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Journal of Chromatography A, 982 (2002) 267–273
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Simple method for the determination of trace levels of pesticides in honeybees using matrix solid-phase dispersion and gas chromatography

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Received 3 June 2002; received in revised form 16 September 2002; accepted 17 September 2002

Abstract

A simple multiresidue method for the determination of insecticides in honeybees is described. The developed method is based on the matrix solid-phase dispersion technique. A total number of 12 insecticides (azinfos-methyl, buprofezin, chlorpyriphos, chlorpyriphos-methyl, diazinon, ethion, fenitrothion, fipronil, methidathion, phosalone, pirimicarb, propoxur) used on flowering fields are determined by this method. The method uses Florisil and silica as dispersing agents, alumina and silica as cleanup adsorbents and a low polarity solvent system to elute pesticide residues from the honeybee samples. The insecticides were quantified using capillary gas chromatography with a nitrogen–phosphorus detector. The method has shown good recovery (70–110%) for various levels of spiked samples (0.01–1.0 mg/kg). The relative standard deviations were in the range of 2–8% for all pesticides studied. The limits of detection were in the range of 0.005–0.05 mg/kg. The procedure can be applied for the determination of residues of low-polarity and medium polarity pesticides in honeybee samples.

Keywords: Matrix solid-phase dispersion; Honeybees; Extraction methods; Pesticides

1. Introduction

The extensive use of pesticides to improve agricultural productivity has resulted in the wide distribution of these compounds in the environment. Honeybees are good bioindicators of environmental contamination with toxic substances. The insects are in close contact with pesticides during their ''work'' on flowering plants and therefore can be used to evaluate environmental contamination with pesticides and their metabolites. A consequence of the treatment of pesticides on crops can be poisoning of bees, bumblebees, ladybirds and other useful insects, especially that some pesticides are classified as being dangerous to them.

Insecticides are the main group of pesticides that have caused incidents of poisoning of honeybees in Poland in recent years. This was the main reason for developing a simple, sensitive and reliable method for determining their residues in honeybee samples. A few analytical procedures for determination of pesticides in bees has been published in the last few years. Sample extraction is usually based on liquid–

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0021-9673/02/$ – see front matter
PII: S0021-9673(02)01505-4

liquid extraction (LLE) [1–3], solid-phase extraction (SPE) [4,5] and supercritical fluid extraction (SFE) [6]. However, for complex matrices such honeybees, extraction of pesticides is complicated due to a large amount of organic matter in the sample, which affects the results of the analysis. A substantial amount of wax in extracts causes problems with chromatographic detection because of blocking of the active sites in liners and columns [6]. Therefore, extensive cleanup is required before gas chromatographic determination. Gel permeation chromatography (GPC) [7–9] is usually used for this purpose.

The development in recent years of the matrix solid-phase detection (MSPD) method and its successful application to extract pesticide residues and for cleanup of extracts from a variety of matrices [10–14] has encouraged us to use the method for the analysis of honeybees. The proposed method is based on the use of Florisil and silica as dispersing agents, alumina and silica as cleanup adsorbents, and a solvent system of low polarity to isolate insecticides from honeybee samples. The method allows for the determination of trace levels of residues.

2. Experimental

2.1. Chemicals and materials

Analytical reference standards were supplied by Dr. Ehrenstorfer Laboratory (Germany). Standard stock solution of various concentrations: (ethion, 0.08 μg/ml; diazinon, pirimicarb, chlorpyrifos, chlorpyrifos-methyl, fenitrothion, methidathion, 0.1 μg/ml; phosalone, buprofezine, 0.2 μg/ml; azinfos-methyl, 0.3 μg/ml; fipronil, 0.4 μg/ml; propoxur, 0.5 μg/ml), were prepared in acetone and stored at 4 °C. Standard working solutions were prepared by appropriate dilution of the stock solution with a *n*-hexane–acetone (9:1) mixture.

The solid-phase materials used were: Florisil PR (Floridon, USA), Silica gel 60 (70-230 mesh, Merck) and neutral alumina (70-230 mesh, activity IB supplied by Merck). All adsorbents were activated by heating overnight at 150 °C before use, allowed to cool and stored in a well-closed flask. Freshly activated adsorbents were used for this study. Anhydrous sodium sulfate analytical reagent-grade used for drying was heated for 4 h at 500 °C. All solvents (acetone, *n*-hexane, diethyl ether) were of analytical quality.

Honeybee samples used for spiking and blank studies were taken from experimental beehives that were found to be free of pesticides. They were stored at −20 °C until analyzed.

2.2. Extraction procedure

2.2.1. Procedure A

A sample of bees (0.5 g; about five insects) was homogenized with 1.5 g of activated Florisil in a glass mortar to obtain a semi-dry, homogenous mixture of sample dispersed on the solid-phase. The mixture was placed in a glass column (30 cm×10 mm I.D.) plugged with a silanized glass-wool and containing 2 g of activated silica at the bottom. The column head was covered with 1.5 g of anhydrous sodium sulfate and was lightly tapped to remove air bubbles. The prepared column was prewashed with 15 ml of *n*-hexane and the eluate was discarded. The analytes were eluted with solvents of increasing polarity in 15-ml fractions: *n*-hexane–diethyl ether (9:1), *n*-hexane–diethyl ether (8:2), *n*-hexane–ethyl acetate (7:3). Elution was performed by gravity flow. The three fractions were combined and concentrated to about 1 ml using a vacuum evaporator with a temperature programmed bath (40 °C). The final volume of the eluates was adjusted to 2 ml by the addition of a *n*-hexane–acetone (9:1) mixture and subjected to analysis by GC with nitrogen–phosphorous detector (NPD).

2.2.2. Procedure B

The sample of bees (0.5 g; about five insects) was homogenized with 1.5 g of activated silica in a glass mortar to obtain a semi-dry, homogenous mixture. The mixture was placed in a glass column (30 cm×10 mm i.d.) plugged with a silanized glass-wool and containing 1 g of activated alumina at the bottom. The column head was covered with 1.5 g of anhydrous sodium sulfate. The column prepared in this way was prewashed with 15 ml of *n*-hexane and the eluate was discarded. The analytes were eluted with solvents of different polarity in 15 ml fractions: *n*-hexane–diethyl ether (7:3), *n*-hexane–ethyl acetate (7:3). The two fractions were then combined and

concentrated to about 1 ml using a vacuum evaporator. The final volume was adjusted to 2 ml by addition of a *n*-hexane–acetone (9:1) mixture.

Spiked samples were prepared by injecting the standard mixture to honeybees by syringe. The samples were allowed to stand at room temperature for 0.5 h and then treated in the same manner as described above.

2.3. Gas chromatography

A Hewlett-Packard HP 6890 Plus gas chromatograph, equipped with HP 7683 autosampler, NPD, split–splitless injector, and an EPC system, was used. The injector port temperature was 220 °C and the detector temperature 320 °C. Flow-rates were: carrier gas (helium) 1.9 ml/min; hydrogen 3 ml/min; air 60 ml/min, make up (nitrogen) 6 ml/min. A HP-1 capillary column (30 m×0.32 mm, 0.25 μm film thickness) was used. The column was protected by a guard column of 0.5 m in length and of the same type and dimension. The sample (2 μl) was injected in the splitless mode. The oven temperature program was as follows: initial temperature of 100 °C, hold for 2 min, increase to 130 °C at 30 °C/min and then to 260 °C at 8 °C/min and hold for 4 min.

The GC was controlled by a personal computer using Chemstation software (Hewlett-Packard).

3. Results and discussion

An analytical method for the determination of pesticide residues in a complex matrix such as bees requires isolation of residues from the matrix and cleaning of the extracts before chromatographic determination. In this study these two steps are combined in a single step. The sample is dispersed over a large surface area of an activated solid-phase and becomes part of the extraction column. In this way the surface area of the dispersed sample exposed to the solvent is increased. Both analytes and matrix interferences are retained on the solid-phase material. In general, various components of the matrix can influence efficiency of analyte recovery. However, an appropriate solvent system allows the elution of analyte free of matrix components. This can be done either by an additional washing step to remove interferences or by leaving the matrix components adsorbed on the column. In the case of honeybee samples the major problem is the presence of large amounts of interfering waxes [6]. However, prewashing of the MSPD column with *n*-hexane proved sufficient to remove non-polar waxes. In our previous paper on the MSPD method [15], toxic chlorine containing solvents were used. Contrary to this, various mixtures of *n*-hexane, diethyl ether and ethyl acetate were chosen for this study. The best solvent systems for our purpose were found to be *n*-hexane–diethyl ether (9:1), (8:2), (7:3) and *n*-hexane–ethyl acetate (7:3) mixtures. Consecutive elution of the MSPD column with solvents of increasing polarity in 15 ml fractions gave the best results: eluates free of chromatographic interferences were obtained. The fractions were first collected and analyzed separately under the same chromatographic conditions for the optimization of the elution scheme. Then they were combined and analyzed together. This approach gave the best results: non-polar and moderately polar pesticides were recovered almost quantitatively with good reproducibility using both Procedures A and B (Table 1). It must be stressed that elution of pesticides with solvents of gradually increasing polarity is by far better than use of a single polar solvent system in term of sample clean-up.

Two different procedures (A and B) were compared to each other in respect to their suitability for extraction of pesticides from honeybee samples. Known amounts of pesticides were added to the samples. Because of a matrix effect [16], curves obtained using calibration standards in the matrix extract were different from those obtained in a pure solvent. For this reason, calibration curves used for quantitation were generated from the standards in the blank sample extract (Fig. 1). In this way, determination errors caused by the matrix effect were reduced. However, it was found that the standard curves obtained for standards in the matrix extracts obtained according to the procedures A and B were practically identical. The calibration curves were plotted for each pesticide to determine the linearity range and the detection limit. The calibration plots constructed from peak height versus concentration of pesticides were linear for all compounds tested in the range 0.01–1.0 μg/ml. Correlation coefficients for

Table 1
Recoveries of pesticides from the spiked samples (100 μl/0.5 g) in the individual fractions (n=5)

Solvent system	Procedure A		Procedure B	
	Compound	Recovery ±RSD (%)	Compound	Recovery ±RSD (%)
n-Hexane–diethyl ether (9:1)	Chlorpyrifos-methyl	87.0±4.0		
	Chlorpyrifos	80.7±5.0		
	Ethion	90.5±2.9		
n-Hexane–diethyl ether (8:2)	Diazinon	79.4±3.3		
	Fenitrothion	83.8±3.4		
	Methidathion	12.8±3.1		
	Buprofezine	80.5±4.2		
	Phosalone	39.0±5.5		
n-Hexane–diethyl ether (7:3)			Diazinon	95.0±3.0
			Chlorpyrifos-methyl	81.1±6.2
			Fenitrothion	78.9±7.2
			Chlorpyrifos	75.2±5.3
			Methidathion	49.1±5.7
			Buprofezine	90.0±3.5
			Ethion	95.5±3.6
			Phosalone	11.1±6.0
n-Hexane–ethyl acetate (7:3)	Propoxur	79.2±3.1	Propoxur	67.1±5.1
	Diazinon	18.0±3.6	Pirimicarb	85.5±5.4
	Fipronil	90.6±3.8	Fipronil	78.3±3.4
	Methidathion	82.0±3.4	Methidathion	50.0±4.8
	Azinfos-methyl	75.6±4.6	Azinfos-methyl	79.2±6.0
	Phosalone	40.2±4.1	Phosalone	31.1±6.6

the standard curves of the 12 extracted pesticides ranged from 0.9985 for pirimicarb to 0.9999 for fipronil and methidathion (linear regression analysis, n=4).

The limits of detection (LODs) and limits of quantitation (LOQs) defined as a response three or ten times the average height of the background noise, respectively, were obtained by using matrix-matched standards in order to obtain more reliable results. LOQs varied between 0.015 and 0.15 μg/g (depending on the particular pesticide) for both Procedures A and B. The LODs, LOQs and correlation coefficients values obtained for pesticides studied are given in Table 2.

Recovery experiments were performed in order to study the accuracy of the method. Due to its selectivity, GC/NPD system was used to measure recoveries from spiked samples. However, for some compounds (e.g. azinfos-methyl, fipronil, propoxur, phosalone) the NPD sensitivity is lower than for the other compounds studied. The honeybee samples free from residues were spiked at three levels and the recoveries of pesticides were studied. All analyses were carried out in five replicates. Spiked levels were settled according to the detector sensitivity to particular pesticides in the range of 0.04–1.0 μg/g. Pesticides were quantified by the external standard method. The two procedures gave relatively high recoveries for the pesticides tested (70–110%) with the reproducibility (calculated as relative standard deviation) varying from 2 to 8% for different pesticides. These values indicate a good performance of the method. The recovery was satisfactory for all compounds except for pirimicarb (zero recovery with Procedure A) and phosalone (small recoveries with Procedure B). A general conclusion can be drawn, that the lower were pesticide spiked levels, the higher the recoveries were observed. Recoveries were more variable (as indicated by RSD values) at lower pesticide concentrations and rather for Pro-

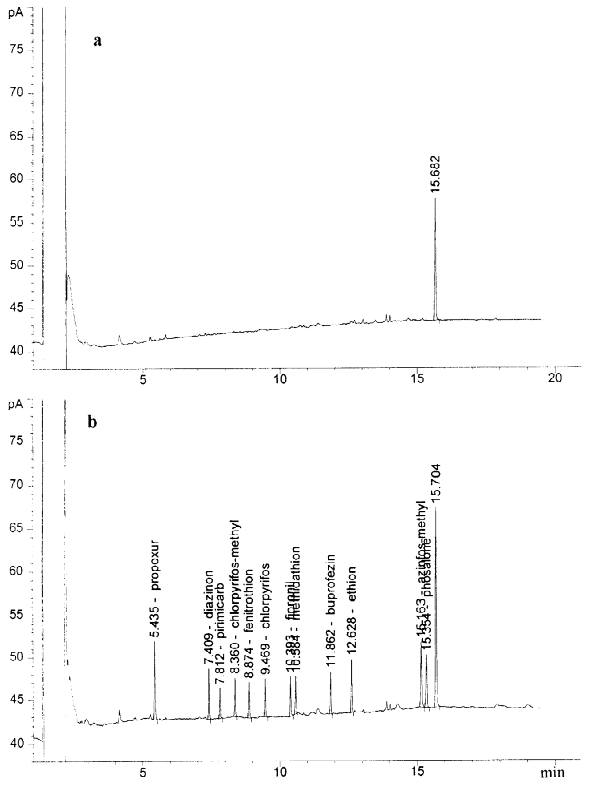

Fig. 1. GC–NPD chromatograms: (a) blank sample of pesticide-free honeybees; (b) standard mixture of pesticides prepared in matrix.

cedure B compared with Procedure A. Results of recovery experiments are presented in Table 3.

The extraction efficiency of MSPD method was compared with this obtained by using traditional liquid extraction based on method recommended for non-fatty animal products by the Dutch General Inspectorate for Health Protection [17], which was previously used in our laboratory. In this method the sample extraction with acetone and acetonitrile was followed by cleanup with Florisil column. The results showed that MSPD gives comparable results to the traditional method, which gave good recoveries and extracts clean enough for GC–NPD analysis. However, in some cases MSPD method proved superior to the traditional one, particularly at low pesticide concentration due to the presence of interferences that were not completely removed.

The most striking advantage of MSPD over liquid extraction is the possibility to confirm results of analysis of some pesticides, e.g. azinfos-methyl, chlorpyrifos, chlorpyrifos-methyl, fenitrothion, ethion, and especially fipronil, by electron capture detection (ECD). This would not be possible with extracts obtained by traditional method because of high background and numerous interfering peaks in the ECD gas chromatograms. The MSPD extraction is more selective than the traditional one.

The performance of clean-up based on various

Table 2
Detection (LOD) and quantification (LOQ) limits, and correlation coefficients of the calibration curves

Compound	LOD (μg/g)		LOQ (μg/g)		Correlation coefficient*
	A	B	A	B	
Propoxur	0.045	0.045	0.15	0.15	0.9997
Diazinon	0.005	0.005	0.015	0.015	0.9999
Chlorpyrifos-methyl	0.01	0.015	0.03	0.05	0.9999
Pirimicarb	–	0.01	–	0.03	0.9985
Fenitrothion	0.01	0.01	0.03	0.03	0.9996
Chlorpyrifos	0.01	0.01	0.03	0.03	0.9998
Fipronil	0.02	0.02	0.06	0.06	0.9999
Methidathion	0.01	0.01	0.03	0.03	0.9999
Ethion	0.005	0.005	0.015	0.015	0.9998
Buprofezine	0.02	0.02	0.06	0.06	0.9998
Azinfos-methyl	0.04	0.04	0.12	0.12	0.9997
Phosalone	0.03	0.045	0.09	0.15	0.9993

*Correlation coefficients from linear regression analysis ($n=4$).

Table 3
Recoveries of pesticides on various spike standard levels for the Procedures A and B (n=5)

Compound	Spiked level (μg/g)	Procedure A		Procedure B	
		Mean recovery (%)	RSD (%)	Mean recovery (%)	RSD (%)
Propoxur	0.2	109.0	4.9	104.6	5.4
	0.5	97.2	3.9	100.2	4.8
	1.0	75.4	3.8	104.0	4.7
Diazinon	0.04	107.0	5.0	104.3	3.2
	0.10	85.0	3.5	100.4	3.6
	0.20	73.2	3.4	96.3	3.1
Pirimicarb	0.04	–	–	100.0	5.7
	0.10	–	–	93.4	5.5
	0.20	–	–	88.9	4.8
Chlorpyrifos-methyl	0.04	100.9	4.0	100.0	6.8
	0.10	100.9	2.8	87.5	7.3
	0.20	75.9	3.0	79.0	6.2
Fenitrothion	0.04	108.0	3.8	98.3	7.5
	0.10	97.4	2.9	91.0	7.1
	0.20	80.0	2.7	77.6	6.6
Chlorpyrifos	0.04	108.0	5.9	96.2	5.7
	0.10	93.6	5.6	87.0	4.9
	0.20	72.2	6.0	77.9	4.8
Fipronil	0.16	102.8	4.6	102.1	4.0
	0.40	102.8	4.0	99.0	3.6
	0.80	89.8	4.9	86.8	3.8
Methidathion	0.04	110.0	3.6	110.0	5.7
	0.10	100.2	4.0	99.1	4.3
	0.20	99.0	3.9	84.4	4.0
Buprofezine	0.08	96.9	5.9	96.7	3.2
	0.20	95.6	4.8	87.5	2.5
	0.40	86.9	5.3	89.0	2.5
Ethion	0.032	102.0	3.0	98.3	3.4
	0.080	95.3	2.3	94.7	2.8
	0.160	90.2	2.8	95.3	2.6
Azinfos-methyl	0.12	110.0	6.6	112.0	6.7
	0.30	94.1	6.1	100.4	6.8
	0.60	76.1	6.8	88.4	5.9
Phosalone	0.08	109.0	6.9	46.1	7.5
	0.20	103.1	7.4	50.6	6.4
	0.40	95.7	5.0	39.0	6.1

adsorbents, including silica, Florisil, alumina and mixtures of them, was also investigated. The best recoveries were obtained using activated silica and Florisil. The additional 2-g sorbent bed at the bottom of the MSPD column provided extra fractionation and cleanup of the sample dispersed on a solid-phase from polar matrix components. As a result, the eluates were sufficiently pure for GC determination of pesticides at low concentration levels, because coextractives were not present in the final extract. GC–NPD chromatograms for blank and spiked honeybee samples showed no matrix interferences in the retention time window.

In summary, the MSPD technique allows for the successful extraction, followed by GC determination of 12 insecticides in honeybee samples at the 0.03–1.0 μg/g level. A remarkable advantage of the presented method is that the isolation and purification are combined into one step. The time required for sample preparation, extraction and cleanup by the traditional method was much longer compared with MSPD technique. The procedure is simple, requires only small volumes of organic solvents and gives clean extracts ready for GC detection.

The results obtained are based on spiked samples. Incurred samples were not available at the time when this research was performed. Since the method cannot be fully validated with spiked samples only, efforts to collect and analyze real honeybee samples, to confirm the applicability of the MSPD method for the pesticide residue analysis, are in progress.

Acknowledgements

Skillful technical assistance by Mrs. Teresa Janowicz, Mrs. Teresa Raciborska and Mrs. Teresa Reszec is gratefully acknowledged.

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