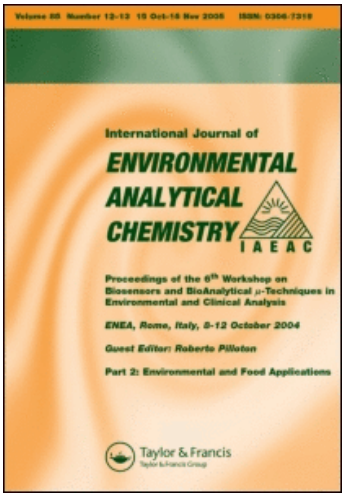

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DETERMINATION OF AMITRAZ IN HONEY BY FIRST-DERIVATIVE SPECTROPHOTOMETRY

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A new method for the determination of 1,5-bis(2,4-dimethylphenyl)-3-methyl-1,3,5-triazopenta-1,4-diene (Amitraz) in honey by derivative spectrophotometry is proposed. Amitraz is extracted from aqueous honey solutions with CCl_4 and it is determined between 0.1 and 1.5 $\mu g.ml^{-1}$ from the first-derivative spectrum by measuring the peak amplitude at 332 nm ($^1D_{332}$), as the vertical distance from the peak to the baseline.

INTRODUCTION

The disease *varroasis* is caused by the mite *Varroa Jacobsoni*. It first appeared in Europe in 1976. The mite multiplies itself in *Mellifera Apis*, in the larvae and adults of both sexes. In a beehive infected by one bee, the activity decreases and the disorganization of the beehive increases. Eventually the bees leave the beehive when the queen dies. Nowadays *varroasis* is the disease that most affects domestic bees. This disease causes more losses than all other diseases combined.

The treatment of the disease is based on the use of a pesticide that shows a high activity to *V. Jacobsoni* and a low toxicity to bees. However, the use of pesticides leads to a residue problem in honey, pollen, jelly, etc.; consequently, it is necessary to propose new methods for residue determinations.

Phenothiazine, thymol, chlorobenzylate, bromopropylate and Amitraz are pesticides used for the treatment of *varroasis*. Amitraz shows activity against *V. Jacobsoni*.[1–3] It can be used in mixtures with other pesticides, such as Fenazox,[4] Tetradifon[5] or Folbex VA and formic acid.[6] It has a low toxicity for bees,[7,8] and is therefore, often used.

References to Amitraz determination in apicultural products are scarce and most of these involve time-consuming sample preparation.[9–11] Derivative spectrophotometry is an analytical technique of great utility for extracting both qualitative and quantitative information from spectra composed of unresolved bands and has demonstrable advantages for the solution of specific analytical problems. A characteristic of this technique is that the differentiation discriminates against broad bands, emphasizing sharper features to an extent that increases with increasing derivative order.[12]

Honey solutions show UV absorption spectra with very broad bands that can be discriminated by using derivative spectrophotometry, while the Amitraz band is

enhanced. This discrimination is better if a previous liquid–liquid extraction is made.

In this paper data on the chemical behaviour of Amitraz are presented and a derivative spectrophotometric method with previous extraction into tetrachloromethane is proposed. The method is applied for determining Amitraz in honey.

EXPERIMENTAL

Apparatus

A Beckman Instruments DU-50 spectrophotometer connected to an IBM PC-XT fitted with Beckman Data Leader Software and a Olivetti DM 282 printer was used for all absorbance measurements.

Reagents

1,5-bis(2,4-dimethylphenyl)-3-methyl-1,3,5-triazopenta-1,4-diene (Amitraz) in ethanolic solution ($0.1\ g.l^{-1}$) was prepared from technical Amitraz supplied by Schering.

An ammonia buffer (pH 9.2) solution was prepared by dissolving 13 g of ammonium chloride, 175.5 g of sodium chloride and 100 ml of 2.5 M ammonia and diluting with water to 1 litre.

All other solvents and reagents used were of analytical grade.

Recommended Procedure

Weigh out about 1 g of honey spiked with Amitraz and dissolve it in 10 ml of ethanol–water (1:1). Transfer this solution and the washings to a 150-ml separatory funnel. Add 20 ml of ethanol, 10 ml of buffer solution and dilute with water to 100 ml. Shake the solution vigorously with 10 ml of CCl_4 for 2 min. After phase separation, the organic phase is centrifuged and an aliquot is transferred to a 1.0-cm cell. Absorption spectra of the samples are recorded with a scan speed of 750 nm/min between 220 and 400 nm against CCl_4. The spectra are smoothed through the use of 15 experimental points and the first derivative with $\Delta\lambda = 4$ nm is recorded. The Amitraz content is determined from the first-derivative spectrum by measuring the peak amplitude at 332 nm ($^1D_{332}$) (vertical distance from the peak to the baseline).

The calibration graph was established with Amitraz standard solutions, prepared in the same way, in the range $0.1–1.5\ \mu g.ml^{-1}$ in water by using five experimental points and one replicate at each point.

RESULTS AND DISCUSSION

The solubility of Amitraz in several solvents at room temperature was determined

by Wittemberger's method.[13] The reagent is very soluble in dimethylformamide ($>890\,g.l^{-1}$), dichloromethane ($>800\,g.l^{-1}$), acetonitrile ($>600\,g.l^{-1}$), carbon tetrachloride ($>500\,g.l^{-1}$), methylisobutylketone ($>340\,g.l^{-1}$), soluble in methanol ($48.0\,g.l^{-1}$) and ethanol ($30.4\,g.l^{-1}$), and relatively insoluble in water ($0.06\,g.l^{-1}$). The $0.1\,g.l^{-1}$ ethanolic Amitraz solution is stable for at least 48 h. The $0.01\,g.l^{-1}$ Amitraz ethanol–water (1:1) solutions are stable for at least 90 min. A smaller ethanol content decreases the stability of the samples.

The absorption spectra of $0.01\,g.l^{-1}$ solutions of Amitraz in ethanol–water (1:1) show an absorption maximum at 286 nm in slightly acid, neutral and basic media ($pH>3$). The absorbance at 286 nm is practically constant for pH values higher than 5. In a strongly acid ($pH<3$) medium, the absorption maximum disappears as a result of the analyte degradation to a 2,4-dimethylaniline derivative.[14]

Extracto-spectrophotometric Study

Aqueous honey solutions show strong absorption in the UV spectrum. When the aqueous honey solutions are shaken with carbon tetrachloride, the larger part of the honey components remain in the aqueous phase, as can be seen from Figure 1. However, under the same conditions Amitraz is extracted quantitatively. Also, the extraction preconcentrates the Amitraz, yielding better sensitivity in the final determination.

The extraction of Amitraz in different organic solvents was tested (Figure 2). The results show that Amitraz is extracted in all organic solvents studied. The best results are obtained with CH_2Cl_2, $CHCl_3$ and CCl_4. In this paper, we have carried out the extracto-spectrophotometric study in CCl_4 and CH_2Cl_2. In these solvents, Amitraz shows absorption maxima at 292 nm and 289 nm, respectively.

Since Amitraz solutions in ethanol–water are used, the effect of this organic solvent on the efficiency of the extraction has been tested. The results showed that for both CCl_4 and CH_2Cl_2 the efficiency is maximal with 20–30% of ethanol in the aqueous phase. We have chosen 25% ethanol, and 1 M NaCl was added for good phase separation.

The efficiency of the extraction is pH dependent. Figure 3 shows that the extraction in CCl_4 is maximal for $pH \geqq 6$. Analogous results were obtained for CH_2Cl_2. The pH was set at 9.2 by adding 10 ml of ammonia buffer solution.

The effect of the phase-volume ratio was studied for 1:1 to 10:1 ($V_w:V_o$) ratios. The extraction efficiency was 97% for a 1:1 phase-volume ratio and 94% for a 10:1 ratio. In consequence, multiple extraction is not necessary. A phase-volume ratio of 10:1 was chosen in all further work. A shaking time of 2 min is sufficient for complete extraction.

In CCl_4 ($V_m/V_o=10:1$), Beer's law is obeyed for 0.1–1.5 μg/ml of Amitraz in water (Figure 4a). The molar absorptivity at 292 nm is $2.22\times10^4\,l.mol^{-1}.cm^{-1}$. The optimum concentration range, evaluated by Ringbom's method is 0.3–1.0 $\mu g.ml^{-1}$ and the relative error (95% confidence level) for $0.5\,\mu g.ml^{-1}$ is $\pm1.3\%$ ($n=8$). In CH_2Cl_2 (289 nm), analogous results were obtained, but a poor value was found for the relative error ($\pm3.7\%$).

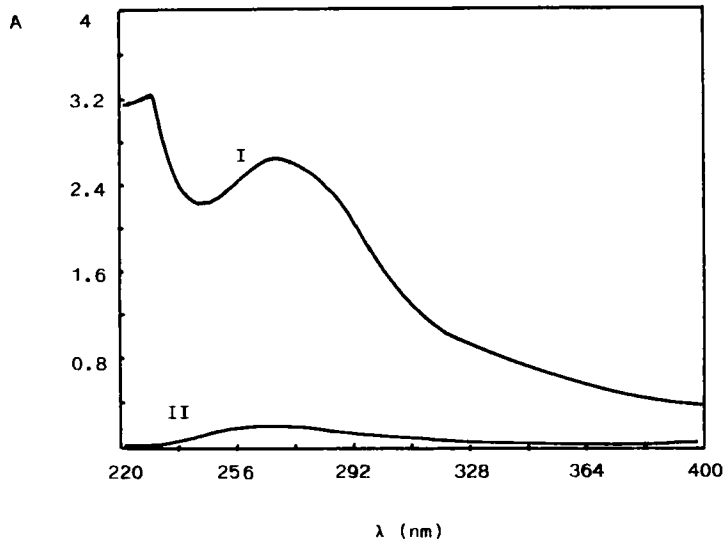

Figure 1 Effect of the extraction on the honey components. I: Aqueous honey solutions. II: Honey extract in CCl4.

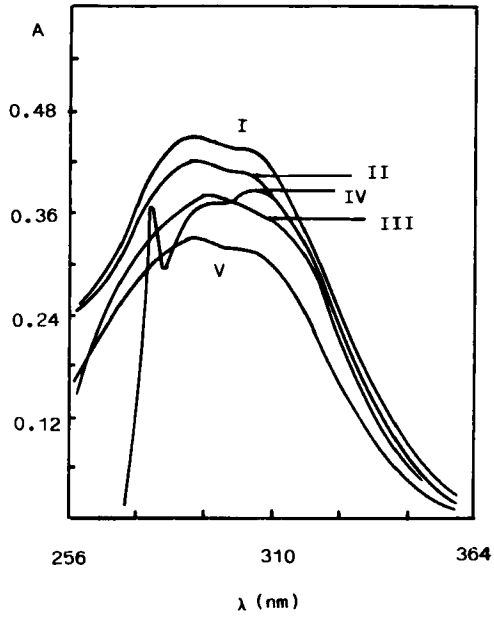

Figure 2 Absorption spectra of Amitraz in organic solvents. I: Dichloromethane. II: Chloroform. III: Tetrachlormethane. IV: Toluene. V. Ethyl acetate.

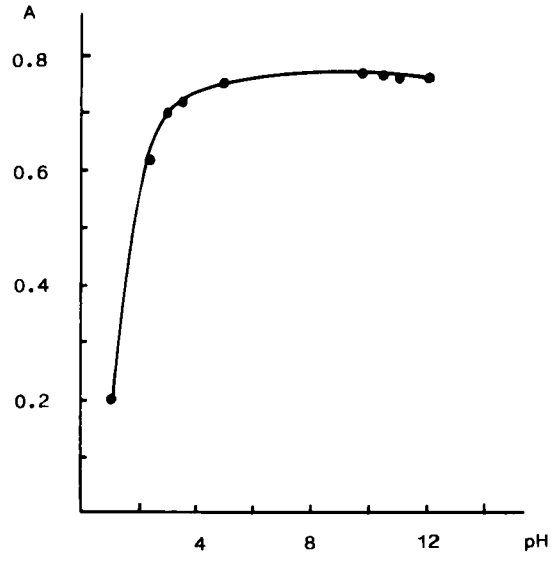

Figure 3 Effect of pH on the efficiency of the extraction in CCl4.

Derivative Spectrophotometry

First-derivative spectra of Amitraz and CCl_4 and CH_2Cl_2 were obtained through the Data Leader Beckman Software. Absorption spectra of the samples (prepared as described before) were recorded with a scan speed of 750 nm/min between 390 and 260 nm. Because of the noise level on the first-derivative spectra, a smoothing function was used, based on a Savitzky and Golay method;[15] it was operated on the absorption spectra by use of 15 experimental points, considered as optimum.

From the smoothed spectra, the first-derivative spectra were obtained with $\Delta\lambda = 4$ nm (Figure 4b). The algorithm used by Data Leader Software for obtaining the *n*th derivative is a variation of a Savitzky–Golay smoothing function.

Calibration graphs were constructed by measuring the amplitude of the first-derivative spectra at 332 nm (in CCl_4) and at 325 nm (in CH_2Cl_2). Straight lines were obtained for 0.1–1.5 μg/ml of Amitraz in water. Statistical data on the calibration graphs, the precision of the methods, determination limits and detection limits are summarized in Table 1.

Application

The methods have been tested for determining Amitraz in honey samples by using both CCl_4 and CH_2Cl_2.

First, it was verified that Amitraz in honey is extracted quantitatively ($\geqq 94\%$ for 10:1 V_w/V_o), by shaking once with CCl_4 or CH_2Cl_2 and that the larger part of the honey components is separated. The efficiency of extraction was tested for different amounts of honey: 5 μg of Amitraz are extracted quantitatively when the honey amount is 0.5–2.0 g.

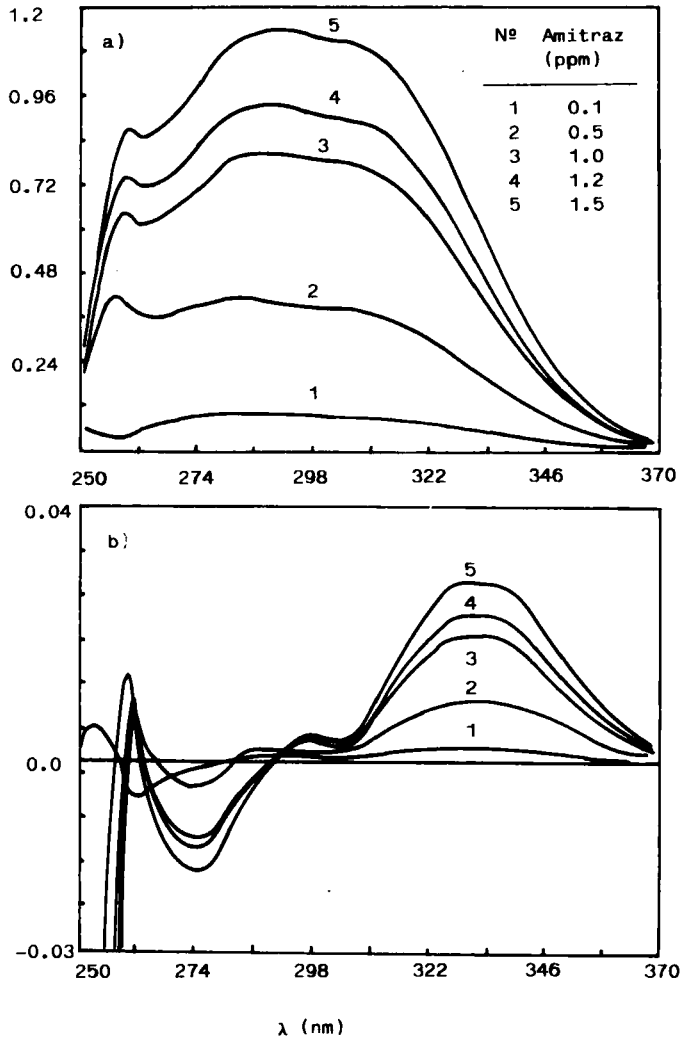

Figure 4 Influence of the Amitraz concentration on: (a) the absorption spectra; and (b) first-derivative spectra in CCl_4.

Table 1 Spectrophotometric characteristics of Amitraz determination obtained from five experimental points ($n=2$ per point)

Parameters	CCl_4	CH_2Cl_2
Equation ($n=5$)	$^1D_{332}=4\times10^{-4}+0.018\,C$	$^1D_{325}=12\times10^{-4}+0.020\,C$
Regression lineal	0.9992	0.9987
Per cent error	±0.67	±4.03
Determination limit (ppm)	5.6×10^{-2}	9.9×10^{-4}
Detection limit (ppm)	1.7×10^{-2}	3.0×10^{-4}

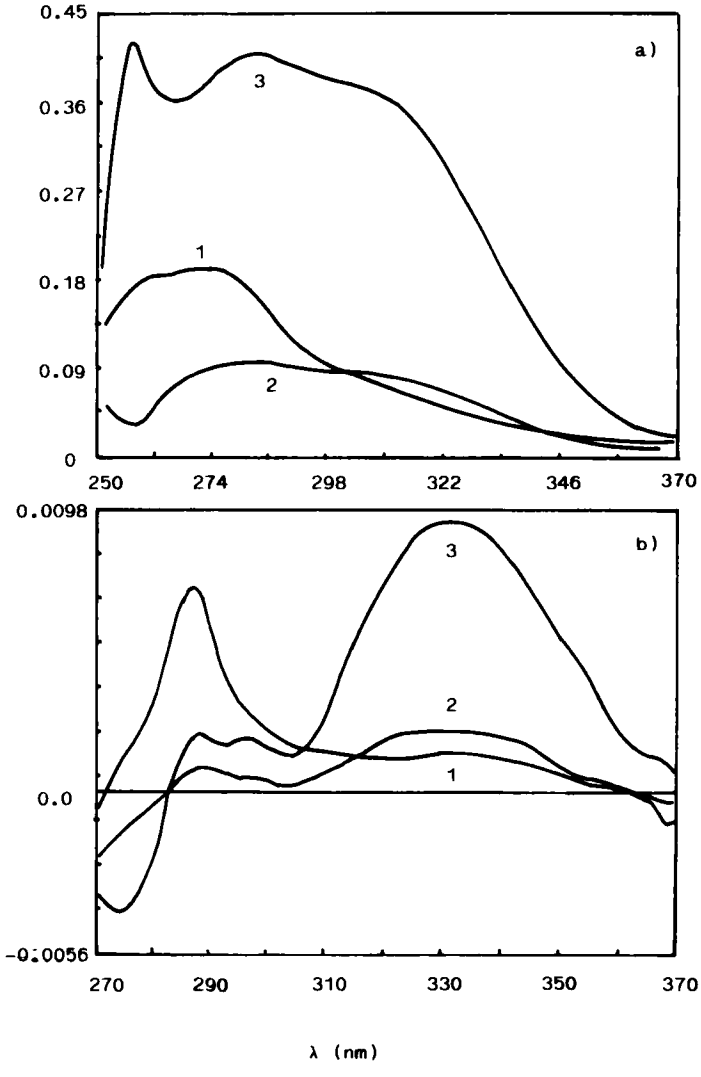

Figure 5 Comparison of Amitraz and pure honey spectra: (a) Zero-order derivative spectra; (b) First-derivative spectra. (1) 1.0 g of pure honey; (2) Pure honey containing 0.1 ppm of Amitraz; (3) Pure honey containing 0.5 ppm of Amitraz.

However, the organic solvents do not provide a sufficiently specific separation of Amitraz to permit the direct determination of the analyte by using zero-order derivative spectra as is observed in Figure 5a, because of the background of pure honey in CCl_4 at 292 nm. First-derivative spectra (Figure 5b) decrease the background of honey at 332 nm. When CH_2Cl_2 is used, similar results are obtained. In consequence, the use of derivative spectrophotometry improves the determination of Amitraz in honey.

Table 2 Determination of Amitraz in honey by using the recommended procedure[a]

Amitraz added (*μg/ml*)	*Honey I*		*Honey II*	
	Found[b] (*μg/ml*)	*Per cent recovery*	*Found*[b] (*μg/ml*)	*Per cent recovery*
—	<0.05[c]	—	<0.05[c]	—
0.1	0.11	110	0.12	120
0.5	0.48	96	0.46	92
1.0	0.98	98	0.91	91
1.2	1.15	96	1.15	96
1.5	1.47	98	1.41	94

[a]Blank honeys I and II contained less than 0.05 ppm of Amitraz.
[b]Mean of three analyses.
[c]Determination limit.

Finally, the utility of the first-derivative method was tested for two different types of honey spiked with varying amounts of Amitraz, using CCl_4 and CH_2Cl_2. The best results were obtained with CCl_4; the results are summarized in Table 2. The effect of the amount of honey on the determination of 5.0 μg of Amitraz was tested for 0.5–2 g of honey. In all cases, the relative error was less than 10%. The detection limit has a sufficiently low value for determining the residue of the pesticide in honey.

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