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Assessment of Pesticide Residues in Honey Samples from Portugal and Spain

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Fifty samples of honey collected from local markets of Portugal and Spain during year 2002 were analyzed for 42 organochlorine, carbamate, and organophosphorus pesticide residues. An analytical procedure based on solid-phase extraction with octadecyl sorbent followed by gas chromatography−mass spectrometry (GC−MS), for organochlorines, and by liquid chromatography−atmospheric pressure chemical ionization-mass spectrometry (LC−APCI-MS), for organophosphorus and carbamates, has been developed. Recoveries of spiked samples ranged from 73 to 98%, except for dimethoate (40%), with relative standard deviations from 3 to16% in terms of repeatability, and from 6 to 19% in terms of reproducibility. Limits of quantification were from 0.003 to 0.1 mg kg^{-1}. Most of the pesticides found in honey were organochlorines. Among them, γ-HCH was the most frequently detected in 50% of the samples, followed by HCB in 32% of the samples and the other isomers of HCH (α-HCH and β-HCH) in 28 and 26% of the samples, respectively. Residues of DDT and their metabolites were detected in 20% of the samples. Of the studied carbamates, both methiocarb and carbofuran were detected in 10% of the samples, pirimicarb in 4% and carbaryl in 2%. The only organophosphorus pesticides found were heptenophos in 16%, methidathion in 4%, and parathion methyl in 2% of honey samples. Results indicate that Portuguese honeys were more contaminated than Spanish ones. However, honey consumers of both countries should not be concerned about the amounts of pesticide residues found in honeys available on the market.

INTRODUCTION

Pesticides play a beneficial role in agriculture, because they help to combat the variety of pest that destroy crops, even though small amounts of pesticide residues remain in the food supply, constituting a potential risk for the human health, because of their sub-acute and chronic toxicity (*1*). The most widely used pesticides are organophosphorus and carbamates, which have almost completely replaced organochlorine pesticides (*2*). The extensive distribution of these groups of pesticides causes bees that have been fed on contaminated blossom to transfer pesticide residues into honey and finally to the consumer (*1*, *2*).

Organochlorine pesticides have been restricted or banned in agriculture since 1978 in North America and Europe because of their persistence and bioaccumulation in the environment. However, these pesticides are still frequently found in soil, from which they continue to cycle through the environment, as soil is a potential source to the atmosphere by way of volatilization and to water, plants, and animals by their movement via runoff (*3*, *4*). Different studies demonstrated the bioaccumulation of organochlorine from contaminated soil to aerial and root tissues of different plants (*5*) and to organisms (*6*, *7*), which can bioconcentrate these fat-soluble pesticides at 10−1000 times the level found in the surrounding environment.

The presence of pesticide residues in honey has impelled the need for setting up monitoring programs to determine the proper assessment of human exposure to pesticides making possible to take policy decisions in the interest of health hazard (*8*). Different national regulations have established maximum concentrations of pesticide residues (MRLs) permitted in honey, but the lack of homogeneity causes problems in international marketing and trade. As an example, Germany, Italy, and Switzerland have set MRLs for amitraz, bromopropylate, coumaphos, cyamizole, flumetrine, and fluvalinate, which oscillate between 0.01 and 0.1 mg kg^{-1} in Germany, between 5 and 500 mg kg^{-1} in Switzerland, and are of 10 mg kg^{-1} in Italy (*9*). Up to now, maximum limits of pesticide residues in honey are not included in the *Codex Alimentarius* (*10*). The European Union (EU) legislation has regulated the MRLs for three acaricides: amitraz, coumaphos, and cyamizole, which are 0.2, 0.1, and 1 mg kg^{-1}, respectively (*11*). The U. S. Environmental Protection Agency (*12*) has established MRLs

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for amitraz (1 mg kg^{-1}), coumaphos (0.1 mg kg^{-1}), and fluvalinate (0.05 mg kg^{-1}).

A multiresidue method able to detect as many pesticides as possible, in a relatively short time period, is crucial for an efficient monitoring program (*8*, *9*, *13*). Generally, these methods are based on the traditional liquid−liquid extraction (LLE) or solid-phase extraction (SPE). LLE main advantage is simplicity but employs a large amount of toxic solvent and is a time-consuming procedure. Much less toxic solvents are consumed by SPE, which also offers a save in sample preparation time. However, this technique has the disadvantage of being unable to handle large sample volumes. Both, LLE (*14*−*16*) and SPE (*17*−*19*) have been selected in various multiresidue methods for extracting organochlorine, organophosphorus, carbamate, and pyrethroid pesticides in honey.

The detection of pesticides is accomplished by gas chromatography (GC) or liquid chromatography (LC). Until now, GC has been the most widely used technique, because its high separation power and availability of selective detectors as electron capture (ECD), nitrogen phosphorus (NPD), and mass spectrometry (MSD) detectors. In recent years, LC has emerged as an excellent alternative technique, especially for polar and thermolabile pesticides, which are not directly determinable by GC. Mass spectrometry (MS) employing atmospheric pressure ionization (API) is becoming the detection system of choice for liquid chromatography (LC), because its versatility, high selectivity, and spectral evidence of individual solutes (*20*, *21*).

As it has been previously reviewed (*13*, *22*), pesticide residues programs for monitoring honey are still scarce. Most studies concentrate efforts to determine residues of acaricides that are used to control *Varroa jacobsoni*, a parasitic mite that affects honeybee colonies (*8*, *23*−*25*). Depending on the regulation of each country and beekeepers practices, the most often detected acaricides are bromopropylate, coumaphos, and fluvalinate. Only a few studies have been focussed on pesticides used for crop protection and introduced into hives by contaminated bees and wax (*14*, *26*, *27*). Most samples analyzed in Jordan during 1995 contained residues of organochlorine pesticides such as α-HCH, *β*-HCH, and lindane, and only some of them were contaminated by organophosphorus pesticides. Pyrethroids and nitrogen-containing pesticides were not found in any sample (*26*). In contrast, compared to the previous report, levels and frequency of organophosphorus and carbamate pesticides were relatively higher in honey samples analyzed in India from 1993 to 1997 (*27*).

The first aim of this study is to extend the extraction method previously proposed (*19*) to determine twenty eight organophosphorus and five carbamates by LC/APCI/MS and nine organochlorines by GC−MS. Validation and optimization of the SPE procedure is presented in terms of recoveries, precision, and limits of quantification. The method was applied to monitor 50 honey samples from various floral origins collected in local markets of Portugal and Spain during year 2002.

MATERIALS AND METHODS

Reagents and Chemicals. Pesticide standards were purchased from Sigma-Aldrich (Madrid, Spain) (see **Tables 1** and **2**). Methanol (HPLC-grade), petroleum ether, dichloromethane, hexane, and ethyl acetate (organic trace analysis) were obtained from Merck (Darmstadt, Germany). Stock solution of each pesticide were prepared at 1000 mg L^{-1} in methanol and then stored at 4 °C. The carbamate and organophosphorus stock solutions were stored for 3 months, and the organochlorine solutions were stored for 1 year. Working solutions were prepared daily by appropriate dilution of aliquots obtained from stock solution in methanol. Deionized water (<18 M cm resistivity) was obtained from a Milli-Q SP Reagent Water System (Millipore, Bedford, MA). C_{18} solid phase (particle diameters of approximately 55 μm and pore diameter 60 Å) was acquired from Análisis Vínicos (Tomelloso, Spain).

Table 1. SIM Conditions for Determining Pesticides by LC–APCI-MS

time	ion (*m*/*z*)	pesticide	frag/(V)	dwell time (ms)
0.0–7.0	208	monocrotophos	30	98
	214	dimethoate		
	272	vamidothion		
	284	phosphamidone		
7.0–9.0	143	carbaryl	80	199
	163	carbofuran		
9.0–11.0	138	paraoxon	60	199
	166	pirimicarb		
11.0–12.3	235	heptenophos	40	400
12.5–14.5	157	fosmet	30	199
	287	methidathion		
14.5–17.5	167	methiocarb	40	199
	248	parathion methyl		
17.5–19.5	329	malathion	30	400
19.5–21.5	262	fenitrothion	40	400
21.5–26.0	185	azinphos ethyl		400
26.0–31.0	169	quinalphos	40	98
	185	fenoxycarb		
	262	parathion ethyl		
	319	phenthoate		
31.0–37.0	153	fonofos	50	98
	275	diazinon		
	361	coumaphos		
	263	fenthion		
37.0–41.0	169	foxim	40	132
	338	phosalone		
	372	pyrazophos		
41.0–45.0	302	chlorpyriphos methyl	60	400
45.0–51.0	207	profenofos	40	400
51.0–60.0	304	pirimiphos ethyl	70	98
	330	chlorpyriphos ethyl		
	351	bromophos		
	451	temephos		

Table 2. SIM Conditions of Organochlorine Pesticides Detected by GC-MS

			selected ions, *m*/*z* (average relative intensities, %)		
pesticides	*tr*(min)	mol weight	quantitation ion	confirmation ion 1 deg	confirmation ion 2 deg
α-HCH	12.93	288	181 (100)	109 (70)	219 (90)
HCB	13.33	288	284 (100)	282 (54)	286 (80)
β-HCH	13.84	288	109 (100)	181 (85)	219 (70)
γ-HCH	14.18	288	181 (100)	109 (64)	219 (90)
Aldrin	19.44	362	263 (100)	261 (60)	265 (70)
pp′-DDE	25.62	316	246 (100)	318 (70)	316 (56)
pp′-DDD	28.08	318	235 (100)	237 (64)	165 (40)
op′-DDT	29.09	352	235 (100)	237 (66)	165 (30)
pp′-DDT	31.74	352	235 (100)	237 (65)	165 (60)

Sampling. Twenty four honey samples were collected from different local markets of Coimbra (Portugal), all of them were of multi flower origin. Twenty six honey samples were taken from local markets of Valencia, those samples were from different floral origins, thyme, multi flowers, rosemary, heather, lavender, orange blossom, lemon, acorn, and eucalyptus. Four of them, V22−V25 (see **Table 5**), were ecological honeys. Both Portuguese and Spanish honeys were locally produced. These samples were stored in their original containers (always glass jars) at room temperature in a dark place.

Extraction Procedure. Honey (5 g) was mixed with 50 mL of water and agitated by a stir bar for 10 min. At the same time, 0.5 g of C_{18} sorbent was introduced into a 100 × 9 mm ID glass chromatography column with a coarse frit No. 2 and covered with a plug of silianized

glass wood at the top. The solid phase was preconditioned by passing 10 mL of methanol and 10 mL of water with the aid of a vacuum pump to avoid dryness. The sample was passed through the solid phase, after that, the retained pesticides were eluted by passing first 10 mL of ethyl acetate, followed by 4 mL of methanol, and then 1 mL of dichloromethane. The eluate was evaporated to 0.5 mL, using a gentle steam of nitrogen, and transferred quantitatively with methanol into a 1-mL volumetric flask, obtaining a final extract in 100% methanol. For the analysis, 5 μL was injected into the LC−MS system, and 1 μL into the GC−MS system.

Samples of honey for determining the limits of quantification (LOQs), recovery and precision were "pesticide free" and different from the samples studied. Recovery experiments were carried out by spiking honey samples (5 g) with volumes between 50 and 100 μL of pesticide working mixtures at appropriate concentrations in methanol. Prior to sample analysis by the proposed method, the spiked samples were let stand at room temperature for 3 h to achieve the solvent evaporation and the pesticide distribution in the honey.

Liquid Chromatography−Mass Spectrometry. The equipment used was a Hewlett-Packard (Palo Alto, CA) HP-1100 Series LC-MSD system equipped with a binary solvent pump, an autosampler, and a mass selective detector (MSD) consisting of a standard API source that can be configured as APCI. An HP Chemstation software version A.06.01 was used for LC-MS control and signal acquisition.

The chromatographic separation was carried out on a Luna C_{18} column (250 × 4.6 mm I.D., particle size 5 μm) protected by a Securityguard cartridge C_{18} (4 × 2 mm I.D.), both from Phenomenex (Madrid, Spain). The methanol/water gradient selected to separate compounds at a flow rate of 0.8 mL min^{-1} was 65% of methanol, which was increased linearly to 70% of methanol in 30 min, then increased to 80% of methanol in 20 min, and held at 80% of methanol for 10 min. Return to the initial conditions was carried out in 10 min.

The APCI interface in negative ionization mode was operated at 400 °C vaporized temperature, 6 bar pressure of nebulizer gas, 8 L min^{-1} drying gas flow-rate, 350 °C drying gas temperature, 4000 V capillary voltage, and 25 μA corona current discharged. Full-scan LC-MS chromatograms were obtained by scanning from *m*/*z* 100 to 400 with a scan time of 0.75s. Time-scheduled selected-ion monitoring (SIM) of the most abundant ion of each compound was used for quantification as it is shown in **Table 1**.

Gas Chromatography−Mass Spectometry. GC 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 splitless was opened after 60 s. A fused silica capillary column (30 m × 0.25 mm I.D., 0.25 μm) with chemically bonded phases DB-5 was used. The oven temperature was as follows: initial temperature of 150 °C, held for 1 min, increased to 230 °C at 3 °C min^{-1}, held for 5 min, and then increased to 250 °C at 3 °C min^{-1} and held for 15 min. The MS ionization potential was 70 eV, and the temperatures were as follows: ion source 250 °C, transfer line 200 °C, and analyzer 230 °C. Analysis was performed in SIM mode monitoring specific ions of each analyte as it is shown in **Table 2**. The most intense ion was used for quantification and the second and third ion for confirmation. Identification criteria was based on (a) the chromatographic retention data, and (b) the relative peak heights of the three characteristic masses in the sample peak that must be within ±20% of the relative intensity of these masses in the mass spectrum of the standard analyzed in the GC/MS system.

RESULTS AND DISCUSSION

Organophosphorus and Carbamates Analysis. A multiresidue method previously reported to analyze twenty two organophosphorus pesticides (*19*) in honey was adapted for the analysis of thirty-three pesticides, five of which are carbamates, and the others organophosphorus. As reported previously, the organophosphorus and carbamates gave intense mass spectra under negative ionization mode conditions (*19*, *28*). The calibration curves constructed were linear over the range of interest. The correlation coefficient were >0.995.

Table 3. Limits of Quantifications (LOQs) and Mean Recovery with Relative Standard Deviations (RSDs) of the Studied Pesticides by LC–APCI-MS and GC–MS

			mean recovery, % ± RSDs, % (n = 5)			
			under repeatability conditions		under reproducibility between days conditions	
peak number	pesticide	LOQ mg kg^{-1}	LOQ	5 × LOQ	LOQ	5 × LOQ
1	vamidothion	0.05	94 ± 9	93 ± 10	95 ± 10	93 ± 15
2	dimethoate	0.1	40 ± 12	42 ± 9	42 ± 15	45 ± 12
3	phosphamidone	0.01	92 ± 6	95 ± 8	91 ± 10	90 ± 11
4	carbofuran	0.02	90 ± 8	89 ± 9	88 ± 16	91 ± 12
5	monocrotophos	0.07	95 ± 9	92 ± 11	97 ± 13	90 ± 11
6	carbaryl	0.005	95 ± 7	93 ± 10	92 ± 12	97 ± 11
7	pirimicarb	0.02	75 ± 10	78 ± 12	77 ± 14	81 ± 15
8	paraoxon	0.01	82 ± 9	83 ± 11	79 ± 12	78 ± 14
9	heptenophos	0.03	89 ± 7	89 ± 9	92 ± 10	90 ± 12
10	methidathion	0.03	90 ± 9	92 ± 10	92 ± 11	97 ± 10
11	fosmet	0.08	86 ± 11	85 ± 12	87 ± 15	92 ± 12
12	parathion methyl	0.01	80 ± 9	83 ± 11	77 ± 12	80 ± 11
13	methiocarb	0.01	83 ± 8	85 ± 9	80 ± 11	81 ± 9
14	malathion	0.03	90 ± 10	92 ± 10	93 ± 17	91 ± 10
15	fenitrothion	0.02	88 ± 11	90 ± 9	85 ± 15	89 ± 9
16	azinphos ethyl	0.03	75 ± 9	74 ± 13	73 ± 17	72 ± 13
17	fenoxycarb	0.01	86 ± 8	85 ± 13	88 ± 19	87 ± 13
18	phenthoate	0.02	75 ± 9	77 ± 10	71 ± 16	76 ± 10
19	parathion ethyl	0.01	91 ± 11	93 ± 13	89 ± 17	96 ± 13
20	quinalphos	0.05	78 ± 12	79 ± 7	75 ± 18	77 ± 7
21	fenthion	0.03	95 ± 15	96 ± 14	97 ± 19	98 ± 14
22	fonofos	0.01	74 ± 14	73 ± 10	78 ± 16	72 ± 10
23	diazinon	0.02	90 ± 11	88 ± 9	92 ± 15	85 ± 9
24	coumaphos	0.02	92 ± 16	89 ± 14	94 ± 19	90 ± 14
25	foxim	0.01	79 ± 12	79 ± 10	77 ± 15	75 ± 10
26	phosalone	0.02	89 ± 9	90 ± 9	93 ± 11	91 ± 10
27	pyrazophos	0.03	86 ± 8	89 ± 7	89 ± 12	94 ± 8
28	chlorpyriphos methyl	0.04	88 ± 10	87 ± 9	87 ± 15	87 ± 15
29	profenofos	0.03	76 ± 13	78 ± 10	74 ± 16	80 ± 13
30	pirimiphos ethyl	0.01	83 ± 11	85 ± 10	80 ± 12	83 ± 13
31	bromophos	0.01	78 ± 13	79 ± 12	80 ± 19	82 ± 15
32	temephos	0.05	73 ± 11	75 ± 11	75 ± 12	77 ± 15
33	chlorpyriphos ethyl	0.03	87 ± 12	85 ± 9	83 ± 14	84 ± 12
34	α-HCH	0.003	97 ± 6	90 ± 7	93 ± 9	87 ± 10
35	HCB	0.008	92 ± 3	85 ± 9	90 ± 18	87 ± 12
36	β-HCH	0.005	95 ± 8	93 ± 5	92 ± 12	98 ± 9
37	γ-HCH	0.004	88 ± 9	91 ± 7	87 ± 12	95 ± 10
38	aldrin	0.008	79 ± 3	83 ± 7	82 ± 6	82 ± 10
39	pp′DDE	0.02	90 ± 8	93 ± 4	92 ± 11	95 ± 8
40	pp′DDD	0.02	91 ± 7	89 ± 6	87 ± 10	87 ± 9
41	op′DDT	0.02	98 ± 9	98 ± 8	96 ± 12	97 ± 11
42	pp′DDT	0.02	89 ± 8	93 ± 9	90 ± 12	91 ± 12

The precision and accuracy of the procedure obtained by analysis of five spiked honey samples at two concentration levels (the limits of quantification (LOQs) and 5 times the LOQ) are summarized in **Table 3**. Recoveries ranged from 73 to 95% with RSDs from 6 to 16% in terms of repeatability (intraday precision), and from 9 and 19% in terms of reproducibility (interday precision). Only dimethoate recovery was lower than 50%. The LOQs, also listed in **Table 3**, varied from 0.005 to 0.1 mg kg^{-1}. These values correspond to the lowest concentration of compound that gives a response that can be quantified with an interassay RSD of less than 20%. Sensitivity was good enough to ensure a reliable determination. An example of a typical LC−MS chromatogram of a sample spiked at LOQs levels of the thirty-three studied pesticides is shown in **Figure 1A**. Some pesticides coeluted at the same retention time and various peaks from the matrix are observed in the initial part of the chromatogram as it is shown in the chromatogram of an unspiked sample (**Figure 1B**), consequently, the use of individual ion chromatogram of each pesticide enabled the selective identification and quantification of doubtful peaks.

Organochlorine Analysis. The extraction method was also extended to determine nine organochlorine pesticides. Preliminary experiments were carried out to find the best eluent for

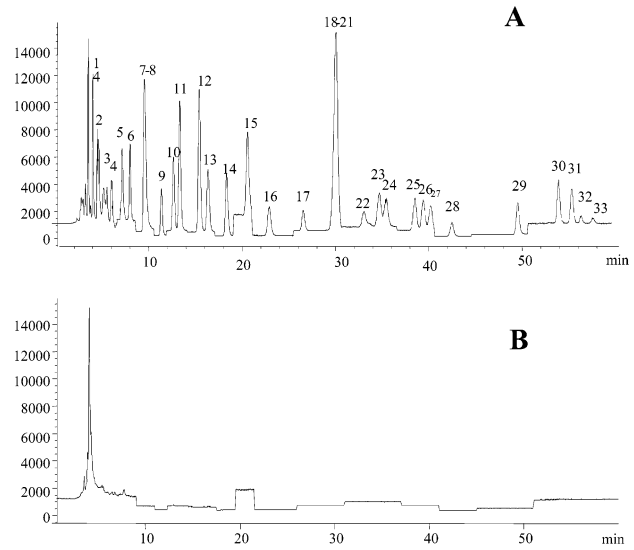

Figure 1. LC–APCI-MS chromatograms of (A) untreated honey sample spiked at 5 times the LOQ (Peak identification as **Table 3**) and (B) a non spiked honey.

organochlorine pesticides from solid phase. Methanol, hexane, petroleum ether, and the previously tested eluent (ethyl acetate, methanol, and dichloromethane) were evaluated as elution solvents. As it is summarized in **Table 4**, satisfactory results were obtained with most of the solvents tested. These results are in accordance with a previous published paper that uses SPE with C_{18} and hexane for the extraction of organochochlorines in honey (*18*). However, the selected elution (ethyl acetate, methanol, and dichloromethane) was preferred because of the high recoveries obtained without extracting large quantities of interferences and the possibility to perform a simultaneous extraction of organochlorine, organophosphorus, and carbamate pesticides. The detector response was linear in the concentration range between LOQ and 100 times the LOQ and correlations were better than 0.999. **Table 3** gives recoveries of honey samples obtained by quintuplicate analysis of spiked honeys at two concentration levels (LOQs, and 5 times LOQs). The mean

Table 4. Recoveries (%)[a] and Repeatability (RSD) of Organochlorine Pesticides from Honey Samples Spiked at 0.1 mg kg^{-1} of Each Pesticide Using Different Solvents[b]

pesticides	hexane	petroleum ether	MeOH	10 mL EA + 4 mL MeOH + 1 mL DCM
α-HCH	68 ± 8	97 ± 8	98 ± 8	97 ± 6
HCB	79 ± 9	104 ± 7	90 ± 8	92 ± 3
β-HCH	72 ± 8	101 ± 10	91 ± 7	95 ± 8
γ-HCH	79 ± 7	106 ± 10	87 ± 10	88 ± 9
Aldrin	58 ± 6	68 ± 10	58 ± 6	79 ± 3
pp′-DDE	84 ± 8	78 ± 7	74 ± 7	90 ± 8
pp′-DDD	82 ± 7	99 ± 7	92 ± 7	91 ± 7
op′-DDT	84 ± 8	77 ± 8	85 ± 8	98 ± 9
pp′-DDT	98 ± 7	77 ± 7	88 ± 7	89 ± 8

[a] Each value is the mean of five determinations. [b] DMC, dichloromethane; EA, ethyl acetate; MeOH, methanol.

recoveries for GC determined pesticides were from 79 to 98% with within-day RSDs between 3 and 9%, and day-to-day RSDs between 6 and 18%. LOQs ranged from 0.003 to 0.02 mg kg^{-1}. **Figure 2A** illustrates a chromatogram of a honey sample spiked at LOQs levels and **Figure 2B** shows a chromatogram of unspiked honey.

Monitoring Study. Table 5 shows the results obtained after analyzing 50 honey samples. Of the 24 samples analyzed in Portugal, pesticide residues were detected in 23 (95%) samples. γ-HCH was the most frequently detected pesticide and at the highest concentration; 16 (66%) samples were contaminated at levels ranging from 0.07 to 4.31 mg kg^{-1}. HCB was detected in 13 (54%) samples in the range of 0.01−0.27 mg kg^{-1}. Other HCH isomers, α-HCH and β-HCH, were detected in 12 (50%) samples at concentrations between 0.06 and 0.28 mg kg^{-1}, and 11 samples (46%) at concentrations between 0.08 and 3.49 mg kg^{-1}, respectively. Once DDT is released into the environment, it begins to degrade and can be found in two other forms, DDE and DDD. DDE is DDT's main metabolite and also the most persistent one. However, DDD is found as a breakdown product and was also independently used as a pesticide. DDE was detected in 6 (25%) samples at 0.02−0.658 mg kg^{-1}. DDD was

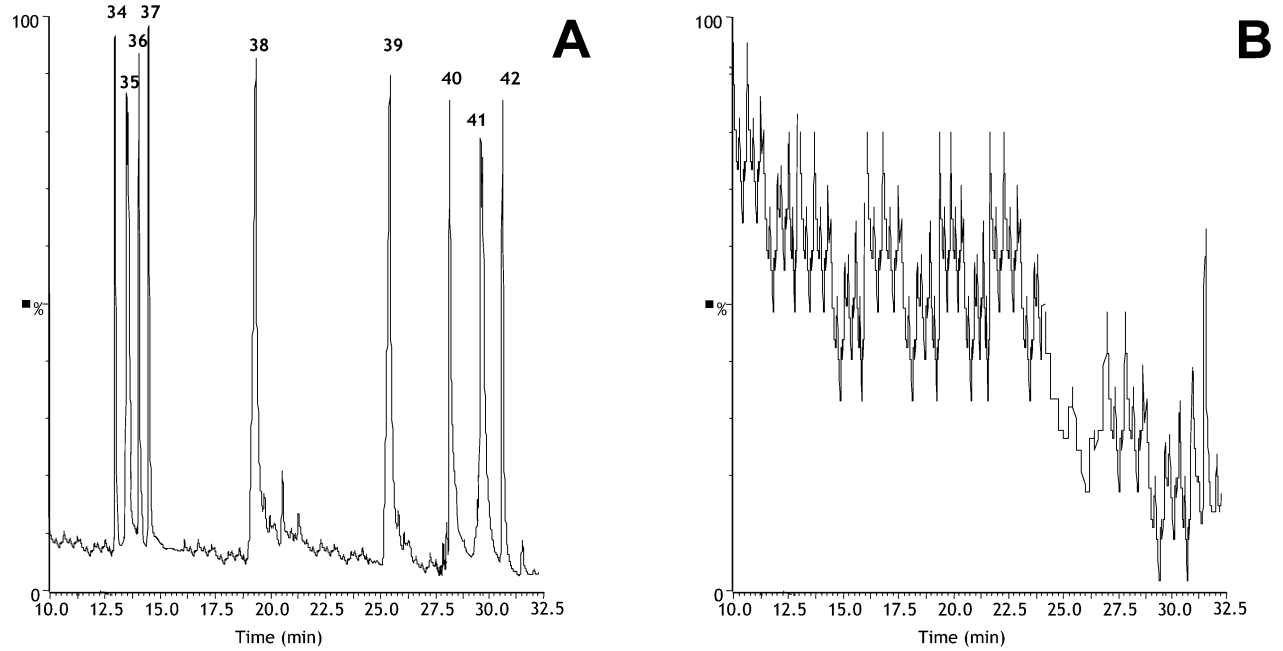

Figure 2. GC–MS chromatograms of (A) untreated honey sample spiked at 5 times the LOQ. (Peak identification as **Table 3**), and (B) a non spiked honey.

Table 5. Pesticide Concentration in Honeys Taken in Portuguese and Spanish Markets Expressed as mg kg^{-1} [a,b]

	α-HCH	HCB	β-HCH	γ-HCH	pp′-DDT[c]	heptenophos	carbofuran	pirimicarb	methidathion	parathion methyl	methiocarb	carbaryl
P1		0.06	0.18		0.268	0.08						
P2		0.06			0.033		0.11					
P3			0.21	1.34	0.027	0.11						
P4	0.1			0.07	0.07	0.05						
P5	0.13		0.08	3.01		0.06						
P6		0.04		1.39	0.658							
P7	0.12	0.01		1.78	0.09	0.08		0.02				
P8	0.04	0.05	3.49		0.06	0.06			0.05			
P9	0.22		0.3	1.96	0.112	0.23				0.01		
P10	0.11	0.27	0.15			0.06						
P11		0.05		1.12	0.06							
P12												
P13	0.09	0.02	0.15								0.01	
P14			0.16									
P15			0.2									
P16	0.28			0.05			0.05					
P17	0.23			2.1	0.06							
P18				1.56								
P19		0.03	0.55	4.31								
P20	0.13			0.18								
P21	0.06	0.17		0.1								
P22		0.02		0.78							0.027	
P23	0.19	0.04		1.06								
P24		0.17	1.75	0.13				0.071				
V1				0.11								
V2		0.01									0.023	0.016
V3		0.01					0.645					
V4												
V5				0.89								
V6				0.05								
V7				2.24					0.03		0.02	
V8		0.03		0.45								
V9											0.021	
V10			0.23									
V11												
V12												
V13												
V14												
V15				0.09								
V16				0.06			0.02					
V17							0.063					
V18												
V19												
V20												
V21												
V22	0.08											
V23			0.12						0.025		0.025	
V24	0.03			1.83					0.068		0.003	
V25				0.77								
V26												

[a] Each value is the mean of three replicate analysis. Each replicate was injected twice. [b] RSDs were ranged from 5 to 20% [c] p,p′-DDT is the sum of p,p′-DDT and its metabolites p,p′DDE and p,p′-DDD expressed as DDT.

found in 2 samples (8%) at 0.06 and 0.07 mg kg^{-1}. The isomer of DDT, pp′-DDT, was detected in 2 samples (8%) at concentrations of 0.06 and 0.07 mg kg^{-1}, and the other isomer, op′-DDT, in only one (4%) sample at 0.06 mg kg^{-1}. Of the 33 organophosphorus pesticides studied, only three of them were detected. Heptenophos was the most commonly detected in 8 (33%) samples at concentration ranging from 0.05 to 0.23 mg kg^{-1}. Just one sample was contaminated with parathion methyl at 0.01 mg kg^{-1} (4%), and a different one (4%) with methidathion at 0.05 mg kg^{-1}. A total of six samples (29%) were contaminated by carbofuran, methiocarb, and pirimicarb in the range from 0.01 to 0.11 mg kg^{-1}.

Spanish honeys were less contaminated than the Portuguese ones. Of the 26 honey samples from Spain, 16 (61%) samples were contaminated with at least one pesticide. γ-HCH was found in the greatest number of samples; 9 samples (35%) were contaminated at levels from 0.05 to 2.24 mg kg^{-1}. Three samples (11%) contained HCB in a range of 0.01−0.03 mg kg^{-1}. α-HCH was found in 2 samples (8%) at levels of 0.03 and 0.08 mg kg^{-1} and β-HCH was detected at 0.12 and 0.23 mg kg^{-1}. Residues of DDT and their metabolites were not detected in the analyzed samples. The only organophosphorus pesticide found was methidathion in 3 samples, (11%) at levels between 0.025 and 0.068 mg kg^{-1}. The most frequently detected carbamate was methiocarb, which was found in 5 samples (19%) at concentrations from 0.003 to 0.025 mg kg^{-1}, followed by carbofuran in 3 samples (11%) at concentrations from 0.02 to 0.645 mg kg^{-1}. Only one sample was contaminated with carbaryl at 0.016 mg kg^{-1}. Residues of more than one pesticide were found in honeys from both countries. Three honeys from Spain contained 2 pesticide residues, three honeys contained 3 pesticides and one honey contained 4 pesticides. In one Portuguese honey was found residues of 2 pesticides. However,

Table 6. Estimated Daily Intakes (EDIs)[a] and ADIs of Pesticide Residues Found in Honey

		Portugal		Spain	
pesticides	ADI (mg kg^{-1} body weight per day)	EDI (mg kg^{-1} body weight per day)	ADI (%)	EDI (mg kg^{-1} body weight per day)	ADI (%)
DDT	20	2.79×10^{-3}	1.39×10^{-2}	n.d.	
heptenophos	10	1.24×10^{-3}	1.24×10^{-2}	n.d.	
carbofuran	10	2.7×10^{-4}	2.7×10^{-3}	1.14×10^{-3}	1.14×10^{-2}
pirimicarb	20	1.5×10^{-4}	7.5×10^{-4}	n.d.	n.d.
methidathion	5	8.55×10^{-5}	1.71×10^{-4}	4.8×10^{-5}	9.6×10^{-4}
parathion methyl	20	1.7×10^{-5}	8.5×10^{-5}	n.d.	
methiocarb	1	7.03×10^{-5}	7.03×10^{-3}	8.4×10^{-5}	8.4×10^{-3}
carbaryl	10			2.52×10^{-5}	2.52×10^{-4}

[a] EDI was calculated using the equation EDI = (Σc) ($C\ N^{-1}D^{-1}K^{-1}$), where Σc is the sum of the pesticide residues concentrations in the analyzed samples (μg kg^{-1}), C is the mean annual intake per person (0.9 kg per person approximately) (*30*), N is the total number of samples analyzed, D is the number of days in a year, and K is the mean body weight, which was considered 60 kg.

nine samples contained 3 residues of pesticides and 10 samples were contaminated by 4 or more different pesticides.

Special mention should be made to the four ecological honeys taken in Spain (V22 to V25) that present high pesticide residue content, showing that they are not so ecological. The four samples contained organochlorine residues (isomers of HCH) at concentrations ranging from 0.03 to 1.83 mg kg^{-1}, and two of them, V23 and V24, also presented methidathion and methiocarb residues between 0.003 and 0.068 mg kg^{-1}. In many cases, pollution of honey is caused by pesticide application in the surrounding area or by environmental contamination, and not by the beekeepers practices, resulting in the unavoidable presence of toxic substances. The pesticide residue determination could be a helpful tool to establish the safety and the quality of the honeys.

Organochlorines were the most frequently detected pesticides in both countries. Although the use of DDT, HCH, and HCB has been banned in Europe for decades, the results obtained could be expected, because those pesticides and their metabolites have been extensively used and are still present in the environment, owing to their high persistence. Organochlorines are lipophilic substances and consequently are soluble and stable in beewax. Therefore, an amount of these substances gradually migrates from wax into the stored honey. On the contrary, carbamates, which are hydrophilic, are easily found in honey (*8*). The presence of more carbamates than organophosphorus pesticides in honey points out the last years tendency of changing application habits.

It is difficult to compare our result with those of other monitoring programs from other countries, because there are only a few of them published, and the range of pesticides considered is different. In a monitoring study conducted to determine 50 pesticide residues in 26 honeys from Jordan from 1994 to 1995 (*26*), 86% of the honeys analyzed were contaminated with organochlorine pesticides, with β-HCH, α−HCH and lindane (γ-HCH) being the most frequently found, and only 14% were contaminated with organophosphorus, as dichhlorvos, bromophos methyl, fenitrothion and mevinphos. Unlike the previous report, the study performed in 27 honey samples from India from 1993 to 1995 (*27*) showed that all samples were contaminated by organophosphorus, mainly DDVP, chlorpyriphos, monocrotophos, dimethoate, and fenitrothion. Carbofuran and carbaryl contaminated 55% of the honey samples. All honey samples studied were also contaminated with organochlorines, but the amount of residues found was much lower than that of organophosphorus and carbamates. Of 177 honey samples analyzed from 1988 to 1990 in Lugo (Spain), 38% were contaminated with azinphos methyl, coumaphos, diazinon, ethion, methamidophos and phosalone (*29*).

To evaluate the toxicological significance of human exposure to the pesticide residues found, **Table 6** compares the estimated contribution of honey consumed to the intake of these substances with the acceptable daily intakes (ADI) established by the FAO/WHO organization. No ADI for HCH or HCB have been published. The ADI of a pesticide is the amount of that pesticide that can be ingested daily by a human being during an entire lifetime without an appreciable risk to the health. Daily intakes of the pesticides are much lower than the ADIs, which shows that honey consumed has a minimal contribution to toxicological risk.

GC and LC coupled with MS have demonstrated to be valuable techniques for the detection and quantification of pesticides in monitoring programs, which are designed to cover a wide range of pesticides in honey samples. The results obtained from honeys of Portugal and Spain show the importance of implement monitoring programs in honey samples. Organochlorine pesticides as HCH and their metabolites and HCB were found in the greatest number of samples, and in some of them at a relevant concentration levels. Other pesticides such as the heptenophos, methidathion, parathion methyl, pirimicarb, methiocarb, and carbofuran were also detected. The samples labeled as ecological honeys presented a high level of pesticide residues, demonstrating that sometimes these type of designations should be carefully considered, especially when the surrounding environment is not controlled. The calculation of estimated daily intakes from these data showed that the contribution of honey to dietary intakes were much lower than ADIs.

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