CHAPTER II.1.

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

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CHAPTER II.1.

Development and Validation of an Analytical Method for the Detection of Estrogens in Water

Summary

In this chapter the development and validation procedure (Belac) of an analytical method that enables routine analysis of four environmental estrogens at concentrations levels below 1 $ng.l^{-1}$ in estuarine water samples are described. The method includes extraction of water samples using solid phase extraction discs and detection with gas chromatography (GC) with multiple mass spectrometry (MS-MS) in electron impact (EI) mode. The targeted estrogens included α- and β-estradiol (αE2, βE2), estrone (E1) and 17α-ethinylestradiol (EE2), which are all known environmental endocrine disruptors. Method performance characteristics, such as trueness, recovery, calibration, precision and 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 8 $ng.l^{-1}$. αE2 was only detected once. Concentrations of βE2 and EE2 were below the LOQ.

1. 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 in reproductive fitness [López de Alda et al., 2000 and 2001, Mol et al., 2000, Snyder et al., 2001]. 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 used in birth control pills and for the management of menopausal syndromes and cancers [López de Alda et al., 2000 and 2001, Snyder et al., 2001].

The compounds α-estradiol (αE2), β-estradiol (βE2) and estrone (E1) are natural female sex hormones produced by humans, mammals and other vertebrates [Belfroid et al., 1999, Vethaak et al., 2002]. These estrogens are lipophylic, fat-soluble molecules. They are excreted either unchanged, but mainly as water-soluble inactive polar glucuronates or sulphate conjugates [Ternes et al., 1999 and 1999b]. Under experimental conditions these conjugates are quickly hydrolysed, leading to the free hormones or their metabolites [Vethaak et al., 2002, Lintelmann et al., 2003]. Based on current evidence, degradation in the environment is expected to take several days when circumstances are optimal, or be far slower under less ideal circumstances [Vethaak et al., 2002, Fine et al., 2003].

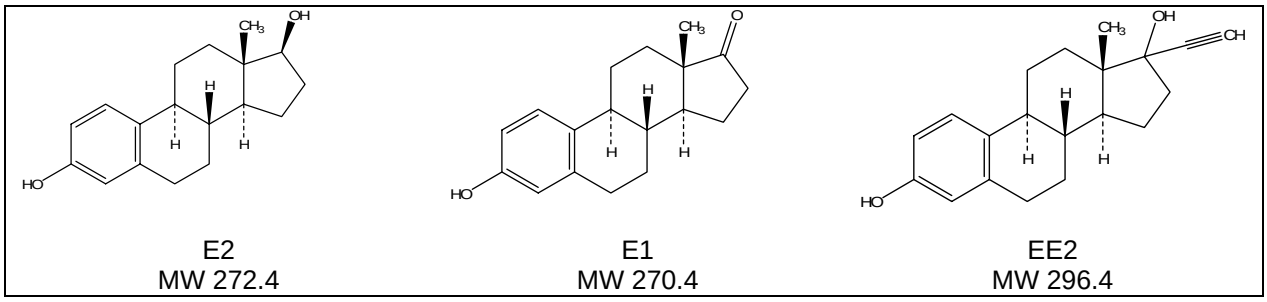

Figure II.1.1.
Chemical structure and molecular weight (MW) of the hormones estradiol (E2), estrone (E1) and ethinylestradiol (EE2)

Estrogens enter environmental compartments directly or after they have passed through wastewater treatment plants (WWTPs) [López de Alda et al., 2001b, Ingrad et al., 2003]. Once in the environment they can undergo degradation or transfer processes or they can be distributed between the environmental compartments water, sediment, suspended matter and biota [Vethaak et al., 2002, Lintelmann et al., 2003]. 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 and which depending on the respective source; enter the environment by different pathways [Fine et al., 2003, Lintelmann et al., 2003]. WWTPs remove the estrogens from the water by degradation or adsorption to the sludge. However, adsorbed estrogens may re-enter the aqueous phase if the sewage sludge is used as fertilizer. Additionally, transport of hormones via bank filtration from contaminated surface water to groundwater, as well as the filtration of waste waters directly from leakage in drains may also occur [Ternes et al., 1999b, López de Alda et al., 2000, DEPA 2001].

Besides natural estrogens, synthetic steroids, a group that mainly consists of oral contraceptives as well as steroids used for substitution therapy during menopause, are known environmental pollutants [Lintelmann et al., 2003]. The synthetic compound ethinylestradiol is the main active component of the contraceptive pill taken by women. This compound has no natural source [Vethaak et al., 2002]. Next to contraception, the uses of estrogens can be categorised into 3 main groups: the management of (post)menopausal syndromes, physiological replacement therapy in deficiency states and the treatment of cancers [López de Alda et al., 2000].

The chemical structure of the estrogens considered in this study is presented in figure II.1.1. They all have a polycyclic structure with an -OH group on C_3, a -CH_3 group on C_{13} and different constituents on C_{17}. Although these compounds can be degraded biologically, they have been detected in WTP effluents and surface water at nanogram per litre ($ng.l^{-1}$) levels [Larsson et al., 1999, Thomas et al., 2001, Fine et al., 2003].

A number of studies have demonstrated that these concentrations are significant for an endocrine disruptor, as research has shown that male fish exposed to $ng.l^{-1}$ levels of these estrogens, will exhibit estrogenic responses, such as vitellogenin (VTG; precursor to yolk, a female-specific protein) production [Purdom et al., 1994, Harries et al., 1996, Janssen et al.,

1997, Panter et al., 1998, Larsson et al., 1999], intersex [Tyler et al., 1998] and the presence of testicular oocytes [Thomas et al., 2001]. 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 an earlier onset of puberty in humans [Salomons et al., 1998, DEPA, 2001]. In order to evaluate the potential risk of this group of endocrine disruptors, the occurrence and the environmental exposure to these compounds needs to be documented. Unfortunately, chemical analysis of these compounds in environmental matrices is a difficult task, because of the matrix complexity and their low environmental concentrations [López de Alda et al., 2001b].

The occurrence of estrogens in wastewaters has received increasing interest during the last years. However, little is known about the presence of these compounds in estuarine water. For this, the aim of this study was to develop an extraction method that allows the determination of low concentrations of a number of environmental estrogens and validate this method with water samples from the Scheldt estuary. This estuary is situated in one of the most heavily populated regions of Europe, with a highly diversified industrial activity [Xiao et al., 2001] and for this an example for other estuaries. The four target estrogens included the natural estrogens αE2, βE2 and E1, and the synthetic estrogen EE2. Although the natural hormones are excreted primarily as 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 [Fine et al., 2003, Sharpe, 1998]. Ethinylestradiol (EE2) was also selected because this compound has a greater potency as endocrine disruptor in comparison with the natural hormones. For valid interpretation of environmental data, for example those described in this doctoral work (**chapters II.2.** and **III**) validation of the method (e.g. according to the criteria of Belac, which is the Belgian Accreditation Structure) 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. Also method performance characteristics and the stability of the compounds are well described. Moreover, it is the first to provide data on the occurrence of these estrogens along the Scheldt estuary.

2. Materials and Methods

2.1. Chemicals

Standards of both natural and synthetic hormones were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA) or Steraloids Inc. (Newport, RI, USA). Equilinine (EQ) and deuterated estrone (E1-D4) were used as procedure internal standards, ethinyltestosterone (ET) was used as a GC-MS reference standard and androsterone (And) was used as a derivatization standard. Stock standard solutions of 200 $ng.\mu l^{-1}$ of the analytes were prepared in ethanol (EtOH). Working solutions of each analyte or mixtures were prepared in various 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 like acetone, water, n-hexane, chloroform and EtOH were purchased from VWR (Merck, Darmstadt, Germany).

2.2.Quality assurance

Before every sample analysis a dilution series (0.1, 0.25, 0.5, 0.75 and 1 ng) of standard mixture of the target estrogens was injected. These standards were used to check the operation conditions of the GC-EI-MS-MS apparatus. When samples of the Scheldt estuary were analyzed, the range of calibration standard concentrations spiked in ultrapure water was 0.25, 0.5, 1.25, 2.5 and 5 $ng.l^{-1}$. 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 $ng.l^{-1}$ prior to extraction. After Speedisk^{TM} extraction 10 ng ET and prior to derivatization 10 ng And were added.

2.3. The Scheldt estuary

The river Scheldt originates in northern France (Saint Quentin) at about 350 km upstream of Vlissingen in the Netherlands where the river discharges in the North Sea (Figure II.1.2.). The estuarine zone, which is the interface between the river Scheldt and the North Sea

is about 70 km long and extends from the North Sea to the Dutch-Belgian border near Bath [Baeyens et al., 1998]. The downstream stretch from the city of Ghent (Belgium) to the North Sea is under tidal influence and is named the Sea Scheldt. The Sea Scheldt is further divided into the Lower Sea Scheldt, stretching from the Dutch Belgian border to Antwerp, and the Upper Sea Scheldt, stretching from Antwerp to the upstream boundary at Ghent [Meire et al., 2005, Van Damme et al., 2005]. The Dutch part of the Scheldt estuary is called the Western Scheldt.

For the Endis-Risks project, eight locations in the Scheldt estuary were sampled. Four of the sampling stations are representative for the major freshwater inputs into the estuary: the Antwerp harbour site, the drainage canal at Bath, the canal Ghent-Terneuzen at Terneuzen and the riverine sampling station at the Dutch-Belgian border, Schaar van Ouden Doel (respectively S22, S09, S04 and S15 on Figure II.1.2.) [Steen et al., 2001].

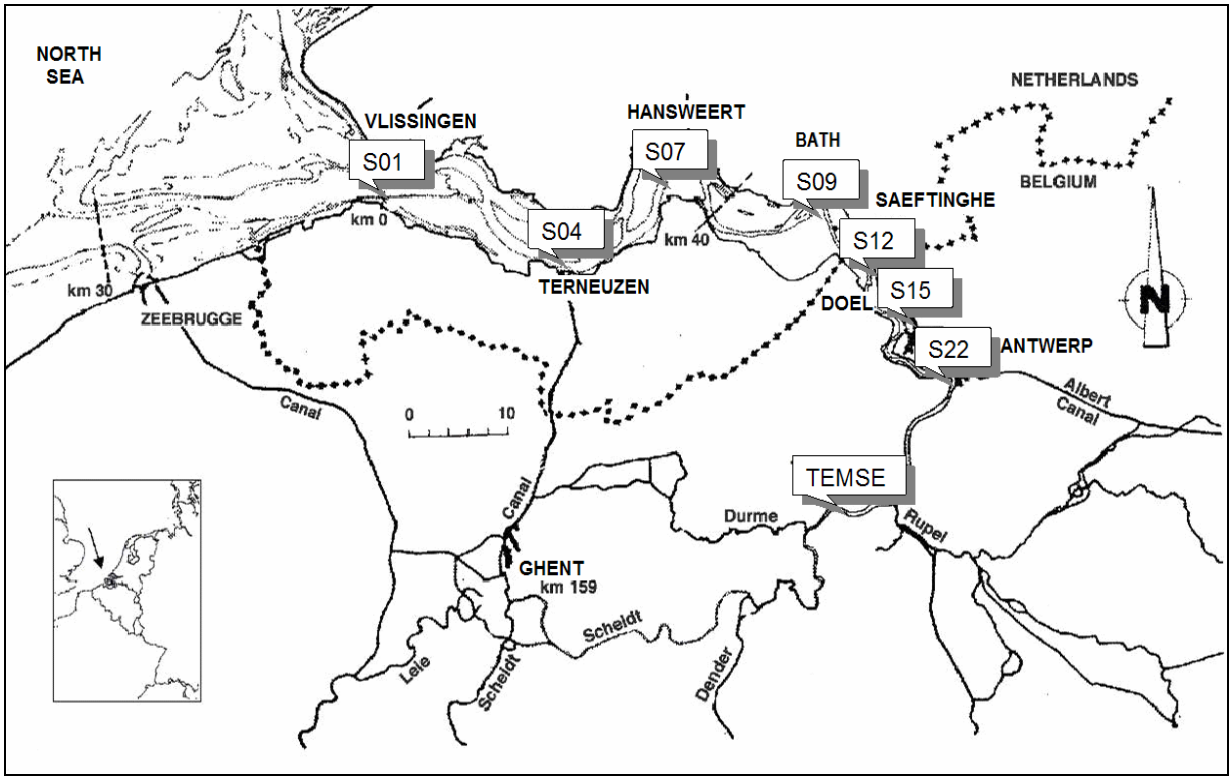

Figure II.1.2.

Map of the Scheldt estuary with location of the different sampling sites: Vlissingen (S01), Terneuzen (S04), Hansweert (S07), Bath (S09), Saefthinghe (S12), Doel (S15), Antwerp (S22) and Temse.

River discharge in the Scheldt estuary is largely dependent on rainfall, and is the highest during winter (average 180 $m^3.s^{-1}$) and the lowest in summer (60 $m^3.s^{-1}$) [Baeyens et al., 1998]. The major tributaries of the estuary are the Rupel, the Durme and the Dender (Figure II.1.2.). The relatively small river discharge of 100 $m^3.s^{-1}$ is strongly dominated by the large intertidal exchange volume of approximately 1 billion m^3. For this, the Scheldt estuary is characterized as a long and well mixed estuary with large intertidal areas and relatively stable salinity zones which are maintained in more or less the same position throughout a tidal cycle. Turbidity in the water of the Scheldt estuary is high, with 7.5 x 10^5 tons.$year^{-1}$ of fluvial fine sediments and 9 x 10^4 tons.$year^{-1}$ of marine suspended matter entering the system, which accumulate in the maximum turbidity zone, upstream of Antwerp. Further details about other physical, chemical and biological parameters of the Scheldt estuary are described in Heip (1989), Baeyens et al., (1998), Van Eck et al., (1991), Soetaert and Herman (1995), Baeyens et al., (1998), Meire et al., (2005) and Van Damme et al., (2005).

Four aspects make the Scheldt estuary distinct from other estuaries: (1) the Scheldt has a tide-governed estuary due to the low river flow resulting in long residence times; (2) the upper estuary receives large inputs of biodegradable organic matter inducing anoxic conditions in the water column during summer; (3) a considerable number (and direct supply) of contaminants occur in the upper estuary as a result of the diverse industrial activities around Antwerp and upstream activities around Ghent and (4) the anoxic zone, the area of maximum contaminant input and the zone of maximum turbidity coincide geographically, making it very difficult to distinguish between their individual effects on the chemical distribution and behaviour [Baeyens et al., 1998, Salomons et al., 1998].

The catchment area of the Scheldt is approximately 20 000 km^2. In this area about 10 million people are living with Vlissingen, Ghent, Antwerp and Brussels as large industrial cities in the vicinity. Large efforts for industrial and municipal waste water treatment has been undertaken in the last years in Flanders, but still untreated waste water is discharged directly or indirectly (via the Zenne) into the estuary with the city Brussels as an example [Van Damme et al., 2005].

From an ecological point of view is the Scheldt estuary an important passing, hibernating and feeding area for waterbirds and a nursery area for fish and shrimps [Heip 1988, Soetaert and Herman 1995, Bayens et al., 1998, Salomons et al., 1998, Van Damme et

al., 2005]. Unfortunately, the Scheldt estuary covers one of the most polluted estuaries in the world and is affected by man's activities, as a large amount of domestic and industrial waste in released into the river. The Western Scheldt (the Dutch part of the Scheldt estuary), excluding the shipping channels, is also recognized as a protection zone under the EU Habitats directive (92/43/EC).

2.4. Sampling

Samples from the Scheldt estuary were collected using the Research Vessel (RV) Belgica (Figure II.2.1.). Three times a year from December 2002 through to July 2005, water, sediment and suspended matter samples were taken at eight sampling points (Figure II.1.2.). This sampling strategy was based on the occurrence of three cohorts (spring, summer, and winter) of the estuarine mysid *N. integer* (Crustacea:Mysidacea) in the estuary as described by Mees et al. (1994). Campaigns were performed in December 2002, March and June 2003, February, May, September and November 2004 and February and July 2005. Further details about these campaigns can be found in the respective boarding reports (online available on www.vliz.be/projects/endis).

Water samples were taken at each sampling site using Teflon-coated Go-Flo water samplers (General Oceanics Inc., Miami, Florida, USA) at a depth of 4 to 5 m (hydrostatic pressure activated) considering tidal movements in the estuary. These water sampling bottles avoid sample contamination at the surface, internal contamination, loss of sample on the deck and exchange of water from different depths. The samples were, depending on the application, immediately extracted on board or transferred to pre-rinsed amber bottles, acidified in order to prevent microbial degradation during transport and stored in the dark at 4°C. The binding of the targeted estrogens to glassware was not investigated, based on the results of Fürhacker et al., [1999] who found that βE2 does not absorb significantly to glass bottles.

2.5. Extraction

Prior to extraction, the pH of the water samples was adjusted to 7 using solutions of HCl or NaOH (1M). Extraction of the water samples was performed using Bakerbond Speedisk^{TM} Octadecyl-bonded silica (C_{18}XF), 50 mm (J.T. Baker, Deventer, The

Netherlands). Extraction was performed using the manufacturer's guidelines. In short, the discs were placed on a Speedisk^{TM} extraction station (J.T. Baker, Deventer, The Netherlands) 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 disk, the disk was rinsed twice with 10 ml ultrapure water. When the sample was drawn through the disk, it was dried under vacuum for at least 30 minutes. 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.

2.6. Clean-up

The Speedisk^{TM} extracts were vaporised in bulb flasks of 100 ml to dryness using a rotavapor (Büchi, Flawil, Switzerland), reconstituted with 500 µl chloroform and used for Solid Phase Extraction. Silica (Si, 500 mg, 10 ml, Sopachem nv, The Netherlands) cartridges were placed on an adsorbex SPU (VWR, Darmstadt, Germany) and conditioned twice with 2.5 ml n-hexane under vacuum. Before the samples were added on the cartridges, 5 ml n-hexane was added to the samples in the bulb flasks, mixed well and transferred onto the cartridges. After the samples were drawn through the cartridges, another 5 ml of n-hexane was added to the bulb flasks and transferred onto the cartridges. Under the Si-cartridges a NH_2-cartridge (100 mg, 1 ml, Sopachem nv, The Netherlands) (to retain humic acids and other interferences) was placed 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 in a centrifugal evaporator system (Gyrovap, Howe and Co., London, UK) at 60°C to dryness. To improve the stability of the target estrogens, the analytes were derivatized in the hydroxyl- and keto-groups of the steroid ring. After derivatization with 25 µl of a mixture of N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA), NH_4I and ethanethiol (1h at 60°C) [Impens et al., 2002] the samples were analysed by GC-EI-MS-MS. As the GC-EI-MS-MS apparatus is used for other routine analyses, in some cases, extracts or derivatized extracts needed to be short-time stored at 4°C in the dark. Experiments were performed to evaluate the shelf-life of extracts in EtOH or derivatized extracts.

2.7. GC-EI-MS-MS analysis

All GC-EI-MS-MS chromatographic measurements were performed with a Thermofinnigan Trace GC 2000 (Austin, TX, USA) Gas Chromatograph fitted with a Polaris ion trap mass spectrometer and a Finnigan MAT AS2000 autosampler. The separations were performed using a BPX-5 (SGE Inc., Austin, TX, USA) (25m x 0,22 mm I.D.) fused silica capillary column with 5% phenyl liquid phase (film thickness 0,25μm). Glass injector liners (10.5 cm x 3 mm) were supplied by SGE Inc. The injector, ion source and transfer line temperature were respectively 250°C, 200°C and 275°C. The temperature program was as follows: initial temperature 100°C, directly ramped at 17°C.min^{-1} to 250°C. Second ramp at 2°C.min^{-1} to 268°C and finally ramped at 30°C.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 as calibration gas. A volume of 1 μl of sample was injected with a split-splitless injector (split flow 20 ml.min^{-1}, splitless time 1 min). The EI spectra were obtained in Electron Impact Mode at 70 eV.

2.8. Data processing, analyte identification and quantification

The data processing was performed using Xcalibur 1.3. software (Thermofinnigan, Austin, TX, USA). In the environmental samples, the targeted estrogens were identified using the following criteria: the chromatographic peaks of the diagnostic ions from the unknown and the standard had to elute at the same relative retention time. Secondly, 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 [2002/657/EC]. 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.

3. Results and Discussion

3.1. Performance of the GC-EI-MS-MS method

Present detection methods for natural and synthetic estrogens in water are based on either chromatographic or *in vitro* techniques, such as the yeast estrogen screen (YES) and the recombinant yeast assay (Rya) [Garcia-Reyero et al., 2001, López de Alda et al., 2001b, Snyder et al., 2001, Thomas et al., 2001]. Gas chromatography-mass spectrometry (GC-MS) is a commonly used technique whereas liquid chromatography-mass spectrometry (LC-MS) has gained in popularity over the last few years [Belfroid et al., 1999, Ternes et al., 1999a and b, Kelly et al., 2000, López de Alda et al., 2000 and 2001a/b, Mol et al., 2000, Ingrad et al., 2003]. The advantage of LC is that steroids can be determinated without derivatization. In addition, both GC and LC are more specific in comparison with biological tests [López de Alda et al., 2000, Ingrad et al., 2003].

The clean-up and GC techniques used in this study were based on the extended experience in the lab with detection of anabolics in animal matrices using this separation technique. Due to the complexity of the matrix, multiple MS was selected. By interpreting the relative retention time and the precursor and product ions in the obtained 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 through additions of known amounts (6 times 6 concentrations levels, 0.25, 0.5, 1.25, 2.5 and 5 ng.l^{-1}) of the target analytes to ultrapure water as described in the material and methods section. The tolerances used for the relative retention time of the target analytes are described in table II.1.1.

Table II.1.1.

Permitted tolerances for the relative retention time of the selected estrogens (n=36)

Analyte	EQ	E1-D4
αE2	0.89±4.05E-2	0.98±1.69E-4
βE2	0.93±4.25E-2	1.02±1.70E-4
E1	0.91±4.14E-2	1.00±1.95E-4
EE2	1.04±4.76E-2	1.14±3.77E-4

It can be noticed that the standard deviation on the relative retention time is lower in the case of E1-D4 as internal standard. In Full Scan MS, the spectrum was characterised by a base peak corresponding to [M+72] or [M+144] according to the 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 II.1.2.

Table II.1.2.

Optimized GC-EI-MS-MS conditions (Internal standards are marked in italic).

Analyte	Precursor Ion (m/z)	Collision Energy (eV)	Product Ions (m/z)
αE2	416	1.00	326, 285
βE2	416	1.00	326, 285
E1	414	1.00	399, 324, 309
EE2	425	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*

Conform to the European Criteria 2002/657, 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. This either from standard solutions or from spiked calibration standards, at comparable concentrations, measured under the same conditions, with tolerances as shown in table II.1.3. Only when both criteria were fulfilled, quantitative analysis of the results was performed.

Table II.1.3.

Maximum permitted tolerances (%) for relative ion intensities using GC-MS²

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

3.2. 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 prior to 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 dark circumstances. Adjustment of pH 2 was performed using 2 ml HCl 6M in order 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 the degradation of βE2 into E1 [Kelly et al., 2000].

Detection of the natural and synthetic hormones in environmental samples requires analytical methods which allow the reliable determination of these compounds at the low $ng.l^{-1}$ concentration level. In most cases, these methods consist of an extraction and pre-concentration step followed by GC or LC detection. In literature, extraction is mostly performed using solid phase extraction (SPE) using cartridges or discs impregnated with different sorbents e.g. C_{18}, graphitised carbon black or styrenedivenylbenzene (SDB) [Sharpe et al., 1998, Belfroid et al., 1999, Kelly et al., 2000, López de Alda et al., 2001b, Nakamura et al., 2001, Quintana et al., 2004]. In this study extraction discs were preferred over normal cartridges because cartridges can clog easily when used for environmental samples due to colloidal material and suspended particles [Kelly et al., 2000]. In addition, these discs provide a large surface area, low levels of recipient contamination [Sharpe et al., 1998, Mol et al., 1999, Kelly et al., 2000] and they are ideal for on board extractions. 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 their presumed longer evaporative concentration time of the extract [Mol et al., 1999].

Preliminary experiments revealed that water samples with a pH range of 2 to 7 gave the best recoveries for the targeted compounds. Nevertheless, pH 7 was preferred because at acid pH, humic acids in the environmental samples are strongly retained on the sorbent. As a consequence, a yellowish extract, due to a high content of humic acids, results in interferes of

the GC-EI-MS-MS analysis [Quintana et al., 2004]. No filtration step was added to the protocol since log K_{ow} values of the target estrogens are in the range of 3 to 4. This indicates that the target analytes have high affinity for suspended matter [Sharpe et al., 1998] and filtration could cause significant losses. However, Lopez and co-workers [2001] demonstrated that a filtration step does not lead to significant losses of the target analytes [Thomas et al., 2001]. In our procedure, filtered particulates on the disc and estrogens adsorbed to the sorbent are ultimately washed with organic solvents. Prior to extraction, the discs were washed with acetone and MeOH in order to clean the disk and to remove any potentially interferences.

3.3. Validation

Because no certified reference material was available, the trueness of the analytical method was assessed through the recovery of additions of a standard mixture of the target analytes in ultrapure water as described before. Using the European criteria 2002/657, the directive for the control of analytical methods for matrices of animal origin, the trueness of the method has to be in the interval -50 % to +20 % for a mass fraction of ≤ 1000 ng.l^{-1}. This European directive was used, because no guidelines for environmental analysis are available. As shown in table II.1.4, all mean recoveries fall within this range.

Table II.1.4.

Trueness of the quantitative method: Mean (0.25 up to 5 ng.l^{-1} in ultrapure water) recovery ± stdev (%) (n=36)

Analyte	EQ	E1-D4
αE2	105±20	107±22
βE2	104±25	103±27
E1	108±21	107±18
EE2	102±21	103±27

Five-point calibration curves 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 and E1-D4 respectively. In literature, correlation coefficients higher than 0.99 are reported for the same target compounds [López de Alda et al., 2000, Isobe et al., 2003, Quintana et al., 2004]. 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} [López de Alda et al.,

2001b]. Consequently, 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 recovery percentage of 105±18 % when EQ was used for quantification. An average recovery percentage of 105±20 % was obtained with E1-D4 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 obtained recoveries were within the same order of magnitude as those reported by other authors [López de Alda et al., 2000 and 2001b, Isobe et al., 2003, Quintana et al., 2004]. However, in literature, recoveries of the same target compounds in aqueous samples were investigated using additions of 75 $ng.l^{-1}$ [Quintana et al., 2004] and 10 $\mu g.l^{-1}$ [López de Alda et al., 2000 and 2001b] which is high considering the low $ng.l^{-1}$ environmental levels of the selected estrogens reported in this study. The coefficients of variation for the repeated analysis of the series of fortified ultrapure water were respectively 17.14 (EQ) and 19.05 (E1-D4) %. Different procedures for the limits of detection (LODs) and quantification (LOQs) are reported in literature. These limits can be experimentally estimated from the injection of serially diluted standard solutions [Ternes et al., 1999, López de Alda et al., 2000] 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, while the limit of quantification (LOQ) is set at three times the LOD [Belfroid et al., 1999]. In the present study the lowest concentration of the calibration curve, i.e. 0.25 $ng.l^{-1}$ was preferred as LOQ. This was chosen in accordance with preliminary tests and literature where LOQs of 1 to 3 $ng.l^{-1}$, depending on the target estrogen, are reported [Quintana et al., 2004]. This LOQ can be extrapolated to the analysis of fairly clean waters, such as drinking water, groundwater or surface water. In case of more complex samples, e.g. estuarine samples, the sensitivity gets compromised by the matrix effect [López de Alda et al., 2001a]. For this, future experiments will be conducted on the determination of the matrix on the detection of the target compounds in this study.

3.4. Stability of the compounds

The European Criteria 2002/657 state that the stability of the analyte in solvent during storage, in matrix during storage and/or sample preparation and in extract during storage and/or analysis should be tested. Stock solutions of 200 $ng.\mu l^{-1}$ are prepared in EtOH and stored in the dark at 4 °C. Working solutions are 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. Similar, matrix stability was not tested as samples were always extracted within one hour after sampling.

Because the GC-EI-MS-MS apparatus used in this study is also used for other routine analysis, the stability of extracts and derivates was studied after short-term (4 weeks) storage in the dark at 4°C. A one-way ANOVA or Kruskal-Wallis test was applied on the peak area ratios of the target analytes and the internal standards to detect significant effects of the short-term storage. With EQ as internal standard, no significant degradation was observed when EtOH or derivatized extracts were stored for up to four weeks (ANOVA, Kruskal-Wallis, $p>0.05$). No significant degradation was observed after storage of the derivatized extracts with E1-D4 (ANOVA, Kruskal-Wallis, $p>0.05$). In case of the EtOH extracts, prior to derivatization, a significant effect of storage during 4 weeks was observed for EE2, αE2 and βE2 (ANOVA $p=0.01$, Mann-Whitney, $p=0.029$ and 0.047). This could not be explained by stability of the GC system as this would have resulted in the same trend being observed when using EQ for quantification. Most likely, storage of extracts affects the stability of E1-D4 and not the stability of the derivatized E1-D4. For this reason extracts were analysed as soon as possible and if storage is necessary, derivatized and EQ was preferred for quantification.

3.5. Estuarine water analysis

The developed analytical method was applied to water samples collected from the Scheldt estuary (B-Nl) in 2002 through to 2004 (1 sample in each sampling point). Figure II.1.3. shows the chromatogram and spectrum obtained, from the analysis of a 2 l water sample of the Scheldt estuary (6.3 $ng.l^{-1}$, May 2004 campaign, Antwerp, S22).

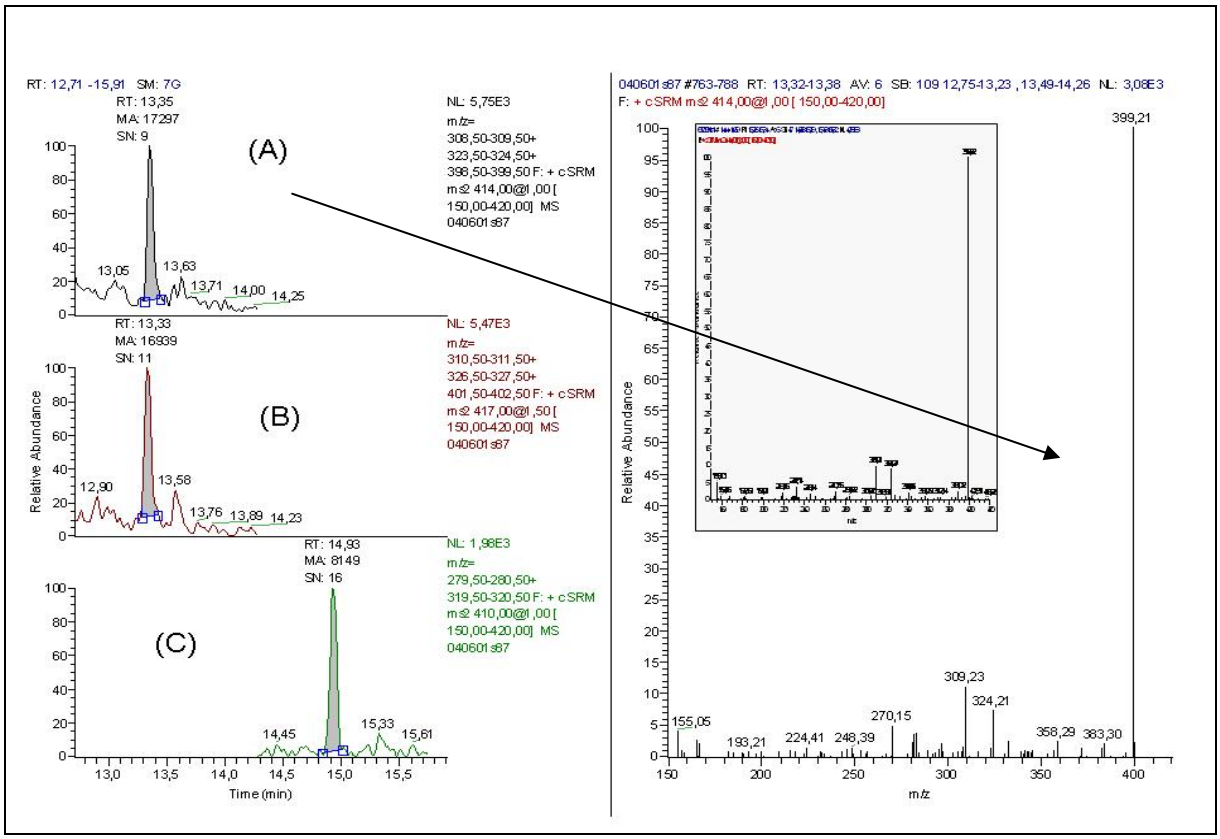

Figure II.1.3.

Chromatogram (shaded zones and spectrum of the analysis of an estuarine water sample taken from the Scheldt estuary (May 2004 campaign at Antwerp site). Peak Identification : (A) E1; (B) E1-D4; (C) EQ. (Inset is the spectrum of a standard mixture of 10 ng E1).

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 most upstream side of the estuary whereas αE2 was only detected once (June 2003) at 2 sites downstream at concentrations near the LOQ. Levels of βE2 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 could be observed up to now. Similar levels of contamination for the target estrogens were previously reported within the same order of magnitude in the Dutch part of the Scheldt estuary [Belfroid et al., 1999] and in surface water elsewhere in the world [Sharpe et al., 1998, Garcia-Reyero et al., 2001, López de Alda et al., 2001b, Isobe et al., 2003].

4. Conclusion

This study showed that the combined use of speedisk extraction and gas chromatography (GC) coupled to multiple mass spectrometry (MS^2) enabled the detection and quantification of estrogens in estuarine and marine water samples at the low $ng.l^{-1}$ level. Quantification limits of 0.25 $ng.l^{-1}$ were achieved with sample volumes of 2 l. This method was validated according to the laboratory quality assurance criteria (after 2002/657/EC). Application of the procedure to Scheldt estuary samples revealed E1 concentration up to 8 $ng.l^{-1}$.