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DETERMINATION OF PESTICIDES RESIDUES IN HONEY BY USING DIFFERENT EXTRACTION METHODS AND GAS CHROMATOGRAPHY WITH ELECTRON CAPTURE DETECTION

[41]

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ABSTRACT

In this study three analytical methods for the extraction of pesticide residues in spiked honey samples were evaluated. They have been applied to identification and quantification of targeted thirty five pesticides from different chemical groups and the possibility of identification of any other eluting compounds. Such methods are based on liquid-liquid extraction (LLE) with different organic solvents; ethyl acetate (method_1), n-hexane (method_2) and (petroleum ether: ethyl acetate 80:20 v/v) (method_3), followed by clean-up with florisil and quantification by gas chromatography-electron-capture detection (GC-ECD). Recoveries of the applied spiked samples were ranged from (64.86 – 113.01 %), (64.67 – 111.28 %) and (67.48 – 107.82 %) with the three mentioned methods, respectively. The best results were obtained from ethyl acetate method.

INTRODUCTION

The persistence organic pollutants (POPs) constitute one of the most serious dangerous by the environmental contaminants. The occurrence of pesticide residues in the food chain has already been reported in several studies. The environmental contamination by persistent pesticide residues has been widely documented in soil, plants, water, milk, and biological fluids (**Pico, *et al* 1995**). The POPs has a lipophilic nature, so it can be 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 (**Fernandez, *et al* 1995**). It can be present in honey by direct treatment of plants and/or migration from wax to honey. Since honeybees travel for long distances and come close to many plants, so honey may be an easily accessible environmental pollution indicator (**Marzycka, 2002**). Monitoring of pesticide residues in bee products is necessary to consumer health (**Fernandez, *et al* 2002**). Honey is a natural product that must be free of any chemical contaminants and safe for human consumption, because it is traditionally used in child, old and ill people foods and its quality must be proved (**Tsipi, *et al* 1999**). However, to date European Union (EU) legislation has established the maximum residue limits (MRL) in honey for only three acaricides, namely amitraz, coumaphos and cymiazole as, 0.2, 0.1 and 1 µg/g respectively (**Herrera, *et al* 2005 and Council Directive/EC 2001**). Many methods have been reported for the determination of pesticides in honey. Most used methods for pesticide residues determination is based on liquid–liquid extraction (LLE) performed with water non-miscible solvents, such as ethyl acetate, petroleum ether, or *n*-hexane and dichloromethane (**Tsipi, *et al* 1999**). After LLE extraction a clean-up with different adsorbents may be necessary, florisil or silica gel or activated carbon (**Jimenez, *et al* 1998**). GC–ECD has been widely applied as the preferred technique for the identification and quantification of pesticide residues. The purpose of this work was to develop a rapid, sensitive and easy method for the analysis of 35 pesticide residues with GC-ECD in honey spiked samples.

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MATERIALS AND METHODS

1- Chemicals

The tested thirty five pesticides standards belong different chemical groups were organophosphorous as trichlorofon, carbamate as furan, [organochlorine as (alpha, beta, gamma and delta) HCH, (o,p and p,p) DDT, o,p-DDE, p,p-DDD, (alpha and beta) endosulfan, aldrin, endrin, dieldrin, heptachlor and heptachlor-epoxide], (triazine as atrazine), (triazinone as metrbuzine) (triazole as Propconazol and Epoxiconazol), (neonicotinoid as acetamiprid)and (pyrethroids as tetramethrin, lampada-cyhalothrin, permithrin, cyfluthrin, cypermethrin, fenvalerate and deltamethrin) were obtained from Dr. Ehrenstofer GmbH Germany. Petroleum ether, diethyl ether, *n*-hexane, and ethyl acetate (for pesticides residue analysis grade) were obtained from Merck Co. Stock solution of each pesticide was prepared separately at 0.5 mg/ml in *n*-hexane-acetone (95:5, v/v). Standard solutions were prepared at 10 µg/ml, and then stored at 4°C. Working solutions were prepared between 0.2 to 2.0 µg/ml. Deionized water was prepared from a Milli-Q system (Millipore, Bedford, MA, USA). Florisil 60-100 mesh was obtained from Merck (Germany), and activated in an oven at 150 °C for 12 h, cooled in a desiccator, and a portion was deactivated to 2% with water. Sodium sulfate anhydrous analytical grade was obtained from Merck (Germany).

2- Apparatus

Rotary vacuum evaporator from Buchi provided with water bath (France), the centrifuge MIKRO-22R from Hettich GmbH (Germany) and high-speed vortex type: pv-1 form Grant-bio Cambridge Ltd (England) was used. Gas Chromatography used was Agilent 6890 series, with Electron capture detector specifies for organochlorine pesticides and HB-5 capillary column (30m × 0.25mm) coated with a 0.25 µm thick film of 5% phenylmethylpolysiloxane was used for separation of the used pesticides.

3- Recoveries

For recovery studies, 0.25 ml of the working solution containing tested pesticides mixture was added to 5 g of honey, and allowed to stand for 15 min before extraction. Three replicates in a series of measurements were used. Blank sample was also considered.

4- Extraction methods

4.1- Method_1 (ethyl acetate)

Five grams of honey was dissolved with 50 ml 4% aqueous solution of sodium sulfate, shaked vigorously and extracted with three portions of ethyl acetate 3 × 20 ml. When emulsion formed it was broken by centrifugation at 3000 rpm for 10 min. The organic phase was dried by anhydrous sodium sulfate.

4.2- Method_2 (n-hexane)

Five grams of honey was dissolved with 10 ml of deionized, water shaked vigorously and extracted with three portions of 20 ml n-hexane. When emulsion formed it was quickly broken by centrifuging at 3000 rpm for 10 min. The organic phase was dehydrated by anhydrous sodium sulfate.

4.3- Method_3 (petroleum ether: ethyl acetate 80:20 v/v)

Five grams of honey was heated in water bath at 35°C for 15min and dissolved with 50 ml of deionized water, shake vigorously and extracted with 3 × 20 ml of (petroleum ether : ethyl acetate 80:20 v/v) then shaken by magnetic stirring for 15 min. When emulsion formed it was broken by centrifugation at 3000 rpm for 10 min. The organic phase was dried by filtering on anhydrous sodium sulfate, and concentrated to 1 ml.

5- Clean-up procedure

The concentrated extract was loaded onto a mini-column filled with 2 g florisil and 1g anhydrous sodium sulfate, prerinsed with 10 ml n-hexane. The elution was performed with 25 ml of 5 % of diethyl ether in n-hexane. The eluate was concentrated to dryness in 5 ml glass tube and redissolved in 1ml of *n*-hexane for analysis.

6- Gas chromatography with electron-capture detector

Gas Chromatography Agilent 6890 series containing auto-sampler Agilent 7883 injector, with 63Ni Electron capture detector was used for quantification the tested pesticide residues and a fused silica capillary column HB-5 (30m × 0.25mm × 0.25µm) was used for the separation. Chromatographic conditions were adjusted as follows:

The temperature program applied was 120 °C held for 1 min and programmed at 20 °C/min to 180 °C held for 2 min and programmed at 5 C/min to 220 °C, held for 5 min and finally programmed at 3 °C/min to 245 °C, held for 30 min. The injection was carried out with split/splitless injection port at 270 °C, and injection volume was 1 µl. The detector temperature was 290 °C. Gases used were: Nitrogen as carrier gas at 2.5 ml/min, with mode constant flow + make-up flow at combined flow 60.0 ml/min. The external standard method was used for quantifications by comparing peak areas of the standard with the peaks of extracts at the same retention time.

7- Statistical analysis procedures

The data were subjected to statistical analysis by two-way ANOVA test using SPSS software for windows version 10.0. Statistical significant differences between obtained recoveries from all methods were carried by Duncan's multiple range (L.S.R_D.) $p \leq 0.05$.

RESULTS AND DISCUSSION

The major objective of this study was the optimization of the extraction methods to reduce the baseline noise in order to the merits of the GC-ECD method in identification and quantitation of pesticide residues in honey.

The choice of solvent(s) is one of the most crucial decisions to be made when developing a multiresidue method for the determination of pesticides. Solvents with high polarity, such as dichloromethane, acetone and ethyl acetate or their mixtures, should be considered in order to increase the extraction efficiency accordingly (**Zhen, *et al* 2006**).

With the view to obtain a more adequate methods for optimization of the extraction and quantification method of the pesticide residues in honey, three liquid-liquid extraction methods by using ethyl acetate, n-hexan and mixture of (petroleum ether : ethyl acetate 80 : 20 v/v) were evaluated. Such evaluations were included the GC conditions, extraction method and statistical parameters. The GC-ECD Parameters and conditions observed achieved good separation of thirty five pesticides belongs to different chemical groups within < 45 min and no interference was observed in honey samples when the chromatographic parameters are carried out as illustrated in **Fig. (1)**, this is in agreement with (**Tsipi, *et al* 1999**) who reported that the matrix interference during analysis of honey in the GC-ECD system was limited and the ECD detector response for target compounds was linear in the concentration range 0.2 to 40 µg/L.

Calibration curves were constructed from peak areas versus pesticide concentrations. Limits of detection (LODs) of the followed methods for the studied pesticides were calculated by weighing 5.0g sample, final volume to 1.0ml and injection 1.0µl (**Zhen *et al* 2006**).

Data in **Table (1)**, show the results of recoveries and standard deviation obtained from quantitative analysis of 35 pesticides in spiked honey samples with different amounts (0.2-2.0 µg/kg). The studied methods of analysis showed differences in the number of the detected pesticides. Method_1 was detect thirty three pesticides with recoveries percent ranged from (64.86 – 113.01) %, and it can't detect two pesticides dicofol and metrbuzine. The other methods were detected thirty one pesticides with recovery percent ranged from (64.67 – 111.28) and (63.48 – 107.82) % with method_2 and method_3 respectively. It can't detect both of trichlorofon and furan while dicofol was detected, but the recovery percent was less than 50% by these two methods.

Statistical data obtained from the three tested methods indicated that the mean difference in mean of total recovery percents was significant at the level (0.05) between methods. The mean of total recovery percents for the three tested methods were 82.78, 77.69 and 81.25% with method 1, 2 and 3 respectively. Each method was in separate statistical group a, b and c **Table (2)**.

Recoveries were assessed, by comparing chromatograms of calibration standard with final extracts of the spiked honey samples with the same calibration standards, (**Tahboub, *et al* 2006**).

Finally ethyl acetate method achieved the best results with respect to extraction efficiency with thirty three pesticides among the studied thirty five tested pesticides This data is in agreement with the finding of (**Blasco, *et al* 2004**) who reported that the best result for fortification levels between (10 – 100 µg/kg), were obtained from extracting with ethyl acetate.

CONCLUSION

In brief all of the three tested methods are suitable for the determination pesticide residues in honey, each of these methods can be used depending on available chemical facilities. The GC-

Table 1. Retention times, fortification levels, detection limits, mean percentages of recovery and standard deviation of 35 pesticides by three different methods of extraction in honey (n=3)

No.	Pesticides	Retention time (s)	Fortification level µg/kg	Limit of detection mg/kg	Mean percentage of Recovery ± S.D.		
					Method_1	Method_2	Method_3
1	Trichlorofon	1.22	0.5	0.05	66.30 ± 1.02	0.00	0.00
2	Furan	1.26	6.0	0.04	64.86 ± 0.67	0.00	0.00
3	Dicofol	1.69	8.0	0.01	0.00	16.14 ± 1.49	46.76 ± 0.74
4	Linuron	3.37	30	0.05	98.15 ± 1.84	82.76 ± 0.62	88.0 ± 0.80
5	Alpha-HCH	6.84	0.8	0.004	68.01 ± 1.48	64.67 ± 2.54	67.48 ± 0.35
6	Atrazin	7.39	80	0.02	109.75 ± 2.55	103270 ± 1.10	105.62 ± 0.7
7	Beta-HCH	7.5	2.0	0.001	86.28 ± 0.97	85.91 ± 0.58	98.86 ± 0.77
8	Gama-HCH	7.67	1.0	0.001	70.88 ± 0.73	67.97 ± 0.52	69.43 ± 0.40
9	Delta-HCH	8.32	1.0	0.005	87.01 ± 0.56	75.35 ± 0.54	88.34 ± 0.12
10	Metrbuzin	9.29	2.0	0.005	0.00	68.20 ± 0.85	63.10 ± 0.32
11	Venclozolin	9.46	2.0	0.01	88.29 ± 0.92	80.16 ± 0.26	91.47 ± 0.98
12	Heptachlor	9.7	1.0	0.08	70.18 ± 1.70	67.06 ± 0.17	59.97 ± 1.54
13	Aldrin	10.78	1.6	0.005	113.01 ± 1.60	111.28 ± 2.88	107.82± 1.89
14	Heptachlor- epoxide	11.09	1.0	0.09	66.04 ± 1.37	98.89 ± 2.01	88.69 ± 0.54
15	Alpha-Endosulfan	12.25	2.0	0.05	85.59 ± 0.36	80.98 ± 1.30	89.60 ± 0.99
16	P,P-DDE	13.3	2.0	0.01	85.67 ± 1.12	83.69 ± 0.77	92.68 ± 0.96
17	Dieldrin	14.2	2.0	0.01	87.65 ± 1.26	84.53 ± 1.41	90.99 ± 1.02
18	O,P-DDD	14.51	2.0	0.01	89.27 ± 2.09	88.77 ± 0.9	78.75 ± 1.02
19	Endrin	15.02	2.0	0.01	86.71 ± 1.51	85.39 ± 0.78	95.26 ± 0.25
20	Beta-endosulfan	15.41	2.0	0.05	87.62 ± 1.97	90.10 ± 0.14	99.0 ± 0.29
21	O,P-DDT	16.01	2.0	0.01	88.92 ± 1.10	85.21 ± 0.90	90.29 ± 0.61
22	Propconazol	17.55 , 17.9	12	0.01	99.89 ± 2.0	95.32 ± 1.22	101.07± 0.22
23	P,P-DDT	17.68	0.4	0.01	96.41 ± 1.12	98.26 ± 0.22	107.08± 2.11
24	Epoxiconazol	19.61	4.0	0.01	96.75 ± 1.20	96.57 ± 1.26	94.50 ± 2.71
25	Acetamiprid	20.5	2.0	0.06	88.87 ± 0.94	0.00	0.00
26	Bromppropelate	20.8	4.0	0.05	90.66 ± 1.74	92.22 ± 0.28	99.29 ± 1.45
27	Tetramethrin	21.19	40	0.09	102.80 ± 0.50	90.12 ± 1.86	88.07 ± 1.15
28	Tetradifon	21.29	2.0	0.05	95.26 ± 0.55	90.90 ± 1.53	93.42 ± 0.97
29	Lampada-cyhalothrin	22.42 , 24.96	6.4	0.01	88.09 ± 1.21	88.89 ± 0.25	95.40 ± 1.18
30	Permithrin	27.27 , 27.75	40	0.01	99.60 ± 0.94	99.27 ± 0.35	103.70± 1.92
31	Prochloraz	28.06	8.0	0.01	74.81 ± 0.21	90.86 ± 2.01	71.65 ± 0.84
32	Cyfluthrin	29.74, 30.18, 30.5, 30.72	20	0.01	92.98 ± 0.38	86.90 ± 0.15	90.66 ± 1.39
33	Cypermethrin	31.06, 31.59, 31.91, 32.14	20	0.05	89.75±0.84	90.04± 0.12	95.99±1.03
34	Fenvalerate	35.86 , 37.29	20	0.01	91.37 ± 0.79	91.34 ± 1.24	97.49 ± 1.17
35	Deltamethrin	41.39	10	0.05	90.0 ± 0.73	88.31 ± 0.12	93.41 ± 1.36

Method_1 (ethyl acetate), Method_2 (n-hexane) and Method_3 (petroleum ether : ethyl acetate 80:20 v/v)

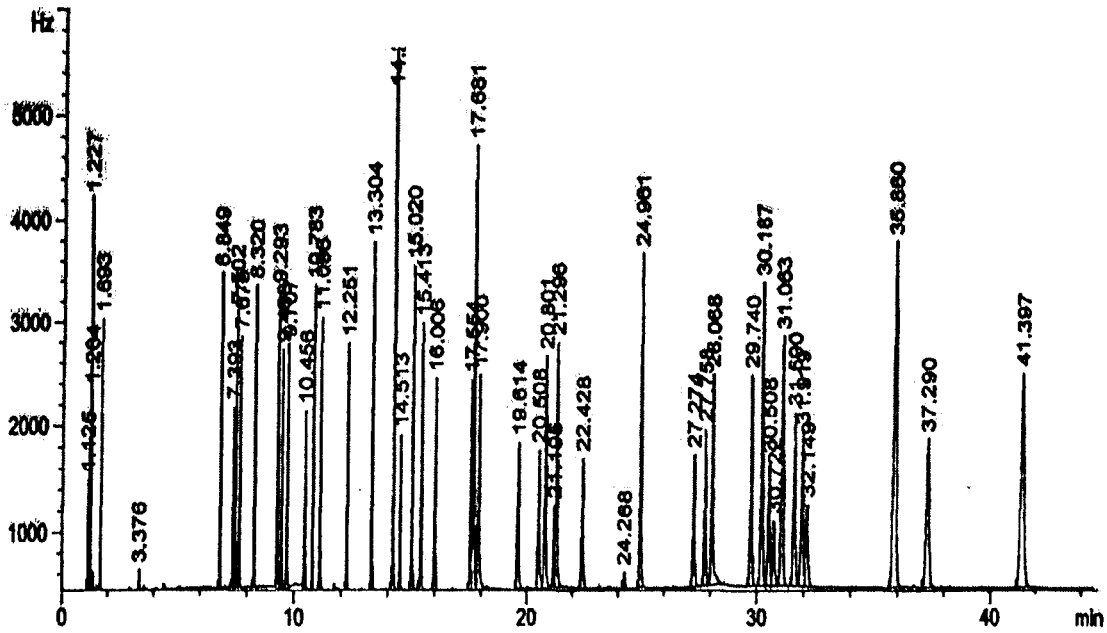

Fig.1. GC-ECD chromatogram of mixed pesticides standard solution

Table 2. Mean recoveries for three methods and standard error

Method	Mean ± S.E.	group
Method_1	82.78 ± 0.112 bc	a
Method_2	77.69 ± 0.112 ac	b
Method_3	81.25 ± 0.112 ab	c

ECD parameters observed a good separation of thirty five pesticides belongs to different chemical groups within less than 45 minutes. It is agreed that ECD is more sensitive. Sensitivity of the method is also based on other factors such as detector sensitivity to the compound which expressed as the concentration factor, extraction method, volume injected into GC and mode of injection.

Regarding the potential of pesticides on human health and in environmental sources (water, soil and plant ..etc.), have led to establish a wide range of monitoring and risk assessment programs by governments and federal research centers. So, the monitoring of pesticides pollution in honey is very important and it can be use as an indicator to environmental pollution.

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تقدير متبقيات المبيدات في عسل النحل بإستخدام طرق مختلفة للإستخلاص والتقدير بالكروماتوجرافي الغازي المزود بكاشف صائد الإلكترونات

[٤١]

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١- قسم بحوث متبقيات المبيدات وتلوث البيئة-المعمل المركزي للمبيدات-مركز البحوث الزراعية-وزارة الزراعة-الدقي-مصر

الموجـــز

إستهدفت هـــذه الدراســـة إجراء تقييم لثلاثة طرق لإستخلاص وتقدير متبقيات المبيدات في عسل النحل، حيث تم تطبيقها لتقدير عدد ٣٥ مبيد تتبع مجاميع كيميائية مختلفة مع إمكانية فصل المشابهات المختلفة وأي مركبات أخري موجودة في العينات. تضمنت المبيدات المستخدمة في الدراسة عدة مجاميع كيميائية مختلفـــة والتي منها مجموعـــة المركبات الفوسفورية العضوية ويمثلها مركب التراي كلوروفون، مجموعة مركبـــات الكاربامـــات ويمثلهـــا مركب الفيـــوران ، مجموعـــة المبيـــدات الكلورونيـــة العضوية ويمثلها مركبات (الفا، بيتا، جاما ودلتا)-هكساكلوروهكسان، (أرثو, بارا وبارا,بارا)-ددت ، أرثو,بارا-دداي ، بارا,بارا-ددد، (الفا و بيتا)-إندوسلفان، ألدرين، داي الدرين ، هبتاكلور وهبتاكلور-إيبوكسيد ، مجموعة تراي آزين ويمثلها مركب أترازين ، مجموعـــة تراي آزينـــون ومنها مركب ميتريبوزين ، مجموعة تراي آزول منها مركبي بروبكونازول و إيبوكسي كونازول ومجموعـــة مركبات البيروثرويدات العضوية ومنها مركبات لمدا سيهالوثرين، بيرمثرين، سيفلوثرين، سيبرمثرين، فينفالبرات، دلتامثرين.

اعتمدت هـــذه الطـــرق علي تقنية الاســـتخلاص بالتوزيع بين سائلين وذلك بإستخدام مذيبات عـــضوية مختلفة هي الإيثيل اسيتات (الطريقة الأولي)، الهكسان العادي (الطريقة الثانية) ومخلوط (بتـــروليم ايثـــر : إيثيل اسيتات ٨٠:٢٠ حجم/حجم) (الطريقة الثالثـــة)، ويلي ذلك إجراء التنقية بإستخدام عمود الفلوروسيل ثم التقدير بإستخدام جهـــاز الكرومـــاتوجرافي الغـــازي الملحق به كاشف الإلتقاط الإلكتروني. اشارت النتائج الي ان الطريقة الأولي كانت افضل الطرق المستخدمة حيث تراوحت نسب الاسترجاع لهذه المركبات مـــن عينات العسل المضاف اليها تركيزات مختلفة من هذه المبيدات فـــي المـــدي مـــن (٦٤,٨٦-١١٣,٠١٪)، (٦٤,٦٧ - ١١١,٢٨٪) و (٦٧,٤٨ - ١٠٧,٨٢٪) بالنسبة للطريقة الأولي والثانية والثالثة علي الترتيـــب كذلك اشارت النتائج الي انه يمكن إستخدام اي مـــن هذه الطرق في الكشف عن متبقيات هذه المبيدات في العسل تبعا للمواد الكيميائية المتاحة.

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أ.د مصطفـــى أبـــو زهو