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Trace determination of primary nerve agent degradation products in aqueous soil extracts by on-line solid phase extraction–liquid chromatography–mass spectrometry using ZrO_2 for enrichment

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ABSTRACT

A method for determination of the primary nerve agent degradation products ethyl-, isopropyl-, isobutyl-, cyclohexyl- and pinacolyl methylphosphonic acid in aqueous soil extracts has been developed utilizing on-line solid phase extraction-liquid chromatography and mass spectrometry (SPE–LC–MS). Four different stationary phases (ZrO_2, TiO_2, polymeric mixed mode anion exchange and porous graphitic carbon) were investigated for their suitability as SPE materials in the on-line SPE–LC–MS setup. Zirconium dioxide was chosen due to its high affinity for the alkyl methylphosphonic acids (AMPAs), and its compatibility with LC–MS. Aqueous soil extracts were acidified with 0.1% acetic acid and aliquots of 300 μL were injected on a 2 mm × 10 mm ZrO_2 column. Separation of the analytes was performed on a reversed phase column with acetonitrile/water gradient and 15 mM ammonium acetate. Method validation was performed with the analytes added to an aqueous extract of a loam soil, and the AMPAs could be determined at concentrations as low as 0.05–0.5 μg L^{-1}. The method was linear ($R^2 > 0.995$) from the limit of quantification (LOQ) to 100 × LOQ, and the within assay repeatability was below 10% and 5% relative standard deviation at LOQ and 50 × LOQ, respectively. The developed method was employed for determination of the AMPAs which had been added to the aqueous extracts of five different soil types from cultivated and uncultivated areas. The obtained recoveries showed that the analytes could be determined at the sensitivities achieved in the method validation in four of the extracts. For the first time, we have demonstrated a method capable of detecting primary nerve agent degradation products at sub ppb levels in the aqueous extracts of various soils. The method requires no sample preparation after soil extraction other than pH adjustment of the aqueous extract.

1. Introduction

The organophosphorous compounds known as nerve agents are the most lethal type of chemical warfare agents currently known. All development, stockpiling and use of the compounds are prohibited by the Chemical Weapons Convention (CWC) [1], except within research activities that are declared and in accordance with the convention. In cases of deliberate or unintentional spread of nerve agents, efficient and sensitive techniques for measurement of the compounds or their degradation products are important. After being released into the environment, the nerve agents degrade by hydrolysis to their corresponding alkyl methylphosphonic acids (AMPAs) as shown in Fig. 1. These degradation products are specific for each nerve agent and do not have any natural sources, and hence they are valuable markers for the release of nerve agents. The AMPAs may undergo further hydrolysis by loss of the O-alkyl group, resulting in the non-specific methyl phosphonic acid (MPA). This process is very slow in water, but more pronounced when the AMPAs are adsorbed to soil [2]. High sensitivity in determination of the primary hydrolysis products may therefore be essential in order to give forensic prove of the spread of nerve agents.

Soil has been utilized as sample matrix for verification of the release of chemical warfare agents on several occasions [3–7]. The highly water soluble AMPAs can be extracted from soil in neutral [8,9] or alkaline [10,11] aqueous solutions. Reversed phase (RP) liquid chromatography connected to mass spectrometry (LC–MS) with electrospray ionisation (ESI) [12–15] and gas chromatography (GC)–MS [10,16,17] are most frequently employed for determination of the AMPAs in aqueous soil extracts. The latter technique

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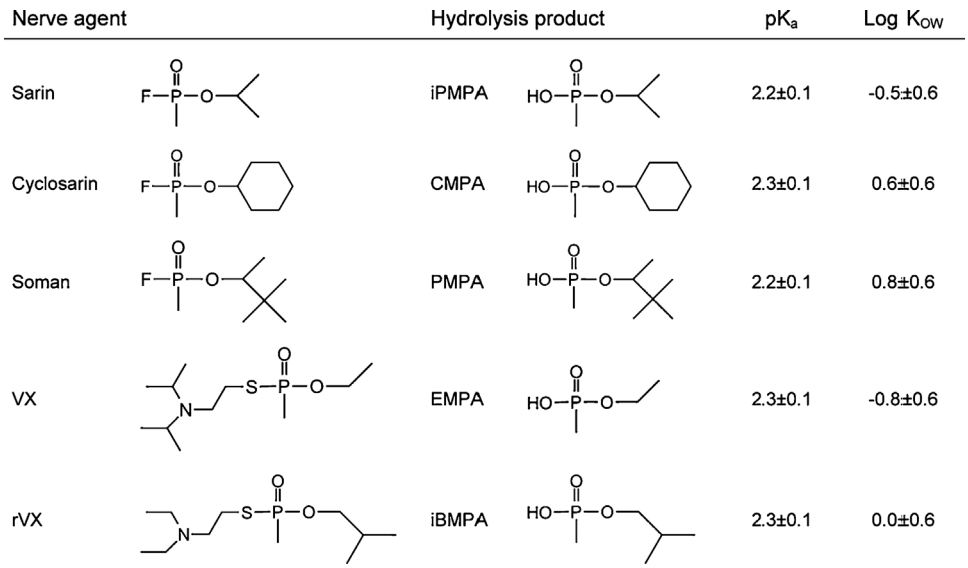

Fig. 1. Structure of selected nerve agents and their primary hydrolysis products. The pK_a and log K_{ow} values were calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02 (© 1994–2012 ACD/Labs).

requires derivatisation of the AMPAs to their respective phosphonate esters prior to determination. Due to their ionic character (pK_a 2.2–2.3), capillary electrophoresis (CE) [18,19] and ion exchange chromatography [20] have been employed for determination of the AMPAs in aqueous soil extracts as well. If the AMPAs are present at low ppb levels, analyte enrichment prior to instrumental determination is required. Aqueous soil extracts often contain, however, high amounts of organic and inorganic components possibly interfering with both analyte enrichment and instrumental determination. Methods for determination of AMPAs in aqueous soil extracts have therefore in most cases included procedures for removal of interfering compounds with low degree of enrichment [9,11,12,21,22].

The aim of the present study was to develop a method for trace determination of AMPAs in aqueous soil extracts. Hence an enrichment step was considered necessary, and the performance of four commercially available solid phase extraction (SPE) columns has been explored for this purpose. The criteria for choice of column material were high recovery of the analytes and compatibility in an on-line SPE–LC–MS system. The efficiency of the aqueous extraction of the AMPAs from different soil types has been examined by others [8,13,22,23] and was not investigated in this work. The stationary phases investigated were porous graphitic carbon (PGC), a polymeric mixed mode anion exchange (MAX) sorbent, ZrO_2 and TiO_2. The PGC sorbent has been successfully employed in on-line SPE–LC–MS for determination of AMPAs in water samples at sub ppb levels [24]. The MAX column was included for investigation due to reported high recoveries by off-line SPE in combination with GC–MS for determination of AMPAs in aqueous samples [25]. Zirconium dioxide exhibits Lewis acid properties and has affinity to strong Lewis bases like the AMPAs when dissociated [26]. Kanaujia et al. explored the enrichment of several AMPAs with zirconia coated silica particles and found that the analytes were selectively extracted in the presence of carboxylic acids [27]. Also, zirconia coated stir bar [28] and zirconia hollow fiber membrane [29] have been used for extraction of the AMPAs from water samples. Titanium dioxide displays Lewis acid properties similar to ZrO_2 [30]. No study has been reported for enrichment of AMPAs on TiO_2, but the material has been extensively used for selective enrichment of other organophosphates [31–33]. Other stationary phases have been used for enrichment of the AMPAs from aqueous matrices, like strong anion exchange (SAX) columns [17,22,34]. Kanaujia et al. found that the efficiency of SAX was lower compared to using MAX, however [25]. Retention based on RP interactions is not suited due to the polarity of the AMPAs. Hydrophilic–lipophilic balanced polymers have been used for isolation of the AMPAs from aqueous matrices after acidifying the samples to protonate the analytes [12,16]. The recoveries obtained for ethyl methylphosphonic acid (EMPA) were below 35% with this technique though. Retention based on hydrophilic interactions has also been utilized for enrichment of the AMPAs [35], as well as the use of molecularly imprinted polymers [12,21]. These two techniques require a change from aqueous to organic solvent prior to analyte enrichment, and are therefore not suited for direct determination of the AMPAs in aqueous extracts.

As a consequence of the SPE column screening, ZrO_2 was chosen for preconcentration of the AMPAs (see Section 3.1). Zirconium dioxide is characterized by several surface properties, and can act both as an anion- and cation exchanger depending on pH [36]. More importantly in this context, ZrO_2 can undergo ligand exchange processes as shown below [26].

$$Zr(OH)(H_2O) + L_1^- \leftrightarrows Zr(OH)L_1^- + H_2O \quad (1)$$

$$Zr(OH)(H_2O) + L_1^- \leftrightarrows Zr(H_2O)L_1 + OH^- \quad (2)$$

$$Zr(OH)L_1^- + L_2^- \leftrightarrows Zr(OH)L_2^- + L_1^- \quad (3)$$

$$Zr(OH)L_1^- + OH^- \leftrightarrows Zr(OH)_2^- + L_1^- \quad (4)$$

The ligand exchange behavior originate from the presence of strong Lewis acid sites on the surface of unsaturated Zr(IV), and occurs when a Lewis base (L^-) is present in the solution. Organophosphates like the AMPAs are strong Lewis bases due to their electronegative phosphonate groups, and this is the reason for ZrO_2 having high affinity for the AMPAs. Process 1 is expected to be the dominant for ligand adsorption because hydroxide ions are more tightly bound by zirconia compared to water molecules, but process 2 will contribute at low pH [26]. The adsorbed Lewis base can be displaced by introducing a second solute Lewis base (L_2^-) from an added salt or buffer [26] as shown in process 3. Also, by pH increase L_1^- can be displaced by the hydroxide ion which is a strong Lewis base itself [37] (process 4 and reversion of process 2).

Table 1
Properties of the selected soils. The values of TOC and CEC refer to dry matter, and the soil type is according to USDA classification.

Soil number	Soil type	TOC (%)	pH	CEC (meq/100 g)	Water content (w/w %)
A	Loam	2.3	7.2	31	10.5
B	Loamy sand	1.8	5.5	10	11.3
C	Sandy loam	1.0	6.2	7	8.6
D	Sand	0.7	5.1	4	4.2
E	Clay	1.6	7.1	27	4.4

In the present work, we report for the first time an automated SPE–LC–MS method for trace determination of primary nerve agent degradation products in aqueous soil extracts. The analytes were preconcentrated on the ZrO_2 SPE column, followed by RP–LC separation and ESI–MS in negative mode. The developed method was employed for determination of five AMPAs (Fig. 1) in the aqueous extracts of five different soils from cultivated and uncultivated areas.

2. Experimental

2.1. Chemicals and solutions

Pinacolyl methylphosphonic acid (PMPA, 97%), EMPA (98%) and MPA (98%) were purchased from Sigma–Aldrich Chemie GmbH, Steinheim, Germany. Isopropyl methylphosphonic acid (iPMPA), isobutyl methylphosphonic acid (iBMPA, 1000 μg mL^{-1} in methanol) and cyclohexyl methylphosphonic acid (CMPA, 1000 μg mL^{-1} in methanol) were delivered by Cerilliant Corporation, Round Rock, TX, USA. Ammonium formate (98%) was purchased from BDH Laboratory Supplies, Dorset, UK. Acetonitrile (ACN, 99.9%), ammonium acetate (AA, 98%), ammonium carbonate (AC) and ammonium hydroxide (25%) were delivered by Merck KGaA, Darmstadt, Germany. Methanol (LC–MS grade), formic acid (98%) and acetic acid (99%) were obtained from 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.

Stock solutions of EMPA, iPMPA and PMPA were prepared at 0.5 mg mL^{-1} by diluting 25 mg of the neat agents in 50 mL ACN. Further dilutions were made in ACN or type I water, while the final working solutions were prepared in type I water or in aqueous soil extracts. The working solutions were prepared to contain no more than 1% ACN. All solutions were stored at 4 °C until use. A solution of 3.1% (v/v) acetic acid was prepared in type I water. From this, 50 μL was added to 1.5 mL of the samples directly in the autosampler vials (final concentration 0.1%, v/v).

2.2. Soil samples and extraction procedure

The soil types that were subjected to aqueous extraction are listed in Table 1, and were obtained from LUFA Speyer, Germany. The soils were sampled at a depth of 0–20 cm from various cultivated (soil A–C) and uncultivated (soil D and E) areas. All soils were dried at room temperature until sieveable, then sieved to a grain size of 2 mm and characterised by the supplier. The total organic carbon (TOC), pH and cation exchange capacity (CEC) of each soil are listed in Table 1. Classification of the soils is given on the basis of the particle size distribution, according to the United States Department of Agriculture (USDA).

Soil extraction was performed according to a recommended procedure for determination of CWC related chemicals [38]. Aliquots of 5 g soil were weighed into 30 mL fluorinated ethylene propylene tubes (Nalge Nunc International, Rochester, NY, USA) and extracted twice with 5 mL type I water. The tubes were shaken for 10 min at 2000 rpm on a Multi Reax test tube shaker (Heidolph Instruments, Schwabach, Germany) and centrifuged at 3200 × *g* for 5 min on a Centra CL3R from IEC (Needham heights, MA, USA). The supernatants were combined in 15 mL polyethylene sample tubes from Sarstedt AG & Co. (Nümbrecht, Germany), and a second centrifugation was performed at 6200 × *g* for 30 min on a Heraeus Megafuge 1.0R (DJB Labcare, Newport Pagnell, UK). If not otherwise described, the supernatant was filtered through a Millex PVDF 0.22 μm filter (Millipore, Carrigtwohill, Co. Cork, Ireland) and added 0.1% (v/v) CH_3COOH.

2.3. Instrumental configuration

An Ultimate 3000 RS LC (Dionex Corporation, Idstein, Germany) was coupled to a MicroTof-Q II mass spectrometer (Bruker Daltonics, Bremen, Germany). A schematic diagram of the final 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 temperature of 35 °C. The loading flow (P_1) was delivered from a DGP-3600M dual gradient pump via a WPS-3000 autosampler with variable volume split-loop injection and a 500 μL sample loop. Solvents delivered by P_1 were (A) type I water; (B) 40 mM AC and 0.75% (v/v) NH_4OH in water/ACN (60/40); (C) 2% (v/v) CH_3COOH in water/ACN (96/4). The LC flow was delivered from channel 2 of the DGP-3600M pump (P_2). Solvents delivered by P_2 were (A) type I water; (B) acetonitrile; (C) 200 mM AA. Preconcentration was performed on a ZrO_2 column (2 mm × 10 mm, 3 μm) from ZirChrom Separations, Inc., Anoka, MN, USA. Separation was achieved with a Nucleodur Pyramide C_{18} column (2 mm × 100 mm, 1.8 μm) from Macherey-Nagel GmbH & Co. KG, Düren, Germany. The 0.2 μm pre-filter was from Thermo Fisher Scientific Inc., Bellefonte, PA, USA.

Aqueous soil extracts of 300 μL were loaded onto the ZrO_2 column with 100% (A) at 300 μL min^{-1}, delivered by P_1. After 3 min, the switching valve was shifted to "Inject" position and the ZrO_2 column was backflushed with 15 mM AA at 200 μL min^{-1}, eluting the AMPAs onto the separation column. At the same time, the pre-filter was backflushed to waste from P_1 for removal of

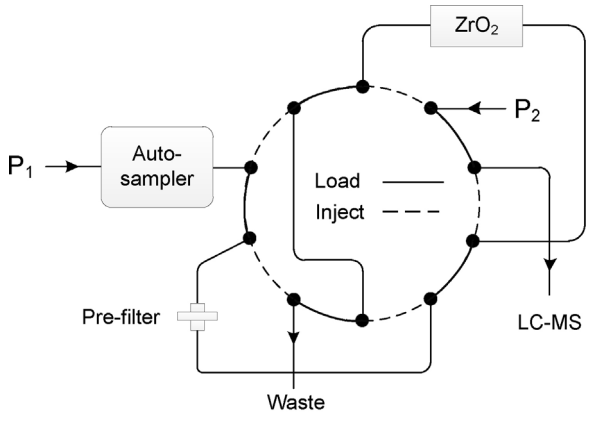

Fig. 2. Diagram of the on-line SPE–LC–MS setup (see text for details).

particles at the filter inlet. This pre-filter backflush procedure was first described by Svendsen et al. [39] and is slightly modified in the present setup for use without a third pump. At 4 min, the valve was switched back to "Load" position for gradient separation of the AMPAs and reconditioning of the ZrO_2 column. Gradient elution from P_2 was 0% (B) at 3–4 min, 0–50% (B) in 4–12 min, 50–90% (B) in 12–14 min and 90% (B) at 14–16.5 min. Eluent (C) was 7.5% throughout the analysis, ensuring a constant concentration of 15 mM AA. After returning to start gradient conditions, the column was equilibrated for 11 min, giving an injection-to-injection cycle time of 28 min. During gradient separation, the ZrO_2 column was re-conditioned by P_1 with 100% (B) at 4–9.5 min and 50% (A)/50% (C) at 10–15.5 min. Finally, the preconcentration column was flushed with 100% (A) prior to next injection.

The ESI was operated in negative ionisation mode with a capillary voltage of 3500 V and an end plate offset of −500 V. The collision cell energy was 5.0 eV and collision RF peak-to-peak voltage was 150 V. Nitrogen for nebulising gas (1.2 bar) and drying gas (8.0 L min^{-1}, 200 °C) was provided by a high purity generator (Domnick Hunter, Durham, UK). Compressed N_2 (purity 6.0) from AGA AS, Oslo, Norway, was used as collision gas. Mass spectra were acquired in the *m/z* range 50–500, and quantitative calculations were performed with peak areas of the extracted quasi molecular ions $[M-H]^-$ ± 5 mDa.

2.4. Solid phase extraction

Four columns with different stationary phases were investigated for preconcentration of the AMPAs from aqueous samples: Hypercarb PGC (2.1 mm × 10 mm, 5 μm) from Thermo Fisher Scientific Inc.; Oasis MAX (2.1 mm × 20 mm, 30 μm) from Waters Corporation, Milford, MA, USA; ZrO_2 (Section 2.3) and TiO_2 (2 mm × 10 mm, 5 μm) from Zirchrom. The performance of the SPE columns was first investigated with the setup as described in Fig. 2, but without separation column. Instead, the "Waste" and "LC–MS" outlets were connected to the MS during "Load" and "Inject", respectively, via the second switch valve in the flow manager. In this way, both potential breakthrough of the analytes during sample loading and the desorption rate could be measured. Three of the AMPAs (EMPA, iPMPA and PMPA) at a concentration of 20 μg L^{-1} in type I water and aqueous extracts of Soil A were used for investigation of the performance of the columns. Optimisation of the washing procedure for the ZrO_2 and TiO_2 columns was performed with the MS in the *m/z* range 300–4000, to measure the signal of eluted humic and fulvic acids.

The performance of the different SPE columns in the complete on-line SPE–LC–MS setup was investigated with EMPA, iPMPA and PMPA added to an aqueous extract of Soil A at 20 μg L^{-1}. The soil extract was divided in two parts, and one part was filtered (0.22 μm) and added 0.1% (v/v) CH_3COOH. The other part of the extract was eluted through a 2.5 mL Ba/Ag/H anion precipitation cartridge (Dionex Corporation, Sunnyvale, CA, USA). The setup and procedure for anion precipitation were as described in a former study [24], except that no $CaCl_2$ was added prior to treatment. The pH was measured before and after treatment with an Orion 2-Star pH meter from Thermo Fisher Scientific Inc. The SPE columns were investigated according to the analytical procedure described in Section 2.3, but with use of a Nucleodur Gravity C_{18} separation column (2 mm × 100 mm, 1.8 μm) from Macherey-Nagel GmbH & Co and with 2% ACN in the loading solvent and as start gradient. When using the PGC column for SPE, the switching valve remained in "Inject" position during gradient separation. Aliquots of 300 μL were injected on the SPE columns, and recoveries were calculated by comparing the obtained peak areas with those where the same amounts of AMPAs in type I water were injected (n = 4).

2.5. Method validation

Method validation was performed with the analytes added to aqueous extracts of Soil A. The linearity was investigated at six concentration levels, namely at 1, 10, 25, 50, 75 and 100 times the limits of quantification (LOQs). The SPE–LC–MS method repeatability was investigated at LOQ and 50 × LOQ by performing six analyses of one extract subsequently (within assay), and by injecting a freshly made extract for six consecutive days (between assay).

Recoveries of the AMPAs were investigated when added to the aqueous extracts of the different soil types shown in Table 1, at concentrations of 50 × LOQ. Three extractions were performed for each soil type and each subsequently spiked extract was analysed two times. The recoveries were calculated by comparing the obtained peak areas with those where the AMPAs were added to type I water (n = 6). Possible ion suppression was investigated by continuously introducing the AMPAs after the separation column when analysing the soil extracts. A t-piece was mounted between the separation column and ESI–MS, and coupled to a 500 μL syringe from Hamilton Bonaduz AG (Bonaduz, Switzerland). The syringe was mounted on a KDS100 syringe pump from KD Scientific (Holliston, MA, USA), and the AMPAs were introduced as a 25 μg mL^{-1} solution (50 μg mL^{-1} for EMPA) at 5 μL min^{-1}.

3. Results and discussion

Aqueous soil extracts vary a lot in composition depending on the characteristics of the extracted soil. For example, agricultural soils give high amounts of organic compounds in the aqueous extract, possibly interfering with further sample preparation steps. In an earlier study, we employed the PGC column in on-line SPE–LC–MS for trace determination of AMPAs in natural water samples [24]. The method worked well also for the aqueous extract of a sandy soil of low organic content, but lower recoveries were observed when handling agricultural or clay soils (results not shown). Therefore, we wanted to explore alternative stationary phases for on-line SPE–LC–MS in order to achieve the highest possible robustness and sensitivity in determination of the AMPAs in a wide range of aqueous soil extracts.

3.1. Screening of stationary phases for SPE

Four different stationary phases, including the PGC, were investigated as SPE materials in an on-line SPE–LC–MS setup for determination of expected low concentrations of the AMPAs in aqueous soil extracts. The SPE material of choice must be able to isolate the highly polar AMPAs from aqueous extracts possibly containing high amounts of interfering compounds such as humic and fulvic acids. Rapid desorption of the analytes should subsequently be achieved using an eluent that is MS friendly and compatible with the separation step. Moreover, if there are co-extracted contaminants from the soil extracts that are not eluted together with the analytes, these should be washed out from the SPE column prior to subsequent injections.

In the former study, we combined PGC SPE with hydrophilic interaction LC (HILIC). The higher amount of organic solvent in HILIC compared to RP separation gives higher sensitivity in ESI–MS due to the enhanced ionisation efficiency [40]. However, since the SPE column must be equilibrated with the same amount of organic solvent prior to HILIC, the method is more prone to analyte breakthrough during preconcentration. Hence, in the present study we have investigated the performance of the different SPE columns with mobile phase conditions suited for RP separation. First, each of the columns was investigated for retention and desorption of the AMPAs without separation column. Then, the SPE columns

were compared for their recoveries of AMPAs from an aqueous soil extract when mounted in the on-line SPE–LC–MS setup.

3.1.1. Porous graphitic carbon

The analytes were introduced in a loading mobile phase containing 2% ACN, suitable as start gradient conditions for separation by RP interactions. Full retention of the AMPAs was achieved when solved in type I water and with 3 min sample loading time (corresponding to 33 column volumes). Backflush desorption was easily achieved by introduction of 10 mM AA in the mobile phase containing 2% ACN. Hence, the PGC column showed good compatibility with separation based on RP. The compounds from the soil that showed retention on the PGC column when injecting an aqueous extract of soil A (300 μL) were eluted by introduction of 10 mM AA in backflush mode. Thus, no reconditioning of the column was needed prior to the next injection, except for equilibrating with the loading mobile phase.

3.1.2. Mixed mode anion exchange column

The polymeric MAX material exhibit both strong anion exchange and hydrophobic interaction properties. Thus, adsorption and desorption of the analytes are governed by pH, ion strength and the amount of organic modifier. The column showed full retention of the AMPAs when injected in type I water, but desorption of the analytes was slow when using mobile phase additives suitable for LC–MS determination. Various H_2O/ACN compositions and amounts of ammonium formate and formic acid were investigated for eluting the analytes. Long desorption time was observed especially for PMPA (15–20 column volumes) and 5–10% carryover was seen for the compounds between successive injections (results not shown). Because of slow desorption of the analytes with LC–MS friendly solutions and high carry-over, further investigation was not performed with the MAX column.

3.1.3. ZrO_2 and TiO_2

The adsorption of Lewis bases like the AMPAs on ZrO_2 is pH dependent [37] and retention should be achieved at acidic to neutral conditions. Desorption is obtained by introducing a Lewis base in the form of hydroxyl ions or other anions of an added salt or buffer, competing for the adsorption sites on zirconia. When the AMPAs were solved in type I water, they were completely retained on the ZrO_2 column after eluting with 40 column volumes of 2% ACN. Two additives were investigated for desorption of the AMPAs, namely AA (pH 7) and AC (pH 9). With 15 mM of both additives in 2% ACN, complete desorption of the analytes was obtained after eluting with 3–4 column volumes in backflush mode. No significant difference was seen in the desorption rate whether AA or AC was used. Due to better chromatography for the early eluting AMPAs on the RP separation column, AA was preferred as additive. However, to fully re-establish the retention of the AMPAs in subsequent injections, the ZrO_2 column needed to be conditioned in acidic solution. For this purpose, acetic acid was used. With 0.1% CH_3COOH in the loading mobile phase, more than twice the desorption volume was needed compared to loading with H_2O/ACN only. Therefore, the column was conditioned with 1% CH_3COOH prior to injection, while sample loading was performed in H_2O/ACN. In addition, the samples were adjusted to pH 3.5–4 by adding 0.1% CH_3COOH.

The TiO_2 column behaved similar to the ZrO_2 column with respect to adsorption and desorption of the AMPAs at different ACN concentrations and type of additive used. Thus, no further method development was performed for this column. In addition to the AMPAs, the performance of the secondary nerve agent degradation product, MPA, was investigated on the ZrO_2 and TiO_2 columns. Complete retention was achieved on both columns, but desorption of the compound was very slow when using AA or AC as mobile phase additives. Since MPA was not considered essential for determination of the use of nerve agents, no further investigation was performed with this compound.

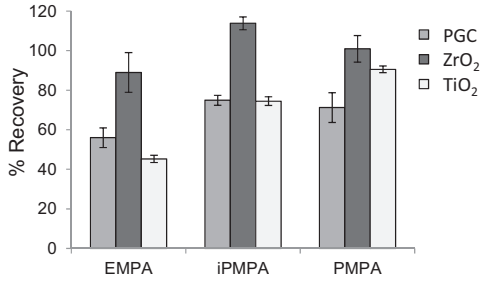

Fig. 3. Recoveries of the AMPAs from an aqueous extract of soil A by on-line SPE–LC–MS with PGC, ZrO_2 and TiO_2 as SPE columns, given as mean values ± SD (n = 4).

When aqueous soil extracts were introduced on the ZrO_2 and TiO_2 columns, many of the compounds with retention on the stationary phases were not completely eluted by introduction of 15 mM AA. When the columns were washed with 50 mM AC in 50% ACN between injections, a continuous signal for m/z 500–4000 was measured (maximum at m/z 1000–1200), probably caused by eluted humic and fulvic acids. The compounds were most effectively eluted from the columns with ACN concentrations between 30% and 60%. This is consistent with what has been found for retention of aromatic carboxylic acids at different ACN concentrations on ZrO_2 and TiO_2 [41,42]. The addition of 50 mM AC (pH 9) was more effective for eluting the compounds compared to adjusting the pH to 11 with NH_4OH. Inorganic phosphate, however, adsorbs strongly to ZrO_2 and is reported to be removed only under alkaline conditions [43]. The washing solution was therefore added 40 mM AC and then adjusted to pH 10 with 0.75% v/v NH_4OH.

3.1.4. Recoveries from different SPE columns

To find the most suitable SPE material for the current application, the recoveries of EMPA, iPMPA and PMPA on the PGC, ZrO_2 and TiO_2 columns were compared. The analytes were added at 20 μg L^{-1} each to an aqueous extract of Soil A. Extraction was performed according to the procedure described in Section 2.2. The columns were mounted in the on-line SPE–LC–MS setup as shown in Fig. 2, and aliquots of 300 μL were injected. The analysis conditions were as described in Section 2.3, except that a separation column with a slightly different C_{18} stationary phase was used, and 2% ACN was added in the loading and start gradient mobile phase. The samples that were injected on the ZrO_2 and TiO_2 columns were acidified with 0.1% CH_3COOH prior to analysis. For the PGC column, it was found in an earlier study that removal of inorganic anions from the aqueous samples significantly improved the recoveries of AMPAs [24]. The soil extract was therefore treated with a precipitation column on Ba-, Ag- and H-form to remove major inorganic anions prior to injection on the PGC column. The pH in the extract was 8.2, and was lowered to pH 3.7 and 3.8 after addition of acetic acid and treatment by the anion precipitation column, respectively. The recoveries obtained from the different SPE columns are presented in Fig. 3.

The recoveries of EMPA and iPMPA were significantly higher with use of the ZrO_2 column compared to the two others. The recovery above 100% (114 ± 3%) for iPMPA is not fully understood, but may be due to ion reinforcement from interfering compounds. Also for PMPA, the highest recovery was obtained with the ZrO_2 column, though less evident. When using the ZrO_2 and TiO_2 columns for preconcentration, the addition of acetic acid was sufficient for preparing the samples. Hence, these two columns offered

Table 2
Method validation data.

		EMPA	iPMPA	iBMPA	CMPA	PMPA
LOD ($\mu g\,L^{-1}$)		0.5	0.3	0.05	0.3	0.05
LOQ ($\mu g\,L^{-1}$)		1.5	0.9	0.15	0.6	0.15
Linearity (R^2), LOQ-100 × LOQ		0.996	0.998	0.997	0.996	0.998
Repeatability (%RSD), $n = 6$						
Within assay	LOQ	8	1	8	8	5
	50 × LOQ	2	3	3	1	4
Between assay	LOQ	13	12	22	12	13
	50 × LOQ	9	5	12	8	9

an advantage over the PGC column in terms of less labor demanding and less expensive sample preparation prior to SPE–LC–MS. In conclusion, the ZrO_2 column was chosen for further investigations due its high recoveries and minimal need for sample preparation.

3.2. Method optimisation

In the screening tests of the SPE columns, a C_{18} separation column with non-polar endcapping was used. At the lowest recommendable amount of organic modifier (2% ACN), the retention of EMPA and iPMPA on the C_{18} column was still low, giving poor refocusing of these compounds. Therefore, a separation column with polar endcapping was chosen, which was stable and functioning in 100% aqueous mobile phase systems. When starting with pure aqueous and 15 mM AA mobile phase, better refocusing of the more polar AMPAs was achieved, giving more symmetric and higher peaks.

The loading capacity of the ZrO_2 column is an important issue as a higher injection volume will increase the sensitivity of the method. The autosampler was configured for variable volume split-loop injection with 500 μL as the highest injection volume possible. With 500 μL injected, the loading volume needed for complete elution of the sample from the injection loop was 750 μL, which corresponds to 33 void volumes of the ZrO_2 column. When the AMPAs were solved in type I water with 0.1% CH_3COOH (500 μL injected at 300 $\mu L\,min^{-1}$), breakthrough occurred after eluting with 70–110 column volumes in the order PMPA < iPMPA < EMPA. With the analytes solved in an extract of Soil A with 0.1% CH_3COOH (500 μL injected), the breakthrough volume was reduced to 30 column volumes for the least retentive compound. Hence, breakthrough of PMPA occurred before the analytes were completely introduced on the ZrO_2 column. Reducing the loading flow to 200 $\mu L\,min^{-1}$ did not increase the breakthrough volume. To ensure high method robustness and repeatability, an injection volume of 300 μL was chosen. The loading time and loading flow rate was set to 3 min and 300 $\mu L\,min^{-1}$, respectively, which correspond to a loading and wash volume of approximately 40 column volumes. At these conditions, and with cleaning and regeneration of the ZrO_2 column between each injection, no reduction in retention of the AMPAs was found after injecting more than fifty aqueous soil extracts. The stability of the ZrO_2 column was confirmed by comparing peak areas of the analytes measured in spiked aqueous extract of soil A at different times during method validation. It was, however, observed that the electrospray ion source should be cleaned regularly due to the deposition of what were probably salts of inorganic ions.

Fig. 4 shows the extracted ion chromatograms (EICs) of an aqueous extract of Soil A with the AMPAs added at concentrations of fifty times the LOQs (determined in Section 3.3). No memory effects were observed for the analytes when introducing a blank sample immediately after the spiked soil extract. The negative ion ESI–MS spectra were dominated by the deprotonated ions at the low collision cell energy of 5.0 eV. The accurate mass measurements of the time-of-flight (TOF) MS provide high selectivity in determination of the AMPAs without employment of tandem MS.

3.3. Method validation

The method validation was performed with the AMPAs solved in the aqueous extract of Soil A to represent an authentic sample matrix. Data from the method validation are summarised in Table 2. The limits of detection (LODs) were determined as the concentration of the analytes giving a signal intensity for the quasi molecular ions of 200–300 counts at three repeated injections when extracted at an accuracy of ± 5 mDa. This is four to five times the signal height of the arbitrary baseline noise present when extracted at this high mass accuracy. The signal of CMPA was disturbed by a background contaminant with a mass difference of 20 mDa, giving a bias in the measured *m/z* at low intensities. Due to this interference, the LOD of CMPA needed to be set at a concentration giving an intensity of approximately 900 counts. Extracted ion chromatograms at the determined LODs are shown in Fig. 5. Prior to the present study, LODs have not been reported for determination of the AMPAs in soil extracts by LC–MS. Lagarrigue et al. employed transient isotachophoresis preconcentration and CE separation coupled to ESI–MS for determination of the five AMPAs in soil extracts with reported LODs of 4–70 $\mu g\,L^{-1}$ [18]. Nassar et al. have obtained LODs for EMPA, iPMPA and PMPA of 25–50 $\mu g\,L^{-1}$ in aqueous leachates of soil samples using CE with electrokinetic injection and UV detection [19]. Compared to what was achieved by the CE techniques,

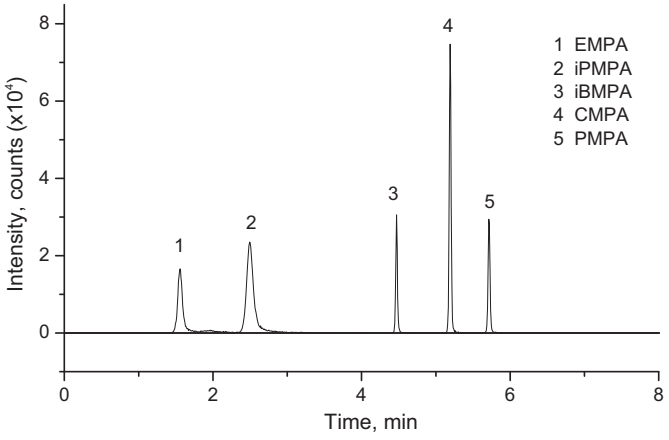

Fig. 4. EICs ($[M-H]^- \pm 5$ mDa) from on-line SPE–LC–MS determination of the AMPAs, added to an extract of a loam soil at concentrations of 50 × LOQ. An aliquot of 300 μL was loaded on the (2 mm × 10 mm) ZrO_2 column, and gradient separation with 0–90% ACN (15 mM AA) was performed on the (2 mm × 100 mm) C_{18} column with polar endcapping.

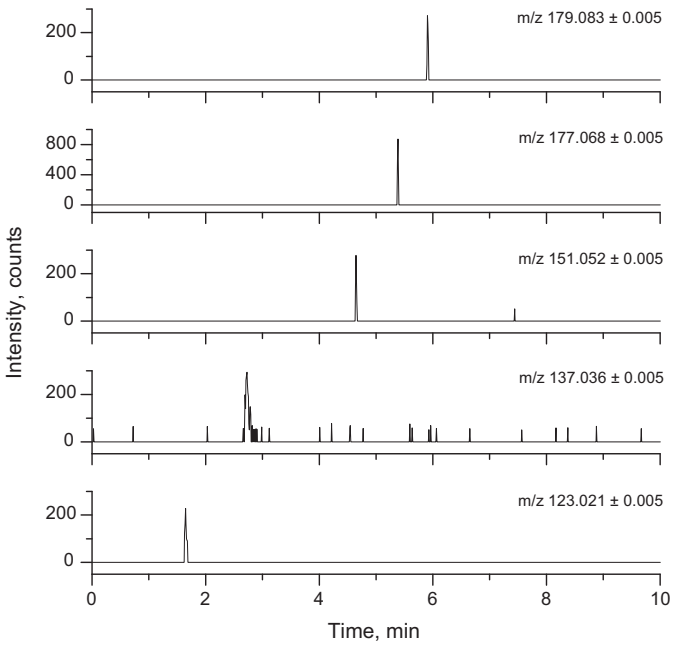

Fig. 5. EICs ($[M–H]^-$ ± 5 mDa) of the AMPAs at the determined LODs in an aqueous extract of a loam soil. From top: PMPA, CMPA, iBMPA, iPMPA and EMPA.

the obtained LODs with the current method are lower by a factor of at least 40.

The LOQs were calculated as three times the LODs except for CMPA, where LOQ was set at two times the LOD. Linearity was investigated in the range of LOQ to 100 × LOQ, and high linear correlation ($R^2 > 0.995$) was found for all compounds. Good within assay repeatability was obtained both at LOQ (<10% RSD) and at 50 × LOQ (<5% RSD). The somewhat higher between assay variability was probably caused by between day variations in the ESI–MS response. It may also be due to minor adsorption of the AMPAs to colloidal materials in the soil extracts, varying between days [23,44]. Internal standard could be used to correct for variations in instrumental response and matrix behavior if quantitative determination is of high importance. The main focus in the present study was on method sensitivity, and hence the obtained between day repeatability was considered acceptable.

No traces of the AMPAs were observed when blank aqueous extracts of the five soil types listed in Table 1 were analysed. Hence, the analytes were added to the extracts at 50 × LOQ for investigation of the recoveries from the on-line SPE–LC–MS procedure. Table 3 shows that recoveries higher than 85% were obtained for iPMPA, iBMPA, CMPA and PMPA, except when analysing the extract of soil E (clay). The recoveries of EMPA were significantly lower compared to the other compounds for all soil types. This is contrary to what was observed for the relative retention of EMPA, iPMPA and PMPA on the ZrO_2 column in Section 3.2. Since EMPA eluted near the column void, observed reduced recoveries were probably caused by ion suppression. Higher recovery of EMPA was found from the extract of soil A when screening for SPE stationary phases (Fig. 3) using the Gravity C_{18} column. The Pyramide C_{18} separation column chosen for the final method gave a more symmetric peak and improved signal height of EMPA. However, the change of separation column may also have resulted in co-eluting compounds with EMPA, giving more ion suppression. The recoveries from soil B, C and D were in the same range as that of the extract (of soil A) used in method validation. This means that the method sensitivity described in Table 2 could be expected also for these samples. The recoveries from the clay soil were 38–95% compared to what was obtained from the extract of soil A, and hence the corresponding poorer method sensitivity could be expected. Clay soils contain large amounts of minerals with colloidal properties (<0.001 mm) that can be distributed in the water phase when performing aqueous extraction. The lower recoveries obtained from soil E may be caused by analyte adsorption to these colloidal minerals [23,44]. No correlation was seen between the recoveries from the aqueous extracts and the organic content of the soils (Table 1). This indicates that the enrichment of AMPAs on ZrO_2 is not vulnerable to high amounts of organic matter in the extracts.

Table 3
Recoveries of the AMPAs (added at 50 × LOQ) from aqueous soil extracts by on-line SPE–LC–MS, given as %recovery ± SD ($n = 6$).

Soil extract	EMPA	iPMPA	iBMPA	CMPA	PMPA
A	48 ± 1	92 ± 3	104 ± 3	96 ± 1	94 ± 4
B	55 ± 5	89 ± 3	104 ± 7	100 ± 3	101 ± 3
C	68 ± 3	103 ± 5	103 ± 3	98 ± 4	99 ± 3
D	38 ± 2	87 ± 7	91 ± 5	95 ± 5	93 ± 7
E	18 ± 1	46 ± 3	72 ± 4	91 ± 4	66 ± 8

To investigate possible ion suppression, the AMPAs were continuously introduced after the separation column when analysing blank samples of the soil extracts. A significant suppression of the EMPA signal was observed in the region where this compound eluted, and most severe when analysing the extract of soil E. The signal in front of the chromatogram was dominated by a broad, tailing peak of the sulphate ion overlapping with EMPA. The degree of signal suppression of EMPA could be correlated to the intensity of the sulphate peak. Sulphate is known to be retained on ZrO_2 [45] and is among the major inorganic anions in soil. Coeluting sulphate was also observed by Zhou and Lucy in on-line SPE–LC determination of phosphonic diacids in water samples, using ZrO_2 for SPE [46]. In that case, the problem was partially solved by increasing the loading time to wash out most of the sulphate ions prior to LC separation. This was not possible for determination of the AMPAs since the sulphate ion had stronger retention on ZrO_2 compared to the analytes.

The relationship between the obtained LODs in the aqueous soil extracts and the sensitivity for determination of the AMPAs in soil is dependent on the aqueous extraction efficiency. In a comprehensive study by Kataoka et al. including 21 different soil types, recoveries of the AMPAs after aqueous extraction varied from approximately 20% and up to 100% [23]. Others have reported recoveries of the AMPAs from soil in the range of 45–96% [8,13,20,22]. Provided a conservative estimate of 20% aqueous extraction recovery from soil, the sensitivity of the present method would be in the range of 0.5–6 ng g^{-1} for soil A–D and 0.8–13 ng g^{-1} for soil E. The injection volume of the aqueous soil extract was 300 μL. Hence, a soil sample of 1 g is sufficient to attain the amount of extract needed for determination of the AMPAs at the obtained method sensitivity. Detection limits for determination of AMPAs in soil have been reported only a few times; Kataoka et al. have employed aqueous extraction of EMPA, iPMPA and PMPA from soils followed by derivatisation and GC–MS determination after treating the extracts different ways, achieving LODs of 0.1–0.2 μg g^{-1} [9,47]. In 1994, Black et al. reported the presence of iPMPA amongst other compounds in authentic soil samples from Iraq at levels from 200 ng g^{-1} and down to 6 ng g^{-1} [4]. The samples were subjected to aqueous extraction followed by solvent change and derivatisation prior to GC–MS determination, where single ion monitoring (SIM) was necessary for identification of iPMPA at these low levels. Certainly, the use of SIM, for example with a quadropole MS, would have enhanced the sensitivity also for the present method. The high resolution full scan MS used in our setup, however, makes it possible to screen for AMPAs of structures others than those employed in the method validation without compromising the

sensitivity. Moreover, the current sample preparation procedure after soil extraction (addition of acetic acid) is much faster and less labor demanding compared to the methods using GC–MS determination. This makes our fully automated SPE–LC–MS procedure well suited for screening aqueous soil extracts for the presence of primary nerve agent degradation products.

4. Conclusions

We have for the first time demonstrated an on-line SPE–LC–MS method capable of determining primary nerve agent degradation products at sub ppb levels in aqueous soil extracts. Zirconium dioxide was chosen for preconcentration of the AMPAs from aqueous soil extracts rather than TiO_2, PGC and MAX due to high recoveries, compatibility with LC–MS and minimal need for sample preparation prior to analysis. The strong Lewis acid sites on ZrO_2 make it able to retain the AMPAs, even when solved in soil extracts containing high amounts of organic and inorganic interferences. The analytes could be desorbed with the addition of an LC–MS friendly additive such as AA, and this made the ZrO_2 stationary phase applicable in an automated SPE–RP–LC–MS setup. By washing and reconditioning the ZrO_2 column between each injection, no reduction in retention of the analytes was seen after injecting more than fifty soil extracts. Detection limits of 0.05–0.5 $\mu g\,L^{-1}$ were achieved for the AMPAs in an aqueous extract of a loam soil. No more than 1 g of the soil is needed to achieve this sensitivity (300 μL extract injected). The only sample preparation needed after soil extraction was the addition of 0.1% acetic acid. Hence, the established method is well suited for screening aqueous soil extracts for the presence of primary nerve agent degradation products.

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