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Determination of acaricides in honey by high-performance liquid chromatography with photodiode array detection

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Abstract

Rapid analytical methods are described to control quality of honeys, concerning residues of acaricides applied in hives to prevent *Varroa jacobsoni* infestation. A liquid–liquid extraction with hexane–propanol-2–ammonia (60 ml:30 ml:0.28%) was used for the simultaneous analysis of coumaphos, bromopropylate, amitraz and fluvalinate. For thymol, one clean up on a solid-phase extraction C_{18} (500 mg, 6 ml) column was performed; for rotenone, a liquid extraction with dichloromethane was realised. Quantitative recoveries obtained with honey were satisfactory and were superior to 80%. All acaricides are identified by reversed-phase high-performance liquid chromatography with photodiode array detection. Quantification limits obtained were below maximal residue limits when these exist.

Keywords: Honey; Food analysis; Pesticides; Coumaphos; Bromopropylate; Amitraz; Fluvalinate; Thymol; Rotenone

1. Introduction

With the introduction by beekeepers of the European bee *Apis mellifera* into Indonesia, the area where *Apis cerana* was endemic, the parasite *Varroa jacobsoni* adapted to this new host was disseminated by contact throughout a great deal of countries including the European Union. The effects of this mite on *Apis mellifera*, used for its high honey production capacity are quite different from those on *Apis cerana*. *Apis mellifera* is very susceptible to the parasitic mite *Varroa jacobsoni* and this external parasite affects bees at all stages of development. The transportation of infested live bees by beekeepers has led to the sudden and widespread diffusion of this mite. At the present time no country has eradicated it or has succeeded in restricting its spread and varroa disease is one of the most serious diseases of honeybees.

Acaricides are widely used to control *Varroa jacobsoni* in hives. Acaricides such as coumaphos (*O*-3-chloro-4-methyl-2-oxo-2*H*-chromen-7-yl *O*,*O*-diethylphosphorothioate), bromopropylate (isopropyl 4,4′-dibromobenzilate), amitraz [*N*-methylbis (2,4-xylyliminomethyl)amine], and fluvalinate [(*RS*)-α-cyano-3-phenoxybenzyl *N*-(2-chloro-α,α,α-trifluoro-*p*-tolyl)-=D-valinate] are currently employed by beekeepers in Europe and particularly in France in different application forms. These acaricides belong to four different chemical families (organophosphor-

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us, benzilate, amidine and pyrethroid, respectively). The use of pesticides inside beehives implies a risk of contamination of honey and other hive products (wax for example), so various methods [1–9] have been developed for the determination of acaricide residues in honey samples, mainly gas chromatography (GC) methods [2–5,7–12]. Liquid chromatography (LC) has been used for fluvalinate analysis of honey [6,13]. Clean up on C_{18} cartridges [2,3,6–8] or Florisil [1,4,5,13] or a mixture of silica gel with activated charcoal for coumaphos and fluvalinate analysis [9] have been employed. Supercritical fluid extraction (SFE) [10,13] and solid-phase microextraction (SPME) [12] have been applied to the analysis of fluvalinate and amitraz, respectively, in honey. For amitraz analysis, hydrolysis of amitraz with the formation of 2,4-dimethylaniline which is derivatized with heptafluorobutyric acid anhydride has been employed for a GC determination [1].

This paper describes a rapid multiresidue method for the evaluation of coumaphos, bromopropylate, amitraz and fluvalinate residue levels in honey samples that involves only a liquid-phase extraction and direct analysis by high-performance liquid chromatography (HPLC) with UV detection.

No maximal limit of residues was determined on honey. Two norms were defined in the No. 2377/90/EEC regulation [14] for coumaphos and amitraz (and its metabolite, 2,4-dimethylaniline): 0.1 and 0.2 mg kg^{-1}, respectively. In other countries like Switzerland for example, the norms for acaricides in honey are: 0.05 mg kg^{-1} for coumaphos, 0.1 mg kg^{-1} for bromopropylate, 0.2 mg kg^{-1} for amitraz and its metabolite and 0.05 mg kg^{-1} for fluvalinate.

Other molecules are employed in apiculture like essential oil components (thymol for example) which are not officially authorized in France, in contrast to Switzerland and Italy. However, thymol (a phenol derivative) is more and more used within in situ preparations in France because thymol has a good efficacy against *Varroa* and is well tolerated by bees. Therefore, it offers an alternative to synthetic acaricides. Each treatment with thymol may induce residues in wax, which can migrate from the wax comb into honey [15] and then jeopardize the quality of the honey. Residues of thymol are without danger for the consumer as long as they do not exceed 50 mg kg^{-1} of honey [15]. So these residues pose no health hazard, but they might affect the honey taste. This would contravene honey legislation, which forbids any addition to honey that changes its natural taste. For this reason, in Switzerland a maximal residue limit value of 0.8 mg kg^{-1} has been fixed. An extraction method in honey was developed by Bogdanov et al. [15] using C_{18} cartridges. The determination of thymol residues was realised by gas chromatography with mass spectrometric (MS) detection [8] or with flame ionisation detection (FID) [15]. We develop in this paper a simple and fast determination by liquid chromatography.

Some beekeepers used also rotenone as acaricide in hives. Few studies were realised on residues of rotenone in honey [16]. But papers exist for other matrices like fruits, vegetables, animal tissues [17] and water [18] and HPLC or GC determinations were described. We develop a method to control the residues in honey because the use of rotenone is not allowed in France.

2. Experimental

2.1. Materials and reagents

A Büchi rotary evaporator equipped with a water bath was purchased from Fisher Bioblock Scientific (Illkirch, France). A solid–liquid extraction system, fitted with a vacuum pump from Fisher Scientific Labosi (Elancourt, France) was used. The centrifuge used was a J2-21M/E from Beckman (Gagny, France) and the T25 Ultra-Turrax blender was from Fisher Scientific Labosi.

All solvents used (acetone, hexane, propanol-2, dichloromethane and methanol) were of ultra-pure-for-pesticides-analysis grade and were bought from Merck Eurolab-Prolabo (Fontenay-sous-Bois, France). Ethanol (95%, v/v, Rectapur), ammonia (RP Normapur for analysis) and orthophosphoric acid (85% min, RP Normapur for analysis) were purchased to Merck Eurolab-Prolabo. Acetonitrile was of HPLC grade and was obtained from Merck Eurolab-Prolabo and we used laboratory-distilled water. Sodium sulfate (reagent grade anhydrous) and Bakerbond solid-phase extraction (SPE) C_{18} extraction columns (500 mg, 6 ml) used for clean up were purchased from Fisher Scientific Labosi.

The acaricides coumaphos, bromopropylate, amitraz, fluvalinate and rotenone were obtained from CIL Cluzeau Info Labo (Sainte-Foy-La-Grande, France) and thymol from Sigma–Aldrich (Saint-Quentin-Fallavier, France). Standard solutions of coumaphos, bromopropylate, amitraz, fluvalinate and thymol were prepared at a concentration of 100 mg l^{-1} in acetone and kept at −20 °C. Solutions are stable and can be stored for 1 year at −20 °C. The solutions were diluted to the required concentrations with acetone before use and stored at +4 °C for 1 month without degradation. For rotenone, methanol was used for standard solutions and kept at +4 °C for only 1 week. Standards were stored in dark bottles as thymol and rotenone are decomposed by exposure to light.

2.2. Extraction and clean up procedures

2.2.1. Simultaneous analysis of coumaphos, bromopropylate, amitraz and fluvalinate

A 20-g amount of honey was mixed for 2 min in an Ultra-Turrax blender with a mixture of *n*-hexane (60 ml), propanol-2 (30 ml) and 0.28% of ammonia because amitraz is very instable in acid environment [19]. The pH of this mixture was 8. The solution was filtered through a paper filter and the same operation was realised once. The Ultra-Turrax was rinsed with 40 ml of *n*-hexane. The combined extracts were transferred to a separating funnel (500 ml). Distilled water with 0.28% of ammonia (50 ml) was added. The separating funnel was shaken vigorously and the filtrate was allowed to separate into two phases. The aqueous phase (inferior) was discarded and the operation was repeated twice with 50 ml of basic distilled water (pH 10). The *n*-hexane phase was filtered through a layer of anhydrous sodium sulfate (ca. 10 g) placed in a funnel plugged with a paper filter. The combined extract was concentrated by evaporation to dryness under reduced pressure in rotary evaporator using a 35–40 °C water bath. The residue obtained was dissolved in 1 ml of acetone and was ready for HPLC analysis after filtration on 0.45 μm nylon filter.

2.2.2. Thymol analysis

The extraction procedure Bogdanov et al. [15] was applied with some differences. A HPLC determination was used after extraction. The solution (20 ml) of honey (10 g) in ethanol–water (80:20, v/v) was centrifuged at 27 200 *g* during 20 min and at room temperature. The clean up on SPE C_{18} was realised (conditioning cartridges: 6 ml of ethanol and 6 ml of water) and the elution was performed with 2 ml of acetone. The extract was directly analysed by HPLC–UV after filtration on a 0.45 μm nylon filter.

2.2.3. Rotenone analysis

A 10-g amount of honey was mixed for 2 min in an Ultra-Turrax blender with dichloromethane (30 ml). The solution was filtered through a paper filter and the same operation was realised once again. The Ultra-Turrax and the paper filter were rinsed with 15 ml of dichloromethane. The combined extract was concentrated by evaporation to dryness in rotary evaporator using a 35–40 °C water bath. The residue was dissolved in 5 ml of methanol and was injected into the HPLC–UV system after filtration on a 0.45 μm nylon filter.

2.3. Instrumentation

HPLC was performed on a Hewlett-Packard 1100 apparatus from Agilent (Karlsruhe, Germany) containing a Rheodyne Model 7725 injector with a 20 μl loop and a photodiode array detector.

2.4. Chromatographic conditions

2.4.1. Simultaneous determination of coumaphos, bromopropylate, amitraz and fluvalinate

The liquid chromatographic column was a LiChrospher 100 RP-18, 5 μm, 250×4.0 mm I.D. from Merck Eurolab-Prolabo. The mobile phase (pH 9) was acetonitrile–water (80:20, v/v) with 0.28% ammonia at a flow-rate of 1 ml min^{-1}. The compounds were analysed under optimal conditions, so different wavelengths were used for each analyte: 313 nm, 233 nm, 289 nm and 254 nm, respectively, for coumaphos, bromopropylate, amitraz and fluvalinate. An external calibration was employed.

2.4.2. Determination of thymol

Chromatography was performed on a C_{18} Hypersil 120 BDS column, 5 μm, 250×4.0 mm I.D. from Merck Eurolab-Prolabo. The mobile phase was a

Table 1
Gradient elution for determination of thymol in honey

Time (min)	Acetonitrile (%)	0.05 *M* Orthophosphoric acid (%)
0	40	60
7	46	54
8	60	40
11	60	40
20	40	60

linear gradient elution of acetonitrile–0.05 *M* orthophosphoric acid (Table 1). The flow-rate and monitoring wavelength were 1 ml min^{-1} and 277 nm, respectively. An external calibration was employed.

2.4.3. Determination of rotenone

The liquid chromatographic column was a C_{18} Hypersil 120 BDS column, 5 μm, 250×4.0 mm I.D. from Merck Eurolab-Prolabo. The mobile phase was acetonitrile–water (60:40, v/v). The flow-rate and monitoring wavelength were 1 ml min^{-1} and 293 nm, respectively. An external calibration was used.

3. Results and discussion

3.1. Analytical characteristics

Calibration curves were constructed from peak areas versus acaricide concentrations. Good linearity was observed ($r=0.999$, at least) for all molecules.

Limits of detection (LODs) were calculated by a signal-to-noise ratio of 3:1 and limits of quantification (LOQs) were calculated by a signal-to-noise ratio of 10:1 [20]. The recovery of acaricides was checked by adding 0.5 ml of each acaricide standard solution at different levels to untreated samples of honey. Blank samples from the same honey without fortification were treated and analysed at the same time with spiked honey samples. Honey samples were spiked just before analysis. The acaricides were extracted and cleaned up by SPE (for thymol) as described in Section 2.2.2 and the sample solutions were analysed by HPLC–DAD under the optimal conditions described in Section 2.4. Each solute was spiked at three different concentrations and five repetitions were carried out for each fortification level. Profiles are shown in Figs. 1–3. The recoveries ranged from 86.2 to 109.3% and the relative standard deviations (RSDs) varied from 1.7 to 8.8% (Table 2).

These results indicate that the three present methods can be applied on samples of honeys from market. Furthermore, these methods allow the determination at levels (Table 3) lower than residues tolerances: 0.1, 0.2 and 0.8 mg kg^{-1} for coumaphos, amitraz and thymol, respectively.

3.2. Analysis of coumaphos, bromopropylate, amitraz and fluvalinate

HPLC analysis was chosen for the simultaneous determination of acaricides. The mobile phase must be set at pH 9 by adding 0.28% of ammonia because of the instability of amitraz. Each analyte showed a different absorbance: 313, 233, 289 and 254 nm for coumaphos, bromopropylate, amitraz and fluvalinate, respectively, and multiresidue determination was performed at these wavelengths. In Fig. 1, a good separation of the four acaricides was achieved in 20 min and no interference was observed in honey samples when chromatographic parameters presented in Section 2.4.1 are carried out. So this method permits to analyse rapidly four acaricides simultaneously without clean up. Acaricides are stable in acetone solution and the extract does not damage the HPLC column (400 samples can be analysed on one column). So HPLC–DAD was used to quantify residues of acaricides because the determination was more simple and rapid than GC: one detector instead of two for GC [an electron-capture detection (ECD) and a nitrogen–phosphorus detection (NPD) system according to the chemical families], the analysis was shorter (20 min for the simultaneous analysis of four acaricides) instead of some hours for GC (ECD for bromopropylate and fluvalinate and NPD for amitraz and coumaphos, respectively) and an internal cali-

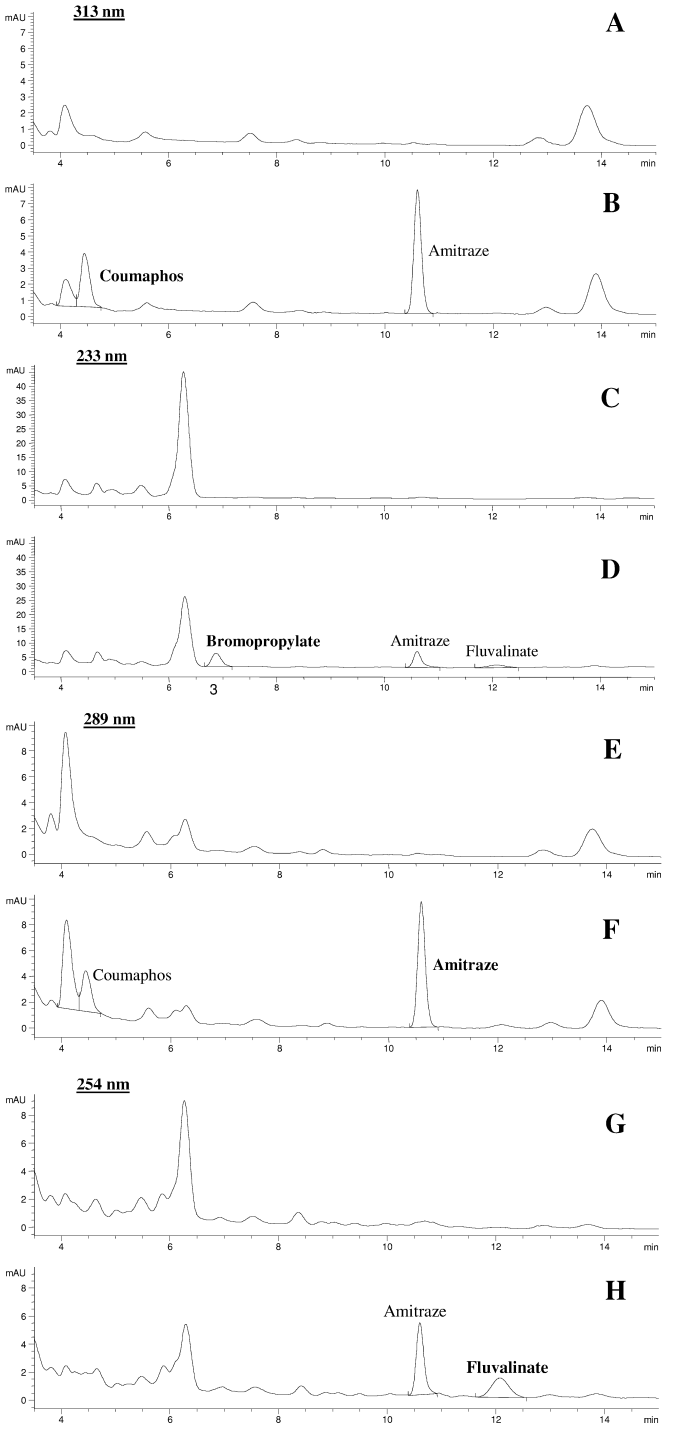

Fig. 1. (A) Chromatogram of a blank extract of honey at 313 nm; (B) chromatogram of honey spiked with 0.05 mg kg^{-1} of acaricides at 313 nm; (C) chromatogram of a blank extract of honey at 233 nm; (D) chromatogram of honey spiked with 0.05 mg kg^{-1} of acaricides at 233 nm; (E) chromatogram of a blank extract of honey at 289 nm; (F) chromatogram of honey spiked with 0.05 mg kg^{-1} of acaricides at 289 nm; (G) chromatogram of a blank extract of honey at 254 nm; (H) chromatogram of honey spiked with 0.05 mg kg^{-1} of acaricides at 254 nm.

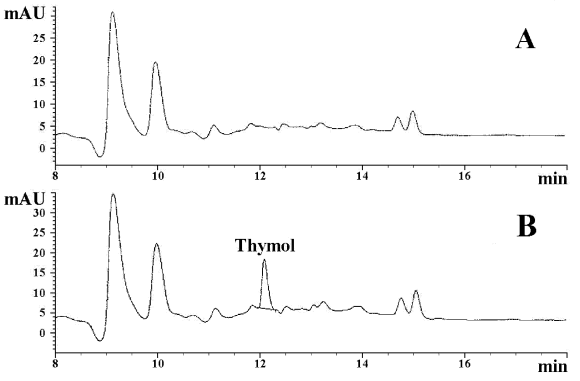

Fig. 2. (A) Chromatogram of a blank extract of honey at 277 nm; (B) chromatogram of honey spiked with 1 mg kg^{-1} of thymol at 277 nm.

bration was not necessary for the quantitative analysis.

This method is used for all honeys even the geographic origins are different. The extraction must to be realised at basic pH by adding 0.28% of ammonia to the mixture of solvents and in the distilled water. So, the efficiency of the extraction is pH dependent because amitraz is instable in acidic medium. Furthermore, the pH of honey is acid (included between 3.2 and 4.5) and amitraz degradation was virtually complete within 15 days [11].

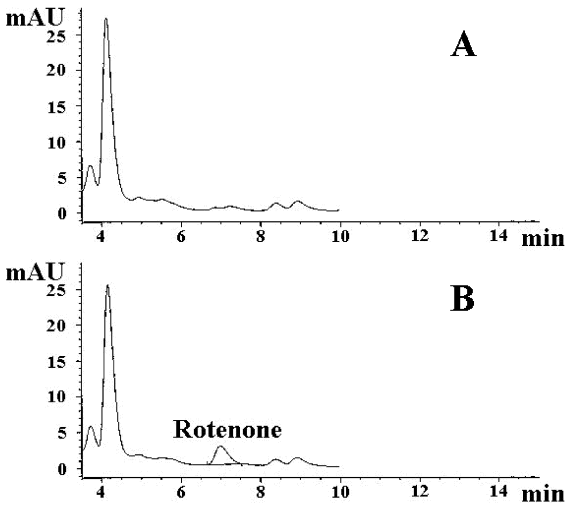

Fig. 3. (A) Chromatogram of a blank extract of honey at 293 nm; (B) chromatogram of honey spiked with 0.5 mg kg^{-1} of rotenone at 293 nm.

Table 2
Recoveries obtained for acaricides at various fortification levels

Acaricide	Fortification level (mg kg^{-1})	Mean recovery* (%)	RSD (%)
Amitraz	0.5	93.8	3.0
	0.125	96.2	4.7
	0.05	98.5	3.8
Coumaphos	0.5	86.2	2.7
	0.125	89.8	4.1
	0.05	101.4	4.0
Bromopropylate	0.5	93.0	3.4
	0.125	94.2	5.2
	0.05	96.7	3.3
Fluvalinate	0.5	93.2	2.0
	0.125	98.7	6.3
	0.05	102.8	7.1
Thymol	1	101.7	1.7
	0.5	101.0	3.5
	0.2	101.0	8.8
Rotenone	1	92.3	5.8
	0.5	91.0	3.3
	0.1	109.3	4.1

*$n=5$.

We observed the degradation of amitraz in fortified honey stored in laboratory at various temperatures (4, 20 and 40 °C). The results showed that the higher the temperature the more rapid was the degradation. No residue was observed at the end of 3, 15 and 30 days, respectively, for 40, 20 and 4 °C conditions (Fig. 4).

Table 2 shows the average values of five replicates of recoveries obtained for the pesticides added to honeys, using the procedure described above. Each standard solution (mixture of four acaricides in acetone) was injected and the calibration range was linear from 0.5 to 30 mg l^{-1} ($r=0.999$, at least for each acaricide). The estimated detection limits for extracts obtained from a honey sample of 20 g and a final volume of 1 ml were 0.005 mg kg^{-1} for

Table 3
LODs and LOQs of the different acaricides used in apiculture

Acaricide	LOD (mg kg^{-1})	LOQ (mg kg^{-1})
Amitraz	0.0015	0.005
Coumaphos	0.005	0.015
Bromopropylate	0.0015	0.005
Fluvalinate	0.003	0.01
Thymol	0.06	0.2
Rotenone	0.01	0.05

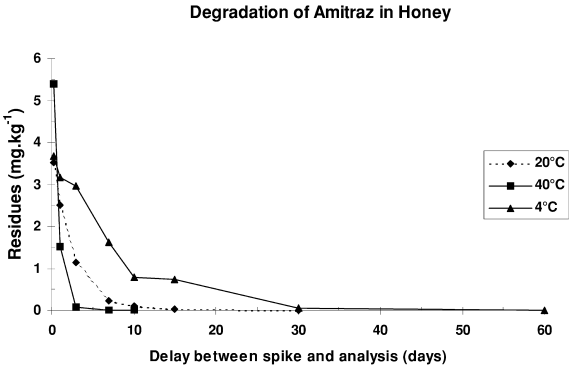

Fig. 4. Degradation of amitraz in honey stored at various temperatures.

coumaphos, 0.003 mg kg^{-1} for fluvalinate and 0.0015 mg kg^{-1} for amitraz and bromopropylate.

The analysis of 320 honey samples collected in 3 years from beehives in diverse areas of France (national sampling) with information about the treatments applied [21] shows that amitraz and bromopropylate were not present in amounts above the determination limit and three for fluvalinate and three others for coumaphos were detected. The levels of pesticide residues are low especially when treatments are properly carried out. Two exceptions are the samples with high concentrations of coumaphos (0.26 and 0.11 mg kg^{-1}) which were collected from hives which received a greater number of treatments than required or the doses used were higher than necessary. For fluvalinate the concentrations are 0.01, 0.023 and 0.026 mg kg^{-1}. Residues are detected when beekeepers do not respect the recommendations of use, either by applying a greater number of treatments than required or by using doses higher than necessary.

3.3. Analysis of thymol and rotenone

Thymol and rotenone were rapidly determined by HPLC–UV (Figs. 2 and 3). These molecules are decomposed by exposure to light. Standards were stored in dark bottles. We observed no degradation for thymol in acetone. For rotenone the standard solutions are weekly prepared because rotenone is instable in methanol. The degradation of the rotenone dissolved in methanol and stored at +4 °C in darkness was related to its concentration [16].

For thymol, determination HPLC has advantages in comparison to GC determination: an internal standard and drying on anhydrous sodium sulfate after SPE with C_{18} cartridges are not necessary contrary to GC determination and the liquid chromatography analysis needs less time (20 min) instead of 50 min for GC analysis [15]. Furthermore chromatograms show no interferences (Fig. 2).

Table 2 shows the average values of five replicates of recoveries obtained for thymol and rotenone added to honeys, using the procedures described above. Each standard solution in acetone (for thymol) or methanol (for rotenone) is injected and the calibration range was linear from 0.5 to 10 mg l^{-1} for thymol and from 0.0255 to 10 mg l^{-1} for rotenone (r=0.999, at least for each molecule). The estimated detection limits were 0.06 and 0.01 mg kg^{-1} for thymol and rotenone, respectively.

4. Conclusion

A HPLC multiresidue method has been developed for determination of the acaricides coumaphos, bromopropylate, amitraz and fluvalinate in honey using reversed-phase chromatography with UV detection. This method is very rapid and highly sensitive. For sample extraction, these methods described in this study were applied to honeys from various floral origins with satisfactory recoveries. These three methods combined with HPLC permit the determination of acaricides residues at levels close to residues tolerances.

In conclusion, reversed-phase HPLC provides a rapid, simple and economical alternative to GC for the separation and determination of acaricides. The utilization of the C_{18} column, in conjunction with a UV detection system (DAD), has allowed us (i) to obtain detection limits comparable with those obtained by GC using electron-capture, nitrogen–phosphorus or flame ionisation detection and (ii) to identify in one analysis residues of coumaphos, bromopropylate, amitraz and fluvalinate.

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