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Determination of alkylphenols and 17β-estradiol in fish homogenate. Extraction and clean-up strategies

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ABSTRACT

The determination of target analytes such as nonyl- and octylphenols and 17β-estradiol in fish homogenate require of solid–liquid extraction step. In this work microwave-assisted extraction (MAE) and focused-ultrasound liquid extraction (FUSLE) were studied as two different alternatives for extraction of the target compounds in zebrafish (*Danio rerio*) homogenate. In this work solid phase extraction (SPE) using 5-g and 10-g Florisil cartridges and gel permeation chromatography (GPC) were studied for the clean-up of the MAE and FUSLE extracts due to the non-selective extraction step. Although good recoveries were obtained both for SPE (106% and 126% range) and GPC (79% and 100% range) clean-up procedures, cleaner chromatograms were obtained after SPE and finally 5-g Florisil cartridges were tested since no improvement was observed when 10-g Florisil cartridges were used. Under optimized clean-up conditions, MAE and FUSLE provided comparable results for 4nOP and NP, while more accurate results were obtained for 4tOP and E2 after FUSLE. Finally, the method was applied to the determination of alkylphenols and 17β-estradiol in zebrafish homogenate that had been exposed to known concentrations of the target analytes. In the case of alkylphenols two different isomers of nonyl- and octylphenol (4-(3′,6′-dimethyl-3′-hepthyl)phenol, 363-NP, and 4-(3′-methyl-3′-hepthyl)phenol, 33-OP) were studied.

1. Introduction

The interest of analytes such as alkylphenols and estrogenic compounds has increased in the last decades due to their possible effects as endocrine disruptor compounds (EDCs) once they enter the environmental ecosystems. Actually, alkylphenols such as nonyl- and octylphenols (NPs and OPs, respectively) have been included as priority pollutants by the European Water Framework Directive (WFD) and estrogens such as 17β-estradiol (E2) as emerging pollutants [1].

NPs and OPs are important intermediates in the production and degradation of their polyethoxylate surfactants, which have a wide variety of industrial, agricultural and household applications [2–12]. Because of their properties as EDCs, the use and sale of products containing more than a 0.1% of nonylphenol ethoxylates or NPs has been forbidden in the European Union (EU) since 2005 [13] and worldwide actions have been taken in order to restrict their use [2]. Not only are these alkylphenols interesting because of their endocrine disruptive properties but also due to their wide distribution and high concentrations in the environment [2]. Under the synthesis procedures used, the technical NP is a mixture of more than 20 isomers, especially para-substituted, with different alkylic chains [14–16]. Recently, several works have demonstrated that the estrogenic activity depends on the structural features of the isomers and, thus, it is necessary to study the specific activity of each isomer [17].

The use of steroid hormones such as E2 in the fattening of animals has been described since 1950 but such use was forbidden by the EU [18–20] due to the risks on human health [6,21,22].

The most important source of EDCs such as NPs, OPs and E2 to the environment is through urban or industrial inputs [2]. EDCs accumulate in wastewater treatment plants (WWTPs) due to their incomplete elimination during wastewater treatment [2–4,23,24]. This way, the water cycle has become a priority environmental issue [22]. Although EDC concentrations have often been measured in wastewater effluents, no conclusive association has been proven

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yet between environmentally relevant concentrations and estrogenic effects. In this sense, the measurement of those analytes *in vivo* experiments is necessary to understand their estrogenic activity [25]. Therefore, it is necessary to develop trustful and robust methods for the determination of such analytes in biological tissues such as fish homogenate.

The analysis of target analytes like the ones mentioned above requires of extraction and clean-up steps prior to chromatographic separation and analysis as reviewed elsewhere [26–29]. In the case of the extraction step, Soxhlet is often used [10,30] although it requires of high extraction volumes and long extraction periods. Recently, other extraction techniques such as sonication [31,32] or accelerated solvent extraction (ASE) [33–35] have also been used for the determination of such target analytes from biota samples in order to minimise solvent consumption and analysis time. In a similar trend, microwave-assisted extraction (MAE) [36–42] or focused-ultrasound solid–liquid extraction (FUSLE) [43–46] have also been successfully applied to the extraction of organic pollutants from environmental matrices such as sediment or biota. However, none of the extraction techniques mentioned above is selective and a clean-up step is necessary in order to eliminate compounds, mainly lipids, that can interfere during the chromatographic separation and analysis of the analytes of interest [30–32,47]. Solid-phase extraction (SPE) using Flosiril, silica, alumina, aminopropyl silica or diol cartridges and/or gel permeation chromatography (GPC) have been mostly studied with these clean-up purposes [30–32,35,47].

The aim of the present work was to study two of the steps, the extraction and clean-up, of the analysis of NPs, OPs and E2 in zebrafish homogenate. This work is part of a project where the estrogenic activity of NP and OP isomers is being studied in both *in vivo* and *in vitro* experiments. Thus, the developed method was applied to the determination of (4-(3′,6′-dimethyl-3′-hepthyl)phenol, 363-NP, and 4-(3′-methyl-3′-hepthyl)phenol, 33-OP) and E2 in zebrafish homogenate.

2. Experimental

2.1. Reagents and materials

4-*tert*-octylphenol (4tOP) was supplied by Supelco (Walton-on-Thames, UK); 4-n-octylphenol (4nOP, 99%), 4-*n*-nonylphenol-2,3,5,6-d_4 (4nNP-d4, 98%) and 17β-estradiol-16,16,17-d_3 (E2-d3, 98%) by Aldrich (Steinheim, Germany); nonylphenol technical mixture (NPs, Pestanal®) by Fluka (Steinheim, Germany) and 17β-estradiol (E2, ≥98%) by Sigma (Steinheim, Germany). 5000 mg/L stock solutions of analytes were individually prepared in methanol and stored in amber vials at −20 °C. Dilutions of the stock solutions (50 mg/L) were performed for sample spiking and calibration purposes.

N,O-bis(trimethylsilyl)trifluoroacetamide with 1% of trimethylchlorosilane (BSTFA11% TMCS, Sylon BFT, 99:1) was purchased from Supelco (Walton-on-Thomas, UK) and pyridine (99.5%) from Alfa Aesar (Karlsruhe, Germany).

Methanol (MeOH, HPLC grade, 99.9%), ethyl acetate (EtOAc, HPLC grade, 99.8%), dichloromethane (DCM, HPLC grade, 99.8%) and acetone (HPLC grade, 99.8%) were supplied by LabScan (Dublin, Ireland).

5 g and 10 g Florisil® cartridges were obtained from Supelco (Walton-on-Thames, UK).

Zebrafish homogenate was prepared as follows: after eliminating the tail and the fins of each zebrafish, samples of each experimental group were homogenized adding 20% ultra pure water in a Potter S homogenizer (B.Braun, Melsungen, Germany) held in an ice-water cooled bath with 4–5 strokes.

1 g of fish homogenate was fortified after addition of 10 μg of NPs and 2 μg of 4tOP, 4nOP and E2 and covered in acetone in order to obtain a slurry, which was stirred overnight. Acetone and not methanol was preferred for homogenization of the fortified sample since it is more easily evaporated. Acetone was gently evaporated at room temperature and in a hood. The fortified fish homogenate obtained was kept in the refrigerator at −20 °C for a month before extraction.

EtOAc and *n*-hexane used during the synthesis of individual isomers were supplied by Panreac (Barcelona, Spain) and anhydrous ligroin and anhydrous diethyl ether by Aldrich (Steinheim, Germany).

Magnesium, crystal of iodine, 1-bromobutane, 3-methyl-1-bromobutane, 2-butanone and BF_3-Et_2O complex were supplied by Aldrich.

Calcium chloride, ammonium chloride, anhydrous sodium sulfate were supplied by Panreac.

TLC silica gel sheets (0.040–0.063 nm) were supplied by Merck (Darmstadt, Germany).

2.2. Microwave-assisted extraction

MAE experiments were performed with the MDS-2000 closed microwave solvent extraction system (CEM, Matthews, NC, USA) equipped with a 12-sample tray and pressure feedback/control. The MAE procedure used was optimized before for the determination of polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), phthalate esters (PEs) and nonylphenols (NPs) in sediment and biota samples [36,37]. Briefly, approximately 0.03–0.1 g of fish homogenate were accurately weighed and quantitatively transferred to the Teflon lined extraction vessel. 350 ng of 4nNP-d_4 and E2-d_3 and 5 mL of acetone were added to the sample and the extraction vessel was closed. Extractions were performed at 21 psi for 15 min at 504 W (80% of the maximum irradiation power). When the irradiation period was completed, samples were removed from the microwave cavity and were allowed to cool to room temperature before opening. The supernatant was filtered through PTFE filters (25 mm, 5 μm, Waters), which had been previously washed with the extraction solvent. The extract was concentrated to ∼0.5 mL using nitrogen blow-down evaporation after the addition of ∼1 mL of *n*-hexane. The concentrated extract was submitted to the clean-up step (SPE or GPC).

2.3. Focused-ultrasound solid–liquid extraction

0.03–0.1 g of fish homogenate were accurately weighed and transferred to the Teflon lined extraction vessel. 350 ng of 4nNP-d_4 and E2-d_3 and 5 mL of acetone were added and the mixture was exposed to ultrasonic irradiation (Sonopuls HD 2070, 20 Hz, 70 W, Bandelin electronic GMBH & Co. KG, Berlin, Germany) under 45% power for 2 min and 5 cycles, with the titanium tip (MS73, diameter 3 mm, Bandelein) of the probe immersed 1 cm from the upper surface of the slurry. Instrumental conditions were fixed according to a previous work of the research group [44]. The supernatant was filtered though PTFE filters (25 mm, 5 μm, Waters). The extract was concentrated to ∼0.5 mL using nitrogen blow-down evaporation after the addition of ∼1 mL of *n*-hexane. The concentrated extract was submitted to the clean-up step (SPE or GPC).

2.4. Solid phase extraction

SPE cartridges (5 g or 10 g Florisil) were conditioned with 10 mL or 20 mL of *n*-hexane, respectively. Next, the extracts were loaded on top of the Florisil cartridges and the analytes were eluted with different volumes of ethyl acetate (see Section 3) in order to minimise the elution of interfering compounds and maximise the

recovery of alkylphenols and E2. Finally, the extracts were concentrated to dryness under a gentle stream of nitrogen, previous transfer to 2 mL amber vials, and submitted to a derivatisation step before GC–MS analysis.

2.5. Gel permeation chromatography

GPC clean-up of the extracts was carried out in a HP 1100 Series liquid chromatograph (Agilent Technologies, Avondale, PA, USA) coupled to a diode array (DAD) and a fluorescence detector (FLD) and equipped with an automatic injector and a fraction collector. 150 μL of fish homogenate extract or a solution of target analytes was injected into an Envirosep ABC (350 mm × 21.2 mm) gel permeation column (Phenomenex®, Torrance, CA, USA). Dichloromethane was used as mobile phase at a flow rate of 5 mL/min. The detectors were set at 254 nm for the DAD and 250 nm (excitation) and 410 nm (emission) for the FLD, respectively.

By means of the fraction collector, from 15.0 min to 18.0 min of the elution profile and at every 0.3 min, a fraction (~1.5 mL) was taken in a vial. The collected fractions were evaporated to dryness, previous transfer to 2 mL amber vials, before derivatisation and GC–MS analysis.

2.6. Derivatisation

Concentrated extracts were re-dissolved in 125 μL of pyridine and 25 μL of BSTFA + 1% TMCS were added. The mixture was shaken in a vortex and sonicated at 80% of power and 9 cycles for 10 min in a Bandelin Sonoplus HD 2070 ultrasound system with a BR 30 Cup booster [48].

2.7. Gas chromatographic–mass spectrometric detection

The derivatised analytes were analyzed in a 6890N gas chromatograph (Agilent Technologies, Avondale, PA, USA) equipped with an Agilent 5973N electron impact ionisation mass spectrometer and a 7683 Agilent autosampler. 2 μL of the derivatised extract was injected in the splitless mode at 280 °C into a HP5 MS (30 m × 0.25 mm, 0.25 μm) capillary column. The following oven temperature program was used for the separation of the analytes: 100 °C (5 min), temperature increase at 10 °C/min to 200 °C, a second increase of 5 °C/min up to 240 °C, and a final increase of 20 °C/min to 300 °C where it was finally held for 2 min. Helium (99.9995%, Carburos Metálicos, Barcelona, Spain) was used as carrier gas at a constant flow of 1 mL/min. The transfer line temperature was maintained at 290 °C, and the ion source and quadrupole at 230 °C and 150 °C, respectively. Measurements were performed both in the scan (50–525 *m*/*z*) and in the SIM (Selected Ion Monitoring) modes. The *m*/*z* values followed for each analyte are the following 4tOP (207, 278), NPs (193, 179), 4nOP (179, 278), NP-d_4 (183, 296), E2 (416, 285), E2-d_3 (419, 285). First ion was used as quantifier and the second one as qualifier.

2.8. Synthesis of 4-(3′-methyl-3′-hepthyl)phenol (33-OP) and 4-(3′,6′-dimethyl-3′-hepthyl)phenol (363-NP)

2.8.1. Synthesis of 3-methylheptan-3-ol and 3,6-dimethylheptan-3-ol

3-methylheptan-3-ol and 3,6-dimethylheptan-3-ol were synthesised according to Ruβ et al. [49]. Magnesium (2.01 g, 82.3 mmol), diethyl ether (10 mL) and crystal of iodine were put in a two-necked flask with reflux condenser, a calcium chloride tube and dropping funnel. In order to activate magnesium, the mixture was heat smoothly. 1-Bromobutane (8.9 mL, 82.3 mmol) or 3-methyl-1-bromobutane (10.72 mL, 82.3 mmol) dissolved in anhydrous diethyl ether (30 mL) was added slowly. When the addition was finished the mixture was heated to 40 °C for 1 h. Then, the reaction mixture was cooled to 0 °C. A solution of 2-butanone (6.7 mL, 74.0 mmol) in anhydrous diethyl ether (20 mL) was added slowly. Then, the reaction mixture was heated to 40 °C for 1 h. Subsequently, the resulting octanol or nonanol was protonated at 0 °C with a mixture crushed ice in water (20 mL) and ammonium chloride (30 mL, 10%). The organic layer was separated, and the aqueous phase was extracted with diethyl ether (2 × 50 mL). The combined organic phases were washed with aqueous sodium bisulfite (3 × 50 mL, 40%) and with saturated aqueous sodium bicarbonate (3 × 50 mL). The combined organic extracts were dried (Na_2SO_4) and concentrated in vacuo. 3-Methylheptan-3-ol (9.2 g, 86%) and 3,6-dimethylheptan-3-ol (10.26 g, 86%) were obtained as oils (see Appendix A for NMR data).

2.8.2. Synthesis of 4-(3′-methyl-3′-hepthyl)phenol (33-OP) and 4-(3′,6′-dimethyl-3′-hepthyl)phenol (363-NP)

In a dry two-necked flask, a solution of 3-methylheptan-3-ol (1.01 g, 7.8 mmol) or 3,6-dimethylheptan-3-ol (1.23 g, 8.52 mmol) and phenol (1.5 g, 15.9 mmol) in anhydrous ligroin (150 mL) was heated under argon at 60 °C. Then, $BF_3.Et_2O$ complex (1.2 mL, 10.38 mmol) was added slowly and the resulting mixture was stirred for 1 h at 60 °C. Crushed ice and water (150 mL) were added and stirring for 30 min more. The organic layer was separated and was washed with water (7 × 30 mL). The combined organic extracts were dried (Na_2SO_4) and concentrated in vacuum. Flash column chromatography (silicagel, *n*-hexane/AcOEt 15/1) afforded 4-(3′-methyl-3′-hepthyl)phenol (33-OP) (1.12 g, 70%) 4-(3′,6′-dimethyl-3′-hepthyl)phenol (363-NP) (1.32 g, 70%) as oils (see Appendix A for NMR data).

2.9. Exposure of zebrafish to 363-NP, 33-OP and 17β-estradiol

Two exposure experiments were carried out. In both experiments, 5 days post-fertilization (dpf) dpf to 14 dpf fish were fed Sera Micron (Sera) three times daily. At 14 dpf, zebrafish were fed an alternating diet of live Artemia or Cyclop Eeze (Argent) and Sera Vipan (Sera) in the mornings and only Sera Vipan at midday and afternoons. Fish were maintained in a temperature-controlled room at 28 °C with a 14-h light/10-h dark cycle. Ammonium concentrations were controlled daily.

The aim of the first experiment was to test different concentrations of commercial NP in order to select a sublethal dose for the second experiment. Newly fertilized eggs of zebrafish were collected and immediately transferred to Petri dishes (50 eggs per dish) containing embryo water (0.4 mg/L methylene blue and 0.4 mg/L ampicillin in 1500 μS water). At 1 day dpf eggs (50 per experimental group) were moved to aerated open-circuit 11 L glass tanks and exposed to different concentrations (50, 250, 500 μg/L) of commercial NP (Sigma–Aldrich, Seelze, Germany) for 4 weeks. Then, zebrafish were kept in the same aquaria with clean water for additional 2 weeks. Concentrations of commercial NP were selected based on previous experiments with zebrafish [50,51]. E2 at 10 ng/L was used as positive control [52] and dimethylsulfoxide (DMSO) at 0.01% (v/v) was used as vehicle and as a negative control. All the eggs exposed to 250 and 500 μg/L NP died after one day exposure. The survival rate of fish exposed to 50 μg/L NP, E2 and DMSO were 48%, 66% and 91%, respectively. Therefore, a 50 μg/L dose was selected for the second experiment with NP and OP isomers. At 6 weeks whole body samples were taken from E2 and DMSO control groups (10 and 18, respectively) for fish homogenate chemical analysis. All fish exposed to the commercial NP mixture were used for other analyses and therefore they were not included in the present study.

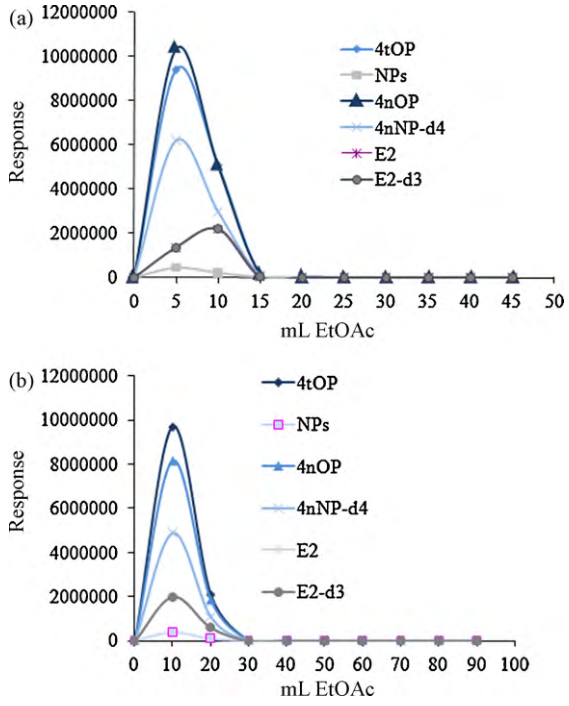

Fig. 1. Elution profiles of target analytes and deuterated analogues during the elution with EtOAc from (a) 5-g Florisil and (b) 10-g Florisil cartridges.

In the second experiment, newly fertilized eggs of zebrafish were collected and immediately transferred to Petri dishes (50 eggs per dish) containing embryo water. At 1 day post-fertilization (dpf) eggs (250 per experimental group) were moved to aerated 38 L glass tanks and exposed to 50 μg/L 363-NP and 33-OP and to DMSO at 0.01% for 4 weeks and then for additional 2 weeks in clean water. Water (5 L) was changed every 24 h. At 6 weeks whole body samples were taken from 363-NP, 33-OP and DMSO groups (51, 34, and 40, respectively) for fish homogenate chemical analysis.

3. Results and discussion

3.1. Optimization of the clean-up step

In order to optimize the clean-up step of the determination of alkylphenols and E2 in fish homogenate, two approaches were studied: SPE using Florisil cartridges and GPC.

Flosiril, silica, alumina, aminopropyl silica or diol cartridges have been mostly used during SPE clean-up of target analytes in biota samples [30–32,35,47]. In this work, 5-g and 10-g cartridges were studied due to previous experience of the research group [37]. In a first step, the elution volume necessary for the quantitative recovery of the target analytes was also studied. After conditioning with *n*-hexane, cartridges were loaded with 0.5 mL of *n*-hexane containing 350 ng of the target analytes and elution was performed with EtOAc based on previous results [36,53]. 5-mL and 10-mL fractions were collected in the case of 5-g and 10-g Florisil cartridges, respectively. Elution volumes up to 45 mL and 90 mL were studied for 5-g and 10-g Florisil cartridges, respectively. The study was repeated in triplicate for each of the cartridges used. As it can be observed from Fig. 1a and b, volumes higher than 15 mL and 30 mL did not enhance the recovery of the target analytes from the 5-g and 10-g Florisil cartridges, respectively.

In order to quantify the recoveries obtained during the clean-up step using 5-g and 10-g Florisil cartridges and GPC, non-fortified fish homogenate was extracted under FUSLE conditions and the extract was divided into three equal volume aliquots that, after evaporation to dryness using a gentle stream of N_2 and reconstitution in an appropriate solvent, were fortified with 350 ng of the target analytes and submitted to SPE or GPC clean-up. Non-fortified FUSLE extracts were processed in parallel for blank correction. Fig. 2 shows the recoveries ($n = 3$) obtained for the target analytes after Florisil clean-up with 5-g and 10-g cartridges, as well as after GPC, after correction with the corresponding deuterated analogues (alkylphenols using 4nNP-d_4 and E2 using E2-d_3) that had been added just after FUSLE extraction, correcting the evaporation and clean-up steps. As it can be observed, good recoveries were obtained for 5-g Florisil (106–126%), 10-g Florisil (110–120%) and GPC (79–100%) clean-up. Relative standard deviations (RSD) lower than 15% were obtained in all the cases. Blank samples of non-spiked fish homogenate showed no signal at the retention time of the target analytes in the SIM mode, thus recoveries higher than 100% could not be attributed to the presence of interferences.

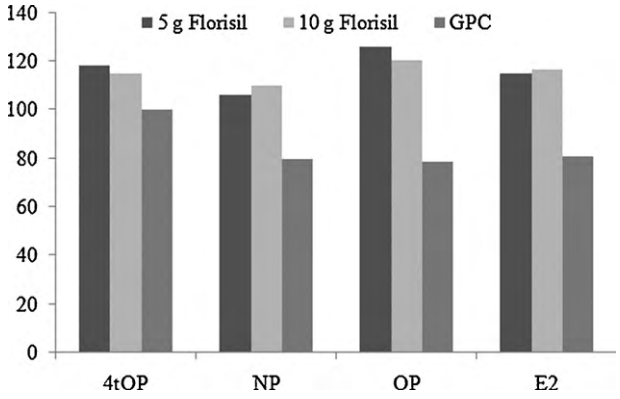

Fig. 2. Recoveries obtained for a fish homogenate extracted fortified with target analytes after FUSLE extraction and submitted to different clean-up conditions: 5-g Florisil, 10-g Florisil and GPC.

Finally, the SIM chromatograms obtained for the extracts obtained after 5-g Florisil (Fig. 3a), 10-g Florisil (Fig. 3b) and GPC (Fig. 3c) clean-ups show that Florisil based SPE provided cleaner chromatograms than GPC and that the chromatograms obtained for 5-g or 10-g Florisil extracts were similar. Besides, according to the analysis of variance of the results obtained for the 5-g and 10-g Florisil cartridges, no significant differences were obtained ($F_{calc} < F_{crit} = 7.71$, for a 95 confidence interval). Therefore, 5-g Florisil cartridges were finally chosen.

3.2. MAE versus FUSLE

MAE and FUSLE were studied as two different alternatives for the solid–liquid extraction of alkylphenols and E2 from fish homogenate samples. Working conditions were chosen from previously developed methods in our research group [36,37,44]. Fish homogenate was fortified with target analytes and aged for 1 month. Different aliquots were processed using FUSLE and MAE procedures. Both MAE and FUSLE extracts were submitted to SPE clean-up using 5-g Florisil cartridges and elution with 15 mL EtOAc as optimized before. Non-fortified fish homogenate was also processed in parallel for blank correction. Average recoveries ($n = 3$) and standard deviations obtained after correction with the corresponding blanks and deuterated surrogates are included in Fig. 4. Comparable ($F_{calc} = 1.5–5.9 < 39$) relative standard deviations (RSD) were obtained for MAE (7–25% range) and FUSLE (10–31%). RSD values in the 20% range are found in the bibliography for similar analyses [31,32] According to Student t-test and for a 95% interval of confidence, no significant differences were obtained for the recoveries obtained for 4nOP and NPs after FUSLE and MAE ($t_{calc} = 1.14–2.34 < t_{crit} = 2.78$), while more accurate results were

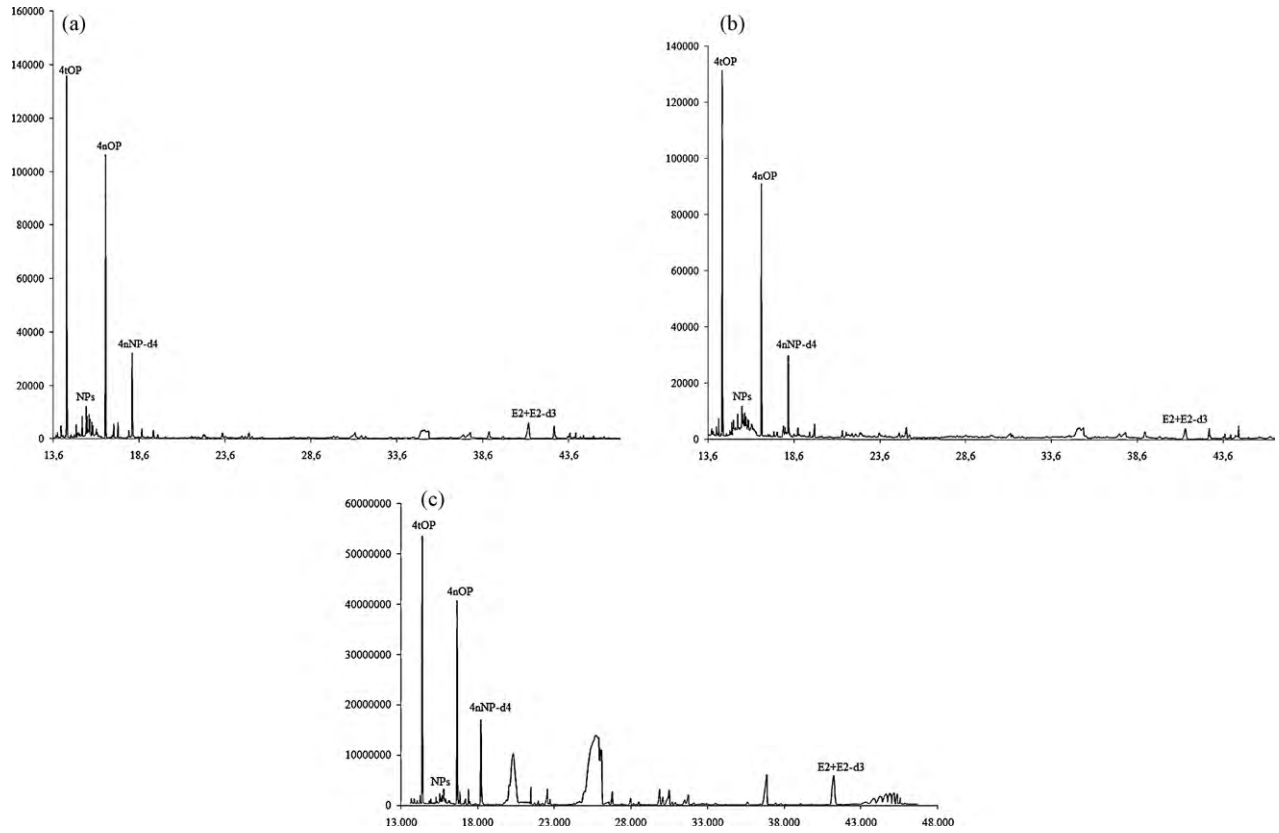

Fig. 3. SIM chromatograms obtained for fish homogenate fortified with target analytes and surrogates after (a) SPE clean-up with 5-g Florisil cartridges, (b) SPE clean-up with 10-g Florisil cartridges and (c) GPC clean-up.

obtained for 4tOP and E2 after FUSLE. The results obtained for 4tOP and E2 after MAE exceed by far the 100% value, indicating the possibility of some kind of interference for these two analytes. It should be kept in mind that the clean-up optimization was carried out using FUSLE extracts and it seems that MAE extracts needed further purification. Thus, the analysis of real samples was performed under FUSLE extraction.

Limits of detection were calculated as the average signal ($n = 3$) plus three times the standard deviation of reagent blank samples. The values obtained were 2 ng (4tOP), 827 ng (NPs), 0.04 ng (nOP) and 1.5 ng (E2), similar to those obtained in the literature [7,8,32,34,54].

Finally, the optimized method was applied to the determination of 363-NP, 33-OP and E2 in zebrafish homogenate. Concentrations of 1.8 μg/g (363-NP), 33 μg/g (33-OP) and 0.35 μg/g (E2) were obtained.

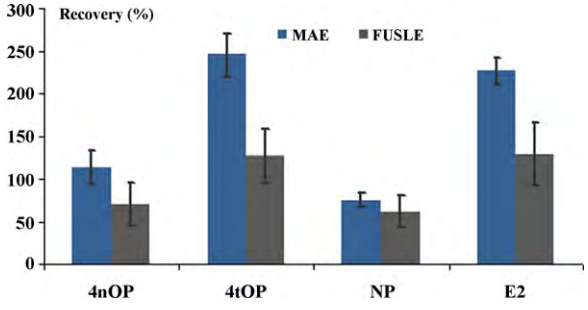

Fig. 4. Comparison of the average ($n = 3$) recovery percentages obtained for 4nOP, 4tOP, NPs and E2 after MAE and FUSLE of a fortified zebrafish homogenate.

4. Conclusions

Two different alternatives both for extraction and clean-up of alkylphenols and E2 in zebrafish homogenate were studied. While SPE provided cleaner extracts than GPC for FUSLE, MAE provided recoveries that exceeded the 100% extraction yield for certain analytes (4tOP and E2), probably due to inadequate clean-up of MAE extracts in 5-g Florisil cartridges. The developed method was applied to the determination of octyl- and nonylphenol isomers and E2 in zebrafish homogenate exposed to the cited endocrine disruptors.

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Appendix A.

A.1. NMR data of 4-(3′-methyl-3′-hepthyl)phenol (33-OP) and 4-(3′,6′-dimethyl-3′-hepthyl)phenol (363-NP).

A.1.1. 4-(3′-methyl-3′-hepthyl)phenol (33-OP)

^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 0.66$ (t, $J = 7.4$ Hz, 3H, C$\underline{H}_3$-CH_2-CH_2), 0.82 (t, $J = 7.3$ Hz, 3H, C$\underline{H}_3$-CH_2-C), 0.90–0.97 (m, 1H, C-C$\underline{H}_2$), 1.07–1.15 (m, 1H, C-C$\underline{H}_2$), 1.21 (s, 3H, C-C$\underline{H}_3$), 1.23–1.28 (m, 2H, CH_3-C$\underline{H}_2$-C), 1.44–1.49 (m, 2H, C-C$\underline{H}_2$-C$\underline{H}_2$), 1.50–1.71 (m, 2H, C-

$C\underline{H}_2$-$C\underline{H}_2$), 4.45 (broad s, 1H, OH), 6.76 (d, J = 8.5 Hz, 2H, $H_{2,6}$), 7.13 (d, J = 8.5 Hz, 2H, $H_{3,5}$). ^{13}C NMR ($CDCl_3$) δ = 8.6 (-CH_2-CH_2-$\underline{C}H_3$), 14.0 (CH_2-$\underline{C}H_3$), 23.4 (-CH_2-$\underline{C}H_2$-CH_3), 23.6 ($\underline{C}H_3$-C), 26.4 (-$\underline{C}H_2$-CH_2-CH_3), 35.6 (CH_3-$\underline{C}H_2$-C), 40.3 (CH_3-CH_2-$\underline{C}$), 42.8 (C-$\underline{C}H_2$-CH_2), 114.6 ($C_{2,6}$), 127.6 ($C_{3,5}$), 140.3 (C_4), 152.9 (C_1).

A.1.2. 4-(3′,6′-dimethyl-3′-hepthyl)phenol (363-NP)

^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.66 (t, J = 7.4 Hz, 3H, $C\underline{H}_3$-CH_2), 0.80–0.83 (m, 6H, $(C\underline{H}_3)_2CH$), 0.84–0.88 (m, 1H, $(CH_3)_2CH$-$C\underline{H}_2$), 0.97–1.04 (m, 1H, $(CH_3)_2CH$-$C\underline{H}_2$), 1.21 (s, 3H, C-$C\underline{H}_3$), 1.38–1.45 (m, 1H, $(CH_3)_2C\underline{H}$), 1.47–1.51 (m, 1H, C-$C\underline{H}_2$), 1.52–1.57 (m, 1H, CH_3-$C\underline{H}_2$-C), 1.61–1.67 (m, 1H, C-$C\underline{H}_2$), 1.68–1.71 (m, 1H, CH_3-$C\underline{H}_2$-C), 4.62 (broad s, 1H, OH), 6.76 (d, J = 8.6 Hz, 2H, $H_{3,5}$), 7.13 (d, J = 8.6 Hz, 2H, $H_{3,5}$). ^{13}C NMR ($CDCl_3$) δ = 8.6 (C-CH_2-$\underline{C}H_3$), 22.6 (($\underline{C}H_3)_2CH$), 23.6 (C-$\underline{C}H_3$), 28.7 (($CH_3)_2\underline{C}H$), 33.2 (($CH_3)_2CH$-$\underline{C}H_2$), 35.7 (CH_3-$\underline{C}H_2$-C), 40.3 (CH_3-CH_2-$\underline{C}$), 40.6 (C-$\underline{C}H_2$-CH_2), 114.7 ($C_{2,6}$), 127.6 ($C_{3,5}$), 140.3 (C_4), 152.9 (C_1).

References

[1] European Parliament, Directive 2000/60/EC (2000) Contract n° SSPI-CT-2003-502492.
[2] R. Céspedes, S. Lacorte, A. Ginebreda, D. Barceló, Environ. Pollut. 153 (2008) 384.
[3] E.-J. Ko, K.-W. Kim, S.Y. Kang, S.-D. Kim, S.-B. Bang, S.-.Y. Hamm, D.-W. Kim, Talanta 73 (2007) 674.
[4] C. Höhne, W. Püttmann, Environ. Sci. Pollut. Res. 153 (2008) 405.
[5] D. Li, M. Dong, W.J. Shim, U.H. Yim, S.H. Hong, N. Kannan, Chemosphere 71 (2008) 1162.
[6] H.-.B. Moon, S.-.P. Yoon, R.-.H. Jung, C. Minkyu, Chemosphere 73 (2008) 880.
[7] F. Ferrara, F. Fabietti, M. Delise, A. Piccioli Boca, E. Funari, Environ. Sci. Technol. 35 (2001) 3109.
[8] T.L. Keith, S.A. Snyder, C.G. Naylor, C.A. Staples, C. Summer, K. Kannan, J.P. Giesy, Environ. Sci. Technol. 35 (2001) 10.
[9] F. Ferrara, F. Fabietti, M. Delise, E. Funari, Chemosphere 59 (2005) 1145.
[10] S.A. Snyder, T.L. Keith, S.L. Pierens, E.M. Snyder, J.P. Giesy, Chemosphere 44 (2001) 1697.
[11] R. Gadzała-Kopciuch, J. Ričanyová, B. Buszewski, J. Chromatogr. B 877 (2009) 1177.
[12] J. Ričanyová, B. Gadzała-Kopciuch, K. Reiffováa, B. Buszewski, Crit. Rev. Anal. Chem. 39 (2009) 11.
[13] EC, Directive 2003/53/EC (2003) Directive 2003/53/EC.
[14] A.S. Russ, R. Vinken, I. Schuphan, B. Schmidt, Chemosphere 60 (2005) 1624.
[15] B. Thiele, V. Heinke, E. Kleist, K. Guenther, Environ. Sci. Technol. 38 (2004) 3405.
[16] R. Espejo, K. Valter, M. Simona, Y. Janin, P. Arrizabalaga, J. Chromatogr. A 976 (2002) 335.
[17] K. Guenther, E. Kleist, B. Thiele, Anal. Bioanal. Chem. 384 (2006) 542.
[18] EC, Directive 81/602/EEC (1981) Official Journal of the European Communities L222.
[19] EC, Directive 88/146/EEC (1988) Official Journal of the European Communities L125.
[20] EC, Directive 96/22/EC (1996) Directive 96/22/EC.
[21] H. Noppe, B. Le Bizec, K. Verheyden, H.F. De Brabander, Anal. Chim. Acta 611 (2008) 1.
[22] P. Pothitou, D. Voutsa, Chemosphere 73 (2008) 1716.
[23] M. Moeder, C. Martin, J. Harynuk, T. Górecki, R. Vinken, P.F.X. Corvini, J. Chromatogr. A 1102 (2006) 245.
[24] J. Jacson, R. Sutton, Sci. Total Environ. 405 (2008) 153.
[25] R. Gibson, C.R. Tyler, E.M. Hill, J. Chromatogr. A 1066 (2005) 33.
[26] S.D. Richardson, Anal. Chem. 79 (2007) 4295.
[27] S.D. Richardson, Anal. Chem. 80 (2008) 4373.
[28] C.J. Koester, Anal. Chem. 77 (2005) 3737.
[29] G. Ying, Chromatogr. Sci. Ser. 93 (2006) 1241.
[30] J. Wang, M. Dong, W.J. Shim, N. Kannan, D. Li, J. Chromatogr. A 1171 (2007) 15.
[31] Y.G. Ahn, J.H. Shin, H.Y. Kim, J. Khim, M.K. Lee, J. Hong, Anal. Chim. Acta 603 (2007) 67.
[32] M.A. Mottaleb, S. Usenko, J.G. O'Donnell, A.J. Ramirez, B.W. Brooks, C.K. Chambliss, J. Chromatogr. A 1216 (2009) 815.
[33] I. Schmitz-Afonso, J.E. Loyo-Rosales, M.d.l.P. Avilés, B.A. Rattner, C.P. Rice, J. Chromatogr. A 1010 (2003) 25.
[34] A. Wenzel, W. Böhmer, J. Müller, H. Rüdel, C. Schröter-Kermani, Environ. Sci. Technol. 38 (2004) 1654.
[35] R. Gadzala-Kopciuch, A. Filipiak, B. Buszewski, Talanta 74 (2008) 655.
[36] L. Bartolomé, E. Cortrázar, J.C. Raposo, A. Usobiaga, O. Zuloaga, N. Etxebarria, L.A. Fernández, J. Chromatogr. A 1068 (2005) 229.
[37] P. Navarro, E. Cortazar, L. Bartolomé, M. Deusto, J.C. Raposo, O. Zuloaga, G. Arana, N. Etxebarria, J. Chromatogr. A 1128 (2006) 10.
[38] C. Basheer, J.P. Obbard, H.K. Lee, J. Chromatogr. A 1068 (2005) 221.
[39] P.N. Carvalho, P.N.R. Rodrigues, F. Alves, R. Evangelista, M.C.P. Basto, M.T.S.D. Vasconcelos, Talanta 76 (2008) 1124.
[40] N. Fidalgo-Used, E. Blanco-González, A. Sanz-Medel, Anal. Chim. Acta 590 (2007) 1.
[41] J. Hernández-Borges, M.A. Rodríguez-Delgado, F.J. García-Montelongo, Chromatographia 63 (2006) 155.
[42] S. Jayaraman, R.J. Pruell, R. McKinney, Chemosphere 44 (2001) 181.
[43] A. Errekatxo, A. Prieto, O. Zuloaga, A. Usobiaga, N. Etxebarria, L.A. Fernández, Anal. Bioanal. Chem. 392 (2008) 1471.
[44] P. Navarro, N. Etxebarria, G. Arana, Anal. Chim. Acta 648 (2009) 178.
[45] M. Gallego-Gallegos, M. Liva, R.M. Olivas, C. Cámara, J. Chromatogr. A 1114 (2006) 82.
[46] J. Sanz-Landaluze, L. Bartolomé, O. Zuloaga, L. González, C. Dietz, C. Cámara, Anal. Bioanal. Chem. 384 (2006) 1331.
[47] S. Meier, J. Klungsoyr, S. Boitsov, T. Eide, A. Svardal, J. Chromatogr. A 1062 (2005) 255.
[48] A. Vallejo, A. Usobiaga, I. Martinez-Arkarazo, A. Prieto, N. Etxebarria, O. Zuloaga, L.A. Fernández, J. Sep. Sci. 33 (2010) 104.
[49] A.S. Ruβ, R. Vinken, I. Schuphan, B. Schmidt, Chemosphere 60 (2005) 1624.
[50] R.L. Hill, D.M. Janz, Aquat. Toxicol. 63 (2003) 417.
[51] F.X. Yang, Y. Xu, Y. Hui, Comp. Biochem. Physiol. C 142 (2006) 77.
[52] F. Brion, C.R. Tyler, X. Palazzi, B. Laillet, J.M. Porcher, J. Garric, P. Flammarion, Aquat. Toxicol. 68 (2004) 193.
[53] M.D. Hernando, M. Mezcua, M.J. Gómez, O. Malato, A. Agüera, A.R. Fernández-Alba, J. Chromatogr. A 1047 (2004) 129.
[54] Z. Long, L. Hong, J. Jie, Chin. J. Anal. Chem. 35 (2007) 983.