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Title: OCCURRENCE OF COMMON PLASTIC ADDITIVES AND CONTAMINANTS IN MUSSEL SAMPLES: VALIDATION OF ANALYTICAL METHOD BASED ON MATRIX SOLID-PHASE DISPERSION

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Abstract: A new matrix solid-phase dispersion (MSPD) extraction methodology, combined with high-performance liquid chromatography equipped with a diode-array detector, was developed and validated for the simultaneous determination of 10 compounds in mussels from Galician Rias (Spain). These pollutants are compounds commonly used for plastic production as additives, as well as common plastic contaminants. The compounds selected were bisphenol-A, bisphenol-F, bisphenol-S, nonylphenol-9, nonylphenol, diethyl phthalate, dibutyl phthalate, di-2-ethylhexyl phthalate, dichlorodiphenyltrichloroethane, dichlorodiphenyldichloroethane, and dichlorodiphenyldichloroethylene. The parameters affecting the MSPD extraction efficiency such as the type of sorbent, mass sample-sorbent ratio, and extraction solvent were optimised . The proposed method provided satisfactory quantitative recoveries (80—100%), with relative standard deviations lower than 7%. In all cases, the matrix-matched calibration curves were linear in the concentration range of 0.32–120.00 µg/kg, with quantification limits of 0.25—16.20 µg/kg. The novel developed MSPD-high-performance liquid chromatography methodology provided good sensitivity, accuracy, and repeatability for quality control analysis in mussels.

Highlights

- A novel approach for endocrine-disrupting chemicals determination in mussels.
- Development of a matrix solid phase dispersion methodology for mussel samples.
- Simultaneous separation and quantification of 11 EDCs by HPLC-DAD.
- Limits of quantification ranged between 0.25-16.20 µg/Kg.

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OCCURRENCE OF COMMON PLASTIC ADDITIVES AND CONTAMINANTS IN MUSSEL SAMPLES: VALIDATION OF ANALYTICAL METHOD BASED ON MATRIX SOLID-PHASE DISPERSION

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Abstract

A new matrix solid-phase dispersion (MSPD) extraction methodology, combined with high-performance liquid chromatography equipped with a diode-array detector, was developed and validated for the simultaneous determination of 10 compounds in mussels from Galician Rias (Spain). These pollutants are compounds commonly used for plastic production as additives, as well as common plastic contaminants. The compounds selected were bisphenol-A, bisphenol-F, bisphenol-S, nonylphenol-9, nonylphenol, diethyl phthalate, dibutyl phthalate, di-2-ethylhexyl phthalate, dichlorodiphenyltrichloroethane, dichlorodiphenyldichloroethane, and dichlorodiphenyldichloroethylene. The parameters affecting the MSPD extraction efficiency such as the type of sorbent, mass sample-sorbent ratio, and extraction solvent were optimised. The proposed method provided satisfactory quantitative recoveries (80–100%), with relative standard deviations lower than 7%. In all cases, the matrix-matched calibration curves were linear in the concentration range of 0.32–120.00 μg/kg, with quantification limits of 0.25–16.20 μg/kg. The novel developed MSPD-high-performance liquid chromatography methodology provided good sensitivity, accuracy, and repeatability for quality control analysis in mussels.

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1. Introduction

Plastic production has increased significantly since the 1950s, reaching 359 million tonnes in 2018. Due to mismanagement of plastic waste, estimates suggest that between 4.8 million tonnes to 12.7 million tonnes of plastics entered the ocean in 2010 (Jambeck et al., 2015). An added problem is the fact that plastics can be fractionated into millimetre-sized particles called microplastics (MP), usually with a size of <5 µm (Ivleva, Wiesheu & Niessner, 2016), where MP are recognised as emerging contaminants due to fact that the smaller microplastics as well as nanoplastics (from 0.001 µm to 0.1 µm) could potentially penetrate the capillaries of organs (Yoo, Doshi, & Mitragotri, 2011). Dumping of plastics in the ocean has led to the ingestion of microplastics by aquatic animals, and to the presence of microplastics in common fisheries and aquaculture commodities. The composition of microplastics and their capability to sorb (either absorb or adsorb) and desorb chemicals such as dichlorodiphenyldichloroethane (DDD), dichlorodiphenyldichloroethylene (DDE), and dichlorodiphenyltrichloroethane (DDT), which are known to be persistent, bioaccumulative, and toxic substances (PBTs), from the surrounding environment could pose a food safety threat.

Microplastics tend to accumulate in the gastrointestinal tract of aquatic animals, where plastics smaller than 1.5 µm can cross the gastrointestinal barrier. Thus, certain food commodities consumed whole are of special interest in terms of dietary exposure to microplastics and associated contaminants (Garrido et al., 2020). The consumption of invertebrates that feed by filtration, such as bivalve molluscs (mussels, clams, oysters, etc.), seems the most likely route of exposure (Li et al., 2019). Wild mussels are complex samples, rich in water, proteins, vitamins, and minerals, where their composition is affected by their environment. Wild mussels have been commonly used as bioindicators for monitoring pollutants in coastal waters due to their extensive distribution, easy sampling, abundance, their ventilation of large volumes of water for nutrition, respiration, and excretion, low mobility, and ecological and economic importance (Chiesa et al., 2018).

Several countries are building regulatory frameworks to reduce the production and use of plastics, as well as the impact of plastic composition in food safety. For instance, the European Union (EU) has adopted different directives and regulations in recent years, such as the new EU Directive 2018/852 (European Parliament, 2018), prohibiting single-use packaging and waste. The EU legislation also set limits for bisphenol-A (BPA) in food-contact plastic materials,

97 establishing a specific migration limit of 0.05 mg of BPA per kilogram of food (European
98 Commission, 2018). For *bis*(2-ethylhexyl) phthalate (DEHP) and dibutyl phthalate (DBP), the EU
99 legislation specifically established limits in products intended for food contact of 1.5 mg/kg for
100 DEHP and 0.3 mg/kg for DBP in its Commission Regulation (EU) N^{o} 10/2011 (European
101 Commission, 2011). Since February 2015, the production of phthalate esters has been
102 forbidden, unless authorisation has been granted for a specific use, although these compounds
103 may still be imported in consumer products (European Commission, 2015).

104 The effects of exposure to the specific compounds mentioned above have been studied, where
105 these compounds are known to be endocrine disruptor chemicals (EDCs) or PBTs (bisphenol-A,
106 bis(2-ethylhexyl) phthalate, nonylphenols (NPs), polychlorinated biphenyls (PCBs), and
107 organochlorine pesticides), but others such as bisphenol-F (BPF), bisphenol-S (BPS), and diethyl
108 phthalate (DEP) are still to be evaluated (European Commission, 2008; EFSA 2017). To
109 understand the magnitude of the problem and the possible implications of these chemicals in
110 the environment and in food safety, it is necessary to develop standardised analytical
111 methodologies to promote consistency in the results obtained. The most used analytical
112 methodologies for the determination of these compounds are gas chromatography (GC)
113 (Filipkowska & Lubecki, 2016; Sánchez-Avila et al., 2011; Wang et al., 2010) and liquid
114 chromatography (LC) (Yang et al., 2014; Salgueiro-González et al., 2016; Luo et al., 2018). High-
115 performance liquid chromatography (HPLC) coupled with different detectors (diode-array
116 detector (DAD) and/or mass detector (MS)) provides advantages over the GC methods, such as
117 simple sample preparation without the need for derivatisation techniques. The liquid
118 chromatography detection method has previously been used for the analysis of EDCs in food
119 samples such as oil (Xian et al., 2017), eggs (Song et al., 2019), hotpot seasoning (Dong, Zeng &
120 Bai, 2018), fish (Tran et al., 2019), and mussels (Ocharoen et al., 2018). The most frequently
121 applied extraction techniques are solid-phase extraction (Azzouz et al., 2019; Filipkowska &
122 Lubecki, 2016; Yang et al., 2014), Soxhlet extraction (Movahedinia, Salamat & Kheradmand,
123 2018), sonication (Blackburn, Kirby & Waldock, 1999), and pressurised liquid extraction
124 (Salgueiro-González et al., 2016). However, these extraction methodologies generally require a
125 clean-up step to reduce the presence of interferents during the subsequent chromatographic
126 determination. Matrix solid-phase dispersion (MSPD) is an effective alternative technique to
127 traditional methods for sample preparation. It offers several advantages such as simplicity and
128 flexibility. This methodology integrates disruption, homogenisation, extraction, and clean-up of
129 the sample into one step (Barker et al., 2007; Qi, 2010; García-Mayor et al., 2012). Sample
130 preparation methods based on MSPD have been efficiently applied to the determination of a

wide variety of micropollutants (PCBs, DEPH, ethoxylates, pesticides, etc.,) in complex biological matrices such as mussels (Carro et al., 2017; Rodríguez-González et al., 2015; Rombaldi, 2015; Wianowska & Gil, 2019). However, to date, MSPD has not been applied for the simultaneous extraction of endocrine disruptors, including different groups of compounds, (bisphenols, phthalates, etc.,) from food samples.

The aim of this work is to develop an effective, simple, and reliable multi-residue analytical methodology based on a matrix solid-phase dispersion extraction technique (MSPD) coupled to high-performance liquid chromatography with a diode-array detector (HPLC-DAD) for the quantitative extraction and simultaneous determination of several EDCs, including common plastic additives (bisphenols (BPA, BPF, and BPS), phthalate esters (DBP, DEHP, and DEP), alkylphenols (NP and NP-9), and other organic pollutants such as organochlorine pesticides (DDD, DDE, and DDT) in wild raw mussel samples. The chemicals studied were selected because they are commonly present in microplastics and some of them are known to be highly potent EDCs that are environmentally persistent (McCombe, 2020) and have already been detected in wild raw mussels (Salgueiro-González et al., 2016; Suaréz et al., 2013), indicating their potential for bioaccumulation.

2. Material and methods

2.1. Chemicals

All reagents were of analytical grade unless specified otherwise. Water (18 MΩ/cm) was purified with a Milli-Q water system (Millipore Ibérica, Madrid, Spain). Bisphenol-A (BPA) (purity ≥ 99%), bisphenol-F (BPF) (purity ≥ 98%), bisphenol-S (BPS) (purity 98%), diethyl phthalate (DEP) (purity ≥ 99%), dibutyl phthalate (DBP) (purity 99%), *bis*(2-ethylhexyl) phthalate (DEHP) (purity ≥ 99.5%), nonylphenol (NP) (purity ≥ 99%), 9-*n*-nonylphenol (NP-9) (purity ≥ 99%), dichlorodiphenyldichloroethane (DDD), dichlorodiphenyldichloroethylene (DDE), and dichlorodiphenyltrichloroethane (DDT) were supplied by Sigma-Aldrich (Madrid, Spain). Stock standard solutions (1000 mg/L) of each analyte were prepared in acetonitrile (ACN), except for the nonylphenols, which were prepared in methanol (MeOH). The standard solutions were stored in dark bottles at 4 °C until use and remained stable for at least three months. Organic solvents (acetonitrile, methanol, and hexane) and sodium hydroxide were purchased from Scharlab (Madrid, Spain). The solid-phase materials used for MSPD were Florisil from Sigma-Aldrich (Madrid, Spain), anhydrous sodium sulfate (Na_2SO_4) from Panreac (Barcelona, Spain), and washed sea sand (0.25–0.30 mm) from Symta (Madrid, Spain). The silanised glass wool used herein was supplied by Panreac (Barcelona, Spain). All solutions

collected from the MSPD procedure were filtered through 0.22 µm Teflon membrane disc filters from Merck (Madrid, Spain).

2.2. Mussel sample collection and preparation

Mussels (*Mytilus galloprovincialis*), which are common species from Arousa stuary in Galicia (Northwest, Spain), from aquaculture production were used as the raw material. A total of 30 live specimens, representing this type of species, were purchased from a local market. The samples were immediately transported to the laboratory, protected from possible contamination, and refrigerated for sample preparation. The samples were dissected with a clean scalpel blade to separate the mussel from the shell. The whole raw mussels were then homogenised and stored at 4 °C prior to the analyses. Measures were taken to avoid contamination by the equipment used for sample collection, transportation, and preparation.

2.3. Matrix solid-phase dispersion procedure

The analytes were extracted from the biomatrix by performing the MSPD procedure with spiked and blank mussel samples. The optimal extraction process was as follows: A 0.1 g sample of homogenised raw mussels was accurately weighed and spiked with 100 µL of stock standard solution containing the analytes at the desired concentration (for instance, NP and NP-9 were spiked at 120 mg/L and the rest of the analytes (BPS, BPF, BPA, DEP, DBP, DDD, DDT, DDE, and DEHP) at 40 mg/L in MeOH/H_2O (85:15, v/v). The mix was maintained at room temperature for 20 min to allow the solution to equilibrate with the mussel matrix. The spiked mussel sample was poured into a glass mortar containing 0.5 g of Florisil (dispersing sorbent, <200 mesh), 0.5 g of Na_2SO_4 (anhydrous agent, purity ≥99%), and 0.2 g of washed sea sand. To achieve complete disruption and dispersion of the biosample on the solid support, the mixture was blended for approximately 10 min using a glass pestle, until an apparently dry and homogeneous material was obtained. The homogenised mixture was then packed into a glass cartridge with a plug of silanised glass wool at the bottom, which retained the entire sample. A small amount of Na_2SO_4 (0.001–0.002 g) was placed on top of the mixture, just enough to cover it. To ensure homogeneous packing of the column, care was taken to pour the material into the tube in several portions and remove the air pockets inside the material. The sample was first conditioned using 1 mL of acetonitrile. The analytes were optimally eluted from the MSPD column dropwise by gravity, with 9 mL of methanol/acetonitrile (30:70, v/v), in three 5 min static extraction steps. The flow rate was constant at approximately 0.6 mL/min. The extracts were collected and evaporated to dryness under a nitrogen stream at room temperature. Finally, the residue was reconstituted in 400 µL of MeOH/H_2O (85:15, v/v) and an

aliquot of 20 µL was injected into the chromatographic system. The corresponding blank sample was prepared by following the same procedure, excluding the spiked analytes from the MSPD mixture. In addition, a solvent blank was prepared to check for background contamination due to the use of laboratory plastic material.

2.4. HPLC-DAD conditions

Chromatographic separation of the analytes was performed using an Agilent Technologies model 1200 series liquid chromatograph (Agilent Technologies, Germany) equipped with an on-line degasser, a quaternary pump, autosampler, and a photo-diode array detector (DAD). The analytes were separated on an ACE 5 C18-PFP HPLC column (150 × 4.6 mm, 5 µm) from Symta (Madrid, Spain). The column and auto sampler were maintained at room temperature. The mobile phase consisting of a mixture of Milli-Q ultrapure water (solvent A) and acetonitrile (solvent B) was set to a gradient programme: 0–30 min: 45–80% B; 30–31 min: 80–100% B; and 100% B for 9 min, at a flow rate of 0.8 mL min^{-1}. Subsequently, the column was reconditioned with 45% B under isocratic conditions for 10 min. The sample injection volume was 20 µL. All compounds were successfully separated within 40 min. To achieve the maximum sensitivity, quantitative measurements of the peak areas were performed by selecting the optimum detection wavelength for each compound. All analytes were therefore quantified at 210 nm. Quantification was performed using external calibration and peak area measurements.

3. Results and discussion

3.1. Optimisation of conditions for HPLC-DAD analysis

Preliminary studies were performed using a chromatography Eclipse Plus C18 3.5 µm (100 x 4.6 mm) column for separation of the selected compounds. Different mobile phases comprising water (eluent A) and acetonitrile or methanol (eluent B) at different ratios (20, 30, 50 and 70%) were tested. In this preliminary study, the signals of the analytes in the chromatograms were not satisfactorily resolved.

To improve the feasibility for elution of all analytes, a new column, an ACE 5 C18-PFP HPLC 5 µm (150 × 4.6 mm), having a slightly modified composition and length, was tested. Different eluent gradient profiles using water (solvent A) and different ratios of methanol or acetonitrile (solvent B) were tested to obtain adequate separation and resolution of the analytes. Finally, a gradient programme was selected, which consisted of 45% ACN initially, followed by an increase to 80% ACN in the first 30 min. The composition of the eluent was increased to 100%

ACN for 1 min, and kept stable at 100% ACN for 4 min. The system was allowed to equilibrate for 10 min at 45% of ACN. The resulting total run time was 40 min. The flow rate was modified between 0.5 and 1.0 ml/min. Because higher flow rates did not significantly improve either the sensitivity or retention time, a flow rate of 0.8 ml/min was selected. The injection volume was varied from 15 to 25 µL. A final volume of 20 µL was selected to increase the sensitivity without any loss of resolution.

In order to achieve the optimum analytical signal for each analyte, the absorption with multi-wavelength detection at 210, 230, 250, 254, and 280 nm using DAD was evaluated by integrating the peaks manually. The maximum areas for all analytes was achieved at 210 nm. The chromatogram corresponding to HPLC-DAD separation of a standard mixture of the compounds under the optimum conditions is shown in Fig. 1. Separation of the eleven compounds was achieved within 35 min.

3.2 Optimisation of MSPD extraction procedure

Selection of the most favourable extraction conditions is crucial for achieving adequate efficiency of the MSPD procedure. Therefore, the dispersing sorbent, the sample mass/sorbent ratio, and the type and volume of the eluent were optimised to obtain clean final extracts and to achieve the highest analyte recoveries and the lowest matrix interferences from mussel samples. Three types of dispersing sorbents (Florisil, Alumina, and C-18) at three loadings (0.5, 1.0, and 1.5 g) were tested with an initial mussel sample mass of 0.5 g and a fixed amount of 0.5 g of Na_2SO_4 and 0.2 g of washed sea sand to improve the sample dispersion. The amount of mussel sample (0.5, 0.3, and 0.1 g) was then optimised using the optimum 0.5 g of Florisil as a clean-up/dispersing sorbent, 0.5 g of Na_2SO_4, and 0.2 g of washed sea sand. Finally, 0.1 g of mussel sample, 0.5 g of Florisil as a dispersing sorbent, 0.5 g of Na_2SO_4, and 0.2 g of washed sea sand were chosen as the optimal MSPD mixture. In these mentioned studies, the mussel sample was spiked with 40 ppm of BPS, BPF, BPA, DEP, DBP, DDD, DDT, and DDE, and 120 ppm of NP-9, and dispersed with the corresponding amount of Florisil, Na_2SO_4, and washed sea sand. The homogenised mixtures in each assay were then transferred to a glass cartridge to prepare the MSPD columns by following the procedure described in Section 2.3. Thereafter, the column was conditioned with 1 mL of methanol and the analytes were extracted with 9 mL of methanol as the starting solvent. Subsequently, the type and volume of the eluent was optimised. The selection of an adequate eluent to desorb the analytes from the MSPD column has an important role in this extraction procedure. A set of assays to test the feasibility of several solvents with different polarity (ACN, MeOH) and mixtures (MeOH/H_2O (80/20) and

MeOH/ACN (80/20, 50/50, 40/60, 30/70, 20/80)) was carried out. Different volumes (3, 6, 9, 12, and 15 mL) of eluent that afforded the elution of all analytes were tested in static cycles of 5 min using 3 mL of eluent per cycle. Prior to the elution process, in all cases, the column was conditioned with 1 mL of the test eluent. The obtained extracts were treated as indicated in Section 2.3 before injection into the HPLC system, and analysed by following the procedure outlined in Section 2.4. From the tests, it was concluded that 9 mL was the most efficient eluent volume for recovering the analytes. Figure 2 shows the results for these experiments using 9 mL of eluent in consecutive 5 min static extraction cycles (3 mL per cycle). When methanol was used as the eluent, the recovery for all analytes was in the range of 70–96%, except for DEP, for which extraction was not possible. When the methanol/water mixture (80:20, v/v) was used, the recovery was 70–100% for most analytes. However, DEP and NP-9 were not extracted at all. The use of acetonitrile as the eluent allowed extraction of all the analytes, with recoveries of approximately 100% for some of them, such as DEP, DBP, and NP, and recoveries ranging between 54 and 82% for BPS, BPF, BPA, DDT, DDD, DDE, and DEHP. In this assay, it was also possible to detect NP-9 with a recovery of approximately 30%. When methanol/acetonitrile mixtures were used, it was not possible to obtain quantitative recovery of all the analytes in any case, and some of them were not isolated (DEP with 80:20 methanol/acetonitrile; NP-9 with 50:50, 40:60, 30:70, 20:80 methanol/acetonitrile). Considering the results (Fig. 2), and to develop a methodology capable of simultaneously determining the greatest number of analytes with quantitative recovery, the mixture of 30:70 methanol/acetonitrile was selected as the eluent for method validation. In this case, the recovery for all detected analytes was in the range of 80–100%. Based on the results obtained by HPLC-DAD analysis, it can be concluded that 0.1 g of mussel sample, 0.5 g of Florisil, 0.5 g of Na_2SO_4, 0.2 g of washed sea sand, and 9 mL of 30:70 methanol/acetonitrile eluent are the most favourable extraction conditions. These conditions afforded the highest intensity chromatographic analyte peaks, indicating a greater percentage recovery of the analytes.

In order to further improve the recovery of the analytes, the possibility of using an additional washing step was evaluated using 0.01 M sodium hydroxide or hexane. For these assays, 3 mL of 0.01 M sodium hydroxide solution or hexane was added to the cartridge to eliminate possible matrix interferences, and elution with methanol/acetonitrile 30:70 was then accomplished. These additional washing steps did not improve the previously obtained results, and in the case of sodium hydroxide, even made it impossible to analyse the samples due to hydrolysis of the phthalic acid esters.

Figure 3 presents a comparison of the chromatograms for an enriched mussel sample (where 30 mg/L of nonylphenols and 10 mg/L of bisphenols, phthalates, and pesticides were added) and an unenriched mussel sample (blank sample) subjected to the developed method. The chromatogram of a standard solution at the concentration level of the spiked samples is included in Fig. 3. Signals corresponding to DBP and DEHP were observed in the chromatogram of the blank. Hence, the quantification of these analytes was carried out by subtracting the blank signal from the sample signal. The peaks of both analytes were confirmed by mass spectrometry.

3.3. Method validation

The developed MSPD method was validated in terms of linearity, precision, recovery, and the limits of detection and quantification, using spiked mussel samples. Calibration plots were constructed by preparing spiked mussel samples in triplicate, where the samples contained increasing concentrations of each compound, in the corresponding range (Table 1). The results showed good linearity for all analytes, with correlation coefficients (R^2) of 0.9873–0.9991.

The precision was calculated in terms of the intra-day repeatability (n = 3) and inter-day reproducibility (three successive days) from triplicate assays at three spiking levels (between 2.00 and 120.00 µg/kg). The lowest spiking level for each analyte was always higher than the corresponding LOQ, which allowed correct quantification of its recovery. The intra-day repeatability, expressed as the relative standard deviation (RSD), ranged from 0.8% to 6.9%. The inter-day repeatability was lower than 6.6% for all analytes. These results demonstrate that the analytical method is sufficiently accurate. As shown in Table 1, good recoveries (80–100%) were obtained for all investigated compounds, except for nonylphenol-9. The non-quantification of NP-9 is caused by the ill-defined chromatographic peak. The peak of NP-9 was broken down into smaller associated peaks, forming a wide peak without an observable clear maximum (Fig. 1).

The limits of detection (LODs) and quantification (LOQs) were calculated at signal-to-noise ratios of 3 and 10, respectively, by applying the following equations: LOD = 3.3 × σ/S and LOQ = 10 × σ/S, where σ is the standard deviation of 3 samples spiked at the experimental estimated LOQ, and S is the slope of the corresponding calibration curve. The obtained results were calculated according to the FDA Guidance for Industry (FDA, 2015). The LODs of the detected analytes were between 0.06 and 6.00 µg/kg, and the LOQs were between 0.25 and 16.22 µg/kg (see Table 1).

4. Conclusions

The novel developed method proposed in this research illustrates the potential of combining MSPD extraction with HPLC-DAD for the analysis of traces of endocrine disruptors in mussel samples. This MSPD-HPLC-DAD method provides good sensitivity, accuracy, and repeatability for quality control analysis in real seafood samples. For most of the analytes, satisfactory recoveries of 80–100% were achieved, with an RSD of less than 7% and LODs and LOQs of 0.06–6.00 µg/kg and 0.25–16.22 µg/kg, respectively. Advantageously, the proposed methodology requires a small amount of mussel sample (0.1 g) for extraction and does not require an additional clean-up stage, as it offers the possibility to carry out sample extraction and clean-up steps at the same time. These advantages make the method a promising alternative to other time-consuming and multi-step analytical procedures. Furthermore, to the best of our knowledge, there is no documentation of methods implementing MSPD and HPLC-DAD for the sensitive simultaneous analysis of this set of compounds in mussel samples.

The determination of plastic additives and plastic contaminants, derived from the contamination of production waters, in fishery and aquaculture products requires further research. The developed method constitutes a significant advance in terms of simplicity, efficiency, and sensitivity, allowing realisation of the first potential simultaneous determination of ten chemical compounds from microplastics that are commonly present in mussel samples.

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References

Azzouz, A., Palacios Colón, L., Souhail, B., & Ballesteros, E. (2019). A multi-residue method for GC-MS determination of selected endocrine disrupting chemicals in fish and seafood from European and North African markets. *Environmental Research, 178,* 108727.

Barker, S. A. (2007). Matrix solid phase dispersion (MSPD). *Journal of Biochemical and Biophysical Methods, 70(2),* 151–162.

Blackburn, M. A., Kirby, S. J., & Waldock, M. J. (1999) Alkylphenol polyethoxylate residues in the UK aquatic environment. *Marine Pollution Bulletin, 38,* 109–118.

Carro, N., Vilas, L., García, I., Ignacio, M., & Mouteira, A. M. (2017). Optimization of a method based on micro-matrix solid-phase dispersion (micro-MSPD) for the determination of PCBs in mussel samples. *Analytical Chemistry Research, 11,* 1–8.

Chiesa, L. M., Nobile, M., Malandra, R., Panseri, S., & Arioli, F. (2018). Occurrence of antibiotics in mussels and clams from various FAO areas. *Food Chemistry, 240,* 16–23.

Dong, H., Zeng, X., & Bai, W. (2018). Solid phase extraction with high polarity Carb/PSA as composite fillers prior to UPLC-MS/MS to determine six bisphenols and alkylphenols in trace level hotpot seasoning. *Food Chemistry, 258,* 206-213.

EFSA. (2017). Bisphenol A. Food ingredients and packaging. *European Food Safety Authority*. URL https://www.efsa.europa.eu/en/topics/topic/bisphenol. Accessed 03.10.19.

European Commission. (2011). Commission Regulation (EU) N^{o} 10/2011 of 14 January 2011 on plastic materials and articles intended to come into contact with food Text with EEA relevance. *Official Journal of the European Union, 12*, 1–89.

European Commission. (2015). Commission Regulation (EU) No 2015/326 of 2 March 2015 amending Annex XVII to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) as regards polycyclic aromatic hydrocarbons and phthalates. *Official Journal of the European Union, 58*.

European Commission. (2018). Commission Regulation (EU) N^{o} 2018/213 of 12 February 2018 on the use of bisphenol A in varnishes and coatings intended to come into contact with food and amending Regulation (EU) No 10/2011 as regards the use of that substance in plastic food contact materials (Text with EEA relevance). *Official Journal of the European Union, 41*.

European Commission. (2008). Phthalates in school supplies. *Health and Food Safety*. URL https://ec.europa.eu/health/scientific_committees/opinions_layman/en/phthalates-school-supplies/about-phthalates-school-supplies.htm#24. Accessed 03.10.19.

European Parliament. (2018). Directive (EU) 2018/852 of the European Parliament and of the Council of 30 May 2018 amending Directive 94/62/EC on packaging and packaging waste. *Official Journal of the European Union, 150,* 141–154.

Garrido Gamarro, E., Ryder, J., Elvevoll, E. O., & Olsen, R. L. (2020). Microplastics in fish and shellfish - a threat to seafood safety?. *Journal of Aquatic Food Product Technology*, *29 (4),* 417-425.

FDA. (2015). VICH GL49 Studies to evaluate the metabolism and Residue Kinetics of veterinary Drugs in Food-Producing Animals: Validation of Analytical Methods used in Residue Depletion Studies. Food and Drug Administration, Center of Veterinary Medicine. URL https://www.ema.europa.eu/en/vich-gl49-studies-evaluate-metabolism-residue-kinetics-veterinary-drugs-food-producing-animals. Accessed 10.09.19.

Filipkowska, A., & Lubecki, L. (2016). Endocrine disruptors in blue mussels and sediments from the Gulf of Gdańsk (Southern Baltic). *Environmental Science and Pollution Research, 23(14)*, 13864–13876.

FDA. (2015). VICH GL49 Studies to evaluate the metabolism and Residue Kinetics of veterinary Drugs in Food-Producing Animals: Validation of Analytical Methods used in Residue Depletion Studies. Food and Drug Administration, Center of Veterinary Medicine. URL https://www.ema.europa.eu/en/vich-gl49-studies-evaluate-metabolism-residue-kinetics-veterinary-drugs-food-producing-animals. Accessed 10.09.19.

García-Mayor, M. A., Gallego-Picó, A., Garcinuño, R. M., Fernández-Hernando, P., & Durand-Alegría, J. S. (2012). Matrix solid-phase dispersion method for the determination of macrolide antibiotics in sheep's milk. *Food Chemistry, 134(1)*, 553–558.

Ivleva, N. P., Wiesheu, A. C., & Niessner, R. (2016). Microplastic in Aquatic Ecosystems. *Angewandte Chemie International Edition, 56(7)*, 1720–1739.

Jambeck, J. R., Geyer, R., Wilcox, C., Siegler, T. R., Perryman, M., Andrady, A., Narayan, R. & Law, K. L. 2015. Plastic waste inputs from land into the ocean. *Science, 347(6223)*, 768-771.

Li, J., Lusher, A. L., Rotchell, J. M., Deudero, S., Turra, A., Brate, I. L. N., Sun, C., Hossain, M. S., Li, Q., Kolandhasamy, P., & Shi, H. (2019) Using mussel as a global bioindicator of coastal microplastic pollution. *Environmental Pollution, 244*, 522-533.

Luo, Z., Lu, J., Li, H., Tu, Y., Wan, Y., & Yang, Z. (2018). Air-assisted liquid-liquid microextraction integrated with QuEChERS for determining endocrine-disrupting compounds in fish by high-performance liquid chromatography–tandem mass spectrometry. *Food Chemistry, 260*, 174–182.

417 McCombe Roark, A. (2020). Endocrine Disruptors and Marine Systems. Reference Module in
418 Earth Systems and Environmental Sciences. Elsevier.

419 Movahedinia, A., Salamat, N., & Kheradmand, P. (2018). Effects of the environmental
420 endocrine disrupting compound benzo[a]pyrene on thyroidal status of abu mullet (Liza abu)
421 during short-term exposure. *Toxicology Reports, 5*, 377–382.

422 Ocharoen, Y., Boonphakdee, C., Boonphakdee, T., Shinn, A. P., & Moonmangmee, S. (2018).
423 High levels of the endocrine disruptors bisphenol-A and 17β-estradiol detected in populations
424 of green mussel, Perna viridis, cultured in the Gulf of Thailand. *Aquaculture, 497*, 348-356.

425 Qi, X. (2010). Development of a matrix solid-phase dispersion-sonication extraction method for
426 the determination of fungicides residues in ginseng extract. *Food Chemistry, 121(3)*, 758–762.

427 Rodríguez-González, N., González-Castro, M. J., Beceiro-González, E., & Muniategui-Lorenzo, S.
428 (2015). Development of a Matrix Solid Phase Dispersion methodology for the determination of
429 triazine herbicides in mussels. *Food Chemistry, 173*, 391–396.

430 Rombaldi, C., de Oliveira Arias, J. L., Hertzog, G. I., Caldas, S. S., Vieira, J. P., & Primel, E. G.
431 (2015). New environmentally friendly MSPD solid support based on golden mussel shell:
432 characterization and application for extraction of organic contaminants from mussel tissue.
433 *Analytical and Bioanalytical Chemistry, 407(16)*, 4805–4814.

434 Salgueiro-González, N., Turnes-Carou, I., Viñas, L., Besada, V., Muniategui-Lorenzo, S., López-
435 Mahía, P., & Prada-Rodríguez, D. (2016). Occurrence of alkylphenols and bisphenol A in wild
436 mussel samples from the Spanish Atlantic coast and Bay of Biscay. *Marine Pollution Bulletin,*
437 *106(1-2)*, 360–365.

438 Sánchez-Avila, J., Fernandez-Sanjuan, M., Vicente, J., & Lacorte, S. (2011). Development of a
439 multi-residue method for the determination of organic micropollutants in water, sediment and
440 mussels using gas chromatography–tandem mass spectrometry. *Journal of Chromatography A,*
441 *1218(38)*, 6799–6811.

442 Song, N-E, Lee, J. Y., Mansur, A. R., Jang, H. W., Lim, M.-C., Lee, Y., Yoo, M., Nam, T. G. (2019).
443 Determination of 60 pesticides in hen eggs using the QuEChERS procedure followed by LC-
444 MS/MS and GC-MS/MS. *Food Chemistry, 298*, 125050.

445 Suárez, P., Ruiz, Y., Alonso, A., & San Juan, F. (2013). Organochlorine compounds in mussels
446 cultured in the Ría of Vigo: Accumulation and origin. *Chemosphere, 90 (1)*, 7-19.

447 Tran, T.A.M., Malarvannan, G., Hoang, T. L., Nguyen, V. H., Covaci, A., & Elskens, M. (2019).
448 Occurrence of organochlorine pesticides and polychlorinated biphenyls in sediment and fish in
449 Cau Hai lagoon of Central Vietnam: Human health risk assessment. *Marine Pollution Bulletin,*
450 *141*, 521-528.

451 Wang, J., Shim, W., Yim, U., Kannan, N., & Li, D. (2010). Nonylphenol in bivalves and sediments
452 in the northeast coast of China. *Journal of Environmental Sciences, 22(11)*, 1735–1740.

453 Wianowska, D., & Gil, M. (2019). New insights into the application of MSPD in various fields of
454 analytical chemistry. *TrAC Trends in Analytical Chemistry, 112*, 29-51.

455 Xian, Y., Wu, Y., Dong, H., Guo, X., Wang, B., Wang, L. (2017). Dispersive micro solid phase
456 extraction (DMSPE) using polymer anion exchange (PAX) as the sorbent followed by UPLC–
457 MS/MS for the rapid determination of four bisphenols in commercial edible oils. *Journal of*
458 *Chromatography A, 1517*, 35-43.

459 Yang, Y., Lu, L., Zhang, J., Yang, Y., Wu, Y., & Shao, B. (2014). Simultaneous determination of
460 seven bisphenols in environmental water and solid samples by liquid chromatography–
461 electrospray tandem mass spectrometry. *Journal of Chromatography A, 1328*, 26–34.

462 Yoo, J., Doshi, N., & Mitragotri, S. (2011). Adaptive micro and nanoparticles: temporal control
463 over carrier properties to facilitate drug delivery. *Advanced Drug Delivery Reviews, 63*, 1247.

464

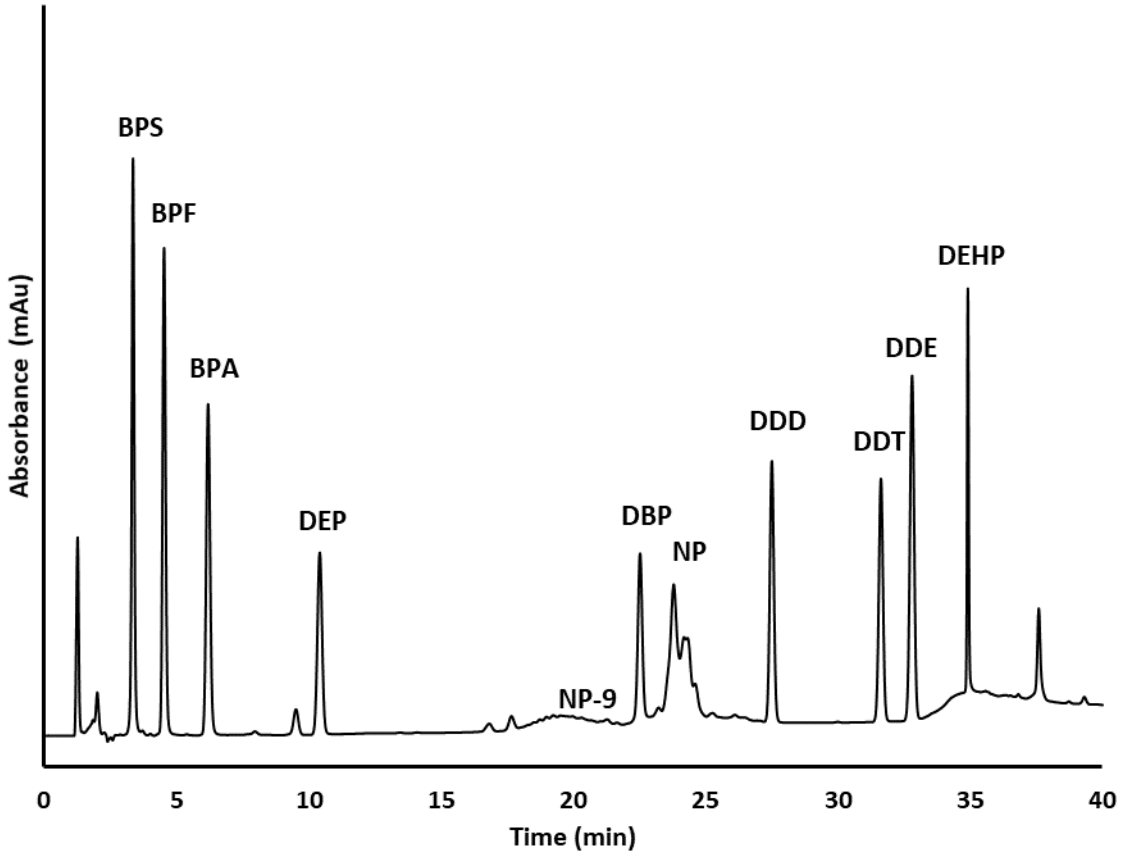

Fig. 1. Chromatogram obtained at 210 nm for standard mixture of EDCs (10 mg/L of BPS, BPF, BPA, DEP, DBP, DDD, DDT, DDE, and DEHP, and 50 mg/L of NP and NP-9) using HPLC-UV-DAD with MeOH/H_2O (85/15, v/v). The optimum experimental conditions are described in the text.

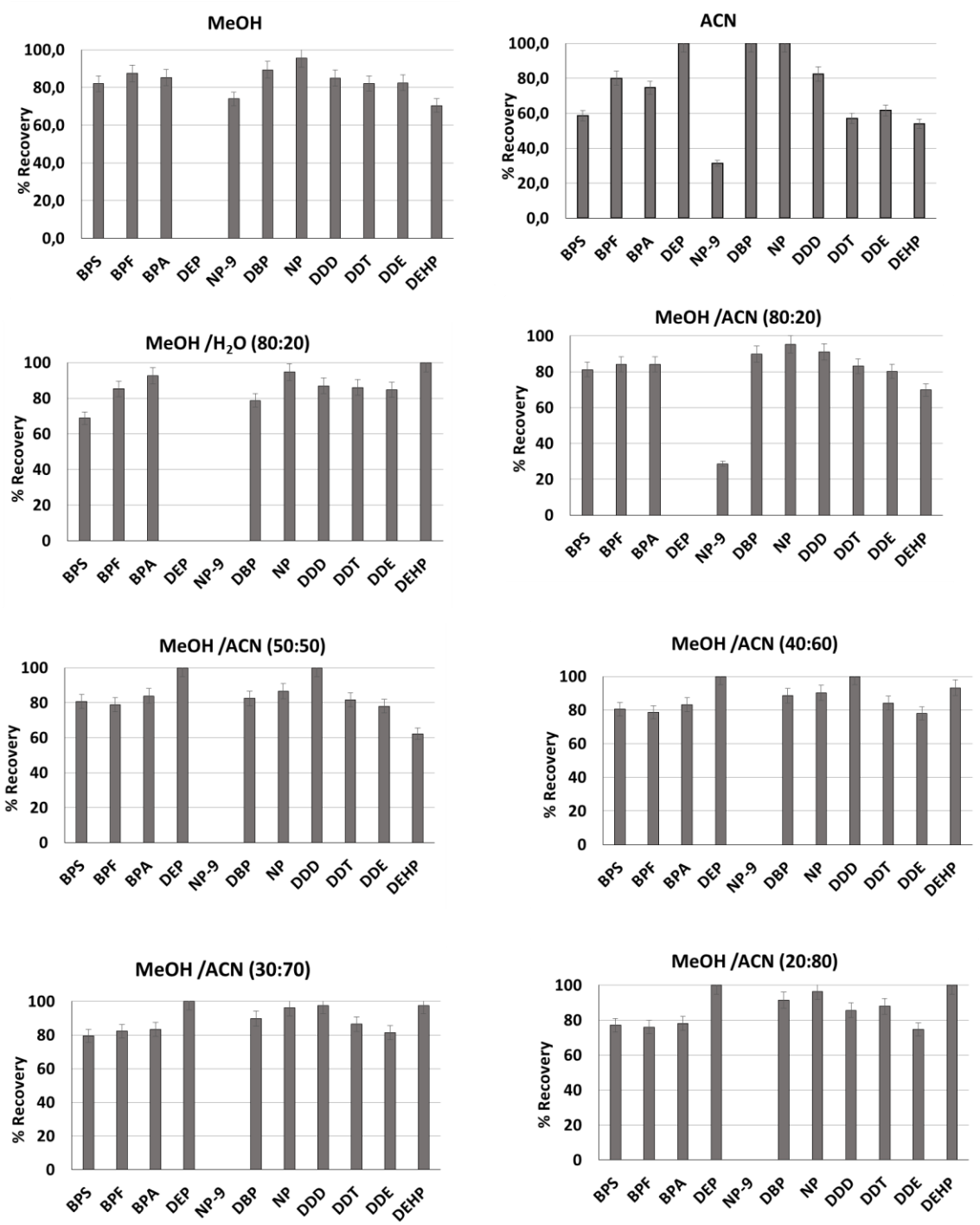

Fig. 2. Elution percentages of target analytes on MSPD columns using 9 mL of different eluents in consecutive 5 min static extraction cycles (3 mL per cycle).

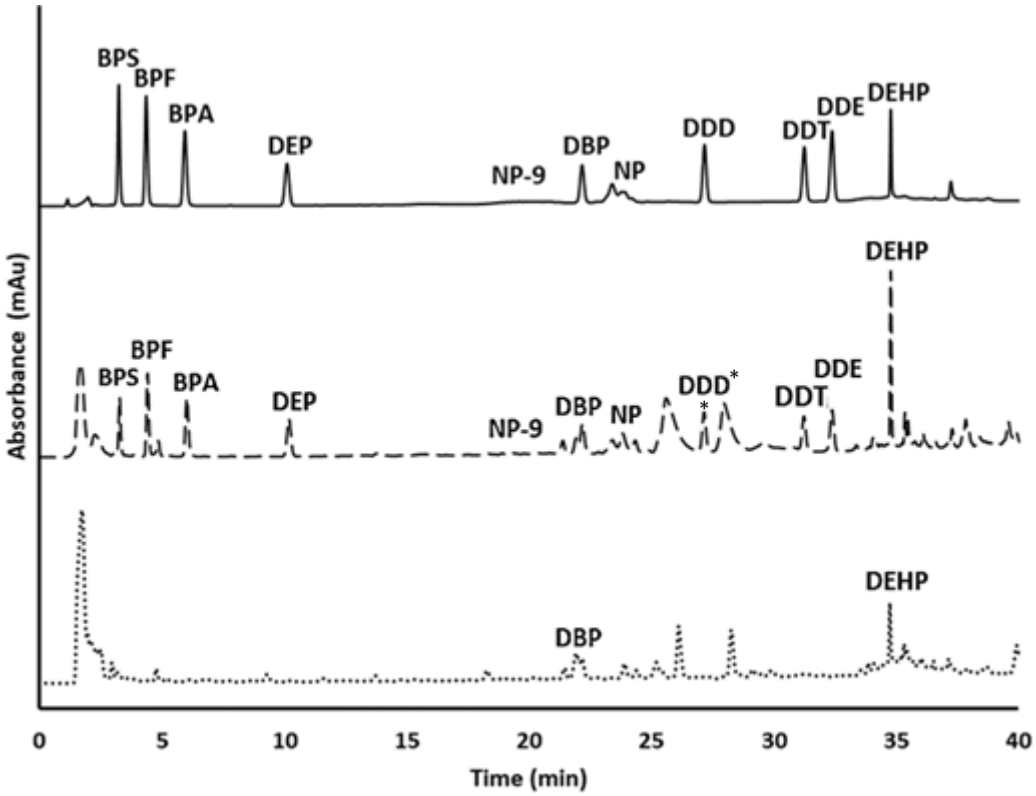

Fig. 3. Chromatograms of a standard solution of 10 mg/L BPS, BPF, BPA, DEP, DBP, DDD, DDT, DDE, and DEHP and 30 mg/L of NP-9 and NP (—), spiked mussel sample with same concentration of compounds (---), and blank mussel sample (…) at 210 nm.

Table 1. Analytical characteristics of target EDCs from spiked mussel samples

Analyte	Concentration range (µg/kg)	Regression equation	R^2	Spiking level (µg/kg)	Intra-day Recovery ± RSD (%)	Inter-day Recovery ± RSD (%)	LOD (µg/kg)	LOQ (µg/kg)
BPS	2.00-6.00	y =59.792C - 0.9654	0.9922	40.00	82.6 ± 1.9	82.2 ± 2.0	0.29	1.12
				20.00	81.7 ± 2.5	81.1 ± 3.0		
				2.00	79.3 ± 3.2	78.5 ± 4.1		
BPF	0.80-6.00	y =76.526C + 8.712	0.9953	40.00	82.7 ± 1.8	82.0 ± 0.9	0.10	0.34
				20.00	80.2 ± 2.1	83.5 ± 1.7		
				1.20	83.6 ± 3.5	82.1 ± 3.4		
BPA	0.32-2.00	y = 110.26C - 3.810	0.9942	40.00	87.2 ± 1.5	86.2 ± 1.8	0.07	0.25
				20.00	85.3 ± 1.9	87.0 ± 2.0		
				0.40	85.6 ± 3.7	81.5 ± 5.4		
DEP	3.20-6.00	y = 51.2C - 11.823	0.9918	40.00	97.8 ± 3.9	96.3 ± 6.4	0.82	2.74
				20.00	104.0 ± 5.1	100.0± 5.4		
				3.20	102.3 ± 6.9	101.1 ± 6.6		
DBP	6.00-32.00	y = 42.235C - 64.875	0.9913	40.00	89.7 ± 2.9	90.7 ± 2.7	1.65	5.52
				20.00	90.1 ± 3.5	91.4 ± 3.8		
				12.00	89.1 ± 4.1	90.5 ± 4.5		
NP	28.00-60.00	y = 15.586C - 70.458	0.9873	120.00	96.9 ± 1.5	97.3 ± 1.8	6.00	16.22
				60.00	97.5 ± 3.9	98.6 ± 3.7		
				28.00	96.9 ± 4.5	96.8 ± 4.7		
DDD	20.00-60.00	y = 70.598C + 325.94	0.9982	40.00	98.0 ± 1.2	101.0 ± 1.4	2.45	10.23
				28.00	98.0 ± 2.3	99.5 ± 2.8		
				20.00	97.9 ± 4.1	102.0 ± 3.9		
DDT	0.60-6.00	y = 76.956C + 10.985	0.9958	40.00	86.2 ± 0.8	86.5 ± 1.1	0.18	0.43
				20.00	86.0 ± 1.3	85.9 ± 3.5		
				2.00	86.8 ± 2.3	87.1 ± 5.1		
DDE	0.40-6.00	y = 92.893C + 2.8236	0.9973	40.00	83.5 ± 2.0	82.8 ± 1.2	0.06	0.25
				20.00	82.5 ± 3.5	82.6 ± 2.3		
				2.00	85.3 ± 4.2	83.9 ± 5.6		
DEHP	2.00-12.00	y = 105.721C + 32.712	0.9991	40.00	102.0 ± 1.5	101.4 ± 1.3	0.42	1.87
				20.00	102.3 ± 2.3	96.7.0 ± 4.8		
				2.00	98.7 ± 4.8	105.3 ± 5.2		

Table 1

Table 1

Analyte	Concentration range (µg/kg)	Regression equation	R^2	Spiking level (µg/kg)	Intra-day Recovery ± RSD (%)	Inter-day Recovery ± RSD (%)	LOD (µg/kg)	LOQ (µg/kg)
BPS	2.00-6.00	y=59.792C-0.9654	0.9922	40.00 20.00 2.00	82.6 ± 1.9 81.7 ± 2.5 79.3 ± 3.2	82.2 ± 2.0 81.1 ± 3.0 78.5 ± 4.1	0.29	1.12
BPF	0.80-6.00	y=76.526C+8.712	0.9953	40.00 20.00 1.20	82.7 ± 1.8 80.2 ± 2.1 83.6 ± 3.5	82.0 ± 0.9 83.5 ± 1.7 82.1 ± 3.4	0.10	0.34
BPA	0.32-2.00	y=110.26C-3.810	0.9942	40.00 20.00 0.40	87.2 ± 1.5 85.3 ± 1.9 85.6 ± 3.7	86.2 ± 1.8 87.0 ± 2.0 81.5 ± 5.4	0.07	0.25
DEP	3.20-6.00	y=51.2C-11.823	0.9918	40.00 20.00 3.20	97.8 ± 3.9 104.0 ± 5.1 102.3 ± 6.9	96.3 ± 6.4 100.0± 5.4 101.1 ± 6.6	0.82	2.74
DBP	6.00-32.00	y=42.235C-64.875	0.9913	40.00 20.00 12.00	89.7 ± 2.9 90.1 ± 3.5 89.1 ± 4.1	90.7 ± 2.7 91.4 ± 3.8 90.5 ± 4.5	1.65	5.52
NP	28.00-60.00	y=15.586C-70.458	0.9873	120.00 60.00 28.00	96.9 ± 1.5 97.5 ± 3.9 96.9 ± 4.5	97.3 ± 1.8 98.6 ± 3.7 96.8 ± 4.7	6.00	16.22
DDD	20.00-60.00	y=70.598C+325.94	0.9982	40.00 28.00 20.00	98.0 ± 1.2 98.0 ± 2.3 97.9 ± 4.1	101.0 ± 1.4 99.5 ± 2.8 102.0 ± 3.9	2.45	10.23
DDT	0.60-6.00	y=76.956C+10.985	0.9958	40.00 20.00 2.00	86.2 ± 0.8 86.0 ± 1.3 86.8 ± 2.3	86.5 ± 1.1 85.9 ± 3.5 87.1 ± 5.1	0.18	0.43
DDE	0.40-6.00	y=92.893C+2.8236	0.9973	40.00 20.00 2.00	83.5 ± 2.0 82.5 ± 3.5 85.3 ± 4.2	82.8 ± 1.2 82.6 ± 2.3 83.9 ± 5.6	0.06	0.25
DEHP	2.00-12.00	y=105.721C+32.712	0.9991	40.00 20.00 2.00	102.0 ± 1.5 102.3 ± 2.3 98.7 ± 4.8	101.4 ± 1.3 96.7.0 ± 4.8 105.3 ± 5.2	0.42	1.87

Figure 1

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Fig. 1

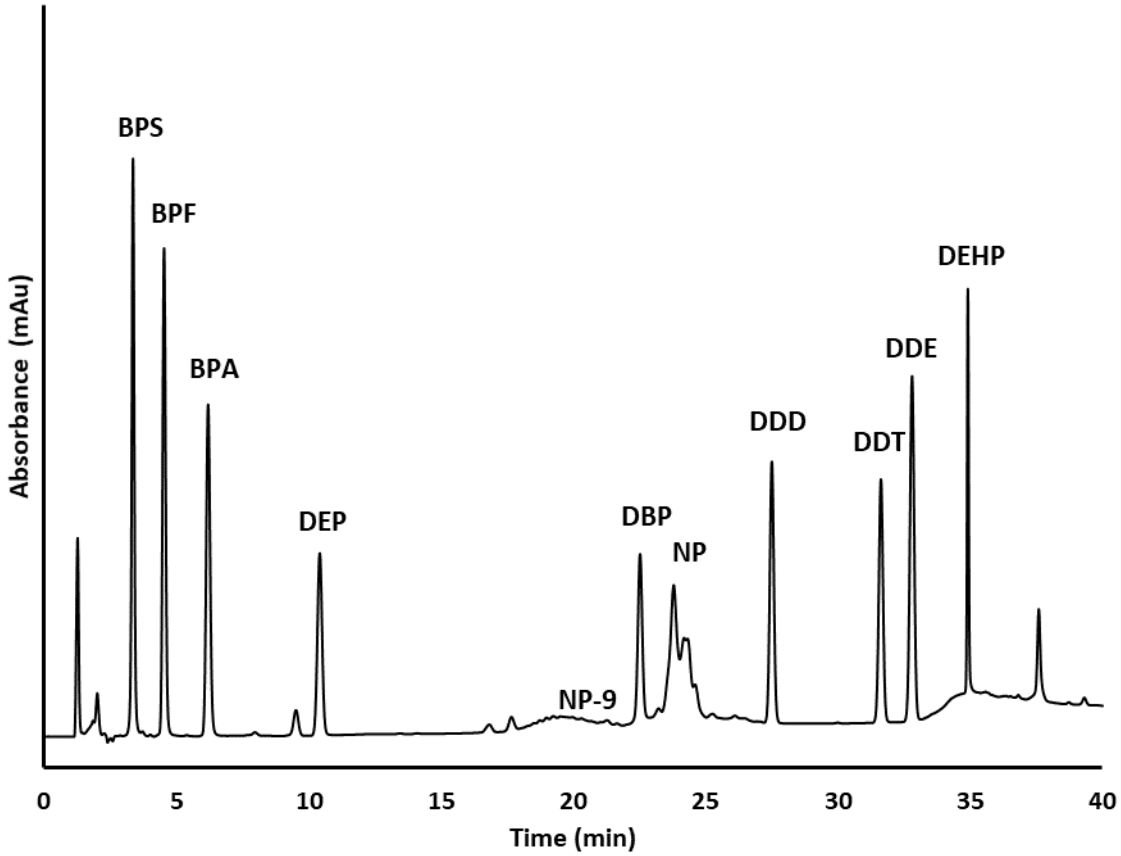

Figure 2

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Fig.2

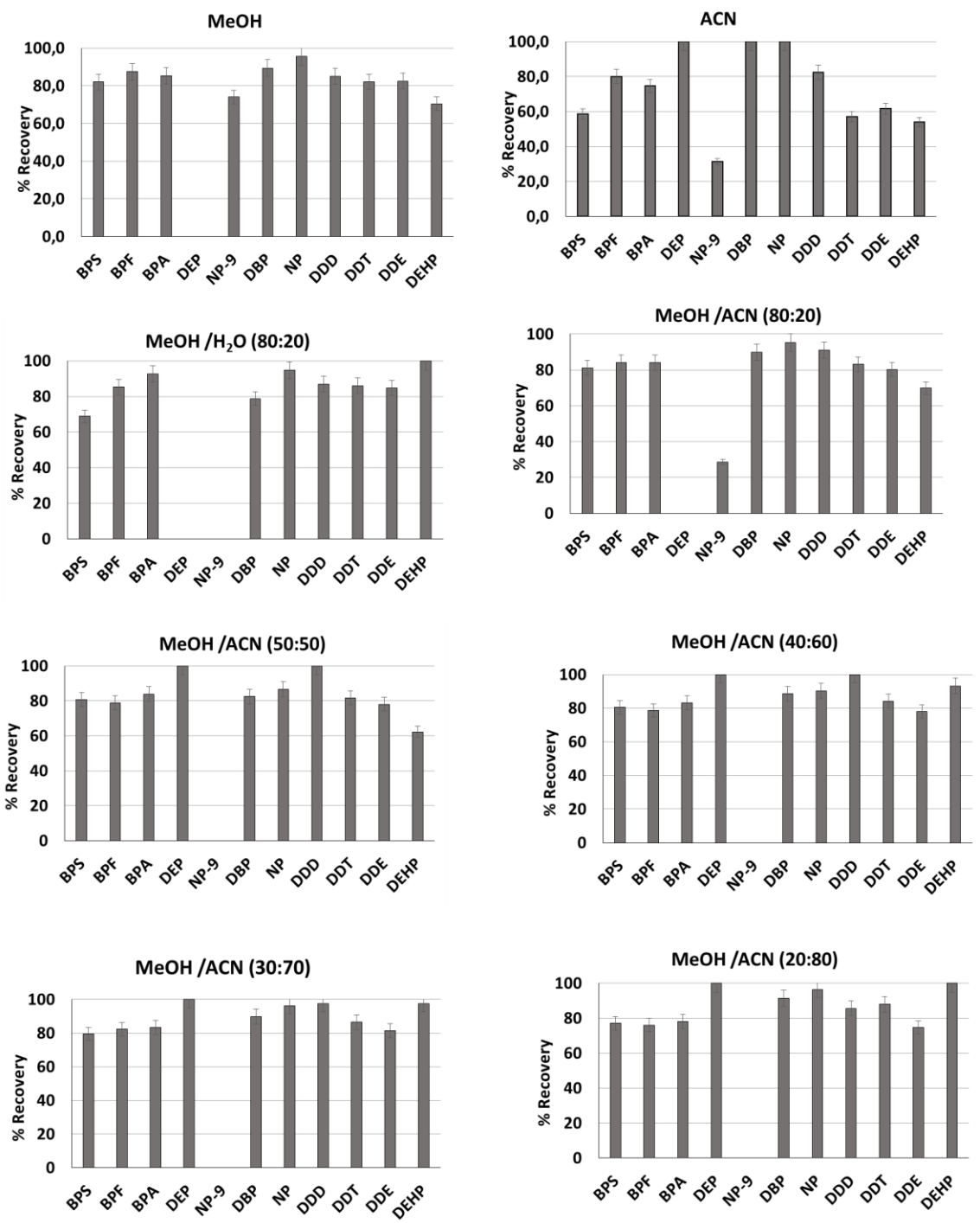

Figure 3
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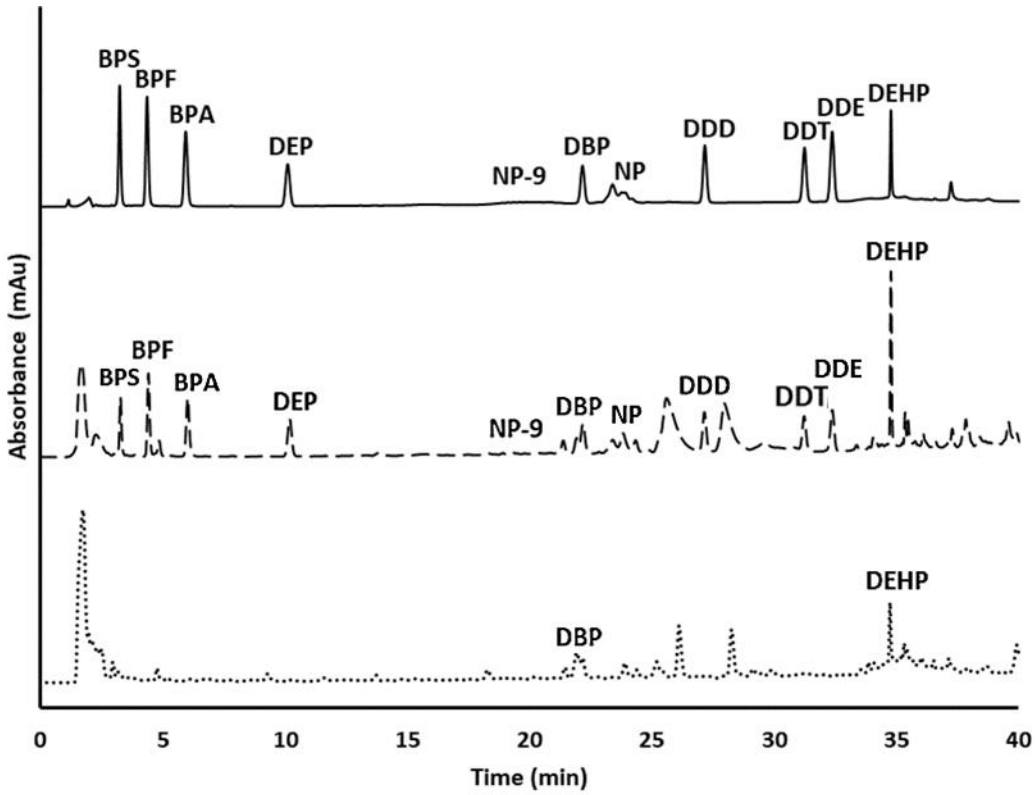
Fig. 3



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