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Determination of organochlorine pesticide residues in honey from the central zone of Portugal and the Valencian community of Spain☆

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Abstract

In this study nine organochlorine pesticide residues (α-, β-, and γ-hexachlorocyclohexane (HCH), hexachlorobenzene (HCB), aldrin, *p*,*p*′-DDE, *p*,*p*′-DDD, *o*,*p*′-DDT, and *p*,*p*′-DDT) in forty nine samples of honey collected from markets of Portugal and Spain during 2001 and 2002, respectively, were evaluated. For this evaluation, three analytical procedures were studied. The analytical procedure, based on LLE extraction with ethyl acetate followed by gas chromatography–electron-capture detection (GC–ECD) for quantification, and mass spectrometry (GC–MS) for confirmation, has been selected. Recoveries of spiked samples ranged from 68%, for β-HCH, and 126% for *p*,*p*′-DDT, for fortification levels between 10 and 100 μg/kg, and 64%, for α-HCH, and 143% for γ-HCH for fortification levels between 20 and 200 μg/kg. Limits of quantification, using GC–ECD, were from 0.01 and 0.10 mg/kg, and limits of detection between 0.001 and 0.02 mg/kg. Fourteen Valencian samples were contaminated, containing residues of HCB or/and HCH isomers. The frequency of detection was 56% for Spanish samples. In Portugal, 23 samples were contaminated, what means 95.8%. In Spanish samples, concentrations range from nd to 0.03 mg/kg for HCB, and nd to 2.24 mg/kg for HCH-total. The mean concentration and standard deviation were 0.017 ± 0.011 mg/kg for HCB, and 0.579 ± 0.747 mg/kg for HCH-total, contributing the γ isomer with the highest values. The samples from Portugal showed higher levels. Levels of HCB ranged from nd to 0.39 mg/kg. HCH-total ranged from nd to 4.86 mg/kg, and DDT-total from nd to 0.658 mg/kg. Mean concentration and standard deviation were 0.09 ± 0.116 mg/kg for HCB, 1.357 ± 1.30 mg/kg for HCH-total, and 0.143 ± 0.193 mg/kg for DDT-total.

Keywords: Organochlorine; Pesticides

1. Introduction

The occurrence of organochlorine compounds in the food chain has already been reported in several studies. This class of organic compounds constitutes one of the most important groups of dangerous organic contaminants. The environmental contamination by persistent organochlorine pesticide (OCPs) residues has been widely documented in several countries, such as Portugal and Spain in medicinal plants, water, milk, and biological fluids [1–4]. Due to its lipophilic nature, OCPs enter into the food chain by accumulating in fats, but can also be present in non-fatty products, even those which have not been treated directly with them [5]. They can be present in honey because of the plant treatment or by migration from wax to honey. Since honeybees travel long distances and come close to many plants, honey may be an easily accessible environmental pollution indicator [6,7]. Pesticide determination in bee products is necessary to monitor contamination and guarantee consumer health [7]. Honey is a natural product that must be free of any chemical

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contaminants and safe for human consumption, because in some countries is traditionally used in child, old and ill people and its quality must be proved [8].

Many methods have been reported for the determination of pesticides in honey. However, these samples pose substantial analytical problems, particularly to high percentage of sugar [7] or, in some cases, intensive coloration due to pigments. Most methods used for OCPs are based on liquid–liquid extraction (LLE) performed with water non-miscible solvents, such as ethyl acetate [9], petroleum ether [10], or *n*-hexane [11,12], dichloromethane [13], or miscible solvents, such as methanol [8,14]. Solid-phase extraction (SPE) with C_{18} cartridge [8], Florisil [11], polystyrene-divinylbenzene sorbent copolymer [15], solid-phase microextraction (SPME) [16] has also been applied to honey samples. Sometimes, after LLE extraction a clean-up with different adsorbents may be necessary, Florisil [10,12,14] or silica or activated carbon and silica gel [13]. GC–ECD has been widely applied as the preferred technique for the identification and quantification of OC pesticides [8–17] due its high sensitivity to molecules that contains electronegative atoms, but requires subsequent confirmation by GC–MS in mode electron impact in which molecules are bombarded by high energy, 70 eV [7].

The purpose of this work was to develop a rapid and liquid–liquid extraction method for the analysis of nine organochlorine pesticide residues, α-, β-, and γ-hexachlorocyclohexane (HCH), hexachlorobenzene (HCB), aldrin, *p*,*p*′-DDE, *p*,*p*′-DDD, *o*,*p*′-DDT, and *p*,*p*′-DDT) in honey followed by GC–ECD and GC–MS, and evaluate the level of contamination with OCP residues.

2. Experimental

2.1. Chemicals

Pesticide standards were purchased from Dr. Eherstörfer (Augsburg, Germany). Pethroleum ether, diethyl ether, *n*-hexane, and ethyl acetate (for residue analysis) were obtained from Carlo Erba (Milan, Italy). Stock solution of each pesticide was prepared separately at 500 mg/l in *n*-hexane, except for β-HCH, which was prepared in *n*-hexane-acetone (95:5, v/v), and γ-HCH, which was supplied at a concentration of 10 μg/ml in cyclohexane. Standard solutions were prepared at 10 mg/l, and then stored at 4 °C. Working solutions were prepared between 0.2 μg/ml for HCB and 2 μg/ml for *p*,*p*′-DDT.

Deionized water was prepared from a Milli-Q system (Millipore, Bedford, MA, USA). Florisil was obtained from Fluka (USA), and activated at 300 °C/3 h in a furnace, cooled in a dessicator, and deactivated to 2% with water.

2.2. Apparatus

One rotary vacuum evaporator from Heidolph VV 2001 (Kelheim, Germany) and two mechanical shakers for separatory funnels (Agitelec, J. Toulemond, Paris; Edmund Bühler 7400 Tubigen KL2, Germany) were used. Glass minicolumns, 100 mm × 8 mm i.d., obtained from Normax (Portugal) were used.

2.3. Sampling

A total of forty-nine honey samples were purchase in different local markets, 25 from Valencian community in Spain, and 24 from central zone of Portugal, collected in November 2002 and during 2001, respectively. Honey samples were provided to the markets from the beekeeper associations of these respective areas ensuring that they were provided in the zone of study.

2.4. Recoveries

For recovery studies, in method 1, 0.25 ml of a working solution containing between 0.2 μg/ml for HCB and 2 μg/ml for *p*,*p*′-DDT were added to 5 g of honey, and allowed to stand for 15 min before extraction, for three replications. For method 2, a similar procedure was followed, adding 0.2 ml of the working solution to 4 g of honey. In method 3, 0.25 ml were added to 5 g. For method 1, another fortification level was evaluated. In this case, 0.5 ml of the working solution containing between 0.2 μg/ml for HCB and 2 μg/ml for *p*,*p*′-DDT were added to 5 g of honey, over three replications.

2.5. Extraction and clean-up procedures

2.5.1. Method 1

Five grams of honey was dissolved with 50 ml 4% aqueous solution of sodium sulphate and extracted with three portions of ethyl acetate (20, 15, and 15 ml). When emulsion is formed it was broken centrifuging at 3000 rpm for 10 min. The organic phase was filtered by anhydrous sodium sulphate, and concentrated to 2.5 ml for analysis, in graduated centrifuge tube, under nitrogen.

2.5.2. Method 2

Four grams of honey was dissolved with 25 ml of deionized water and extracted with three portions of 15 ml light petroleum by mechanical shaking at 55 rpm for 15 min. When emulsion is formed it was quickly broken centrifuging at 3000 rpm for 10 min. The organic phase was filtered by anhydrous sodium sulphate, and concentrated to 1 ml for analysis. The concentrated extract was loaded onto a minicolumn filled with Florisil (2 g) and anhydrous sodium sulphate (1 g), prerinsed with 10 ml light petroleum. The elution was performed with 25 ml of 5% of diethyl ether in petroleum ether. The eluate was concentrated to dryness in graduated centrifuge tube and redissolved in 500 μl of *n*-hexane.

2.5.3. Method 3

Five grams of honey was dissolved with 10 ml of deionized water and extracted with 3 × 5 ml of *n*-hexane by magnetic

stirring for 15 min. When emulsion is formed it was broken centrifuging at 3000 rpm for 10 min. The organic phase was filtered by anhydrous sodium sulphate, and concentrated to 1 ml. The concentrated extract was loaded onto a minicolumn filled with Florisil (2 g) and anhydrous sodium sulphate (1 g), pre-washed with 10 ml *n*-hexane. The pesticides were eluted from the column with 25 ml of 15% of diethyl ether in *n*-hexane. The eluate was concentrated to 1 ml, under a steam of nitrogen.

2.6. Gas chromatography with electron-capture detector

A Carlo Erba Mega HRGC 5300 equipped with ^{63}Ni electron-capture detector was used for quantification. One fused silica capillary column (30 m × 0.25 mm × 0.25 μm with chemically bonded phase DB-5 (J&W Scientific)) was used. One microliter (μl) of sample was injected in the splitless mode and the splitter was opened after 60 s. Chromatographic conditions were at temperature 280 °C for the detector, 220 °C for the injector, and 150 °C held for 1 min and programmed at 10 °C/min to 210 °C held for 1 min and programmed at 3 °C/min to 230 °C, held for 5 min and finally programmed at 3 °C/min to 250 °C, held for 3 min. Gases used were: carrier gas helium N60 carrier at 2 ml/mim, split valve 100 ml/min, purge valve 2 ml/min, make-up gas, nitrogen at 120 kPa. One integrator Spectra-Physics 4270 (Darmstadt, Germany) was used to integrate peak areas. Quantifications were made accordingly Lino et al. [1], using the external standard method, comparing peak areas of the standard with the peaks of extracts at the same retention time.

2.7. Gas chromatography with mass spectrometry

GC confirmatory analysis was carried out on a Trace GC–MS 2000 (Thermo Finnigan, Manchester, UK) system with Xcalibur software-based data acquisition. The injector temperature was 220 °C and the detector one was 280 °C. Sample was injected in the splitless mode and the splitter was opened after 60 s. A fused silica capillary column (30 m × 0.25 mm × 0.25 μm with chemically bonded phase DB-5) was used. This temperature was 150 °C held for 1 min and programmed at 10 °C/min to 210 °C held for 1 min and programmed at 3 °C/min to 230 °C, held for 5 min and finally programmed at 3 °C/min to 250 °C, held for 3 min. The MS ionization potential was 70 eV, the temperatures were as follows: ion source 250 °C, transfer line 200 °C and analyser 230 °C. Analysis was performed in the selected-ion monitoring (SIM) mode monitoring specific ions of each analyte as it is shown in Table 1. The most intense ion was used for quantification and the second and third ion for confirmation.

3. Results and discussion

3.1. Remarks on methods

The ECD system was linear in the range between LOQ and 100 LOQs and correlations were better than 0.994, except for β-HCH, that was 0.972.

Repeatibility and reprodutivity were calculated making three replicate determinations at limit of quantification levels in the same day with relative standard deviations (R.S.D.s) of 5–15%, and in five days with a R.S.D. of 7–14%.

The MS detector was linear in the concentration range between LOQ and 100 times LOQ and correlations were better than 0.999. Repeatibility and reprodutivity were calculated as it has been previously described with R.S.D.s from 6 to 9% and 12 to 15%, respectively.

With a view to obtaining a more adequate method for the quantification of the organochlorine pesticides three methods were evaluated. Untreated samples and fortified honey samples in the range of 10–100 μg/kg were analysed by three different methods, using LLE with different solvents, ethyl acetate, light petroleum, or *n*-hexane. When light petroleum or *n*-hexane was used, a clean-up procedure with Florisil was also applied. Recoveries and relative standard deviation obtained with the three methods are shown in Table 2. The best results, for fortification levels between 10 and 100 μg/kg, were obtained dissolving honey in sodium sulphate and extracting with ethyl acetate (method 1). Results obtained for dissolution in water, extraction with petroleum ether, and clean-up with Florisil were not adequate. Recovery values are very low for α-HCH, β-HCH, *o*,*p*′-DDT and *p*,*p*′-DDT, and very high for HCB. Using *n*-hexane, as extraction solvent, also the recoveries, for all compounds,

Table 1
SIM conditions of organochlorine pesticides detected by GC–MS

Peak number	Pesticides	t_R (min)	Molecular mass	Quantitation ions (*m*/*z*)	Confirmation ions no. 1 (*m*/*z*)	Confirmation ions no. 2 (*m*/*z*)
1	α-HCH	12.93	288	181	109	219
2	HCB	13.33	282	284	282	286
3	β-HCH	13.84	288	109	181	219
4	γ-HCH	14.18	288	109	181	219
5	Aldrin	19.44	362	263	261	265
6	*p*,*p*′-DDE	25.62	316	246	318	316
7	*p*,*p*′-DDD	28.08	318	235	199	165
8	*o*,*p*′-DDT	29.09	352	235	270	272
9	*p*,*p*′-DDT	31.74	352	235	270	272

Table 2
Recoveries obtained with the three methods in honey [mean ± R.S.D. (%) (n = 3)]

Peak no.	Pesticide	Fortification level (μg/kg)	Method 1	Method 2	Method 3
1	α-HCH	25	82 ± 10	25 ± 12	235 ± 23
2	HCB	10	110 ± 8	702 ± 1012	153 ± 17
3	β-HCH	50	68 ± 21	43 ± 15	131 ± 21
4	γ-HCH	25	82 ± 10	84 ± 35	137 ± 16
5	Aldrin	50	111 ± 31	75 ± 34	128 ± 5
6	*p,p′*-DDE	50	77 ± 6	73 ± 36	150 ± 25
7	*p,p′*-DDD	50	86 ± 16	75 ± 38	140 ± 15
8	*o,p′*-DDT	50	85 ± 22	51 ± 24	170 ± 25
9	*p,p′*-DDT	100	126 ± 17	53 ± 25	168 ± 37

are inconsistently higher, probably due to matrix effects. The method 1 was also applied to spiked samples between 20 and 200 μg/kg. Table 3 gives recoveries of honey samples spiked at two levels, R.S.D., LODs, and LOQs. The mean recoveries vary between 68%, for β-HCH, and 126%, for *p,p′*-DDT for fortification levels between 10 and 100 μg/kg, and 64%, for α-HCH, and 143% for γ-HCH for fortification levels between 20 and 200 μg/kg. R.S.D.s ranged from 6 and 31% for the first fortification level, and from 1 and 23% for the second one. The LODs and LOQs obtained by GC–ECD ranged from 0.001 to 0.02 mg/kg and from 0.01 to 0.10 mg/kg, respectively, whereas by GC–MSD, the LODs were from were 0.003–0.01 mg/kg and the LOQs 0.01–0.04 mg/kg.

3.2. Application to real samples

This study shows that 14 Valencian samples were contaminated, containing residues of HCB or/and HCH isomers. The frequency of detection was 56% for Spanish samples. In Portugal, 23 samples were contaminated, what means 95.8%. Only aldrin residues were not detected in Portuguese samples.

Table 4 shows the results obtained with Portuguese and Valencian samples. In Spanish samples, concentrations range from nd to 0.03 mg/kg for HCB, and nd to 2.24 mg/kg for HCH-total. The mean concentration and standard deviation were 0.017 ± 0.011 mg/kg for HCB, and 0.579 ± 0.747 mg/kg for HCH-total, contributing the γ isomer with the highest values, 0.72 ± 0.76 mg/kg. Mean levels of α- and β-HCH isomers were 0.055 ± 0.035 mg/kg, and 0.175 ± 0.078 mg/kg, respectively. Samples from Portugal showed higher levels. Levels of HCB ranged from nd to 0.39 mg/kg. HCH-total ranged from nd to 4.86 mg/kg. Figs. 1 and 2 show the chromatograms of spiked sample and Portuguese sample obtained by GC–ECD and GC–MS, respectively. Mean concentration and standard deviation were 0.09 ± 0.116 mg/kg for HCB, 1.357 ± 1.30 mg/kg for HCH-total, contributing the γ isomer with the highest values, 1.30 ± 1.117, 0.656 ± 1.05 mg/kg for β-HCH, and 0.141 ± 0.072 mg/kg for α-HCH. *p,p′-DDT* is released into the environment and begins to degrade and can be found in isomers, *o,p′*-DDT, and analogous, *p,p′*-DDE, its main metabolite and the most persistent one, and *p,p′*-DDD. DDT-total ranged from nd to 0.658 mg/kg and its mean concentration and standard deviation were 0.143 ± 0.193 mg/kg, presenting DDE the highest mean level, 0.186 ± 0.25 mg/kg. *p,p′*-DDD and *p,p′*-DDT were found both at 0.065mg/kg, with SD 0.007. *o,p′*-DDT was at 0.06 ± 0 mg/kg.

Table 4
Mean concentration ± R.S.D. (mg/kg) and frequency of detection (%) in Portuguese and Valencian honey samples

Pesticides	Mean concentration ± S.D.		Frequency of detection	
	Spain	Portugal	Spain	Portugal
α-HCH	0.055 ± 0.035	0.141 ± 0.072	8	50
HCB	0.017 ± 0.011	0.09 ± 0.116	12	54.2
β-HCH	0.175 ± 0.078	0.656 ± 1.05	8	45.8
γ-HCH	0.72 ± 0.76	1.30 ± 1.17	36	66.7
p,p′-DDE	nd	0.186 ± 0.25	0	25
p,p′-DDD	nd	0.065 ± 0.007	0	8.3
o,p′-DDT	nd	0.06 ± 0	0	4.2
p,p′-DDT	nd	0.065 ± 0.007	0	8.3
HCH-total	0.579 ± 0.747	1.357 ± 1.30	48	91.6
DDT-total	nd	0.143 ± 0.193	0	41.75

Frequency of detection was also lower in Valencian samples. Alfa and β-HCH were found in 2 samples (8%), HCB in 3 (12%), and γ isomer in 9 (36%), and HCH-total in 12 (48%). In Portuguese samples, HCH isomers presented the highest frequency of detection, 16 (66.7%), 12 (50%), and 11 (45.8%) samples, for γ-, α-, and β-HCH, respectively. HCB was detected in 13 (54.2%) samples. Among DDT, isomers and analogous, DDE was the most frequently detected, 6 (25%) samples, followed by *p,p′*-DDD and *p,p′*-DDT, both

Table 3
Recoveries obtained at two fortification levels, LOD, and LOQ for method 1 in honey [mean ± R.S.D. (%) (n = 3)]

Pesticide	Fortification level (μg/kg)	Method 1	Fortification level (μg/kg)	Method 1	LOQ (mg/kg) GC–ECD	LOD (mg/kg) GC–ECD	LOQ (mg/kg) GC–MS	LOD (mg/kg) GC–MS
α-HCH	25	82 ± 10	50	64 ± 6	0.03	0.004	0.01	0.003
HCB	10	110 ± 8	20	78 ± 10	0.01	0.004	0.02	0.006
β-HCH	50	68 ± 21	100	74 ± 10	0.06	0.001	0.01	0.002
γ-HCH	25	82 ± 10	50	143 ± 1	0.05	0.001	0.01	0.003
Aldrin	50	111 ± 31	100	75 ± 23	0.06	0.005	0.02	0.006
p,p′-DDE	50	77 ± 6	100	80 ± 6	0.06	0.01	0.04	0.01
p,p′-DDD	50	86 ± 16	100	91 ± 19	0.06	0.01	0.04	0.01
o,p′-DDT	50	85 ± 22	100	81 ± 12	0.06	0.02	0.04	0.01
p,p′-DDT	100	126 ± 17	200	71 ± 2	0.10	0.01	0.04	0.01

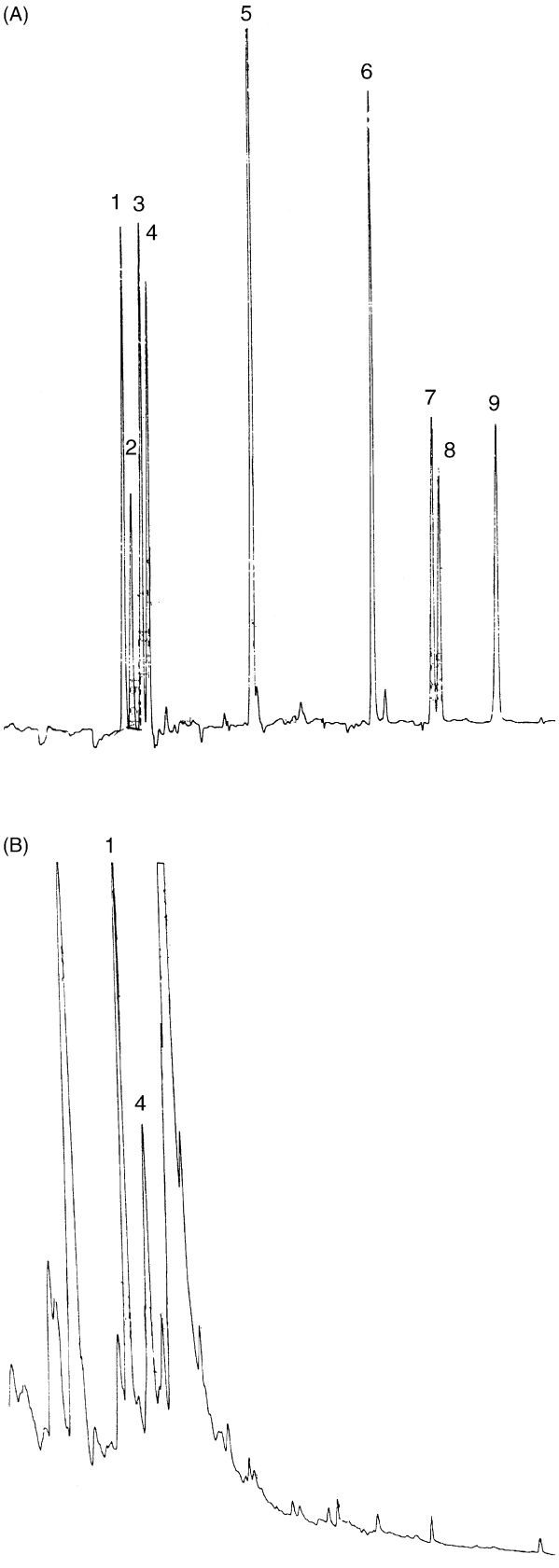

Fig. 1. GC–ECD chromatograms of (A) untreated honey sample spiked at five times the LOQ (peak identification as Table 2), and (B) a P14 honey sample that contains 0.27 mg/kg of α-HCH and 0.05 mg/kg of γ-HCH.

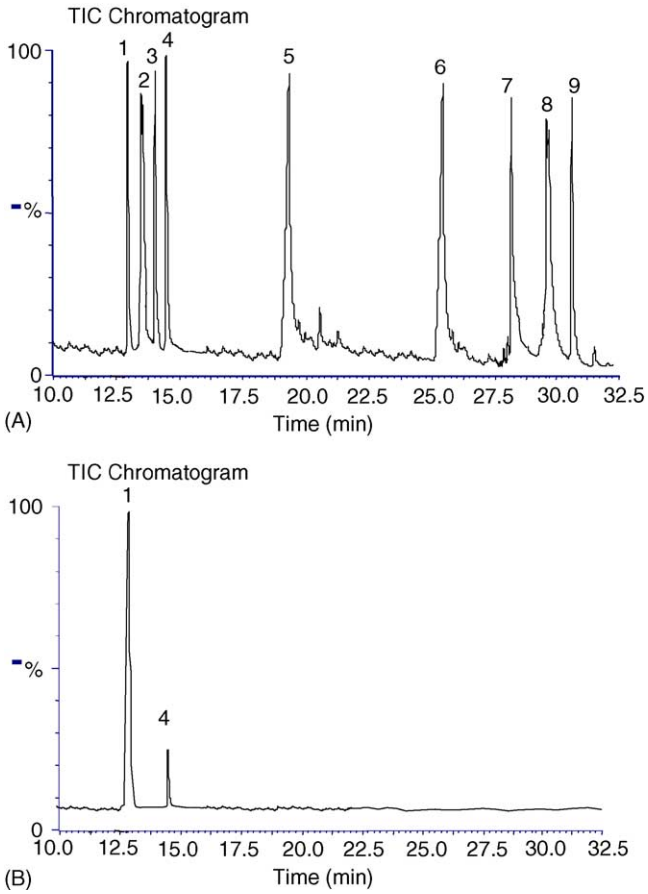

Fig. 2. Total ion current GC–MS chromatograms of (A) untreated honey sample spiked at five times the LOQ (peak identification as Table 1), and (B) a P14 honey sample that contains 0.27 mg/kg of α-HCH and 0.05 mg/kg of γ-HCH.

with 2 (8.3%), and *o,p′*-DDD with 1 (4.2%) sample. DDT-total was detected in 10 (41.75%) samples, while 22 (91.6%) samples had HCH-total.

According to European Union (EU) Regulations, honey as a natural product, must be free of any chemical contaminants and safe for human consumption [18]. On this basis, only 12 samples are agreed with this regulation, one from Portugal, and eleven from Spain.

4. Conclusions

The present procedure, which involves a rapid and liquid–liquid extraction with ethyl acetate and GC–ECD and GC–MS, requires less solvent than the traditional ones and a small amount of sample (4 g), with the consequent reduction in co-extracts, and provides satisfactory recoveries, repeatability and reproducibility.

The described method has used to evaluate contamination of honey samples from central zone of Portugal and Valencian community in Spain, and it was clear than levels of OCPs studied are higher in Portuguese samples.

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