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Determination of various estradiol mimicking-compounds in sewage sludge by the combination of microwave-assisted extraction and LC–MS/MS

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ABSTRACT

In this work, we present the development and application of a microwave assisted extraction followed by liquid chromatography–tandem mass spectrometry methodology (MAE-LC–MS/MS) for the determination of various estradiol-mimicking compounds in sewage sludge samples. For the purification of the MAE extracts, we have employed a solid phase extraction (SPE) clean-up procedure, previously optimised. The entire method provides recoveries between 71.7% and 103.1%, with relative standard deviation lower than 11.1% and limits of detection ranging from 0.6 to 3.5 ng g^{-1}. The developed method was applied to samples from three wastewater treatment plants (WWTPs) located in Las Palmas of Gran Canaria (Spain), two of which had a conventional activated sludge treatment (AST), whereas the third treatment plant had an advanced membrane bioreactor treatment (MBR). All of the analytes in the study, including (nonylphenol (NP), octylphenol (OP), and some of their ethoxylated chains AP$_n$EOs ($n \leq 7$), 17β-estradiol (E2), estriol (E3), 17α-ethynylestradiol (EE) and bisphenol-A (BPA)), were found in almost all samples in concentrations ranging from 0.9 to 710.2 ng g^{-1}.

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1. Introduction

Endocrine systems involve complex mechanisms that regulate and coordinate bodily functions and maintenance of homeostasis. Secretory glands release hormones, which act as chemical messengers that interact with receptors in target cells to trigger specific responses, such as the induction of hormone responsive genes [1]. Therefore, there are various target organ sites in which an environmental agent could disrupt endocrine function, especially in the early stages of life [2].

All humans and animals can excrete oestrogens from their bodies, which are introduced into the environment through sewage discharge and animal waste disposal. These compounds may interfere with the normal functioning of endocrine systems, thus affecting reproduction and development in wildlife and humans [3]. In this sense, the steroids of environmental interest due to their endocrine disruption potential are mainly natural oestrogens (17β-estradiol (E2), oestrone (E1) and estriol (E3)) and synthetic oestrogens included in the contraceptive compositions (such as ethynylestradiol (EE) or mestranol (MeEE)).

In addition to steroid hormones, non-steroidal substances of widely diverse chemical structures mimic the oestrogen action. This diversity makes it difficult to predict the estrogenicity of xenobiotics based solely on structural analysis [4]. This is the case of the so-called emerging contaminants, previously unrecognised pollutants that are employed in everyday life [5], such as surfactants (e.g., alkylphenols ethoxylated (AP$_n$EOs)), plasticisers (e.g., bisphenol-A (BPA)), pharmaceuticals, personal care products, and gasoline additives.

Due to the highly lipophilic behaviour of many endocrine disrupting compounds (EDCs), including the more oestrogenic compounds (Table 1), it is expected that these chemicals tend to associate strongly to particulate matter, and therefore to the sediments [6–8]. Thus, the study of these contaminants in wastewater sludge has gained importance in revealing the real fate of EDCs in the environment (it has been reported that over 60% of more estrogenic EDCs are associated to particulate matter and sediments [9–11]); nevertheless, the number of studies involving these contaminants in the solid phase fraction remain much lower than that of the dissolved phase fraction.

The determination of these substances in sewage sludge has importance because wastewater treatment plants (WWTPs) are a sink for organic compounds, and therefore can be viewed as a potential emissary of these substances to the environment if their removal is not complete. In addition, during the past decade, the use of sewage sludge from wastewater treatment plants as an organic amendment has become common practice in Europe to mitigate the low productivity or profitability of several agriculture soils [12], which facilitates the "arrival" of these pollutants to humans through the food chain [13].

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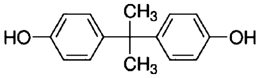
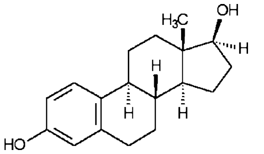
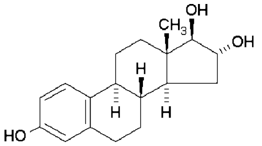
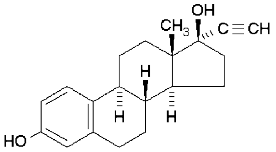
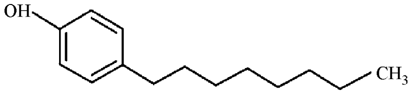
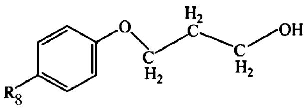
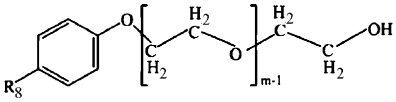
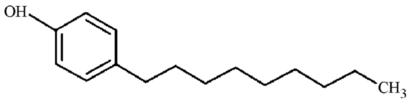
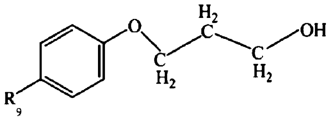
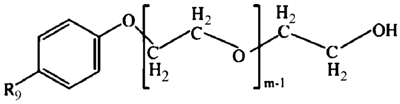
Table 1
Physiochemical properties of the compounds investigated.

Chemical name	Chemical structure	Molecular weight	Water solubility[a] (mg L^{-1} at 20 °C)	Log K_{ow}[a]
Bisphenol-A (BPA)		228.0	120	3.32
17β-Estradiol (E2)		272.4	13	3.94
Estriol (E3)		288.4	13	2.81
17α-Ethynylestradiol (EE)		296.4	4.8	4.15
4-Octylphenol (OP)		206.0	12.6	4.12
Octylphenol monoethoxylate (OP_1EO)		250.0	8.0	4.10
Octylphenol polyethoxylate ($OP_{2-7}EO$)		–	–	–
4-Nonylphenol (NP)		220.0	1.57	4.48
Nonylphenol monoethoxylate (NP_1EO)		264.0	3.02	4.17
Nonylphenol polyethoxylate ($NP_{2-7}EO$)		–	–	–

[a] Data taken from Refs. [6,7].

The main objectives of this work are the development and application of a simple, rapid and sensitive methodology for the determination of various estradiol-mimicking compounds in sewage sludge samples (Table 1). The method was applied to samples collected from three WWTPs located on the island of Gran Canaria (Spain). Two of the WWTPs had a conventional activated sludge treatment (AST), whereas the third treatment plant had an advanced membrane bioreactor treatment (MBR).

For the extraction of analytes from the sludge samples, we employed a microwave assisted extraction (MAE) technique followed by a solid phase extraction (SPE) clean-up step. To our knowledge, there are only a few studies that employ this technique for the extraction of a mixture of EDCs in this type of sample (e.g. [14,15]), despite that this method offers high extraction efficiencies that employ low volumes of organic solvents and short extraction times. The detection and quantification of analytes was made by high performance liquid chromatography with a triple quadrupole mass spectrometer (LC–MS/MS) detection system, which facilitated the correct and unambiguous identification of each analyte with increased sensitivity.

2. Experimental

2.1. Chemicals and reagents

All of the IGEPAL mixtures and standards were acquired from Sigma–Aldrich (Madrid, Spain). Individual compounds were used as standards (≥98% of purity) for short ethoxylated chains AP_nEOs ($n \leq 2$), 17β-estradiol, estriol, 17α-ethynylestradiol and bisphenol-A. The stock solutions (1000 μg mL^{-1}) of alkylphenols, steroidal hormones, and bisphenol-A were prepared by dissolving appropriate amounts of the commercial products in methanol and then storing the solutions in glass-stoppered bottles at −18 °C prior to use. Nonylphenol monoethoxylate, nonylphenol diethoxylate, octylphenol monoethoxylate and octylphenol diethoxylate were directly purchased in stock solutions (1 mL) at 10 μg mL^{-1} in acetone and were also stored at −18 °C.

Long-chain AP$_n$EOs ($n \geq 3$) were only available in technical mixtures. IGEPAL CO210 and CO520 contained a range of NP$_n$EO oligomers between 3 and 7 ethoxy units (EO), whereas IGEPAL CA210 and CA520 contained the same EO range of OP$_n$EO oligomers. Stock solutions (1000 μg mL^{-1}) of long-chain alkylphenolic ethoxylated surfactants were also prepared by dissolving appropriate amounts of each mixture into methanol and were stored in glass-stoppered bottles at −18 °C.

LC–MS-grade methanol and water, used to dissolve the standards and to prepare the mobile phases, were purchased from Panreac Química (Barcelona, Spain). HPLC-grade glacial acetic acid and ammonium acetate were used to prepare the mobile phase and were obtained from Scharlau Chemie S.A. (Barcelona, Spain). Ultra-high-quality water, obtained by a Milli-Q (Millipore, Bedford, MA, USA) water purification system, was used in the solid phase extraction protocol and to dilute samples. The 0.45-μm syringe-driven filter employed for the purification of the extract solution was provided by Scharlau Chemie S.A. (Barcelona, Spain).

The cartridges (6 mL) employed in this study were Sep-Pak Vac C$_{18}$ (500 mg) from Waters (Milford, MA, USA). A Varian Vac Elut 20 SPE Manifold (Varian Inc., CA, USA) coupled to a Sartorius vacuum pump was used for the extractions.

2.2. Instrumentation

Analysis was performed by reversed phase liquid chromatography coupled to a triple quadrupole mass spectrometer equipped with an electrospray interface (LC–ESI-MS/MS). The apparatus was composed of a Varian 320-MS TQ Mass Spectrometer (Varian Inc., CA, USA) equipped with a Varian HPLC system consisting of a binary pump, autosampler and temperature controlled column compartment. The microwave oven used for the extraction was a multiwave with a 6 EVAP rotor and 6 MF100 vessels (Anton Paar, Graz, Austria).

2.3. Sample collection

All samples were taken from three WWTPs located in the northeast of Gran Canaria Island (Spain), which is the most populated region of the island and hosts more than half a million people, with a population density that exceeds 800 persons km^{-2} [16,17]. In addition, much of the small industrial activity on the island was concentrated in this area.Two of the three treatments plants in the study had a conventional activated sludge treatment, whereas the third presented an advanced membrane bioreactor treatment. Sludge samples from the AST plants were taken after they are dewatered from the drying tanks, whereas samples from the MBR plant were taken directly from the output of the centrifuge employed for dewatering the sludge.

Samples were collected quarterly from July 2009 to July 2010 from the first AST plant (AST1) and bimonthly from July 2010 to January 2011 from the second AST (AST2) plant and MRB plant. Once collected, the samples were placed in glass-stoppered flasks and were stored at −18 °C prior to their analysis.

2.4. Preparation of spiked samples

Raw sludge samples (blanks), directly taken from the sludge thickener output of AST1, were spiked with each solution of IGEPAL mixtures and individual standards in methanol to obtain a final concentration of 10 and 100 ng g^{-1} of each analyte (quantification and quality control). Then, the samples were stirred to homogenise and air-dried for 12 h in the dark at room temperature. These sludge samples were previously analysed to verify that the samples did not contain any quantity of the analytes in the study. This procedure was similar to those employed by other authors [18,19].

2.5. MAE–SPE procedure

For the MAE procedure, 1 g of the spiked sludge was transferred to the polytetrafluoroethylene (PTFE) vessels. Then, 5 mL of extractant agent (methanol) was added to the sample, and the vessels were appropriately closed and placed symmetrically in a rotor. Once the rotor was placed in the microwave oven, a power of 300 W for 10 min duration was used for the satisfactory extraction of all analytes, including the more hydrophobic compounds. Under these conditions, the vessels reached a temperature close to the solvent boiling point due to the high dielectric constant ($\varepsilon' = 32.6$) and dissipation factor ($\tan\delta = 0.64$) of methanol [20].

After the completion of the extraction process, the vessels were cooled for 10 min in the presence of the microwave fan and then for another 10 min at room temperature outside the microwave oven before the vessels were opened. The extract solutions were filtered through 0.45-μm syringe filters and were diluted with an optimised volume of Milli-Q water. Before the SPE clean-up step was performed, the diluted extract was vigorously shaken for 5 min to ensure a well-mixed solution.

The SPE protocol employed in this work had been previously optimised by our research group [21]. In summary, Waters Sep-Pak C$_{18}$ cartridges were activated with 3 × 5 mL of methanol and 3 × 5 mL of Milli-Q water. Samples were loaded at a flow rate of ~10 mL min^{-1}, and immediately after this process, the cartridges were washed with 2 × 5 mL of a water/methanol 9/1 (v/v) solution and dried under vacuum for 5 min. Then, the retained analytes were eluted with 2 × 1 mL of methanol at a flow rate no longer than 1 mL min^{-1}. The extracts obtained were placed in a glass vial until ready for analysis.

2.6. LC–ESI-MS/MS analysis

2.6.1. Chromatographic conditions

Chromatographic separation was performed on a Pursuit XRs Ultra-C$_{18}$ reversed phase column (2.8 μm particle size, 50 mm × 2 mm) from Varian Inc. (CA, USA). The mobile phase consisted of water (solvent A) and methanol (solvent B), both with 0.1% (v/v) glacial acetic acid and 15 mM ammonium acetate to promote the ionisation of each analyte into the electrospray ionization (ESI) and to form ammonium adducts for the compounds that would be detected in the ESI+ mode. Gradient elution consisted of a 30:70 (v/v) methanol:water solution for 9 min, followed by an increase in methanol to 100% over 10 min. The injection volume was 10 μL and the flow rate was 200 μL min^{-1} for 10 min. The temperature in the column compartment was set to 40 °C.

2.6.2. MS/MS conditions

Multiple reaction monitoring (MRM) parameters were optimised for the subsequent quantitative analysis. Precursor ions included $[M+NH_4]^+$ for AP$_{1-7}$EOs in positive ion mode (ESI+) and $[M-H]^-$ for APs, steroidal hormones, and BFA in negative ion mode (ESI−). This procedure was conducted using a 1 mL syringe pump (Hamilton Company, Reno, NV, USA), employing a continuous flow rate of 20 μL min^{-1}. Each standard or mixture was prepared as 10 mg L^{-1} in methanol. Using the Hamilton syringe, 0.1 mL of each solution was taken up and the remaining 0.9 mL of the syringe volume was filled with the mobile phase. The composition of the mobile phase depended on the ionisation of each analyte in ESI.

Table 2
Characteristic of ESI/MS/MS parameters for each compound studied.

Compound	m/z Precursor[M+NH$_4$]$^+$	m/z Precursor[M−H]$^-$	Cone[a](V)	Fragment ions[b]	Ion mode
NP$_1$EO	282.3	–	30	265.3 (6)[c], 127.1 (8)	ESI+
NP$_2$EO	326.3	–	30	183.1 (9)[c], 121 (20)	ESI+
NP$_3$EO	370.3	–	32	353.3 (8)[c], 227.1 (11)	ESI+
NP$_4$EO	414.5	–	32	397.4 (8)[c], 271.2 (13.5)	ESI+
NP$_5$EO	458.6	–	48	441.5 (12)[c], 315.2 (15.5)	ESI+
NP$_6$EO	502.6	–	52	485.5 (13.5)[c], 359.3 (17)	ESI+
NP$_7$EO	546.7	–	56	529.6 (14.5)[c], 403 (18)	ESI+
OP$_1$EO	268.1	–	30	251.1 (6)[c], 113 (7.5)	ESI+
OP$_2$EO	312.3	–	30	183.0 (9.5)[c], 121 (19.5)	ESI+
OP$_3$EO	356.4	–	32	339.4 (8)[c], 227.1 (14)	ESI+
OP$_4$EO	400.4	–	32	383.4 (10)[c], 271.2 (14)	ESI+
OP$_5$EO	444.5	–	48	427.5 (12)[c], 315.2 (15.5)	ESI+
OP$_6$EO	488.5	–	52	471.5 (13.5)[c], 359.3 (17)	ESI+
OP$_7$EO	532.8	–	52	516.6 (15)[c], 403.3 (18)	ESI+
NP	–	218.7	−64	105.7 (20.5)[c]	ESI−
OP	–	204.7	−72	134 (16.5)[c], 106 (19.5)	ESI−
BPA	–	226.7	−60	211.7 (17.5)[c]	ESI−
E2	–	271.1	−60	183.5 (14.5)[c], 145.2 (20.5)	ESI−
E3	–	287.2	−67	171.0 (16.5)[c], 145.2 (19.5)	ESI−
EE	–	295.3	−71	159.5 (17.5)[c], 145.2 (21.5)	ESI−

[a] Capillary voltage.
[b] Collision energy in brackets.
[c] Fragment ion used for quantitation (MRM).

Ionisation in the ESI source was achieved using nitrogen as a nebuliser and drying gas. For the optimisation of the syringe pump injections for MS/MS, the housing and desolvation temperature were set to 60 °C and 250 °C, respectively. However, to obtain a strong signal for each analyte, the desolvation temperature was set to 200 °C during the first 4 min and was increased linearly to 350 °C until the end of the chromatographic run. The drying and nebulising gas pressures were fixed at 30 psi and 65 psi, respectively. The capillary voltage was set to 4.5 kV in positive mode (ESI +) and −3 kV in negative mode (ESI−). The shield voltage was maintained at −600/600 V (ESI+/ESI−) and the cone voltage was optimised for each individual compound (Table 2). Collision induced dissociation (CID) was conducted with argon as the collision gas at a fixed pressure of 2 mTorr. The fragment ions obtained for each compound and the collision potential are displayed in Table 2.

2.7. Statistical analysis

The experimental designs for the optimisation of MAE conditions were performed using the Statgraphics Plus software, version 5.1 (Manugistic, Rockville, MD, USA). The statistical analysis of the partial and bivariate correlations was done using the SPSS 17.0 program.

3. Results and discussion

3.1. EDCs in sewage sludge matrices: Backdrop of extraction techniques

Classical approaches for the extraction of EDCs in solid matrices were major based on Soxhlet extraction and steam-distillation, employed almost exclusively in the 1980s and 1990s with polar or non-polar solvents. However, both techniques make the analysis procedure excessive time consuming (up to 48 h) and moreover, require large amounts of hazardous organic solvents (between 50 and 300 mL) [21].

With the perspective to palliate these shortcomings presented by the classical methodologies, new extraction approaches have been developed and applied to the extraction of this kind of organic pollutants. Among all of the techniques that have been employed, one of the most widely used for the EDCs isolation from sewage sludge matrices is the ultrasonic extraction [22–26]. The most common solvents employed for the extraction of these substances by using ultrasonic irradiation are acetone, hexane, dichloromethane, water and mixtures of them in different proportions (see Table 3).

Although sonication is considerably faster than Soxhlet extraction, it also requires relative large volumes of toxic and expensive organic solvents (30–60 mL). Therefore, more efficient techniques such as PLE (also known as pressurized fluid extraction (PFE) or accelerated solvent extraction (ASE)) [12,27,28], microwave assisted extraction [15] or, in a lesser extent, supercritical fluid extraction (SFE) [29,30], have also been reported for the extraction of EDCs in this kind of samples.

PLE offers a great reduction in solvent consumption (between 15 and 30 mL) and provides a faster sample processing and a higher level of automation than the techniques cited above [27,28]. PLE also offers the advantage that only two variables need to be optimised: extraction time and temperature, since the solvents chosen for PLE can be the same as that used in the Soxhlet extraction or sonication extraction [28].

For its part, SFE presents several advantages such as rapid extraction, low solvent requirement, low cost and high extraction efficiencies. Among all the solvents used in SFE, pure CO_2 is the most popular due to its low critical properties, chemical inertness, low toxicity and cost, and its ability to solvate a wide range of organic compounds including those having high molecular mass [31]. Nevertheless, low recoveries for polar compounds (e.g. E3, BPA or long-chain APEOs) have been shown with this solvent. The lack of extraction efficiency for these solutes can be overcome by addition of modifiers or co-solvents to the carbon dioxide. Thus, methanol or water is the most commonly used solvent modifier in SFE [20,29].

During the last decade, microwave energy has been investigated and widely applied in analytical chemistry to accelerate sample digestion, to extract analytes from different matrices and in chemical reactions [31]. MAE is an efficient extraction technique for solid samples, which is applicable to thermally stable compounds. Since its development, MAE has became a viable alternative to conventional techniques due to it presents substantial improvements over other sample preparation techniques such as shorter extraction time, lower amount of solvent and, principally, the ability to perform multiple extractions simultaneously [15].Obviously, this ability is associated with small problems of repeatability and

Table 3
Methods for the determination of endocrine-disrupting compounds (EDCs) in solid samples.

Compounds	Matrix	Sample pretreatment	Extraction technique	Characteristics (solvent)	Recoveries (%)	LOD ($ng\,g^{-1}$)	Instrumental Analysis	Refs.
NP, NPEOs	Sewage sludges	1% Formaldehyde	Soxhlet	MeOH	66–88	–	LC–FD	[15]
NP, $NP_{1-2}EOs$	WWTP sludges	Lyophilization, sieving	Ultrasonic	Hexane	93–117	189–751	GC–MS	[22]
Estrogens	Sewage sludges and sediments	Freeze-drying	Ultrasonic	MeOH; acetone	57–121	0.6–1.2	GC–MS/MS	[23]
NP, $NP_{1-2}EOs$, BPA	WWTP sludges	Homogenized, dried (60 °C)	Ultrasonic	MeOH:H_2O mixture	84–107	1.9–14	GC–MS	[24]
Estrogens	Sewage sludges	Lyophilization, sieving	Ultrasonic	MeOH; acetone	65–107	0.6–9.1	LC–DAD–FD	[25]
Estrogens	Sludges, sediment, agriculture soil	Freeze-drying	Ultrasonic	Acetonitrile:ethylacetate (5:1)	60–109	2.8–16.8	LDTD–MS/MS	[26]
APs, $AP_{1-15}EOs$	Amended soil	Air-drying, sieving	PLE	Acetone:hexane (1:1)	36–110	0.3–30	LC–MS	[12]
Estrogens	Sewage sludges	Lyophilization	PLE	MeOH:acetone (1:1), MeOH:H_2O (1:1)	81–100	0.15–26	LC–MS/MS	[27]
Estrogens	Sewage sludges	Freeze-drying, sieving	PLE	MeOH:H_2O (80:20)	86–126	0.3–1.5	LC–MS/MS	[28]
NP, NPEOs	Sewage sludges	1% Formaldehyde	MAE	DCM–MeOH (30:70)	61–91	1820–2860	LC–FD	[15]
EDCs	Sewage sludges	Air-drying, sieving	SFE	CO_2	–	–	GC–MS; LC–FD	[29]
$NP_{1-17}EOs$	Sewage sludges	Air-drying, sieving	SFE	CO_2	86–105	500	LC–FD	[30]

Abbreviations: DAD, diode array detection; DCM, dichloromethane; FD, fluorescence detection; GC, gas chromatography; LDTD, laser diode thermal desorption; MeOH, methanol.

reproducibility, which are usually solved with the proper use of the equipment, such as placing the duplicates symmetrically in the rotor, filling the empty space into the rotor with blanks containing the same amount of sludge and solvent (to maintain the same temperature in all vessels), making sure that the vessels are tightly sealed to prevent loss of solvent, etc.

Table 3 summarises some methodologies for the extraction and determination of EDCS employing the techniques cited above.

3.2. *Optimisation of the MAE–SPE procedure*

To carry out the optimisation of the MAE–SPE parameters, we opted for the utilisation of a factorial design strategy. The application of this statistical method allows for a decrease in the number of assays required and obtain the influence of each variable on the extraction process and the variable correlations to each other [31,32]. The performance of the factorial design was conducted in two different stages, a screening phase to determine the influence of each variable on analyte recovery and a response surface design to achieve the optimum values of the variables, which have a greater influence on the extraction efficiency.

3.2.1. *Preliminary assays*

The SPE protocol employed in this study had been previously optimised [33]. However, prior to beginning the optimisation of the parameters that affect the microwave extraction (extractant volume, irradiation time and power), it was necessary to evaluate the conditions of the SPE clean-up and pre-concentration step.

First, we checked the effects of the dilution of the MAE methanol extract in several methanol/Milli-Q water relationships (1/2.5, 1/5, 1/10, 1/20 and 1/50 (v/v)). These methanol extracts were obtained under the following conditions: 10 mL of methanol was added to 1 g of spiked samples (10 $\mu g\,g^{-1}$) placed in the PTFE vessels, and then, a power of 200 W was employed for 10 min. These results were compared to those obtained by the spiked Milli-Q water sample (100 mL) at the same concentration.

Peak areas obtained for all methanol/Milli-Q ratios were similar to those observed for the spiked Milli-Q water sample for almost all compounds. However, low retention efficiencies in the SPE cartridges were observed for $AP_{3-7}EOs$ when the ratios of 1/2.5 and 1/5 (v/v) were studied. From 1/10 to 1/50 (v/v) dilution ratios, the peak areas obtained for the ethoxylated compounds were similar to those observed for the sample of Milli-Q water, suggesting that in these dilution levels, there was no appreciable competition between the methanol fractions and the solid phase employed. This phenomenon could be explained if an increase in polarity of the alkylphenolic ethoxylated chains with the increasing number of ethoxylated units is considered [31]. As a result, a methanol/water ratio of 1/20 (v/v) was chosen for the dilution of the MAE extract.

3.3. *Optimisation of microwave assisted extraction parameters*

3.3.1. *Screening phase*

This study was performed on 1 g of sample (containing 500 $ng\,g^{-1}$ of each analyte) that were duplicated and randomised. In this stage of the MAE optimisation, a 2^3 factorial design (three variables at two levels) has been employed. The experimental parameters under study were the extractant volume (5 and 10 mL), irradiation time (5 and 15 min) and power (100 and 300 W). These variables were selected because they have a greater influence on the extraction of the analytes. Irradiation time and power have a direct influence on the energy transfer to the sample, whereas the extractant volume is important because, in many instances, the amount of analyte extracted is proportional to the volume employed. By applying this factorial design, it was possible to determine the different correlations among variables and their influence on recovery (Table 4).

As seen in Table 4, the variable that individually exerts the greatest influence on analyte recoveries is the extraction time, closely followed by the extraction power. By contrast, the volume of methanol used in the extraction appears to have little influence on the analyte recoveries. This behaviour was observed in almost all of the analytes in the study because, in a few cases, the extraction power appears to exert a greater influence over the extraction efficiency than the extraction time (i.e., nonylphenol (NP) and 17α-ethynylestradiol (EE)). Due to the low influence of the methanol volume on the recoveries obtained, we select the lowest volume of solvent, which was 5 mL. Thus, we achieve a higher level of pre-concentration and reduced the amount of solvent employed. Lower methanol volumes were not estimated due to the difficulties in removing the solvent from the PTFE vessels for the subsequent filtration through the 0.45 μm filters.

The strongest correlations for all the compounds were achieved for the interaction between the extraction time and the microwave

Table 4
Partial and bivariate correlations between the variables under study. The maxima correlations are +1 and −1.

	Volume (mL)	Power (W)	Time (min)	Volume × power	Volume × time	Time × power
E3	−0.017	0.393	0.519	−0.017	−0.020	0.905
BPA	0.125	0.318	0.621	−0.123	−0.041	0.579
E2	−0.156	0.692	0.537	−0.031	−0.028	0.802
EE	0.059	0.401	0.773	−0.100	−0.120	0.567
OP_1EO	0.206	0.505	0.533	−0.131	0.025	0.551
OP_2EO	0.104	0.412	0.801	−0.006	−0.019	0.721
OP_3EO	0.003	0.499	0.845	−0.128	−0.101	0.702
OP_4EO	0.172	0.204	0.427	0.056	−0.074	0.626
OP_5EO	0.220	0.427	0.444	−0.082	−0.078	0.591
OP_6EO	0.100	0.412	0.620	−0.023	−0.031	0.826
OP_7EO	0.058	0.371	0.672	−0.150	0.021	0.755
OP	0.238	0.712	0.902	−0.033	−0.100	0.777
NP_1EO	0.193	0.201	0.638	0.099	−0.043	0.648
NP_2EO	−0.020	0.301	0.522	−0.110	−0.018	0.650
NP_3EO	0.057	0.401	0.609	−0.045	−0.031	0.783
NP_4EO	0.150	0.355	0.492	0.162	−0.126	0.903
NP_5EO	0.201	0.241	0.401	0.002	−0.010	0.712
NP_6EO	−0.033	0.425	0.631	−0.140	0.099	0.735
NP_7EO	0.201	0.320	0.669	−0.099	−0.029	0.605
NP	0.098	0.801	0.767	−0.152	−0.014	0.889

power (Table 4). These two variables are going optimised together using another 3^2 factorial design with the duplication of central points, where the recovery is the dependent variable.

3.3.2. *Response-surface design*

A 3^2 factorial design with two variables (power and irradiation time) and three levels (5, 7.5 and 10 min and 100, 200 and 300 W, respectively) have been employed to optimise the two parameters that showed the greatest influence in the analyte recoveries: power and extraction time. This design consisted of 12 randomly distributed runs distributed into three blocks with a central point for each block. All runs were performed in duplicate.

Fig. 1 shows the response surface obtained for nonylphenol. This figure has been made using the polynomial fits of the results obtained by using the 3^2 factorial design. The results were similar for the remaining compounds, although there exist certain nuances for some of these.

By studying the response surface of all of the compounds, it is observed that, with increasing the extraction power and time, the peak area of analyte extracted increase. However, this increase was more pronounced for less polar substances (“higher slope”) than for the more polar compounds (“lower slope”). This behaviour correlate well with the physico-chemical characteristics of each analyte in the study because the more polar compounds are less bounded to the sludge than the less polar; therefore, they are more easily removed from the matrix. This explains why, by using “soft” conditions (100 W and 5 min), relatively good recoveries (>65%) were obtained for BPA, E3 and long chain AP_nEOs ($n \geq 5$), whereas, in the same conditions, recoveries lower than 40% were obtained for the less polar compounds (NP, OP and EE). Higher times and powers were not employed because of the decreasing recovery for some compounds, probably due to volatilisation or degradation of these analytes.

Summarising, the higher recoveries of the MAE–SPE method were obtained with 1 g of sludge sample at 10 min of time, 300 W of extraction power, 5 mL of methanol as extractant solvent and a dilution level of 1/20 methanol/Milli-Q (v/v). Fig. 2 shows a flow scheme of the extraction and determination procedure of the analytical method. Fig. 3 shows a multiple reaction monitoring chromatograms containing all of the compounds under study. All transitions shown correspond to a spiked sample (100 ng g^{-1}) after the whole MAE–SPE process.

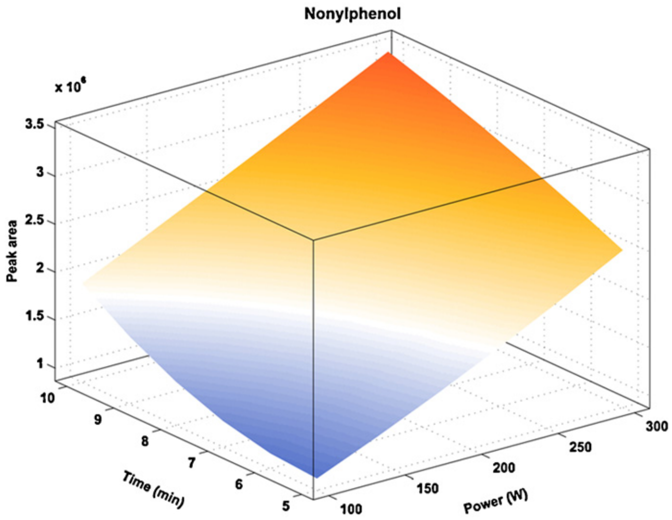


Fig. 1. Response surface for the effect of power and time on the extraction of nonylphenol (NP).

3.4. *Matrix effects*

In the analysis of WWTPs sludge samples, the matrix composition, despite the high sensitivity and low chemical noise in LC–MS/MS systems, exerts a great influence on the analyte signal. Low analyte signal could be obtained as the result of co-eluting compounds that impair ionisation [34].

To explain the phenomenon in sludge samples, we evaluated the relative signal suppression caused by the matrix effects by using the algorithm published by Vieno et al. [35] (Eq. (1)).

$$\frac{A_s - (A_{sp} - A_{usp})}{A_s} \times 100 \qquad (1)$$

where A_s corresponds to the peak area of the analyte in pure standard solution, A_{sp} corresponds to the peak area in the spiked matrix extract, and A_{usp} corresponds to the matrix extract of a real sample. Relative signal suppressions, ranging from 18% to 31%, were obtained. Greater signal suppression was detected for hydrophobic compounds, especially for APs, $AP_{1-2}EO$, E_2 and EE. The results obtained are in agreement with those reported in similar studies [36].

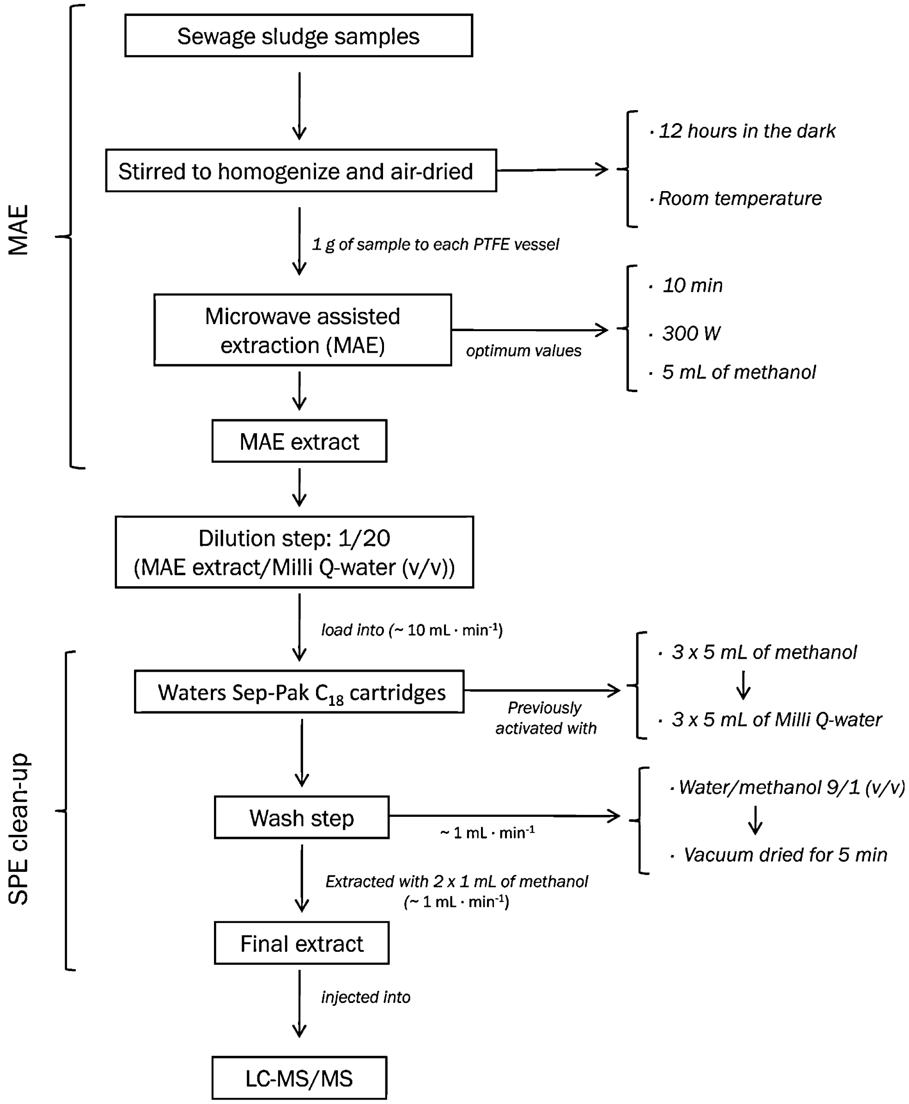


Fig. 2. Flow scheme of the optimisation protocol of the analytical method.

3.5. Quantification and quality control

The performance of the method was evaluated by the estimation of the linearity, sensitivity, precision and recovery. Calibration curves were obtained for spiked raw sludge samples at six concentration levels of each compound, using duplicate analysis of each one. The range of linearity was between 1 and 750 ng g^{-1}. The correlation coefficients were higher than 0.992 in all cases.

Limits of detection (LOD) and quantification (LOQ) were defined and determined to equal the concentration of analyte that yielded a signal to noise ratio of 3 and 10, respectively. Both limits were low enough to determine the presence of all EDCs under consideration in real sewage sludge samples (Table 5). Relative standard deviations (RSDs) and recoveries were calculated at two different levels (10 and 100 ng g^{-1}) from six spiked raw sludge samples at the same concentration. As seen in Table 5, recoveries were greater than 71% for all of the compounds, and RSDs were lower than 12% in all cases.

These results were similar to those obtained by other advanced extraction methodologies, such as pressurised liquid extraction PLE [15,37] or supercritical-fluid extraction SFE [38]. In comparison to the conventional extraction methodologies, our results also showed similar results in terms of recoveries and RSDs [15,23,39] but with much lower solvent requirements and analysis time.

3.6. Determination of EDCs in sewage samples

The developed method was applied in routine analysis to samples from three WWTPs of Gran Canaria Island (Spain). Table 6a and b list the concentrations of all the compounds included in the study from each of the WWTPs and every sampling conducted.

In all of these samples, alkylphenol mono- and di-ethoxylates were invariably the most abundant ethoxylates, with levels (on dry weight basis) ranging from 193.7 to 710.2 ng g^{-1}, and from 57.9 to 279.3 ng g^{-1} for the NP$_{1-2}$EO and OP$_{1-2}$EO, respectively. However, for the increased number of ethoxylated chains, the concentrations observed for each of the alkylphenolic ethoxylated compounds significantly decreased. Thus, compounds containing more than 5 ethoxy units were only found in some samples. These results are in good agreement with previous studies [40].

The primary degradation product of AP$_n$EOs, nonylphenol and octylphenol (OP), were detected in almost all samples in concentrations ranging from 6.3 to 110.1 ng g^{-1} and from 2.5 to 35.4 ng g^{-1}, respectively. These concentrations are considerably lower than those reported in other publications [15,40]. This phenomenon may be explained by the absence of large industrial areas in which these surfactants are used. Another observation was the higher concentrations observed for NP and NP$_n$EOs with respect to those observed for OP and their ethoxylated. This pattern, reported in

Table 5
Analytical parameters for the determination of EDCs under study using MAME-SPE procedure.

Compound	10 ng g^{-1} [a]		100 ng g^{-1} [a]		LOD ng g^{-1} [b]	LOQ ng g^{-1} [c]
	Recovery (%)	RSD[d] (%)	Recovery (%)	RSD[d] (%)		
E3	99.7	7.1	102.1	5.1	1.2	4.0
BPA	102.5	9.5	92.4	11.1	0.7	2.3
E2	75.9	5.4	71.7	5.1	1.5	5.0
EE	72.5	7.5	78.2	3.9	0.9	3.0
OP_1EO	82.6	6.0	88.1	5.1	2.3	7.6
OP_2EO	79.3	6.3	82.2	6.2	2.4	7.9
OP_3EO	88.8	9.6	83.9	8.8	3.1	10.2
OP_4EO	75.1	3.8	80.9	3.1	1.5	5.0
OP_5EO	95.3	10.0	90.4	6.7	0.8	2.6
OP_6EO	98.1	4.0	97.2	7.0	0.9	3.0
OP_7EO	92.9	5.8	97.0	2.7	1.1	3.6
OP	80.0	10.2	85.1	6.6	2.2	7.3
NP_1EO	87.1	6.1	90.0	4.9	3.3	10.9
NP_2EO	82.1	6.1	81.1	9.1	0.8	2.6
NP_3EO	88.6	2.4	87.9	9.5	0.8	2.6
NP_4EO	84.5	5.7	80.1	7.1	0.6	2.0
NP_5EO	92.9	9.1	90.1	8.4	1.2	4.0
NP_6EO	100.2	7.5	98.3	6.3	1.5	5.0
NP_7EO	99.1	6.7	103.1	5.9	0.8	2.6
NP	85.1	4.8	77.7	4.1	3.5	11.6

[a] $n = 6$.
[b] Limit of detection.
[c] Limit of quantification.
[d] Relative standard deviation.

each sampling and in each WWTP included in the study, is perfectly consistent with the overall production of these substances, given that approximately 80% of total alkylphenolic ethoxylates production employ nonylphenol as raw material [2].

Regarding the steroid hormones, it has been observed that the largest concentration detected in all samples corresponded to 17β-estradiol. 8This natural oestrogen has been found in all of the samples in levels varying from 2.2 to 95.7 ng g^{-1}. In turn, synthetic oestrogen 17α-ethynylestradiol was also found in almost all of the samples but in concentrations slightly lower than those observed for E2. Estriol has also been detected in a few samples and in concentrations close to the limit of detection. As previously observed [4], E3 tends to be less abundant due, at least in part, to its lower affinity to solids, despite it is known to be the main metabolite of the highly abundant E2. Thus, the concentration observed for E3 range from 1.8 ng g^{-1} to 9.5 ng g^{-1}. The levels of bisphenol-A in the samples were from 1.4 ng g^{-1} to 54.9 ng g^{-1}. Moreover, BPA was present in a large number of samples, particularly in all the samples from AST plants and only in one sample from MBR plant.

The results obtained in this work correlated well with those described by Ying and Kookana [6]. In that work, they studied the sorption coefficients of all of the EDCs studied in this work,

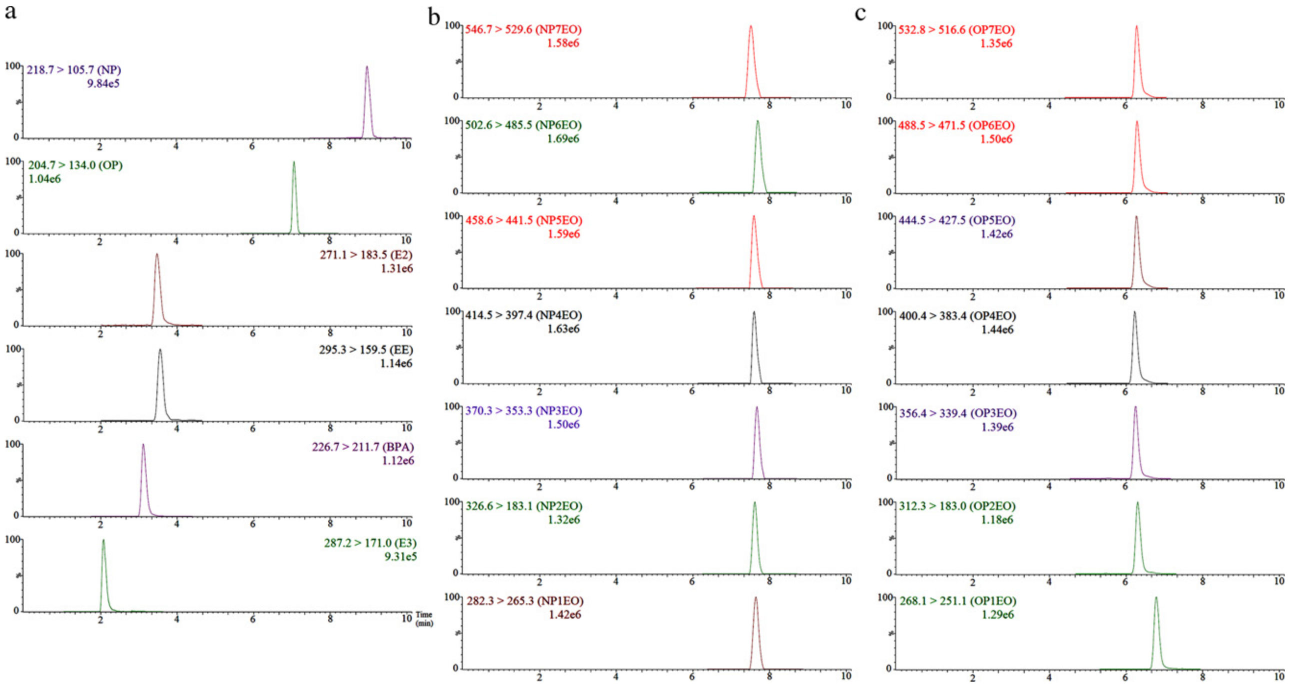


Fig. 3. MRM chromatograms of a spiked sample (100 ng g^{-1}) with all analytes after MAE–SPE process: (a) BPA, NP, OP and oestrogens; (b) nonylphenol polyethoxylated compounds; (c) octylphenol polyethoxylated compounds.

Table 6
Concentrations levels of each analyte in: (a) AST1 and (b) AST2 and MBR treatment plants.

AST1[a]					
Compounds	July 2010	October 2009	January 2010	April 2010	July 2010
NP	59.7 ± 27.1	110.1 ± 4.5	71.9 ± 3.9	99.7 ± 7.7	72.1 ± 13.7
$NP_{1-2}EO$	679.2 ± 50.3	710.2 ± 51.2	512.4 ± 70.5	700.5 ± 60.1	582.6 ± 19.8
$NP_{3-4}EO$	330.2 ± 12.8	250.6 ± 30.0	110.6 ± 17.5	235.8 ± 12.5	311.8 ± 61.7
$NP_{5-7}EO$	30.2 ± 0.9	91.5 ± 9.9	10.2 ± 1.0	80.9 ± 3.4	51.0 ± 19.0
OP	18.0 ± 2.6	35.4 ± 5.9	9.5 ± 0.7	33.8 ± 15.0	16.5 ± 5.9
$OP_{1-2}EO$	237.3 ± 9.1	279.3 ± 13.5	137.3 ± 18.3	112.7 ± 20.2	202.7 ± 5.3
$OP_{3-4}EO$	200.9 ± 6.9	110.1 ± 7.0	20.3 ± 6.5	73.9 ± 6.2	147.0 ± 27.0
$OP_{5-7}EO$	<LOD[b]	45.1 ± 4.7	<LOD[b]	<LOD[b]	<LOD[b]
BPA	11.6 ± 2.0	27.2 ± 3.7	11.6 ± 0.7	54.9 ± 8.1	19.9 ± 0.8
E2	75.3 ± 17.5	55.3 ± 6.0	75.8 ± 12.8	92.7 ± 11.2	100.4 ± 6.0
E3	<LOD[b]	<LOD[b]	<LOD[b]	6.1 ± 0.4	<LOD[b]
EE	18.6 ± 2.0	22.3 ± 5.1	18.6 ± 5.5	40.1 ± 9.0	21.5 ± 1.5

Compounds	AST2[a]				MBR[a]			
	July 2010	September 2010	November 2010	January 2011	July 2010	September 2010	November 2010	January 2011
NP	19.0 ± 1.5	41.2 ± 6.2	28.5 ± 0.6	30.4 ± 2.7	6.3 ± 0.3	20.1 ± 7.3.5	8.8 ± 7.1	6.9 ± 0.5
$NP_{1-2}EO$	251.9 ± 12.2	527.8 ± 25.5	233.9 ± 17.3	200.7 ± 14.8	221.5 ± 18.3	390.3 ± 40.8	200.3 ± 20.3	193.7 ± 22.0
$NP_{3-4}EO$	165.1 ± 8.6	79.9 ± 0.4	170.0 ± 5.9	95.0 ± 3.0	126.9 ± 9.7	139.0 ± 9.4	78.2 ± 8.8	64.4 ± 21.7
$NP_{5-7}EO$	<LOD[b]	12.6 ± 1.1	44.9 ± 5.3	9.5 ± 0.2	<LOD[b]	35.7 ± 0.4	5.5 ± 1.0	<LOD[b]
OP	4.3 ± 0.2	7.3 ± 0.6	5.5 ± 0.4	10.1 ± 0.3	<LOD[b]	2.8 ± 1.2	7.9 ± 0.6	2.5 ± 0.8
$OP_{1-2}EO$	105.2 ± 0.5	172.4 ± 40.1	90.0 ± 0.8	72.9 ± 2.5	73.9 ± 4.2	65.9 ± 21.5	116.2 ± 12.0	57.9 ± 6.6
$OP_{3-4}EO$	48.3 ± 3.0	90.3 ± 4.7	12.6 ± 9.0	21.8 ± 0.5	<LOD[b]	6.1 ± 0.1	62.5 ± 18.1	11.9 ± 4.1
$OP_{5-7}EO$	<LOD[b]	<LOD[b]	<LOD[b]	<LOD[b]	<LOD[b]	<LOD[b]	<LOD[b]	<LOD[b]
BPA	5.9 ± 2.1	10.1 ± 0.2	1.4 ± 0.1	5.8 ± 0.6	<LOD[b]	1.5 ± 0.6	<LOD[b]	<LOD[b]
E2	23.6 ± 11.5	66.9 ± 3.9	15.2 ± 0.8	18.1 ± 3.1	2.2 ± 0.5	12.9 ± 2.1	4.4 ± 1.1	9.3 ± 1.7
E3	<LOD[b]	9.5 ± 1.0	<LOD[b]	1.8 ± 0.3	<LOD[b]	<LOD[b]	<LOD[b]	<LOD[b]
EE	8.6 ± 0.9	48.1 ± 0.6	3.3 ± 0.1	7.1 ± 0.5	<LOD[b]	2.1 ± 1.3	0.9 ± 0.5	<LOD[b]

[a] Mean and standard deviation of two determinations.
[b] Concentration below of the limit of detection.

concluding that the affinity of these compounds to adsorb on a solid decreased in the following order: NP > OP > EE > E2 > E1 > E3 > BPA. BPA is most mobile, oestrogens are moderately mobile, and alkylphenols are least mobile in the soils. Fig. 4 shows a chromatogram of a real sewage sludge sample from AST 2 (sampling from July 2010).

The concentration level of these xenobiotics in the sludge depends on various factors, such as the discharge of industrial wastewaters in the WWTP influent, the population size of the area being served by the plant, and the type of treatment and sludge employed.

We observed that the highest concentrations from each WWTP in the study were correlated in some way with the volume of wastewater treated by each one. Levels observed in AST1 (20,000 m^3 day^{-1}) were higher than those observed for AST2 (835 m^3 day^{-1}), the later was higher than those observed for MRB (700 m^3 day^{-1}). The higher concentration detected in AST2 with respect to those detected in MRB, despite the similar volume treated and the proximity between both WWTPs (~6 km), leaves us, in the absence of a more detailed study of the nature of water input to each plant and the nature of the sludge employees, with a glimpse of the greater efficiency of removal that can be achieved by the advanced membrane bioreactor treatment.

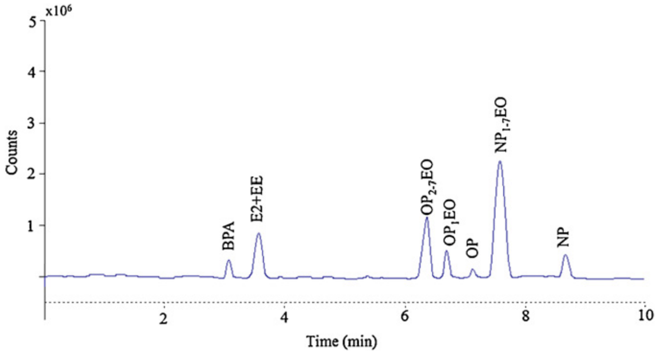


Fig. 4. Total ion current (TIC) chromatogram of a real sewage sludge sample (from AST2 July 2010).

4. Conclusions

In this work, a trace analytical method based on microwave-assisted extraction and SPE clean-up (MAE–SPE) combined with LC–MS/MS was developed for the accurate and precise determination of important EDCs in sewage sludge samples. MAE procedure demonstrate that the proposed method provides a viable alternative to other extraction methodologies in real sewage sludge samples, because it reduces amounts of time and extractant required, thus lowering the cost considerably. The best extraction conditions involve 5 mL of methanol as the extractant solvent, an extraction power of 300 W and an extraction time of 10 min. The optimum values of these parameters were achieved by using a factorial design strategy.

Reasonably low LODs and LOQs were reached through the use of a previously optimised solid phase extraction clean-up and concentration steps. This procedure was a key step for the quantitative determination of the target compounds because it eliminated the interferences present in the sludge samples, thereby reducing the well-known matrix effect and providing a direct path to increase the method sensitivity. Moreover, the use of LC–MS/MS working in multiple reaction monitoring provided the analytical methodology required for identification power to determine the selected EDCs in this kind of sample.

To the best of our knowledge, this is the first work that has reported the concentration levels of these compounds in sewage sludge samples from the Canary Island (Spain). By applying this method to sludge samples collected between July 2009 and January

2011 from three WWTPs of Gran Canaria Island, it was possible to determine the presence of all compounds in almost all or the samples analysed with concentrations on the order of ng g^{-1}. The results showed that the selected EDCs tended to associate with the activated sludge, especially the more hydrophobic compounds, and that these pollutants were not eliminated during the biodegradation processes.

Finally, the results obtained demonstrate that the optimised methodology represents a powerful tool in the evaluation of the behaviour of the EDCs in sewage sludge samples.

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