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ORIGINAL PAPER

Optimisation of stir bar sorptive extraction and in-tube derivatisation–thermal desorption–gas chromatography–mass spectrometry for the determination of several endocrine disruptor compounds in environmental water samples

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Abstract The analysis of organic pollutants in environmental water samples requires a pre-concentration step. Pre-concentration techniques such as stir bar sorptive extraction (SBSE) have gained popularity since they minimise the use of toxic organic solvents and can be considered as green analytical techniques. Similar to other pre-concentration techniques, one of the problems when SBSE is used is the matrix effect, which often occurs during the analysis of environmental water samples such as estuarine or wastewater samples. The present work studied the matrix effect during SBSE coupled to in-tube derivatisation–thermal desorption (TD)–gas chromatography–mass spectrometry for the determination of several endocrine disruptor compounds, such as alkylphenols, bisphenol A, estrogens and sterols, in environmental water samples, after optimisation of the major variables affecting the determination. Variables such as the addition of methanol or an inert salt to the donor phase, the extraction temperature, the volume of the donor phase, the stirring rate and the extraction time were studied during the SBSE optimisation. In the case of the in-tube derivatisation and TD step, the volume of the derivatisation reagent (*N*,*O*-bis(trimethylsilyl)triufloroacetamide with 1% of trimethylchlorosilane (BSTFA+1% TMCS)) and the cryo-focusing temperature were fixed (2 μL and −50 °C, respectively) according to a consensus between maximum signal and optimal operation conditions. Good apparent recovery values (78–124%) were obtained for most of the analytes in Milli-Q water, except for 4-*tert*-octylphenol (4-tOP), which showed apparent recovery values exceeding 100%. Precision (n=4) was in the 2–27%, and method detection limits were in the low nanogrammes per litre level for most of the analytes studied. The matrix effect was studied using two different approaches. On the one hand, Milli-Q water samples were spiked with humic acids, and apparent recovery values were studied with and without correction with the corresponding deuterated analogue. On the other hand, estuarine water and wastewater samples were spiked with known concentrations of target analytes, and apparent recoveries were studied as explained above. In general, the matrix effect could be corrected with the use of deuterated analogues, except for 4-tOP and nonylphenols for which [$^2H_4$]-*n*-nonylphenol did not provide good corrections.

Keywords Endocrine disruptor compounds · Environmental water samples · Stir bar sorptive extraction · Optimisation · Matrix effect

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Introduction

In the complex process of harmonizing laws, directives and actions within the European Union countries, the European Water Framework Directive (WFD, 2000/60) is probably

the most important international legislation introduced for many years in the water management and protection field. This Directive considers water management from a wide perspective, looking for the prevention of any future deterioration of water bodies, as well as the protection and improvement of the state of marine ecosystems, in order to obtain "a good state" of water bodies [1]. According to the WFD, the good state of the aquatic bodies is obtained when the concentration of the priority substances in water, sediments and biota are below the established Environmental Quality Standards (EQSs), which have only been fixed for water. All the European Union member states should implement management plans in their river basins, which include monitoring programmes. However, the WFD does not specify the analytical methods that have to be used, so there is an urgent need to develop monitoring tools and analytical methodology able to provide improved chemical and biological data at a lower cost in order to respond to the challenges of the various tasks involved in each type of monitoring [2].

Most of the analytes in the WFD list are organic pollutants (hydrocarbons, organochlorine compounds, organic solvents, pesticides and chlorophenols), although four toxic metals (mercury, nickel, cadmium and lead) and one organometallic compound (tributyltin (TBT)) have also been included. Some pharmaceuticals, hormones and endocrine disruptor compounds (EDCs) have also been included as emerging contaminants in the WFD list due to their presence in environmental waters, the threat they pose on drinking water sources and the concern about their possible estrogenic and other kind of effects [3]. EDCs, defined as "exogenous substances that alter function(s) of the endocrine system and consequently cause adverse health effects in an intact organism, or its progeny, or (sub)-populations" [4], can have synthetic and biological origin and include hormones, insecticides, phytoestrogens or industrial chemicals. EDCs are often detected in wastewater treatment plants (WWTPs) effluents and their receiving environments since not all micropollutants are completely removed in such plants [5, 6]. In fact, effluents from several WWTPs have been reported to be estrogenic to fish [7].

The determination of contaminants at trace levels in water samples requires of both an extraction and a pre-concentration step. Traditionally, liquid–liquid extraction (LLE) [8, 9], as well as solid-phase extraction (SPE) [8–10], has been employed to this aim. Recently, and due to the importance reached by miniaturisation in the analytical chemistry field, other techniques such as solid-phase microextraction (SPME) [11], stir bar sorptive extraction (SBSE) or supported liquid membrane (SLM) extraction have attained great importance in the pre-concentration of analytes from water samples [12–18]. These techniques minimise the use of organic solvents (green chemistry) and analysis time, and improve the sensitivity of the global analytical method [8].

SBSE could be described as a liquid–liquid microextraction due to the analytes in the aqueous phase preconcentrate in the polydimethylsiloxane (PDMS) phase. In SBSE, stir bars (so-called "twisters") are coated with a PDMS layer typically 0.5–1-mm thick. Due to the chemical and hydrodynamical conditions at the solution/stir bar interface, the process is kinetically governed until a steady state is attained, where the extraction efficiency is governed by the distribution or partition coefficient of the target analyte between both phases ($K_{PDMS,w}$) and their respective volumes. The larger amount of PDMS present in SBSE compared to SPME (around 50–250 times higher) outcomes on higher recoveries and handling larger sample amounts. The thermal stability of PDMS allows desorbing the analytes thermally in the hot injection port of a gas chromatograph or, alternatively, chemically into an organic solvent. The earliest published reviews on SBSE [19–21] cover more deeply the physico-chemical features of PDMS and highlight the fact that the sorption process is essentially a liquid–liquid partition and, thus, not only the surface area but also the total amount of the extraction phase is important in sorptive extraction. On the contrary, the most recent reviews [8, 21–24] cover in more detail the analytical applications in several fields like environmental analysis, biological fluids or food analysis. At present, only PDMS-coated stir bars are commercially available, and this represents one of the main SBSE drawbacks, since due to the non-polarity of the PDMS polymer, polar compounds are poorly extracted.

In the case of polar analytes, derivatisation reactions are used in order to improve extraction efficiency and/or chromatographic response.

Different derivatisation strategies can be employed when SBSE is coupled to gas chromatography (GC): in situ, on-stir bar or post-extraction [25]. Both in situ acylation (normally acetylation) and in-tube silylation are the most widely used derivatisation techniques. In the first approach (in situ acetylation with anhydride acid acetic at basic sample pH), the derivatisation of analytes containing phenolic moieties has been performed, e.g. chlorophenols and alkylphenols (APs) [26–29] and estrogens and xenoestrogens [30–34]. This approach improves both the extraction efficiency, because log $K_{o,w}$ increases, and sensitivity in the GC analysis, e.g. the in situ derivatisation of BPA with anhydride acid acetic exhibited approximately a 100-fold higher sensitivity than the method without derivatisation [33]. Silylation can be used to derivatise a wide range of functional groups, such as aromatic and aliphatic alcohols, carboxylic acids, amines and amides. Since silylating agents are very sensitive to traces of water and

other protic sorbents, derivatisation is performed post-extraction. *N*-(tert-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (MTBSTFA) has been applied for in-extract derivatisation of phenolic compounds and acidic pharmaceuticals and herbicides [35]. Because of its high volatility, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) was selected for in-tube derivatisation [26]. The responses obtained for APs by SBSE and thermal desorption (TD)-GC-mass spectrometry (MS) without derivatisation, with in situ acetylation and in-tube silylation have been compared by Kawaguchi et al. [26]. Both derivatisation approaches, in situ acetylation and in-tube silylation, improved the sensitivity as compared to without derivatisation. Between the two derivatisation approaches, in situ acetylation provided better sensitivity for the compounds with hydrophilic properties because the affinity of the compounds for the PDMS phase increases, whereas in-tube silylation provided better results for the analytes with hydrophobic properties [26]. When in-tube desorption is applied, a small glass capillary tube containing the derivatisation reagent is placed with the stir bar in the desorption chamber (see Fig. 1). In the second case, the derivatisation reagent is added to the organic solvent after stir bar desorption. In-tube derivatisation has been used for the determination of APs [26], 17β-estradiol (E2) [34, 36, 37] and sterols [36].

Extraction techniques such as SBSE suffer of matrix effect when applied to real samples such as environmental water samples. In such cases, isotopically labelled analogues are often used in order to minimise such effects [25]. In the present work, different variables affecting extraction and desorption steps during SBSE coupled to in-tube derivatisation-TD-GC-MS were optimised for the determination of APs (octyl- and nonylphenols), bisphenol A (BPA), hormones and sterols in estuarine and wastewater samples. The effect of organic matter was also studied using two different approaches. On the one hand, Milli-Q water samples were spiked with humic acids, and apparent recovery values were studied with and without correction with the corresponding deuterated analogue. On the other hand, estuarine water and wastewater samples were spiked with known concentrations of target analytes, and apparent recoveries were studied as explained above.

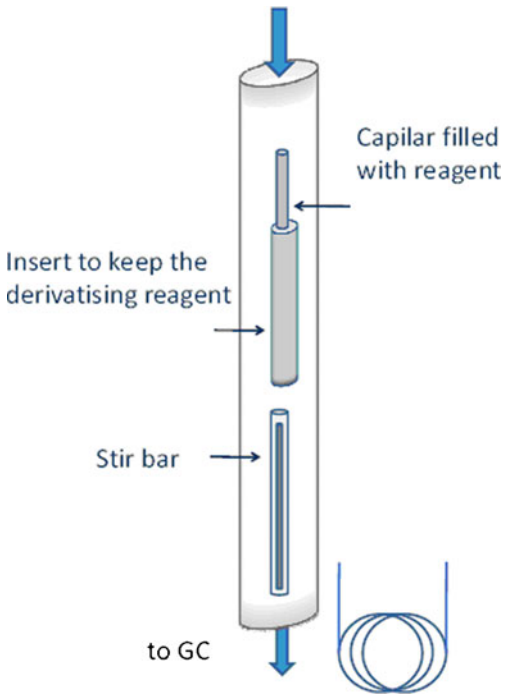


Fig. 1 Scheme of the desorption tube that contains the stir bar together with a capillary filled with 2 μL of BSTFA

Experimental

Cleaning procedure

No detergent was used during the cleaning of glass in order to avoid possible interferences produced by the detergent residues. Therefore, the material was rinsed with abundant water and then sonicated under clean acetone during at least 30 min or maintained in a clean acetone bath overnight. Afterwards, the material was rinsed with Milli-Q (<0.057 S/cm, Milli-Q model 185, Millipore, Bedford, MA, USA) water. Finally, the glass material was dried in an oven at 450 °C for at least 4 h and stored.

Chemicals and reagents

n-Octylphenol (nOP), [$^2H_4$]-4*n*-nonylphenol ([$^2H_4$]-NP), bisphenol A (BPA), diethylstilbestrol (DES), cis-androsterone (ADT), equilin (EQ), [$^2H_4$]-equilin ([$^2H_4$]-EQ), 17β-estradiol (E2), [$^2H_3$]-17β-estradiol ([$^2H_3$]-E2), testosterone (TT), 19-norethisterone (NT), progesterone (PG), coprostan-3-ol (COP), cholesterol (CHL), [$^2H_6$]-cholesterol ([$^2H_6$]-CHL), stigmastanol (STOL) and equilenin (EQN) were obtained from Sigma-Aldrich (Steinheim, Germany). Nonylphenol technical mixture (NPs, Pestanal®) was purchased from Fluka (Steinheim, Germany), [$^2H_{16}$]-biphenol A ([$^2H_{16}$]-BPA) and 4-tert-octylphenol (4-tOP) from Supelco (Walton-on-Thames, UK) and estrone (E1), mestranol (MeEE2), 17β-ethynylestradiol (EE2) and estriol (E3) from Riedel-de Haën (Seelze, Germany; see Table 1). Stock solutions for each compound and deuterated analogues were dissolved individually in anhydrous methanol in order to prepare approximately 5,000 mg/L solutions, except for EQ, [$^2H_4$]-EQ, COP, STOL, CHL and [$^2H_6$]-CHL, which were prepared in dichloromethane. Besides, MeEE2 was prepared in acetonitrile. EQN was obtained directly at 100 mg/L in acetonitrile. Chemical standards

Table 1 Studied analytes including CAS number, chemical structures, derivatised analogues and *m/z* values of fragment ions

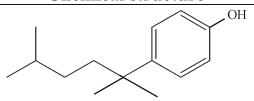
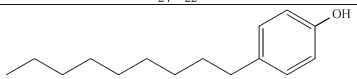
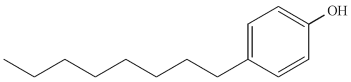
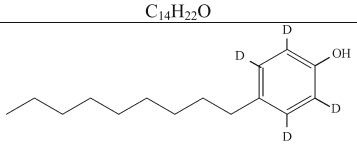
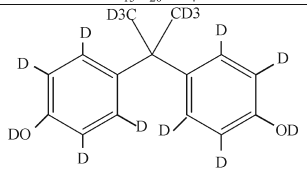
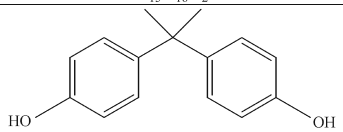
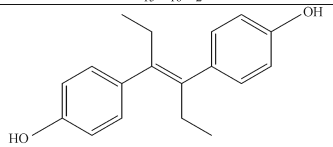
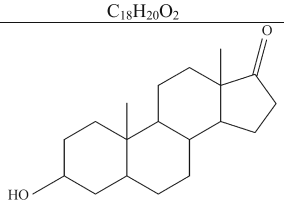
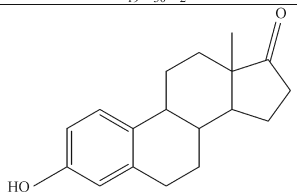
Analyte	CAS number	Chemical structure	Reacting funcionality	m/z
4-tert-Octylphenol (4-tOP)	140-66-9	 $C_{24}H_{22}O$	-OH	207, 208[a]
Nonylphenols mixture (NPs)	104-40-5	 $C_{15}H_{24}O$	-OH	193, 221[a]
Octylphenol (4-nOP)	1806-24-4	 $C_{14}H_{22}O$	-OH	179, 278[a]
[$^2H_4$]-Nonylphenol ([$^2H_4$]-NP)	358730-95-7	 $C_{15}H_{20}OD_4$	-OH	183, 296
[$^2H_{16}$]-Bisphenol A ([$^2H_{16}$]-BPA)	96210-87-6	 $C_{15}D_{16}O_2$	2 x (-OD)	368, 386
Bisphenol A (BPA)	80-05-7	 $C_{15}H_{16}O_2$	2 x (-OH)	357, 358[b]
Diethylstilbestrol (DES)	56-53-1	 $C_{18}H_{20}O_2$	2 x (-OH)	412, 383[b]
cis-Androsterone (ADT)	53-41-8	 $C_{19}H_{30}O_2$	-OH	272, 347[d]
Estrone (E1)	53-16-7	 $C_{18}H_{22}O_2$	-OH	342, 257[d]

Table 1 (continued)

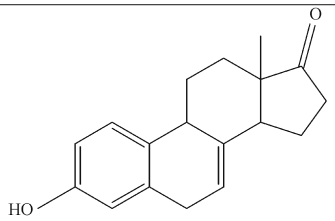
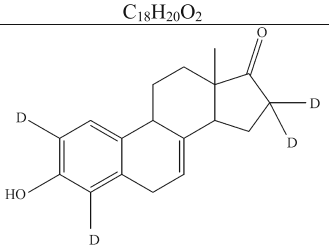
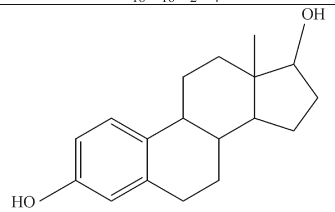
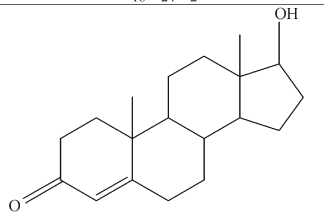
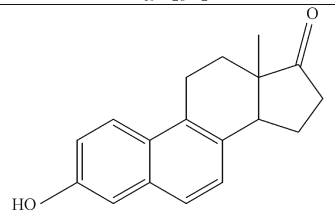
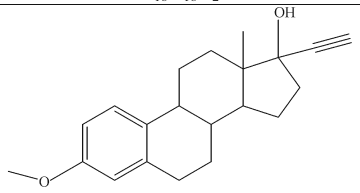
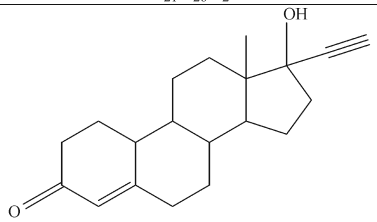
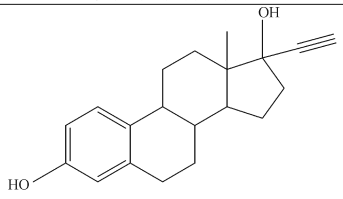
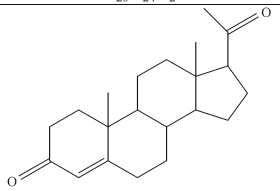
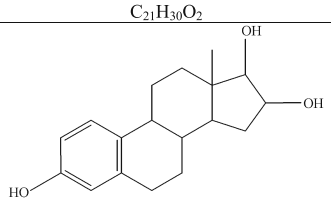
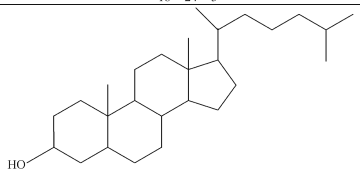
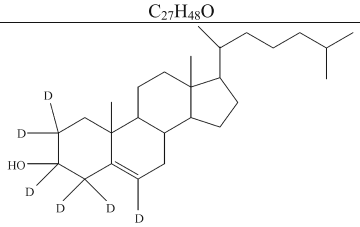
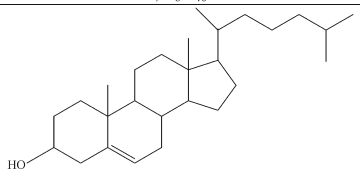
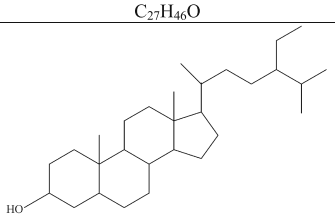
Analyte	CAS number	Chemical structure	Reacting funcionality	m/z
Equilin (EQ)	474-86-2	 $C_{18}H_{20}O_2$	-OH	340, 216[d]
[$^2H_4$]-Equilin ([$^2H_4$]-EQ)	285979-79-5	 $C_{18}H_{16}O_2D_4$	-OH	344, 257
17β-Estradiol (E2)	50-28-2	 $C_{18}H_{24}O_2$	2 x (-OH)	416, 285[c]
Testosterone (TT)	58-22-0 57-85-2 (propionate ester)	 $C_{19}H_{28}O_2$	-OH and =O [f]	432, 417
Equilenin (EQN)	517-09-9	 $C_{18}H_{18}O_2$	-OH	338, 295[c]
Mestranol (MeEE2)	72-33-3	 $C_{21}H_{26}O_2$	-OH	367, 382[c]
19-Norethindrone (NT)	68-22-4	 $C_{20}H_{26}O_2$	-OH and =O [f]	442, 427

Table 1 (continued)

Analyte	CAS number	Chemical structure	Reacting funcionality	m/z
17α-Ethynyl estradiol (EE2)	57-63-6	 $C_{20}H_{24}O_2$	2 x (-OH)	425, 359[c]
Progesterone (PG)	57-83-0	 $C_{21}H_{30}O_2$	2 x (=O)[f]	458, 443
Estriol (E3)	50-27-1	 $C_{18}H_{24}O_3$	3 x (-OH)	504, 345[c]
Coprostan-3-ol (COP)	360-68-9	 $C_{27}H_{48}O$	-OH	370, 355[e]
[$^2H_6$]-Cholesterol ([$^2H_6$]-CHL)	92543-08-3	 $C_7D_6H_{40}O$	-OH	464, 374
Cholesterol (CHL)	57-88-5	 $C_{27}H_{46}O$	-OH	329, 368[e]
Stigmastanol (STOL)	19466-47-7	 $C_{29}H_{52}O$	-OH	473, 488[e]

[a]Corrected with [$^2H_4$]-nonylphenol

[b]Corrected with [$^2H_{16}$]-bisphenol A

[c]Corrected with [$^2H_3$]-17β-estradiol

[d]Corrected with [$^2H_4$]-equilin

[e]Corrected with [$^2H_6$]-cholesterol

[f]Not derivatised under in-tube derivatisation

were stored at 4 °C in the dark, and stock solutions were stored at −20 °C. One hundred milligrammes per litre dilutions were prepared in anhydrous methanol weekly. Dilutions at lower concentrations were prepared daily, according to the experimentation.

Dichloromethane (HPLC grade, 99.8%) and anhydrous methanol (HPLC grade, 99.9%) were obtained from Labscan (Dublin, Ireland) and acetonitrile (HPLC, 99.9%) from Panreac (Barcelona, Spain). Sodium chloride was obtained from Merck (Darmstadt, Germany).

N,*O*-Bis(trimethylsilyl)triufloroacetamide with 1% of trimethylchlorosilane (BSTFA+1% TMCS, Sylon BFT, 99:1) was used as the derivatising reagent and was purchased from Supelco. Stir bars (10×0.5 mm) were obtained from Gerstel (Mülheim an der Ruhr, Germany). Humic acids (technical grade) were obtained from Fluka.

Sampling

Estuarine water samples were collected from the estuaries of Bilbao and Urdaibai (Bay of Biscay, Spain) in May 2010. Wastewater samples were collected at the WWTP of Bakio in May 2010 and at WWTP of Galindo in March 2011 (Bay of Biscay, Spain). Samples were collected in pre-washed amber bottles and carried to the laboratory in cooled boxes. Samples were filtered through 0.45-μm cellulose filters (Whatman, Kent, UK) and kept in the fridge at 4 °C before treatment, which was performed within 48 h.

Stir bar sorptive extraction from water samples

The pre-concentration of the analytes from water samples was performed using 10×0.5-mm stir bars. Twenty grammes of NaCl were directly weighed into a 125-mL extraction vessel and diluted with 100 mL of Milli-Q or natural water in order to obtain a 20% (*w*/*v*) solution. Then, a clean stir bar was added, and the solution was stirred until complete dissolution of the salt. Afterwards, deuterated analogues were added, and the sorption of the analytes was carried out at room temperature and 600 rpm for 15 h. Once the sorption step was over, the stir bar was removed and rinsed with Milli-Q water in order to eliminate salt residues, and finally, dried with a clean tissue.

In-tube derivatisation-TD-GC-MS

The derivatisation and TD were simultaneously performed using a thermal desorption unit (TDU; Gerstel) connected to a CIS-4 injector (Gerstel). The TDU unit has a MPS2 autosampler (Gerstel) able to handle 98 coated stir bars.

Stir bars were placed into a TD tube together with a disposable glass capillary tube (32 mm length, 0.0111″ ID, 0.0300″ OD, accuracy 1%) filled with 2 μL of BSTFA which was placed inside an insert on top of the stir bar (see Fig. 1).

Desorption and simultaneous derivatisation of the target analytes was carried out as follows: 30 °C (0.50 min), temperature increase at 150 °C/min to 300 °C (held for 10 min). During the desorption step, compounds were introduced in the splitless mode at 75 mL/min into a CIS-4 injector that contained a deactivated liner filled with quartz wool and kept at −50 °C using liquid nitrogen (Air Liquide, Madrid, Spain) for cryo-focusing. Once the desorption step was over, the CIS-4 unit was programmed to increase at 12 °C/s to 300 °C, where it was held for 4 min.

The TDU was installed in an Agilent 6890 coupled to an Agilent 5975 electron impact ionization MS system (Agilent Technologies, Palo Alto, CA, USA) and with a HP-5MS (30 m×0.25 mm, 0.25 μm, Agilent) capillary column.. The following temperature program was used for the separation of the analytes: 50 °C (5 min), temperature increase at 10 °C/min to 220 °C and a second increase of 3.20 °C/min up to 300 °C, where it was finally held for 5 min. Helium (99.9995%, Carburos Metálicos, Barcelona, Spain) was used as carrier gas at a constant flow of 1.5 mL/min. The transfer line temperature was maintained at 310 °C and the ion source and quadrupole at 230 °C and 150 °C, respectively. Measurements were performed in the scan (50–525 *m*/*z*) and in the selected ion monitoring (SIM) modes. The *m*/*z* values for each analyte are summarised in Table 1. The first ion was used as quantifier and the second one as qualifier.

Figures of merit

In the absence of certified reference materials (CRMs), the figures of merit or the performance characteristics of the method were estimated using spiked Milli-Q or environmental (estuarine water or wastewater) samples.

Linearity was studied using Milli-Q water samples spiked in the method detection limit (MDL) 500-ng/L range. MDLs were estimated using spiked (25 ng/L) effluent aliquots (n=5) according to US Environmental Protection Agency (EPA) revised guidance for MDL calculation (http://www.epa.gov/waterscience/methods/det/rad.pdf).

Extraction efficiency, defined as the amount of analyte extracted to the PDMS phase, was calculated by comparing the responses obtained after the desorption of stir bars that had been in contact with Milli-Q water spiked at 500 ng/L and the responses obtained after the direct introduction of 50 ng of the target analytes. For the direct introduction of the analytes in the CIS-4 unit, 50 ng of target analytes in MeOH were evaporated in an insert under a gentle stream of nitrogen. A 2-μL capillary tube filled with BSTFA+1% TMCS was placed on top of the insert and submitted to TD.

Apparent recovery, defined as the recovery obtained after correction with the corresponding deuterated analogue, was calculated using Milli-Q water samples spiked at 100 ng/L.

Matrix effect

Two different approaches were used to study the matrix effect of the developed method.

In the first case, different amounts of humic acids (0, 5, 10, 20, 40, 60, 80, 100 and 120 mg/L) were dissolved in 100-mL Milli-Q water aliquots that were then spiked at 100 ng/L of the target analytes. Experiments were performed in duplicate.

In the second approach, 100-mL aliquots of different environmental samples (estuarine and wastewater) were spiked at 100 ng/L.

Results and discussion

Optimisation of variables affecting SBSE

SBSE has been previously used for the determination of APs [36–38], BPA [33, 36, 37], estrogens [34, 36, 37] and steroids [36, 37]. However, few of those works [36, 37] perform a simultaneous determination of such a large amount of analytes. Besides, extraction conditions can differ from work to work [34, 36, 38], and thus, a thorough optimisation of some of the variables affecting SBSE should be performed. In this work, NaCl and MeOH addition, sample volume, extraction temperature and time, and agitation speed were studied using spiked (100 ng/L) Milli-Q water samples. pH adjustment is usually studied when analytes have acidic/basic properties. However, target analytes occur in the non-ionic form at the pH of natural waters according to the pKa values (9.7–15.1). Besides, Quintana et al. [35] proved that pH was not significant during the determination of APs. Therefore, pH was not studied in the present work. In the case of the acceptor phase, 10×0.5-mm stir bars were used due to their availability in our laboratory.

In general, the addition of an inert salt increases the response for polar analytes (log $K_{o,w}$<3.5) since the ionic strength of the water solution increases, modifying the solvating effect of water [35]. Therefore, solubility in water is reduced, and extraction efficiency is improved [39]. For hydrophobic analytes (log $K_{o,w}$>3.5), however, it has been observed that, in most cases, the addition of an inert salt reduces the extraction efficiency. In fact, salt addition could cause an "oil effect" that promotes the movement of non-polar analytes to the water surface, minimizing the affinity with the PDMS phase. Due to the increase of electrostatic, or pair ion-pairing interactions, the ability of the compounds to move to the PDMS phase is reduced [40, 41]. Other authors attribute this decrease to the increase of viscosity, which reduces the extraction kinetics of the compounds [35].

As a general rule, MeOH addition avoids adsorption of the analytes into the glass walls of the vials for compounds with high log $K_{o,w}$ (>5.0), while it increases solubility of the compounds in the solution and decreases $K_{PDMS,\ w}$ for compounds with lower log $K_{o,w}$ values.

The effect of MeOH and NaCl addition during SBSE of target analytes was studied by means of a central composite design (CCD). The equation that defines the central composite design is

$$f = 2^{(k-p)} + 2k + n_0 \quad (1)$$

where f is the number of experiments to be performed, k is the number of variables, p the number of fractions of the complete design and n_0 the number of runs of the central point. The ranges used for this study were between 0% and 20% for NaCl and MeOH. The design matrix was fitted to the following non-linear equation using the Statgraphics program (STATGRAPHICS, Centurion XV):

$$Y = B_0 + B_1X_1 + B_2X_2 + B_{11}X_1^2 + B_{22}X_2^2 + B_{12}X_1X_2 \quad (2)$$

where Y is the response (logarithmic of the chromatographic peak area), B_{ij} are the adjusting parameters, and X_1 and X_2 the percentage of NaCl and MeOH, respectively. Correlation coefficients in the range of 0.85 were obtained. Most of the analytes showed similar response surfaces (built using only significant parameters (p<0.05)) to EE2, plotted in Fig. 2. As it can be observed, in general terms,

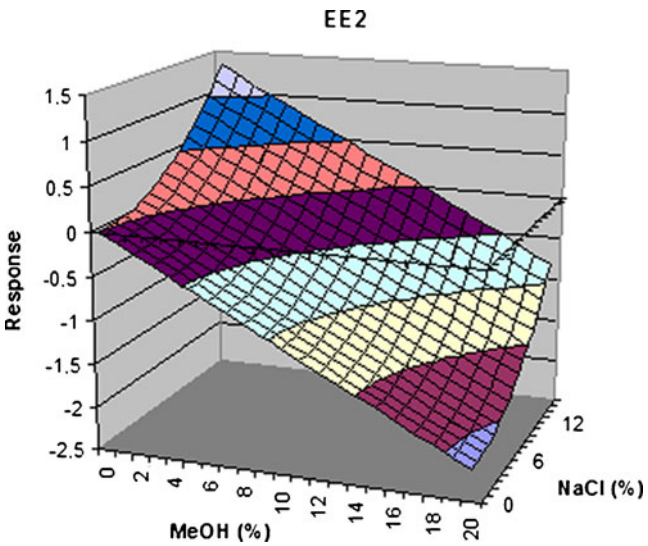


Fig. 2 Response surface of EE2 for the optimisation of MeOH (percent) and NaCl (percent) additions

the addition of NaCl had a positive effect. Similar results were obtained by Almeida and Nogueira [42] for steroid sex hormones, although a negative effect was observed for E1. No significant effect of NaCl addition has been observed in the bibliography for TT, E3 and NT [38, 43]. Consequently, NaCl was fitted at 20% (*w*/*v*) for the rest of the experiments.

In the case of MeOH addition, a negative effect on the extraction efficiency for most of the analytes was observed. Thus, it could be concluded that the presence of MeOH increased water solubility, whereas it decreased $K_{PDMS,w}$ of the target analytes. Therefore, no MeOH was added in further experiments.

In the case of sample volume, although higher sample volumes decrease extraction efficiency [44], an increase in the mass of the analyte may increase chromatographic response [45]. Average responses (n=3) obtained for 30 and 100 mL aliquots were compared by means of one factor analysis of variance (ANOVA) for a 95% confidence interval. Similar to the bibliography, where little effect of sample volume has been observed for polar analytes [46], sample volume was not significant for most of the analytes except for APs, BPA and ADT, which showed increased signals for higher sample volumes. In order to maximise the response for the latter analytes and due to the availability and low cost of the samples studied (estuarine and wastewater), 100 mL aliquots were used in further experiments.

Extraction temperature is studied during SBSE since it has two opposite effects. While at elevated temperature the extraction equilibrium is reached faster [47–49], $K_{PDMS,w}$ partition coefficient, and thus extraction efficiency, becomes lower [49]. The stirring rate is also evaluated because it can accelerate the extraction and, thus, increase responses at a fixed extraction time. This fact is explained by the decrease of the thickness of the boundary layer between the stir bar and the solution [49]. However, high agitation speeds can suppose lack of homogeneity and bubble formation, and these effects can affect the response. In this sense, extraction temperature and agitation speed were studied at two different levels: 25 °C and 40 °C, and 600 and 1,000 rpm, respectively. Extraction temperature had no significant effect, and lower agitation speeds yielded better extraction efficiency for all the target analytes, except for sterols, which showed no significant effect. Therefore, both variables were fitted at the lowest values studied, room temperature and 600 rpm.

Finally, the extraction time was studied under optimised conditions. In order to obtain the extraction time profile, different extraction times (15, 30, 60, 90, 120, 180, 240, 300, 360 and 1,140 min) were examined in duplicate. The time profiles obtained for 4-tOP, E2 and MeEE2 are included in Fig. 3a–c, respectively. The results showed that in most cases, more than 20 h was not enough to reach equilibrium. Overnight (15 h) extraction was finally chosen.

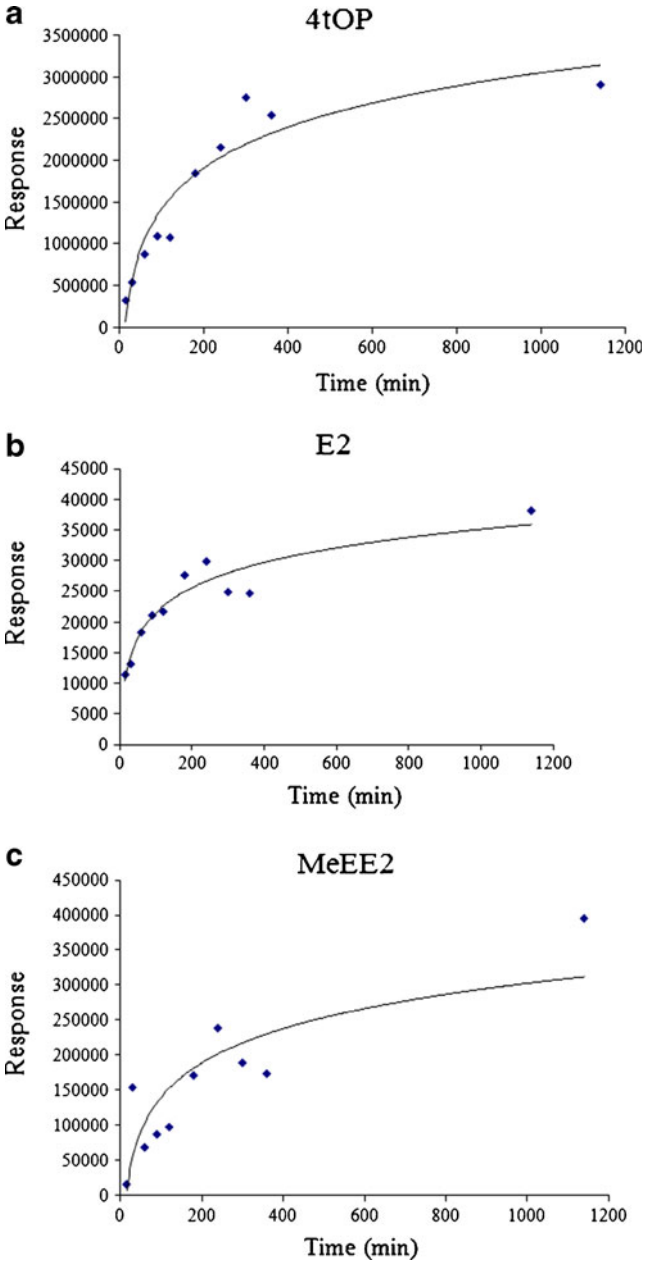


Fig. 3 Time profile for 4-tOP (**a**), E2 (**b**) and MeEE2 (**c**). Average response (n=2) is plotted

In-tube derivatisation-TD-GC-MS optimisation

During in-tube derivatisation-TD-GC-MS, the volume of the derivatisation reagent (BSTFA+1% TMCS) and the cryo-focusing temperature were studied. Desorption temperature and time were fixed at high values (300 °C and 10 min, respectively) in order to guarantee complete desorption of the target analytes from the stir bar. Actually,

when stir bars were submitted to a second desorption (after refilling of the capillary tube with BSTFA+1% TMCS), signals lower than 1% were obtained for all the compounds in comparison with the first desorption, and thus, no further conditioning of the stir bars was necessary.

Different volumes of derivatisation reagent (0.5–20 μL) were tested. Higher derivatisation reagents provided higher chromatographic responses, but when volumes higher than 2 μL were repeatedly introduced in the instrument, blockage of the CIS-4 unit and overpressures occurred. Thus, derivatisation reagent volume was fixed at 2 μL.

Cryo-focusing temperature is one of the most usually studied variables during TD. Since TD of stir bar is long (up to 10 min in most cases), a focusing of the analytes is necessary previous to the introduction of the analytes into the chromatographic column. Lower cryo-focusing temperatures yielded better results, but once again, cryo-focusing temperatures lower than −50 °C caused blockage of the CIS-4 unit, and thus, −50 °C was further used.

All target analytes except TT, PG and NT were derivatised under proposed conditions. Besides, E1 can be artifactly produced from the oxidation of EE2 when the latter is derivatised with BSTFA+1% TMCS in the absence of a solvent such as pyridine [50]. However, during in-tube derivatisation, the formation of E1 was less than 1% and was considered negligible. The absence of the non-derivatised species was checked in chromatograms obtained in the scan mode.

Figures of merit

Since PG, TT and NT are not derivatised in-tube, they have been excluded from the validation section. E3 was excluded as well since the calibration results were incoherent.

Squared correlation coefficients in the range of 0.972–0.998 were obtained for the calibration curves built in the MDL 500-ng/L range. MDLs were in the 0.8–84 (see Table 2). The MDLs estimated were in the same range as those obtained for SPE-derivatisation-GC-MS [51], better that those obtained by microextraction by packed sorbent (MEPS) [52] but worse than using LC-MS-MS [53–55]. It should be highlighted that the present work performs a simultaneous determination of 15 analytes.

Experimental extraction efficiency values are included in Table 2 and compared to theoretical extraction efficiency values, calculated using the KowWIN software program (Syracuse Research Corp., Syracuse, NY, USA), which is based upon a log $K_{o,w}$ calculator. Good correlation was obtained between the theoretical and experimental values for some of the analytes studied, except for the 4-nOP, E1, E2, MeEE2, STOL and CHL.

Good apparent recovery values for Milli-Q water (see Table 2) were obtained in most of the cases (62–124%), except for 4-tOP, which showed values that significantly exceeded 100%. The unsatisfactory results obtained for 4-tOP could be attributed to a different sorptive capacity of 4-tOP and [$^2H_4$]-NP. As can be observed for both the

Table 2 Figures of merit of SBSE in-tube derivatisation-TD-GC-MS

Analyte	Log $K_{o,w}$	Theoretical efficiency (%)	Experimental efficiency[a] (%)	Apparent recovery[b] (Milli-Q at 100 ng/L; %)	RSD (%)	MDL (ng/L)
4-tOP	5.28	97.9	79	147	26	11
NP	6.19	99.7	103	124	10	21
4-nOP	4.12	76.0	46	99	12	13
BPA	3.43	39.2	20	105	5	0.8
DES	5.07	96.6	80	110	9	10
ADT	3.75	57.4	53	97	12	25
E1	3.69	54.0	5	62	20	35
EQ	3.52	44.3	50	100	2	5.3
EQN	3.76	58.0	–[c]	78	27	70
E2	4.13	76.4	28	108	15	16
MeEE2	5.18	97.3	7	83	14	84
EE2	4.52	88.8	55	101	19	7
COP	10.22	100	99	98	8	42
STOL	10.2	100	56	103	23	50
CHL	9.85	100	32	103	15	56

[a] Amount extracted to the PDMS phase

[b] Recovery after correction with the corresponding deuterated analogue

[c] Not determined

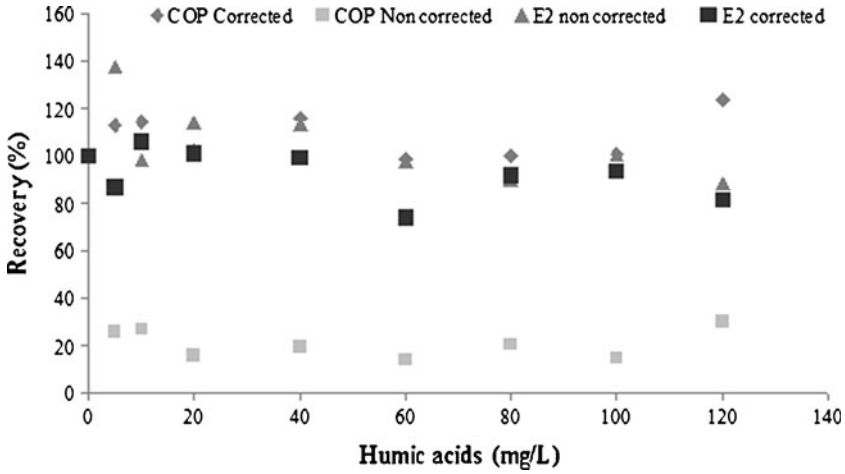


Fig. 4 Influence of humic acids on non-corrected and corrected recovery for E2 and COP

theoretical and experimental extraction efficiencies of 4-tOP and 4-nOP (see Table 2), branched and linear APs show different extraction capacity. Since the deuterated analogue used for correction is linear [$^2H_4$]-NP, it seems not to be the best deuterated analogue for correction of branched 4-tOP.

Finally, repeatability (n=4) was studied using four Milli-Q water samples spiked at 100 ng/L, and values in the 2–27% range were obtained, similarly to the literature [51–54].

Effect of organic matter and application of develop method to real samples

Like in many other sample preparation techniques for trace analysis, the efficiency of SBSE can be strongly affected by the complexity of the matrix involved [25, 56, 57]. In environmental water samples, substantial levels of dissolved organic matter may interfere with the extraction of the target compounds to the PDMS phase, and therefore, the extraction yield may drastically change from sample to sample.

One way to minimise matrix effect is based on the use of deuterated analogues from the very beginning of the analytical procedure, as it has been done in this work. If the extraction efficiency decreases similarly for both the analyte and the deuterated analogue signal, deuterated analogues may compensate for the matrix effect.

In a first approach, different amounts of humic acids were added to spike Milli-Q water, and chromatographic peak areas were normalised to 100% with and without correction with the corresponding deuterated analogue. The behaviour of the analytes could be divided into three groups.

In a few cases (E2 and EE2), no significant influence of humic acid addition was observed (see Fig. 4 for E2). Moreover, there was not a significant difference when correction with the corresponding deuterated analogue was performed.

In the case of 4-nOP, BPA, DES, ADT, E1, EQ, MeEE2, COP, CHL and STOL, a significant decrease was observed in the presence of humic acids, but this decrease was fairly well corrected with the corresponding deuterated analogue for COP (see Fig. 4).

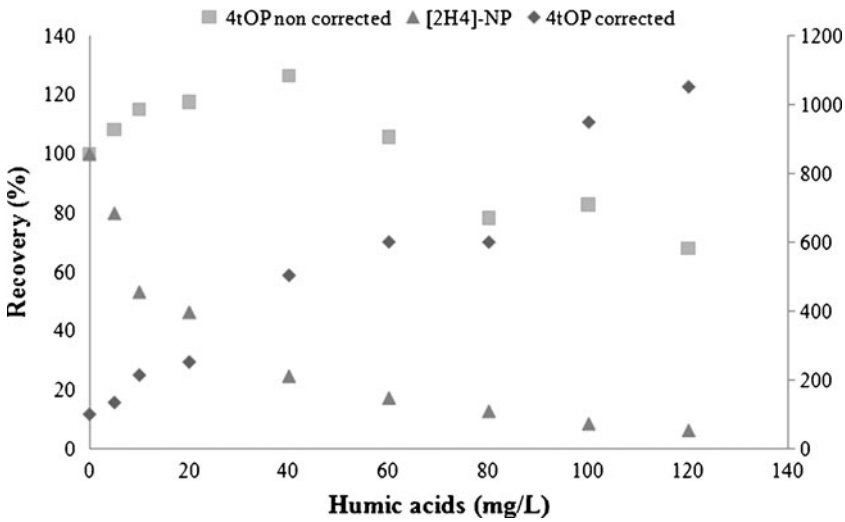


Fig. 5 Influence of humic acids on non-corrected (primary axis) and corrected recoveries for 4-tOP (secondary axis). Comparison with the behaviour of [$^2H_4$]-NP (primary axis)

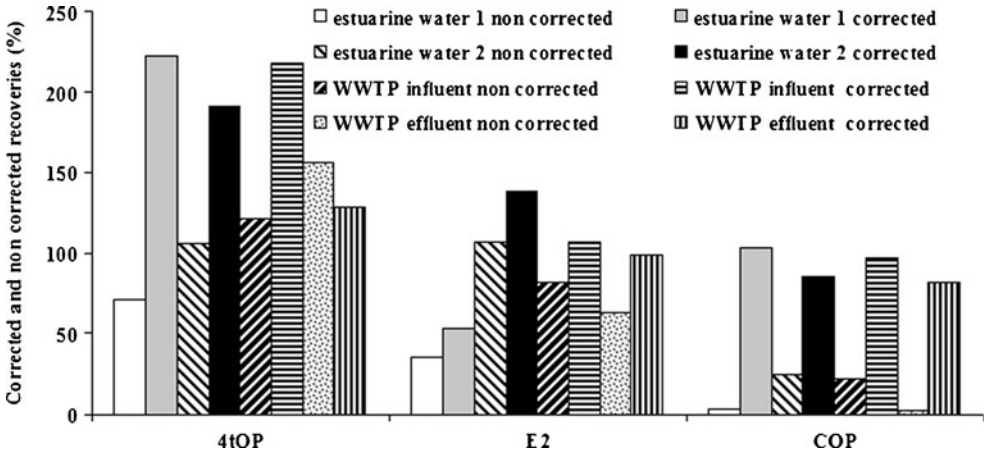


Fig. 6 Corrected and non-corrected extraction recovery values for spiked estuarine and wastewater samples

In the case of 4-tOP and NPs, a decrease of extraction efficiency was observed in the presence of humic acids (see Fig. 5), but it could not be corrected with the use of [$^2H_4$]-NP. As it can be observed in Fig. 5, [$^2H_4$]-NP extraction efficiency suffers a higher decrease than the studied 4-tOP and NPs, and thus, apparent recoveries for 4-tOP and NPs exceeded 100%.

In order to guarantee that this lack of correction was due to a different behaviour of NPs and 4-tOP with respect to the deuterated analogue and not due to an interference introduced by the present of organic matter, blanks of humic acid water solutions were performed. However, as no presence of target APs was observed, we concluded that the lack of correction was due to a different behaviour and not to the presence of interferences. As mentioned above for apparent recoveries in Milli-Q water, linear and branched APs show a different extraction capacity onto the PDMS polymer, but in the presence of organic matter, this difference increases. Thus, the unsatisfactory results obtained for 4-tOP and NPs cannot only be attributed to a difference in extraction capacity onto PDMS but also to a different interaction with organic matter of branched and linear APs.

In a second approach, estuarine and wastewater samples were spiked at known concentrations, and recoveries were calculated, with and without correction with the corresponding deuterated analogue (see Fig. 6). In general, the results obtained are similar as those obtained during the humic acids simulation experiments. [$^2H_4$]-NP does not work as a good analogue for 4-tOP and NPs since recoveries exceed 100%. Sterolic compounds show a significant influence of organic matter, but it is corrected using [$^2H_6$]-CHL. Recoveries for E2 and EE2 are not affected by the presence of organic matter and, for the rest of the compounds, better results are obtained after correction as a general rule.

The optimised method was applied to influent and effluent samples collected at the WWTP of Galindo (Bilbao, Spain). Results obtained are summarised in Table 3. Most of the target analytes were found under the corresponding MDL values, except for ADT, BPA, EQ, E1, E2, CHL and COP. Since influent and effluent samples were collected the same day, it is not possible to discuss about whether higher or lower concentrations are found for treated and non-treated samples. Finally, similar concentrations have been found in the literature [5, 37, 58].

Conclusions

A method has been developed to determine a variety of EDCs in environmental water samples based on techniques

Table 3 Concentrations (nanogrammes per litre) and standard deviations of the analytes in the influent and effluent of the WWTP from Galindo in April 2011

Compound	Galindo wastewater	
	Influent	Effluent
4tOP	<MDL	<MDL
NP	<MDL	<MDL
4nOP	<MDL	<MDL
BPA	23.0±0.3	59±8
DES	<MDL	<MDL
ADT	137±65	3±1
E1	77±13	64±1
EQ	14±17	18±0
E2	<MDL	21±11
EQN	<MDL	<MDL
MeEE2	<MDL	<MDL
EE2	<MDL	<MDL
COP	608±62	244±39
CHL	191±38	166±12
STOL	<MDL	<MDL

that minimise solvent consumption both during the extraction and derivatisation steps. Several variables affecting the analytical procedure, such as NaCl and MeOH addition, sample volume, extraction temperature and time, agitation speed, derivatisation reagent volume and cryo-focusing temperature have been studied and successfully optimised. It should be underlined that compounds such as TT, NT and PG were not in-tube derivatised, and that the determination of E3 was not possible since it gave no coherent calibration curves. Finally, the effect of organic matter on extraction efficiency is corrected for most of the analytes studied, except for 4-tOP and NPs, where the correction using [$^2H_4$]-NP is not properly working due to the different behaviour of the target analytes and the deuterated analogue. Another alternative analogue should be chosen, branched, in order to compensate matrix effects of 4-tOP and NPs.

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