Journal of Chromatography A, 655 (1993) 95–99
Elsevier Science Publishers B.V., Amsterdam

CHROM. 25 056

Supercritical fluid extraction of fluvalinate residues in honey. Determination by high-performance liquid chromatography

J. Atienza* and J.J. Jiménez

S.I.A. Laboratory, P.O. 172, 47080-Valladolid (Spain)

J.L. Bernal and M.T. Martín

Department of Analytical Chemistry, Faculty of Sciences, Valladolid (Spain)

ABSTRACT

A method for the analysis of fluvalinate residues in honey from beehives treated with this product to prevent varroatosis is described. The method involves supercritical fluid extraction with carbon dioxide and further analysis by high-performance liquid chromatography on a C_{18} reversed-phase column, acetonitrile–water (80:20) as mobile phase and detection at 254 nm. This method is simpler than the one in which extraction with organic solvents, thin-layer chromatography and gas chromatography is used.

INTRODUCTION

Varroatosis is an external form of parasitosis caused by the mite *Varroa jacobsoni* which affects bees at all stages of development. It is regarded as a severe disease since it results in massive losses in bee colonies that in turn results in occasional dramatic economic losses. Most chemical treatments used against this parasitic mite include bromopropilate, coumaphos, amitraz, chlordimeform or fluvalinate —the last being the most widely employed for the purpose because of its high effectiveness [1].

Spanish law sets no maximum allowable limit for fluvalinate concentration in honey. However, the law does specify maximum permitted concentrations for other products, such as corn forage (3 mg/kg), citric and stone fruits (1 mg/kg), tomato and pepper (0.5 mg/kg), corn grains (0.10 mg/kg) and cotton seed (0.05 mg/kg), as well as a generic concentration of 0.01 mg/kg for all other vegetable products.

The determination of fluvalinate in honey is usually performed by using methods that involve prior extraction of the compound with acetonitrile–hexane or benzene–isopropanol mixtures and subsequent cleaning by means of a Florisil or octadecylsilane column or, alternatively, isolation by thin-layer chromatography. The final extract obtained is typically analysed by gas chromatography with an electron-capture or nitrogen–phosphorus detector [2–10]. The use of gas chromatography poses the problem that fluvalinate decomposes readily by heating, so it has been quantified by applying GC–MS to its degradation products [11]. The degradation resulting from the thermal lability of the analyte can be overcome by using high-performance liquid chromatography (usually with UV detection), although the sensitivity achieved by HPLC

* Corresponding author.

0021-9673/93/$06.00

is somewhat lower than that provided by GC [3,12].

Supercritical fluids, particularly carbon dioxide, which is highly efficient for extractions from complex matrices [13–15], are gaining increasing popularity as replacements for standard solvent extraction techniques.

In this work we present the results of a comparison of the standard extraction–TLC–GC procedure and a supercritical fluid extraction (SFE)–HPLC–UV method developed in our laboratory. The applicability of the newly developed procedure for the analysis of honey from beehives treated with fluvalinate is also presented.

EXPERIMENTAL

Reagents

The following organic solvents, which were of residue analysis grade and supplied by Scharlau (Barcelona, Spain), were used: methanol, ethanol, isopropanol, chloroform, dioxane, toluene, carbon tetrachloride, benzene, acetone and dichloromethane.

HPLC-grade acetonitrile purchased from Panreac (Barcelona, Spain) was also used.

Ultrapure water obtained from a Milli-Q plus apparatus (Millipore, Milford, MA, USA) was employed throughout.

Fluvalinate, coumaphos, chlordimeform, amitraz and bromopropilate certified purity pesticide standards were purchased from Chemservice (West Chester, PA, USA).

Finally, C-60 grade carbon dioxide (purity = 99.9999%) was provided by Carburos Metálicos (Madrid, Spain).

Gas chromatographic system

The gas–liquid chromatographic set-up used consisted of a Hewlett-Packard (HP) 5890 chromatograph (Avondale, PA, USA) equipped with an HP 7673A sample autoinjector, an electron-capture and a nitrogen–phosphorus detector using argon–methane and helium, respectively, as auxiliary gas, and a 30 m × 0.25 mm × 0.25 μm DB5 capillary column from J&W Scientific (Folsom, CA, USA) employing helium at a flow-rate of 0.6 ml/min as carrier gas. The assembly was controlled by an HP 3396A integrator. The temperatures of the injection port and detector were 200 and 300°C, respectively, and the temperature programme used was as follows: initial temperature, 125°C for 5 min; temperature gradient, 2.5°C/min; final temperature, 270°C for 15 min. An injected volume of 5 μl was employed throughout.

HPLC system

The HPLC system used was composed of a ConstaMetric 4100 pump fitted with four eluent ways, an AutoMetric 4100 autosampler, a SpectroMonitor 3200 UV–visible detector and a membrane degasser, all of which were supplied by LDC Analytical (Riviera Beach, FL, USA). Data were obtained and processed by means of a computerized system developed in our laboratory that controlled the entire set-up. The operating conditions employed were as follows: 15 cm × 4.6 mm Novapak C_{18} chromatographic column from Waters–Millipore (Milford MA, USA); mobile phase, acetonitrile–water (80:20) containing 14 ml/l 0.01 *M* HAcO; flow-rate, 1.5 ml/min; injected volume, 20 μl; wavelength, 254 nm.

Extraction with organic solvents–TLC

Fluvalinate was extracted from a honey sample of 150 g that was treated with four 75-ml portions of benzene–isopropanol (7:3, v/v). Each mixture was stirred mechanically for 20 min. The extracts were joined and evaporated to dryness under a nitrogen atmosphere at 35°C. The residue obtained was dissolved in 0.5 ml of methanol.

The extract was cleaned by two-dimensional thin-layer chromatography using glass plates covered with a 0.5-mm layer of Kiesegel 60 (Merck, Darmstadt, Germany), onto which 200 μl of the extract in methanol were placed. The plates were developed with chloroform–ethanol (1:1 v/v) in one direction and with toluene–carbon tetrachloride (4:1, v/v) in the other. Once the plates were dried, the fluvalinate spot (detected by a 254-nm UV lamp) was scraped and brought into contact with 1 ml of methanol for 24 h in order to ensure complete dissolution. Then, the solution was filtered through PTFE

with 0.5 μm pore size (MFS, Dublin, CA, USA). The processed sample was ready for the determination of fluvalinate.

Extraction with supercritical carbon dioxide

The extraction was carried out with a Hewlett-Packard 7680A supercritical fluid extractor using CO_2 as extractant under the following optimal working conditions: fluid density, 0.45 g/ml; working pressure, 138 bar; extraction chamber temperature, 70°C; flow-rate, 0.8 ml/min; dynamic extraction time, 20 min; analyte collection over a trap packed with stainless-steel balls at a nozzle temperature of 75°C with 1 ml of methanol.

Before the extraction step the sample needs a pretreatment: 20 g of honey were mixed with an amount of water equivalent to about 20% of the resulting mixture. Then, cellulose powder (Aldrich, Steinheim, Germany) was added in a proportion equivalent to 10% of the original honey sample. The mixture was homogenized by stirring and the preparation was frozen at −40°C and lyophilized in Telstar equipment (Barcelona, Spain). A fraction of 2 g of lyophilizate was treated with 25 μl of benzene–isopropanol (7:3, v/v) as organic modifier for CO_2, thereby being made ready for extraction.

RESULTS AND DISCUSSION

Stability of fluvalinate

In order to check the stability of the analyte, several solutions taken from an available formulation, Mavrick (Sandoz, Basle, Switzerland), were prepared. After filtration, they were analysed by HPLC at different times after preparation.

As can be seen in Fig. 1, metabolite peaks started to appear soon after the elution began. Such peaks were virtually the only ones observed in chromatograms run after 12 days.

Consequently, taking into account the phytosanitary procedure used to apply fluvalinate and the acidity and diastatic activity of honey, the analyte will hardly be encountered as such in this natural product.

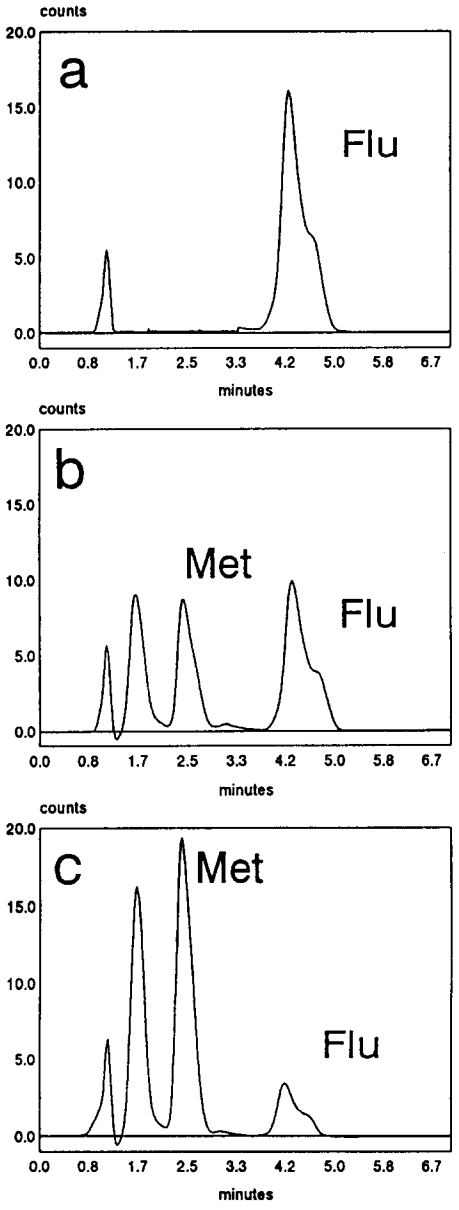

Fig. 1. Chromatograms obtained by HPLC–UV of a commercial preparation of fluvalinate (Mavrick) in methanol. The fluvalinate concentration in 10 mg/l. (a) One day after preparation. (b) Six days after preparation. (c) Ten days after preparation. Flu = Fluvalinate; Met = evaluated metabolite.

On the other hand, the chromatograms of fluvalinate samples attained by the use of GC show a greater number of compounds of decomposition than the HPLC ones.

Thin-layer chromatographic clean-up

The optimal operational conditions for the application of this procedure were established by assaying several kinds of plates: aluminium covered with alumina or cellulose and glass covered with cellulose or silica gel. The last one was finally chosen. Even more important was the choice of the eluent used to isolate fluvalinate from other acaricides, such as bromopropilate, amitraz, coumaphos, chlordimeform, etc., potentially present in honey. For this purpose we assayed methanol, acetonitrile, toluene, dichloromethane, benzene, isopropanol, dioxane, acetone, ethanol and chloroform, which were used on plates onto which 9 μg of standard in methanol had been previously placed.

Pure non-polar eluents failed to elute any of the products. On the other hand, polar eluents elute fluvalinate, bromopropilate and coumaphos in the front, whereas eluents of intermediate polarity (benzene, dichloromethane) resulted in an intermediate situation.

Based on the results provided by the pure eluents and the polarity of the compounds involved, a test set with binary mixtures of the solvents was carried out. None of the mixtures in question allowed complete separation by one-dimensional TLC. On the basis of the partial separations achieved, the use of the two-dimensional procedure was chosen, which allowed fluvalinate to be isolated from the other acaricides and chromatographic interferents present in honey (Fig. 2).

Extraction with supercritical CO_2

Table I lists the results obtained in the extraction of fluvalalinate from fortified honeys using the SFE–HPLC method. The recovery was less than the fortification level. Recovery from honey samples fortified at 0.5 mg/kg was 94% and at 10 mg/kg 53%; relative standard deviation was about 1.4–3.0%.

Table II shows the recoveries of fluvalinate obtained after the organic solvent extraction–TLC–GC procedure was applied to fortified honeys. The recovery was 88% at a fortification level of 0.5 mg/kg and 49% at 10 mg/kg, with relative standard deviations of 2.8 and 6.1%, respectively.

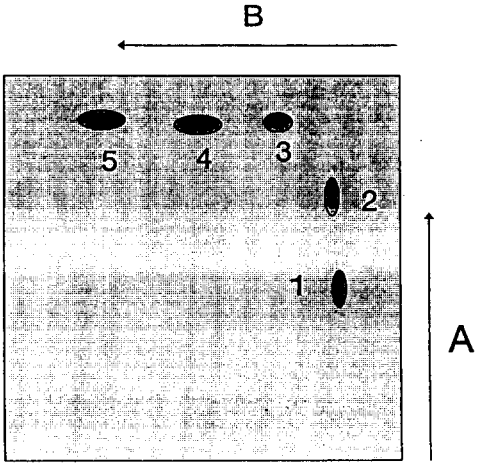

Fig. 2. Chromatogram obtained by two-dimensional thin-layer chromatography by the use of the elect eluents. 1 = Amitraz; 2 = chlordimeform; 3 = coumaphos; 4 = bromopropilate; 5 = fluvalinate. (A) First eluent, chloroform–ethanol (1:1). (B) Second eluent, toluene–carbon tetrachloride (4:1).

TABLE I

RECOVERIES (%) AND STANDARD DEVIATIONS (σ_{n-1}) OF FLUVALINATE OBTAINED AFTER SFE–HPLC OF FORTIFIED HONEYS ($n = 5$)

Fortified amount (mg/kg)	SFE–HPLC	
	Recovery (%)	σ_{n-1}
10	53	1.6
5	80	1.6
1	84	1.5
0.5	94	1.3

TABLE II

RECOVERIES (%) AND STANDARD DEVIATIONS (σ_{n-1}) OF FLUVALINATE OBTAINED AFTER STANDARD SOLVENT EXTRACTION–TLC–GC OF FORTIFIED HONEYS ($n = 5$)

Fortified amount (mg/kg)	TLC–GC	
	Recovery (%)	σ_{n-1}
10	49	3.0
5	71	2.7
1	77	2.5
0.5	88	2.5

The linear range for fluvalinate response of the HPLC–UV system was 0.1–5 mg/l. The detection limit of fluvalinate, calculated from successive dilutions of a standard, was about 0.06 mg/l. So, the detection limit for SFE–HPLC analytical procedure was 0.02 mg fluvalinate per kg of honey.

Application of the SFE–HPLC procedure

The analysis of sixteen honey samples collected from beehives in the provinces of Zamora, Valladolid and Salamanca, all of them treated with fluvalinate in different doses, and six commercial available honey samples shows that fluvalinate was not present in amounts above the detection limit, according to the high degradation observed on standard solutions.

On chromatograms of honey extract the metabolite peak labelled "Met" in Fig. 1 was detected. The amount of fluvalinate in honeys was determined on the assumption that the metabolite concentration is related to the initial certified fluvalinate concentration by using temporally degraded fluvalinate certified standards.

On the above basis, fluvalinate occurs in all honeys except two commercial samples. The fluvalinate concentration range was 0.029–0.750 mg/kg in beehive honeys and 0.055–0.215 mg/kg in commercial honeys.

CONCLUSIONS

SFE of fluvalinate residues from a complex polar matrix such as honey is better than extraction with organic solvents and avoids the need for a clean-up step. On the other hand, the HPLC technique gives simpler chromatograms than GC.

Two-dimensional TLC can be used for clean-up purposes as part of the standard extraction method since it allows the prior isolation of other acaricides that may accompany fluvalinate in the sample.

Fluvalinate as phytosanitary product has only been detected in properly preserved laboratory fortified honey samples. It has never been detected in beehive honey owing to its rapid degradation, which results in several chromatographic peaks that can be ascribed to some of its natural metabolites.

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