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# Determination of rotenone residues in raw honey by solid-phase extraction and high-performance liquid chromatography

J.J. Jiménez[a,*], J.L. Bernal[a], M.J. del Nozal[a], M. Novo[a], M. Higes[b], J. Llorente[b]

[a]*Department of Analytical Chemistry, Faculty of Sciences, University of Valladolid, Prado de la Magdalena s/n, E-47005 Valladolid, Spain*

[b]*Regional Apiculture Centre, SIA Junta de Comunidades de Castilla La Mancha, Marchamalo, E-19180 Guadalajara, Spain*

**Abstract**

A method for determining residues of the insecticide rotenone in raw-honey by high-performance liquid chromatography (HPLC) is described. To extract the residues, organic solvents such as ethyl acetate, *n*-hexane/dichloromethane and solid-phase extraction with octadecylsilane cartridges or Florisil packed columns were tested. Determination was carried out by reversed-phase HPLC using acetonitrile–buffer phosphate (pH 7) (60:40, v/v) as mobile phase and detection at 210 nm. Although the data showed that the two extraction methods were able to isolate the pesticide residues, the extraction on octadecylsilane cartridges was preferred due to its simplicity and higher recovery. Recoveries depended strongly on the fortification level for the two extraction procedures. Practical determination limits of 0.015 mg/kg were obtained. In the analysis of honeys, from beehives treated with rotenone at therapeutical doses for 1 month, residual amounts below 0.2 mg/kg were found. 

*Keywords:* Honey; Food analysis; Extraction methods; Rotenone; Pesticides

## 1. Introduction

Rotenone is an insecticide of botanical origin that seems to have good efficacy, in field studies, against *Varroa Jacobsoni* mite that affects the colonies of honey bees, because of that its application in a big scale is being considered, which implies that it is necessary to know the adequate dosage and also if, after the beehive treatment, residues of the insecticide can appear in honey. To select the doses field studies must be done, spraying different quantities of the insecticide inside the beehive, testing the death rate of the mites and collecting raw honey to check that residues are below a certain level, for that it is necessary to have a reliable method to evaluate the residues.

This insecticide is usually determined in waters due to its toxicity for fish [1–3]; to extract the residues solid-phase extraction with ODS cartridges [1,4], liquid–liquid extraction with *n*-hexane–dichloromethane [3] or even direct injection [2] have been proposed. The determination is usually made by high-performance liquid chromatography (HPLC) with UV detection and using mixtures of water–acetonitrile, with different pH values as mobile phase [2,5–7].

For pesticide residue analysis in honey several methods have been proposed, previously it is necessary to extract the analytes from the matrix, which is more complex when raw-honey is treated. To isolate the compounds liquid–liquid extraction with ethyl acetate or solvent mixtures [8–12] has been proposed although solid-phase extraction using ODS [10,13] or Florisil [14] cartridges has been also recom-

*Corresponding author.

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mended.

Taking into account the above considerations, in this work several ways of extraction of rotenone residues on raw-honey samples have been tested with the aim of finding one that leads to acceptable recoveries with less disturbances. The determination is carried out by reversed-phase HPLC with UV detection modifying the parameters to achieve a good separation between the front and the rotenone peak. The selected procedure is applied to analyze rotenone residues in samples directly collected from beehives treated with rotenone for 1 month.

## 2. Experimental

### *2.1. Reagents*

HPLC-grade methanol and acetonitrile, and residue analysis grade *n*-hexane, ethyl acetate, dichloromethane and acetone were provided by Lab-Scan (Dublin, Ireland). Florisil of 60–100 mesh was purchased from Baker (Deventer, Netherlands), and octadecylsilane (500 mg) cartridges from Waters (Milford, MA, USA). Distilled water was further purified by passing it through a Milli-Q apparatus (Millipore, Bedford, MA, USA). Sodium dihydrogenphosphate, disodium hydrogenphosphate, sodium chloride and hydrochloric acid of analytical grade were purchased form Panreac (Barcelona, Spain).

Rotenone certified standards were obtained from Promochem (Wessel, Germany). Stock solutions and dilutions were made in methanol.

### *2.2. Preparation of raw-honey samples*

Raw-honey samples, that usually contain extraneous matter such as bees and bee-wax, were centrifuged at 4000 *g* for 15 min to separate the honey. The honey portion was then filtered through a glass plate.

Honey samples were spiked just before analysis. For this purpose, a rotenone solution in methanol (0.1 ml) was added to the corresponding honey–water mixtures (see sections below).

Some experiments were also repeated on commercial honey, free of extraneous matter, to compare some results.

### *2.3. Extraction of rotenone with organic solvents*

A sample amount of 2 g was placed in a threaded glass tube and mixed with 15 ml of water and 2×15 ml of *n*-hexane–dichloromethane (50:50, v/v). The sample was extracted by sonication for 20 min with a sonicator from Selecta (Barcelona, Spain) operated at 43 kHz, and then, the organic phase was separated by centrifugation at 2500 *g* by 10 min and collected. The two organic portions were combined and the solvent was evaporated in a rotary evaporator (Büchi, Plawil, Switzerland) under vacuum at 30°C. The dry residue was dissolved in 3 ml of methanol and filtered through a 0.50 μm pore-size PTFE membrane prior to chromatographic analysis.

### *2.4. Extraction of rotenone by octadecylsilane cartridges*

A honey amount of 2 g mixed with 25 ml of water was passed through the cartridge. Cartridges were conditioned by successive elution of 10 ml of methanol, and 10 ml of water, by a gentle evacuation with the aid of a pump. Then, the sample was eluted at a flow-rate of about 10 ml/min. The cartridge was never allowed to dry during these steps. After the sample has passed through, water was removed from the cartridge by pulling nitrogen through it for ca. 30 min. Then, the pump was disconnected and 3 ml of methanol was poured into the cartridge. The stationary phase was left to be soaked with the methanol for 2 min, and afterwards it was slowly eluted and collected for its injection in HPLC.

### *2.5. HPLC system*

The chromatographic system consisted of a ConstaMetric 3200 liquid chromatography pump from LDC Analytical (Riviera Beach, FL, USA) equipped with a Rheodyne model 7125 injector, and a HP1050 UV detector from Hewlett-Packard (Willmington, DE, USA). Data were collected and integrated by a Cl-4100 Milton-Roy (Dublin, Ireland) integrator. The operating conditions were as follows: a 250×4.6 mm I.D. Hypersil column from Shandon (Cheshire, UK), acetonitrile–buffer phosphate (pH 7) (60:40, v/v) as mobile phase at a flow-rate of 1 ml/min,

injection volume 20 μl. Detection was performed at 210 nm.

## 3. Results and discussion

### *3.1. Mobile phase composition*

When the extract was injected in the mobile phase currently used for rotenone residue analysis in water samples (acetonitrile–water, 70:30, at pH 7) it was not possible to separate the front due to the matrix from the rotenone peak. Decreasing the acetonitrile percentage to 60%, at the same pH value, the rotenone peak appeared later (the retention time changed from 5.1 to 10.5 min) but the coextracted substances from the matrix still made the rotenone determination difficult. If hydrochloric acid is added to get lower pH, near pH 3, the rotenone peak was retained for more time (till 12.3 min) but the interfering peaks still disturbed. The best results were achieved keeping pH 7 but using buffer phosphate, then the matrix front does not interfere with the rotenone peak, and the results are more reproducible.

To verify the selectivity of the extraction-determination procedures, extractions of blank honey samples were made and then injected in the HPLC system. Fig. 1 shows the chromatograms obtained for the solvent extraction with *n*-hexane–dichlorome-

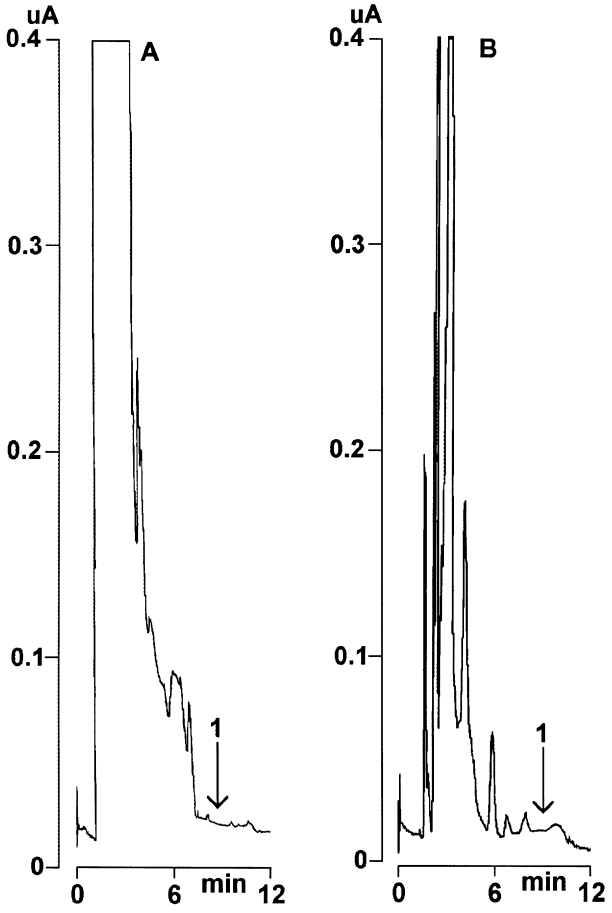


Fig. 1. Chromatograms obtained from extracts of honey without rotenone. (a) Solid-phase extraction on ODS, (b) extraction with *n*-hexane–dichloromethane (50:50). Peak 1=Retention time for rotenone.

thane (50:50) and the solid-phase extraction on ODS cartridges. As it can be seen there are not interfering peaks eluting at the same retention time as rotenone.

Under the best conditions, the inter-day reproducibility for the peak area of an standard of 0.1 mg/l in terms of relative standard deviation was 5.9% ($n=7$). Similar values were obtained when honey extracts were injected.

### 3.2. Stability of solutions

It could be observed that the degradation of the rotenone dissolved in methanol and stored at 4°C in darkness was related to its concentration. Two months after their preparation the initial concentrations for the standards of 0.1; 1; 10 and 100 mg/l fell nearly 50%, 30%,10% and 1% respectively, because of that the stock solutions must not be used for periods higher than 1 month and the working solutions must be prepared weekly.

### 3.3. Liquid–liquid extraction

Ethyl acetate, *n*-hexane–dichloromethane (50:50, v/v), *n*-hexane–acetone (50:50 and 80:20, v/v) were assayed to extract rotenone from the samples. The best results were obtained for ethyl acetate and *n*-hexane–dichloromethane, which are shown in Table 1. The recoveries obtained with *n*-hexane–acetone mixtures were always lower than those ones obtained with ethyl acetate or *n*-hexane–dichloromethane. The recoveries achieved with *n*-hexane–acetone were lower than 45% for concentrations below 5 mg/kg.

It could be also observed that the recoveries were lower for spiking concentrations of 0.1, 1 and 5 mg/l. Usually the precision was higher when higher fortification levels were used.

We think that those lower recoveries obtained for the 0.1, 1 and 5 mg/l fortification levels can be related to the major influence of the extraneous matter in the filtered raw honey because when a commercial honey sample was spiked with the same quantities of rotenone the recoveries increased from about 60% to 80%.

These recoveries were achieved after two extractions with organic solvent. If a raw-honey sample spiked with 1 mg/kg of rotenone was extracted only once with the organic solvents the recoveries decreased to about 44% and 50% for ethyl acetate and *n*-hexane–dichloromethane, respectively. When three extractions were carried out the recoveries obtained were 66% and 64% respectively ($n=7$).

Table 1
Recoveries obtained in the solvent extraction of rotenone on honey samples spiked with different concentrations ($n=7$)

| Concentration (mg/kg) | Mean recovery (%) | Precision RSD (%) |
|---|---|---|
| *n*-Hexane–dichloromethane (50:50, v/v) | | |
| 0.1 | 61 | 6.3 |
| 1 | 59 | 5.2 |
| 5 | 74 | 6.1 |
| 10 | 91 | 5.0 |
| 25 | 95 | 6.1 |
| 50 | 102 | 3.8 |
| Ethyl acetate | | |
| 0.1 | 59 | 8.0 |
| 1 | 64 | 8.3 |
| 5 | 70 | 7.0 |
| 10 | 91 | 5.8 |
| 25 | 97 | 3.7 |
| 50 | 102 | 3.8 |

The addition of NaCl (0.5–1 g) to enhance the recovery does not give better results because the NaCl increased enormously the presence of co-extracted substances whose peaks overlap the rotenone signal giving anomalous results (recoveries higher than 400% and RSDs near 90%).

In Fig. 2 some chromatograms are shown. As it can be appreciated ethyl acetate gave dirtier chromatograms than those obtained using other solvents, it can be also observed the effect of the NaCl addition.

On the other hand, the peak width and shape of rotenone in Fig. 2a, b and c are different as a consequence of the co-extraction of carbohydrates which were built-up in the chromatographic system, mainly in the injection loop and the chromatographic column. This implied a continuous increase of the pressure and a drop of the efficiency of the column, which explains the different chromatographic peaks. So it is advisable to rinse the column with water at the end of every working day.

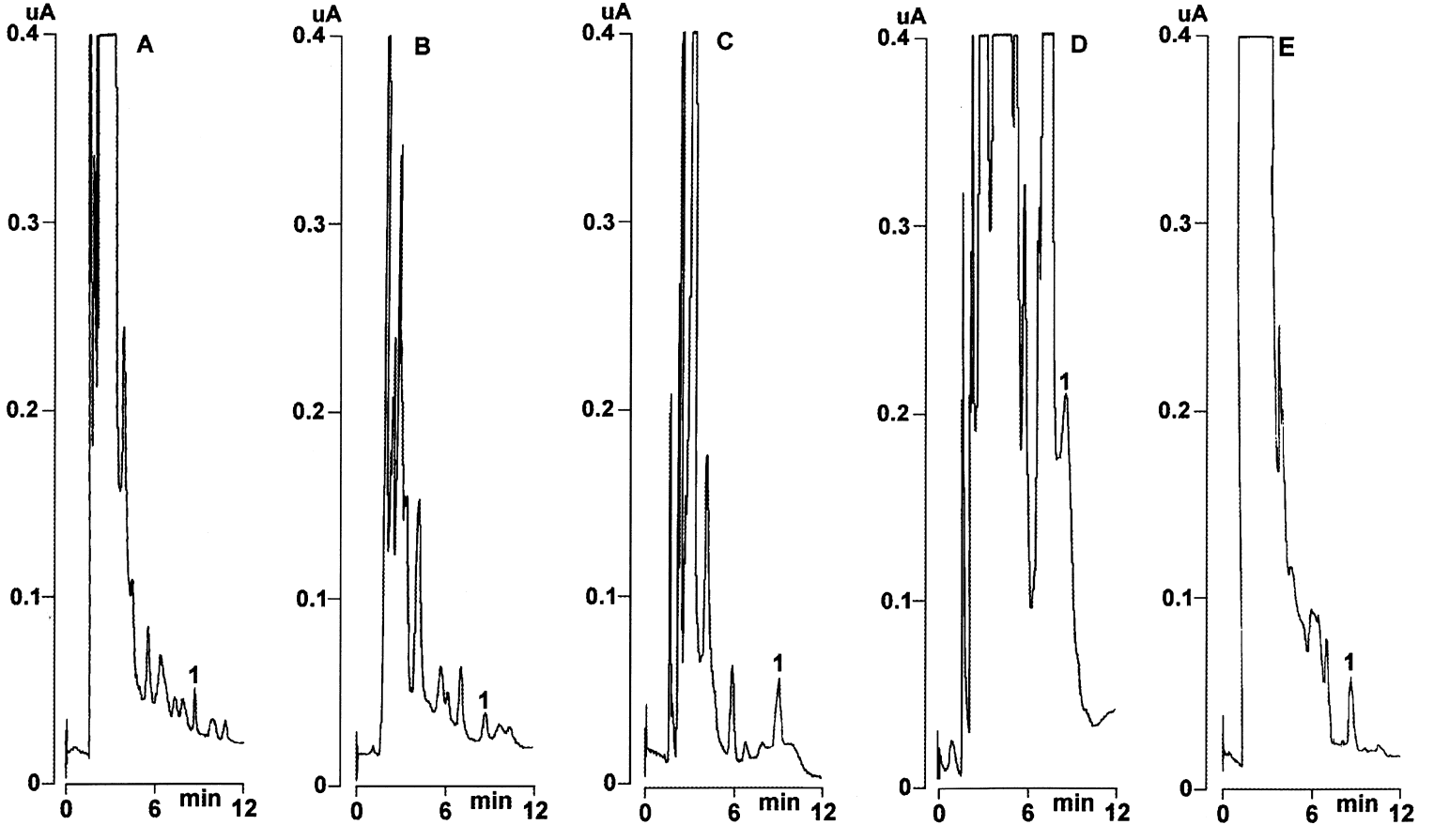


Fig. 2. Chromatograms obtained applying liquid–liquid and solid-phase extraction to honeys containing 1 mg/kg of rotenone (attenuation 256): (a) ethyl acetate, (b) *n*-hexane–acetone (50:50), (c) *n*-hexane–dichloromethane (50:50), (d) *n*-hexane–dichloromethane (50:50)+NaCl, (e) solid-phase extraction on octadecylsilane cartridges. Peak 1=Rotenone.

Table 2
Recoveries and precision obtained in the solid-phase extraction on ODS cartridges for honey samples spiked with different concentrations ($n=7$)

| Concentration (mg/kg) | Mean recovery (%) | Precision RSD (%) |
|---|---|---|
| 0.1 | 80 | 6.9 |
| 1 | 78 | 6.0 |
| 5 | 83 | 6.5 |
| 10 | 90 | 3.3 |
| 25 | 86 | 6.2 |
| 50 | 98 | 5.2 |

### *3.4. Solid-phase extraction*

Firstly, a solid-phase extraction on Florisil was assayed. The recoveries obtained were not satisfactory because they increased gradually from 15% to 98% when the amount of the added standard increased from 0.1 to 50 mg/kg, which can be attributed to a strong interaction between rotenone and the sorbent. Moreover, the precision was very bad with RSDs ranging from 100 to 4%, respectively.

The results obtained applying solid-phase extraction on ODS columns are shown in Table 2. As can be observed the recovery on ODS cartridges was satisfactory for concentrations higher than 10 mg/kg, obtaining lower percentages when the concentration of rotenone in the sample decreases. If commercial honey samples are spiked and submitted to the solid-phase extraction procedure the recoveries are higher (about 90% for concentrations of 0.1, 1 and 5 mg/kg), the same as in the solvent extraction procedure. A chromatogram is shown in Fig. 2e.

### *3.5. Analytical characteristics*

At 210 nm, the calibration range was linear from 0.1 mg/l to 30 mg/l ($r^2=0.999$, at least). To measure lower concentrations, there was also an acceptable linearity between 0.02 and 2 mg/l ($r^2=0.98$, at least). The estimated detection limits for extracts obtained from a honey sample of 2 g and a final volume of 3 ml were always lower than 0.01 mg/l. This concentration gave a signal/noise ratio at least of 15, whereas that one for the standard directly injected was near 40. That means that for the samples the limit of detection is near 0.015 mg/kg.

As rotenone spectrum has another maximum at 294 nm, if this wavelength is used to measure the eluting peaks the linearity range is now 1–30 mg/l and the detection limit for the extracts is near 0.1 mg/l, ten times higher as a consequence of the lower molar absorptivity at 294 nm.

The chromatogram profile at 294 nm is similar to that obtained at 210 nm, obviously the height peak was reduced notably, nevertheless the baseline stability seems to be better, which can be advantageous in determining high quantities of insecticide.

### *3.6. Application to samples from treated beehives*

An aqueous suspension (10 g/l) of the commercial product Agri3000, which contains 3% rotenone, was weekly sprayed into two beehives for 1 month. The honey yielded along the month was collected and analyzed.

In one of those beehives the average concentration found was 0.12 mg/kg ($n=4$), while in the other beehive the concentration was 0.10 mg/kg.

## 4. Conclusions

Solvent extraction with *n*-hexane–dichloromethane (50:50, v/v) or solid-phase extraction on ODS cartridges are the most adequate alternatives to extract rotenone residues from raw honey. Nevertheless, this later is more useful when a great number of samples must be analyzed.

In the chromatograms a great front of coextracted substances always appears, a mobile phase of acetonitrile–phosphate buffer at pH 7 (60:40) allows adequate quantification of the rotenone.

In honey samples from beehives treated with therapeutical doses of rotenone the residues are always below 0.2 mg/kg.

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