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Liquid chromatography quadrupole time-of-flight mass spectrometry quantification and screening of organophosphate compounds in sludge

R. Celano [a,b], I. Rodríguez [a,*], R. Cela [a], L. Rastrelli [b], A.L. Piccinelli [b]

[a] Departamento de Química Analítica, Nutrición y Bromatología, Instituto de Investigación y Análisis Alimentario (IIAA), Universidad de Santiago de Compostela, Santiago de Compostela 15782, Spain
[b] Dipartimento di Farmacia, Università degli Studi di Salerno, Fisciano 84084, Italy

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ABSTRACT

For the first time, we assess the performance of liquid chromatography (LC) quadrupole time-of-flight (QTOF) mass spectrometry (MS) for the selective quantification of eight organophosphate compounds (OPs), used as plasticizers and flame retardants additives, in sludge from urban sewage treatment plants (STPs). Moreover, the usefulness of accurate, full scan MS and MS/MS spectra to screen and to confirm the presence of additional OPs, without using reference standards, in sludge samples is discussed. Matrix solid-phase dispersion (MSPD) was used as a sample preparation technique. Under optimized conditions, MSPD provided quantitative recoveries for the group of targeted analytes, requiring just 15 mL of solvent and integrating extraction and clean-up processes in the same step. For these species, the achieved limits of quantification (LOQs) varied between 2 and 50 ng g^{-1} and the efficiency of electrospray ionization (ESI) did not change significantly between pure standards and sludge extracts. Among targeted OPs, tri (chloroisopropyl) phosphate (TCPP), tributoxyethyl phosphate (TBEP) and triphenyl phosphate (TPP) were ubiquitous in sludge. The average concentrations of TCPP and TBEP stayed above 700 ng g^{-1}, whereas the mean value for TPP was 67 ng g^{-1}. Full scan, accurate spectra provided relevant clues for the screening of additional OPs, using a database containing just their empirical formulae and exact molecular weights; however, the occurrence of in-source fragmentation processes hampered the detection and correct identification of those species which did not render the expected $[M+H]^+$ molecular ion, as was the case of 2-ethylhexyl-diphenyl phosphate (EHDPP).

1. Introduction

Organophosphate esters (OPs) are high production volume chemicals, mainly used as plasticizers and flame retardants additives in furniture, upholstery and building materials. Consequently, they have been reported in air and particulate matter from indoor environments [1,2]. OPs are also ubiquitous in the aquatic media, where they are introduced through urban sewage water [1]. Some OPs (e.g. tri(chloroisopropyl) phosphate, TCPP) display limited biodegradation at sewage treatment plants (STPs) and can be accumulated in sludge [3]. Assuming that around 50% of the sludge generated at STPs is disposed as a fertilizer in agriculture fields [4], evaluation of OPs discharges in the environment requires not only determining their dissolved concentrations, at the outlet stream of STPs, but also addressing their levels in sludge. This latter issue becomes particularly concerning after having reported (1) significant uptakes of polar OPs by vegetable roots and (2) their capability to migrate from roots to leaves [5]; thus, the risk of OPs introduction in the human food web through livestock animals is not negligible. Additionally, the phase out of polybrominated diphenyl ethers (PBDEs) might entail an increase in the amounts of OPs incorporated in upholstery and building materials to meet regulated flammability standards [6,7].

Most of the OPs are amenable to gas chromatography (GC) separation, with very low limits of quantification (LOQs) provided by the nitrogen-phosphorus detector (NPD); on the other hand, the sensitivity of this system largely varies depending on the state of the active element in the NPD detector, which requires frequent replacement [8]. GC–MS, using electron ionization (EI), has also some drawbacks such as (1) the excessive fragmentation of trialkyl OPs, which lead to ions with low *m/z* ratios resulting in a limited selectivity, and (2) the poor ionization of tributoxyethyl phosphate (TBEP) [8]. The problems mentioned above have been overcome with positive chemical ionization (PICI), combined with single MS [9], or tandem mass spectrometry (MS/MS) [2,10]. Another option for OPs analysis is liquid chromatography (LC) followed by MS/MS, based on triple quadrupole (QqQ) mass spectrometers. LC–MS/MS allows the determination of tri- and di-substituted OPs, attaining very low detection limits for sewage water analysis [11–13]; however, its performance has not been evaluated with sludge samples.

* Corresponding author. Tel.: +34 881814387; fax: +34 881814468.
E-mail address: isaac.rodriguez@usc.es (I. Rodríguez).

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With regard to the sample preparation process, approaches for OPs extraction from sludge should provide high extraction yields and enough selectivity to avoid interferences and signal suppression, or enhancement, in the determination step. Usually, such problems are related to variations in the efficiency of the injection process, between pure standards and extracts from complex matrices, and changes in the yield of electrospray ionization (ESI), for GC and LC–MS based methods [2], respectively. To date, the proposed sample preparation strategies for OPs determination in sludge involve a hard extraction step (using high temperatures, pressures and multiple cycles), based on non-selective pressurized liquid extraction (PLE) [14,15] or Soxhlet [16], followed by extensive clean-up of the raw extract with normal-phase sorbents plus gel permeation chromatography, ending with GC–EI-MS detection. Although effective in terms of recoveries, these approaches are time and solvent consuming.

The aim of this research was to develop a novel and advantageous analytical procedure, suitable to investigate the presence of OPs residues in sludge samples. Matrix solid-phase dispersion (MSPD) was selected as an extraction technique considering its low cost, reasonable selectivity [17,18] and previous successful applications dealing with emerging compounds extraction from sludge [19,20]. Although MSPD has been already proposed for OPs extraction from dust [21] and biota [22], its performance for the most complex sludge matrix has not been investigated, yet. OPs were determined by LC using, for the first time, a hybrid quadrupole time-of-flight (QTOF) MS system, as an alternative to QqQ instruments. The quantitative possibilities of such system for targeted OPs determination in sludge samples are discussed. Furthermore, the information contained in accurate, scan MS spectra were used to screen the presence of additional OPs, which had not been included in the quantitative method, in sludge samples. The reliability of tentative identifications derived from this post-target analysis strategy, without using reference standards, and its capability to detect residues of novel organophosphorus flame retardants in sludge, are also discussed.

2. Experimental

2.1. Standards, solvents and sorbents

Standards of TPrP (internal standard, IS), TiBP, TBP, TCEP, TDCP, TBEP, TPP and TPPO were obtained from Sigma–Aldrich (Milwaukee, WI, USA). TCPP, as technical mixture of isomers, was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Full-names of these targeted analytes are compiled in Table 1. Tri(2-ethylhexyl) phosphate (TEHP), 2-ethylhexyl-diphenyl phosphate (EHDPP) and diphenyl phosphate (DPP) standards, also purchased from Sigma–Aldrich, were used to confirm their tentative identification in sludge, derived from accurate MS and MS/MS spectra (post-target analysis). However, they were not considered during the optimization of the quantitative analytical procedure. Individual standards of each compound were prepared in methanol and stored at −20 °C. Diluted solutions and mixtures of OPs were made in acetonitrile and acetone. Calibration standards, containing increasing concentrations of the eight targeted OPs and a fixed amount of the IS (300 ng mL^{-1}), were prepared in acetonitrile:water (1:1) and used for a maximum of one week.

Formic acid, acetonitrile (HPLC gradient quality), *n*-hexane and acetone (trace analysis grade) were supplied by Merck (Darmstadt, Germany). Ultrapure water was obtained in the laboratory from a Milli-Q Gradient A-10 system (Millipore, Billerica, MA, USA).

Diatomaceous earth and the C18 sorbent were provided by Sigma–Aldrich. Silica bonded to ethylenediamine-N-propyl groups (PSA) and graphitized carbon were purchased from Supelco (Bellefonte, PA, USA). All sorbents were employed as received, without any further clean-up. Empty polypropylene syringes (15 mL capacity) and 20 μm polyethylene frits were acquired from International Sorbent Technology (Mid Glamorgan, UK).

2.2. Samples and sample preparation

Non-digested sludge samples (primary, biological and mixtures of both) were obtained from different STPs located in Galicia (Northwest Spain). After reception at the laboratory, they were maintained at −20 °C and lyophilized at the beginning of this study. Freeze-dried samples were stored, at 4 °C, in amber glass vessels. Their total carbon and nitrogen contents varied between 20–40% and 2–7%, respectively. A reference material of sludge, BCR-088, was purchased from the Institute for Reference Materials and Measurements (Geel, Belgium).

MSPD conditions were optimized with a pool of primary and biological sludges (TOC 30%) fortified with targeted OPs at the 1000 ng g^{-1} level. Spiked samples were prepared by mixing an accurately weighed amount of sludge with a standard solution of OPs in acetone. The slurry was manually blended and left in the hood for 2 days (protected from direct exposure to sun light) in order to allow acetone removal. The spiked samples were stored for 5–6 days, at 4 °C, before extraction. Recoveries, provided by the optimized MSPD method, were evaluated with individual samples of primary and biological sludges spiked at different concentration levels.

Freeze-dried sludge samples (0.5 g) were mixed and dispersed with 2 g of C18 in a glass mortar, with a pestle, for 5 min. Then, the blend was transferred to a polypropylene syringe containing 1 g of PSA as clean-up sorbent. Analytes were recovered passing 15 mL of acetonitrile through the packed syringe. After the addition of TPrP (IS), the extract was evaporated (a gentle stream of nitrogen at room temperature was used) and adjusted to a final volume of 1 mL. The concentrated acetonitrile extracts were diluted with ultrapure water (1:1) before injection in the LC–QTOF-MS system.

Table 1
LC–MS/MS determination parameters, linearity and limits of quantification (LOQs) of the LC–QTOF-MS instrument.

Compound	Abbreviation	Retention time (min)	$[M+H]^+$ ions (*m*/*z*)	Quantification ion (*m*/*z*)	Other product ions (*m*/*z*)	Collision energy (eV)	Linearity, R^2 (5–1000 ng mL^{-1})	LOQs[a] (ng mL^{-1})
Tri(2-chloroethyl) phosphate	TCEP	7.72	284.9615	124.9995	98.9843; 160.9761	12	0.998	4
Triphenyl phosphine oxide	TPPO	10.55	279.0934	201.0460	173.0510; 77.0392	27	0.993	0.5
Tri(chloroisopropyl) phosphate	TCPP	14.30	327.0086	98.9843	174.9919; 250.9997	8	0.999	3
Tri(dichloroisopropyl) phosphate	TDCP	17.93	430.8884	98.9843	208.9527; 320.9187	15	0.999	3
Tri-iso-butyl phosphate	TiBP	18.36	267.1724	98.9843	155.0446; 211.1091	10	0.994	5
Triphenyl phosphate	TPP	18.56	327.0781	215.0254	153.0690; 77.0392	30	0.995	2
Tri-n-butyl phosphate	TBP	18.58	267.1724	98.9843	155.0446; 211.1091	10	0.998	15
Tributoxyethyl phosphate	TBEP	19.84	399.2514	199.0723	98.9843; 143.0102	27	0.996	4
Tripropyl phosphate (IS)	TPrP	12.21	225.1252	98.9843	141.0310; 183.0789	12	–	–

[a] LOQs without considering the sample preparation.

2.3. Determination conditions

Compounds were determined using a LC–ESI–QTOF-MS system acquired from Agilent (Wilmington, DE, USA). The LC instrument was an Agilent 1200 Series, consisting of an autosampler, two isocratic high pressure mixing pumps, a vacuum degasser unit and a chromatographic oven. The QTOF mass spectrometer was an Agilent 6520 model, furnished with a Dual-Spray ESI source.

Compounds were separated in a Luna C18 column (100 mm × 2 mm, 3 μm) acquired from Phenomenex (Torrance, CA, USA) and connected to a C18 (4 mm × 2 mm) guard cartridge from the same supplier. Ultrapure water (A) and acetonitrile (B), both 0.1% in formic acid, were used as mobile phases applying the following gradient: 0–2 min, 35% B; 17 min, 85% B; 18–30 min, 100% B; 31–38 min, 35% B. The mobile phase flow was 0.2 mL min^{-1}, the injection volume for standards and sample extracts was 10 μL and the column temperature was set at 30 °C.

Nitrogen (99.999%), provided by a high purity generator (ErreDue srl, Livorno, Italy), was used as a nebulizing (35 psi) and drying gas (330 °C, 10 L min^{-1}) in the ESI source. The QTOF instrument worked in the 2 GHz Extended Dynamic Range resolution mode (mass resolution 5000 at *m/z* values of 120) and compounds were ionized in positive ESI, applying a capillary voltage of 3500 V. A mass reference solution (Agilent calibration solution A) was continuously infused in the source of the QTOF system, through the second nebulizer, employing the ions with *m/z* 121.0509 (purine) and 922.0098 (HP-921) for recalibrating the mass axis. The Mass Hunter Workstation software was used to control the LC–ESI–QTOF-MS system and to process the obtained data.

Precursor ($[M+H]^+$) ions for targeted compounds were obtained using a fragmentor voltage of 130 V. Collision energies were optimized with the aim of generating several products from each precursor. Accurate product ion scan (MS/MS) spectra were acquired in the range of *m/z* values from 70 to 500 units, considering a time window of 3 min centered in the retention time of each analyte. Full scan MS spectra (*m/z* range 100–1700 units) were simultaneously acquired to the MS/MS ones. Acquisition rates in MS and MS/MS modes were set at 1.4 spectra s^{-1}, with each spectrum being the combination of 9600 transients. Selective LC–MS and LC–MS/MS chromatograms were extracted with a mass window of 20 ppm around the $[M+H]^+$ and the most intense product ion of each OPs, respectively. The MS/MS mode was employed for quantification purposes, whereas LC–MS chromatograms were used, in the post-target analysis strategy, to screen the presence of nine additional OPs in real-life sludge samples.

2.4. Matrix effects, MSPD extraction efficiency and samples quantification

Potential matrix effects (ME) occurring in the ESI source were calculated as follows: $ME=[(A_{se}-A_{be})/A_s]\times 100$, where A_{se} is the response (peak area without IS correction) measured for a targeted compound in the spiked extract from sludge, A_{be} is the response for the same compound in an un-spiked extract of the same sludge, and finally, A_s is the response for a standard solution containing the same concentration of the analyte [23]. Thus, ME values around 100% point out to little differences between the efficiency of ESI ionization for sludge extracts and standard solutions.

The yield of the MSPD extraction was calculated as the ratio between the corrected responses (analyte peak area/IS peak area) measured for spiked sludge samples and extracts from the same matrix fortified after the extraction step, multiplied by a factor of 100.

The overall recoveries (R) of the procedure were defined as follows: $R=[(C_s-C_b)/C_t]\times 100$. Being C_s the concentration measured in the extract from a spiked sample, C_b is the concentration in the extract from a non-spiked fraction of the same sludge and C_t is the concentration added to the sample. C_s and C_b were determined using calibration curves obtained for standard solutions prepared in acetonitrile:water (1:1). As discussed further, the MSPD procedure provided overall recoveries above 70% for the eight targeted analytes; therefore, their levels in sludge were calculated by comparison with calibration solutions containing increasing concentrations of these OPs (5–1000 ng mL^{-1}) and TPrP (300 ng mL^{-1}) as IS.

3. Results and discussion

3.1. LC–QTOF-MS determination parameters

LC conditions were optimized to achieve the best possible resolution between the two isomeric tributyl phosphates (TiBP and TBP). In addition to the mobile phase gradient, the peak shapes of OPs and the resolution between TiBP and TBP were affected by the injection solvent. Fronting peaks for the earlier eluting compounds (TCEP and TPPO) and co-elution of tributyl phosphates were observed for standards in acetonitrile; thus, an acetonitrile:water (1:1) mixture was selected as injection solution.

ESI and MS/MS parameters were evaluated in order to (1) maximize the responses for the $[M+H]^+$ ion of each OP and (2) obtain, at least, two intense product ions in their MS/MS spectra. Optimal LC–QTOF-MS determination conditions are compiled in Table 1. The LC–MS extracted chromatograms for a standard solution (50 ng mL^{-1}) of targeted OPs are provided as supplementary information (Fig. S1). The chromatographic trace for tributyl phosphates (*m/z* 267.1724) displayed a significant baseline disturbance at the retention time of TBP, which was also noticed in the LC–MS/MS mode (figure not shown). Such a disturbance was observed even for simulated injections of empty vessels (Fig. S1), and it was noticed with different LC columns. Replacement of ACN by methanol and formic acid by ammonium acetate as organic mobile phase and modifier; respectively, did not overcome the problem. Thus, the origin of TBP contamination was attributed to ultrapure water, as previously reported [24]. However, the intensity of the TBP contamination remained mostly unchanged after passing the mobile aqueous phase through a C18 solid-phase extraction (SPE) membrane and varied only slightly among different ultrapure water samples. Thus, leaching of TBP from plastic components (e.g. pipes between phase reservoirs and pumps) in the LC system cannot be excluded. Although identification of the TBP contamination source requires a deeper study, a small column, placed before the injection valve, might serve to retain TBP coming from LC pipes and/or pumps.

The instrumental LOQs of the LC–QTOF-MS system, operated in the MS/MS mode, were established as the concentration of each compound providing a peak area 10 times higher than the standard deviation of the chromatographic baseline for an injection blank. They varied between 0.5 and 4 ng mL^{-1}, except in the case of TBP (LOQ 15 ng mL^{-1}) (Table 1). These LOQs are 5–10 times higher than those reported by our group for same compounds using a triple quadrupole LC–MS/MS system [12]. On the other hand, they remained below LOQs reported for GC–EI-MS (20–50 ng mL^{-1}) and GC–PICI-MS/MS (4–200 ng mL^{-1}) [10].

Linearity of LC–QTOF-MS responses was evaluated in the range of concentrations from 5 ng mL^{-1} (20 ng mL^{-1} for TBP) to 1000 ng mL^{-1}, using TPrP as a IS (300 ng mL^{-1}). Determination coefficients (R^2) of the obtained graphs stayed above 0.994 (Table 1).

3.2. Optimization of MSPD conditions

Starting MSPD conditions were adopted from previous studies dealing with emerging pollutants extraction from sludge [20,25].

In brief, lyophilized samples (0.5 g) were dispersed with 2 g of diatomaceous earth and loaded into a polypropylene syringe containing PSA (1 g) followed by graphitized carbon (0.25 g), as clean-up sorbents. Acetonitrile and acetone (25 mL) were selected as elution solvents on the basis of their affinity for OPs [21]. Extracts in acetonitrile were concentrated to 1 mL and diluted with ultrapure water (1:1) before LC–QTOF-MS analysis. Those in acetone were evaporated to dryness and reconstituted with 2 mL of acetonitrile:water (1:1). Under above conditions, TPP could not be recovered from the spiked pooled sludge matrix.

Removal of the carbon clean-up layer overcame the above problem, at the expense of increasing the visual complexity (color) of the extracts. Thus, the potential benefit of introducing a washing step in the extraction protocol was evaluated [22,26]. To this end, MSPD cartridges were first rinsed with 10 mL of n-hexane, and then analytes eluted using either acetone or acetonitrile. Rinsing and elution fractions were injected in the LC–QTOF-MS system after solvent exchange when required. Although OPs were not eluted in the rising fraction, this extra step exerted a minor improvement in the selectivity of the extraction; furthermore, exhaustive drying of the MSPD syringe was required after n-hexane rinsing and before acetonitrile elution due to the immiscibility of both solvents. Therefore, neither the washing step nor the carbon clean-up layer was included in the MSPD extraction protocol. Comparison between the relative extraction efficiencies of acetone and acetonitrile revealed similar responses for most targeted OPs (Fig. S2); however, acetonitrile extracts displayed a less intense color than those in acetone. Taking this observation into account, and considering also that dryness evaporation was not required for acetonitrile extracts, this solvent was selected to continue with optimization of extraction conditions.

The effect of the dispersant in the performance of the extraction was assessed in terms of efficiency and selectivity. Diatomaceous earth, used in the initial extractions and regarded as an inert material allowing to mechanically disrupt the sample and to increase the surface of sludge in contact with the elution solvent, was compared to C18, the original dispersant reported by Barker et al. [27] for MSPD. The alkyl chains in the C18 sorbent are supposed to solubilize and to retain certain components of the sludge matrix, improving the efficiency and the selectivity of the process [27]. As shown in Fig. 1, equivalent extraction efficiencies, ranging from 89% to 105%, were obtained in both cases. ME, calculated as defined in Section 2.4, varied from 82% to 126% for samples dispersed with diatomaceous earth; whereas, they stayed between 84% and 108% for C18 (Table 2). Also, completely transparent extracts were obtained with C18, whereas, those from samples dispersed with diatomaceous earth displayed a pale yellowish appearance and a slight turbidity after water dilution, requiring a filtration step before injection in the LC–QTOF-MS system. Likely, in case of C18 dispersion some lipophilic components of sludge remained, within the MSPD syringe, trapped due to interactions with C18 chains, which resulted in cleaner extracts and lower matrix effects during ESI ionization process. Thus, C18 was used as a dispersant in further extractions.

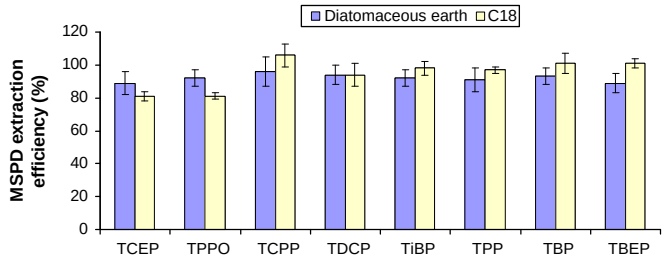

Fig. 1. Efficiency of MSPD extraction as function of the dispersant sorbent, $n=4$ replicates.

Table 2
Evaluation of matrix effects (ME, %) as function of the dispersant sorbent, $n=6$ replicates.

Compound	ME (%) ± SD	
	Diatomaceous earth	C18
TCEP	100 ± 2	104 ± 6
TPPO	110 ± 2	103 ± 6
TCPP	114 ± 9	92 ± 3
TDCP	96 ± 7	91 ± 4
TiBP	126 ± 5	93 ± 2
TPP	91 ± 5	84 ± 3
TBP	82 ± 10	99 ± 2
TBEP	126 ± 3	108 ± 2

Table 3
Overall recoveries of the developed method ($n=4$ replicates) and limits of quantification (LOQs, ng g^{-1}) of the analytical procedure referred to lyophilized sludge.

Compound (ng g^{-1})	Recoveries (%) ± SD				LOQs
	Primary sludge		Biological sludge		
	1000 ng g^{-1} [a]	300 ng g^{-1} [a]	1000 ng g^{-1} [a]	100 ng g^{-1} [a]	
TCEP	86 ± 3	101 ± 6	92 ± 3	95 ± 9	16
TPPO	94 ± 2	85 ± 4	95 ± 3	88 ± 2	2
TCPP	87 ± 7	93 ± 12	87 ± 7	123 ± 5	12
TDCP	70 ± 5	113 ± 7	82 ± 1	102 ± 6	12
TiBP	96 ± 4	111 ± 3	89 ± 1	85 ± 4	18
TPP	76 ± 2	81 ± 3	84 ± 2	69 ± 3	6
TBP	96 ± 5	83 ± 1	100 ± 2	96 ± 3	50
TBEP	109 ± 5	117 ± 13	110 ± 7	n.e.[b]	18

[a] Added concentration.
[b] Not evaluated.

The minimum volume of acetonitrile required for the quantitative extraction of targeted compounds was established by collecting consecutive fractions (5 mL each) from the MSPD cartridge. Above 75% of the responses measured for all targeted compounds corresponded to the 1st fraction; however, some compounds were still noticed in the 3rd fraction, data not given. Thus, 15 mL was adopted as the working acetonitrile extraction volume.

3.3. Performance of the method

The overall recoveries of the optimized procedure were evaluated with primary and biological sludge samples. Providing that (1) MSPD achieved quantitative extraction yields (Fig. 1) and (2) the efficiency of ESI ionization underwent small variations between pure standards and sample extracts (Table 2), absolute recoveries were assessed against standard solutions, prepared in acetonitrile:water (1:1). For each sludge sample, un-spiked ($n=3$) and spiked ($n=4$) fractions, at two different concentration levels, were processed. The attained overall recoveries are compiled in Table 3. In the case of primary sludge, they varied from 70%, for TDCP, to 117%, for TBEP, with standard deviations remaining below 13%. For biological sludge, recoveries ranged from 69%, for TPP, to 123%, for TCPP, with standard deviations below 9%. For this latter matrix, the recovery for TBEP at the lower addition level (100 ng g^{-1}) could not be evaluated since its native concentration in the matrix (around 1800 ng g^{-1}) was significantly higher than the added level (Fig. 2). Recoveries compiled in Table 3 are better than those reported by Chen and Bester [15] (from 57% to 96%) for same compounds, considering PLE extraction followed by a multistep clean-up approach, requiring around 200 mL of different organic solvents per sample, versus 15 mL used in this research.

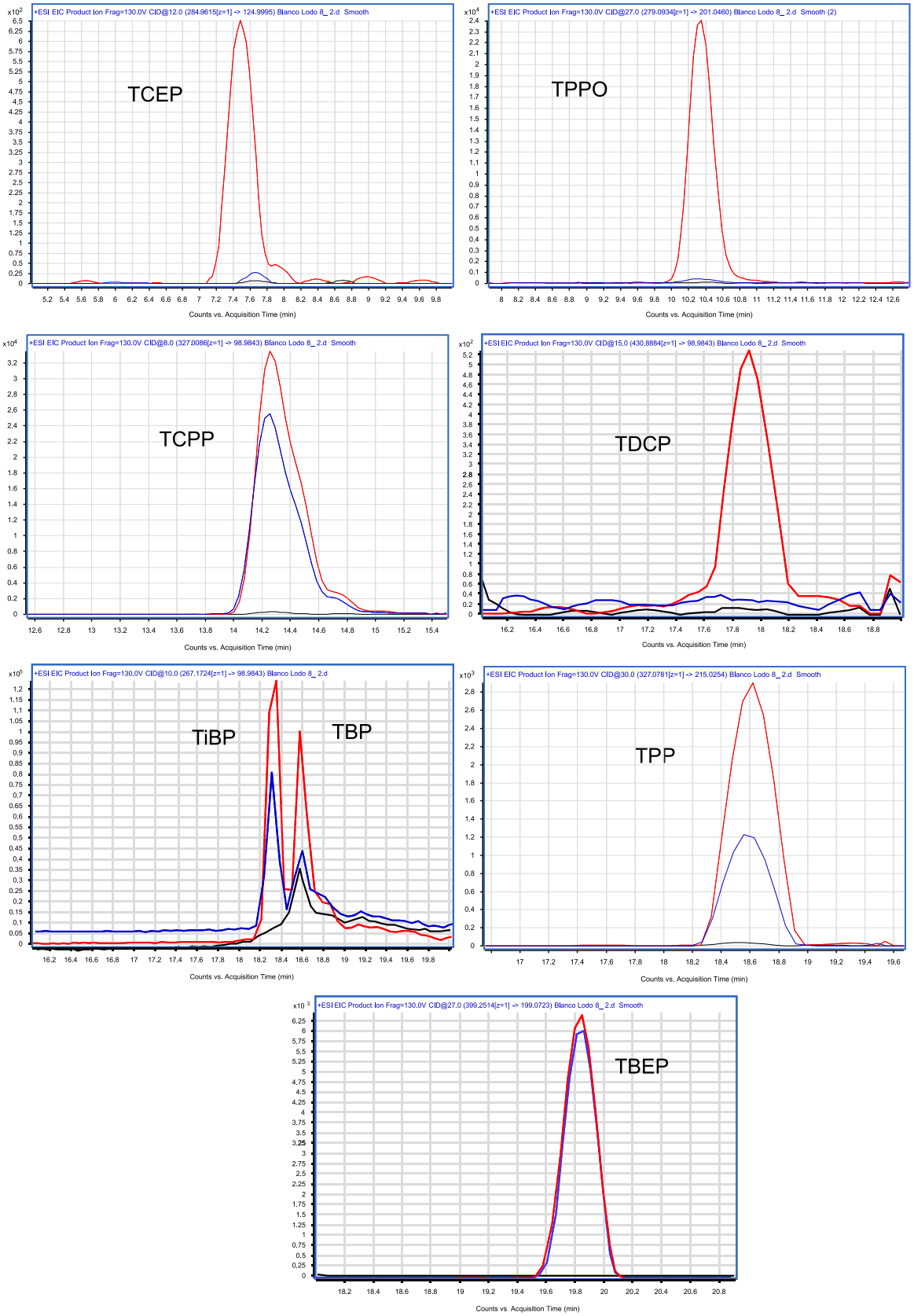

Fig. 2. LC–MS/MS chromatograms for a procedural blank (black), a non-spiked sludge sample (code 2, Table 4, blue) and same matrix fortified with target OPs at 100 ng g^{-1} (red). (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.)

The limits of quantification (LOQs) of the overall method varied from 2 ng g^{-1}, for TPPO, up to 50 ng g^{-1}, for TBP (Table 3). Procedural blanks did not contain traces of OPs, with the exception of the already commented contamination problem for TBP (Fig. 2) which cannot be attributed to the sample preparation process. Thus, for the rest of OPs, the attained LOQs were controlled by

sensitivity of the LC–QTOF-MS instrument, the sample intake and the final extract volume. In the previous studies, the achieved LOQs varied from 10 ng g^{-1}, for TPP, to 100 ng g^{-1}, for TCPP, using PLE followed by GC–MS [15]. Following a very similar methodology, Marklund et al. [14] calculated LOQs in the range of values from 0.5 to 15 ng g^{-1}; nevertheless, they highlighted the presence of TBP and TiBP at the 20 ng g^{-1} level in procedural blanks.

3.4. Real samples quantification

Table 4 summarizes the concentrations of targeted OPs in 11 freeze-dried sludges and a reference material of the same matrix (BCR-088). With regards to sludge obtained from local STPs (codes 1-11), TCPP, TBEP and TPP were quantified in all samples, with maximum levels above 1000 ng g^{-1} for the first two congeners. In the case of TPP, the measured concentrations remained below 150 ng g^{-1}. Their arithmetic mean concentrations (sample codes 1-11) were 758 ± 379 ng g^{-1} (TCPP), 744 ± 437 ng g^{-1} (TBEP) and 67 ± 30 ng g^{-1} (TPP), which are similar to the levels reported in sludge samples from Sweden (n=11 STPs) [14]. On the other hand, the mean concentration of TCPP is lower than 5000 ng g^{-1}, reported as the average value of this flame retardant in sludge from several (n=20) German STPs [16]. The BCR-088 sludge material contained similar levels of most OPs to the rest of samples compiled in Table 4. The exception was TCEP, which was present at higher level in BCR-088.

The concentrations of TCPP, TBEP and TPP in samples from years 2005 to 2010 (codes 1-6) were similar to levels measured in sludges collected in 2013 (codes 7-11). However, TDCP and TiBP were more frequently detected in the latter group of samples (Table 4). This trend, which requires additional confirmation, might be a consequence of the phase out of other flame retardants, such as PBDEs, and agrees with the proposed increase in OPs consumption [7,28].

3.5. Post-target screening of additional OPs

In addition to the product ion (MS/MS) spectra of preselected targeted OPs, the LC–QTOF-MS instrument acquires and records full scan, accurate MS spectra throughout LC chromatograms. These spectra allow searching for additional pollutants, not included in the quantitative procedure, providing that they are co-extracted from sludge together with target analytes. Hence, this latent information can be useful to detect the use and potential accumulation of novel OPs in the previously processed sludge samples. Tentative identifications derived from this post-target strategy require additional confirmation, using product ion scan MS/MS spectra (which are obtained in a 2nd injection, preferably considering different collision energies), and/or retention time comparison with pure standards, when available [29].

In order to assess the reliability of this strategy, a database (Table 5) with the empirical formulae and the exact molecular weights of nine OPs, non-included in the quantitative method but previously reported in environmental samples [2,6,28], was built. It is highlighted that reference standards of these compounds were not injected in the LC–QTOF-MS system. The Mass Hunter software was used to search for their $[M+H]^+$ ions (automated search of sodium and ammonium adducts is also possible) in the LC–MS chromatograms of samples compiled in Table 4, within a mass interval of 10 ppm around their theoretical values. This software extracts the accurate LC–MS chromatograms and compares the experimental MS spectra of detected peaks with the theoretical

Table 4
Concentrations (ng g^{-1}) of targeted OPs in freeze-dried sludge samples, n=3 replicates.

Code	Type	Sampling year	Concentration (ng g^{-1}) ± SD							
			TCEP	TPPO	TCPP	TDCP	TiBP	TPP	TBP	TBEP
1	P.S.	2005	n.d.	n.d.	1184 ± 83	n.d.	n.d.	54 ± 1	n.d.	909 ± 45
2	B.S.	2005	n.d.	n.d.	396 ± 36	n.d.	137 ± 3	83 ± 2	n.d.	1786 ± 36
3	P.S.	2010	n.d.	3 ± 1	700 ± 200	32 ± 6	n.d.	66 ± 13	n.d.	810 ± 110
4	B.S.	2010	n.d.	n.d.	780 ± 180	n.d.	n.d.	52 ± 3	n.d.	527 ± 16
5	B.S.	2010	n.d.	n.d.	583 ± 35	n.d.	n.d.	54 ± 5	n.d.	213 ± 19
6	B.S.	2010	n.d.	n.d.	270 ± 35	n.d.	n.d.	47 ± 2	n.d.	516 ± 19
7	M.S.	2013	22 ± 1	2.0 ± 0.1	381 ± 14	25 ± 3	115 ± 14	58 ± 1	n.d.	1200 ± 250
8	M.S.	2013	n.d.	2.1 ± 0.1	919 ± 38	13 ± 1	58 ± 2	38 ± 3	n.d.	736 ± 45
9	M.S.	2013	n.d.	n.d.	888 ± 77	n.d.	49 ± 6	86 ± 10	n.d.	391 ± 16
10	M.S.	2013	n.d.	n.d.	670 ± 80	n.d.	41 ± 36	50 ± 6	n.d.	562 ± 6
11	M.S.	2013	n.d.	2.2 ± 0.2	1570 ± 80	40 ± 6	55 ± 6	144 ± 6	n.d.	532 ± 32
12	BCR-088		1650 ± 150	6 ± 1	517 ± 15	n.d.	48 ± 2	117 ± 26	124 ± 3	800 ± 48

n.d., not detected; P.S., primary sludge; B.S., biological sludge; M.S., mixture of primary and biological sludge.

Table 5
Database of OPs investigated in sludge using a post-target screening strategy.

Name	Abbreviation	CAS number	Formula	Mass[a]
Tri(4-butylphenyl) phosphate	TTBPP	78-33-1	$C_{30}H_{39}O_4P$	494.2586
Tri(4-methylphenyl) phosphate	TMPP	78-32-0	$C_{21}H_{21}O_4P$	368.1177
Tri(2,3-dibromopropyl) phosphate	TDBPP	126-72-7	$C_9H_{15}Br_6O_4P$	691.5808
2-ethylhexyl-diphenyl phosphate	EHDPP	856800-52-7	$C_{20}H_{27}0_4P$	362.1647
Tripentyl phosphate	TPP	2528-38-3	$C_{15}H_{33}O_4P$	308.2116
Trihexyl phosphate	THP	2528-39-4	$C_{18}H_{39}O_4P$	350.2586
Diethylhexyl phosphate	DEHP	298-07-7	$C_{16}H_{35}O_4P$	322.2273
Diphenyl phosphate	DPP	838-85-7	$C_{12}H_{11}O_4P$	250.0395
Tri(2-ethylhexyl) phosphate	TEHP	78-42-2	$C_{24}H_{51}O_4P$	434.3525

[a] Monoisotopic molecular weights.

(calculated) ones. Then, a normalized score (0–100), which combines mass accuracy, isotopic pattern and spacing among ions in the $[M+H]^+$ cluster, is calculated. A score of 100 represents a perfect match between the empirical and the theoretical spectrum.

LC–MS chromatograms for all samples compiled in Table 4 contained a well-defined peak at m/z 435.3598 Da (retention time 31.61 min), and half of them showed also a signal at m/z 251.0468 Da (retention time 23.18 min). The MS spectra of both peaks fitted (calculated scores above 95%) with the theoretical ones of TEHP and DPP, respectively. Fig. 3 shows the extracted ion LC–MS chromatograms and the experimental MS spectra (average peak spectrum after background correction) for both peaks in non-spiked sludge samples. The superposed boxes represent the calculated spectra of TEHP and DPP (Fig. 3). Differences between calculated and experimental masses of the most intense ion in MS spectra remained below 1 ppm in both cases.

The identity of TEHP was confirmed from its experimental MS/MS spectrum, and by injection of a pure standard of this compound (Fig. S3). The MS/MS spectrum of the peak at 23.18 min was also coherent with the structure of DPP; however, its retention time did not agree with the relatively high polarity, and thus, poor retention expected for DPP in C18 LC columns [12]. In fact, the retention time for a pure standard of DPP, under conditions reported in Section 2.3, turned to be 4.2 min. The 2nd possibility was that the peak at 23.18 corresponds to EHDPP, assuming that during ESI ionization the 2-ethylhexyl moiety (C_8H_{16}) is replaced by one atom of hydrogen. In such a case, the MS spectrum of EHDPP will render the $[M+H-C_8H_{16}]^+$ ion ($C_{12}H_{12}O_4P$, 251.0468 Da), instead of the $[M+H]^+$ one ($C_{20}H_{28}O_4P$, 363.1720 Da). This second hypothesis was confirmed with the MS/MS spectrum and the retention time for a standard solution of EHDPP (Fig. 4).

Although the performance of the MSPD procedure was not validated for TEHP and EHDPP, a semi-quantitative evaluation of their levels in sludge was performed assuming that, as occurred for the targeted OPs, quantitative recoveries are attained for both compounds and that their ESI ionization efficiencies were similar between standards and sludge extracts. Under these considerations, TEHP levels ranged between 20 and 100 ng g^{-1}, whereas EHDPP varied from not detected up to 30 ng g^{-1}.

4. Conclusions

LC–QTOF-MS, in the MS/MS mode, provides instrumental LOQs low enough to allow the quantification of eight OPs in sludge from urban STPs, with accurate product ion spectra permitting the unambiguous identification of these targeted OPs. When LC–QTOF-MS detection is combined with the mild extraction conditions employed in the optimized MSPD method, quantitative recoveries and limited ESI matrix effects were observed. Consequently, targeted analytes could be quantified by comparison against pure standard solutions. Another significant advantage of the described procedure is the reduction in the consumption of organic solvents versus previously published methodologies.

Accurate full scan MS spectra, provided by the LC–QTOF-MS instrument, render valuable clues to investigate the presence of additional OPs, not considered during method development, in real samples. However, preliminary identifications derived from this post-target screening approach require additional confirmation using authentic standards, since some OPs might undergo in-source fragmentation and then, their molecular ions are not obtained.

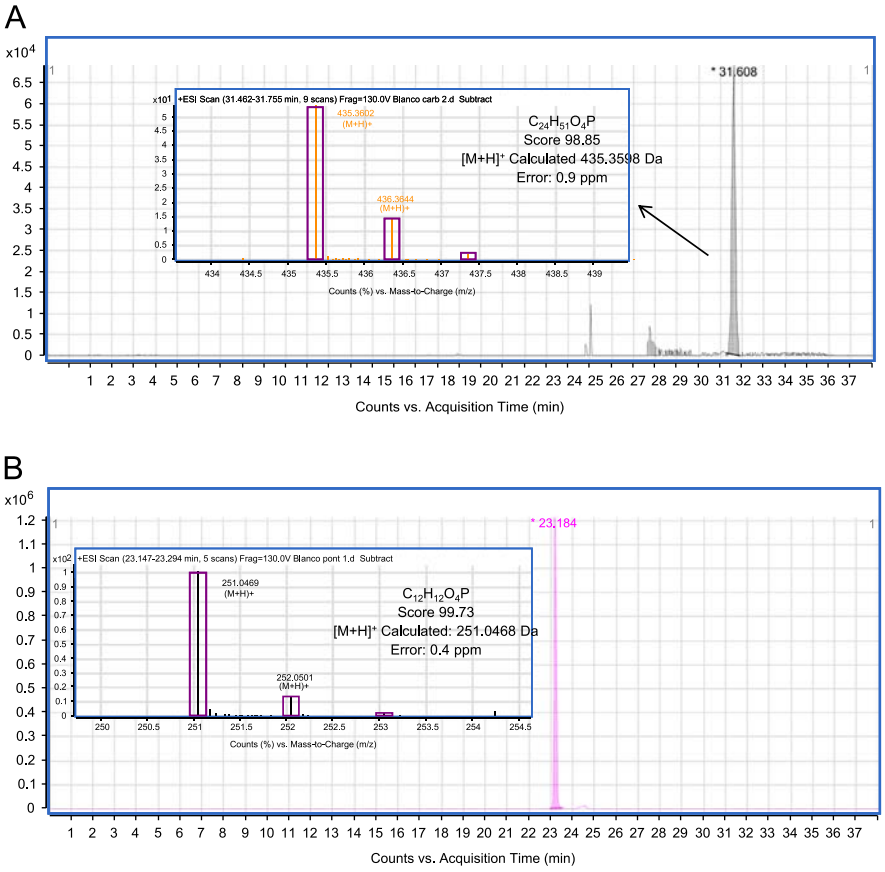

Fig. 3. Extracted LC–MS chromatograms and detail of empirical MS spectra for compounds tentatively identified as TEHP (A) and DPP (B) in non-spiked sludge samples.

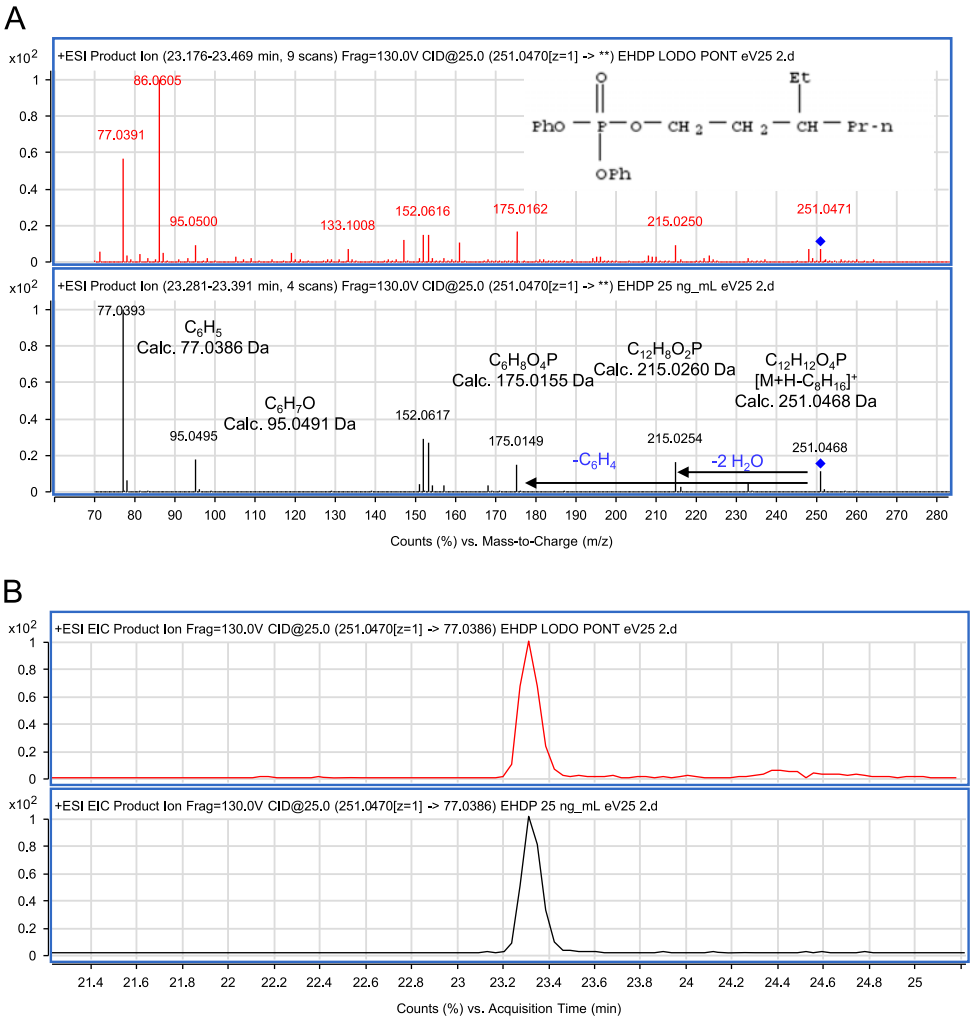

Fig. 4. MS/MS spectra (A) and LC–MS/MS chromatograms (B) for a sludge sample (red) and a standard of EHDPP (black). (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.)

Analysis of un-spiked sludge samples confirmed the accumulation of significant levels of TCPP, TBEP and TPP in this matrix. Moreover, TEHP was also ubiquitous in sludge.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.10.024.

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