Title

On-line solid phase extraction - liquid chromatography - mass spectrometry for trace determination of nerve agent degradation products in water samples

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Keywords

On-line SPE-LC-MS; Porous graphitic carbon; Hydrophilic interaction liquid chromatography; Alkyl methylphosphonic acid; Diethyl phosphate

Abbreviations

OPCW: Organisation for Prohibition of Chemical Weapons; AMPAs: alkyl methylphosphonic acids; EMPA: ethyl methylphosphonic acid; IMPA: isopropyl methylphosphonic acid; PMPA: pinacolyl methylphosphonic acid; DEP: diethyl phosphate; AF: ammonium formate; PGC: porous graphitic carbon

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Abstract

Three primary nerve agent degradation products (ethyl-, isopropyl- and pinacolyl methylphosphonic acid) have been determined in water samples using on-line solid phase extraction - liquid chromatography and mass spectrometry (SPE-LC-MS) with electrospray ionization. Porous graphitic carbon was employed for analyte enrichment followed by hydrophilic interaction chromatography. Diethylphosphate was applied as internal standard for quantitative determination of the alkyl methylphosphonic acids (AMPAs). By treating the samples with strong cation-exhange columns on Ba, Ag and H form, the major inorganic anions in water were removed by precipitation prior to the SPE-LC-MS determination. The AMPAs could be determined in tap water with limits of detection of 0.01-0.07 μg L^{-1} with the $[M-H]^-$ ions extracted at an accuracy of ± 5 mDa. The within and between assay precisions at analyte concentrations of 5 μg L^{-1} were 2-3%, and 5-9% relative standard deviation, respectively. The developed method was employed for determination of the AMPAs in three natural waters and a simulated waste water sample, spiked at 5 μg L^{-1}. Recoveries of ethyl-, isopropyl- and pinacolyl methylphosphonic acid were 80-91%, 92-103% and 99-106%, respectively, proving the applicability of the technique for natural waters of various origins.

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1 Introduction

The organophosphorous compounds known as nerve agents are extremely toxic to humans by disrupting the mechanism for transfer of nerve messages to organs. The nerve agents are classified as chemical weapons, and all development, stockpiling and use of the compounds are banned by the Organisation for Prohibition of Chemical Weapons (OPCW) [1]. Yet, such compounds have been employed in both military conflicts and terrorism and still pose a threat against civilians [2]. When released into the environment and in contact with water, the nerve agents first degrade to their corresponding alkyl methylphosphonic acids (AMPAs) (Fig. 1). Further degradation by loss of the alkyl groups results in the less informative methylphosphonic acid, but this reaction is very slow in water [3,4]. Hence, if the intact nerve agents are not determined, their primary degradation products are the most likely (and the most informative) markers to be found in water samples.

Fig. 1 in approximately here

For determination of the AMPAs in aqueous matrices, liquid chromatography combined with electrospray ionisation mass spectrometry (LC-ESI-MS) has been extensively applied [5-11]. The ionic character of the AMPAs (pKa 2-3, [4]) makes them suitable for separation by capillary electrophoresis as well [12-14]. Owing to its sensitivity and selectivity, gas chromatography (GC) with MS detection has also been widely employed for determination of the compounds [15-18]. The latter technique requires more sample preparation, however, since the AMPAs must be isolated from the aqueous matrix and transformed to their respective phosphonic esters prior to GC-MS determination.

When only trace amounts of the AMPAs are present in the sample, enrichment of the analytes prior to determination may be necessary. Solid phase extraction (SPE) has been the major technique for preconcentration and isolation of the AMPAs. Isolation of the deprotonated AMPAs on strong anion exchange cartridges has been widely applied [17-20], while also mixed-mode anion exchange has

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been utilized [16]. SPE based on reversed phase (RP) interactions has been performed as well, using acidification of the sample to protonate the AMPAs prior to extraction [15,21]. Le Moullec et al. have applied SPE with molecularly imprinted polymers (MIPs) and obtained good recoveries of AMPAs [21,22], but a prerequisite for the extraction was a change from aqueous to organic solvent. Isolation of the AMPAs from urine samples has also been performed on non-bonded silica [9,23]. However, since adsorption is due to strong polar interactions, the analytes had to be percolated in an excess of organic solvent prior to extraction. For enhanced sensitivity and ease of application, automated on-line SPE-LC-MS techniques are highly advantageous [24]. Some alkyl phosphonic diacids have been determined in water samples using a zirconia preconcentration column coupled on-line to an ion chromatography (IC) column and an RP column in series, followed by conductivity detection [25]. Katagi et al. employed on-line RP SPE-LC-MS/MS for determination of three AMPAs in various matrices after solvent change and derivatisation of the analytes prior to the SPE-LC-MS procedure [26]. However, no on-line technique for determination of the underivatised AMPAs has been published to our knowledge.

Here we report for the first time an automated on-line SPE-LC-MS technique for the determination of AMPAs in aqueous matrices. The analytes were preconcentrated on a porous graphitic carbon (PGC) column and separated by hydrophilic interaction liquid chromatography (HILIC) on a zwitter-ionic column. To improve the robustness of the method, major inorganic anions naturally occurring in water were removed by precipitation and adjustment of pH prior to extraction. Analytes investigated were ethyl methylphosphonic acid (EMPA), isopropyl methylphosphonic acid (IMPA) and pinacolyl methylphosphonic acid (PMPA), which are primary hydrolysis products of the nerve agents VX, sarin and soman, respectively (Fig. 1).

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PGC is known for its unique properties, providing retention by both hydrophobic and polar interactions [27-29], and has been employed for separation of AMPAs in several studies [30-32]. In 2000, Hennion reviewed PGC for SPE applications with emphasis on its capability to retain very polar and water soluble compounds [33]. Since then it has been employed for trapping various polar analytes like anatoxin-a [34], herbicides [35] and degradation products of atrazine [36] in water. Moreover, strong retention of polar analytes on PGC has been observed both with pure water and with high amounts of organic solvents in the mobile phase [37]. When evaluating PGC for separation of the AMPAs, Mercier et al. found that the compounds were totally retained with pure water as the mobile phase, but effectively eluted by the introduction of a carboxylate anion [31]. If the target analytes are retained on PGC with high amounts of organic solvent in the mobile phase, and eluted when introducing a carboxylate anion, this makes the on-line coupling with HILIC separation favourable. HILIC has been established as a valuable complementary approach to RPLC for the separation of polar compounds, and the applicability of HILIC in environmental analysis has recently been discussed in several reviews [38-40]. Separation of the highly polar AMPAs by HILIC has been demonstrated as well [9,23]. A well-known advantage of HILIC is the enhanced ionisation efficiency in ESI due to the high amounts of organic solvent in the mobile phase, resulting in increased sensitivity. In the present study, PGC and HILIC have successfully been combined in an on-line SPE-LC-MS method for trace determination of AMPAs in water samples.

2 Experimental

2.1 Chemicals and solutions

Ethyl methylphosphonic acid (EMPA, CAS 1832-53-7, 98%), pinacolyl methylphosphonic acid (PMPA, CAS 616-52-4, 97%), dibutyl phosphate (CAS 107-66-4, 97%) and diphenyl phosphate (CAS 838-85-7, 99%) were purchased from Sigma-Aldrich Chemie GmbH Steinheim, Germany. Isopropyl methylphosphonic acid (IMPA, CAS 1832-54-8) was delivered by Cerilliant Corporation, Round Rock, TX, USA, and diethyl phosphate (DEP, CAS 598-02-7, 99.5%) was produced by ChemService, West

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Chester, PA, USA. Ammonium formate (AF, 98%) was purchased from BDH Laboratory Supplies, Dorset, UK. Acetonitrile (99.9%), sodium chloride (99.5%), sodium hydrogen carbonate (99.5%), potassium sulphate (99.0%) and calcium chloride (anhydrous) were delivered by Merck KGaA, Darmstadt, Germany. Polyethylene glycol 300 (PEG300) was produced by Fluka Chemie GmbH, Buchs, Switzerland. Laboratory type I water (classified according to the American Society of Testing and Materials, D1193-91) was delivered in-house by Maxima ultra pure water system from ELGA, Marlow, UK.

A stock solution of the AMPAs was prepared at 0.5 mg mL^{-1} by diluting 25 mg of the neat agents in 50 mL acetone. Further dilutions were made in type I water and the final dilutions into working solutions were made in tap water or type I water. An internal standard (IS) stock solution was prepared by diluting 12.5 mg of DEP in 25 mL acetone, and this was further diluted in type I water to a 200 µg L^{-1} working solution. All solutions were stored at 4 °C.

2.2 Instrumentation

The SPE-LC-MS analyses were performed on an Ultimate 3000 RSCL (Dionex Corporation, Idstein Germany), coupled to a MicroTof-Q II mass spectrometer (Bruker Daltonics, Bremen, Germany). A schematic diagram of the setup for sample loading and chromatographic separation is shown in Fig. 2. The SPE-LC system was located inside an FLM-3100 flow manager supported with two 10-ports two position micro switching valves (only one used in the final method), and with a constant temperature of 30 °C. Loading flow (P1, Fig. 2) was delivered from a DGP-3600M dual gradient pump (2 x 10-2500 µL min^{-1}) via a WPS-3000 autosampler with variable volume split-loop injection and 500 µL (max volume) sample loop. Micro flow was delivered from channel 2 of the DGP-3600M pump (P2) via an electronic flow splitter (1:6). Solvents delivered by P1 were A) type I water, B) acetonitrile, and solvents delivered by P2 were A) acetonitrile, B) type I water, C) 200 mM AF. Preconcentration was performed on a Hypercarb column (2.1x10 mm, 5 µm) from Thermo Scientific, Bellefonte, PA,

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USA, and separation was achieved with a ZIC®-HILIC column (1x150 mm, 3.5 µm, 200 Å) from Merck SeQuant AB, Umeå, Sweden.

Fig. 2 in approximately here

The sample was loaded onto the PGC column from P1 via the 500 µL sample loop, starting with 100% A (type I water) at 400 µL min^{-1}. From the moment of start sample loading, a 2 min linear gradient changed the mobile phase composition of the loading flow from 100% A to 15% A/85% B. After 7 min, the switching valve shifted to "Inject" position and the PGC column was backflushed with gradient elution from P2, starting with 85% A, 10% B, 5% C at 40 µL min^{-1}. The gradient was 85-60% A in 0-6 min, followed by 60% A for 6-15 min. C was 5% during the whole run, ensuring a buffer concentration of 10 mM. At 15 min, the switching valve was shifted to "Load" for conditioning of the PGC column with 100% H_2O prior to next injection.

The ESI was operated in negative ionisation mode with a capillary voltage of 3500 V and end plate offset of -500 V. The collision cell energy was 5.0 eV and collision RF peak-to-peak voltage was 170. Nitrogen for nebulizing gas (0.6 bar) and drying gas (6.0 L min^{-1}, 200 °C) was provided by a high purity generator (Domnick Hunter, Durham, UK), while compressed N_2 (purity 6.0) was used as collision gas. Mass spectra were acquired in the *m/z* range of 50-500, and quantitative calculations were performed with peak areas of the extracted quasi molecular ions $[M-H]^-$ ± 5 mDa.

2.3 Method development

The elution order of the AMPAs on PGC was investigated with the cartridge mounted between the injection port and MS, and with solvents delivered from P2. Measurements of the elution volume were performed in backflush mode and with the outlet of the PGC cartridge connected to the ESI-MS. The AMPAs were dissolved in type I water at 5 µg L^{-1}, and 500 µL was loaded. Subsequently, the

ACN/H_2O composition was changed to the conditions at desorption. Isocratic elution was performed with ACN/H_2O compositions varying from 7 to 95% H_2O with 10 mM AF in the mobile phase and from 10 to 95% H_2O with 20 mM AF. The elution volumes were determined as the times where the quasi molecular signal of the analytes decreased below 1000 counts (90-95% of complete desorption). The sample loading capacity was investigated with the PGC cartridge mounted in frontflush mode without separation column. Thus, the analyte signals were monitored both during sample loading and desorption. The mobile phase consisted of type I water at the time of start sample loading, and was subsequently changed to 90% ACN with a 2 min gradient. At 7 min, 10 mM AF was introduced in the mobile phase for desorption. The breakthrough volumes were found by injecting a 10 µg L^{-1} solution of AMPAs at volumes increasing stepwise from 100 to 500 µL.

Recoveries of the AMPAs from the solutions where inorganic ions were added to type I water were investigated with the setup and conditions as described in Section 2.2, and with a sample volume of 500 µL. Sodium chloride (100 mg L^{-1} Cl^-), potassium sulphate (100 mg L^{-1} SO_4^{2-}) and sodium hydrogen carbonate (100 mg L^{-1} HCO_3^-) were added in separate aliquots to water containing the AMPAs at 5 µg L^{-1}. Recoveries were calculated by comparing the obtained peak areas with those where the same amounts of AMPAs in type I water only were injected (n=2). Only one injection was performed from each solution containing high concentration of inorganic ions, to avoid severe contamination of the PGC column.

2.4 Precipitation of inorganic anions

The On guard Ba/Ag/H 2.5 mL cartridges for precipitation of anions were delivered by Dionex Corporation, Sunnyvale, CA, USA. The capacity of each cartridge was 2.2-2.6 meq for Ba and Ag, and 0.8 meq for H. A disposable syringe (Omnifix 20 mL, B. Braun Melsungen AG, Melsungen, Germany) was attached to the cartridge, and a Millex PVDF 0.22 µm filter (Millipore, Carrigtwohill, Co. Cork. Ireland) was attached to the outlet for removal of colloidal forms of the precipitates. The syringe and

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cartridge was mounted in vertical position to a Syringe pump (SAGE instruments, Cambridge, MA, USA) and the washing and sample solutions were passed through by positive pressure at 3 and 1.5 mL min^{-1}, respectively. Prior to application, the samples (10 mL) were added 50 µL of a 400 mM $CaCl_2$ solution (2 mM Ca^{2+} added) to improve the displacement of Ba ions. Conditioning of the cartridge prior to sample application was performed with 15 mL of type I water. The first 7 mL eluted were discarded and the next 2 mL were collected for analysis. From this, 1.50 mL was transferred to an auto sampler vial and 40 µL of a 200 µg L^{-1} IS solution was added.

For determination of the discard volume, three replicate cartridges were loaded with a solution spiked with the AMPAs at 50 µg L^{-1}, and with 2 mM $CaCl_2$. Fractions of 1 mL each of the eluate were collected for analysis from 5 to 10 mL eluted. From these, 50 µL was analysed by the SPE-LC-MS method. Possible loss of analytes during anion precipitation was investigated by comparing peak areas in samples where the AMPAs were added to the water (5 µg L^{-1}) before and after being treated with the precipitation column. The samples were analysed according to the settings in Section 2.2 (500 µL loaded, n=6).

2.5 Method validation

Method validation was performed with the analytes added to tap water collected over a period of five days. The tap water samples were kept in borosilicate glass 3.3 (Duran) bottles with screw caps having Teflon gaskets (Schott, Mainz, Germany), and stored at 4 °C. Linearity was investigated at six concentration levels, of 0.2, 1.0, 5, 12 and 20 µg L^{-1} as well as at the calculated quantification limits (LOQs). Additional samples at 3, 9 and 16 µg L^{-1} were prepared for EMPA and IMPA, due to the limited linear range of these compounds. Weighted linear regression was performed when calculating the correlation coefficients. Within assay repeatability was investigated with six replicates at two concentration levels, at LOQ and 5 µg L^{-1}, and each replicate was individually treated with an anion precipitation column prior to SPE-LC-MS. For investigation of the between assay repeatability,

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the analytes were dissolved in tap water (5 µg L^{-1}) collected at six separate days and analysed the respective day. The sample for robustness testing was prepared by adding sodium chloride (200 mg L^{-1} Cl^-), potassium sulphate (200 mg L^{-1} SO_4^{2-}) and sodium hydrogen carbonate (100 mg L^{-1} HCO_3^-) into the same aliquot of tap water, containing the AMPAs at 5 µg L^{-1}. Six replicates of the sample were treated with precipitation columns and analysed. Recoveries were calculated by comparing the obtained peak areas with those where the AMPAs were added to tap water which had been pretreated with an anion precipitation column (n=6).

2.6 Spiked natural waters and simulated waste water

Three natural water samples were collected for recovery tests: (1) river water from Skarselva, collected in a rural recreation area (clear water), (2) river water from Akerselva, collected in the city of Oslo (this sample was weakly yellowish, indicating moderate amounts of humic substances) and (3) water from a rain pool, collected on the roadside of a suburban city street. The rain water was yellowish and contained considerable amounts of suspended solids. All samples were collected in 500 mL borosilicate glass 3.3 (Duran) bottles (Schott, Mainz, Germany) filled to the top, and stored at 4 °C. Particles in the water were allowed to settle for 24 hours and the mid top layers were subsequently selected for analysis. The pH levels (determined with pH-indicator strips from Merck KGaA) were measured to approximately pH 6 in the three samples. A simulated waste water sample was prepared by adding 500 mg L^{-1} PEG300, 200 mg L^{-1} Cl^-, 200 mg L^{-1} SO_4^{2-} and 100 mg L^{-1} HCO_3^- to tap water (pH 8). Spiked samples were prepared by adding the AMPAs at 5 µg L^{-1} to aliquots of 100 mL. Three replicates of each spiked sample were pre-treated with the precipitation columns as described in section 2.4 and subjected to SPE-LC-MS determination (two injections of each replicate). One blank of each collected water sample was prepared and analysed for possible traces of the AMPAs or DEP. Recoveries were calculated by comparing the obtained peak areas (IS corrected) with those where the AMPAs were added to tap water at 5 µg L^{-1} (n=6).

3 Results and discussion

Because of their very hydrophilic character, PGC was explored as a possible sorbent for extraction of AMPAs from aqueous matrices. The PGC material has shown the ability to retain polar analytes even with high amounts of organic solvents in the mobile phase [37], while retained compounds like the AMPAs have been eluted by the introduction of carboxylate anions [31]. An elution step with high amounts of organic modifier makes it highly compatible with HILIC, which is very well suited for separation of polar compounds like the AMPAs. Hence, the combination of PGC and HILIC has been investigated for determination of AMPAs in an on-line SPE-LC-MS setup. Unless otherwise mentioned, all method development was performed with the 2.1x10 mm preconcentration column and the 1x150 mm separation column described in Chapter 2.2.

3.1 Retention of AMPAs on PGC

3.1.1 Retention behaviour

When eluted through the PGC column with H_2O/ACN (90/10) and 10 mM AF, the retention order of the AMPAs was PMPA > IMPA > EMPA, which was expected for retention based on hydrophobic interactions. With only type I water as the mobile phase (no AF), complete retention of the analytes was obtained. Total retention was maintained when ACN was introduced in the mobile phase, even up to 95%, and no breakthrough was observed after elution with 100 column volumes of ACN/H_2O (95/5). Thus, electrostatic interactions between the negatively charged AMPAs and the surface of PGC were obviously also taking part. The interactions involved in retention of anions on PGC have been discussed in several papers [27-29], but West et al. recently stated that the explanation models are still somewhat vague [27]. Further exploration of the reason for this phenomenon is beyond the scope of this work, but apparently strong anion interactions are taking part between the AMPAs and PGC. Desorption of the compounds was achieved by introducing AF into the mobile phase with the anion acting as competitor for the adsorption sites. Mercier et al. found that the formate ion had higher elution strength for the AMPAs on PGC compared to acetate [31]. Hence, AF was chosen in

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the present study for desorption from the PGC column and for chromatographic separation of the analytes on the HILIC analytical column.

3.1.2 Desorption volume

The flow through the PGC column was reversed for desorption as backflushing generally gives lover desorption volumes compared to frontflushing. Fig. 3 shows how the desorption volume of EMPA and PMPA varied as a function of the ACN/H_2O composition with 10 and 20 mM AF in the mobile phase. Approximately two column volumes were needed for desorption of the analytes with 30-70% water in the mobile phase. Almost no reduction in the desorption volume was seen when increasing the buffer concentration from 10 to 20 mM within this range, even though desorption of the AMPAs was dependent on the presence of formate ions. At water contents below 30%, which are typical for HILIC separations, the desorption volume increased progressively for both compounds. A somewhat lower desorption volume with 20 mM AF concentration (approximately 20% reduction at 10% H_2O) was observed. Similar trends were seen for PMPA at water contents above 70%, while no significant change was observed for the more polar EMPA.

Fig. 3 in approximately here

3.1.3 Loading capacity

The loading capacity of the preconcentration column is an important issue as a higher loading volume will increase the sensitivity of the method. The maximum loading volume investigated was 500 µL, however, since the autosampler was not configured for larger injection volumes. When dissolved in type I water, a sample volume of 500 µL could be introduced with full retention of the AMPAs. With tap water as the sample matrix, PMPA was still fully retained with 500 µL sample loaded while breakthrough occurred for EMPA and IMPA at loading volumes of approximately 200 and 300 µL, respectively. A reduction in the loading flow from 400 to 200 µL min^{-1} gave no significant

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improvement in the loading capacity. Elfakir et al. showed that PGC was able to retain inorganic anions present in water, while the inorganic cations had virtually no retention [41]. Hence, it was assumed that the loss of retention of the AMPAs when dissolved in tap water was caused by inorganic anions competing for the adsorption sites on PGC. Major anions of concern in most fresh water samples are chloride, sulphate and hydrogen carbonate, and the effect of the presence of these ions was investigated by adding 100 mg L^{-1} of each into separate aliquots of type I water, containing the AMPAs at 5 µg L^{-1}. Table 1 gives the recoveries of the AMPAs from the different matrices when 500 µL was loaded on the PGC cartridge. It was evident that the retention, and hence recoveries, of EMPA and IMPA was severely affected by all of the anions. Addition of the divalent sulphate ions resulted in complete breakthrough of the compounds and also severely affected the recovery of PMPA. Since the levels of 100 mg L^{-1} of these ions must be expected in some environmental samples, removal or reduction of the ions prior to PGC SPE-LC-MS would be of great advantage. Otherwise, the injection volume would have to be reduced (or adsorbent bed increased) when using PGC for preconcentration.

Table 1 in approximately here

3.2 Removal of inorganic anions

Removal of selected inorganic anions by the use of precipitation columns is commonly used for matrix elimination in ion chromatography [42]. In the present study, removing all the major inorganic anions naturally occurring in water prior to PGC SPE was considered highly desirable. For this purpose a strong cation-exchange cartridge consisting of a layered styrene-based sulfonate resin on Ba, Ag and H form was preferred. The Ba form resin removes sulphate by on-column precipitation while the Ag form removes halides and phosphate [43]. The H form resin is located at the end in order to trap soluble silver and other cations in the sample matrix. Thus, the H form resin also lowers

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the pH of alkaline samples and thereby shifts the equilibrium of weak acids like the hydrogen carbonate towards its protonated form.

For free Ba and Ag ions to react with the anions in solution, displacing cations must be present in the sample. The Ag^+ ions are easily displaced by both monovalent and divalent cations naturally occurring in water. However, the displacement of Ba^{2+} ions is more dependent on divalent cations, which may not be present at sufficient amounts for complete sulphate removal. Slingsby and Pohl presented a solution to the problem by adding extra calcium to the sample as a divalent displacing cation in the form of $CaCl_2$ [42]. Chloride was chosen as counter ions since these can be removed by precipitation with the Ag resin if desired. It was our experience as well that addition of $CaCl_2$ helped increasing the sulphate removal when treating water with the Ba/Ag/H column (results not shown). Hence, all samples were added 2 mM $CaCl_2$ prior to precipitation column treatment.

The amount of sample needed for precipitation cartridge equals the discard volume and the volume collected for SPE-LC-MS analysis (2 mL). The first part of the sample will be diluted with residual wash water as it flows through the resin bed. The minimum volume to be discarded should then be set when no dilution occurs, and this was determined by collecting and analysing successive fractions of the eluted sample (results not shown). The discard volume was determined to be 7 mL, which required a total applied volume of at least 9 mL. Co-precipitation of some analytes may occur when treating samples with the Ba and Ag columns [43]. Possible loss of analytes was therefore investigated using tap water containing the AMPAs at 5 µg L^{-1}. Recoveries from the precipitation columns (n=6) were 99 ± 3% for EMPA, 90 ± 2% for IMPA and 91 ± 2% for PMPA. Losses of analytes of ≤ 10% were considered acceptable and highly preferable compared to the low recoveries obtained from non-treated samples containing inorganic anions. The time consumed for each sample in this single precipitation cartridge setup was approximately 15 min. If many samples are to be handled this way, parallel treatment on manual or automated SPE systems may be performed.

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3.3 Column selection and system dimensions

A large diversity in HILIC stationary phases is found, and thereby also a considerable variation in resolution of polar analytes [44-46]. Three silica-bound HILIC stationary phases were investigated for separation of the AMPAs in preliminary studies: ZIC®-HILIC, TSKgel Amide-80 and Polar-100, containing functional groups of sulfoalkylbetaine, carbamoyl and 2-ethoxy-hydroxy, respectively. Good resolution was achieved both with the ZIC®-HILIC and TSKgel Amide-80 column, while the Polar-100 column gave almost no retention of the AMPAs in HILIC mode (70-90 % ACN). Slightly better peak symmetry and separation between EMPA and IMPA were accomplished with the sulfoalkylbetaine stationary phase compared to carbamoyl. Hence, the ZIC®-HILIC column was employed in the present study.

Initially a setup with a 1x10 mm PGC SPE column (loading volume 100 µl) and a 0.3x150 mm ZIC®-HILIC column was investigated. High separation efficiency was achieved with this setup, but repeatability of the peak areas between injections was very poor. When changing to a 1x150 mm chromatographic column with the same stationary phase, highly repeatable peak areas were achieved between successive injections. The poor repeatability found with the 0.3 mm i.d. column setup may therefore be caused by the relatively large volume of AF free eluent, present in the PGC column at the time of start elution, and that is forced through the separation column. Even though no large variations in retention time or peak symmetry was observed, the conditions for electrospray ionisation may have varied between analyses. The larger i.d. column would be more tolerable against the volume of buffer free eluent, with faster re-establishment of stable AF concentration. Hence, the dimension ratio between SPE and LC column of $1^2/0.3^2$ may not be compatible with the solvents and flow rate used. Instead, a setup with a 2.1x10 mm PGC column and the 1.0x150 mm chromatographic column was explored, with a ratio of dimensions of the SPE and LC column of $2.1^2/1.0^2$.

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3.4 On-line SPE-LC-MS conditions

Sample loading was performed with type I water as the mobile phase, with subsequent change of the ACN/H_2O composition to the analytical column start gradient conditions within 2 min. It was important that the PGC column was in equilibrium with the separation start gradient at the moment of desorption, otherwise peak broadening occurred. Due to the gradient delay, 7 min was needed to assure homogeneous mobile phase composition at the time of sample desorption with a loading flow rate of 400 µl min^{-1}. As shown in section 3.2, the desorption volume increased progressively for increasing ACN contents from 70% to 93%. Isocratic elution with 70-80% ACN was therefore examined, but abandoned due to peak broadening. A higher concentration of AF was favourable for desorption, but considerable ion suppression was observed in the ESI-MS at buffer concentrations of 20 mM or higher. A better approach was to keep the buffer concentration below 20 mM and the ACN content above 85%. This allowed refocusing of the analytes on the HILIC column prior to ACN/H_2O gradient elution. The best chromatographic performance and signal intensity were achieved with 10 mM AF and a start gradient of 90% ACN. However, a gradient starting with 85% ACN was preferred as this gave better repeatability of the peak areas between injections. The reason for the better repeatability with higher amounts of water in the mobile phase is not fully understood, but it may be caused by faster incorporation of the water plug and establishment of stable AF concentration. Possible carry over in the system was examined by analysing samples of 10 µg L^{-1} AMPAs followed by blank samples. No memory effects were observed. Fig. 4 shows the extracted ion chromatograms (EICs) of a tap water sample with the AMPAs added at 5 µg L^{-1}. The negative ion ESI-MS spectra were almost exclusively dominated by the deprotonated ions. Neither adduct ions nor cluster formations commonly observed in positive ESI was observed, and the collision cell energy was set low (5.0 eV) to prevent the typical fragmentations by loss of alkene [47]. The accurate mass measurements of the TOF (commonly within ± 2 mDa) provide high specificity in determination of the AMPAs without employment of tandem MS. Hence, full scan mass spectra are acquired, which allows for screening for all compounds of interest.

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Fig. 4 in approximately here

After running the 15 min LC gradient, a relatively long column re-equilibration time of 25 min (15 column volumes) was needed prior to subsequent injections. The need for larger equilibration volumes in HILIC compared to RPLC when performing gradient elution is emphasized in the manufacturer guidelines [48], and has also been experienced for ZIC-HILIC columns by others [49,50]. Since preconcentration could be performed during column equilibration, this gave an injection-to-injection cycle time of 33 min.

3.5 Choice of internal standard

Several concerns have to be taken into account when looking for a proper IS for the current setup. The compound must give full retention on PGC at the conditions described, be easily desorbed by the addition of buffer, and show adequate chromatographic behavior by HILIC. For this purpose, isotopically labelled standards constitute an obvious but expensive choice. Diethyl phosphate (DEP), dibutyl phosphate and diphenyl phosphate were therefore investigated because they are less expensive and have similar chemical characteristics as the AMPAs. An important prerequisite is low pKa of the compounds (< 1 [51]), which ensure complete deprotonation at pH 3 and higher. Full retention was obtained for all three compounds on PGC with 500 µL loaded, even when dissolved in tap water that was not treated with the anion precipitation column. This was valid also when the mobile phase composition was changed to 90% ACN. Thus, the retention strength of the dialkyl/aryl phosphates on PGC was seemingly equal to or higher than that of PMPA. Similar to the AMPAs, desorption was achieved by introducing the formate ion into the mobile phase. DEP showed the best peak symmetry at the conditions described in Section 3.4, and also eluted in the retention time window of the AMPAs. Hence, DEP was preferred as IS in the present study. Environmental samples

should be examined for background levels of DEP, however, since recent works have suggested that the compound may occur from degradation of organophosphorous insecticides [52,53].

3.6 Method validation

The validation was performed with the AMPAs dissolved in tap water, which represents an authentic sample matrix. Data from the method validation are summarised in Table 2 where linearity and repeatability are given both with and without IS correction. The limits of detection (LODs) were determined as the concentration of the analytes giving a signal intensity for the quasi molecular ions of approximately 200 counts when extracted at an accuracy of ± 5 mDa. This is approximately three times the signal height of the arbitrary baseline noise present when extracted at this high mass accuracy. Extracted ion chromatograms at the determined LODs are shown in Fig. 5.

The obtained LODs of 0.01-0.07 μg L^{-1} compare favourably to those achieved by off-line SPE methodologies and GC-MS determination in single ion monitoring mode (0.08-0.14 μg L^{-1} [17,18]). Katagi et al. achieved LODs between 1 and 20 μg L^{-1} for the three AMPAs in river water by on-line SPE-LC-MS/MS with fast atom bombardment ionisation after solvent change and derivatisation of the compounds [26]. No reported LODs are found for determination of the AMPAs in water by off-line SPE in combination with LC-MS determination. However, Mawhinney et al. obtained LODs between 0.03 μg L^{-1} (PMPA) and 0.24 μg L^{-1} (EMPA) in urine after solvent change and SPE on non-bonded silica followed by LC-MS/MS determination on a triple quadropole instrument, indicating the applicability of their method for environmental samples [9].

Fig. 5 in approximately here

The LOQs were calculated as three times the LODs, and linearity of the method was investigated from LOQ to 20 μg L^{-1}. PMPA showed linear response over the measured range, while the curves for

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EMPA and IMPA were nonlinear at concentrations above 9 and 12 µg L^{-1} (4.5 and 6 ng on-column), respectively. When analyses were performed with direct injection (1 µL) on the separation column, the upper linear range of EMPA and IMPA was approximately 5 ng on-column. Hence, it was shown that the limited linear range of these compounds was a result of the ESI-MS response and not loss of analytes during preconcentration. For autosamplers of the present type with variable volume injection, quantitation of EMPA and IMPA at higher concentrations could easily be performed by reducing the loading volume on the PGC column. Alternatively, polynomial calibration could be used, which gave R^2 of 0.999 (IS corrected) for both EMPA and IMPA from LOQ to 20 µg L^{-1}. If linear calibration is employed, the high correlation coefficients within the linear range indicate that a three point calibration curve is sufficient for most purposes. All repeatability tests gave residual standard deviation (RSD) values well below 10% with the between assay repeatability at 5 µg L^{-1} as low as 2-3%. Only arbitrary differences were found when comparing the repeatability and linear regression coefficients calculated with and without IS correction. This proves good volumetric control in sample preparation and stable signal response of the ESI-MS system both intraday and during the six day period of the between assay repeatability test. Still, application of IS will always provide more reliable quantitative determinations as instability in the ESI-MS signal may occur occasionally.

Table 2 in approximately here

Method robustness was investigated by adding extra amounts of the three major ions naturally occurring in water. Chloride and sulphate ions were added at 200 mg L^{-1} each, which is close to the maximum EU recommended levels in drinking water (250 mg L^{-1}) [54]. Hydrogen carbonate was added at a level of 100 mg L^{-1}, which is within the typical range of hard water. These amounts were added in addition to the ions already present in the tap water (not determined). As a worst case scenario all ions were added to one sample, containing the AMPAs at 5 µg L^{-1}. The quantitative recoveries obtained for all three analytes proved high method robustness when treating the sample

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with an anion precipitation column prior to SPE-LC-MS. Interestingly, the recoveries were even higher than those obtained when the AMPAs were added to tap water only (Section 3.2). This may be due to less co-precipitation of the analytes when the sample contains higher levels of hydrogen carbonate [42].

3.7 Application to natural waters and simulated waste water

The method was employed for determination of the AMPAs in three natural water samples as well as a simulated waste water sample. The analysed blank samples did not show any traces of AMPAs or DEP. Hence, each sample was added the analytes at 5 µg L^{-1} and determined in accordance with the optimised procedure. The pH values after anion precipitation were measured to approximately pH 5 in all four samples, which was a decrease of about one pH unit for the natural waters and three units for the simulated waste water. Thus, the major part of hydrogen carbonate in the samples was protonated to carbonic acid (pKa 6.4). Excellent recoveries (Table 3) were obtained for PMPA, proving the high retention of this compound on PGC. The least retentive compound (EMPA) showed recoveries of 80% and higher. Some reduction in recoveries of IMPA and EMPA was observed from the rain and river waters collected in the city compared to the clear river water collected from a rural recreation area. The two former samples had a weak yellow discolouration which may be attributed to humic matter, being responsible for the loss of EMPA and IMPA on the PGC column. It must be assumed that the fulvic and humic acids containing deprotonated carboxyl groups at pH 5 [55] compete with the adsorption sites on PGC, similar to the formate ions responsible for elution of the AMPAs. A higher background signal was observed in the two urban samples compared to the two others, supporting this hypothesis. Recoveries from the simulated waste water sample (91-99%) showed that the addition of PEG300 (500 mg L^{-1}) in addition to the salts had a weak influence on the retention of EMPA and IMPA. In general, the overall recoveries of 80-106% prove that the method is applicable for determination of AMPAs at trace levels in most water samples of interest.

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Table 3 in approximately here

4 Conclusions

Trace determination of AMPAs in aqueous samples by on-line SPE-LC-MS has been successfully demonstrated using PGC for analyte enrichment and HILIC for chromatographic separation. Sample volumes of 500 µL were loaded on the PGC column followed by a change of solvent composition to 85% ACN/15% H_2O. Backflush desorption and chromatographic separation was performed by introducing 10 mM AF in the mobile phase, followed by ACN/H_2O gradient elution. By treating the samples with strong cation-exhange columns on Ba, Ag and H form, the major inorganic anions naturally occurring in water were removed by precipitation prior to SPE-LC-MS determination.

To our knowledge, this is the first on-line SPE-LC-MS technique reported for determination of underivatised AMPAs. EMPA, IMPA and PMPA could be determined from 0.07, 0.04 and 0.01 µg L^{-1} in tap water, respectively. The method showed very good within assay repeatability (2-3% RSD) and between assay repeatability (5-9% RSD) at analyte concentrations of 5 µg L^{-1}, with DEP as IS. Recoveries from three natural waters and a simulated waste water sample, spiked with the AMPAs at 5 µg L^{-1}, were 80-91% for EMPA, 92-103% for IMPA and 99-106% for PMPA. This proves that the method is applicable for fast determination of AMPAs at trace levels in water samples of most origins.

Acknowledgements

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Table 1 Recoveries of the AMPAs (5 µg L^{-1}) from type I water which was added 100 mg L^{-1} of inorganic anions in separate solutions. The samples were analysed as described in Section 2.2, with loading volumes of 500 µL.

	% recovery (n=1)		
Ions added	EMPA	IMPA	PMPA
HCO_3^-	24	41	109
Cl^-	19	39	83
SO_4^{2-}	nd	nd	17

Table 2 Data from method validation.

		EMPA	IMPA	PMPA
LOD (µg L^{-1})		0.07	0.04	0.01
Linear range (µg L^{-1})		0.2 - 9	0.12 - 12	0.03 - > 20
With IS correction				
R^2		0.996	0.997	0.997
Repeatability (RSD), n=6				
Within assay	LOQ	3.8	7.2	4.3
	5 µg L^{-1}	3.1	2.1	1.9
Between assay, 5 µg L^{-1}		5.3	6.1	8.4
Recovery [a] (% ± SD), n=6		103 ± 3	99 ± 4	102 ± 6
Without IS correction				
R^2		0.993	0.999	0.998
Repeatability (RSD), n=6				
Within assay	LOQ	3.4	6.6	5.8
	5 µg L^{-1}	2.3	2.9	3.4
Between assay, 5 µg L^{-1}		6.5	8.6	7.7

[a] Tap water containing the AMPAs at 5 µg L^{-1}, which was added 200 mg L^{-1} Cl^-, 200 mg L^{-1} SO_4^- and 100 mg L^{-1} HCO_3^-

Table 3 Recoveries of the AMPAs from natural waters and a simulated waste water sample, spiked at 5 µg L^{-1}.

	% recoveries ± SD (n=6)			
	River water rural area	River water urban area	Rain water suburban street	Simulated waste water
EMPA	91 ± 1	83 ± 2	80 ± 3	91 ± 2
IMPA	103 ± 5	92 ± 6	95 ± 4	95 ± 3
PMPA	106 ± 3	100 ± 4	99 ± 2	99 ± 5

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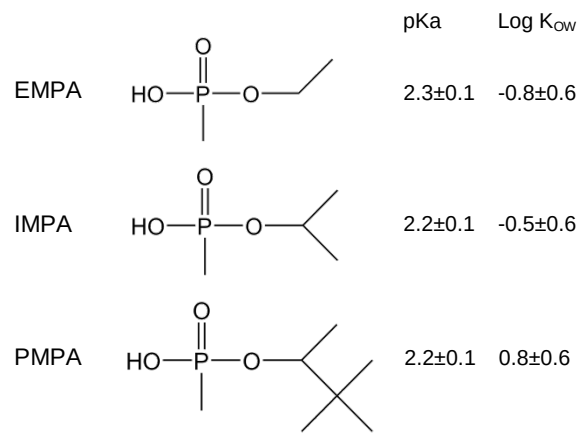

Fig. 1

Structure of the studied alkyl methylphosphonic acids, together with the pKa and Log K_{ow} values, calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02 (© 1994-2012 ACD/Labs).

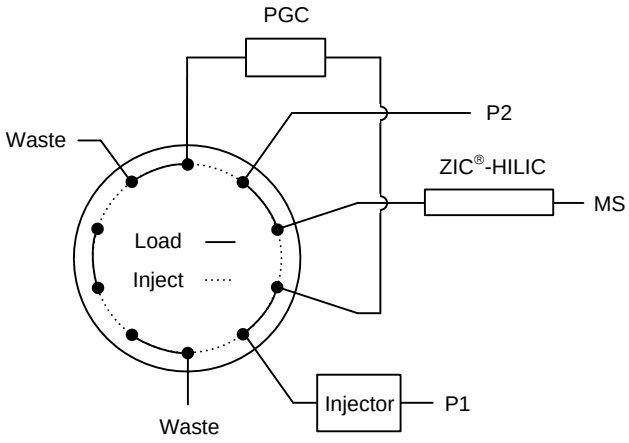

Fig. 2

Schematic diagram of the on-line SPE-LC-MS setup (see text for details).

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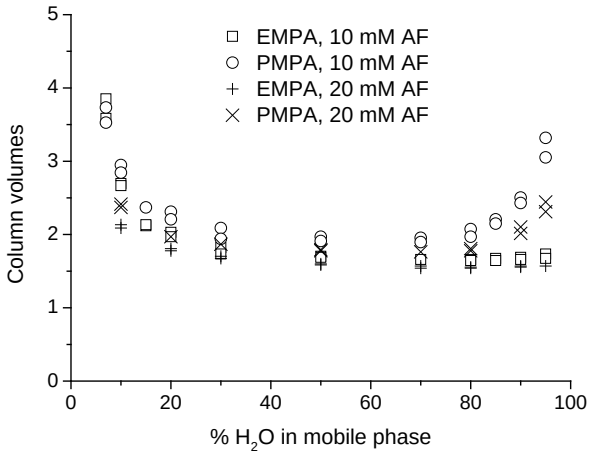

Fig. 3

Desorption volumes of EMPA and PMPA from PGC as a function of the ACN/H_2O composition with 10 and 20 mM AF in the mobile phase. The measured void volume of the 2.1x10 mm PGC column was 27 µL.

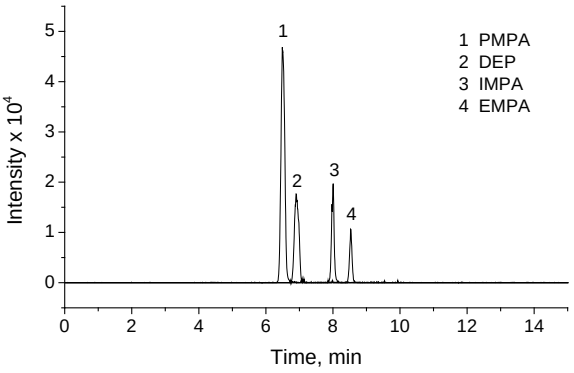

Fig. 4

EICs from on-line SPE-LC-MS determination of the AMPAs with DEP as IS ($[M-H]^-$ *m/z* 123.021, 137.036, 153.031, 179.083 ± 0.005). The analytes were added to tap water at 5 µg L^{-1}, and 500 µL were loaded on the PGC column. Gradient elution of ACN/H_2O was performed (85-60% ACN), with 10 mM AF in the mobile phase.

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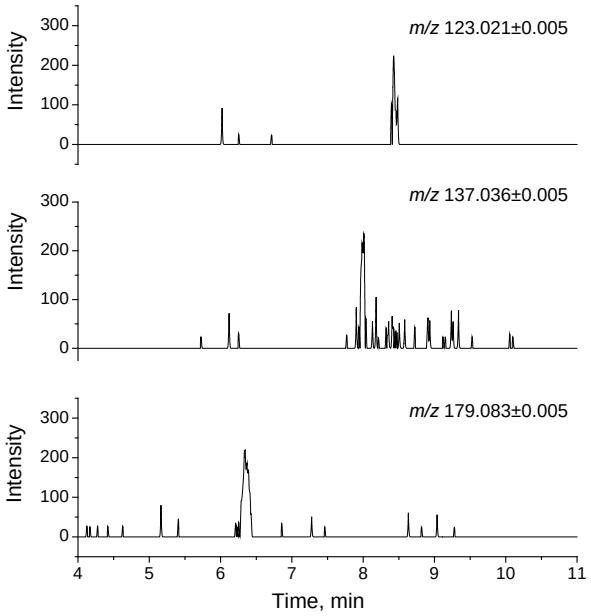

Fig. 5

EICs ($[M-H]^-$ ± 5 mDa) of EMPA (top), IMPA (middle) and PMPA (lower) at the LODs of 0.07, 0.04 and 0.01 μg L^{-1}, respectively.

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