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Feasibility of an on-line restricted access material/liquid chromatography/tandem mass spectrometry method in the rapid and sensitive determination of organophosphorus triesters in human blood plasma

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Abstract

A rapid on-line solid phase extraction/liquid chromatography/tandem mass spectrometry (SPE/LC/MS/MS) method using restricted access material (RAM) was developed for the simultaneous determination of eight organophosphorus triesters in untreated human blood plasma. In a process involving column-switching techniques, the analytes were enriched on the RAM column, separated using a C-18 analytical column and detected with LC/MS. Tandem mass spectrometry was used to characterize and quantify the analytes. To elucidate the fragmentation pathway of a number of the analytes, MS^3 experiments using an ion trap mass spectrometer were performed. The matrix effects associated with using APCI and ESI interfaces were investigated. The recoveries obtained were in the range 60–92% (R.S.D. $< 6\%$), with estimated detection limits between 0.2 and 1.8 ng/ml of plasma, and the total analysis time was 27 min.

Keywords: Restricted access material; Organophosphorus triesters

1. Introduction

Organophosphorus triesters are used as additives, i.e. not chemically bound, to act as flame retardants and/or plasticizers in a wide range of products. Volatilization and leaching of these compounds into indoor environments [1–3] and their presence in blood donor plasma [4], at concentrations as high as 1 μg/g [5], have been reported. There is scant information in the literature about the impact of organophosphorus triesters on human health. However, they have been reported to have diverse biological activities depending on their structure. For instance, triphenyl phosphate inhibits human blood monocyte carboxylesterase [6], tris(2-chloroethyl) phosphate has neurotoxic and carcinogenic effects in rats [7] and a variety of arylated phosphorus esters can induce toxic responses in reproductive systems [8,9].

To determine a target compound in complex matrices such as plasma or urine, several preparative steps are usually required prior to analysis. Liquid–liquid extraction is one of the techniques commonly

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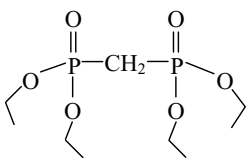
employed for this purpose. However, it is laborious, time- and solvent-consuming, and gives relatively poor reproducibility. Alternatives such as microporous membrane liquid–liquid extraction [4] and robotic liquid handling workstations [10,11] have been developed to overcome these problems, but despite this solid phase extraction (SPE) is still the most popular sample preparation technique to date.

In this study a selective SPE, termed restricted access material (RAM), was used on-line prior to LC/MS for sample clean up and analyte pre-concentration. RAMs allow the direct and repetitive injection of untreated complex matrices, such as environmental [12–14] and biological samples [15–18]. The sample clean up relies on two simultaneous chromatographic separation mechanisms: size exclusion chromatography, which excludes macromolecules such as plasma proteins, and reversed phase interaction, allowing the retention of small target molecules [19].

LC/MS is a widely used technique for the fast and sensitive characterization and quantification of analytes in complex matrices such as biological fluids. Electrospray mass spectrometry is a popular ionization technique, partly because of its ability to analyze large and non-volatile molecules such as proteins [20]. However, electrospray systems are susceptible to signal suppression by the sample matrix, which is a significant drawback [21,22]. Several factors have been proposed as possible causes of this problem, including column overload [22], as well as co-elution of matrix components and analytes [22–24]. A number of possible methods have been suggested to compensate for, or prevent, matrix signal suppression, including the use of internal standards [23], the addition of various additives or buffers to the solution or mobile phase



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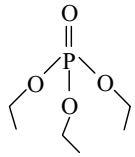
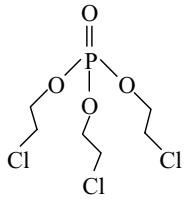
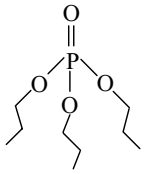


Triethyl phosphate



Tris(2-chloroethyl) phosphate



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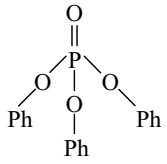

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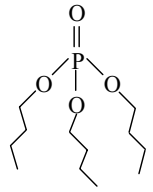
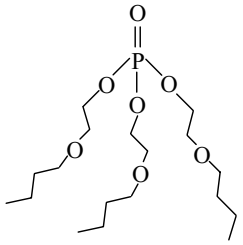


Tris(n-butyl) phosphate



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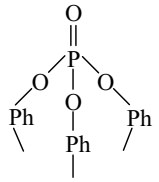
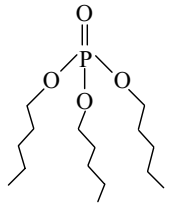


Tritolyl phosphate



Tris(n-pentyl) phosphate

Fig. 1. Structures of the compounds studied.

[25] and the use of valves to divert unwanted fractions and thus reduce the build up of matrix components in the ionization source [26]. However, the most direct and common means of reducing signal suppression is sample clean up prior to LC/MS analysis [21,22].

The aim of this study was to develop a rapid and sensitive method for the determination of low concentrations of the organophosphorus triesters shown in Fig. 1 (including the more polar triethyl phosphate and tris(2-chloroethyl) phosphate, which are difficult to extract using liquid–liquid extraction methods) in human blood plasma. The method developed also provides an unequivocal identification of the analytes using collision-induced dissociation (CID) spectra.

2. Experimental

2.1. Chemicals

Methanol was purchased from BDH (Poole, England), acetonitrile from Merck (Darmstadt, Germany) and formic acid from Riedel-de Haën (Seelze, Germany). Tetraethyl ethylenediphosphonate, triethyl phosphate, tris(2-chloroethyl) phosphate, tris(*n*-propyl) phosphate, triphenyl phosphate, tris(*n*-butyl) phosphate, tris(2-butoxyethyl) phosphate, tritolyl phosphate, tris(*n*-pentyl) phosphate and diphenyl methyl phosphate were purchased from Aldrich (Milwaukee, WI, USA). All the solvents and compounds, except for the internal standard diphenyl methyl phosphate with a purity of exceeding 99%, were of analytical grade (>98%). Stock solutions containing 1 μg/μl of each compound in acetonitrile were prepared and used throughout the experiments for sample preparation.

2.2. On-line SPE/HPLC system

The analytical system used consisted of two pumps (Varian 9012, California, USA and Shimadzu LC-10AD, Kyoto, Japan), two six port injection valves (Rheodyne), a column switch valve (Valco Instruments Co., Inc.), a C-18 HPLC column (5 μm, 250 mm × 4.6 mm i.d., Alltech), a restricted access material column (25 μm, 25 mm × 4.0 mm i.d., LiChrospher® RP-18 ADS, Merck) and a UV spectrophotometric detector (Shimadzu SPD-6A). Data were acquired and processed using ELDS software (Chromatography Data System, Sweden, version 1.1).

The procedure for extracting organophosphorus triesters from plasma samples involved two steps, as shown in Fig. 2. Samples were injected using injection valve no. 3 (500 μl injection loop) while valve no. 5 was in the position shown in Fig. 2a. The auxiliary pump flushed the sample matrix through the RAM trap, delivering 1 mM formic acid in water with a flow rate of 0.6 ml/min for 5 min. After sample loading, valve no. 5 was rotated through 60° as shown in Fig. 2b. The analytes were then eluted and separated by the HPLC analytical column using a 22-min gradient from 70 to 100% methanol followed by isocratic for 5 min. Both the methanol and the water delivered by the HPLC pump contained 1 mM of formic acid and the flow rate was 0.8 ml/min. The external reference and the internal standard were injected in the analysis mode using valve no. 4 and a 20 μl injection loop. One RAM cartridge was used throughout the whole study and only a slight increase in the pressure was observed while performing the extraction step.

2.3. LC/MS detection

The mass spectrometer used in this study was a triple quadrupole Quattro Micro (Micromass, Manchester, UK). When using +ESI, the operating parameters were: capillary 3 kV, cone 25 V, extractor 2 V, RF lens 0.3 V, source temperature 130 °C, desolvation temperature 275 °C and desolvation gas (N_2, Aquilo nitrogen generator, The Netherlands) flow rate 900 l/h. When using +APCI the parameters were set to: corona 2.0 μA, cone 30 V, extractor 2 V, RF lens 0.3 V, source temperature 130 °C, APCI probe temperature 275 °C, desolvation gas (N_2, Aquilo nitrogen generator, The Netherlands) flow rate 100 l/hr and cone gas (N_2, Aquilo nitrogen generator, The Netherlands) flow rate 10 l/h. The pressure of argon (AGA, Sweden) in the collision cell was 4×10^{-3} mbar. Data were acquired and processed using MassLynx version 3.5 from Micromass. The *m*/*z* isolation width was set to 1 and the dwell times for the monitored product ions were 0.20 s.

In order to clarify the structure of certain fragments, MS^3 experiments were performed using a LCQ ion trap mass spectrometer (Finnigan Mat, CA, USA) with +ESI under the following conditions: sheath gas (N_2,

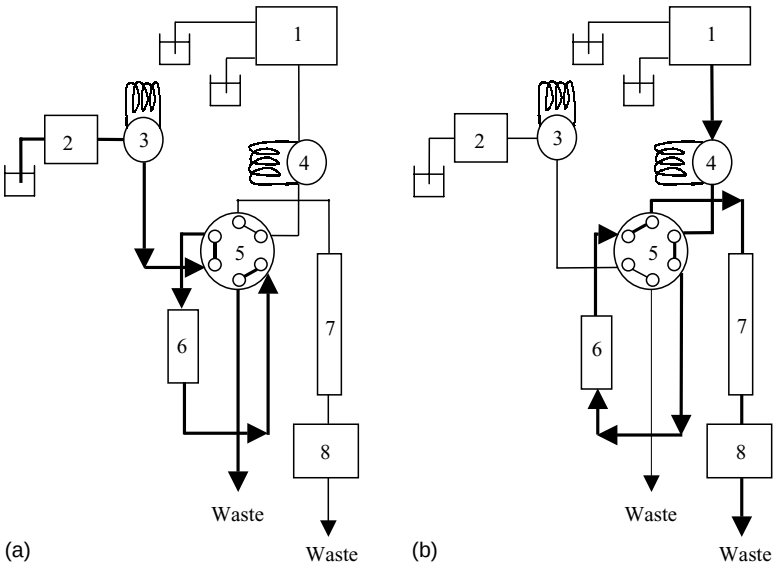


Fig. 2. Schematic diagram of the on-line SPE/HPLC system used for the extraction of organophosphorus triesters from human blood plasma. Parts (a) and (b) illustrate the loading mode and the analysis mode, respectively. (1) HPLC pump; (2) auxiliary pump; (3, 4) injection valves; (5) column switch valve; (6) RAM column; (7) HPLC column; and (8) detector.

Aquilo nitrogen generator, The Netherlands) flow rate 90 arbitrary units, auxillary gas (N_2, Aquilo nitrogen generator, The Netherlands) flow rate 70 arbitrary units, spray voltage 4 kV, capillary temperature 200 °C and capillary voltage 18 V. Data acquisition was performed using Xcalibur version 1.1.

2.4. Matrix effects and quantitative analysis

Post-column infusion experiments were performed by infusing (flow rate of 5 μl/min) a 10 ng/μl standard solution of triethyl phosphate and diphenyl methyl phosphate into blank plasma extract through out the analysis. The infusion syringe pump (Micromass, Manchester, UK) was placed after the analytical column (no. 7 in Fig. 1).

For the quantitative analysis, an external reference was obtained by injecting 20 μl of a 0.5 ng/μl solution of all the organophosphorus triesters prepared in 50% methanol–water. The same solution was used to spike plasma and plasma extracts. To evaluate recoveries and matrix effects, quantitative analysis was performed by calculating the ratios between the peak area of the analytes and the internal standard (diphenyl methyl phosphate) in the sample, and comparing them to the ratios obtained from the external reference, with the external reference being injected after every second extraction.

3. Results and discussion

3.1. Matrix effects and selection of the ionization source

The use of RAM greatly simplifies sample pretreatment and allows the injection of untreated plasma in on-line SPE/LC/MS, providing a combined enrichment and clean up of the sample. Recently it has been reported that even after clean up or column switching some matrix effects still occur [22,27–29]. To assess this possibility and to determine the most appropriate method for analyzing organophosphorus triesters in plasma, both ESI and APCI ionization techniques were evaluated. In negative ionization weak signals were observed for the analytes. On the contrary, both techniques, in the positive ionization mode, can be considered suitable for the LC/MS detection of organophosphorus triesters. However, the use of positive ESI resulted in the highest signal to noise ratios, as

Table 1
Comparison between +ESI and +APCI ionizations. Percentage recoveries obtained from spiked plasma are compared with injection of standard solution into blank plasma extract ($n = 3$)

Organophosphorus triester	+ESI			+APCI		
	Spiked plasma	Analytes injected + blank plasma extract	LOD (ng)[a]	Spiked plasma	Analytes injected + blank plasma extract	LOD (ng)[a]
Tetraethyl ethylenediphosphonate	20 ± 0.5	19 ± 0.3	0.01	91 ± 2.0	90 ± 1.9	0.3
Triethyl phosphate	20 ± 0.6	19 ± 0.4	0.01	93 ± 2.1	92 ± 2.0	0.1
Tris(2-chloroethyl) phosphate	19 ± 0.5	19 ± 0.5	0.1	92 ± 2.2	93 ± 2.1	0.4
Tris(*n*-propyl) phosphate	77 ± 2.0	75 ± 1.8	0.01	89 ± 1.9	90 ± 2.0	0.05
Triphenyl phosphate	101 ± 3.2	97 ± 2.9	0.2	83 ± 1.5	82 ± 1.6	0.5
Tris(*n*-butyl) phosphate	110 ± 3.8	105 ± 3.4	0.01	85 ± 1.7	84 ± 1.6	0.08
Tris(2-butoxyethyl) phosphate	108 ± 3.5	102 ± 2.8	0.1	77 ± 1.1	79 ± 1.2	0.1
Tritolyl phosphate	90 ± 2.9	95 ± 2.2	0.1	62 ± 0.9	63 ± 1.0	0.2
Tris(*n*-pentyl) phosphate	104 ± 3.0	103 ± 3.1	0.02	80 ± 1.4	81 ± 1.6	0.07

[a] The instrumental LOD values, estimated at S/N = 3, are based on the injection of 20 μl of a 0.5 ng/μl standard solution.

shown by the instrumental limit of detection (LOD) values listed in Table 1. In a preliminary study using +ESI, in which spiked plasma samples were extracted, very low recoveries were obtained for the early eluting compounds. To determine whether the calculated low recoveries resulted from matrix effects or insufficient extraction, the standard solution used for spiking plasma samples was injected simultaneously into a blank plasma extract using valve no. 4 (Fig. 2). The results were then compared to those derived from the extraction of spiked plasma (Table 1). This comparison suggests that the suppression of the recovery is mainly due to matrix effects rather than insufficient extraction. An identical procedure was performed using +APCI and lower signal suppressions were observed in this case.

To further investigate the possible matrix effects in +ESI, one of the compounds (triethyl phosphate) that had a low recovery with +ESI (but not with +APCI) and the internal standard (diphenyl methyl phosphate) were infused into blank plasma just before ionization (see Section 2 for details). The chromatograms were collected using both +ESI and +APCI in the full scan mode and UV detection (220 nm) and are compared in Fig. 3. In the case of triethyl phosphate ionized by +ESI, an enormous reduction in the signal was observed as soon as the extracted plasma reached the MS detector. In a comparison of the single MS spectra (with no CID) of a reference with a spiked sample, no differences in the fragments or metal adduct abundances were observed. This suggests that the signal suppression observed when using +ESI is probably due to a competition in ion formation between the analyte and co-extracted endogenous compounds. The low recoveries calculated for the compounds eluted in the first part of the +ESI chromatogram are most likely due to this matrix suppression effect. Even though an internal standard was employed to compensate for differences in ionization efficiencies, it was not an ideal solution, since the signal at the retention time of the internal standard (approximately, 14 min) was not suppressed by the matrix to the same extent as signals in the first part of the chromatogram. For both compounds, the +APCI ionization was much less strongly affected by the matrix when compared to the +ESI ionization, and the signal was significantly more stable.

An attempt to reduce the matrix effect by using less retentive RAMs was made. However, the use of C-4 and C-8 RAMs, instead of C-18, not only increased the matrix effect but also significantly decreased the recoveries of the first eluting compounds. In addition, in order to stabilize the pH conditions in the first part of the chromatogram, acidic water was used to load the sample and wash the RAM cartridge.

3.2. CID fragmentation of organophosphorus triesters

The fragmentation patterns of a number of organophosphorus compounds have been investigated by several authors using CID (also referred to as collision

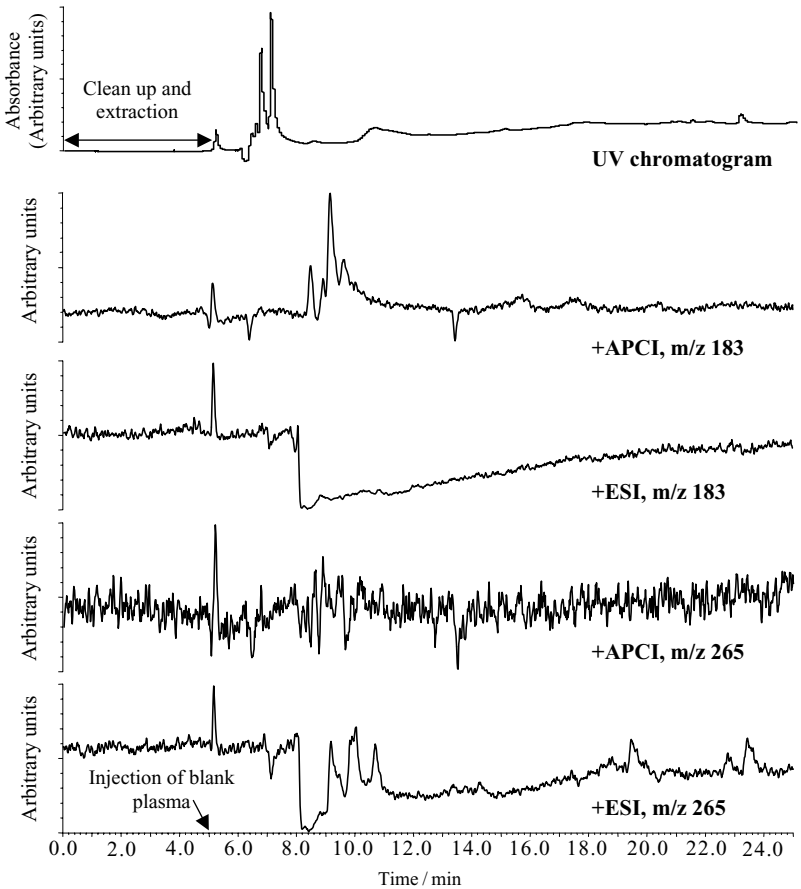


Fig. 3. UV and extracted ion chromatograms of post-column infusion of triethyl phosphate (*m*/*z* 183) and diphenyl methyl phosphate (*m*/*z* 265) into extract of blank plasma. The compounds were ionized using +APCI and +ESI.

activated dissociation or decomposition, CAD) [30–40]. Most of these studies focused on pesticides, chemical warfare agents or their metabolites. The information reported in the literature to date is based on alkylated phosphorus esters and, to the best of our knowledge, there is no published information on the CID fragmentation of di- and tri-arylated organophosphorus triesters.

In this study, substantial differences in the stability of the protonated molecules of organophosphorus triesters were observed. The fragments obtained and their relative abundances are reported in Table 2. In agreement with a previous report [33], the CID mechanism in all the alkyl-substituted phosphorus esters (with alkyl chains longer than ethyl) was dominated by three successive McLafferty rearrangements. Trialkyl phosphorus esters went through a successive, neutral loss of their aliphatic chains, as the corresponding alkenes, at relatively low fragmentation energies, until only protonated phosphoric acid (*m*/*z* 99) was left (Table 2). Similar behavior was observed for tris(2-chloroethyl) phosphate (Fig. 4a). In the CID process of tris(2-chloroethyl) phosphate, ion *m*/*z* 125 was not generated from the fragmentation of ion *m*/*z* 161, which suggests that the protonated phosphoric acid monoethenyl ester (*m*/*z* 125) was probably formed by the simultaneous loss of a chloroethyl group and hydrochloric acid. In the case of tris(2-butoxyethyl) phosphate (Fig. 4b), the fragmentation pathway was dominated by the loss of positively charged butoxyethyl ions *m*/*z* 101. The formation of ion *m*/*z* 143 through loss of a butyl from

Table 2
List of the organophosphorus triesters in their elution order from the C-18 column. The fragments were obtained at two different collision energies and their relative abundances (>10%) reported. Three fragments with the optimum collision energy were determined for each compound for their further use in SRM mode

Organophosphorus triester MH^+	Daughter scan collision energy (16 eV)		Daughter scan collision energy (25 eV)		SRM	
	Fragment (*m*/*z*)	Relative abundance (%)	Fragment (*m*/*z*)	Relative abundance (%)	Fragment (*m*/*z*)	Collision energy (eV)
Tetraethyl ethylenediphosphonate (*m*/*z* 303)	303	78	219	17	275	16
	275	100	191	68	191	22
	247	83	173	100	173	28
	229	22				
	219	48				
	201	50				
	191	23				
	173	32				
Triethyl phosphate (*m*/*z* 183)	127	17	99	100	155	10
	99	100			127	10
					99	16
Tris(2-chloroethyl) phosphate (*m*/*z* 285)	223	31	125	21	161	16
	161	93	99	100	125	16
	125	89			99	19
	99	100				
Diphenyl methyl phosphate (*m*/*z* 265)	265	100	215	18	189	25
	153	10	189	73	171	22
			171	48	153	22
			165	17		
			159	13		
			153	100		
			152	73		
			143	43		
			141	60		
			127	15		
			109	55		
			95	22		
			94	12		
			91	72		
Tris(*n*-propyl) phosphate (*m*/*z* 225)	99	100	99	100	183	7
					141	10
					99	16
Triphenyl phosphate (*m*/*z* 327)	327	100	327	40	251	25
			251	46	215	25
			233	47	153	25
			229	13		
			228	17		
			215	58		
			171	15		
			153	100		
			152	67		
			95	16		

Table 2 (*Continued*)

Organophosphorus triester MH^+	Daughter scan collision energy (16 eV)		Daughter scan collision energy (25 eV)		SRM	
	Fragment (*m*/*z*)	Relative abundance (%)	Fragment (*m*/*z*)	Relative abundance (%)	Fragment (*m*/*z*)	Collision energy (eV)
Tris(*n*-butyl) phosphate (*m*/*z* 267)	99	100	99	100	211	7
					155	10
					99	16
Tris(2-butoxyethyl) phosphate (*m*/*z* 399)	299	24	101	100	299	16
	199	60	99	23	199	16
	101	100			101	19
Tritolyl phosphate (*m*/*z* 369)	369	100	369	56	166	25
			279	12	107	31
			261	27	91	34
			256	13		
			243	32		
			181	15		
			179	11		
			166	100		
			165	26		
			153	13		
			109	15		
			107	25		
			91	83		
Tris(*n*-pentyl) phosphate (*m*/*z* 309)	99	100	99	100	239	7
					169	10
					99	16

the last butoxyethyl group is also noteworthy. In the fragmentation pathway of tetraethyl ethylenediphosphonate (Fig. 4c) two different sequences appeared. The sequence of *m*/*z* 275 to 247 to 219 and finally to 191 was generated by the loss of ethyl groups. The ion *m*/*z* 229 was probably generated by an internal rearrangement leading to the hypothetic structure illustrated in Fig. 4c. Further loss of ethyl groups from this ion generated ions *m*/*z* 201 and 173.

The fragmentation pattern of the di- and tri-arylated phosphorus esters is more complex and difficult to interpret. In the case of triphenyl phosphate, several other peaks in addition to the expected fragments arising from the loss of aryl (*m*/*z* 251) and protonated phenol (*m*/*z* 95) appeared in the spectrum (Fig. 5a). Identification of these peaks, including the base peak *m*/*z* 153, is not possible on the basis of simple assumptions. Fragment *m*/*z* 233 can be interpreted, by analogy with Kuivalainen et al. [31], as the product of a phenol loss through an intramolecular electrophilic

Table 3
Recoveries of organophosphorus triesters extracted from spiked (10 ng) plasma samples using C-18 RAM column and LC/APCI/MS/MS detection ($n = 7$)

Analyte	Recovery (%)	R.S.D. (%)	LOD (ng/ml)[a]
Tetraethyl ethylenediphosphonate	92	5.2	0.6
Triethyl phosphate	92	1.6	0.4
Tris(2-chloroethyl) phosphate	92	4.7	0.8
Tris(*n*-propyl) phosphate	89	0.9	0.2
Triphenyl phosphate	82	3.7	1.8
Tris(*n*-butyl) phosphate	84	2.9	0.2
Tris(2-butoxyethyl) phosphate	75	4.9	0.6
Tritolyl phosphate	60	4.8	0.8
Tris(*n*-pentyl) phosphate	79	3.1	0.4

[a] The method LOD values, estimated at S/N = 3 for the fragments with the weakest signal, are based on the extraction of 0.5 ml spiked plasma.

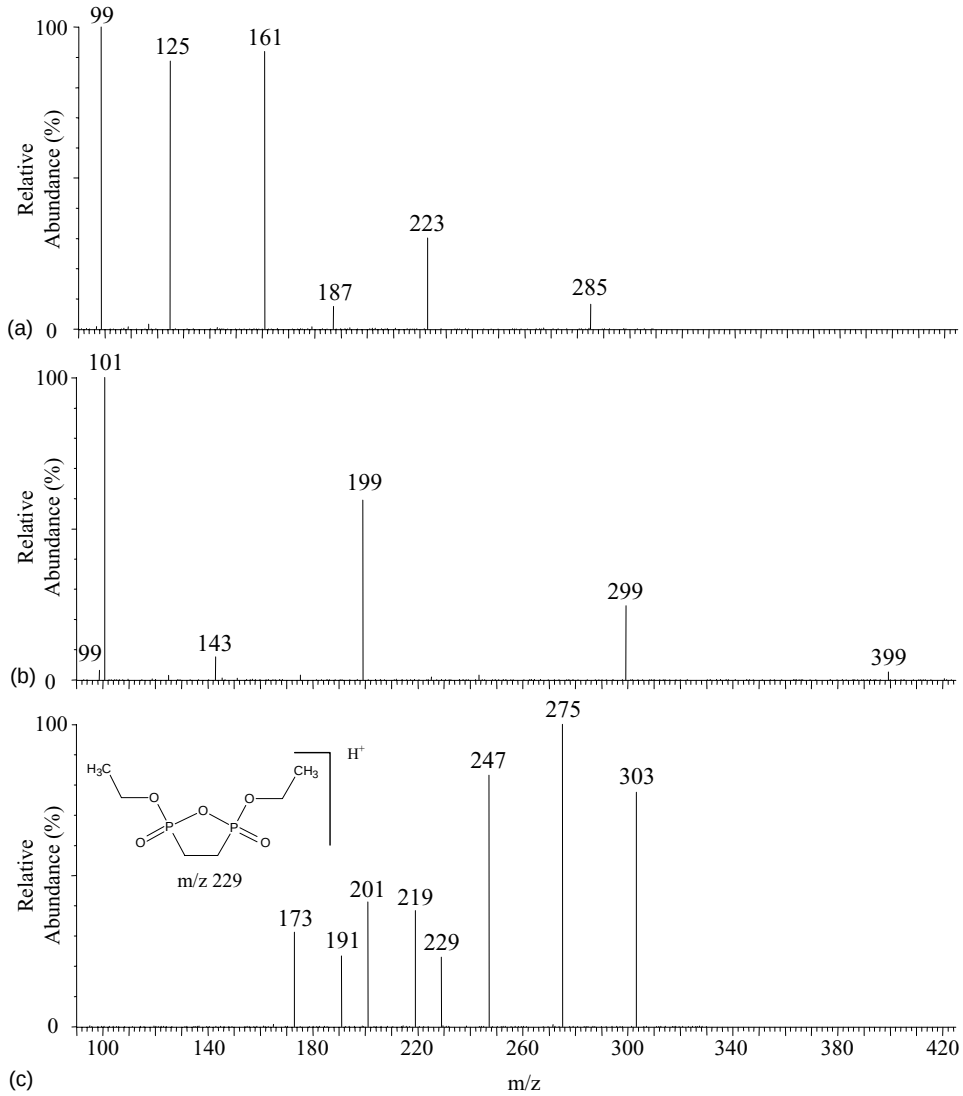


Fig. 4. CID spectra of (a) tris(2-chloroethyl) phosphate *m*/*z* 285 (scan range *m*/*z* 90–310); (b) tris(2-butoxyethyl) phosphate *m*/*z* 399 (scan range *m*/*z* 90–425); and (c) tetraethyl ethylenediphosphonate *m*/*z* 303 (scan range *m*/*z* 90–330). The collision energy was set to 16 eV.

aromatic substitution. In our case, this four-center intermediate is probably stabilized by the presence of a second aromatic ring on the fragment. Ion *m*/*z* 215 is also most likely related to the structures suggested by Kuivalainen et al., but with two aromatic rings. Similar behavior has been observed in the spectra of diphenyl methyl phosphate and tritolyl phosphate. The possibility of tolyl fragments rearranging into tropylium, which then condense into other aromatic structures, most likely explains the presence of a base peak with an even-numbered *m*/*z* ratio (*m*/*z* 166) in the spectra of tritolyl phosphate. The nature of these fragments is of considerable interest and will be the object of further study.

3.3. Recovery calculations and quality parameters

A LC/APCI/MS/MS method using selective reaction monitoring (SRM) was developed for the analysis of organophosphorus triesters. The characteristic

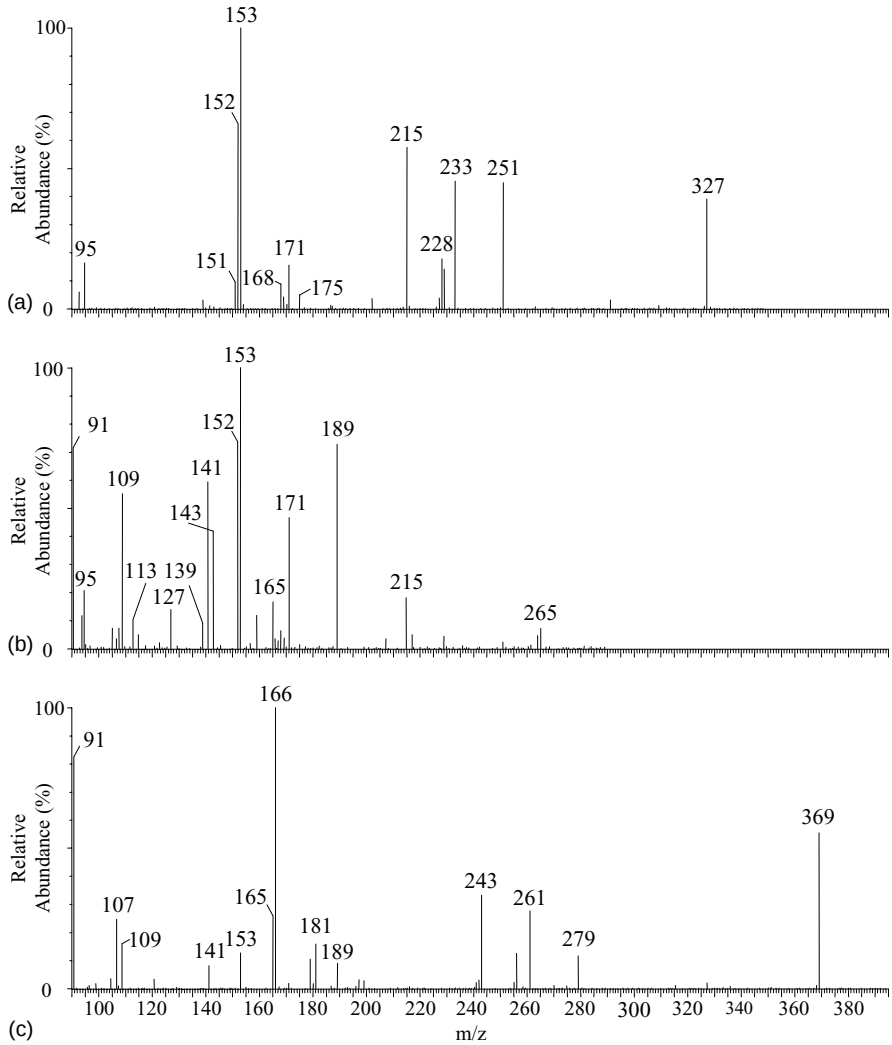


Fig. 5. CID spectra of (a) triphenyl phosphate *m*/*z* 327 (scan range *m*/*z* 90–350); (b) diphenyl methyl phosphate *m*/*z* 265 (scan range *m*/*z* 90–290); and (c) tritolyl phosphate *m*/*z* 369 (scan range *m*/*z* 90–400). The collision energy was set to 25 eV.

fragmentation pattern of each compound was investigated using MS/MS in a range of collision energies. In order to assign the peaks unequivocally, after selection of the protonated molecule for the CID process, at least three fragments were selected. Finally, in order to obtain the lowest possible LOD, the optimum collision energy giving the best signal to noise ratio was determined for the three selected fragments by acquiring spectra in the SRM mode for a collision energy step of 3 eV between 4 and 40 eV. The fragments obtained at two different collision energies and their relative abundances as well as the optimized conditions for the SRM mode are summarized in Table 2. Typical SRM chromatograms obtained from the extraction of a human plasma sample spiked with 10 ng of organophosphorus triesters are shown in Fig. 6. The recoveries calculated for this sample, using the optimized SRM mode, are presented together with the method detection limits in Table 3.

Linearity was investigated for two compounds: one aromatic, triphenyl phosphate, and the other non-aromatic, triethyl phosphate. These compounds

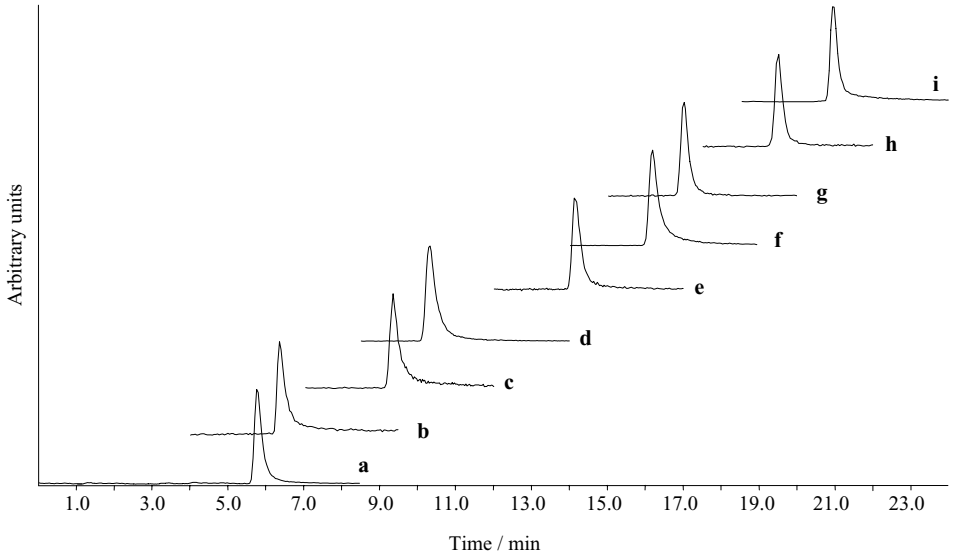


Fig. 6. LC/+APCI/SRM chromatograms obtained from a human plasma sample spiked with 10 ng of the investigated organophosphorus triesters. (a) Tetraethyl ethylenediphosphonate and triethyl phosphate; (b) tris(2-chloroethyl) phosphate; (c) diphenyl methyl phosphate; (d) tris(*n*-propyl) phosphate; (e) triphenyl phosphate; (f) tris(*n*-butyl) phosphate; (g) tris(2-butoxyethyl) phosphate; (h) tritolyl phosphate; and (i) tris(*n*-pentyl) phosphate. The peaks were normalized to 100%.

showed linearity in the ranges 1–100 and 0.5–100 ng, respectively, with correlation coefficients equal to 0.999.

4. Conclusions

A rapid and sensitive method for the determination of traces of organophosphorus triesters in human blood plasma has been developed. The use of RAM avoids extensive and time-consuming sample treatments. The use of an APCI ion source, operating in the described conditions, has been shown to be more robust when compared with ESI. Characterization and unequivocal identification of the analytes was obtained by CID spectra. The simplicity, speed and reproducibility of this method make it suitable for epidemiological studies in order to evaluate the occurrence of such contaminants in exposed people.

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