Column-switching system with restricted access pre-column packing for an integrated sample cleanup and liquid chromatographic–mass spectrometric analysis of alkylphenolic compounds and steroid sex hormones in sediment

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Abstract

A novel methodology based on column switching LC–MS, using restricted access material (RAM), for an integrated sample clean-up and analysis of endocrine disrupting compounds in sediment samples is described. The use of RAM precolumns, that combines size exclusion and reversed-phase retention mechanisms, enables fast on-line clean-up of sediment extracts and sensitive determination of alkylphenolic compounds, bisphenol A and steroid sex hormones at low ppb level (LODs=0.5–5 ng/g). Different LiChrospher ADS RAM precolumns (Merck, Germany) with C_4, C_8 and C_{18}, respectively, modification of inner pore surface were tested. ADS C_4 precolumns gave the best results in terms of recovery, selectivity and sensitivity, eliminating efficiently matrix components and consequently reducing ion suppression effects. Except for the most polar compounds, all compounds exhibit complete recovery with RSD from 0.87 to 15%. A complete analysis, including efficient pressurized liquid extraction (PLE) of target compounds, on-line clean-up, chromatographic separation and MS detection takes approximately 2 h, which is a significant improvement in comparison to the methods reported previously.
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1. Introduction

Due to the low detection limits required and complexity of matrices, quantitative analysis of endocrine disruptors (EDs) in solid environmental samples (sediment, soil and sludge) is cumbersome and a challenging task [1,2]. Extraction and clean-up protocols used are generally time and labor consuming, and they often constitute the bottleneck of the analytical method. Furthermore, the growing number of samples to be analyzed in laboratories carrying out monitoring studies requires employment of high-

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throughput and fully automated analytical techniques. Recent developments of sample handling techniques are directed, from one side, toward automatization and on-line coupling of sample preparation units and detection systems and from another toward development of more selective sorbents, such as immunosorbents, molecular imprinted polymers and restricted access materials (RAM).

RAMs are bifunctional sorbents tailored for the fractionation (clean-up) of samples into macromolecular matrix components and low molecular target analytes. Term "restricted access" expresses the limited accessibility of macromolecular matrix components to adsorption sites of well-defined porous supports [3]. Size-exclusion separation of unwanted matrix components is a result of topographical restriction achieved either by a physical diffusion barrier (small pore diameter) or by a chemical diffusion barrier (chemically bonded phase). Low-molecular-weight analytes, able to access active adsorption centers (alkyl chains or ion-exchange groups) at the inner pore surface, are retained by a reversed-phase, affinity or ion pair mechanism, respectively [4,5].

RAMs have been successfully applied for direct extraction and enrichment of hydrophobic low molecular mass analytes from biological fluids carrying a high load of proteins (plasma, blood, urine, saliva, supernatants of cell cultures and tissue) and from food samples (milk, food homogenates) [6,7]. However, in environmental analysis RAMs were seldom applied. The tailor-made RAMs have been successfully applied for the separation of humic substances interfering in the analysis of triazines [8], acidic herbicides [9] and polar fungicides [10] in river and groundwater and for high-throughput flow immunoassay of triazines [11].

In this paper, we describe a new analytical method for the determination of EDs in sediments, based on dual column LC–MS, using restricted access precolumn packing. To the best of our knowledge, this is the first application of RAM precolumns in the analysis of EDs in environmental samples.

This study focuses on the determination of two groups of EDs. The first included phenolic compounds: bisphenol A (BPA), nonyl- and octylphenol ethoxylates (NPEOs and OPEOs), nonyl- and octylphenoxy carboxylates (NPECs and OPECs) and

nonyl- and octylphenol (NP and OP) and corresponding halogenated (chlorinated and brominated) derivatives. The second group included the natural estrogen estradiol and its main metabolites, estriol and estrone, the synthetic estrogens ethynylestradiol and diethylstilbestrol, the natural hormone progesterone and the synthetic progestogens norethindrone and levonorgestrel.

The specific objective of this work was to optimize the operational parameters of RAM–LC–MS system, which included the selection of RAM precolumns (with C_4, C_8 or C_{18} modification, respectively), and the switching times as defined by the matrix elution profile, breakthrough time of analytes and analyte elution profile.

2. Experimental

2.1. Materials and standards

All solvents (water, acetonitrile, methanol and dichloromethane) were HPLC grade and were purchased from Merck (Darmstadt, Germany). Analytical grade formic acid and sodium acetate were from Panreac (Barcelona, Spain).

Pure standards of natural and synthetic estrogens and progestogens were purchased as powders from Sigma (St. Louis, MO, USA). High purity standard of BPA (>99%) was supplied by Aldrich (Milwaukee, WI, USA).

The APEO standards used in this study were laboratory-synthesized nonylphenol monoethoxylate (NP_1EO), nonylphenol diethoxylate (NP_2EO), octylphenol monoethoxylate (OP_1EO) and octylphenol diethoxylate (OP_2EO) [12]. Additionally, technical mixtures of polyethoxylated surfactants alkylphenol polyethoxylates Findet 9Q/22 and Findet S8Q/21, respectively, corresponding to $NP_{10}EO$ and OP_9EO, where *n* is an average number of ethoxy groups, were from Kao (Barcelona, Spain).

Alkylphenoxy carboxylates (NP_1EC and OP_1EC) and alkylphenoxy ethoxy carboxylates (NP_2EC and OP_2EC) were synthesized according to the method described by Marcomini et al. [13]. High purity (98%) 4-heptylphenol (which was used as the internal standard), 4-*tert.*-octylphenol (OP) and technical

grade 4-nonylphenol (NP) were obtained from Aldrich (Milwaukee, WI, USA).

BrNP was synthesized using elemental bromine according to the method described by Reinhard et al. [14]. ClNP was prepared by chlorination of nonylphenol using sulfuryl chloride according to the method of Stokker et al. [15] $BrNP_1EC$ and $ClNP_1EC$ were synthesized by reacting brominated and chlorinated nonylphenol, respectively, with chloroacetic acid in the presence of sodium hydride and dimethylformamide as a solvent. These two synthesized compounds rendered $BrNP_1EO$ and $ClNP_1EO$ by reduction with lithium aluminum hydride in ether solution. $BrNP_2EO$ and $ClNP_2EO$ were synthesized by reacting BrNP and ClNP, respectively, with 2-(2-chloroethoxy)ethanol in the presence of NaOH in water. Finally, $BrNP_2EC$ and $ClNP_2EC$ were obtained from $BrNP_2EO$ and $ClNP_2EO$, respectively, by oxidation with Jones reagent [14].

2.2. Sediment collection

The sediment used in all spiking experiments was a grab sample of river sediment from the Cardener River (Catalonia, NE Spain), collected upstream of known sources of pollution. Sample was wrapped into aluminum foil, stored at 4 °C, transferred to the laboratory, then frozen at −20 °C and finally lyophilized. The lyophilized samples were ground and homogenized using a mortar and pestle and then sieved through a 125-μm sieve.

Freeze-dried sediment was spiked with 100 ng/g of the composite standard solution of alkylphenolic compounds and steroid sex hormones 72 h before analysis, and analyzed by applying the method described below, together with a blank sample.

2.3. Pressurized liquid extraction

Extractions were carried out using a Dionex ASE 200 (Dionex, Idstein, Germany). The extraction cell (11 ml) was half-filled with Na_2SO_4 (pre-washed with methanol and baked at 400 °C). Five grams of sediment were then placed into the extraction cell and carefully mixed with Na_2SO_4. Additionally, any void space was filled with Na_2SO_4. Extraction conditions were as follows: mixture of acetone–methanol (1:1, v/v) was used as extraction solvent,

temperature of 50 °C, pressure 1500 p.s.i., the heating time 5 min, two cycles of static extraction, the time of each static cycle was 5 min. As a final step, the cell was purged with gaseous nitrogen. The total volume of extract was ~20 ml. The extracts were concentrated to an approximate volume of 0.5 ml using a rotary vacuum evaporator at 30 °C and filtrated through 1-μm PTFE filter units and finally reconstituted with methanol to a final volume of 1 ml.

In order to compare the performances of the RAM–LC method and the conventional SPE cleanup, extracts obtained by PLE, were concentrated to an approximate volume of 1 ml using a rotary vacuum, redissolved in 100 ml of HPLC water and subsequently purified by SPE using LiChrolut C_{18} cartridges (Merck, Darmstadt, Germany), as described elsewhere [12].

2.4. Equipment and instrumental set-up

The modular LC–LC system consisted of two gradient pumps connected to an OSP-2A solid-phase extraction unit (Merck, Darmstadt, Germany) with an automatic six-port switching valve. The first HPLC system consisted of an HP 1100 autosampler having a 100-μl loop and an HP 1090 A LC binary pump, both from Hewlett-Packard (Palo Alto, CA, USA). The second HPLC pump was a Merck LC 6200A. Instrumental set-up and the sequence of different working steps (sample injection, fractionation, analyte transfer and analytical separation) were as described elsewhere [6,16].

In coupled column analysis, a 25×4 mm I.D. column packed with 25 μm LiChrospher ADS (alkyl diol silica), from Merck, was used as a precolumn and a 5-μm, 250×4 mm I.D. C_{18} reversed-phase column (LiChrospher 100 RP-18), proceeded by a guard column (4×4 mm, 5-μm) of the same packing material, both from Merck, was used as an analytical column.

2.5. LC–LC conditions

The time schedule for the extract clean up and analyte transfer onto the LC column is shown in Table 1. The injection volume was set at 25 μl and the flow-rate through both columns was 1 ml/min.

Table 1
Column switching program

Pump 1 (Merck 6200A)			Pump 2 (HP 1090)		
Time (min)	Solvent	Event	Time (min)	Solvent	Event
0–4.9	H_2O–ACN (70:30, v/v)	Analytical column conditioning	0–5	H_2O–ACN (95:5, v/v)	Sample loading into the RAM precolumn
5–8.9	H_2O–ACN (70:30, v/v) (NI)	Analyte transfer to the analytical column	5–10	H_2O+0.5% HCOOH	RAM precolumn cleaning
	H_2O–methanol (70:30, v/v) (PI)		10–40	ACN	
9–45	Gradient elution	LC separation	40–45	H_2O–ACN (95:5, v/v)	RAM precolumn conditioning

ACN, acetonitrile; PI, positive ionization; NI, negative ionization.

PLE extract was directly injected into the chromatographic system and introduced into RAM pre-column by a stream of mobile phase H_2O–acetonitrile (95:5, v/v). After a clean-up time of 5 min, the six-port switching valve was activated and the analyte was transferred, in back-flush mode, from RAM pre-column to the analytical column using a mobile phase H_2O–acetonitrile (70:30, v/v) (initial conditions for analytical separation). When the transfer was finished, the switching valve was activated and the analytes were separated in a conventional manner. Simultaneously, RAM pre-column was rinsed and conditioned. All steps were performed automatically.

Chromatographic separation of compounds detected under NI conditions was performed under gradient elution condition using acetonitrile (A) and water (B). Gradient was held linear at 30% A for 1 min and then linearly increased to 60% A in 6 min, increased to 75% A in 5 min, increased to 80% A in 7 min and kept isocratic for 10 min. Compounds detected under PI conditions were separated using solvent system containing methanol–water and the same gradient program.

2.6. Mass spectrometric detection

Detection was carried out using an HP 1040 M diode array UV–Vis detector coupled in series with an LC–MSD HP 1100 mass selective detector, equipped with an atmospheric-pressure ionization (API) source and electrospray (ESI) interface. The operating parameters were as follows (PI/NI): drying gas flow 12/11 l/min; drying gas temperature 375/325 °C; nebulizer pressure 55/60 p.s.i.; capillary voltage 3500/5000 V and fragmentation voltage 60/100 V.

Diagnostic ions used for the analysis of APEOs, halogenated APEOs and progestogens in PI mode were those corresponding to $[M+Na]^+$. Ions monitored were m/z 321 (norethindrone), m/z 335 (levenorgestrel), m/z 337 (progesterone), m/z 287 (NP_1EO), m/z 331 (NP_2EO), m/z 273 (OP_1EO), m/z 317 (OP_2EO), m/z 321/323 ($ClNP_1EO$), m/z 365/367 ($ClNP_2EO$ and $BrNP_1EO$, respectively) and m/z 409/411 ($BrNP_2EO$).

Estrogens, BPA, APs and halogenated NPs were detected under NI conditions as $[M–H]^-$, while for AP_1ECs, AP_2ECs and corresponding halogenated derivatives base ions (at the fragmentor voltage of 100 V) corresponded to $[M–CH_2COOH]^-$ and $[M–CH_2CH_2OCH_2COOH]^-$, respectively. Ions monitored were: estrone (m/z 269), estradiol (m/z 271), estriol (m/z 287), ethynylestradiol (m/z 295), diethylstilbestrol (m/z 267), m/z 227 (BPA), m/z 205 (OP, OP_1EC and OP_2EC, respectively), m/z 219 (NP, NP_1EC and NP_2EC, respectively), m/z 253/255 (ClNP, $ClNP_1EC$ and $ClNP_2EC$, respectively) and m/z 297/299 (BrNP, $BrNP_1EC$ and $BrNP_2EC$, respectively).

3. Results and discussion

3.1. Optimization of a column-switching system

As a first step of this study, a comparative study employing three types of LiChrospher ADS precolumns, with different chemical modification of the pore (internal) surface (bonded alkyl chains C_4, C_8 or C_{18}) was performed in order to determine their performance in decreasing matrix content and interference encountered in the trace analysis of EDs in sediment extracts. The method development of a

column-switching technique consisted of several experiments that were performed in order to determine the proper valve-switching times. To successfully apply a RAM precolumn for the fractionation of complex samples, the elution profile of the sample matrix, as well as elution profile of the analytes should be recorded, and according to them, the duration of the fractionation event optimized. To record the matrix elution profile raw sediment extract was injected into the RAM precolumn, which was directly coupled to a DAD–MS detection system and flushed with H_2O–acetonitrile (95:5, v/v). It was found that the precolumn with a low hydrophobicity, packed with ADS C_4 material, is much more efficient in eliminating matrix components from sediment extracts than more hydrophobic C_8 and C_{18} precolumns, respectively. Using an ADS C_4 matrix elution was achieved within 2.5 min. From the other side, it was found that C_8 and C_{18} precolumns have advantage with respect to long breakthrough time (>15 min for all compounds, including the most polar ones), while when using C_4 precolumn (see Table 2), the breakthrough time for the most polar compound (OP_1EC) was short (5.5 min).

Table 2
Validation parameters of RAM–LC–MS integrated method using LiChrosphere ADS C_4 precolumn (Merck, Germany)

Compound	Breakthrough time (min)	Recovery (%)	RDS (%) ($n=3$)
OP_1EC	5.5	62	5.5
NP_1EC	8.0	77	2.5
OP_2EC	8	94	0.87
NP_2EC	12	97	1.7
Estriol	10	94	2.5
BPA	>15	98	2.4
Halogenated AP_1EC	>15	96	3.2
Halogenated AP_2EC	>15	97	4.1
Estradiol	>15	104	3.6
Estrone	>15	101	2.5
Ethynylestradiol	>15	98	7.1
Diethylstilbestrol	>15	97	5.7
Norethindrone	>15	96	2.3
Levenorgestrel	>15	103	4.1
Progesterone	>15	102	4.0
APEOs	>15	96	2.8
Halogenated APEOs	>15	97	3.1
APs	>15	102 (OP)	8.5 (OP)
		99 (NP)	8.8 (NP)
Halogenated APs	>15	83 (ClNP)	12 (ClNP)
		81 (BrNP)	15 (BrNP)

Once the fractionation step is terminated, the valve is switched and the analytes are transferred to the analytical HPLC column with a mobile phase with the stronger elution power. Problems arise from the fact that the optimal mobile phase for analyte transfer is a mobile phase with high content of organic modifier (acetonitrile, methanol etc.), which is in conflict with the requirements of subsequent HPLC analysis. Therefore, to prevent (or minimize) disturbance of the phase system used for subsequent RP separation the proportion of organic modifier in the mobile phase used for elution should be equal or lower then that used for the separation step. Thus, analytes concentrate on the top of the analytical column resulting in so-called peak compression and narrow profile. Furthermore, the back-flush elution also results in smaller zones as compared to front-flushed elution.

The elution profile of the analyte transfer was recorded by connecting the RAM precolumn directly with the DAD–MS detection system, bypassing the analytical column. From the profiles, shown in Fig. 1, it is clear that the faster desorption and hence transfer of analytes is accomplished using a precolumn of low hydrophobicity (ADS C_4). Using the same mobile phase as in the separation step (acetonitrile–H_2O, 30:70, v/v) a narrow elution profile is obtained and complete desorption is accomplished within 2.5 min. When using ADS C_8 and C_{18} columns, that strongly retained analytes, transfer required a larger volume of mobile phase and unacceptably broad peaks were obtained for some compounds. The most hydrophobic compounds (i.e. NP and halogenated NPs) eluted slowly, as shown in the inset of Fig. 1B.

Compounds detected under PI conditions exhibit a reduced MS response when using a mixture of acetonitrile and water as the elution solvent. Significantly higher signal intensity was obtained using a protic solvent (e.g. methanol). Thus, for APEOs, halogenated APEOs and progestogens transfer of analytes from RAM column to the analytical column, and subsequent chromatographic separation, was performed with mobile phase containing methanol–water (30:70, v/v). The efficiency of this mobile phase to elute target compounds from precolumn was comparable to the acetonitrile–water solvent mixture.
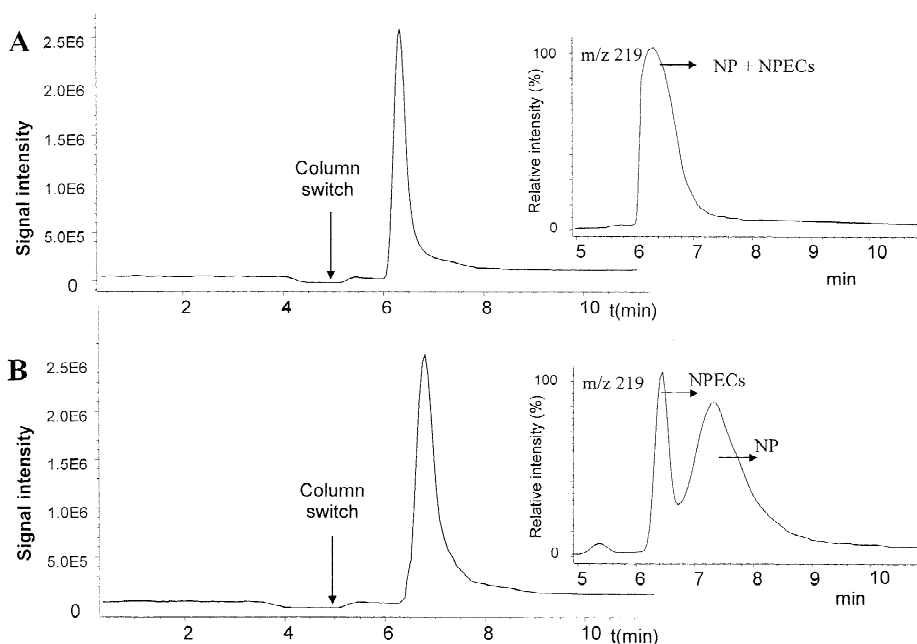
Fig. 1. Elution profile of analyte transfer. (A) LiChrospher ADS C_4 precolumn; (B) LiChrospher ADS C_{18} precolumn. Insets: extracted traces for m/z 219 characteristic for NP, NP_1EC and NP_2EC.

To summarize, the best results in terms of re-covery of target compounds and selectivity were obtained using a LiChrospher ADS C_4 precolumn. Recoveries were calculated by comparing the peak areas obtained by LC–MS and RAM–LC–MS, respectively. Except for the most polar compounds, all compounds exhibit high recoveries with RSD from 0.87 to 15% (Table 2). With the C_4 precolumn very good fractionation of matrix components and analytes was achieved, and good transfer of hydro-phobic compounds, but the fractionation step, due to short breakthrough time for the most polar com-pounds, was limited to a maximum of 5 min. Hydrophobic precolumns (C_8 and C_{18}) retained the target analytes well, but the transfer of the most hydrophobic compounds (APs and halogenated APs) was poor, as was the removal of matrix components.

3.2. LC separation and MS detection

With a 30-min gradient elution, chromatographic separation and peak shape obtained using RAM–LC system were comparable to those obtained by direct HPLC analysis, confirming the efficiency of analyte

transfer. No additional band broadening was ob-served because the elution power of the starting mobile phase was low enough to enable efficient in-line enrichment and thus high separation quality. Good linearity was found in the concentration range studied (0.1 to 25 μg/ml) for all compounds, with correlation coefficients (r^2) higher than 0.990 in all cases. Chromatogram showing the separation of alkylphenolic compounds and BPA, detected under Ni conditions, using a RAM (ADS C_4)–LC inte-grated system is given in Fig. 2. The variation of the capillary voltage provided the possibility to increase the fragmentation of carboxylic alkylphenolic com-pounds. When the voltage is increased to 100 V, the base peak, with high absolute intensity, for OP_1EC and OP_2EC is m/z 205, for NP_1EC and NP_2EC m/z 219, for $ClNP_1EC$ and $ClNP_2EC$ m/z 253/255 and for $BrNP_1EC$ and $BrNP_2EC$ m/z 297/299. Therefore, these compounds were monitored using the same m/z channels as APs and halogenated APs, respec-tively, increasing the relative instrument dwell time and enhancing sensitivity. Specific issues regarding the MS detection of estrogens and progestogens [17] and alkylphenolic compounds and their halogenated
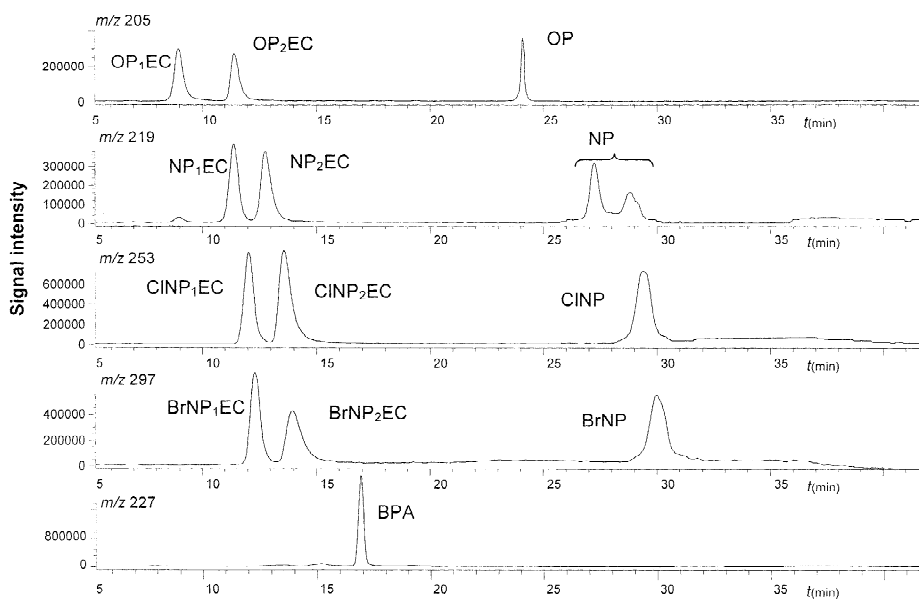
Fig. 2. Reconstructed ion chromatograms, obtained under NI conditions, corresponding to alkylphenolic compounds and bisphenol A, obtained by RAM–LC–MS method.

derivatives [12,18] are discussed in details elsewhere.

3.3. Matrix effect

Suppression of the analyte signal, caused by high concentration of matrix components, is one of the problems to be solved when analyzing EDs by MS. Humic substances and other macromolecular matrix components, eliminated by size-exclusion mechanism in the fractionation step using a RAM, are only a part of matrix that can interfere in the analysis of target compounds and induce ion suppression effect. Among nonvolatile solutes, inorganic salts, such as sulfates and phosphates are found to cause most ionization suppression [19], but the effect is also applicable to any nonvolatile compound, including co-eluting analyte.

Efficiency of RAM in reducing matrix content, and consequently reducing the suppression of the ionization process in the ESI–MS interface was evaluated by experiments based on comparative analysis of the same sediment extracts, injected into LC–MS system with and without additional clean-up. As a measure of ion suppression, the intensity of 4-heptylphenol (4-HP), which was used as an inter-

nal standard, was recorded in raw PLE extract, in extracts after clean-up using SPE and RAM precolumn, respectively. The signal intensity of 4-HP was reduced by 35% in the sediment extract obtained by PLE, under optimized conditions [20], and directly injected into LC–MS system. The signal reduction was limited to 10.5% when an off-line SPE was applied as a clean-up method and to only 8.3% using on-line RAM clean up.

Furthermore, the application of conventional methodology (SPE clean-up) to the field samples evidenced occurrence of important interferences co-eluting with target analytes and partially overlapping peaks of interest, as shown in Figs. 3 and 4 for estriol and estradiol, respectively. Clean up using RAM C_4 precolumn was found to efficiently remove polar interferences appearing at the beginning of the chromatogram. The RAM precolumn improved the resolution between the analytes and impurities sufficiently to trace the analytes present at low concentration levels. As a consequence, LODs for estriol and estradiol were approximately one order of magnitude lower than those obtained with SPE clean up.

And finally, the main advantage of using on-line clean-up is significant reduction in total analysis time
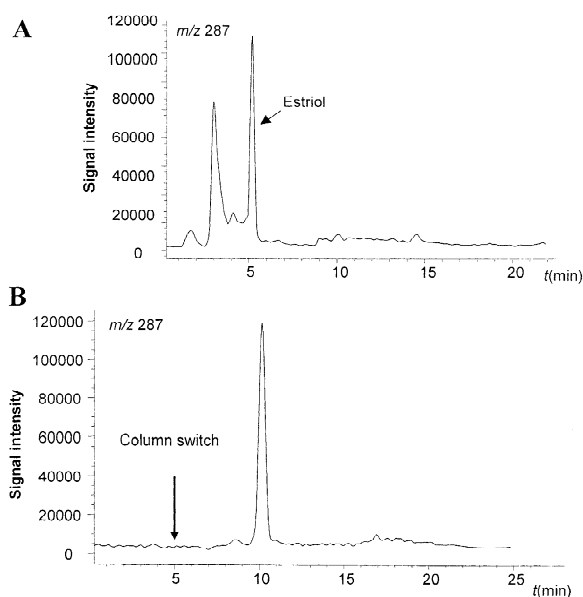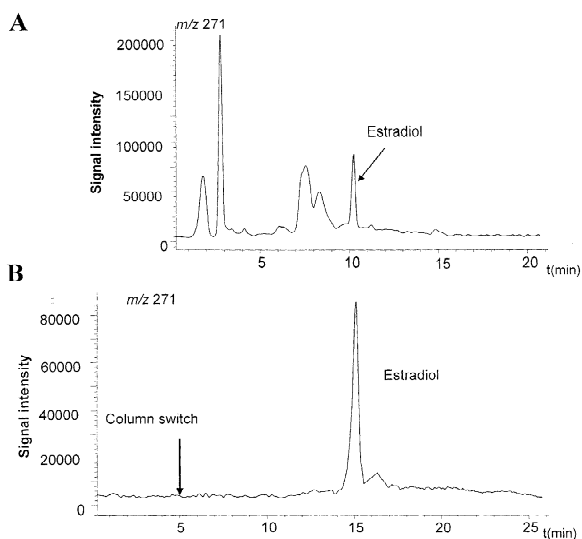
Fig. 3. Reconstructed ion chromatograms of SIM channel m/z 287 corresponding to $[M–H]^-$ of estriol obtained under NI conditions of spiked river sediment. (A) PLE-off line SPE–LC–MS; (B) PLE–RAM–LC–MS.

Fig. 4. Reconstructed ion chromatograms of SIM channel m/z 271 corresponding to $[M–H]^-$ of estradiol obtained under NI conditions of spiked river sediment. (A) PLE-off line SPE–LC–MS; (B) PLE–RAM–LC–MS.

(2 vs. >12 h) and reduction in sample manipulation since all clean-up steps are performed with continuously pumping devices, resulting in fully automated system and high sample throughput.

3.4. Method validation

The established protocol, PLE followed by RAM–LC–MS was validated by the triplicate analysis of spiked sediment samples. The sediment used as matrix was from the Cardener River (Catalonia, NE Spain), and it was collected at a sampling point upstream of known sources of pollution. Nevertheless, small concentrations of APs were found, and chromatogram traces obtained for spiked samples were subtracted for chromatograms of a blank sample analyzed by a parallel assay.

The detection limits (LOD) of combined PLE–RAM–LC–MS procedure, achieved by the extraction of 5 g of river sediment, were calculated as the minimum amount of a compound present in a sample that produces a signal-to-noise ratio of 3, based on an injection of a 25-µl aliquot of the final 1 ml extract. For alkylphenolic compounds LODs ranged from 0.5 to 2 ng/g, for BPA LOD was 0.5 ng/g, and for steroid sex hormones as follows: 0.5 ng/g (progestogens); 1 ng/g (estriol and diethylstilbestol); 2 ng/g (estradiol); 5 ng/g (estrone); 5 ng/g (ethynylestradiol). Generally, LODs achieved using RAM–LC were lower than those previously reported, obtained using conventional off-line clean up [21–23]. The overall precision of the analysis was satisfactory with RSD of triplicate measurements falling between 9.6 and 19%.

4. Conclusions

The newly developed PLE–RAM–LC–MS procedure allows efficient extraction of alkylphenolic compounds and steroid sex hormones from sediment samples and provides high sensitivity for their detection due to the reduced MS background noise. The method permits simultaneous trace analysis of APEOs, APECs, APs, and their halogenated derivatives and steroid sex hormones in less than 2 h (for two injections, in PI and NI mode, respectively), which is a significant improvement in comparison to

the methods reported previously. The use of a dual column LC system with restricted access precolumn packing allows efficient and rapid clean-up of extracts, thus reducing the concentration of interferences and minimizing matrix-induced ion suppression effects.

The best results in terms of selectivity and sensitivity were obtained using a RAM column of low hydrophobicity (LiChrospher ADS C_4). It has been shown that a restricted access precolumn efficiently separates high-molecular matrix components (humic substances), polar impurities and inorganic salts.

Acknowledgements

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