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Study of semi-automated solid-phase extraction for the determination of acaricide residues in honey by liquid chromatography

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Abstract

A solid-phase extraction (SPE) method followed by a reversed-phase high-performance liquid chromatography (HPLC) procedure is reported for the assay of a wide polarity range acaricide residues in honey. After selection of suitable chromatographic and detection conditions, most steps of the SPE procedure that may affect to the recovery were investigated. Honey sample was buffered at pH 6 and then applied to the preconditioned C_{18} sorbent. A washing step was performed with 1 ml of a mixture of tetrahydrofuran (THF)–phosphate buffer (10:90, v/v) and finally, the analytes were eluted with 1 ml of THF. The extract was evaporated to dryness, reconstituted in mobile phase and chromatographed on a reversed-phase C_{18} column with diode array detection. The recoveries of the more polar acaricides were higher than 80% and 60–70% for the more apolar ones. Limits of detection obtained ranged from 1 to 200 ng/g.

Keywords: Solid-phase extraction; Honey; Acaricides

1. Introduction

Varroosis is a bee disease caused by the mite *Varroa Jacobsoni* which endangers beekeeping all over the world. In order to prevent economic losses beekeepers treat their colonies with acaricides. The use of these acaricides inside beehives implies a risk of direct pollution of honey and other hive products, therefore maximum residue levels (MRLs) on honey has been fixed in many countries in order to protect consumers.

Traditionally, the analysis of acaricides is carried out by means of gas chromatography, employing electron-capture, nitrogen–phosphorus or mass detectors [1–13]. Liquid chromatography is less used [14–18], as far as we know there is only one multiresidue method for analysing acaricides (coumaphos, bromopropylate, 4,4′-dibromobenzophenone and fluvalinate) residues in honey by high-performance liquid chromatography (HPLC) using diode array detection (DAD) [15]. Sample clean-up for these methods is based on steam distillation [1], liquid–liquid extraction (LLE) [2,8–12,16–18], solid-phase extraction (SPE) [3–5,13–15], supercritical fluid extraction [7] or solid-phase microextraction [6]. Although SPE procedures have

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been widely used before, no systematic studies have been carried out, so the aim of this work was to study in more detail the SPE process of acaricides.

Tested acaricides were tau-fluvalinate, flumethrin, chlorfenvinphos, coumaphos, amitraz, chlordimeform, bromopropylate and cymiazole, as well as some of their degradation products. These substances cover a wide range of polarities, whereas the multiresidue SPE methods developed until the moment are only useful for more apolar acaricides (coumaphos, bromopropylate and fluvalinate [4,5,13,15]).

After selection of suitable chromatographic and detection conditions, most steps of the SPE procedure that affect the recovery, such as the sample pH and the composition of the elution and washing liquids were investigated, using aqueous standard samples. The selectivity of the procedure was checked in spiked honey samples and the method performance in real samples was tested in honey samples from different botanical and geographical origin.

2. Experimental

2.1. Reagents and chemicals

Amitraz and its degradation products [2,4-dimethylaniline (DMA), 2,4-dimethylphenylformamide (DMF) and *N*-(2,4-dimethylphenyl)-*N′*-methylformamidine (DPMF)] were kindly supplied by Schering Agrochemicals (Hauxton Cambride, UK) and chlorfenvinphos, bromopropylate and cymiazole by Ciba-Geigy (Basel, Switzerland). 4,4′-Dibromobenzophenone (BBP), tau-fluvalinate, deltamethrin, coumaphos and chlordimeform were from Cromlab (Barcelona, Spain) and flumethrin was from Bayer (Barcelona, Spain). Stock standard solutions of these compounds were prepared in acetonitrile at a concentration of 1000 μg/ml and were stored at 4°C in the dark.

Methanol, acetone, acetonitrile, tetrahydrofuran (THF), hexane, dichloromethane and toluene were of HPLC grade from Romil (Harvehill, UK). The water used in all experiments was purified on a Milli-Q system from Millipore (Bedford, MA, USA). Sodium acetate, acetic acid, phosphoric acid, sodium hydrogen and dihydrogenphosphates, sodium carbonate, sodium hydrogencarbonate, sodium hydroxide and triethylamine (TEA) were of analytical quality from Merck (Darmstadt, Germany). Buffers were: 0.05 *M* HCl (pH 1.36), 0.1 *M* H_3PO_4–NaH_2PO_4 (pH 2.0), 0.1 *M* HAc–NaAc (pH 4.0), 0.1 *M* NaH_2PO_4–Na_2HPO_4 (pH 6.0–8.0) and 0.1 *M* $NaHCO_3$–Na_2CO_3 (pH 10.0).

2.2. Materials and equipment

The SPE cartridges used were Bond Elut (Varian, Barcelona, Spain) octadecyl (C_{18}, 100 mg). Extraction was performed with ASPEC XL equipment from Gilson (Villiers le Bel, France). HPLC was used for the determination of the recovery of the acaricides eluted from the cartridges.

The HPLC–UV–Vis system consisted of two LKB (Barcelona, Spain) 5140 pumps, a high-pressure mixer LKB 2152-400, a HPLC controller LKB 2152, a Rheodyne (Cotati, CA, USA) 7125 sample injector with a fixed loop of 50 μl, and a Waters (Barcelona, Spain) 484 UV–Vis detector. A reserved-phase Nova-Pak C_{18} column (Waters) (150×3.9 mm I.D., 4 μm) was used together with a Waters Nova-Pack C_{18} pre-column (20 mm×3.9 mm I.D., 4 μm). The effluent was monitored at 210 nm. The system operated at room temperature and the flow-rate was 1 ml/min. Binary gradient conditions were used: first an isocratic step from 0 to 1.5 min with acetonitrile–0.01 *M* TEA (pH 6.1 with 0.75 *M* H_3PO_4) (30:70, v/v) and then a linear gradient was applied arriving at 100% acetonitrile in 15 min.

The HPLC–DAD system consisted of a Hewlett-Packard series 1100 system, equipped with a vacuum degasser, a quaternary pump, an autosampler and a DAD system, connected to HP ChemStation software. A Zorbax SB-C_{18} column (15 cm×4.6 mm I.D., 5 μm) was used together with a Waters Nova-Pack C_{18} pre-column (20 mm×3.9 mm I.D., 4 μm). The injection volume was 50 μl. The system operated at room temperature and the flow-rate was 1 ml/min. Due to polar interference peaks from honey, the linear gradient was smoothed for honey determination, arriving at 100% acetonitrile in 23 min instead of 15 min.

2.3. Extraction procedure

SPE studies in aqueous standard samples were performed as follows: the C_{18} SPE cartridge was activated with 2 ml of methanol and 1 ml of the tested aqueous buffer in each case; then, 1 ml of buffered sample with the buffer tested in each case of 0.5 μg/ml of the acaricides was passed through the cartridge; and the analytes were eluted with 1 ml of a convenient liquid.

For spiked honey samples a multifloral commercialized honey was heated at 40°C in a bath at least for 20 min and then allowed to stand at room temperature for 10 min. After that, 0.5 ml of a known concentration solution of the studied acaricides was added to 10 g of honey. The mixture was mechanically stirred with a blender in order to ensure homogenisation and let stand at room temperature until complete solvent evaporation.

SPE studies on spiked honey samples were carried out as follows: 0.5 g of spiked honey were dissolved in aqueous buffer (1 *M*, pH 6.0), brought up to 5 ml and sonicated during 5 min; 4 ml of this solution were passed through a C_{18} SPE cartridge, previously conditioned with 2 ml of methanol and with 1 ml of phosphate buffer (1 *M*, pH 6.0). The cartridge was washed with 1 ml of THF–aqueous buffer (1 *M*, pH 6.0) (10:90, v/v), passing 6 ml of air through it for drying, and then the acaricides were eluted with 1 ml of pure THF.

Evaporation of the extracts (1 ml) and reconstitution in low volumes of mobile phase (100–200 μl) was necessary in order to reach an adequate preconcentration of acaricides that allowed one to obtain limits of detection (LODs) low enough to satisfy the MRLs for these residues. Extracts from all studies were evaporated to dryness at 40°C under a nitrogen stream with the aid of a Zymark (Barcelona, Spain) Turbo Vap LV evaporator, reconstituted with 100 or 200 μl of mobile phase containing 5 μg/ml of an internal standard (deltamethrin).

3. Results and discussion

The studied acaricides include compounds from five families (Fig. 1): pyrethroids (tau-fluvalinate and flumethrin), organophosphorus (chlorfenvinphos and coumaphos), amidines (amitraz and chlordimeform), benzylates (bromopropylate) and tiazolydines (cymiazole), as well as some of their degradation products presents in honey: three main degradation products of amitraz (DMA, DMF and DPMF [24]) and a bromopropylate oxidation product (BBP).

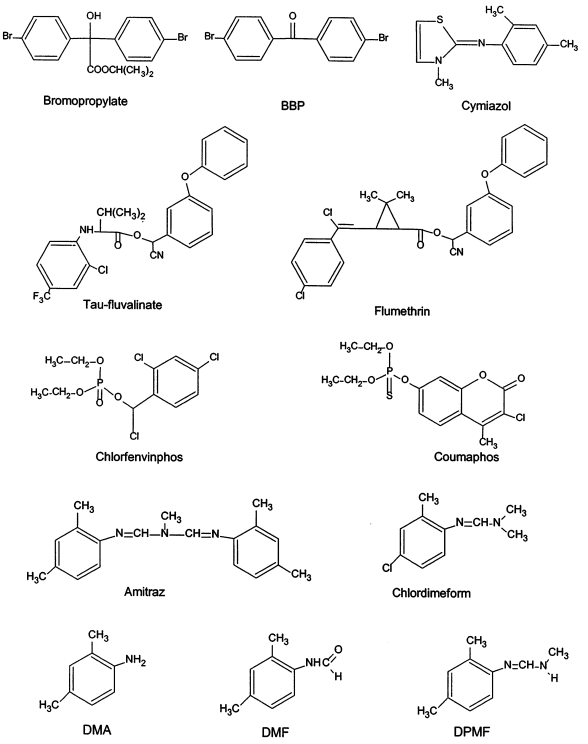

Fig. 1. Chemical structures of the studied analytes.

3.1. HPLC separation

Electrochemical and fluorimetric detection were first checked, but none of the tested acaricides (DMA, DMF, DPMF, chlorfimeform, cymiazole, coumaphos, BBP, bromopropylate, amitraz and fluvalinate) was electroreducible at the mercury electrode and only amitraz and cymiazole were oxidized at the glassy carbon electrode. Only DMA, DMF and coumaphos showed a little native fluorescence. Thus, for multiresidue purposes, spectrophotometric detection was used, first using 210 nm as a general wavelength and later more specific

values for each acaricide with the aid of a diode array detector.

Due to the wide range of polarities of the studied compounds (log $P_{\text{octanol/water}}$=0.11–5.5 [19,20]), their elution was performed under binary gradient conditions. Moreover, the effect of mobile phase pH was investigated (Fig. 2), showing that in order to improve the resolution between more polar analytes, pH values had to be higher than 6. The selected value was 6.1 since increasing pH values produced more drift in the baseline.

3.2. SPE studies in aqueous standard samples

Honey is a water-soluble matrix, therefore apolar C_{18} cartridges were selected for SPE. It is also the most commonly used by other authors. Recoveries were evaluated using aqueous standard samples of 0.5 μg/ml as a function of elution liquid, pH and concentration of aqueous buffer used to dissolve the sample.

Initially recoveries from aqueous standard samples buffered at pH 6 (0.1 *M*) were studied using elution solvents of different polarities. This pH value was selected since some of the compounds are unstable in acidic and basic media [21,22]. Different behaviours were observed depending on the compound polarity.

For the most polar compounds (Fig. 3A), recovery

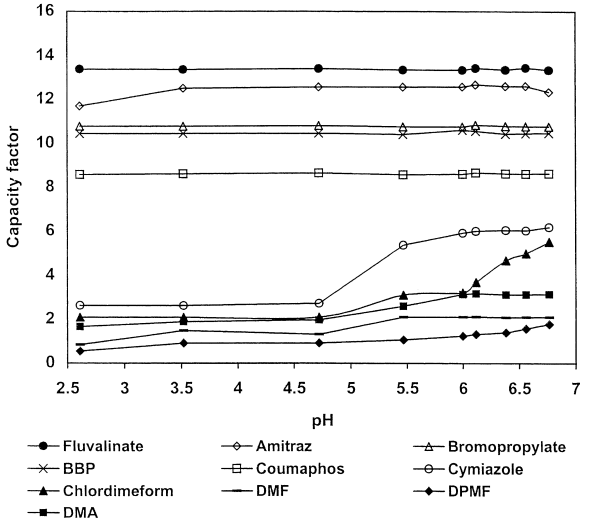

Fig. 2. Capacity factor of the acaricides as a function of the pH of the mobile phase.

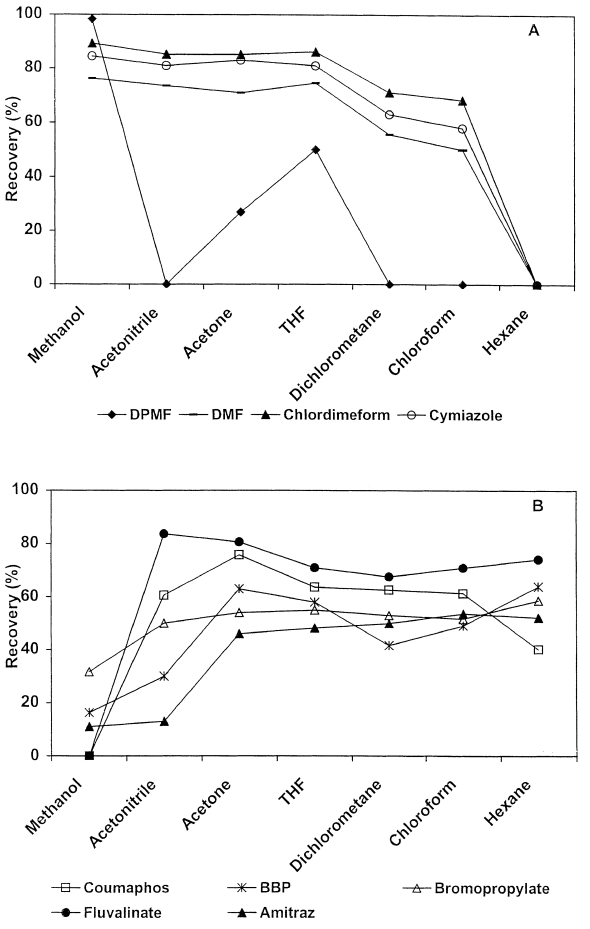

Fig. 3. Means values (n=3) of the recoveries corresponding to the SPE of acaricides from aqueous standard solutions as a function of the elution solvent.

decreased with decreasing solvent polarity, being zero with hexane (this eluent is widely used in the literature for the most apolar acaricides). The most polar analytes have amine groups, so they have probably ionic secondary interactions with the residual free silanols on the silica surface that hexane could not disrupt. In the case of DPMF these secondary interactions are so strong that also acetonitrile, and in some extract acetone, are unable to disrupt them. Otherwise, for the more apolar compounds (Fig. 3B) recoveries improve when solvent polarity decreases due to apolar interactions on modified silica surfaces. THF was selected as the elution solvent due to its ability to disrupt both types

of interactions, getting good recoveries for all the studied compounds.

The recovery of DMA was null with all tested solvents due to losses during the evaporation step of the extract after SPE even at room temperature. Thus, the developed method is not adequate for DMA which, fortunately, does not often occur in honey [23]. No losses of the others acaricides were found in this step even when evaporation was carried out at 40°C.

Ionic interactions between analytes and residual silanol groups can be avoided by the addition of a competitive cation such as ammonia or triethylamine to the organic eluent or alternatively, using a buffer of high ionic strength [24]. As many analytes studied are not stable in basic media, the use of ammonia or triethylamine cannot be a good choice. On the other hand, improvements of the recoveries of DPMF, DMF and cymiazole were observed when diluting the sample in a buffer with higher ionic strength (1 *M* instead of 0.1 *M*).

Behaviours of acaricides as a function of the pH to which the sample was buffered were very different (Fig. 4). The recoveries of DPMF, DMF, chlordimeform and cymiazol improved when pH increased being maximum at pH 6 and then decreasing slightly. This behaviour could be explained by the ionic interactions of these substances with the adsorbent that can be disrupted by pH changes. At very acid pH values the residual silanols are uncharged and interactions with protonated and charged analytes are weakened (especially for DPMF and cymiazol). In contrast at basic pH values the amine groups of the analytes are uncharged and ionic interactions with the unprotonated silanol are also more difficult (this is also the case of fluvalinate). Amitraz recovery improved when pH increased probably due to its rapid degradation in acidic medium. Something similar could happen with coumaphos that are unstable in basic media. No differences in bromopropylate and BBP recoveries were detected as a function of the pH.

As a compromise value, pH 6.0 was selected, taking into account that good recoveries were obtained for all compounds and their stability.

A suitable selection of washing and elution solvents will provide the cleanest samples in the SPE process, and therefore, a better selectivity. Fig. 5

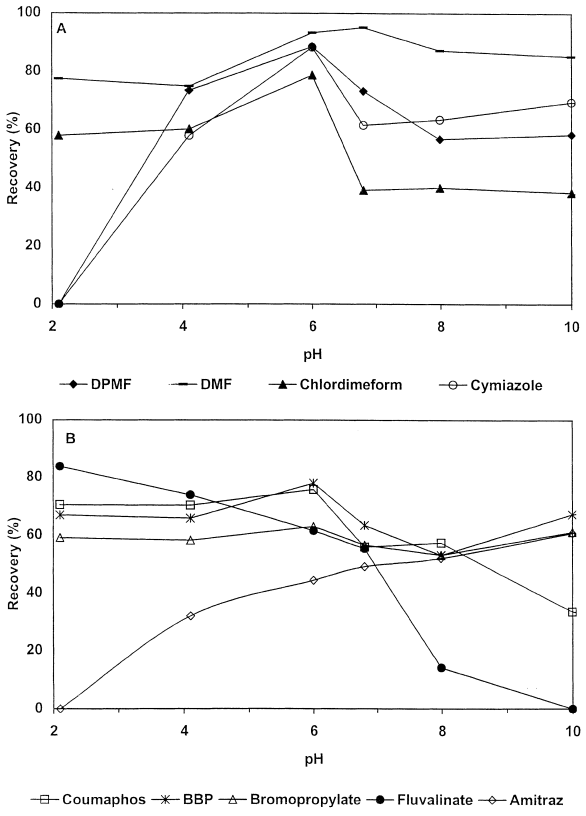

Fig. 4. Means values ($n=3$) of the recoveries from aqueous standard samples as a function of the pH of the buffer used to fix the sample pH.

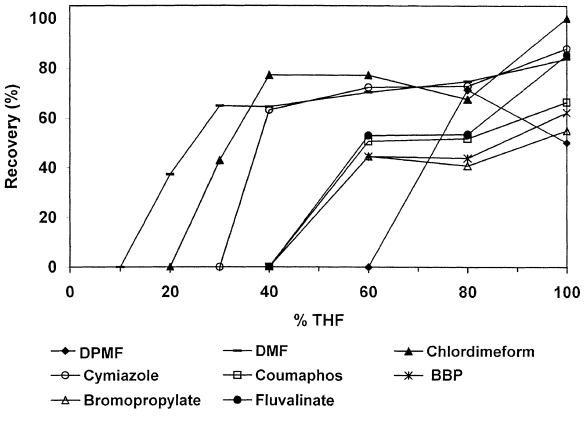

Fig. 5. Means values ($n=3$) of the recoveries of acaricides from aqueous standard samples versus the composition of the elution binary mixture (1 ml of THF–aqueous buffer).

shows the elution profiles for each acaricide obtained by testing recovery versus % THF in the elution liquid (a binary mixture of THF and aqueous buffer). Depending on the analyte polarity, its affinity for the solid phase is different. Whereas DMF starts to elute from a % THF in the eluent between 10 and 20%; coumaphos, BBP, bromopropylate, amitraz and fluvalinate elution is null until percentages greater than 40%. Thus, a suitable washing liquid that eliminates some interferences from the honey matrix but not elute the analytes, would not contain more than 10% of THF (1 ml of THF–buffer, pH 6.0, 10:90, v/v). For the complete elution of all analytes pure THF is necessary, since a lower percentage of THF in the elution solvent leads to losses in recoveries.

3.3. Validation of the method for the analysis of acaricide residues in honey

Some residue methods for the determination of amitraz in honey have proposed to analyse only the active ingredient amitraz, even though it is completely degraded in honey within 2 weeks. In a previous work [23] we concluded that the quick amitraz degradation in honey ($t_{1/2}$=12–52 h) takes place through a 1:1 stoichiometric reaction, producing 1 mol of DMF and 1 mol of DPMF for each mol of amitraz. Thus, a good estimation of the total residue can be estimated by only measuring DMF or DPMF. In the literature, there are no determination methods for these compounds in honey, however there is one for its determination in beeswax [25].

Two new acaricides (chlorfenvinphos and flumethrin) were added to the study at this stage and the SPE method optimised with aqueous standard samples was applied to spiked honey samples. The quantity of honey in diluted sample was studied to prevent a collapse of the solid phase by passing through the cartridge different quantities of honey spiked with 0.5 μg/g of acaricides. Recovery decreased with amounts higher than 0.75 g, so a maximum of 0.5 g was selected.

Method selectivity was tested analysing honey samples free from residues and spiked samples (Fig. 6). No interfering peaks were found for any acaricide, except for DPMF (t_R=5.9 min) for which a honey matrix peak co-eluted, so its determination is not possible. Thus, the quantitation of amitraz with the proposed method is only possible by analysing DMF.

Recoveries of the analytes from spiked honey at three different concentration levels, ranging from 0.015 to 0.1 μg/g, were examined (Table 1). The signals from acaricides for blank honey extracts spiked with analytes were different from those for standard solutions in pure solvent, showing the presence of a matrix effect; then recoveries were determined by using matrix matched standards. The mean absolute recoveries for the most polar compounds were higher than 80%, whereas the recoveries of apolar compounds ranged from 60 to 70%. The average precision of the analytical method at three concentrations levels, expressed by the coefficient of variation, was estimated by measuring the within-day repeatabilities at these three concentration levels, being in all cases lower than 8%.

Linearity was checked fortifying honey blank samples at six concentration levels from 0.007 to 0.5 μg/g. Linear ranges found for each analyte were: 0.08–0.5 μg/g for chlordimeform, 0.05–0.5 μg/g for cymiazole, 0.03–0.5 μg/g for chlorfenvinphos and 0.007–0.5 μg/g for coumaphos, BBP, bromopropylate, fluvalinate and flumethrin. The limits of detection (LODs) and quantitation (LOQs) were calculated as the concentration corresponding to the mean signal of honey blanks plus three times (for LOD) or 10 times (for LOQ) the standard deviations of the blanks (n=10).

The performance of the method for real samples was tested analysing 30 honeys of different botanical and geographical origin. These real honey samples were supplied by a local beekeepers association (Bizkaiko Erlezainen Elkartea, Bilbao, Spain) and a food research institute (Departamento de Mieles y Derivados del Laboratorio Agroalimentario de Extremadura, Caceres, Spain). In all the analysed real honey samples, no acaricide residues were detected, so the good quality of these honeys in relation to the presence of acaricide residues could be confirmed. The results obtained with these analyses led us to think that the proposed method is selective for the determination of all the acaricides studied except for DPMF, DMF and chlordimeform. In some honeys, peaks at the same retention time of these three analytes were observed, but in all cases their ab-

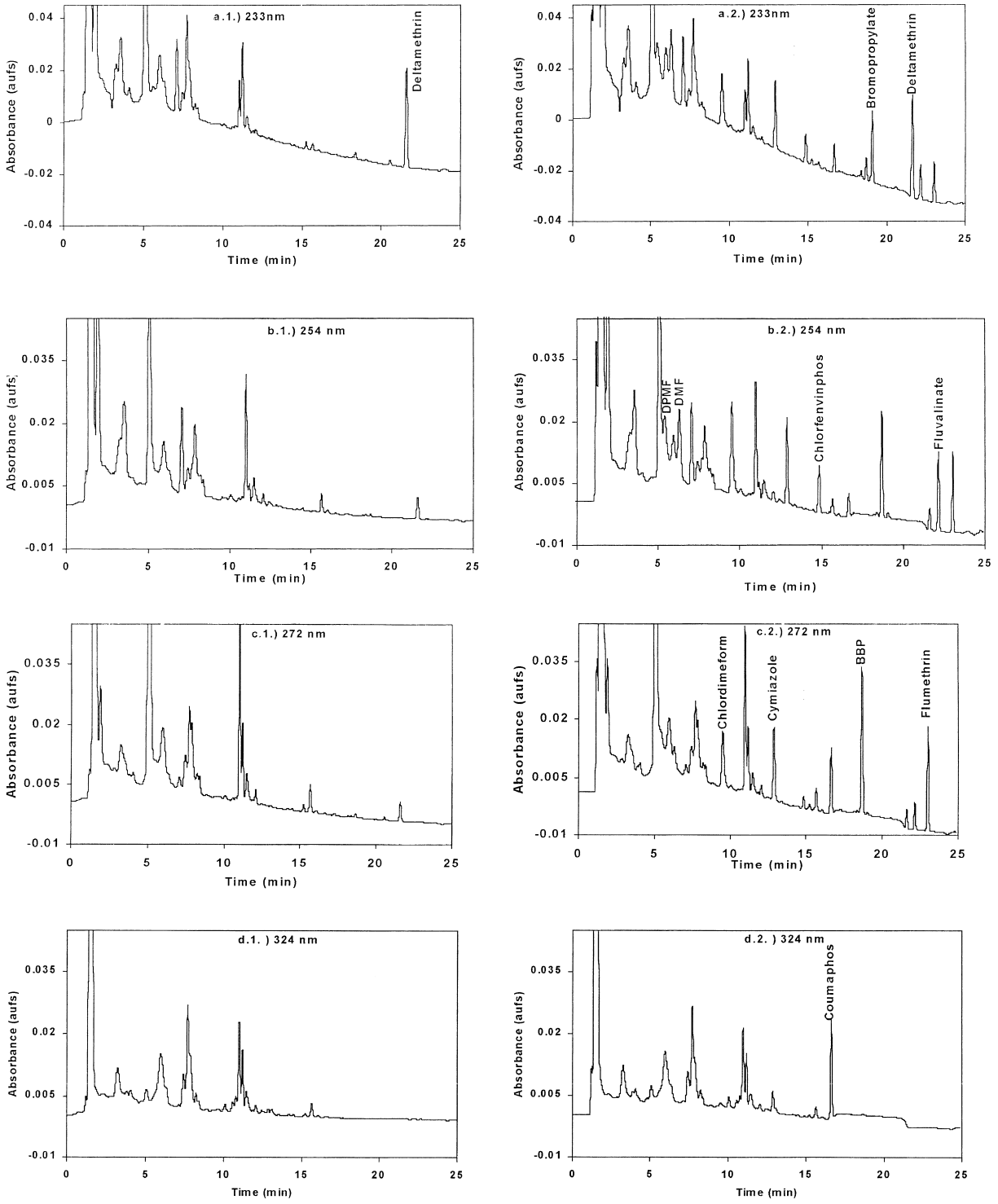

Fig. 6. Typical chromatograms obtained by coupling the semi-automated SPE preparation to HPLC–DAD using deltamethrin (5 μg/g) as an internal standard, for blank honey: (1) and honey spiked with 0.5 μg/g of each acaricide (2) at 233 nm (a), 254 nm (b), 272 nm (c) and 323 nm (d).

Table 1
Validation results: limits of detection (LODs) and quantitation (LOQs) (μg/g of honey), average of recoveries at three concentrations levels (0.015, 0.04 and 0.1 μg/g) and average of precision as RSD (%) for the different acaricide residues determination in honey ($n=3$ determinations)

Acaricide residue	LOD in honey (μg/g)	LOQ in honey (μg/g)	Amount added (μg/g)	Recovery (%)	Precision (RSD, %)
DMF	0.2	0.5	0.5	85±2	3.0
Chlordimeform	0.03	0.08	0.1	100±4	3.9
Cymiazole	0.02	0.05	0.04–0.1	83±3	3.2
Chlorfenvinphos	0.01	0.03	0.04–0.1	84±1	1.2
Coumaphos	0.001	0.003	0.015–0.1	76±6	7.6
BBP	0.003	0.008	0.015–0.1	63±3	5.5
Bromopropylate	0.002	0.005	0.015–0.1	69±5	7.9
Tau-fluvalinate	0.004	0.01	0.015–0.1	68±3	3.9
Flumethrin	0.002	0.005	0.015–0.1	71±3	5.0

sorbance spectra were different from those corresponding to these residues.

4. Conclusion

A multiresidue method for the detection of acaricide residues in honey was developed. Residues of DMF, chlordimeform, cymiazole, chlorfenvinphos, coumaphos, BBP, bromopropylate, fluvalinate and flumethrin can be detected, whereas this method is not suitable for DMA and DPMF. The proposed method involves diluting the honey in water, a clean up step with an SPE column and analysis by HPLC–DAD. This method provides the simultaneous determination of acaricide residues in honey with acceptable recoveries and repeatabilities and limits of detection low enough in order to satisfy European MRLs. In addition, DAD allows the confirmation of the identity of possible residues.

In comparison with multiresidue analytical procedures previously reported, the proposed method is able to detect a larger number of acaricides, being the limits of detection similar to the achieved obtained before. Most SPE methods previously reported are suitable for bromopropylate, coumaphos and fluvalinate, whereas the method described here can be also detect other more polar compounds also used in the hive.

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References

[1] M.B. Taccheo, M. De Paoli, C. Spessotto, Pestic. Sci. 23 (1988) 59.
[2] B. Neri, A. Ubaldi, D. Barchi, A. Di Llullo, R. Cozzani, Ind. Aliment. 31 (1992) 748.
[3] M. De Paoli, M. Taccheo, Pestic. Sci. 34 (1992) 61.
[4] M.A. Fernández, M.T. Sancho, J. Simal, J.M. Creus, J.F. Huidobro, J. Simal, J. Food Prot. 60 (1997) 78.
[5] S. Bogdanov, V. Kilchenmann, A. Indorf, J. Apic. Res. 37 (1998) 57.
[6] J.J. Jiménez, J.L. Bernal, M.J. del Nozal, M.T. Martín, A.L. Mayorga, J. Chromatogr. A 829 (1998) 269.
[7] J.J. Jiménez, J. Atienza, J.L. Bernal, J. High Resolut. Chromatogr. 18 (1995) 367.
[8] S.M. Waliszewski, V.T. Pardio, K.N. Walizewski, A. Ochoa, R. Infanzon, J. Sci. Food Agric. 77 (1998) 149.
[9] J.J. Jiménez, J.L. Bernal, J. Atienza, Chromatographia 42 (1996) 130.
[10] M.A. García, M.I. Fernández, C. Herrero, M.J. Melgar, Bull. Environ. Contam. Toxicol. 56 (1996) 881.
[11] D. Bachavora, P. Demirev, N. Mollova, Y. Blagoeva, Dolk. Bolg. Akad. Nauk. 41 (1988) 75.
[12] R. Cozzani, M. Di Pietrogiacomo, Ind. Aliment. 28 (1989) 608.
[13] M.A. Fernández, J. Simal, Analyst 118 (1993) 1519.
[14] M. Taccheo, M. De Paoli, C. Spessotto, Pestic. Sci. 25 (1989) 11.
[15] D. Blanco, A. Castaño, M. Megido, M.D. Gutiérrez, Chromatographia 39 (1994) 602.
[16] J.J. Berzas, M.C. Mahedero, J.A. Olibares, F. Salinas, Int. J. Environ. Anal. Chem. 43 (1991) 187.

[17] N.W.M. Van Buren, J. Mariën, H.H.W. Velthuis, R.C.H.M. Oudejans, Environ. Entomol. 21 (1992) 860.
[18] P. Cabras, M. Melis, L. Spanedda, J. AOAC Int. 76 (1993) 92.
[19] C. Tolim, The Pesticide Manual, The British Crop Protection and Royal Society of Chemistry, 1994.
[20] CCE, IPCS, International Chemical Safety Cards, ICSC: 0124 Chlordimeform, 1994.
[21] E. Corta, A. Bakkali, L.A. Berrueta, B. Gallo, F. Vicente, Talanta 48 (1999) 189.
[22] E. Corta, A. Bakkali, A. Barranco, L.A. Berrueta, B. Gallo, F. Vicente, S. Bogdanov, Talanta 52 (2000) 169.
[23] E. Korta, A. Bakkali, L.A. Berrueta, B. Gallo, F. Vicente, V. Kilchenmann, S. Bogdanov, J. Food Agric. Chem., in press.
[24] K.C. Van Horne, in: Sorbent Extraction Technology, Analytichem International Varian, Cambridge, 1990, p. 28;
K.C. Van Horne, in: Sorbent Extraction Technology, Analytichem International Varian, Cambridge, 1990, p. 65.
[25] E. Korta, A. Bakkali, L.A. Berrueta, B. Gallo, F. Vicente, S. Bogdanov, J. Chromatogr. A, submitted for publication.