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On-line two-dimensional liquid chromatography–tandem mass spectrometric determination of estrogens in sediments

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ABSTRACT

The development of an on-line system for the simultaneous determination of α-estradiol, β-estradiol, estrone and 17α-ethynylestradiol in river sediments is described. The analytes were extracted from sediments by microwave-assisted extraction. A crude extract was directly analysed by a heart-cutting two dimensional high-performance liquid chromatography–ion trap-tandem mass spectrometry with an atmospheric pressure photoionization source operating in the positive mode. The method shows excellent performance in terms of accuracy, precision, and sensitivity. The accuracy of each estrogen was in the range of 98.8–107.1%. Intra-batch and inter-batch precisions were in the range of 6.2–7.0% and 8.3–9.5%, respectively. The limits of detection ranged from 90 to 250 pg g^{-1}. A significant reduction in the total analysis time and a reduction in sample manipulation are the main advantages of the proposed method. Finally, the method was applied on real sediment samples.

1. Introduction

Sample preparation is a crucial step for establishing a selective and sensitive separation method for trace analysis in complex matrices. In recent years, there has been an increasing level of interest in the development and use of high-throughput and fully automated analytical methods for the efficient separation and analysis of environmental, biological and natural product samples. Among them, two-dimensional separation systems have become powerful tools in the separation of such complex samples. Various 2D-chromatographic configurations have been described in the literature [1–3] that allow full automation of 2D-separations with the absence of a manual sample transfer from the first separation dimension to the second.

Both natural and synthetic estrogens belong to the group of endocrine disrupting compounds (EDCs) known for their adverse effect on wildlife, and possibly even on humans, at trace concentration levels [4–6]. Consequently, methods employing high separation efficiency in conjunction with highly specific and sensitive detection are required.

On-line SPE connected to liquid chromatography has been primarily employed for the determination of estrogens in water [7–11] and sediment [12] samples. Salvador et al. analysed estrogens in sewage influent and effluent using an on-line SPE–LC system and, moreover, described the possibility of performing a derivatization step directly during SPE [13]. Mitani et al. used fully automated in-tube solid phase microextraction (SPME) and reported up to a 90-fold increase in the method's sensitivity when compared to the direct injection method [14]. Although on-line SPE is quite universal and capable of multiple analyte analysis, this technique mainly uses the first column as a trapping mechanism. On the other hand, heart-cutting 2D-chromatography effectively utilizes the selectivity and separation efficiency available from both chromatographic columns in order to improve separation. Nguyen et al. used a heart-cutting 2D-chromatography column set-up for the determination of four native estrogens in human cerebrospinal fluid in order to improve a poor separation of dansylated estrogens. Dansylated estriol and estrone were separated in the first dimension by an amide-C18 column while dansylated 17β-estradiol and 17α-ethynylestradiol were separated in the second dimension using two C18 columns connected in series [15].

Tandem mass spectrometry (MS/MS), mostly using electrospray ionization (ESI) operated in the negative mode, has become a dominant technique for the detection of estrogens due to its high sensitivity and specificity. However, ESI is not suitable for less polar estrogens with low proton affinity or with weak acid properties. Moreover, ESI suffers from ion suppression when complex matrices are analysed [16–18]. This issue was overcome with atmospheric pressure photoionization (APPI), which was introduced as an innovative ionization source for LC–MS systems in 2000 [19]. The fundamentals of photoionization have been described in detail in earlier reviews [20–22]. It is obvious from the literature data

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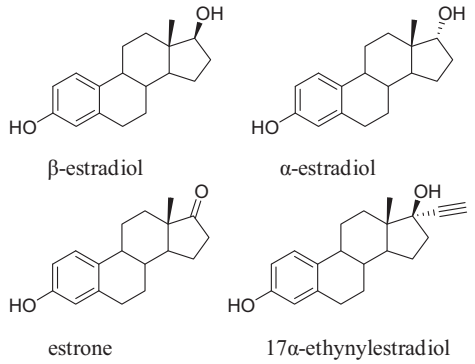

Fig. 1. The structures of selected estrogens.

that APPI provides superior performance in the ionization of neutral compounds and generates more reproducible signals. Furthermore, ionization is less susceptible to ion suppression caused by matrix effects or salt buffers when compared to ESI or atmospheric pressure chemical ionization (APCI) [23,24].

The aim of the work presented in this article was to develop a method for the determination of four priority estrogens – β-estradiol, α-estradiol, estrone, and 17α-ethynylestradiol (their structures are given in Fig. 1) in river sediment samples based on a heart-cutting on-line two-dimensional liquid chromatography–tandem mass spectrometry. The method was proposed to achieve these goals: (1) to minimize sample treatment employing an on-line system; (2) to separate estrogens from each other; (3) to apply atmospheric pressure photoionization; and (4) to find the best conditions for the use of an APPI source in terms of solvent composition, additive, and dopant. Furthermore, these optimized conditions were intended to be employed in chromatographic separation on the second column in order to provide high sensitivity for the determination of estrogens by 2D-LC–MS/MS.

Although ESI, APCI, and APPI sources were previously used for the analysis of estrogens [10,11,25–28], inconsistent data in terms of evaluating the performance of API sources were found. Therefore, the use of both positive and negative ion modes in ESI, APCI and APPI sources for the detection of estrogens was compared.

2. Experimental

2.1. Chemicals

Estrone (E1) (min. 99.5%), α-estradiol (α-E2) (min. 98.8%), β-estradiol (β-E2) (min. 98.9%), and 17α-ethynylestradiol (EE2) (min. 99.4%) were purchased from Riedel de Haën (Seelze, Germany). Internal standard [2,4,16,16-H_4]-β-estradiol (β-E2-d_4) (purity 95–97%) was obtained from Cambridge Isotope Laboratories (Andover, MA, USA). Each compound was dissolved in MeOH to make a stock solution at a concentration of 50 μg ml^{-1} and stored at 5 °C. Working solutions were prepared by mixing and diluting the stock solutions with methanol prior to use. Acetonitrile (ACN), methanol (MeOH) and 2-propanol (IPA) were obtained from Riedel-de Haën (LC/MS CHROMASOLV® grade, Seelze, Germany). Acetic acid, formic acid, ammonium formate, and ammonium hydroxide solution were purchased from Fluka Chemie (puriss p.a., eluent additive for LC–MS grade, Buchs, Switzerland). De-mineralized water was purified using a Milli-Q-RG (Millipore, Bedford, MA, USA).

2.2. Sample collection and preparation

River sediments sampling was performed at 10 sites (two samples per site, each collected sample was analysed in triplicate) along the Svratka river (Brno, Czech Republic) in the summer of 2010. All samples were pre-treated as described in detail earlier [29].

The sediments were extracted with 10 ml of MeOH:water (95:5, v/v) mixture for 10 min. A high pressure microwave extractor (Milestone, Sorisole, Italy) was used. After cooling, the extract was filtered stepwise through 0.7 μm glass-fibre (25 mm in diameter) and 0.2 μm PTFE (25 mm in diameter) syringe filters (Cronus, SMI-Labhut Ltd, Glouchester, UK) and evaporated to dryness using a rotary evaporator at 40 °C. The residue was then dissolved in 0.2 ml of water:MeOH (1:1, v/v) mixture and a 50 μl aliquot was injected into the chromatographic system.

2.3. 1D and 2D-high performance liquid chromatography

The on-line 2D-LC system consisted of an Agilent 1100 chromatographic system (Agilent, Waldbronn, Germany, equipped with a vacuum degasser, quaternary pump, autosampler and a column thermostat) and a Knauer HPLC 100 pump (Dr. Ing. Herbert Knauer GmbH Berlin, Germany, equipped with a vacuum degasser) and two columns connected via an electronically controlled six-port two-position switching valve (valve 1, part of the column thermostat). An additional six-port valve (valve 2, part of the mass spectrometer) was used to divert the flow of the mobile phase to the mass spectrometer only during the analytes' elution. A schematic of the column configuration and valve switching is shown in Fig. 2.

The development of the on-line 2D separation consisted of several steps. Firstly, a comparative study of five columns used in the first dimension (1D, column 1) – Luna Phenyl-hexyl 50 mm × 2.0 mm, 3 μm particle size; Luna C8 50 mm × 2 mm, 3 μm particle size; Luna C18 50 mm × 2 mm, 3 μm particle size; Gemini MercuryMS Phenyl-hexyl 10 mm × 2.0 mm, 3 μm particle size; and Gemini MercuryMS C18 10 mm × 2.0 mm, 5 μm particle size (all products of Phenomenex, Torrance, CA, USA) was performed. The mobile phase consisted of water or 0.1% (v/v) formic acid as solvent A and MeOH or ACN as solvent B. The chromatographic column, mobile phase, its flow rate, and mobile phase gradient profile were optimized in order to effectively separate estrogens from the matrix components in the first dimension.

The following columns – Pursuit Diphenyl 75 mm × 2.0 mm, 3 μm particle size (Varian, Palo Alto, CA, USA); Luna C18(2) 100 mm × 2.0 mm, 3 μm particle size; Gemini C6-Phenyl 100 mm × 2.0 mm, 3 μm particle size; Kinetex PFP, 100 mm × 2.1 mm, 2.6 μm particle size (Phenomenex, Torrance, CA, USA); XBridge Phenyl 100 mm × 2.1 mm, 3.5 μm particle size; XBridge Shield RP18, 150 mm × 2.1 mm, 3.5 μm particle size; XBridge C18, 100 mm × 2.1 mm, 3.5 μm particle size (Waters, Milford, MA, USA); Zorbax Eclipse Plus C18, 100 mm × 2.1 mm, 3.5 μm particle size; Zorbax SB-Phenyl 100 mm × 2.1 mm, 3.5 μm particle size (Agilent, Palo Alto, CA, USA) were employed in the second dimension (2D, column 2). A mobile phase consisting of 0.1% (v/v) formic acid and MeOH was used. The mobile phase gradient profile, mobile phase flow rate, and column temperature were optimized to achieve the estrogens' separation from each other and from matrix components in as short analysis time as achievable. In addition to the separation, necessary attention was paid to the preservation of the peaks' symmetry and the quantitative transfer of analytes from the first to the second dimension. These requirements were achieved by optimizing the mobile phase's gradient profile and flow rates in both dimensions and by optimizing the switching times of valve 1.

In the optimized procedure, a Luna C8(2) (50 mm × 2.0 mm, 3 μm particle size) was used as column 1 and an XBridge Shield

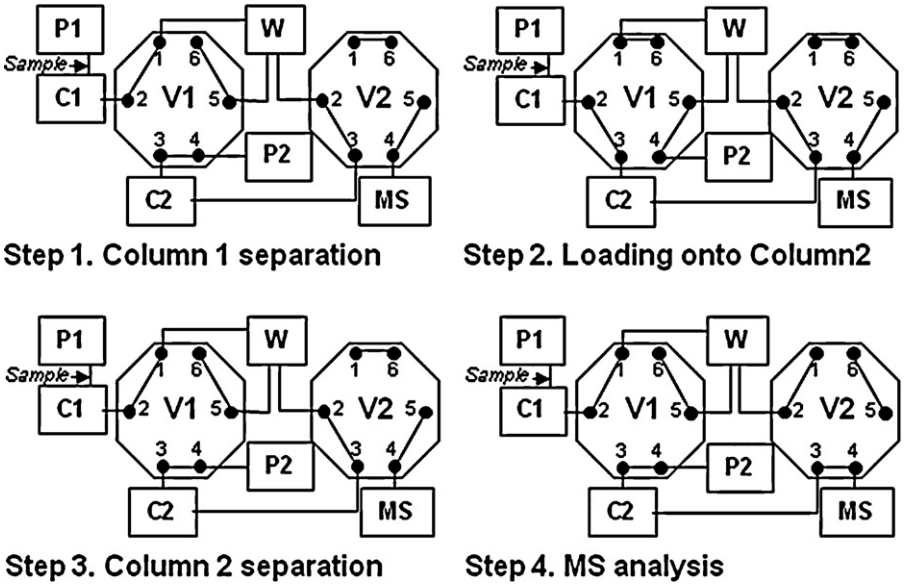

Fig. 2. The schematic diagram of the heart-cutting 2D-HPLC–MS/MS system. P1: pump 1, C1: column 1, V1: switching valve 1, P2: pump 2, C2: column 2, V2: switching valve 2, MS: mass spectrometer, W: waste.

RP18 (150 mm × 2.1 mm, 3.5 μm particle size) was used as column 2. The mobile phase consisted of 0.1% formic acid in water (solvent A) and MeOH (solvent B). The columns' oven temperature was maintained at 45 °C. The time schedule for column 1, column 2, valves switching, flow rates, and mobile phase gradient profiles is shown in Table 1.

2.4. Tandem mass spectrometry

A Finnigan LCQ Advantage MAX (Thermo Fisher Scientific, San Jose, CA, USA) ion-trap mass spectrometer, equipped with ESI, APCI, and an APPI interface was used. A KDS model 100 Series (KD Scientific, Holliston, MA, USA) syringe pump was used for dopant infusion via a T-piece into the ion source if required. Instrument control and data analysis were performed using XCalibur (version 2.0.7) software. The mass spectrometer optimization was performed in three separate steps: (1) determination of the best ionization source and polarity in conjunction with the mobile phase used; (2) optimization of the ion-source parameters (e.g. capillary and vaporizer temperature, gas flow rates, ion transport voltages, and in-source collision voltage); and (3) optimization of the compound-dependent parameters (e.g. collision energy). The effect of both mobile phase and API source (APCI, APPI, and ESI) on the ionization efficiency of each estrogen was investigated in both positive and negative modes by direct injection of 5 ng of each compound into a mobile phase. The mobile phase consisted of a modifier (water, 0.01–0.3% (v/v) formic acid or acetic acid, 0.01–0.3% (w/v) ammonium formate, and 0.005–0.1% (v/v) ammonium hydroxide) and an organic solvent (MeOH, ACN, or IPA) in the ratio of 1:1 (v/v). The flow rate was 0.25 ml min^{-1}. Dopant-assisted APPI was tried to improve efficiency in both positive and negative modes. Toluene, anisole, and acetone infusion into the source and its optimal flow rate were tested. The best conditions in terms of the highest intensity found for an individual API source were selected for further optimization of the ion-source and compound-dependent parameters by continuous infusion of 1 μg ml^{-1} of each compound into the mobile phase. The optimized MS conditions of the used API sources are given in Table 2. Quantitative analysis was performed by a full scan MS/MS mode, two specific product ions were monitored per compound. The compound-dependent parameters are summarized in Table 2.

2.5. Method validation

The method was validated by the following set of parameters: linearity, accuracy, precision, and sensitivity.

Calibration curves were built from triplicate analysis of the sediment extracts (previously probed not to contain the target analytes) spiked with the standard mixture of the analytes. Five concentrations (0.28, 0.5, 1, 5, and 10 ng g^{-1} for α-E2 or β-E2; 0.48, 0.7, 1, 5, and 10 ng g^{-1} for E1; and 0.84, 1, 1.6, 5, and 10 ng g^{-1} for EE2) were prepared for all analytes and each level contained 1 ng g^{-1} of internal standard. The calibration curves were constructed based on the peak area ratios of analytes and internal standard versus the analyte concentration. Un-weighted linear regression analysis was used.

The limit of detection (LOD) was estimated as at least 3 × standard deviation of the blank measurement (sb)/slope of cal-

Table 1
The chromatographic conditions and column switching program.

Column 1				Column 2		
Time (min)	*F* (ml min^{-1})	*B* (%) MeOH	Event	Time (min)	*B* (%) MeOH	Event
0–2	0.75	40	Majority matrix removing	0–5	100	Cleaning
2–3	0.20	60	Conditioning	5–10	60	Conditioning
3–10	0.20	60 → 100	1D-LC separation Valve 1 open at 8.25 min Valve 1 close at 9.45 min	10–20	60 → 100	2D-LC separation Valve 2 open at 14 min Valve 2 close at 18 min
10–1717–20	0.750.75	100 40	Cleaning Conditioning	Flow rate 0.3 ml min^{-1}		

Table 2
The optimized conditions of three API sources operated in the positive and negative mode used for the determination of estrogens. Values in brackets give parameters valid in the negative mode.

Parameter	β-E2, α-E2	E1	EE2
ESI mode			
Capillary temp. (°C)	350 (350)	350 (350)	350 (350)
Sheat/auxiliary gas flow rate (units)	38/21 (44/14)	44/18 (34/24)	28/41 (36/42)
Spray current (μA)	4.5 (3.2)	4.5 (3.2)	4.5 (3.2)
Capillary voltage (V)	8 (−18)	22 (−10)	30 (−14)
Tube lens offset (V)	12 (−24)	5 (−12)	24 (−12)
In-source CID (V)	8 (10)	8 (10)	8 (10)
APCI mode			
Vaporiz. temp. (°C)	450 (375)	450 (375)	450 (375)
Capillary temp. (°C)	250 (150)	250 (150)	250 (150)
Sheat/auxiliary gas flow rate (units)	36/45 (36/29)	57/54 (60/55)	38/29 (26/39)
Spray voltage (kV)	10 (10)	10 (10)	10 (10)
Capillary voltage (V)	6 (−16)	3 (−4)	24 (−21)
Tube lens offset (V)	15 (−25)	10 (−45)	15 (−60)
In-source CID (V)	6 (8)	6 (8)	6 (8)
APPI mode			
Vaporiz. temp. (°C)	400 (375)	400 (375)	400 (375)
Capillary temp. (°C)	200 (175)	200 (175)	200 (175)
Sheat/auxiliary gas flow rate (units)	35/46 (46/14)	33/13 (42/36)	46/27 (28/54)
Capillary voltage (V)	18 (−16)	30 (−10)	22 (−4)
Tube lens offset (V)	10 (−22)	25 (−24)	25 (−15)
In-source CID (V)	8 (10)	8 (10)	8 (10)
Parent ion (m/z)	255 (271)	271 (269)	279 (295)
Product ions (m/z)	133 + 159 (145 + 183)	157 + 197 (145 + 183)	133 + 159 (145 + 185)
Relative collision energy (%)	46 (50)	44 (48)	42 (51)

Q = 0.3; ion scan 1/200 s.

ibration curve (b). Similarly, the limit of quantification (LOQ) was estimated as at least 10 × standard deviation of the blank measurement/slope of calibration curve. The calculated LODs and LOQs of the method were experimentally checked by analysis of five sediment samples spiked on the concentration levels of LOD and LOQ.

Accuracy (or recovery) of the whole method was evaluated by analysing five replicate sediment samples spiked with a concentration of 1 ng g^{-1} of each standard. The recovery was determined by comparison of the mean result for five analyses to the nominal concentration value. The recovery ranging from 90 to 110% was acceptable.

The precision was evaluated at the concentrations of LOQ (0.8, 0.48, and 0.84 for E2, E1, and EE2, respectively), 2, and 10 ng g^{-1}. The intra-batch precision was evaluated, based on analysing five sediment samples spiked on the same day. Inter-batch precision was assessed by measuring five replicates per concentration level on three consecutive days. The intra-batch and inter-batch precisions were expressed as relative standard deviations (RSDs). A precision as RSD less than 15% was acceptable except for the LOQ, where it should not exceed 20% [30].

The transfer efficiency of each analyte from the first to the second dimension was measured by the analysis of three standard solutions at a concentration of 20 pg μl^{-1}.

3. Results and discussion

3.1. APPI-mass spectrometry

All analysed estrogens were detected in the negative mode in the form of their de-protonated $[M-H]^-$ ions. In positive mode, the protonated molecule $[M+H]^+$ was the most abundant ion in the case of E1 while the $[M+H-H_2O]^+$ fragment ion was the most abundant in the cases of β-E2, α-E2 and EE2. In-source fragmentation of EE2 led to the occurrence of two further $[M+H-C_2H_2]^+$ and $[M+H-C_2H_2-H_2O]^+$ ions, but the most abundant dehydrated ion was selected as the parent ion. The APPI-MS and MS/MS product spectra acquired in the positive mode as well as proposed structures of significant product ions are given in Fig. 3.

The influences of the mobile phase composition and dopant on the ionization efficiency of analytes under reverse-phase HPLC condition were studied. In negative mode, the highest intensities of the analytes were achieved when 0.1% (v/v) of ammonium hydroxide in 2-propanol was used. A mobile phase consisting of 0.1% (v/v) formic acid in methanol showed the highest abundances in the positive mode. The influence of mobile phase composition on the ionization efficiency of the APPI ion source operated in both positive and negative modes is shown in Table 3. A significant decrease in signal intensity was observed when ammonium formate or ammonium hydroxides were used in the positive mode. Formic acid, acetic acid or ammonium formate as a mobile phase modifier, negatively affected the ionization efficiency in the negative mode. The optimization of MS detection in respect to the separation step was performed as well. Propanol provided the best results in MS operated in the negative mode, but its disadvantage is a high column backpressure.

A dopant assisted-APPI was chosen to improve the ionization efficiency of APPI ionization source. Toluene, anisole, and acetone were infused into the source and their optimum flow rates were tested to provide a higher ionization efficiency of the analytes. In positive mode, the highest ionization efficiency of all analytes was achieved by an infusion of toluene at a flow rate of 0.030 ml min^{-1}. In negative mode, this goal was achieved by an infusion of toluene at a flow rate of 0.035 ml min^{-1}. Neither toluene nor acetone affected both positive and negative full-scan MS spectra of estrogens. On the contrary, anisole, when used as a dopant in the positive mode-operated MS [31], strongly affected the mechanism of ion formation when charge exchange reactions led to the occurrence of the major cation radical $[M]^{+\bullet}$ ion in the spectra of all estrogens.

3.2. Comparison of three API interfaces

The performance of three atmospheric pressure ionization (ESI, APCI, and APPI) sources operated in both positive and negative mode for the determination of four estrogens was evaluated. ESI and APCI sources operated in both positive and negative mode gave the best ionization efficiencies when the same mobile phases as for

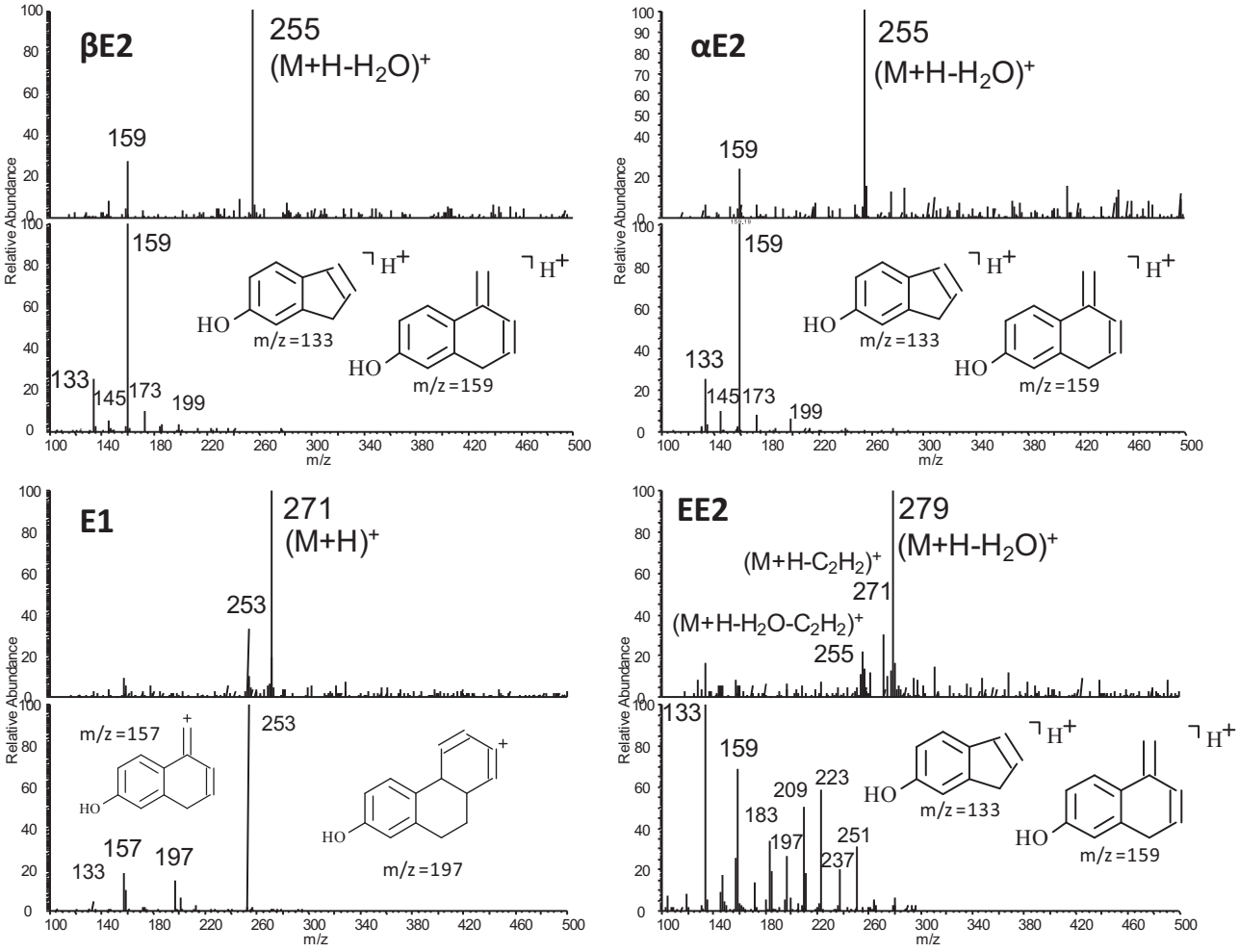

Fig. 3. The APPI-MS and MS/MS spectra of estrogens acquired in the positive mode.

APPI were used. The instrument quantification limits (IQLs) of the three studied ion sources are given in Table 4. Inferior ionization efficiencies were found when the ESI operated in the positive mode was used. Dopant-assisted APPI operated in the positive mode gave the best results not only in terms of sensitivity but the optimized mobile phase composition was finally applicable also for the chromatographic separation.

Table 3
The influence of the mobile phase composition on the ionization efficiency of estrogens using atmospheric pressure photoionization (APPI) source operated in positive and negative mode. The ionization efficiencies were normalized using the highest value as 100%.

	ACN		MeOH		IPA	
	0.1% HCOOH	H_2O (%)	0.1% HCOOH	H_2O (%)	0.1% HCOOH	H_2O (%)
APPI (+)						
β-E2	21	18	100	78	30	15
α-E2	23	15	100	76	34	17
E1	24	14	100	75	28	16
EE2	23	17	100	70	38	16
	ACN		MeOH		IPA	
	0.1% NH_3	H_2O (%)	0.1% NH_3	H_2O (%)	0.1% NH_3	H_2O (%)
APPI (−)						
β-E2	26	21	67	58	100	83
α-E2	25	19	65	54	100	81
E1	31	24	64	52	100	84
EE2	31	20	55	48	100	85

Table 4
The instrument quantification limits (IQLs)[a] for all studied atmospheric pressure ion sources.

	ESI (−)[b] (pg)	APCI (+)[c] (pg)	APCI (−)[b] (pg)	APPI (+)[b,d] (pg)	APPI (−)[c,d] (pg)
β-E2	200	50	200	20	200
α-E2	200	50	200	20	200
E1	200	100	200	50	100
EE2	300	200	300	80	500

[a] IQLs as 10 S/N.
[b] Mobile phase in negative mode: 0.1% (v/v) ammonium hydroxide: IPA (1:1, v/v), flow rate 0.3 ml min^{-1}.
[c] Mobile phase in positive mode: 0.1% (v/v) formic acid: MeOH (1:1, v/v), flow rate 0.3 ml min^{-1}.
[d] Toluene was used as dopant in case of APPI source.

3.3. Optimizing 2D-HPLC conditions

A simple 2D-LC system was designed to consist of two six-port two-position switching valves and two independently operated chromatographic columns. Co-elution of the matrix components with analytes as well as column overload may result in difficulties in qualitative and quantitative characterization of complex samples. Therefore, selecting optimal 2D-LC conditions are critical for a successful analysis.

3.3.1. 1D-chromatography

Lower capacity and efficiency in the separation of sediment constituents were the main drawback for both of the Gemini

MercuryMS columns and thus they received no further consideration.

The same elution order (β-E2 → α-E2 → EE2 → E1) and similar retention factors were observed for the tested 1D columns when acetonitrile as a mobile phase was used. The selectivity was slightly different which mainly affected the separation of E1 and EE2. When methanol as a mobile phase was used and the separation was carried out using C8 or C18 columns, a completely different elution order (E1 → β-E2 → EE2 → α-E2) was observed. Methanol as a mobile phase was much more efficient in the separation of the matrix components from analytes than acetonitrile regardless of the column used as was demonstrated by the separation of EE2 (Fig. 4).

The flow rate of the mobile phase was optimized to achieve as good a separation of matrix components from target analytes as possible. It was found that a high amount of matrix components was removed when a flow rate of the mobile phase was increased from 0.20 to 0.75 ml min^{-1} at the beginning of analysis (the upper value of flow rate was limited by column backpressure). On the other hand, the higher flow rate was not compatible with the separation in the second dimension. Therefore, a flow rate gradient was employed in the first dimension.

Better separation of analytes from the matrix components in 1D-chromatography results in a lower matrix load onto the 2D column and thus avoids potential column overload. Similarly, a narrow heart-cutting time window results in a lower introduction of the matrix onto the second column. Column overload can affect the stability of retention time of analytes that is crucial for the quantitative transfer of analytes between both columns. The stability of retention times was determined by analysis of 20 spiked sediment extracts. The RSDs were found to be in the range of 1.1–1.3% and the time-window was adjusted according to this finding. No problem with loss of analytes during transfer step was observed. Furthermore, a high matrix load can affect the lifetime of a column resulting in deteriorated resolution and/or discrepancies in retention times. Nevertheless, none of these issues were encountered in our case. The lifetime was estimated to be at least 200 injections.

3.3.2. 2D-chromatography

The object of the 2D-chromatography was not only the removal of rest matrix components from analytes, but also the separation analytes from each other due to a qualitative and a quantitative analysis. The chromatographic separation was demanded because the same parent and product ions are involved in MS detection in the case of α-E2 and β-E2. The pair of E1/EE2 had to be also resolved because a fragment ion of EE2 has the same $m/z = 271$ as a parent ion of E1.

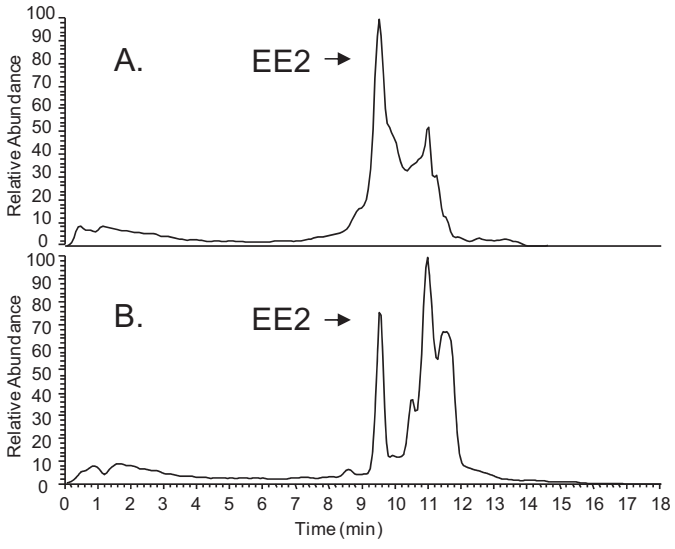

Fig. 4. The 1D-chromatograms-TIC–MS/MS of sediment sample enriched with 17α-ethynylestradiol on level of 10 ng g^{-1}. Data was obtained using ACN (A) or MeOH (B) as mobile phase. Column was Luna C8(2) 50 mm × 2 mm, 3 μm particle size.

From the two suitable systems found, the first one employed Luna C8 50 mm × 2 mm, 3 μm particle size as column 1 (mobile phase consisted of 0.1% (v/v) formic acid and ACN) and Zorbax SB-Phenyl 100 mm × 2.1 mm, 3.5 μm particle size as column 2 (mobile phase consisted of 0.1% (v/v) formic acid and MeOH). The second one used Luna C8 50 mm × 2 mm, 3 μm particle size as column 1 (mobile phase was 0.1% (v/v) formic acid and MeOH) and XBridge Shield RP18, 150 mm × 2.1 mm, 3.5 μm particle size as column 2 (mobile phase was 0.1% (v/v) formic acid and MeOH). The latter was superior in terms of qualitative and quantitative analyses due to a higher efficiency in the separation of matrix components from analytes (data not shown).

Both qualitative and quantitative mass spectrometric analyses in full-scan mode of an analyte in a complex matrix present an analytical challenge emphasized even more by the fact that a low-resolution mass spectrometer is employed. A large amount of matrix components may result in the suppression of analytical signal and poor data quality. Fig. 5 shows mass spectra obtained in analysis of an estrogens-enriched sediment by 1D-LC and 2D-LC methods acquired in full-scan and MS/MS mode. Through inspection of these spectra, it is evident that there is a significant improvement in the mass spectra obtained with the 2D-LC method. The reconstructed ion 1D-LC and 2D-LC chromatograms acquired in selected ion monitoring (SIM) or MS/MS mode are shown in Fig. 6. The quantitative data demonstrated that signal suppression/enhancement was decreased by the use of the 2D-LC–MS/MS method. The achieved quantitative improvement in signal suppression (+)/enhancement (−) was about +15%, +14%, +30%, and −15% for β-E2, α-E2, EE2, and E1, respectively. A substantial improvement in precision was also achieved. Recoveries of estrogens obtained by 1D-LC and 2D-LC analyses of three estrogen-enriched sediment samples are shown in Table 5. The quantitative analysis of estrogens using 1D-LC–SIM-MS is not possible. The accurate peak integration is extremely difficult due to the high load of matrix components and a poor selectivity of the detection. As a result, precision higher than 15% was observed. A substantial improvement in the precision of analysis was achieved using 2D-chromatography (data acquired in SIM mode). Nevertheless, recoveries were still out of range except for EE2. The benefits of 2D-chromatography were clearly demonstrated, even though a tandem mass spectrometry was used. Suitable choice of parent to product ion transitions in MS/MS should have led to substantial data improvement. Nevertheless, 1D-HPLC–MS/MS analyses were still not sufficient in terms of recovery. The influence of matrix components was still evident. Only the coupling of an effective 2D-chromatography and a selective tandem mass spectrometry resulted in a rapid progress in data quality.

3.4. Method validation

The method validation was determined with sediment samples enriched to the required concentration level with a standard solution of each estrogen because corresponding reference materials were not available at the time. All used sediment samples were previously examined not to contain target analytes. Parameters of validation (recovery, linear range and correlation coefficients, precisions, LODs, and LOQs) are given in Table 6.

The accuracy in the range 90–110% and a precision lower than 15% were adopted for the method validation. The precision on LOQ level lower than 20% was acceptable. Agreement of HPLC retention

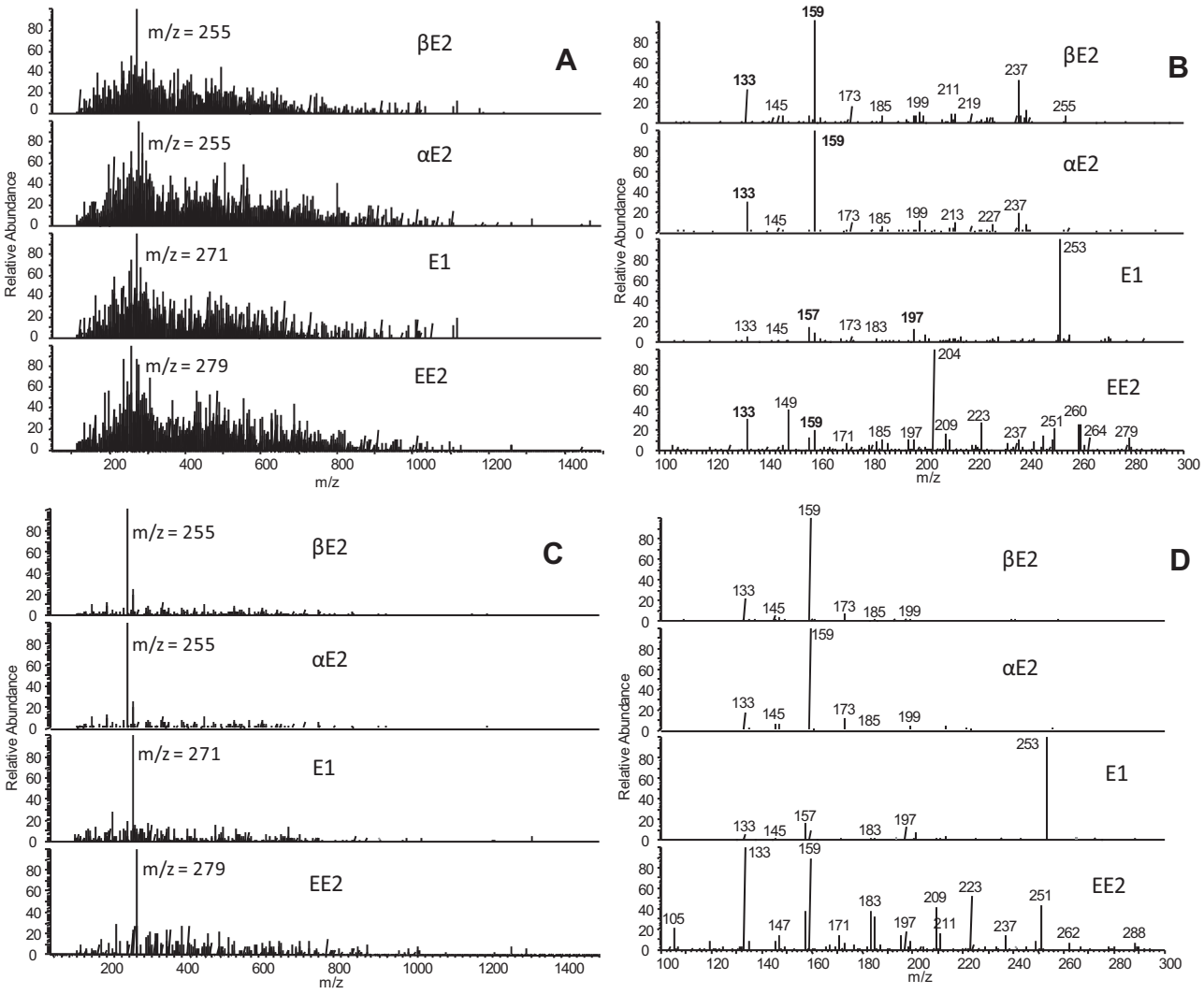

Fig. 5. The full MS and MS/MS spectra obtained using 1D-LC–MS and 2D-LC–MS. Sediment sample was spiked on level of 1 ng g^{-1}. (A) 1D-LC–MS; (B) 1D-LC–MS/MS; (C) 2D-LC–MS; (D) 2D-LC–MS/MS. β-E2: β-estradiol, α-E2: α-estradiol, E1: estrone, EE2: 17α-ethynylestradiol.

times within 1.5% and relative abundance of the two product ions within 20% were the criteria used to identify the analytes.

Calibration curves of each analytes showed excellent linearity over the entire range with correlation coefficients higher than 0.99.

The recovery of only transfer step was measured by triplicate analysis of a standard solution of each estrogen. The recovery was 96.5% for β-E2, 97.3% for α-E2, 99.5% for E1, 98.7% for EE2, and 96.2% for β-estadiol-d_4 with RSDs 4.9%, 4.5%, 4.6%, 4.7%, and 4.8%, respectively.

The use of deuterated β-estradiol covered the assessment of the recoveries of the extraction process, transfer step, estimation of ion suppression/enhancement, losses during sample manipulation, etc. The recovery of the whole analytical method based on measuring five times a standard solution of β-estadiol-d_4 was 94.3% with RSD 6.6%. The recovery of the whole analytical method based on measuring five different sediment samples enriched with β-estadiol-d_4 (1 ng g^{-1}) was 92.8% with RSD 7.1%. Deuterated β-estradiol was used as the internal standard for all target estrogens because no other isotope-labelled estrogens were available at the time.

The accuracy of each estrogen was in the range of 98.8–107.1%. Precision was evaluated at concentration levels distributed over the linear range. Intra- and inter-batch precisions for all compounds, expressed as RSDs, were 6.2–7.0% and 8.3–9.5%, respectively.

Estimated LODs ranged from 90 to 250 pg g^{-1} and LOQs were in the range 280–840 pg g^{-1}. Higher LOD and LOQ found for EE2 were mainly the result of the behaviour of the analyte during the ionization and fragmentation process. When estimated LODs were experimentally checked, the signal-to-noise ratio (S/N) was 7:1, 8:1, 7:1, and 5:1 for β-E2, α-E2, E1, and EE2, respectively. Similarly, S/N on LOQ concentration levels were 26:1, 24:1, 26:1, and 25:1 for β-E2, α-E2, E1, and EE2, respectively. The criteria were 3:1 for LOD and 10:1 for LOQ.

3.5. Analysis of real sediment samples

The optimized method was applied to the analysis of four estrogens in sediments. Twenty samples collected from 10 different locations (2 samples per location) along the Svratka river were analysed, but no occurrence of estrogens was observed for these samples. The same samples were also analysed according to our earlier approach [29] and the same negative results were also obtained. In comparison to the present negative results, E1 (1.01–2.37 ng g^{-1}), β-E2 (1.15–1.84 ng g^{-1}), α-E2 (1.35 ng g^{-1}), and EE2 (1.63 ng g^{-1}) were found in Svratka sediments sampled and analysed in autumn 2006. A microwave-assisted extraction followed by off-line SPE and HPLC–ESI(-)MS/MS analysis was employed for the determination of estrogens in this case [29].

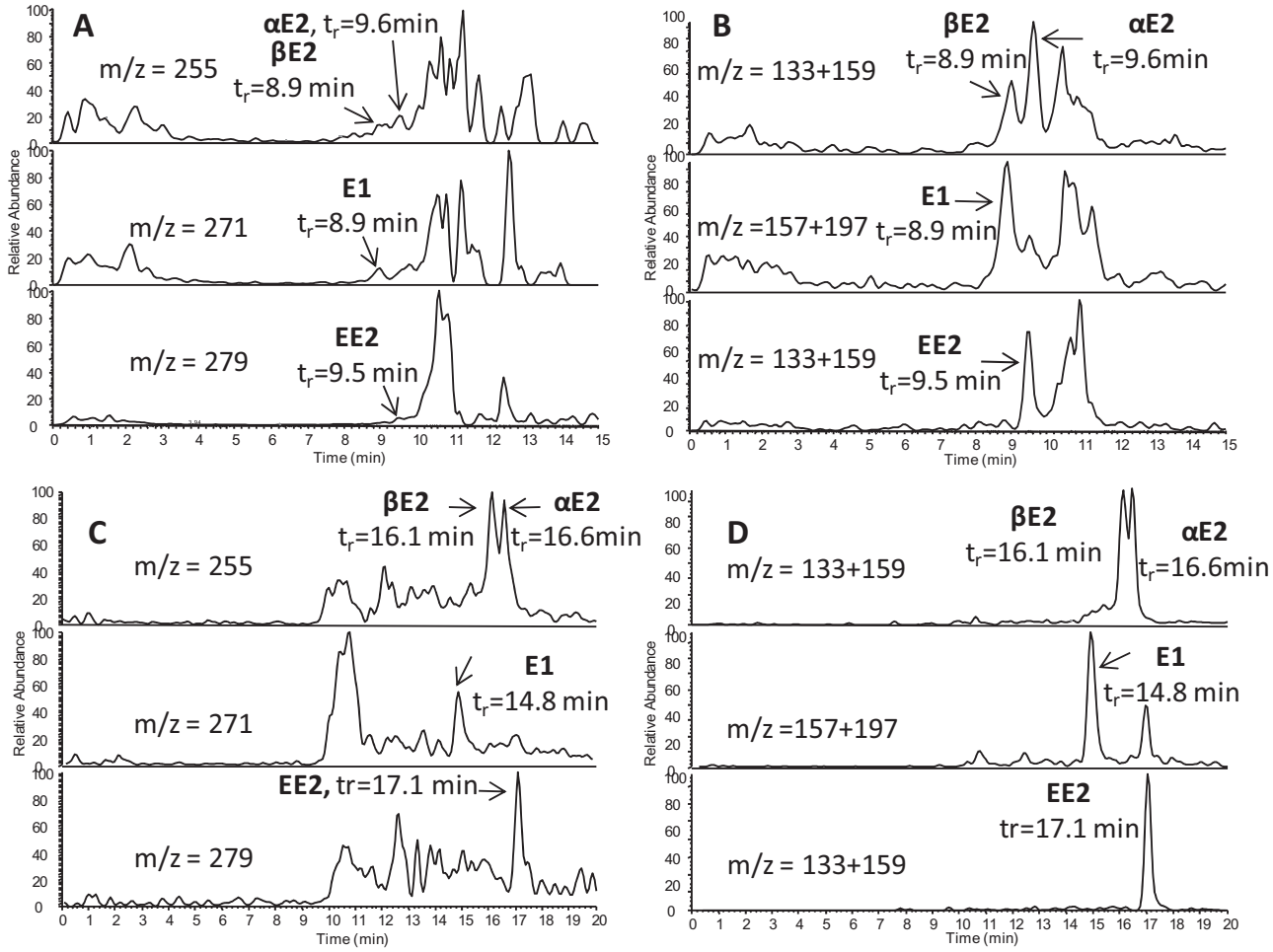

Fig. 6. The 1D-LC and 2D-LC chromatograms acquired in SIM-MS and MS/MS modes. The sediment sample was spiked on level of 1 ng g^{-1}. (A) 1D-LC–MS; (B) 1D-LC–MS/MS; (C) 2D-LC–MS; (D) 2D-LC–MS/MS. β-E2: β-estradiol, α-E2: α-estradiol, E1: estrone, EE2: 17α-ethynylestradiol.

Table 5
The comparison of absolute recovery (%) obtained with LC–MS and LC–MS/MS analysing spiked sediment samples on level of 1 ng g^{-1} (data were obtained during the method development). Signal response is expressed as a percentage of that obtained from a standard solution. Values in brackets give the relative standard deviation (n = 3).

	Recovery of 1D-LC (%)		Recovery of 2D-LC (%)	
	SIM-MS	MS/MS	SIM-MS	MS/MS
β-E2	n.q.[a]	91.0 (10.8)	113.5 (9.8)	105.7 (7.3)
α-E2	n.q.	87.1 (10.8)	114.7 (10.5)	102.1 (6.6)
E1	n.q.	69.5 (10.9)	114.1 (9.7)	99.2 (6.9)
EE2	n.q.	121.7 (12.3)	109.4 (8.9)	106.5 (6.8)

[a] n.q. = not quantifiable, recovery out of range of 90–110% and RSD higher than 15%.

4. Conclusion

The heart-cutting 2D-LC–MS/MS system for the simultaneous determination of four estrogens in river sediment samples was developed and validated. This method has been based on the microwave-assisted solvent extraction followed by two-dimensional liquid chromatography with tandem mass spectrometric detection. The recoveries for each compound ranged from 98.8 to 107.1% and the intra- and inter-batch precisions represented as RSDs ranged from 6.2 to 7.0% and 8.3 to 9.5%, respectively. The LODs were in the range of 90–250 pg g^{-1}. The 2D-LC chromatograms clearly demonstrated a significant decrease in matrix components content compared to the 1D-LC analysis. A significant

Table 6
The analytical performance of proposed method.

	t_r (min)	Recovery[a] (%)	Linear range (ng·g^{-1})	r^2	LOD[b] (ng g^{-1})	LOQ[c] (ng g^{-1})	Intra-batch precision (%)[d]			Inter-batch precision (%)[e]		
							LOQ[f]	2 (ng g^{-1})	10 (ng g^{-1})	LOQ[f]	2 (ng g^{-1})	10 (ng g^{-1})
β-E2	16.1	106.4 (7.1)	0.16–10	0.9925	90	280	7.0	6.9	6.6	9.2	9.1	8.7
α-E2	16.6	103.3 (6.8)	0.16–10	0.9974	90	280	6.5	6.5	6.3	9.5	9.3	8.6
E1	14.8	98.8 (7.0)	0.4–10	0.9958	180	480	6.3	6.2	6.2	8.5	8.3	8.4
EE2	17.1	107.1 (6.7)	0.64–10	0.9919	250	840	6.7	6.7	6.5	9.0	9.1	8.7

[a] Recovery of whole analytical procedure, RSDs are given in parentheses (n = 5).
[b] Limit of detection as 3 × standard deviation of blank measurement/slope of calibration curve.
[c] Limit of quantification as 10 × standard deviation of blank measurement/slope of calibration curve.
[d] n = 5.
[e] n = 15.
[f] LOQ for β-E2 and α-E2 was 0.28 ng g^{-1}; LOQ for E1 was 0.48 ng g^{-1}; LOQ for EE2 was 0.84 ng g^{-1}.

reduction in total analysis time and reduction in sample manipulation represent the main advantages of this method.

A relatively less sensitive 3D-ion trap mass spectrometer was employed for the detection of estrogens. If a triple quadrupole or other similar mass spectrometer systems were used, lower detection limits would likely to be achieved. Consequently, smaller portion of sample would be needed, resulting in a lower load of matrix components into the 2D-chromatographic system.

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