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Contamination of Honey with Organophosphorus Pesticides

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Pesticide residues in food are of great importance in the evaluation of food quality. They are normally minimal in honey but it is very important to know their level. Pesticides might be introduced into honey by bees that fed on nectar or pollen from contaminated blossom and to control Varroa mite (*Varroa jacobsoni)* with the organophosphorus pesticides, so that one might expect that there will be residues in honey.

Apiculture in Lugo (NW Spain) is in a state of expansion. In 1989, there were 32500 hives, representing an increase of 9% on the year 1987 and accounting for 6.7% of the Spanish total. Honey production in that year was 218.0 tonne, up to 11.2% as compared with the preceding year (Fernández 1990). The expense in pesticides in the county of Lugo (NW, Spain) in the years 1988-90 was of 698 ptas (4 $)/Ha of land cultivate that which represent a 21% of the total wornout in Spain of that which only 105 ptas (0.75 $)/Ha return to insecticides. If it compare with the expense of other country from Europe (Germany, France, Holland) should represent a 22%, 21% and 11% respectively in pesticides and a 6%, 5.6% and 3% as for insecticides (Vilariño 1993). The analytical methods available for the determination of organophosphorus pesticides in honey (coumaphos, malathion,...) used against Varroatose are numerous (Thrasyvoulou and Pappas 1988; Taccheo et al. 1989; Stricker et al. 1989; Gnadinger 1989; García et al. 1994) but there are less methods about organophosphorus pesticides determination in honey used to control insects on numerous field crops (Danielyan et al. 1976; Orbaek 1988). These analytical methods, first, require extraction with different solvents: water-acetone, dichloromethane, benzene-isopropanol, acetone-petroleum ether and n-pentane, followed by purification of the organic layer and finally chromatographic determination. Gas-Liquid

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chromatography (GLC) is one of the most important analytical techniques used in pesticide residue analysis. Two advantages are its sensitive and specific detector systems and the ability to separate mixtures of analytes in the columnn. Until recently, GLC of pesticides has been conducted using packed columns containing a large variety of liquid phases and supports. In the last years capillary columns have improved significantly and the fused silica GLC columns are more flexible and offer an easier of manipulation and installation than found with glass capillary columns. The determination of organophosphorus pesticides in honey with glass capillary columns and GL-mass spectrometry (MS), GC-nitrogen phosphorus and electron capture, GC-electron capture, GL-flame photometric detector has been realized.

The aim of this work was to set up a residue method with a pesticide extraction procedure for honey with acetonitrile-water and chromatographic determination of the levels of the pesticide residues in honey from Lugo (NW Spain).

MATERIALS AND METHODS

The study was carried out on 177 samples of honey, provided by beekeepers, from the county of Lugo (NW Spain) depicted in Figure 1. The samples were unpasteurised, although some of them had been heated to facilitate their extraction. They were collected in 1988, 1989 and 1990. Physicochemical analysis were realized on some samples (Herrero 1993).

Solvents used in the study were analytical grade and freshly distilled prior to use or pesticide residue grade. All inorganic chemicals were analytical reagents (AR). Florisil, 60-100 mesh (Sigma Chemical Co.) activated in a 130 ^{o}C oven overnight and Sep-pak cartrigde (Waters No 51960) were used. Pesticide standards (Supelco, Inc. Kit No. 52) of azinphosmethyl, coumaphos, diazinon, dichlorvos, dimethoate, disulfoton, ethion, fenchlorphos, malathion, methamidophos, mevinphos, naled, oxydemetonmethyl, phorate and phosalone were used.

Samples were mixed, and sub-samples (100 g) were extracted with a mixture of acetonitrile-water (300 mL, 2+1 by volume). The liquid after filtration was again extracted with hexane (100 mL) for transferring of residues and concentrated to volume of 5 mL. A further cleanup with a Florisil column or a Florisil Sep-pak was made on the samples of honey. The column was washed with hexane and the residues were eluted with 200 mL of hexane or 10 mL for Sep-pak. Each eluate was concentrated to 2 mL in Kuderna-Danish concentrator and collected in a 2 mL vial. The solvent was evaporated to dryness at room temperature under nitrogen stream. The dried sample was taken up with an appropriate volume (1 mL) of hexane.

The residues of 15 pesticides were determined by gas-liquid chromatography using a Hewlett-Packard, Model 5890A with nitrogen-phosphorus detector (NPD), equipped with a bore fused silica capillary column (HP-101) containing methylsilicone fluid as non polar stationary phase (12 m length, 0.2 mm i.d. and 0.2 μm film thickness). The GLC conditions are given below:
Carrier gas flow (nitrogen), 1.4 mL min^{-1}; auxiliary gas flow (nitrogen 50), 36 mL min^{-1} and the flow rates of hydrogen and air were 3.5 and 100

mL min^{-1} respectively. The injection port temperature was 200 °C and the temperature of the detector, 250 °C. The column had an initial temperature of 60 °C (1 min) progressively increased to a final temperature of 240 °C for 1 min with a ramp of 30 °C min^{-1}.
Two µL of sample extract were injected by splitless injection technique with the split closed for 0.5 minutes using the operating conditions indicated above.
In the estimation of various residues from pesticides, their metabolites often have identical retention times. In order to avoid errors resulting from this fact, the identities of organophosphorus pesticides were confirmed by GLC using a Perkin-Elmer Autosystem fitted with ^{63}Ni electron capture detector and capillary column (HP-5) containing phenyl methylsilicone as non polar stationary phase (25 m lengh, 0.2 mm i.d. and 0.33 µm film thickness). The GLC conditions are given below:
Carrier gas flow (nitrogen 50) and auxiliary gas flow, 30 mL min^{-1}. The injection port temperature was 200 °C and the temperature of detector, 300 °C. The column had an initial temperature of 60 °C (1 min) progressively increased to a temperature of 180 °C for 1 min with a ramp of 20 °C min^{-1}, followed by 10 °C min^{-1} to 240 °C for 2 min, and then 5 °C min^{-1} to 250 °C for 1 min.
Standards were prepared by successive dilution of a 1000 mg L^{-1} solution of pesticides in methanol to obtain the following solutions : 100-400 µg mL^{-1} for coumaphos, methamidophos and phosalone; 40-100 µg mL^{-1} for azinphosmethyl, oxidemetonmethyl; 0.3-20 µg mL^{-1} for diazinon, dichlorvos, dimethoate, disulfoton, ethion, fenchlorphos, malathion, mevinphos, naled, and phorate.
A pesticide recovery study was carried out by fortifying honey samples. Pesticide standards were added to the homogenized honey samples before the first solvent extraction step and they were analyzed by application of the previously described method. The recovery assays were replicated three times. Fortification levels and recoveries are indicated in table 1.
The linearity of response of the NPD for standard pesticide solution was investigated as well as the minimum detectable level (MDL), which is defined as the concentration giving a signal twice the noise. These values are shown in table 1.

RESULTS AND DISCUSSION

Pesticide residue extraction was achieved using acetonitrile-water (2:1 v/v), because other solvents (acetone, hexane,...) extract natural components of honey which interfere in the chromatograms of the 15 pesticides. Besides acetonitrile-water allows the recovery a greater number of polar and non polar pesticides.
Cleanup as described, is necessary because the honeys are rich in interfering factors. Furthermore, if the samples are numerous, this cleanup method permits that the GC stage to be faster by removing those substances with long retention times and if there multiple pesticide residues it permits good separation and identification of the pesticides.

The recoveries of the pesticide residues were in the range from 83 to 100%, except for two pesticides oxydemetonmethyl and mevinphos with 78 and 76% respectively. Table 1 shows the average values of three

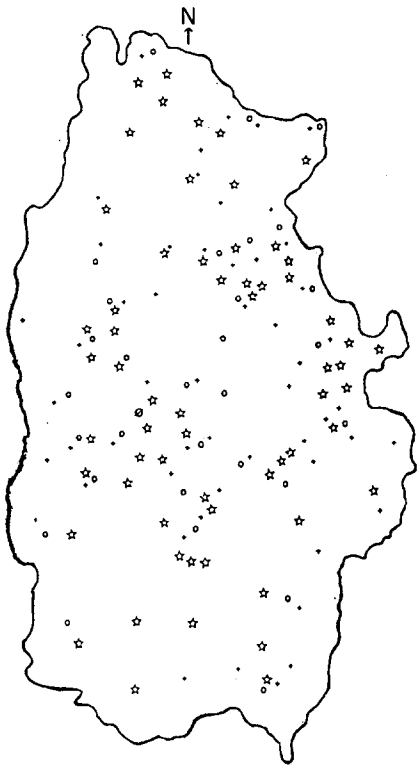

Figure 1.- Locations where honey samples where collected per year o = 1988, + = 1989, = 1990.

Table 1. Pesticide recovery from honey fortified and minimum detectable levels.

Pesticide	Minimun detectable level, ng g^{-1}	Fortification level, μg g^{-1}	Rec.[a] (%), SD
Azinphosmethyl	0.800	2	85 ± 4.2
Coumaphos	2.000	3	91 ± 3.1
Diazinon	0.030	1	84 ± 2.4
Dichlorvos	0.030	1	83 ± 4.3
Dimethoate	0.070	1	94 ± 4.8
Disulfoton	0.006	1	100 ± 2.3
Ethion	0.200	1	100 ± 1.5
Fenchlorphos	0.020	3	92 ± 2.1
Malathion	0.030	2	92 ± 2.2
Methamidophos	2.300	5	86 ± 4.3
Mevinphos	0.050	1	76 ± 5.0
Naled	0.200	8	100 ± 4.3
Oxidemetonmethyl	0.300	2	78 ± 3.5
Phorate	0.009	2	100 ± 6.1
Phosalone	2.000	4	92 ± 3.6

[a] Mean values of three replicates.

replicates of recoveries obtained for the pesticides added to honeys, using the procedure described above with manual Florisil columns. We may compare our results with those given in multiresidue methods of phosphate residues (McMahon and Burke 1978) in food. It can be seen that similar results of recoveries were obtained for coumaphos, diazinon, ethion, malathion whereas we obtained better results for others pesticides with manual Florisil columns.

The individual pesticides are identified by comparison of retention data obtained in two detectors of different selectivity (NP and EC) and two capillary columns, HP-101 and HP-5 respectively.

The NPD was linear for all the tested organophosphorus pesticides over the range 0.3-20 $\mu g\ mL^{-1}$ for diazinon, dichlorvos, dimethoate, disulfoton, ethion, fenchlorphos, malathion, mevinphos, naled, and phorate; 40-100 $\mu g\ mL^{-1}$ for azinphosmethyl, oxidemetonmethyl; 100-400 $\mu g\ mL^{-1}$ for coumaphos, methamidophos and phosalone. The minimum detectable levels (MDL) were determined and found to be different, because this chemical class of pesticides exhibits a great diversity in its chemical structures and properties (polarity, volatility,...). These values of MDL were 0.006 and 0.009 ng g^{-1} for disulfoton and phorate respectively; 0.02 ng g^{-1} for fenchlorfos; 0.03 ng g^{-1} for diazinon, dichlorvos and malathion; 0.05 and 0.07 ng g^{-1} for mevinphos and dimethoate respectively; 0.2 ng g^{-1} for ethion and naled; 0.3 ng g^{-1} for oxidemetonmethyl; 0.8 ng g^{-1} for azinphosmethyl; 2 ng g^{-1} for coumaphos, methamidophos, and phosalone.

Results of the minimum detectable levels in honeys have been reported for coumaphos (Thrasyvoulou and Pappas 1988; Taccheo et al 19897; Anderson and Wojtas 1986), diazinon (Anderson and Wojtas 1986), dimethoate (Barker et al. 1980), ethion (Klein et al. 1986), malathion (Thrasyvoulou and Pappas 1988) and methamidophos (Drescher and Fiedler 1983). The minimum detectable levels in honeys for azinphosmethyl, dichlorvos, disulfoton, fenchlorphos, mevinphos, naled, oxidemetonmethyl, phorate and phosalone are reported in this work for the first time (table 1).

A hundred seventy seven honey samples were collected in several years as follows, 36 in 1988, 68 in 1989 (García et al 1992) and 73 in 1990. Table 2 shows the results of the pesticide residue analysis in these honeys. It can be seen that only six pesticides were detected: azinphosmethyl, coumaphos, diazinon, ethion, methamidophos and phosalone. The results obtained show that some pesticide residues were detected in 69 samples of the 177 studied, these values are shown in Table 2. Coumaphos, which is the pesticide used against Varroa mite appeared with the most frequency (14%), whereas the other pesticides used in vegetables pest were observed in less samples: ethion (13%), phosalone (10%), methamidophos (9%), diazinon (4%) and azinphosmethyl (2%). The other 108 samples were free from any detectable trace of pesticides.

If results are analysed per year, the observed pesticide residues are of

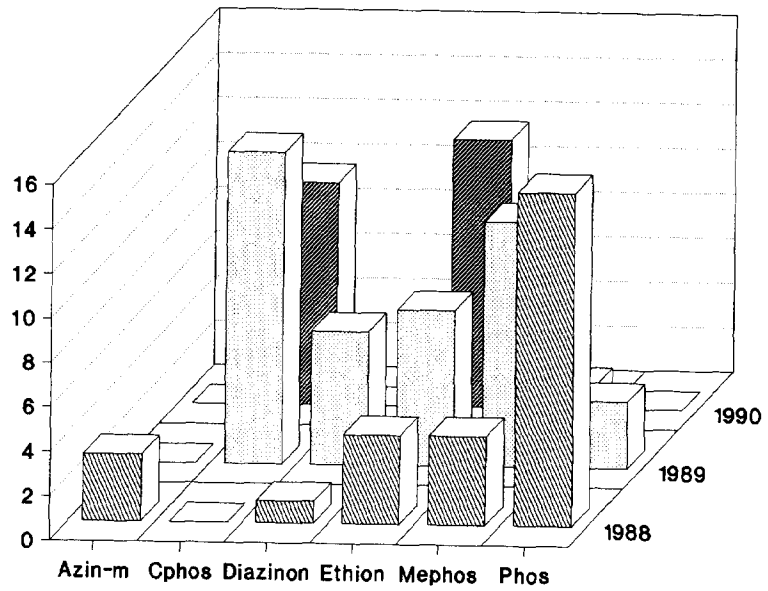

Figure 2.- Tridimensional Histogram of Organophosfphorus Pesticide Residue per year. Azin-m = Azinphosmethyl; Cphos = Coumaphos; Mephos = Methamidophos; Phos = Phosalone.

Table 2.- Pesticide Residues Concentrations in 69 honey samples (μg/kg)

Pesticide	Appearances	Range	Mean	SD
Azinphosmethyl	3	2-13	5.7	5.2
Coumaphos	24	1-53	6.0	10.3
Diazinon	7	17-116	41.3	31.4
Ethion	23	1-8	3.0	1.6
Methamidophos	16	4-25	8.6	5.5
Phosalone	18	3-37	10.3	8.7

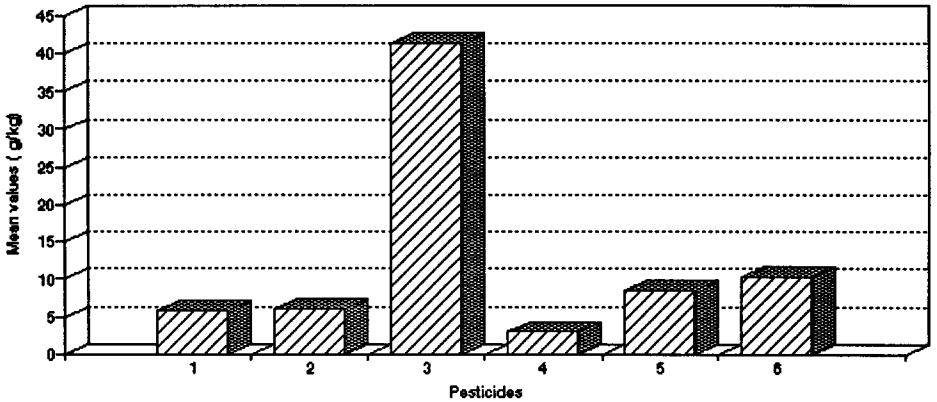

Figure 3.- Concentrations ($\mu g\ Kg^{-1}$) of azinphosmethyl, coumaphos, diazinon, ethion, phosalone and methamidophos in 69 honey samples contaminated.

different nature; in 1988 phosalone was the most frequent, it appears in 15 samples of 27 contaminated samples; in 1989 was coumaphos with 14 samples of 41 contaminated samples; in 1990 was ethion with 12 samples of 23 samples contaminated. Tridimensional histogram of frequency appearance of pesticides per years are shown in Figure 2.

The levels of pesticide residues are low, generally they are between 1-6 ug kg^{-1}, they are shown in Figure 3, specially when treatments properly are carried out. An exception is the sample with a high concentration of diazinon (116 $\mu g\ kg^{-1}$), which was collected from hives near to a wheat field fumigated with a large quantity of diazinon. Another exceptions, for example coumaphos 53 $\mu g\ kg^{-1}$, are produced due to that farmers and beekeepers do not always conform to the directions for use, either applying a greater number of treatment than required or using doses higher than necessary or their hives are near to the fumigated field crops .

In conclusion, the data presented here provide evidence that capillary column GC with NPD detection can be used reliably and advantageously for regulatory determination of organophosphorus pesticide residues in honey. The described method allows quantitative extraction of the organophosphorus pesticides used to control pests in the field crops from Lugo (Informe Agrario 1991, Consellería de Agricultura. Xunta de Galicia). It can be concluded from the present study that the contamination of honey is not prevalent in Lugo. The most residue levels are very low (<6 ppb). Finally, it must be pointed out that the maximun residue levels (MRL) in honey have not been established by the Spanish legislation and the residue levels obtained in this study are very small compared to the MRL of the Spanish regulations for vegetable products (B.O.E. Boletín Oficial del Estado, 4-11-1989).

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