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Determination of Phosphoric Acid Mono- and Diesters in Municipal Wastewater by Solid-Phase Extraction and Ion-Pair Liquid Chromatography−Tandem Mass Spectrometry

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The first analytical method for the determination of 13 phosphoric acid mono- and diesters from aqueous samples is presented. The method consists of solid-phase extraction (SPE) and ion-pair liquid chromatographic separation with tri-*n*-butylamine coupled to electrospray ionization tandem mass spectrometry in the negative ion mode. Due to a lack of pure standards, only 3 of the 13 esters could be quantified. SPE recoveries ranged from 71 to 112% for di-*n*-butyl phosphate, diphenyl phosphate, and di-(2-ethylhexyl) phosphate (DEHP) with limits of quantification from 7 to 14 ng/L for 100-mL samples. At analyte concentrations ≥1 μg/L, aqueous samples can be analyzed by direct injection without extraction. In municipal wastewater, six diesters and one monoester were unambiguously identified by comparison with synthesized reference material. DEHP showed highest concentrations of 60 and 5 μg/L in raw and treated wastewater, respectively. The detection of monoethylhexyl phosphate was confirmed by LC−Q-TOF-MS analysis, and it was found at a concentration level comparable to DEHP. Laboratory degradation tests show that phosphoric acid diesters can be formed as intermediates in the microbial degradation of trialkyl phosphates that are being used as flame retardants and plasticizers.

Phosphoric acid triesters that are used as organophosphorus flame retardants (OPFR) and plasticizers are a class of chemicals with a high consumption volume (∼190 000 tons/year worldwide)[1] and have been determined in many environmental samples.[2−5] Recently, a first method was developed for the determination of 11 OPFR, phosphoric acid triesters, bisphosphates, and triphenylphosphine oxide from aqueous samples using LC−MS/MS.[6]

In addition to phosphoric acid triesters, some phosphoric acid diesters, such as di-*n*-butyl phosphate (DnBP) and di-(2-ethylhexyl) phosphate (DEHP), and also monoesters, such as mono-(2-ethylhexyl) phosphate (MEHP), are industrially produced and used, among other applications, for metal extraction, as plasticizers, and in the textile industry.[7,8] The estimated annual consumption of these chemicals in 1992 was of ∼1000 tons of DEHP and MEHP (worldwide)[7] and 200−350 tons of DnBP (in Western Europe).[8] In addition to being intentionally produced, phosphoric acid diesters may also originate from hydrolysis of triesters, as shown for tri-*n*-butyl phosphate (TnBP) upon irradiation in the nuclear industry[9,10] and as assumed for the microbial degradation of triphenyl phosphate.[11]

For these phosphoric acid diesters, no analytical method is available that would allow their determination from wastewater and other aqueous samples. Their detection from water is hampered by the high polarity and acidity of the free phosphoric acid moiety (p*K*a = 1−2[7,8]). The latest analytical method for the determination of diesters from water dates back to the 1980s and uses solid-phase extraction on XAD resins and GC analysis after derivatization with diazobutane for phosphoric acid diphenyl and dicresyl ester only.[11] Only recently has the suitability of LC−MS for the determination of diphenyl phosphate (DPhP) from biological fluids been shown.[12]

Here, the first LC−MS/MS method for the determination of a set of 13 analytes, 7 phosphoric acid diesters and 6 phosphoric acid monoesters, from municipal wastewater samples is presented. Due to the high polarity and acidity of the analytes, ion-pairing with tri-*n*-butylamine (TrBA) is employed for extraction and

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Table 1. LC−ESI-MS/MS Parameters for the Determination of Phosphoric Acid Mono- and Diesters by MRM in the Negative Ion Mode

phosphate	acronym	t_R (min)	molec. anion (CV),[a] m/z (V)	product ions (CE), m/z (eV)	proposed formula	Tr1/Tr2[b]
monochloropropyl	MCPP	4.4	173/40	137/8	$[M - H - H^{35}Cl]^-$	3.8
			175/40	137/8	$[M - H - H^{37}Cl]^-$	
mono-isobutyl, mono-*n*-butyl	MiBP MnBP	4.7	153/50	79/14	$[PO_3]^-$	
monophenyl	MPhP	5.1	173/20	79/12	$[PO_3]^-$	
monobutoxyethyl	MBEP	7.4	197/50	79/16	$[PO_3]^-$	
dichloroethyl	DCEP	7.9	221/25	35/8	$[^{35}Cl]^-$	2.4
			223/25	37/8	$[^{37}Cl]^-$	
dichloropropyl	DCPP	11.4	249/30	35/8	$[^{35}Cl]^-$	2.2
			251/30	37/8	$[^{37}Cl]^-$	
di-isobutyl	DiBP	13.3	209/25	79/22	$[PO_3]^-$	3.2
				153/14	$[M - H - C_4H_8]^-$	
di-*n*-butyl	DnBP	14.0	209/25	79/22	$[PO_3]^-$	1.8
				153/14	$[M - H - C_4H_8]^-$	
diphenyl	DPhP	14.6	249/35	93/26	$[PhO]^-$	11
				155/20	$[M - PhOH]^-$	
monoethylhexyl	MEHP	15.8	209/50	79/14	$[PO_3]^-$	
di-benzyl	DBzP (IS)	18.8	277/45	79/32	$[PO_3]^-$	
dibutoxyethyl	DBEP	20.0	297/50	79/24	$[PO_3]^-$	4.1
				197/18	$[M - H - C_6H_{14}]^-$	
diethylhexyl	DEHP	23.4	321/25	79/26	$[PO_3]^-$	7.0
				209/22	$[M - H - C_8H_{16}]^-$	

[a] Molecular anion $[M - H]^-$ and cone voltage (V). [b] Relative intensity of the two fragment anions.

chromatography, which has previously proven suitable for very acidic aromatic sulfonates.[13]

This new method is applied to municipal wastewater samples to obtain, first, data on the occurrence of phosphoric acid di- and monoesters in this kind of wastewater and, second, to check if this class of industrial chemicals may have some environmental relevance. Finally, the method is used to investigate if phosphoric acid mono- and diesters can be formed by mixed bacterial cultures upon degradation of the respective nonionic triesters that occur in municipal wastewater due to their use as OPFR and plasticizers.[6]

EXPERIMENTAL

Reagents and Chemicals. The following phosphoric acid di- and monoesters are available as pure reference compounds: DPhP (CAS No. 838-85-7), DEHP (CAS No. 298-07-7), MPhP (CAS No. 701-64-4), DBzP from Aldrich (Steinheim, Switzerland), and DnBP (CAS No. 107-66-4) from Fluka (Steinheim, Switzerland). Other analytes for qualitative analysis were prepared by chemical hydrolysis from the triesters as described below. Full names, structures, and acronyms of all analytes are given in Table 1. Tributylamine (TrBA, puriss plus >99.5%) was obtained from Fluka (Buchs, Switzerland). All other chemicals were received as described previously.[6]

Chemical Hydrolysis of Trisubstituted Phosphate Esters. By a base-catalyzed hydrolysis[14,15] of trichloropropyl phosphate (TCPP), tri-*n*-butyl phosphate (TnBP), tri-isobutyl phosphate (TiBP), tributoxyethyl phosphate (TBEP), trichloroethyl phosphate (TCEP), and tris(ethylhexyl) phosphate (TEHP), those phosphoric acid mono- and diesters were obtained that were not commercially available. In a 5-mL, screw-cap glass tube, 2 mmol of the parent compound was dissolved in 2 mL of 2 M NaOH in water/methanol (20/80). The tube was closed and heated to 100 °C for 2 h, allowed to cool, neutralized with a small excess of aqueous HCl (~4.5 mmol), and then diluted (~2−4-fold) with methanol/water (50/50). An aliquot of these solutions was directly injected into the LC−MS system without any attempt to isolate the hydrolysis products. From most of the triesters, except for the chloroalkyl phosphates, a good yield of the diester with some monoester was obtained.

Wastewater Samples. A municipal wastewater treatment plant (WWTP) with a capacity of 240 000 m^3/d at dry weather flow, involving activated sludge treatment with enhanced nutrient removal (tertiary treatment) was investigated. Composite samples (24-h) of the influent, primary effluent, and tertiary effluent were collected in August 2004. Tertiary effluent samples were taken with a 24-h delay to account for the hydraulic retention time of the wastewater in the WWTP. All samples were filtered through 0.45-μm membrane filters (cellulose acetate; Sartorius, Goettingen, Germany) and analyzed immediately.

Instrumentation. A HP1100 (Agilent Technologies, San Jose, CA) liquid chromatographic system consisting of a membrane degasser, binary high-pressure gradient pump, autosampler, and column thermostat was used. The system was interfaced to a Quattro LC triple-stage quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with a Z-spray electrospray interface. Nitrogen was provided by a nitrogen generator (model 75-72, Whatman, Haberville, USA) and used as drying and nebulizing gas. Argon (99.999%) was used as collision gas. Exact mass measurements were performed with a Waters Alliance 2695 HPLC system coupled to a Quattro Micro quadrupole-time-of-flight (Q-TOF) instrument with an electrospay interface (Waters, Eschborn, Germany).

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LC−MS/MS Analysis. *Liquid Chromatography.* Separation of analytes was carried out on a Luna 3-μm Phenyl-Hexyl 2 × 150 mm column (Phenomenex, Eschborn, Germany) at a flow rate of 0.20 mL/min and column temperature of 45 °C. Eluent A was MeOH/water (20/80), and B was MeOH/water (95/5), both containing 1 mM TrBA and 1 mM acetic acid. The gradient was as follows: 0 min, 25% B; 0.5 min, 40% B; 15 min, 48% B; 16 min, 100% B; 18 min, 100% B; 19 min, 25% B; and 28 min, 25% B.

Mass Spectrometry. Analytes were detected by electrospray ionization tandem mass spectrometry (ESI-MS/MS) operated in the negative ion mode with multiple reaction monitoring (MRM). The capillary voltage was 3.0 KV, source temperature was 120 °C, desolvation temperature was 200 °C, nebulizer gas flow was 100 L/h, and the drying gas flow was set to 700 L/h. Argon pressure in the collision cell was kept at 1.0×10^{-3} bar for MS/MS measurements. Individual MS/MS parameters for each compound are shown in Table 1.

Samples subjected to SPE were quantified by the standard addition procedure over the extracts: 50 μL of a standard solution containing increasing concentration of analytes was added to 200-μL aliquots of each extract.

Exact mass determination with the Q-TOF instrument was performed with a capillary voltage of 3.0 KV, a cone voltage of 30 V, a source temperature of 120 °C, desolvation temperature of 300 °C, cone gas flow of 50 L/h, and desolvation gas at 500 L/h. When operated in MS/MS, the collision gas (Ar) pressure was kept at 1.0×10^{-3}, and the product ion spectra were recorded at collision energies of 10, 20, and 30 eV. Aqueous phosphoric acid (0.06%) was used as the mass lock reference.

Solid-Phase Extraction. Solid-phase extraction (SPE) was performed with an Autotrace SPE Workstation (Zymark, Hopkinton, MA). Three SPE cartridges were initially tested: Oasis HLB (60 mg; Waters, Milford, MA); Lichrolut EN (200 mg); and Lichrolut RP-18 (500 mg), both from Merck (Darmstadt, Germany).

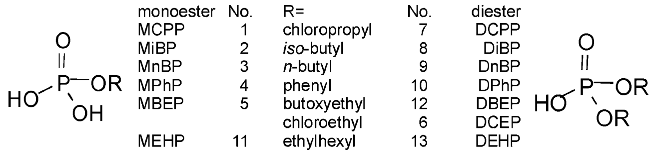
Extraction at Acidic pH. Samples were acidified to pH 2, and SPE cartridges were sequentially conditioned with 5 mL of MeOH and 5 mL of ultrapure water (pH 2). A 100-mL volume of sample was then passed through at a flow rate of 10 mL/min and rinsed with 2.5 mL ultrapure water (pH 2), and the cartridges were dried for 30 min (N_2) and eluted with the appropriate volume of MeOH.

Ion-Pair Extraction. For ion-pair solid-phase extraction (IP-SPE), 11 mL of a buffer solution (2% MeOH, 50 mM TrBA, pH 5 with formic acid) was added to 100 mL of the samples, agitated, and allowed to stand for ~30 min prior to extraction. Cartridges (Lichrolut RP-18, 500 mg) were conditioned with 5 mL of MeOH, 5 mL of ultrapure water, and 2 × 5 mL of buffer solution. The sample (100 mL + 11 mL of buffer) was passed through the cartridge, washed with 2.5 mL of buffer and 1 mL of ultrapure water, dried for 30 min (N_2), and eluted with 2 × 2 mL of MeOH.

The SPE extracts were concentrated in a Turbovap II nitrogen concentrator (Zymark) to ~0.3 mL, spiked with 40 μL of IS solution (5 μg/mL), and diluted to 1 mL with ultrapure water.

Method Testing and Evaluation. Preliminary experiments for selection of the appropriate SPE cartridge were performed with treated wastewater at three spike levels (0, 0.4, and 2 μg/L).

For the determination of recoveries and for validation of the final method, 100-mL aliquots of ultrapure water were spiked at two different levels (0.05 and 1.0 μg/L), and WWTP influent and effluent were spiked at 4 μg/L. Recovery experiments were performed in triplicate. Another aliquot of each sample type was extracted, and the same amounts of analytes were added to the extracts to detect matrix effects.[6,16]



monoester	No.	R=	No.	diester
MCPP	1	chloropropyl	7	DCPP
MiBP	2	*iso*-butyl	8	DiBP
MnBP	3	*n*-butyl	9	DnBP
MPhP	4	phenyl	10	DPhP
MBEP	5	butoxyethyl	12	DBEP
		chloroethyl	6	DCEP
MEHP	11	ethylhexyl	13	DEHP

Figure 1. Structures and acronyms of the 13 phosphoric acid mono- and diesters covered in this study.

Biodegradation Tests. Microbial batch degradation tests using activated sludge of a municipal wastewater treatment plant as inoculate were performed with three triesters (TiBP, TPhP, or TEHP) in 2.5-L glass bottles containing 2 L of deionized water, 50 μg/L of one of the triesters, 10 mg/L of fresh sludge, and a phosphate buffer.[17] Additionally, 50 mg/L of powdered milk was added once a week as an additional carbon source. The open bottles were stirred in the dark without active aeration. A poisoned control (1 mg/L of Hg(II)) was performed for each of the trialkyl phosphates.

Samples (~40 mL) were taken at variable intervals, filtered through 0.45-μm membrane filters (cellulose acetate; Sartorius, Goettingen, Germany), and stored frozen until being analyzed by LC−MS/MS (without SPE). Phosphoric acid di- and monoesters were determined as described above, and trialkyl phosphates, as described previously.[6] The activity of the microorganisms was controlled by determining the removal of powdered milk by dissolved organic carbon (DOC) detection with a HighTOC analyzer (Elementar, Hanau, Germany).

RESULTS AND DISCUSSION

Determination by LC−ESI-MS/MS. Phosphoric acid di- and monoesters (Figure 1) have one or two highly acidic functional groups ($pK_a = 1-2^{7,8}$) that render these compounds very polar and difficult to retain in reversed-phase LC. Ion-pair reversed-phase liquid chromatography with volatile tributylamine (TrBA) has been shown to be well-suited for the LC−MS determination of highly acidic aromatic sulfonates[13] by providing strong chromatographic retention of acidic analytes and being sufficiently volatile to enable coupling with electrospray ionization MS.[18] It appeared promising to use this approach also for the LC−MS analysis of phosphoric acid mono- and diesters. Indeed, a good separation of the commercially available three diester standards and the 10 di- and monoesters synthesized in this work was obtained with 1 mM TrBA (Figure 2). DCPP exhibits three peaks (one of them hardly visible in Figure 2), which likely reflect three of the isomers of TCPP in the technical mixture, from which the DCPP was synthesized.

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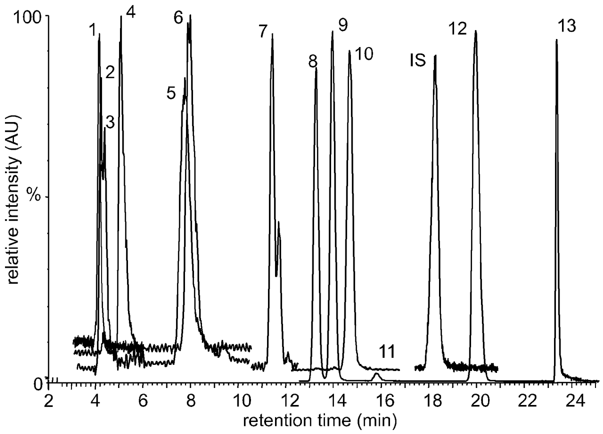

Figure 2. Ion-pair LC−ESI-MS chromatogram (MRM) of a mixture containing the target analytes and the internal standard (IS). Analytes described in Figure 1, MRM transitions given in Table 1.

The fragmentation behavior of the molecular anions of the di- and monoesters in the MS/MS mode was studied to elaborate selective and sensitive transitions for their MRM determination. The fragmentation of the molecular anions of the nonchlorinated dialkyl phosphates (DiBP, DnBP, DBEP, and DEHP) is dominated by a McLafferty rearrangement, yielding the monoalkylated anion and a subsequent loss of the neutral alcohol to form the phosphite anion at *m/z* 79.[9,19] Correspondingly, the monoalkyl esters (MiBP, MnBP, MBEP, and MEHP) show only one intense transition from the molecular anion to the phosphite anion. For DBEP, the ether bond in each butoxyethyl ester group gives rise to additional losses of butanol (−74 Da) to produce the anions *m/z* 223 from the molecular anion (*m/z* 297) and *m/z* 123 from *m/z* 197 (Figure 3a).

The molecular anions of the chloralkyl esters show a completely different fragmentation from their nonchlorinated counterparts. For DCPP (Figure 3b) and DCEP, formation of the chloride anion (*m/z* 35) is the most intense transition. Additionally HCl elimination occurs from DCPP (to *m/z* 213); this fragmentation is the only one observed for MCPP.

The product ion mass spectra of the diaryl phosphates DPhP (Figure 3c) and DBzP are dominated by the production of the phenolate (*m/z* 93) and benzoate (*m/z* 107) anions, respectively, accompanied by the formation of phosphite (*m/z* 79). Elimination of phenol from the anion of DPhP results in the formation of *m/z* 155 (Figure 3c). As for the monoalkyl esters, the monoaryl ester MPhP exhibits the phosphite anion (*m/z* 79) as the only intense fragment. For the sensitive determination of the phosphoric acid diesters, the two most intense transitions were selected for MRM detection, whereas for the monoesters, only one transition was available (Table 1).

Solid-Phase Extraction. Three different SPE cartridges (Lichrolut EN, Oasis HLB and Lichrolut RP18) were tested for their suitability to extract the three phosphoric acid diesters that are commercially available as pure standards (DnBP, DPhP, and DEHP). Because of the very high elution volume required (>8 mL) Lichrolut EN was excluded in an early stage of this study. Oasis HLB and Lichrolut RP18 were further tested with acidified (pH 2) samples, parallel to ion-pair extraction with TrBA and RP18 material at neutral pH. Recoveries were determined by comparing the analyte response obtained by spiking the analytes into samples before SPE (R_2) and into SPE extracts (R_3).[6,16] Satisfactory recoveries (75−123%) were obtained for DnBP and DPhP under all three conditions. For DEHP, however, only the ion-pair extraction provided a reasonable recovery (109%). Therefore, ion-pair extraction using Lichrolut RP18 was favored in this work. IP-SPE is expected to be even more advantageous with respect to the more polar monoesters of phosphoric acid.

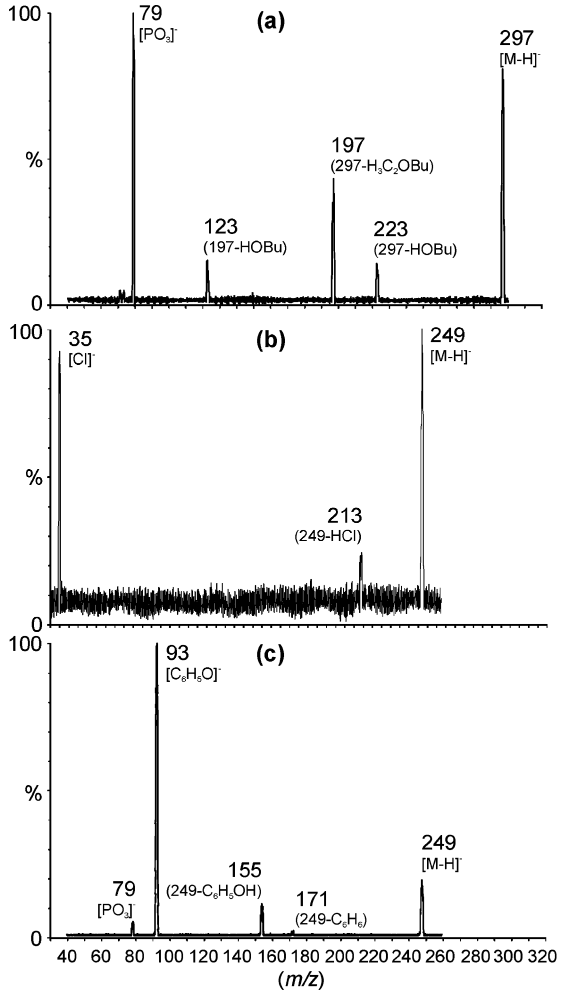

Figure 3. Negative ion ESI-MS/MS product ion spectra of (a) DBEP, (b) DCPP, and (c) DPhP.

Analytical Performance and Matrix Effects. After further optimization, good recoveries were obtained for the three diesters (71−112%) using ion-pair SPE with TrBA from pure water and treated and even untreated municipal wastewater (Table 2). No breakthrough occurred for any of the three compounds from ultrapure and treated municipal wastewater when 100 mL volumes were extracted.

The developed LC−MS/MS method shows good linearity ($R^2 \geq 0.9981$) and repeatability (RSD ≤ 5.3%; Table 2). Calculated limits of quantification (LOQ; S/N > 10) range from 7 to 14 ng/L for the three analytes after extraction of 100 mL of pure water samples spiked at the 50 ng/L level, which is adequate for the analysis of most aqueous samples (Table 2). LOQs could not be experimentally determined from real wastewater samples because of their high inherent concentration of the analytes.

Compared to the only previous method dating back to the 1980s and employing GC/MS to determine diphenyl and dicresyl

Table 2. Method Validation Data for the Ion-Pair SPE-LC−MS/MS Method

			% recoveries (RSD)[c]				LOQs[d](ng/L)	
compd	linearity[a] (R^2)	precision[b] (RSD)	pure water (0.05 μg/L)	pure water (1 μg/L)	WWTP effluent (4 μg/L)	WWTP influent (4 μg/L)	LC−MS/MS[e]	SPE-LC−MS/MS[f]
DnBP	0.9991	5.3	80 (13%)	97 (7%)	93 (2%)	71 (8%)	780	14
DPhP	0.9981	1.9	88 (13%)	98 (6%)	81 (1%)	85 (8%)	460	7
DEHP	0.9987	1.3	82 (13%)	76 (14%)	112 (8%)	71 (10%)	810	14

[a] Six-point calibration with pure water standards (2−500 μg/L) without SPE. [b] 20 μg/L pure water standard ($n = 5$). [c] Determined by the standard addition procedure; $n = 3$. [d] S/N ≥ 10. [e] Direct injection of 100 μL of treated wastewater. [f] 50 μL injection of an SPE extract of pure water spiked at the 50 ng/L level.

phosphoric acid esters from water,[11] this method provides orders of magnitude lower detection limits from much smaller sample volumes without the need of derivatization. Furthermore, this LC−MS method can be used to directly analyze wastewater samples without preceding SPE when analyte concentrations exceed ~1 μg/L (Table 2).

Matrix effects that occur during electrospray ionization may alter the signal intensity of an analyte in ESI-MS analysis of real samples.[20] Matrix effects are reliably detected by comparing the response factors obtained from standard addition of the analytes into a wastewater extract (R_2) and into pure water (R_1) as described previously.[6,16] The relative response (R_2/R_1) of the three diesters was reduced to ~80% in treated municipal wastewater and to 50−70% in untreated municipal wastewater. Therefore, a reliable quantification of phosphoric acid diesters from aqueous environmental samples using LC−MS with electrospray ionization requires standard addition. This was also the case for phosphoric acid triesters[6] and is common for many other compounds for which stable isotope labeled standards are not available.[21]

Application to Real Samples. This ion-pair SPE ion-pair LC−ESI-MS/MS method was used to investigate which of the three commercially available and the newly synthesized phosphoric acid mono- and diesters may occur in the influent, primary effluent, and tertiary effluent of a WWTP; however, only those three diesters for which pure reference standards were available (DEHP, DnBP and DPhP) could be quantified. A total of six analytes, five phosphoric acid diesters (DiBP, DnBP, DPhP, DBEP and DEHP), and one monoester (MEHP; Figure 4a) could be identified in the influent of one WWTP.

The concentration of four of the esters in the WWTP influent was high enough to detect them by directly injecting the sample into the LC−MS system without any enrichment (Figure 4b). DPhP and DBEP were, however, hardly detectable by direct injection. None of the other seven di- and monoesters included in this analytical procedure was detected in any of the three samples.

Five of the six analytes that were detected in municipal wastewater fulfill all criteria set by the European Union for a positive identification in residue analysis using the agreement in retention times, the number of precursor/product-ion pairs, and their relative intensity between the analytes and the reference compounds[22] (Table 3). However, since MEHP, as all phosphoric acid monoesters, shows only one fragment ion, its positive identification required the confirmation of its exact mass. This was achieved by using a Q-TOF MS with a mass resolution of 5000 ($m/\Delta m$ at fwhm). The determined masses of the molecular anion, its one product ion, and the difference between the two ions show <5 mDa deviation from the expected values (Table 4). Moreover, of the possible formulas proposed by the Masslynx software, only the combination of $(C_8H_{18}O_4P)^-$ for the molecular anion, $(PO_3)^-$ for the product ion and $(C_8H_{18}O)$ for the difference between the two detected ions is a consistent combination. All other suggested formulas for any of these three masses did not correspond to the formula proposals of the other two ions (Table 4). Therefore, now all six detected phosphoric acid mono- and diesters, including MEHP, can be considered positively identified.[22]

Of those three analytes for which pure reference material was available, DEHP showed the highest concentration in the untreated wastewater (61 μg/L), and concentrations of DnBP and DPhP were about 1 and 2 orders of magnitude lower (Table 3). Due to the lack of pure reference materials, no concentration could be calculated for MEHP, DiBP, and DBEP. Assuming, however, that their sensitivity in MRM detection would be comparable to the other three analytes, the concentration of MEHP would be similar to DEHP in the raw wastewater (~65 μg/L; Table 3). The concentration of DiBP would fall in a range of 100 ng/L to 1 μg/L, whereas the concentration of DBEP would be 1 order of magnitude lower. Thus, MEHP and DEHP appear to occur in

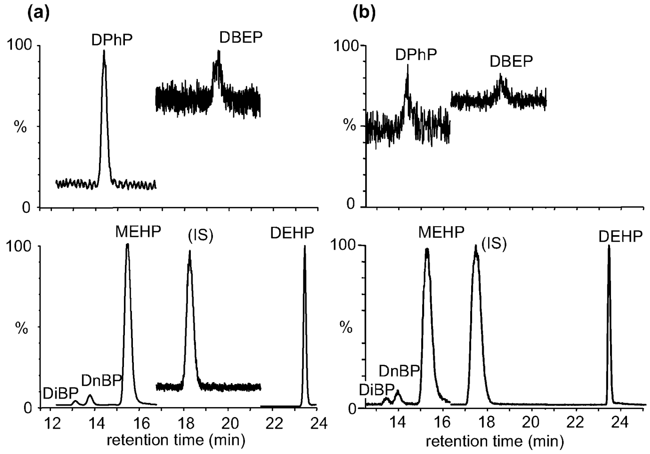

Figure 4. LC−MS/MS chromatogram (MRM) of untreated municipal wastewater: (a) after ion-pair SPE of 100 mL of sample (50 μL injected) and (b) direct injection (no SPE) of the same sample (100 μL injected). MRM transitions as in Table 1.

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Table 3. Data for Phosphoric Acid Mono- and Diesters Determined in Single Composite Samples of a WWTP

	influent			primary effluent			tertiary effluent			
compd	found Tr1/Tr2[a]	AU (RSD)[b]	μg/L (SD)[c]	found Tr1/Tr2[a]	AU (RSD)[b]	μg/L (SD)[c]	found Tr1/Tr2[a]	AU (RSD)[b]	μg/L (SD)[c]	Tr1/Tr2 tolerance[d]
D*n*BP	1.9	6136 (2)	1.6 (0.1)	1.9	5442 (7)	1.8 (0.1)	1.7	196 (26)	0.027 (0.006)	1.8 ± 0.36
DPhP	10	1583 (9)	0.18 (0.01)	9.7	1226 (8)	0.20 (0.01)	11	358 (8)	0.050 (0.005)	11 ± 5.5
DEHP	6.3	62348 (13)	61 (5)	6.7	74019 (7)	62 (1)	7.2	3447 (11)	4.7 (0.2)	7.0 ± 2.1
D*i*BP	3.3	1993 (3)		3.3	1778 (6)		3.0	349 (8)		3.2 ± 0.8
MEHP		67151 (3)			75305 (7)			14903 (3)		
DBEP	3.5	275 (12)		4.6	121 (17)			nd[e]		4.1 ± 1.0

[a] Relative intensity of the two fragment anions found experimentally in the samples. [b] AU, area units; RSD, relative standard deviation (%) for $n = 3$. [c] SD, standard deviation for $n = 3$. [d] Relative intensity tolerance range for positive identification according to ref 22. [e] Not detected.

Table 4. LC−Q-TOF MS Results Obtained for the Compound Identified as MEHP in a Wastewater Sample: Data for the Molecular Anion, the Product Ion, and the Difference between the Two[a]

anion	mass (m/z)	proposed formula[b]	calcd (m/z)	error (mDa)	no. of P
molecular	209.0938	$\mathbf{C_8H_{18}O_4P}$	**209.0943**	**−0.5**	1−2
		$C_6H_{16}N_3O_3P$	209.0929	0.9	
		$C_9H_{17}N_5O_2$	209.0959	−2.1	
		$C_{11}H_{16}NOP$	209.0970	−3.2	
		$C_7H_{19}N_2O_1P_2$	209.0973	−3.5	
product	78.9623	H_2NPS	78.9646	−2.3	0−2
		$\mathbf{PO_3}$	**78.9585**	**3.8**	
difference	130.1315	$C_{16}H_{16}N_3$	130.1344	−2.9	0−2
		$\mathbf{C_8H_{18}O}$	**130.1358**	**−4.3**	

[a] The only consistent combination of the three masses is highlighted in bold. [b] Formulas proposed using the following conditions: mass tolerance of ± 5 mDa, element composition, C = 0−20, H = 0−40, N = 0−5, O = 0−6, and S = 0−1; and the conditions for the number of P as given in the last column.

higher concentration than the previously detected phosphoric acid triester OPFR in the same WWTP.[6]

Primary wastewater treatment does not seem to reduce the concentration of the phosphoric acid esters, whereas their amount appears to be strongly reduced by the secondary and tertiary treatment (Table 3). Even though more than 90% removal is obtained for DEHP, its final concentration in the treated wastewater still amounts to 4.7 μg/L. A part of this concentration decrease may be due to the formation of MEHP, for which only a 75% removal was calculated in this one pair of samples on the basis of its signal areas. These first data on the occurrence of phosphoric acid mono- and diesters in municipal wastewater treatment suggest that these organic compounds are relevant contaminants of municipal wastewater and that the extent of their removal appears to be incomplete.

Biodegradation Assay. Of the five phosphoric acid diesters detected here in municipal wastewater, only DnBP, MEHP, and DEHP are known to be used as technical products,[7,8] whereas the origin of DiBP, DPhP, and DBEP was unclear. However, it has previously been hypothesized that di- and monoester may be formed as biodegradation intermediates of phosphoric acid triesters.[11,23]

To test this hypothesis, the degradation of TPhP, TiBP, and TBEP was studied in an aerobic laboratory degradation test with activated sludge as inocculum and powdered milk as additional carbon source. Biodegradation was followed by direct injection of aqueous samples into the LC−MS system (Figure 5). Water solubility limited the initial aqueous concentrations to 20 nmol for TPhP, 50 nmol for TiBP, and 5 nmol for TEHP. The concentration of TPhP rapidly decreased within the first days (Figure 5a). Indeed, DPhP was formed as the initial microbial hydrolysis product with a maximum at day 3 that accounted for ~33 mol % of the TPhP. This diester was completely removed within another 4 days. No MPhP was detectable during the biodegradation.

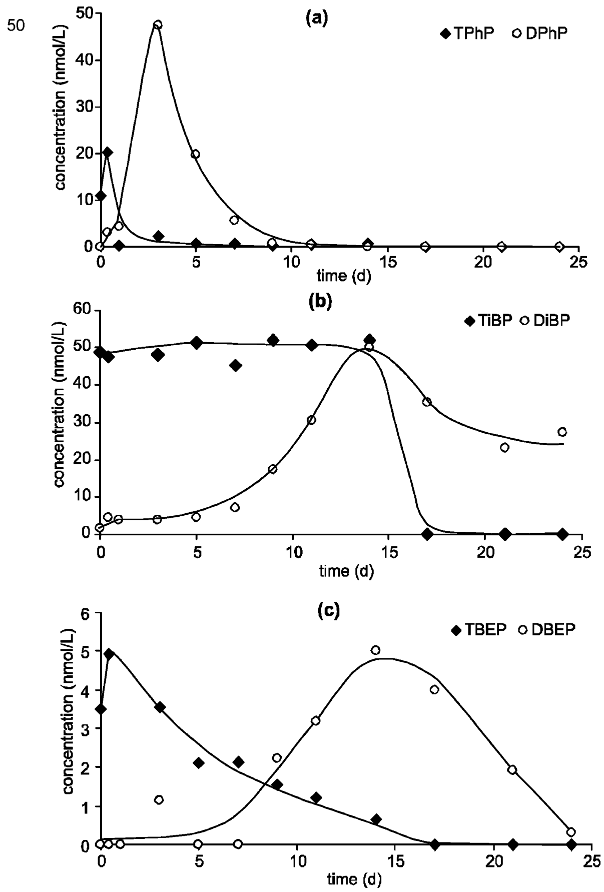

Figure 5. Time course of aerobic microbial degradation of phosphoric acid triesters: (a) TPhP, (b) TiBP, and (c) TBEP. Concentrations in nanomoles per liter, except for DiBP and DBEP (arbitrary scale).

The microbial degradation of the trialkyl esters TiBP and TBEP (Figure 5b, c) was significantly slower. A slower microbial

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transformation of aliphatic triesters as compared to aromatic triesters has previously been observed for sediment leachates under aerobic conditions.[24] Here, 16 days was required for the complete removal of both triesters. As for TPhP, microbial hydrolysis of the triesters to the respective diesters occurred with a maximum concentration at day 14 (Figure 5b,c). These diesters were successively removed to 30 nmol/L of DiBP and to below the LOD for DBEP at day 24 of the experiment. Again, no monoesters could be detected in the course of the triester degradation. This may indicate that the breakdown of phosphoric acid monoesters proceeded faster than their formation from the diesters.

In the poisoned control, no degradation or metabolite formation was observed for TiBP and TBEP, indicating that the hydrolysis of these phosphoric acid triesters was microbially catalyzed. For TPhP, however, a slow hydrolysis to DPhP also occurred in the poisoned control experiment. Parallel to this, ~70% of the powdered milk was mineralized over 24 days. This DOC removal indicated that the poisoned control was not sterile. It has previously been shown, however, that an aqueous solution of TPhP is stable under sterile conditions.[23] Thus, TPhP hydrolysis in this control experiment was likely also due to microbial activity and need not indicate an abiotic hydrolysis.

Although some laboratory degradation studies of phosphoric acid triesters have been performed previously,[23,24] the formation of biodegradation intermediates has not been investigated before. With the new analytical method presented here, it has now been proven that microbial degradation of trialkyl or triaryl phosphates starts with the microbial hydrolysis of one of the ester linkages to form dialkyl or diaryl phosphates, which are then further degraded along a yet unknown pathway. Thus, microbial degradation of phosphoric acid triesters can be a source for phosphoric acid diesters in municipal wastewater.

CONCLUSIONS

An ion-pair SPE procedure followed by ion-pair LC−MS/MS has been developed which allows the determination of 13 phosphoric acid mono- and diesters from wastewater samples. The high sensitivity of the method enables direct injection of water samples if the analyte concentration is in the micrograms-per-liter range. For the first time, five phosphoric acid diesters and one monoester were determined in a WWTP effluent. The identification of these analytes in wastewater samples was confirmed by comparison with commercially available standards and standards synthesized from the triesters.

Pure reference standards were available for only three phosphoric acid diesters. Of these, DEHP exhibited the highest concentration in municipal wastewater (61 μg/L) and showed 90% removal in biological wastewater treatment. MEHP was removed by 75% only. These preliminary data indicate that phosphoric acid diesters and monoesters may occur in municipal wastewater in micrograms-per-liter quantities and that even tertiary treatment does not ensure complete removal of these compounds. Laboratory degradation tests suggest that some of the diesters found in municipal wastewater may originate from the microbial hydrolysis of phosphoric acid triesters that are used as organophosphorus flame retardants. Thus, studies on the fate of OFPR in wastewater treatment and the environment may also consider these microbial hydrolysis products.

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