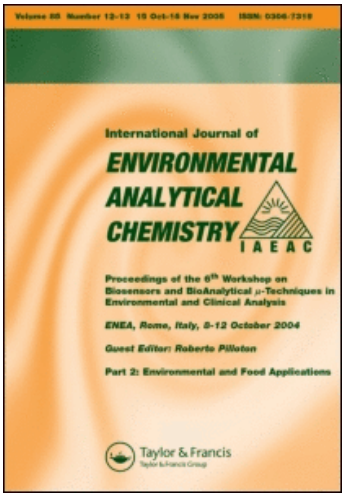

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SIMPLIFIED PROCEDURE FOR ORGANOCHLORINE PESTICIDES RESIDUE ANALYSIS IN HONEY

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A simplified method for analysing organochlorine (OC) pesticides residues in honey was devised. It uses capillary column gas chromatography.

It implies dissolving honey in water. The solution thus obtained is extracted by petroleum ether. The extract is purified on a Florisil micro-column. The recovery for the eleven OC pesticides studied was better than 90% for three levels of fortification. The LOQs lie between 0.27 and 0.48 ng/g.

This technique was applied to 28 samples of honey coming from various geographical areas. p,p'-DDE was the only compound detected in 24 samples (85.7%) and its mean concentration was 0.58 ng/g. The richest p,p'-DDE samples came from Third World countries where OC pesticides were, in the recent past, still in heavy use.

KEY WORDS: Honey bee, capillary gas chromatography, organochlorine pesticides.

INTRODUCTION

The contamination of the Tunisian environment by persistent organochlorine (OC) pesticide residues has been widely documented. These residues have been detected by our team as well as by other workers in mother milk[1-4] and human blood, hen and falcon eggs[5,6] and mussels[7]. Moreover, in 1988, Tunisia, like many other African and Middle East countries, suffered a dramatic acridian (locust) invasion which led to heavy HCH sprayings (2 to 25 kg/ha) over a total area of 346,357 ha scattered over the whole country[8]. Since honeybees (*Apis mellifera L.*) travel long distances and come close to many dusted surfaces and plants, honey may be a useful and easily accessible environmental pollution indicator.

Besides, in Tunisia, by tradition, honey is a food usually served to infants, old and sick persons. Therefore, it seems important to check the quality of honey[9], especially after the extensive HCH use against locusts. Some sources say that "no other food can contain so many different known toxicants as honey"[10]. The situation in this regard worsened recently

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because of the sprayings against the parasitic mite *Varroa* of the honeybee that are performed in hives[11]. Moreover, in Germany, honeybee and honey are included in the German Environment Specimen Banking Project[12]. Honey was also used to monitor the Tchernobyl blast[13] and served previously as radioactivity[14–16] as well as a heavy metals[17–19], PCBs[20] and pesticides[21–25] bioindicators.

Even though various workers have reported pesticide residues in honeybees, honey and wax, it is found that there are only few analytical methods dealing with these kind of samples. A recent review of chromatographic methods for pesticide analysis in foods[26] does not even mention honey.

One can essentially spot analytical methods that are used simultaneously for both honey and fatty or non fatty foods and consist of two purification steps. The first one is either a partition step of pesticides and co-extracted compounds between two solvents of different polarity or sulfuric acid treatment of the extract. The second step involves an adsorption chromatography on Florisil, charcoal or silica gel[27–29]. However, Fernandez *et al.*[30] have recently published an analytical procedure that can be applied to honey only. These authors use 25 g of honey and achieve a mean recovery of 89.6% for seven OC pesticides.

In the present paper, a modified method for OC pesticide residues determination is proposed. Compared to earlier techniques, it deals with 4 g of honey only. Thus, it reduces the rate of coextrated compounds without affecting the detection limit. Moreover, it is simple and has a single purification step using adsorption chromatography on a Florisil micro-column. Its extraction and purification yields are discussed with respect to the recovery. This method was duly assessed with the help of 28 honey samples coming from various parts of the world.

A preliminary assessment of the p,p′-DDE contamination of the treated samples is discussed.

EXPERIMENTAL

Chemicals

The solvents n-hexane, petroleum ether (40–60°C) and diethyl ether (pesticide grade) were obtained from Merck (Darmstadt, Germany). The water used for sample preparation was twice distilled and it was also triple-extracted with n-hexane. Anhydrous sodium sulfate, 12-60 mesh (analytical-reagent grade) was purchased from Fluka (Buchs, Switzerland), heated at 300°C and stored in a 130°C oven. Florisil (60-100 mesh) was obtained from Merck, activated at 650°C and retreated at 130°C for 5 h before use. OC pesticide standards were obtained from Polyscience (Niles, Illinois, USA). Purities of the individual standards ranged from 97 to 99%. The pesticides, listed in the order in which they appear in the chromatograms (Figure 1), are (1) α-HCH, (2) γ-HCH, (3) Aldrin, (4) Heptachlor epoxyde, (5) o,p′-DDE, (6) Dieldrin, (7) p,p′-DDE, (8) o,p′-DDD, (9) p,p′-DDD, (10) o,p′-DDT and (11) p,p′-DDT.

A stock solution of the standard mixture containing 500 μg/ml of each pesticide was prepared in n-hexane. However, because of their relatively low ECD response, DDD and

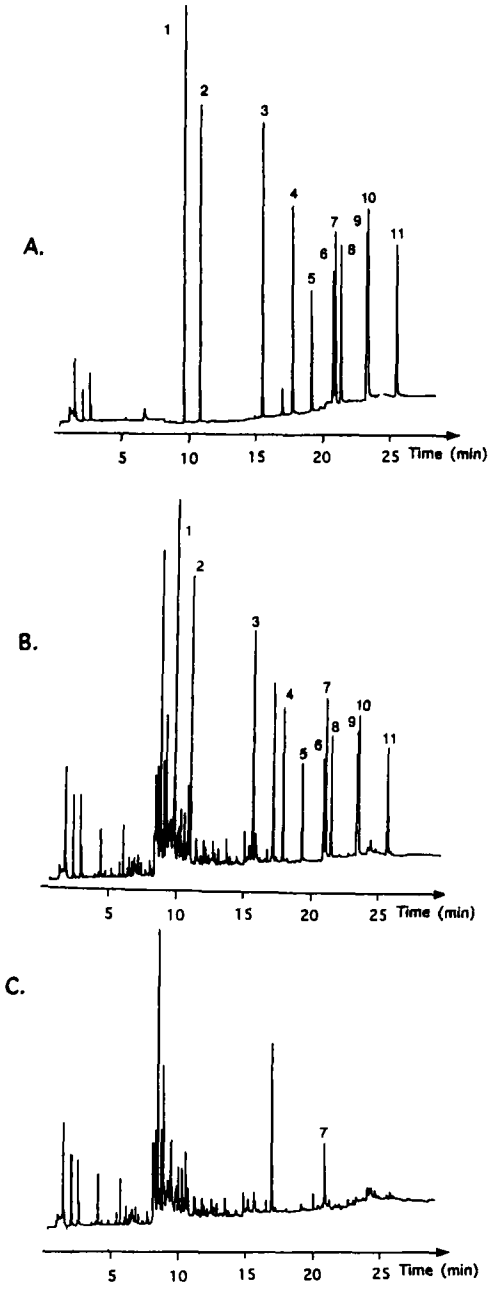

Figure 1 Capillary-GC-ECD chromatograms of (A) organochlorine pesticide standards, (B) spiked honey sample and (C) unspiked honey sample.
Peak identification: 1 = α-HCH; 2 = γ-HCH; 3 = aldrin; 4 = heptachlor epoxyde; 5 = o,p′-DDE; 6 = dieldrin; 7 = p,p′-DDE; 8 = o,p′-DDD; 9 = p,p′-DDD; 10 = o,p′-DDT; 11 = p,p′-DDT.
Column: 25 m × 0.32 mm I.D.; 0.12 μm OV-101. For chromatographic conditions, see text.

DDT isomers were in solutions of 1000 µg/ml. The solutions were further diluted to obtain fortifying and GC calibration standard solutions for all pesticides.

Sample collection

All our samples came from general food stores except for the Siberian honey which was a gift from Barnaoul Agricultural Station and the sample of Madagascar which was donated by a French apicultor. The French sample came from a shop selling only "biological" and "organic" agricultural products in Vézelay in Burgundy.

Extraction

Four grams of honey were weighted into a 100 ml flask. 25 ml of bi-distilled water were added to the flask and shaken vigorously with a vortex until the honey was dissolved. 15 ml of petroleum ether were used to extract the mixture by agitation on a rotary mixer for 15 min at 55 rpm. The content of the flask was transferred into a 100 ml separatory funnel and the phases allowed to separate. An emulsion was usually formed between the petroleum ether and water/honey layers, but it was easily broken by drawing off the aqueous layer and vigorously shaking the remaining petroleum ether layer. All samples with persisting emulsion were centrifuged for 10 min at 3000 rpm. The aqueous layer was transferred into a flask and extracted with two further 15 ml portions of petroleum ether. The combined organic extracts were dried over anhydrous sodium sulfate and evaporated to 1 ml in a Kuderna-Danish (K-D) evaporator fitted with a 10 ml graduated glass tube.

Clean-up

A 0.5 cm layer of silanized glass wool was placed in a chromatographic micro-column 30 × 0.8 cm I.D., equipped with a porous glass septum and a teflon stopcock. The column was packed with 2 g of Florisil and topped with 2 cm anhydrous sodium sulfate. The packed column was washed with 10 ml of petroleum ether (discarded) and the 1 ml extract was added, just before the top of the column wash reached the sodium sulfate layer. The tube that contained the extract was rinsed with 0.5 ml of petroleum ether and added to the column. The extract was eluted with 25 ml of 5% diethyl ether in petroleum ether. The eluate was evaporated just to dryness in a micro K-D concentrator and the residue dissolved in 500 µl of n-hexane.

Chromatographic analysis

Following the Florisil clean up, 2 µl of extract were injected onto the column of a gas chromatograph (GC 121 DFL, Delsi, France) equipped with a ^{63}Ni electron capture detector (ECD). Two columns were used: column I, fused silica capillary column, 25 m × 0.32 mm

I.D., coated with 0.12 μm film thickness of OV- 101 (Delsi; France); column II, wide bore fused silica capillary column, 15 m × 0.53 mm I.D., coated with 0.5 μm film thickness of SPB- 608 (Supelco SA, Switzerland). The column I was used as the primary analytical column. The data presented in this paper were obtained using this column. Column II was used as a confirmatory column.

The operating conditions were as follows: Injector temperature 240°C, detector temperature 300°C; oven temperature for column I: initial 55°C for 2 min, programmed to 160°C at 40°C/min, followed by 3°C/min to 250°C; for column II: initial 150°C for 4 min, programmed to 280°C at 16°C/min, final 280°C for 15 min; carrier gas: hydrogen with a column head pressure for column I of 80 KPa and for column II of 120 KPa; detector make-up gas was nitrogen at a flow rate of 50 ml/min; sample injection volume 2 μl; injection mode: splitless for 1 min. Chromatograms were recorded and peaks integrated with an ICR-1B (Delsi) instrument.

When a sample was analysed, the data system first identified the analyte by comparing the retention time of the suspect to the retention time generated by the calibration standard and the fortified blank. The presence of any residue was confirmed only if it emerged on the second column.

Linear regression of ECD responses with respect to the peak height of five levels of each standard were used to calculate the amount of pesticides in each sample.

Recovery

Pesticides recovery were investigated by adding known volumes of the mixed fortifying standard solution in hexane to a sample of honey. In order to achieve a reasonable simulation of the true situation because the honey is insoluble in n-hexane, the fortification process was carried out as follows: 20 ml of a n-hexane standard solution was added to 4 g of honey sample in 100 ml flask which was sealed and its contents stirred for 15 min. After this step, the solvent was evaporated by an air stream at ambient temperature. Afterwards, the spiked sample was analysed by application of the previously described method.

Recoveries were estimated at three different fortification levels for all pesticides (Table 2). Three replicates at each fortification level and three check samples were analysed. The percent recovery for each analyte was calculated and corrected for background concentration measured in the unfortified sample.

RESULTS AND DISCUSSION

Analytical discussion

The proposed technique was used to analyse the eleven OC pesticides listed in Table 1. These compounds include some insecticides already detected in many Tunisian samples of human milk and blood[2–4] and others have been found only occasionally (e.g. Aldrin, Heptachlor epoxyde).

Table 1 Relative retention time, regression linear coefficient (R), limit of detection (LOD), and limit of quantification (LOQ) of the studied compounds

Compound	*Relative retention time*[a] *I*[b]	*II*[b]	*R*	*LOD (μg/l)*	*LOQ (μg/Kg)*
α-HCH	0.83	0.62	0.9958	0.80	0.27
γ-HCH	0.89	0.70	0.9977	0.72	0.28
Aldrin	1.00	1.00	0.9980	0.93	0.23
Heptachlor epoxyde	1.09	1.15	0.9922	0.97	0.25
o,p′-DDE	1.12	1.24	0.9934	1.10	0.30
Dieldrin	1.15	1.36	0.9965	1.25	0.30
p,p′-DDE	1.17	1.35	0.9934	1.00	0.28
o,p′-DDD	1.19	1.39	0.9915	1.75	0.40
p,p′-DDD	1.29	1.52	0.9940	1.50	0.38
o,p′-DDT	1.29	1.52	0.9951	1.75	0.40
p,p′-DDT	1.32	1.67	0.9897	2.00	0.45

[a]Relative to aldrin. Retention times of aldrin are 10.87 and 10.85 min on column I and II respectively.
[b]Column I (OV-101); column II (SPB-608). For operating conditions, see text.

Table 2 Recovery of OCPs from spiked honey

Compound	*Spiked level (ng/g)*	*% Recovery (% RSD)*[a]	*Spiked level (ng/g)*	*% Recovery (% RSD)*[a]	*Spiked level*	*% Recovery (% RSD)*[a]
α-HCH	1	92 (8.7)	10	92.15 (4)	15	95 (5.8)
γ-HCH	1	93.5 (9.2)	10	93.5 (3.1)	15	94.9 (6.9)
Aldrin	1	80 (7.5)	10	72 (2.4)	15	93.9 (4.3)
Heptachlor epoxyde	1	95.2 (9.9)	10	84.5 (3.5)	15	89.3 (3.3)
o,p′-DDE	1	96.4 (9.6)	10	85.8 (3.5)	15	93.1 (3)
Dieldrin	1	101.6 (9.1)	10	96.5 (12.7)	15	96.4 (7.4)
p,p′-DDE	1	115.2 (13.02)	10	98.9 (4.9)	15	89.2 (8.8)
o,p′-DDD	2	105 (7.1)	20	91.4 (5.6)	30	96.2 (8.2)
p,p′-DDD	2	89.6 (2.7)	20	81.2 (7.4)	30	90.8 (5.9)
o,p′-DDT	2	102.6 (8.8)	20	86 (5.6)	30	84.1 (3.1)
p,p′-DDT	2	99 (8.3)	20	92.9 (11.7)	30	93.8 (7.8)

a: For three trials

The chromatographic behaviour of the eleven pesticides on the two columns used is not the same as shown by their relative retention times in Table 1 due to the different phase polarities. For the chromatographic parameters chosen, column I achieves, in 25 minutes, a good separation of all the compounds studied with a resolution greater than 1 except for p,p′-DDE and dieldrin which, however, exhibit a resolution better than 98%.

With column II, the analysis lasts only 15 minutes but p,p′-DDT and o,p′-DDT coelute. On the other hand, the p,p′-DDE and dieldrin elution order is reversed with respect to column I.

The ECD response linearity was assessed with respect to the peak height for all the OC pesticides studied in a concentration span 0,5–30 ng/g with 2 μl injections. The correlation coefficients of the corresponding calibration curve are satisfactory (R> 0.99).

Table 3 Average percent recovery (n = 5) of the studied insecticides on a Florisil microcolumn using various eluents

Compound	*Eluent*		
	Petroleum ether	*Petroleum ether-5% diethylether*	*Petroleum ether-10% diethylether*
α HCH	96	98	100
γ HCH	13	97	98
Hep. Epoxyde	0	99	99
Dieldrin	0	98	98.2
p,p′-DDE	99.3	99.8	99.8
p,p′-DDT	9	96.5	99

The limit of detection (LOD) was calculated from the expression:

$$LOD = a + 3\,\sigma_{x/y}$$

where a is the intercept of the calibration curve and $\sigma_{x/y}$ is the standard deviation of the fitting[31]. The results obtained are shown in Table 1. The limits of quantification (LOQ) for an actual sample were determined in the same way but using a chromatogram background of non-spiked honey sample. The estimated values are 2 to 3 times higher than the LOD. LOQ are within the range 0,23–0,45 ng/g.

The extracts cleanup on a 2g Florisil micro-column was performed with three eluant mixtures; namely: petroleum ether (PE), PE with 5% diethyl ether and PE with 10% diethyl ether. The recoveries of the pesticides studied when using 25 ml of eluent are given in Table 3. These results show that 25 ml of PE do not elute all the solutes. However, if PE contains at least 5% of diethyl ether, the recovery of all the pesticides is close to 100%. When using a mixture of PE with 10% of diethyl ether, then, 15 ml are enough to achieve a complete recovery (100%) of the whole set of the pesticides of interest.

However, this particular mixture has not a good clean-up yield. Fair results are obtained with 25 ml of PE blended 5% of diethyl ether as shown on the chromatograms of Figure 1. In such conditions, some of the peaks appearing on the chromatogram of the unspiked sample of Figure 1-c may display the retention times of some studied insecticides. They were not quantified, however, for two reasons:

a) The peak intensity was lower than the LOQ (e.g. p,p′-DDT)
b) The peak cannot be checked on the second confirmatory column (e.g. HCHs and aldrin)

Application

The described method was applied to the analysis of the eleven OC pesticides studied. p,p′-DDE was found in 24 samples out of the 28 examined (85,7%) (Table 4). The mean concentration value of that residue was 0,58 ng/g. p,p′-DDE—one of the metabolites of p,p′-DDT—is far less toxic than its precursor. Bees carrying sizeable loads of p,p′-DDT may have been killed. Moreover, intake, metabolism and/or excretion of pollutants may change

Table 4 p,p′-DDE residues (ng/g) in commercial honeys from various countries

Sample	*Year*	*Residue*	*Sample*	*Year*	*Residue*
Tunisia			Canada		
T1	1990	0.76	C1	1991	0.40
T2	1990	0.57	C2	1991	0.44
T3	1990	0.78	C3	1991	0.42
T4	1990	0.38	Italy		
T5	1990	N.D	I1	1990	0.48
T6	1990	0.35	I2	1990	0.36
T7	1991	1.00	I3	1990	0.37
T8	1991	0.45	France		
T9	1991	0.49	F	1990	0.37
T10	1991	0.58	Egypt		
T11	1991	0.79	E1	1992	0.66
T12	1991	1.20	E2	1992	0.66
Venezuela			E3	1992	1.10
V1	1990	0.45	Madagascar		
V2	1990	0.46	M	1993	N.D
V3	1990	0.45	South China[b]		
Siberia[a]			Ch	1993	N.D.
S	1992	N.D			

a: Altaï mountains; b: Canton region.
N.D: not detected.

with environment and exposure of the insect. Physical, chemical and ecological phenomena interplay and may explain the kinetics of transfer and the different levels of p,p′-DDE and its precursor[32]

Three samples coming from Third World countries where the heavy use of some OC compounds was only recently interrupted exhibited residue concentrations equal or higher than one ng/g. They originate from Tunisia (samples T7 and T12) and from Egypt (sample E3). The two p,p′-DDE richest samples came also from these two countries. The residue contents of the French, Canadian, Italian and Venezuelan samples were lower than the mean value. It is well known that OC pesticides in the industrialized countries were banned in the early 1970s and as a consequence, their residues in the ecosystem are dwindling[33–35]. The case of the Venezuelan honey is closer to the Chinese, Madagascar and Siberia ones, which are p,p′-DDE free probably because all these countries use little or no expensive agrochemicals at all.

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