Determination of hexamethylphosphoramide and other highly polar phosphoramides in water samples using reversed-phase liquid chromatography/electrospray ionization time-of-flight mass spectrometry

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ABSTRACT

The widely used solvent hexamethylphosphoramide (HMPA) and its biological (metabolic) and chemical (abiotic) phosphoramide-based oxidation products may cause adverse health effects through occupational exposure and intake of contaminated groundwater. However, no current methods exist for the separation and the detection of the many polar HMPA oxidation products. Thus, we developed a new RPLC/ESI–TOF–MS method and further investigated the chromatographic performances of two columns (i.e., XTerra Phenyl and XBridge Phenyl). In addition, the impact of (forced) acid hydrolysis for optimized chromatographic performance of the XTerra Phenyl column is investigated. The XTerra Phenyl column showed the best separation of the less polar major metabolic oxidation products pentamethylphosphoramide and hydroxymethyl-pentamethylphosphoramide, however, only after treating the column with formic acid (acid-treated). The XTerra column separated most of the investigated HMPA oxidation products (11 of 16 compounds) in a single chromatographic run. In contrast, the XBridge Phenyl column requires one method for the less polar and another method for the more polar oxidation products. However, this results in an overall better separation performance of the XBridge Phenyl column, especially for the less polar major abiotic oxidation products hydroxymethyl-pentamethylphosphoramide and formyl-pentamethylphosphoramide, as well as for 11 highly polar oxidation products ($R_S > 1.5$). The RPLC/ESI–TOF–MS method presented and validated in this study is the first analytical method that can be used to separate and detect HMPA (LOD 0.10 μM without preconcentration) and all of its oxidation products.

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1. Introduction

Hexamethylphosphoramide (HMPA) is a polar aprotic organophosphorus compound which is highly soluble in both aqueous and organic phases. These unique properties led to its extensive application as a solvent for gases, organic and organometallic compounds, as a polymerization catalyst, UV inhibitor in plastic, processing solvent for aromatic polyamide fiber, and for many other purposes [1,2]. The widespread use of HMPA has raised potential health concerns due to occupational exposure, for which skin absorption and inhalation are the major uptake mechanisms [3], as well as due to contaminated groundwater, which has been found in the U.S. [4]. The acute toxicity of HMPA is low to moderate [3], but it has been classified as a possible human carcinogen (Group 2B) by IARC [5]. Furthermore, HMPA and several of its oxidation products were shown to be mutagenic in *Drosophila*, which possesses metabolizing enzymes similar to those in mammals, such as cytochrome P-450 [6].

The oxidative metabolism of HMPA was proposed to proceed via sequential *N*-demethylation. Pentamethylphosphoramide (PMPA), tetramethylphosphoramide (TetMPA), and trimethylphosphoramide (TriMPA) were detected in the excreta of mice, rats, and house flies through thin layer chromatography (TLC) coupled with infrared (IR) spectroscopy as well as gas chromatography in combination with a flame photometric detector [3,7,8]. The generation of formaldehyde during demethylation led to the assumption that HMPA is initially oxidized to the mutagen hydroxymethyl-pentamethylphosphoramide (HM-PMPA), which then decomposes to produce formaldehyde and PMPA [6,8]. However, none of the applied analytical methods were capable of HM-PMPA detection. As minor metabolites, formylated oxidation products were detected in rat urine via TLC and IR spectroscopy [9].

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Using gas–liquid partition chromatography coupled with IR and proton magnetic resonance spectroscopy as well as mass spectrometry, non-oxygenated and formylated reaction intermediates were identified from the abiotic oxidation of HMPA by permanganate [10], an oxidizing agent that is frequently used for drinking water, waste water, and groundwater treatment [11]. Again, it was speculated that HM-PMPA was the precursor of formyl-pentamethylphosphoramide (formyl-PMPA). However, the applied methods were not capable of detecting any of the mutagenic hydroxymethylated reaction intermediates.

An analytical method based on liquid chromatography had not been developed, until we showed for the first time in a recent study, using reversed-phase liquid chromatography (RPLC) in combination with electrospray ionization time-of-flight mass spectrometry (ESI–TOF–MS), that hydroxymethylated as well as minor amounts of carboxylated intermediates are formed from the oxidation of HMPA by aqueous permanganate (Fig. 1) [12]. However, chromatographic separation was only performed for the comparatively less polar products formed during the initial stage of the oxidation experiment. In addition, we also successfully used this method for identification of HMPA hydrolysis products [13].

Thus, the objective of this paper was to develop and evaluate an RPLC/ESI–TOF–MS method that is capable of separating and identifying all products of abiotic, as well as the proposed products of biotic HMPA oxidation. In the course of this development, we investigated the impact of (forced) acid hydrolysis on separation characteristics as a means of method optimization. After testing several different RPLC columns containing both polar and non-polar stationary phases, only two columns revealed satisfactory chromatographic separation: an XTerra Phenyl and an XBridge Phenyl column. The XTerra Phenyl column is a first-generation organic–inorganic hybrid, produced from tetraethylsiloxane and methyltriethylsiloxane monomers, with a 2-phenylpropyl ligand bonded to the surface and a surface coverage of $2.47 \,\mu mol/m^2$ [14,15]. The XBridge Phenyl column is a second-generation hybrid, based on tetraethylsiloxane and bis(triethoxysilyl)ethane monomers, with a surface-bonded 1-phenylhexyl ligand and a surface coverage of $3.0 \,\mu mol/m^2$ [14].

2. Experimental

2.1. Chemicals, reagents and materials

Hexamethylphosphoramide (99.1% purity; *only compound commercially available for this project*) was purchased from MP Biomedicals (Solon, OH, USA). Pentamethylphosphoramide (PMPA), TetMPA and TriMPA (each 97% purity) were custom synthesized and generously provided by E. I. du Pont de Nemours and Company (Wilmington, DE, USA). $KMnO_4$ (certified ACS), $Na_2S_2O_3$ (99.8% purity), and acetonitrile (99.9% purity) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Formic acid (98% purity) was purchased from Sigma–Aldrich (Steinheim, Germany). Puradisc 25 TF filters with a $0.25 \,\mu m$ pore size PTFE membrane were obtained from Whatman (Sanford, ME, USA). The water used in the preparation of standards, reaction media, and mobile phases was obtained from a Barnstead NANOpure system D8991 (Dubuque, IA, USA).

2.2. Sample preparation

To synthesize the analytes that had previously been detected or suggested to be produced from both biotic and abiotic HMPA oxidation, HMPA was oxidized by aqueous permanganate, since this reaction was shown to lead to the formation of singly and multiply hydroxymethylated, formylated, and less methylated reaction intermediates [12]. In a 1-L Pyrex media glass bottle, an 800 mL solution containing 2.5 mM HMPA and 60 mM $KMnO_4$ in DI water was prepared. The bottle was sealed with a PTFE-faced silicone septum and stored in the dark at 22 °C without agitation. Samples were collected after 10 min, 6 h, and 72 h. For the early-stage oxidation (i.e., 10 min) sample (which contained the less polar, less oxidized compounds), 80 μL of the reaction solution were quenched by adding 7.92 mL of 6 mM $Na_2S_2O_3$ (1:100 dilution). For the mid- (i.e., 6 h) and late-stage oxidation (i.e., 72 h) samples (which contained the more polar, more oxidized compounds), 800 μL of the reaction solution were quenched by adding 7.2 mL of 60 mM $Na_2S_2O_3$ (1:10 dilution). The lower dilution for the mid- and late-stage oxidation samples was applied due to decreased detector sensitivity for the more polar analytes. All quenched samples were vortexed until brown flocculates developed. Polytetrafluoroethylene (PTFE) filters were pre-flushed with 5 mL of quenched sample solution. Subsequently, 1.6 mL sample solution was filtered and analyzed immediately.

2.3. HPLC chromatography

Analyses were performed on an Agilent 1100 Series liquid chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with a vacuum degasser, a binary pump, an autosampler, and a temperature-controlled column compartment. Masshunter software (B.02.00) was used for instrument control, data acquisition, and analysis.

For the XBridge Phenyl column, two methods were developed, one using isocratic elution with A (0.1% formic acid) and B (acetonitrile) at a ratio of 85:15 (XBridge method 1), and one using gradient elution with 0–3 min, 0% B; 3–10 min, 0–15% B; 10–18 min, 15% B, 18–30 min, 0% B (XBridge method 2). For all samples, the injection volume was 5 μL, the flow rate was 1.2 mL/min, and the temperature was controlled at 30 °C.

Two pristine (150 and 250 mm long) XTerra Phenyl columns were initially tested under the same conditions as the XBridge Phenyl column. However, both columns revealed impractically long retention times, and substantially longer retention times than an aged, extensively-used 150 mm XTerra column that had been used previously [12]. It was assumed that the stationary phase in the aged column had been altered due to hydrolysis reactions of siloxane bonds (for details, see Section 3). To test this hypothesis, the pristine 150 mm XTerra Phenyl column was stored in 100% of 0.1% formic acid (pH 2.7) for two months, and six more months in 0.01% formic acid/acetonitrile (98:2, pH 3.1). After storage of the 150 mm XTerra Phenyl column in acidic (formic acid) solution, separation of all samples was carried out using gradient elution: 0–10 min, 0% B; 10–12 min, 0–5% B; 12–20 min, 5% B, 20–30 min, 0% B. The injection volume was 5 μL, the flow rate was 0.5 mL/min, and the temperature was controlled at 30 °C.

2.4. Mass spectrometry

The HPLC was connected to an Agilent G3250AA MSD TOF system (Agilent Technologies, Wilmington, DE, USA) with an electrospray ionization (ESI) source (ESI–TOF–MS). The capillary, fragmentation, and skimmer voltages were 800 V, 130 V, and 60 V, respectively. The temperature of the drying gas (N_2) was 350 °C, the flow rate of the drying gas was 12 L/min, and the pressure of the nebulizer gas (N_2) was 50 psi. The mass analyzer was calibrated from m/z 90 to 600 in positive ionization mode using 10 reference ions.

2.5. Calibration, LOD, LOQ and precision

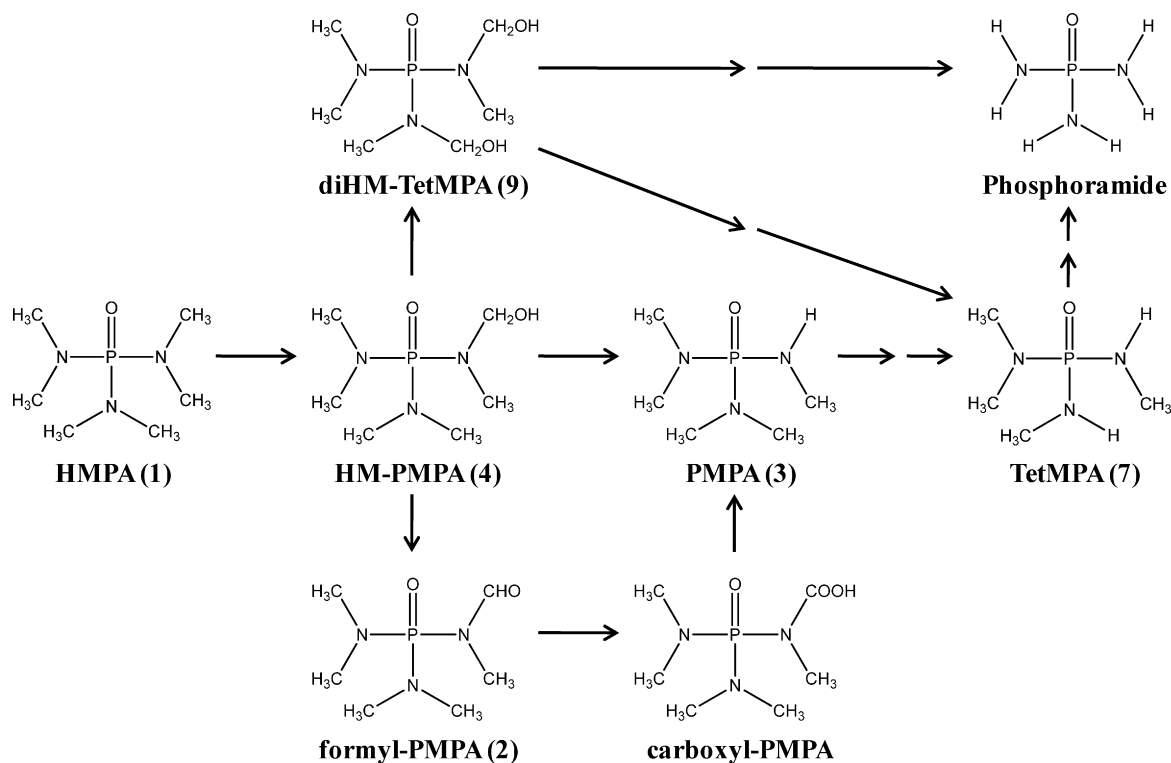Stock solutions containing the four available reference compounds (HMPA, PMPA, TetMPA, and TriMPA) were prepared

Fig. 1. Degradation pathway for HMPA oxidation by aqueous permanganate.

and diluted to appropriate concentrations for the generation of ten-point calibration curves. Each concentration was injected in triplicates, using the XBridge Phenyl column and XBridge method 1. The limits of detection (LOD) and quantification (LOQ) were determined at a signal-to-noise ratio (S/N) of 3 and 10, respectively. The inter-day variability, expressed through the relative standard deviation (%RSD), was determined to assess the precision of the analytical method. For this purpose, one mixed standard solution was analyzed on six consecutive days.

2.6. Theoretical calculations

Retention times (t_R), retention time factors (k), and chromatographic resolution (R_S) were calculated for all compounds. R_S was calculated using the peak width at the peak base.

To determine analyte polarity, dipole moments were calculated using the Gaussian 03 software package. All molecular structures were initially optimized to a ground state (verified by absence of imaginary frequencies), and their dipole moments in aqueous phase were determined at the B3LYP/6-311++G(d,p) level of theory including the IEFPCM solvation model.

3. Results and discussion

3.1. Reversed-phase liquid chromatography

Initially, different columns were tested for chromatographic performance, however, with dissatisfactory results. A Supelcosil C_8 column (Supelco, 150 mm × 4.6 mm I.D., 5 μm), an Allure Biphenyl column (Restek, 150 mm × 3.2 mm I.D., 5 μm), and two Atlantis HILIC Silica columns (Waters, 100 mm × 4.6 mm I.D., 3 μm, and 50 mm × 4.6 mm I.D., 5 μm) showed peak broadening and poor separation performance. An XTerra Phenyl column (Waters, 250 mm × 4.6 mm I.D., 3.5 μm) revealed impractical (i.e., too long) retention times. Satisfactory chromatographic performance was achieved using an XBridge Phenyl (Waters, 250 mm × 4.6 mm I.D.,

3.5 μm) and an XTerra Phenyl column (Waters, 150 mm × 2.1 mm I.D., 5 μm), latter one, however, only after chemical (formic acid) treatment as discussed below. These two columns were then used for method optimization.

For the XBridge Phenyl column, it was generally observed that decreasing acetonitrile concentrations in the mobile phase led to increasing retention times, as expected for a reversed phase stationary phase. Using the isocratic method at 0.1% formic acid/acetonitrile (85:15) (Fig. 2a), three of the initial and less polar HMPA oxidation products, HM-PMPA, formyl-PMPA, and PMPA, were separated with a resolution R_S between 0.95 and > 1.5 (Table 1). The fact that the retention time for PMPA was 0.22 min higher than for HM-PMPA and 0.22 min lower than for formyl-PMPA (Table 1) confirms that 15% acetonitrile was the optimal concentration in the mobile phase. At higher acetonitrile concentrations, PMPA and formyl-PMPA were not separated, at lower acetonitrile concentrations, PMPA and HM-PMPA were not separated. In addition, an increase in column temperature [16] of up to 50 °C only led to a worse separation performance (data not shown).

The acetonitrile concentration of 15%, however, resulted in co-elution or poor separation of four of the more oxidized and polar tetramethylphosphoramide derivatives (peaks 6–9 in Fig. 2a), as indicated by their low R_S values between 0.18 and 1.12 (Table 1). Thus, in addition to the isocratic method, a gradient method with an initially lower acetonitrile concentration was developed.

Application of the gradient method to the less polar oxidation products (Fig. 2b) enabled separation of peaks 6 and 7. However, peak 5 was still not baseline-separated, and peaks 8 and 9 remained unresolved. Furthermore, two of the initial oxidation products i.e., PMPA (peak 3) and HM-PMPA (peak 4) were not separated. Thus, unless it is the specific objective to separate formyl-hydroxymethyl-tetramethylphosphoramide (formyl-HM-TetMPA, peak 6) and tetramethylphosphoramide (TetMPA, peak 7), isocratic elution is the preferred method for separation of the less polar products of HMPA oxidation.

Table 1
TOF-MS characteristics, retention times (t_R), retention time factors (k), and chromatographic resolution (R_S) for HMPA and its detected oxidation products. Relative standard deviation of t_R and k < 1% ($n = 5$) for all compounds and all methods.

| Compound	Peak no.	Base peak	Base peak formula	Theoretical m/z	Observed m/z	Error (ppm)	XBridge t_R (min)	Method 1 k	R_S	XBridge t_R (min)	Method 2 k	R_S	(acid-treated) XTerra[a] t_R (min)	k	R_S
HMPA	1	[M+H]$^+$	$C_6H_{19}N_3OP^+$	180.1260	180.1261	0.6	7.75	2.69	>1.5	12.86	4.85	>1.5	17.43	19.50	>1.5
formyl-PMPA	2	[M+H]$^+$	$C_6H_{17}N_3O_2P^+$	194.1053	194.1053	0.0	4.38	1.09	0.95	12.40	4.64	>1.5	9.42	10.08	0.67
PMPA	3	[M+H]$^+$	$C_5H_{17}N_3OP^+$	166.1104	166.1110	3.6	4.16	0.98	1.01				6.95	7.17	>1.5
HM-PMPA	4	[M+H]$^+$–H$_2$O	$C_6H_{17}N_3OP^+$	178.1104	178.1106	1.1	3.94	0.87	>1.5	10.43	3.74	>1.5	8.71	9.24	>1.5
formyl-TetMPA	5	[M+H]$^+$	$C_5H_{15}N_3O_2P^+$	180.0896	180.0899	1.7	3.19	0.52	1.18				2.95	2.47	0.29
formyl-HM-TetMPA	6	[M+H]$^+$–H$_2$O	$C_6H_{15}N_3O_2P^+$	192.0896	192.0902	3.1	3.07	0.46	0.53				2.95	2.47	0.00
TetMPA	7	[M+H]$^+$	$C_4H_{15}N_3OP^+$	152.0947	152.0949	1.3	3.02	0.44	1.12	9.81	3.46	>1.5	2.26	1.66	>1.5
HM-TetMPA	8	[M+H]$^+$–H$_2$O	$C_5H_{15}N_3OP^+$	164.0947	164.0948	0.6	2.90	0.38	0.18				2.45	1.88	0.73
diHM-TetMPA	9	[M+H]$^+$–H$_2$O	$C_6H_{17}N_3O_2P^+$	194.1053	194.1052	−0.5	2.86	0.36					2.83	2.32	0.94
formyl-TriMPA	10	[M+H]$^+$	$C_4H_{13}N_3O_2P^+$	166.0740	166.0739	−0.6				6.41	1.91	>1.5	1.38	0.63	0.60
TriMPA	11	[M+H]$^+$	$C_3H_{13}N_3OP^+$	138.0791	138.0794	2.2				5.56	1.65	>1.5	1.24	0.46	0.79
formyl-DiMPA	12	[M+H]$^+$	$C_3H_{11}N_3O_2P^+$	152.0583	152.0584	0.7				3.93	0.79	>1.5	1.06	0.25	0.43
DiMPA	13	[M+H]$^+$	$C_2H_{11}N_3OP^+$	124.0635	124.0635	0.8				3.39	0.54	>1.5	0.98	0.15	
formyl-MonoMPA	–	[M+H]$^+$	$C_2H_9N_3O_2P^+$	138.0427	138.0423	−2.9				2.75	0.25	>1.5	non-retained		
MonoMPA	–	[M+H]$^+$	$C_1H_9N_3OP^+$	110.0478	110.0473	−4.5				2.57	0.17	>1.5	non-retained		
Phosphoramide	–	[M+H]$^+$	$H_7N_3OP^+$	96.0321	96.0319	−2.1				2.34	0.06	>1.5	non-retained		

[a] Stored in 100% of 0.1% formic acid (pH 2.7) for two months, then six more months in 0.01% formic acid/acetonitrile (98:2, pH 3.1).
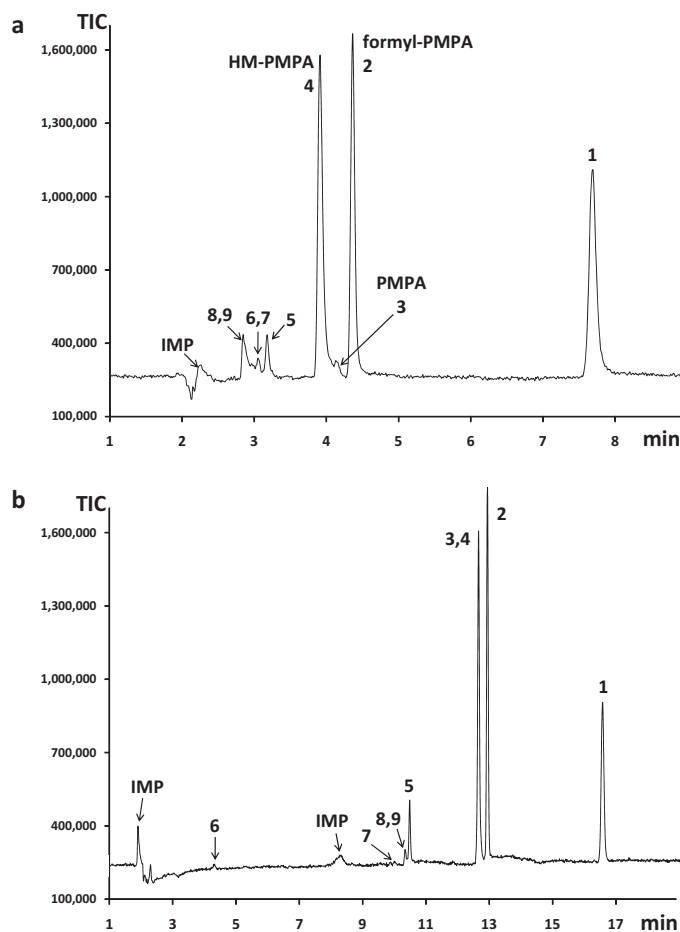
Fig. 2. XBridge Phenyl chromatogram of HMPA and its less polar oxidation products (after 10 min of reaction with permanganate) using (a) the preferred method 1 and (b) method 2. The compound names related to the peak numbers are listed in Table 1 (IMP: impurity from Teflon filter; UP: unidentified peak).

The chromatograms in Fig. 3 reveal that, after 6 h of reaction with permanganate, no hydroxymethylated intermediates are present, probably due to their faster oxidation or decomposition kinetics compared to the accumulating formylated and non-oxygenated compounds. Using gradient elution, baseline separation of all detected oxidation products was achieved (Table 1; Fig. 3a). Phosphoramide, the end product of HMPA oxidation [12] (detected after 72 h of reaction with permanganate), was slightly retained with a retention time of 2.34 min compared to a void time of 2.1 min. A stronger analyte retention through increased solvent polarity could not be achieved since the mobile phase consisted of solely water. Application of isocratic elution, the method of choice for the less polar oxidation products, resulted in worse separation of the more polar oxidation products (Fig. 3b). Thus, gradient elution is the preferred method for separation of the more polar products of HMPA oxidation.

For column comparison, separation of the four phosphoramide-based reference compounds was initially attempted with an XTerra Phenyl column of the same dimensions as the XBridge Phenyl column (250 mm × 4.6 mm I.D., 3.5 μm), the differences only being a different structure of the surface-bonded phenylalkyl group and a slightly lower surface coverage as noted in the Introduction. In contrast to the XBridge column, however, decreasing acetonitrile concentrations in the mobile phase led to decreasing retention times for the pristine XTerra Phenyl column, a behavior typical for normal phase chromatographic conditions. Furthermore, even under the fastest possible chromatographic conditions of
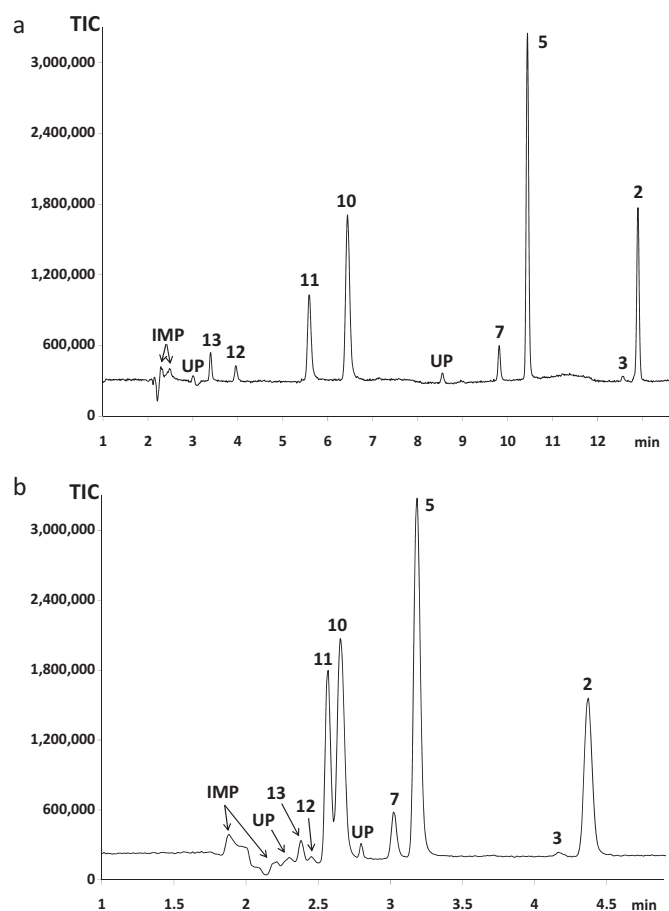
Fig. 3. XBridge Phenyl chromatogram of HMPA and its more polar oxidation products (after 6 h of reaction with permanganate) using (a) the preferred method 2 and (b) method 1. The compound names related to the peak numbers are listed in Table 1 (IMP: impurity from Teflon filter; UP: unidentified peak).

Fig. 4. Chromatogram of (a) HMPA and its less polar oxidation products (after 10 min of reaction with permanganate) and (b) the more polar HMPA oxidation products (after 6 h of reaction with permanganate) using the acid-treated XTerra Phenyl column. The compound names related to the peak numbers are listed in Table 1 (UP: unidentified peak).

0% acetonitrile and a flow rate of 1.3 mL/min (which was not further increased due to a back pressure of 330 bar), retention times were impractically long, with 48 min for PMPA and 319 min for HMPA. A difference in the chromatographic performance between the XBridge Phenyl and the XTerra Phenyl columns can be expected since the different structure of the phenyl-containing ligands of the two columns may lead to differences in solvation and retention properties. However, the magnitude of the observed differences for the investigated phosphoramide-based compounds was substantially larger than the one reported by Kiridena and co-workers [14]. They compared the separation characteristics of an XBridge Phenyl column and an XTerra Phenyl column using an acetonitrile-water mobile phase (acetonitrile ≥10%) for 70 analytes, including reduced and oxidized hydrocarbons as well as nitro, amino, and N-heterocyclic compounds, but no (highly polar) organophosphorus compounds. In their study, it was observed that the chromatographic performance of the two columns was quite similar under reversed-phase separation conditions.

A shorter, pristine XTerra Phenyl column was also used (150 mm × 2.1 mm I.D., 5 μm), which showed the same (normal-phase-like) chromatographic behavior as the longer XTerra Phenyl column and still resulted in impractically long retention times (HMPA 116 min at 0% acetonitrile, 0.5 mL/min, and 300 bar). Since the analytes had been successfully separated with an aged XTerra Phenyl column, it was hypothesized that the stationary phase in the aged column had undergone hydrolysis, resulting in altered chromatographic behavior. Thus, the impact of column storage in acidic solution on chromatographic performance to simulate a
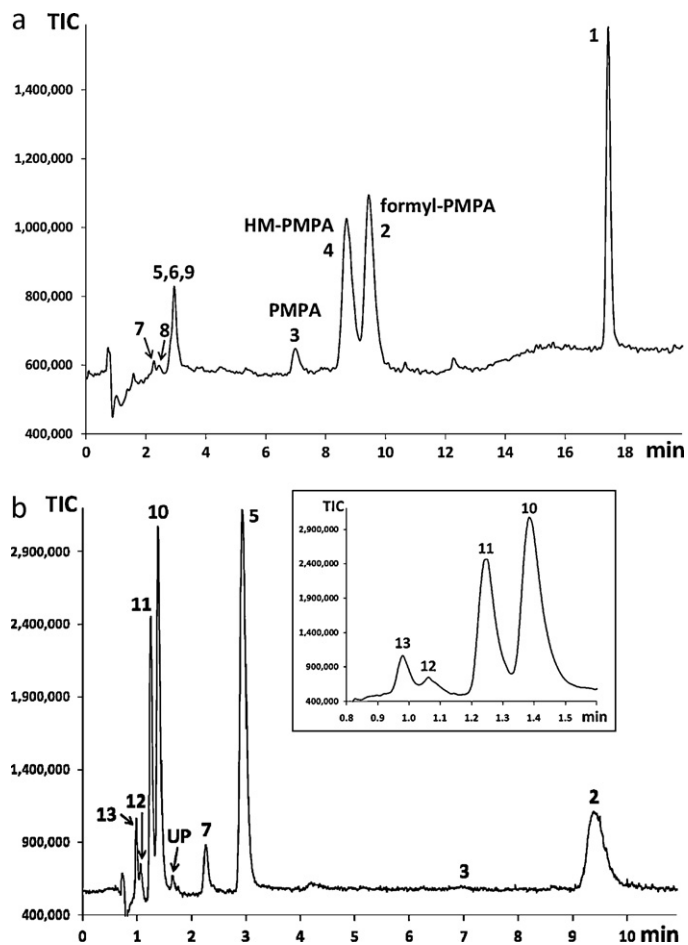
potential ageing process was investigated by flushing with 100% of 0.1% formic acid (pH 2.7) followed by two months storage. Storage at low pH greatly decreased retention times (e.g., HMPA 11.3 min at 2% acetonitrile, 0.5 mL/min), and also reversed the effect of acetonitrile concentration in the mobile phase, with decreasing acetonitrile concentrations in the mobile phase now leading to increasing retention times, as observed for the XBridge Phenyl column. The chromatographic performance of the modified XTerra Phenyl column after two months of storage in acidic solution is reported elsewhere [12].

We suspect that hydrolysis of the stationary phase altered the surface composition since it has been shown that acids can catalyze the hydrolysis of siloxane bonds (Si–O–Si) to produce surface silanol groups (Si–OH) [17–19]. These silanol functional groups would increase the polarity of the stationary phase, thereby improving wettability and enabling the expected reversed phase chromatographic performance. Collection of the hydrolysate from the column and analysis it via ESI–TOF–MS was attempted, however, without success.

The modified XTerra Phenyl column was then stored for six months in a slightly higher pH solution of 0.01% formic acid/acetonitrile (98:2, pH 3.1) and frequently used for HMPA analyses. During this time, the retention times continued to decrease (HMPA 11.3 min to 10.0 min at 2% acetonitrile, 0.5 mL/min), indicating further hydrolysis. Based on the superior separation obtained with this acid-treated column, we decided to use it for the method

Table 2
Linear regression data, linear range, limit of detection (LOD), limit of quantification (LOQ), and inter-day variability for the reference compounds.

Analyte	HMPA	PMPA	TetMPA	TriMPA
Linear regression equation	$y = 1,680,000x + 66,000$	$y = 1,190,000x + 196,000$	$y = 834,000x + 194,000$	$y = 597,000x + 244,000$
R^2	0.9991	0.9980	0.9995	0.9988
Linear dynamic range (μM)	0.34–30	0.48–15	0.66–15	0.93–15
LOD (μM)	0.10	0.14	0.20	0.28
LOQ (μM)	0.34	0.48	0.66	0.93
Inter-day variability (%RSD, $n = 6$)	7.0	6.9	8.6	10.7

development reported here (Table 1, Fig. 3a and b), and the storage conditions were changed to DI water/acetonitrile (50:50) to minimize hydrolysis.

For the modified XTerra Phenyl column, one gradient method was developed and applied to all samples. The initial HMPA oxidation products HM-PMPA and formyl-PMPA were not completely baseline-separated ($R_S = 0.67$, Fig. 3a), but good separation was achieved for PMPA ($R_S > 1.5$). Poor separation, however, was observed for the tetramethylphosphoramides (peaks 5–9) when hydroxymethylated derivatives were present. When only non-oxygenated and formylated compounds were present (Fig. 3b; after 6 h of reaction with permanganate), the two tetramethylphosphoramide compounds (peaks 5 and 7) were completely baseline-separated. With increasing analyte polarity (decreasing number of methyl substituents), the chromatographic performance deteriorated. Both TriMPA (peak 11) and dimethylphosphoramide (DiMPA, peak 13) were not fully separated from their respective formylated derivative (peaks 10 or 12, $R_S = 0.60$ and 0.43, respectively). The very polar methylphosphoramide (MonoMPA), formyl-methylphosphoramide (formyl-MonoMPA), and phosphoramide were not retained on the modified XTerra Phenyl column, and stronger retention could not be achieved due to the absence of acetonitrile in the mobile phase (i.e., mobile phase composition 100% of 0.1% formic acid) (see Fig. 4).

For the two XBridge methods as well as the XTerra method, it can be seen that the elution order was mainly determined by the number of methyl substituents, i.e., HMPA had the highest retention time, followed by the pentamethylphosphoramides, then the tetramethylphosphoramides, and so forth. This behavior indicates that hydrophobic interactions between the methyl groups and the surface-bonded alkylphenyl ligands were a major retention mechanism.

Within one group of phosphoramides with the same number of methyl substituents, however, differences in the elution order were observed as a function of mobile phase composition. When acetonitrile concentrations were $\leq$9% on the XBridge Phenyl column and $\leq$10% on the XTerra Phenyl column, the general elution order was –H, –CH$_2$OH, –CHO (for instance, PMPA, HM-PMPA, formyl-PMPA). At higher acetonitrile concentrations, the general elution order –CH$_2$OH, –H, –CHO was observed on both columns. Quantum chemical calculations of the dipole moments revealed that the order of polarity for the pentamethylphosphoramides is PMPA (6.2 Debye) > HM-PMPA (4.7 Debye) > formyl-PMPA (1.6 Debye). Thus, at low acetonitrile concentrations, the XBridge and XTerra Phenyl columns show the expected chromatographic performance under reversed-phase conditions, while at higher acetonitrile concentrations, the elution order of PMPA and HM-PMPA was unexpectedly inverted. This indicates that mechanisms other than polar interactions may also contribute to the retention of phosphoramide compounds with the same number of methyl substituents.

3.2. Time-of-flight mass spectrometry

Capillary, fragmentation, and skimmer voltages as well as drying gas flow rate and nebulizer gas pressure were initially opti-

mized to maximize detector sensitivity for the phosphoramide compounds. The calibration curves for the four available reference compounds HMPA, PMPA, TetMPA, and TriMPA ($R^2 \geq 0.998$, Table 2), which revealed a linear range of approximately two orders of magnitude, were obtained by plotting peak areas (i.e., total ion currents (TIC) integrated over time) against concentration. The slopes of the linear regression equations revealed that the detector sensitivity decreased with increasing polarity of the analytes (i.e., decreasing degree of methylation). This is likely due to a decreased affinity of more polar compounds for the ESI droplet surface, where excess charge exists, and/or decreasing ion evaporation rates from the ESI droplets with increasing analyte polarity or solvation energy [20]. Moreover, the linear range decreased with increasing analyte polarity, while LOD and LOQ (0.10–0.28 μM and 0.34–0.93 μM, respectively) increased with increasing analyte polarity. The inter-day variabilities for all four reference compounds showed an acceptable precision of the method (6.9–10.7% RSD).

Identification of HMPA oxidation intermediates was performed via TOF–MS accurate mass measurement. Table 1 lists measured observed mass-to-charge ratios (m/z) and errors with respect to the theoretical masses for all detected compounds, including di- and monomethylated phosphoramides as well as phosphoramide (detected after 72 h of reaction with permanganate). Fragmentation of all structures occurred at the P–N bond, in agreement with previous observations for cyclophosphamide, N,N',N''-triethylenephosphoramide, and N,N',N''-triethylenethiophosphoramide [21].

It is noted that HMPA N-oxide and its rearrangement product ((bis(dimethylamino)phosphinyl)oxy)dimethylamine, which have been detected after HMPA oxidation by m-chloroperoxybenzoic acid [22], have the exact same mass as HM-PMPA and possibly the same fragmentation pattern. Thus, the chromatographic peak corresponding to m/z 178.1104 (see Table 1) could not be unambiguously assigned to any particular compound based on ESI–TOF–MS analysis alone. However, after sample storage at room temperature for ca. two weeks, it was observed that this analyte had disappeared, and equimolar amounts of formaldehyde (for method details, see Ref. [12]) and PMPA had been produced. Thus, it was concluded that this detected m/z corresponded to HM-PMPA, which is a well-known formaldehyde generator [12,23], while ((bis(dimethylamino)phosphinyl)oxy)dimethylamine does not generate formaldehyde [22].

Analyses for carboxylated oxidation products in negative ionization mode were not performed since these were shown to be produced in minor amounts only [12].

4. Conclusion

A novel RPLC/ESI–TOF–MS method was developed, which is the first and currently only method that can be used to separate and detect HMPA and its biotic and abiotic oxidation products. Whereas the use of several different columns failed to achieve satisfactory chromatographic performance, only an XBridge Phenyl and an XTerra Phenyl column were capable of separating the majority of the phosphoramide-based analytes. The advantage of the

XBridge Phenyl column is a better separation performance through the use of two elution methods, especially for the very polar oxidation products with low degree of methylation, as well as for the less polar oxidation products HM-PMPA and formyl-PMPA, which are the major initial products of abiotic oxidation by permanganate. Furthermore, the column can be used for separation of phosphoramide-based compounds without chemical modifications. The advantage of the formic acid-treated XTerra Phenyl column is that one elution method can be applied for all stages of oxidation, and that the less polar oxidation product PMPA is baseline-separated from the oxygenated HM-PMPA and formyl-PMPA. This can be advantageous for biological systems, where formylated intermediates are only minor oxidation products [6,9].

The pristine XTerra Phenyl column showed a substantially different chromatographic behavior from the XBridge Phenyl column, with acetonitrile concentrations in the mobile phase having an opposite effect on retention times as well as revealing impractically long retention times. Only after storage in a low-pH formic acid solution, the chromatographic performance of the XTerra Phenyl column was altered in a way suitable for phosphoramide analysis, most likely due to hydrolysis of the siloxane bonds in the stationary phase.

Acknowledgments

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