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Targeted quantification and untargeted screening of alkylphenols, bisphenol A and phthalates in aquatic matrices using ultra-high-performance liquid chromatography coupled to hybrid Q-Orbitrap mass spectrometry

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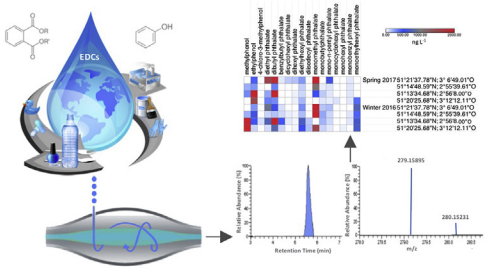
HIGHLIGHTS

- A reduced risk on false-positive results of phthalate contamination.
- Holistic monitoring of a broad range of known and unknown plasticizers.
- The simultaneous quantification of alkylphenols, phthalates and primary phthalate metabolites.

GRAPHICAL ABSTRACT



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ABSTRACT

Plasticizers and other plastics additives have been extensively used as ingredients of plastics and are as a result thereof easily released in the aquatic environment, due to different physical diffusion processes. In this context, a dedicated method was developed for the simultaneous quantification of 27 known and a virtually unlimited number of unknown alkylphenols, Bisphenol A and phthalates in 2 aquatic matrices, i.e. sea- and freshwater. To this extent, a novel instrumental HESI-UHPLC-HRMS (heated electro-spray ionization ultra-high performance liquid chromatographic high resolution mass spectrometric) method was devised for the simultaneous analysis of 7 phenols (i.e. 6 alkylphenols and Bisphenol A) and 20 phthalates within 10 min. Thereafter, a solid-phase extraction protocol was statistically (95% confidence interval, $p > 0.05$) optimized based on experimental designs. The method was proven fit-for-purpose through a successful validation at environmentally relevant nanomolar concentrations. Analytical precautions were taken for minimizing false-positive results to suppress in-house contamination. The method demonstrated an excellent analytical performance across all known plasticizers and plastics additives for sea- and freshwater, revealing good linearity ($R^2 > 0.99$, $n = 39$), stable recoveries (98.5–105.8%), satisfactory repeatability (RSD < 8%, $n = 54$) and reproducibility (RSD < 10%, $n = 36$).

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Subsequently, a novel analytical strategy was devised for the tentative identification of unknown plasticizers and plastics additives using specific in-house determined fragments incorporated in a Python code. The applicability of the analytical platform was demonstrated by measuring 24 seawater samples. Interestingly, 16 out of 27 known plasticizers, plastics additives and primary metabolites could be quantified while the untargeted analysis uncovered 1042 compounds, whereof 5% (n = 46) could be assigned a plasticizer-plastics additive chemical identity, providing evidence for the severe plastic contamination status of our marine environment.

1. Introduction

The amount of plastic waste that enters our ocean is currently estimated at 4.8 to 12.7 million tons per year. Even more, this cumulative quantity of plastic waste is predicted to increase by an order of magnitude by 2025 [1,2]. As a result of this widespread global contamination of plastics in the marine environment, plasticizers and plastics additives are leaching and inevitable entering marine waters [3]. Indeed, these compounds can be easily released either directly or indirectly, through manufacturing or metabolisation processes as a result of the weak physical bonding with plastic polymers. The most common components that have been used to alter the physical properties of plastics are alkylphenols (APs), bisphenol A, and phthalates (phthalic acid esters, PAEs) [4,5]. APs haven mainly been used in industrial and household applications, covering more than 80% of the total alkylphenolic production [6]. For Bisphenol A, a production of 6.8 billion kg has been reported in 2013 [7]. PAEs are globally synthetized at approximately 6.0 million metric tons per year [8,9]. PAEs are not only used as plasticizer in the polymer industry, but are also used to improve the performance quality of cosmetics, detergents, adhesives, food package materials, personal care products, fragrances, medical devices and lubricants [10,11]. Nowadays, the analysis of APs, Bisphenol A and PAEs in the aquatic environment has received relatively little to almost no attention, especially in comparison to pesticides and pharmaceuticals [12–15]. Nevertheless, the abundance of APs, Bisphenol A and PAEs has recently prompted significant public and mass media interest because of severe known and unknown adverse ecological effects, and possible impact of indirect exposure to human health. For example, bisphenol A causes developmental and reproductive effects in aquatic species, such as zebra fish, frogs and swordtail fish [16,17]. In addition, PAEs can cause severe toxic effects in fish, invertebrates and amphibians [18]. In zebra fish, low doses of diethylhexyl phthalate (DEHP) mainly interfere with steroidogenesis and oocyte growth, while higher doses affect the oocyte maturation [19]. In adults, the disturbance of sex-hormone levels has been observed at low concentrations of di(2-ethylhexl)phthalate, resulting in severe consequences, including infertility, gynecological disorder, diabetes type 2 and pregnancy-induced hypertension [15].

In spite of the plethora of adverse effects that have been noticed for APs, Bisphenol A, PAEs and their metabolites, only a limited number of these compounds have been included in target lists by regulatory bodies that are responsible for monitoring water quality status, such as the European Watch list, Norman, Reach, Clean Water Act, and OSPAR [20]. In order to further improve environmental quality standards (EQS), it is obvious that an increased number of APs and PAEs should be included in monitoring programs, warranting the requirement of sensitive and reliable analysis methods.

Up until now, studies have mainly reported the occurrence of intact plasticizers and plastics additives in freshwater environments, whereas data for marine environments are rare [21]. Investigated fresh water environments for plasticizer contamination include raw wastewater, groundwater, riverine water, and drinking water [11,22,23]. These four major freshwater bodies receive their contamination load primarily from local anthropogenic activities, resulting in a local contamination profile. To acquire a complete overview of the environmental contamination with plasticizers and plastics additives, it is evident that the marine environment should be monitored as well. Furthermore, metabolite and degradation products should also be included in monitoring strategies, since they display similar biochemical activities as their parent compounds. At present, only a limited number of studies have reported on the determination of phthalate metabolites in the aquatic environment [24,25].

Therefore, this study presents a new analytical platform for simultaneous quantification of 27 known plasticizers, i.e. phenols (n = 7, 6 alkylphenols and Bisphenol A) and PAEs (n = 20, 11 diphthalates and 9 mono-phthalates) complemented by an untargeted approach for plasticizer metabolite and degradation product detection in the marine environment. To establish this innovative platform, a solid-phase extraction (SPE) and ultra-high-performance liquid chromatographic high-resolution mass spectrometric method (UHPLC-HRMS) were developed and validated for targeted quantification of the selected phenols and PAEs in marine waters. The fitness-for-purpose of this method for marine water monitoring (targeted and untargeted) was demonstrated by measuring a number of samples originating from the Belgian Part of the North Sea (BPNS). The relevance of samples originating from the BPNS is high, as the latter is located near the English Channel, which is known to be world's busiest seaway and is ranked among the most highly affected marine ecosystems on earth [26,27].

2. Materials and methods

2.1. Chemicals and reagents

In this study, 27 target compounds were considered (Table 1 and Table A.1), which were purchased from Accustandard (New Have, CT, USA) and Sigma Aldrich (St. Louis, MO, USA). The target compounds were selected based on relevant literature, and covered 3 different classes, i.e. 7 phenols, 11 di-phthalates and 9 mono-phthalates [28–30]. The selected internal deuterated standards comprised of 2 phenols, i.e. 2-chlorophenol-d_4 and phenol-d_5; and 2 phthalates, i.e. dicyclohexyl phthalate-3,4,5,6-d_4 and diethyl phthalate-3,4,5,6-d_4. Primary stock solutions and mixed standards, reaching concentrations between 1 and 1000 ng μL^{-1}, were prepared in optima grade acetonitrile. The solutions were stored in amber glass bottles at −20 °C. The organic solvents were of optima UHPLC-MS grade, purchased at Fisher Scientific (Loughborough, UK). Reference seawater was prepared according to ASTM D-1141 [31], using inorganic salts supplied by Sigma Aldrich (St. Louis, MO, USA), i.e. NaCl, Na_2SO_4, MgCl.6(H_2O), $CaCl_2$.2(H_2O), $SrCl_2$.6(H_2O),

Table 1
Summary of the instrumental performance and method validation characteristics for alkylphenol and phthalate analysis with SPE-UHPLC-HR-Q-Orbitrap-MS (MDL = method detection limit (n = 39), MQL = method quantification limit (n = 39), SD = standard deviation (recovery and repeatability: n = 54 and within lab reproducibility: n = 18)). The mass spectra of the target compounds were mainly characterized in full-scan as $[M-H]^-$, $[M+H]^+$, and $[M+H]^+$ for phenols, di-phthalates and mono-phthalates, respectively. IS represents the internal standard that was used for correcting variation in the target analyte response.

Compound	Elemental formula	IS	t_r (min)	Accurate mass (*m/z*)	MDL (ng L^{-1})	MQL (ng L^{-1})	Recovery +SD (%)	Repeatability +SD (%)	Within lab reproducibility +SD (%)
Phenols									
2-methyl phenol	C_7H_8O	a	4.99	107.04879	75	100	101.3 ± 3.6	3.6 ± 3.2	6.0 ± 4.7
4-ethylphenol	$C_8H_{10}O$	a	5.08	121.06453	10	25	102.2 ± 3.5	3.4 ± 4.1	4.9 ± 3.0
4-isopropyl phenol	$C_9H_{12}O$	a	5.19	135.08024	150	200	102.5 ± 1.7	1.6 ± 1.3	3.8 ± 2.0
4-chloro-3-methylphenol	C_7H_7ClO	a	5.19	141.01004	10	25	100.4 ± 5.0	4.7 ± 2.0	5.5 ± 1.5
2,5-dichloro phenol	$C_6H_4Cl_2O$	b	1.75	160.95545	25	50	103.7 ± 3.7	3.5 ± 3.8	7.0 ± 3.8
3,4,6-trichlorophenol	$C_6H_3Cl_3O$	a	4.80	194.91693	10	25	104.2 ± 2.6	2.4 ± 1.1	2.4 ± 1.0
Bisphenol A	$C_{15}H_{16}O_2$	a	5.05	227.10737	25	50	101.8 ± 7.8	5.2 ± 3.5	8.0 ± 1.3
Phenol-d_5 (a)	C_6HOd_5		3.77	98.06591					
Chlorophenol-d_4 (b)	C_6HCld_4		1.47	131.01945					
Di-phthalates									
Dimethyl phthalate	$C_{10}H_{10}O_4$	c	5.06	195.06502	25	50	105.8 ± 6.4	6.2 ± 6.8	9.2 ± 2.1
Diethyl phthalate	$C_{12}H_{14}O_4$	c	5.26	223.09621	25	50	105.2 ± 8.1	7.7 ± 2.0	8.9 ± 2.0
Dibutyl phthalate	$C_{16}H_{22}O_4$	c	5.76	279.15863	5	10	101.0 ± 4.6	4.6 ± 2.6	9.9 ± 1.8
Diamyl phthalate	$C_{18}H_{26}O_4$	d	6.12	307.18981	5	25	103.5 ± 9.7	9.5 ± 3.4	9.7 ± 2.2
Benzyl butyl phthalate	$C_{19}H_{20}O_4$	d	5.70	313.14282	10	25	102.4 ± 8.3	7.7 ± 3.4	9.6 ± 2.0
Dicyclohexyl phthalate	$C_{20}H_{26}O_4$	d	6.18	331.18972	5	20	102.2 ± 5.2	5.1 ± 1.1	6.2 ± 0.5
Dihexyl phthalate	$C_{20}H_{30}O_4$	d	6.51	335.22098	10	20	105.4 ± 7.3	6.9 ± 3.1	7.6 ± 3.2
Dibenzyl phthalate	$C_{22}H_{18}O_4$	d	5.64	347.12718	5	20	103.0 ± 6.8	7.1 ± 1.8	8.6 ± 1.5
Diethylheyxl phthalate	$C_{24}H_{38}O_4$	d	7.52	391.28348	20	25	105.8 ± 1.8	5.8 ± 1.8	7.7 ± 1.7
Dinonyl phthalate	$C_{26}H_{48}O_4$	d	8.00	419.31473	25	25	105.2 ± 5.8	5.8 ± 4.1	7.5 ± 2.6
Diisodecyl phthalate	$C_{32}H_{54}O_4$	d	8.71	447.34688	25	50	102.6 ± 3.9	2.7 ± 2.3	9.3 ± 1.5
Diethyl phthalate-d_4 (c)	$C_{12}H_{10}O_4d_4$		5.05	227.12100					
Dicyclohexyl phthalate-d_4 (d)	$C_{20}H_{22}O_4d_4$		6.26	335.21476					
Mono-phthalates									
Monomethyl phthalate	$C_9H_8O_4$	c	1.00	181.04953	20	25	101.2 ± 6.4	6.2 ± 3.5	9.9 ± 3.3
Monoethyl phthalate	$C_{10}H_{10}O_4$	c	1.02	195.06497	20	25	99.5 ± 5.1	5.1 ± 6.1	5.4 ± 6.6
Monobutyl phthalate	$C_{12}H_{14}O_4$	c	2.35	223.09615	5	25	98.5 ± 1.8	2.5 ± 1.1	3.2 ± 1.6
Mono-n-pentyl phthalate	$C_{13}H_{16}O_4$	d	4.12	237.11174	20	25	102.6 ± 7.9	7.7 ± 5.0	9.7 ± 3.5
Monocyclohexyl phth.	$C_{14}H_{16}O_4$	d	4.04	249.11170	5	10	99.2 ± 7.0	7.0 ± 4.4	7.9 ± 4.9
Monohexyl phthalate	$C_{14}H_{18}O_4$	d	4.24	251.12736	20	25	103.3 ± 5.2	5.0 ± 1.9	7.4 ± 4.4
Monobenzyl phthalate	$C_{15}H_{12}O_4$	d	4.02	257.08033	5	10	98.7 ± 4.4	4.4 ± 2.2	3.4 ± 1.3
Monoethylhexyl pht.	$C_{16}H_{22}O_4$	d	4.35	279.15879	25	50	101.8 ± 4.9	5.3 ± 2.3	4.5 ± 1.6
Mono-isonyl phthalate	$C_{17}H_{23}O_4$	d	5.30	292.16691	20	25	102.7 ± 3.0	2.9 ± 1.6	5.9 ± 2.8

KCl, $NaHCO_3$, KBr, H_3BO_3 and NaF. Ultrapure water was obtained by usage of a purified-water system (Millipore).

2.2. Instrumentation

Chromatographic separation of target compounds was executed using a UHPLC system, consisting of an UltiMate 3000 XRS pumping system, coupled to an UltiMate 3000 RS column compartment and autosampler (Dionex, Amsterdam, The Netherlands). Separation of the target compounds was carried out using a Hypersil Gold column (1.9 μm, 100 × 2.1 mm) (Thermo Fisher Scientific, San-Fransisco, USA) at a temperature of 45 °C based on gradient elution. The mobile phase consisted of a mixture of water (Eluent A) and acetonitrile (Eluent B) both containing 0.1% ammonium hydroxide, pumped at a flow rate of 300 μL min^{-1}. The linear gradient program was as follows: 0–1 min, 5% B; 1–2 min, 5–40% B; 2–2.3 min, 40–90% B; 2.3–6.1 min, 90–96% B; 6.1–8 min, 96% B and 8–10 min, 5% B. The injection volume was 10 μL. Additionally, a Hypersil Gold trap column (1.9 μm, 50 × 2.1 mm) (Thermo Fisher Scientific, San-Fransisco, USA) was placed between the UHPLC pump and the injection valve for retarding phenols and PAEs originating from the mobile phase and analytical instrument.

The detection of target compounds was carried out using a Q-Exactive™ Benchtop HRMS (Thermo Fisher Scientific, San-Fransisco, USA) fitted with a Heated Electrospray Ionization (HESI-II) source. Analysis was realized through full-scan events with following optimal operating conditions for positive and negative ionization (polarity switching mode); auxiliary gas flow 30 arbitary units (a.u.), sweep gas flow 2 a.u., discharge current (−) 3.5 kV, capillary temperature 250 °C and heater temperature 350 °C. Optimal MS parameters of the Q-Exactive™ were an S-lens Radio Frequency (RF) level of 70, a resolution of 70,000 FWHM (Full Width at Half Maximum) at 1 Hz, and an *m/z* scan-range of 60–900 Da. Moreover, balanced scans were applied by targeting the automatic gain control (AGC) to $5e^5$ ions and a maximum injection time of 50 ms. Calibration of the instrument was carried out by infusing calibration mixtures for the positive and negative ion mode (LTQ Velos ESI positive and negative ion calibration solution, Thermo Fisher Scientific).

Tentative identification of unknowns, that are related to the backbone of plasticizers and plastics additives, was obtained by combining the full-scan events at a resolution of 70,000 FWHM with an additional Parallel Reaction Monitoring (PRM) HRMS event at a resolution of 17,500 FWHM and optimal Collision Energy (CE) of 20 eV.

2.3. Sample preparation and extraction

2.3.1. Statistical experimental designs for the optimization

A statistical workflow, consisting of 3 experimental designs, was used to efficiently optimise sample preparation and solid-phase extraction (SPE) [21]. First, 14 parameters that could affect the extraction efficiency were selected based on literature (see Table A.2) [32–34]. The significant parameters were determined by

a three-level fractional factorial resolution IV experimental design (n = 18 experiments) and retained for further optimization. Second the optimal composition of the solid phase eluents was achieved using a simplex lattice mixture design (n = 10 experiments, Table A.3.) optimizing the percentage of organic solvents, i.e. methanol (CH_3OH), acetonitrile (CH_3CN) and methyl-tert-butylether ($C_5H_{12}O$). The more apolar solvent metyl-tert-butylether was tested in a later phase, to assure that adding this solvent would not improve recovery of the target compounds in line with previous work [35,36]. Third, the selected significant parameters were optimised through response surface modelling (RSM), using a box-behnken design (n = 15 experiments). All the experiments were performed using reference seawater that was spiked with 200 ng L^{-1} of each target compound prior to sample preparation and extraction. The above-mentioned experimental designs were selected, evaluated and modelled by JMP 12.0 (SAS Institute Inc, Cary, USA). Moreover, the designs were optimised using the summarized normalized area, thereby acknowledging the high number of analytes and ensuring equal compound contribution. Appropriate designs were selected by maximizing the Chi-efficiency score and minimizing the number of experiments. Thereafter, responses were statistically evaluated by one-way analysis of variance (ANOVA) at a confidence interval of 95% (p-value < 0.05). Finally, optimal extraction settings, yielding the highest response, were calculated using a generalised reduced gradient non-linear algorithm and RSM.

2.3.2. Final protocol

Grab samples of 0.5 L were acidified to pH 3 using 1 M HCl and stored in dark amber glass bottles at 4 °C. Upon extraction, samples were brought to room temperature. Afterwards, samples were spiked with a mixture of deuterated internal standards, i.e. 100 ng L^{-1} for the deuterated phthalates and 400 ng L^{-1} for the phenols. Subsequently, Oasis® HLB cartridges (6 cc, 500 mg sorbent, 60 μm particle size; Waters) were conditioned with 6 mL 5% CH3CN diluted in ultrapure water and 7 mL ultrapure water under vacuum. Next, samples were drawn through the cartridges under vacuum (10 mL min^{-1}), followed by a washing step of 8 mL ultrapure water and applying a vacuum (20 min) to remove residual water drops. Afterwards, elution was executed by using 9 mL of 0.1% formic acid in CH_3CN. The extracts were vaporized under a mild stream of nitrogen at a temperature of 40 °C until dry. Consequently, the extracts were reconstituted in 150 μL of CH_3CN/H_2O (95/5, v/v), centrifuged at 2430 g. Finally, supernatants were transferred into LC-MS vials prior to analysis.

2.4. Method validation

The optimised UHPLC-HRMS method was validated on reference seawater to evaluate its fitness-for-purpose. Currently, there is a lack of specific criteria for validating analytical methods for monitoring organic micropollutants in the marine environment. At the time of execution, the only available European guideline for evaluating the water status was CD 2009/90/EC [37], which stipulates that reported concentrations can have a maximal uncertainty of 50% or must be below environmental quality standards (EQS). At present, no EQS are available on the abundance of APs, Bisphenol A and PAEs in the aquatic environment, except for DEHP [38]. Detection limits should be 30% below the EQS. Therefore, additional performance criteria in analytical method validation were consulted as stricter guidelines, i.e. CD/2002/657 [39], Eurachem guidelines [40] and review articles [41,42]. The analytical evaluation criteria included the empirical method detection (MDL) and quantification limit (MQL), linearity, specificity and selectivity, trueness, and precision. The MDL, MQL and linearity were examined by establishing a 13-point matrix-matched calibration curve in threefold at relevant environmental concentrations (0, 5, 10, 20, 25, 50, 75, 100, 200, 400, 600, 800 and 1000 ng L^{-1}). For a limited number of target compounds, i.e. mainly the di-phthalates (and not the mono-phthalates), concentrations were detected up to 1000 ng L^{-1} [28–30]. To evaluate the specificity, selectivity, trueness and precision, seawater was spiked at 1.5, 2.0 and 2.5 times the MQL-level in 6-fold. This procedure was repeated on 3 different days and by 2 operators. Additionally, 20 non-spiked reference seawater samples were analysed as blanks.

A cross-validation on fresh tap water was performed in parallel to assess the matrix-versatility of the presented method. To do this, a 14-point matrix-matched calibration curve was constructed twice to investigate linearity. To evaluate the specificity, selectivity, trueness and precision, the freshwater samples were spiked by 1.5 times the MQL-level (n = 18).

2.5. Data analysis

The targeted processing of full-scan data, including identification and quantification of targeted compounds, was executed by XCalibur 4.0 software (Thermo Fisher Scientific). Identification of a compound was realized by use of the accurate mass of the pseudo-molecular parent ion (mass deviation ≤ 3 ppm), the C isotope pattern and the retention time relative to that of the internal standard (deviation ≤ 2.5%), all being investigated from the corresponding reference standard. Compound Discoverer 2.1 (Thermo Fisher Scientific) was applied for the untargeted data interpretation, characterizing detected ions in terms of accurate mass (*m/z*), retention time, and peak intensity. Parameters for automated peak alignment, noise removal, peak extraction and deconvolution are presented in Table A.4. The assignment of characteristic fragments to the untargeted data was processed by an own written code in Python (Version 2.7.), that included the neutral losses and characteristic fragments (mass deviation ≤ 3 ppm) as determined for the APs, Bisphenol A and PAEs. During untargeted screening of seawater samples, compounds were tentatively identified using the tier 3 confidence level (according to the Chemical Analysis Working Group & Metabolomics Standards Initiative) [43].

2.6. Study area and sampling

The applicability of the SPE followed by UHPLC-HESI-HRMS was demonstrated by quantifying grab samples, collected at four different locations; i.e. 51°21′37.78″N; 3° 6′49.01″O (MOW1), 51°20′25.68″N; 3°12′12.11″O (HZ), 51°14′48.59″N; 2°55′39.61″O (Akust39) and 51°13′34.68″N; 2°56′8.00“O (HO), in the Belgian Part of the North Sea (BPNS) during two different periods of the year, i.e. winter 2016 (November 25th) and spring 2017 (April 10th). A map of the sampling locations can be consulted in Fig A1. To this end, 0.5 L grab samples were taken in threefold at a depth of 3 m, using Niskin bottles [44]. Upon arrival in the lab, grab samples were acidified to pH 3 using 1 M HCl and stored in dark amber bottles at 4 °C prior to extraction.

3. Results and discussion

3.1. Method development

3.1.1. Liquid chromatography

GC generally limits the analysis of higher molecular PAEs (ester side-chains containing more than 5 carbons) due to their intermediate volatility (see Table A1). Moreover, time-consuming derivatization steps have shifted the analysis of PAEs to LC in recent years, particularly to UHPLC. UHPLC has been proposed as a

superior technique for profiling multiple phenols and PAES as compared to conventional HPLC [45]. In general, UHPLC offers a better resolution (5 - fold), speed (10 - fold), sensitivity (analyte specific) and reduced solvent consumption (5-fold) for analytical determinations as opposed to HPLC [45,46]. Therefore, UHPLC was the platform of choice for targeting a broad range of low and high molecular PAEs. UHPLC separation methods for PAEs are however scarce [47], and has already been proven to be very challenging for AP analysis (because of their high volatility - see Table A.1). Hence, the optimization of the UHPLC conditions - including stationary phase, flow rate, mobile phase composition, additives, column temperature, and injection volume - were studied in detail by evaluating the inter-linked resolution ($R_{s,minimal} = 0.28$ and $R_{s,optimal} = 10.00$), chromatographically symmetric peak shape ($A_{s,minimal} = 1.50$ and $A_{s,optimal} = 1.00$) and potential interfering background of the 27 target analytes. Moreover, interfering background peaks of diethyl hexyl and dinonyl phthalate were observed in almost every analytical run (Fig. 1.). Fig. 1 depicts the varying area (intensity) of the interfering background peaks (uncoloured area of Fig. 2) of diethyl hexyl and dinonyl phthalate. Moreover, interfering background peaks of diethyl hexyl and dinonyl phthalate were observed in almost every analytical run (Fig. 1.). Fig. 1 depicts the fluctuating area (intensity) of the interfering background peaks (not coloured area of Fig. 2) of the of diethyl hexyl and dinonyl phthalate. Therefore, a number of analytical precautions were taken to minimize false positive results and favour reliable quantification. First, a trap column was placed between the UHPLC pump and the injection valve for retarding any PAEs and phenol contaminations originating from the analytical instrument and eluent. This is exemplified in Fig. 2, representing the chromatographic delay of the interfering background peaks (uncoulored area in Fig. 2) of diethyl hexyl and dinonyl phthalate as compared to the target analytes (colored area in Fig. 2)." Without the use of this trap column, the varying background contamination (depicted in Fig. 1 for diethyl hexyl and dinonyl phthalate) of the analytical instrument and eluent would interfere with the analysis of the compounds of interest originating from the samples. Second, the eluent acetonitrile - instead of the conventional methanol - was selected to minimize transesterification of target and untargeted PAEs into the primary mono-methyl phthalate. Controlling the degree of transesterification results in better quantification of target PAEs and identification of untargeted PAEs. Indeed, this transesterification has been observed when methanol was combined with formic acid at the high prevailing temperatures and voltages of the ionization source [48]. Furthermore, instability of the retention times (within a retention time window of 1.5 min) were observed for the APs using formic acid because the pH of the mobile phase was near the pKa of the compounds. Therefore, ammonium hydroxide was selected as mobile phase additive, having the supplementary benefit of an enhanced ionization rate for the alkylphenols.

The results of UHPLC optimization can be consulted for the standards, blanks and spiked samples in Figs. A.2., A.3., A.4., A.5, A.6., A.7., A.8., A.9. and A.10.

3.1.2. Ionization and full-scan mass spectrometry

Reliable and accurate quantification was achieved by optimizing the HESI and HRMS conditions upon evaluation of the overall peak intensity of the target compounds. The specific suspected pseudo-molecular ions for a salt matrix were not observed in full-scan, i.e. adducts of $[Na]^+$, $[K]^+$ and $[NH_4]^+$ [49]. Instead, the mass spectra of the target compounds were mainly characterized in full-scan as $[M+H]^+$, $[M+H]^+$ and $[M-H]^-$ for di-phthalates, mono-phthalates and phenols, respectively. The abundant pseudo-molecular ions and their corresponding ^{13}C-isotope were selected for accurate identification and quantification (Table 1). Remaining full-scan MS parameters, i.e. the resolving power and AGC target, were optimized. The resolving power was determined by optimizing the balance between sufficient number of data points across the chromatographic peak and a minimal mass deviation. Improving the mass accuracy (achieved by a higher resolving power) resulted in a better selectivity and consequently exclusion of isobaric matrix interferences, which contributed towards unambiguous identification and accurate quantitation. However, increasing the resolving power also resulted in less data points across the chromatographic peak, which negatively affected the repeatability and sensitivity. Therefore, a resolving power of 70,000 FWHM was retained to acquire sufficient data points across the chromatographic peak (>10) but at the same time accommodate sufficiently high mass accuracies (mass deviations < 3 ppm) [50]. The optimal AGC target was set at $5e^5$ ions, as this setting demonstrated the lowest mass deviation (<3 ppm) at MQL-level.

3.1.3. Extraction procedure

Preliminary experiments (See Table A.4.) demonstrated that 2 of the 11 commercially available SPE cartridges were appropriate for target compound clean up (i.e. Oasis™ HLB and Strata X™, based on the highest recovery, number of analytes, best reproducibility and lowest contamination in the blanks). These 2 cartridges were retained for the first step of the three-step statistical workflow for optimizing the phenol and PAE extraction procedure.

As described in the material and methods, first, the statistical significance of 14 extraction parameters on the phenols (i.e. AP and Bisphenol A) and PAE (i.e. mono- and di-phthalates) recovery was determined using a three-level fractional factorial resolution IV experimental design (Table A.5.). Nine parameters were significant (p-value < 0.05) for the di-phthalates, whereas for the mono-phthalates and phenols, respectively, 2 and 3 parameters were found significant. Significant parameters included filter step, pH, type of cartridge, volume of the equilibration solvent, loading volume, wash volume, elution solvent, elution solvent additive, volume of the elution solvent and evaporation temperature. The individual significance can be consulted in Table A.5. After the screening phase, the following significant parameters were fixed: pre-treatment, type of cartridge, conditioning solvent, and additive (based on the optima) and pH, loading volume and evaporation temperature (based on optima and technical limitations). The other significant parameters were optimized in later steps.

Based on our initial findings and different elution solvents reported in literature [35,51], the elution solvent was further optimized in a 2nd step using a simplex lattice mixture design, which pointed towards the use of 100% CH_3CN instead of a $C_5H_{12}O$-CH_3OH-CH_3CN mixture (Fig. A.11). This is in line with literature, as CH_3CN facilitates the elution of PAEs tightly adsorbed to the sorbent phase by undergoing lower surface tension/interactions with the sorbent [8]. Recently Jeong et al. [52] demonstrated that the surface tension/interaction of organic compounds to Oasis HLB™ in aquatic matrices is mainly dominated by physisorption and enhanced by chemisorption. Chemisorption is mainly driven by π-π interactions between the sorbent and the target compounds, due to the aromatic structure of the target compounds. This π-π interaction is however impeded when using acetonitrile [53].

In the last step, the equilibration, wash and elution volume of solvent were optimized using RSM (Fig. A.12) for providing maximal extraction efficiency. The final and optimized extraction procedures are reported above.

3.1.4. Analytical precautions

The use of plastic as glass-substitute was tested for the potential contamination with PAEs, as recommended in literature [48].

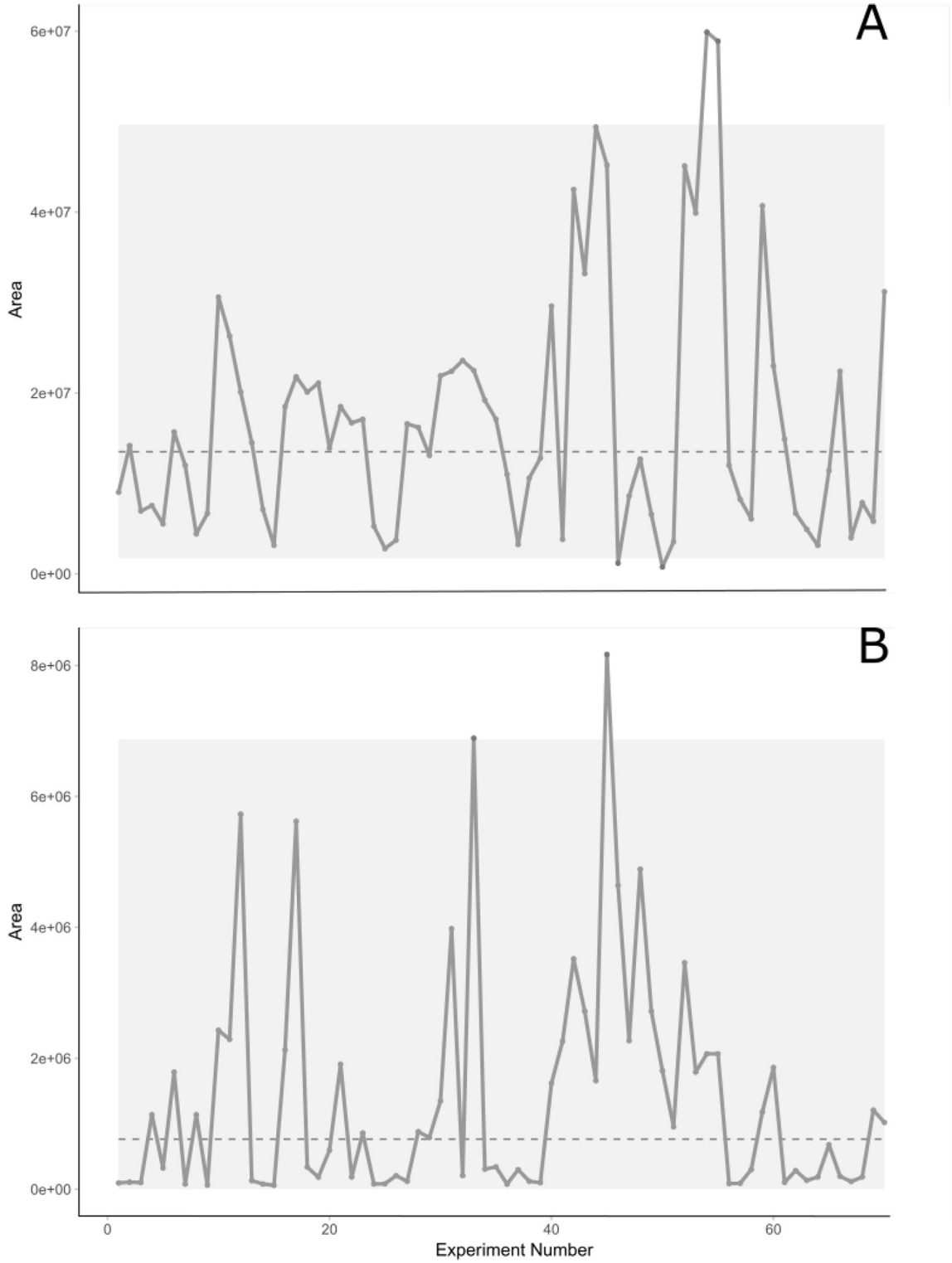

Fig. 1. Control chart of the interfering background that was delayed by using a trap column, i.e. (a) di-ethyl hexyl phthalate and (b) dinonyl phthalate. The dotted line represents the mean, and the grey shaded area represents the area between the upper and lower central limit.

Therefore, a home-made database of 51 PAEs was used (Table A.7.), including potential contaminants that have been reported during quantitative analysis [5,54]. No significant difference ($p > 0.05$) was observed between glass and polypropylene micropipette tips, and none of the contaminants from the home-made database were detected. A significant loss ($p < 0.05$) of high molecular PAEs, i.e. diamyl, benzyl butyl, dibenzyl, and diisodecyl phthalate, was however observed during the evaporation of the eluent in glass, which was not the case for polypropylene falcon tubes (TPP, Switzerland). Prospectively, the polypropylene falcon tubes were selected as material of choice.

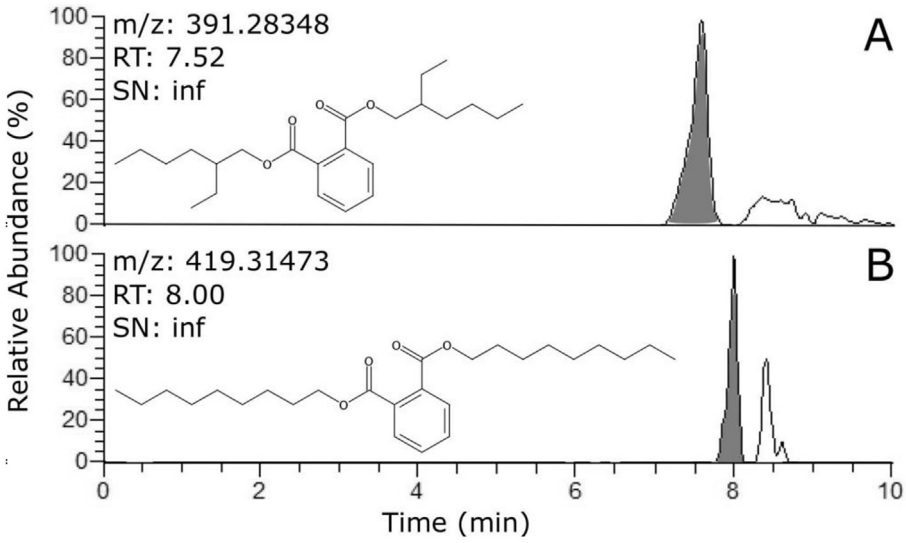

Fig. 2. UHPLC-HRMS chromatograms of the target compounds (coloured) that are separated from the interfering background (not coloured), i.e. (a) di-ethyl hexyl phthalate and (b) dinonyl phthalate.

3.2. Method validation

3.2.1. Limits of detection and quantification (MDL and MQL)

Determining MDLs and MQLs of target analytes when using HRMS gives rise to new challenges. Traditionally, MDLs and MQLs are estimated by theoretical or empirical calculations based on signal-to-noise ratios. Signal-to-noise ratios are, however, often of infinite magnitude when using HRMS, resulting in virtually infinitely low MDLs and MQLs. These unrealistic estimations stress the need of new strategies based on more practical criteria. Therefore, validation criteria for measuring emerging micropollutants in the aquatic environment were combined and refined, i.e. CD 2002/657/EC (food safety), CD 2009/90/EC (water monitoring) and Eurachem 2016 (general guidelines) as previously described by Vergeynst et al. [55]. In brief, the MDL was determined using a multi-injection statistical methodology commonly applied for trace analysis. Using the mean value and standard deviation of biological replicate extractions provides a statistically valid approach to discriminate the differences between a low-level analyte (near MDL) and the combined uncertainties in both the analyte and background measurements, and the uncertainty in the sampling process. The MDLs, determined by using the latter statistical tool, were practically confirmed by spiking reference seawater at MDL level. Thereby, an additional confirmation criteria was used for approving the reliability of the MDL, i.e. the presence of the ^{13}C-isotope and $^{13}C/^{12}C$-ratio of each target compound at the concentration investigated. The presence of the ^{13}C-isotope and C^{13}/C^{12} ratio has been frequently used as an additional confirmatory tool in omics studies for enhancing analytical accuracy [19,56]. Moreover, as long as the ^{13}C-isotope with the corresponding ^{12}C-isotope of the target compound is detectable, the presence of the compound can be undoubtedly confirmed. If the ^{13}C-isotope is no longer detectable, the presence and identity of the target analyte is questionable. The latter criterium was also used to fine tune the MDLs. Furthermore, the determination of the $^{13}C/^{12}C$-ratio also enables to determine the number of carbon atoms present in the target compound. As a consequence, matching experimental and theoretical number of carbon atoms of the target compound provides sufficient evidence for its presence in aquatic samples and the reliability of the MDLs [19]. The MQL on the other hand is regarded as the smallest quantity of a target compound that can be detected in a sample with an RSD below 20% of at least 3 independent measurements using spiked reference blank samples. This 20% criterium has been indicated in many regulations (such as 2002/657/EC, 2009/90/EC and CD 2013/39/EU) as the maximal allowed variation that can been considered as reliable [20–22,57]. Ultimately, considering the above-mentioned approaches, the MDLs for phenols, di-phthalates and mono-phthalates ranged respectively from 10 to 150 ng L-1, 5–25 ng L^{-1} and 5–25 ng L^{-1}, whereas the MQLs ranged respectively from 25 to 200 ng L^{-1}, 10–50 ng L^{-1} and 10–50 ng L^{-1}. The MQLs attained are sufficiently low, based on the only available EQS in literature for DEHP, i.e. 1.3 μg L^{-1} in surface waters [23].

3.2.1.1. Specificity and selectivity. No detectable residues of exogenous APs, Bisphenol A and PAEs at their accurate mass and specific retention time were observed in reference seawater used as a blank (Table 1 and, Figs. A.5., A.6., A.7., A.8., A.9. and A.10.). Similar conductivity and salinity were noticed between reference and real seawater, which can be consulted in Table A.8. Spiking the target analytes to the blanks resulted in a significant increase, taking into account a maximal RSD of 20% (Table A.9.), confirming the selectivity of the optimised method for the 27 target compounds. The latter were identified based on their accurate mass and relative retention time, i.e. the ratio between retention time of the analyte and its deuterated internal standard. Moreover, the target low and high molecular phthalates were respectively corrected by using the diethyl phthalate-3,4,5,6-d_4 and dicyclohexyl phthalate-3,4,5,6-d4. The specific deuterated internal standard that was used for quantification of every target compound can be consulted in Table 1. The observed retention time deviations (<0.05 min) and observed mass deviations (<1 ppm) confirm the excellent instrumental stability for the developed UHPLC-HRMS method. In addition, all procedural blanks were in fact fully blank at the retention time of interest of the target peak, implying that the analytical precautions (see section 3.1.4.) taken were successful.

3.2.1.2. Linearity. Weighted linear regression models (Table A.10.) indicated good linearity ($R^2 > 0.99$) and no lack of fit (95% confidence interval, F-test, p-value > 0.05) [39].

3.2.1.3. Trueness and precision. The recovery ranged for all compounds between 98.5 and 105.8%, with RSDs below 10% (n = 70,

independent extractions at 3 different days). These recoveries outperform these reported in related literature, ranging in aquatic matrices from 91.8 to 118% [58]. The precision, encompassing the repeatability and within-laboratory reproducibility complied to the Horwitz equation. The RSDs of repeatability and within-laboratory reproducibility ranged for all the target compounds, respectively, from 1.6 to 9.5% and 2.4–9.9% (Table 1 and Table A.9.). Comparing our results to reported literature, recovery and within-laboratory reproducibility ranges respectively from 4.1 to 17% and 4.7–12%, our precision can be considered as good [47].

3.2.2. Cross validation on freshwater

The scope of the method was extended and versatility was indicated by performing a cross-validation on fresh tap water samples (Table A.11. and Table A.12.). Tap water was used to evaluate whether the presence of e.g. free chlorine affected the method performance because of matrix effects [59]. When comparing the performance characteristics of freshwater and seawater, similar results were obtained for both matrices. These validation results (inclusive cross validation) suggest that the developed analytical method is robust and applicable to a broad spectrum of aquatic matrices, ranging from very salty to fresh aquatic water.

3.3. Analytical strategy for the identification of unknown plasticizers

As the analytical targeted platform was developed on a HR-Q-Orbitrap-analyzer, this also enables the detection of untargeted plasticizer degradation products and metabolites. To elucidate the typical fragmentation profiles and identify characteristic fragments of both the phenols (i.e. alkylphenols and Bisphenol A) and PAEs, the commercially available target standards (prepared in ultrapure water) were fragmented at 20 eV, allowing the simultaneous detection of the pseudo-molecular ion and its associated fragments. Lower collision energies (<20 eV) resulted in little to no fragmentation of pseudo-molecular ions, whereas higher collision energies (>20 eV) in the absence of the pseudo-molecular ions.

3.3.1. Alkylphenols

Although no truly specific fragments were detected for the branched alkyl substituted phenols, intermediate [M-H-CH3]- and predominant [M-H-CH4]- fragments were observed. The observed fragments result from the stepwise loss of a methyl radical (resulting in the intermediate fragment) and a hydrogen radical (leading to the predominant fragment) by the branched alkyl substituted phenols. Similar fragments $[M\text{-}H\text{-}CH4]^-$ have also been observed for phenols and structurally related compounds when using high resolution mass spectrometry [60,61]. For the chlorinated APs though, the neutral loss of HCl was noticed resulting in the $[M\text{-}H\text{-}HCl]^-$ fragment. Furthermore, bisphenol A was characterized by the presence of a fragment with *m/z* 133.066 Da, assigned as $[M\text{-}H\text{-}C_6H_6O]^-$. This fragment resulted from the cleavage of the phenyl-alkyl bond followed by the α-cleavage of the ether group. The above-mentioned neutral losses and characteristic fragments were in line with previous fragmentation studies [9,62] and were included in the Python code for tentative identification of unknown AP metabolites or degradation products.

3.3.2. Phthalates

The typical fragmentation profiles that were obtained for the protonated pseudo-molecular PAEs $[M+H]^+$ are summarized in Fig. 3. The left branch depicts the characteristic peaks associated with the fragmentation of $[M+H]^+$. A first step comprised the elimination of the placeholders, i.e. $[R_1\text{-}H]$ and $[R_2\text{-}H]$, leading to a McLafferty rearrangement product with *m/z* 167.033 Da [63]. Elimination of placeholders has previously been proposed for propyl and high molecular esters [64]. The McLafferty rearrangement is followed by the loss of water $[-H_2O]$, resulting in the protonated phthalic anhydride with *m/z* 149.023 Da. Subsequently, carbonyl [-CO] is eliminated leading to the formation of protonated benzoic acid with *m/z* 121.029, eventually followed by the loss of oxoketene leading to *m/z* 65.039. This carbonyl loss was not observed for all PAEs. Indeed, for some compounds, the direct generation of *m/z* 65.039 occurred. The right branch represents the specific fragments obtained for placeholders R_1 and R_2, which was only observed for high molecular di-phthalates. For this group, one placeholder was eliminated, followed by the loss of water, resulting in the remaining protonated placeholder. All the afore-mentioned ions (see also Fig. 3) were considered specific for the PAEs and incorporated in the Python code to enable tentative identification of unknown phthalate metabolites and degradation products. The strength of the proposed approach lies within the use of the high resolution (70,000 FWHM) of the MS and the simultaneous detection of 4 different fragmentation ions within a specific ratio.

3.4. Application to seawater samples

To demonstrate the applicability of the developed and validated UHPLC-HRMS method, 24 seawater samples were analyzed in the BPNS, i.e. both targeted quantification and untargeted screening for plasticizers was performed.

During targeted analysis, 2 APs and 14 PAEs were detected (Table 2). The highest concentrations were observed for ethylphenol, methylphenol and dibutyl phthalate in the harbor of Oostende (HO). Furthermore, Bisphenol A was not detected at any of the locations, although this was expected due to its extensive use in products and applications and previous reports on its widespread occurrence in human biofluids [65]. In addition to the quantified parent phthalates, also mono-phthalates (i.e. primary phthalate metabolites) were ubiquitously detected at all sampling locations. This may be attributed to the metabolic transformation (and excretion) from aquatic species or human excretion. Since primary phthalate metabolites have been appointed as relevant biomarkers for PAE exposure, both in aquatic organisms [66] and humans [67], our results suggest that phthalate contamination is widely distributed across different trophic levels. Ultimately, it can be concluded that, the developed and validated HRMS platform, compared to other aquatic screening methodologies [13,68,69], minimized as first the false-positive rate caused by in-house phthalate contamination both for targeted quantification and unknown screening.

For unknown screening purposes, data of the full-scan analysis at a resolution of 70,000 FWHM were subjected to the extraction of relevant unknown components (Compound Discoverer 2.1), which resulted in the detection of 1042 unique unknown components for both polarity modes combined. To elucidate the chemical identity of these unknowns, each extracted component was fragmented by using the PRM scan mode. The generated fragments were screened - using our newly written Python code - on their agreement with characteristic fragments and neutral losses obtained from the commercially available alkylphenols and PAEs (Table A.13.). In total, 5% ($n = 46$) of the unknowns - at the confidence level of Tier 3 according to the Chemical Analysis Working Group & Metabolomics Standards Initiative [43] - could be tentatively identified as plasticizer, i.e. 7 as phenol and 20 as PAE. The following characteristic fragments could be assigned for the phenols: 5 times $[M\text{-}H\text{-}HCl]^-$, 3 times $[M\text{-}H\text{-}CH_3]^-$ and 13 times $[M\text{-}H\text{-}CH_4]^-$. For the PAEs, the following specific fragments were detected for almost every unknown assigned a PAE structure: *m/z* 167.033, 149.023, 121.029 and 65.039. The MS/MS spectra can be consulted in Fig. A.13.

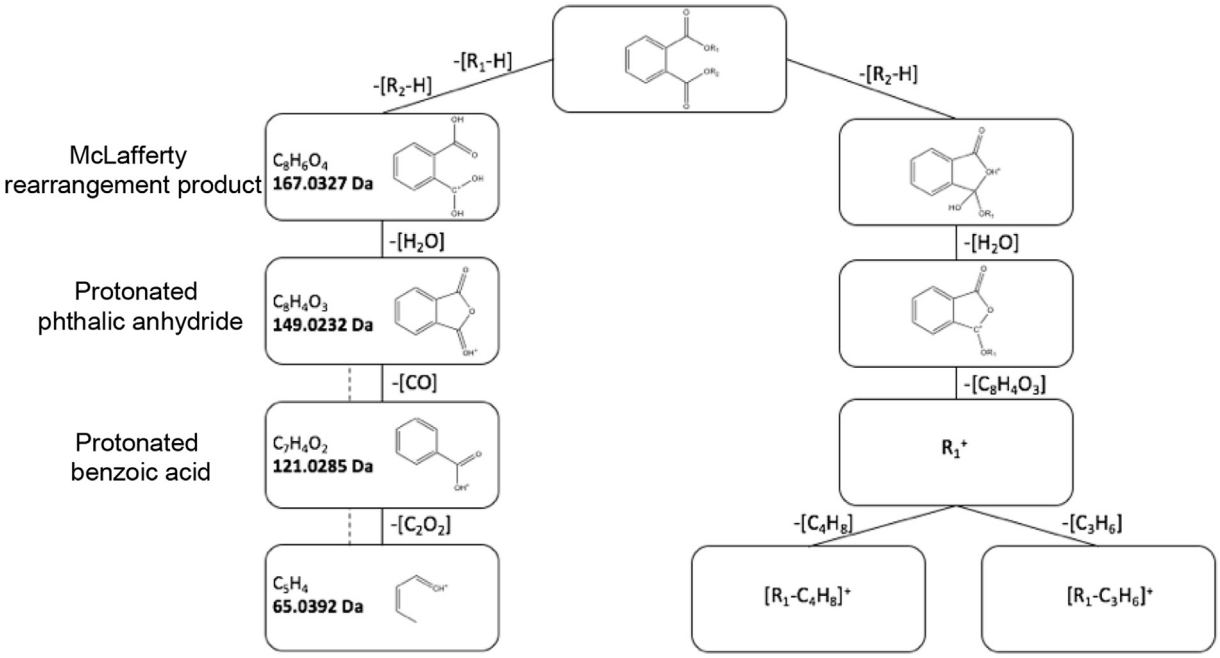

Fig. 3. The observed fragmentation patterns for the phthalates in ultrapure water (R1 and R2 represent the placeholders of the plasticizer) at a CE of 20 eV.

Table 2
Detailed quantified concentrations with the associated standard deviations in ng L^{-1} of the grab samples taken at 4 different locations in the BPNS (51°21′37.78″N; 3° 6′49.01″O (MOW1), 51°20′25.68″N; 3°12′12.11″O (HZ), 51°14′48.59″N; 2°55′39.61″O (Akust39) and 51°13′34.68″N; 2°56′8.00“O (HO)) and 2 different time points (for each time point and each location investigated in threefold, n = 3). Only compounds with concentrations above the MQL, for at least one of the sampling locations or time points, were incorporated in this table.

Grab samples	Sampling Winter 2016				Sampling Spring 2017			
	MOW1	HZ	Akust39	HO	MOW1	HZ	Akust39	HO
Methylphenol	< MDL	< MDL	63 ± 47	< MDL	< MDL	2302 ± 509	< MDL	6737 ± 2468
ethylphenol	328 ± 150	1518 ± 113	43 ± 3	2508 ± 243	407 ± 440	112 ± 23	593 ± 345	469 ± 159
diethyl phthalate	159 ± 3	336 ± 371	< MDL	235 ± 111	27 ± 3	43 ± 64	56 ± 62	753 ± 95
dibutyl phthalate	< MDL	308 ± 297	496 ± 83	2645 ± 250	77 ± 11	791 ± 242	205 ± 35	1502 ± 401
diamyl phthalate	< MDL	< MDL	< MDL	< MDL	< MQL	< MQL	< MQL	< MQL
benzyl butyl phthalate	< MDL	< MDL	< MDL	< MDL	79 ± 70	105 ± 29	60 ± 91	343 ± 283
dicyclohexyl phthalate	67 ± 93	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL
dihexyl phthalate	< MDL	37 ± 9	< MDL	< MDL	< MDL	23 ± 2	< MDL	< MDL
dibenzyl phthalate	< MDL	< MQL	< MQL	< MDL	< MDL	< MDL	< MDL	< MQL
diethylhexyl phthalate	269 ± 151	80 ± 54	66 ± 41	100 ± 71	298 ± 145	524 ± 156	218 ± 55	766 ± 314
diisodecyl phthalate	< MDL	< MQL	< MDL	< MDL	< MQL	< MDL	< MQL	108 ± 49
monomethyl phthalate	235 ± 161	< MDL	< MDL	< MDL	2542 ± 226	158 ± 2	1604 ± 127	< MDL
monobutyl phthalate	< MDL	73 ± 28	53 ± 30	165 ± 28	26 ± 10	109 ± 14	292 ± 37	192 ± 8
mono-n-pentyl phthalate	< MQL	< MDL	< MQL	< MDL	58 ± 170	25 ± 42	< MDL	138 ± 100
monobenzyl phthalate	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	< MDL	58 ± 1
monoethylhexyl phthalate	< MDL	399 ± 98	< MDL	< MDL	740 ± 391	674 ± 115	423 ± 53	656 ± 123

Finally, during untargeted screening of seawater samples, all target analytes were detected based on the aforementioned ions, confirming that fragmentation occurred similarly in a saline aqueous matrix.

4. Conclusions

A novel analytical SPE-UHPLC-HR-Q-Orbitrap™-MS method was developed and successfully validated for the simultaneous detection and quantification of 27 known plasticizers and plastics additives in sea and freshwater. Validation demonstrated excellent performance, i.e., stable recoveries ranging from 98.5 to 105.8%, satisfactory repeatability (RSD < 8%, n = 54) and reproducibility (RSD < 10%, n = 36). The empirical MQL in aquatic matrices for the phenols, di-phthalates and mono-phthalates ranged respectively from 25 to 200 ng L-1, 10–50 ng L^{-1} and 10–50 ng L^{-1}. These low MQLs for a broad range of physico-chemical diverse target compounds (log P ranging from 1.1 to 9.9) are vital for the environmental application of this novel method. Indeed, the presented analytical method is the first fulfilling the current need, i.e. the simultaneous quantification of APs, Bisphenol A, PAEs and their primary metabolites at environmental relevant concentrations. The analytical platform also enables simultaneous holistic monitoring of unknown plasticizers by making use of accurate mass data on known characteristic AP fragments and newly discovered PAE fragmentation patterns. Comparing our innovative rapid HRMS platform to other aquatic screening methodologies, the developed platform minimized as a first in its kind the false-positive rate caused by in-house phthalate contamination both for targeted quantification and unknown screening.

In conclusion, our newly developed analytical platform, facilitating the monitoring of a broad range of known and unknown plasticizers and plastics additives, may contribute to national and international legislation, such as the European Water Framework Directive, resulting in better regulations on environmental quality standard levels. Even more, holistic environmental fingerprinting may also contribute to fundamental insights in ocean health, and potential threats on aquatic organisms and humans.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2018.10.045.

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