CHAPTER II.4.

Multi-analyte approach for the determination of ng.l^{-1} amounts of steroid hormones in unidentified aqueous samples

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CHAPTER II.4.

Multi-analyte Approach for the Determination of ng.l^{-1} amounts of Steroid Hormones in Unidentified Aqueous Preparations

Summary

Since the 70s, many analytical methods for the detection of illegal growth promotors, such as thyreostats, anabolics, β-agonists and corticosteroids have been developed for a wide range of matrices of animal origin, including meat, fat, organ tissue, urine and faeces. The aim of this study was to develop an analytical method for the determination of ng.l^{-1} levels of estrogens, gestagens, androgens (EGAs) and corticosteroids in aqueous preparations (i.e. drinking water, drinking water supplements), commercially available on the 'black' market. For this, extraction was performed with Bakerbond C_{18} speedisk, a technique commonly used in environmental analysis. After fractionation, 4 fractions were collected using a methanol:water gradient program. Gas Chromatography coupled to Electron Impact multiple Mass Spectrometry (GC-EI-MS-MS) screening for the EGAs was carried out on the derivatized extracts. For the detection of corticosteroids, Gas Chromatography coupled to Negative Chemical Ionization Mass Spectrometry (GC-NCI-MS) was used after oxidation of the extracts. Confirmation was done by Liquid Chromatography coupled to Electrospray Ionization multiple Mass Spectrometry (LC-ESI-MS-MS). The combined use of GC and LC coupled to MS enabled the identification and quantification of anabolics and corticosteroids at the low ng.l^{-1} level. This study demonstrated the occurrence of both androgens and corticosteroids in different commercial aqueous samples.

1. Introduction

Steroid hormones are steroids which act as hormones. They can be divided into different groups: corticosteroids (glucocorticosteroids, mineralocorticosteroids) and estrogens, gestagens and androgens (EGAs) [Courtheyn et al., 2002, Impens et al., 2002]. This large group of estrogenic compounds is legally used in human and veterinary medicine. However, besides their use under regulated conditions, they are also illegally used in animal fattening and aquaculture because of their possibility to increase weight gain and to reduce the feed conversion ratio, which is the average feed intake in relation to the weight gain. In addition, their synergetic effects and their possibility to reduce nitrogen retention and to increase the water retention and fat content have been reported in literature [Antignac et al., 2001 and 2004, Courtheyn et al., 2002, Hidalgo et al., 2003]. Illegal growth promotors are mostly injected, resulting in injection sites in which high concentrations (mostly esters) can be found [De Wasch et al., 1998 and 2003]. Also via the feed, animals can be treated with EGAs [Impens, 2002].

The improper or illegal use of these compounds may result in drug residues in food products produced of these animals. To protect consumer's health, the European Union requires that all veterinary drugs are evaluated [EC/2377/90], and establishes Maximum Residue Limits (MRLs) of these compounds in specific edible matrices, i.e. muscle, fat, organ tissue, milk and eggs. The illegal use of steroid hormones in livestock breeding and aquaculture is banned within the European Union as described by 96/22/EC. Surveillance for the presence of residues of veterinary drugs in food-producing animals and foods is regulated by 96/23/EC. Consequently, the Federal Agency for the Safety of the Food Chain (FAVV-AFSCA) controls the illegal use of these compounds. For analytical method validation and interpretation of the results, criteria are established as described in the European Criteria EC/2002/657.

Nowadays, the presence of steroid hormones in animal matrices is not a new issue. The illegal use of veterinary medicines is monitored both by injections sites as by analysis of urine, faeces, fat, muscle and organ tissue (e.g. kidney, thyroid gland). In this sense, the need to develop highly sensitive and specific analytical methods for the determination of these compounds in a wide variety of matrices of animal matrices has increased due to the wide variety of illegal applications of steroid hormones.

As reported in literature, many novel approaches have been developed for the detection of steroid hormones in veterinary matrices like faeces, urine, liver, meat, fat, hair, milk, feed and injection sites [Stolker et al., 2000, Noben et al., 2002, Antignac et al., 2001 and 2004, De Wasch et al., 2003, Hooijerinck, 2003, Cherlet et al., 2004, Gratacós-Cubarsí et al., 2006]. However, as unidentified, probably illegal used aqueous preparations (i.e. drinking water or supplements) with suspected growth promoting properties may still being found on farms, research on developing multi-disciplinary strategies for the multi-residue analysis of these aqueous solutions is needed.

The present study was based on a previously described multi-disciplinary approach for the detection of estrogens in water samples [Noppe et al., 2006a, **chapter II.3.**]. The major goals of this study were to develop and to apply a multi-residue strategy to identify and quantify a large number of steroid hormones in aqueous samples and to use this method for the routine detection of ng l^{-1} levels of these compounds in a wide variety of 'unknown' aqueous preparations. For this, different chromatographic techniques, i.e. GC and LC coupled to MS were used.

2. Materials en methods

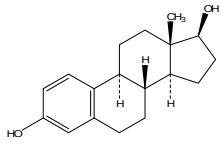
2.1. Chemicals

Standards of the natural and synthetic hormones were purchased from Sigma-Aldrich (St Louis, MO, USA), Steraloids (Newport, RI, USA) or were gifts from various sources. All solvents used for extraction and clean-up of the samples were of analytical grade and were purchased from Merck (VWR, Darmstadt, Germany) or Acros (Acros organics, Fairlawn, New Jersey, USA).

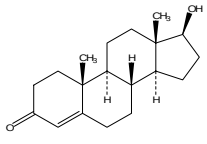
Primary stock standard solutions of the targeted steroid hormones were prepared individually in ethanol (EtOH) at a concentration of 200 ng.μl^{-1}. The working solutions of the mixtures at various concentrations were prepared by appropriate dilution of the stock solutions in EtOH for subsequent spiking of aqueous preparations. All standard solutions were stored at 4 °C in the dark following the quality assurance instructions of Belac accreditation (EN17025).

Table II.4.1.

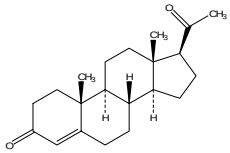
Structures and diagnostic ions of the investigated anabolic steroids (internal standards are marked in italic).



Estradiol (E2)



Testosterone (T)



Progesterone (P)

Compound	MW	Full Scan MS	Prec. ion	Product ions MS²	Spike (ng l^{-1})
β-zeranol (bZ)	322.4	307-335-389-433	433	295-309-323-337-389-415	50
hexestrol (HEX)	270.4	163-179-191-207	207	163-179-191	50
diethylstilbestrol (DES)	268.4	217-383-397-412	412	217-383-396-397	50
dienestrol (DE)	266.3	379-381-395-410	410	379-381-395	50
β-boldenone (bBOL)	286.4	206-325-415-430	206	163-175-183-191	75
α-boldenone (aBOL)	286.4	206-325-415-430	206	163-175-183-191	75
ethinyl estradiol (EE2)	296.4	232-285-425-440	425	193-231-281-283-303-323-407	50
fluoxymesterone (FMT)	336.4	319-407-462-552	552	319-407-462	125
α-zeranol (aZ)	322.4	307-335-389-433	433	295-309-323-337-389-415	50
17β-nortestosterone (bNT)	274.4	182-194-403-418	418	182-247-287-313-327-328-403	50
methyl boldenone (MeBol)	300.4	206-339-429-444	444	191-206-283-297-312-339-354-429	75
17α-nortestosterone (aNT)	274.4	182-194-403-418	418	182-247-287-313-327-328-403	50
norgestrel (NG)	312.4	194-301-316-456	456	301-316-337-366-427	50
chlorandrosteendione (ClAD)	320.4	429-449-456-464	464	234-339-359-429-449	125
methyl testosterone (MT)	302.4	301-341-356-446	446	251-301-314-341-356	50
methanedriol (MAD)	304.5	253-268-343-358	253	155-169-183-197-211	125
acetoxy progesterone (AP)	372.5	208-366-441-456	456	208-351-366-428-441	2500
norethandrolone (NE)	302.4	287-300-356-446	446	287-299-300-356	50
methyl androstandiol (MeAD)	306.5	255-270-345-435	435	199-213-255-345	125
ethyl estrandiol (EED)	306.0	157-241-331-421	331	145-185-199-241	50
medroxyprogesterone acetate (MPA)	386.5	222-380-455-470	470	222-237-365-380-455	500
melengestrol acetate (MeLA)	396.5	375-467-480-482	482	337-376-377-454-467	2500
megestrol acetate (MeGA)	384.5	363-453-468-470	468	323-363-440-453	1250
chlormadinon acetate (CMA)	404.9	437-453-473-488	488	363-383-437-453-473	500
caproxy progesterone (CP)	428.6	208-366-441-456	456	208-351-366-428-441	2500
chlortestosterone acetate (ClTA)	364.8	401-421-436-438	436	230-385-401-421	2500
androstadiendione (ADD)	284.0	206-323-413-428	428	191-206-222-323-413	125
equilenine (EQ)	266.3	280-305-395-410	410	280-294-305-320-381-395	125
ethinyl testosterone (ET)	312.4	301-316-441-456	456	299-301-316-351-441	125
methyl nortestosterone (MeNT)	288.4	287-342-417-432	432	285-287-300-342	125
1-dehydroprogesterone (1-DhP)	312.4	235-351-441-456	456	206-235-250-351-441	125
6-dehydroprogesterone (6-DhP)	312.4	171-351-441-456	456	171-249-351-366-441	125
16β-methyl progesterone (16b-MeP)	328.5	171-367-457-472	472	171-302-367-382-457	125
androsterone (And)	290.4	239-329-419-434	434	239-329-344-419	125

2.2. Chemical analysis

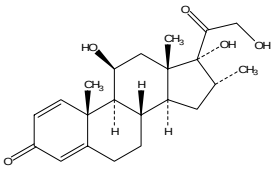
The target hormones in this study, their structure, molecular weight, parent and product ions are summarized in tables II.4.1. and II.4.2. This selection was based on the extended experience of the Laboratory of Chemical Analysis with residue-analysis of these compounds in matrices of animal origin [Impens, 2002].

2.2.1. Sample extraction and clean-up

Of the aqueous preparations, 100 to 500 ml (depending on the characteristics of the sample) were diluted to 1 l with ultrapure water, and subsequently spiked with internal standard (see tables II.4.1. and II.4.2., 125 ng.l^{-1} for EGAs and 40 ng.l^{-1} for corticosteroids). When needed, samples were filtered through Whatman filter paper (GF/C Ø 47 mm, Merck, Darmstadt, Germany) prior to extraction in order to avoid clogging of the sorbent. Subsequently, filters were washed with MeOH to prevent for losses of the compounds of interest. The extraction method for aqueous preparations performed in this study was based on a method developed for the extraction of estrogens from environmental water samples using Bakerbond Speedisk^{TM} Octadecyl-bonded silica (C_{18}XF), 50 mm (J.T. Baker, Deventer, The Netherlands) as previously described by Noppe et al., 2005 and 2006a [See also **chapter II.1.** and **II.3.**].

Table II.4.2.

Chemical structure and diagnostic ions of the investigated corticosteroids (internal standard in italic), spiked concentrations 10 and 40 ng.l^{-1}.



Dexamethasone (Dxm)

Compound	MW	GC-NCI	LC-ESI	
		Full Scan MS	Full Scan MS	Product ions MS²
dexamethasone (Dxm)	392,5	295-310-311-312	451	361-391
betamethasone (Btm)	392,5	295-310-311-312	451	361-391
prednisolone (prolon)	360,4	177-297-298-299	419	329-359
methyl prednisolone (Mprolon)	374,5	177-312-313-314	433	343-373
flumethasone (Flm)	376,5	313-314-328-329	469	379-409
fluorometholone (Fml)	410,5	295-310-311-330	435	255-355-375
isoflupredone (IFP)	378,4	281-282-296-297	437	347-377

2.2.2. Analytical procedure EGAs

After extraction and HPLC fractionation [Noppe et al., 2006a] using a water:methanol gradient programme (See **chapter II.3.**), samples for the analysis of EGAs were derivatized with a mixture of MSTFA, ethanethiol and ammoniumiodide [Impens et al., 2002]. Chromatographic analysis for the EGA's was performed by GC-EI-MS-MS.

All chromatographic and spectrometric analyses were performed using a Trace GC 2000 Gas Chromatograph fitted with a Polaris ion trap mass spectrometer (Thermo Finnigan, Austin, TX, USA) with a Carlo Erba autosampler AS2000 (Thermo Finnigan, Austin, TX, USA). Helium (99.99 % purity, Air Liquide, France) was used as carrier gas at a flow rate of 1 $ml.min^{-1}$. FC43 (Perfluorotributylamine) (Ultra Scientific, North Kingstown, USA) was used as calibration gas. A volume of 1 µl was injected (spit flow 60 $ml.min^{-1}$, splitless time 1 min).

Separation of the target analytes was performed on a BPX-5 (SGE Inc., Austin, TX, USA) (25 m x 0.22 mm I.D.) fused silica capillary column with 5 % phenyl liquid phase (film thickness 0.25 µm). Injector, ion source and transfer line temperature were respectively 250 °C, 200 °C and 275 °C. Temperature program: initial 100 °C; ramp at 17 $°C.min^{-1}$ to 250 °C; ramp at 2 $°C.min^{-1}$ to 300 °C (hold 1.30 min). The spectra were obtained in Electron Impact (EI) mode at 70 eV.

2.2.3. Analytical procedure corticosteroids

For the chromatographic analysis of the corticosteroids, extracts were after fractionation analysed by GC-NCI-MS and if needed confirmed by LC-ESI-MS-MS. For the GC analysis, the targeted extract was taken to dryness and reconstituted in a mixture of 50 µl acetonitrile and 50 µl of an oxidation reagent. The latter consisted of 1g potassiumdichromate and 10 ml 10% aqueous sulphuric acid. After 10 min at 60 °C (± 2°C), extraction was carried out using 100 µl aqueous sodium carbonate (10 %), 800 µl water and 3 ml of a n-hexane-dichloromethane mixture (2:1). This mixture was centrifuged and frozen. The organic layer was taken to dryness and reconstituted in 50 µl toluene.

Gas chromatographic analyses were performed in electron impact mode with a Finnigan Trace Gas Chromatograph coupled to a PolarisQ ion trap mass spectrometer and a Finnigan MAT A200S autosampler (Thermofinnigan, Austin Texas, USA). Separations were conducted on a BPX-35 fused-silica capillary column, 25 m x 0.22 µm ID; 0.25 µm film thickness, 35 % phenyl polysilphenylene-siloxane liquid phase (moderately polar) (SGE Inc., Austin Texas, USA). A sample volume of 1 µl of sample was injected with a split-splitless injector (split flow 20 $ml.min^{-1}$, splitless time 1 min). The column was held at 90 °C (1 min), ramped at 90 $°C.min^{-1}$ to 270 °C, ramped at 3 $°C.min^{-1}$ to 300 °C (1 min) . The injector, the ion source and transferline temperature were respectively 250, 200 and 275 °C. Helium was used as carrier gas at a flow rate of 1 $ml.min^{-1}$. FC43 was used as a calibration gas. The ion trap was equipped with the variable damping gas option that provided a control of the helium damping gas and the ammonium (NH_3 VLSI 0.2 kg x 0.4 S Din8, quality 5.2., Air products, Vilvoorde) gas flow in the ion trap. This flow was set at respectively 0.3 and 1.4 $ml.min^{-1}$. Spectra were obtained in the full scan mode.

For the LC-ESI-MS-MS analysis, the extract was taken to dryness and reconstituted in 100 µl of 0.2 % aqueous acetic acid and 0.2 % acetic acid in acetonitrile (20:80). The LC system consisted of a Finnigan surveyor Autosampler plus and a Finnigan surveyor MS pump plus coupled to a Finnigan LTQ linear ion trap mass spectrometer equipped with an Electrospray Ionization (ESI) source, which was in the negative mode (Thermo Electron, San José, CA, USA). Chromatographic separation was achieved using a Thermo hypercarb column (100 x 2.1 mm, 5 µm particle size, Thermo electron, San José, USA). The mobile phase consisted of 0.2 % aqueous acetic acid (A) and 0.2% acetic acid in acetonitrile (B). The gradient started with 20% A: 80% B for 18 minutes and subsequently increased to 100 % B. At 22.10 minutes the initial gradient conditions were restored until 26 min. Mobile phase flow was set at a flow rate of 0.3 $ml.min^{-1}$. The sample tray was maintained at 15 °C, whereas the column was maintained at 35 °C. Spectra were obtained using MS-MS scan mode. Of the extracts, 10 µl was brought on column.

2.2.4. Data interpretation

Prior to sample analysis standard mixture of the targeted compounds was injected in order to check the operation conditions of the chromatographic devices. All data were processed using Xcalibur® software (Thermo Electron, San Jose, USA).

3. Results and discussion

3.1. Extraction and clean-up procedure

Due to the 'unknown' status of the aqueous preparation and the suspected low concentrations levels, sample volumes as large as possible, depending on the characteristics of the sample, were processed in order to attain the preconcentration factors needed for a quantitative analysis. For this, up to 500 ml sample (or a certain amount diluted to 1 l with ultrapure water) was used for speedisk extraction, a technique commonly used in environmental analysis (See also **chapter II.1.**).

Also due to the 'unknown' state of these samples fractionation was performed in order to obtain clean extracts that can be used for chromatographic analysis. As described earlier [**chapter II.3.**] fractionation of the extracts is an advisable approach to get rid of interfering peaks and background noise in the chromatogram. Based on the extended experience of the laboratory with the detection of hormone steroids in matrices of animal origin (i.e. faeces, urine, meat, fat) and the use of HPLC-fractionation as clean-up technique for extracts of these matrices, it was known that of the 4 fractions obtained, the targeted corticosteroids (see table II.4.2.) were within the first collected fraction and the targeted EGAs (see table II.4.1.) were collected within the other 3 fractions.

3.2. Method validation

Because no guidelines for the analysis of 'unknown water samples' exist, the European Criteria 2002/657, which are the criteria for analytical residue methods for matrices of animal origin were used in the present study. Compounds were identified based on relative retention time and the ion ratio of the precursor/product ions in the obtained spectrum. The described multi-residue method for the detection of steroid hormones in 'unknown water samples' is a semi-quantitative method. Because no blank 'unknown water sample' was available, the specificity of this method was assessed by the analysis of blank and fortified ultrapure (which was used when the samples were diluted) and tap water samples. For this, blank water samples were fortified with steroid hormones in the range of 50 to 2500 $ng.l^{-1}$ (see table II.4.1.), depending on the target compound and based on preliminary experiments. No interferences could be observed using both GC-NCI-MS and LC-ESI-MS-MS. According to

the European Criteria 2002/657 the minimum number of identification points (IPs) for steroid hormones is set at four. For the targeted EGAs, each precursor ion counts for 1 IP and each product ion counts for 1.5 IPs. As can be seen in table II.4.1. each targeted EGA has at least 2 product ions. For the targeted corticosteroids using GC-NCI-MS, 4 precursor ions (isotope ions included) were selected each counting for 1 IP. When the samples were analyzed with LC-ESI-MS-MS, 1 precursor ion and at least 2 product ions were selected each counting for respectively 1 and 1.5 IPs. When the criteria for both the relative retention time and the ion ratio (IPs) were fulfilled, the concentration of the steroid compound was estimated using standard mixture injections or fortified blank samples.

3.3. Chromatographic analysis

3.3.1. EGAs

Fractions of EGAs were analysed using Gas Chromatography coupled to ion trap Mass Spectrometry (GC-MS-MS) in the electron impact (EI) mode. It should be added that, for screening purposes for EGAs, multiple MS is preferred above MS. Although the latter results in a higher intensity, the selectivity is insufficient when taken into account the possible matrix interferences and the low levels of interest in animal or water matrices. Figure II.4.1. shows the chromatogram and spectrum obtained from the extraction of 1 l of an unknown aqueous sample. In this sample medroxyprogesterone acetate (MPA) or 6α-methyl-3,20-dioxopregn-4-en-17-yl acetate was detected (> 4 IPs), which is a synthetic progestagen. The concentration of 40 $ng.l^{-1}$ was determined using standard mixture injections.

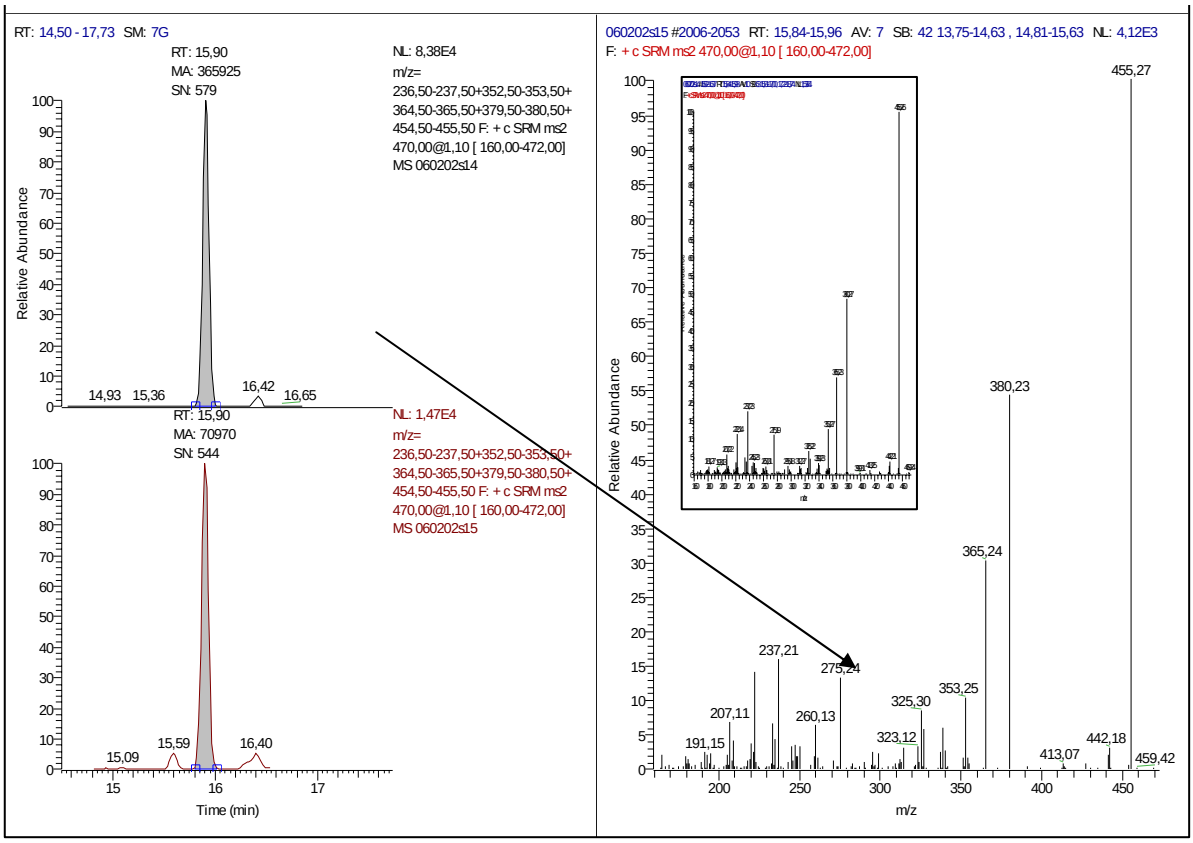


Figure II.4.1.

Chromatograms (shaded zones = peak area) and spectrum of medroxyprogesterone acetate (MPA) in (A) an unknown water sample and (B) standard mixture (2 ng on column).

3.3.2. Corticosteroids

In the first place, GC-NCI-MS was used to analyse the fraction for the targeted corticosteroids because it is a better technique when matrix interference is expected (See Table II.4.2. for precursor and product ions). However, using GC-NCI-MS, it is known that by-products and interfering compounds can complicate proper interpretation of the chromatographic analysis and less complex sample preparation [Antignac et al., 2004, Cherlet et al., 2004]. It is known that the differentiation between dexamethasone (Dxm) and betamethasone (Btm), which differ only in the configuration of the methylgroup on C_{16}, is not always clear [De Wasch et al., 2001, Deventer and Delbeke, 2003, Taylor et al., 2004]. Only when there is no matrix interference or apparatus contamination, distinction between Dxm and Btm can be made through the ratio of both peaks as shown in figure II.4.2.

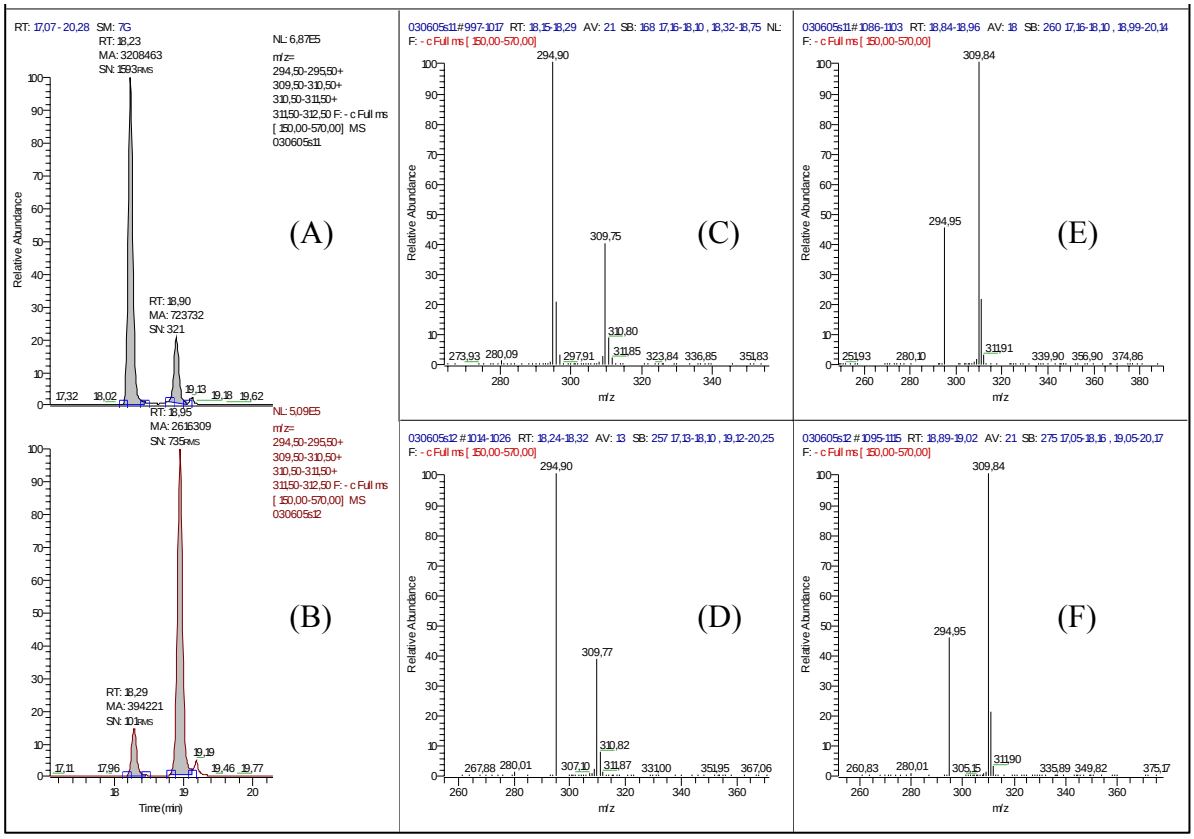


Figure II.4.2.

Chromatograms (shaded zones=peak areas) of (A) dexamethasone (Dxm) and (B) betamethasone (Btm) and spectra of both peaks of Dxm (C and E) and both peaks of Btm (D and F).

As can be seen in figure II.4.3. for an 'unknown' water preparation, 2 peaks were obtained in the chromatogram, both with the same relative retention time and with the same products and ion ratios in their corresponding spectrum. This indicates that the field of application of GC-NCI-MS is limited to screening purposes because different compounds can lead to the same derivative. Above this, LC does not require a derivatization step and as such, enables direct measurements of corticosteroids [Antignac et al., 2004]. For this, to obtain better selectivity in order to confirm the unambiguous identity of suspected Dxm or Btm, a second injection on a new device, Liquid Chromatography coupled to a LTQ linear ion trap Mass Spectrometer was performed. This device offers more sensitivity due to the novel ion trap, dual detector and ion ejection technologies.

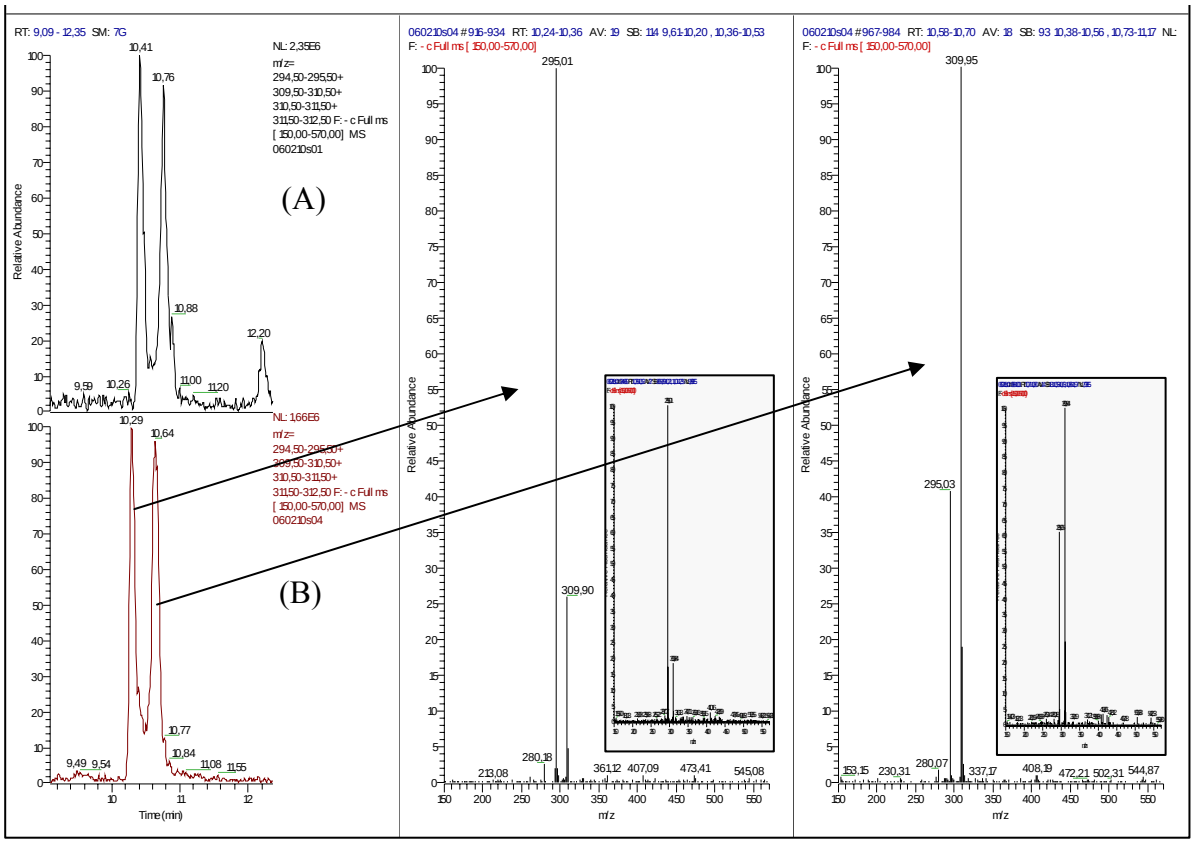


Figure II.4.3.

Chromatograms and spectra of dexamethasone (Dxm) and betamethasone (Btm) in (A) standard mixture (1 ng on column) and (B) an unknown water preparation after analysis with GC-NCI-MS. Insets are standard mixture spectra.

Interpreting the results of the GC-NCI-MS analysis (Figure II.4.3.) it can be concluded that Dxm or Btm is suspected, although a clear distinction between these two compounds is not possible. After addition of Dxm to the sample and LC-ESI-MS analysis (Figure II.4.4.) it was concluded that the sample contained betamethasone (9α-fluoro-11β, 17α,21-trihydroxy-16α-methylpregna-1,4diene-3,20-dione) at 50 ng.l^{-1} (4 IPs). This synthetic glucocorticosteroid has a widespread application in human and veterinary medicine.

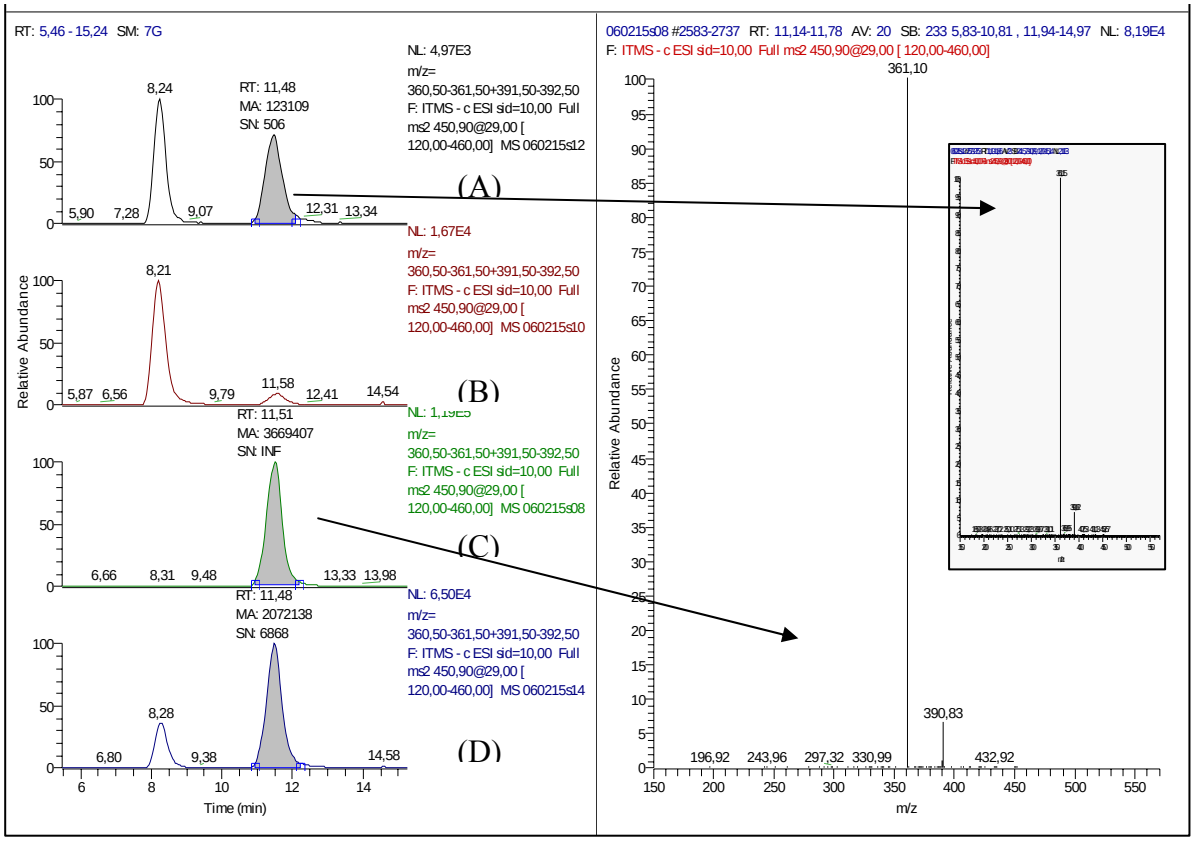


Figure II.4.4.

Chromatograms of (A) standard mixture (1 ng on column), (B) a fortified ultrapure water sample (40 ng.l^{-1}), (C) an unidentified aqueous water sample and (D) the same extract fortified with Dxm. On the right side the spectrum of betamethasone (Btm) of the 'unidentified' water sample is given (Inset is the spectrum of Btm from the standard mixture).

4. Conclusion

In this investigation, a routine multi-analyte approach for the screening of estrogens, gestagens and androgens (EGAs) and corticosteroids in unidentified aqueous preparations is described. With this method, a large group of steroid hormones at ng.l^{-1} levels can be detected, which fits into the inspection services strategy to control the abuse of EGAs and corticosteroids for animal fattening purposes.