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Multi-residue analytical method for the determination of endocrine disruptors and related compounds in river and waste water using dual column liquid chromatography switching system coupled to mass spectrometry

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ABSTRACT

The present study describes a novel, fully automated method, based on column switching using EQuan™ columns for an integrated sample preconcentration and liquid chromatography coupled to tandem mass spectrometry (LC–LC–MS/MS). The method allows the unequivocal identification and quantification of the most relevant environmental endocrine disruptors compounds (EDCs) and compounds suspected to be EDCs, such as natural and synthetic estrogens and their conjugates, antimicrobials, parabens, bisphenol A, alkylphenolic compounds, benzotriazoles, and organophosphorus flame retardants, in surface river water and wastewater samples.

Applying this technique, water samples were directly injected into the chromatographic system and the target compounds were concentrated into the loading column. Thereafter, the analytes were transferred into the analytical column for subsequent detection by MS–MS (QqQ). A comparative study employing three types of columns, with different chemical modifications, was performed in order to determine the optimal column that allowed maximum retention and subsequent elution of the analytes. Using this new optimized methodology a fast and easy online methodology for the analysis of EDCs in surface river water and wastewater with low limits of quantification (LOQ) was obtained. LOQs ranged from 0.008 to 1.54 ng/L for surface river water and from 0.178/0.364 to 12.5/25.0 ng/L (except for alkylphenol monoethoxylates) for effluent/influent waste water. Moreover, employing approximately 1 h, a complete analysis was performed which was significant improvement in comparison to other methods reported previously.

This method was used to track the presence and fate of target compounds in the Ebro River which is the most important river in Spain whose intensive agricultural and industrial activities concentrate mainly close to the main cities in the basin, deteriorating soil and water quality.

1. Introduction

EDCs are a group of exogenous substance that interfere with the endocrine system and disrupt the physiological function of hormones [1]. These compounds can act in a low dose in a variety of organisms producing development disorders such as sexual problems, feminizing of males or masculine effects on females and infertility [2]. Some of these contaminants, with different structures and properties, are found in a high variety of products commonly used in the daily life (detergents, in personal care products such as cosmetics, pharmaceuticals and in different industrial formulations). Consequently they are detected in the aquatic environment, being waste water treatment plants (WWTPs) effluents and run-off from farmlands the main sources for their introduction into the aquatic environment [3–7]. The importance of these contaminants lays is their estrogenicity, specially of natural estrogens that have been shown to exert estrogenic effects in fish at very low concentration in water (below ng/L) [8,9] becoming the major contributors to endocrine-disrupting activity in sewage water and surface water [10] and in consequence, dramatic effects on the aquatic organisms [11–15].

Natural estrogens, such as estradiol (E2) and its main metabolites estriol (E3) and estrone (E1), are excreted from the human

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body in urine as conjugates (basically sulfates and glucoronides), which are largely biologically inactive. However, steroids in the free, deconjugated state have also been detected in sewage effluents [16], implying that deconjugation occurs prior to and/or during wastewater treatment [17–19].

Other important group of EDCs are alkylphenolic ethoxylates (APEOs) that are widely used in diverse cleaning and industrial applications [20,21]. Nonylphenol ethoxylates (NPEOs) account for approximately 80% the total APEOs that reach WWTP where the biotransformation products are formed, including mono- and diethoxylates and carboxylates formed in aerobic environments and alkylphenols (APs), such as nonylphenol (NP) and octylphenol (OP) formed in anaerobic conditions [22]. These transformation products are found to be widespread in the aquatic environment [23,24], especially in waters impacted by WWTPs [25].

Due to their estrogenic activity, both alkylphenolic compounds and estrogens have been included in diverse list of EDCs. NP and OP have been listed as priority hazardous substances in the field of water policy by the European Community Water Framework Directive 2000/60/EC and the final European Union decision No. 2455/2001/EC while estradiol and ethinylestradiol are proposed to be included on a revised list of priority substances (proposal COM(2011) 876).

This study also includes some well-known EDCs such as bisphenol A (BPA) that is mainly used in production of polycarbonate plastics and epoxy resins and other chemicals with potential endocrine disrupting properties, such as parabens, organophosphorus flame retardants, some antimicrobials, triclorocarban (TCC) and triclosan (TCS), and anticorrosive agents benzotriazoles. Parabens are extensively applied as preservatives (usually as mixtures) in a large number of products including cosmetics and toiletries such as shampoos, skin care products and toothpastes [26,27]. Parabens most commonly present in consumer products are methylparaben (MeP) and propylparaben (PrP). Although these products are readily biodegradable under aerobic conditions, their high consumption amounts and continuous introduction into the environment may lead to a pseudo-persistent situation [28]. The organophosphorus flame retardants, such as tris(butoxyethyl) phosphate (TBEP), tris(chloroisopropyl) phosphate (TCCP) and tris (2-chloroethyl) phosphate (TCEP) are used in a large variety of consumer products as a flame retardants and plasticizers, antifoaming agents and additives. Anticorrosives included in this study, 1H-benzotriazole (BT), toxic to the aquatic organisms [21,29–31], and tolytriazol (TT) widely used in cooling and hydraulic fluids, in antifreezing products, or in dishwasher detergents. Finally, the list of target compounds includes caffeine as a marker of urban wastewaters [32].

There are several analytical methodologies already available in the literature for the determination of EDCs in surface river water and wastewater [33–38]. Most of these methods cover only one or two groups of compounds containing similar polarities [39–42], similar structures [43–47] or similar activities [48–50]. Several studies proposing multi-residue methods for the determination of broad range of EDCs are published [51,24]; however they generally use multiple extraction methods and/or LC eluent systems [52] resulting therefore in tedious and time consuming method.

The aim of this work was to develop a robust, fast and high-throughput method for the simultaneous determination of all above listed EDCs and related compounds in river and wastewater using Equan™ Direct Injection Technology. Specifically the objective was to optimize LC–LC–MS/MS parameters, which included the selection of EQuan™ preconcentration column 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

Pure standard of the target estrogens E2, E1, E3, ethinylestradiol (EE2), diethylstilbestrol (DES), estriol 3-sulfate (E3-3S), estradiol 17-glucuronide (E2-17G), estrone 3-glucuronide (E1-3G), estriol 16-glucuronide (E3-16G), TCS, MeP, ethylparaben (EtP), PrP, benzylparaben (BeP), BPA-d_{16}, 4-tert-octylphenol (OP), OP-d_2, 4-tert-octylphenol-3,5 d_2-diethoxylate (OP_2EO-d_2), triphenyl-d_{15}-phosphate, caffeine and caffeine C_{13} were purchased from Sigma–Aldrich (St. Louis, MO, USA). TCC, BeP, BPA and TT, TBEP, TCEP were supplied by Aldrich (Milwaukee, WI, USA). NP, NP-d8, octylphenol mono- and dicarboxylate (OP_1EC and NP_1EC), octylphenol mono- and diethoxylate (OP_1EO and NP_1EO), octyl- and nonylphenol diethoxylate (OP_2EO and NP_2EO), NP1EO d_2 were purchased from Dr. Ehrenstorfer (Germany). E2-d_5, E1-d_4, EE2-d_4, E1-3S-d_4, were also obtained from CDN Isotopes Pointe-Claire, Quebec, Canada. BT, TCCP, etinylhidroxibenzoate C_{13}, BT ring d_4 were purchased from Fluka (Buchs, Switzerland).

Individual stock solutions of the analytes were initially prepared at 1 mg/mL in methanol and subsequently diluted in order to obtain an appropriate analyte concentration. Standard mixtures of various compound classes were prepared in methanol at different concentrations by appropriate dilution of individual stock solution.

Solid phase extraction (SPE) cartridges (3 mL, 3 mg, HLB) were obtained from Waters Corp. (Millford, MA). All the solvents (water and methanol) were HPLC grade and were purchased from Merck (Darmstadt, Germany), and ammonium formiate and acetic acid were from Panreac (Barcelona, Spain). Nitrogen for drying 99.995% of purity was from Air Liquide (Spain).

2.2. Sample collection

In order to apply our new analytical methodology we analyzed three different matrices water samples, surface water and influent and effluent water.

A total of 22 water samples were collected from the Ebro River basin (NE Spain) during a sampling campaign in 2010. The surfaces waters corresponded to sampling points with agricultural or industrial activities or near big cities. Influents and effluents waters of six major WWTPs in the basin were also analyzed.

The following samples were taken: ARG, downstream Pamplona WWTP (WWTP1); NAJ, an important agricultural wine area, upstream Logroño WWTP (WWTP2); EBR4, downstream to WWTP2; EBR 5, downstream Tudela WWTP (WWTP3); GAL2 in the agricultural area and HUE, inside the city of Zaragoza, receives the effluents from several industrial areas, both sampling points upstream Zaragoza WWTP (WWTP4); EBR6 downstream WWTP4; SEG, downstream Lleida WWTP (WWTP5); EBR8 and EBR9 the last before the Ebro reaches the sea with rice fields, both downstream Tortosa WWTP (WWTP6).

Water samples were collected in an amber glass bottles and transported to the laboratory under cooled conditions (4 °C). Upon reception, samples were filtered through 0.45 μm nylon and stored at 4 °C.

2.3. Analytical method

The Thermo Scientific EQuan™ system for online sample preconcentration and analysis consisted of a triple quadrupole (QqQ) MS with an electrospray ionization source (ESI), two LC quaternary pumps (Finnigan Surveyor L-Pump) and two LC columns, one for preconcentration of the sample and the second for the analytical separation. A 6-port divert valve was programmed by data system to control the loading and eluting of two LC columns. Schematic

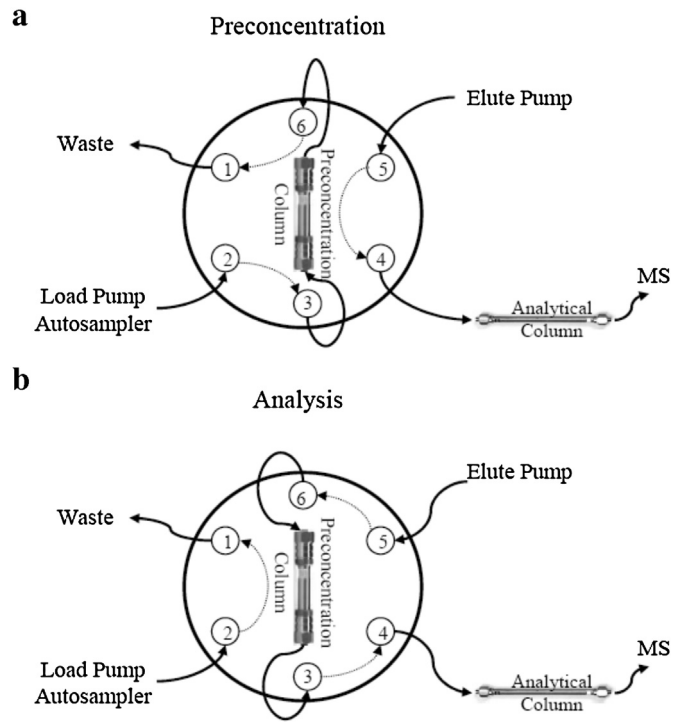

Fig. 1. Schematic EQuan™ system. (a) Position 1 (load position), for loading the sample onto the loading column. (b) Position 2 (inject position), for eluting the compounds retained on the loading column onto the analytical column.

EQuan™ system is shown in the Fig. 1. A Hypersil GOLD™ Aqua 20 × 2.1 mm, 12 μm particle from Thermo Scientific, 250 × 4 mm I.D C_{18} reversed-phase column (LiChrospher 100 RP-18) from Merck, were used as preconcentration and analytical columns respectively.

2.3.1. LC–LC conditions

The LC–LC conditions for the water sample preconcentration and subsequent separation of the target compounds onto the analytical column is shown in Table 1. The injection volume was set at 5 mL in the case of surface river water and 2 mL for waste water. The flow-rate through loading column was 1.75 mL/min during the charge step. In the transfer step its flow rate down to 0.3 mL/min, the preconcentration column was cleaned and conditioned at flow-rate ranged between 0.5 and 1.0 mL/min, while during all the analysis time the flow-rate the eluting column was 0.3 mL/min.

One thousand milliliters samples were spiked with 100 μL of a 250 μg/L solution of surrogate standards and the water samples were directly injected into the chromatographic system and the target compounds were concentrated into the loading column by a stream of mobile phase (aqueous: organic solvent (98:2, v/v)). Thereafter, the six-port switching valve was activated and the analytes was transferred from preconcentration column to the analytical column using the same mobile phases than previous step through both columns. When the transfer was finished, the switching valve was activated and the analytes were separated in a conventional manner. Simultaneously, the preconcentration column was rinsed and conditioned. All the steps were performed automatically.

Chromatographic separation of compounds detected under NI conditions was performed under gradient elution condition using water (A) and methanol (B). The initial condition was 50% B, then the gradient was linearly increased to 70% B in 2 min, increased to 100% B in 6 min and kept isocratic for 6 min. Compounds detected under PI conditions were separated using the same gradient program using solvent system containing water–methanol

Table 1
The LC–LC conditions.

		Pump 1				Pump 2	Elute pump						
		Injection volume	Load pump Surface river water: 5 mL Effluent water: 2 mL Influent water: 2 mL										
		Preconcentration column:	20 × 2.1 mm, 12 μm Hypersil GOLD aQ			Analytical column5 μm, 125 × 2 mm i.d., C18 reversed-phase column							
							Purospher STAR RP-18						
		Solvent A	NI: water PI: water (20 mM ammonium formiate, 0.1% acetic acid)			Solvent A:	NI: water PI: water (20 mM ammonium formiate, 0.1% acetic acid)						
		Solvent B	NI: methanol PI: methanol (20 mM ammonium formiate, 0.1% acetic acid)			Solvent B:	NI: methanol PI: methanol (20 mM ammonium formiate, 0.1% acetic acid)						
Valve position	Start time (min)	Flow (mL/min)	Gradient	A%	B%	Description	Flow (mL/min)	Gradient	A%	B%	Description		
Load	0:00	1.75	Step	98	2	Sample loading into the EQuan™ column	0.3	Step	50	50	Analytical column conditioning		
Load	3:25[a]	1.75	Ramp	98	2	Sample loading into the EQuan™ column	0.3	Step	50	50	Analytical column conditioning		
Inject	3:50	0.2	Ramp	50	50	Analyte transfer to the analytical column	0.3	Ramp	50	50	Analyte transfer to the analytical column		
Load	5:50	1	Ramp	50	50	EQuan™ column cleaning	0.3	Ramp	30	70	LC separation		
Load	11:50	1	Ramp	–	100	EQuan™ column cleaning	0.3	Ramp	–	100	LC separation		
Load	17:50	0.5	Ramp	50	50	EQuan™ column cleaning	0.3	Ramp	–	100	Analytical column cleaning		
Load	22:50	0.5	Ramp	98	2	EQuan™ column conditioning	0.3	Ramp	50	50	Analytical column conditioning		
Load	24:50	1	Step	98	2	EQuan™ column conditioning	0.3	Ramp	50	50	Analytical column conditioning		

PI, positive ionization; NI, negative ionization.
[a] For influent and effluent water this time was reduced at 1:25 (in order to adapt this time with sample loading loop because the injection volume was 2 mL), consequently the total time was reduced too.

Table 2
The optimized MS/MS parameters for SRM analysis.

Abbreviation	Name	Internal standard [a]	Precursor ion (*m*/*z*)	SLens (Hz)	SRM1 (*m*/*z*)	Collision energy (eV)	SRM2 (*m*/*z*)	Collision energy (eV)
Negative ionization								
Natural and synthetic estrogens and conjugates								
E2	Estradiol	Estradiol d_5	271	148	145	43	183	42
E1	Estrone	Estrone d_4	269	120	145	38	143	54
E3	Estriol	Estrone d_4	287	144	171	41	145	40
EE2	Ethinylestradiol	Ethinylestradiol d_4	295	126	145	41	159	33
DES	Diethylstilbestrol	Estrone d_4	267	108	237	26	251	29
E1-3S	Estrone 3-sulfate	Estrone d_4 sulfate	349	132	269	32	145	58
E3-3S	Estriol 3-sulfate	Estrone d_4 sulfate	367	138	287	33	171	53
E2-17G	Estradiol 17-glucuronide	Estrone d_4 sulfate	447	129	271	32	325	27
E1-3G	Estrone 3-glucuronide	Estrone d_4 sulfate	445	137	113	21	269	39
E3-16G	Estriol 16-glucuronide	Estrone d_4 sulfate	463	179	287	32	113	30
Antimicrobials/disinfectants								
TCC	Triclorocarban	Etinylhidroxibenzoate C_{13}	313	88	160	17	126	26
TCS	Triclosan	Etinylhidroxibenzoate C_{13}	287	73	35	34	37	34
Preservatives								
MeP	Methylparaben	Etinylhidroxibenzoate C_{13}	151	70	92	23	136	16
EtP	Ethylparaben	Etinylhidroxibenzoate C_{13}	165	78	92	24	137	16
PrP	Propylparaben	Etinylhidroxibenzoate C_{13}	179	87	92	24	136	17
BeP	Benzylparaben	Etinylhidroxibenzoate C_{13}	227	88	92	25	136	16
Plasticizer								
BPA	Bisphenol A	Bisphenol A d_{16}	227	125	212	21	133	27
Alkylphenolic compounds								
OP	Octylphenol	Octylphenol d_2	205	109	133	30	134	20
NP	Nonylphenol	Nonylphenol d_8	219	105	133	28	147	29
OP_1EC	Octylphenol monocarboxylate	Nonylphenol monocarboxylate d_2	263	82	205	20	106	31
NP_1EC	Nonylphenol monocarboxylate	Nonylphenol monocarboxylate d_2	277	72	133	45	219	21
Positive ionization								
OP_1EO	Octylphenol monoethoxylate	Nonylphenol monoethoxylate d_2	268	52	113	10	57	7
NP_1EO	Nonylphenol monoethoxylate	Nonylphenol monoethoxylate d_2	282	56	127	10	71	12
OP_2EO	Octylphenol diethoxylate	Octylphenol diethoxylate d_2	312	68	183	6	121	20
NP_2EO	Nonylphenol diethoxylate	Octylphenol diethoxylate d_2	326	81	183	6	121	16
Anticorrosives								
BT	1H-Benzotriazole	1H-Benzotriazole d_4	120	76	65	21	92	15
TT	Tolytriazol	1H-Benzotriazole d_4	134	78	77	24	79	17
Organophosphorus flame retardants compounds								
TBEP	Tris(butoxyethyl) phosphate	Triphenyl-d_{15}-phosphate	399	121	299	10	199	13
TCCP	Tris(chloroisopropyl) phosphate	Triphenyl-d_{15}-phosphate	327	87	99	26	81	55
TCEP	Tris(2-chloroetyhl) phosphate	Triphenyl-d_{15}-phosphate	287	89	99	23	225	12
Chemical biomarker								
Caff	Caffeine	Caffeine C_{13}	195	73	138	17	110	20

SRM: selected reaction monitoring.
[a] Internal standard applied for the identification and quantification of each analyte.

both phases with 20 mM of ammonium formiate and 0.1% of acetic acid.

2.3.2. Mass spectrometric detection

Detection was carried out using a mass spectrometer TSQ Vantatge, equipped with an ESI turbo spray interface. The operating parameters were as follows for negative and positive ionization (NI/PI) respectively: spray voltage 2500/3000 V, sheath gas pressure 40/40 (N_2), auxiliary gas pressure 20/20 (N_2), ion sweep gas pressure 0.5/0.5 (N_2) and transfer tube temperature 270/300 °C. The precursor and product ions of individual target compounds were obtained by tuning after direct injection of 1 ppm. The optimized MS/MS parameters for SRM analysis of the analytes are given in the Table 2.

Natural and synthetic estrogens and conjugates, antimicrobials/disinfectants, preservatives, BPA, and the alkylphenolic compounds (OP, NP, OP_1EC and NP_1EC) were detected under NI conditions as $[M-H]^-$. Diagnostic ions used for the analysis of anticorrosives, organophosphorus flame retardants compounds and the chemical biomarker caffeine, in PI mode were those corresponding to $[M+H]^+$, while for the alkylphenolic compounds (OP_1EO, OP_2EO, NP1EO and NP_2EO) the adduct precursor ion $[M+NH_3]^+$ was analyzed.

2.3.3. Offline sample preparation and detection

In order to compare the performances of the LC–LC method and conventional SPE preconcentration methodology, which have been widely used for the analysis of target compounds in water samples, an off-line sample preparation was applied [53].

Water sample preconcentration was performed by SPE using HLB (3 mL, 3 mg) cartridges. Different volumes were taken depending on the type of the sample: 500 ml for surface river water, 200 mL and 100 mL for effluent and influent waste water respectively. Samples were spiked with 100 μL of a 250 μg/L solution of surrogate standards.

The SPE cartridges were subsequently conditioned (at a flow rate of 1 ml/min) with 5 ml of methanol followed by 5 ml of water. Cartridges were eluted, at the same flow rate, with 2 × 5 ml of methanol and the eluents were evaporated with a gentle stream of nitrogen and reconstituted to a final volume of 1 ml with methanol. The same

Table 3
Comparative study employing three types of EQuan™ columns.

Compound	Hypersil GOLD Aqua		Hypersil GOLD PFP		Hypersil GOLD PEP	
Abbreviation name	Recovery (%)	(% RSD, $n=3$)	Recovery (%)	(% RSD, $n=3$)	Recovery (%)	(% RSD, $n=3$)
E2	61	11	66	11	41	14
E1	71	4	64	7	43	9
E3	101	5	102	4	62	3
EE2	73	15	57	11	51	3
DES	59	9	59	9	39	5
E1-3S	95	4	85	5	73	8
E3-3S	25	6	39	10	105	7
E2-17G	74	12	71	12	60	8
E1-3G	62	8	85	4	76	7
E3-16G	55	16	80	11	110	9
TCS	80	5	46	1	14	8
TCC	54	8	53	4	19	4
MeP	67	10	24	9	65	27
EtP	84	5	60	2	56	9
PrP	78	1	56	4	44	1
BeP	57	4	45	4	23	13
BPA	99	7	75	6	65	7
OP	88	2	83	7	50	3
NP	73	12	57	8	49	6
OP_1EC	54	5	51	3	40	2
NP_1EC	79	1	59	3	46	1
OP_1EO	28	6	9	7	4	7
NP_1EO	58	8	23	6	64	8
OP_2EO	47	5	62	7	44	4
NP_2EO	32	6	63	12	38	1
BT	89	10	54	11	102	9
TT	65	8	47	8	95	7
TBEP	62	7	32	7	32	7
TCPP	54	5	36	12	39	3
TCEP	47	5	40	7	45	8
Caff	80	10	60	10	10	9

RSD: relative standard deviation.

LC–MS/MS analysis described before was applied and the volume injected was 10 μL.

3. Results and discussion

3.1. Optimization of the LC–LC conditions, LC separation and MS detection

As the first step of this study, the load flow was optimized, testing flows between 1 and 3 mL/min and calculating the time required to evacuate the sample injection loop (2–5 mL). The charging time was tested too, considering the load flow and the volume of sample injected. A critical point was the transfer time, when both columns were connected. Transfer time had to be reduced to the maximum in order to obtain the optimum conditions where the target compounds had been transferred onto the analytical column and the sample matrix remained in the preconcentration column. The percentage of organic solvent supported by the EQuan column without the elution of the target compounds was very low, not giving a possible clean up step with higher percentage of organic mobile phases than 5% and subsequently higher concentrations of impurities removed.

As the last step of optimization, a comparative study employing three types of columns Hypersil GOLD™ 20 × 2.1 mm, 12 μm particle size, from Thermo Scientific, with different chemical modifications was performed in order to select the optimal column that allowed maximum retention and subsequent elution of the analytes: an Hypersil GOLD™ Aqua, specially indicated to retain analytes injected with high percentage of aqueous solution; an Hypersil GOLD™ PEP, for the retention of polar and non-polar analytes; an Hypersil GOLD™ PFP, with a modification that resolve mixtures of halogenated compounds and non-halogenated polar aromatic compounds. Table 3 shows a comparison of recoveries (%) obtained with these three types of columns.

Chromatographic separation and peak shape obtained using LC–LC system were comparable to those obtained by direct LC analysis, with a 25 min gradient elution, confirming the efficiency of analyte transfer.

Compounds detected under PI conditions exhibited a reduced MS response when a mixture of water and methanol was used as the elution solvents. Significantly higher signal intensity was obtained using formic acid as a modifier. In addition, for APEOs, a second modifier was necessary for the formation of ammonium adduct required for detection by MS–MS in chromatographic systems, loading and eluting pump.

3.2. Method validation

In order to evaluate this new methodology, a validation study was made. 100 mL of different water matrices: surface river and effluent and influent waters were spiked with a mixture of target compounds to final concentrations of 2.5, 25 and 250 ng/L, except for the alkylphenolic compounds OP_1EO and NP_1EO where the final concentration were 1 μg/L due to their higher LOD/LOQs. Recoveries, LODs and LOQs were evaluated using the spiked samples.

LODs and LOQs of the target compounds in surface water and influent and effluent water were calculated by a signal-to-noise ratio of 3 and 10, respectively (the ratio between intensity of signal of each compound obtained under SRM conditions and intensity of noise in a spiked sample). The level of the spiked sample selected for the determination of LODs and LOQs was chosen based on the spiked sample concentration nearest to these limits.

Relative recoveries were calculated by comparing the peak areas obtained by LC–MS/MS and LC–LC–MS/MS respectively. For all

Table 4
Validation parameters of LC–LC–MS/MS integrated method using Hypersil GOLD™ 20 × 2.1 mm, 12 μm EQuan column.

Compound	Instrumental parameters					Surface river water		Effluent water		Influent water	
Abbreviation name	r^2	LOD (pg)	LOQ (pg)	Repeatability (% RSD, $n=6$)	Reproducibility (% RSD, $n=6$)	Relative recovery (% RSD, $n=6$)	LOD/LOQ (ng/L)	Relative recovery (% RSD, $n=6$)	LOD/LOQ (ng/L)	Relative recovery (% RSD, $n=6$)	LOD/LOQ (ng/L)
E2	0.995	0.17	0.56	5.5	8.9	94 (5.2)	0.037/0.12	75 (7.2)	0.59/1.9	58 (15)	5.4/18
E1	0.998	0.23	0.77	3.3	8.5	98 (3.1)	0.050/0.17	110 (2.8)	0.14/0.45	100 (2.8)	0.14/0.47
E3	0.992	0.78	2.6	3.6	8.1	65 (13)	0.17/0.56	51 (4.5)	2.3/7.8	58 (3.7)	3.1/10
EE2	0.996	0.06	2.1	7.3	14	104 (5.0)	0.14/0.47	85 (2.2)	3.8/12	99 (12)	4.2/14
DES	0.991	0.21	0.68	5.5	12	54 (5.6)	0.043/0.14	56 (2.4)	1.2/4.0	100 (7.1)	2.7/8.9
E1-3S	0.996	0.017	0.058	5.1	9.1	103 (9.4)	0.0038/0.013	79 (11)	0.21/0.71	59 (9.9)	0.35/1.2
E3-3S	0.997	0.14	0.45	12	21	96 (19)	0.030/0.010	53 (20)	0.75/2.5	57 (13)	0.99/3.3
E2-17G	0.991	2.0	6.7	4.0	14	128 (15)	0.46/1.5	52 (14)	2.9/9.6	52 (19)	4.2/14
E1-3G	0.991	0.24	0.80	11	14	111 (16)	0.056/0.18	81 (11)	0.96/3.2	49 (9.5)	3.1/10
E3-16G	0.996	0.29	0.97	4.0	6.7	100 (13)	0.059/0.19	73 (10)	2.0/6.6	66 (16)	7.5/25
TCS	0.993	0.73	2.5	4.8	11	62 (11)	0.17/0.58	105 (6.1)	1.5/5.0	88 (17)	2.1/6.9
TCC	0.992	0.16	0.52	4.6	10	74 (7.8)	0.036/0.12	82 (6.5)	0.14/0.47	59 (4.0)	0.18/0.61
MeP	0.993	0.86	2.9	5.2	7.7	72 (5.6)	0.20/0.68	59 (7.2)	0.59/1.9	77 (10)	1.5/5.0
EtP	0.994	1.3	4.4	1.9	8.1	116 (1.8)	0.27/0.89	112 (9.0)	1.2/4.0	92 (5.2)	1.4/4.8
PrP	0.998	0.10	0.34	2.4	4.5	100 (2.2)	0.021/0.069	126 (3.9)	0.35/1.2	125 (5.5)	0.94/3.1
BeP	0.994	0.14	0.48	1.8	7.3	95 (3.5)	0.031/0.10	103 (6.5)	0.12/1.1	119 (4.7)	1.0/3.5
BPA	0.991	0.50	1.7	8.3	10	95 (3.8)	0.11/0.39	110 (19)	0.69/2.3	112 (7.6)	1.4/4.6
OP	0.995	0.63	2.1	4.9	9.2	93 (8.6)	0.14/0.46	104 (2.6)	1.6/5.4	96.1 (7.9)	2.9/9.6
NP	0.991	0.06	0.21	5.1	8.8	70 (5.2)	0.013/0.043	64 (6.4)	0.30/1.0	74 (14)	0.71/2.4
OP_1EC	0.990	0.31	1.1	5.1	9.9	101 (8.3)	0.065/0.22	52 (1.6)	0.64/2.1	68 (5.2)	0.87/2.9
NP_1EC	0.994	0.17	0.56	4.1	7.5	103 (5.5)	0.034/0.12	110 (6.7)	0.078/0.26	64 (3.5)	0.11/0.36
OP_1EO[a]	0.990	80	267	9.1	14	82 (8.7)	17/56	65 (7.5)	20/68	44 (10)	33/109
NP_1EO[a]	0.993	297	990	8.5	12	75 (16)	62/208	76 (8.5)	125/417	67 (12)	187/625
OP_2EO	0.998	0.049	0.16	2.3	5.3	106 (2.5)	0.011/0.035	83 (15)	0.092/0.30	83 (16)	0.20/0.67
NP_2EO	0.998	0.062	0.21	2.0	4.4	107 (23)	0.013/0.044	105 (13)	0.11/0.37	95 (14)	0.12/0.42
BT	0.991	0.34	1.1	5.5	9.6	86 (5.3)	0.072/0.24	76 (6.7)	0.16/0.52	82 (7.6)	0.54/1.8
TT	0.998	0.062	0.21	4.4	7.9	78 (9.8)	0.013/0.044	85 (3.6)	0.10/0.34	67 (11)	0.21/0.69
TBEP	0.998	0.011	0.037	2.4	6.5	87 (3.4)	0.0024/0.0079	101 (5.1)	0.053/0.18	99 (5.2)	0.082/0.28
TCPP	0.993	0.013	0.044	3.9	7.4	95 (5.3)	0.0025/0.0084	106 (12)	0.063/0.21	137 (11)	0.55/1.8
TCEP	0.994	0.15	0.51	2.0	6.7	90 (12)	0.034/0.11	65 (4.8)	0.098/0.33	65 (8.9)	0.36/1.2
Caff	0.991	0.10	0.34	4.3	8.3	115 (4.9)	0.021/0.071	76 (7.3)	0.18/0.50	135 (12)	0.23/0.78

RSD: relative standard deviation.
[a] Spiked sample concentration was 1 μg/L.

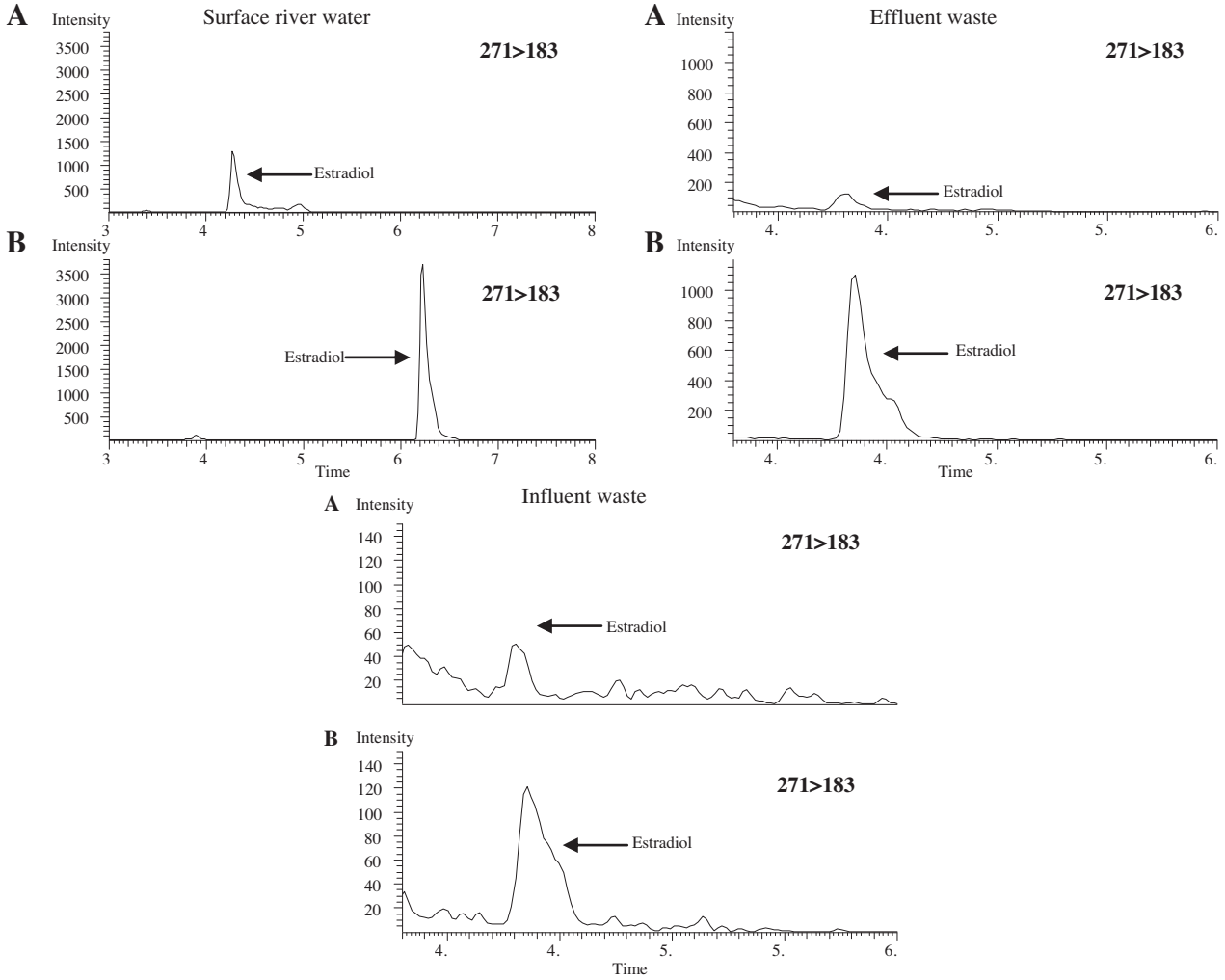

Fig. 2. Reconstructed ion chromatograms of MS/MS (271 > 183) corresponding to $[M-H]^-$ of estradiol obtained under NI conditions of spiked samples at level concentration of 250 ng/L. (A) offline SPE-LC–MS/MS; (B) online LC–LC–MS/MS. *Differences in retention times between chromatograms can be explained due to the different LC methodologies applied.

the matrices blank samples were analyzed in triplicate. A summary of quality assurance and quality control data are showed in Table 4 where the relative recoveries for different water matrices were calculated with the samples spiked at 250 ng/L except for the alkylphenolic compounds OP_1EO and NP_1EO where the final concentration were 1 μg/L, while the data for other two concentration levels (2.5 and 25 ng/L) are shown in the Supporting information (Table 3).

Identification and confirmation criteria of selected analytes were based on the following restrictions: (1) retention time for all monitored transitions for a given analyte should be maximized simultaneously ±1 s, with signal-noise ratio ≥3 for each; and (2) the ratio between the two monitored transitions should be within 15% of the theoretical value (calculates upon standards). Quantification was carried out adding isotopic compounds of the different families analyzed as surrogates.

The instrumental parameters presented good linearity in the concentration range studied (2.5–3000 ng/L) for all compounds, with correlation coefficients (r^2) higher than 0.990 in all cases. Intraday study showed values below 6.00% except for E3-3S (12.3%), E1-3G (10.9%), OP1EO (9.06%) and NP1EO (9.06%). Interday study also had shown acceptable results (below 14.0% except for the same compounds appointed above). In addition, a sensitive instrumental limits of detection and quantification (LODs and LOQs), between (0.0111–2.01 pg and 0.0369–6.68 pg), except for the alkylphenolic compounds OP_1EO and NP_1EO were obtained. Higher values for alkylphenolic compounds with LOQ values of 267 and 990 pg respectively, were due to mass detection, because these molecules had lower affinity to form ammonium adduct, that provided a good fragmentation in the MS system, instead of hardly fragmentable sodium adducts.

Method LODs obtained for natural and synthetic estrogens and conjugates ranged between 0.0038–0.46 ng/L, 0.14–3.8 ng/L and 0.14–7.5 ng/L for surface river water, effluent and influent wastewater, respectively. In the case of antimicrobials, disinfectants and preservatives LODs of 0.021–0.27 ng/L, 0.12–2.0 ng/L and 0.18–7.5 ng/L were obtained, respectively. BPA showed limits of 0.11, 0.69 and 1.4 ng/L. For alkylphenolic compounds (with exception of OP_1EO and NP_1EO) LODs of 0.011–0.14 ng/L, 0.078–1.6 ng/L and 0.11–2.9 ng/L were calculated, respectively. Compound belonging to the families of anticorrosives and organophosphorus flame retardant method LODs ranged between 0.0024–0.072 ng/L, 0.053–0.16 ng/L and 0.082–0.55 ng/L, respectively for river water, effluent and influent wastewater. For the chemical biomarker caffeine LODs of 0.021 ng/L, 0.18 ng/L and 0.23 ng/L were obtained in the same matrices.

3.3. Online/offline methods comparison

3.3.1. Online/offline recoveries comparison

A new online methodology for the determination of EDCs in waters samples was developed in order to improve the classical SPE off-line methodology, minimizing the matrix effects and consequently reducing LODs. Moreover the total time of analysis was reduced too; applying online preconcentration methodology no significant sample manipulation was required and the time of analysis was 25 min instead of 6 h in case of conventional off-line SPE followed by LC–MS–MS.

The off-line recoveries were evaluated spiking water samples (500 ml for surface river water, 200 mL and 100 mL for effluent and influent waste water respectively) with a mixture of target compounds to a final concentrations of 250 ng/L, except for the alkylphenolic compounds OP_1EO and NP_1EO where the final concentration were 1 μg/L. These recoveries were compared with the ones obtained by on-line analysis of the spiked samples at the same concentration level of 250 ng/L. The results showed that in general the on-line methodology was capable of improving the recoveries for all the target compounds analyzed. For example, in the case of natural and synthetic estrogens and conjugates the off-line recoveries ranged between 54–104%, 51–110% and 49–100% for surface river water, effluent and influent wastewater, respectively and were generally higher than applying the off-line methodology. In the case of alkylphenolic compounds, significant improvement in recoveries was obtained by EQuan technology. For example for OP recoveries of 90–100% were obtained with on-line method vs. 20–40% with off-line SPE.

Table 5
(a) Concentration values (ng/L) of different families of target compounds in surface river water (A) and wastewater (B) in the Ebro basin during 2010 campaign. (b) Concentration values (ng/L) of different families of target compounds in surface river water (A) and wastewater (B) in the Ebro basin during 2010 campaign.

	E2	E1	E3	E1-3S	E3-3S	E1-3G	E3-16G	TCS	MeP	EtP	PrP	BeP
(A)												
ARG	1.9	4.6	nd	1.0	nd	nd	nd	0.4	12	13	15	0.31
NAJ	nd	0.4	nd	nd	nd	nd	nd	2.0	4.0	2.6	7.8	1.1
EBR4	1.7	1.4	nd	nd	nd	nd	nd	0.3	3.0	nd	0.5	0.16
EBR5	1.1	0.6	nd	nd	nd	nd	2.04	nd	9.7	3.7	0.8	0.33
GAL2	1.5	nd	nd	nd	nd	nd	nd	0.1	27	4.9	1.7	nd
HUE	nd	4.9	4.81	nd	nd	nd	nd	nd	1.4	1.7	1.0	0.61
EBR6	1.4	0.7	nd	nd	nd	nd	nd	nd	7.7	nd	2.0	nd
SEG	1.4	0.8	nd	nd	nd	nd	3.08	nd	6.1	2.1	0.68	nd
EBR8	1.2	nd	nd	nd	nd	nd	nd	nd	3.4	nd	1.4	nd
EBR9	0.9	0.3	nd	nd	nd	nd	nd	nd	4.8	nd	1.3	nd
(B)												
WWTP1 In	23	24	nd	18	nd	nd	nd	22	25	204	19	nd
WWTP1 Out	5.0	15	nd	2.6	nd	nd	nd	5.9	nd	6.0	24	1.4
WWTP2 In	nd	8.4	nd	26	nd	19	nd	72	157	814	913	nd
WWTP2 Out	2.9	0.5	nd	3.5	nd	nd	nd	15	40	4.5	15	1.3
WWTP3 In	nd	nd	nd	12	7.8	nd	nd	nd	34	160	16	nd
WWTP3 Out	6.6	20	nd	8.5	5.8	nd	nd	nd	13	nd	nd	nd
WWTP4 In	nd	14	nd	8.6	17	nd	nd	26	93	51	41	nd
WWTP4 Out	4.5	1.3	nd	1.5	8.5	nd	nd	nd	13	6.8	10	1.7
WWTP5 In	nd	11.4	nd	nd	nd	nd	nd	10	9.0	14	20	nd
WWTP5 Out	5.1	4.2	nd	1.0	nd	nd	nd	16	26	nd	2.7	nd
WWTP6 In	24	39	nd	15	nd	nd	nd	8.4	40	70	54	nd
WWTP6 Out	4.3	1.2	nd	0.8	6.3	nd	nd	9.8	17	nd	5.4	nd

	BPA	OP	NP	NP1EC	OP2EO	NP2EO	BT	TT	TBEP	TCCP	TCEP	Caffeine
(A)												
ARG	61	4.4	nd	575	1.7	17	275	568	54	210	44	151
NAJ	28	2.9	2.1	86	2.2	13	18	49	21	27	3.5	54
EBR4	12	5.3	6.5	105	2.6	12	121	182	49	229	12	60
EBR5	10	4.1	3.2	54	2.7	13	76	346	37	56	10	95
GAL2	28	4.4	21	16	8.3	275	38	29	146	255	39	43
HUE	29	3.4	12	420	4.0	55	126	308	95	122	58	419
EBR6	19	4.1	nd	66	2.5	12	55	146	100	74	9.4	152
SEG	5.7	3.1	15	72	2.4	13	63	132	80	43	10	357
EBR8	nd	3.7	5.4	32	2.4	11	43	59	53	55	9.3	139
EBR9	nd	3.4	4.2	32	2.4	9.4	32	44	63	75	19	75
(B)												
WWTP1 In	13,063	60	651	2595	75	288	1954	4901	4414	1635	588	48,563
WWTP1 Out	608	26	nd	2419	55	180	1115	2388	367	4868	757	172
WWTP2 In	592	57	83	1096	581	2819	25,418	37,760	2657	12,307	146	82,559
WWTP2 Out	109	56	32	1817	36	67	3303	1169	276	36,594	243	108
WWTP3 In	164	178	755	1513	74	350	720	8500	2150	69	21	2100
WWTP3 Out	50	21	85	3257	44	182	695	7588	2033	356	165	477
WWTP4 In	220	28	94	503	68	124	741	1286	2218	494	45	29,424
WWTP4 Out	236	64	nd	2263	38	65	644	3311	2950	1599	246	399
WWTP5 In	159	351	707	445	148	1995	425	3242	853	941	275	32,571
WWTP5 Out	67	21	nd	622	54	168	446	2105	561	5021	954	176
WWTP6 In	202	24	56	601	75	697	483	2944	308	2906	581	29,071
WWTP6 Out	57	10	nd	1778	nd	130	877	1853	814	5244	634	109

EE2, DES, E2-17G, TCC, OP1EC, OP1EO and NP1EO were not detected in any sample.

3.3.2. Online/offline matrix effects comparison

Suppression of the analyte signal, caused by high concentration of matrix components, is one of the problems to be solved when analyzing EDCs by MS/MS. Nonvolatile solute, inorganic salts such as sulfates and phosphates are found to cause most ionization suppression [54], but the effect is also applicable to any volatile compound, including co-eluting analyte.

The results showed that online preconcentration methodology allowed improving the efficiency in terms of minimizing the matrix effects, as compared with an offline technique. Reconstructed ion chromatograms of MS/MS detection corresponding to $[M-H]^-$ of estradiol in off-line SPE-LC–MS/MS, on-line LC–LC–MS/MS conditions are shown in the Fig. 2. The figures showed that the signal intensity of estradiol was reduce by 66%, 89% and 60% for surface river water, effluent and influent wastewater, respectively, applying the conventional SPE preconcentration off-line methodology. Particularly in influent waste water the application of off-line SPE preconcentration to the filed samples evidenced occurrence of important interferences, coeluting either with target analytes or affecting to the ion suppression.

3.4. Analysis of river and waste water samples

As a part of the validation procedure, the method developed was applied to the analysis of target compounds in 22 water samples (10 surface river water and 12 wastewaters from 6 WWTPs (influent/effluent)) collected in the Ebro River basin during a sampling campaign in 2010, as are shown in Table 5. Majority of target compounds were detected in both wastewater and river water, at concentrations ranging from below ng/L or low ng/L as in case of hormones, triclosan and paraben up to several hundreds of ng/L in river water or low μg/L level in WWTP influents in case of alkylphenolic compounds, organophosphate flame retardants and benzotrizoles. Generally satisfactory elimination is observed in WWTP with exception of some compounds, such as NP1EC that are found at higher concentrations in effluent waters than in influents due to their formation as intermediary products in the transformation of long chain NPEOs. Overall, the methodology develop was successfully applied and therefore confirmed the importance of developing rapid and efficiency methodologies in order to detect EDCs and related compounds at low levels in river and wastewater matrices.

4. Conclusions

The newly developed LC–LC–MS/MS procedure allowed efficient preconcentration of EDCs from surface river water and wastewater samples and provided high sensitive for their detection due to the reduced MS/MS background noise. The method allows simultaneous trace analysis of EDCs belonging to different families and with different structures and properties in less than 1 h (for two injections, in NI and PI mode, respectively), with minimum sample pretreatment (filtration), which was a significant improvement in comparison to the methods reported previously. The use of a dual column LC system with a six port valve reduced the interferences, and improved the analysis parameters obtaining an enhanced optimization. The best results in terms of selectivity and sensitivity were obtained using an EQuan column, Hypersil GOLD™ Aqua, specially indicated to retain analytes injected with high percentage of aqueous solution.

Applying this new methodology low LODs (pg/L to low ng/L) were reached for all target compounds and matrices, obtaining acceptable recoveries and an important reduction in matrix effects with respect to the conventional SPE preconcentration methodology.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2013.04.0281.

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