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Simultaneous determination of the endocrine disrupting compounds nonylphenol, nonylphenol ethoxylates, triclosan and bisphenol A in wastewater and sewage sludge by gas chromatography–mass spectrometry

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Abstract

An integrated analytical method for the simultaneous determination of 4-*n*-nonylphenol (4-*n*-NP), nonylphenol monoethoxylate (NP1EO), nonylphenol diethoxylate (NP2EO), bisphenol A (BPA) and triclosan (TCS) in wastewater (dissolved and particulate phase) and sewage sludge was developed based on gas chromatography–mass spectrometry. Chromatographic analysis was achieved after derivatization with bis(trimethylsilyl)trifluoroacetamide (BSTFA). Extraction from water samples was performed by solid-phase extraction (SPE). The optimization of SPE procedure included the type of sorbent and the type of the organic solvent used for the elution. Referred to solid samples, the target compounds were extracted by sonication. In this case the optimization of the extraction procedure included the variation of the amount of the extracted biomass, the duration and the temperature of sonication and the type of the extraction organic solvent. The developed extraction procedures resulted in good repeatability and reproducibility with relative standard deviations (RSDs) less than 13% for all the tested compounds for both types of samples. Satisfactory recoveries were obtained (>60%) for all the compounds in both liquid and solid samples, except for 4-*n*-NP, which gave recoveries up to 35% in wastewater samples and up to 63% in sludge samples. The limits of detection (LODs) of the target compounds varied from 0.03 (4-*n*-NP) to 0.41 $\mu g\,l^{-1}$ (NP2EO) and from 0.04 (4-*n*-NP) to 0.96 $\mu g\,kg^{-1}$ (NP2EO) for liquid and solid samples, respectively. The developed methods were successfully applied to the analysis of the target compounds in real samples.

Keywords: Wastewater analysis; Sludge; Endocrine disruptors; Phenolic compounds; Silyl derivatization; SPE

1. Introduction

A great number of recent studies indicate the widespread occurrence of several synthetic organic compounds in wastewater and sewage sludge and significant research effort has been devoted to their identification, as well as to the investigation of their fate and toxicity in wastewater treatment systems [1–3]. Among these compounds, nonylphenol (NP), nonylphenol ethoxylates (NP_nEOs, where n indicates the number of ethoxy units), triclosan (TCS) and bisphenol A (BPA) present significant research interest due to their extended use in several consumer and personal-care products and their toxicological and physicochemical properties.

Nonylphenol polyethoxylates represent an important group of non-ionic surfactants that are widely used worldwide in a number of commercial and household formulations, including detergents, cosmetic products, water-based paints, inks and textiles [2]. Throughout the wastewater treatment, due to biodegradation, the hydrophilic ethoxylate chain of these compounds is shortened and as a result they are transformed to monoethoxylate (NP1EO) and diethoxylate (NP2EO). NP1EO and NP2EO are further biodegraded to completely deethoxylated NP, which is more lipophilic and toxic one and more resistant to biodegradation compared with the long chain ethoxylates [4]. NP, NP1EO

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and NP2EO have been reported to cause a number of estrogenic responses on aquatic organisms and thus they have been classified as endocrine disruptors (EDCs) by several organizations [2]. Nonylphenol, which has been listed as a priority pollutant in the Water Framework Directive [5], is a mixture of different branched and linear chain isomers (*ortho*-, *meta*- or *para*-), with the most common ring isomers being the *para* isomers (4-NPs), represented by 4-*n*-nonylphenol, as a recent article denotes [6]. Although early studies supported that the branched alkyl chain in the compound is more estrogenic than the linear (4-*n*-NP) [7], recent studies have been shown that 4-*n*-NP is also a strong estrogenic [8–12] and as a result its specific detection presents scientific interest.

BPA is a monomer used in the production of polycarbonates and epoxy resins from which a wide variety of products are generated such as coating of food and drink packages, dental sealants and baby-milk bottles, powder paints and optical lenses [13]. It was shown that BPA, when present in food can linings, can leach into the product and acquire estrogenic activity. In addition to its weakly estrogenic activity, BPA has been shown to possess some antiandrogenic activity [14].

TCS is a broad spectrum antimicrobial agent that, is widely used in personal-care products such as soaps, toothpastes, cosmetics, skin creams and deodorants, as well as in textiles such as sportswear, shoes and carpets [15]. According to its physicochemical properties, TCS is hydrolytically stable, relatively nonvolatile and hydrophobic [16]. According to the literature, TCS blocks lipid biosynthesis by specifically inhibiting the enzyme enoyl-acyl carrier protein reductase [17]. Moreover, recent studies have suggested that TCS has potentially weak androgenic and antiestrogenic potency [18,19].

Due to their widespread use of the aforementioned compounds, an adequate analytical method for the simultaneous detection of these compounds in wastewater and sewage sludge is needed in order to determine their concentrations in the wastewater treatment systems and derive safe conclusions for their fate and possible threat during final disposal. Up to date several extraction procedures have been developed for the isolation of nonylphenols, BPA or TCS from environmental samples. For liquid samples the most common method used is solid-phase extraction [20–25], but liquid–liquid extraction has also been reported [26]. Recently methods utilizing the direct solid-phase microextraction (SPME) of 4-*n*-NP and BPA for water samples have been also reported [27,28]. Regarding solid samples, several extraction methods have been used, including pressurized liquid extraction [20,29,30], microwave assisted extraction [31], Soxtec extraction [32] and sonication [33]. The chromatographic determination of the compounds in the extracts is achieved either by liquid chromatography [20,24,25,30,32–34] or gas chromatography [20–23,26,29] with mass spectrometric detection. Despite the high number of available analytical methods, to the best of our knowledge, none of the published studies reports the simultaneous qualitative and quantitative determination of 4-*n*-NP, NP1EO, NP2EO, TCS and BPA in wastewater and sewage sludge using an integrated method, as fast and cheap, as possible. There is one method developed by Patrolecco et al. [35] for the simultaneous determination of EDCs compounds by liquid chromatography—fluorescence detection, but the mixture of the compounds consisted of 4-NP (technical grade), NP1EO, NP2EO and BPA (TCS was not included) and the method refers to river sediment samples. Moreover, despite the fact that, due to their hydrophobicity, these compounds tend to accumulate on the particulate phase of wastewater, most available analytical methods aim to their determination only in the dissolved phase of wastewater. Ignoring the adsorption phenomena on the suspended particulate matter that may take place, it is possible to underestimate the concentrations of these compounds in influent and effluent wastewater.

According to the above observations from the literature review, the purpose of this study was to develop, optimise and fully validate a simple, fast and precise integrated analytical method for the simultaneous determination of 4-*n*-NP, NP1EO, NP2EO, BPA and TCS in the dissolved and particulate phase of wastewater, as well as, in sewage sludge. SPE and GC–MS were used for liquid samples, while sonication and SPE were applied in the case of solid samples before the GC–MS analysis. Trimethylsilylation was chosen as the derivatization procedure for all samples before the chromatographic analysis. Different extraction cartridges and organic solvents were examined for the efficient extraction of the target compounds from wastewater samples. Regarding solid samples, four key parameters in sonication procedure, including the amount of the extracted biomass, the temperature, the duration of sonication and the type of organic solvent, were carefully studied and optimized. Finally, the developed methods were applied to wastewater, particulate matter and sewage sludge samples collected from different wastewater treatment plants (WWTPs) in the island of Lesvos (Greece), so as to prove their applicability to natural samples. The sample collection was part of a more extensive monitoring program in Greece.

2. Experimental

2.1. Chemicals and standards

All the organic solvents used included methanol (MeOH), dichloromethane (DCM), hexane, ethyl acetate, acetonitrile (ACN) and acetone were of HPLC grade (Merck, Darmstadt, Germany) and were used as received. Pyridine and ultra-pure HCl (30%), used for derivatization, were purchased by Carlo Erba-SDS (Val de Reuil, France) and Merck, respectively.

Silica-based bonded C18 (Sep-Pak, 6 ml, 500 mg) and poly(divinylbenzene-*co*-*N*-vinylpyrrolidone) (Oasis HLB, 6 ml, 200 mg) cartridges were supplied by Waters (Milford, MA, USA). Styrene-divinylbenzene (EnviChrom P, 6 ml, 500 mg) and hydroxylated styrene-divinylbenzene (Isolute ENV+, 6 ml, 200 mg) cartridges were obtained from Supelco (Bellefonte, PA, USA) and IST (Mid Glamorgan, UK), respectively.

bis(Trimethylsilyl)trifluoroacetamide (BSTFA) and bis(trimethylsilyl)acetamide (BSA) were also supplied by Supelco. Analytical standards of 4-*n*-NP, NP1EO, NP2EO and TCS were supplied by Dr. Ehrenstorfer (Ausburg, Germany). BPA and the deuterated [$^2H_{16}$]bisphenol A (BPA-d_{16}), used as internal standard, were purchased from Fluka (Buchs, Switzerland). All

compounds were used without further purification (minimum percent purity >97%). Stock solutions of individual compounds were prepared in methanol at 1000 mg l^{-1} and kept at −18 °C. The stock solutions were used to regularly prepare working standard solutions for calibration and spiking experiments. HPLC grade water was prepared in the laboratory using a Milli-Q/Milli-RO Millipore System (Milford).

2.2. Sampling

Wastewater samples for the spiking procedure were collected in 1 l pre-cleaned amber glass bottles from the outlet of the Campus of University of the Aegean wastewater treatment plant. A portion of the collected samples was analyzed prior to have being spiked, to determine possible background concentrations of these compounds. A similar procedure was applied during method development in sewage sludge samples.

Following successful development, the method was applied for the determination of the target compounds to wastewater treatment plants' influents, effluents, their particulate matter and sewage sludge samples taken from different WWTPs in the island of Lesvos (City of Mytilene, Hospital of Mytilene and University Campus). Sampling was performed in March 2006. Soon after the sampling, the wastewater samples were immediately filtered and stored in the dark at 4 °C [20]. Their extraction was performed within 48 h of their arrival. Sludge samples were oven dried at 40 °C [32], grinded using a mortar and pestle and stored at −18 °C until their analysis. As for particulate matter, 10 ml of wastewater sample (influent and effluent) were filtered, the filters were oven dried until constant weight and stored at −18 °C until their analysis. The solid samples (particulate matter and sludge) were analyzed within 5 days.

2.3. Extraction of wastewater samples

Solid-phase extraction was used as the isolation technique of the target compounds from wastewater samples. The developed procedure is a modification of the previously described method of Castillo and Barcelo [36], used for the isolation of non-ionic polyethoxylated surfactants in tannery wastewaters. A volume of 100 ml of wastewater sample was filtered through a pre-ashed (4 h, 400 °C) glass fiber filter (GF/F, pore size 0.7 μm, Whatman, Brentford, Middlesex, UK). For the spiking experiments, blank samples were spiked with the five compounds at a final concentration of 0.5 μg l^{-1} each. The spiked samples were mixed in an ultrasonic bath for 10 min to ensure efficient distribution of the compounds in the solution and afterwards they were allowed to equilibrate for 10 min prior to extraction. Isolation of the compounds from the spiked wastewater samples was performed using C18 cartridges fitted on a vacuum apparatus (Alltech, Deerfield, IL, USA). The cartridges were conditioned by 2 × 3.5 ml of methanol and 2 × 3 ml of Milli-Q grade water at a flow rate of 0.5 ml min^{-1}. The samples were passed through the cartridge with a flow rate of 10 ml min^{-1}. In order to remove any interference, the cartridges were washed with 4 × 2.5 ml Milli-Q grade water and then dried under vacuum for 60 min. The compounds were eluted with 4 × 2 ml of mixture of DCM–hexane (4:1). The eluates were evaporated to dryness, under a gentle stream of nitrogen at 40 °C. An amount of 50 ng of internal standard (BPA-d_{16}) was added into the vials and further evaporation to dryness was accomplished [21]. Finally, the dried residues were subjected to derivatization reaction.

2.4. Extraction of solid samples

Extraction of the target compounds from solid samples (suspended particulate matter of the wastewater and sewage sludge) was performed by sonication. Initially, an appropriate volume of wastewater was filtered through pre-ashed GF/F filters and the filters placed in 60 °C until constant weight. The same drying procedure was applied to sludge samples. For the method development and validation experiments, the dried samples were spiked with methanolic solution of the five compounds (100 ng each) and left in a fume cupboard for 30 min to remove the organic solvent. The sonication was carried out once at 50 °C for 30 min using 8 ml of mixture of methanol (5 ml) and Milli-Q grade water (3 ml) as the extraction solvent. The supernatant was collected after centrifuging and diluted to a final volume of 100 ml using Milli-Q grade water, after which, was directly extracted, according to the aforementioned SPE method using C18 cartridges.

2.5. Derivatization procedure

To the vials containing the dried residues and the internal standard, a volume of 50 μl of BSTFA plus 50 μl of pyridine was added. The vials were closed and completely mixed for 1 min using a vortex system. The derivatization reaction was performed at 65 °C for 20 min. The derivatives were allowed to cool to room temperature and subjected to GC–MS analysis.

2.6. GC–MS analysis

For the quantitative analysis a Hewlett-Packard gas chromatograph 5890 Series II connected to a Hewlett-Packard mass spectrometer HP5971 MSD was used (Palo Alto, CA, USA). The separation of the compounds was achieved by using a DB5MS capillary column (60 m) with a film thickness of 0.25 μm and internal diameter of 0.32 mm (Supelco). The carrier gas was helium and maintained at a constant flow rate of 0.9 ml min^{-1}. A sample volume of 1 μl was injected in splitless mode at an inlet temperature of 280 °C. The column temperature, after preliminary experiments, was programmed as followed: at 80 °C for 1 min, from 80 to 220 °C at 15 °C min^{-1} and from 220 to 280 °C at 5 °C min^{-1}. The MS transfer line temperature was maintained at 280 °C, whereas the ion source temperature was 180 °C.

For the qualitative analysis, the full scan mode was used, monitoring the mass range from 50 to 400. Moreover, apart from the mass spectrum, the relative retention times of each compound was used as additional tool for the confirmation of the presence of the compounds in unknown samples, since this parameter presented excellent reproducibility (<0.15% for all the compounds). Quantitative analysis was carried out using selected ion moni-

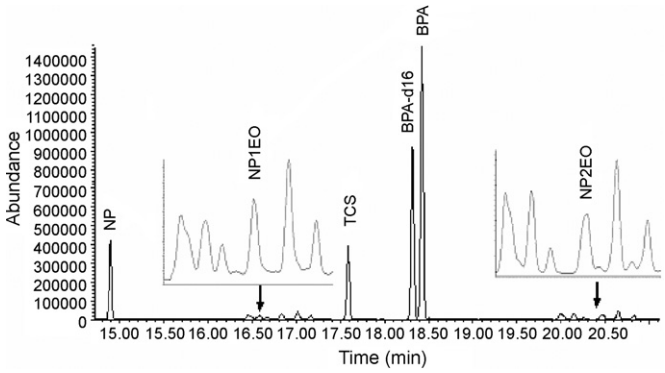

Fig. 1. Chromatogram of a standard solution containing 1 mg l^{-1} of the target compounds and the IS (BPA-d_{16}) in SIM mode.

toring (SIM) mode. For each compound, the most abundant ions were selected from its spectrum. The chosen ions for SIM were 179 and 292 for 4-*n*-NP, 237, 251, 265 and 293 for NP1EO, 200, 247 and 362 for TCS, 368 and 369 for the internal standard (BPA-d_{16}), 357 and 358 for BPA and 309 and 295 for NP2EO. Due to the fact that NP1EO and NP2EO are isomeric mixtures that are separated by GC and the signals of these isomers resulted in numerous peaks in the chromatogram, the analytical parameter for these compounds was the sum of the peak areas (Fig. 1). The selected ions of the target compounds after trimethylsilylation are in agreement with those reported elsewhere [21,22,32,37].

2.7. Statistical analysis

The results from the method development and the validation experiments were compared for any significant differences using one-way analysis of variance (ANOVA) (SPSS for Windows, Version 11.0, SPSS, 2001).

3. Results and discussion

3.1. Derivatization procedure

As referred in the open literature [38] non-ionic surfactants with a short polyethoxylate chain, such as nonylphenol (NP) and NP1EO, can be determined directly by GC due to higher volatility compared with the higher degree of ethoxylation non-ionic surfactants. TCS also has been analyzed by GC–MS without derivatization [39]. From this point of view, in order to achieve shorter analysis times, preliminary experiments were performed to examine if all of the tested compounds could be sufficiently analyzed without derivatization. However, analysis without derivatization resulted in poor chromatographic peaks, with inadequate precision, mainly for BPA, TCS and NP2EO, due to their low volatility, indicating that the derivatization procedure is essential in order to achieve the highest possible sensitivity of determination.

From the several derivatization methods, such as acylation [29], alkylation [40] and silylation [6,41] that have been used for the GC–MS analysis of phenolic compounds, trimethylsilylation was chosen in the present study. Preliminary experiments were conducted in order to select which silylation reagent between BSTFA and BSA was more suitable (produced higher peak areas with adequate repeatability). During the derivatization procedure both BSTFA and BSA were used without the addition of any other reagent. In the vials containing the dry residues a volume of 50 μl of derivatization reagent was added and the residues were derivatized for 20 min at 65 °C. The derivatives were allowed to cool at room temperature. A volume of 450 μl of isooctane:acetone in mixture (99:1) was added and sonication took place for 10 min. After sonication, the derivatives were subjected to GC–MS analysis. According to the results, BSTFA gave both narrower and higher chromatographic peaks and more precise retention times of the compounds than BSA. As reported, BSTFA reacts very fast with compounds containing hydroxyl groups, producing derivatized compounds with high volatility, adequate stability and solubility and it is enough volatile to elute very quickly near the void volume and therefore it has been used successfully by many authors for the derivatization of the target compounds [21,26,32,37,42]. BSTFA acts faster and more completely than BSA because its leaving group is trifluoroacetamide and not acetamide like BSA. Moreover, it can act as its own solvent. Although the use of BSTFA resulted in better chromatographic peaks compared with BSA, underivatized forms of some compounds were also observed in the chromatograms indicating that complete derivatization cannot be achieved using this reagent alone.

In order to improve the degree of derivatization of the compounds, different volumes of BSTFA (from 50 to 200 μl) were tested. Results showed that the degree of derivatization was not affected by the volume of the derivatization reagent. Thus, the smallest volume (50 μl) of the silylation reagent was chosen and the use of another reagent together with BSTFA was examined, so as to achieve complete derivatization of the target compounds. The use of pyridine was examined using a derivatization method that was previously described [21], with some modifications. A volume of 50 μl of BSTFA plus 50 μl of pyridine were added to the dry residues and derivatization was performed for 20 min at 65 °C. The derivatives were allowed to cool at room temperature and subjected to GC–MS analysis. Full scan analysis of derivatized standard solutions ensured that the procedure was complete and the repeatability of the procedure was significantly improved (RSD ≤ 13%) using pyridine as a catalyst for the derivatization reaction. The use of pyridine resulted in almost two-fold peak areas for NPs and three-fold for TCS and BPA. The use of pyridine together with BSTFA for the analysis of endocrine disrupting compounds has been reported by two other groups, as well [21,22].

3.2. Optimization of the extraction procedure

3.2.1. Extraction on different cartridges

The optimization of the extraction procedure of the target compounds from wastewater samples included the type of the sorbent and the type of the organic solvent for the elution. Using recovery experiments in wastewater samples, four types of cartridges were investigated for their extraction efficiency: C18, EnviChrom P, Isolute ENV+ and Oasis HLB. Before the SPE procedure, the pH of the samples was measured and it was always

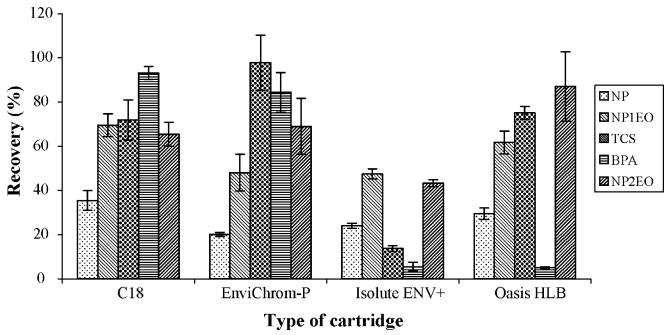

Fig. 2. Mean recoveries (%) of the target compounds in wastewater using different types of SPE cartridges.

between 7.0 and 7.5. Liu et al. [21] studied the effect of pH on extraction efficiencies of similar target analytes and the results showed that extraction recovery for all the target compounds remain relatively similar at pH 4–8. Moreover, Cai et al. [43] studied the effect of pH of water samples on the extraction efficiencies of 4-*n*-NP, 4-*tert*-OP and BPA using multiwalled carbon nanotubes. They found that the pH of sample solutions in the range of 3–11 has no significant influence on the recoveries of 4-*n*-NP and 4-*tert*-OP, but the recoveries of BPA were dramatically decreased at pH above 8. On the basis of their experimental results, pH in the range of 5–8 was chosen as the pH of the sample solutions [43].

Among the four types of cartridges, C18 cartridges resulted in sufficient absolute recoveries (>65%) for most of the compounds from the spiked wastewater sampes (Fig. 2) and the results were comparable with those reported by other authors [20,21,32]. 4-*n*-NP was the only compound for which low recoveries were obtained (35%) probably due to sample matrix effects or interference of 4-*n*-NP with ambiguous derivatization byproducts, which led to signal suppression of the compound during GC–MS analysis. The obtained recoveries for 4-*n*-NP are in accordance with those reported by other authors that use C18 or Oasis HLB cartridges and final determination with GC–MS [21,32]. In a recent study on the determination of 4-*n*-NP in wastewater samples by GC–MS, C18 cartridges were also used, but the reported high recoveries were the relative recoveries (using the standard addition method and surrogate correction) [6]. In another work on the determination of 4-*n*-NP in water samples by LC–MS [24], the reported absolute recoveries from spiked UHQ water were also low (approximately 50%) for all the tested cartridges (C18, Oasis HLB and LiChrolut EN), denoting the difficulty in extraction of this compound. It should be noted that the high recoveries of NPs reported by others authors usually include spiking in distilled water (and not wastewater), correction of the recovery by using isotope-labeled internal standards and final determination by LC–MS, regardless the type of the cartridge material [25,30,34]. Nevertheless, this results show that future experiments should be conducted in order to improve the recovery rates of 4-*n*-NP or NPs in general.

EnviChrom-P cartridges led to significant reduction of the recoveries of both 4-*n*-NP and NP1EO, whereas the recoveries of the rest of the compounds remained high. Isolute ENV+ cartridges were appeared to be completely improper for the extraction of BPA under these experimental conditions, giving recoveries less than 5% for this compound. It was shown that Isolute ENV+ could be used successfully for the extraction of BPA only if acidification of the sample is preceded [44]. This also applies to the other compounds, where lower recoveries from Isolute ENV+ were obtained, compared with C18 and EnviChrom-P cartridges. Using Oasis HLB cartridges, the extraction of BPA was again insufficient, while the recoveries of the other compounds varied almost in the same levels like on C18 cartridges. Due to the fact that the C18 cartridges resulted in sufficient recoveries for the majority of the compounds and taking into account their lower cost, they were chosen for the isolation of the target compounds from wastewater samples in the present study.

3.2.2. Elution with different solvents

As mentioned in Section 2.3, the extraction procedure used in this study based on that developed by Castillo and Barcelo [36], who used a mixture of DCM–hexane (4:1) for the elution of nonylphenol ethoxylates from C18 cartridges. In order to shorten and simplify the procedure, DCM and hexane were separately examined for their elution efficiency. The experiments were conducted in triplicate.

Both DCM and hexane did not improve the recovery of 4-*n*-NP, which remained low compared with the mixture of DCM–hexane. Elution with DCM resulted in recoveries higher than 100% for NP1EO, TCS and NP2EO indicating probably co-elution of impurities or other substances from the matrix when this solvent is used, giving rise to matrix effects in GC–MS determination. In contrast, elution with hexane did not significantly affect the recovery of both NP1EO and NP2EO compared with the mixture, but the recovery of TCS was significantly reduced from 72 (elution with DCM–hexane) to 31% (elution with hexane). Regarding to BPA, its recovery was significantly decreased from 93% when the elution was accomplished with DCM–hexane to 57 and 4.8% when DCM and hexane were used, respectively. The above results confirmed the appropriateness of the mixture of DCM–hexane for the elution not only of nonylphenol ethoxylates, which were examined in the study of Castillo and Barcelo [36], but, also for TCS and BPA.

Since the single use of both DCM and hexane as elution solvents was rejected, methanol and ethyl acetate were also examined. For the majority of the target compounds both solvents resulted in similar to DCM–hexane recoveries, with no statistically significant difference between the results, except for 4-*n*-NP, for which the recovery was significantly decreased using methanol (28%). According to these findings, the mixture of DCM–hexane was chosen as the organic solvent for the simultaneous elution of NPs, TCS and BPA.

3.3. Extraction of solid samples

In order to maximize the potential of sonication, which was chosen as the extraction method of the target compounds from particulate matter on filters, four key parameters affecting the extraction procedure were studied in detail. These parameters included the amount of the extracted biomass (10–30, 100–160

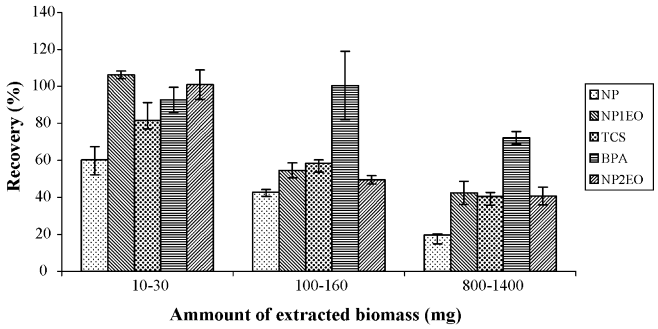

Fig. 3. Effect of the amount of biomass on the mean recoveries (%) of the target compounds in particulate matter.

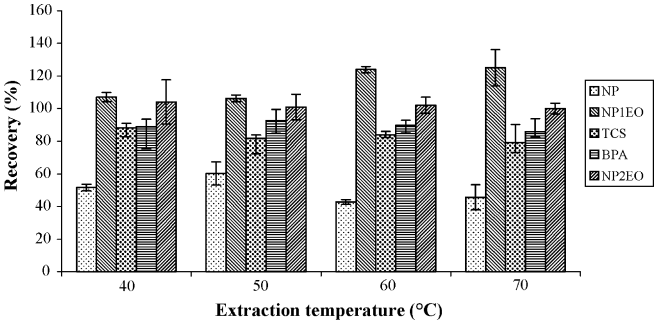

Fig. 4. Effect of the extraction temperature on the mean recoveries (%) of the target compounds in particulate matter.

and 800–1400 mg), the extraction solvent (methanol, acetonitrile and acetone), the duration of sonication (15, 30, 40 and 60 min) and the temperature of sonication (40, 50, 60 and 70 °C). All the experiments were repeated at least in triplicate and the optimum value of each parameter was chosen. Furthermore, the optimum method was applied in spiked sewage sludge samples so as to confirm the appropriateness of the selected values of each parameter to that type of substrate, as well. Blank samples were also analyzed in all cases. The results of the optimization experiments are shown in Figs. 3–6.

The extraction of up to 30 mg of biomass resulted in satisfactory recoveries, which were ranged between 60 and 100% for all the tested compounds (Fig. 3). Extraction of higher amounts of biomass affected the extraction efficiency giving lower recoveries (<60%) for almost all the compounds, probably due to the fact that the volume of the organic solvent used for the extraction remained constant to 5 ml, although the amount of biomass was increased and, as a result, it was not high enough so as to extract the compounds to a greater extent. Moreover, matrix effects during the GC–MS determination could be increased. The only compound for which the increase of the amount of biomass did not actually affect its recovery was BPA. The recovery of the compound remained high for up to 160 mg of biomass, probably denoting the appropriateness of BPA-d_{16} as internal standard for BPA determination. This might also occur due to the lower hydrophobicity of BPA compared with the other target compounds. The log K_{ow} for BPA is 3.4 while for the other compounds is higher than 4 (log K_{ow}: NP, 4.48; NP1EO, 4.17; NP2EO, 4.20; TCS, 4.78) [35] and thus BPA might not be bound so strongly on the particulate matter, like the other compounds, resulting in easier removal from the solid matrix into the extraction solvent. However, when higher amounts of biomass were extracted (800–1400 mg), a reduction of about 30% in the recovery of BPA was observed. In a recent review on the determination of estrogens in environmental samples, Gomes et al. [45] supported that sludge is the "dirtiest" environmental sample, so 10-fold lower amount than sediment should be used for the extraction of estrogens. From this set of experiments, a sample mass range between 10 and 30 mg was chosen as the amount of the extracted matrix.

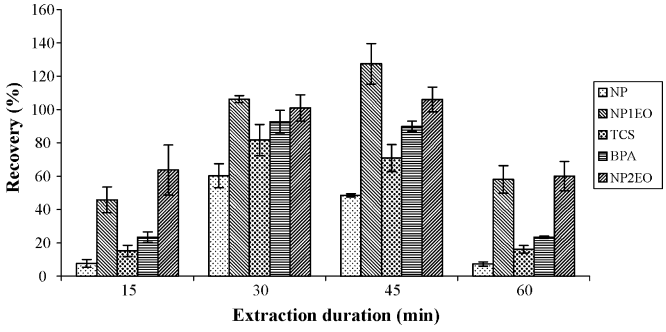

Fig. 5. Effect of the extraction time on the mean recoveries (%) of the target compounds in particulate matter.

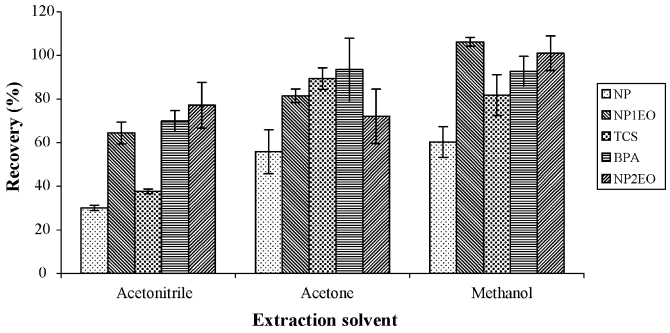

Fig. 6. Mean recoveries (%) of the target compounds in particulate matter after the extraction with different organic solvents.

The increase of the temperature had no significant influence on the recoveries of the target compounds (Fig. 4). Among the four examined temperatures slightly better recoveries were observed for all the compounds at 50 °C. It should be noted that even if the boiling point of methanol is 65 °C [46], no evaporation of the solvent was observed during the extraction at 70 °C, due to the addition of water (3 ml), which increased the boiling point of the mixture. From this set of experiments, 50 °C temperature value was chosen as the best temperature for the extraction of the target compounds.

On the contrary, the duration of the extraction affected the recoveries of the target compounds (Fig. 5). When the biomass samples extracted for 15 min, the recoveries were ranged from 7 to 63% indicating that the time was not enough for the sufficient extraction of the target compounds. Increasing the duration of the extraction from 15 to 30 or 45 min, the recoveries of the compounds were significantly improved. The recoveries achieved at these two sonication periods were similar for almost all the compounds. Longer extraction duration (60 min) resulted

Table 1
Analytical characteristics of the optimized GC–MS method for the determination of endocrine-disrupting compounds in wastewater and sewage sludge: calibration equations and coefficients of determination (R^2) for concentrations ranged from 0.10 to 10 mg l^{-1} for each analyte and internal standard concentration 0.50 mg l^{-1}, and method limits of detection and quantification

Compound	Calibration equation	R^2	LOD (μg l^{-1})	LOQ (μg l^{-1})	LOD (μg g^{-1})	LOQ (μg g^{-1})
4-*n*-NP	$y = 7.177x - 0.035$	0.9994	0.03	0.11	0.04	0.13
NP1EO	$y = 1.094x + 0.028$	0.9969	0.34	1.13	0.49	1.61
TCS	$y = 1.347x - 0.002$	0.9968	0.13	0.42	0.15	0.49
BPA	$y = 1.909x - 0.023$	0.9982	0.14	0.48	0.56	1.84
NP2EO	$y = 0.289x - 0.003$	0.9909	0.41	1.34	0.96	3.17

in reduction of the recoveries, probably due to the degradation of the compounds [47]. According to the results of these experiments, a sonication time of 30 min was selected for the optimized method.

Among the three water miscible organic solvents that were examined for their extraction efficiency, both acetone and methanol were proved the most appropriate for the extraction of the target compounds from the particulate matter, while acetonitrile resulted in lower recoveries compared with those obtained by the other two solvents (Fig. 6). Although the recoveries obtained using acetone were not statistically different from those observed with methanol, the repeatability of the results was slightly better in the case of methanol with RSDs ranged from 2.0 to 12%, whereas for acetone the RSDs ranged between 4 and 18%. Accordingly, methanol was chosen as the organic solvent of choice for the simultaneous extraction of the target compounds.

The optimized procedure (20 mg of biomass spiked with 100 ng of each compound extracted with 8 ml of mixture of methanol–water at 50 °C for 30 min) was repeated in triplicate to confirm the optimization results and satisfactory recoveries (53–116%) for all the compounds were obtained. Furthermore, the developed method was applied to sewage sludge samples, which were collected from the University Campus wastewater treatment plant (WWTP). The samples were oven dried at 60 °C, homogenized and a portion of about 20 mg spiked with the target compounds and extracted with the optimized procedure. This recovery experiment was repeated three times. The recoveries in spiked sewage sludge samples ranged between 51 (for 4-*n*-NP) and 102% (for NP2EO) with adequate repeatability (RSD < 15%). The results indicated that the developed method can be applied successfully for the extraction of the target compounds, not only from loaded filters, but from sewage sludge samples as well, giving the advantage of using only one method for the simultaneous determination of NPs, TCS and BPA in both particulate matter of wastewater and sludge.

Compared with other studies [33], the developed extraction method is much faster, since satisfactory recoveries are obtained for all the compounds after 30 min of sonication time. Moreover, very small volumes of organic solvent are used (only 5 ml of methanol) lowering the cost and shortening even more the time of analysis, because the samples can be subjected directly to SPE for the clean up step. Thus, the rotary evaporation, which normally is used when large volumes of organic solvents are used for extraction and often leads to losses of the most volatile compounds [24], can be avoided. Furthermore, the proposed method in this study could be characterized as an "environmental friendly" method, since low volumes of toxic organic solvents are needed to be disposed.

3.4. Validation of the method

An internal instrument calibration was carried out with BPA-d_{16} as internal standard for concentrations ranged from 0.10 to 10 mg l^{-1} for each analyte, with three replicates per concentration. BPA-d_{16} was present at a concentration of 0.50 mg l^{-1} in every standard solution. As shown in Table 1, the response of MS detector was linear for all the target compounds with coefficients of determination, $R^2 > 0.99$.

For the determination of the method LOD, 100 ml of wastewater and about 20 mg of particulate matter were extracted and then spiked with 100 ng of BPA-d_{16}. The LOD of each compound for the two types of samples was determined as three times the standard deviation of six independent replicate analyses. Limits of quantification (LOQs) were determined as 3.3 times of LODs. For the wastewater samples, the obtained LODs ranged from 0.03 (4-*n*-NP) to 0.41 μg l^{-1} (NP2EO), whereas for solid samples the LODs varied between 0.04 (4-*n*-NP) and 0.96 μg g^{-1} (NP2EO). Accordingly, the LOQs varied from 0.11 (4-*n*-NP) to 1.34 μg l^{-1} (NP2EO) for wastewater samples and from 0.13 (4-*n*-NP) to 3.17 μg g^{-1} (NP2EO) for solid samples. The achieved LODs and LOQs are adequate for environmental monitoring of the target compounds and low enough, taking into account the complexity of the samples and the low sample amounts used. Both for wastewater and solid samples, the LODs achieved in the present work were at similar levels or lower than those obtained in previous studies with GC–MS. Agüera et al. [20] determined for urban wastewater samples a LOD value of 1 μg l^{-1} for TCS. Ballesteros et al. reported LOQs of 20 and 150 ng l^{-1} for 4-*n*-NP and BPA, extracting a 500 ml sample [6]. Hernando et al. achieved a low LOD of 26.5 ng l^{-1} for BPA in wastewater [22]. Petrovic et al. [33] obtained LODs for NP1EO and NP in sludge samples equal to 25 and 5 μg kg^{-1}, respectively, whereas according to Meesters and Schröder [29], the obtained LODs in spiked sewage sludge samples for both NP and BPA determined by GC–MS as acetates were calculated to 0.125 mg kg^{-1}.

For both types of samples, precision was assessed by performing repeatability and reproducibility experiments. For repeatability experiments, six replicates of a sample (either wastewater or sewage sludge) were spiked at a level of 100 ng of the target compounds and analyzed during 1 day ($n = 6$, intra-day preci-

Table 2
Precision data of the extraction procedures for the two types of substrates

Compound	Wastewater		Sewage sludge	
	Intra-day precision RSD (%), $n=6$	Inter-day precision RSD (%), $n=3$, $k=3$	Intra-day precision RSD (%), $n=6$	Inter-day precision RSD (%), $n=3$, $k=3$
4-*n*-NP	4.1	12	2.0	3.2
NP1EO	5.2	7.3	9.2	9.3
TCS	2.8	6.4	4.4	4.5
BPA	5.0	5.6	4.0	5.5
NP2EO	3.3	9.1	11	13

Level of spike for each analyte: 100 ng.

Table 3
Mean recoveries (%) and standard deviations ($n=6$) of the target compounds in spiked wastewater samples

Compound	Concentration level		
	0.5 $\mu g\,l^{-1}$ recovery (%)	1 $\mu g\,l^{-1}$ recovery (%)	3 $\mu g\,l^{-1}$ recovery (%)
4-*n*-NP	31.2 ± 1.1	35.5 ± 4.4	32.4 ± 3.8
NP1EO	60.0 ± 9.2	69.5 ± 5.1	67.9 ± 4.0
TCS	80.0 ± 3.6	71.9 ± 9.1	86.7 ± 14.9
BPA	86.7 ± 5.6	93.2 ± 3.0	87.7 ± 2.6
NP2EO	60.3 ± 18	57.1 ± 5.4	53.6 ± 12.6

sion). For reproducibility experiments three replicates ($n=3$) of wastewater or sludge samples spiked at the same level as above were analyzed at 3 different days ($k=3$) over a period of 1 week (inter-day precision). Precision data of the extraction procedure for the two types of samples are given in Table 2. The results had shown satisfactory intra and inter-day precision of the analytical procedure, both for wastewater and biomass samples. RSDs were less than 12% for all the compounds in both substrates, indicating the good precision of the developed extraction methods for both types of samples and the advantage of the applied internal standard method.

In order to evaluate the trueness of the method, recovery experiments were performed. To accomplish this, wastewater (100 ml) and biomass samples (about 20 mg) were spiked at three fortification levels for each compound. The results are given in Tables 3 and 4. The recoveries ranged between 31.2 and 93.2% and between 47.6 and 106%, for wastewater (Table 3) and sludge samples (Table 4), respectively. Statistical analysis of the results had shown that there is no significant difference between the recoveries at the three fortification levels. As regards 4-*n*-NP, since the repeatability for this compound is very good (RSD < 4.5%), its low recovery should be accepted [48] and the method can be applied, correcting the results with the recovery rate. As mentioned before, more experiments should be conducted for increasing the recovery of the 4-*n*-NP determination.

Table 4
Mean recoveries (%) and standard deviations ($n=6$) of the target compounds in spiked sludge samples

Compound	Level of spiking		
	50 ng recovery (%)	100 ng recovery (%)	200 ng recovery (%)
4-*n*-NP	54.5 ± 5.8	62.7 ± 8.1	47.6 ± 2.7
NP1EO	99.5 ± 6.4	106 ± 2.1	106 ± 6.8
TCS	76.6 ± 1.3	85.5 ± 9.6	70.6 ± 0.0
BPA	101 ± 7.1	96.3 ± 4.1	87.2 ± 1.0
NP2EO	87.6 ± 8.3	101 ± 7.9	86.3 ± 12.2

Regarding the solid samples, the recoveries of the target compounds obtained using sonication for their isolation from solid samples, are comparable with those achieved by other extraction methods such as pressurized liquid extraction [20,29], Soxtec extraction [32], but the proposed method is much easier and faster and it requires simpler and cheaper instrumentation than the aforementioned techniques.

3.5. Environmental levels

The developed methods were successfully applied to wastewater, particulate matter and sewage sludge samples taken from different WWTP, as mentioned before, in order to monitor the levels of the target compounds in the environment. The results are given in Table 5. The concentrations for 4-*n*-NP are reported as they were determined and they were not corrected for recovery. According to the results (Table 5), most of the target compounds were determined in all samples at concentrations lower than those referred in other studies for WWTP samples [25,31,33,43]. In influent samples, the levels of the compounds were ranged between <LOD (4-*n*-NP) and 9.07 (TCS) $\mu g\,l^{-1}$ and between <LOD (BPA) and 37.0 (TCS) $\mu g\,g^{-1}$ for wastewater and particulate matter, respectively, whereas in effluent samples, the concentrations varied from <LOD (NP) to 5.22 (NP1EO) $\mu g\,l^{-1}$ and from <LOD (4-*n*-NP) to 103 (NP1EO) $\mu g\,g^{-1}$ for wastewater and particulate matter, respectively. Sewage sludge samples were collected and analyzed only from WWTP of the City of Mytilene and the levels of the target compounds ranged from 0.11 (4-*n*-NP) to 2.89 (NP2EO) $\mu g\,g^{-1}$. Since considerable concentrations of the compounds were detected in particulate matter, this substrate should also be analyzed in order to avoid underestimation of the results.

Table 5
Levels of the target compounds in wastewater, particulate matter and sewage sludge samples from different WWTPs

	Influent		Effluent		Sewage sludge ($\mu g\,g^{-1}$)
	Filtrate ($\mu g\,l^{-1}$)	Particulate matter ($\mu g\,g^{-1}$)	Filtrate ($\mu g\,l^{-1}$)	Particulate matter ($\mu g\,g^{-1}$)	
WWTP of Mytilene City					
4-*n*-NP	0.04	0.46	0.07	1.87	0.11
NP1EO	0.75	10.3	0.56	13.9	1.01
TCS	0.28	10.1	0.43	31.2	1.84
BPA	0.16	3.75	0.22	10.4	0.62
NP2EO	0.68	11.1	1.05	29.6	2.89
WWTP of the Hospital					
4-*n*-NP	<LOD	0.05	<LOD	0.14	–
NP1EO	2.63	9.49	5.22	103	–
TCS	9.07	37.0	1.12	16.1	–
BPA	1.01	0.66	0.13	0.77	–
NP2EO	3.60	22.0	3.43	66.1	–
WWTP of the University					
4-*n*-NP	0.09	0.05	<LOD	2.17	–
NP1EO	0.79	1.46	<LOD	11.1	–
TCS	0.79	1.05	0.23	22.4	–
BPA	<LOD	<LOD	<LOD	<LOD	–
NP2EO	1.75	3.78	0.68	1.12	–

4. Conclusions

An efficient and accurate integrated method for the simultaneous determination of 4-*n*-NP, NP1EO, NP2EO, TCS and BPA in wastewater (filtrate and particulate matter) and sewage sludge samples was developed. During method development various key parameters were carefully studied.

For liquid samples, sufficient isolation of almost all the compounds from the matrix was obtained using C18 cartridges and 8 ml of mixture of dichloromethane–hexane (4:1) as the elution solvent, whereas for solid samples sufficient extraction of the target compounds was performed by sonication of about 20 mg of biomass in 30 min at 50 °C using only 5 ml of organic solvent (plus 3 ml of water), reducing the time and the cost of analysis.

The proposed methods show satisfactory precision, good recoveries for almost all the tested compounds and adequate limits of detection for environmental monitoring, taking into account the complexity of the matrices and the small amounts of the extracted samples (100 ml of wastewater and about 20 mg of biomass). Performance of the method was demonstrated by its application to samples from various WWTPs. The target compounds were detected in all of the samples.

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