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Determination of the fungicide vinclozolin in honey and bee larvae by solid-phase and solvent extraction with gas chromatography and electron-capture and mass spectrometric detection

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Abstract

Methods for the determination of residual vinclozolin in honey and bee larvae are proposed. The fungicide can be extracted with an *n*-hexane–acetone (70:30, v/v) mixture, or by passage through ODS cartridges which requires no subsequent clean-up. Vinclozolin is quantified by capillary gas chromatography with electron-capture and mass spectrometric detection. Recoveries from spiked samples exceed 90%, with a relative standard deviation of 3.5–4.5%.

Keywords: Honey; Environmental analysis; Vinclozolin; Pesticides

1. Introduction

Vinclozolin is an agrochemical fungicide with a long tradition in farming applications. Its efficiency to control ascospheriosis in honey bee hives is currently being assessed and this necessitates measuring its concentration in different hive compartments.

Methods for the determination of vinclozolin in hive products have not been described till now. The fungicide has so far been quantified in soil, plants, wine and water, by extraction with organic solvents of low to medium polarity or by solid-phase extraction (SPE) using octadecylsilane (ODS) as absorbent. Depending on the complexity of the particular sample, a clean-up step may be required prior to the chromatographic determination, which is usually carried out by gas chromatography–electron-capture detection (GC–ECD) or reversed-phase high-performance liquid chromatography with ultraviolet detection [1–9].

This paper reports a method for the determination of vinclozolin in honey and bee larvae. Samples are extracted with an *n*-hexane–acetone mixture or with SPE on ODS cartridges. Two clean-up procedures, chromatography on Florisil and on ODS columns, were assayed after the solvent extraction. Vinclozolin was determined in the extracts by capillary GC with ECD and mass spectrometric detection in the electron impact mode (EI–MS).

2. Experimental

2.1. Reagents

Chromatographically pure vinclozolin standards were obtained from Riedel de Haën (Seelze, Hannover, Germany). Residue analysis grade acetonitrile, methanol, dichloromethane, acetone and *n*-hex-

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ane were supplied by Lab-Scan (Dublin, Ireland). Ultrapure water was obtained by using a Milli-Q apparatus from Millipore (Bedford, MA, USA). Florisil of 60–100 mesh was purchased from Baker (Deventer, Netherlands). Octadecyl 500 mg Bond Elut cartridges from Analytichem International (Harbor City, CA, USA) were used for SPE and clean-up. Disposable syringe filter units of 0.5 μm pore size were obtained from Microfiltration Systems (Dublin, CA, USA). Perchloric acid (60%) and potassium sulphate were supplied by Panreac (Barcelona, Spain).

2.2. General instrumentation

A Turbo-vap evaporator equipped with a thermostated water bath and a nitrogen supply was purchased from Zymark (Hopkinton, MA, USA). A CL/Samplextract solid–liquid extraction system, fitted with a vacuum pump, was supplied by Cromlab (Barcelona, Spain). The centrifuges used were obtained from Kokusan (Tokyo, Japan) and the rotary mechanical shakers were from Selecta (Barcelona, Spain).

2.3. Fortification of honey and larvae samples

Honey samples were spiked as follows: 50 g of honey were heated at 35°C for 15 min and then supplied with 0.1 ml of a vinclozolin solution of known concentration in acetone. The mixture was homogenized by vigorous shaking and stored at 4°C in the darkness prior to analysis. Each spiked sample was used for a maximum of ten days, after which it was discarded.

Larva samples were spiked immediately prior to extraction. For this purpose, 1 g of sample was ground in the extraction vessel and 50 μl of a vinclozolin solution in acetone was added. The mixture was stirred with a rod and allowed to stand for 15 min prior to extraction.

2.4. Extraction of honey and larvae with n-hexane–acetone

The procedure used to extract vinclozolin with an organic solvent from the two types of sample was similar. Thus, a sample amount of 1–5 g was placed in a threaded glass tube and mixed with 30 ml of organic solvent (plus 2 ml of water for honey samples, in order to get a better dispersion of the sample). A mixture of two solvents (*n*-hexane and acetone) in variable proportions was assayed as extractant. The fungicide was extracted by shaking for 20 min. Then, the organic phase was separated by centrifugation at 5000 *g* for 5 min and then collected. The sample was again extracted with 30 ml of solvent and the above-described procedure was repeated. The two portions collected were combined and the solvent was evaporated under a gentle nitrogen stream at 35°C. Finally, the resulting residue was dissolved in 2 ml of acetone with sonication and passed through a PTFE filter of 0.50 μm pore size.

2.5. Solid-phase extraction of honey

A honey amount of 1–5 g was mixed with 100 ml of water and the mixture homogenized by mechanical shaking for 15 min. Simultaneously, ODS cartridges were conditioned by successive elution of 10 ml of methanol, 10 ml of acetone and 10 ml of water, by a gentle evacuation with the aid of a pump, the cartridge was never allowed to dry during this step. Then, the 100 ml sample was eluted at a rate of about 10 ml/min and the cartridge dried with nitrogen for about 30 min. Finally, the cartridge was eluted by gravity with 2 ml of acetone.

2.6. Solid-phase extraction of larvae

A sample amount of 1 g was placed in a threaded glass tube, ground and extracted with 100 ml of hot water (40°C) by shaking for 30 min. A 2-ml volume of acetonitrile was then added to the aqueous extract and the mixture was filtered through porous glass plate with the aid of gentle vacuum. The ODS cartridges used were conditioned as described in Section 2.5, and then the aqueous sample was eluted at a rate of about 10 ml/min. Finally, the cartridge was dried with a nitrogen stream for about 30 min and eluted by gravity with 2 ml of acetone.

2.7. Octadecylsilane clean-up

ODS cartridges (500 mg) were also conditioned as described in Section 2.5. With the cartridge still wet,

the solvent extract (honey or larvae) collected in 2 ml of acetone was eluted by gravity, followed by a further 2 ml of pure acetone that was combined with the previous portion to obtain a final volume of 4 ml.

2.8. Florisil clean-up

Honey and larva solvent extracts were also cleaned up by passage through a Florisil-packed glass column. Florisil was previously conditioned by heating at 120°C for 4 h. The column, 10×1 cm I.D., was prepared from Florisil (about 5 g) slurry in *n*-hexane and compacted with the aid of a rod. Once ready, the column was loaded with 2 ml of extract and eluted by gravity with 30 ml of *n*-hexane–dichloromethane (1:1, v/v); care was taken to prevent the column from drying at any time. Subsequently, the eluate was evaporated under a nitrogen stream at 35°C and the residue was dissolved in 2 ml of acetone.

2.9. Gas chromatography–mass spectrometry

A Hewlett-Packard (Avondale, PA, USA) 5890 Series II gas chromatograph, equipped with an HP7673 autosampler and a 30 m×0.25 mm capillary column, coated with a 0.25-μm thick film of 50% phenylmethylpolysiloxane (called DB 17, J & W Scientific, Folsom, CA, USA), was directly interfaced to an HP5989A mass spectrometer. The oven temperature programme was as follows: initial temperature, 50°C; held for 1 min; 6°C/min ramp to 220°C and finally 20°C/min ramp to 275°C, then held for 2 min. The carrier gas (He) pressure programme was as follows: initial pressure, 21 kPa; a 682.6 kPa/min ramp to 276 kPa, held for 0.1 min, then a 682.6 kPa/min ramp to 35 kPa, and finally a 2.3 kPa/min ramp to 99 kPa and held for 5 min. Splitless injections (1 μl) were performed at 200°C and the purge valve was on at 1 min. The working conditions for the mass spectrometer were as follows: transfer line temperature, 280°C; ion source temperature, 200°C; quadrupole temperature, 100°C; scan range, 65–400 u. The electron multiplier voltage was always kept 400 voltage units above autotune.

2.10. Gas chromatography–electron-capture detection

GC–ECD was performed on an HP5890 gas chromatograph equipped with an HP7673 autosampler (both from Hewlett-Packard) and a 60 m×0.25 mm capillary column coated with 0.25 μm of 50% phenylmethylpolysiloxane (called 007-17, Quadrex Scientific, Surrey, UK). The oven temperature programme was as follows: initial temperature, 80°C; held for 1 min; 20°C/min ramp to 160°C; 2°C/min ramp to 241°C, and finally 20°C/min ramp to 275°C, then held for 20 min. The carrier gas (He) flow-rate was 0.7 ml/min, measured at 80°C. Splitless injection of a 1 μl volume was carried out at 200°C and the purge valve was on at 1 min. The detector temperature was 300°C and argon–methane (90:10) was used as the auxiliary gas.

3. Results and discussion

Table 1 shows the recovery and precision achieved by applying the above-described solvent extraction procedure to honey and larva samples spiked with 25 mg/kg vinclozolin. As can be seen, the fungicide was incompletely extracted by *n*-hexane and its recovery increased with increasing proportions of acetone in the mixture. Higher recovery from both honey and larva samples was achieved with *n*-hexane–acetone (70:30, v/v). The recoveries obtained with higher proportions of acetone were similar, but the number and concentration of co-extracted substances increased, particularly with honey, no matter which clean-up procedure was used, column chromatography with ODS or Florisil. Table 1 shows the results obtained with ODS clean-up, which were quite similar to those for Florisil. At the assumed optimal *n*-hexane–acetone ratio (70:30, v/v), vinclozolin recoveries from 1 and 5 g of honey were 99% and 98%, respectively, and that from 1 g of larvae was 95%. The lower recovery from larvae samples could be attributed to the binding of the vinclozolin to sample proteins. As regards precision, the relative standard deviation (R.S.D., $n=5$) was about 4.5% with both ODS and Florisil.

The extracts from honey samples were cleaner than those from larvae, the performance of both

Table 1
Recovery of vinclozolin from honey and larvae samples, spiked with 25 mg/kg, by solvent extraction with hexane–acetone at different proportions and ODS clean-up ($n=5$)

Sample amount:	Honey				Larvae	
	1 g		5 g		1 g	
Extractant solvent (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)
Hexane (100)	45	4.2	42	4.8	48	4.4
Hexane–acetone (90:10, v/v)	64	4.4	63	4.4	67	4.5
Hexane–acetone (80:20, v/v)	81	4.6	82	4.7	78	4.3
Hexane–acetone (70:30, v/v)	99	4.7	98	4.6	95	4.7
Hexane–acetone (60:40, v/v)	99	4.8	98	4.6	96	4.5
Acetone (100)	99	4.4	98	4.4	95	4.7

R.S.D.: relative standard deviation.

clean-up procedures being similar. Fig. 1 shows typical chromatograms of honey extracts obtained with the mass spectrometer and the electron capture detectors. Larva extracts exhibited a large number of chromatographic peaks that decreased on clean-up. Fig. 2 shows an amplified view of the chromatograms for a larva extract, both untreated and after clean-up with Florisil or ODS. As can be seen, both clean-up procedures decreased the number and height of the chromatographic peaks, without clearly discerning a procedure as being the most adequate. As can be seen in Fig. 3, some compounds exhibited a negative response to the ECD, which required using a slower temperature gradient in the GC–ECD system to prevent one of these compounds from co-eluting with vinclozolin.

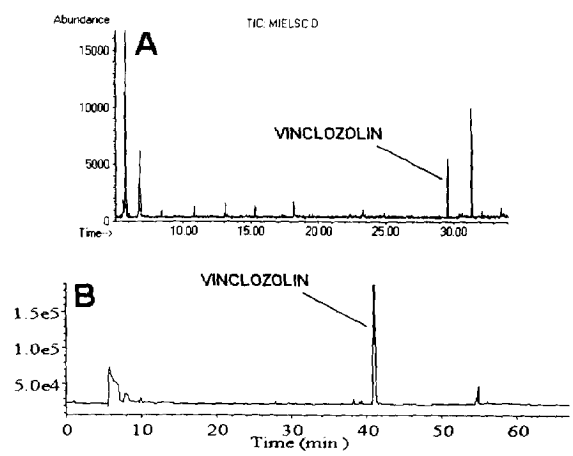

Fig. 1. Chromatograms of a honey extract obtained by extraction with hexane–acetone (70:30, v/v) and ODS clean-up. (A) Total ion chromatogram by EI–MS (scan mode) detection. Vinclozolin concentration in the extract, 15.3 mg/l. (B) Electron capture detection. Vinclozolin concentration in the extract, 22.5 mg/l.

The most relevant compounds in the larva extracts were identified by MS, which revealed the dominant presence of fatty compounds, whose origin was ascribed to wax residues from hive combs adhering to the larvae. Such compounds may be responsible for the negative peaks provided by the ECD. The nature of the compounds and their retention times (in minutes) in the GC–MS system were as follows: pentadecanoic acid, methyl ester (23.55); hexadecanoic acid, methyl ester (26.85); hexadecanoic acid (27.96); 9-octadecenoic acid, methyl ester (29.90); 12?,15?-octadecadienoic acid, methyl ester (30.11); 9?,12?,15?-octadecatrienoic acid, methyl ester (30.48); 9-octadecenoic acid (30.76) and 9,12,15-hexadecatrienoic acid, methyl ester? (31.25). The concentration of most of these compounds in the extract decreased on clean-up with ODS or Florisil, and the latter completely removed the acidic compounds.

Honey and larvae samples spiked with vinclozolin, at 25 mg/kg, were also extracted by SPE procedures without subsequent clean-up. The recoveries from honey samples were similar to those from the solvent extraction procedure, so for 1 and 5 g samples, the recoveries were 96 and 98%, with R.S.D. values of 3.5 and 3.2% ($n=5$), respectively. For larvae samples, the recovery was somewhat lower (91% for 1 g sample) and the R.S.D. was 3.6% ($n=5$). The precision achieved by the SPE procedure was better than that provided by solvent extraction.

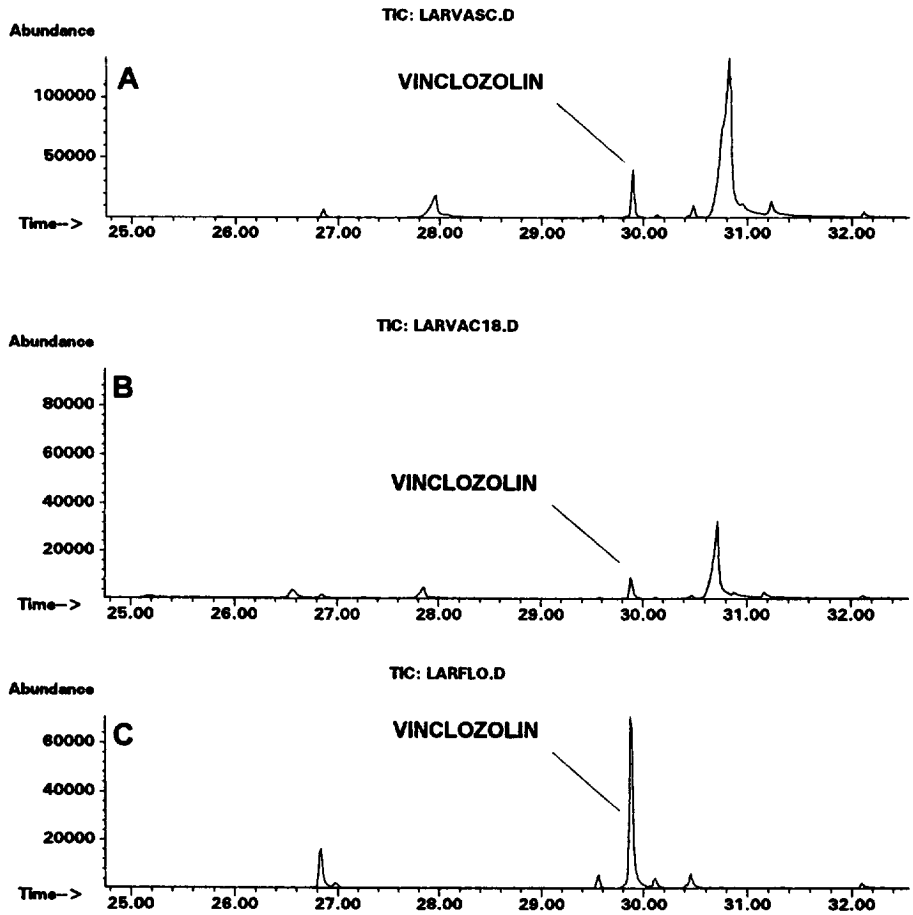

Fig. 2. Chromatograms of a larva extract obtained by extraction with hexane–acetone (70:30, v/v), EI–MS (scan mode). (A) Without clean-up, (B) with ODS clean-up and (C) with Florisil clean-up.

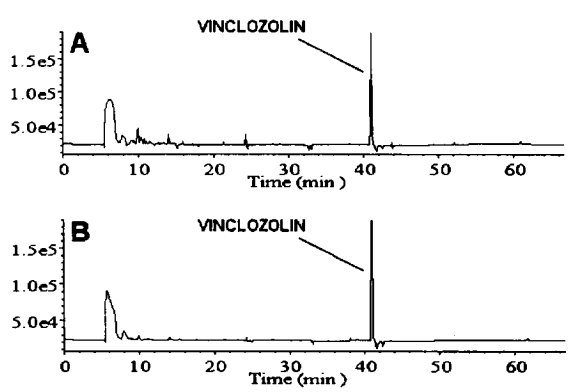

Fig. 3. Chromatograms of a larva extract obtained by extraction with hexane–acetone (70:30, v/v) and ECD, (A) without clean-up and (B) with Florisil clean-up.

The chromatograms for a solvent extract from larvae, subjected to no clean-up, and another extract collected over ODS, are shown in Fig. 4. SPE proves to be more selective than solvent extraction. The chromatograms obtained for the honey and larva extracts by using SPE exhibited a smaller number of peaks of co-extracted compounds in both detection systems. Negative peaks were not observed in ECD.

SPE of acaricides from water-diluted honey is currently a widely used procedure. So, we tried to develop a procedure of this type for the extraction of larvae, based on the solubility of vinclozolin in water (3.4 mg/l at 20°C). A temperature of 40°C was chosen in order to increase the fungicide solubility, to avoid degradation phenomena and to facilitate the handling of the material. The larva aqueous extracts

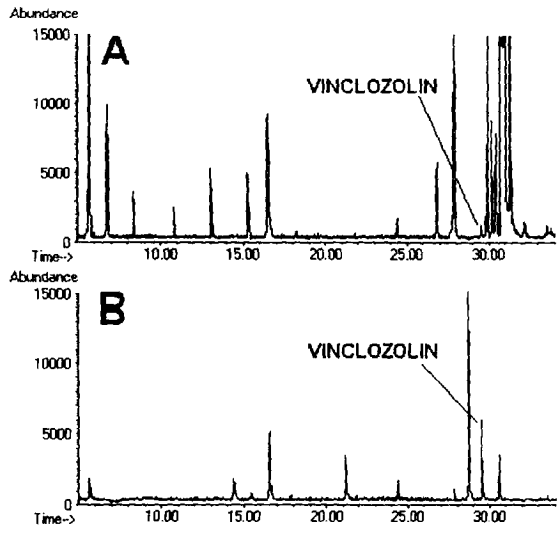

Fig. 4. Chromatograms of a larva extract by EI–MS (scan mode). (A) Hexane–acetone (70:30, v/v) extraction and (B) SPE.

thus obtained showed a turbidity or emulsion, which was not broken by centrifugation at 5000 *g*, and hindered filtration of the solid residue. In order to facilitate filtration on a glass plate, the addition of three different substances were assayed, *viz.* acetonitrile (2 ml), potassium sulphate (1 g) and perchloric acid (2 ml). The results obtained with the last two were even worse. With potassium sulphate, filtration was similarly cumbersome and virtually no vinclozolin was recovered, whereas with perchloric acid, filtration was quite fluid but the recovery was below 30% and the resulting chromatogram included several small peaks that suggested the occurrence of hydrolysis. On the other hand, acetonitrile facilitated the filtration and resulted in quantitative recovery. Direct elution of the extract, without filtering, through the cartridges was not possible, as they were clogged with unsolved residues from ground larvae.

Table 2 gives the vinclozolin concentrations obtained with both extraction procedures, as applied to real honey and larva samples from a hive treated with the fungicide. The results provided by both procedures seems to be concordant.

As regards the detection systems, Fig. 5 shows the linearity of the detector response (peak height) with the mass amount, 0.5, 5, 10, 25, 50, 75 and 100 mg/l for ECD and MS in the scan and SIM modes (quantitation of the ion *m/z* 212 and monitoring of

Table 2
Results obtained on honey and larva real samples by solvent extraction with hexane–acetone (70:30, v/v) with ODS clean-up, and SPE ($n=2$)

	Vinclozolin concentration (mg/kg)	
	Solvent extraction	Solid phase extraction
Honey	26.1	25.3
	3.5	3.1
	12.2	13.0
	10.7	9.8
Larvae	10.1	9.9
	30.4	30.8
	4.2	4.3
	11.1	10.9

the ions *m/z* 198 and 285 as qualifiers). MS detection in scan mode has been preferentially used in this work, carrying out the calibration graphs with concentrations in the 5–75 mg/l range. The coefficient of correlation (r^2) of the linear fitting was, at least, 0.996. Recovery and precision data for the extraction procedures were obtained with it.

The detection limit varied between standards and extracts from real samples. At a signal-to-noise ratio of 3, the detection limit for standards was 0.04, 2 and 0.09 mg/l with ECD, MS–scan and MS–SIM, respectively. On the other hand, the detection limit

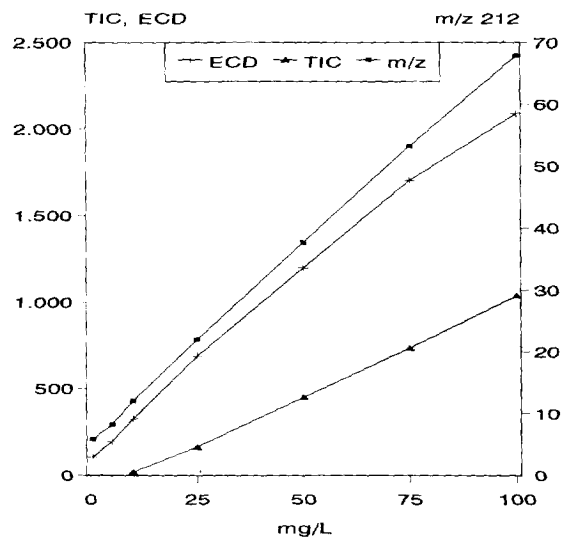

Fig. 5. Linearity of the response (peak height) for ECD and MS in the scan (TIC) and SIM (*m/z* 212) modes.

for spiked solvent extracts from honey blanks was 0.08, 2 and 0.1 mg/, and was 0.4, 3 and 0.1 mg/l for larvae blanks. Considering a sample amount of 5 g and a final volume of 2 ml, the detection limits were in the 0.03–0.8 mg/kg and 0.04–1.2 mg/kg ranges for honey and larvae samples, respectively, according to the determination technique.

4. Conclusions

The SPE procedure using ODS cartridges is more selective than the extraction with organic solvent and provides simpler chromatograms, with good recovery and reproducibility. A clean-up procedure, based on Florisil or ODS, is advised for the solvent extracts. The honey matrix is simpler and its extracts contain fewer co-extracted compounds relative to the larva matrix.

The proposed solvent and SPE procedures for vinclozolin on spiked honey and larva samples seemingly provide similar results on real samples.

Both ECD and MS can be used to determine vinclozolin in the extracts, with acceptable detection limits. It should be noted that fatty acids may co-elute with vinclozilin and cause interferences.

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