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Synthesis and use of deuterated fenamiphos and its metabolites as internal standards for mass spectrometric analysis in water

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Abstract

The applications of mass spectrometry (MS) are increasing in environmental analysis, pesticides measurement in particular. To take full advantage of the capabilities of MS it is important to have available labeled standards that enhance the performance of the quantitative analysis, increasing its accuracy and precision. We synthesized a series of deuterated internal standards corresponding to fenamiphos (Ia) and some of its metabolites, using rapid procedures and inexpensive materials. The use of 3-fluoro-3-(heptafluoropropyl)-2-(nonafluorobutyl)-*cis*-oxaziridine (IV) was particularly attractive for the preparation of oxidative metabolites of fenamiphos (Ia), analytical and deuterated standards. We used these compounds for the analysis of these pollutants in water. We used solid-phase extraction, testing several materials, and supercritical fluid extraction techniques. The analysis was done by gas chromatography–mass spectrometry, with the selected ion monitoring (SIM) technique, obtaining good results with the oxidative metabolites of compound Ia (compounds IIa and IIIa). For the more polar metabolites Ib, IIb and IIIb, liquid chromatography–mass spectrometry analysis in the SIM mode gave the best result.

Keywords: Environmental analysis; Water analysis; Fenamiphos; Pesticides; Organophosphorus compounds

1. Introduction

MS has been recognized as the reference method for identification of various pollutants, and pesticides in particular, and has been proposed as a validating technique on account of its high sensitivity in determining compounds on the basis of their chemico-physical characteristics [1]. MS is widely used by the EPA in the USA and in Europe for an increasing number of quantitative applications. One reason is the limited availability of suitable internal standards (I.S.) that enhance the quantitative analysis of pesticides by MS, greatly increasing its accuracy and the precision.

GC–MS and LC–MS in the SIM mode permit compounds labeled with stable isotopes to be used as I.S., providing good accuracy and reproducibility, in view of the very similar properties to those of the substances to be analyzed. I.S. are used mainly to compensate for the sample loss during clean up and to correct the deviations of injection volumes and variation in detector response.

In a previous paper [2] ethofumesate and its metabolites were analyzed in water using penta-deuteroethyl ethofumesate as I.S. As part of a project aimed at monitoring organophosphorus agrochemical in the environment [3], we were also interested in the

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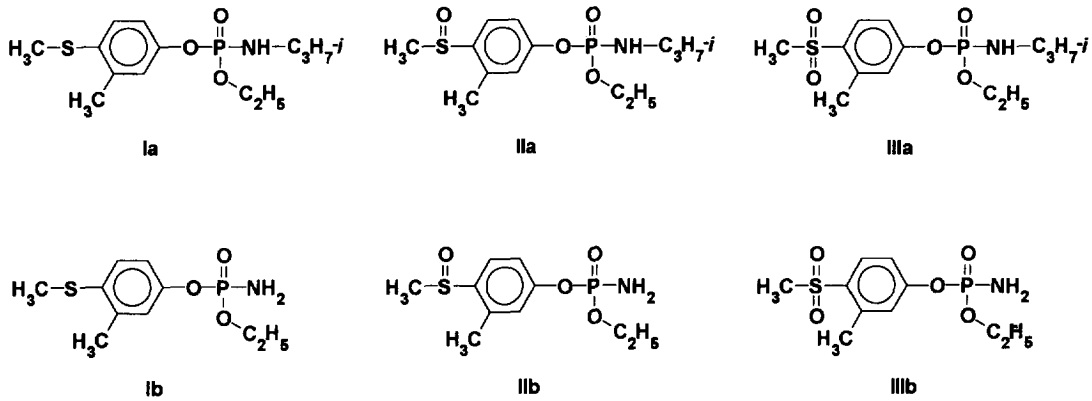

Fig. 1. Formulae of the analysed compounds.

water analysis of (1-methylethyl)phosphoramidic acid ethyl 3-methyl-4(methylthio)phenyl ester Ia (fenamiphos, Fig. 1) [4] and its chief metabolites. Fenamiphos, a phosphoramido ester pesticide widely used in Europe [1] for the control of soil and leaf nematodes [5], is a systemic nematicide, active against ecto-endo parasitic, free living, cyst-forming and root-knot nematodes.

Analysis of metabolites is another important task in the quantitative water analysis of pesticide residues in view of their toxicological importance even at low concentrations. The metabolism of fenamiphos includes hydrolysis [6] and dealkylation [7,8] of the phosphoramido group to form 3-methyl-4-methylthiophenol and phosphoramidic acid ethyl 3-methyl-4(methylthio)phenyl ester Ib (des-isopropyl fenamiphos), respectively. Other important metabolic pathways involve oxidative elaboration of the thioether function directly on Ia or on the metabolic product Ib to give the corresponding sulfoxides IIa,b and sulfones IIIa,b [6] (Fig. 1).

Oxidation of sulfides to sulfoxides and sulfones is a very important pathway in the metabolism of biologically active compounds such as natural products, drugs, and other xenobiotics [9,10]. Many examples of sulfur oxygenation in pesticide metabolism are reported [11]. Some pesticides, for instance fenamiphos [12], are rapidly transformed in water due to environmental processes, and this can produce products that already may be present in the water sample. In these cases the use of labeled I.S. is of particular importance to avoid mistakes due to the procedure.

The analysis of fenamiphos [3] involves the use of solid-phase extraction (SPE) with Carbopack B or C_{18} and HPLC with an UV detector [13], though fluorimetric, electrochemical conductivity and MS with a thermospray interface have also been used as HPLC detectors [14–16]. For GC analysis, several detectors have been employed [17–19]. MS has also been used [20–22] with an ion trap in the chemical ionization (CI) mode and with electron impact (EI) ionization, or with CI pulsed positive–negative detection.

Some authors consider permanganate oxidation as a preliminary step in the simultaneous determination of fenamiphos and its oxidative metabolites [23,24], and as I.S. for quantitative analysis O-phenyl dimethyl thiophosphinate and O-2-naphtyl dimethyl thiophosphinate were found to be suited for organophosphorus pesticides in general [25].

To prepare analytical and deuterated standards of fenamiphos and its oxidative metabolites, we needed an easy and efficient procedure. Preparation of these products through direct oxidation of pesticide Ia and the related synthesized compound Ib appeared particularly convenient.

A wide range of oxidants has been employed for sulfide oxygenation, but few of them are highly selective and able to stop at the sulfoxide without significant overoxidation to sulfones [26]. Recently, 3-fluoro-3-(heptafluoropropyl)-2-(nonafluorobutyl)-

cis-oxaziridine IV has been shown to work as a new, effective, neutral, and aprotic oxidizing agent [27–31]. Aliphatic and aromatic sulfides have been oxidized chemoselectively to the corresponding sulfoxides and sulfones [32]. Since the reaction conditions were notably mild we decided to study the oxygenation of organophosphorus agrochemical Ia,b by oxaziridine IV, to obtain metabolites and the corresponding deuterated analytical standards. Then we used these compounds for the analysis of these pollutants in water by MS with the selected ion monitoring (SIM) technique in order to enhance the performance of quantitative analysis of this pesticide and its metabolites in water. For pesticide recovery SPE was preferred to liquid–liquid extraction (LLE), as it offers several advantages [33,34]. Several materials were tested as extractive phases and compared with the result given by the SFE technique.

2. Experimental

2.1. Chemicals and materials

Perfluoro-*cis*-2,3-dialkyloxaziridine IV was easily prepared from perfluoro tri-*n*-butylamine as previously reported [35,36]. Oxaziridine IV has a pungent odor but no hazard has ever been experienced; it has to be handled carefully, wearing gloves and measuring it out under a hood.

Flash chromatography for purification of the synthesized products was done with silica gel 60 (63–200 μm) and TLC were run on silica gel 60 F_{254} plates from Merck (Darmstadt, Germany), using distilled solvents. Commercially available reagent grade solvents were employed without purification. Fenamiphos standard was purchased from Dr. Ehrenstorfer (Augsburg, Germany).

C_{18} (Insolute) extraction cartridges (500 mg, 6 ml) were obtained from International Sorbent Technology (New Road, Hengoed, Mid Glamorgan, UK), LiChrolut extraction cartridges (500 mg, 6 ml) from Merck, ENVIChromP, ENVICarb (120–400 mesh) and ENV+ extraction cartridges (500 mg, 6 ml) from Supelco (Bellefonte, PA, USA). Chem-Elut and sodium sulfate sorbing phases were purchased from Varian (Humboldt, CA, USA) and from Merck. The solvents used for synthesis and extraction were specific for pesticide residue analysis and were purchased from Merck and Carlo Erba (Milan, Italy).

2.2. Synthesis

(1-Methylethyl)phosphoramidic acid ethyl 3-methyl-4(methylthio)phenyl ester (Ia). Compound Ia was synthesized as reported in reference [37] and the analytical data are corresponding with those reported.

Pentadeuteroethyl (1-methylethyl)phosphoramidic acid ethyl 3-methyl-4(methylthio)phenyl ester (Ia-d_5). To a cooled (0 °C) solution of phosphorous oxychloride (5 mmol, 750 mg) in dry ether (20 ml), a solution of 3-methyl-4-methylsulfenylphenol (5 mmol, 770 mg) and triethylamine (5 mmol, 500 mg) in the same solvent (20 ml), was added dropwise under vigorous stirring. The reaction mixture was stirred for 4 h at 0 °C then treated with sodium ethylate (5 mmol of sodium in 2 ml of ethanol-d_5). After a further 2 h the resulting suspension was filtered and treated at 0 °C with isopropylamine (10 mmol) in ether (20 ml). The mixture was stirred for 2 h then the suspension was washed twice with water, and the organic layer was dried on anhydrous sodium sulfate and concentrated in vacuo. Flash chromatography (silica gel; chloroform–methanol, 95:5) afforded pure Ia-d_5 (43% yield): m.p. 44–46 °C; ^{31}P NMR and MS are reported on Table 1 and Fig. 3 respectively.

Phosphoramidic acid ethyl 3-methyl-4(methylthio)phenyl ester (Ib). Compound Ib was synthesized as reported in reference [37] and the analytical data corresponded to those reported.

Table 1
Chemical and analytical data of the deuterated standards of fenamiphos and its metabolites

d_5 Standard	% Yield	Isotopic purity	^{31}P NMR
Ia-d_5	43	>99%	4.15
IIa-d_5	>98	>99%	3.98
IIIa-d_5	93	>99%	3.70
Ib-d_5	40	>99%	5.50
IIb-d_5	92	>99%	5.51
IIIb-d_5	95	>99%	5.00

Isotopic purity was determined by GC–MS analysis as reported in Section 2.3.

Pentadeuteroethyl phosphoramidic acid ethyl 3-methyl-4(methylthio)phenyl ester (Ib-d_5). To a cooled (0 °C) solution of phosphorous oxychloride (5 mmol, 750 mg) in dry ether (20 ml), a solution of 3-methyl-4-methylsulfenylphenol (5 mmol, 770 mg) and triethylamine (5 mmol, 500 mg) in the same solvent (20 ml) was added dropwise under vigorous stirring. The reaction mixture was stirred for 4 h at 0 °C then treated with sodium ethylate (5 mmol of sodium in 2 ml of ethanol-d_5). After a further 2 h the suspension was filtered and the resulting solution was treated at 0 °C with dry ammonia. The mixture was stirred for 2 h, then the suspension was washed twice with water, and the organic layer was dried on anhydrous sodium sulfate and concentrated in vacuo. Flash chromatography purification (silica gel; chloroform–methanol, 95:5) afforded pure Ib-d_5 (40% yield): m.p. 65–68 °C; ^{31}P NMR and MS are reported on Table 1 and Fig. 3 respectively.

General procedure for the preparation of sulfoxide metabolites and the corresponding deuterated standards.

Sulfides I oxidation to the corresponding sulfoxides II [37] using an equimolecular amount of oxaziridine IV was performed following the general procedure reported below: a cooled solution (0 °C) of sulfide (1 mmol) in chloroform (15 ml) was treated with oxaziridine IV (1.16 mmol, 524 mg) under N_2, and the reaction mixture was stirred for 10 min. The volatile materials were removed in vacuo to give pure sulfoxide in quantitative yield.

(1-Methylethyl)phosphoramidic acid ethyl 3-methyl-4(methylsulfinyl)phenyl ester (IIa) [38]: yield >98%; mp 65–67 °C; ^{1}H NMR (C^2HCl_3) δ: 1.14, 1.19 (dd each, $J_{H,H}$=6.0 Hz, $J_{H,P}$=0.5 Hz, $(CH_3)_2CH$, 6H), 1.34 (dt, $J_{H,H}$=8.0 Hz, CH_3CH_2OP, 3H), 2.37 (s, CH_3Ar, 3H), 2.68 (s, CH_3SO, 3H), 3.14 (m, NH, 1H), 3.35–3.50 (m, $CH(CH_3)_2$, 1H), 4.16 (dq, $J_{H,H}=J_{H,P}$=8.0 Hz, CH_3CH_2OP, 2H), 7.12 (m, H-6, 1H), 7.28 (m, H-2, 1H), 7.90 (d, ^3J=8.5 Hz, H-5, 1H); ^{31}P NMR (C^2HCl_3) δ: 3.95 (brs); MS *m/z* (ra %): 319 (M^+, 42), 304 (100), 276 (7), 234 (6), 196 (27), 150 (19), 122 (68), 80 (25).

Pentadeuteroethyl (1-methylethyl)phosphoramidic acid ethyl 3-methyl-4(methylsulfinyl)phenyl ester (IIa-d_5): yield >98%; oil; ^{31}P NMR and MS are reported in Table 1 and Fig. 3 respectively.

Phosphoramidic acid ethyl 3-methyl-4(methylsulfinyl)phenyl ester (IIb): yield 94%; analytical data corresponded to those previously reported [37].

Pentadeuteroethyl phosphoramidic acid ethyl 3-methyl-4(methylsulfinyl)phenyl ester (IIb-d_5): yield 93%; mp 118–122 °C; ^{31}P NMR and MS are reported in Table 1 and Fig. 3 respectively.

General procedure for the preparation of sulfone metabolites and the corresponding deuterated standards.

A cooled solution (0 °C) of sulfoxide (1 mmol) in CF_3CH_2OH (15 ml) was treated with oxaziridine IV (1.16 mmol, 524 mg) under N_2, and the reaction mixture was stirred for 30 min. The volatile materials were removed in vacuo to give pure sulfone in quantitative yield.

(1-Methylethyl)phosphoramidic acid ethyl 3-methyl-4(methylsulfonyl)phenyl ester (IIIa): yield 94%; analytical data corresponded to those previously reported [37].

Pentadeuteroethyl (1-methylethyl)phosphoramidic acid ethyl 3-methyl-4(methylsulfonyl)phenyl ester (IIIa-d_5): yield 98%; m.p. 83–85 °C; ^{31}P NMR and MS are reported in Table 1 and Fig. 3 respectively.

Phosphoramidic acid ethyl 3-methyl-4(methylsulfinyl)phenyl ester (IIIb): Yield 95%; analytical data corresponded to those previously reported [37].

Pentadeuteroethyl phosphoramidic acid ethyl 3-methyl-4(methylsulfinyl)phenyl ester (IIIb-d_5): yield >98%; m.p. 85–88 °C; ^{31}P NMR and MS are reported in Table 1 and Fig. 3 respectively.

2.3. Instrumental analysis

Melting points were determined on a Kofler hot-stage apparatus and were not corrected. Identities and quantities of products synthesized were established by MS, ^{1}H NMR and ^{31}P NMR. ^{1}H and ^{31}P NMR spectra were recorded with a Bruker AC 250 spectrometer. TMS (internal) and phosphoric acid (external) were used as standards and C^2HCl_3 as solvent. Chemical shifts are reported in ppm. DIS-MS spectras of the synthesized products were obtained on a VG TS 250 instrument by EI at 70 eV and purity was determined by GC–MS in EI ionization analysis, performed with a Finnigan ITD instrument.

Water analysis of fenamiphos Ia and its metabolites IIa and IIIa was performed by GC–MS tech-

niques. The samples extracted were analyzed by GC HP 5890 with a MSD detector HP 5971 (Hewlett-Packard, Palo Alto, CA, USA). The GC oven was fitted with a Mega (Legnano, Italy) SE 52 column (15 m×0.25 mm I.D). The best chromatographic performances were achieved using a deactivated liner and inlet pressure programming. The pressure of helium, the carrier gas, was initially kept at 80 kPa for 2 min then lowered to 30 kPa at 680 kPa/min for the remaining time. The injector was in the splitless mode at 260 °C. The oven was programmed as follows: starting with 100 °C (for 3 min), increased to 200 °C at 30 °C/min and then to 260 at 10 °C/min. The detector temperature was held at 280 °C and the electron energy was at 70 eV. The SIM ions selected for analysis are reported in Table 2.

For HPLC–electrospray MS (HPLC–ESP-MS) analysis of the most polar metabolites Ib, IIb and IIIb the eluent was delivered by a gradient system from Waters 616 Pumps controlled by a Waters 600S controller from Waters-Millipore (Milford, MA, USA). The LC eluent conditions varied from 25:75 to 60:40 (v/v) methanol–water in 20 min at 0.3 ml/min. The column was 15 mm×2.1 mm I.D., packed with 5 mm particles from Zorbax, Rockland Technologies (Nuenen, Netherlands) coated with a cyanopropyl stationary phase. This LC gradient system was connected to a VG Platform ESP from Fisons Instruments (Manchester, UK) equipped with a Megaflow ESP coaxial flow probe [39]. After LC separation, the sample is introduced into the ESP source together with a nebulizing gas, which flows directly through the probe tip, maximizing the efficiency of nebulization. A drying gas is added to remove the solvent before entry into the analyser, and to facilitate the desolvation.

The pneumatically assisted ESP using the VG Platform instrument was used at a flow-rate of 0.3 ml/min and source temperature 150 °C. Data acquisition was performed in positive mode, quantifying the corresponding $[M+Na]^+$ ion (Table 4) of each analyte in SIM scan mode, at an extraction voltage of 20 V. Full-scan acquisition was between m/z: 60 and 400, with a scan time of 1.5 s, to obtain spectral information.

Table 2
SIM ions chosen for each analyte in GC–MS, and the limit of detection obtained

Compound	Ions (protonated)	Ions (deuterated)	LOD (pg)
Ia	217–303	293–308	4.5
IIa	304–319	309–324	4.5
IIIa	292–320	293–325	9

2.4. Extraction of fenamiphos (Ia) and its metabolites IIa and IIIa

2.4.1. Sample preparation

One hundred ml of mineral water samples at pH 7 were spiked with 2 $\mu g\ l^{-1}$ of fenamiphos and its metabolites. These compounds were successively extracted from the water matrix with different SPE cartridges (C_{18} or LiChrolut or ENV+ or ENVI-ChromP or ENVICarb) and the SFE technique. All extracts were evaporated under a nitrogen stream and diluted in ethyl acetate for GC analysis.

2.4.2. Solid-phase extraction

(i) C_{18}: the cartridges were conditioned, without vacuum, with 5 ml of ethyl acetate and 5 ml of methanol. They were then transferred to a Supelco vacuum apparatus and the samples were added. At the end of sample passage the phase was dried under vacuum for 15–20 min and eluted with 5 ml of ethyl acetate without vacuum.

(ii) LiChrolut (ethylbenzene–divinylbenzene), ENV+ and ENVIChromP (styrene–divinylbenzene): the cartridges were conditioned at atmospheric pressure with 5 ml of methanol and 5 ml of distilled water. The samples were added following the same procedure as C_{18} and eluted with 6 ml of methanol (two 3 ml fractions separated by a pause of 3 min).

(iii) ENVICarb (graphitized nonporous carbon): The cartridges were conditioned, without vacuum, firstly with 5 ml of an 8:2 mixture of dichloromethane–methanol, secondly with 5 ml of methanol and thirdly with a 10 ng ml^{-1} solution of ascorbic acid in 0.01 *M* HCl. The samples were added as described for the other phases and eluted with the 8:2 mixture of dichloromethane–methanol [40].

2.4.3. Supercritical fluid extraction

All extractions were carried out on a ISCO CO2-SFX 220 model, spiking mineral water with the analytes (2 $\mu g\ l^{-1}$) and collecting 1 ml of the water

onto a sodium sulfate or Chem-Elut filled extraction cartridge. The trapping solvent was ethyl acetate or dichloromethane, gently heated to avoid ice formation at the end of the restrictor. After the extraction was complete, the I.S. were added and, after slight preconcentration, the sample was analyzed in GC–MS. The various extraction conditions tested for optimization of the procedure are reported on table IV.

2.5. Sample preparation and extraction of the polar metabolites Ib, IIb and IIIb

One hundred ml of ground water samples acidified to pH 4.5 were spiked at 0.5 $\mu g\ l^{-1}$ with a fenamiphos metabolites mix. These compounds were extracted from the water matrix with ENV+ cartridges (styrene–divinylbenzene).

SPE was performed using an automatic sample preparation with extraction column (ASPEC) XL, from Gilson (Villiers-le-bel, France). The system was fitted with an external 306 LC pump for the dispensing of samples through the SPE cartridges, and with an 817 switching valve for the samples selection.

The cartridges were conditioned at positive pressure, with 5 ml of methanol and 5 ml of water. After adding the sample at a flow of 10 ml/min, the cartridges were dried for 20 min in a Baker SPE 12 G apparatus with the vacuum set at 100 kPa. Elution with 9 ml of methanol was done in two steps with a pause of 5 min, to improve recovery. After addition of the deuterated standards and careful evaporation of the solvent until a fixed volume of 500 μl, and the addition of 500 μl of water, 20 μl were injected into the LC–ESP-MS, using SIM conditions.

3. Results

3.1. Synthesis of the analytical standards

Phosphoramidates Ia,b and the corresponding deuterated standards were obtained by reaction of 3-methyl-4-methylsulfenylphenol with phosphorodichlorhydric acid O-alkyl ester and the desired amine (Fig. 2). The phosphorodichlorhydric acid

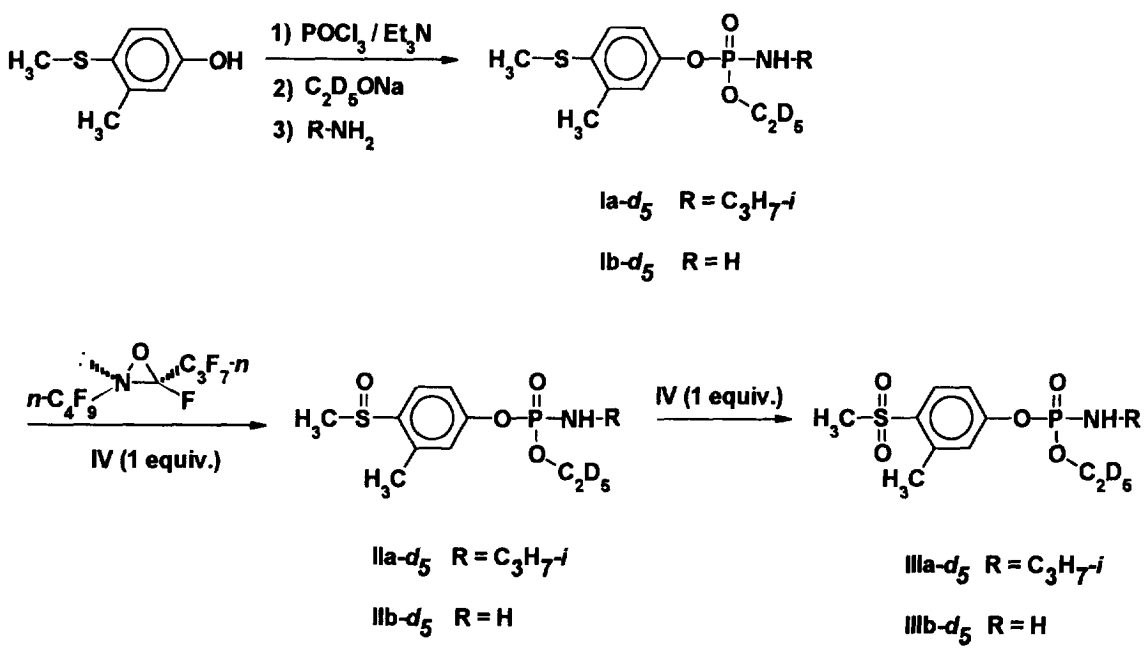

Fig. 2. Scheme of synthesis of deuterated fenamiphos and its metabolites.

O-alkyl ester was prepared using ethanol-d_5 in the synthesis of deuterated standards.

Oxygenation of methylthio substituted phosphoroamidates Ia,b and the corresponding d_5 deuterated standards, with one and two equivalents of oxaziridine IV afforded the corresponding sulfoxides IIa,b and sulfones IIIa,b in quantitative yields (Fig. 2). Selective attack at sulfur was observed in both cases without any oxidation at nitrogen (to give hydroxylamino derivatives) as shown by proton NMR analysis.

With our method the oxidation products were isolated in pure form by simply removing, under reduced pressure, the solvent, any excess reagent, and the volatile perfluoro *cis*-5-aza-4-nonene, obtained as coproduct from oxidation.

All the deuterated standards were obtained with very high isotopic purity, as reported in Table 1 were the yields obtained and ^{31}P NMR analysis are also summarized. Mass spectra of the deuterated standards synthesized are reported on Fig. 3.

3.2. MS analysis

Fig. 4A shows the reconstructed ion chromatogram of the GC–MS analysis of fenamiphos (Ia) and metabolites IIa and IIIa. The partial overlapping of metabolites IIa and IIIa does not affect the measurement of the peak areas, since the two compounds have separate ion traces, as reported in Table II. All the compounds presented good linearity at low concentration (LOD<9 pg ml^{-1}) and a SIM chro-

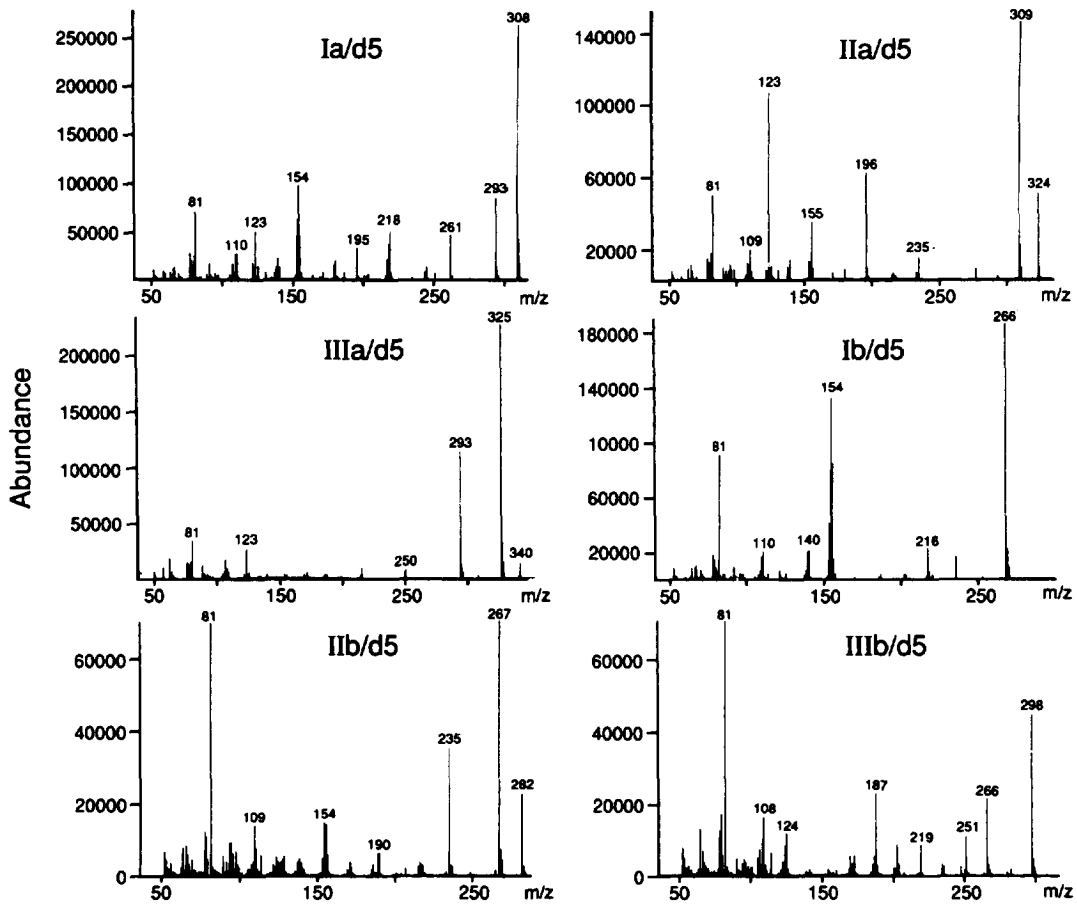

Fig. 3. Mass spectra of deuterated fenamiphos and its metabolites obtained by GC–MS analysis as reported under Section 2.

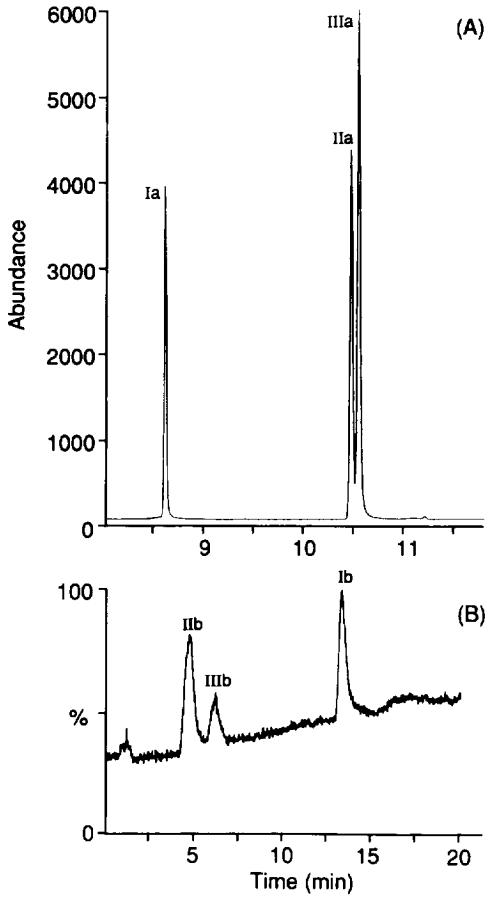

Fig. 4. GC reconstructed chromatogram of fenamiphos (Ia), and metabolites IIa and IIIa (A) and LC reconstructed chromatogram of metabolites Ib, IIb and IIIb (B). GC–MS and LC–MS analysis were performed by injecting 1 ng of each analyte, as reported in the Section 2.

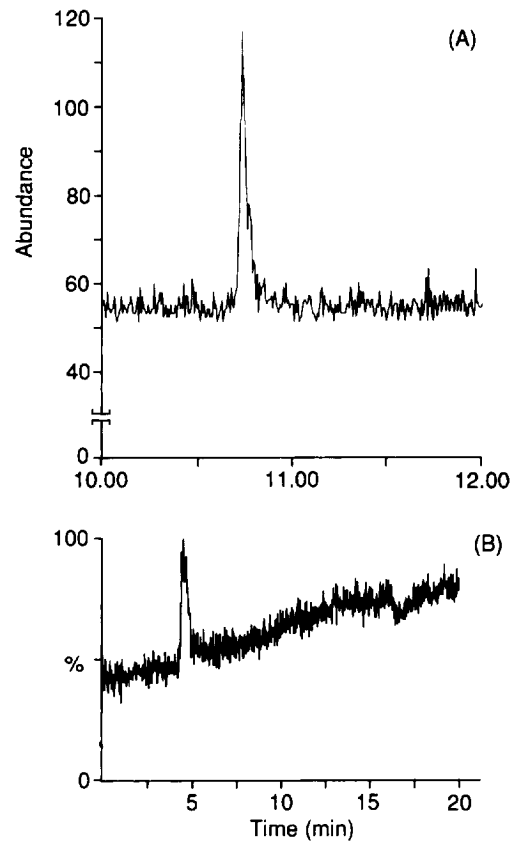

Fig. 5. GC–MS-SIM chromatogram of ion m/z 304 for compound IIa (A) and LC–MS-SIM chromatogram of ion m/z 300 for compound IIb (B), obtained by analysis at the LOD concentration reported in Table 2 and Table 4.

matogram of fenamiphos (Ia) at concentration next to LOD is shown in Fig. 5A.

For these compounds good GC calibration curves (Fig. 6A) were obtained in GC–MS analysis in SIM condition, injecting 1 μl of each solution at different concentrations obtained by successive dilution from a solution of 2 ng μl^{-1}, with a fixed concentration (5000 pg μl^{-1}) of each deuterated standard. The use of a pressure programming, as reported in the experimental part, was more straightforward than the constant flow or constant pressure procedure, resulting in a notable increase of linearity. In fact, from constant pressure to pressure programming, r^2 increased as follows: from 0.987 to 1.000 for fenamiphos, from 0.989 to 0.999 for its sulfoxide and from 0.995 to 1.000 for its sulfone; this increase was possibly due to a better peak shape observed, resulting in a better definition of area to be calculated.

Although the des-isopropyl fenamiphos (Ib) analyzed in GC–MS gave a good peak at high concentration, the degradation products of fenamiphos lacking the isopropyl moiety (compounds Ib, IIb and IIIb) presented some analytical difficulties in GC–MS analysis. They gave high LOD, considerable memory effects and a lack of linearity in the lower range of the calibration curve. We therefore tested LC–ESP–MS analysis and the relative reconstructed chromatogram is reported in Fig. 4B, while SIM chromatogram of compound IIb at concentration next

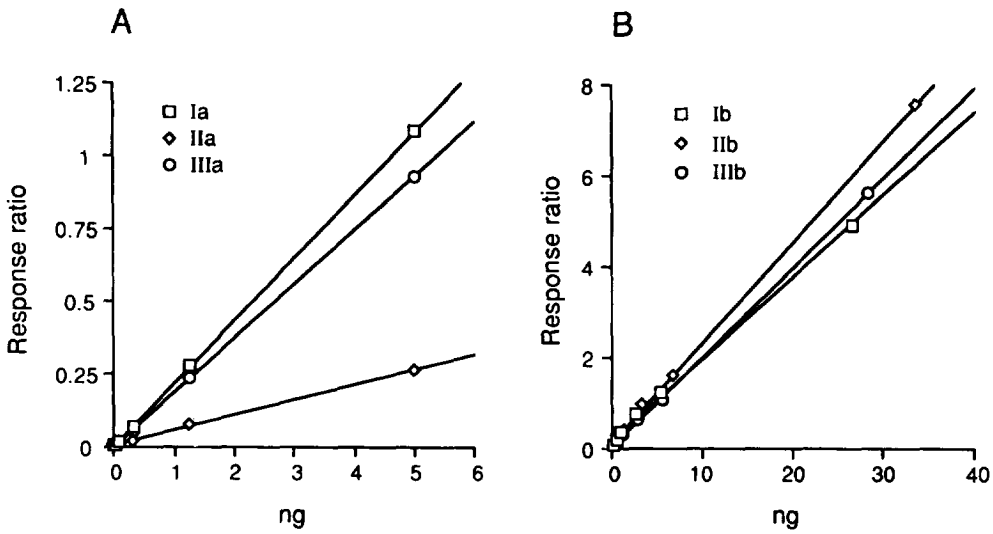

Fig. 6. Calibration graphs of fenamiphos (Ia), and metabolites IIa and IIIa obtained injecting different amounts from 0 to 5 ng in GC–MS (A), and of compound Ib, IIb and IIIb obtained injecting different amounts from 0.3 to 35 ng in LC–MS. Experimental conditions are reported in the text. Compound Ia: regression equation $y=2.17x-8.17\cdot10^{-5}$; $r^2=1.000$. Compound IIa: regression equation $y=0.52x+1.14\cdot10^{-3}$; $r^2=0.999$. Compound IIIa: regression equation $y=1.86x-3.71\cdot10^{-4}$; $r^2=1.000$. Compound Ib: regression equation $y=1.82x+1.21$; $r^2=0.996$. Compound IIb: regression equation $y=2.23x+7.62\cdot10^{-1}$; $r^2=0.999$. Compound IIIb: regression equation $y=1.97x+2.22\cdot10^{-1}$; $r^2=1.000$.

to LOD is shown, as example, in Fig. 5B. The LOD were calculated using a signal-to-noise ratio of 3 and the values obtained for the studied compounds, are reported in table IV.

The calibration curves and equations for the compounds Ib, IIb and IIIb analyzed in LC–MS are shown in Fig. 6B. These calibration curves were obtained monitoring the $[M+Na]^+$ ion of each metabolite in SIM conditions and the system was linear using six points from 0.3 to 35 ng injected.

One of the advantages of electrospray ionization is the possibility of CID (collisional induced dissociation), which means that the molecule can be fragmented by increasing the extraction voltage in the transport zone of the system. As an example, Fig. 7 reports the spectra of compound IIIb-d_5, at different extraction voltages.

At low potential, for instance 20 V (Fig. 7), all compounds remain practically in the molecular form, being detected as $[M+H]^+$ (*m/z* 299 for compound IIIb-d_5) in the positive mode. On account of its acidity the molecule also forms an adduct with sodium, present as an impurity of the organic solvent employed, resulting in the formation of a base peak in the spectra corresponding to the $[M+Na]^+$ ion (*m/z* 321).

Increasing the extraction voltage gives a higher molecule fragmentation and structural information that could be useful for identifying the compound in a real environmental sample. The presence on the molecule of a deuterated moiety in compound IIIb-d_5 permits the identification of the main molecular fragment as the ions at *m/z* 113 and 266, that were then identified respectively as the $[H_2NP(O)OC2^2H_5]^+$ ion and the ion derived from the loss of the ethyl-d_5 radical.

3.3. Water recoveries

Table 3 sets out the recoveries of fenamiphos Ia and the metabolites IIa and IIIa obtained with the different extraction techniques. SPE provided good recoveries for all compounds, except that C_{18} did not offer good results for fenamiphos. As expected, considering its polarity, fenamiphos was recovered better with resins and graphitized carbon phases than with C_{18}. The good recoveries and high standard deviation obtained for the recovery of metabolites IIa

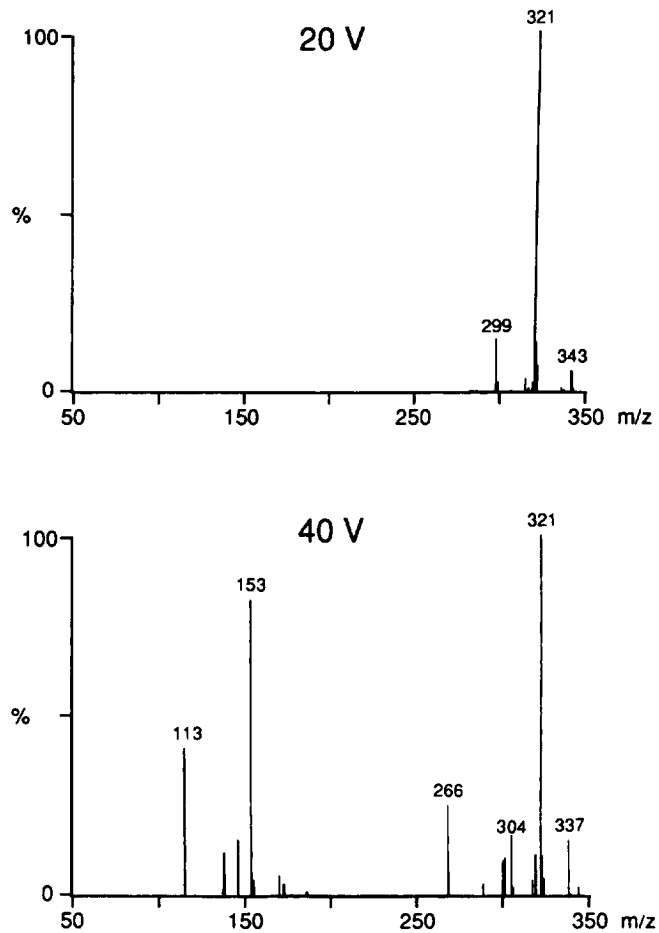

Fig. 7. Spectra of the compound IIIb-d_5, obtained at different extraction voltages in LC–MS as reported in the Section 2.

Table 3
Recovery (S.D. %) by SPE with different phases, and by SFE of fenamiphos (Ia) and its metabolites IIa and IIIa

Extraction	Compound		
	Ia	IIa	III a
C_{18}	46 (± 2)	107 (±32)	79 (±22)
ENVICarb	80 (±15)	97 (±14)	90 (±20)
LiChrolut	74 (± 7)	121 (±16)	92 (±10)
ENVIChromP	79 (± 4)	145 (±10)	101 (± 2)
ENV+	90 (±14)	124 (±15)	99 (±14)
SFE[a]	90 (± 3)	95 (± 9)	88 (± 1)

[a] Test 6 is considered for SFE: conditions as reported in Table 4; $n=4$.

and IIIa might be attributed to their possible rapid transformation in water [12].

Since SFE is gaining importance in environmental analysis because of its advantages (no solvents, faster sample preparation and greater selectivity) we tried to extract fenamiphos and its metabolites IIa and IIIa directly from spiked water samples. SFE provided good results for all these compounds and it is shown in Table 4 using various extraction conditions. An increase in temperature or pressure caused a remarkable increase of the recoveries. However at 80 °C we found that there was an increase of water extracted with the supercritical fluid, and this caused some problems due to formation of ice at the end of the restrictor. For this reason we chosen the temperature of 60 °C. Furthermore, it was useful to add methanol

Table 4
SFE conditions tested for extraction optimization and obtained results

Compound Ia	50 (±6)	41 (±4)	36 (±5)	71 (±7)	112 (±15)	90 (±3)	118 (±25)
Compound IIa	54 (±7)	42 (±8)	30 (±7)	82 (±7)	112 (±14)	95 (±9)	85 (±19)
Compound IIIa	55 (±5)	44 (±5)	36 (±6)	73 (±8)	127 (±31)	88 (±1)	103 (±22)
Trapping solvent	AcOEt						CH_2Cl_2
Modifier	–	–	–	–	–	MeOH (100 μl)	–
Pressure (p.s.i.)[a]	2500	5000	7500				
Temperature (°C)	60	60	40	60	80	60	60

[a] 1 p.s.i.=6894.76 Pa.

to the cell, because recovery improved while the standard deviation decreased (Table 4, Test 6): from ±15–20% to the value obtained for compounds I, II, III as reported in Table 3.

On the base of the result obtained for fenamiphos Ia and the metabolites IIa and IIIa, SPE of the most polar metabolites Ib, IIb and IIIb was performed using the extracting phase ENV. This SPE provided, for all these polar compounds, more than 80% recovery with good standard deviation values as reported in Table 5.

4. Discussion

The synthesis of deuterated I.S. was obtained by easy and rapid procedures, using inexpensive materials and obtaining high or nearly quantitative yields. Some general comments can be made on the reactions described. Yields of isolated sulfoxides and sulfones were invariably high, often quantitative. The oxidation results obtained prove further [32,37] that oxaziridine IV can oxidize sulfides to sulfoxides quantitatively and without detectable overoxidation to sulfones. This is a particularly interesting property for a reagent which at the same time is sufficiently reactive to oxidize sulfoxides to sulfones in quantitative yield when used in stechiometric amounts.

Table 5
SIM ions used in LC–MS analysis, LOD obtained and recovery (±S.D. %) by SPE of the most polar metabolites Ib, IIb and IIIb

Compound	SIM ion (d_5)	LOD (ng)	Recovery %[a]
Ib	284 (289)	0.1	81.0 (±10.2)
IIb	300 (305)	0.1	82.3 (±10.5)
IIIb	316 (321)	0.1	81.3 (±13.0)

[a] Extraction by SPE was performed with ENV+ as reported in Section Section 2; $n=4$; the LC–MS analyses of the extracts are reported in the Section 2experimental section.

In conclusion, the procedure employed seems particularly attractive for the preparation of oxidative metabolites, analytical and deuterated standards of biochemical, toxicological and environmental compounds in view of the high chemical yields, the simplicity of the workup, and the possibility of handling oxaziridine IV without particular precautions.

This work confirms the potency of MS for residue analysis of pesticides, due to its high selectivity and sensitivity in the identification of peaks. The use of deuterated compounds as I.S. in the quantitative analysis of fenamiphos and its metabolites in water might be an important task for the future, with the possibility to eliminate the artefact due to product degradation during analytical procedure [12].

Good recovery was obtained for all the products with both SPE and SFE. The results obtained using SFE suggest that this method should be considered quick and affordable. We are not aware of other studies using this SFE approach, probably due to the formation of ice in the restrictor affecting the analysis length and reproducibility. We have anyway noticed that a slight warming of collecting solvent with water is enough to overcome the trouble without significant analyte losses (this would not be suggested with more volatile substances). SFE also showed a lower standard deviation, compared to SPE.

A problem with SFE can be the sensitivity of the analytical method employed, since low volume is used in the conditions described. On the other hand SPE allows the use of larger volume and a range of different materials can be employed to obtain extraction optimization.

As expected, LC–MS provided better results for the most polar compounds, while GC–MS proved to be more sensitive and therefore more useful for isopropyl-moiety compounds.

Although the main application of the electrospray technique was, at the beginning, the analysis of proteins and biomolecules of high molecular mass, applications to the environment performed in the last years demonstrate the high potential of this technique in this field [39,41]. This work confirms the capability of this technique to analyze very polar and small compounds like pesticides or their corresponding metabolites, as reported here for those not amenable to GC–MS, the more polar metabolites of fenamiphos. Although the samples extracted were spiked with 0.5 $\mu g\ l^{-1}$, the sensitivity of this technique would allow easily to determine these compounds at 0.1 $\mu g\ l^{-1}$, according to the CEE Drinking Water Directive.

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