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Automated sample clean-up and fractionation of chlorpyrifos, chlorpyrifos-methyl and metabolites in mussels using normal-phase liquid chromatography

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Abstract

An automated method based on normal-phase LC has been developed for the sample clean-up of mussel extracts prior to gas chromatographic analysis of residues of chlorpyrifos, chlorpyrifos-methyl and their metabolites chlorpyrifos-methyl-oxon and 3,5,6-trichloro-2-pyridinol. Pesticides were extracted by means of a high speed blender using acetonitrile–acetone (90:10, v/v). The extract obtained was filtered and concentrated using rotavapor and the residue was dissolved in hexane. One ml of the hexanoic extract was injected on the silica-gel column, using hexane as mobile phase. Pesticides and metabolites were eluted in fat-free fractions with different mixtures of hexane–ethyl acetate. Diode array detection allowed monitoring on-line the elution of lipids. Purified extracts were analyzed by GC using nitrogen–phosphorus detection for quantitation and MS for confirmatory purposes. The method is fully automated from the injection of the extract to the collection of fractions, which are directly injected into the GC system. In this way, neither further clean-up nor solvent exchange were necessary prior to GC analysis. Recoveries obtained from fortified mussel samples at two concentration levels —100 and 20 ng g^{-1} for parent pesticides and 200 and 40 ng g^{-1} for metabolites— were higher than 90%. Limits of detection of the whole procedure of analysis were lower than 1 ng g^{-1} for parent pesticides and than 10 ng g^{-1} for metabolites. This method has been successfully applied to bioconcentration studies with mussels exposed to chlorpyrifos. Chlorpyrifos and its metabolic derivative 3,5,6-trichloro-2-pyridinol were detected and confirmed by MS in analyzed samples. © 1997 Elsevier Science B.V.

Keywords: Sample preparation; Environmental analysis; Pesticides; Organophosphorus compounds; Chlorpyrifos

1. Introduction

Currently, the use of organochlorine pesticides has been banned or restricted after evidence of their toxicity, persistence and bioaccumulation in environmental matrices. So, substitutes of the organochlorine pesticides such as organophosphorus pesticides (OPPs) are being used in large amounts in the European Union (EU) and the USA. Total turnover of OPPs has increased around the world, having been detected in ground and drinking waters [1], natural surface waters [2], marine organisms [3] and foods [4,5].

A recent FAO report [6] on the assessment of the state of the environment quality of the Mediterranean sea indicates the environmental risks of OPPs to the marine environment. Therefore, fast and reliable methods are needed for the determination of organophosphorus pesticide residues in marine organisms for environmental monitoring.

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On the other hand, transformation products of OPPs are often more toxic than parent pesticides. These compounds are biotransformed by different reactions that take place within the aquatic organisms such as a variety of hydrolyses and oxidations. The phosphorothioates are bioactivated in fish and other animals by oxidative desulphuration to the corresponding P=O derivatives, which are more toxic than the parent pesticide, inhibiting acetylcholinesterase activity [7–11]. The metabolic routes of the organophosphorus compounds in fish and other aquatic organisms are illustrated with two organophosphorus insecticides widely used (chlorpyriphos and chlorpyrifos-methyl) in Fig. 1. Studies on the fate of OPPs in fish demonstrate this metabolic transformation [12,13]. So, there is also a great interest in the determination of these metabolites in marine organisms.

Determination of pesticide residues in fatty samples such as marine organisms by gas chromatography (GC) requires the elimination of interfering compounds (mainly lipids) from the extracts before sample injection into the chromatographic system. Even small amounts of lipids can cause damage of the column and contamination of the detector.

Techniques for fatty food clean-up prior to GC determination of pesticide residues have been reviewed by Walters [14]. Official methods of analysis for several organochlorine and organophosphorus pesticide residues in fat-containing foods, including

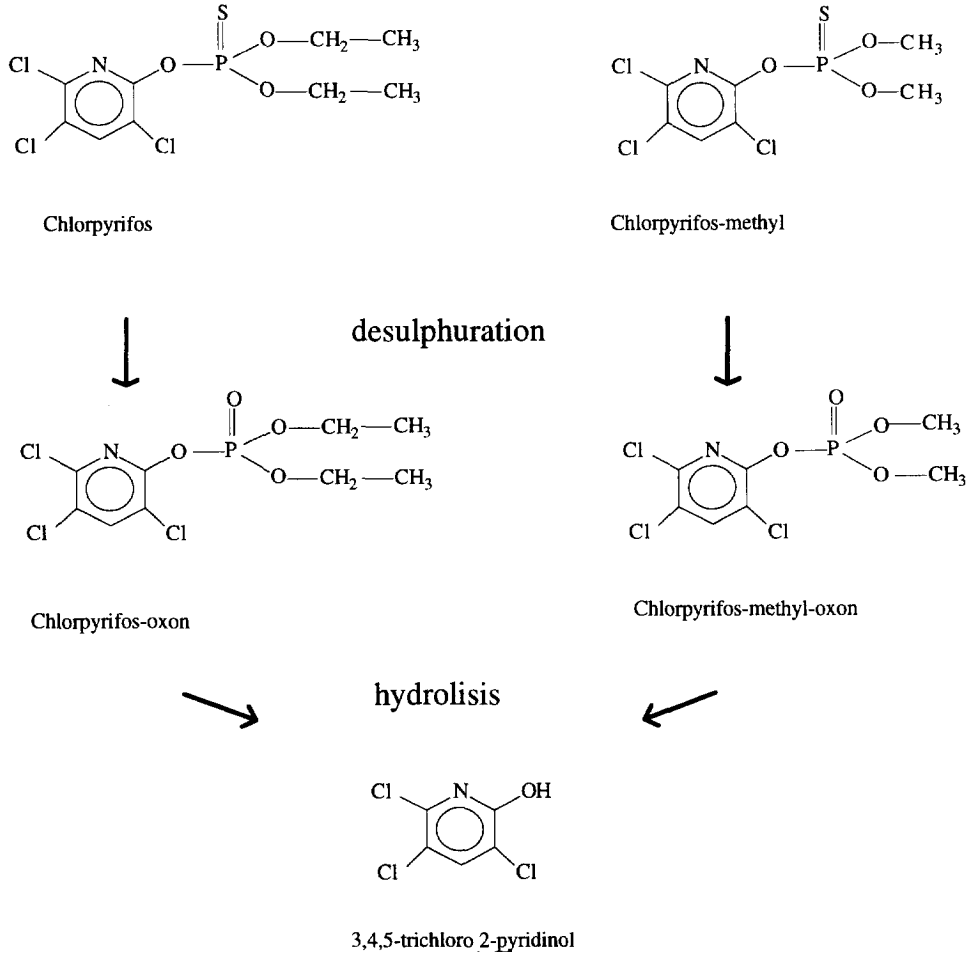

Fig. 1. Biotransformation of parent organophosphorus pesticides (chlorpyrifos and chlorpyrifos-methyl) in aquatic organisms.

fish, use adsorption chromatography on Florisil to remove residual fat from sample extracts after partial clean-up by partitioning between aqueous acetonitrile and petroleum ether [15–17]. These procedures are time consuming and give low recoveries for some pesticides [18].

Gel permeation chromatography (GPC) has also been adapted to lipid sample clean-up for the separation of pesticides and PCBs from fish extracts. Adequate clean-up for GC analysis was achieved in most cases. However, some overlap of the large lipid chromatographic band with the pesticide fraction typically occurs [14]. On the other hand, GPC does not give fat-free fractions because it allows all components of a given size to pass, whether polar or apolar. Thus, the clean-up effect is hardly visible in the gas chromatogram when not using a selective detector. Although the use of a nitrogen–phosphorus selective detector reduces the presence of interfering peaks, the introduction of fats to the gas chromatograph can damage the columns and contaminate the detector. For these reasons, a further clean-up step is often needed [19]. Recently, GPC has been used for the analysis of OPPs and derivatives in processed foods, oxon derivatives from OPPs giving very variable recoveries in different matrices [20]. Currently, laborious clean-up processes based on official methods are still applied in the multi-residue OPPs analysis in fatty matrices [4,21,22].

The major difficulty in the analysis of OPPs and their metabolites in marine organism samples concerns the wide polarity range for both pesticides and lipids present in the matrix. The effectiveness of silica-gel for the separation of lipids from pesticides and the usefulness of modifiers with different polarities in normal-phase chromatography [23] make the normal-phase LC with silica-gel an adequate technique for the clean-up of this type of samples. Thus, Gillespie and Walters [24] introduced a procedure by means of semipreparative normal-phase LC on silica to separate organochlorine pesticides from butterfat matrix. Adsorption column chromatography and normal-phase HPLC with silica-gel have been successfully used for clean-up of mollusc samples prior to the GC determination of OPPs [25,26]. The above methods in most cases improve the laborious clean-up procedures usually applied for the analysis of OPPs in fatty samples. However, fully automated clean-up procedures would be very helpful to minimize the analysis time and to avoid human errors associated to sample handling. In this sense, HPLC and solid phase extraction are very suitable for the development of automated techniques [27,28].

The present paper describes an automated sample clean-up system based on normal-phase HPLC using a silica gel column for the efficient clean-up and fractionation of the organophosphorus pesticides chlorpyrifos [O,O-diethyl O-(3,5,6-trichloro-2-pyridinol) phosphorothioato, CAS RN 2921-88-1] and chlorpyrifos-methyl [O,O-dimethyl O-(3,5,6-trichloro-2-pyridinol] phosphorothioato, CAS RN 5598-13-0) and their metabolites chlorpyrifos-methyl-oxon and 3,5,6-trichloro-2-pyridinol in molluscs. These two pesticides are widely used in Spain as insecticides [commercial products. Dursban, Polmix (chlorpyrifos), Dowelanco Iberica, Reldan E (chlorpyrifos-methyl)] in citrus crops, vegetables, grains and stores of agricultural products. The elution pattern of matrix lipids using solvents with different polarity is monitored on line by diode array detection and quantified by gravimetry and colorimetry. Fat-free fractions containing pesticides and metabolites are subsequently injected directly in the gas chromatograph using selective nitrogen–phosphorus (GC–NPD) and mass spectrometry (GC–MS) detection.

2. Experimental

2.1. Reagents

Reference materials from Dr. S. Ehrenstorfer (Promochem, Wesel, Germany) with a purity >93–99% were used for the preparation of standards of chlorpyrifos and chlorpyrifos-methyl in ethyl acetate. Standard solutions of 10 $\mu g\ ml^{-1}$ of chlorpyrifos-methyl-oxon in ethyl acetate and 3,5,6-trichloro-2-pyridinol in acetonitrile were purchased from Dr. S. Ehrenstorfer. Working solutions were prepared by dilution with ethyl acetate. Ethyl acetate, acetonitrile and acetone for pesticide residues analysis were purchased from Scharlau (Barcelona, Spain). Anhydrous sodium sulfate was obtained from Baker (pesticide residue quality, Deventer, Netherlands)

and was purified for 18 h at 300°C before use. Celite was purchased from Merck (Darmstadt, Germany).

2.2. LC instrumentation

A schematic representation of the LC system is shown in Fig. 2. It was constructed from the following equipment: HPLC Pump: Master 305 piston pump, Gilson (Middleton, USA); Peristaltic pump: LKB (Bromma, Sweden). Solvent flex tubing (solvent resistant), Elkay (Galway, Ireland); Valves: two 6-way high-pressure valves, VICI Valco, Europe Instruments (Schenkon, Switzerland), electronically controlled; Sampler: injection valve Rheodyne (Cotati, CA, USA) with 1.0-ml loop; Column: 150×3.9 mm I.D. packed with 4 μm silica Nova-pak (Waters, Milford, MA, USA); Detector: 2140 Rapid Spectral Detector LKB; Fraction collector: 2212 Helirac, LKB (Bromma, Sweden); Mobile phases: *n*-hexane and *n*-hexane–ethyl acetate mixtures; Flow-rate: 1 ml min^{-1}.

2.3. GC instrumentation

GC analysis was performed on a Hewlett–Packard 5890 series II (Avondale, USA) with NPD detection, equipped with an HP 7673 autosampler. Splitless injections of 2 μl (purge off time: 1 min) were performed on a fused-silica HP Ultra 2 capillary column coated with crosslinked 5% phenyl methyl silicone with a length of 25 m×0.25 mm I.D. and a film thickness of 0.33 μm. Helium was used as carrier gas at a flow-rate of 0.5 ml min^{-1} as well as make-up gas at a flow-rate of 30 ml min^{-1}. The detector temperature was 270°C. The oven temperature was programmed as follows: 90°C for 1 min, at 30°C min^{-1} to 180°C, at 4°C min^{-1} to 270°C with a final hold for 20 min.

GC–MS was performed with a Hewlett–Packard 5890 series II which was equipped with a HP 7673 autosampler and a MSD 5971 mass-selective detector. Splitless injections of 2 μl (purge off time: 0.75 min) were performed into a fused-silica Ultra 2 capillary column coated with crosslinked 5% phenyl methyl silicone with a length of 25 m×0.25 mm I.D. and a film thickness of 0.33 μm. Helium was applied as carrier gas at a flow-rate of 0.7 ml min^{-1}. The oven temperature was programmed as follows: 90°C for 1 min, at 10°C min^{-1} to 140°C, at 5°C min^{-1} to 270°C with a final hold for 10 min.

Mass detector characteristics were as follows: electron impact (70 eV) mode with electron multi-

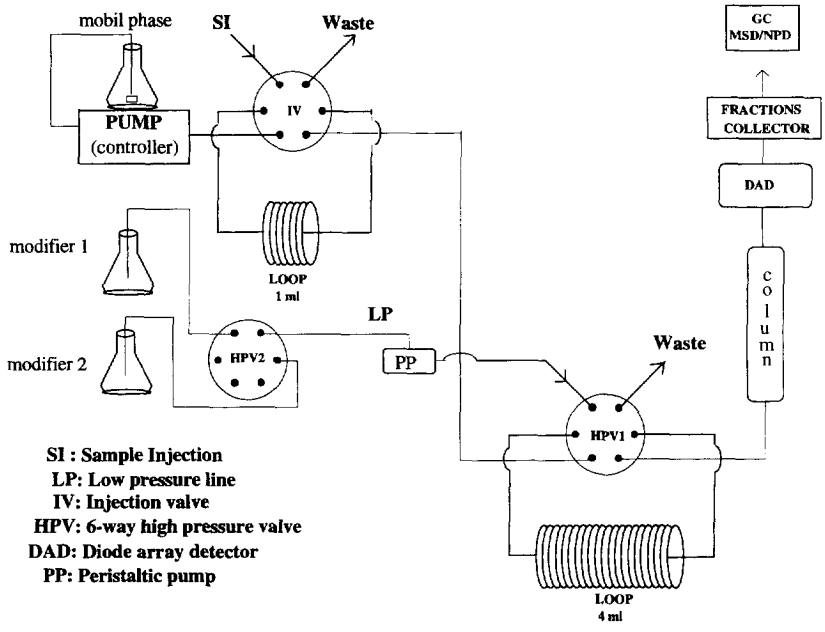

Fig. 2. Scheme of the automated LC system.

plier voltage of 1700; temperature of transfer line was 280°C. Analysis were carried out in selected ion monitoring mode using the single ions listed in Table 1.

2.4. Procedure

2.4.1. Preparation of samples

Mussels (*Mytilus galloprovincialis*) were collected alive, directly from the sea (western Mediterranean coast 40°N, 0°35'E), and also from an experimental aquaria where they were treated with 1 mg l^{-1} of chlorpyrifos to carry out bioconcentration and biodegradation studies. After sampling they were stored at −20°C. The mussels were thawed at room temperature and the soft tissue was removed from the shell before analysis. Non-treated mussels were used as a blank and for spiking in the optimization of the procedure and in recovery experiments. Treated mussels were used to confirm the presence of chlorpyrifos and metabolites in real samples

2.4.2. Extraction and pre-concentration

Wet mussel tissue (50 g) was homogenized by a domestic chopper and mixed with anhydrous sodium sulphate and 5 g of Celite. A 750-ml volume of acetonitrile–acetone (90:10; v/v) was added and the triturate was mixed in a high speed blender for 3 min by means of a Ultraturrax T 25 (Janke and Kunkel, Mannheim, Germany).

After filtering through filter paper twice (firstly with gentle vacuum and secondly by gravity) the extract was pre-concentrated and evaporated to dryness at 65°C using a vacuum rotary evaporator Heidolph (Selecta, Düsseldorf, Germany).

The final residue was dissolved in 5 ml of *n*-hexane.

Table 1
Selected ions for the single monitoring mode (MS-SIM)

Compound	Selected ions
Chlorpyrifos	197–125
Chlorpyrifos-methyl	286–125
Chlorpyrifos-methyl-oxon	270–109
3,5,6-Trichloro-2-pyridinol	197–71

2.4.3. Automated clean-up procedure

The mobile phase (*n*-hexane) was set at a flow-rate of 1 ml min^{-1} and a volume of 1 ml of the hexanoic extract was injected onto the column using the 6-way injection valve (IV). Diode array detection (DAD) was used at 280 nm for monitoring lipids elution and 1-ml fractions were collected by means of the fraction collector. During the first 4 min neutral lipids were eluted from the column. Then, a volume of 4 ml of modifier solvent (hexane–ethyl acetate, 99:1, v/v) was injected using the valve HPV1 in order to increase lightly the polarity of the mobile phase. The parent pesticides were eluted free of fat in fraction 8. After 12 min a volume of 4 ml of ethyl acetate was injected using the valve HPV1, eluting the pesticide metabolites in fraction 14 and the polar lipids in fraction 15. The peristaltic pump (PP) (2 ml min^{-1}) was used for loading the HPV1 loop (4 ml) with modifier solvents through a low pressure line. The valve HPV2 allowed to change from modifier 1 (hexane–ethyl acetate, 99:1, v/v) to modifier 2 (ethyl acetate). The whole procedure was completely controlled from the HPLC pump (Fig. 2).

Table 2 shows the events that take place during the automated sample clean-up procedure.

2.4.4. GC analysis

The fat-free fractions containing pesticides and metabolites were injected directly into the GC system using selective NP and MS detection (see Section 2.3). Quantitation was carried out by means of external standard method.

2.4.5. Determination of the fat

The fat content in the extract and HPLC fractions was determined by gravimetry or by a colorimetric method for total lipids [29], respectively. The colorimetric method used is based in the sulphophosphovainilline reactivity. Triacylglycerides calibrator solution (Sigma, St. Louis, MO, USA) was used as a standard in the range 125–1000 μg.

3. Results and discussion

In order to study the elution pattern of compounds, 1-ml standard solution of pesticides and metabolites (1 μg ml^{-1} each) was injected on to the column

Table 2
Events that take place during the automated sample clean-up procedure

Step	Time (min)	Activated device	Event(s) that take(s) place
1	0	IV	1 ml of extract is injected and initiates the controller pump for the further execution of the programme
2	0	Fraction collector HPV1[a] HPV2 Peristaltic pump	Collection programme is started HPV1 in load position HPV2 in position 1 (load solvent modifier 1) PP is loading 4 ml loop with modifier solvent 1 at 2 ml min^{-1}
3	4	HPV1	HPV1 is switched to inject position. Solvent modifier 1 is carried to the column
4	8	HPV1 HPV2	HPV1 is switched on load position HPV2 is switched on position 2 (load solvent modifier 2)
5	12	HPV1	HPV1 is switched to inject position. Solvent modifier 2 is carried to the column
6	16	HPV1 HPV2	HPV1 is switched to load position HPV2 is switched to position 1 (load solvent modifier 1)
7	20	None	System ready for next cycle

[a] HPV=high pressure valve.

using hexane as mobile phase. Previous data gave the elution of neutral lipids during the first 4 min in mussel analysis [26], so, in a first attempt, 4 ml ethyl acetate was injected at minute 4. Results obtained by GC showed that all the analytes eluted in fraction 6–7 (1 fraction per minute). The application of this procedure to fortified mussel extracts (50–60 mg ml^{-1} raw lipids) showed the elution of 1 mg of fat in fractions 6–7 as the polar lipids were eluted together with organophosphorus compounds due to the high polarity of the eluent used.

In a second approach, a solvent modifier with lower eluotropic strength was used. The injection of 4 ml hexane–ethyl acetate (99:1, v/v) at 4 min eluted the parent pesticides in fraction 8. Due to the higher polarity of metabolites, another injection of 4 ml ethyl acetate at 12 min (minimum time required to re-load the 4 ml loop in HPV1) was necessary. The two metabolites eluted together in fraction 14. The application of this procedure to fortified mussel extracts showed that fat appeared only in fractions 1–4 and 15. Therefore, the analytes were obtained in fat-free fractions as shown in Fig. 3. The fat content

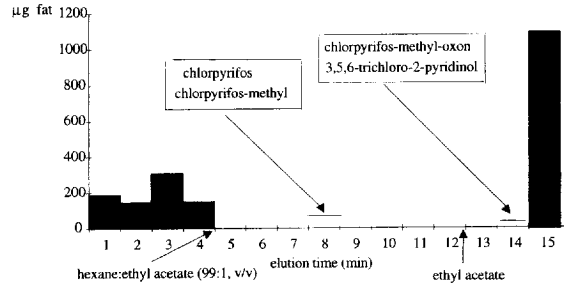

Fig. 3. Elution patterns of lipids and pesticides after automated silica-gel clean-up using hexane as mobile phase. Arrows below the x-axis indicate the injection from 4 ml loop of modifier 1 and 2. Sample contains 50 mg ml^{-1} of lipids.

Table 3
Recoveries after pre-concentration of spiked mussels extracts[a] by Kuderna–Danish or rotavapor

Compound	Recoveries[b] (%)	
	Kuderna–Danish[c]	Rotavapor
Chlorpyrifos	105 (5)	96 (4)
Methyl-chlorpyrifos	99 (6)	93 (3)
Methyl-chlorpyrifos-oxon	88 (8)	85 (9)
3,5,6-Trichloro-2-pyridinol	40 (17)	83 (10)

[a] Fortification level: 0.1 $\mu g\ ml^{-1}$ for parent pesticides and 0.2 $\mu g\ ml^{-1}$ for metabolites.
[b] Average and relative standard deviation, $n=3$.
[c] For methodology see [26].

in every fraction was determined by colorimetry and monitored by DAD; thus, the on-line detection allowed a rapid optimization of the procedure. A visual monitoring (yellow color) was even feasible.

The procedure, including pre-concentration with Kuderna–Danish at 80°C, evaporation to dryness under gentle stream of nitrogen at 40°C (according to a previous method for OPPs analysis in mussels [26]), dissolution of residue in hexane, and the optimized automated clean-up, was applied to mussel extracts (acetonitrile–acetone, 90:10, v/v) fortified at levels of 0.1 $\mu g\ ml^{-1}$ for parent pesticides and 0.2 $\mu g\ ml^{-1}$ for metabolites. The metabolite 3,5,6-trichloro-2-pyridinol was not recovered quantitatively, although the recoveries of the other analytes were satisfactory (Table 3). Losses of 3,5,6-trichloro-2-pyridinol were probably due to the high temperature used in the Kuderna–Danish system, producing the volatilization and/or degradation of this compound. As a consequence, another method for the evaporation of the extract was checked. The use of rotavapor at 65°C led to satisfactory recoveries and precisions (relative standard deviation lower than 10% for all the analytes) as shown in Table 3.

Consequently, the concentration of extracts was performed by a rotavapor at a maximum temperature of 65°C to avoid losses and/or degradation of metabolites. Moreover, the fractions containing 3,5,6-trichloro-2-pyridinol had to be injected before 24 h to prevent losses that took place possibly due to degradation.

The overall analytical procedure, including extraction with acetonitrile–acetone, evaporation in rotavapor, application of the automated silica-gel clean-up and final determination by GC–NPD, was applied to mussel extracts fortified at two concentration levels (20 and 100 $ng\ g^{-1}$ for parent pesticides; 40 and 200 $ng\ g^{-1}$ for parent metabolites). Recoveries were satisfactory at the two levels assayed for all the compounds, with relative standard deviations for 4 replicates lower than 11% (Table 4).

Figs. 4 and 5 show the NPD and MS chromatograms obtained from the direct injection in GC of the fractions containing pesticides (fraction 8 for parent pesticides, and fraction 14 for metabolites). The chromatograms were very clean and, as revealed the additional analysis, no fat was present in the extracts. This allowed us to obtain very low detection limits, which is of great importance especially in the analysis of metabolites which can be found in real samples at very low concentration levels. Limits of

Table 4
Recoveries after application of the overall analytical procedure to mussel samples fortified at two levels and limits of detection in MS and NP

	Recoveries[a] (%)		LOD[b] ($ng\ g^{-1}$)	
	Level 1[c]	Level 2[d]	NP	MS
Chlorpyrifos	98 (6)	104 (8)	0.5	4
Chlorpyrifos-methyl	96 (5)	101 (6)	1	4
Chlorpyrifos-methyl-oxon	91 (11)	95 (6)	5	12
3,5,6-Trichloro-2-pyridinol	88 (7)	90 (5)	8	16

[a] Average and relative standard deviation, $n=4$.
[b] LOD=limits of detection.
[c] 100 and 200 $ng\ g^{-1}$ of pesticides and metabolites, respectively.
[d] 20 and 40 $ng\ g^{-1}$ of pesticides and metabolites, respectively.

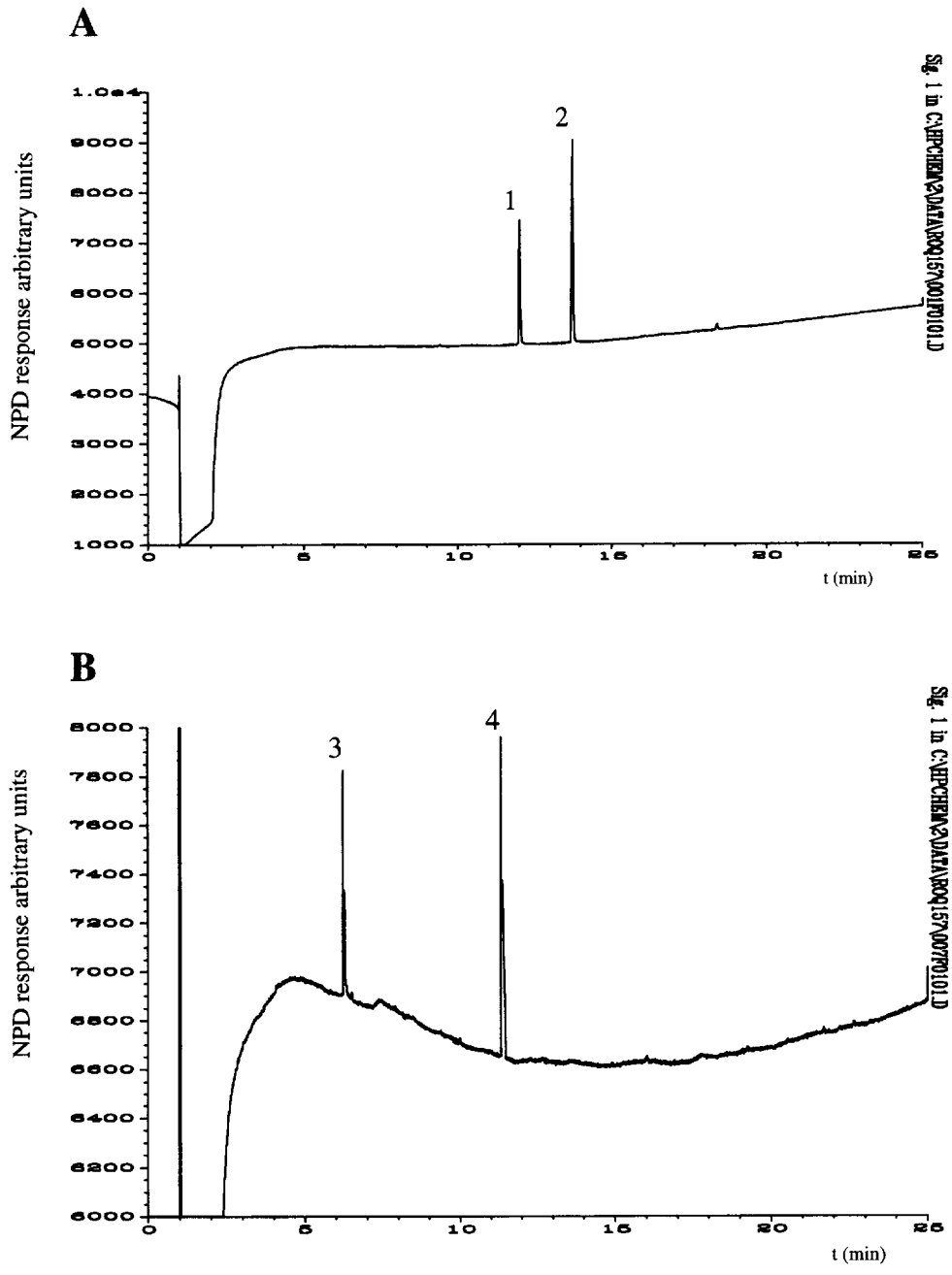

Fig. 4. (B) NPD–gas chromatograms from mussels tissues fortified with 40 ng g^{-1} of chlorpyrifos-methyl-oxon (4) and 3,5,6-trichloro-2-pyridinol (3) and (A) with 20 ng g^{-1} of chlorpyrifos (2) and chlorpyrifos-methyl (1).

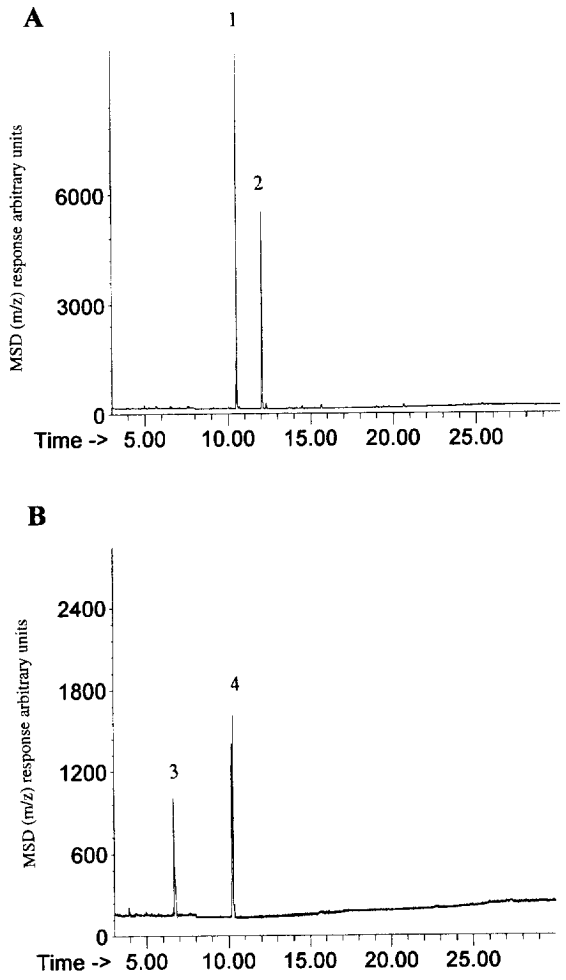

Fig. 5. (B) MS–gas chromatograms from mussels tissues fortified with 200 ng g^{-1} of chlorpyrifos-methyl-oxon (4) and 3,5,6-trichloro-2-pyridinol (3) and (A) with 100 ng g^{-1} of chlorpyrifos (2) and chlorpyrifos-methyl (1). Ions represented: chlorpyrifos 197–125, chlorpyrifos-methyl 286–125, chlorpyrifos-methyl-oxon 270–109, 3,5,6-trichloro-2-pyridinol 197–71, time in minutes.

detection were calculated from chromatograms corresponding to mussels fortified at 10 ng g^{-1} of pesticides and 25 ng g^{-1} of metabolites using a signal-to-noise ratio of three (Table 4). The procedure allows the detection of pesticides at ng g^{-1} level using NPD.

Finally, the developed procedure was applied to mussel samples belonging to an experimental study about bioconcentration of OPPs in these organisms. Mussels were exposed to chlorpyrifos along 35 days in sea water aquaria, at a concentration of 1 mg l^{-1}. After that time, the analysis performed to samples (n=3) composed by 6 specimens (in order to avoid the variability from biological samples) by using the procedure developed in this paper revealed that both chlorpyrifos and its metabolite 3,5,6-trichloro-2-pyridinol were present in the samples. They were quantified by GC–NPD (49±7 μg g^{-1} chlorpyrifos, 0.10±0.01 μg g^{-1} metabolite) and confirmed by GC–MS. In this way, the biotransformation of chlorpyrifos by desulphuration and hydrolysis in mussels was proved. Besides, this biotransformation can be evidence because no degradation of chlorpyrifos in water can be expected, as the water solution is renewed each 4 days and the degradation route is only possible in living organisms.

4. Conclusions

The method proposed in this paper allows the automated clean-up by normal-phase LC of mussel extracts prior to the gas chromatographic determination of chlorpyrifos, chlorpyrifos-methyl and their metabolites chlorpyrifos-methyl-oxon and 3,5,6-trichloro-2-pyridinol. The application of this system has several advantages: the procedure is completely automated and the LC separation can be monitored on line by DAD, thus rendering a fast and reliable optimization of the system for any fatty matrix; the clean-up time is 14 min and the whole procedure, including extraction, clean-up and GC determination allows the analysis of about 16 samples per day; the efficiency of the clean-up procedure joined to the sensitive determination by GC–NPD led to low detection limits for both the parent pesticides and metabolites, which make the method very suitable for laboratory and environmental studies; as well LC fat-free fractions are injected directly to GC which may finally lead to a fully automated LC–GC procedure in the near future. The use of one LC column for 2 months does affect the retention of pesticides and lipids.

Acknowledgments

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