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Multiresidual method for the gas chromatographic analysis of pesticides in honeybees cleaned by gel permeation chromatography

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Abstract

The analysis of several organophosphorus and carbamate pesticide residues in the bodies of honeybees using gas chromatography (GC) and gel permeation chromatography (GPC) clean-up is described. Freeze-dried or lyophilized insect samples were blended with diatomaceous earth (Extrelut) then underwent elution with methylene chloride. This extraction method has shown good recovery on various spike standard levels. Samples are cleaned up by GPC with a Bio Beads SX 3 column and a cyclohexane–ethylacetate (1:1) eluant. Organophosphorus and carbamate compounds are quantified using capillary gas chromatography. Good linearity ranges were observed for all compounds. The extraction process was rapid and results were good, despite the complexity of the matrix on which it was applied. It allowed a reduction both in cost and the consumption of solvents, thereby safeguarding the health of the analyst and the environment. Environmental monitoring using bees was confirmed to be a valid procedure.

Keywords: Honeybees; Pesticides

1. Introduction

Despite the fact that scientific research concentrates on the development of increasingly selective active ingredients or ones having a lower environmental impact and attempts to reduce their amount, the quantity of pesticides used in agriculture is still too high, hence the importance of environmental monitoring. The etiology of honeybees allows a minute exploration of the ground; for this reason they are used to evaluate the presence of environmentally polluting substances [1]. Despite their high toxicity, organophosphorus and carbamate pesticides still make up the majority of pesticides used in agriculture. The bee is a good direct indicator of insecticides because it responds with an intense and extensive mortality to this type of treatment; the insect is not sensitive to other kinds of pollutants but is nonetheless exposed to them, and provides information as a collector of residues [2,3]. In order to use the bee as a bioindicator it was necessary to apply a multiresidual analytical method which would allow all the main pesticides sought to be detected with just one process. However, given the complexity of the bee matrix, it was likewise necessary to apply a method for purifying the sample, so to eliminate artifacts. Some methods used for the extraction of pesticides from complex matrices have

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required liquid–liquid separation procedures [4] which entail both considerable dexterity and a high consumption of solvents. Other methods [5–10] involve the extraction of the main pesticides in the solid phase (SPE); this technique is inadequate, as it does not yield many pesticides. Attempts to combine gel permeation with GC and HPLC were made in the 1970s [11] and thereafter abandoned. We tried once again to apply this technique, which would solve some of the above problems; we decided extraction of the sample adsorbed on diatomaceous earth (Extrelut) [12] with purification of the extract by gel permeation chromatography (GPC) [13–21]. The procedure should enabled a good recovery of all the main pesticides we were seeking, and the extraction should have been clean enough to be analyzed through gas chromatography with selective and non-selective detectors.

2. Materials and methods

2.1. Chemicals and samples

All the solvents (acetone, ethylacetate, *n*-hexane and dichloromethane) were of analytical-grade for the analysis of pesticide residues (Carlo Erba Reagenti, Milan, Italy).

Standard organophosphorus and carbamate insecticides at 98.0 and 99.9% purity were used (SI Dr Ehrenstorfer, Augburg, Germany). Standard stock solutions (1 mg/ml) were prepared by dissolving pure pesticide in acetone. Appropriate aliquots of stock solutions were diluted with acetone to make organophosphorus and carbamate mixtures. Solutions containing each active component, both singly and in combination, were prepared.

The bee samples used to develop the method came from environmental monitoring stations located in the area around Bologna. The bees were analyzed according to previously adopted procedures [4] and were found to be pesticide-free.

The samples were freeze-dried at −20°C (Drywinner Heto 1.0–60/CT 60 Cooling Trap, Denmark) in order to avoid any putrefaction which might degrade the sample. Freeze-drying also facilitates the crushing of sample, which in turn makes them easier to analyze.

2.2. Extraction of the sample adsorbed in the solid phase

The sample, consisting of 3 g of lyophilized bees, was broken up in a mortar and added to 20 g of diatomeous earth (Extrelut-Refill pack for column fillings Extrelut® NT20-Merck, Darmstadt, Germany). The resulting mixture was put into a column (Pre-packed columns Extrelut® NT20 for extraction of lypophilic compounds; Merck, Darmstadt, Germany) and extracted after 10 min by means of elution with 100 ml of dichloromethane. Extraction was complete after about 20 min with the recovery of 70 ml of extract. The extract was then dried in a rotary evaporator at a reduced pressure and at a temperature below 40°C [22]. The residue was collected with 1 ml of cyclohexane–ethylacetate (1:1) for subsequent purification in GPC.

2.3. Gel permeation chromatography

A glass column for GPC (45×1 cm) was filled with 200–400 mesh Biobeads SX resin (Lab Service Analytica S.r.l., Bologna, Italy).

To gel the resin a 1:1 mixture of cyclohexane–ethyl acetate was used; this mixture also constituted the mobile phase. The procedure devised consisted of injection of the sample (1 ml) in a column and collection of an intermediate fraction (15 ml) of eluate. The flux of the hexane–ethylacetate phase (1:1) was regulated at 1 ml/min.

To decrease the volume of the sample containing the pesticides it was placed in a rotary evaporator at a reduced pressure and at a temperature below 40°C [22]. The residue was collected with 2 ml of acetone for the purposes of GC analysis.

2.4. Gas chromatography

A Carlo Erba 8000 Top equipped with a nitrogen phosphorus detector (NPD) system (290°C) (CE Instruments, Milan, Italy) and AS 800 autosampler (CE Instruments, Milan, Italy) was used to analyze the pesticides in honeybee samples. The instrument has a fused silica capillary column SPB-608™ 30 m, 53 μm I.D., 0.50-μm film thickness (Supelco, Bellefonte, PA, USA). Helium was used as the transport gas, with a flow speed of 15 ml/min. The sample

was injected using the splitless method. Temperatures were programmed as follows: injection at 60°C, increasing to 120°C with increases of 50°C/min; from 120 to 250°C with increases of 10°C/min followed by isothermal conditions for 20 min.

The extract obtained from the bee sample was dissolved in acetone up to 2 ml and 1 μl was injected into the gas chromatograph by means of the autosampler. The gas chromatographic signal was processed by the operating software of the instrument (Borwin 6.11, Jasco, Japan).

A pesticide-free bee extract obtained as previously described was injected into other gas chromatographs with different selection detectors. This experiment, which served as a means for further evaluating the quality of the extract, was carried out using a FISON gas chromatograph mod. GC 8000 with an FID detector (CE Instruments, Milan, Italy) equipped with a SE52, 25 m, 0.32 μm I.D. column (Mega, Milan, Italy) the injection (1 μl) was made using the on-column method. Temperatures were programmed as follows: injection at 70°C, progressively increasing to 100°C with increments of 50°C/min; then to 330°C with increments of 7°C/min. The temperature of the detector was 350°C, a Carlo Erba gas chromatograph mod. 4160 with an ECD detector (CE Instruments, Milan, Italy) equipped with an OV1, 25 m, 0.32 μm ID column (Supelco) was used; the injection (1 μl) was made using split modality 1:20. Temperatures were programmed as follows: injection at 120°, progressively increasing to 250°C with increments of 10°C/min. The injector had a temperature of 240°C while the detector was set at 290°C. An HP gas chromatograph mod. GCD 1800 C with MS detector equipped with an HP5, 30 m, 0.25 mm I.D. column (Hewlett Packard, USA) was also used. Helium was used as the transport gas with a flow speed of 0.8 ml/min. Temperatures were programmed as follows: injection at temperatures rising from 60 to 120°C with progressive increments of 50°C/min, 120 to 250°C program rate 4°C/min. The injector had a temperature of 280°C while the detector was set at 290°C.

3. Results and discussion

To obtain a clean extract, we combined diatomaceous earth – generally acknowledged to be effective in the extraction of pesticides from samples of animal or vegetable origin [24] – with a suitable solvent (dichloromethane in our case) and polystirene-divinylbenzene resin; various studies [13–21] have suggested the latter, whose structural features are similar to those of Bio Beads SX-3, for cleaning complex samples from which interfering compounds must be eliminated. The steric exclusion mechanism underlying this technique allows the molecules in the sample to be separated according to size. This eliminates the problems associated with other techniques, which discriminate molecules by their chemical properties (solid phase extraction with different stationary phases). By purifying the sample in GPC

Table 1
Detection limit of organophosphate and carbamate pesticides and correlation coefficients of the calibration curves ($N=5$)[a]

Pesticides	Detection limit (μg/l)	Correlation coefficient	Coefficient of variation (%)
Methamidophos	29.5	0.9986	7.6
Heptenophos	2.9	0.9995	3.1
Omethoate	1.0	0.9971	4.0
Diazinon	1.1	0.9966	3.1
Fonofos	1.1	0.9973	1.9
Dimethoate	5.7	0.9689	6.3
Paraoxon ethyl	1.8	0.9970	4.4
Chlorpyrifos methyl	2.0	0.9990	3.4
Parathion methyl	1.6	0.9974	5.1
Pirimiphos methyl	1.3	0.9996	3.1
Malathion	1.3	0.9998	3.5
Parathion ethyl	2.1	0.9978	2.5
Pirimiphos ethyl	1.4	0.9964	5.0
Bromophos methyl	1.2	0.9988	7.5
Quinalphos	1.8	0.9996	4.9
Phentoate	1.6	0.9995	4.1
Methidathion	1.0	0.9951	3.1
Vamidothion	25.5	0.9995	6.8
Triazophos	2.6	0.9996	9.0
Phosmet	79.0	0.9805	8.9
Phosalone	5.5	0.9976	4.6
Pirazophos	5.9	0.9987	5.5
Azinphos methyl	65.2	0.9990	7.0
Coumaphos	5.2	0.9988	6.8
Pirimicarb	1.0×10^3	0.9969	3.3
Ethiofencarb	1.2×10^3	0.9889	2.5
Methiocarb	6.2×10^2	0.9871	6.5
Carbaryl	5.3×10^2	0.9998	4.3
Fenoxycarb	2.5×10^3	0.9052	6.8

[a] Experimental conditions are reported in the text.

we were able both to separate the analytes from the matrix in a satisfactory manner and to recover the main pesticides having different chemical properties, but similar molecular weights.

The purification mechanism underlying this technique distinguishes three fractions of eluate at the outlet of the column: waste (matrix: initial 16 ml), collect (pesticides: subsequent 15 ml) and wash (final 10 ml).

The distinction between the fraction containing the matrix and the one containing the pesticides is not clear-cut: in the second fraction (collect) a small quantity of matrix was still found, but did not interfere with the analysis of our sample. The complete elimination of the matrix would, in fact, have compromised the recovery of pesticides of higher molecular weights.

3.1. Determination of pesticide residues by gas chromatography

3.1.1. Calibration

In accordance with good laboratory practice, a calibration curve was plotted for each pesticide to determine the linearity range and the detection limit. These curves, in the form of straight lines, were obtained by injecting acetone solutions of the active components at different decreasing concentrations of the pesticides into the gas chromatograph; peaks were considered detectable until they were three times the height of the background noise. The regression coefficients of the lines for five measurements are listed in Table 1.

The NPD detector was shown to be optimal for determining the majority of the organophosphates we

Table 2
Average recovery of organophosphate and carbamate pesticides extracted from the bee matrix ($N=5$)[a]

Pesticides	Amount of pesticide added to bee samples (μg/g)	Recovery (%)	Coefficient of variation (%)
Methamidophos	0.40	92.9	6.0
Heptenophos	0.74	80.9	5.5
Omethoate	0.32	81.6	6.2
Diazinon	0.44	80.2	8.1
Fonofos	0.51	83.6	8.1
Dimethoate	0.34	94.8	9.0
Paraoxon ethyl	0.60	94.2	9.2
Chlorpyrifos methyl	0.63	87.2	3.5
Parathion methyl	0.34	86.3	3.8
Pirimiphos methyl	0.33	76.0	4.7
Malathion	0.48	87.5	3.0
Parathion ethyl	0.50	85.4	4.0
Pirimiphos ethyl	0.51	74.9	2.9
Bromophos methyl	0.41	79.1	4.0
Quinalphos	0.73	78.3	5.5
Phentoate	0.48	70.9	7.2
Methidathion	0.34	95.5	1.9
Vamidothion	0.33	106.8	18.2
Triazophos	0.30	100.4	9.8
Phosmet	0.36	96.1	23.6
Phosalone	0.33	83.2	20.2
Pirazophos	0.33	79.7	7.4
Azinphos methyl	0.33	87.5	15.6
Coumaphos	0.37	91.3	20.3
Pirimicarb	33.6	38.7	2.1
Ethiofencarb	39.3	48.6	0.4
Methiocarb	32.8	46.6	36.9
Carbaryl	35.6	97.5	11.3
Fenoxycarb	33.3	58.4	7.2

[a] Experimental conditions are reported in the text.

analyzed. With regard to dimethoate, it was found that linearity was maintained only at high concentrations (up to 0.1 mg/l, $r=0.9989$). In the mixture analyzed, the retention time of fosmet was such that there was a partial overlap of peaks and therefore, at lower concentrations, integration became imprecise; this was reflected in a lower correlation coefficient ($r=0.9805$).

The NPD detector instead showed poor sensitivity in the determination of carbamates [23]. The attempt to find carbamates with an NPD detector was justified by the need to determine different families of the main pesticides in the same sample, without using other extractive and/or instrumental techniques.

3.1.2. Determination of pesticides

To assess the quantity of pesticides recovered using this method of extraction and purification, 1 ml of acetone solution containing a mixture of pesticides was added to a sample (3 g) free of pesticides. The mixtures consisted of (a) 24 phosphoric esters in concentrations of around 1 ng/μl and (b) five carbamates in concentrations of around 100 ng/μl.

The recoveries and the coefficient variations calculated for five measurements are reported in Table 2.

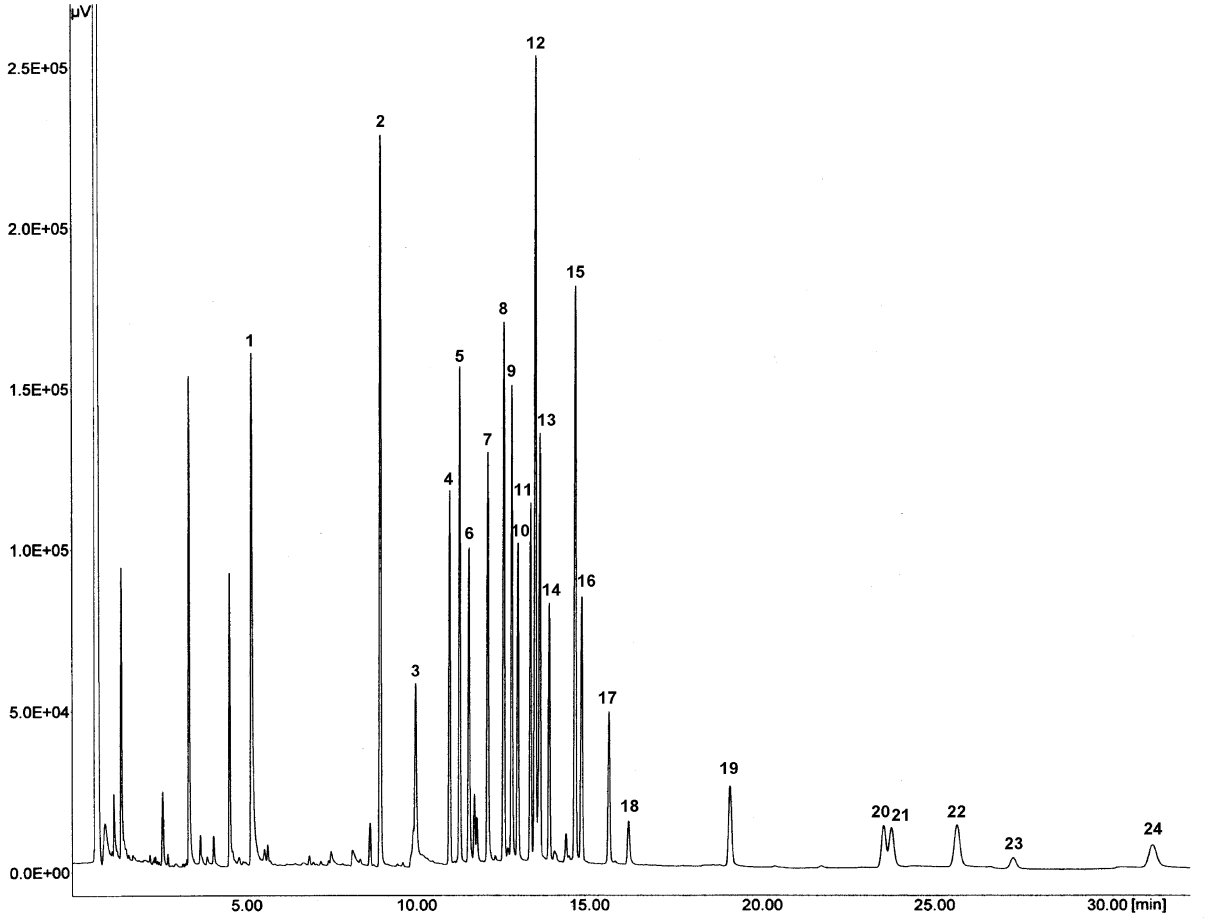

Fig. 1. Chromatogram obtained from a bee extract containing phosphorus esters and purified by GPC (NPD detector, as reported in the text). (1) Methamidophos, (2) heptenophos, (3) omethoate, (4) diazinon, (5) fonofos, (6) dimethoate, (7) paraoxonethyl, (8) chlorpyrifosmethyl, (9) parathionmethyl, (10) pirimiphosmethyl, (11) malathion, (12) parathionethyl, (13) pirimiphosethyl, (14) bromophosmethyl, (15) quinalphos, (16) phenthoate, (17) methidathion, (18) vamidothion, (19) triazophos, (20) phosmet, (21) phosalone, (22) pirazophos, (23) azinphosmethyl, (24) coumaphos.

Recovery was highly satisfactory for organophosphates; recovery percentages vary between 70.9% for phentoate and 106.8% for vamidothion, with an average recovery value of 86.4% and an imprecision (repeatability expressed as coefficient of variation) of 8.6%. These percentages were lower for carbamates, varying between 38.7% for pirimicarb and 97.5% for carbaryl with an average recovery value of 58.0% and an imprecision (repeatability) of 6.7%. The coefficients of variation are below 10% for most of the pesticides (Table 2), indicating a good repeatability of the method.

Figs. 1 and 2 show examples of two chromatograms obtained from the extraction and purification of a bee sample to which, in the first case (Fig. 1), an acetone mixture of phosphoric esters in concentrations of around 1 ng/μl and, in the second case (Fig. 2), a mixture of carbamates in concentrations of about 100 ng/μl was added. The chromatographic peaks lend themselves easily to interpretation. The remaining matrix components may be seen in an area of the chromatogram (in the first 5 min) where they do not cause interference with any of the pesticides.

These results suggest that an extract with these features could also be analyzed with non-selective detectors, which would not be possible with a non-purified extract. The sample treated as previously described was therefore analyzed both with a flame ionization detector (FID) and with an electon capture detector (ECD).

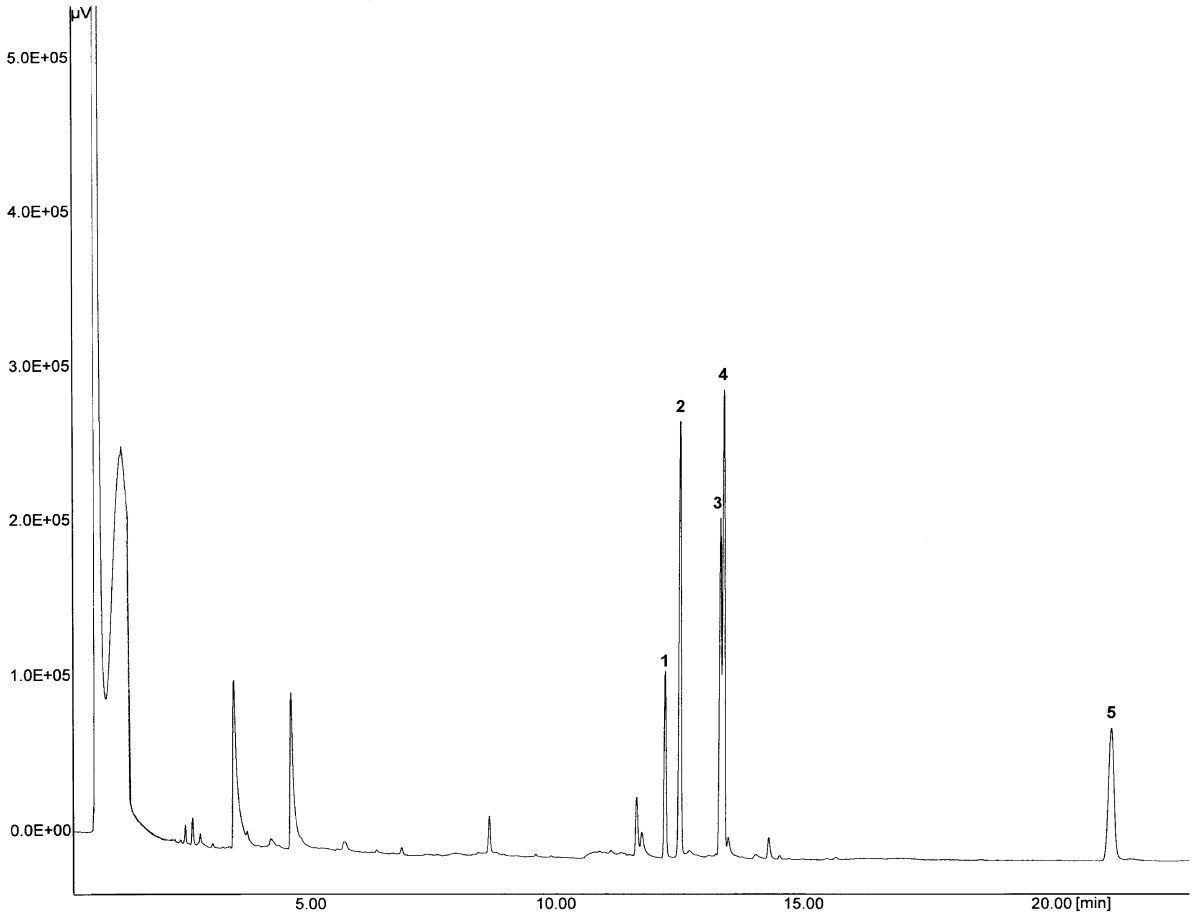

Fig. 2. Chromatogram obtained from a bee extract containing carbamates and purified by GPC (NPD detector, as reported in the text). (1) Pirimicarb, (2) ethiofencarb, (3) methiocarb, (4) carbaryl, (5) fenoxycarb.

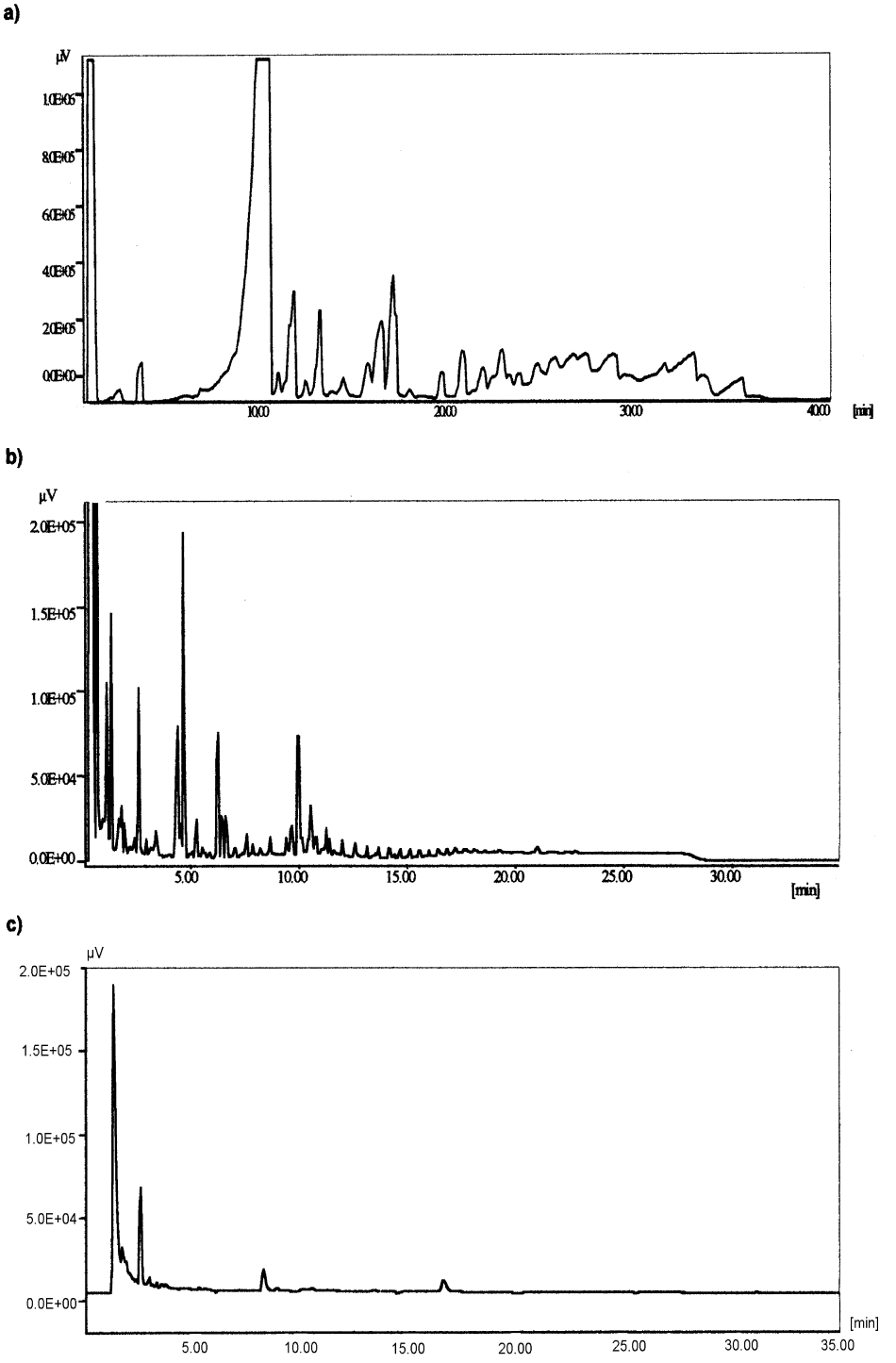

Fig. 3. Chromatogram derived from a pesticide-free bee extract (a) unpurified (FID detector), (b) purified in GPC (FID detector), (c) purified in GPC (ECD detector).

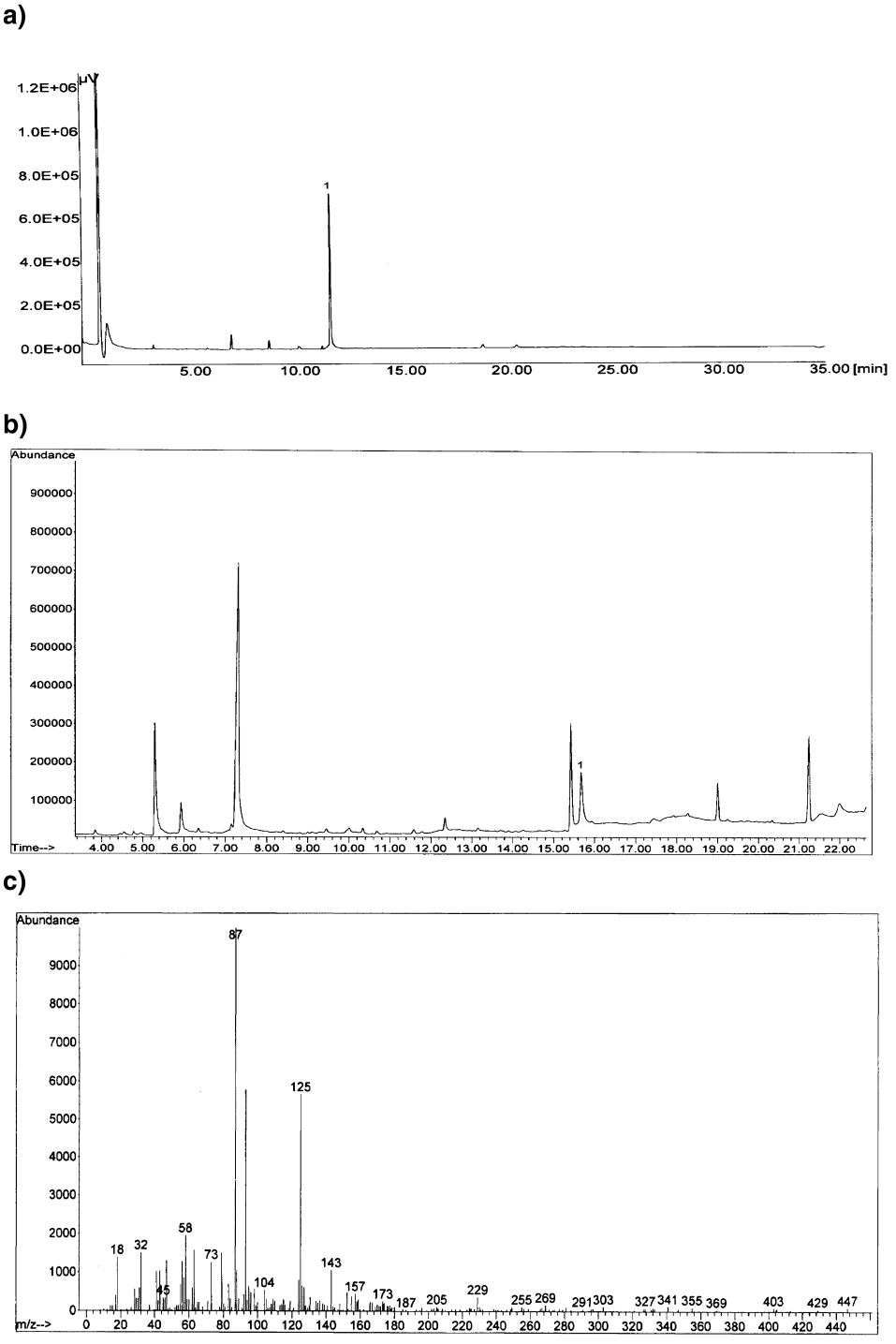

Fig. 4. (a) Chromatogram derived from a real bee sample (NPD detector); (b) Sim analysis for ions 87, 93, 125, 79, see (c). (1) Dimethoate. Experimental conditions are reported in the text.

Fig. 3a–c compares the chromatograms derived from the same pesticide-free sample and analyzed with different detectors. The chromatogram derived from a non-purified extract in GPC (Fig. 3a) would be impossible to interpret.

The chromatogram obtained with the purified sample and analyzed in GC with an FID detector (Fig. 3b) displays a series of equidistant peaks indicating the presence of aliphatic compounds of increasing molecular weight. This characteristic is found in each non-contaminated sample of bee extract and may be attributed to a lipid component present in the body of the bee, which we assumed to be wax. Comparison with a chromatogram obtained from beeswax under the same conditions confirmed our hypothesis.

The same extract analyzed using the gas chromatograph provided with an ECD detector shows a chromatogram free from significant artifacts (Fig. 3c).

This method was applied to the real samples and the structure of the peaks in the chromatogram was confirmed by GC–MS as shown in Fig. 4 for a bee sample containing dimethoate.

4. Conclusions

The procedure developed in this study successfully combined a rapid extractive technique with a higly effective method for purifying the sample. We were able to identify a fair number of pesticides by submitting the sample only to a single extraction and purification treatment, with evident savings in terms of time and cost. In addition, a reduction in the use of solvents is to be welcomed as it helps safeguard both the operator's health and the environment – an important aspect for a project which aims to determine the quality of the environment in order to identify possible dangers to human beings and nature.

Processing a sample to obtain an extract that can be analyzed with different types of detectors means that environmental monitoring using bees can be extended and the possibility of determining other micropollutants is currently being studied. Particular attention is being focused on the determination of benzo(*a*)pyrene, as an indicator of various types of combustion pollution, and of other pesticides used in phytosanitary treatment (organochlorides, pyrethroids, triazides, methylcarbamates, etc.).

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