ORGANOCHLORINE PESTICIDES IN SEVERAL TYPES OF ROMANIAN HONEY

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Scopul acestui studiu îl constituie determinarea poluanţilor organici persistenţi (OCP), α-, β-, γ-, δ- hexaclorciclohexan (HCH) în unele tipuri de miere din regiunea Neamţ şi evaluarea nivelului de contaminare cu reziduuri de OCP utilizând metoda GC/MS. Caracteristicile metodei, precum: liniaritate, recuperare, precizie şi limitele joase de detecţie şi cuantificare, utilzând date de validare sunt prezentate. Metodologia analitică propusă a fost aplicată analizei pesticidelor ţintă din probe de miere colectate dintr-o zonă poluată din regiunea Neamţ. Metoda prezintă aspecte originale referitoare la partea de extracţie şi clean-up a probelor de miere.

The purpose of this work is to determine OCPs, α-, β-, γ-, δ- hexachlorocyclohexane (HCH) in some types of Romanian honey from Neamt region and evaluate the level of contamination with OCPs residues using GC/MS method. The method characteristics such as: linearity, recovery, precision and lower limits of detection and quantification, using validation data are presented. The proposed analytical methodology was applied to the analysis of target pesticides in honey samples collected from a polluted area in Neamt region. The methods contains original aspects concerning the extraction and the clean-up stepss.

Keywords: organochlorine pesticides, GC/MS, food analysis

1. Introduction

Ideally, risk assessments for chemicals, should consider all sources (plant protection products, veterinary drugs, human medicines), pathways (food, drinking water, residential, occupational) and routes (ingestion, dermal, inhalation) of exposure that could contribute to a person's total exposure [1]. The combined toxicity of two or more compounds can take three possible forms: dose-addition, response-addition or interaction. The term pesticides cover herbicides, fungicides and insecticides.

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Persistent organic pollutants (POPs) such as polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs) have been found in food since about half a century. Although most of these contaminants have been phased out, residues are still being found, emphasizing the persistent character of these POPs [2], [3]. Honey is an exported product of Romania and according to EEC regulations, honey as a natural product must be free of any chemical contaminants for human consumption [4]. While the nutritional and quality aspects of honey are very important, safety of honey is also critical, as it determines the consumer acceptance. Contamination of persistent organochlorine pesticides (OCPs) is still significant in certain regions, honey representing a potential indicator for the degree of contamination.

In literature, high presence of OCPs have been found in several kinds of honeys from different European countries [5-9], even if the organochlorine pesticides have been restricted or banned for agriculture since 1978 in the USA and Europe. These pesticides have been restricted due to their persistence and bioaccumulation in the environment. Continuous exposure of the bees on the influence of various sorts of chemical compounds has also an impact on the quality of produced honey. The routes of honey contamination with pesticides are direct and indirect, the direct one being in connection with beehives treatments with pesticides in the environment, respectively [10].

Due to its lipophylic nature, OCPs enter into the food chain by accumulating in fats, but they can also be present in non-fatty products, even those which cannot be treated with them [11]. It has to be emphasized that honey may also be an environmental pollution indicator for air and soils [12], [13].
In order to assess pesticide residual levels in honey and their compliance with quality standards fixed by UE or National regulations, several methods have been developed. It is very important in pesticides detection and quantification to comprise minimum extraction and clean-up steps for an efficient method.

The occurrence of different kinds of contaminants [14] among which pesticide compounds in the food chain in Romania has been already reported [15], organochlorine pesticides being still significant contaminants in Romanian food samples. Between 2001 and 2006, the results from a monitoring program [16] indicated in Romania the presence of organochlorine pesticide residues, the mean residues levels of total α-, β-, γ-, δ- HCH (hexachlorocyclohexane) varying between 0.044 mg/kg (2001) to 0.024 mg/kg (2006). Determination of these pesticides in honey from certain Romanian regions is important for prevention, control and reduction of pollution as well as for occupational health and epidemiological studies.

Various methods have been reported for the determination of OCPs in honey. Chromatographic methods with MS detection are capable of identifying analytes in the full scan MS method; all ions produced in the MS are employed in

confirmation and quantification of targeted analytes. An advantage of the full scan method over SIM method is the capability of quantification of targeted analytes and simultaneous identification of other eluted compounds.

The purpose of this work is to determine OCPs, α-, β-, γ-, δ- HCH in some types of Romanian honey and evaluate the level of contamination with OCPs residues. In this work, a convenient and fast analytical method for simultaneous identification and quantification of four organochlorine pesticides employing GC/MS in full scan is presented. GC and MS parameters were optimized for baseline resolution and high sensitivities combined with the identification of targeted pesticides, respectively. The performance of the method was evaluated by analyzing the 4 pesticides mentioned before in local honey samples from Neamt region, Romania.

2. Experimental

2.1. Materials and apparatus

Pesticides standards. Pesticides standards, α-, β-, γ-, δ- HCH and quintozene as internal standard were purchased from Sigma Aldrich and most of them were > 99% certified purity. N-hexane, ethyl acetate and acetone were of pesticide grade (Merck, Germany). Concentrations of standard solutions were corrected by the certified purity of the standards, whenever below 99%. Individual stock standard solutions of pesticides were prepared by dissolving 500 mg/L of each compound in n-hexane, except for β-HCH which was prepared in n-hexane-acetone (95:5, v/v). Standard solutions were prepared at a concentration of 10 mg/L and stored in the refrigerator. Working solutions were prepared between 0.2 µg/mL and 20 µg/mL.

All other chemicals were of analytical purity.

A Varian GC Saturn 3900 MS ion trap mass detector was employed, consisting of a Varian CP-3900 gas chromatograph coupled with a Saturn 2200 mass spectrometer. The data system contains the software required for calibration and data processing for qualitative and quantitative analysis. One rotary vacuum evaporator Heidolph Laboratory 402 (Kelheim, Germany) was used. C18 mini-packed columns (100mm x 8mm i.d.) were obtained from Merck. The column used in separations was a factor four capillary VF-5ms 30m x 0.25 mm.

Sampling. A total of 20 samples were purchased from local markets in Neamt area. Honey samples were provided from the beekeepers associations of this region. The samples were stored in their original containers, at 10 ^{0}C in a dark place until their analysis.

2.2. Methods

Extraction and clean up procedures

10 g of honey diluted with 10 mL methanol-water (30:70, v/v) in order to obtain a better sample homogenization prior to analyte extraction were passed through a C18 packed-column. The column was washed with 10 mL of methanol-water (70:30, v/v) and the pesticides were eluted with 10 mL of mixture hexane-ethyl acetate (50:50, v/v). The polarity of the extracting solvent is a challenge between an acceptable recovery and a good measurement. The use of hexane-acetone mixture increases the solvent polarity and the recovery of polar analyte, but decreases the stability of the baseline due to co-extracted substances.

Gas chromatography / mass spectrometry method

The operating conditions were as follows: injection volume: 1 µL; injector temperature: 250 ^{0}C; oven temperature: 180 ^{0}C; detector temperature: 220 ^{0}C and the flow rate of the carrier gas (helium) was maintained to 1 mL/min. The ionization potential was 70 eV. The temperature program of the column was: 150 ^{0}C held for 1 min then programmed at 3 ^{0}C/min rate to 230 ^{0}C, held for 5 min and finally programmed at 3 ^{0}C/min to 250 ^{0}C, held for 5 min. The MS temperature was as follows: ion source 250 ^{0}C, transfer line 200 ^{0}C and analyzer 230 ^{0}C. Analysis were performed in full scan FS mode, simultaneously monitoring specific ions of each analyte as it follows: α-HCH (quantification ion m/z 181; confirmation ions m/z 109 and 219); β-HCH (quantification ion m/z 109; confirmation ions m/z 181 and 219); γ-HCH (quantification ion m/z 109, confirmation ions m/z 181 and 219); δ-HCH (quantification ion m/z 109, confirmation ions m/z 181 and 219). The compounds are identified by their retention times and the qualifier and quantifier fragment ions (m/z).

Validation

Linearity of the method was proved by running the final extracts of the honey samples in triplicates at ten spiking concentrations. The limit of detection (LOD) for each pesticide was determined from injections of the standards and it was defined as approximately three times the standard deviation. The limit of quantification (LOQ) was defined as approximately 10 times the standard deviation. Recoveries were estimated by comparing chromatograms of calibration standards with extracts of spiked samples. The response factor of the standard pesticides relative to the internal standard quintozene were carried out by injecting 1 µL of OCPs mixture together with the internal standard in a concentration range between 0.2 – 20 µg/L for each pesticide and 1 µg/L internal standard. The response factor was calculated based on the equation: RF = peak area of the pesticide standard/peak area of the internal standard.

3. Results and discussion

Many methods have been reported for the determination of pesticides in honey [17]. Some of these methods following the classical analytical procedures for the determination of pesticides in food employ usually time consuming clean-up steps that make them impractical for routine analysis, being necessary to develop specific and rapid methods for the determination of organochlorine pesticide residues in this substrate.

Pesticides in honey are usually extracted by treating the sample with organic solvents, or in solid phase using C18 cartridges or Florisil ones after diluting the honey samples with water. Clean-up is obligatory in order to remove the interferences like lipids, pigments and carbohydrates, including gel permeation chromatography, liquid-liquid partitioning, solid phase extraction and adsorption chromatography. Most methods for OCPs analysis are based on liquid-liquid extraction performed with water non-miscible solvents such as ethyl acetate, petroleum ether, n-hexane, dichoromethane, or miscible solvents such as methanol. Solid phase extraction with C18 cartridges, Florisil, polystyrene-divinylbenzene sorbent copolymers, solid phase microextraction (SPME) are used in pesticides determination in honey samples. GC-ECD has been applied as preferred technique for the identification and quantification of OCPs being confirmed by GC/MS in electron impact mode in which molecules are bombarded by high energy, 70 eV. Most of the methods employ MS in selected ion monitoring mode (GC/SIM-MS) in which sensitivity is improved.

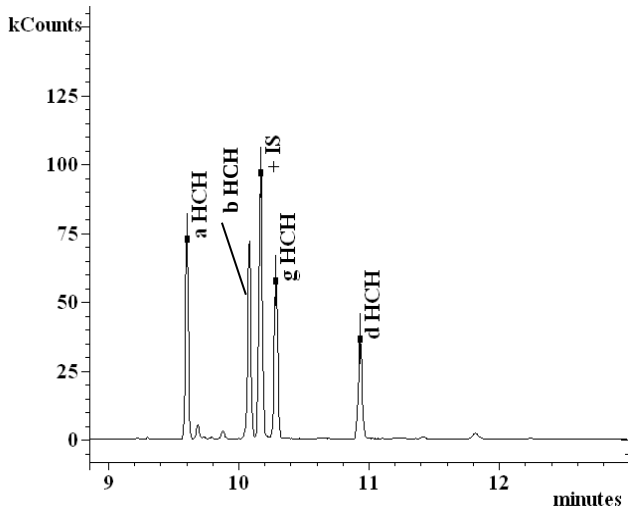

Fig. 1. A full-scan GC/MS chromatogram for a spiked honey sample at 10µg/kg for the four pesticides determined

Recovery experiments, linearity range, accuracy and precision, detection limits and quality assurance

Chromatographic methods of analysis with MS detection are capable of identifying analytes, but the confirmation of targeted analytes is a major concern. In full scan MS method, all ions produced in the MS are employed in confirmation and quantification of targeted analyte. Quantification and identification by MS in full scan method is achieved through the selection of at least three ions related to the targeted analyte, one ion being assigned for quantification and the other two for confirmation.

Recoveries were determined by adding the pesticides to a honey sample at a final concentration of 20µg/kg and analyzing the samples using the proposed method. Recoveries greater than 75% were obtained for all four studied pesticides. Blank analyses were performed in order to calculate the limit of detection and the limit of quantification. The accuracy was determined in terms of relative standard deviation RSD% by the analysis of 3 replicates of spiked samples at three concentration levels at 5, 10 and 15 µg/kg. The precision (in terms of repeatability) without an autosampler has a value less than 10%. Spiked recoveries ranged from 75 to 95% and the positive results obtained in the honey samples were confirmed by comparing the retention times, identifying the main ions in relation to those of a pesticide standard mixture.

Table 1

Linear regression equations and linearity range for organochlorine pesticides investigated

Pesticide	Regression equation	Correlation coefficient	Linearity range, µg/kg
α - HCH	$y = 0.2405x \pm 0.2848$	0.9952	0.2 – 20
β - HCH	$y = 0.231x \pm 0.2243$	0.9993	0.2 – 20
γ - HCH	$y = 0.242x \pm 0.2951$	0.9954	0.2 – 20
δ - HCH	$y = 0.2191x \pm 0.2083$	0.9954	0.2 – 20

The regression results are based on three replicates at ten concentrations in the range 0.2-20 µg/kg. The GC/MS system was linear in the same range, with correlation coefficients between 0.995 and 0.999. Repeatability and reproducibility were calculated making 5 replicate determinations in the same day with relative standard deviation RSDs of 10-20 %. The analyte identification was based on the relative retention times to the internal standard used, ion chromatograms and intensity ratios of the monitored ions.

Table 2

Recovery values and precision of the proposed method

Pesticide	Mean recovery, n=5
α – HCH	90(8)
β – HCH	90(5)
γ – HCH	95(6)
δ – HCH	93(5)

Recovery experiments were carried out in triplicate at three fortification levels of 5, 10 and 15 µg/kg, by adding known volumes of pesticide standards in hexane to homogenized honey samples and the samples were analyzed according to the proposed method. Uncertainties of recoveries reported as R.S.D. values (precision) varied between 5 and 8.

Table 3

Values of limit of detection and limit of quantification for the analyzed pesticides

Pesticide	LOD, limit of detection, µg/kg	LOQ, limit of quantification, µg/kg
α – HCH	0.04	0.135
β – HCH	0.06	0.211
γ – HCH	0.04	0.122
δ – HCH	0.06	0.209

The limit of detection (LOD) for each pesticide was determined from injections of the standards and it was defined as approximately three times the standard deviation. The limit of quantification (LOQ) was defined as approximately 10 times the standard deviation.

Table 4

Detected organochlorine pesticides in three kinds of Neamt honey

Pesticide	Neamt 1, µg/kg	Neamt 2, µg/kg	Neamt 3, µg/kg
α – HCH	0.46 ± 0.11	0.43 ± 0.15	0.55 ± 0.02
β – HCH	0.16 ± 0.10	0.19 ± 0.05	nd
γ – HCH	0.85 ± 0.13	0.56 ± 0.25	0.78 ± 0.47
δ – HCH	nd	nd	nd

Each value represents the mean of three replicates. Each replicate was injected twice. R.S.D. values ranged between 0.02 and 0.47. The method was evaluated by analyzing three kinds of Neamt honey samples from regions with increased concentrations of organochorine pesticides in the environment. These samples revealed the presence of α – HCH and γ – HCH in all of them, δ – HCH was found in neither of them and β – HCH was found in only two kinds of tested Neamt honeys.

As it can be seen from Table 4, the values of organochlorine pesticides are very low. Even this, organochlorine pesticides were the most frequently detected pesticides in this region. Also the use of this kind of pesticides has been banned in Europe for decades the results obtained could be expected, because those pesticides have been extensively used and are present in the environment.

4. Conclusions

This procedure involves a rapid extraction with a mixture of hexane-ethyl acetate (50:50, v/v) and GC/MS quantitative analysis requires small amounts of

honey samples (10 g) and 10 mL of solvent mixture providing satisfactory recoveries, repeatability and reproducibility. The full-scan methods are less sensitive to matrix in comparison to ECD, multi ion criteria making them more reliable than SIM with three ion criteria. The method contains original aspects concerning the extraction and the clean-up steps.

This method was used to evaluate honey contamination in Romania (Neamt region) being clear that levels of studied OCPs are in good agreement with EU regulations. Control samples revealed small amounts of organochlorine pesticide residues in honey of Neamt provenience; this being a good sign in what concerns the good quality of this product. The preliminary results of this study show that there is not a significant contamination source for honey in this region. More extensive studies will be done in other Moldavian areas in order to conclude if it is possible to consider honey as an indicator of pesticides in the environment.

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