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ORIGINAL PAPER

**Herlinde Noppe · Katia De Wasch · Sofie Poelmans
Nathalie Van Hoof · Tim Verslycke · Colin R. Janssen
Hubert F. De Brabander**

Development and validation of an analytical method for detection of estrogens in water

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Abstract An analytical procedure enabling routine analysis of four environmental estrogens at concentrations below 1 ng L^{-1} in estuarine water samples has been developed and validated. The method includes extraction of water samples using solid-phase extraction discs and detection by gas chromatography (GC) with tandem mass spectrometry (MS–MS) in electron-impact (EI) mode. The targeted estrogens included 17α- and 17β-estradiol (aE2, bE2), estrone (E1), and 17α-ethinylestradiol (EE2), all known environmental endocrine disruptors. Method performance characteristics, for example trueness, recovery, calibration, precision, accuracy, limit of quantification (LOQ), and the stability of the compounds are presented for each of the selected estrogens. Application of the procedure to water samples from the Scheldt estuary (Belgium – The Netherlands), a polluted estuary with reported incidences of environmental endocrine disruption, revealed that E1 was detected most frequently at concentrations up to 7 ng L^{-1}. aE2 was detected once only and concentrations of bE2 and EE2 were below the LOQ.

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H. Noppe · K. De Wasch · S. Poelmans · N. Van Hoof
H. F. De Brabander (✉)
Laboratory of Chemical Analysis,
Department of Veterinary Public Health and Food Safety,
Faculty of Veterinary Medicine,
Ghent University, Salisburylaan 133,
9820 Merelbeke, Belgium
E-mail: hubert.debrabander@ugent.be

T. Verslycke
Biology Department, MS#32,
Woods Hole Oceanographic Institution,
Woods Hole, MA 02543, USA

C. R. Janssen
Laboratory of Environmental Toxicology and Aquatic Ecology,
Faculty of Bioscience Engineering, Ghent University,
J. Plateaustraat 22, 9000 Ghent, Belgium

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Introduction

The occurrence of endocrine-disrupting chemicals in the environment has led to a growing awareness that both animals and humans may be adversely affected, leading to cancer, reproductive tract disorders, reduced sperm counts, and reduction of reproductive fitness [1–4]. From the large group of substances that are suspected or known to be endocrine disruptors, the natural and synthetic estrogens are reported as compounds with high potent estrogenic properties. The latter are used in birth-control pills and for management of menopausal syndromes and cancers [1, 3].

The compounds 17α-estradiol (aE2), 17β-estradiol (bE2) and estrone (E1) are natural female sex hormones produced by humans, mammals, and other vertebrates [5, 6]. These estrogens are lipophilic, fat-soluble molecules. They are excreted unchanged or, mainly, as water-soluble inactive polar glucuronates or sulfate conjugates [7]. Under experimental conditions these conjugates are quickly hydrolyzed, leading to the free hormones or their metabolites [5, 8]. On the basis of current evidence, degradation in the environment is expected to take several days when circumstances are optimum, or to be far slower in less ideal circumstances [5, 9]. Estrogens enter environmental compartments directly or after they have passed through wastewater treatment plants (WTP) [10, 11]. Once in the environment they can undergo degradation or transfer processes or can be distributed between the environmental compartments water, sediment, suspended matter, and animals [5, 8]. A quantitatively important source of natural estrogens is livestock husbandry. These animals are often kept at one site, which results in sewage and manure that contains high concentrations of sex steroids which, depending on the source, enter the

environment by different pathways [8, 9]. WTP remove the estrogens from the water by degradation or by adsorption on sludge. Adsorbed estrogens may, however, re-enter the aqueous phase if the sewage sludge is used as fertilizer. Transport of hormones via bank filtration from contaminated surface water to groundwater, and the filtration of waste water directly from leakage in drains may also occur [3, 12, 13]. Besides natural estrogens, synthetic steroids, a group that mainly comprises oral contraceptives and steroids used for substitution therapy during the menopause, are known environmental pollutants [8]. The synthetic compound 17α-ethinylestradiol is the main active component of the contraceptive pill taken by women. This compound has no natural source [5]. Next to contraception, the uses of estrogens can be categorized into three main groups—management of (post)menopausal syndromes, physiological replacement therapy in deficiency states, and the treatment of cancers [3]. The chemical structures of the estrogens considered in this study are presented in Fig. 1. All have a polycyclic structure with an –OH group on C3, a $-CH_3$ group on C13, and different substituents on C17. Although these compounds can be degraded biologically, they have been detected in WTP effluents and surface water at nanogram per liter levels [9, 14, 15]. A number of studies have demonstrated that these concentrations are significant for endocrine disruptors, because research has shown that male fish exposed to low nanogram per liter levels of these estrogens give estrogenic responses, for example vitellogenin (VTG; precursor to yolk, a female-specific protein) production [14, 16–19], intersex [20], and the presence of testicular oocytes [21]. It has been hypothesized that the occurrence of these substances is linked with a decline in sperm counts, in the increasing incidence of breast cancer and testicular cancer, and earlier onset of puberty in humans [12, 22]. To evaluate the potential risk of this group of endocrine disruptors, the occurrence and environmental exposure to these compounds must be documented. Unfortunately, chemical analysis of these compounds in environmental matrices is a difficult task, because of the complexity of the matrix and their low environmental concentrations [10].

Although the occurrence of estrogens in wastewaters has received increasing interest in recent years, little is known about the presence of these compounds in estuarine water. The aim of this study was therefore to develop an extraction method enabling determination of low concentrations of several environmental estrogens and to validate the method with water samples from the Scheldt estuary. This estuary, which is situated in one of the most heavily populated regions of Europe, with highly diversified industrial activity [23], is an example for other estuaries. The four target estrogens included the natural estrogens aE2, bE2 and E1, and the synthetic estrogen EE2. Although the natural hormones are excreted primarily as the conjugated forms, this method was developed for analysis of the free forms, because conjugated estrogens are expected to be relatively short-lived in the environment [9, 24]. Ethinylestradiol was also selected because this compound has a greater potency as an endocrine disruptor than the natural hormones. For valid interpretation of important environmental data, for example those in this study, validation of the method is necessary. This study provides a description of the analytical method, based on existing derivatization and GC techniques, but using a less common extraction technique. Method performance characteristics and the stability of the compounds are also described in detail. This is the first study to provide data on the occurrence of these estrogens in the Scheldt estuary.

Materials and methods

Chemicals

Standards of the natural and synthetic hormones were obtained from Sigma–Aldrich (St Louis, MO, USA) or Steraloids (Newport, RI, USA). Equilinine (EQ) and deuterated estrone (E1–D4) were used as procedure internal standards; ethinyltestosterone (ET) was used as GC–MS reference standard and androsterone (And) was used as derivatization standard. Stock standard solutions of the analytes were prepared at 200 ng μL^{-1} in EtOH. Working solutions of each analyte or mixtures were prepared at different concentrations by appropriate dilution of the stock solution in EtOH. All solutions were stored at 4°C in the dark. HPLC-grade methanol (MeOH) was obtained from Acros organics (Fairlawn, NI, USA). Pro-analysi-grade solvents acetone, water,

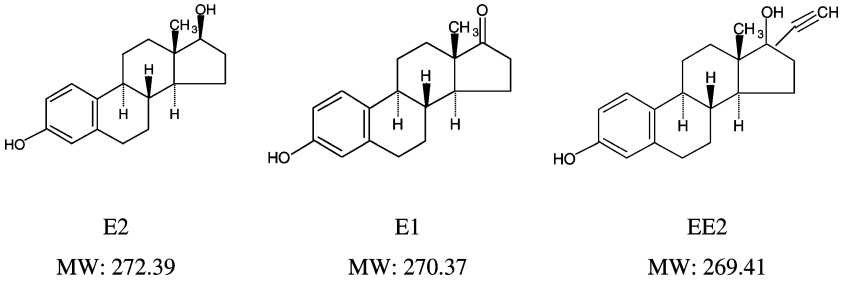

Fig. 1 Chemical structures and molecular weights (MW) of the hormones estradiol (*E2*), estrone (*E1*), and ethinylestradiol (*EE2*)

n-hexane, chloroform, and ethanol (EtOH) were purchased from VWR (Darmstadt, Germany).

Calibration standards

Before every sample analysis a dilution series (0.1, 0.25, 0.5, 0.75, and 1 ng) of standard mixtures of the target estrogens was injected. These standards were used to check the operating conditions of the GC–EI-MS–MS apparatus. When Scheldt samples were analyzed, the range of calibration standard concentrations was 0.25, 0.5, 1.25, 2.5, and 5 ng L^{-1} spiked in ultrapure water. With a final extract volume after derivatization of 25 μL, the extract concentration equivalent to the lowest spiked concentration was equal to the second lowest calibration standard, 0.1 ng on column. The procedure internal standards (EQ and E1–D4) were added to every sample at a concentration of 5 ng L^{-1} before extraction. After Speedisk extraction 10 ng ET and before derivatization 10 ng And were added.

Environmental sample collection, sample preparation, and preservation

Samples are taken three times a year (spring, summer and winter) in the Scheldt estuary, using the research vessel Belgica (MUMM) during the duration (2002–2006) of the ENDIS–RISKS project (http://www.vliz.be/projects/endis). Seven sampling locations were chosen in accordance with running national and international sampling programs and other monitoring surveys in the Scheldt estuary (Fig. 2). Water was sampled using a Teflon-coated Go-Flo bottle at a depth of 3 m at each site taking into consideration tidal movements in the estuary. A sample volume of 2 L was extracted immediately on board the sampling ship to avoid addition of chemical preservatives.

Extraction

Before extraction, the pH of the water samples was adjusted to 7 using solutions of HCl or NaOH (1 mol L^{-1}). Extraction of the water samples was performed using Bakerbond Speedisk octadecyl-bonded silica (C_{18}XF), 50 mm (JT Baker, Deventer, The Netherlands). Extraction was performed using the manufacturer's guidelines. In brief, the discs were placed on a Speedisk extraction station (JT Baker) and preconditioned by passing 20 mL acetone and 20 mL MeOH through the discs at a flow rate of 10 mL min^{-1}. Before adding the sample to the disc, the disc was rinsed twice with 10 mL ultrapure water. When the sample had been drawn through the disc, the disc was dried under vacuum for at least 30 min. Elution was performed using 5 mL acetone and 15 mL MeOH (which was used to rinse the sample bottles). Extracts were stored at 4°C in the dark until clean-up before the final analysis.

Clean-up [25]

The Speedisk extracts were vaporized to dryness in 100-mL bulb flasks by use of a Rotavapor (Büchi, Flawil, Switzerland), reconstituted with 500 μL chloroform and used for solid-phase extraction. Silica (Si, 500 mg, 10 mL, Sopachem, The Netherlands) cartridges were placed on an Adsorbex SPU (VWR) and conditioned twice with 2.5 mL *n*-hexane under vacuum. Before the samples were applied to the cartridges, 5 mL *n*-hexane was added to the samples in the bulb flasks, mixed well and the mixture was transferred on to the cartridges. After the samples had been drawn through the cartridges another 5 mL *n*-hexane was added to the bulb flasks and transferred on to the cartridges. Under the Si cartridges an NH_2 (100 mg, 1 mL, Sopachem) cartridge was placed (to retain humic acids and other interfer-

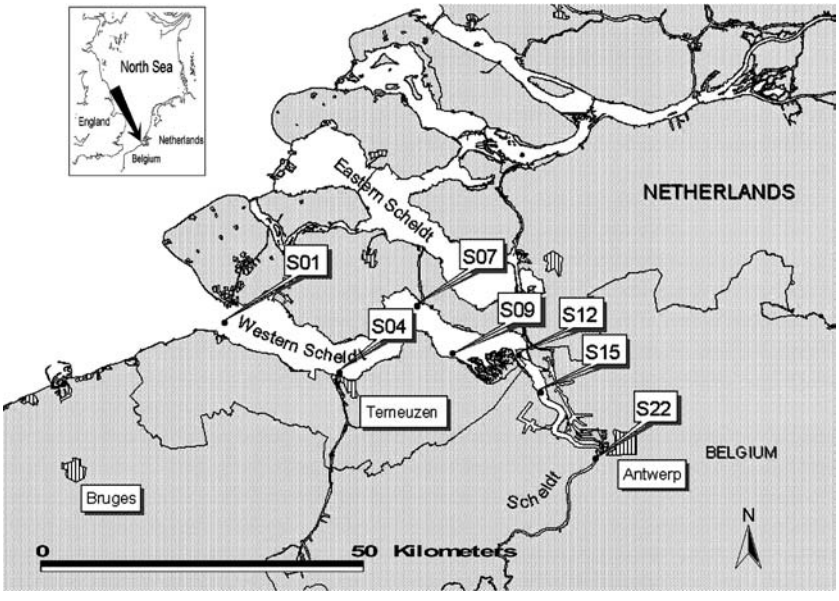

Fig. 2 Map of the Scheldt estuary with location of the different sampling sites: S01 Vlissingen (VLIS), S04 Terneuzen (TERN), S07 Hansweert (HANS), S09 Bath (BATH), S12 Saefthinghe (SAEF), S15 Doel (DOEL) and S22 Antwerp (ANTW)

ences) and both were rinsed with 5 mL *n*-hexane. Elution was performed with 5 mL chloroform–acetone (4:1). These extracts were dried and reconstituted with 300 μL EtOH. This was passed to a GC–MS vial and again evaporated to dryness, at 60°C, in a centrifugal evaporator system (Gyrovap, Howe, London, UK). To improve the stability of the target estrogens, the hydroxyl and keto groups of the steroid ring were derivatized. After derivatization with 25 μL of a mixture of MSTFA (*N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide), NH_4I, and ethanethiol (1 h at 60°C) the samples were analyzed by GC–EI-MS–MS. Because the GC–EI-MS–MS apparatus was used for other routine analyses, occasionally extracts or derivatized extracts had to be stored for a short time at 4°C in the dark. Experiments were performed to evaluate the shelf-life of EtOH solutions of the extracts and derivatized extracts.

GC–EI-MS–MS analysis

All GC–EI-MS–MS chromatographic measurements were performed with a Thermofinnigan (Austin, TX, USA) gas chromatograph fitted with a Trace GC 2000 ion-trap mass spectrometer and a Finnigan MAT AS2000 autosampler. Compounds were separated on a 25 m × 0.22 mm i.d, film thickness 0.25 μm, BPX-5 (5% phenyl liquid phase) fused silica capillary column (SGE, Austin, TX, USA). Glass injector liners (10.5 cm × 3 mm) were supplied by SGE. The injector, ion source, and transfer-line temperatures were, respectively, 250, 200, and 275°C. The temperature program was: initial temperature 100°C, directly ramped at 17° min^{-1} to 250°C, second ramp at 2° min^{-1} to 268°C, and finally ramped at 30° min^{-1} to 300°C. Helium was used as carrier gas at a flow rate of 1 mL min^{-1} and perfluorotributylamine (PFTBA), also known as FC43, was used as calibration gas. Sample (1 μL) was injected with a split–splitless injector (split flow 20 mL min^{-1}, splitless time 1 min). The EI spectra were obtained in EI mode at 70 eV.

Data processing, analyte identification and quantification

Data processing was performed using Xcalibur 1.3 software (Thermofinnigan). The estrogens were identified in the environmental samples when the following criteria were fulfilled: the chromatographic peaks of the diagnostic ions from the unknown and the standard had to elute at the same relative retention time. Second, the ratio between the selected ions had to be the same in both the sample and the standard with a tolerance between 20 and 50% depending on the intensity of the ion [26]. Sample analyses were acquired in triplicate and the average of the three results is reported. Quantification of the estrogens was done by calculating a linear regression equation for the peak area ratios of the target analyte and the internal standard of the spikes. By application of the equation to the sample data, the concentration of the analytes in the samples was calculated. An internal calibration was performed using EQ and E1–D4. All statistical data processing was performed using SPSS 11.0 software.

Results and discussion

Performance of the GC–EI-MS–MS method

Current detection methods for natural and synthetic estrogens in water are based on either chromatographic or in-vitro techniques, for example the yeast estrogen screen (YES) and the recombinant yeast assay (Rya) [2, 10, 21, 27]. Gas chromatography–mass spectrometry (GC–MS) is a commonly used technique and liquid chromatography–mass spectrometry (LC–MS) has gained in popularity over the last few years [1, 3, 4, 6, 7, 10, 11, 13, 28]. The advantage of LC is that steroids can be determined without derivatization. In addition, both GC and LC are more specific than biological tests [3, 11]. In this study, the clean-up and GC techniques used were based on extended experience in the laboratory with detection of anabolics in animal matrices using this separation technique. Because of the complexity of the matrix, tandem MS was selected. By interpreting the relative retention time and the precursor and product ions in the mass spectrum, this method was very specific for the analytes in this study. Because no certified reference material was available, criteria for relative retention time were assessed by recovery of additions of known amounts of the target analytes to ultrapure water as described in the ''Material and methods'' section. The tolerances used for the relative retention times of the target analytes are described in Table 1. It can be noticed that the standard deviation of the relative retention time is lower when E1–D4 is used as internal standard. In full scan MS the spectrum was characterized by a base peak corresponding to [M + 72] or [M + 144], in accordance with derivatization with a mixture of MSTFA, ethanethiol, and NH_4I. The most abundant ion was chosen as precursor ion for MS–MS. The optimised GC–EI-MS–MS conditions are shown in Table 2. To conform with European Criteria 2002/657 [26] the relative intensity of the product ions, expressed as a percentage of the intensity of the most intense ion, needs to correspond to those of the standard, whether

Table 1 Permitted tolerances for the relative retention time of the selected estrogens

Analyte	EQ	E1–D4
aE2	$0.89 \pm 4.05\times10^{-2}$	$0.98 \pm 1.69\times10^{-4}$
bE2	$0.93 \pm 4.25\times10^{-2}$	$1.02 \pm 1.70\times10^{-4}$
E1	$0.91 \pm 4.14\times10^{-2}$	$1.00 \pm 1.95\times10^{-4}$
EE2	$1.04 \pm 4.76\times10^{-2}$	$1.14 \pm 3.77\times10^{-4}$

Table 2 Optimized GC–EI-MS–MS conditions

Analyte	Precursor ion (m/z)	Collision energy (eV)	Product ions (m/z)
aE2	416	1.00	326, 285
bE2	416	1.00	326, 285
E1	414	1.00	399, 324, 309
EE2	456	1.15	407, 323, 303, 281, 231, 193
EQ	410	1.00	395, 320, 280
E1–D4	417	1.00	402, 327, 312
ET	456	1.05	441, 351, 316, 301
And	434	0.85	419, 329

from standard solutions or from spiked calibration standards, at comparable concentrations, measured under the same conditions, with tolerances as shown in Table 3. Only when both criteria were fulfilled was quantitative analysis of the results performed.

Performance of the extraction method

Samples were handled and processed in such a way that there was a maximum probability of detecting the analytes of interest. The amber sample bottles were rinsed with MeOH and ultrapure water before sample addition. Water samples of the Scheldt estuary were taken by using Go-Flo water samplers that open automatically, activated by hydrostatic pressure at a specified depth. The advantage of these water samplers is that sample contamination at the surface, internal spring contamination, loss of sample on the deck and exchange of water from different depths is avoided. When necessary, water samples were stored at 4°C in the dark. Adjustment to pH 2 was performed using 2 mL 6 mol L^{-1} HCl to avoid microbiological degradation of the estrogens. It has been reported that storage of water samples for more than one week without acidification resulted in degradation of bE2 into E1 [28]. Detection of the natural and synthetic hormones in environmental samples requires analytical methods which enable reliable determination of these compounds at low ng L^{-1} levels. Such methods usually consist of an extraction and pre-concentration step followed by GC or LC detection. In the literature, extraction is mostly performed by solid-phase extraction (SPE) using cartridges or discs impregnated with different sorbents, e.g. C_{18}, graphitized carbon black, or styrene–divinylbenzene (SDB) [6, 10, 24, 28–30]. In this study extraction discs were preferred to normal cartridges, because cartridges can clog easily when used for environmental samples, because of colloidal material and suspended particles [28]. In addition, these discs have a large surface area, low levels of contamination [4, 24, 28], and they are ideal for on-board extraction. The last is very important because it can prevent degradation and contamination of the target compounds during transportation. One possible drawback of the extraction discs over cartridges is the presumed longer evaporative concentration time of the extract [4]. Preliminary experiments revealed that water samples with a pH range of 2–7 gave the best recoveries for the target compounds. Nevertheless, pH 7 was preferred because at acid pH, humic acids in the environmental samples are strongly retained by the sorbent. As a consequence a yellowish extract, because of its high humic acid content, results in interference with GC–EI-MS–MS analysis [30]. No filtration step was added to the procedure because log K_{ow} values of the target estrogens are in the range of 3–4. This indicates that the target analytes have high affinity for suspended matter [24] and filtration could cause significant losses (although López and co-workers demonstrated that a filtration step did not lead to significant losses of the target analytes [21]). In our procedure, filtered particulates on the disc and estrogens adsorbed by the sorbent are ultimately washed with organic solvents. Before extraction, the discs were washed with acetone and MeOH to clean the disc and to remove any potential interferences.

Table 3 Maximum permitted tolerances (%) for relative ion intensities using $GC–MS^2$ [26]

Relative intensity	StdevSD
> 50	± 20
> 20 to 50	± 25
> 10 to 20	± 30
≤ 10	± 50

Validation

Because no certified reference material was available, the trueness of the analytical method was assessed by recovery of a standard mixture of the target analytes added to ultrapure water as described in the "Materials and methods" section. European criteria 2002/657 [26], the directive for control of analytical methods for animal matrices, stipulates the trueness of the method must be in the interval −50% to +20% for a mass fraction of ≤ 1,000 ng L^{-1}. This European directive was used, because no guidelines for environmental analysis were available. As shown in Table 4, all mean recoveries fall within this range. Five-point calibration plots were constructed using triplicate injections of extracts obtained from the fortified ultrapure water samples as described in the "Materials and methods" section. Analysis of the results demonstrated the concordance of the response with a linear model. The mean correlation coefficients were 0.96 ± 0.01 and 0.95 ± 0.01 using EQ

Table 4 Trueness of the quantitative method

Analyte	EQ	E1–D4
aE2	105 ± 20	107 ± 22
bE2	104 ± 25	103 ± 27
E1	108 ± 21	107 ± 18
EE2	102 ± 21	103 ± 27

Mean (0.25 up to 5 ng L^{-1} in ultrapure water) recovery ± SD (%).

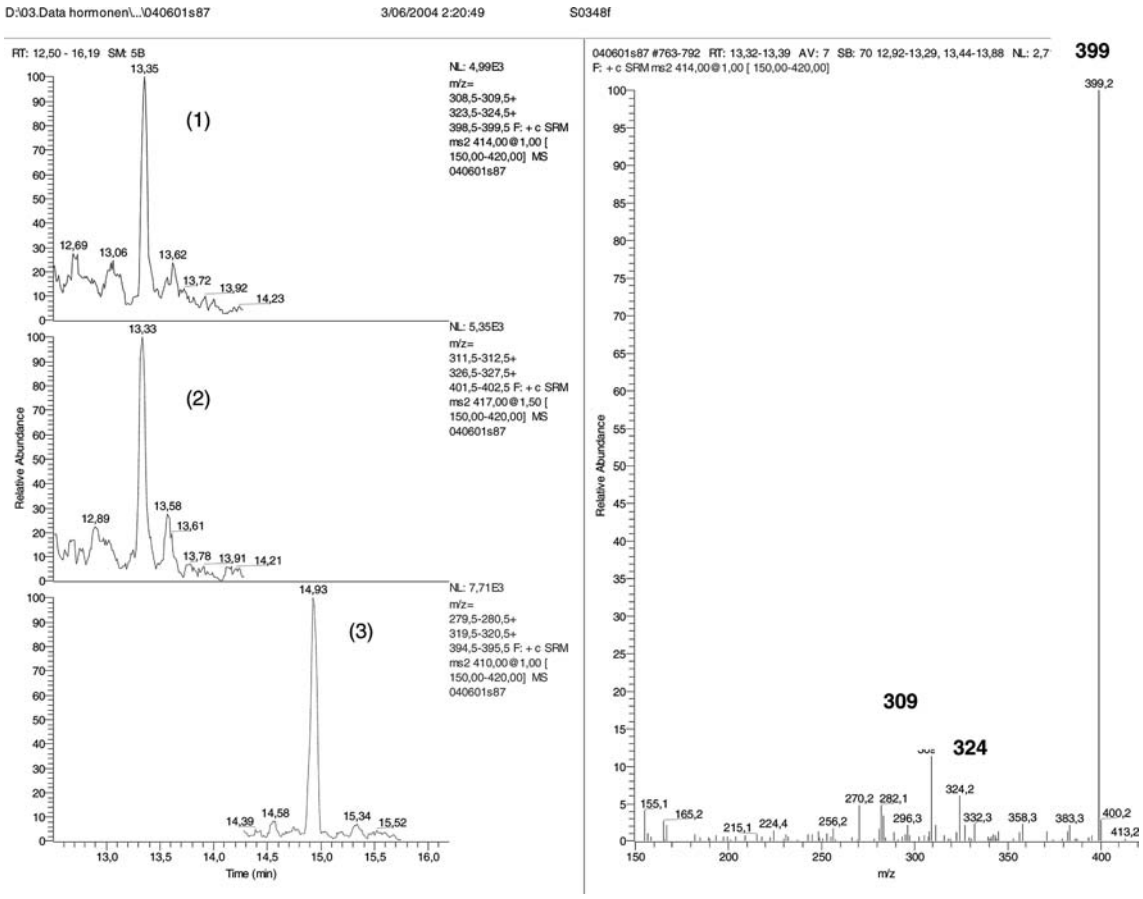

Fig. 3 Chromatograms and spectrum from analysis of an estuarine water sample taken from the Scheldt estuary (May 2004 campaign at Antwerp site). Peak identification: E1 (*1*); E1–D4 (*2*); EQ (*3*)

and E1–D4, respectively. In the literature correlation coefficients higher than 0.99 are reported for the same target compounds [3, 30, 31]. However, in most of these studies, linearity is tested using standard mixtures or with fortified water samples at concentrations ranging from 25 ng L^{-1} to 10 µg L^{-1} [1]. This could explain the lower correlation coefficients reported in this study. All correlation coefficients were not significantly different for all four target analytes and for both internal standards (analysis of variance (ANOVA), Kruskal–Wallis, $P > 0.05$). The method precision and accuracy were satisfactory, with an average percentage recovery of $105 \pm 18\%$, when EQ was used for quantification. An average percentage recovery of 105 ± 20% was obtained when E1–D4 was used as internal standard for quantification. The recovery was independent of the spiked concentration (ANOVA, Kruskal–Wallis, $P > 0.05$) and the target analyte (ANOVA, Kruskal–Wallis, $P > 0.05$). The recoveries obtained were within the same order of magnitude as those reported by other authors [1, 3, 30, 31]. In the literature, however, recoveries of the same target compounds from aqueous samples are reported to be 75 ng L^{-1} [30] and 10 µg L^{-1} [3, 10] which is high considering the low ng L^{-1} environmental levels of the selected estrogens reported in this study. The coefficients of variation for replicate analysis of the series of fortified ultrapure water were 17.14 (EQ) and 19.05 (E1–D4). Different procedures for determination of the limit of detection (LOD) and the limit of quantification (LOQ) are reported in literature. These limits can be experimentally estimated by injection of serially diluted standard solutions [3, 13] or extracts of fortified water samples until the signal-to-noise (s/n) ratio reaches a value of three. Another reported method is to set the LOD at three times the noise level of the baseline in the chromatogram, and the LOQ is set at three times the LOD [6]. In this study the lowest concentration of the calibration curve, i.e., 0.25 ng L^{-1} was preferred as LOQ. This was in accordance with preliminary tests and literature LOQ of 1–3 ng L^{-1}, depending on the target estrogen are reported [30]. This LOQ can be extrapolated to the analysis of fairly clean waters, for example drinking water, groundwater, or surface water. For more complex samples, e.g. estuarine samples, the sensitivity is compromised by matrix effects [1]. For this, future experiments will be conducted to determine the effect of the matrix on the detection of the target compounds in this study.

Stability of the compounds

European Criteria 2002/657 [26] state that the stability of the analyte in solvent during storage, in the matrix

during storage and/or sample preparation, and in the extract during storage and/or analysis should be tested. Stock solutions of 200 ng μL^{-1} were prepared in EtOH and stored in the dark at 4°C. Working solutions were obtained by dilution of the stock solutions in EtOH and were renewed before every batch of samples. For this reason the stability of the target compounds in solvent was not considered problematic and therefore not investigated in this study. Similarly, the matrix stability was not tested because samples were always extracted within one hour of sampling. Because the GC–EI-MS–MS equipment used in this study was also used for other routine analysis, the stability of extracts and derivatives was studied after short-term (4 weeks) storage in the dark at 4°C. A one-way ANOVA or Kruskal–Wallis test was applied to the peak area ratios of the target analytes and the internal standards to detect significant effects of short-term storage. With EQ as internal standard, no significant degradation was observed when EtOH or derivatized extracts were stored for up to 4 weeks (ANOVA, Kruskal–Wallis, $P > 0.05$). No significant degradation was observed after storage of the derivatized extracts with E1–D4 (ANOVA, Kruskal–Wallis, $P > 0.05$). For EtOH extracts before derivatization a significant effect of storage for 4 weeks was observed for EE2, aE2, and bE2 (ANOVA $P = 0.01$, Mann–Whitney, $P = 0.029$ and 0.047). This could not be explained by stability of the GC system, because this would have resulted in the same trend being observed when using EQ for quantification. Most probably, storage of extracts affects the stability of E1–D4 and not the stability of the derivatized E1–D4. For this reason extracts were analyzed as soon as possible and if storage was necessary, derivatized, and EQ was preferred for quantification.

Estuarine water analysis

The developed analytical method was applied to water samples collected from the Scheldt estuary (B-Nl) from 2002 to 2004 (one sample in each sampling point). Figure 3 shows chromatograms and spectrum obtained from analysis of a 2-L water sample from the Scheldt estuary (3 ng L^{-1}, May 2004 campaign, Antwerp). Detected concentrations of the target hormones in the water samples were in the low ng L^{-1} range. Of the four hormones measured in this study, E1 was detected most frequently. The highest concentration of E1, 8 ng L^{-1} was measured in December 2002. E1 was most frequently detected in the post upstream side of the estuary whereas aE2 was detected once only (June 2003) at two sites downstream at concentrations near the LOD. Levels of bE2 and EE2 were below the LOQ. The temporal and spatial patterns of the different compounds were irregular. In geographical positions along the Scheldt estuary a trend could be observed. The target estrogens were most concentrated in Antwerp, the most upstream site. However, no seasonal trends have yet been observed. Similar levels of contamination with the target estrogens have previously been reported within the same order of magnitude in the Dutch part of the Scheldt estuary [6] and in surface water elsewhere in the world [10, 21, 24, 27, 31].

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