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Improved sample treatment for the determination of bisphenol A and its chlorinated derivatives in sewage sludge samples by pressurized liquid extraction and liquid chromatography–tandem mass spectrometry

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ABSTRACT

A selective, sensitive, robust and accurate method for the determination of bisphenol A (BPA) and its chlorinated derivatives in sewage sludge samples using liquid chromatography–tandem mass spectrometry (LC–MS/MS) is presented. Prior to instrumental analysis, an extraction procedure using pressurized liquid extraction (PLE) was carried out in order to obtain the highest recoveries and improve sensitivity. After LC separation, the MS conditions, in negative atmospheric pressurized chemical ionization (APCI) mode, were individually optimized for each analyte to obtain maximum sensitivity in the selected reaction monitoring (SRM) mode. The use of two reactions for each compound allowed simultaneous quantification and identification in one run. The analytes were separated in less than 6 min. BPA-d_{16} was used as internal standard. The limits of detection of the method ranged from 4 to 8 ng g^{-1} and the limits of quantification from 14 to 26 ng g^{-1}, while inter- and intra-day variability was under 6% in all cases. Due to the absence of certified materials, the method was validated using matrix-matched calibration and a recovery assay with spiked samples. Recovery rates ranged from 97.7% to 100.6%. The method was satisfactorily applied for the determination BPA and its chlorinated derivatives in sewage sludge samples collected from wastewater treatment plants (WWTPs) located in the province of Granada (Spain). The sludge samples came from a conventional activated sludge (AS) plant and from a membrane bioreactor (MBR) pilot plant.

1. Introduction

The impact on human health and environment of endocrine disrupting chemicals (EDCs), even at ng L^{-1} levels, is increasingly becoming an important focus for scientific research [1]. EDCs encompass a wide variety of synthetic and natural chemicals that have the ability to mimic hormones and might, therefore, interfere or disrupt normal hormonal functions [2]. Exposure to EDCs has become a highly controversial public health issue. Although sexual differentiation has been the major endpoint for the toxicological assessment of EDCs, concern with these substances also stems from their potential to affect reproductive, metabolic, immune and development functions, growth, behaviour and memory [3]. Effects of EDCs are associated with reduced fertility, congenital malformations of the reproductive tract, and increased incidence of cancer in estrogen-responsive tissues [4]. Recent studies are focused on anthropogenic EDCs, such as synthetic hormones used as contraceptives, a variety of pharmaceuticals and personal care products, as well as large amounts of industrial chemicals, with bisphenol A (BPA), PCBs, dioxins, pesticides, phthalates, alkylphenols and alkylphenol ethoxylates being of special importance [5].

In recent years, most attention has focused on exposure to BPA, a widely used industrial plasticizer with known estrogenic properties. Over 2.000 million tons/year of BPA are used in the manufacture of epoxy resins and polycarbonate plastics, which are, in turn, used in a wide variety of domestic products [6]. BPA is present in dental fillings, plastic food and water containers, baby bottles, food wrap, as well as in the lining of beverage and food cans, presenting a large number of routes for human exposure. Numerous studies have confirmed leaching of BPA from food containers, and detectable levels of BPA have been found in a wide range of packaged foods [7] being oral exposure the primary source of human exposure to BPA [8]. BPA also accounts for most estrogenic activity that leaches from landfills into the surrounding ecosystem; effluent from industrial activity, including treatment of leachate, may serve as an additional route of human exposure, particularly if it finds its way into aquatic species [9]. Wastewater containing BPA is also a source of contamination of aquatic environments from where BPA could reach ground

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waters, rivers, streams and, eventually, drinking water, resulting in a continuous low-level exposure to BPA [6,7]. Even the low levels of BPA found in aquatic ecosystems pose a serious threat to aquatic life [10].

On the other hand, due to its highly lipophilic behaviour, BPA also tends to strongly associate with particulate matter and can, therefore, be found in solid matrices, such as sewage sludge produced during wastewater treatment [11,12]. This represents a serious hazard because the use of sewage sludge as organic amendment of soils has become a common practice in Europe since the last decade, facilitating the "arrival" of these pollutants to humans through the food chain [13]. Given the ubiquity of BPA in human environments, it is not surprising that exposure to BPA is virtually universal. It is also known that BPA easily reacts with sodium hypochlorite – used as bleaching agent in paper factories and in water disinfection – to produce chlorinated derivatives of BPA (mono-, di-, tri- and tetra-chlorobisphenol A) (ClBPAs) that can be released into the environment [14]. BPA chloroderivatives, especially tri- and tetra-chlorobisphenol A, also represent a serious concern, because in both, in-vitro and in-vivo studies, they have proved to be even more active than BPA in competing with 17β-estradiol for human estrogen receptors-α and -β (ER-α and ER-β) binding sites [15], even at lower concentrations than BPA [14,16]. Furthermore, using human breast carcinoma MCF7 cells, it was determined that ClBPAs had greater potential to stimulate growth than BPA [17], which was also confirmed in in-vivo experiments performed with ovariectomized female rats. In addition to their strong estrogenic activity, BPA and their chloroderivatives caused a significant increase in the weight of the uterine tissue and endometrium, leading to an increased risk of cancer. Chronic exposure to ClBPAs even at very low doses may cause more uterotrophic activity than BPA [17]. In chicken and frogs, it was reported that ClBPAs inhibited the binding of 3,3′, 5-triiodothyronine (T3) to transthyretin (TTR), responsible for the plasma transport of thyroid hormone, more strongly than BPA [18].

On the other hand, the effects of chlorination on the acute toxicity of BPA and its derivatives have raised concerns because they have not been clarified yet. It is known that ClBPAs can photodegrade, producing more toxic oxygen reactive species. Acute cytotoxicity of ClBPAs was increased by UVB and UVC irradiation [19]. These results are interesting, because ClBPAs, which return to the environment from wastewater treatment after a slow and even failed biodegradation [20,21], will be exposed to sunlight that will enhance their cytoxicity by the generation of photodegradation subproducts.

Moreover, it is known that BPA is metabolized to glucuronide in rat liver [22] and the metabolites are rapidly eliminated in the faeces and urine, but ClBPAs are degraded slowly and accumulated through the food chain in the human body where they act as persistent EDCs.

There is a growing need to determine the fate of EDCs in the environment, since it has been reported that EDCs with high estrogenic activity have a great tendency to associate with particulate matter and sediments [23]. However, although there are data regarding the levels of BPA in treated sludge, the same is not true for its chlorinated derivatives, and this information is required to determine which processes could improve the removal of these EDCs from wastewaters in WWTPs. Furthermore, the determination of the partition coefficient between the solid and liquid phases in biological treatment units will certainly help to explain the fate and behaviour of BPA and its derivatives in WWTPs. Since the studies involving the determination of the dissolved phase fraction of these contaminants are much more numerous than those involving the determination in solid matrices, it seems reasonable to develop selective, sensitive and robust analytical methods for determination of these substances in treated sludge.

Until the 1990s, traditional approaches for the extraction of EDCs in solid matrices were based almost exclusively on Soxhlet extraction and steam-distillation. However, these techniques make the analysis procedure excessively time consuming (up to 48 h) and require large amounts of hazardous organic solvents [24]. To overcome these limitations, new extraction approaches have been developed for the extraction of organic pollutants. One of the most widely used techniques for sewage sludge matrices is ultrasound-assisted extraction (USE) [25–28]. Although this technique is considerably faster than Soxhlet extraction, it also requires relative large volumes of toxic and costly organic solvents. More efficient techniques have therefore been developed such as pressurized liquid extraction (PLE) [29–31] or microwave-assisted extraction (MAE) [32]. PLE is a very efficient technique that can be applied to thermally stable compounds. It offers important improvements over other techniques including shorter extraction time, lower amount of solvent, higher level of automation and the ability to perform multiple extractions simultaneously [33]. Moreover, although there are several methods for the determination of BPA in sludge matrices, almost all of them include a solid-phase extraction (SPE) procedure after the extraction process; this improves preconcentration of the analytes and help reduce the matrix effect. However, these extraction methodologies lead to a long, tedious and expensive analytical process without a clear improvement of the final extracts. It is important to highlight that there are not methods for the determination of each and every chlorinated BPA in sewage sludge. Table 1 summarizes the most relevant methods available.

The main objective of the present work is to develop a rapid, robust, sensitive and accurate method for the determination of BPA and its chlorinated derivatives in sewage sludge samples using PLE followed by LC–MS/MS analysis. In addition, the efficiency of the SPE procedure to reduce the matrix effect is also evaluated. The method will allow the analysis of a larger number

Table 1
Analytical methods for the determination of BPA and derivatives in sewage sludge.

Analyte	Analytical technique	LOQ	Reference
BPA	USE–SPE–GC–MS	130 ng g^{-1} (LOD)	[35]
BPA	USE–SPE–GC–MS	108 ng g^{-1}	[25]
BPA	Selective pressurized liquid extraction–GC–MS	35.7 ng g^{-1}	[37]
BPA	USE–SPE–GC–MS	1.84 μg g^{-1}	[27]
BPA	USE–SPE–LC–MS/MS	9.8 ng g^{-1} (LOD)	[28]
BPA	MAE–SPE–LC–MS/MS	2.3 ng g^{-1}	[32]
BPA	USE–SPE–UHPLC–MS/MS	0.7 ng g^{-1}	[36]
BPA and Cl_4-BPA	Soxhlet–SPE–LC–MS/MS	0.15 ng g^{-1} (BPA) 0.03 ng g^{-1} (Cl_4-BPA)	[34]

LOQ, limit of quantification; LOD, limit of detection; SPE, solid-phase extraction; LC, liquid chromatography; MS, mass spectrometry; USE, ultrasound extraction; MAE, microwave assisted extraction; GC, gas chromatography, UHPLC, ultra high performance liquid chromatography.

of samples in a short time. Finally, this method will allow the development of further research on the environmental occurrence, contamination pathways, fate and risk assessment of this important group of EDCs.

2. Materials and methods

2.1. Chemicals and reagents

Analytical grade standards of BPA, tetrachlorobisphenol A (Cl_4-BPA) and deuterated bisphenol A (BPA-d_{16}) – used as internal standard – were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mono-, di- and trichloro-bisphenol A (Cl-BPA, Cl_2-BPA, Cl_3-BPA) were synthesized in our laboratory [38]. Individual standard solutions of compounds (200 μg mL^{-1}) were prepared in methanol and stored at −20 °C. These solutions were prepared fresh monthly. Working standard mixtures were prepared by diluting the individual stock solution in methanol or in the initial mobile phase immediately before use. They were stored at 4 °C and prepared fresh weekly. All solutions were stored in amber glass bottles. Water (18.2 MΩ cm) was purified using a Milli-Q system from Millipore (Bedford, MA, USA). LC–MS grade water and methanol – used for the preparation of standards and mobile phases – were purchased from Fluka (St. Louis, MO, USA). Disodium hydrogen phosphate and citric acid, as well as sodium hydroxide for the preparation of McIlvaine buffer solution [39] were obtained from Panreac (Barcelona, Spain). Ammonia (> 25%), acetonitrile, ethyl acetate, dichloromethane, hexane and acetone were purchased from Merck (Darmstadt, Germany). The SPE cartridges were LiChrolut RP-18 (500 mg, 3 mL) from Merck (Darmstadt, Germany). Sample extracts were filtered through a 0.45 μm cellulose acetate disk filter (Millipore) prior to analysis.

2.2. Instrumentation and software

Extraction procedure was performed in a Dionex Accelerated Solvent Extractor, ASE® 200 (Sunnyvale, CA, USA), equipped with a solvent controller. The cell tray holds 24 sample cells and 4 rinse tubes. The vial tray holds 26 collection vials and 4 rinse vials. ASE® 200 whole operation cycles as well as the control of modules (air and nitrogen pressure, temperature, solvent and rinse procedures) can be controlled from the ASE® 200 front panel.

A Crison 2000 digital pH-meter with a combined glass-Ag/AgCl (KCl 3 M) electrode (Crison Instruments S.A, Barcelona, Spain) was used for pH measurements. A vortex-mixer (Yellow line, Wilmington, NC, USA), a Hettich Universal 32 centrifuge (Tuttlingen, Germany), and a Memmert oven (Schwabach, Germany) were also used. Statgraphics software package was used for statistical treatment of data.

2.3. Sample collection and storage

Samples of sewage sludge were collected from two WWTPs located in the province of Granada (Spain). The samples were kept in amber glass bottles and decreased biological activity was achieved by adding 1% (*v*/*v*) formaldehyde. Once in the laboratory, samples were centrifuged at 3634 × *g* for 15 min and the solid components recovered, dried in a heater at 60 °C to constant weight and finely ground (≤ 1.41 mm). The samples were stored in the dark at 4 °C until analysis.

2.4. Preparation of fortified samples

Due to the absence of certified materials, blank samples for recovery studies were spiked at different concentrations by adding 1 mL of a methanolic standard solution containing the different analytes under study to 1.0 g of dry sewage sludge sample. This volume allows the analytes to come in contact with the whole sample. In order to attain sorption equilibrium, the mixtures were shaken for 10 min and were then left to stand for 24 h at room temperature in the dark before analysis.

2.5. Basic procedure

Dried samples of 1.0 g sewage sludge were weighted and transferred into an 11 mL stainless steel extraction cell of the Dionex extractor. Ethyl acetate was used as extraction solvent. The operating conditions were: extraction temperature, 100 °C; extraction pressure, 1000 psi; preheating period, 2 min; static extraction period, 8 min; number of extraction cycles, 3; solvent flush, 30% of the cell volume and nitrogen purge, 75 s. Final extraction volume was approximately 15 mL. The extracts were evaporated to dryness at 50 °C under a stream of nitrogen and 500 μL of the initial mobile phase containing the internal standard were added to dissolve the residues. The obtained extracts were centrifuged again at 3634 × *g* for 30 min, and supernatants were directly injected into the LC system.

2.6. Liquid chromatographic–mass spectrometric analysis

Detection and quantification of the analytes were performed using an Agilent 1200 series (Agilent Technologies Inc., Palo Alto, CA, USA) LC system equipped with a binary pump, a vacuum membrane degasser, a thermostated column compartment, an automatic autosampler and an automatic injector. The LC system is coupled "on line" to an API 2000 (Applied Biosystems, Foster City, CA, USA) triple quadrupole mass spectrometer system that can use either atmospheric pressurized chemical ionization (APCI) or electrospray ionization (ESI) interfaces. Analyst software version 1.5.2 was used for instrument control, data acquisition and analysis.

Chromatographic analysis was performed using a Gemini C_{18} column (100 × 2.0 mm i.d., 3 μm particle size) and a C_{18} guard column, both supplied by Phenomenex (Torrance, CA, USA). The flow rate was 350 μL min^{-1}, the column was maintained at 40 °C and the injection volume was 40 μL. A gradient mobile phase consisting of 0.025% (*v*/*v*) ammoniacal aqueous solution (solvent A) and 0.025% (*v*/*v*) ammonia in methanol (solvent B) was used. Gradient conditions were: 0.0–3.5 min, 60% B; 3.5–4.0 min, 60–100% B; 4.0–5.0 min, 100% B and back to 60% in 1.0 min. The total run time was 6 min, and the post-delay time for reconditioning the column with 60% B was 4 min.

The mass spectrometer (MS) was operated with APCI ionization in negative ion mode. The tandem mass spectrometer was operated in multiple reaction monitoring (MRM) mode and Q1 and Q3 quadrupoles were set at unit mass resolution. Mass spectrometric conditions were optimized for each compound by continuously infusing the standard solutions (50 μg mL^{-1}). The ion source temperature was maintained at 350 °C. The IonSpray voltage was set at −3 kV. Nitrogen was used as curtain gas at 30 psi and as ion source gas 1 and 2 at 50 and 30 psi, respectively; collision gas was air at 10 psi. The horizontal and vertical positions of the interface were 3 mm.

Additional parameters that were optimized included declustering potential (DP), focusing potential (FP), entrance potential (EP), collision energy (CE), collision cell exit potential (CXP) and dwell time, in order to obtain the maximum sensitivity with the highest amount of product ions available, as well as the two most sensitive MRM transitions (one used for quantification and the other for confirmation).

Table 2
Selected MRM transitions, optimized voltages, dwell times and retention times of the target compounds.

Compound	Transitions	Dwell time (ms)	DP (V)	FP (V)	EP (V)	CE (V)	CXP (V)	Retention time (t_R, min)
BPA	227.2→212.2[a] 227.2→132.9[b]	685.7	−41	−255	−10	−30	−30	6.01
Cl-BPA	261.1→182.1[a] 261.1→210.0[b]	600.0	−45	−170	−10	−40	−18	5.01
Cl_2-BPA	295.1→244.1[a] 295.1→215.2[b]	428.6	−42	−240	−9	−30	−25	4.17
Cl_3-BPA	329.1→250.1[a] 329.1→278.0[b]	471.4	−46	−150	−10	−47	−31	1.94
Cl_4-BPA	365.0→314.2[a] 365.0→286.1[b]	214.3	−50	−260	−10	−41	−31	1.34
BPA-d_{16}	241.2→142.0[a]	428.6	−43	−160	−11	−32	−20	5.78

DP, declustering potential; FP, focusing potential; EP, entrance potential; CE, collision energy; CXP, collision cell exit potential.

[a] MRM transition used for quantification.

[b] MRM transition used for confirmation.

For quantification, the most abundant transition was selected to obtain the maximum sensitivity. The interscan delay was set at 5 ms. In terms of sensitivity, the most influential parameters were DP and CE. Only one transition was selected for the internal standard because this substance is an isotopically labelled compound that is unlikely to be found in environmental samples.

Table 2 lists the optimized parameters for BPA and BPA-d_{16} as well as the mass transitions and retention times.

3. Results and discussion

3.1. Liquid chromatographic separation

Preliminary studies were carried out to optimize chromatographic separation and signal intensity using a standard mixture of compounds (100 ng g^{-1}). A Gemini C18 liquid chromatography column (100 × 2 mm i.d., 3 μm particle size) from Phenomenex (Torrance, CA, USA) and an Acquity UPLC column (100 × 2.1 mm i.d., 1.7 μm particle size) from Waters (Mildford, MA, USA) were evaluated. Although both columns offered similar resolution for all compounds, the Gemini C18 column was chosen because the Acquity column generated pressures close to the maximum allowed by the system.

3.1.1. Effect of the interface and mobile phase on the development of the chromatographic method

The ESI interface in negative mode and a mixture of pure methanol and water, as mobile phase, were used as initial conditions [40]. Nonetheless, the results obtained were not satisfactory because BPA showed a very low response. The MS interface was then changed to APCI and different additives were used in mobile phases to improve ionization processes. The use of 1% (*v*/*v*) aqueous acetic acid as solvent A [41] was evaluated, because acid mobile phases help to suppress the ionic mobility of the analytes, therefore, ensuring appropriate retention on the stationary phase [42]. However using these conditions, the sensitivity for the detection of BPA did not improve. Then, alkaline additives were also studied because ionization of phenolic compounds (acid groups) is better achieved under basic conditions. A 20 mM solution of ammonium formiate, a mixture of 5 mM of acetic acid and 5 mM triethylamine, and a 0.025% (*v*/*v*) solution of ammonia were assayed and the best results were obtained using ammonia, which was also added to methanol (solvent B) at the same concentration.

3.1.2. Effect of column temperature, flow rate and injection volume

In general, chromatograms of BPA and its chlorinated derivatives showed good peaks separation. However, some parameters were optimized in order to obtain strongest responses, and shorter times of analysis. First, the column temperature was optimized. Considering the manufacturer's specifications, temperatures from 30 to 50 °C were studied. Good peak separation was observed with all temperatures and there were no significant changes in sensitivity or peak shapes with the increase of temperature. However, temperatures > 40 °C provided significantly shorter retention times. This was selected as the optimal temperature. Then, the effect of flow rates from 0.2 to 0.5 mL min^{-1} was assayed. Although according to the product specifications, the maximum flow for the Gemini C18 column is 0.5 mL min^{-1}, pressure increased significantly at flow rates over 0.35 mL min^{-1}. Results demonstrated that the influence of the flow rate was more important than temperature and a significant improvement in resolution, intensity of peaks and marked reduction in retention times were observed with higher flow rate. A flow rate of 0.35 mL min^{-1} was therefore chosen as the optimal flow rate. Finally, injection volumes from 5 to 40 μL (the highest acceptable injection volume) were assayed and 40 μL was selected as optimal. Although a slightly peak broadening was observed, a marked increase in sensitivity without loss of resolution was obtained. Fig. 1 shows a standard chromatogram for the studied analytes using the optimized conditions.

3.2. Optimization of pressurized liquid extraction (PLE)

BPA has a relatively high octanol–water partition coefficient (log K_{ow} = 2.2–3.8) [43], characteristic of hydrophobic compounds. This entails poor hydrosolubility and a high tendency to sorb to organic material of the sludge matrix [44]. Sorption of BPA to sludge is mainly a physical process, which occurs rapidly when BPA comes into contact with sludge [45]. These characteristics made necessary an exhaustive optimization of the extraction process that would make possible a quantitative and selective recovery of the EDCs from sludge samples. Parameters such as pH, extraction solvent and the most influential variables affecting the PLE procedure were optimized. Samples spiked with 200 ng g^{-1} were used for optimization. The initial PLE conditions were temperature, 75 °C; pressure, 1500 psi; static extraction time, 5 min; two cycles; purge time, 120 s, and flush volume, 30% [30]. The values of the optimization are shown in the Supplementary material.

3.2.1. Effect of extraction pH

BPA and its chlorinated derivatives have phenolic groups that are weak acids; therefore, pH could have an influence in the extraction step. Mixtures of aqueous buffer at different pH and methanol (1:4) were evaluated. McIlvaine buffer solution [39] was selected because it covers a wide range of pH values (from 2 to 8). pH values from 10 to 13 were adjusted with NaOH solutions. Given the low water solubility of BPA and its chlorinated derivatives, a mixture with a higher proportion of organic solvent was used. We observed that recoveries were pH-dependent but were very low

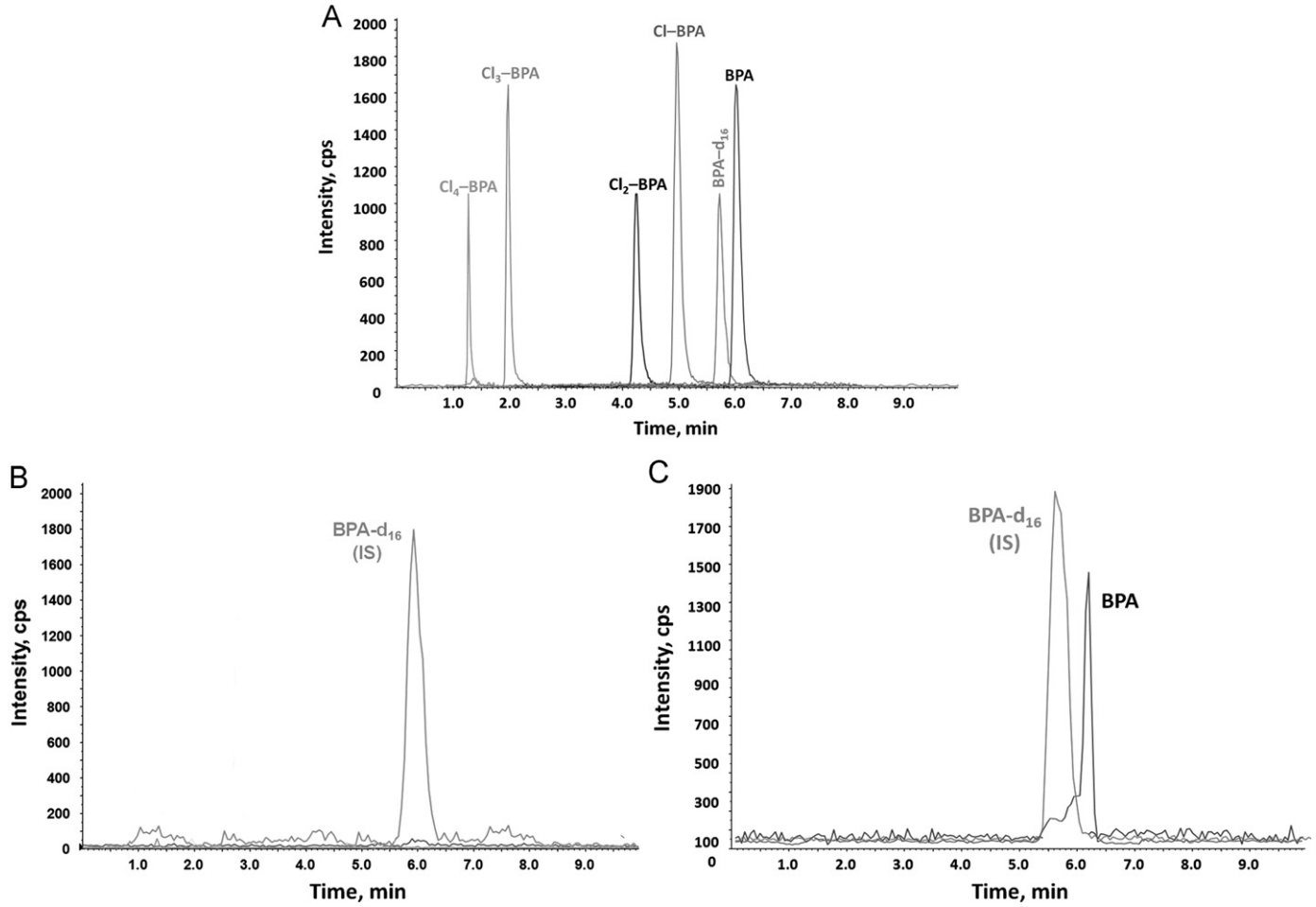

Fig. 1. MRM mode chromatograms of: (A) A standard mixture of BPA and its chlorinated derivatives (200 ng g^{-1} of each compound). (B) Not contaminated (blank) sewage sludge sample. (C) Contaminated sewage sludge sample with BPA. Flow rate: 0.35 mL min^{-1}, temperature: 40 °C and injection volume: 40 μL.

(< 65%) in all cases. These recoveries were even lower at pH values where the ionized form of analyte was more abundant. The reason for this is that the dissociated form could not be efficiently extracted using a mixture with a high percentage of organic solvent. There is a pH interval (2–6) within which the recoveries of all analytes are optimal. On the other hand, the low recoveries showed that the optimization of extraction solvent was necessary.

3.2.2. Effect of extraction solvent

Different extraction solvents were tested. Since we presumed that given the low water solubility of analytes, the presence of water in the extraction solvent could be the responsible for the observed low recoveries, only organic solvents were assayed. Solvents were slightly acidified with formic acid prior to extraction assays. Acetone, ethyl acetate, methanol, dichloromethane and a mixture of acetone–hexane (50:50, *v*/*v*) were evaluated. It was difficult to choose only one optimal solvent that would work for all analytes, but we observed that pure methanol instead the aqueous mixture increased the recoveries in about 20%. Nonetheless, ethyl acetate was chosen as compromise solvent because it provided higher recoveries (> 80%) than methanol for all studied compounds. Dichloromethane and acetonitrile provided recoveries < 70%.

3.2.3. Selection of significant PLE variables by Plackett–Burman design

Plackett–Burman design (PB) was used in the preliminary optimization process to determine the most influential factors in PLE, given the multiple parameters involved in this extraction technique. The experimental PB design resulted in 12 experiments plus three replicates for the central point. Variables were examined at two levels, low and high, as well as the intermediate level for the central point. A total of seven variables were analyzed: pressure (600 and 1700 psi), temperature (60 and 160 °C), static time (4 and 12 min), number of extraction cycles (1 and 5), preheating time (0 and 5 min), N_2 purge time (30 and 120 s) and flush (30 and 150%).

The PB design allows the screening of the most influential variables from a large number of variables. The application of the PB design was very useful in this preliminary study to differentiate the more influential variables – which will be further optimized – from those that are not. A 95% confidence interval was used for the statistical evaluation of the results. A minimum *t*-value – indicated by the vertical line in Fig. 2 – was obtained. Variables with higher *t*-values were considered statistically significant factors. Fig. 2 shows the statistically significant effect of each variable.

As shown in Fig. 2, temperature is the most influential parameter in all cases. Flush (%) was not an influential factor and it did not require optimization. It was set at the minimum value (30%), which was high enough to remove the extracted analytes and to clean the system but was low enough to avoid extreme dilutions of the extracts. The other significant parameters, such as pressure, static, preheating and purge times were positive for all compounds, except for BPA where pressure had a negative effect. The number of extraction cycles was the least influential variable.

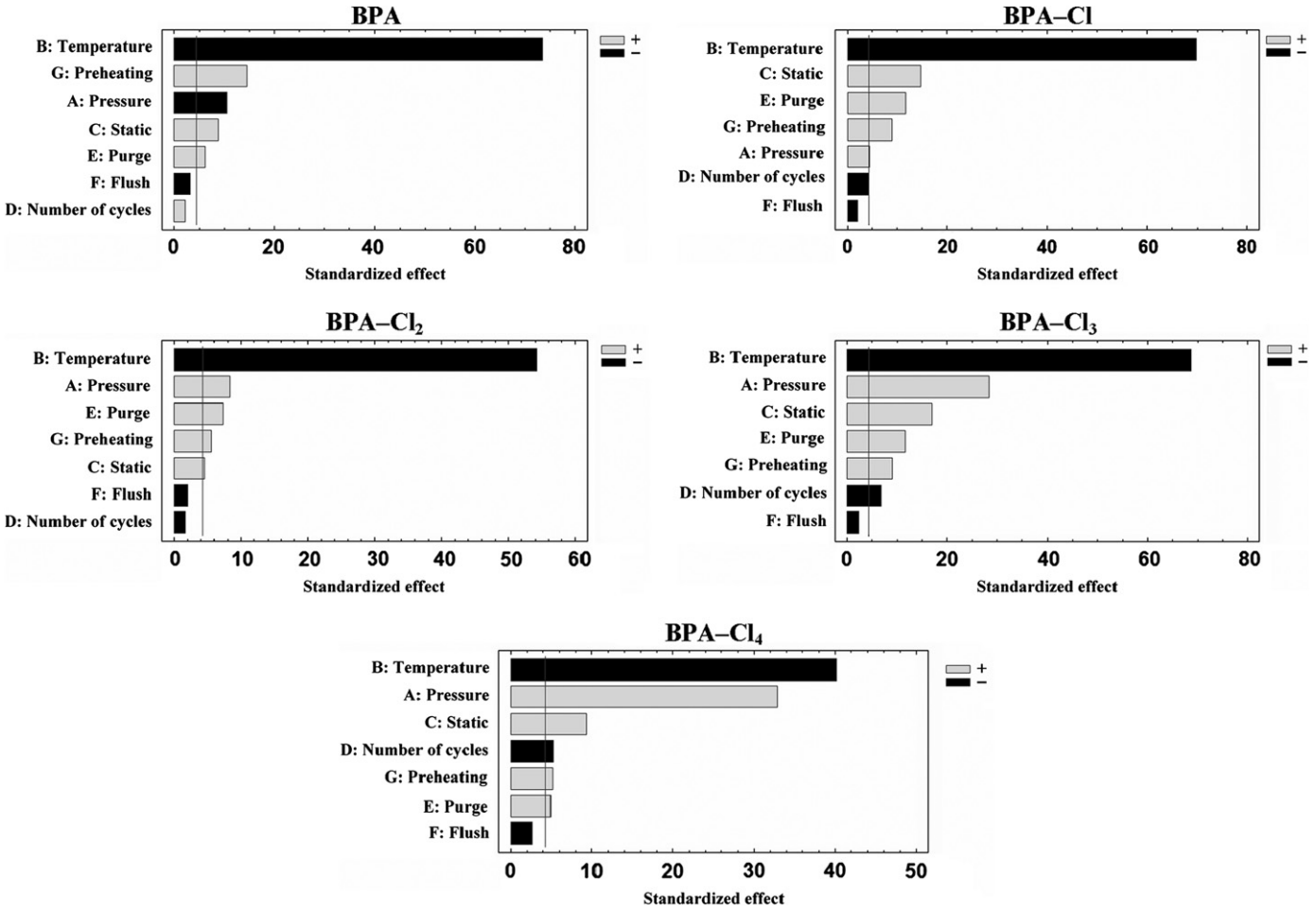

Fig. 2. Standardized main effect Pareto charts for the Plackett–Burman design.

3.2.4. Optimization of temperature and pressure

The first PLE variable that was optimized was temperature. The initial conditions for the other parameters were similar to that established for the central point in the PB design, except for flush value, previously determined. An increase in temperature has a negative effect on the recoveries of analytes, especially in cases of highly chlorinated BPA. A temperature of 100 °C was selected as compromise temperature. Probably, the higher amounts of matrix components that are extracted at higher temperatures affect the extraction of the analytes. This could be a first evidence of significant matrix effects.

Pressure was also optimized but it did not have a pronounced effect on recoveries, except for very high pressures. A value of 1000 psi was selected because values >1000 psi had a negative effect on BPA.

3.2.5. Optimization of extraction time and number of extraction cycles

Since the two variables are closely related and there is a chance of interaction between them, the optimization was done according to a Doehlert experimental design. The Doehlert matrix consisted of nine experiments, including three central points. Five levels for number of extraction cycles (from 1 to 5) and three levels for static time (from 4 to 12 min) were considered. Fig. 3 shows the response surfaces obtained.

To identify the influence of variables on the recovery of each compound, the data were evaluated by ANOVA. The test gave a determination coefficient (R^2) over 0.969, therefore the fitted regression equations explain more than 96.9% of the total variation in the data. Since the P value for the *lack-of-fit* test is >0.05 in all cases, the model appears to be satisfactory for the obtained data at the 95% confidence level. Both parameters, particularly their quadratic terms, resulted statistically significant for all compounds. In general, static time showed a positive influence, whereas the number of extraction cycles had a negative effect. Significant interactions were also observed between the variables. As a compromise solution, a static time of 8 min and three extraction cycles (or 24 min of total extraction time were selected, with recoveries $>85\%$.

3.2.6. Other extraction parameters

Preheating time is usually a fixed parameter that is not considered during the optimization process. However, in this study, the PB design determined that this variable had a positive effect on the extraction process by PLE, especially for BPA. Three different preheating times were evaluated and 2 min was established as the optimal value. Purge also was a significant variable but according to the PB design and as reported by other studies [46], its influence is not crucial. Its value was established at 75 s.

3.3. Method validation

The method performance was evaluated by determining the linearity, sensitivity, accuracy (trueness, repeatability, reproducibility) and matrix effects.

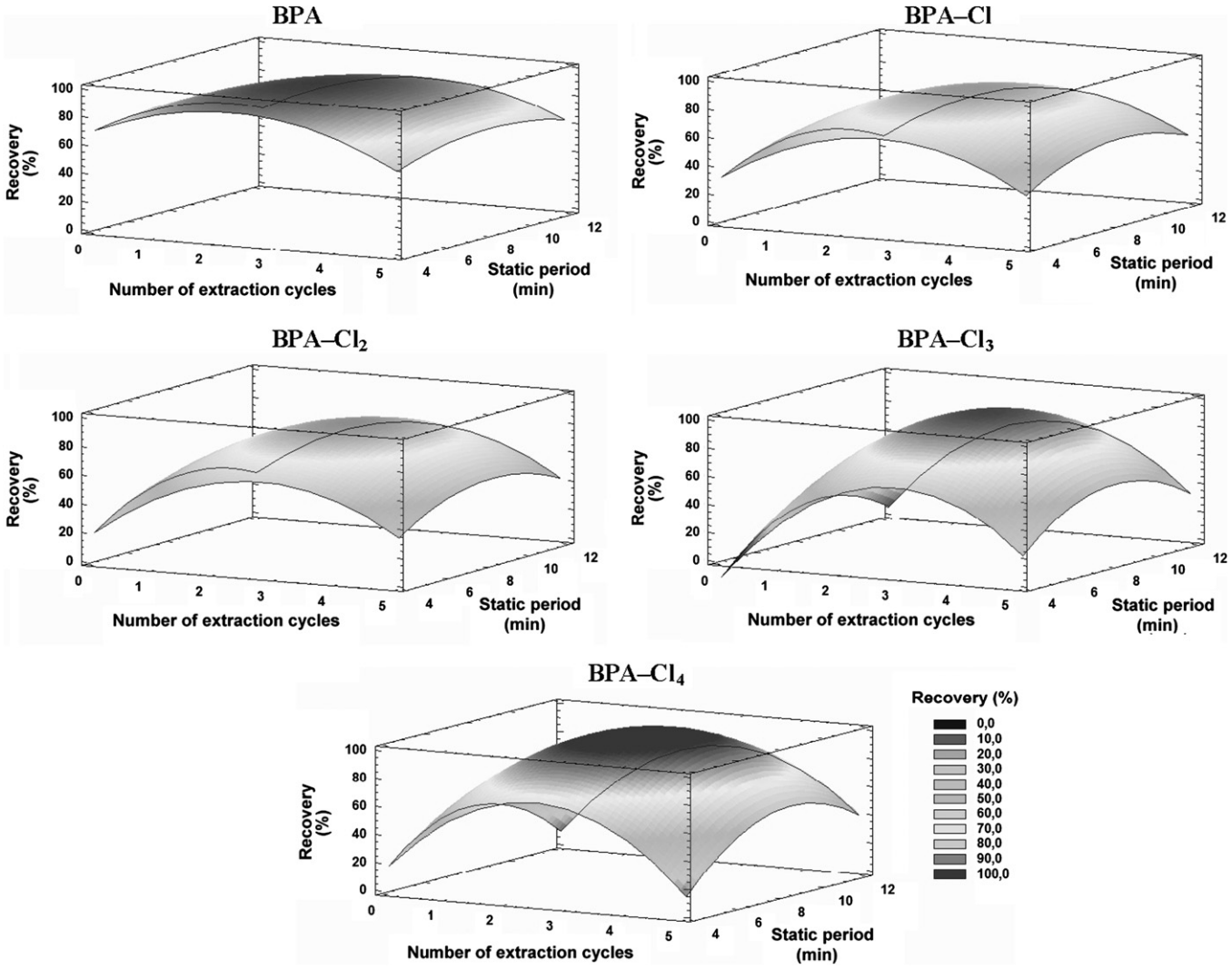

Fig. 3. Response surfaces according to Doehlert experimental design. Recovery of spiked sludge samples (200 ng g^{-1}) is shown as a function of number of extraction cycles and static time.

3.3.1. Linearity

Due to the presence of a strong matrix effect, quantification was performed by using the matrix-matched calibration, based on peak areas. BPA-d_{16}, was used as internal standard. Seven calibration points were generated in the range from the limit of quantification to 1 μg g^{-1} dry weight. Each calibration level was made in triplicate, and analyzed twice. The linearity was quantified by both linear correlation coefficient (R^2) and the lack-of-fit test (P_{lof}) Table 3 shows the main calibration parameters. Linearity for all compounds within this wide concentration range was achieved with R^2 ranging from 99.77% to 99.94% and P values of the *lack-of-fit* test were $>5\%$ in all cases; these facts indicated a good linearity within the stated ranges.

3.3.2. Selectivity

The specificity of the method could be demonstrated by LC–MS/MS analysis of blank sludge samples. Retention times of the analytes showed no interferences after analysis of the blank samples and after analysis of spiked matrices with all studied EDCs. These observations approve the high selectivity of the LC–MS/MS method. A blank chromatogram is also shown in Fig. 1.

Table 3
Analytical and statistical parameters.

Parameter[a]	BPA	BPA-Cl	BPA-Cl_2	BPA-Cl_3	BPA-Cl_4
R^2	99.89	99.94	99.82	99.77	99.80
n	42	42	42	42	42
b (g ng^{-1})	7.3×10^{-3}	1.3×10^{-2}	5.8×10^{-3}	3.8×10^{-3}	1.5×10^{-3}
s_b (g ng^{-1})	5.5×10^{-5}	7.7×10^{-5}	5.5×10^{-5}	4.3×10^{-5}	1.7×10^{-5}
$s_{y/x}$	0.085	0.120	0.086	0.066	0.025
LOD (ng g^{-1})	5	4	7	8	8
LOQ (ng g^{-1})	18	14	23	26	25
LDR (ng g^{-1})	18–1000	14–1000	23–1000	26–1000	25–1000

[a] R^2, determination coefficient; n, points of calibration; b, slope; s_b, slope standard deviation; $s_{y/x}$, regression standard deviation; LOD, limit of detection; LOQ, limit of quantification; LDR, linear dynamic range.

3.3.3. Sensitivity

Two fundamental aspects need to be examined in the validation of any analytical method to determine whether an analyte is present in the sample: the limit of detection (LOD) and the limit of quantification (LOQ). These parameters were determined as the minimum detectable amount of analyte with a signal-to-noise ratio of 3 and 10 for the LOD and LOQ, respectively. Table 3 shows the obtained values.

3.3.4. Accuracy: Precision and trueness

To assure precise quantifications, the precision of the method in terms of intra- and inter-day variability was evaluated at three concentration levels (50, 250 and 500 ng g^{-1}). Precision was determined from triplicate spiked sludge samples at different levels during the same day (repeatability) and in nine successive days (reproducibility). Precision was expressed as relative standard deviation (RSD, %). The values obtained are summarized in Table 4. RSD values fell between 0.7% and 5.7%. Precision data indicated that method is highly reproducible and robust.

Due to the absence of certified materials, a recovery assay to validate the method in terms of trueness was carried out. Blank spiked samples previously analyzed to ensure they did not contain the compounds of interest or that these were below the LOD of the method were used. Trueness was evaluated by determining the recovery of known amounts of tested compounds in sludge samples. Samples were analyzed using the proposed method and the concentration of each compound was determined by interpolation from the standard calibration curve within the linear dynamic range and compared with the amount of analytes previously added to the samples. As is shown in Table 4, the recoveries are close to 100% (97.7% to 100.6%).

3.3.5. Quantification of matrix effects

Depending on the complexity of the samples, the matrix co-extracted with the analytes can modify the signal, leading to ion suppression or enhancement when using these ionization techniques. It has been reported that APCI is less sensitive to matrix effects than ESI [47], however, both ion suppression and enhancement by co-extractive substances from sample have also been observed for LC–MS/MS using APCI [48].

Some operational strategies can be used for the compensation of interference effect caused by the matrix components. Extensive cleanup procedures prior to LC–MS/MS analysis could help to reduce the introduction of matrix components into the analytical system. However, these are sometimes laborious, costly, and cannot eliminate matrix constituents efficiently. Another approach is to dilute the final sample extract as much as possible to be injected into the analytical column. In some instances, this method is effective for eliminating signal suppression, while achieving acceptable sensitivity.

Matrix effect was evaluated by calculating the percentage of signal suppression or enhancement. The peak areas from the analysis of spiked sludge extracts were compared with the ones corresponding to the spiked solvent (mobile phase) at the same concentration. The results of signal suppression in extracts are shown in Fig. 4A.

Fig. 4 shows that chlorinated derivatives of BPA exhibited significant matrix effects, and the ion suppression increases with the degree of chlorination, reaching values $>80\%$ for BPA-Cl_4. However, in spite of strong matrix effects, especially for the chlorinated derivatives, the accuracy obtained was satisfactory for the target EDCs (Table 4) because of the use of matrix matched calibration.

In order to evaluate whether the SPE clean-up reduces effectively the matrix components, a comparison of extracts was performed, one was directly injected to the LC system after the extraction procedure, following to Section 2.5. The results are presented in Fig. 4A (left bars). The second extract was prepared reconstituting an evaporated residue with a small volume of methanol and diluting with water until 500 mL. This aqueous solution was cleaned-up by SPE, following a procedure published elsewhere [41] and obtaining a final extract with the same concentration level than the first one. The results of signal suppression for these extracts are also shown in Fig. 4A (left bars). It was observed that the clean up using SPE procedures reduced the matrix effects, but not as efficiently as expected. The SPE process was not carried out in order to reduce analysis time and operational costs.

Other alternative to overcome this problem is the dilution of the extracts obtained by the extraction of sludge samples. Dilution of samples proved to be an effective approach in cases when the preconcentration of matrix components during sample preparation magnified matrix effect. In some cases, it has been noted that dilution is sufficient to minimize the signal suppression increasing the signal intensity of the analytes, thus making possible to correct the results of quantitative analysis. In this work, a process of sequential dilution (1:1 and 1:4) of extracts of sludge samples were injected into the LC–MS/MS system and the signal intensity was compared to that obtained from non-diluted extract. Dilution led in all cases to a decrease in sensitivity

Table 4
Accuracy of the method. Precision and trueness of target compounds in samples.

Compound	Spiked level (ng g^{-1})	Trueness ($n=54$) Recovery (%)	Precision	
			Intra-day (%)[a] ($n=6$)	Inter-day (%)[a] ($n=54$)
BPA	50	99.4	1.0	1.6
	250	99.5	1.0	1.3
	500	99.4	0.7	0.9
BPA-Cl	50	99.0	1.6	2.4
	250	100.6	1.0	2.0
	500	99.3	1.3	1.7
BPA-Cl_2	50	99.0	2.8	3.2
	250	98.9	3.0	4.0
	500	99.4	1.8	2.4
BPA-Cl_3	50	99.7	3.8	4.9
	250	97.7	2.3	4.6
	500	99.7	1.3	2.5
BPA-Cl_4	50	98.7	4.6	5.7
	250	97.7	3.1	4.9
	500	99.3	2.1	5.0

[a] RSD (%) percentages.

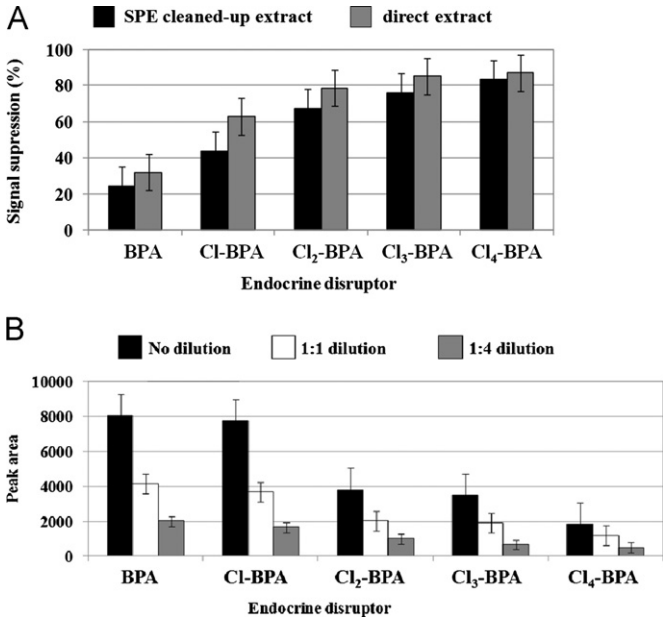

Fig. 4. (A) MS ion suppression of sludge extracts obtained from comparison of injection to LC of extracts with and without previous clean-up by SPE. (B) Sample extracts dilutions for eliminating matrix effects.

Table 5
Method application to samples from different WWTPs and from different treatments.

	Wastewater treatment[b]	Sample label	Concentration (ng g^{-1})[a]				
			BPA	BPA-Cl	BPA-Cl_2	BPA-Cl_3	BPA–Cl_4
Granada	AS	GAS-1[c]	48.3	nd	nd	nd	nd
		GAS-2 [c]	d	nd	nd	nd	nd
		GAS-3[c]	107	nd	nd	nd	nd
		GAS-4[c]	41.4	nd	nd	nd	nd
		GAS-5	d	nd	nd	nd	nd
	MBR	GMB-1[c]	nd	nd	nd	nd	nd
		GMB-2[c]	nd	nd	nd	nd	nd
		GMB-3[c]	45.9	nd	nd	nd	nd
		GMB-4[c]	23.5	nd	nd	nd	nd
		GMB-5	680	nd	nd	nd	nd
		GMB-6	30.8	nd	nd	nd	nd
		GMB-7	nd	nd	nd	nd	nd
		GMB-8	205	nd	nd	nd	nd
		GMB-9	31.1	nd	nd	nd	nd
Motril	AS	MAS-1	32.0	nd	nd	nd	nd
		MAS-2	133	nd	nd	nd	nd
		MAS-3	29.4	nd	nd	nd	nd

Note: nd, not detected (< LOD); d, detected (between LOD and LOQ).

[a] Mean of six determinations.

[b] AS: Activated sludge treatment; MBR: Membrane bioreactor treatment.

[c] Sewage samples produced from parallel treatments of AS and MBR feeded with the same wastewater.

(Fig. 4B), being therefore not a suitable alternative to improve method sensitivity by elimination of matrix effects.

3.4. Application of the method

The developed method was applied for the determination of BPA and its chlorinated derivatives in sewage sludge from two WWTPs located in the province of Granada (South-East, Spain). The sludge samples came from a conventional activated sludge (AS) plant and from a membrane bioreactor (MBR) pilot plant. Concentration values for six replicate samples are shown in Table 5. Fig. 1 also shows an example of chromatograms corresponding to some of the analyzed samples.

None of the analyzed samples contained any of the chlorinated derivatives of BPA, but BPA appeared in several of the analyzed samples. In addition, lower concentrations of BPA were found in the sewage sludge from the MBR pilot plant than in the AS plant, both fed with similar raw wastewater. This could prove the higher efficiency of MBR technology in the elimination of these substances from sludge.

4. Conclusions

The developed method allows extraction and analysis of BPA and its chlorinated derivatives from complex sewage sludge matrices using a sensitive procedure that involves pressurized liquid extraction, and determination and quantification by LC–MS/MS. The obtained data indicate strong MS signal suppression effects, which were not effectively reduced by a SPE clean-up step. The analytical performance of the method was validated, achieving very low LOD (between 4 and 8 ng g^{-1}), high recoveries and precision, which is an important achievement in comparison with other methods involving more clean-up procedures and using less efficient extraction techniques. This method allows for the determination of the levels of BPA and its chlorinated derivatives and it may be used to perform screening studies about the presence and final fate of these substances in the environment, taking into consideration that sludge or sludge-derived compost is used on agricultural land, where they pose a serious environmental threat.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2012.08.045.

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