Food Control 21 (2010) 1307–1311

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Optimization of the liquid–liquid extraction method and low temperature purification (LLE–LTP) for pesticide residue analysis in honey samples by gas chromatography

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ARTICLE INFO

Article history:
Received 31 July 2009
Received in revised form 15 March 2010
Accepted 23 March 2010

Keywords:
Extraction method
Honey
Pesticide
Gas chromatography

ABSTRACT

This work optimized a simple and practical method for identification and quantification of the pesticides chlorpyrifos, λ-cyhalothrin, cypermethrin and deltamethrin in honey samples. The method was based on liquid–liquid extraction and low temperature purification using acetonitrile: ethyl acetate (6.5 mL:1.5 mL) as the solvent for extraction. A final clean up step with 2 g florisil was performed before analysis by gas chromatography using electron-capture-detector. The technique was proven satisfactory with efficiency exceeding 85% and linear chromatographic response for the tested pesticides, ranging from 0.033 to 1.7 μg g^{-1} with correlation coefficients above 0.99. Detection and quantification limits were lower than 0.016 and 0.032 μg g^{-1}, respectively. The proposed method was applied to 11 honey samples. Chlorpyrifos and λ-cyhalothrin residues were found in two samples at concentrations below maximum residue limit (MRL) established for food products. The presence of these compounds was confirmed by mass spectrometry in SIM mode (GC–MS-SIM).

1. Introduction

Honey is traditionally consumed by humans for being considered a product of natural origin and healthy. Honey must therefore remain free of any chemical or biological contaminant to be safe for human consumption. However, some studies have reported the presence of pesticide residues in honey samples (Kadar & Faucon, 2006; Pirard et al., 2007; Rissato, Galhiane, Almeida, Gerenutti, & Apon, 2007). These residues may originate from the treatment of bee hives with acaricides in the control of *Varroa jacobsonie* and *Ascosphera apis*. The most commonly used pesticides are amitraz, cymiazole, bromopropylate, coumafos, flumethrin, fluvalinate, imidacloprid and fipronil (Korta, Bakkali, Berrueta, Gallo, & Vicente, 2001; Rial-Otero, Gaspar, Moura, & Capelo, 2007). Although the regulatory agencies of several countries have established the maximum residue limit (MRL) for some of these pesticides in honey samples, these limits are not included in the Codex Alimentarius (1998).

Indirect honey contamination can occur during pesticide application in agriculture. Pesticide application in crops can contaminate soil, air, water, and the flowers from which bees collect nectar for honey production (Kujawski & Namiesnik, 2008).

Bees and honey may serve as indicators of environmental pollution (Celli & Maccagnani, 2003; Kevan, 1999). High concentrations of pesticide residues lead to high mortality rate of bees, and the honey produced is unfit for human consumption. Rissato and collaborators detected malathion residues in all honey samples analyzed in the region of Bauru (São Paulo, Brazil). Presence of residues of these compounds in the samples was attributed to pesticide application for dengue vector control in the area.

Analysis of pesticide residues in complex matrices consists of four steps: extraction, extract cleaning, identification and quantification of compounds. Among the extraction methods commonly used in honey analysis, are solid phase extraction (Albero, Sánchez-Brunete, & Tadeo, 2004; Blasco et al., 2003), supercritical fluid extraction (Rissato, Galhiane, Knoll, & Apon, 2004), conventional liquid–liquid extraction (Blasco et al., 2004, matrix solid phase dispersion (Fernández, Pico, & Manes, 2002) and solid phase microextraction (Campillo, Penalver, Aguinaga, & Hernandez-Cordoba, 2006). The clean up stage is based on techniques such as gel permeation chromatography and adsorption chromatography (Fernandez, Pico, & Manes, 2002; Rossi et al., 2001). The steps of identification and quantification of pesticide residues are based on gas chromatography (GC) and high performance liquid chromatography (HPLC) (Van der Hoff & Van Zoonen, 1999).

More recently, liquid–liquid extraction and low temperature purification (LLE–LTP) has emerged as an alternative for pesticide

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0956-7135/$ - see front matter © 2010 Published by Elsevier Ltd.
doi:10.1016/j.foodcont.2010.03.006

extraction in water (Vieira, Neves, & Queiroz, 2007) and milk (Goulart, Queiroz, & Neves, 2008). The method is based on the partition of analytes between the aqueous and organic phase resulted from temperature lowering (−20 °C). The advantage of this method is that the sample components are frozen with the aqueous phase, whereas pesticides are extracted by the organic phase.

This work aimed to optimize and validate a method using liquid–liquid extraction with low temperature purification for pesticide residue analysis in honey by gas chromatography. The method was applied for the determination of chlorpyrifos, λ-cyhalothrin, cypermethrin and deltamethrin which are often detected in monitoring studies of food samples. The insecticide chlorpyrifos is one of the most widely used crop protection products in the world, while the pyrethroids are insecticides included in over 3500 registered products, many of which are used in agriculture (Environmental Protection Agency, 2009).

2. Experimental

2.1. Reagents

Stock standard solutions of chlorpyrifos (99.0% w/w), cypermethrin (92.4% w/w) and deltamethrin (99.0% w/w) purchased from Chem Service (West Chester, PA, USA) and λ-cyhalothrin (86.5% w/w) purchased from Syngenta (São Paulo, Brazil) were prepared in acetonitrile to the concentration of 500 mg L^{-1} and stored at 4 °C. Working solutions were prepared from the dilution of stock solutions containing the four pesticides at adequate concentrations (50 mg L^{-1} and 5 mg L^{-1}) in the same solvent. The same procedure was used to prepare the bifenthrin solution (92.2% m/m – FMC Brazil) at 5 mg L^{-1}, used as internal standard.

Ethyl acetate (Merck, Darmstadt, Germany) was used as solvent for trace analysis; and acetonitrile, methanol and hexane from Mallinckrodt/HPLC (Baker, Paris, France). Anhydrous sodium sulfate and sodium chloride (purity greater than 99%) were purchased from Vetec (Rio de Janeiro, Brazil). Florisil (J.B. Barcker) was conditioned with acetonitrile and ethyl acetate and then dried in an oven at 45 °C.

2.2. LLE–LTP optimization

This technique was optimized using the following variables: (1) amount of honey extracted (2) composition of the extraction solvent (3) homogenization techniques (4) amount of florisil used for the clean up step and (5) different ionic strength used. Table 1 describes these variables.

Pesticide-free honey samples (Table 1) were placed in transparent glass vials (22 mL) and heated to 50 °C in water bath to reduce viscosity. The samples were fortified with 100 μL of standard solution containing chlorpyrifos, λ-cyhalothrin, cypermethrin and deltamethrin at 5.0 mg L^{-1} and homogenized. Four mL of aqueous phase and 8.0 mL of extraction solvent were added to the fortified samples (Table 1). The system was homogenized (Table 1); samples were left resting and after phase separation were chilled to −20 °C for 6 h. The organic phase of the biphasic system was removed and cleaned up in a polyethylene column containing florisil (Table 1) and 1.5 g of sodium sulfate. The obtained extract was recovered in 10.0 mL volumetric flask, containing 100 μL of bifenthrin standard solution (5.0 mg L^{-1}) (internal standard) and stored in the freezer until analysis by GC-ECD. Extracts were dried in rotary evaporator and recovered in 100 μL of acetonitrile for GC–MS analysis.

Table 1
Variables evaluated in the univariate optimization of pesticide LLE–LTP in honey samples.

Variables	Levels
Sample amount (g)	1, 2, 3 and 4
Ionic strength	4.0 mL water
	4.0 mL 0.5 mol L^{-1} NaCl solution
Extraction solvent (8.0 mL)	Acetonitrile (8.0 mL)
	Acetonitrile/methanol (6.5 mL/1.5 mL)
	Acetonitrile/ethyl acetate (6.5 mL/1.5 mL)
	Acetonitrile/ethyl acetate (4.0 mL/4.0 mL)
	Hexane/ethyl acetate (4.0 mL/4.0 mL)
Homogenization techniques	Vortex (30 s)
	Ultrasound (10 min)
	Table estirring (40 min)
	Manual
Florisil amount (g)	0, 1 and 2

2.3. Laboratory equipment

The following equipment was used for sample preparation: (1) ultrasonic bath (Unique, São Paulo, Brazil) (150 W and 25 kHz), (2) table estirring (Tecnal TE – 420, São Paulo, Brazil) (175 oscillations per minute at 25 °C) and (3) a vortex.

2.4. GC-ECD

A Shimadzu (CG-2014) gas chromatograph equipped with a electron-capture-detector (ECD) equipped with auto-injector AOC-20I and a HP-5 capillary column (Agilent Technologies), stationary phase of 5% phenyl – 95% dimethyl-siloxane (30 m × 0.25 mm i.d., 0.1 μm film thickness) and nitrogen as carrier gas (1.2 mL min^{-1}). Injector temperature 280 °C and detector temperature 300 °C, initial column temperature of 150 °C, programmed at 20 °C/min to 250 °C, followed by a second rate at 10 °C/min to 290 °C held for 5 min. One microliters of sample was injected into the gas chromatograph with split ratio set at 1:5.

2.5. GC–MS

GC–MS analyses were performed on a Shimadzu GCMS-QP5050A equipped with an AOC-5000 auto-injector and a HP-5 capillary column (Agilent Technologies), with stationary phase of 5% phenyl – 95% dimethyl-siloxane (30 m × 0.25 mm i.d., 0.1 μm film thickness), using helium as carrier gas (1.2 mL min^{-1}). The temperature program is similar to the GC-ECD program. The transfer-interface temperature was 290 °C and a splitless injection was used. The mass detector operated using electron-impact ionization in scan mode (30–600 m/z) and selected ion monitoring mode (SIM). Identification of extract components was carried out by comparing with Wiley spectral data collections (Wiley 330,000), with literature data and also with data of pesticide standard solutions.

2.6. Method validation

LLE–LTP analytical parameters, including selectivity, limit of detection (LOD), limit of quantification (LOQ), method linearity, precision and accuracy were evaluated in compliance with protocols by the major regulatory agencies (Agência Nacional de Vigilância Sanitária, 2000; European Commission, 2000; Instituto Nacional de Metrologia, Normalização e Qualidade Industrial, 2003).

2.7. Technique application on honey samples

The validated method was applied to pesticide residue determination in 11 samples of honey produced in the state of Minas Gerais, Brazil.