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Determination of human metabolites of chlorinated phosphorous flame retardants in wastewater by N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide-derivatization and gas chromatography-high resolution mass spectrometry

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Highlights:

- A method to determine organophosphate (OP) diesters in sewage has been developed
- OP diesters are rapidly silylated showing characteristic mass spectra
- First GC-based method for OP diesters in wastewater extracts
- Bis(chloropropyl) phosphate has been quantified at levels > 60 ng/L for the first time

Non-intrusive approach for assessing human exposure to OP

Abstract

The analysis of wastewater for the determination of human biomarkers of exposure (human metabolites) is a non-intrusive, economic and complementary alternative to the analysis of urine in the monitoring of human exposure to chemicals of concern. This study provides the first gas chromatography-based method for the determination of three metabolites of chlorinated organophosphorous flame retardants (OPFRs): (bis(2-chloroethyl) phosphate, bis(chloropropyl) phosphate and bis(1,3-dichloro-2-propyl) phosphate) in wastewater. A solid-phase extraction procedure based on the use of mixed-mode reversed-phase weak anion exchange sorbents was optimized including a fractionated elution of OPFRs and their metabolites. Analytes derivatization was investigated by comparing two silylating reagents, N-*tert*-butyldimethylsilyl-N-methyltrifluoroacetamide and N-methyl-N-(trimethylsilyl)trifluoroacetamide, the first one providing better results. Determination was performed by gas chromatography-high resolution mass spectrometry with a quadrupole-time-of-flight system (GC-QTOF) in order to improve selectivity. Furthermore, the use of GC-QTOF combined with the specific ion obtained from silylated metabolites (*m/z* 154.9924) can be exploited to screen for other phosphate ester metabolites. Under final conditions, the overall method performance was satisfactory, affording method detection limits ranging from 1.1 to 4.6 ng/L, percentages of recovery from 90% to 110%, and relative standard deviations

below 13%. The analysis of composite raw wastewater samples collected over 24 h in the NW of Spain allowed to quantify, for the first time in this matrix, the metabolite bis(chloropropyl) phosphate at levels over 60 ng/L.

Keywords: phosphorous flame retardants and plasticizers; metabolites; wastewater; human exposure; silylation; gas chromatography-high resolution mass spectrometry

1. INTRODUCTION

Organophosphate esters, particularly triesters, are high-production-volume chemicals generally used as flame retardants and plasticizers (FRs) in a wide range of consumer goods, such as plastics, foams, paints, resins, textiles, electronics and furniture [1, 2]. Non-chlorinated alkyl phosphates are most commonly used as plasticizers, whereas chlorinated derivatives are used as flame retardants, receiving altogether the combined name of organophosphorous FRs (OPFRs) [2]. These compounds are employed as additives, i.e. mixed into the material but not chemically bonded to it, what facilitates their release into the surrounding environment and their further distribution into other environmental compartments [1]. They have been quantified in indoor air and dust [3-5], outdoor air, suspended particulate matter, sewage, surface and ground water, biota, soils and sediments [2, 6-8]. Their ubiquity and special high concentrations in indoor atmospheres entail a continuous exposure to OPFRs by the majority of the population, mainly via dermal absorption, ingestion of dust or contaminated food and inhalation of the most volatile species [9-13], tris(2-chloroethyl) phosphate (TCEP) and tris(chloropropyl) phosphate (TCPP, a technical mixture of four isomers differing in the alkyl chain) [5, 8, 14-16]. Harmful effects of this exposure include dermatitis (TCPP and 1,3-dichloro-2-propyl phosphate (TDCPP)) [17], endocrine disruption (TCEP and

TDCPP) [7, 18] and toxicity towards specific organs (TCEP for the kidney [7]). TDCPP has also been classified as carcinogenic, TCPP as carcinogenic to animals and TCEP as potentially carcinogenic [2, 19]. TCEP and TDCPP are subject to regulations in North America, Europe and Japan [20-22], and the European Chemicals Agency (ECHA) is currently preparing a proposal of restriction of use of these three chlorinated OPFRs [23]. Assessment of human exposure to OPFRs is usually performed through the determination of the precursor compounds and their metabolites in urine. In the case of chlorinated OPFRs, the main metabolites analysed in this matrix are the diesters bis(2-chloroethyl) phosphate (BCEP) for TCEP; bis(chloropropyl) phosphate (BCPP) and bis(chloropropyl) hydroxypropyl phosphate for TCPP; and bis(1,3-dichloro-2-propyl) phosphate (BDCPP) for TDCPP [7, 16]. Although the analysis of urine is the most widely applied strategy in human biomonitoring, it is not exempt from limitations. It is subjected to ethical implications, restricted to a limited number of samples (what implies a limited population coverage) and affected by selection bias. Alternatively, the analysis of wastewater, understood as a pooled sample of urine of a whole community, provides chemical information that may help to understand exposure at the population level. Known as wastewater-based epidemiology (WBE), this methodology was initially implemented to gather information on the consumption of illicit drugs [24], and further extended to estimate human exposure to pesticides [25, 26], phthalates [27] and, very recently, OPFRs [28] through the determination of their human metabolites. An advantage inherent to WBE is that it is a non-intrusive methodology where one sample of wastewater represents thousands of urine samples, thus being inexpensive and more representative of the entire population. The promising results got in large-scale studies [29-31] highlights the need of developing analytical methods than can be applied

worldwide to establish WBE as a complementary tool for the monitoring of chemical exposure.

Although there are several publications dealing with the determination of chlorinated OPFRs in wastewater [1, 6, 8], such application for their metabolites has been performed only in two occasions [28, 30]. Thereby, this study is aimed at optimizing, validating and applying a new analytical method to determine three biomarkers of exposure to chlorinated OPFRs (BCEP, BCPP and BDCPP) in wastewater. Conversely to the extended use of liquid chromatography (LC) - tandem mass spectrometry (MS/MS) for the separation and detection of OPFRs and their metabolites in urine [32-35] and, very recently, wastewater [28], we suggest an alternative gas chromatography (GC) - high resolution mass spectrometry (HRMS) method after derivatization of the analytes to silyl-derivatives. GC-HRMS can afford further selectivity and qualitative information, improving the sensitivity in some cases. Therefore, the final objective is to present a GC-compatible solid-phase extraction (SPE) combined with a GC-based separation that can be applied to quantitatively determine BCEP, BCPP and BDCPP in wastewater. To the best of our knowledge, this is the first time that BCEP is included in a quantitative validated method for the determination of chlorinated OPFR metabolites in wastewater.

2. MATERIAL AND METHODS

2.1. Chemicals and reagents

The structures of the chlorinated organophosphate diesters studied in this work and their precursor triesters are shown in Table S1. Individual standards of BCEP, BCPP (technical mixture of four isomers), BDCPP and their deuterated analogs (BCEP-d8, BCPP-d12 and BDCPP-d10) were supplied by Biozol (Munich, Germany). TCEP,

TCPP (also as technical mixture of four isomers) and TDCPP were supplied by Sigma-Aldrich (San Louis, Mi, USA). Their deuterated analogs (TCEP-d12, TCPP-d18 and TDCPP-d15) were supplied by Wellington Laboratories (Southgate Dr., Ontario, Canada). Mixed stock solutions containing the three diesters, the three diester deuterated analogs (used as surrogate or internal standards, IS), the three triesters or the three triester deuterated analogs were prepared in methanol (MeOH) and in ethyl acetate (EtOAc) and stored in the dark at -20 °C until use.

HPLC-grade MeOH, acetic acid (100%) and ammonia (NH_3) solution in ultrapure water (25%) were supplied by Merck (Darmstadt, Germany). EtOAc, formic acid (95-97%) and NH_3 solution in MeOH (7 N) were supplied by Sigma-Aldrich (San Luis, Mi, USA). Ultrapure water was obtained in the laboratory by purifying demineralized water in a Milli-Q Gradient A-10 system (Merck-Millipore, Bedford, MA, USA). Silylation reagents, N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) and N-*tert*-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) were provided by Sigma-Aldrich (San Louis, Mi, USA).

2.2. Sampling and sample treatment

Composite raw wastewater samples of 24 h were collected at the inlet of an urban wastewater treatment plant (WWTP) that receives mostly domestic wastewater and serves a population of ~136,500 inhabitants in Santiago de Compostela (NW of Spain). Samples were collected in December 2017 and in March 2018 by a Sigma SD900 portable sampler from Hach (Loveland, CO, USA) working in time proportional mode. An aliquot of 120 mL was collected every 10 min from 9.00 a.m. to 9.00 a.m. of the following day. Composite samples were transferred to the laboratory and extracted within 8 h after the end of the sampling.

Aliquots (100 mL) were vacuum-filtered through 0.7 µm glass microfiber filters GF/A (Whatman, Kent, UK) and 0.45 µm cellulose filters (Merck-Millipore, Bedford, MA, USA) and spiked with 20 ng of BCEP-d8, BCPP-d12 and BDCPP-d10. An SPE procedure was developed for the extraction of the organophosphate diesters using the mixed-mode reversed-phase weak anion exchange sorbents Oasis WAX-150 mg (Waters, Milford, MA, USA). Sorbents were subsequently preconditioned with 6 mL of MeOH, 6 mL of ultrapure water and 6 mL of 2% of formic acid in ultrapure water to assure the protonation of the amine groups included in their polymeric structure. Samples were loaded at their natural pH and, after loading, sorbents were washed with 6 mL of ultrapure water and dried under nitrogen for ca. 30 min. A fractionated elution was performed: first, 4 mL of EtOAc were passed through the cartridges to remove interfering chemicals, including the precursor triesters. Then, analytes were recovered with 2 mL of EtOAc:MeOH:NH_3 (83:15:2). Eluates were evaporated to dryness under nitrogen (99.999%), redissolved in 100 µL of EtOAc and filtered through 0.2 µm syringe-driven Nylon filters (Chmlab Group, Barcelona, Spain). An aliquot of 75 µL of each filtered extract was transferred into a glass micro-insert and mixed with 25 µL of MTBSTFA before injection. Samples were processed in triplicate.

2.3. Gas chromatography-high resolution mass spectrometry

Instrumental analyses were performed using a GC-QTOF-MS system comprised of a 7890A gas chromatograph (Wilmington, DE, USA), a 7638B automatic sampler and a 7200 Quadrupole Time-of-Flight (Q-TOF) mass spectrometer from Agilent (Wilmington, DE, USA). Large-volume injections of 10 µL (2×5 µL) were made in solvent vent mode using a 10 µL-syringe and a Programmable Temperature Vaporizer (PTV) injector equipped with an Agilent ultra-inert liner containing glass wool. The

inlet temperature was increased from 60 °C (held for 0.6 min) to 300 °C (15 min) at a rate of 700 °C/min. The flow rate through the split vent was set at 60 mL/min up to 0.6 min. After this time, the split valve was closed for 2 min and opened again with a purge flow of 100 mL/min.

Chromatographic separation was carried out on a HP-5MS type capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness) supplied by Agilent Technologies. Helium (99.9999 %, Praxair, Spain) was used as carrier gas at a constant flow rate of 1.2 mL/min. The oven temperature programme was as follows: 60 °C (held for 2 min) ramped at 15 °C/min to 280 °C (held for 5 min). The total run time was 21.67 min and the solvent delay 6 min. The transfer line, quadrupole and electron impact source were set at 280 °C, 150 °C and 230 °C, respectively.

The mass spectrometer was operated in Electron Ionization (EI) mode at 70 eV and in single MS mode. The TOF analyzer worked in 2-GHz extended dynamic range mode, providing a resolution between 5500 at *m/z* 130.9915 and 9000 at *m/z* 413.9770. The mass axis was automatically recalibrated every 5 injections by infusion of a commercial solution of perfluorotributylamine in the EI source. Full scan high resolution mass spectra were recorded in centroid mode in the range from 50 to 500 *m/z*, at a frequency of 3.33 spectra/s.

Table 1 displays retention times, empirical formulae and accurate *m/z* of the quantifier ions (Q) and qualifier ions (q) of the *tert*-butyl dimethylsilyl (TBDMS) derivatives of BCEP, BCPP, BDCPP and their deuterated analogs and of TCEP, TCPP, TDPP and their deuterated IS. Extracted ion chromatograms (EIC) of Q and q were reconstructed with a symmetric *m/z* window of 20 ppm. For BCPP and BCPP-d10, three peaks, corresponding to the three main isomers occurring in the commercial mixture, were obtained. The EI high-resolution mass spectra of the TBDMS forms of BCEP, BCPP,

BDCPP and their deuterated IS are shown in Figure 1 and Figure S1, respectively. For BCPP and BCPP-d10, only the spectrum of the most intense chromatographic peak is provided.

The criteria for analytes positive identification in the sample was based on the Q/q ion ratio, which should not differ more than a 30% from that given in Table 1, in accordance with the SANTE/11813/2017 guideline.

2.4. Method validation and blank evaluation

Analytes were quantified using their corresponding deuterated analogs (BCEP-d8, BCPP-d12, BDCPP-d10) as IS. Calibration curves were prepared in EtOAc and derivatized with 25% of MTBSTFA (i.e. 25 µL of derivatizing agent to 75 µL of standard solution) prior to injection.

Linearity was assessed by a 10-point calibration curve ranging from the IQL to 1000 ng/mL (IS concentration: 200 ng/mL). Instrumental blanks were run at the beginning of every sequence. Instrumental detection and quantification limits (IDL and IQL) were estimated from the lowest calibration standards as the concentrations providing a signal to noise ratio (S/N) of 3 and 10, respectively. For BCPP, the highest peak, corresponding to the first eluting isomer, was used. Intra-day and inter-day instrumental precision were assessed from the relative standard deviation (%RSD) of six injections of a standard performed over 24 h (intra-day precision) or over four weeks (inter-day precision). Two concentration levels were considered: 10 ng/mL and 100 ng/mL.

Procedural blanks were run together with every set of samples. For BCEP and BCPP, method detection and method quantification limits (MDL and MQL) were calculated as 3 and 10 times, respectively, the standard deviation (SD) of the concentrations in the procedural blanks. For BDCPP, absent in blanks, they were estimated from the

measured concentrations in spiked wastewater samples (n=3), downscaling the levels for which the S/N values are 3 (MDL) and 10 (MQL). Trueness and precision of the whole SPE-GC-HRMS method were evaluated using ultrapure water spiked with 25 ng/L of the three analytes and 200 ng/L of IS, and wastewater spiked with 500 ng/L of analytes and 200 ng/L of IS. Additional aliquots of wastewater spiked only with IS were processed simultaneously. Trueness was expressed as the average recovery from the nominal spiking value, and precision as the %RSD from the average measured concentration (n=3).

2.5. Stability studies in wastewater

The stability of the target organophosphate diester metabolites and their precursor triesters was assessed in raw wastewater stored in the dark at room temperature (22 ± 2 °C) for 48 h. Two batches of experiments were performed (n=3 in each case). In a first batch, samples of 100 mL of wastewater were spiked with 50 ng/mL of TCEP, TCPP and TDCPP, and aliquots of 10 mL were collected at the beginning of the experiment (0 h) and after 2, 6, 24 and 48 h. These aliquots were spiked with 20 ng of the deuterated triesters and the deuterated diesters, filtered through 0.45 µm syringe-driven PVDF filters (Millex®, Merck-Millipore) and extracted as detailed in section 2.2. Triesters were recovered with 4 mL of EtOAc (clean-up step) and the potential diesters formed during the experiment with 2 mL of EtOAc:MeOH:NH_3 (83:15:2). Eluates were collected separately, evaporated to dryness and reconstituted in 100 µL of EtOAc for instrumental analysis. EtOAc extracts containing the diesters were derivatized just before injection. In a second batch of experiments, samples of 100 mL were spiked with 5 ng/mL of BCEP, BCPP and BDCPP and kept at room temperature for 48 h. Aliquots (10 mL) were taken at different times, spiked with 20 ng of the deuterated diesters,

filtered through 0.45 µm syringe-driven PVDF filters and solid-phase extracted as detailed in section 2.2. In this case, only the basified EtOAc fractions were collected, evaporated to dryness, reconstituted in EtOAc and derivatized with MTBSTFA.

3. RESULTS AND DISCUSSION

3.1. Determination conditions

Separation and detection were first attempted by ultra-high performance liquid chromatography-MS/MS with a Waters Acquity UPLC® H system interfaced to a Xevo TQD mass spectrometer through an electrospray ionization source (Waters Corp., Milford, MA, USA). Under optimized detection and separation conditions (separation performed on a Luna® Omega Polar C18 column of 1.6 µm of particle size) the sensitivity was very low and all the analytes showed MDLs higher than 10 ng/mL. We related this observation to the unsatisfactory method performance results observed by Been et al. for BCEP and BCPP [28] and tentatively attributed it to a poor electrospray ionization behaviour of the three analytes. Thus, an alternative procedure based on the use of GC-HRMS was proposed.

Organophosphate diesters contain an acidic hydroxyl moiety that needs to be transformed to decrease their polarity and increase their volatility prior to their determination by GC-MS. Although Shindler et al. used pentafluorobenzyl-bromide to determine several OPFR metabolites in urine by GC-MS/MS [37, 38], we selected two silylation reagents due to their already good known performance and efficiency in improving the detectability of compounds with active H groups: MSTFA and MTBSTFA [39]. The trimethylsilyl (TMS) derivatives and TBDMS derivatives formed, respectively, with each of them provided EI spectra characterized by a base peak at *m/z* 154.9924 that was selected as Q for all the analytes. This ion corresponds to the

phosphate dimethylsilyl ester after losing one methyl (TMS derivatives) or butyl (TBDMS derivatives) group and all alkyl chains by 3 consecutive McLafferty rearrangements (empirical formula $C_2H_8O_4PSi$). Figure 1 compiles the spectra of the TBDMS forms and Figure S2 the spectra of the TMS forms. The ion at *m/z* 154.9924 is characteristic of silylated acidic phosphate esters and could be used to screen for metabolites of other OPFRs or organophosphorous pesticides (see 3.5). The other ions observed in the EI spectra correspond to the mono-alkyl dimethylsilyl ions, selected as q for confirmation purposes, and to the di-alkyl dimethylsilyl ions (Figure 1 and Figure S2). The base peak of the deuterated analogs is at *m/z* 157.005, since it contains two deuterium atoms (Figure S1).

The effect of both temperature and time in the off-line derivatization was assessed with 500 ng/mL standards in EtOAc derivatized with 10% of every derivatizing agent. Neither the temperature (room temperature versus 60 ℃) nor the time (5-30 min) affected the extension of the reaction. For both reagents, the response in aliquots derivatized at room temperature for 5 min were identical to those of aliquots kept at 60 ℃ for 30 min, proving that the derivatization process was very fast. However, the three TBDMS derivatives showed higher signals than the TMS derivatives and, therefore, MSTFA was discarded in favour of the use of MTBSTFA (Figure S3). The percentage of derivatizing agent to be added was then assessed with standards in EtOAc (n=3) and with reconstituted fractions resulting from the evaporation of 2 mL of EtOAc:MeOH:NH_3 (83:15:2), n=3, emulating SPE extracts (see section 3.2). No differences were observed among the standards containing 5%, 10% and 25% (v/v) of MTBSTFA. Yet, the signals of the reconstituted residues containing 5% and 10% of MTBSTFA dropped when compared to the signal of the standards and they were not repeatable, presumably due to the reaction of the derivatizing agent with the variable

quantity of NH_3 remaining in the residue. To ensure a repeatable derivatization of the organophosphate diesters, independently of the partial consumption of MTBSTFA by reaction with NH_3 or with interferences occurring in real extracts, the percentage of derivatizing agent was set at 25% (v/v) (i.e. 25 µL of MTBSTFA plus 75 µL of standard/extract). Extracts and standards were checked to be stable for at least 24 h (data not show), which agrees with the published stability of TBDMS derivatives [40].

The injection of 10 µL of a standard using the PTV injector was compared to the injection of 2 µL in splitless mode (liner: Ultra Inert Liner, single taper, glass wool 900 µL; injector temperature: 300 °C; splitless time: 1 min; split flow: 60 mL/min). The peak height of all analytes increased substantially when injecting large volumes versus when injecting 2 µL (Figure S4). Thus, the injection volume was set at 10 µL.

Once the derivatization and injection conditions were optimized, the temperature gradient was adjusted as to obtain a good separation with a reasonable run time. The final gradient is provided in section 2.3.

3.2. Solid-phase extraction

Since pK_a values of BCEP, BCPP and BDCPP are, respectively, 1.92, 1.86 and 1.83 [41], the three molecules are completely deprotonated at the usual pH of wastewater (typically 7-8 units). Considering this and the methodologies available in the literature for the extraction of organophosphate diesters in urine [37, 38, 42, 43] and water [28, 30], two SPE strategies were assessed: i) acidification of samples to pH 2, extraction on hydrophilic-lipophilic balance reversed-phase polymeric sorbents Oasis HLB 200 mg (Waters), and elution with 3×2 mL of EtOAc; and ii) extraction of samples at their natural pH on mixed-mode reversed-phase weak anion exchange sorbents Oasis WAX 150 mg (Waters), and elution with 3×2 mL of EtOAc:MeOH:NH_3 (58:37:5). It must be

noticed that MeOH was present in the elution solvent because the originally purchased NH_3 solution was prepared in this solvent. In both cases, individual fractions were evaporated to dryness, reconstituted in 100 µL of EtOAc containing 20 ng of IS and derivatized with MTBSTFA (75 µL of extract + 25 µL of MTBSTFA). Preliminary tests extracting 100 mL aliquots of spiked ultrapure water (n=3) showed that none of the analytes was quantitatively recovered from the Oasis HLB sorbents (recoveries lower than 1%), despite the use of similar reversed-phase materials to extract organophosphate diesters in other studies [28, 30, 37, 38]. With the Oasis WAX, 65%-80% recoveries were achieved in the first fraction of basified EtOAc, with less than 1% being recovered in the other fractions. Therefore, we selected the last sorbent and 2 mL of basified EtOAc as elution solvent for subsequent experiments.

Further experiments were performed by extracting 100 mL aliquots of spiked raw wastewater on Oasis WAX cartridges (n=3, elution with 2 mL of EtOAc:MeOH:NH3 (58:37:5), evaporated to dryness and reconstituted in 100 µL of EtOAc). However, a solid residue insoluble in EtOAc was observed in the final extracts. A washing step of the sorbent with (a) 5 mL of ultrapure water:formic acid 98:2 followed by 5 mL of MeOH after sample percolation or (b) 4 mL of EtOAc after sorbent dryness were included in the SPE protocol, but a solid residue was still formed. This was attributed to MeOH-soluble, EtOAc-insoluble anionic interferences swept along with the elution solvent, since the NH_3 used comes from a methanolic solution 7 N that implies ca. 37% of MeOH for a 5% NH_3 elution mixture. To decrease the amount of MeOH, the percentage of NH_3 was reduced to 2% (i.e. 15% of MeOH in the elution solvent) and the volume needed for a quantitative recovery of the analytes was again optimized with spiked ultrapure water samples. Between 98% (for BCEP) and 100% (for BCPP and BDCPP) of the eluted analytes were recovered in a single fraction of 2 mL of

EtOAc:MeOH:NH_3 (83:15:2), therefore selected as elution solvent. Under these conditions, no insoluble residue was observed when extracting real wastewater.

A matter of concern is the putative interference of precursor triesters, usually present in wastewater at high concentration levels. By comparing the responses of a standard of triesters before and after being mixed with 25% (v/v) of MTBSTFA, we verified that ca. 12% of TCEP and 8% of TCPP were degraded with the silylation reagent, giving to the formation of 0.7% and 1.7%, in molar basis, of the corresponding diesters. Conversely, TDCP turned out to be stable. To ensure the complete removal of triesters before the elution of diesters, an SPE of 100 mL of ultrapure water spiked with 100 ng TCEP, TCPP and TDCPP and 20 ng of their deuterated analogs (n=3) was performed following the protocol optimized for the extraction of diesters. Aliquots of 4 mL of EtOAc and 2 mL of EtOAc:MeOH:NH_3 (83:15:2) were consecutively passed through the cartridges, collected separately, evaporated to dryness and reconstituted in 100 µL of EtOAc for instrumental analyses. 100% of the eluted triesters were recovered in the EtOAc fraction, rendered as an effective clean-up step for the determination of diesters in the basified EtOAc fraction. Additionally, a washing step of the sorbent with 6 mL of ultrapure water before drying the cartridges was kept in the final procedure to remove salts and inorganic interferences.

3.3. Method performance

Method performance parameters are displayed in Table 2. The representation of the ratio analyte area/IS area versus analyte concentration fitted a linear model in the range IQL-1000 ng/mL with determination coefficients (R^2) above 0.99. The analysis of instrumental blanks proved the absence of carryover or blank problems in the GC system. IDLs were between 0.38 ng/mL and 1.1 ng/mL, and IQLs between 1.3 ng/mL

and 3.6 ng/mL. Instrumental precision (both intra- and inter-day) at 10 and 100 ng/mL was satisfactory for the three analytes, with %RSD values for six injections of a standard between 0.75% and 8.5%.

MDLs, estimated as 3 times the SD of the procedural blanks for BCEP and BCPP and as the concentration in a sample providing a S/N of 3 for BDCPP, ranged from 1.1 to 4.6 ng/L. MQLs ranged from 4 to 15 ng/L, higher than the MQL reported for BDCPP (0.8 ng/L) by Been et al [30] but better than the MQL reached in their study for BCPP (15.4 ng/L), whereas BCEP could not be measured in that method.

Trueness and precision of the whole SPE-GC-HRMS method, assessed through recovery experiments in ultrapure water and wastewater, were satisfactory for all the compounds: recoveries varied from 90% to 110% in ultrapure water spiked at 25 ng/L and from 90% to 100% in wastewater spiked at 500 ng/L. RSD varied between 3% and 13% in ultrapure water and between 1% and 6% in wastewater. Both recovery and %RSD values were acceptable according to the Commission Decision 2002/657/EC [44].

3.4. Stability of diesters and triesters in wastewater

Stability tests in real wastewater were conducted to assess: (i) the potential formation of BCEP, BCPP and BDCPP from their precursor organophosphate triesters occurring in wastewater; and (ii) the potential decrease in the concentration of BCEP, BCPP and BDCPP due to biodegradation and/or adsorption phenomena. Experiments were performed using unfiltered raw wastewater samples spiked only with triesters (objective (i)) or only with diesters (objective (ii)) and kept at room temperature for 48 h, a period of time longer than in-sewer residence and sampling. Figure 2 displays the responses of the investigated compounds relative to their average responses at time zero. For the

three triesters, the differences between the signals at different times were not statistically significant at the 95% of confidence level, proving that these compounds are stable. This observation is in agreement with previous findings reporting the non-elimination of chlorinated OPFRs during wastewater treatments, in contrast to the partial removal of some non-chlorinated OPFRs [45]. Moreover, the diesters were absent in the basic eluates from the triester stability study, proving that they are not formed from their precursor triesters in wastewater.

In the diesters stability test, only the signal of BCEP underwent a significant increase at 6 h, followed by a slight decrease at 24 h and a stabilization afterwards. Been et al. had previously assessed the stability of some OPFRs metabolites in wastewater, although BCEP was not included in their study [28, 30]. They found that BCPP and BDCPP showed a decrease of 20% in the first 30 min at room temperature, but then appeared to stabilize in partial agreement with the results observed here.

Therefore, the levels of BCEP, BCPP and BDCPP in sewage can be attributed to human metabolism, and they are neither expected to decrease due to biodegradation or adsorption processes nor expected to increase due to microbial or spontaneous hydrolysis of their precursor triesters (usually occurring in sewage [1, 46-48]).

3.5. Analysis of real samples

Composite 24 h raw wastewater samples collected on two consecutive days in March 2018 were analyzed following the validated method. Only BCPP was found in both samples at levels of 63 ng/L and 64 ng/L, what implies population-normalized mass loads of 45 µg/day inhabitant and 47 µg/day inhabitant, respectively (daily flow rates: 97817 m^3/day and 99917 m^3/day; population served by the WWTP: 136,500 inhabitants). These values contrast with the study of Been et al. [30], in which they

assessed the levels of BCPP and BDCPP, among other analytes, in wastewaters from five European cities (Antwerp, Brussels, Geneva, Athens and Vilnius). They found an average concentration of BDCPP between 21 and 52 ng/L, with BCPP being <MQL (i.e. <15.4 ng/L) in all the analyzed samples. However, no Spanish cities were included in their study, a fact that could certainly contribute to the differences observed. It must be noticed that the levels reported in the current work correspond to two single days, and a larger wastewater sampling should be performed in future studies to properly establish an exposure estimation to organophosphate triesters following the WBE principles. Figure 3 shows the EIC of the analytes and the deuterated IS in a standard of 50 ng/mL and in a sample extract (IS level: 200 ng/mL).

The use of HRMS allowed us to screen for the presence of other silylated phosphate esters through the search of the ion *m/z* 154.9924 ($C_2H_8O_4PSi$). The EIC of this ion showed, for all samples, a high peak at 11.14 min that was further identified as dibutyl phosphate (DBP) by the acquisition and analysis of its analytical standard. Besides the characteristic base peak at *m/z* 154.9924, the EI spectrum shows other two ions at *m/z* 211.0556 ($C_6H_{16}O_4PSi$) and *m/z* 267.1184 ($C_{10}H_{24}O_4PSi$) corresponding to the monobutyl and dibutyl dimethyl silyl fragments (Figure S5).

4. CONCLUSIONS

This study provides the first derivatization combined to GC-MS methodology developed for the determination of three chlorinated OPFR metabolites (BCEP, BCPP and BDCPP) in wastewater. Analyte separation and detection is conducted by GC-HRMS after derivatization with MTBSTFA, a silylation reagent that provided an excellent derivatization efficiency even in complex wastewater extracts. The determination by HRMS with a QTOF system demonstrated a good performance in

terms of sensitivity, repeatability and reproducibility, further allowing to screen for other acidic organophosphate esters through the search of the ion *m/z* 154.9924, characteristic of the EI spectra of their silylated forms. The analysis of composite raw wastewater samples of 24 h collected in the NW of Spain showed, for the first time in this matrix, the presence of BCPP at levels over 60 ng/L.

Appendix A. Supplementary material

Declarations of interest: none

Acknowledgements

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Figure Captions

Figure 1. High-resolution mass spectra (EI) of the *tert*-butyl dimethylsilyl (TBDMS) derivatives of BCEP, BCPP and BDCPP.

Figure 1

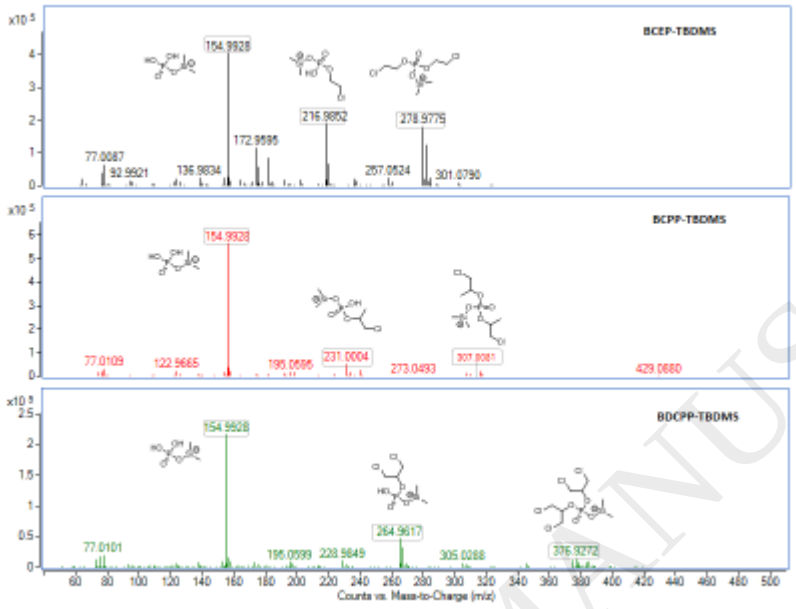

Figure 2. Stability of the investigated organophosphate triesters and diesters in wastewater at room temperature. Responses relative to their average responses at time zero.

Figure 2

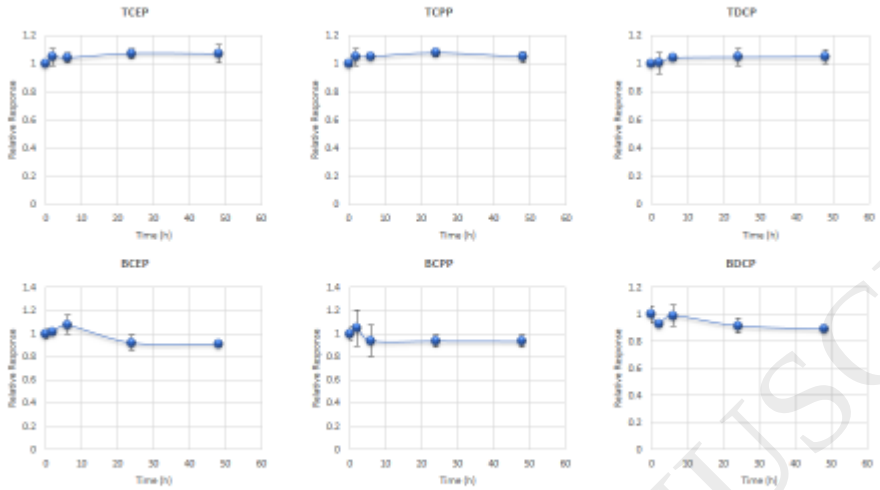

Figure 3. Extracted ion chromatogram of the analytes and the deuterated internal standards (IS) in a standard of 50 ng/mL and in a sample extract (IS level: 200 ng/mL).

Figure 3

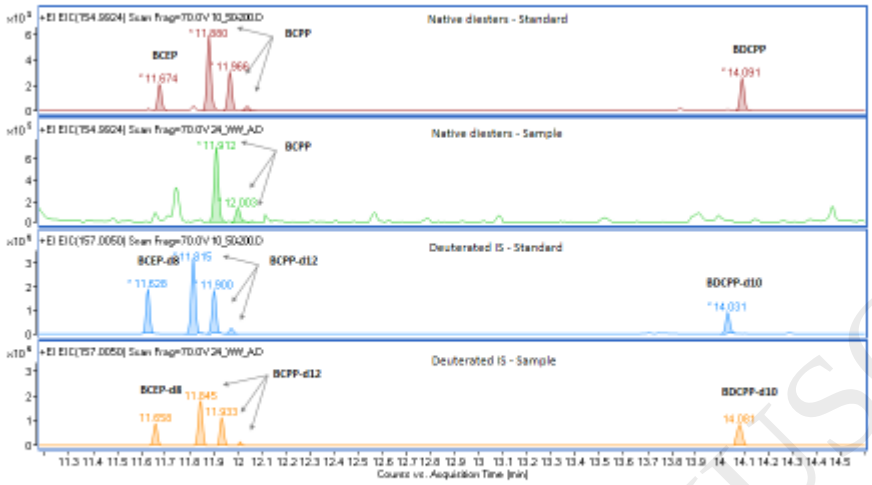

Table 1. Retention times (RT), empirical formulae, accurate *m/z* values of the ions selected as quantifier (Q) and qualifier (q) and ratio between both ions (%).

Analyte	RT	Quantifier ion (Q)		Qualifier ion (q)		q/Q ratio (%)
		Formula	*m/z*	Formula	*m/z*	
BCEP-TBDMS	11.67	$C_2H_8O_4PSi$	154.9924	$C_4H_{11}ClO_4PSi$	216.9847	47
BCPP-TBDMS	11.88/11.97/12.04[a]	$C_2H_8O_4PSi$	154.9924	$C_5H_{13}ClO_4PSi$	231.0004	11[b]
BDCPP-TBDMS	14.09	$C_2H_8O_4PSi$	154.9924	$C_5H_{12}Cl_2O_4PSi$	264.9614	19
BCEP-d8-TBDMS	11.63	$C_2H_6D_2O_4PSi$	157.0050	□	□	□
BCPP-d12 -TBDMS	11.82/11.90/11.97[a]	$C_2H_6D_2O_4PSi$	157.0050	□	□	□
BDCPP-d10-TBDMS	14.03	$C_2H_6D_2O_4PSi$	157.0050	□	□	□
TCEP	11.50	H_4O_4P	98.9842	$C_2H_5ClO_3P$	142.9659	116
TCPP	11.76/11.86/11.94[a]	H_4O_4P	98.9842	$C_2H_6O_4P$	124.9998	105[b]
TDCPP	14.02	H_4O_4P	98.9842	$C_3H_6Cl_2O_3P$	190.9426	51
TCEP-d12	11.45	D_4O_4P	103.0093	□	□	□
TCPP-d18	11.70/11.80/11.88[a]	D_4O_4P	103.0093	□	□	□
TDCPP-d15	14.96	D_4O_4P	103.0093	□	□	□

[a] RT of the three main isomers

[b] q/Q ratio calculated considering the three main isomers

Table 2. Method performance parameters: linearity, intra- and inter-day instrumental precision, instrumental quantification and detection limits (IQL and IDL), trueness, method precision and method detection and quantification limits (MDL and MQL).

Analyte	Linearity	Intra-day precision (%RSD)[b]		Inter-day precision (%RSD)[b]		IDL	IQL
	(R^2)[a]	10 ng/mL	100 ng/mL	10 ng/mL	100 ng/mL	(ng/mL)	(ng/mL)
BCEP	0.9988	6.6	2.6	8	8.5	1.1	3.6
BCPP	0.9988	4.1	0.75	4.1	5.3	0.38	1.3
BDCPP	0.9997	3.4	1.5	2.8	2.5	0.81	2.7

Analyte	Trueness and precision (%R and %RSD)[c]		MDL	MQL
	Ultrapure water (25 ng/L)	Wastewater (500 ng/L)	(ng/L)	(ng/L)
BCEP	110 (13)	100 (1)	1.1	4
BCPP	96 (3)	90 (2)	1.2	4
BDCPP	90 (5)	98 (6)	4.6	15

[a] Determination coefficient for a 10-point calibration curve. Linear range: IQL - 1000 ng/mL

[b] Relative standard deviation (%) for six injections of a standard over 24 h (intra-day precision) or four weeks (inter-day precision)

[c] Average recovery (%R) from the nominal spiking value and %RSD from the average measured concentration; experiments performed in triplicate

Supplementary Material to:

Determination of human metabolites of chlorinated phosphorous flame retardants in wastewater by N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide-derivatization and gas chromatography-high resolution mass spectrometry

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Table S1. Organophosphate diesters considered in this work and their corresponding parent triesters. N.B.: BCPP and TCPP comprise a mixture of isomers, the most abundant one only being shown.

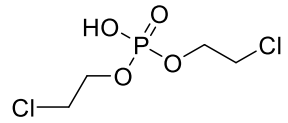
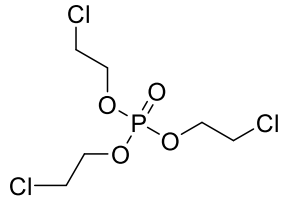
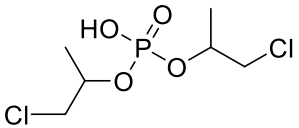
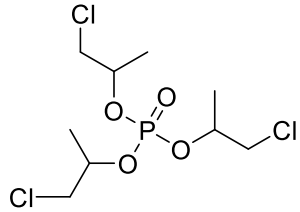
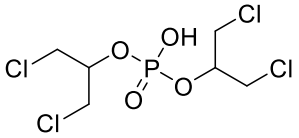
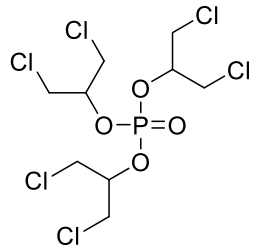
Diester	Structure	Triester	Structure
Bis(2-chloroethyl) phosphate (BCEP)		Tris(2-chloroethyl) phosphate (TCEP)	
Bis(1-chloro-2-propyl) phosphate (BCPP)		Tris(1-chloro-2-propyl) phosphate (TCPP)	
Bis(1,3-dichloro-2-propyl) phosphate (BDCPP)		Tris(1,3-dichloro-2-propyl) phosphate (TDCPP)	

Figure S1. High-resolution mass spectra (EI) of the tert-butyl dimethylsilyl derivatives of BCEP-d8, BCPP-d12 and BDCPP-d10.

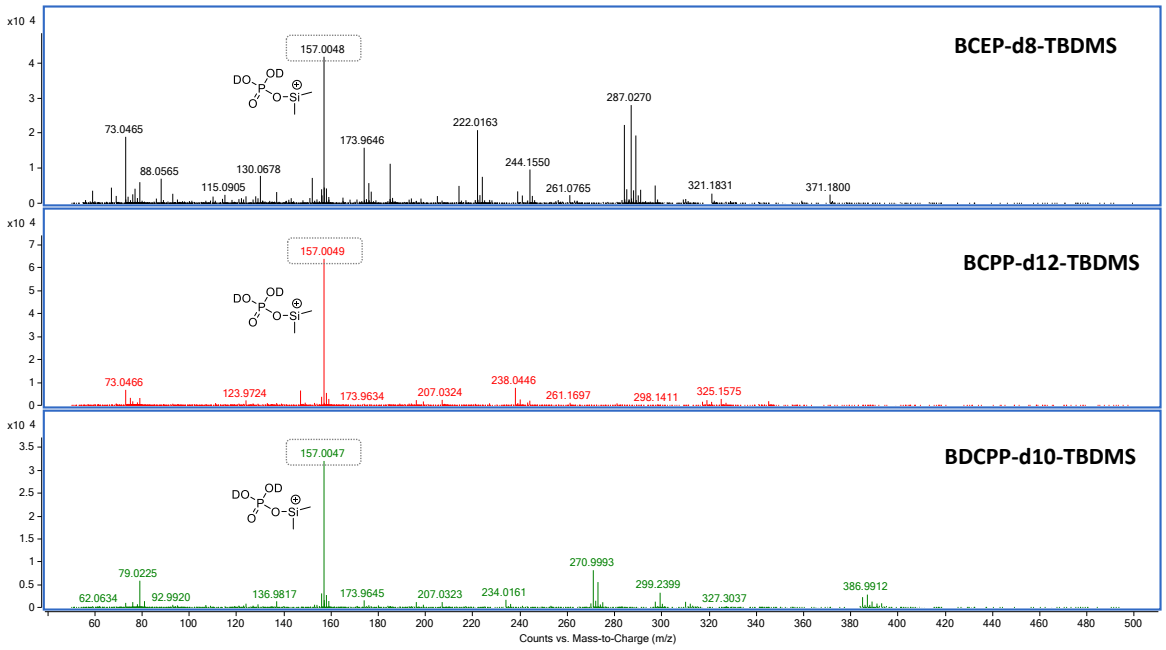


Figure S2. High-resolution mass spectra (EI) of the trimethylsilyl derivatives of BCEP, BCPP and BDCPP.

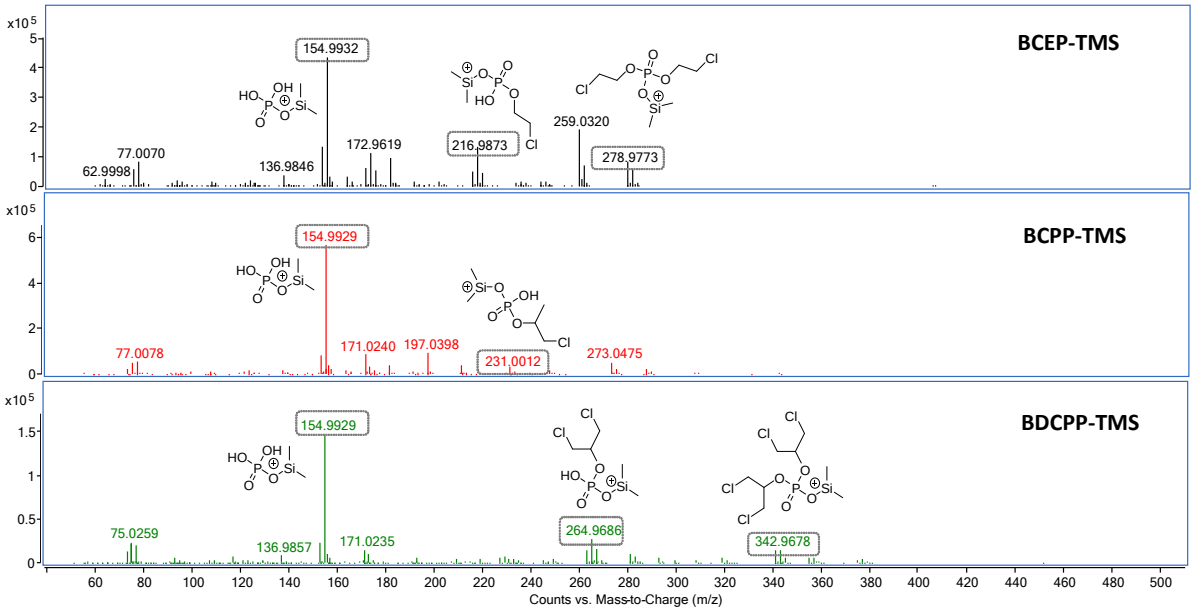


Figure S3. Comparison of the extracted ion chromatogram (EIC) of the three organophosphate diesters in a 100 ng/mL standard derivatized with 25% of MSTFA (green) and with 25% of MTBSTFA (blue). Acquired after a GC separation with the following temperature programme: initially 60 ℃, ramped at 10 ℃/min to 280 ℃ (held for 5 min); injection in splitless mode.

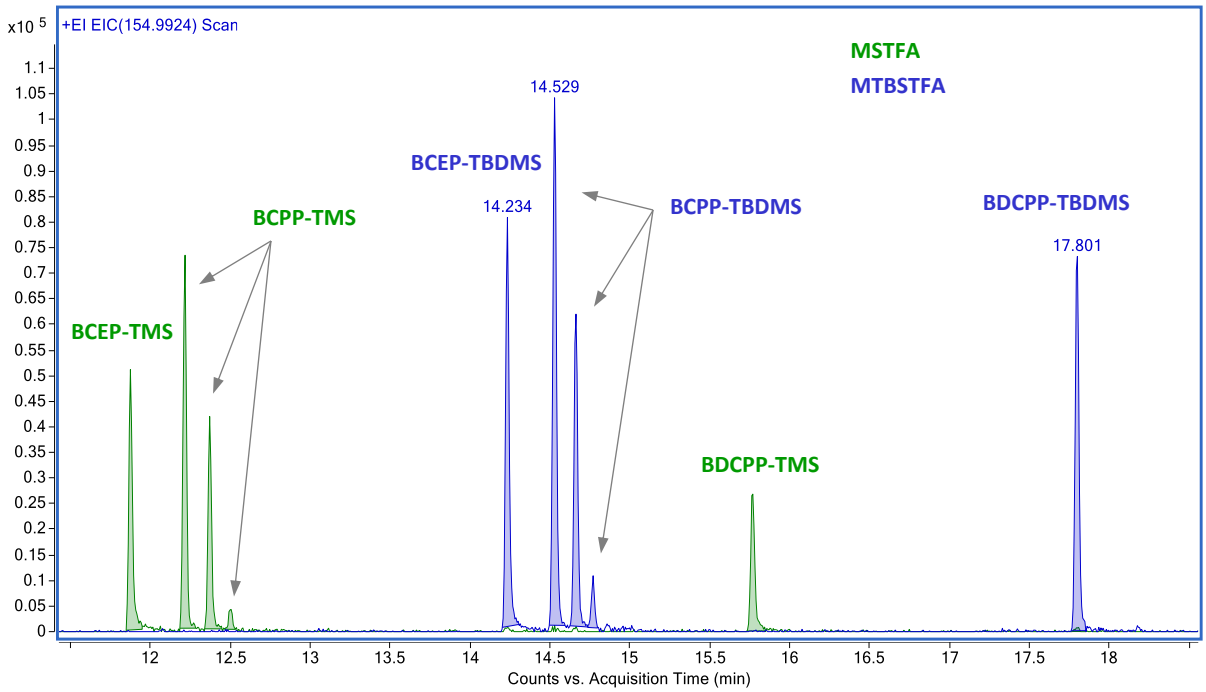


Figure S4. Height of individual peaks for the organophosphate diesters-TBDMS derivatives when injecting 2 µL in splitless mode versus 10 µL in large-volume injection mode. BCPP 1 to 3 represent the area of the three main isomers of BCPP**.**

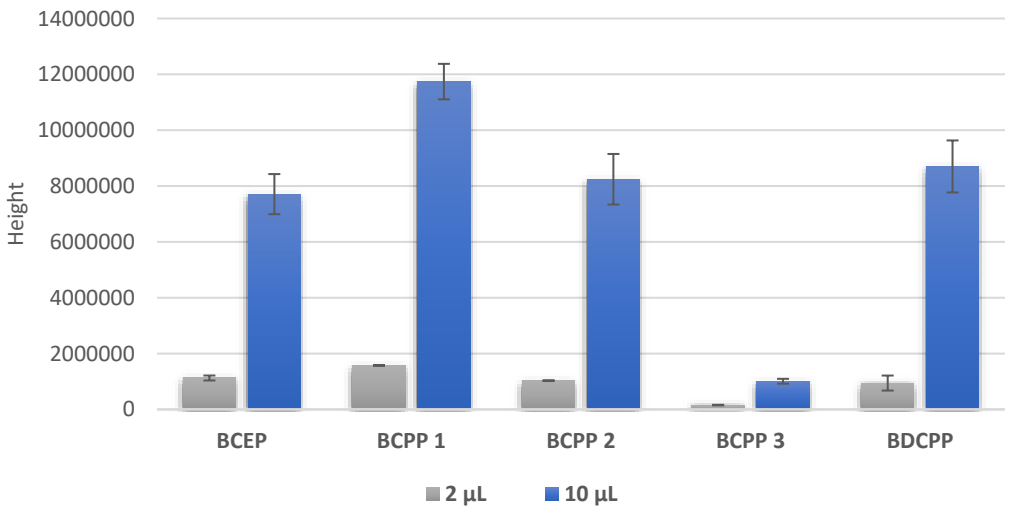


Figure S5. Extracted ion chromatogram (EIC) of *m/z* 154.9924 and EI MS spectra of the peak at 11.14 min in a standard of dibutyl phosphate-TBDMS (upper chromatogram and spectrum) and in a real sample (lower chromatogram and spectrum).