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Liquid chromatography–tandem mass spectrometry analysis of estrogenic compounds in coastal surface water of the Baltic Sea

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Abstract

An analytical method has been developed for the determination of five naturally occurring estrogens (estradiol, estriol, estrone, genistein, daidzein), one synthetic hormone (ethynylestradiol) and three xenoestrogens (4-nonylphenol (NP), 4-*tert*-octylphenol (4-*tert*-OP), bisphenol A (BPA)) in coastal marine waters. The procedure includes a solid-phase extraction of approx. fifty litres of water samples on the solid-phase copolymer Oasis HLB followed by a clean-up on silica. Twenty-five percent aliquots were used for the analytical determination of the analytes using high performance liquid chromatography coupled with electrospray-ionisation tandem mass spectrometry (HPLC–ESI-MS/MS). Calculated extraction recoveries between 52 (4-*tert*-octylphenol) and 91% (nonylphenol) were obtained for the method developed. Matrix interferences occurring during electrospray ionisation were quantified by spiking the extracts prior to the measurements. Method detection limits ranged from 0.02 (estrone) to 1 ng L^{-1} (estriol). The method was applied to determine environmental estrogens in coastal waters of the Baltic Sea. The analyses showed the presence of five compounds at levels between 0.10 (estrone) and 17 ng L^{-1} (ethynylestradiol).

Keywords: Estrogens; Xenoestrogens; Endocrine disruptors; Solid-phase extraction; HPLC–ESI-MS/MS; Matrix interferences; Marine environment; Baltic Sea

1. Introduction

Endocrine disrupting chemicals (EDCs) of either natural or synthetic origin have the ability to interfere with the normal functioning of the endocrine system. Of major concern are estrogenic compounds which have the potential to influence the regulation of development and growth by mimicking endogenous hormones [1,2]. In many well-documented examples the reproduction of wildlife has been adversely affected by EDCs [3–5]. Steroid estrogens have the potential to exert estrogenic effects in the low ng L^{-1} level, whereas alkylphenolic compounds are estrogenic at μg L^{-1} concentrations [4]. The discussion about effects on human health is still controversial [2]. It is hypothesized that EDCs are associated with a decreased male reproductive capacity [6–8]. The substances enter the environment mainly through sewage treatment plant (STP) effluents due to inefficient removal rates during the wastewater treatment process [9]. Accordingly, most of the reported effects are found in the aquatic environment, especially in rivers with a high charge of domestic and industrial wastewaters. The most commonly used analytical technique for EDCs in the past has been gas chromatography with mass spectrometric detection (GC–MS) [9–16]. However, in recent years the combination of solid-phase extraction (SPE) as a fast sample preconcentration and the separation and determination with liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) has become a widely-used tool for determination of estrogens and/or xenoestrogens in environmental samples [17–22]. One drawback of the electrospray mass spectrometry technique during measurements of

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environmental samples is the signal suppression effect, which has a negative influence on the reproducibility and accuracy of the analyses. Various approaches such as a selective extraction procedure followed by an efficient sample clean-up or the use of suitable co-eluting surrogates and standard-addition to eliminate or compensate matrix effects have been discussed [23–27].

Natural and synthetic hormones are frequently detected in sewage treatment plant effluents and receiving surface waters with concentrations ranging from pg to ng L^{-1} [11,17,28], whereas alkylphenolic compounds are found in concentrations up to μg L^{-1} [12,14,29]. In contrast, comparable analytical data for marine environments are scarce. Heemken et al. [30] determined concentrations of alkylphenols in the North Sea ranging from 1 to 84 ng L^{-1}. The natural hormone metabolite estrone could be found at average concentrations of 52 pg L^{-1} in open-ocean water samples from tropical regions by Atkinson et al. [31].

Due to higher dilution in marine waters, the concentrations of EDCs are expected to be low and thus, direct effects of single estrogenic substances are assumed to be of minor or negligible relevance [32]. For this reason, aquatic ecosystems have received only little attention in recent years and thus there is a lack of knowledge concerning the occurrence and fate of these chemicals in marine environments [31]. Insufficient detection limits of analytical procedures for the determination of estrogenic substances in marine waters is another reason why only few data concerning contamination levels in marine environments are available. Especially in the semi-enclosed Baltic Sea, with limited water exchange, the organisms are exposed to a variety of compounds, which could lead to a background exposure, with complex effects such as, e.g., a decreasing fitness of nearshore ecosystems. A histopathological assessment of the gonads of male fish using the eelpout (*Zoarces viviparous*) as sentinel species showed the presence of intersexuality at a number of locations between the Wismar Bay and the Darss Peninsula [33]. It is suggested that this is due to the exposure to endocrine disrupting compounds, but analytical data are lacking.

The most important discharger in this region is the STP of the city of Wismar. The STP was rebuilt between 1996 and 2002 for the optimised cleaning of receiving wastewaters. The municipal STP (90,000 population equivalents) has an average inflow of untreated wastewater in the range of 10,200 m^3/day. It consists of three main treatment steps: a primary mechanical clarification followed by a secondary biological treatment comprising nitrification and denitrification zones and finally a chemical clarification including a phosphate elimination step.

The current work is focussed on the natural and synthetic hormones including 17β-estradiol (E2), estrone (E1), estriol (E3) and 17α-ethynylestradiol (EE2), the phytoestrogens daidzein and genistein, the phenolic substances 4-nonylphenol (NP), 4-*tert*-octylphenol (4-*tert*-OP) and bisphenol A (BPA) and their analytical determination in the Baltic Sea. The main objectives were (i) to develop a method for the simultaneous determination of selected estrogens in coastal waters using LC–MS/MS including an extraction method that allows the preconcentration of high-volume water samples and (ii) to apply this method to samples from different locations situated in the Baltic Sea in order to provide baseline contamination data.

2. Experimental

2.1. Chemicals

Standard substances were purchased from the following companies: E1, E2, E3 and EE2 from Sigma (Seelze, Germany), E2-D_3 from Supelco (Taufkirchen, Germany), NP from Riedel-de Haen (Seelze, Germany), 4-*tert*-OP from Dr. Ehrenstorfer GmbH (Augsburg, Germany), 4-*n*-OP from Promochem (Wesel, Germany), BPA, genistein and daidzein from Fluka (Buchs SG, Switzerland), BPA-D_{16} from Cambridge Isotope Laboratories (Massachusetts, USA). Stock solutions of these substances were prepared in methanol at 1 μg μL^{-1}. Calibration as well as spiking mixtures were diluted from stock solutions and generally contained all analytes including deuterated standards. Methanol SupraSolv® and ammonium acetate Fractopur® were obtained from Merck (Darmstadt, Germany). Dichloromethane and acetone of Picograde® quality were purchased from Promochem (Wesel, Germany). Deionised organic-free water was obtained from a Milli-Q Plus 185™ coupled to an Elix 5™ system (Millipore, Schwalbach, Germany). The solid-phase extraction sorbent Oasis HLB (divinylbenzene-*co*-*N*-vinylpyrrolidone, 60 μm, bulk material) was supplied by Waters (Eschborn, Germany).

2.2. Sampling

High-volume water samples were collected at five locations in the Baltic Sea between 7 and 11 July 2003 and between 26 and 29 July 2004 (Fig. 1) with the research vessel "Ludwig Prandtl". All sampling sites were located in the eastern part of the German Baltic Sea. Several parameters of the water samples (pH, °C, DOC, POC, salinity) were measured and data for both campaigns are given in Table 1. Three locations were situated in the inner coastal waters (Inner Wismar Bay, Eggers Wiek and Salzhaff), where intersex prevalences and histological alterations in eelpouts (*Zoarces viviparous*) were found [33]. The sampling site Inner Wismar Bay was situated directly in the vicinity of the STP of the city of Wismar. The stations Outer Wismar Bay and Darss Peninsula were expected to be reference sites.

2.3. Sample enrichment, extraction and clean-up

Sampling at a water depth of approximately 1.5 m was performed using a Kiel in situ pump (KISP) system developed by Petrick et al. [34]. Surface water was filtered in situ through a glass fibre filter (GF 52, Schleicher &

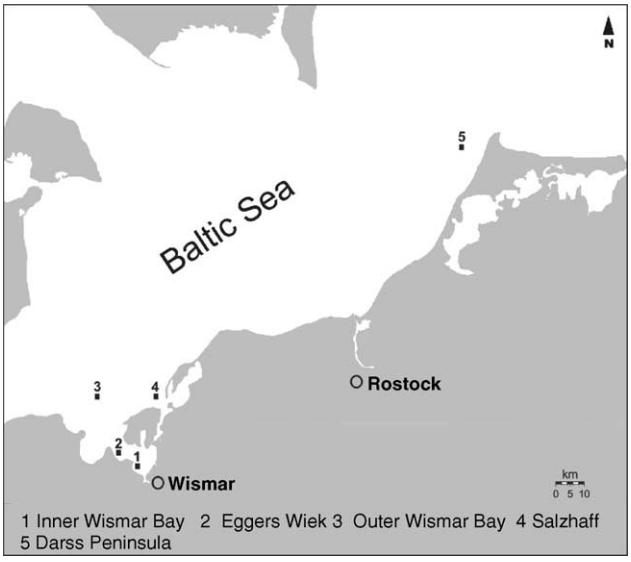

Fig. 1. Location of sampling sites in the German Baltic Sea.

Schuell, Dassel, Germany, 30 cm i.d., pore size 1 μm). For analyte enrichment, two approaches were used: a direct in situ solid-phase extraction as well as experiments on board with water samples temporarily stored in stainless steel containers. For the container and in situ experiments, glass cartridges (4 cm i.d.) were used, which were packed with 4 g Oasis HLB. Prior to the experiments the cartridges were conditioned with 150 mL acetone:methanol (80:20, v/v) followed by 150 mL Milli-Q water.

2.3.1. Container experiments

The filtrate was pumped on board through Teflon tubes and three 49 L stainless steel containers were filled in an alternating manner in order to obtain three samples that were as homogeneous as possible. The samples were extracted with Oasis HLB cartridges using 2 bar excess pressure of nitrogen. At each station, one sample was used for a recovery experiment at a spiking level of 4 ng L^{-1}. With the other two samples a duplicate determination of the environmental analyte concentrations was performed.

2.3.2. In situ experiments

Simultaneous to the container experiments, one in situ sample was taken at each station (except for station Outer Wismar Bay, July 2003). For this purpose, the outlet of the filter holder of the in situ pump system was directly connected to a cartridge which was attached to a flow meter. In approx. 1.5 m water depth, the KISP pumped with an initial flow rate of 300 mL min^{-1} for a period of 3–4 h to obtain an extracted volume similar to those in the container experiments, but depending on the load of clogging substances different volumes in the range between 19 and 104 L were enriched on the sorbent.

2.3.3. Extraction and clean-up

After sampling, the cartridges were stored in darkness at 4 °C until the complete processing in the laboratory. Initially, cartridges were washed with 200 mL of deionised water. Afterwards, the solid-phase material was dried overnight by sucking air through the columns. To avoid contamination, the air was purified with charcoal filters attached to the inlet of the glass columns. Analytes were eluted from the cartridges by primarily using 100 mL of solvent (acetone:methanol (80:20, v/v)), which was drawn into the SPE material, left to infuse for 5 min and then was sucked through the cartridge drop by drop, while another 100 mL of solvent was added. The sample extracts were reduced to 1–2 mL by rotary evaporation and finally reduced to 100 μL under a gentle stream of nitrogen (purity 99.999%). 400 μL of dichloromethane were added to the vial to get a solvent composition of dichloromethane:methanol of 80:20 (v/v). The 500 μL extract was quantitatively transferred to a prepared 3 g silica gel column (1 cm i.d. × 15 cm). Previously, the silica gel (0.063–0.200 mm, Merck, Darmstadt, Germany) had been baked out overnight at 450 °C to remove organic contaminants. Purification of the extracts was accomplished by eluting the column subsequently with 7 mL dichloromethane:methanol (90:10, v/v) followed by another 7 mL with a composition of 80:20 (v/v). The eluate was finally reduced to a volume of 400 μL. A 50 μL aliquot was used for the determination of analyte concentrations. For calculation of signal suppressions during LC–ESI-MS/MS measurements another aliquot of 50 μL was spiked with a defined amount of standard solution. The remaining volume of 300 μL was needed for further and still ongoing investigations in order to analyse the estrogenic potential of these environmental samples.

Table 1
Characteristics of the water samples taken at five different sampling sites in the German Baltic Sea (campaigns July 2004 and July 2003)

Sampling site	Longitude (°E)	Latitude (°N)	Temperature (°C)		pH		DOC (mg L^{-1})[a]		POC (mg L^{-1})[b]		Salinity (‰)	
			2003	2004	2003	2004	2003	2004	2003	2004	2003	2004
Inner Wismar Bay	11°26.4	53°54.5	18.0	19.0	8.1	8.4	5.5	5.0	7.8	9.3	12.0	12.4
Eggers Wiek	11°23.4	53°57.1	18.8	19.5	8.2	8.5	4.8	4.0	3.7	10.8	12.6	12.8
Outer Wismar Bay	11°18.5	54°02.4	18.0	18.8	8.2	8.3	4.1	3.1	2.7	2.8	14.1	11.7
Salzhaff	11°29.4	54°02.4	18.0	18.5	8.2	8.3	4.5	3.7	2.0	3.0	13.4	12.5
Darss Peninsula	12°29.1	54°28.0	16.5	15.5	8.1	8.0	4.5	3.3	2.6	1.9	10.3	9.4

[a] Dissolved organic carbon.
[b] Particulate organic carbon.

2.4. LC–MS/MS analysis

Liquid chromatography was performed on an Agilent Series 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with a degasser, a binary pump, an autosampler and a column oven. The analytes were chromatographed on a C18 polar end-capped reversed-phase column (Synergi^{TM} Hydro-RP) with a particle size of 4 μm, a length of 150 mm and an inner diameter of 2.0 mm. The column was guarded with a precolumn of the same packing material (4 mm × 2.0 mm), both from Phenomenex (Aschaffenburg, Germany). The column oven temperature was set to 23 °C. Injection volume was 10 μL. Samples were analysed in negative and positive ionisation modes.

Chromatography was carried out using the mobile phases A (water) and B (methanol), both containing equal concentrations of ammonium acetate (NH_4Ac). The gradient was performed as follows: 30% B > 90% B (8 min)/90% B > 100% B (15 min)/100% B (5 min)/100% B > 30% B (2 min). The system was re-equilibrated for 5 min between runs. The flow rate of the mobile phase was set to 200 μL min^{-1}. The influence of different NH_4Ac concentrations used as an eluent modifier to enhance ionisation efficiency was investigated at concentrations of 0 mM, 2.5 mM, 5 mM and 10 mM NH_4Ac. Moreover, for negative and positive ionisation mode different levels of ion spray voltages (−3500 V, −4200 V, −4500 V; +5000 V, +5500 V, respectively) were tested. The measurements were carried out by flow injection analysis with an eluent composition of 90% B and an injection volume of 5 μL standard solution or spiked field sample (Section 3.1). Finally, the field samples were analysed using an optimised modifier concentration of 2.5 mM NH_4Ac and an ion spray voltage of −4500 V and +5500 V, respectively.

The LC system was coupled to a triple-stage quadrupole mass spectrometer (API 4000, Applied Biosystems/MDS Sciex, Darmstadt, Germany). Optimisation of the ion source and MS/MS settings was performed by the automatic optimisation function of the MS software (Analyst 1.4, Applied Biosystems) assisted by manual optimisation using infusion with a syringe-pump and flow injection of standard solutions. The relevant instrument settings for each precursor-product ion transition are shown in Table 2. The electrospray ion source (Turbo-Ionspray, Applied Biosystems) was operated at 250 °C. Nitrogen was used as nebulizer, drying, curtain and collision gas. Ion source gas 1 (nebulizer gas) was adjusted to 50 psi and ion source gas 2 (drying gas) to 70 psi. The nitrogen

Table 2
Retention times, MS and MS/MS detection parameters

Analyte	Retention time (min)	Precursor ion (*m*/*z*)	Declustering potential (V)	Product ions (*m*/*z*) (% relative abundance)[a]	Collision energy (V)	Collision cell exit potential (V)
E1	14.7	269.2 $[M-H]^-$	−106	145 (100)	−52	−7
				143 (40)	−78	−7
E2	14.7	271.2 $[M-H]^-$	−121	145 (100)	−58	−9
				183 (90)	−58	−9
E2-D_3	14.7	274.2 $[M-H]^-$	−110	145 (100)	−56	−7
				185 (80)	−58	−13
E3	12.7	287.2 $[M-H]^-$	−111	145 (100)	−62	−7
				171 (120)	−52	−13
EE2	14.5	295.2 $[M-H]^-$	−110	145 (100)	−54	−7
				143 (65)	−74	−11
				159 (45)	−52	−9
				183 (40)	−55	−11
Daidzein	12.4	255.1 $[M+H]^+$	80	199 (100)	37	14
				152 (55)	61	10
Genistein	13.1	271.1 $[M+H]^+$	70	153 (100)	39	10
				91 (95)	63	6
BPA	13.6	227.1 $[M-H]^-$	−70	133 (100)	−32	−7
				212 (185)	−30	−15
BPA-D_{16}	13.5	242.2 $[M-H]^-$	−70	143 (100)	−36	−7
				97 (10)	−34	−3
NP	17.7	219.2 $[M-H]^-$	−70	133 (100)	−42	−7
				117 (15)	−82	−5
4-*tert*-OP	16.6	205.2 $[M-H]^-$	−80	133 (100)	−34	−7
				117 (10)	−82	−7
4-*n*-OP	18.1	205.2 $[M-H]^-$	−70	106 (100)	−28	−5
				–	–	–

[a] Relative abundances of quantifier ions were set to 100%.

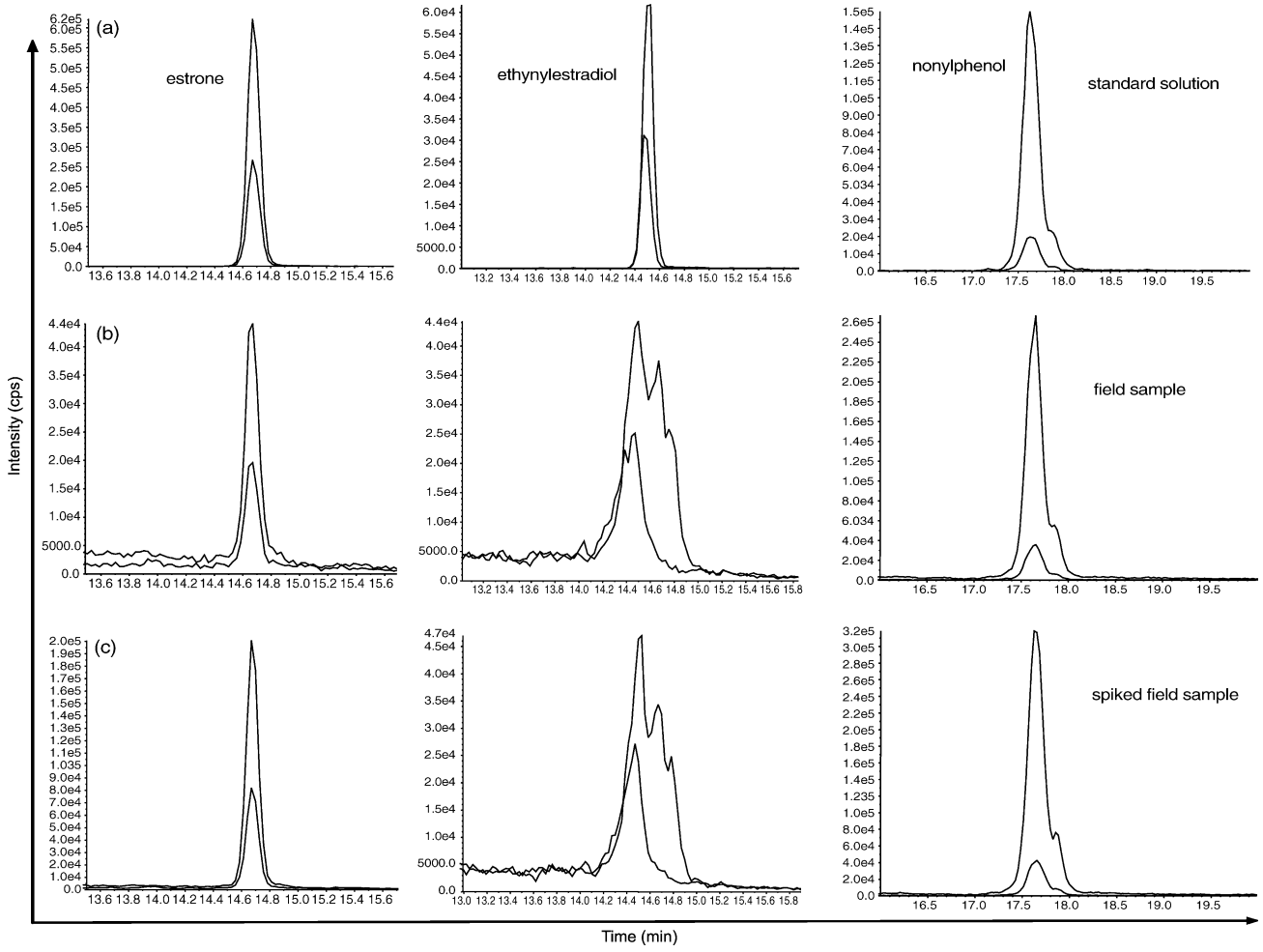

Fig. 2. Chromatograms of three analytes showing quantifier and qualifier (E1, EE2, NP) of (a) a standard solution, (b) a field sample (Inner Wismar Bay) and (c) the corresponding spiked field sample.

settings for curtain gas and collision gas were optimised to 10 and 6 psi, respectively. For quantification the multiple reaction monitoring (MRM) mode was chosen. The dwell time for each precursor-product ion transition was set to 50 ms.

2.5. Data analysis

The base peak selected for quantitation of the analytes investigated corresponds to the deprotonated molecule $[M-H]^-$, whereas the phytoestrogens daidzein and genistein are detected as protonated molecules $[M+H]^+$. The analysed compounds were identified by the following criteria (i) two characteristic precursor-product ion transitions (quantifier and qualifier), (ii) specific ratios of the intensities of the product ions and (iii) specific retention times (Table 2). The monitored precursor-product ion transitions of three compounds (E1, EE2, NP) for (a) a standard solution (approx. 1.4 ng absolute), (b) a field sample (Inner Wismar Bay, 2004) and (c) the corresponding spiked field sample are depicted in Fig. 2. As shown, the quantitation of EE2 in environmental samples was hampered by interfering signals. In order to ensure correct determination, two additional transitions of EE2 were measured. The product ion *m*/*z* 153 was chosen as qualifier due to consistent ratios to the quantifier *m*/*z* 145 concerning field samples.

Quantification was performed using an external eight-point calibration curve covering the range from 50 pg to 5 ng absolute. Within measurement sequences, every 8–10 h, field samples were bracketed with external calibrations to cover possible fluctuations in signal intensity. Calculated concen-

Table 3
Instrumental limits of detection (LOD) and method detection limits (MDL)

Analyte	LOD (pg absolute)	MDL (ng L^{-1})
E1	0.2	0.02
E2	4	0.30
E3	8	1.0
EE2	5	0.45
Daidzein	2	0.43
Genistein	5	0.61
BPA	8	0.04
NP	13	0.12
4-*tert*-OP	6	0.14

trations from the container experiments and in situ samples were corrected for signal suppression. Signal suppression was determined from aliquots of the sample extracts spiked prior to LC–MS/MS analysis using a defined amount of standard solution containing a mixture of all compounds analysed (spiking level 2 ng L^{-1}). The overall method recovery was obtained from non-spiked samples and samples spiked prior the solid-phase extraction with a spiking level of 4 ng L^{-1}. Both, signal suppression and overall method recovery, were assessed by comparing results from spiked and non-spiked samples. Extraction recoveries were calculated from overall method recoveries and signal suppression. The instrumental limit of detection (LOD) and the method detection limit (MDL) were determined as the concentration with a signal-to-noise-ratio (S/N) of 3 (Table 3). The instrumental LOD was calculated by the S/N determined by injecting 10 μL of the lowest calibration concentration. The MDLs were estimated from analyses of field samples with lowest observed concentrations. Observed concentrations with values between MDL and MQL (method quantification limit, S/N = 10), indicated in Table 4, were only determined by the quantifier (S/N between 3 and 10, specific retention time). Possible sample contamination during the sampling and extraction procedure was quantified by blank cartridges, which were handled like the sample columns. In the blanks, only BPA, NP and 4-*tert*-OP could be detected. As the blank concentrations were lower than 20% compared to lowest observed concentrations in field samples, a correction was considered as dispensable. Internal standards (E2-D_3, BPA-D_{16}, 4-*n*-OP) were used for confirmation of recovery results and not for quantitation purposes.

3. Results and discussion

3.1. Performance, recoveries and detection limits of the analytical method

The external eight-point calibration curve using an 1/*x* weighting showed linearity for all analytes in a range from 50 pg to 5 ng absolute with correlation coefficients $r > 0.990$. The repeatability of a tenfold injection of a standard solution (0.5 ng μL^{-1}) from the same vial gave relative standard deviations (RSDs) between 3% (daidzein) and 7% (BPA).

The eluent buffer concentration and the ion spray voltage have an important impact on the ionisation efficiency of analytes as shown in Fig. 3. The optimisation of the buffer concentration from 0 to 10 mM ammonium acetate for an EE2 standard solution (0.34 ng μL^{-1}) with an ion spray voltage of −4200 V is depicted in Fig. 3(a). Increasing NH_4Ac concentrations were related to a decrease in signal intensity. The response of EE2 in a spiked matrix sample (approx. 0.75 ng μL^{-1}) at different buffer concentrations and three ion spray voltages (−3500 V, −4200 V and −4500 V) shows that in contrast to standard solutions higher buffer concentrations and a higher ion spray voltage are required for better signal

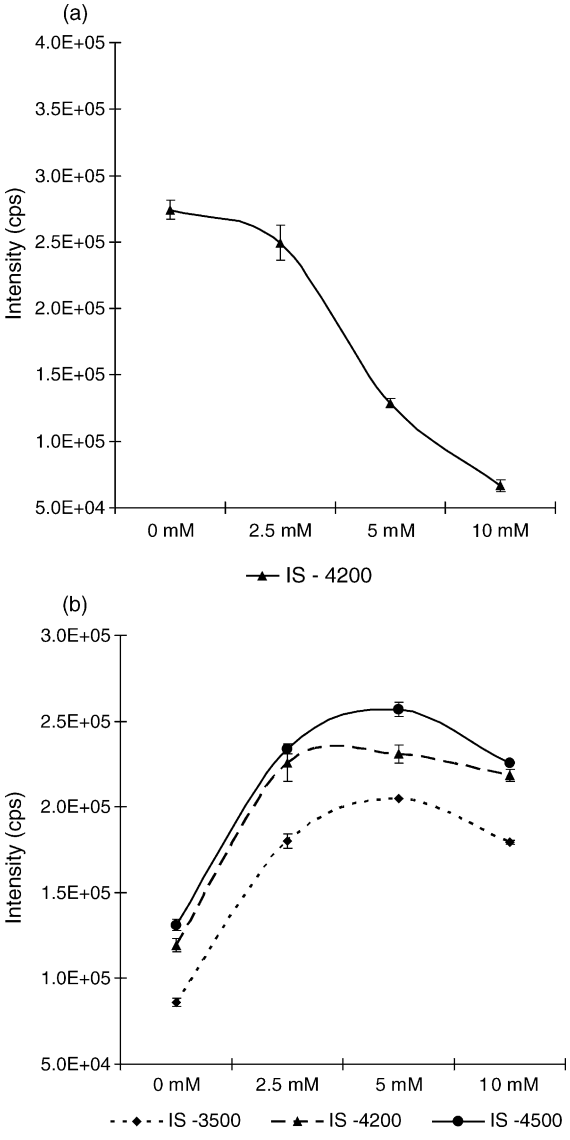

Fig. 3. Influence on signal intensities of EE2 using different modifier (NH_4Ac) concentrations for (a) a standard solution and (b) a spiked field sample. In part (b) the impact of varying ion spray voltages is shown.

intensities (Fig. 3(b)). All analytes showed similar graphs. An optimised modifier concentration of 2.5 mM NH_4Ac and an ion spray voltage of −4500 V for negative ionisation and +5500 V for positive ionisation were chosen.

In Fig. 4, arithmetic means of overall method recoveries, signal suppressions and calculated extraction recoveries of five spiking experiments at the different stations in the Baltic Sea (Sampling July 2004) are depicted. The spiking level was 4 ng L^{-1}. The overall method recoveries of the analytes vary between 10% (genistein) to 81% (NP). Matrix interferences caused by co-eluting components led to signal suppressions in a range of 31% (NP) to 85% (E3). One reason for the significant signal interferences could be the universal extraction method and subsequent clean-up approach taken to allow analysis of a range of compounds simultaneously. The polymer Oasis HLB used is known

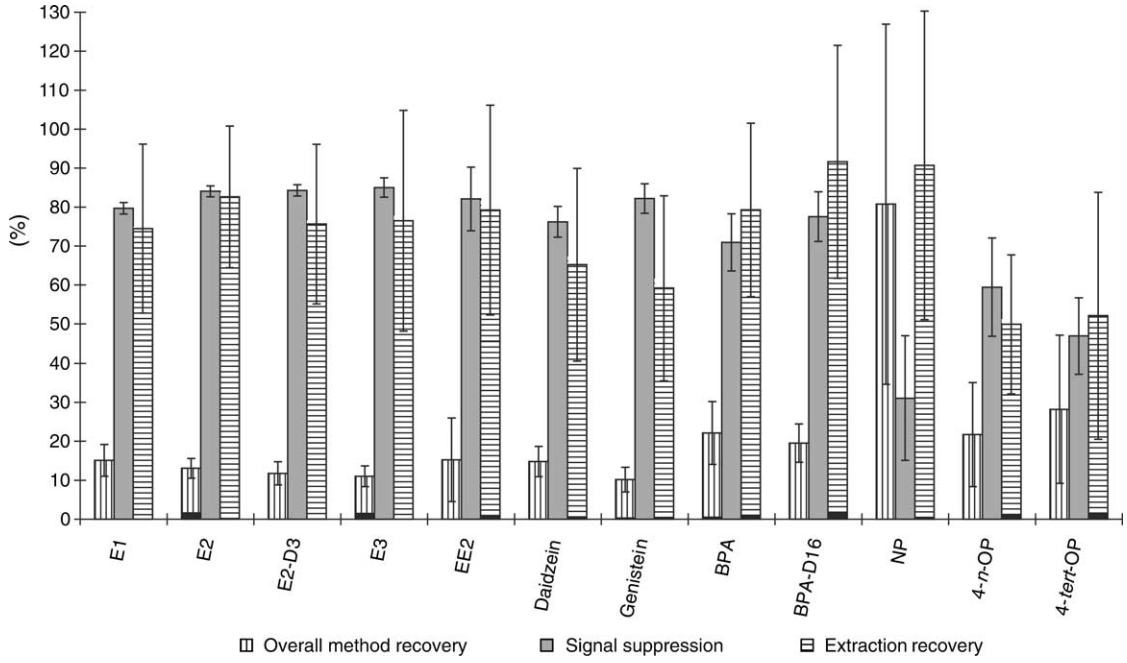

Fig. 4. Recovery results and signal suppressions calculated from five spiking experiments (except for NP ($n = 4$)) during the sampling campaign in July 2004.

as a sorbent which retains a broad spectrum of different substances covering acidic, neutral and basic compounds [26,35]. In order to minimise the pH-dependent amount of co-extracted humic acids, the predominantly weak acidic analytes were extracted without acidification. Previous experiments have shown that recoveries for samples at pH 8.5 (spiked Milli-Q water) were nearly equivalent to those obtained for samples adjusted to pH 2.5 (results not given), which is in agreement with Quintana et al. [16]. The clean-up procedure reduced the amount of co-extracted substances, but not to the extent desirable for the electrospray ionisation technique. Therefore, for each individual sample signal suppression was calculated and quantitation results were corrected.

Extraction recovery was calculated from overall method recovery and signal suppression, which resulted in larger standard deviations (error bars in Fig. 4). Extraction recoveries ranged from 52% (4-*tert*-OP) to 91% (NP). The internal standards for E2, BPA and 4-*tert*-OP showed similar recovery results and thus accomplished their purpose of data confirmation. The powerful technique of tandem mass spectrometry led to instrumental LODs between 0.2 and 13 pg absolute (E1 and NP, respectively). The method developed allows the determination of estrogenic compounds in coastal waters in the range of 0.02 ng L^{-1} (E1) to 1 ng L^{-1} (E3) (Table 3).

3.2. Concentrations of estrogenic compounds in a coastal zone of the Baltic Sea

The occurrence of environmental estrogens in a coastal zone of the Baltic Sea was investigated by the application of the high-volume multi-compound extraction method as described in Section 2.3. Five different sampling sites, covering expected higher and lower contaminated sites, were analysed in the years 2003 and 2004. The concentrations were corrected for signal suppression and are summarised in Table 4. The in situ extractions and container experiments led to similar quantification results and thus, are not listed separately. E2, E3 and the phytoestrogens daidzein and genistein could not be detected. Possible explanations are high removal rates during the sewage treatment, including oxidation and degradation processes. However, the natural hormone E1 was found at all stations. The degradation of E2 to E1 and the fact that E1 is the most abundant estrogen excreted by menstruating and pregnant women are two reasons for the determination of E1 at all sampling sites.

As expected, the Inner Wismar Bay and Eggers Wiek showed the highest concentration levels for the natural (July 2004, E1: 0.53 ng L^{-1}, 0.51 ng L^{-1}, respectively) and synthetic hormones (July 2004, EE2: 17 ng L^{-1}, 8 ng L^{-1}, respectively). These relatively high concentrations of E1 are comparable with data from Atkinson et al. [31], who measured concentrations of E1 in Hawaiian open-ocean water of 52 pg L^{-1} and in lagoon seawater of 1.7 ng L^{-1} by using a radioimmunoassay technique. At much lower concentrations, E1 and EE2 could also be determined at stations expected to be low or not contaminated sites (Darss Peninsula, Outer Wismar Bay). Higher concentrations found for EE2 can probably be explained by its wide use as a contraceptive and its higher persistence compared to natural hormones. However, despite the selectivity of LC–MS/MS, co-extracted sample constituents led to difficulties in quantitation of EE2. An interfering compound with the same parent ion at *m*/*z* of 295, nearly the same retention time (14.5 min, Fig. 2 (b)) and sim-

Table 4
Concentrations of estrogenic compounds at five different sampling sites in a coastal area of the German Baltic Sea for two sampling campaigns (July 2003 and July 2004)

Analyte	Concentration (ng L^{-1})[a]									
	Inner Wismar Bay		Eggers Wiek		Outer Wismar Bay		Salzhaff		Darss Peninsula	
	2003	2004	2003	2004	2003	2004	2003	2004	2003	2004
E1	0.47 (0.45–0.49)	0.53 (0.52–0.53)	0.42 (0.36–0.50)	0.51 (0.54–0.49)	0.33 (0.31–0.35)	0.16 (0.13–0.20)	0.27 (0.27–0.28)	0.34 (0.30–0.37)	0.25 (0.24–0.26)	0.10 (0.08–0.11)
E2	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
E3	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
EE2	3.0 (2.1–3.8)	17.2 (16.5–17.9)	2.4 (<MDL–7.2)	8.0 (<MDL–14.1)	<MDL	2.1 (<MDL–3.9)	1.7[c] (1.6–1.8)[c]	2.9 (1.7–4.0)[c]	2.5 (2.1–3.2)	1.7[c] (1.7–1.8)[c]
Daidzein	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
Genistein	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL	<MDL
BPA	2.5 (1.3–3.6)	5.4 (5.3–5.7)	0.75 (0.61–0.85)	0.96 (0.52–1.8)	0.37 (0.37–0.37)	0.22 (0.11–0.37)	0.22 (<MDL–0.38)	0.35[b] (0.31–0.39)	1.1 (0.67–1.4)	0.47[b] (0.28–0.66)
NP	4.3	6.1[b]	2.5	3.5	3.4	2.5	4.2	4.2	13.8	4.7[b]
	(3.1–6.2)	(5.9–6.3)	(1.3–3.4)	(2.1–5.2)	(3.3–3.6)	(1.6–3.9)	(1.6–6.3)	(2.2–7.5)	(9.4–21.3)	(3.7–5.8)
4-*tert*-OP	0.4 (0.3–0.56)	0.32 (0.10–0.57)	0.60 (0.31–1.1)	0.37 (0.35–0.40)	0.12 (0.11–0.13)	0.23 (0.14–0.36)	0.19 (0.14–0.22)	0.20 (0.14–0.26)	0.45 (0.08–0.95)	0.11[b] (0.04–0.17)

[a] Arithmetic means ($n = 3$), except for Outer Wismar Bay 2003 ($n = 2$); values have been corrected for signal suppression, but not for recovery.
[b] Values have been corrected for one outlier.
[c] Concentrations are between MDL and MQL.

ilar fragmentation patterns (product ions at *m*/*z* of 145, 143 and 183) led to problems in exact peak integration. Ternes et al. [9] as well as Huang and Sedlak [36] reported matrix interferences for EE2 during GC–MS analyses and annotated the risk of overestimating concentration data, but using the selectivity of MS/MS a precise quantification became possible for them. The measurement of four precursor-product ion transitions and correct ratios between quantifier (*m*/*z* 145) and qualifier (*m*/*z* 159) ensured a correct determination. However, the interfering peak resulted in estimated measurement uncertainties in the range of 20–30%.

Also with regard to the phenolic xenoestrogens (BPA, NP, 4-*tert*-OP) the Inner Wismar Bay showed the highest concentrations. Mean concentrations varied between 5 ng (BPA), 6 ng (NP) and 0.3 ng (4-*tert*-OP) per litre (Sampling July 2004). Except for 4-*tert*-OP, the lowest observed concentrations were found at the Outer Wismar Bay (factor 25 for BPA and factor 2 for NP in comparison to the Inner Wismar Bay, July 2004). Similar concentrations determined from in situ and container experiments show that no additional contamination from on board enrichment occurred.

The concentrations of analytes, determined at the Darss Peninsula suggest that there is a nearshore transport of contaminants from the Inner Wismar Bay in the direction of the outer located sampling site. Half-lives of up to 50 days [37] for the more persistent alkylphenols and estimated half-lives of 2–6 days for estrogenic steroids [38] seem to be sufficient to make transport from west to east possible. For this coastal area, a water flow from west to east is confirmed by rates of sediment transportation [39]. Both sampling campaigns (2003 and 2004) showed concentrations in similar ranges (except for EE2 found in the Inner Wismar Bay) and showed similar distribution patterns. Differences in concentrations between the years could be attributed to different charges of STP effluent water and the high variability of water bodies, partly characterised by different values of the parameters shown in Table 1.

Considering the lowest observed effect concentrations of E1 and EE2, which are in the ng and sub-ng L^{-1} range, respectively [40], the measured concentrations are of concern. E1 and EE2, having at least two orders of magnitude higher estrogenic activity compared to known phenolic xenoestrogens, are the target analytes mainly responsible for possible endocrine disrupting effects. In order to allow correlations between observed reproductive disorders in the Wismar Bay and detected concentrations of estrogenic substances, further investigations including chemical analyses and exposure experiments would have to be carried out.

It should be noted that this study is focused on the water phase, whereas for natural steroids and especially for synthetic hormones and phenolic compounds with higher $\log K_{ow}$ (octanol–water coefficient) values sorption on sediment or suspended particulate matter (SPM) could be a significant factor.

4. Conclusions

The analytical method presented allows the simultaneous extraction, identification and quantification of a range of compounds with known estrogenic activity in coastal surface waters. To the authors' knowledge, it is the first time that estrogenic compounds with the focus on natural and synthetic hormones have been detected in a coastal zone of the German Baltic Sea. E1, EE2 and the phenolic xenoestrogens BPA, NP and 4-*tert*-OP were found with concentrations of E1 and EE2 in the range of effect concentrations for aquatic organisms. Significant signal suppressions in electrospray ionisation occurred due to co-extracted matrix constituents and had to be controlled and corrected for by spiking experiments with aliquots of sample extracts. As a follow-up of this study, chemical analyses will be supplemented by the application of bioassays (Yeast-Screen, MCF-7-Bioassay) to assess estrogenic activities of the sample extracts.

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