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ORIGINAL PAPER

Validation and application of an LC-MS/MS method for the simultaneous quantification of 13 pharmaceuticals in seawater

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Abstract Knowledge of the presence of micropollutants such as pharmaceuticals, in coastal areas, is very limited; therefore, the main objective of this study was to optimize and validate a new analytical method for the quantitative analysis of 13 multiclass pharmaceuticals in seawater. Target compounds included antibiotics, non-steroidal anti-inflammatory drugs, β-blockers, lipid regulators and one psychiatric drug. A combination of solid-phase extraction and liquid chromatography coupled with multiple mass spectrometry enabled their detection at the low nanogram per litre level. The limits of quantification varied between 1 and 50 ng L^{-1}, for most components the linearities were more than 0.99 and the recoveries obtained in seawater (95–108%) were satisfactory. This method was applied to seawater and estuarine water samples collected in the Belgian coastal zone, to assess the prevalence of common pharmaceuticals in this marine environment. Seven pharmaceuticals, including compounds of which the presence in marine environments had not been reported earlier, were detected, with salicylic acid and carbamazepine being the most abundant, in concentrations up to 855 ng L^{-1}.

Keywords Pharmaceuticals · Liquid chromatography–tandem mass spectrometry · Validation · Marine environment · Persistence

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Introduction

Pharmaceuticals are the active ingredients of medicinal products used in human and veterinary medicine and include approximately 3,000 different compounds with a large variation in chemical structure, function and behaviour [1, 2]. In Belgium, the consumption of reimbursed pharmaceuticals available at the pharmacy level ranges between 0.001 and 6 tons per year depending on the individual pharmaceutical (Table 1) [3] (H. Beyers, personal communication). Pharmaceuticals for human use are excreted—either in their native form or as metabolites—and discharged into the sewer system [4, 5]. Via sewage, pharmaceuticals reach the wastewater treatment plant (WWTP), where most pharmaceuticals are, according to current literature, not completely removed [6, 7]. As a result of WWTP effluents, land application of sewage sludge, improper disposal and manufacturing processes, pharmaceuticals are introduced into natural aquatic systems [8, 9]. These different pathways may result in a continuous release of pharmaceuticals into the aquatic environment. Consequently, there is growing public and scientific concern regarding the occurrence and potential effects of pharmaceuticals in the aquatic environment.

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Table 1 Chemical structures of the target compounds, their physicochemical properties [6, 27, 28] and the annual consumption at pharmacy level in 2007 in Belgium [3] (H. Beyers, personal communication)

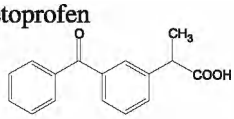
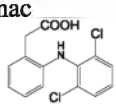
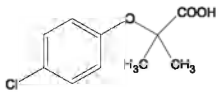
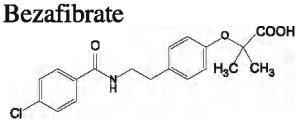
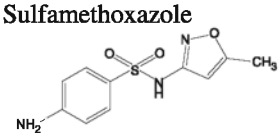
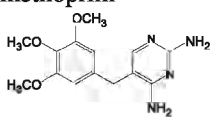
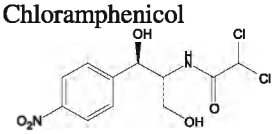
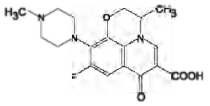
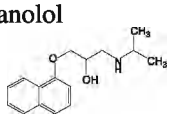
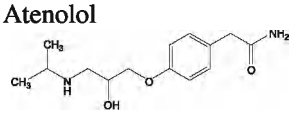
Compounds	Molecular mass	Log K_{ow}	pK_a	Consumption (kg/year)
Non-steroidal anti-inflammatory drugs				
Salicylic acid 	138.12	2.43	3.50	11
Mefenamic acid 	241.28	4.16	4.20	ND
Ketoprofen 	254.28	3.22	4.45	199
Diclofenac 	296.15	3.91	4.15	3,360
Lipid regulators				
Clofibric acid 	214.65	2.58	3	ND
Bezafibrate 	361.83	3.85	3.60	176
Antibiotics				
Sulfamethoxazole 	253.28	0.68	5.70	453
Trimethoprim 	290.32	0.65	6.60	ND
Chloramphenicol 	323.13	1.14	5.5	1

Table 1 (continued)

Ofloxacin 	361.37	0.35	ND	322
Neuroactive compounds				
Carbamazepine 	236.27	2.3	13.90	6,107
β-Blockers				
Propranolol 	259.34	3.03	9.49	2 320
Atenolol 	266.34	0.46	9.16	2 232

ND no data

As pharmaceuticals are designed to exert specific effects at low doses and to resist metabolic degradation, their possible biological activity in aquatic organisms and their persistence cannot be excluded [4, 5]. Moreover, the continuous environmental input of these compounds, and resulting mixtures, may induce unnoticed adverse effects on aquatic and terrestrial organisms [10, 11]. Although several studies have reported standard ecotoxicity data for pharmaceuticals [8, 12, 13], in general their environmental significance, pertaining to environmental effects, is largely unknown [14]. However, a preliminary risk assessment database for common pharmaceuticals with a focus on marine and estuarine environments is nowadays available to provide information on pharmaceutical threats to the environment [6].

Regulatory guidance to assess the presence of pharmaceuticals in the aquatic environment is still lacking. The Water Framework Directive (2000/60/EC) [15] and its daughter directive (2008/105/EC) [16] lay down environmental quality standards for a list of priority substances, but pharmaceuticals are not included. Furthermore, the OSPAR lists of chemicals of priority action and of substances of possible concern include, respectively, two and 19 pharmaceuticals [17]. However, these listed pharmaceuticals are used in fairly small quantities and their occurrence in the environment is limited [9]. Currently, no guidance is established for widely used and widespread occurring pharmaceuticals such as carbamazepine and diclofenac.

Nevertheless, in recent years, numerous monitoring studies have demonstrated the occurrence of pharmaceuticals in aquatic systems. They have been detected in WWTP influents and effluents and in freshwater systems in the nanogram per litre up to the microgram per litre range [18–21]. More rarely, pharmaceuticals have been detected in drinking water [22, 23] and groundwater samples [21, 24], mostly in the nanogram per litre range and occasionally at microgram per litre levels. In contrast to the extensive literature describing the occurrence and persistence of pharmaceuticals in freshwater systems, little attention has been paid to their prevalence and quantification in marine ecosystems [25, 26].

Therefore, the main objective of this study was to develop a quantitative analytical method for pharmaceuticals in seawater. On the basis of data on the current use in Belgium (Table 1), 13 environmentally relevant pharmaceuticals were selected from five different therapeutic classes. These included four antibiotics (sulfamethoxazole, ofloxacin, trimethoprim and chloramphenicol), four non-steroidal anti-inflammatory drugs (NSAIDs) (mefenamic acid, diclofenac, salicylic acid, and ketoprofen), two β-blockers (propranolol and atenolol), two lipid regulators (bezafibrate and clofibric acid) and one psychiatric drug (carbamazepine). The chemical structures of the pharmaceuticals considered in this study and their physicochemical properties are presented in Table 1 [6, 27, 28]. Secondly, an extensive validation study was carried out to demonstrate

the applicability of this analytical approach. To this end, the method developed was applied to marine water samples taken from the North Sea and the Scheldt estuary. In this way, the presence of pharmaceuticals in the Belgian marine environment and their transfer to estuarine and marine ecosystems was examined. This study is part of the INRAM project (http://www.vliz.be/projects/inram), a 4-year project that aims to use an integrated approach to assess the risks of micropollutants in the Belgian coastal zone.

Experimental

Study area and sampling

The study area is located in the three Belgian coastal harbours (Ostend, Nieuwpoort and Zeebrugge), the Scheldt estuary and the offshore coastal area of Belgium. An overview of the study area and the sampling stations is depicted in Fig. 1. Ten sampling stations were selected in three coastal harbours; four in the harbour of Zeebrugge (ZB01–ZB04), three in the harbour of Nieuwpoort (NP01–NP03) and three in the harbour of Ostend (OO02–OO04). In each harbour, one sampling station was representative for the major freshwater inputs into the harbour, whereas the second sampling location represented the water at the harbour mouth and at least one station between these points was sampled as well. An additional station was selected at the Sluice Dock in Ostend (OO01) since at this location aquacultural activities take place. Two stations were sampled in the Scheldt estuary: one station located at the river mouth near Vlissingen, the second more upstream near Antwerp. Six sampling stations were chosen in the Belgian coastal area: three (W01–W03) were located close to the harbour mouths of Ostend, Nieuwpoort and Zeebrugge; the remaining three (W04–W06) were situated more offshore. Four sampling campaigns were carried out: in May and December 2007, April 2008 and June 2009.

Harbour stations were sampled using the *Zeekat*, a rigid inflatable boat. North Sea and Scheldt estuary stations were sampled with the larger research vessels *Belgica*, *Zeeleeuw* and *Scheldewacht*. Water was sampled at each sampling site using Go-Flo® bottles (General Oceanics, Miami, FL, USA) at a depth of 4–5 m. Go-Flo® bottles avoid sample contamination at the surface, internal contamination, loss of sample on the deck and exchange of water from different depths. In accordance with Noppe et al. [29], samples were acidified to prevent bacterial or algal growth and were stored at 4 °C in the dark before analysis.

Fig. 1 Sampling stations in the North Sea (*W01–W06*), the Scheldt estuary (*S01* and *S22*) and in the harbours of Nieuwpoort (*NP1–NP3*), Ostend (*OO01–OO04*) and Zeebrugge (*ZB01–ZB04*)

Reagents and chemicals

Ketoprofen (purity 99.0%), mefenamic acid (purity better than 99.0%), carbamazepine (purity better than 99.0%), diclofenac (purity better than 99.0%), bezafibrate (purity 98.0% or better), salicylic acid (purity better than 99.0%), clofibric acid (purity 97.0%), atenolol (purity 98.0% or better), trimethoprim (purity 98.0% or better), chloramphenicol (purity 99.0% or better), and sulfamethoxazole (purity 99.0%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ofloxacin (purity better than 99.0%) was obtained from ICN Biomedicals (Aurora, OH, USA) and propranolol (purity better than 99.0%) was purchased from Eurogenerics (Brussels, Belgium). The synthetic isobutcar 61 [4-3(isobutylamino-2-hydroxypropoxy)-carbazole] was found to be a very suitable internal standard for this application as it corrected sufficiently for any matrix effects in the sample preparation and mass spectrometry (MS).

Acetone, methanol and formic acid were of analytical grade and were purchased from VWR (Merck, Darmstadt, Germany). High performance liquid chromatography (HPLC)-grade acetonitrile and water were obtained from VWR (Merck, Darmstadt, Germany) and Acros Organics (Fairlawn, NJ, USA), respectively. Primary stock solutions of all individual analytes were prepared in ethanol at a concentration of 1 ng μL^{-1}. Working standard mixture solutions were prepared by appropriate dilution of the stock solutions in ethanol. All solutions were stored at -20°C in the dark.

Extraction and clean-up

The extraction technique developed is based on the method of Gómez et al. [30] and Nebot et al. [31]. Prior to extraction, the pH of the water samples was adjusted to 7± 0.5 using solutions of HCl (1 M) and NaOH (1 M). Seawater samples of 1 L were filtered through a Whatman filter paper (GF/C diameter 47 mm, particle retention 1.2 µm, Merck, Darmstadt, Germany) to avoid clogging of the sorbent. The filters were washed with 2 mL methanol to prevent loss of the compounds of interest. The methanol extract was collected and added to the filtered sample. Prior to extraction, the internal standard isobutcar 61 was added to every sample at a final concentration of 50 ng L^{-1}. Subsequently, solid-phase extraction (SPE) was carried out using Chromabond HR-X cartridges (3 mL, 200 mg, Macherey-Nagel, Düren, Germany). The cartridges were preconditioned with 5 mL methanol and 5 mL Milli-Q water. After they had been loaded with 1 L of the filtered sample pooled with the methanol from the filter washing, the cartridges were rinsed twice with 5 mL Milli-Q water. Subsequently, the cartridges were dried under a vacuum for 30 min. Elution was performed using 5 mL acetone and 2× 5 mL methanol. Next, the extracts were dried using nitrogen and the residues were reconstituted in 300 µL acetonitrile/0.02 M formic acid (50/50) before transfer to iquid chromatography (LC)–MS vials.

Chromatographic instrumentation

Analysis was carried out using HPLC. The apparatus comprised an 1100 series quaternary gradient pump and autosampler (Hewlett Packard, Palo Alto, CA, USA). Chromatographic separation was achieved using a Nucleodur® C_{18} Isis HPLC column (5-µm particle size, 250 mm×4.0 mm, Macherey-Nagel, Düren, Germany). The void volume of the system was 2.5 mL. A filter (porosity 2 µm, 4 mm; Alltech, Waukegan, IL, USA) and a precolumn (Nucleodur® C_{18} Isis, Macherey-Nagel, Düren, Germany) were used to prevent rapid deterioration of the analytical column. The mobile phase consisted of a mixture of 0.02 M formic acid (solvent A) in water and acetonitrile (solvent B). A linear gradient of 0.3 mL min^{-1} was used starting with a mixture of 60% solvent A and 40% solvent B for 5 min. The acetonitrile percentage was increased linearly from 40 to 100% in 20 min. Twenty microlitres was injected onto the LC-MS^n system. Analytes were detected with an LCQ DECA ion trap mass spectrometer equipped with an electrospray ionization (ESI) interface (Thermo Finnigan, San Jose, CA, USA) using the optimized MS parameters described in "Results and discussion". The optimal ionization source working parameters were as follows: sheath gas flow rate, 80 arbitrary units (a.u.); auxiliary gas, 20 a.u.; capillary temperature 350° C; capillary voltage, -14 V; and tube lens offset, 20 V. Chromatograms and spectra were recorded and processed using Xcalibur® 2.0 (Thermo Finnigan, San Jose, CA, USA).

Validation of the method

For environmental sample analysis, rigorous validation procedures are usually not well defined. Similar to reported studies on pharmaceuticals in water [11, 20, 32], we validated the method developed according to Commission Decision 2002/657/EC [33] concerning the determination of analytes in products of animal origin. Also SANCO/825/00 revision 7 [34] was used as a guideline for the validation of this new analytical method. In general, validation includes the evaluation of linearity, specificity, selectivity, recovery, matrix effects and the determination of the limit of quantification (LOQ). Because the method was particularly aimed at examining marine waters, seawater was used to validate the method. On the basis of preliminary results, blank seawater samples contained low levels of some of the target analytes. The calibration curves were corrected for these concentrations. In addition, none of the pharmaceuticals were detected in reagents or Milli-Q water.

Quality assurance

Before and after analysis of a series of samples, a standard mixture (1 ng on column) of the target pharmaceuticals was injected to check the instrument parameters of the LC-ESI-MS^n system. Quality control of the method was performed by analysis of a blank sample, together with a linear calibration curve constructed using 1 L seawater samples spiked with standard solutions at seven concentrations between 1 and 1,000 ng L^{-1}. This was performed for every series of samples. These calibration curves were used for quantification.

Results and discussion

Method development

Extraction of the target pharmaceuticals

Irrespective of the log K_{ow} and pK_a values of the substances studied (Table 1), an extraction procedure aiming at the recovery of all target analytes in an efficient and repeatable manner was developed. To obtain a concentrated extract suitable for analysis, extraction of pharmaceuticals in water is generally executed using SPE [20, 27, 31, 35]. SPE includes the extraction of the analytes by means of a solid sorbent and is usually performed off-line. Preliminary experiments were performed to evaluate the extraction efficacy of different types of SPE sorbents: OASIS HLB (3 mL, 60 mg, Waters, Milford, MA, USA), Strata-x (33 μm polymeric reversed phase, 6 mL, 200 mg, Phenomenex, USA), Bakerbond Speedisk extraction disk (H_2O-phobic and H_2O-philic, J.T. Baker, Deventer, The Netherlands) and Chromabond HR-X (3 mL, 200 mg, Macherey-Nagel, Düren, Germany) cartridges. Of all the cartridges, Chromabond® HR-X exhibited the best performance in simultaneously retaining all the analytes at a pH of 7. Indeed, Zhang and Zhou [36] demonstrated similar extraction recoveries at pH 4.2 and at pH 10.3; therefore, a pH of 7.0 was selected for the water samples to obtain an analytical compromise for the best retention for all analytes. The SPE procedure using Chromabond HR-X cartridges was therefore optimized. In the final extraction protocol, prior to elution, the SPE cartridges were rinsed twice with 5 mL of Milli-Q water. In marine analytical chemistry an additional advantage of the washing step is the removal of remaining sea salt from the cartridge. Higher recovery rates were achieved by including this washing step (data not shown). Optimal elution of the pharmaceuticals was achieved using acetone followed by methanol. The most optimal elution solvent was selected on the basis of analytical characteristics such as peak area, resolution and signal-to-noise ratio.

Detection

Owing to the limited volatility of the pharmaceuticals, LC was the preferred chromatographic technique used in this study to achieve separation of the target analytes. Good chromatographic separation of the compounds under investigation was achieved using a C_{18} Isis reversed-phase LC column as the stationary phase. The retention mechanism of this column is based on steric and hydrophobic interactions and resulted in the optimized separation of all analytes. A mixture of acetonitrile and 0.1% formic acid in water proved superior as opposed to other solvent modifiers such as ammonium acetate.

Mass spectra were obtained using direct infusion of each standard in the mobile phase. The following operational parameters of the MS detector were optimized: MS ion mode, collision energy (eV), isolation width (*m/z*) and activation *Q*. ESI was used as the ionization source in both negative and positive ion mode by injecting the final extract twice. Detection of the negative precursor ion $[M\text{-}H]^-$ was performed for chloramphenicol, salicylic acid, bezafibrate, clofibric acid and mefenamic acid, whereas detection of the positive precursor ion $[M + H]^+$ was performed for the other compounds of interest. Precursor and product ions and collision energies are presented in Table 2. The isolation width was set at 2.0*m/z*, except for chloramphenicol, clofibric acid and diclofenac (3.0*m/z*). For the activation *Q*, the default value of 0.25 was used, except for salicylic acid (0.35). MS^n was performed for all precursor ions and allowed reliable confirmation of the target analytes. Figures 2 and 3 show the chromatograms and spectra obtained for the pharmaceuticals detected in positive and negative ionization mode, respectively (100 ng L^{-1} spiked in seawater).

Validation study

Identification/selectivity

Identification and confirmation of the compounds was performed according to the procedure prescribed by Commission Decision 2002/657/EC [33]. Compounds were identified on the basis of their relative retention time, which is the ratio of the retention time of the analyte to that of the internal standard. In addition, the ion ratios of the precursor and product ions in the spectrum obtained upon chromatographic analysis were taken into account when the peak in the chromatogram had a signal-to-noise ratio of at least 3:1. Commission Decision 2002/657/EC [33] also describes a system of identification points. Detection of precursor and product ions yields, respectively, one and 1.5 identification points. To obtain a minimum of four identification points, MS^3 fragmentation was required for salicylic acid, bezafibrate and mefenamic acid.

Table 2 Precursor and product ions (*m/z*) and collision energy (eV) of the pharmaceuticals and internal standard (isobutcar 61) considered

Compound	Precursor ion	Product ions MS^2 (MS^3)	Collision energy MS^2 (MS^3)
Salicylic acid	137	93 (65)	37 (47)
Mefenamic acid	240	196 (180)	38 (45)
Ketoprofen	255	209; 177	26
Diclofenac	297	278; 250	30
Clofibric acid	213	127; 85	26
Bezafibrate	360	274 (154)	28 (40)
Sulfamethoxazole	254	188; 156; 147	40
Trimethoprim	291	230; 258; 123	40
Chloramphenicol	321	194; 257; 176	28
Ofloxacin	362	318; 344	45
Carbamazepine	237	194; 220; 192	35
Propranolol	260	183; 116; 157	32
Atenolol	267	225; 190; 208; 249	35
Isobutcar 61	313	222; 130; 196	32

Specificity

The specificity of our method was evaluated through the analysis of seawater samples spiked with each compound separately and of seawater samples spiked with a mixture of all compounds at a concentration of 100 ng L^{-1}. The specificity of the analytical approach was confirmed since no interferences were demonstrated by using LC-MS^n as described "Experimental". No other significant peaks with a signal-to-noise ratio of 3 or more were observed at the specific retention times of the target pharmaceuticals, indicating a high specificity of the analytical method.

Linearity and limit of quantification

Linearity was evaluated by seven-point calibration curves (six replicates) in seawater. Seawater samples (1 L) were spiked with a standard mixture obtaining concentrations of 1; 5; 10; 50; 100; 500 and 1,000 ng L^{-1} of the different pharmaceuticals. The mean correlation coefficients (n=6) of the calibration curves were 0.99 or higher for the target analytes, indicating good linearity in the concentration range 1–1,000 ng L^{-1} (Table 3). This is in accordance with the correlation coefficients reported in the literature for the same pharmaceuticals in freshwater [20, 30]. Despite the high salinity of our sample matrix, the linearity of the analytical method in seawater was not affected. To demonstrate the flexibility of our analytical procedure, besides seawater, calibration curves were also constructed in tap water. Correlation coefficients of 0.99 or higher were found for all compounds.

LOQs were determined using spiked matrix samples and were defined as the lowest detectable concentrations of the calibration curves with a signal-to-noise ratio of at least 10 [21, 27, 30, 31]. The LOQs obtained for the target compounds (Table 3) varied between 1 and 50 ng L^{-1} in seawater and were the same in tap water (chromatograms and spectra not shown). These LOQs are considered acceptable and are comparable to previously reported LOQs for the same pharmaceuticals. Indeed, similar LOQs (between 1 and 20 ng L^{-1}) were reported for bezafibrate, clofibric acid, sulfamethoxazole, carbamazepine, propranolol, chloramphenicol and thrimethoprim [32, 37]. Gómez et al. [30] reported comparable LOQs for atenolol and diclofenac.

Recovery and precision

Because no certified reference material was available, recoveries and intermediate precision (samples were measured on different days) were determined using seawater samples spiked with known amounts of the analytes (six replicates of seven concentrations: 1, 5, 10, 50, 100, 500 and 1,000 ng L^{-1}). The intermediate precision of the method was determined by calculating the relative standard deviation (RSD). Table 3 summarizes the recovery and precision results. According to SANCO/825/00 revision 7 [34], typically a recovery within the range 70–110% and a repeatability of RSD≤20% are required. The mean recoveries (in the 95–108% range) were satisfactory for all the target pharmaceuticals. Zhang and Zhou [36] described the increasing extraction efficiency in SPE of carbamazepine, sulfamethoxazole, propranolol and diclofenac owing to the increasing salt concentration. Taking this into account, we can explain the recovery rates of 100% or more of nine pharmaceuticals in this study. Except for bezafibrate and salicylic acid (RSDs of 27%), the analytical method was sufficiently precise for quantitative analysis of the pharmaceuticals (RSDs between 16 and 20%). The RSDs of

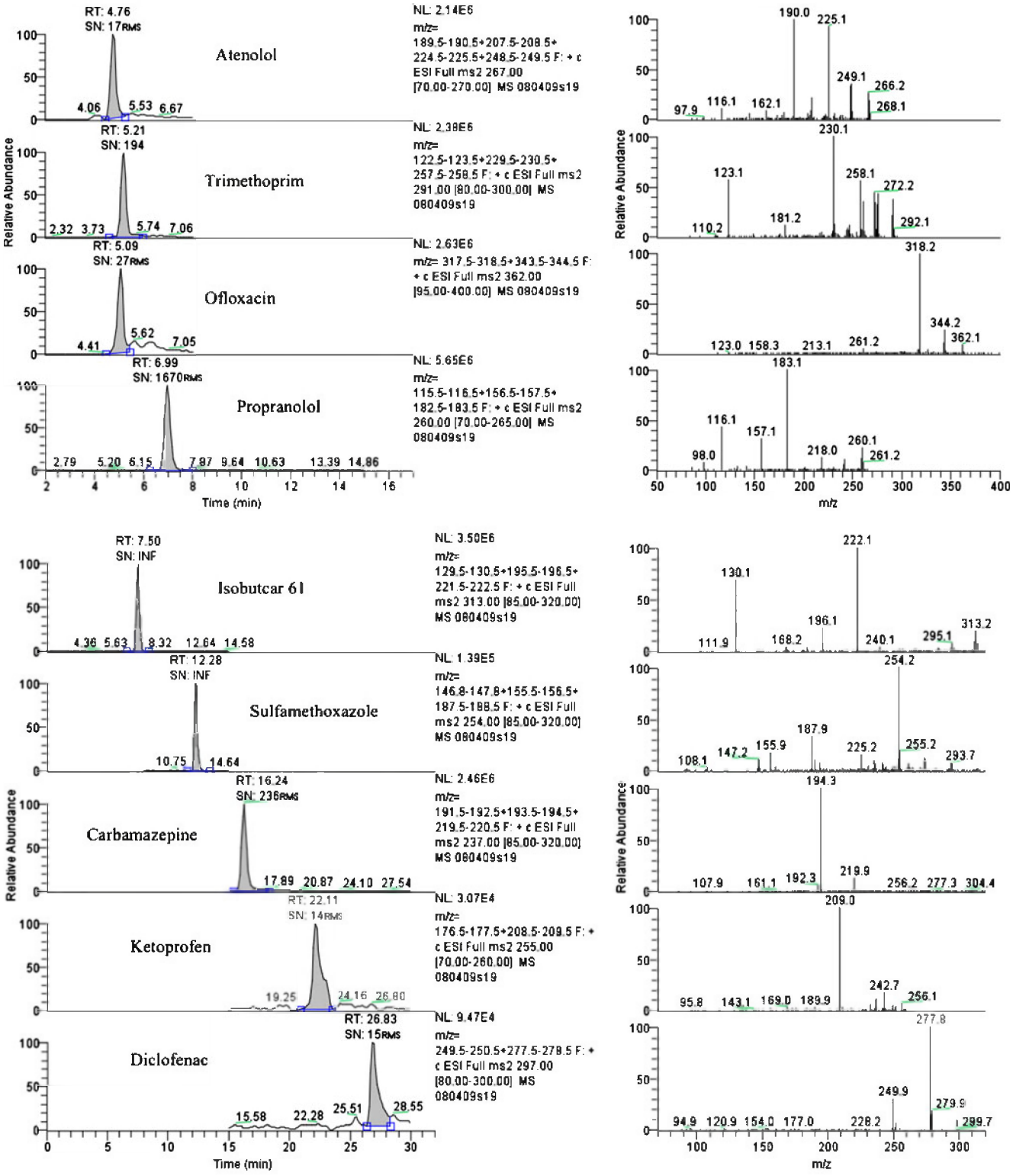


Fig. 2 Chromatograms and spectra of the pharmaceuticals detected in positive ion mode (100 ng L^{-1} spiked in seawater)

bezafibrate and salicylic acid were somewhat higher at the lowest concentrations. This can be attributed to the unavailability of a representative blank sample and consequently to the variation of its presence in unspiked water samples. As can be seen from Table 3, the method developed allows quantification of the target analytes in tap water as well. The recoveries (between 92 and 109%) and RSDs (17% or less) in tap water were satisfactory.

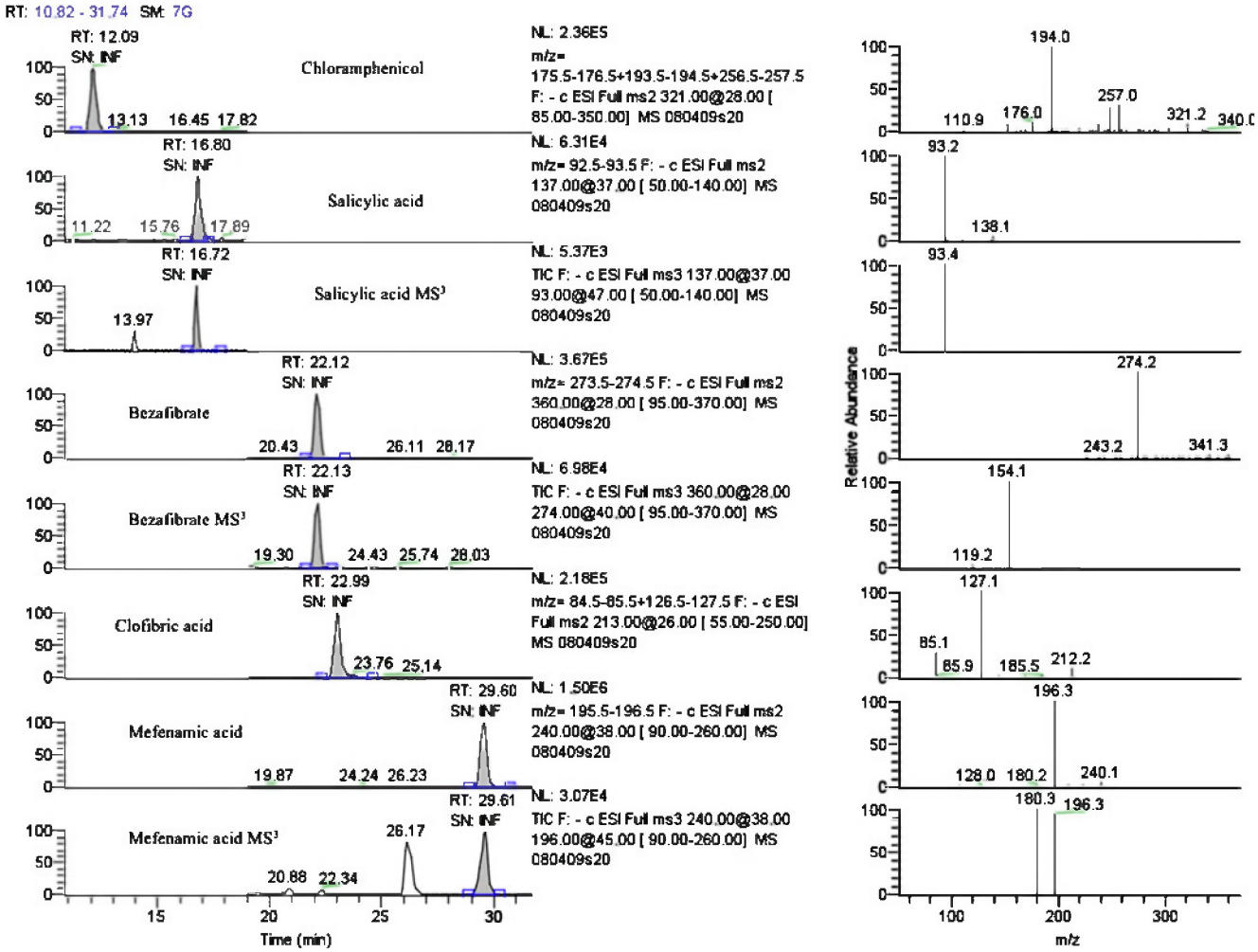


Fig. 3 Chromatograms and spectra of the pharmaceuticals detected in negative ion mode (100 ng L^{-1} spiked in seawater)

Table 3 Validation results: limits of quantification (*LOQ*), correlation coefficients (R^2), recovery and precision in seawater and tap water of the target pharmaceuticals

Compound	LOQ (ng L^{-1})	R^2	Seawater (n=42)		Tap water (n=6)	
			Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Salicylic acid	5	≥ 0.99	108	27	98	14
Mefenamic acid	5	≥ 0.99	95	20	92	14
Ketoprofen	50	≥ 0.99	103	18	109	9
Diclofenac	50	≥ 0.99	101	17	104	14
Clofibric acid	5	≥ 0.99	97	18	94	10
Bezafibrate	5	≥ 0.99	98	27	93	11
Sulfamethoxazole	10	≥ 0.99	100	19	106	17
Trimethoprim	10	≥ 0.99	108	19	96	12
Chloramphenicol	5	≥ 0.99	101	17	102	10
Ofloxacin	50	≥ 0.99	97	18	92	14
Carbamazepine	5	≥ 0.99	101	19	100	5
Propranolol	1	≥ 0.99	107	18	94	7
Atenolol	50	≥ 0.99	104	16	105	14

Matrix effects

Since the ESI part of the mass spectrometer may be subject to signal suppression or enhancement due to coextracted matrix constituents [10], the validation study included the evaluation of matrix effects. Concerning the analysis of seawater samples, these matrix constituents include mainly particulate components, sea salt and other impurities. Despite the thorough optimization of our sample preparation protocol to avoid matrix effects, these were still observed following our analytical procedure. Therefore, the matrix effects were studied by comparing the calibration curves for the different compounds, in seawater and in tap water. In addition, standard mixtures in mobile phase containing concentrations equal to the spiked matrix sample concentrations were brought onto column. A signal enhancement was observed in seawater for several analytes (ofloxacin, propranolol, chloramphenicol, salicylic acid, bezafibrate, clofibric acid). On the other hand, ion suppression effects due to matrix constituents were also observed for a number of analytes (atenolol, trimethoprim, sulfamethoxazole and mefenamic acid). The influence of the matrix was negligible for carbamazepine, diclofenac and ketoprofen. No significant variations in matrix effects between the different samples were observed. The proper correction for matrix effects implies the use of one (labelled) internal standard per analyte. However, the commercial availability of reference standards is rather low, and according to the literature satisfactory results can be obtained using only one or two internal standards to correct for all compounds [11]. Therefore, with every series of samples, calibration curves were prepared in the sample matrix to minimize the matrix effect on the quantification of the analytes.

Application to North Sea samples

The method developed was applied to water samples collected during the INRAM project (see "Study area and sampling"). Six offshore samples, 11 harbour samples and two samples from the Scheldt estuary were collected and analysed. This was repeated four times: in May and December 2007, April 2008 and June 2009. As can be deduced from Table 4, seven different pharmaceuticals were detected. The other pharmaceuticals were not detected at any of the sampling stations. Sulfamethoxazole and trimethoprim were found up to concentrations of 96 and 29 ng L^{-1}, respectively. Other antibiotics could not be detected. The widely used NSAID salicylic acid was detected very often. Salicylic acid, the deacylated, more active form of acetylsalicylic acid, was detected in more than 90% of all samples at a concentration up to 855 ng L^{-1}. The β-blocker propranolol was found in half of the samples at levels up to 24 ng L^{-1}, whereas atenolol was detected only six times up to 293 ng L^{-1}. Bezafibrate was detected at concentrations below 18 ng L^{-1} and residues of the psychiatric drug carbamazepine were frequently found at levels up to 321 ng L^{-1}. Salicylic acid and carbamazepine may be considered as the most relevant compounds for the North Sea and Scheldt estuary since they were detected most often and at the highest concentrations.

Many studies have reported the presence of pharmaceuticals in rivers and in influents and effluents of WWTPs [18–20, 35]. The concentrations observed depend on the therapeutic class. The presence of antibiotics in surface water is generally reported in the low nanogram per litre concentration range, whereas several NSAIDs reach microgram per litre levels. The levels of salicylic acid measured in our study are within the same order of magnitude as those reported in the literature for freshwater samples. Detected concentrations of β-blockers and lipid regulators in the Belgian coastal zone are rather low in comparison with concentrations detected in surface water [18, 38]. On the other hand, carbamazepine occurs at high levels and is detected very often. This can be explained by its low removal efficiency in WWTPs [21, 37]. With regard to marine waters, Buser et al. [39] and Weigel et al. [25, 40] reported the presence of clofibric acid and carbamazepine in the North Sea at concentrations of approximately 1 and 2 ng L^{-1}, respectively. More recent, Togola and Budzinski [41] reported higher concentrations for carbamazepine, diclofenac and ketoprofen in the Mediterranean Sea. However, in general, monitoring data for pharmaceuticals in the marine environment are rather sparse. This may reflect the absence of a method to extract and identify multiclass pharmaceuticals in marine systems [40]. In this study, a novel multiclass analytical method for pharmaceuticals was developed that clearly showed the ubiquitous character of several pharmaceutical compounds in seawater and as a result in the marine environment as well. These findings will assist to further research within the INRAM project, in which risk assessments of the pharmaceuticals detected for the marine environment will be performed.

The concentrations of some pharmaceuticals under investigation show large temporal variations. However, except for salicylic acid at W01 and W02, the same general picture of lower concentrations in the North Sea (W01–W06) in comparison with the harbours and the Scheldt estuary could be noticed. We suggested that, owing to both dilution and degradation, there is little transport from the Scheldt estuary and the harbours to the open sea. Also previous studies on oestrogens, nonyl phenol ethoxylates, and polychlorinated biphenyls reported the limited transport from the Scheldt estuary to the North Sea [42–44]. At sampling locations OO02 and S22, target pharmaceuticals were detected most frequently and at the highest concentrations. OO02 is situated at the mouth of the river Noord-

Table 4 Detected concentrations (ng L^{-1}) of the pharmaceuticals of interest in water sampled in May and December 2007, April 2008 and June 2009 at the different sampling locations in the North Sea, the Scheldt estuary and Belgian harbours (see Fig. 1)

Compound	Station																		
Sampling period	W01	W02	W03	W04	W05	W06	S01	S22	OO1	OO2	OO3	OO4	NP1	NP2	NP3	ZB1	ZB2	ZB3	ZB4
Salicylic acid																			
May 2007	126	26	53	65	18	ND	51	372	246	855	NM	161	44	31	11	48	130	110	41
December 2007	660	276	106	68	26	59	135	229	598	365	234	104	99	94	121	136	142	271	197
April 2008	102	56	62	88	65	49	91	71	203	74	43	67	44	46	NM	114	87	80	146
June 2009	263	412	ND	227	237	60	307	264	481	222	374	ND	306	ND	177	16	312	310	ND
Bezafibrate																			
May 2007	ND	ND	ND	ND	ND	ND	ND	16	5	18	NM	6	ND	ND	ND	ND	ND	ND	ND
December 2007	ND	ND	ND	ND	ND	ND	ND	5	ND	7	12	11	ND	ND	ND	ND	ND	ND	ND
April 2008	8	ND	ND	ND	ND	ND	6	ND	5	9	7	6	ND	ND	NM	6	6	5	ND
June 2009	ND	ND	ND	ND	ND	ND	ND	ND	ND	18	10	ND	ND	ND	ND	ND	ND	ND	ND
Sulfamethoxazole																			
May 2007	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	NM	ND	ND	ND	ND	ND	ND	ND	ND
December 2007	ND	ND	ND	ND	ND	ND	ND	30	ND	ND	39	27	13	ND	ND	ND	ND	ND	ND
April 2008	ND	ND	ND	ND	ND	ND	ND	96	ND	43	21	15	15	ND	ND	ND	ND	ND	ND
June 2009	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Trimethoprim																			
May 2007	ND	ND	ND	ND	ND	ND	ND	ND	ND	15	NM	ND	ND	ND	ND	ND	ND	ND	ND
December 2007	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	17	ND	ND	ND	ND	ND
April 2008	ND	ND	ND	ND	ND	ND	ND	ND	ND	29	ND	ND	ND	ND	NM	ND	ND	ND	ND
June 2009	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	13	ND	ND	ND	ND	ND	ND	ND	ND
Carbamazepine																			
May 2007	18	ND	ND	7	ND	ND	5	321	31	119	NM	16	29	15	ND	11	10	11	11
December 2007	19	ND	ND	10	ND	ND	18	154	29	19	32	30	68	54	37	14	12	17	24
April 2008	16	14	4	12	ND	ND	27	185	30	64	35	36	48	20	NM	30	25	23	20
June 2009	11	ND	ND	7	ND	ND	14	129	21	50	36	20	19	7	ND	10	13	20	16
Propranolol																			
May 2007	ND	ND	ND	ND	ND	ND	ND	22	5	24	NM	3	6	3	ND	1	ND	ND	1
December 2007	1	ND	ND	ND	ND	ND	1	10	3	6	9	9	12	12	7	1	1	4	3
April 2008	ND	ND	ND	ND	ND	ND	3	22	2	21	11	12	3	2	NM	3	3	2	2
June 2009	ND	ND	ND	ND	ND	ND	ND	15	ND	13	3	ND	ND	ND	ND	ND	ND	ND	ND
Atenolol																			
May 2007	ND	ND	ND	ND	ND	ND	ND	89	ND	88	NM	ND	ND	ND	ND	ND	ND	ND	ND
December 2007	ND	ND	ND	ND	ND	ND	ND	293	ND	ND	ND	80	ND	ND	ND	ND	ND	ND	ND
April 2008	ND	ND	ND	ND	ND	ND	ND	188	ND	82	ND	ND	ND	ND	NM	ND	ND	ND	ND
June 2009	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

ND not detected, *NM* not measured

Ede and the canal Bruges-Ostend in the harbour of Ostend (in the middle of the Belgian coastal zone), whereas S22 is located in the Scheldt estuary in Antwerp. Several WWTPs are located close to OO02 and in Flanders the effluents of more than 65 WWTPs are discharged into the Scheldt estuary. Furthermore, the WWTPs with the largest capacity are located near the Scheldt estuary: Deurne, Ghent and Antwerp (approximately 200,000 inhabitant equivalent) (Aquafin, personal communication). It may be concluded that both locations receive major inputs of contaminated industrial and domestic wastewater, probably resulting in the increased presence of the target pharmaceuticals.

Conclusion

In this study, an analytical method for the quantification of important pharmaceuticals in seawater was developed and optimized. A combination of SPE and LC-MS^n enabled the detection and quantification of multiclass pharmaceuticals of widely differing chemical structures in seawater at the low nanogram per litre level. The method was validated according to the laboratory quality assurance criteria developed in accordance with Commission Decision 2002/657/EC and SANCO/825/00 revision 7 [33, 34]. Application of the procedure to North Sea and Scheldt estuary samples confirmed the occurrence of seven pharmaceuticals in the marine environment up to the low microgram per litre level. Frequently detected compounds were salicylic acid, carbamazepine and propranolol. Two antibiotics were detected occasionally: sulfamethoxazole and thrimethoprim. Little transport of pharmaceuticals could be observed from the Scheldt estuary and the harbours to the open sea. In general, it may be concluded that the results of this 2-year monitoring study are quite novel and may provide relevant insights into the field of pharmaceutical analysis in the marine environment.

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