiella, Proteus, Pseudomonas*). Nevertheless, the antimicrobial properties of honey and low percentage of water (<20 %) prevent the growth of some bacteria or their reproduction in honey (Al-Waili et al., 2012).

For centuries, honey bees have been domesticated to live in artificial hives intended for honey production. The first application of antibiotics in beekeeping dates back to the 1940s, aiming to prevent the infection with different types of disease and the growth of parasites that the bees are exposed to. The most common diseases among bees are the American foulbrood, caused by the spore-forming *Paenibacillus larvae*, the European foulbrood, caused by *Malissococcus plutonius,* and the nosemosis, caused by the spores of the microsporidian fungi *Nosema apis* or *Nosema ceranae* (Barganska et al., 2011).

According to the Rapid Alert System for Food and Feed (RASSF), the most used antibiotics in honey and bee products belong to the groups of macrolides, tetracycline and sulphonamides, with special concerns being raised by the toxic antibiotics belonging to the group of nitrofurans and phenicols. The antibiotic residues range from the high concentrations that may be detected a week following the treatment, to the low ones that could be detected even a year after application (Reybroeck et al., 2018). The use of antibiotics in apiculture may not only cause damage to the consumers, but also leads to the disruption of the gut microbiota of treated bees. The tetracycline treatment negatively affects the function of the gut microbiota and subsequently impacts the physiology of their host (Jia et al., 2022).

The Council Directive 96/23/EC regulates the national monitoring plan for honey and honey products in Croatia. With regard to the antibiotic residues in honey, this plan includes the following groups of analytes: antibacterial substances from group B1 and prohibited substances from group A6. The reference points for

action (RPAs) were set in the Commission Regulation (EU) 2019/1871 with the aim of regulating substances for which no MRLs have been established, which is the case for antibiotics in honey. The RPAs have been established in honey only for chloramphenicol and nitrofurans. For other antibacterials in honey, several European countries (Switzerland, Belgium, United Kingdom) have established the action limits, generally between 10 to 50 µg/kg for all antibiotics (Al-Waili et al., 2012). In order to avoid toxicological effects on consumers and to slow down the development of antibiotic resistance, a highly selective, precise and accurate methodology, using QUECHERS extraction procedure and UHPLC/MS/MS technique, was developed. The aim of this paper is to present the validation of the method for the analysis of 36 antimicrobial substances belonging to the groups of quinolones, macrolides, sulphonamides and tetracyclines.

Materials and Methods

Chemicals and standards

All reagents were of analytical, HPLC or LC-MS grade. ULC/MS-CC/SFC grade acetonitrile and methanol were purchased from Biosolve Chimie (Dieuze, France). Honeywell Burdick Jackson (Michigan, USA) supplied acetic acid Puriss. p.a., formic acid LC-MS Ultra, dimethyl sulfoxide Chromasolv plus for HPLC and sodium sulfate ACS. Nitrogen 5.0 and 5.5 were acquired from SOL spa® (Monza, Italy). Ultra-pure water was generated by using the Milli-Q system (Millipore®, Bedford, USA). The QUECHERS Bond Elut d-SPE for drug residues was acquired from Agilent (Agilent Technologies, Santa Clara, USA).

The standards of chlortetracycline hydrochloride, ciprofloxacin, danofloxacin, dapsone, difloxacin hydrochloride, enrofloxacin, erythromycin A dihydrate, flumequine, lincomycin hydrochloride, marbofloxacin, nalidixic acid, oxolinic acid, oxytetracycline hydrochloride, sulfachloropyridazine, sulfadiazine, sulfadimethoxine, sulfadoxine, sulfaguanidine, sulfamerazine, sulfameter, sulfamethazine, sulfamethoxazole, sulfamethoxypyridazine, sulfamonomethoxine, sulfaquinoxaline, sulfathiazole, sulfisoxazole, sulfabenzamide, sulfacetamide, sulfaclozine, Sulfamethizol, sulfamoxole, sulfapyridine, sulfasalazine, sulfatroxazole, sulfisomidine, tetracycline hydrochloride, tylosin tartrate, and sodium chloride ACS were acquired from Merck KGaA (Darmstadt, Germany). Enrofloxacin-D5, sulfamethazine D4 and tetracycline D6 were acquired from Toronto Research Chemicals (North York, ON, Canada).

Equipment

The following equipment was used in sample preparation: Waring Commercial Blender 7011HS (Waring Commercial, CT, USA), IKA® Vortex model MS2 Minishaker (IKA®-WERKE GMBH & CO.KG, Staufen, Germany), multi-tube vortexer (VWR International GmbH, Ulm, Germany), ultrasonic bath Grant (Grant instruments, Cambridge, UK), centrifuge Rotanta 460R (Hettich Zentrifugen, Tuttlingen, Germany), ultracentrifuge (Thermo Fisher Scientific, Waltham MA, USA) and MultiVap 54 automatic evaporation system (LabTech Srl, Sorisole (BG), Italy). The LC-MS/MS system consisted of UHPLC Agilent Technology 1290 Infinity II and Triple Quad LC/MS 6470 mass spectrometer with Jet Stream Technology Ion Source (AJS ESI) (Agilent Technologies, Santa Clara, USA). Instrument control and data processing were performed by using MassHunter Workstation 10.0 software developed by Agilent Technologies, Inc. For chromatography, an Acquity UPLC HSS T3 column (1.8 µm, 2.1x150 mm) was used (Agilent Technologies, Santa Clara, USA).

Standard solution and calibration

Individual standard stock solutions (1 mg/ml) were prepared by using different solvents. Quinolones were deluted in 1 ml 1M NaOH and by adding 9 ml of MeOH, with the exception of flumequine which was prepared by using DMSO. Macrolides, lincosamides, tetracyclines and sulphonamides stock solutions were prepared in methanol, with the exception of sulfadiazine (1M NaOH) and sulfaquinoxaline (1 ml 0.5 M NaOH and 9 ml MeOH). These stock solutions were stored at -20°C. An intermediate stock solution as a mixture of all antibiotic groups was prepared in methanol at 10 µg/ml. Two working solutions were prepared for standard addition at the concentration of 1 µg/ml and 0.1 µg/ml. The working solution of labelled internal standards was prepared in methanol by the using stock solutions of enrofloxacin-D5, sulfamethazine-D4, tylosin-D3 and tetracycline-D6 at the concentration of 2 µg/ml. The intermediate and working standard solutions are stable for one month period.

Sample preparation

Blank honey samples were collected during national monitoring. Floral and chestnut honey were tested to detect the possible differences in the matrix effect. For the purpose of the extraction procedure, 2 g (±0,05) of honey was weighted into a 50 ml polypropylene centrifuge tube and 150 µl of the IS working solution was added to each sample and vortexed. For the calibration purposes, honey samples were spiked at the following

concentrations: 0.1, 0.25, 0.5, 0.75, 1, 2.5, 5, 10 and 20 µg/kg. 10 ml of 1% acetic acid in ACN/H2O (v/v, 80:20) was added to each sample for the extraction process and vortexed for 10 min. The excess water was removed by adding 4 g of Na2SO4 and 1 g of NaCl. Following the centrifugation at 4500 rpm, 6 ml of ACN aliquot was transferred to another tube containing Bond Elut dSPE salts. After the samples were vortexed and centrifuged, 4 ml of supernatant and 50 µl of DMSO was gradually evaporated by using N2 at 40 °C. A dry residue of evaporation was diluted in 0.5 ml of 2:8 ACN/H2O and centrifuged at 10,000 rpm. Prepared extracts were transferred to the HPLC vials.

Chromatography and MS/MS conditions

Chromatography was performed by using Agilent Technology UHPLC 1290 Infinity II and Triple Quad LC/MS 6470 mass spectrometer with Jet Stream Technology Ion Source (AJS ESI). The analytes were separated by using Acquity UPLC HSS T3 column (1.8 µm, 2.1x150 mm) with a specific guard pre-column and thermostated at 40 °C. Samples were cooled in an autosampler at 10°C and the injection volume was 10 µl including a needle wash function. The mobile phase consisted of 0.1% formic acid (A) and LC-MS grade methanol (B) with the following gradient steps: 0–3 min 100% A; 3-10 min to 95% B; 10-13 min held at 95% B; 13-13.5 min to 100% A, completed by equilibration until the 17th min.

Scanning was performed by using the multi reaction monitoring method with at least two transition ions, which, together with the retention time and the ion ratio, have met the criteria for the identification and confirmation of the analyte. The mass spectrometry scanning parameters in positive ionization mode were as follows: gas temperature 150°C, gas flow 11 l/min, nebulizer 35 psi, sheath gas temperature 300°C, sheath gas flow 6 l/min, capillary voltage 4000V, and nozzle voltage 500V. Fragmentor voltages and collision energies were optimized for each analyte (Table 1).

Validation

According to the Commission Implementing Regulation (EU) 2021/808 (European Commission, 2021), the method was validated as a quantitative confirmatory method. Since no MRLs have been established for antibiotics in honey, the limits selected for the method validation ranged from 0.1 µg/kg to 20 µg/kg. The blank samples were fortified in equidistant concentration levels (LCL, 2xLCL, 3xLCL). In order to determine selectivity, trueness, repeatability, within lab-reproducibility and decision limit (CCα), 3 validation series comprising 7 different batches at six different levels were analysed.

Table 1. Mass spectrometry parameters for 36 antibiotic substances

Analyte	Precursor m/z	Product ion m/z			Retention time	Fragmentor (V)	Collision energy (eV)
Fluoroquinolones							
Ciprofloxacin	332	314	288	231	7.4	135	20/20/40
Danofloxacin	358	340	314	82	7.4	135	20/16/48
Difloxacin	400	382	356	299	7.5	135	20/16/32
Enrofloxacin	360	342	316	245	7.4	135	20/16/32
Flumequin	262	244	202	174	9.5	135	10/40/40
Marbofloxacin	363	345	320		7.0	135	20/10
Nalidixic acid	233	215	187	159	9.4	135	10/20/40
Oxolinic acid	262	244	216	172	8.8	135	10/40/40
Sarafloxacin	386	368	342	299	7.6	135	20/20/40
Enrofloxacin-d5	365	347	321	288	7.4	135	20
Macrolides and lincosamides							
Erythromycin A	734	576	158		9.3	135	20/20
Lincomycin	407	359	126		6.9	135	10/20
Tylosin A	916	772	174		9.3	135	20/40
Erythromycin-D6	740	582			9.4	135	20
Tylosin-D3	919	775			9.4	135	20
Tetracyclines							
Chlortetracycline	479	462	444	154	8.17	140	20/20/40
Oxytetracycline	461	443	426	201	7.4	135	6/14/48
Tetracycline	445	427	410	154	7.3	135	10/20/40
Tetracycline-d6	451	433	416		7.4	135	10
Sulfonamides							
Sulfabenzamide	277	156	108	92	8	135	10/20/20
Sulfacetamide	215	156	108	92	5.9	135	10/20/20
Sulfachloropyridazine	285	156	92		7.6	135	12/24
Sulfadiazine	251	108	92		6.5	135	20/28
Sulfadimethoxine	311	156	92		8.5	135	16/36
Sulfadoxine	311	156	108	91	7.9	135	16/28/32
Sulfaguanidine	215	156	92		2.9	135	10/20
Sulfamerazine	265	156	92		7.0	135	12/28
Sulfamethazine	279	186	124	92	7.4	135	12/24/32
Sulfamethizol	271	156	108	92	7.2	135	10/20/20
Sulfamethoxypyridazine	281	156	92		7.4	135	12/35
Sulfamethoxazole	254	156	92		7.7	135	12/24
Sulfamonomethoxine	281	156	126	108	7.8	135	20/20/40
Sulfamoxol	268	156	113	108	7.2	135	20/20/20
Sulfapyridine	250	184	156	108	6.7	135	10/20/20
Sulfaquinoxaline	301	156	108	92	8.6	135	16/28/32
Sulfasalazine	399	156	108	92	9.8	135	20/24/32
Sulfathiazole	256	156	92		6.6	135	12/28
Sulfatroxazole	268	156	108	92	7.7	135	20/20/20
Sulfisomidine	279	124	92		6.4	135	20/40
Trimethoprim	291	123	110	81	7.0	135	40/40/40
Sulfamethazine-d4	283	186	160	124	7.4	135	12

Results and Discussion

Selectivity was tested by analysing 20 blank samples of polyfloral and chestnut honey, where no interfering compounds from the matrix were found in the retention time of the analyte. According to Commission Implementing Regulation (EU) 2021/808 (European Commission, 2021) all validation parameters complied with

the set requirements. Validation parameters were calculated using validation software for comprehensive in-house validation InterVal Plus® and it consists of the following experiments: linearity, specificity/selectivity, repeatability and inter-laboratory reproducibility (Rel sR), decision limit (CCα) and detection capability (CCβ) (Table 2). Honey samples were spiked prior to extraction using a standard mixture of antibiotics and the measured response for analyte corrected by the internal standard was plotted against the expected concentration.The satisfactory linearity of the method was achieved for all analytes in the range from 1 to 20 µg/kg. Due to the high sensitivity of the method for erythromycin, nalidixic acid, tylosin A, and sulphonamides, linearity was tested in the range from 0,1 to 1 µg/kg, where coefficients of determination (R2) ranged from 0,96 to 0,98. According to the fortified samples at the lowest calibration level (LCL), two times LCL and 3xLCL decision limit was calculated according to the CIR 2021/808 for the unauthorized or prohibited pharmacologically active substances as CCα=LCL+k*standard measurement uncertainty at LCL.

Significant matrix effects were noticed during the analysis of different species of honey samples fortified at LCL concentration. It was noticed that slopes of the matrix calibration curves deviate significantly between floral and chestnut honey. The highest deviations were noticed for SSZ, SDX, DAN, SDZ, CLTTC, SGN, SAC, and OXOA with the deviation between 71 and 121 %. According to these findings, specific calibration curves are used for each honey type.

Table 2. Validation parameters calculated according to CIR 2021/808

Analyte		Calibration interval µg/kg	CCα µg/kg	CCβ µg/kg	Recovery [%] at CCα	Rel sR [%] at CCα
Fluoroquinolones						
Ciprofloxacin	CIPRO	1.0 - 20	1.34	1.86	96.6	9.9
Danofloxacin	DAN	1.0 - 20	1.12	1.57	98.7	7.7
Difloxacin	DIFLOX	1.0 - 20	3.67	5.60	101.2	16.4
Enrofloxacin	ENR	1.0 - 20	2.53	3.73	104.1	12.3
Flumequin	FLU	1.0 - 20	2.08	3.06	102.5	11.4
Marbofloxacin	MARB	1.0 - 20	2.16	3.12	99.7	12.3
Nalidixic acid	NALA	0.1 - 20	0.29	0.43	100.1	15.1
Oxolinic acid	OXOA	0.75 - 20	1.15	1.60	102.7	7.8
Sarafloxain	SAR	0.25 - 20	0.61	1.11	100.2	18.4
Macrolides and lincosamides						
Tylosin A	TYL	0.1 - 20	0.54	0.76	101	13.0
Erythromycin A	ERY	0.1 - 20	0.31	0.46	99.8	15.3
Lincomycin	LINC	1.0 - 20	1.83	2.53	100.5	18.5
Tetracyclines						
Tetracycline	TTC	0.25 - 20	0.51	0.70	101.9	11.9
Chlortetracycline	CLTTC	0.5 - 20	1.04	1.58	101.2	13.6
Oxytetracycline	OXYTTC	0.5 - 20	0.83	1.18	98.6	10.3
Sulfonamides						
Sulfabenzamide	SBZ	0.1 - 20	0.22	0.30	100.4	12.4
Sulfacetamid	SAC	0.1 - 20	0.18	0.25	103.4	10.9
Sulfachloropyridazine	SCP	0.1 - 20	0.23	0.32	100.8	12.6
Sulfadiazine	SDZ	0.1 - 20	0.13	0.16	100.6	5.8
sulfadimethoxine	SDM	0.1 - 20	0.13	0.16	99.6	5.7
Sulfadoxin	SDX	0.1 - 20	0.17	0.22	99.3	9.4
Sulfaguanidine	SGN	0.1 - 20	0.70	1.20	99.9	13.1
Sulfamerazine	SMR	0.1 - 20	0.27	0.39	100.2	14.3
Sulfamethazine	SMZ	0.1 - 20	0.23	0.32	100.9	12.5
Sulfamethizol	SMZOL	0.1 - 20	0.18	0.24	99.5	10.6
Sulfamethoxypyridazine	SMP	0.1 - 20	0.20	0.28	100.4	11.6
Sulfametoxazole	SMX	0.25 - 20	0.45	0.58	100.6	9.6
Sulfamonomethoxine	SMM	0.1 - 20	0.25	0.36	100.2	13.5
Sulfamoxole	SMOX	0.1 - 20	0.48	0.76	100	17.8
Sulfapyridine	SPYR	0.1 - 20	0.23	0.33	100.4	12.9
Sulfaquinoxaline	SQX	0.1 - 20	0.16	0.21	100.7	8.6
Sulfasalazine	SSL	1.0 - 20	3.26	5.07	94.2	18.7
Sulfathiazole	STZ	0.1 - 20	0.17	0.22	100.4	9.1
Sulfatroxazole	STROX	0.1 - 20	0.23	0.31	99.6	12.8
Sulfisomidine	SSOM	0.25 - 20	0.30	0.34	98.2	4.1
Trimethoprim	TMP	0.1 - 20	0.38	0.58	100.1	16.5

Conclusion

The proposed screening and confirmation method based on the UHPLC-MS/MS technique was successfully developed and validated for screening and confirmation of quinolones, macrolides, lincosamides, sulphonamides, and tetracyclines. The QUECHERS extraction procedure allows for fast and specific extraction of the selected analytes, ensuring high sensitivity of the method and ability to determine residues in the range from 0.1 to 20 µg/kg. The validation procedure included selectivity, trueness, repeatability, within lab-reproducibility, and decision limit (CCα). In the routine use of the methodology, the results of the analyses shall be considered non-compliant when equal to or above the decision limit for confirmation (CCα).

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POSTER P87

DEVELOPMENT AND VALIDATION OF A CONFIRMATORY LC-MS/MS METHOD FOR 15 ANTIVIRAL DRUGS IN POULTRY MUSCLE

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Abstract

In this work, a method was developed to analyse 15 antiviral drug residues in poultry muscle tissue using liquid chromatography coupled to tandem mass spectrometry. The most challenging aspect of this work was the identification of a suitable and robust analytical column that could provide adequate retention and separation of both highly polar and less polar antiviral drugs in a single chromatographic run. Several different column chemistries were evaluated including reversed-phase, phenyl and various HILIC (hydrophilic interaction chromatography) phases. An amide column operating in HILIC separation mode was the only column that provided adequate retention and best separation of the highly polar analytes. A range of different sample preparation procedures were investigated for selective isolation of antiviral drug residues from muscle tissue. A mixture of acetonitrile:H_2O (80:20, *v/v*) was found to be the most effective extraction solvent, with extraction recoveries ranging from 42% to 81%. This step was followed by a dilution in a methanol/water solution. The method was validated according to the EU 2021/808 guidelines on various species including chicken, duck, turkey and quail. Decision limits (CCα) ranged between 0.12 µg kg^{-1} (arbidol) and 34.7 µg kg^{-1} (favipiravir). The method was applied to a total of
120 retail samples from the Irish market, which were all found to be residue-free.

Introduction

Avian influenza outbreaks represent a growing problem for intensive poultry production and for wild bird species. Different strains of avian flu have been identified, and can spread to humans causing serious illness or fatalities (Lai *et al.* 2016). In the last 20 years, concerns have been raised over the use of antiviral drugs in poultry farming, and over the generation of drug-resistant influenza strains (Wan *et al.* 2013). As a consequence, these drugs are not licensed for treating influenza infections in food producing animals. It is also suggested that the monitoring of antiviral residues in food of animal origin will likely be introduced in newly proposed legislation on EU National Residue Control Plans.

Various screening methods have been developed in the last decade for the analysis of antiviral drug residues in poultry. these fast screening tests can be sensitive, but are unable to provide quantitative results and are often restricted to a limited number of analytes (Zhang *et al.* 2021). as Alternatively, LC-MS/MS (liquid chromatography coupled to tandem mass spectrometry) have been reported in the literature but involve laborious sample preparation procedures (Berendsen *et al.* 2012) or include a limited range of compounds only (Yan *et al.* 2013; Wu *et al.* 2014; Tsuruoka *et al.* 2017). This is mostly due to the different polarity of these drugs, which ranges from highly polar to relatively non-polar.

The main objective of this study was to develop a fast and comprehensive LC-MS/MS method for the confirmatory analysis of influenza antiviral drug residues and their metabolites in poultry muscle. Fifteen antiviral drugs, including compounds ranging from highly polar to non-polar, were included in the method, and a detailed study was performed to assess the suitability of different stationary phases to achieve the satisfactory separation of all analytes over a 13 min chromatographic run. The method was successfully validated according to the 2021/808EU guidelines (EU 2021), and proved to be fit-for-purpose. Further objectives were the application of the method to poultry samples purchased from the Irish market, and the investigation of the stability of the residues in matrix when stored at different temperatures. To the best of our knowledge, this is the first time that such a diverse range of antiviral drugs have been analysed in a single method.

Materials and Methods

Chemicals and standard

Amantadine, rimantadine hydrochloride, acyclovir, ganciclovir, oseltamivir phosphate, ribavirin, zanamivir hydrate, arbidol hydrochloride, favipiravir, peramivir and osetlamivir acid were supplied by Carbosynth (St-Gallen, Switzerland). Viramidine hydrochloride, arbidol sulfoxide, arbidol sulfone, laninamivir, amantadine-d_{15} hydrochloride, arbidol-d_6, oseltamivir-d_3 phosphate, oseltamivir-d_3 acid, ganciclovir-d_5, zanamivir-$^{13}C^{15}N_2$ and ribavirin-$^{13}C_5$ were sourced from Toronto Research Chemicals (North York, Canada). Methanol (MeOH) and acetonitrile (MeCN) were purchased from Romil (Cambridge, UK), while dimethyl sulfoxide (DMSO) was sourced from Sigma Aldrich (Dublin, Ireland). Ultra-pure water was in-house generated using a Merck Millipore system (Cork, Ireland). Formic acid and ammonium acetate were respectively supplied by Honeywell Fluka (Seelze, Germany) and VWR International (Leuven, Belgium). Stock standard solutions of 1mg mL^{-1} were prepared by dissolving each solid standard in MeOH, H_2O or DMSO, depending on their respective solubility. Intermediate, working and internal standards were prepared in MeCN:H_2O (50/50, *v/v*). All solutions were found to be stable for at least three months when stored at -30°C.

Sample preparation

Muscle tissue (5.0 ± 0.05 g) aliquots were weighed into 50 mL polypropylene tubes. A volume of 50 µL of internal standard was added to all samples, and a volume of 100 µL of the working calibrant standard solutions was added to the calibrant samples. A ceramic homogenizer and 20 mL volume of MeCN:H_2O (80:20, *v/v*) were added to each tube, which were then shaken on a vibrational shaker for 1 min. The samples were subsequently centrifuged at 2725 ×*g* at -4°C for 10 min. A 1 mL aliquot of the supernatant was diluted in a 15 mL tube with 0.6 mL volume of MeOH:H_2O (2:1, *v/v*). The tubes were vortexed for 30 sec and filtered through 0.2 µm PTFE syringe filters. A 2µL volume of the filtered samples was injected onto the LC-MS/MS system.

LC-MS/MS analysis

Sample analysis was perfomed using a Waters Acquity I Class UPLC system coupled to a Waters Xevo TQ-XS triple quadrupole mass spectrometer equipped with UniSpray™ and electrospray ionisation probes (Milford, USA). Separation was carried out on a BEH Amide HILIC column (100 mm x 2.1 mm, 2.7 µm) maintained at a temperature of 50°C. The separation was carried out using a binary gradient system including mobile phase (A) 0.1% formic acid and 2 mM ammonium acetate in H_2O and mobile phase (B) 0.1% formic acid and 2 mM ammonium acetate in MeCN:H_2O (95:5, *v/v*) at a 0.3 mL min^{-1} flow rate. The MS source parameters were optimised to achieve satisfactory overall sensitivity (Table 1).

Validation and data analysis

The validation of the method was carried out following the 2021/808 EU guidelines (EU 2021). The selectivity, matrix effects, trueness, repeatability (WLr) and the within-laboratory reproducibility (WLR) were assessed. The validation levels for these unauthorised drugs were selected based on the lowest calibrant level (LCL), depending on the sensitivity of each analyte. The validation levels were 1.0, 2.0 and 3.0 times the LCL. The reproducibility study was carried out using a total of 63 poultry samples, on three different days, with seven samples for each level analysed by three different analysts. The matrix effect study and the selectivity run were performed using 22 and 21 poultry samples, respectively. Chicken, duck, turkey and quail samples were included in the validation process. Measurement uncertainty values were calculated from the combination of the coefficient of variation (CV) of the WLR study at the LCL and the standard error of the absolute recovery. Decision limits (CCα) were calculated following method 3 of the 2021/808 guidelines (LCL + k(one-sided, 99%) x (combined) standard measurement uncertainty at LCL). A stability study was also carried out using samples fortified at four times the LCL and stored at -28°C and -80°C. These samples were tested in five replicates at weeks 0, 1, 3, 5 and 10. The calculated mean concentrations were compared to the concentrations determined at week 0.

Sample survey

A total of 120 poultry meat products were purchased in five different supermarkets in the Republic of Ireland in 2020. The survey samples included 29 non-EU and 91 EU/UK products, and were analysed using the proposed method.

Results and Discussion

Method development

The most sensitive and/or selective transitions were identified during MS optimisation studies, and selected by avoiding non-specific transitions when possible. The transitions chosen were largely in agreement with previously published papers (Chan *et al.* 2011; Berendsen *et al.* 2012). Two different ionization sources, namely, Electrospray and UniSpray™, were evaluated for the analysis of antiviral drugs. In agreement with previous studies (Lubin *et al.* 2017), UniSpray™ was found to be more sensitive than electrospray at lower flow rate and higher aqueous content in the mobile phase. UniSpray™ was therefore advantageous when used in the chromatographic reversed-phase mode, while electrospray was more suitable when carrying out chromatography in HILIC mode.

The antiviral drug class have a broad polarity range (from a log P= -5.8 for zanamivir to a log P= 3.8 for arbidol), as well as the heterogeneity of the analyte selection in terms of acid-base properties, presented a challenge during the chromatographic method development. Various reversed-phase columns were first screened, including C_{18} columns as well as alternative reversed-phase columns such as phenyl-hexyl or PFPP (pentafluorophenyl propyl). The latter was found to give the best retention of the most hydrophilic compounds including zanamivir and ribavirin, due to the phenyl π-π interactions and the fluorine function dipole-dipole interactions. However, chromatographic retention deemed to be unsatisfactory for the most polar compounds, which exhibited retention factors of 0.5, while a factor 2 is required and could only be achieved using heptafluorobutyric acid (HFBA) as an ion-paring agent. Nevertheless, the use of HFBA caused ion suppression through source contamination and therefore was not considered as a viable option.

HILIC was selected as an alternative mode of separation because it is known to provide better retention of highly polar molecules that cannot be retained using reversed-phase chromatographic systems. A range of different stationary phases including PFPP, HILIC-Z and BEH Amide were evaluated in HILIC separation mode. As expected, it was found that phases based on partitioning-based retention mechanism (HILIC-Z and BEH Amide) provided better retention than the ion-exchange based phase (PFPP). The BEH Amide column was finally selected, as it gave better results for early eluting compounds including favipiravir, arbidol and its metabolite. The most suitable mobile phase additives were found to be 0.1% formic acid and 2 mM ammonium acetate in both mobile phases A and B. Besides the retention improvements brought by HILIC, the ion suppression was largely beneficial for polar compounds that were also generally the least sensitive analytes.

The goal of the sample preparation development work was to develop a simple procedure that gave highest recovery of the broad range of analytes while also producing suitably clean extracts. In addition, it was decided to avoid the use of time consuming solid phase extractions. Various extraction procedures were assessed including QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe), QuPPe (Quick Polar Pesticide method) and solvent extractions. QuEChERS was found to be incompatible with polar compounds, while the QuPPe method led to low extraction efficiency for all compounds. The use of MeOH or MeCN:H_2O (80:20, *v/v*) led to better extractability, and the latter was finally selected because it led to more matrix enhancement or less matrix suppression for most of the compounds. Simpler sample clean-up methods were also evaluated including dispersive-solid phase extraction, freezing steps and ultrafiltration, but none of these provided any additional benefits. Instead, a dilution step with a MeOH:H_2O mixture was chosen because it led to overall improved peak shapes and signal-to-noise.

Validation

Table 1. Validation results for antiviral drug residues in poultry muscle tissue.

Analyte	LCL (µg kg^{-1})	Trueness% (CV%)			Matrix effects (%)	C.V. matrix effects (%)	Extraction efficiency (%)	Decision limit (µg kg^{-1})
		1.0 x LCL	2.0 x LCL	3.0 x LCL				
Arbidol Sulfoxide	0.1	99 (7.2)	96 (6.6)	89 (6.5)	59	10.6	81	0.12
Arbidol	0.1	104 (7.5)	104 (7.7)	88 (9.2)	31.9	8.8	78	0.12
Arbidol Sulfone	0.5	101 (12.4)	100 (10.6)	93 (16.3)	28.8	11.2	78	0.65
Acyclovir	0.5	127 (15.6)	125 (22.7)	117 (22.1)	136	29.2	77	0.68
Oseltamivir	0.5	99 (5.5)	97 (6.6)	97 (4.9)	139	3.9	78	0.57
Rimantadine	0.5	94 (4.1)	92 (5.6)	93 (6.6)	61	6.1	79	0.55

Amantadine	1	97 (3.8)	97 (4.5)	97 (2.8)	49	4.4	71	1.1
Zanamivir	2	93 (7.2)	98 (4.4)	100 (3.7)	913	4.9	42	2.3
Peramivir	2	105 (6.2)	104 (6.3)	106 (8.6)	61	8.1	67	2.3
Ganciclovir	2	97 (5.1)	100 (10.6)	100 (4.3)	80	5.5	66	2.3
Viramidine	2	85 (17.2)	90 (21.7)	93 (21.6)	57	18.5	57	2.8
Oseltamivir Acid	2	101 (10.3)	101 (9.6)	103 (11.7)	43	6.9	62	2.5
Laninamivir	10	84 (13.2)	86 (16.2)	88 (11.7)	337	14.6	51	13.1
Ribavirin	25	96 (5.9)	96 (6.0)	98 (5.4)	15.0	6.7	68	28.5
Favipiravir	25	95 (16.6)	95 (20.7)	90 (21.2)	37.8	19.1	68	34.7

LCL = lowest calibration level;. MS parameters: source temperature 150°C; desolvation temperature 600°C; cone gas flow 150 L h^{-1}; desolvation gas flow 1,000 L h^{-1}; capillary voltage 2.5 kV.

Selectivity, matrix effects and linearity

The selectivity of the method was assessed by verifying the absence of interference between analytes, internal standards, and from the poultry muscle sample matrix. No interferences were observed, as this was minimised by both sample preparation procedure and chromatography. The matrix effect study showed results from high suppression (15%) to high enhancement (913%) (Table 1). Matrix effects were compensated by matrix-matched calibration curves and/or internal standards. The coefficients of variation of the matrix effects were therefore below the maximum limits established by the validation guidelines. The linearity was found to be satisfactory, with correlation coefficient $R^2 \geq 0.988$ for all compounds and individual residuals within the ± 20% range from the calibration curve.

Accuracy, trueness and CCα

Accuracy and trueness were assessed following the repeatability and the within-laboratory reproducibility studies. Concerning the repeatability study, trueness values (Table 1) were found to be acceptable (85-109%) as well as the coefficients of variation, which were all below 10.8%. In relation to the reproducibility study, the trueness value range was wider (84-127%), with coefficients of variation ≤22.7%. All results were found to be within the limits recommended by the validation guidelines, except for acyclovir due to more variation in matrix effects. CCα values (Table 1) ranged from 0.12 µg kg^{-1} for arbidol to 34.7 µg kg^{-1} for ribavirin.

Storage stability study

The stability study in matrix at -28°C found the concentrations of the residues to be between 79% and 109% of the initial concentrations after 10 weeks (Figure 1), while at -80°C they were found to be between 85% and 108%. The tolerance fixed was 85%, with results of storage for viramidine and favipiravir at -28°C slightly below this standard. Therefore, a storage temperature of at least -28°C is recommended when freezers able to reach lower temperatures are not available.

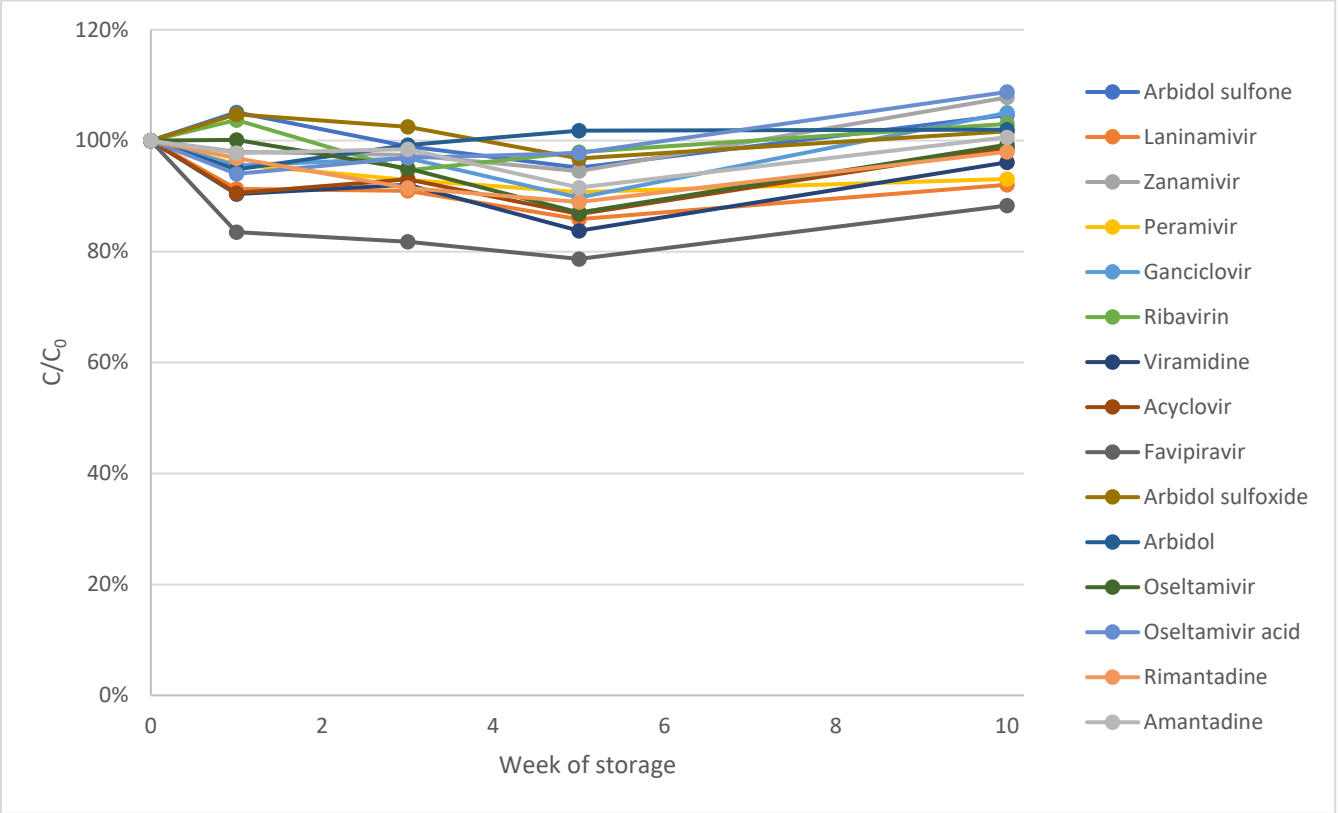

Figure 1. Storage stability of antiviral drugs in poultry muscle tissue at -28°C.

Application to market samples

No trace of antiviral drug residues against influenza were found in the commercial samples tested. This result was in accordance with other findings from research studies on the European market (Berendsen *et al.* 2012).

Conclusions

In this research, a LC-MS/MS method was developed and validated for the confirmatory and quantitative analysis of 15 antiviral drug residues in poultry muscle. The method included highly polar, non-polar, acidic, basic and neutral compounds. The sample preparation procedure consisted of a MeCN-based protein precipitation step, followed by a dilution in a MeOH:H_2O mixture, and was optimised by evaluating various solvents and methods of extraction with different clean-up options. A number of stationary phases were assessed for chromatographic separation including reversed-phase, ion pairing and HILIC chromatography to obtain optimal analyte retention and resolution, and also to minimise matrix effects. Satisfactory results were achieved on a BEH Amide column in HILIC mode. This method is advantageous over current published procedures because it includes a wider range of compounds, and because of its simplicity and cost-efficiency. The high-throughput sample preparation protocol allows a single analyst to process 50 test samples in one day. The validation, which was performed according to the new EU guidelines 2021/808 at levels as low as 0.1 µg kg^{-1}, showed the accuracy of the method in the quantification of the 15 antiviral drug residues. Trueness and precision calculated from the WLR studies were in the ranges 84-127% and 2.8-22.7%, respectively. No residues of the antiviral drugs were found in the 120 samples purchased from the Irish market that were analysed using the proposed method.

Acknowledgements

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POSTER P88

A DECADE IN REVIEW: THE BRAZILIAN OFFICIAL LABORATORY NETWORK AND THE OFFICIAL TESTING FOR RESIDUE & CONTAMINANTS IN FOOD

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Abstract

Since 2009 a critical analysis of the national residue monitoring system inaugurated a period of stronger research, development and innovation in the Brazilian official laboratory network. It showed how investments and clear objectives were essential to reshape an otherwise insufficient national residue control plan. Now, a decade later, it is worthwhile to look back and see what has changed. The scenarios are different, both in terms of capability and challenges. Newly implemented methods allowed the broadening of the NRCP scope. Adjusts in analytical and quality management procedures propitiated a better use of resources, by increasing the number of tests in a single sample. The participation in scientific publications has grown substantially, along with the awareness of what is being developed around the world being turned into a current mindset. Some innovative initiatives, such as the mobile laboratory unit, were started up. The emergency preparedness that was absent in previous years is now a trustworthy reality, with practical cases to study from. The opinion of FVO audits that points to the maintenance of a reliability status observed first a decade ago. This work reviews what was achieved and sets a future outlook

Introduction

It has become common ground how Brazil has established itself as a significant player in the global food trade. Indeed, annual statistics have shown the fundamental importance of agribusiness in the Brazilian economy, especially in the last two decades. In that context, the growing necessity of food safety focused policies has become more observable. In Brazil, the Unified System for Agricultural and Livestock Health Attention (Brasil, 2006) is established and regulates what is necessary for food production, both for domestic consumption and for international trade.

It is in the context of a unified inspection system focusing on food safety and consumer exposure to foodborne risks that a reliable laboratory presence is imperative. The National Agricultural Laboratory Network is MAPA's analytical branch and performs directly within inspection actions in every step of the production chain, in regional, national and international levels. Such network has evolved and changed in many aspects since its inception (de Queiroz Mauricio, 2012), and a substantial part of that work and its results were first presented to the scientific community in an article published in 2009 (Mauricio, 2009). This is a special paper for us, in the sense that it helped strengthen an academic interchange between our laboratories and the rest of the world, and also inaugurated the use of systematically viewing and publishing some milestones from a managerial point of view. The objective of that paper was to assess the results obtained after sound investments, planning and commitment to fulfil science-based necessities with science-based solutions. Ten years later, we intend to revisit some of those performance indicators, observe others, and reflect on what has changed in the period, while checking if predictions made at that time were fully or partially addressed in some way.

Managerial and Technical/Analytical aspects of the Laboratory Network
The different aspects that govern, both strategically and operationally, the Laboratory Network are discussed in their context, impacts and results from a decade of work.

Budget
The General-Coordination of Agricultural Laboratories (CGAL) is responsible for analytical services in 16 different expertise areas. The overall budget to cover expenses and to make investments is defined each year at the Federal Government level. The decade trend is shown in Figure 1.

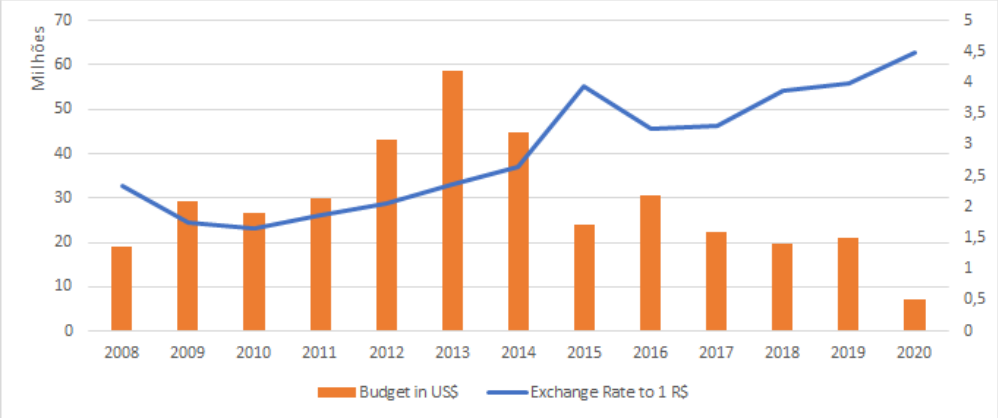

Figure 1. Laboratory specific budget and exchange rate fluctuation since the last published managerial paper (Mauricio, 2009).

The budget growth in the first half of the decade seems to have reached a peak in 2013, which allowed for many vital projects to be carried out. However, since 2015, the country has entered a severe economic recession, which may explain the budget cuts for the federal laboratories. The scenario that once pointed to stating that investments in laboratories bring important returns to the nation's economy, turned into a challenge of doing more with less. The year 2020 alone shows a 62% reduction in comparison with the earlier year, which will definitely hinder many critical actions in the analytical
services. The volatile exchange rate is a further complicating matter, since Brazilian science and research heavily rely on imported products and inputs, and the national currency has been weakened in recent years in comparison to US Dollars. Most high-end instrumentation manufacturers are based in the US and Europe, and so there is a direct impact on how far the laboratories are able to improve or renew their capabilities.

Number of samples and tests
The National Residue and Contaminants Control Plan (NRCP) is based on the *Codex Alimentarius* Guidelines CAC/GL 71-2009 and its amendments, and established by Federal regulation (Brasil, 1999). It follows the monitoring approach based on estimated prevalence, and it covers a multitude of veterinary drugs as well as environmental contaminants. It can be noted from Figure 2 that the participation of official laboratories and external, authorized laboratories in the NRCP has shifted drastically.

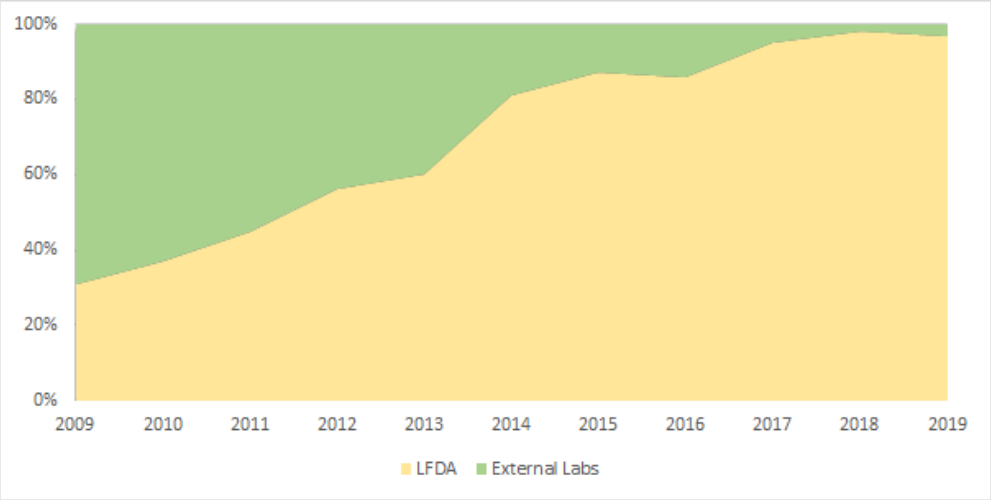

Figure 2 - The growing participation of all Federal Agricultural Laboratories (LFDA) in the Brazilian NRCP, yearly evolving

The philosophy of directing the routine testing to contracted laboratories and leaving space for the official ones to perform as reference laboratories that once was in place, was almost completely switched. There has been a tremendous effort in the official laboratories to implement several methods in order to diminish the dependency on external contractors. The LFDAs now cover basically all of the NRCP, which eliminates the need for them to take part in external audits for the authorization processes, but brings in an impressive workload in number of samples to be tested. Alongside with the monitoring program routine, they are also responsible for method research, development and implementation, validation and performance studies, settling disputes and providing scientific consultancy for decision making. An ISO 17025 based quality system is in place in all six of them, with constant work towards its accredited status maintenance and scope expansion. In order for this work to be carried out with limited staff, a strong capacity building policy must be conducted with commitment from the
central authority as well as the access to automation solutions such Laboratory Information Management System - LIMS, which runs in all of them.

The scope of the NRCP can only be broadened with a suitable laboratorial capability. Number and variety of substances tested for has been following an increasing trend, while monitored species reached a plateau when it was observed that the most relevant ones, both in domestic consumption and international trade, were already covered, given the higher risk they convey. The overall number of samples, however, has been kept quite constant, with a slight decrease in recent years, as shown in Figure 3. The reason for this behaviour is in the development and perfecting of analytical methods. The laboratories evolved from using single residue to multi-residue methods, to multi-class procedures. An excellent example of success with such an approach is in LFDA-RS and its wide-covering testing procedure in milk, which tests for 53 veterinary drug residues. The laboratory is able to report several classes of antimicrobials in a single sample, including: penicillins, β-lactams and macrolides, fluoroquinolones, amphenicols, sulfonamides, and tetracyclines, as well as antiparasitics such as avermectins. This development gave the inspectors the opportunity to gather results from the monitoring of more drugs in less collected
samples. This model has been discussed as a reasonable and achievable way of increasing the benefit-cost ratio for the NRCP, and its expansion to other food matrices is one of the objectives in the expansion of capabilities of the official laboratory network.

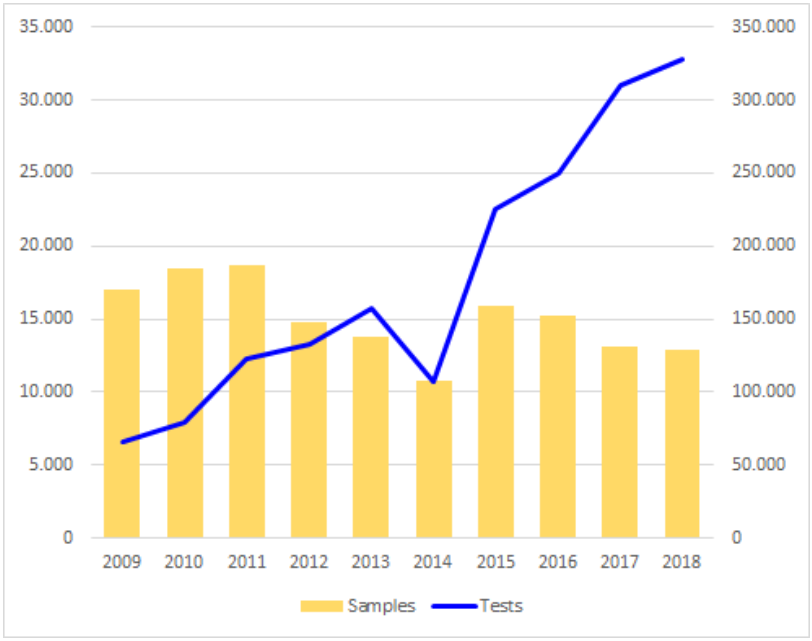

Figure 3. Changes in number of samples in the Brazilian NRCP as a contrast to the increments in total number of tests performed, especially with multi-residue, multiclass methods

The sudden growth between 2014 and 2015 is explained by an approximate 2000 % increment in the number of poultry fat samples tested for Dioxins and more than doubled the amount for Antimicrobial substances tested in bovines. From then on, the levels were known, and mitigation actions began taking place at the inspection level. Likewise, the growth in number of monitored substances comes from the implementation of QuEChERS-based multiresidue method for animal tissue, comprehending Organophosphates, Carbamates, Pyrethroids and several other groups. The overall number of substances tested for, in all matrices considered, is shown in Figure 4.

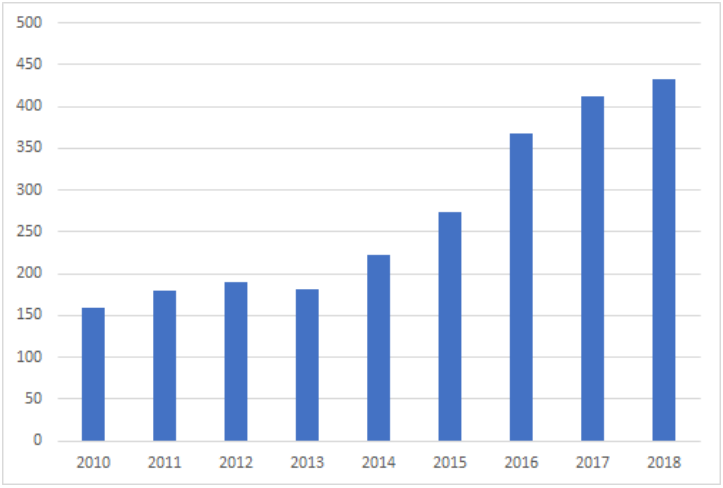

Figure 4. Increasing scope of the Brazilian NRCP in terms of unique substances tested for in all matrices, all species covered annually.

Capabilities in place

State-of-the-art equipment in place has been acknowledged as a crucial need in several of the central level management cycles. The willingness to acquire instrumentation and renew it over time is an indicator that authorities understand that

having appropriate tools is one of the pillars of an efficient laboratory. A lot of the higher end instrumentation in operation today was acquired after 2006, that is, gas or liquid chromatography with triple quadrupole mass spectrometry systems. Until 2009, the whole official laboratory network counted on: 8 LC-MS/MS and 3 GC-

MS/MS, from various manufacturers and mostly applied to veterinary drug residues. From 2010 on, however, this number was elevated to 16 LC-MS/MS and 8 GC-MS/MS in total. These are used in shared instrumental facilities, being applied to vet drugs, pesticides and mycotoxins in a myriad of matrices and products. In addition to one HRMS instrument dedicated to dioxins and PAHs in a number of food matrices, 9 newer, high resolution systems were put in place, including Q-TOF, MALDI-TOF, TripleTOF, Orbitrap and linear ion trap. These instruments have been used for antibiotics screening and for banned substances, such as hormones. Their high resolution performance has added a reliability component for classes of substances whose monitoring is of great interest. Projects are in place to reinforce and explore their potential in, for instance, untargeted analysis. This is part of a greater project that intends to strengthen intelligence-based monitoring at national level. Instrumentation alone is not enough, and so there has been a continuous effort to enhance capacity building and tailor the analytical staff to be able to operate with such techniques. External training with reference centres such as IAEA/Austria, FERA/UK, WUR/Netherlands, as well as the EURL in Almeria, Spain, for fruits and vegetables, to name a few, has been sought; likewise, knowledge transfer within the network has been fostered as much as possible. Training and extended education initiatives for laboratory personnel are known to show concrete results and real applications at a very high rate, and the knowledge acquired by one trainee is largely shared and extended to the rest of the team. These activities are valuable to keep the different laboratory units in harmony and in pace with what is developed in Europe, Asia and North America.

Scientific productivity

CGAL tried to foster the publication of any innovative work performed at the LFDAs since 2009. Unfortunately, because there is no specific budget for participation in symposia and congresses, the presence in national and international level events is less than what would be ideal. Nonetheless, the publication in scientific media has been consistent. An extensive list of peer-reviewed publications in a variety of journals is shown in Table 1. The pursuit of a validation protocol that would demonstrate fitness-for-purpose in the methods utilized in the laboratory network led also to the production of MAPA's Analytical Quality Assurance Handbook (Brasil, 2011), in 2011, as a fruit of collaboration between laboratory experts in each of the five grand areas (veterinary drugs, pesticides, mycotoxins, heavy metals and organic pollutants) with academia. This handbook set the guidelines for a harmonized protocol to be followed by all analytical units and comprises the validation studies and performance levels as well as routine quality controls.

Table 1. Summary of the recent peer reviewed publications in chemical residue and contaminants analyses

Contribution Theme	Reference in Supplement Material

Quality Assurance, Quality Control and Production of Reference Materials	[12], [41,42]
Dyes in fish products	[5]
Mycotoxins in food	[3], [10], [44], [55]
Feed (residues of antibiotics)	[27]
Non-steroidal anti-inflammatory drugs in tissues	[11]
Honey	[49], [54]
Milk adulteration	[8]
Pyrethroids in Milk	[37]
Antimicrobials (several classes) in milk and tissues – confirmatory methods	[23-27], [29], [4], [34-35], [43], [50], [3], [45-46]
Antimicrobials – screening methods	[36], [7], [19], [31], [33]
Antimicrobials – occurrence	[39]
Sedatives and beta-blockers in swine kidney	[15]
Heavy metals in fish and meat	[13], [17], [1], [28], [48], [53]
Avermectins in meat	[51]
Chloramphenicol and other amphenicols	[6], [47]
Sulfonamides in animal tissue and milk	[20-22], [30]
Pesticide residues in food	[14], [32],[16], [9], [38], [18]
Organic pollutants	[52]

Proficiency tests

The LFDA network has its provider for interlaboratory comparisons, PRIMAR. This unit is responsible for the sample production, outreach of participating labs, preparing materials for dispatch, monitoring and receiving results, and analyzing those results to produce a final report per round. All procedures are established under an ISO 17043 guided environment, and at the elaboration of this manuscript, the formal accreditation is underway.

Table 2 presents a summary of the proficiency test rounds coordinated and conducted by PRIMAR in the past decade. Some of these rounds were done with reference materials acquired from other internationally known providers, but others have utilized in house prepared samples, usually naturally incurred - that is, samples with positive quantification results from the monitoring program are stored for a definite time, and later evaluated for a possible material to be distributed as a PT round. One will note that the number of PT rounds provided was larger when there were more external laboratories participating in the NRCP. That was one important tool to monitor their performance, and now that the NRCP is pretty much entirely within the official laboratories, there is a lower number of potential participants in interlaboratory exercises. Additionally, rounds with a very low number of participants refer to activities tailored to investigate the performance of a particular method or laboratory, in directed quality assurance assessments for contracted laboratories, and so certified reference materials have been used as blind samples. For regular exercises, however, we took the initiative to extend PT rounds to other countries in Latin America, with the aid of RILAA and RALACA networks to facilitate communication and invitations.

Table 2. Summary of PT rounds and interlaboratory exercises organized by PRIMAR since 2010

Year	Groups of Analytes	Matrix	Material produced by	Number of participating labs	Reach level
2010	Sulfonamides	Honey	FAPAS	2	MAPA network
2010	Pesticides and PCBs	Fish Oil	FAPAS	1	MAPA network
2010	Aminoglycosides	Honey	FAPAS	8	MAPA network
2010	Macrolides	Honey	FAPAS	2	MAPA network
2010	Sulfonamides	Swine kidney	FAPAS	2	MAPA network
2010	Nitrofurans	Shrimp	FAPAS	3	MAPA network
2010	Tetracyclines	Swine kidney	FAPAS	3	MAPA network
2010	Leucomalachite	Fish muscle	FAPAS	2	MAPA network
2010	Chloramphenicol	Shrimp	FAPAS	2	MAPA network
2011	Avermectins	Bovine muscle	LRM/LFDA-MG	20	National and International
2011	Sulfonamides	Swine kidney	LRM/LFDA-MG	15	National and International
2014	Dioxins	Fish	LDP/LFDA-MG	11	National and International
2015	Ractopamine	Swine Muscle	LRM/LFDA-MG	25	National
2016	Dioxins	Poultry Fat	LDP/LFDA-MG	12	National and International
2016	Avermectins	Milk	LRM/LFDA-MG	1	National

Project Sagres

A crucial milestone in the strengthening process of the LFDA network was Project LANAGRO, which later evolved into Project SAGRES. The name alludes to the legendary Court of Sagres, which would have been an eminent group of experts who worked for the capacity building and nautical technical development for the great expeditions in the fifteenth century in Portugal. The initiative in the LFDA (at that time still called Lanagros) was to bring together researchers and scholars into all residue laboratory facilities with an overall objective to develop, optimize and implement analytical methods, automated processes, and quality assurance tools in a concentrated effort. It was the largest scholarship-based project in Brazilian scientific research and development history, and it produced a mass of knowledge that still shows its fruits.

Between 2008 and 2012, the Projects accomplished: 200 scholarships granted at different specialization levels; 133 validated and accredited methods; a huge leap in the number of substances monitored in the NRCP, from the historical level of 49

analytes until 2005 to 204 in 2012; several papers published in indexed journals with one special edition of FAC published in 2012.

These new validations, extensions of scope and related studies have been accompanied by a strong quality management work, which is evidenced in the number of accredited methods put in routine for official testing: at the time this manuscript was produced, 93 % of a total of 5175 tests or assays are fully accredited under ISO 17025. The remaining is already submitted for approval to the national metrology body, and it is safe to say that current quality management systems are capable of keeping that stream flowing, for the dynamicity of analytical method implementation. These projects were important to continue and even speed up the evolution in the Brazilian network, by working more closely with academia. The fruits of such work are still visible after all these years, as the LFDA reached a reliability status recognized by external audits, such as FSIS and FVO-UE.

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Supplementary Material

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POSTER P89

WHEN THE TWO FRENCH NRLs FOR VMPR JOIN FORCES TO CONSIDER THE IMPLEMENTATION OF REG 2021/808/EU

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Abstract
At a time when the Commission Implementing Regulation (EU) 2021/808 repealing Decisions 2002/657/EC and 98/179/EC and relating to the "*performance of analytical methods for residues of pharmacologically active substances used in food-producing animals and on the interpretation of results as well as on the methods to be used for sampling*" has recently come into force, the two French NRLs in charge of substances of interest have decided to carry out a joint reflection in order to propose a unified implementation methodology at the national level.
A plenary steering committee bringing together the necessary expertise within the two NRLs has been set up. It coordinates five working groups (WGs) involving scientists and quality managers from both structures, to deal with the following issues: WG1_Identification criteria, WG2_Validation of unauthorised and prohibited substances, WG3_Validation of substances with MRL, WG4_Implementation in field laboratories and WG5_Communication.
The work involves the necessary coordination with the national accreditation body with a view to the accreditation of the field laboratories and the NRLs concerned, as well as the synchronization of the programming of the revision of methods and their implementation in the monitoring plans together with the competent authority.
A strict retro-planning allowing the implementation of the new provisions as from the 2023 official plans has therefore been elaborated: it intends to bring all official methods in line with Reg/2021/808 in 2024 subsequently allowing 2 years to the field laboratories to integrate these changes and to be fully operational for the deadline of 10th June 2026.

POSTER P90

THE POTENTIAL FOR CONTAMINATION OF FOOD SAMPLES BY SEMICARBAZIDE FROM STORAGE CONTAINERS

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Abstract

Nitrofuran drugs are banned in food producing animals as they are potential carcinogens, with the reference point for action (RPA) of 0.5 µg kg^{-1}. Semicarbazide (SEM) has been established as the marker residue for the nitrofuran antibiotic nitrofurazone; however, other sources of SEM contamination, such as the breakdown residue of the banned blowing agent azodicarbonamide the sealing gaskets of metal jar lids, present challenges to the monitoring of this metabolite. These problems are generally circumvented by the established method of tissue washes prior to analysis, however, this approach is not suitable for analysis in egg. Recently, due to the challenges brought by COVID-19 pandemic this laboratory was forced to purchase storage containers from new suppliers. In the course of routine testing of nitrofuran metabolites SEM levels between 0.5 – 2.5 µg kg^{-1} were detected in seven out of nine egg samples. The high proportion of suspect samples lead to an investigation, which quickly established all contaminated samples had been stored in newly acquired storage jars. Further testing showed egg samples stored for as little as 30 min in contaminated jars had SEM concentrations above the RPA. This work highlights the importance of establishing the suitability of all materials within the laboratory prior to use.

Introduction

The nitrofuran class of antibiotics have been in use for over 60 years, finding application in the treatment of various infections including urinary tract, topical and *Helicobacter pylori* infection (Le *et al.*, 2021). In addition to their role as antibiotics, they also found use as feed additives, commonly administered to promote growth in avian, porcine and bovine livestock (Vass *et al.*, 2008). It was discovered, however, that the 5-nitrofuraldehyde metabolite had mutagenic and carcinogenic properties (McCalla, 1983). As a result these compounds are now banned in food producing animals within the EU (Commission Regulation, 2010).

It has been previously reported the nitrofuran parent drugs undergo rapid metabolism when administered, resulting in stable protein bound metabolites. These metabolites may be released by hydrolysis and following derivatisation with 2-nitrobenzaldehyde (NBA), detected and quantified by LC-MS/MS (Szilagyi *et al.*, 2006). A minimum required performance limit (MRPL) had been initially established at a value of 1 µg kg^{-1} (CRL Guidance Paper, 2007) however subsequently an RPA has been introduced at a lowered value of 0.5 µg kg^{-1} (Commision Regulation, 2019).

An additional complication in their monitoring is, however, presented in the chemical simplicity of some metabolite structures, specifically the parent drug nitrofurazone and its metabolite semicarbazide (SEM), and its potential environmental presence both as naturally occurring traces in some food producing species, for example in the shells or crab and shrimp (EFSA, 2015), and as by-products of certain plastic manufacturing processes and food production techniques (Commission Directive, 2004; Hoenicke *et al.*, 2004; O'Keeffe *et al.*, 2021). To mitigate the risk of environmental contamination, samples generally undergo a thorough set of washes with a range of organic solvents including MeOH, EtOH and Et_2O, leaving only the protein bound residue. This is not, however, generally carried out on egg samples.

In this work we present how the presence of the SEM metabolite in tested egg samples was not due to administration of the parent drug, but was instead the result of contamination from an environmental source, the sealing gasket of a newly purchased batch of storage containers used within our laboratory. Further investigation was undertaken to determine how quickly samples would be contaminated with levels greater than the current RPA, and what effect the storage conditions of samples in containers would have on the concentrations detected.

Materials and Methods

LC-MS/MS Conditions

Analysis was performed on an Agilent 1290 Infinity system connected to an Agilent 6490 mass spectrometer (Agilent Technologies, USA). The column used was an Agilent Zorbax RRHD Eclipse Plus Phenyl Hexyl 2.1 × 100mm 1.8 µm (Agilent Technologies, Ireland) held at 55°C. A binary gradient separation comprising of 0.5 mM

ammonium acetate / 0.1% formic acid in H_2O: MeOH (95:5, *v/v*) (mobile phase A) and MeOH:MeCN (1:1, *v/v*) (mobile phase B) was used at a flow rate of 0.5 mL/min. The gradient was as follows: (1) 0.0 – 2.2 min: 95% A, (2) 2.2 – 4.0: linear decrease to 53.8% A, (3) 4.0 – 4.5: linear decrease to 20% A, (4) 4.5 – 5.5: 20% A, (5) 5.5 – 6.25: linear increase to 95% A, (6) 6.25 – 8.0: 95% A, for a total run time of 8 minutes. The injection volume was 5 µL and the autosampler was held at 12°C. The mass spectrometer was operated in positive ESI mode, acquiring with dynamic MRM.

Experimental design

Previously established negative egg samples (25 g) were transferred to three identical storage containers. The samples were then shaken and stored upside down, to ensure contact with the foam seal of the lid. Two samples were immediately transferred to a -20°C freezer and the other was stored at room temperature. At each time point the container was inverted and a 1 g sample transferred in duplicate to 50 mL polypropylene tubes, which were immediately stored at -20°C. For the frozen samples, once removed from the freezer the lids were removed and the sample allowed to defrost at room temperature. Once defrosted the sample was treated as per those stored at room temperature. 'Room temperature' samples were sampled at the following time points: 0.25, 0.5, 1, 2, 4 and 8 hours. Frozen samples were transferred at 1 and 4 hour time points. Once sampling was complete, the samples were allowed to reach room temperature, extracted and analysed.

Extraction

All extractions were performed using the analytical method based on the previously published protocol for nitrofuran metabolites (McCracken *et al.*, 2007). Egg samples (1 ± 0.02 g) were transferred to 50 mL polypropylene tubes and fortified with 100 µL of 100 ng/mL $^{13}C,^{15}N_2$-SEM internal standard. 4 mL of water and 0.5 mL 1 M HCl were added, followed by 150 µL of 50 mM NBA. Samples were vortexed and incubated in a waterbath overnight at 37°C. After incubation the samples were buffered with 5 mL 0.1 M K_2HPO_4 and 0.4 mL 1 M NaOH, followed by liquid-liquid extraction with 2 × 5 mL portions of EtOAc. The combined organics were dried and reconstituted in 400 µL MeOH:H_2O (1:1, *v/v*). All samples were quantified against a solvent curve between 0.25 – 5.0 µg kg^{-1} (CCα 0.19 µg kg^{-1}), employing $^{13}C,^{15}N_2$-SEM as internal standard, extracted in the same manner.

Results and discussion

During routine statutory testing within our laboratory seven out of nine tested egg samples in a single analysis contained concentrations of SEM above the RPA of 0.5 µg kg^{-1}. No traces of SEM contamination were detected in any negative or reagent blank samples, nor were there traces in the solvent blank injections in the same analysis.

To account for such a large number of potentially non-compliant samples two possible scenarios were considered; an environmental contamination of the samples during sampling and storage, or due to the limited number of feed suppliers in Northern Ireland, a widespread contamination of avian feed; this situation was particularly worrying given that the affected egg samples were from a number of different producers. It was quickly determined, however, that the environmental contamination scenario was most likely when it was established all affected egg samples had been processed and stored in containers that had only recently been purchased for the laboratory.

The suspicion that the contamination was due to the storage containers used was founded on the knowledge that SEM has previously been observed as a contaminant, either from food production techniques or as a breakdown product from some foam blowing agents (EFSA, 2015). It has previously been argued that SEM is so common an environmental contaminant that it is not an appropriate marker for the abuse of the nitrofuran drug nitrofurazone in agricultural animals (Stadler *et al.*, 2015).

To confirm this contamination was the result of transfer from the storage containers we returned to the additional egg samples from each batch that are retained in the laboratory in their shells in case of potential contamination situations. The samples were processed in a different location, with each egg wiped down with water prior to sampling and mixing. Once cracked and mixed, the samples were transferred to fresh containers of known quality and tested. All previously SEM contaminated egg samples returned compliant results without any traces of SEM present.

To establish the role of the new storage containers in the contamination, we performed an investigation of the levels of SEM contamination within a known negative egg sample stored at both room temperature and -20°C in this new container. It was important to establish the potential levels at both type of storage conditions as samples may be transferred to containers and immediately frozen when received in the laboratory, however, there may be extended periods during defrosting of the samples when contamination may also occur.

The results of the investigation, the SEM concentration in egg samples over time, are shown in Figure 19.

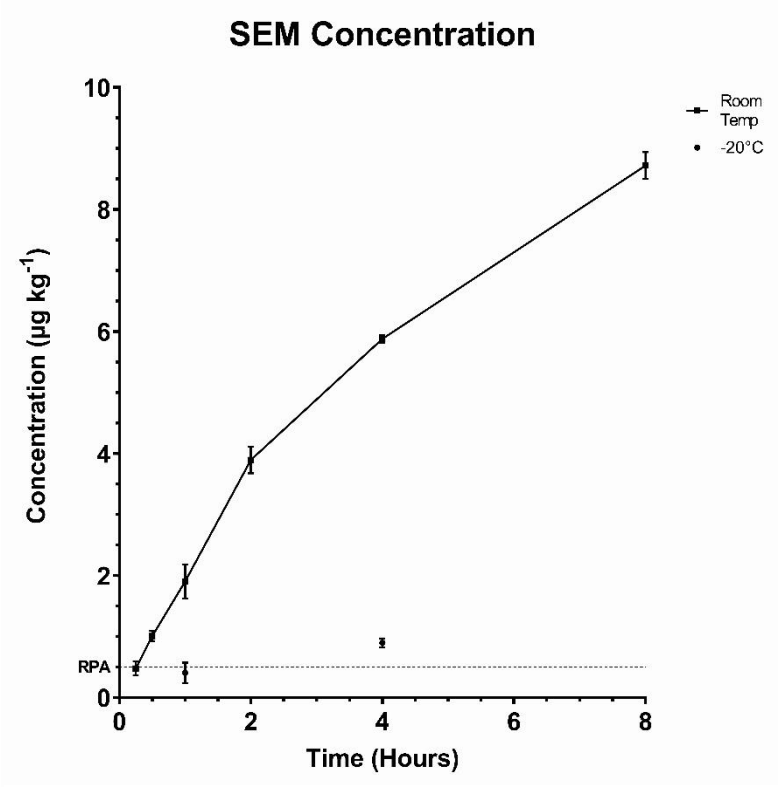
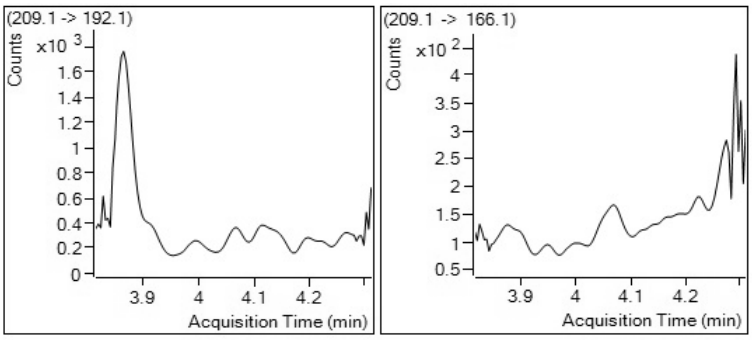


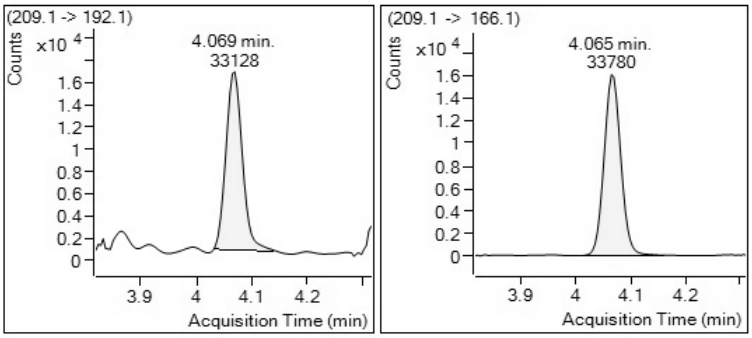
Figure 19. SEM concentration in egg against storage time in both room temperature and -20°C samples

It can be seen that the SEM concentration quickly increased during storage at room temperature, with a concentration just below the RPA present within 15 minutes (0.48 µg kg^{-1}, *n* = 2, RSD = 24%) and had reached twice the RPA within 30 minutes (1.00 µg kg^{-1}, *n* = 2, RSD = 9%). By the 8th hour of the experiment the concentration in the sample had exceeded the RPA by almost 18 times (8.70 µg kg^{-1}, *n* = 2, RSD = 3%). Storage at -20°C decreased the rate at which SEM was incorporated into the samples almost 5 fold, with the sample at the 1 hour time point having concentrations below the RPA (0.42 µg kg^{-1}, *n* = 2, RSD = 42%), while by the 4 hour time point the SEM concentration had reached almost double the RPA (0.80 µg kg^{-1}, *n* = 2, RSD = 8%). See chromatograms below (Figure 20) of the negative, positive controls and the room temperature sample following 30 minutes storage.

A



B



C

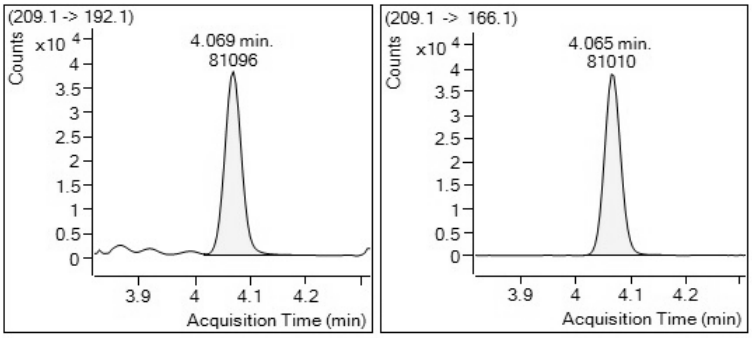


Figure 20. Chromatograms of qualifier (209.1 > 166.1) and quantifier (209.1 > 192.1) for (A) negative control, (B) positive control (0.5 µg/kg) and (C) sample following 30 minutes storage at room temperature

Due to the potential for contamination, a new policy within the laboratory has been established to perform a 'blank' analysis on any new materials used during routine statutory testing. This requires the extraction of previously established negative samples with both old and new resources. Additionally for storage containers we ensure such negative samples are stored in the appropriate conditions for a representative period of time before analysis.

Conclusion

From this work, it is clear the importance of establishing the suitability of any new resources used in the performance of routine sample testing within the laboratory. Although the potential for SEM contamination from blowing agents used in the production of foams in sealing gaskets has been long established, this blowing agent has been banned in the EU for almost 20 years. However, due to the situation created by the on-going COVID-19 crisis, it was necessary to turn to alternative suppliers for our needs. This contamination issue served as a reminder for us not to be complacent and to perform appropriate suitability assessments when new suppliers or resources are used in the laboratory.

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POSTER P91

LOOKING FOR INTERNAL STANDARD CANDIDATES IN LC-MS/MS ANALYSIS OF ANTICOCCIDIALS IN THE FOOD OF ANIMAL ORIGIN

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Abstract
The residues of anticoccidials (coccidiostats) remain on the constant level of occurrence in food of animal origin. Non-compliant samples for anticoccidials (B2b) in 2017 in Europe were reported in horses (0.85%), pigs (0.01%), poultry (0.21%), rabbits (0.65%) and eggs (0.47%). The number of analytes, their diversity of chemical properties and low maximum levels and MRLs require the use of LC-MS/MS technique.
Among this group of compound ionophores coccidiostats (monensin, narasin, salinomycin, lasalocid, maduramycin) seem to be most challenging in LC-MS/MS analysis. High molecular mass (700-900 Da) and hydrophobic structure cause strong retention on reversed-phase liquid chromatography systems and co-elution with matrix constituents. Quantitative analysis is often problematic; matrix effect and lack of labeled internal standard can cause high interlaboratory variability. Thus there is strong need for a new internal standard for those compounds.
In the presented study, synthesized derivatives of monensin and salinomycin (20 different esters of C-1 and C-26) were verified as internal standard candidates. The results showed significant improvement of the precision and accuracy of the LC-MS/MS method for coccidiostat residues in animal muscles, livers, and eggs.

Introduction
Chromatographic techniques coupled with mass spectrometry have become routine in food testing for residues of veterinary drugs, hormones, feed additives, and environmental contaminants. Thanks to these techniques, multi-component methods have been developed, including several hundred compounds in their range. At the same time, it is possible to achieve very low limits of quantification or detection capability CCalpha in demanding matrices such as food samples of animal origin. Methods based on LC-MS/MS and GC-MS/MS can also be used to confirm the abundance of compounds by providing information about the molecule's structure, which makes it an ideal tool in the hands of experienced analysts. The Achilles heel of these methods, however, is quantitative analysis. The matrix effect often hinders good linearity of the LC-MS/MS methods (Olejnik et al. 2013), caused by interference of matrix components with analytes at the mass spectrometer interface. In practice, it isn't easy to eliminate the matrix effect. Still, it can be reduced by modifying the sample preparation step (additional purification of the extract or its dilution) while using labelled internal standards.

The challenge, however, is when labelled internal standards are not available for the analytes being determined. An excellent example of such a situation is the ionophore coccidiostats (monensin, narasin, salinomycin, lasalocid, maduramycin). High molecular mass (700-900 Da) and hydrophobic structure cause strong retention on reversed-phase liquid chromatography systems and co-elution with matrix constituents. Nigericin is used as a standard in many methods to determine coccidiostats residues, but it has a different chemical structure and retention time than the determined compounds (Olejnik et al. 2009).

This study aimed to find alternative compounds that could serve as internal standards in coccidiostat analysis. Monensin and salinomycin derivatives initially synthesised to verify their anti-cancer ability, were used in this study (Huczyński, 2012).

Materials and Methods

Chemical and materials

Maduramicin ammonium, monensin sodium, narasin, nigericin, and salinomycin sodium from Sigma-Aldrich (Germany). Lasalocid sodium was purchased from Dr. Ehrenstorfer Labratories (Germany). Semduramicin sodium was kindly donated by European Union Reference Laboratory in Berlin. Synthesised monensin and salinomycin compounds for internal standards were prepared by the Faculty of Chemistry, Adam Mickiewicz University.

LC-MS/MS conditions

The LC-MS/MS system consisted of Shimadzu Nexera X2 UHPLC coupled with Shimadzu LC-MS 8050 mass spectrometer. The instrument was controlled by LabSolution SP2 software. Separation was performed using Agilent Zorbax Eclipse Plus C18 RRHD 1.8 µm, 2.1 x 50 mm chromatographic column in gradient mode with 0.01 M ammonium formate pH 4.0 (mobile phase A) and acetonitrile (mobile phase B) at the flow rate of 0.6 mL/min. The oven temperature was set to 40 °C; injection volume was 5 µL, and the total analytical run was 8 min.

The analysis was performed using positive and negative heated electrospray ionisation mode (HESI). The parameters of the mass spectrometer were as follows: nebulising gas flow - 3 L/min, heating gas and drying gas flows - 10 L/min both, interface temperature - 300°C, heat block temperature - 400 °C, capillary voltage - −3 kV and 4 kV for negative and positive ionisation mode, respectively. Selected reaction monitoring (SRM) mode was applied; each coccidiostat was analysed using two transitions, and for the internal standard, one transition was monitored. Mass spectrometry conditions are shown in Table 1.

Table 1. Mass spectrometry parameters

Analyte	Precursor ion	Product ions	Q1 Pre Bias (V)	Q3 Pre Bias (V)	Collision energy (eV)
Lasalocid (LAS)	613.3	577.3/377.2	30 30	30/28	34/37
Semduramicin (SEMD)	895.4	851.5/833.7	26	34/32	36/32
Monensin (MON)	693.3	675.4/479.3	34	26/24	40/53
Salinomycin (SAL)	773.4	531.3/431.1	38	40/22	44/52
Maduramicin (MAD)	934.5	647.5/629.4	28	34/32	22/27
Narasin (NAR)	787.4	531.3/431.3	40	40/30	48/53
Monensin AW morph	766	698	20	28	21
Monensin AW morph ammonium adduct	783	698	20	28	27
Monensin AW morph sodium adduct	788	744	20	28	43
Monensin AW morph potassium adduct	804	760	20	30	42
Salinomycin ketone potassium adduct	787	380	22	22	44
Salinomycin-NH-prop ammonium adduct	805	770	22	30	20
Salinomycin-NH-prop sodium adduct	810	302	22	22	55
Nigericin	747.5	703.5	36	38	54

Sample preparation

A 2 ± 0,01 g liver sample was weighed into the polypropylene centrifuge tube. Afterwards, 20 µL of IS solution was added, and the sample was vortex-mixed and allowed to rest for 10 min. Then, 5 mL of 90% acetonitrile was added, and the sample was vigorously mixed using a vortex. Then, 3 ml of hexane was added and centrifuged (3500 rpm, 15 min). The upper layer was discarded, and the procedure was repeated with another portion of 3 ml of hexane. The extract was transferred to falcon tine containing 600 mg of C18 sorbent and 80 mg of PSA sorbent. The sample was mixed and centrifuged (4000 rpm, 10 min). The supernatant was transferred to a clean Falcon tube and put in - 20° C for 1h. The upper layer was collected and evaporated to dryness in a stream of nitrogen at 40° C. Dry residue was dissolved in 250 µL of 50% acetonitrile.

Experiment design

The internal standard solution was prepared by dissolving 10 mg of the new internal standard solution and nigericin in 10 ml of acetonitrile. Afterwards, the working solution of 2.0 µg/mL was prepared. The experiment was performed by preparing a sample batch consisting of liver samples from four different species: horse, pig, cattle, and chicken. Samples were fortified with a solution of the ionophore coccidiostats. Those samples were analysed with and without adding an internal standard solution to evaluate candidates for internal standards samples. The relative standard deviation was calculated for each analyte-internal standard combination to calculate the effect of internal standard influence.

Results and discussion

Table 2. Results for candidates for an internal standard

Results and Discussion

It is worth mentioning that this study is the first published attempt to use monensin and salinomycin

	Relative standard deviation (RSD) from 12 samples					
	SEM	MAD	SAL	MON	NAR	LAZ
No internal standard	14%	27%	10%	11%	12%	24%
Monensin AW morph	10%	22%	8%	10%	15%	34%
Monensin AW morph ammonium adduct	15%	23%	14%	14%	20%	35%
Monensin AW morph sodium adduct	14%	26%	11%	14%	18%	30%
Monensin AW morph potassium adduct	12%	20%	10%	7%	15%	27%
Salinomycin ketone potassium adduct	18%	34%	19%	20%	21%	35%
Salinomycin-NH-prop ammonium adduct	27%	45%	33%	29%	19%	32%
Salinomycin-NH-prop sodium adduct	15%	23%	12%	14%	22%	35%
nigericin	39%	23%	36%	31%	24%	31%

derivatives as internal standards in residue analysis. The results of our experiment confirm that finding a suitable compound for internal standard in the case of ionophore coccidiostats is a challenge (Table 2). Based on data obtained for our work, none of the compounds we analysed can be recommended as a reliable internal standard for all ionophore coccidiostats. Madumanicin and lasalocid were the most difficult analytes. For those two coccidiostats, the calculated values of RSD were the highest. In the case of maduramicin, the RSD was in the range of 20 % to 45%, and for lasalocid, the range was from 24% to 35%. The most promising results were found for salinomycin and monensin. For those analyses, the calculated RSD values were below 10%. That is also interesting; based on our results, in some cases adding internal standards resulted in higher values of RSD. This could suggest that not using the internal standard can be recommended. Although, it should be noted that those results were obtained using 12 samples, and more data is needed to confirm our findings.

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POSTER P92

DEVELOPMENT OF THE METHOD FOR THE DETERMINATION OF AMINOGLYCOSIDE IN FOODS USING LC-MS/MS WITH A ZWITTERIONIC HILIC STATIONARY PHASE

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Abstract
Aminoglycosides (AMGs) are broad-spectrum antibiotics that have bactericidal activity against aerobic bacterial infection and are commonly used as veterinary drugs on food-producing animals and in human medicine. Thus, it is important to monitor residues in food to control AMG use. Many countries have established maximum residue limits (MRL) for aminoglycosides approved for use on animals. AMGs are currently analysed in honey, eggs, milk, tissues, and fluids of food-producing animals for control and monitoring purposes. AMGs are highly polar compounds and show little to no retention in reversed phase columns. Although ion-pairing reagents have been utilised successfully to chromatograph AMGs on C18 columns, when used with liquid chromatography-tandem mass spectrometry (LC-MS/MS) this approach suffers from ion suppression and contamination of the LC and MS/MS systems. The introduction of hydrophilic interaction chromatography (HILIC) provided a more MS-compatible option for the analysis of polar compounds. Here we show the results from the successful evaluation of the new Atlantis Premier BEH Z-HILIC column, which has a zwitterionic sulfoalkylbetaine stationary phase on BEH particles, for the determination of AMGs. The overall performance of a method incorporating the Z-HILIC column was established for AMG residues in a range of representative sample types.

Keywords: Aminoglycosides, LC-MSMS, Screening

POSTER P93

A REAL TIME METABOLOMICS PROFILING APPROACH USING RAPID EVAPORATIVE IONIZATION MASS SPECTROMETRY (REIMS) TO SCREEN FOR ANABOLIC PRACTICES IN A RANGE OF LIVESTOCK TISSUES

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Abstract

Rapid evaporative ionization mass spectrometry (REIMS) was explored for the rapid analysis of a range of tissues (liver, meat, kidney) from treated livestock. In this context, the combination of REIMS with untargeted metabolomics workflow was investigated to identify treated animals on the basis of a modification of indirect metabolites profile. REIMS strategy generates indeed specific lipidic fingerprint patterns which enabled differentiating tissue samples collected from animals treated with steroids, SARMs and β-agonists. This proof of concept research indicates that REIMS implemented in an untargeted-metabolomics workflow can be considered as a promising high-throughput and powerful strategy for real-time tissues classification. Further work should focus on validation and robustness assessment before potential implementation as rapid screening test, at the slaughterhouse or at boarder inspection points, to detect such practice.

Introduction

In order to ensure the food safety and health of consumers, a large-scale research on the presence of prohibited substances and residues of chemical, biological and veterinary medicines products in animals as well as in biological material and food of animal origin is conducted.

Hormonally active substances like steroids, stilbenes, resorcylic acid lactones and corticosteroids may illegally be used in cattle fattening as they increase the weight gain of food-producing animals. Beneficial effects of natural and synthetic steroids related to animal growth promotion and feed conversion efficiency have led to a wide use of these compounds in food producing animals since the 1950s (Guiroy *et al. 2002*). The weight gain can, e.g. be induced by anabolic effects through modulation of estrogen or androgen receptors (e.g. steroids, stilbenes, resorcylic acid lactones) or by modulation of the thyroid function leading to an increased water retention (e.g. thyreostats. A class of non-steroidal synthetic anabolic substances are the so-called selective androgen receptor modulators (SARMs) (Dalton *et al. 1998, 2010*). Like androgenic anabolic steroids (e.g. testosterone) these compounds are ligands and modulators of the androgen receptor, but act in a tissue-selective way causing agonistic effects primarily on muscle and bone and sparing the prostate (Chen *et al. 2005*; Kim *et al. 2013*). In the European Union (EU) the use of hormonally active substances in food-producing animals for growth promoting purposes was banned a long time ago (EC 1988). A conclusion of the Scientific Committee on Veterinary Measures Relating to Public Health (SCVPH) stated as early as in 1999 that the risk originating from residues of hormonally active substances in food of animal origin could not be assessed (SCVPH, *2002*). For specific estrogens like estradiol, even a genotoxic mode of action was discussed (Yager *2015*). Based on the precautionary principle (EC *2002a, 2002b*) to prevent the consumer from encountering potential health risks, certain hormonally active substances were explicitly banned in the EU for use in food-producing animals. To control the zero-tolerance concept of prohibited or nonauthorised drugs residue control is mandatory within the EU (EC 1996, 2004). In the past many illegal anabolic substances found their way from sports doping to livestock production (Vanoosthuyze et al. 1994) and with regard to veterinary practice SARMs may have some misuse potential due to their pronounced anabolic effect in combination with their availability on the black market. However, SARMS have not yet been included in the standard residue control programmes of the European Union. An essential prerequisite would be the availability of test methods validated according to Commission Implementing Regulation (EU) 2021/808 (EC 2021). Since compounds that improve fattening in beef production are allowed in some other parts of the world, their presence in the European Union has to be controlled

For the detection of trace levels of hormones and selective androgenne receptor modulators (SARMs) in tissues, kidney, liver, plasma and urine in slaughter animals, gas chromatography mass spectrometry (GC–MS), provides high sensitivity, specificity and chromatographic resolution(Marchand *et al. 2000*, Impens *et al. 2002*,

Hartman *et al. 1997*, Heitzman *1994*, Gerace *et al. 2011*). However, the derivation process is tedious and time-consuming. Therefore, the development of more straightforward methods is of great interest. Liquid chromatography–electrospray ionization tandem mass spectrometry is now considered efficient technique for residual analysis because of its high selectivity, specificity and sensitivity to residual hormonal chemicals; there is no need for derivatization steps, which reduces the analysis time, eliminates predictable sources of error and decreases the use of hazardous and expensive reagents. the applications of LC–MS and LC–MS/MS to foods of animal origin are limited to several papers that reported the analysis of steroids and SARMs in urine samples and plasma samples (Choi *et al., 2002*; Van Poucke *et al., 2002*; Bean *et al., 199*; Leinonen *et al., 2004*; Schmidt and Mankertz *2018;* Rojas et al., 2017; Cesbron *et al.,* 2017).

Issues encountered in controlling the use of growth promoters in livestock's led over the last past 10 years to the development of innovative untargeted approaches, consisting in the investigation of the physiological effects induced as a consequence of illegal practices. The objective is to reveal biomarkers of exposure that may subsequently be monitored for screening purposes. Such so-called omics strategies mainly referring to the study of changes in mRNA-expression, protein or small molecules profiles, have already proven their relevance using respectively either transcriptomics (Reiter et al. 2007; Riedmaier et al. 2009b, 2009a), proteomics (Cacciatore et al. 2009; Mooney et al. 2009a; Mooney et al. 2009b; Nebbia et al. 2011), or metabolomics (Courant et al. 2009; Pinel et al. 2010; G. Dervilly-Pinel et al. 2012; Gaud Dervilly-Pinel et al. 2015).

Compared to other omics strategies, metabolomics gathered more interest from the residue-control world since it involves analytical platforms similar to those already available in research laboratories in this area. Furthermore, biomarkers evidenced upon metabolomics studies are considered as easier to subsequently monitor since involving targeted analytical strategies close to those already available in corresponding laboratories in charge of the control.

In this study, an innovative analytical technique namely iknife, based on a shotgun MS approach could be a valid aid to obtain fingerprint of hormones in bovine tissues, kidney and liver. The iknife that means „inteligent knife", is the term coined in 2013 by prof. Takats and co-workers to described the coupling between the electrosurgical tool (the knife) and a MS system based on rapid evaporative ionization mechanism (REIMS – rapid evaporative ionization mass spectrometry) (Balog *et al. 2013*). It operate s using an electrosurgical knife, bipolar or laser which creates an aerosol (smoke) when cutting into a samples. The aerosol is evacuated from the sample through a transfer line into the ionisation source of a mass spectrometer where a heated collision source is situated and the ionisation proces occurs (Black *et al. 2017*). REIMS determines the structual lipide profile of sample by the on-line analysis of electrocautery smoke and uses this information for the rapid characterisation of dissected samples. The analysis takes only few minutes and guarantees point-of-control analysis. Originally it was intended for in vivo identification of tissues during surgical interventions. In the last several years there have been several publications about the use of REIMS technology for the analysis of food and all focus on animal tissues discriminate different kind of meat (Balog *et al. 2017*) or fish (Black *et al. 2017*) or identify boar taint in pig fat (Verplanken *et al. 2017*). In 2018 Guitton et al. used REIMS technology for determination ractopamine in pig muscles and in 2019 Rigano and co-workers used iknife with REIMS for authenticity assessment of pistachio samples.

In this study, REIMS was explored to develope a predictive model for accurate high-throughput identification of hormones (nandrolone and estradiol) and selective androgenne receptor modulators (SARMs) in bovine muscle, kidney and liver. The obtaine results demonstrated that REIMS technology can be use for determination of muscle, liver and kidney samples originating from hormones and SARMs – treated bovines.

Materials and Methods

Samples

The experiments were carried out on the tissues and organs of animals derived from other experiments conducted at LABERCA. The animals were given hormones such as nandronol/estradiol, nandronol/estradio +dexomethasone or selective androgen receptor modulators (SARMS). Control muscle, kidney and liver samples were from control animals. In addition, several samples of bovine liver were purchased at the local supermarket and used as part of the validation study. All samples were frozen at - 20°C. Prior REIMS analysis the samples were thawed all night in the fridge.

REIMS analysis

The iKnife hand-held sampling device (Waters, Milford, MA USA) was used to apply a localised high frequency electric current to the surface of each sample, which instantly vaporises molecules from the latter. It consists of a monopolar cutting device with a shortened knife blade approximately 6 mm long and was applied in auto-cut mode in combination with a diathermy electrosurgical generator (Erbe VIO 50 C) (Erbe, Tuebingen, Germany) at a power of 30 W. Sampling was carried out for 3–5 s and, for each sample, ten technical replicates were analysed, thus taking into account repeatability of the analysis. All samples were cut on the return electrode and a venturi gas jet pump driven by nitrogen evacuated the aerosol produced at the sample site towards a heated kanthal coil that was operated at 6.4 W. All analysis was performer in REIMS TOF MS sensitivity mode with continuum data aquisition. A lockmass solution of Leucine Enkaphalin (LeuEnk) (m/z 554.2615) (80 fg/µL) in isopropanol was infused using a Waters Acquity UPLC I-class system (Waters Corporation, Miliford, MA, USA) at a continuous flow rate of 0.2 mL/min for accurate mass corection and to promote the ionisation of lipid – fatty acid and phospholipid. Species and maintant source cleanliness. The mass resolution was approximately 20.000 FWHM over the mass range of interest. The declustering voltage is the DC potential applied between the last electrode of the first stepwave and the differentia pumping aperturę between the first vacuum region and the second vacuum region; this was set at a difference of 100 V. mass spectrometric analysis was performed in negative ionisation mode over a mass range of 50 – 1200 m/z with an integration time 1 s/scan Prior the analysis the mass spectrometer was calibrated using 5 mM sodium formate solution in isopropanol/water mixture (9:1, v/v) at a flow rate of 0.1 mL/min for 5 min. Each tissue sample was cut 10 times for reproducibility with each cut lasting approximetly 3 – 5 s. The enabled multiple locations on each tissue sample to be analysed. The delay between sampling and appearance of a signal was 2 – 3 s, with no carry-over effects visible between each burn and/or sample.

Chemometric data analysis

Multivariate statistical software package LiveID™ (Waters Corporation, Wilmslow, UK) was used as a model builder and recognition tool. In order to generate models from the untargeted profiling REIMS TOF MS data acquired in MassLynx v. 4.1 (Waters Corporation, Wilmslow, UK), the following data treatment steps were performed: lock-mass correction applied using the leucin enkaphalin ion at m/z 554.2647 such as lock mass; all spectra contained within each Bburn event^ termed the region of interest (ROI) were combined to form a single continuum spectrum; adaptive background subtraction (ABS) algorithm was applied to reduce the chemical background in the combined spectra; data resampling (binning to 0.1 Da) was performed to reduce the data dimensionality; and the resulting spectrum was normalized using the TIC. For principal component analysis (PCA), the data was centered using the mean value of the entire data set. For linear discriminate analysis (LDA), the data was centered using the mean values of each model class. In either type, the mean for each m/z bin is subtracted from the values of that bin. Other than normalization and centering, no additional manipulation was performed (for example, scaling). Following data pre-treatment steps, a PCA/LDA model was calculated. First, an unsupervised PCA (Singular Value Decomposition algorithm) transform is applied to the spectral data calculating the scores and loadings; a supervised LDA transform is then applied to the scores calculated by the PCA transform. LDA is a transform that maximises the inter-class variance, while minimising the intra-class variance, resulting in a projection where examples from the same class are projected close to each other and, at the same time, the class centres (means) areas far a part as possible. Although it is not a true regularisation technique, PCA-LDA is found to reduce the chance of over-fitting that may occur with a pure LDA model. During the recognition step, the model transforms spectra acquired from test samples into the associated model-space, after which a classifier decides to which class (if any) the spectra belongs. The model classifier uses a multivariate normal distribution (MVN) for each model class. During the model building phase, these distributions are constructed by transforming the training spectra to generatescores for the n principal components/linear discriminants selected for the model. The number of dimensions in the MVNs is also equal to n. The MVNs produce a like lihood measure for each class, and Bayes' rule is then applied to derive posteriori probabilities.

In silico, fivefold stratified validation was performed to determine the predictive accuracy of the model. The model building data set was divided in five partitions (five fold), each of which contains a representative proportion of each class within it (stratified). Four partitions (80%) of dataset are used to build a model under the same conditions as the original model. This model is used to predict the classifications of the one partition (20%) of the training set that was left out. The cycle was repeated iteratively 5 times and each partition was predicted once by a model trained from the other four. The output of the validation details the total number of correct and incorrect classifications, as well as the number of outliers.

Outliers were calculated according to the Mahalanobis distance to the nearest class center (Mahalanobis *1936*). If this distance is greater than the outlier threshold, the sample is considered an outlier.

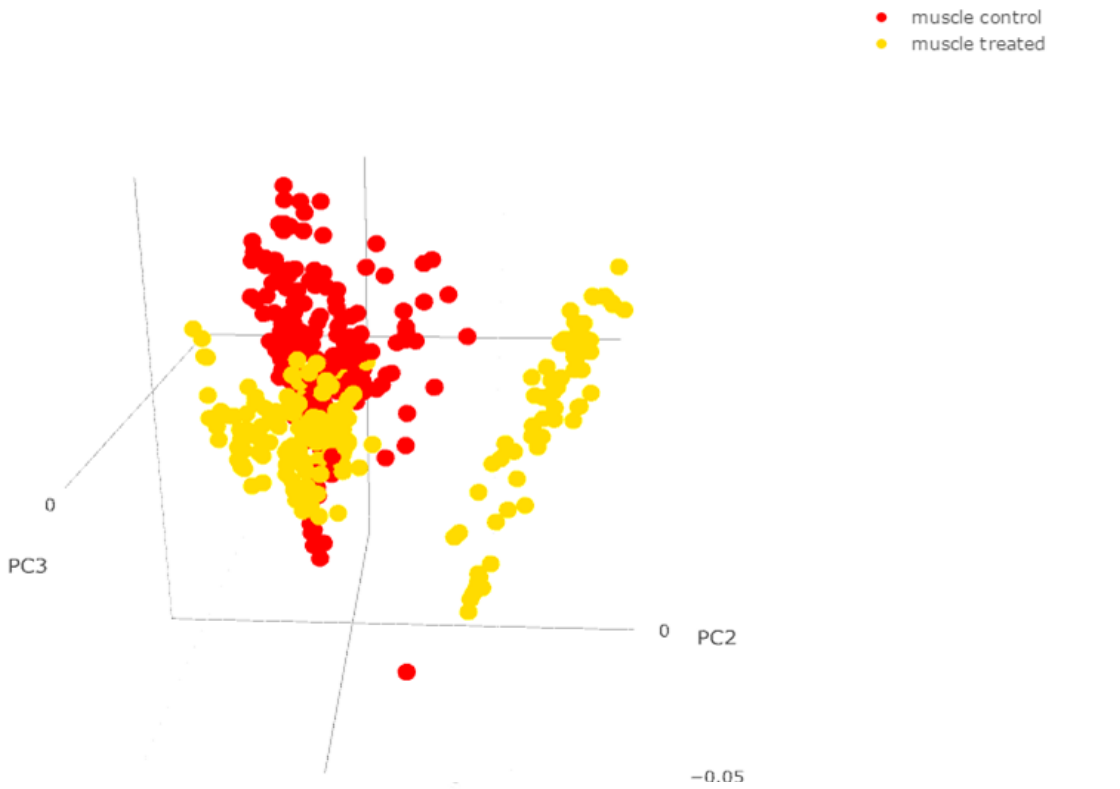
Following iterations of model optimization, an independent validation step was performer using a sample series not included in the training set.

Results and Discussion

REIMS – database training and challenge

The 50 muscles, 50 kidney and 39 liver samples from the control and from the treated populations with SARMs or nandronole/estradiol were analysed using REIMS in negative ionisation mode as a minimum of triplicates on three different days. The mass spectra generated were processed and analysed with the objective of investigating differences in the metabolic feature profiles between the two populations, control and treated bovines. The spectra were binned at 0.1 Da and the region between 600–950 m/z was used to generate a multivariate model with 10 PCA dimensions (for data reduction) followed by one LDA dimension for binary classification. During the model optimisation phase, 10 PCA dimensions were shown to provide the optimum predictive accuracy as determined via the percent correctness score generated using *in silico* stratified five-fold validation following iterative rounds of model training using between 2 and 30 PCA components. The percent variance explained by each PCA dimension was calculated, and greater than 95% of the total variance was shown to be explained with the first 10 components. An outlier threshold of three standard deviations was applied. As illustrated, in Figure 1 a, b and c the model enabled discrimination of the mass spectral profiles corresponding to the muscle, kidney and liver samples from either control or treated animals.

This research indicates that REIMS implemented in an untargeted-metabolomics workflow can be considered as a promising high-throughput and powerful strategy for real-time tissues classification. Further work should focus on validation and robustness assessment before potential implementation as rapid screening test, at the slaughterhouse or at boarder inspection points, to detect such practice.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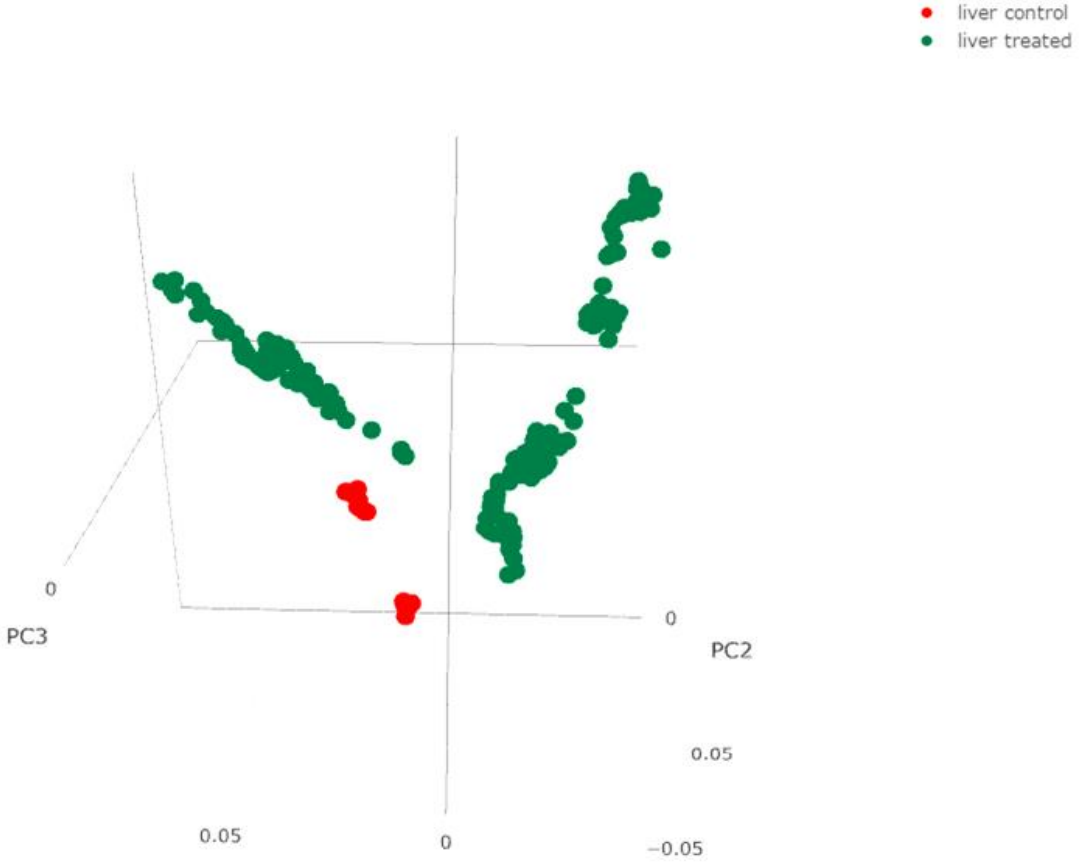
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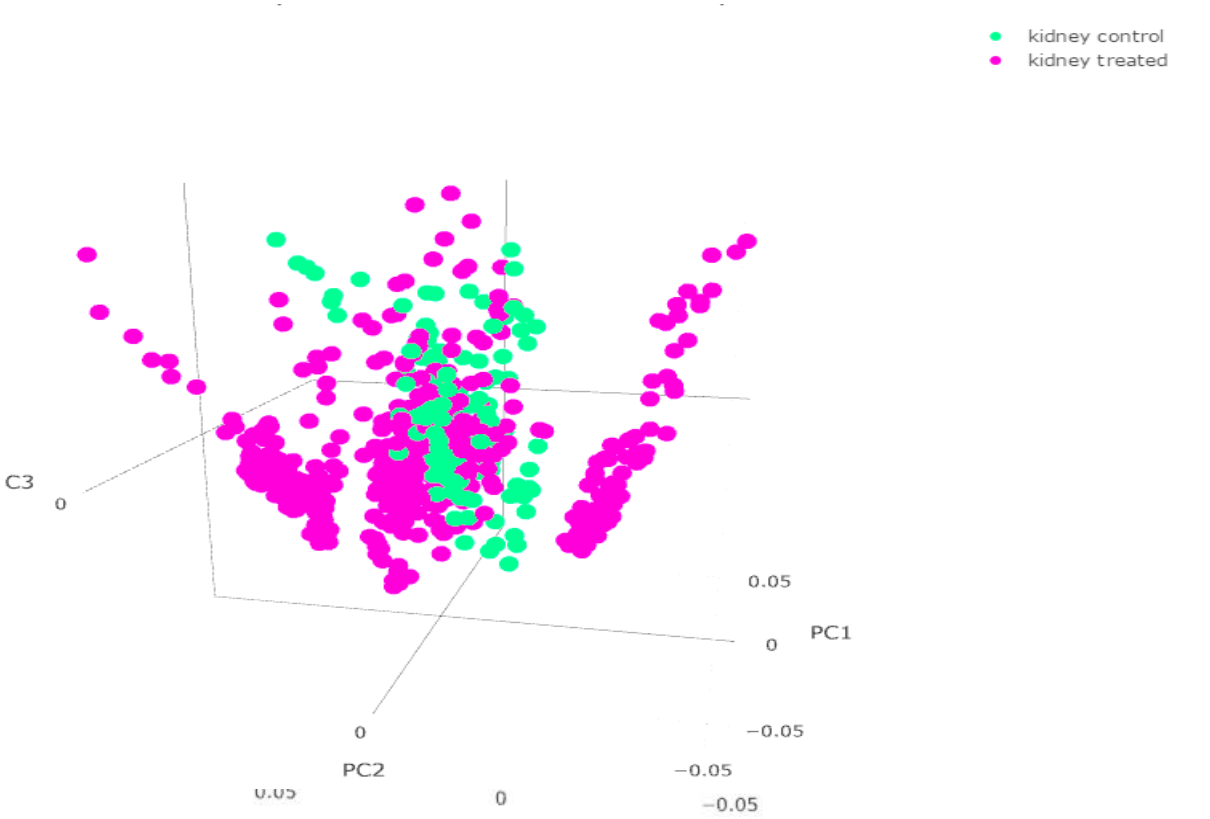

Figure 1. Principal component analysis (PCA), 3D PCA score plot generated in LiveID using 10 PCA dimensions generated from the training set of 280 spectra (150 treated and 130 control) acrross a mass tanged of 600 – 950 m/z with a binning of 0.1 Da.

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