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High-performance liquid chromatographic determination of benomyl and carbendazim residues in apiarian samples[1]

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Abstract

Simple procedures for the extraction and chromatographic determination of benomyl and carbendazim in honey, bees wax, larvae, bees and pollen are proposed. The fungicides were extracted from honey, larvae and bees using ethyl acetate, while methanol was more suitable for wax and pollen samples. Pollen extracts need a further clean-up step with *n*-hexane. The determination is carried out by reversed-phase high-performance liquid chromatography (HPLC) with fluorescence detection. The procedures have been applied to the analysis of benomyl on honey and larvae samples from hives whose bees were nourished with artificial food mixed with benomyl. © 1997 Elsevier Science B.V.

Keywords: Benomyl; Carbendazim; Pesticides

1. Introduction

Ascospheriosis is a serious disease caused by the mite *Ascosphera apis* that affects the larvae of the honey-bee (*Apis mellifera*), resulting in major mortality in the colony and important economic losses, not only to the apiarists but also to the surrounding farmers through decreasing pollination. No effective treatment against this parasitosis has so far been devised until now; however, the fungicides benomyl [methyl-1-(butylcarbamoyl) benzimidazol-2-yl carbamate] and carbendazim [methyl benzimidazol-2-yl carbamate] seem to be two of the most suitable chemicals to control the disease and, moreover, they have a low toxicity for the honey-bees [1].

Several methods can be found in the literature for the analysis of benomyl and carbendazim on vegetal samples, such as vegetables, fruits, oils [2–9], water and wine [10–12], and soils [13]. In all of them, benomyl is determined as carbendazim, its main breakdown product. The most frequently used procedures consist of an extraction with organic solvent, generally ethyl acetate, a clean-up by liquid–liquid partitioning, and subsequent determination by reversed-phase HPLC with UV or fluorescence detection. Recently, an extraction/clean-up procedure for vegetal samples has also been carried out by using cation-exchange cartridges [14].

Analysis procedures for benomyl as such have been reported to evaluate the purity of commercially available agricultural formulations containing ben-

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omyl [15,16]. To our knowledge, the analysis of benomyl and carbendazim on apiarian products has not been described till now.

In this work, simple analysis procedures have been developed for the determination of benomyl and carbendazim on apiarian samples, i.e. honey, bees wax, pollen, larvae and bees, which may contain residues after treating the beehive with the fungicides. Moreover, honey and larvae samples collected on benomyl-treated beehives have been analyzed in order to reveal the temporal evolution of this compound.

According to the procedures commonly used for their determination in vegetables, solvent extraction, with a minimum clean-up (if necessary), and determination by HPLC with fluorescence detection, have been adapted for the analysis of apiarian products in this work. Mass spectrometric detection has been also used to confirm the presence of the analyzed residues in some extracts.

2. Experimental

2.1. Reagents

Residue analysis and HPLC-grade methanol, ethyl acetate, acetonitrile and hexane were purchased from Panreac (Barcelona, Spain).

Hydrochloric acid was supplied by Panreac and ultrapure water was obtained with a Milli-Q plus apparatus from Millipore (Milford, MA, USA).

Benomyl and carbendazim certified standards were provided by Chemservice (West Chester, PA, USA). The formulation Benlate (50% benomyl) was obtained from DuPont (Wilmington, DE, USA).

2.2. Preparation of spiked samples

Fifty grams of honey, placed in a glass vessel, were heated in a water bath at 50°C for 15 min to reduce its viscosity. Then, the honey was allowed to cool at 25°C for 10 min and 1 ml of an aqueous suspension containing benomyl or carbendazim was added to the sample. Subsequently, the mixture was homogenized with a blender.

To analyze the wax samples, an amount of 50 g from a honeycomb was first cut into pieces and then ground in a universal food cutter. The sample was then spiked with 5 ml of an aqueous solution containing the analyte and homogenized by shaking.

As regards the pollen, larvae and bee samples, 10 g were ground in a glass mortar and spiked with 1 ml of the aqueous solution of the respective fungicides. Then, the slurry was homogenized by shaking.

Spiked samples were maintained at room temperature for 24 h to achieve a better accommodation of the fungicide to the matrix and were kept in glass containers at −35°C in darkness until analysis.

2.3. Extraction

For honey, larvae and bee extraction, 3 ml of 0.05 *M* HCl and 15 ml of ethyl acetate were added to 1 g of sample. The mixture was mechanically shaken for 15 min and the liquid phases were separated by centrifugation at 4000 *g* for 10 min. The organic phase was collected and the sample was further extracted with 15 ml of ethyl acetate, separated by centrifugation and combined with the previous one. Finally, 3 ml of 0.1 *M* NaOH and another 15 ml of ethyl acetate were added, shaking again for 15 min, centrifuging for 10 min and collecting the organic phase. The extracts dissolved in ethyl acetate were combined and evaporated under a gentle nitrogen stream at 40°C, and the residue was dissolved in 2 ml of methanol. Finally, the extract was filtered through a 0.50-μm pore size PTFE membrane prior to chromatographic analysis (see flow-chart in Fig. 1).

For the extraction of wax samples, 1 ml of 0.05 *M* HCl and 15 ml of methanol were added to 1 g of sample. The mixture was mechanically shaken for 15 min, and then the liquid phase was isolated from the wax residue by centrifugation. The residue was re-extracted with 15 ml of methanol, and the solvent collected again. Finally, 1 ml of 0.1 *M* NaOH and another 15 ml of methanol were added to repeat the extraction process. The organic phases were joined and then evaporated to dryness. Finally, the residue was dissolved in 2 ml of methanol and filtered.

The extraction procedure for pollen samples was similar to that for wax samples but including a clean-up step in the procedure. The dry residue was reconstituted in 2 ml of methanol and three liquid–

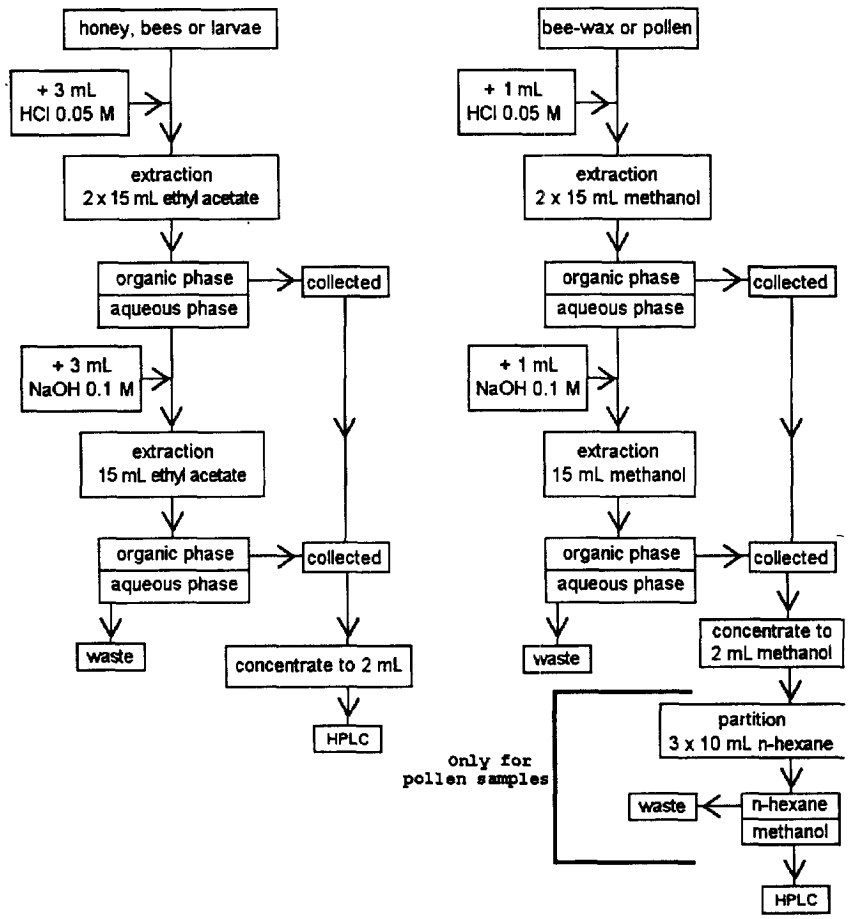

Fig. 1. Flow charts showing the extraction procedures used in the analysis of apiarian products.

liquid partitions were made with 10 ml of *n*-hexane to remove coextracted compounds.

2.4. HPLC determination: fluorescence detection

The chromatographic system was composed of a 510 HPLC pump, a 700 satellite WISP (automatic sampler), and a 470 fluorescence detector, all supplied by Waters (Milford, MA, USA). The chromatographic system was controlled by a Maxima workstation. The operating conditions were as follows: a 150×3.9 mm Novapak-ODS column from Waters; acetonitrile–water (40:60, v/v, pH 4, acidified with HCl) as mobile phase; flow-rate of 1 ml/min; injected volume 20 μl. The column was thermostated at 25°C. The excitation and emission wavelengths used to analyze carbendazim were 285 and 317 nm, respectively. Benomyl was hydrolyzed during the sample preparation steps and determined as carbendazim (retention time 3.9 min).

2.5. Confirmation by HPLC–PB-MS

An electron impact mass spectrometry analysis has been used to verify the identity of carbendazim in the extracts. The chromatographic system consisted of a 5989A MS engine mass spectrometer coupled to an HPLC equipment by a particle-beam interface. The HPLC equipment was composed of a degasser, autosampler and pump (series 1050), all from Hewlett-Packard (Avondale, PA, USA). The working conditions were as follows: a 250×2 mm Spherisorb

ODS-2 column from Sugelabor (Madrid, Spain); mobile phase, water–methanol (45:55 v/v); flow-rate, 0.3 ml/min; injected volume, 5 μl; chamber dissolution temperature, 60°C; helium pressure, 210 kPa; nebulizer adjusted for maximum sensitivity; ion source and quadrupole temperatures, 236 and 100°C, respectively. The carbendazim retention time was 4.1 min. Electron multiplier voltage was maintained at 400 voltage-units above autotune.

3. Results and discussion

3.1. Preliminary extraction assays

Initially, ethyl acetate was used to extract carbendazim from all the different types of samples, according to the procedures mentioned in Section 2.3, and providing acceptable quantitative results for the honey, larvae and bee matrices. However, the results were unsatisfactory on pollen and bees wax as a consequence of the appearance of an emulsion in the extraction tubes. This emulsion probably consisted of fatty compounds because of the sample nature. Centrifugation of the extract enabled the partial separation of the supernatant solvent but the recoveries were poor (lower than 60%). A treatment of the extract with various aliquots of 0.5 *M* NaOH partially broke down the emulsion, but it was insufficient to enhance the recovery and increased the operation time. Using a more polar extractant such as methanol helped to overcome this problem. Pollen analysis required a clean-up with *n*-hexane to decrease the excessive pigmentation of the extract and to reduce the high front observed in the chromatogram.

The experiences made on benomyl spiked apiarian samples revealed that the extraction with organic solvents, without acidification of the sample, did not provide good recoveries (below 80%) due likely to a relatively slow hydrolysis rate to carbendazim in the operation conditions. In fact, the successive injection of extracts containing benomyl denoted a gradual increase in the carbendazim concentration. To raise the hydrolysis-rate and solve this problem, the extractant was acidified with HCl, which also increased the solubility of carbendazim in the aqueous phase. Afterwards, and in order to avoid that carbendazim remained in this phase after the last extraction, NaOH was added to the extraction medium. The results of this procedure were quite good and are detailed in the following paragraphs.

3.2. Analytical procedures

Table 1 shows the recovery and precision ob-

Table 1
Recovery and precision obtained on honey and bees-wax samples after applying the proposed methods ($n=7$)

Sample amount (g)	Fortification (mg/kg)	Carbendazim		Benomyl	
		Recovery (%)	Precision (R.S.D., %)	Recovery (%)	Precision (R.S.D., %)
Honey					
1.0	1.0	97.4	4.1	97.0	4.3
1.0	3.0	99.5	4.2	96.2	4.1
5.0	1.0	97.0	4.0	96.3	4.0
5.0	3.0	95.2	4.2	95.2	3.9
10.0	1.0	94.3	4.3	91.3	4.1
10.0	3.0	93.9	4.7	92.3	3.9
Wax					
1.0	1.0	96.3	4.0	96.3	4.0
1.0	3.0	97.2	3.5	95.9	3.9
5.0	1.0	95.4	4.2	94.3	4.0
5.0	3.0	97.2	3.1	95.2	3.6
10.0	1.0	92.3	4.2	90.8	4.5
10.0	3.0	91.3	4.8	92.0	4.8

R.S.D., relative standard deviation.

tained in the application of the proposed procedures to the honey and wax samples spiked with benomyl and carbendazim. Recoveries were always about 95% or higher for sample amounts of 1 and 5 g, decreasing slightly (about 90%) for an amount of 10 g. The relative standard deviation (R.S.D.) was close to 4% ($n=7$) for the different spiking levels and sample amounts assayed.

To verify the validity of the procedures for the larvae, bees and pollen analysis, smaller sample amounts were used owing to their limited availability. In Table 2 it can be seen that benomyl and carbendazim recoveries were also high, and close to 95%, for 1 and 3 g of sample spiked with 1 and 3 mg/kg. The reproducibility of the results was also about 4% (R.S.D., $n=7$).

On preliminary experiments, very high concentrations of fungicides were found in the apiarian products from beehives treated with Benlate a few days before. In consequence, some laboratory experiences were also made with higher fortification levels, 10 and 50 mg/kg. As can be seen in Table 3, recoveries were above 90% for 1 g of sample and precisions were slightly better at these concentration levels. Fig. 2 shows the chromatograms obtained for honey and wax extracts. The chromatogram from the wax matrix exhibits, with regard to the honey chromatogram, a higher number of chromatographic peaks belonging to the coextracted compounds. The chromatograms for the other types of samples were essentially similar to those from honey.

A standard-addition method was applied on some extracts in order to check the quantitative results achieved by the routine multilevel calibration. Therefore, the dry residue from an extract was dissolved in 5 ml of methanol and spiked successively with 5, 10, 15 and 20 μl of a solution containing 400 mg/l of carbendazim, keeping the final volume constant. The concentration measured by the standard-addition method was 0.95 mg/l, while the conventional calibration provided a value of 1.0 mg/l ($n=5$).

The calibration graphs for the quantitative analysis of carbendazim by fluorescence detection were made in the 0.25–15 mg/l range, always obtaining correlation coefficients (r^2) equal to or bigger than 0.990. The detection limits calculated by spiking extracts of non-treated samples with carbendazim standards, and considering a signal-to-noise ratio of 2, ranged between 0.01 and 0.05 mg/l. Consequently, the limits of the proposed procedures are close to 0.05 mg/kg for a sample amount of 1 g.

The presence of carbendazim in the extracts of samples obtained from treated beehives was confirmed by HPLC–PB-MS in SIM mode. For this

Table 2
Recovery and precision obtained on larvae, bees and pollen samples after applying the proposed methods ($n=7$)

Sample amount (g)	Fortification (mg/kg)	Carbendazim		Benomyl	
		Recovery (%)	Precision (R.S.D., %)	Recovery (%)	Precision (R.S.D., %)
Larvae					
1.0	1.0	97.4	4.0	96.4	4.3
1.0	3.0	98.3	3.7	95.3	4.4
3.0	1.0	98.9	3.6	97.4	3.9
3.0	3.0	96.3	3.5	95.0	4.2
Bees					
1.0	1.0	95.3	3.9	95.2	3.7
1.0	3.0	97.3	4.1	95.9	4.5
3.0	1.0	94.5	4.0	93.7	4.8
3.0	3.0	96.2	3.6	94.2	4.3
Pollen					
1.0	1.0	93.4	3.8	93.9	3.9
1.0	3.0	95.3	3.8	94.3	4.4
3.0	1.0	94.9	4.3	95.2	4.5
3.0	3.0	93.8	4.0	94.7	4.1

R.S.D., relative standard deviation.

Table 3
Recovery and precision obtained on the apiarian products spiked at high concentrations after applying the proposed methods ($n=7$)

Sample amount (g)	Fortification (mg/kg)	Carbendazim		Benomyl	
		Recovery (%)	Precision (R.S.D., %)	Recovery (%)	Precision (R.S.D., %)
Honey					
1.0	10.0	93.1	3.8	92.5	3.8
1.0	50.0	91.3	3.6	91.8	3.7
Wax					
1.0	10.0	92.3	3.5	93.4	3.7
1.0	50.0	90.9	3.7	91.5	3.6
Larvae					
1.0	10.0	94.3	3.6	93.5	3.7
1.0	50.0	92.3	3.5	92.2	3.7
Bees					
1.0	10.0	92.0	3.7	92.7	3.7
1.0	50.0	90.6	3.4	90.7	3.8
Pollen					
1.0	10.0	91.5	3.8	92.2	3.6
1.0	50.0	90.3	3.9	91.6	4.0

R.S.D., relative standard deviation.

purpose, the ions m/z 191 (molecular ion), 159 (base peak) and 105 were monitored (Fig. 3).

3.3. Application to beehive samples

Beehives infected with *Ascosphera apis* were treated with the commercial formulation Benlate, which was given to the beehives with artificial food. Two types of food, liquid and semi-solid, were tested. The first one consisted of an homogeneous preparation containing 50 ml of water, 50 g of honey and 20 g of Benlate, while the semi-solid one was composed of 375 g of sugar, 375 g of honey, 30 ml of water and 20 g of Benlate. Both preparations were delivered to the beehives every 7 days. After giving the artificial food to the beehives, the larvae samples were collected every 2 days, rinsed with water to remove honey and bees-wax residues and frozen until their analysis. Honey samples were also collected.

The carbendazim concentration on 5-day-old larvae after treatment can be seen in Fig. 4. It can be seen that the liquid food provided higher concentrations of fungicide in the larvae. Fig. 5 shows the carbendazim concentration found in 5-day-old larvae for different doses of Benlate added to the liquid food; as expected, increasing the dose of fungicide causes a higher carbendazim concentration in the larvae. The values for carbendazim residues found in the honey collected during the treatment were of 7.3, 13.4 and 17.5 mg/kg according to the dose of fungicide incorporated into the food.

4. Conclusions

Ethyl acetate is shown to be a suitable extractant to analyze benomyl and carbendazim residues in honey, larvae and bee samples, while for bees wax and pollen samples the use of methanol is more appropriate. The recoveries obtained on samples spiked at several concentration levels were higher than 90% and the R.S.D. lower than 5% in all cases.

As deduced from the treatment performed with artificial food containing benomyl, it can be stated that the fungicide concentration in larvae decreases sharply after application, and that there is not an appreciated accumulation when the treatment is repeated.

Fungicide application as a liquid food gives higher levels of the fungicide in the larvae than when given as solid food. To maintain high levels of the fun-

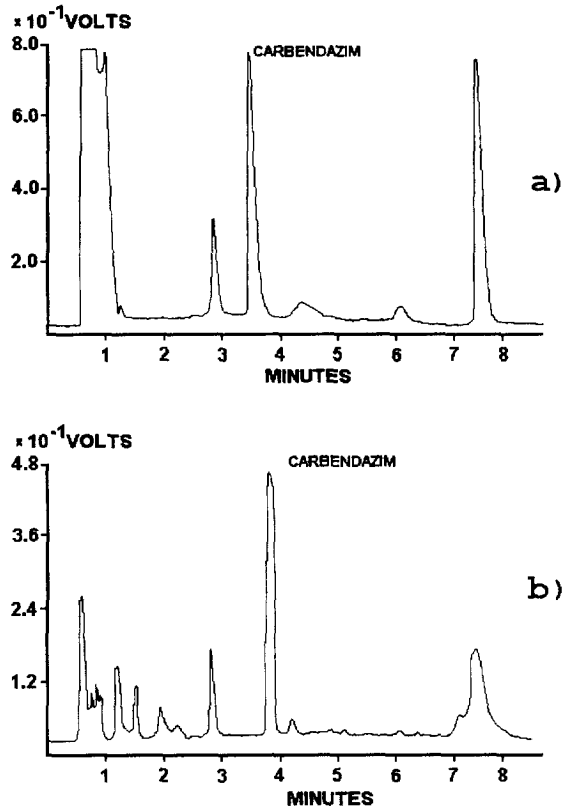

Fig. 2. Chromatograms obtained by the proposed procedures. (a) Honey extract; (b) bees-wax extract.

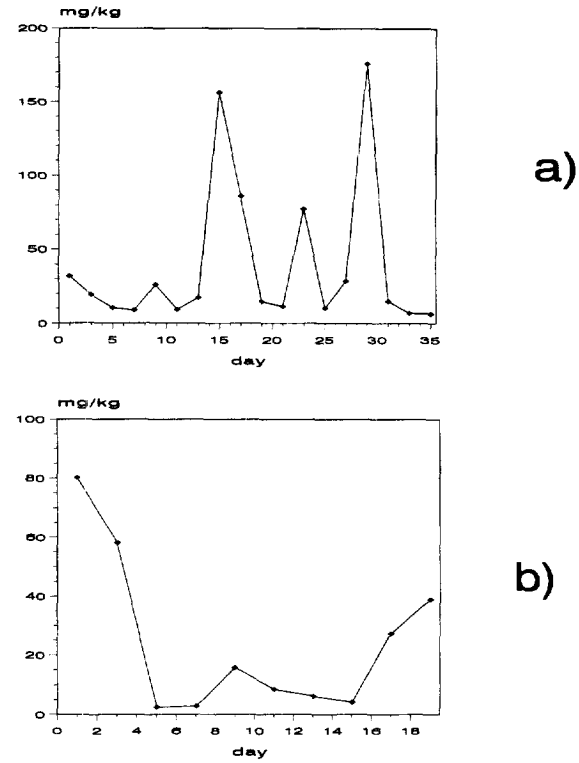

Fig. 4. Concentration of carbendazim in larvae after treating the beehives with two different kinds of food. (a) Liquid food; (b) semi-solid food.

gicide in the larvae it is convenient to repeat the treatment at least every 7 days.

Attention must be paid to the dosage of the commercial product because carbendazim residues are found in the honey.

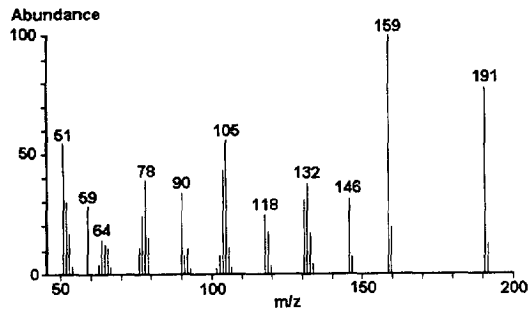

Fig. 3. Electron impact mass spectrum for carbendazim.

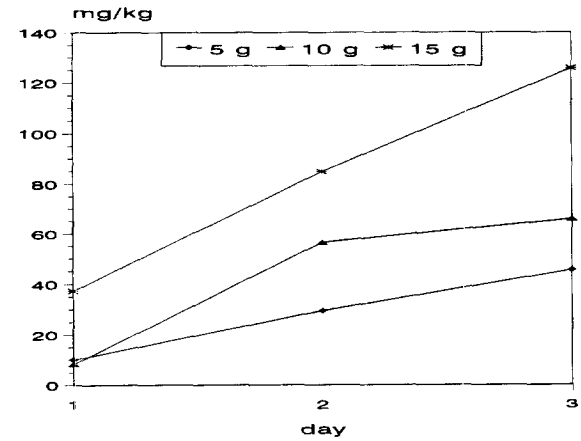

Fig. 5. Concentration of carbendazim in larvae for 3 days, after giving liquid food containing different doses of Benlate to the beehives.

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