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Determination of the main hydrolysis products of organophosphorus nerve agents, methylphosphonic acids, in human serum by indirect photometric detection ion chromatography

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Abstract

For the verification of the use of chemical warfare agents (CWA), sarin, soman and VX, a simple rapid and accurate method which allows us to simultaneously determine their degradation products, isopropyl methylphosphonic acid (IPMPA), pinacolyl methylphosphonic acid (PMPA), ethyl methylphosphonic acid (EMPA) and methylphosphonic acid (MPA), in human serum, was explored by indirect photometric detection ion chromatography (IPD-IC) which employs an anion-exchange column. IC analysis was performed after sample preparation with an Ag^+-form cation-exchange resin cartridge, and the four methylphosphonic acids could be separated well. The proposed conditions are as follows: eluent, 0.5 m*M* phthalic acid–0.1 m*M* Tris (hydroxymethyl) aminomethane–5% acetonitrile; flow-rate, 1.0 ml/min; temperature, 50°C; UV detector, 266 nm. All four methylphosphonic acids were eluted within 30 min with hardly any disturbance by impurities in the serum. Linear calibration curves were obtained for MPA, EMPA and IPMPA in the concentration range from 50 ng/ml to 1 μg/ml and for PMPA from 100 ng/ml to 1 μg/ml. The relative standard deviation for the methylphosphonic acids ranged from 3.8 to 6.9% at 500 ng/ml and the detection limits were 40 ng/ml for MPA, EMPA and IPMPA and 80 ng/ml for PMPA. The method would be suitable for analysis of human serum samples. © 1997 Elsevier Science B.V.

Keywords: Sarin; Soman; VX; Methylphosphonic acids

1. Introduction

Owing to their strong acetylcholinesterase-inhibiting properties, the nerve agents – isopropyl methylphosphonofluoridate (sarin, GB), pinacolyl methylphosphono-fluoridate (soman, GD) and *O*-ethyl *S*-(2-diisopropylaminoethyl)methylphosphonothiolate (VX) – have been feared to become the 'nuclear weapons' of the poor nations, because they can be manufactured by relatively simple chemical techniques, and the raw materials are inexpensive and readily available. Therefore, their use is forbidden by international conventions [1,2]. However, the use of other chemical weapons (CW) – sulphur mustard and *O*-ethyl *N,N*-dimethylamidophosphorylcyanide (tabun) – in the Iran–Iraq conflict has been documented [3] and, furthermore, the Chemical and Biological Defense Establishment in England has proved the use of sarin by Iraq against Kurdish communities in northern Iraq according to the chemical analysis of samples such as soil and metal

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fragments collected from Kurdish villages [4,5]. What is worse, CW were recently used as tools for terrorism and have caused great fear among the population: sarin was used to commit indiscriminate murder in Matsumoto City in 1994 and Tokyo in 1995; and VX was used to commit murder in Osaka in 1994. This emphasizes the need for reliable detection and identification methods for the nerve agents in order to prove their use.

The three nerve agents contain the alkyl methylphosphono moiety in their chemical structures, and they are all readily hydrolyzed to the corresponding alkyl methylphosphonic acids: VX to ethyl methylphosphonic acid (EMPA), sarin to isopropyl methylphosphonic acid (IPMPA) and soman to pinacolyl methylphosphonic acid (PMPA). The alkyl methylphosphonic acids are ultimately hydrolyzed to methylphosphonic acid fairly slowly (Fig. 1) [6–16]. Up to now, no papers have been published on the metabolism of these nerve agents in the human body. However, in the human body, they are also thought to be enzymatically or/and spontaneously hydrolyzed to the methylphosphonic acids (EMPA, IPMPA, PMPA and MPA) according to the pathways outlined in Fig. 1 [17]. Therefore, for unequivocal proof of the use of the nerve agents, it will be very important to have methods available for the determination of the degradation products, methylphosphonic acids.

The nerve agents themselves can relatively easy be directly analyzed using gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS) [18–21]; however, their degradation products, methylphosphonic acids, are polar and non-volatile compounds. Their determination, therefore, has been mainly studied by high-performance liquid chromatography (HPLC) [22], ion chromatography (IC) [13,15,23], capillary electrophoresis [16], high-performance liquid chromatography–mass spectrometry [24] and capillary electrophoresis–mass spectrometry [25] without derivatization, and by HPLC and GC–MS with derivatization [8,10–12,26–35]. Due to its sensitivity and selectivity, GC-MS is thought to be one of the most suitable techniques for their identification, and we have previously reported a GC-MS technique for the determination of EMPA in human serum [35]. However, prior derivatization to volatile compounds, which is often complicated and causes contamination of the ion source of the GC–MS system, is necessary if GC–MS is to be employed. Furthermore, almost all of the determinations were from authentic samples and environmental samples

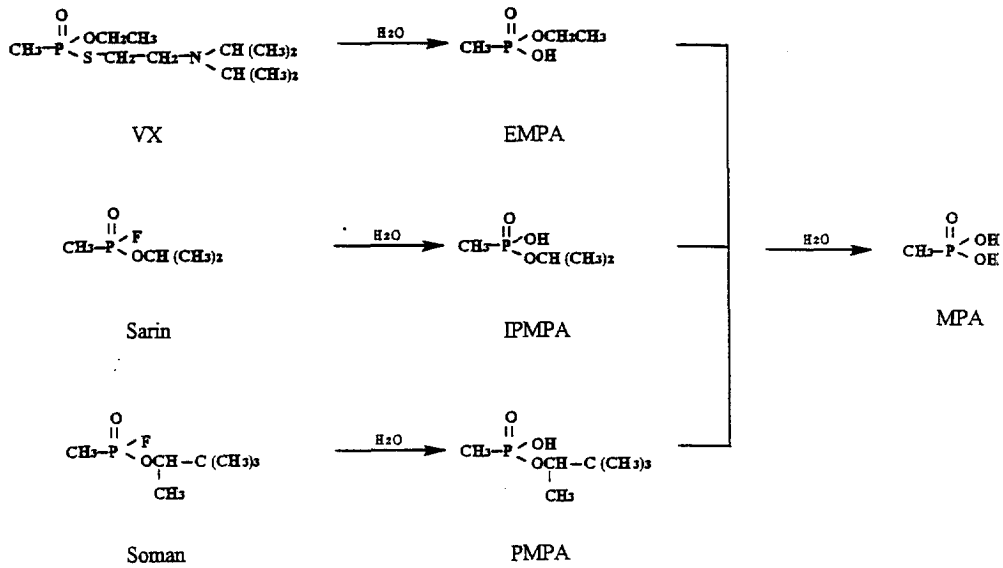

Fig. 1. Generalized hydrolysis pathways for VX, sarin and soman.

such as soil and water, while there are no reports on their determination in biological samples such as human serum and urine, except for that using GC–MS. In this study, we have, therefore, investigated and developed a rapid and sensitive analytical technique using IC for the simultaneous determination of the four methylphosphonic acids in human serum samples.

Ion chromatographic analyses of anions have been usually performed by using an electrical conductivity detector [36]. However, the molecular masses of the methylphosphonic acids are relatively large, and their equivalent ionic conductances would be so small that it would be more suitable to use an indirect photometric detection (IPD) technique. In the IPD, an organic acid which has strong ultraviolet absorption properties is employed in mobile phases, and the reduction of absorbance of the mobile phases, caused by elution of object ions which have no ultraviolet absorption, is detected. The technique is, therefore, generally thought to be effective for the detection of compounds, such as organic acids and phosphoric acid, the equivalent ionic conductances of which are small [37–39]. We, then, compared the sensitivity of EMPA, using both detection techniques in our laboratory, and the IPD technique was proved to be approximately 50 times more sensitive than the electrical conductivity detection technique. (In this study, phthalic acid was used as the organic acid.) Thus, we chose the IPD technique in this study.

2. Experimental

2.1. Materials

Methylphosphonic acid (MPA), ethyl methylphosphonic acid (EMPA) and pinacolyl methylphosphonic acid (PMPA) were purchased from Aldrich (Milwaukee, WI, USA), and isopropyl methylphosphonic acid was synthesized in our laboratory. The standard solutions of the four methylphosphonic acids were prepared in distilled water (1 mg/ml), and adjusted to the appropriate concentration with distilled water or human serum immediately prior to use. Phthalic acid and tris(hydroxymethyl)aminomethane (Tris) purchased from Wako Pure Chemical Industries (Osaka, Japan) were of the highest available purity. Acetonitrile was of HPLC grade, and other chemicals used were of analytical grade. Micro-ultrafiltration units were purchased from Millipore (Dradford, MA, USA). Sep IC-Ag cartridge was obtained from Lida (Kenosha, WI, USA). Mobile phases were prepared by dissolving the appropriate reagents in purified water.

2.2. Ion chromatography (IC)

IPD-IC was performed on a LC-10A liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with an SPD-10A UV detector (Shimadzu) set at 266 nm (polarity being negative) and a CTO-10A temperature-controlled column compartment (Shimadzu) set at 50°C. The analytical column (an anion-exchange column packed with particles of a polyacrylate resin with quaternary ammonium groups on the surface) used was a Shim-pack IC-A3 (column dimension and particle size being 150×4.6 mm I.D. and 5 μm) (Shimadzu). The mobile phase consisted of 0.5 m*M* phthalic acid–0.1 m*M* Tris–5% acetonitrile, and the flow-rate of the mobile phase was 1 ml/min.

2.3. Sample preparation

The analytical procedure was as follows: the serum sample (1 ml) was deproteinized with the micro-ultrafiltration units, the filtrate was extracted with dichloromethane, and the aqueous layer was separated. The aqueous layer was passed through a Ag^+-form cation-exchange resin cartridge (Sep IC-Ag cartridge) and the eluate was evaporated just to dryness under a stream of nitrogen at 80°C. The residue was dissolved into 100 μl of distilled water, and a 10-μl aliquot of the solution was directly injected into the IC (Fig. 2).

Also, for the comparison in optimization of sample preparation, solid-phase extraction and solvent extraction were tested with the following methods.

Extraction with Sep pak C_{18} was conducted as follows: the Sep pak C_{18} cartridge was prewashed successively with 5 ml of methanol, 5 ml of distilled water and 5 ml of 0.01 *M* hydrochloric acid. The sample was adjusted to pH 2 and loaded on the prewashed cartridge. Subsequently, the cartridge was

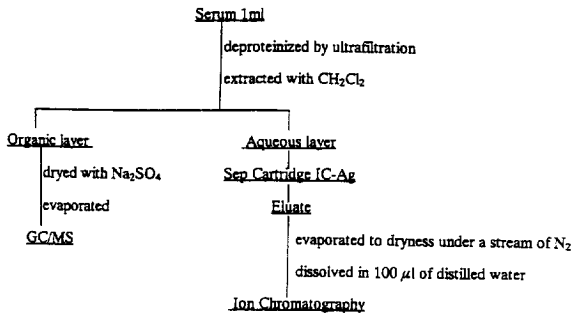

Fig. 2. Procedure for preparation of serum samples.

washed with 1 ml of distilled water. The retained compounds were eluted with 3 ml of methanol.

Extraction with Sep pak QMA and Bond Elut Certify II were accomplished as follows: Sep pak QMA and Bond Elut Certify II were prewashed successively with 10 ml of methanol and distilled water. The samples were loaded on the prewashed cartridges. Subsequently, the cartridges were washed successively with 2 ml of distilled water and 2 ml of methanol. The retained compounds were eluted with 3 ml of 0.01 *M* hydrochloric acid in methanol through the Sep IC-Ag cartridges, which were connected at the bottom of the extraction cartridges for the reduction of chloride.

Solvent extraction was performed according to the method described in our previous paper [35] and the extract was evaporated to dryness under the stream of nitrogen at 80°C. The residue was dissolved into 1 ml of methanol and the methanol solution was passed through a Sep IC-Ag cartridge for the reduction of chloride.

Peak assignments of four methylphosphonic acids were made by comparison of retention times from samples with those from authentic standards.

3. Results and discussion

3.1. Chromatographic conditions for indirect photometric detection IC

It is known that in IC analysis the retention characteristics and the separation of sample ions frequently change depending largely on the pH of the mobile phase. Then, to optimize the separation of the four methylphosphonic acids (EMPA, IPMPA, PMPA and MPA) on an anion-exchange column (Shim-pack IC-A3), suitable mobile-phase composition was explored starting with a mixture of phthalic acid and Tris. The ratios of the Tris concentration to the phthalic acid concentration were varied between 1.0 and 0.2, the pH of the mobile phase was varied, and the k values of the four methylphosphonic acids were measured. As samples, artificial mixtures of the four methylphosphonic acids were used (5 μg/ml). In the indirect photometric detection, the concentration of phthalic acid used in the mobile phase was approximately between 0.5 and 1 m*M*. In this study, we chose 0.5 and 1 m*M* as the concentration of phthalic acid, and compared the results obtained at both concentrations. The four methylphosphonic acids were eluted within 30 min, in the order of polarity: MPA, EMPA, IPMPA and PMPA. As shown in Fig. 3, the smaller ratio of the Tris concentration to the phthalic acid gave a lower pH, larger k values and the better separation. At a phthalic acid concentration of 1 m*M*, the separation between MPA, EMPA and IPMPA was not sufficient, while at a concentration of 0.5 m*M*, baseline separation could be achieved for all four methylphosphonic acids, at a ratio of Tris concentration to phthalic acid concentration below 0.6, and the best result in this eluent system was obtained at a concentration ratio of 0.2. The composition was 0.5 m*M* phthalic acid–0.1 m*M* Tris (referred to as eluent A).

For the serum sample, however, some components were eluted with insufficient resolution. Then, for improvement, the optimum composition was examined by adding small volumes of acetonitrile to eluent A, and finally sufficient resolution was achieved when the ratio of acetonitrile was 5%. The final composition was 0.5 m*M* phthalic acid–0.1 m*M* Tris–5% acetonitrile.

3.2. Sample preparation

To optimize the sample preparation for serum samples, the following three procedures were tested and the results obtained by IPD-IC were compared: direct evaporation to dryness without extraction (procedure A); solid-phase extraction (procedure B); and extraction with acetonitrile (procedure C).

For procedure A, pretreatment for the removal of

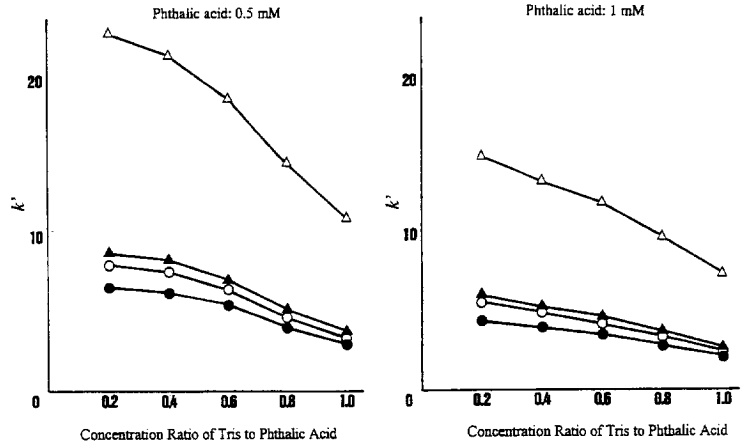

Fig. 3. Effect of eluent composition on the capacity factor (k'). Symbols: ●, MPA; ○, EMPA; ▲, IPMPA; △, PMPA.

chloride is necessary because the serum samples contain high chloride levels. To remove the chloride, sample pretreatment by passing the sample through an Ag^+ cartridge was examined after prewashing the deproteinized serum samples with dichloromethane. The eluate was analyzed according to the method described in Section 2. Deproteinized blank serum samples were used.

In the chromatogram obtained for blank serum samples prepared by directly evaporating to dryness, very large impurity peaks can be seen, which disturbed the detection of methylphosphonic acids; while by passing through the cartridge before evaporating to dryness, the high chloride level was reduced allowing us to determine the four methylphosphonic acids with scarcely any disturbance. Based on the above results, we chose the pretreatment by passing the samples through a Sep IC-Ag cartridge for procedure A.

For the solid-phase extraction of methylphosphonic acids, some solid-phase cartridges such as anion-exchange cartridges [22] and an octadecylsilane cartridge [24] have recently been used successfully. However, the techniques could not be applied to the IC analysis in the present study. Thus, we tested the three kinds of extraction cartridges for procedure B.

The solid-phase extractions with Sep pak C_{18}, Sep pak QMA and Bond Elut Certify II were performed according to the methods described in Section 2. As samples, artificial aqueous solution of four methylphosphonic acids (1 μg/ml) were used. Every resultant extract obtained was evaporated to dryness under a stream of nitrogen at 80°C, and the residues were dissolved into 100 μl of distilled water. The solutions were analyzed by IC and the recoveries of four methylphosphonic acids were compared. With Bond Elut Certify II, as shown in Table 1, relatively high recoveries for all four methylphosphonic acids were obtained, suggesting that Bond Elut Certify II was the best of the three cartridges tested. We, then, chose Bond Elut Certify II as the solid-phase extraction cartridge for procedure B.

Procedure C (extraction with acetonitrile) was performed according to the method described in

Table 1
Recoveries of MPA, EMPA, IPMPA and PMPA by using various solid-phase extraction cartridges

	Recoveries (mean±S.D., n=3) (%)			
	MPA	EMPA	IPMPA	PMPA
Sep Pak C_{18}	23.7±1.9	46.9±3.0	47.9±3.8	50.3±2.6
Sep Pak QMA	89.9±2.9	30.5±2.6	29.4±2.3	18.1±0.6
Bond Elut Certify II	83.3±3.1	57.9±3.9	54.1±3.5	49.7±3.6

Section 2. The extract was evaporated to dryness under a stream of nitrogen at 80°C, and the residues were dissolved into 100 μl of distilled water.

The IC analyses were carried out after the pretreatments according to procedures A, B and C by using standard aqueous sample solution (1 μg/ml) as samples, and the recoveries of the four methylphosphonic acids were compared.

The recoveries obtained are shown in Table 2. Fairly low recovery of MPA was obtained by procedure C and, therefore, procedure C was ruled out. The best recoveries of the four methylphosphonic acids were given by procedure A, suggesting that procedure A should be preferred to procedure B. However, large volumes of samples could be treated and concentrated in a short time by procedure B, while relatively long times were needed in evaporating to dryness by procedure A. To compare procedures A and B in more detail, we treated blank serum samples according to procedures A and B, and IC analyses were subsequently performed.

The chromatograms depicted in Fig. 4a–c were obtained for serum samples from a healthy volunteer which were prepared by procedures A and B, and for an authentic sample, respectively. In the chromatogram in Fig. 4b, one can see a large impurity peak at the retention time of ca. 11 min which would disturb the detection of MPA and EMPA. On the other hand, scarcely any peaks which would interfere with the detection of four methylphosphonic acids appeared in the chromatogram in Fig. 4a, suggesting that procedure A is preferable to procedure B for the preparation of serum samples. Based on the above comparison, in the present study we finally chose procedure A. The serum sample (1 ml) was deproteinized with the micro-ultrafiltration units, and the filtrate was extracted with dichloromethane, and

Table 2
Recoveries of MPA, EMPA, IPMPA and PMPA using various sample preparation techniques

	Recoveries (mean±S.D., n=3) (%)			
	MPA	EMPA	IPMPA	PMPA
Procedure A[a]	93.2±3.7	91.2±3.4	89.3±3.2	82.2±4.1
Procedure B[a]	83.3±3.1	57.9±3.9	54.1±3.5	49.7±3.6
Procedure C[a]	5.4±0.4	57.2±4.2	57.3±4.1	47.4±4.1

[a]See text.

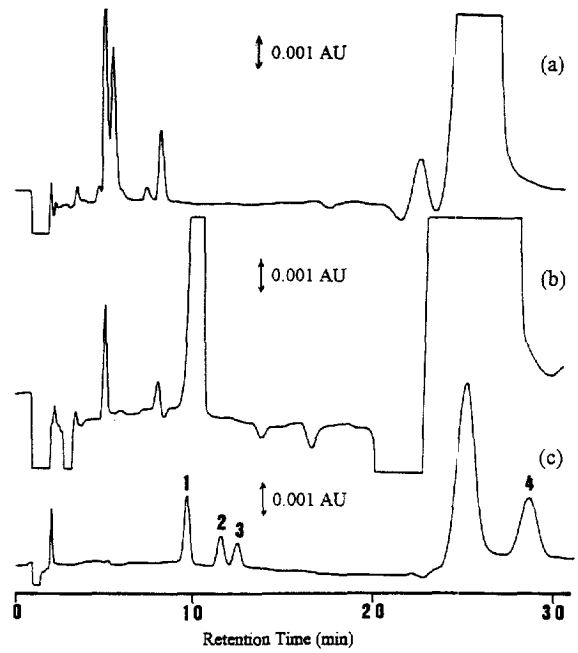

Fig. 4. Chromatograms obtained from a serum sample of a healthy volunteer after sample preparation with: (a) Sep IC-Ag cartridge, and (b) Bond Elut Certify II; and (c) from an authentic sample (5 μg/ml). Peaks: 1, MPA; 2, EMPA; 3, IPMPA; 4, PMPA.

the aqueous layer was separated. The aqueous layer was passed through a Ag^+-form cation-exchange resin cartridge (Sep IC-Ag cartridge) and the eluate was evaporated just to dryness under a stream of nitrogen at 80°C. The residue was dissolved into 100 μl of distilled water, and a 10-μl aliquot of the solution was directly injected into the IC.

Incidentally, when procedure B was applied to samples such as soil and environmental water, the four methylphosphonic acids could be detected without scarcely any disturbance by impurities in samples. Additionally, procedure B has the advantage of availability for preparation and concentration of large volume of sample, which would lead to very sensitive analysis. It suggests that procedure B would be preferable to procedure A for analyses of large volumes of environmental samples.

3.3. IC analysis of fortified serum samples

According to the method developed in the present study, IC analysis was carried out for fortified serum

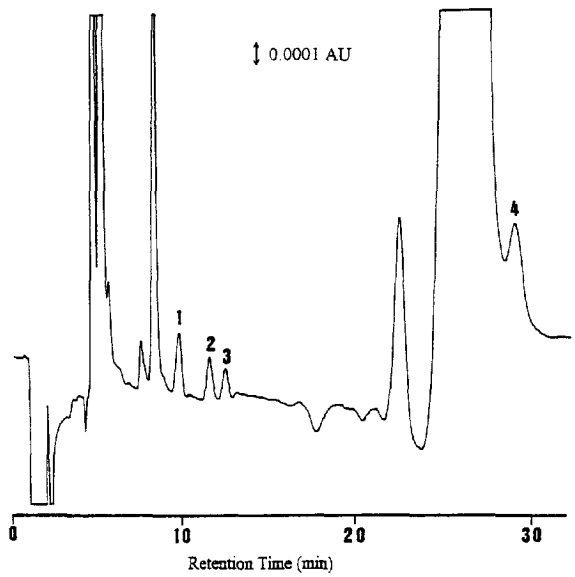

Fig. 5. Chromatogram obtained from a fortified serum sample (250 ng/ml). Peaks: 1, MPA; 2, EMPA; 3, IPMPA; 4, PMPA. Chromatographic conditions are given in Section 2.

samples in which the concentration of all added methylphosphonic acids was 250 ng/ml. As depicted in Fig. 5, all four methylphosphonic acids could be simultaneously detected in approximately 30 min without scarcely any disturbance by impurities in the serum, except that PMPA was eluted with a little interference from a component, which was suspected to be chloride.

The recoveries were 78.1±5.8% for MPA, 85.3±3.9% for EMPA, 84.9±3.3% for IPMPA and 72.9±4.9% for PMPA, respectively ($n=3$), which were a little inferior to that from standard aqueous solution samples.

3.4. Quantitative analysis and detection limits

To see how the methods established here work reliably, quantitative measurements were performed for fortified serum samples where the concentration of the added methylphosphonic acids were varied. The calibration curves constructed from the peak areas showed good linearity throughout the concentration range from 50 to 1000 ng/ml for MPA, EMPA and IPMPA (MPA, $y=15.1x+45.8$, $r^2=0.999$; EMPA, $y=12.8x-66.7$, $r^2=0.999$; IPMPA, $y=14.6x-57.7$, $r^2=0.999$), and from 100 to 1000 ng/ml for PMPA ($y=47.4x-747$, $r^2=0.996$). The detection limits were calculated to be 40 ng/ml for MPA, EMPA and IPMPA, and 80 ng/ml for PMPA at the S/N ratio of 3. The relative standard deviation obtained at the sample concentration of 500 ng/ml was 6.9% for MPA, 4.1% for EMPA, 3.8% for IPMPA, and 6.5% for PMPA ($n=5$).

4. Conclusion

In the present study a simple, rapid and accurate detection method was developed, which allows us to simultaneously detect the degradation products, methylphosphonic acids, by IPD-IC analysis after deproteinizing by ultrafiltration and successively passing through a Sep IC-Ag cartridge for reduction of chloride. In the method, the sample preparation offers relatively good recoveries and high sensitivity of four methylphosphonic acids, and reliable reproducibility of results. Furthermore, by employing solid-phase extraction with Bond Elut Certify II as the sample preparation, the method can be applied to the more sensitive analyses of methylphosphonic acids in soil and environmental water. Thus, it should become a powerful method for the rapid and sensitive detection in biological and environmental samples of the use of the nerve agents such as VX and sarin.

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