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Determination of Aldicarb, Propoxur, Carbofuran, Carbaryl and Methiocarb Residues in Honey by HPLC with Post-column Derivatization and Fluorescence Detection after Elution from a Florisil Column

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ABSTRACT

The aim of this study was to develop an easy analytical method for determining aldicarb, propoxur, carbofuran, carbaryl and methiocarb residues in honey by high performance liquid chromatography with post-column derivatization and fluorescence detection (HPLC/PCD-FL) for routine analysis. A florisil packed column was used in sample preparation step and showed good performance. The recoveries ranged between 72.02 and 92.02% for various spiking levels (50, 100, 200 ng/g). The limits of detection were 4–5 ng/g, depending on different *N*-methylcarbamate pesticides. The extraction method used in the present study was easy, inexpensive, and rapid. Thus, it could be used efficiently in the routine monitoring studies.

Key words: honey, liquid chromatography, post-column derivatization, *N*-methylcarbamate pesticide, residue, florisil

INTRODUCTION

Carbamate pesticides, a class of highly effective commercial pesticides, have been used worldwide since the beginning of 1960s due to their high insecticide and nematocide effects. Their structures are *N*-substituted carbamic acid esters(1). Carbamate pesticides began to replace organochlorine such as DDT and organophosphorous pesticides, due to their low environmental persistence and low toxic effects on mammalians(2,3). However, carbamate pesticides are suspected carcinogens and mutagens(4) because of they are also acetylcholinesterase inhibitors. The increasing use of carbamate pesticides poses a risk to apiculture and human environment. Thus, it is necessary to quantify their residue amounts in foods to prevent harmful effects on animals, human and the environment(4-9). Honey, a natural product of bees, is composed primarily of sugars and water. Quality of honey is directly related to its floral origin and the region of production. Its quality control is performed to monitor residues(10). According to the Directive 96/23/EC, it is necessary to research *N*-methylcarbamate pesticide residues in honey(11). Many methods are available for determination of carbamate pesticide residues, such as ELISA, cholinesterase-based biosensors and chromatography(12,13). For chromatography, different sample preparation methods have been applied to the determination of carbamate pesticides, including liquid-liquid extraction (LLE), solid-phase extraction (SPE), and supercritical fluid extraction (SFE). SPE requires much lower volume in organic solvent usage(14-20). On the other hand, common techniques for determining carbamate pesticides are based on chromatographic techniques, such as gas chromatography (GC), high-performance liquid chromatography (HPLC), liquid chromatography mass spectrometer (LC-MS), GC-MS, LC-MS-MS and GC-MS-MS(21-25). For polar, less volatile and thermally labile pesticides, such as carbamate pesticides, HPLC is obviously the prefered approach because of its high sensitivity and selectivity(25-31). Derivatization reaction is employed mainly to increase sensitivity or selectivity for the detector, particularly fluorescence detector. Among the pre-column and post-column derivatization methods reviewed, post-column method is the most commonly used(32-39).

The carbamate hydrolysis and derivatization reac-

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tion is shown in Figure 1.

In this study, we preferred to use HPLC post-column derivatization and fluorescence detection and aimed to develop a new, simple multiresidue method for determining several *N*-methylcarbamate pesticide residues in honey by using a florisil packed-column. The florisil was used in previous studies(40-44). The method described in this study was rapid and suitable for screening pesticide residues in monitoring programs. Clean-up was performed using activated florisil packed-column as SPE technique. No methods have so far been documented for *N*-methylcarbamate pesticides in honey by HPLC with post-column derivatization and fluorescence detection after elution from activated florisil.

MATERIALS AND METHODS

I. *Biolocigal Materials*

Honey samples were collected from local markets in Turkey and used to develop method for *N*-methylcarbamate pesticide residue analyses. Samples were stored during work at -20°C.

II. *Chemicals, Reagents and Standards*

In this study, all solvents (acetonitrile, methanol, n-hexane) were of analytical grade and obtained from Merck (Darmstadt, Germany). Hydrolysis reagent, thiofluor, [o-phthalaldehyde (OPA)] was supplied from Pickering Laboratories (California, USA). The standards of *N*-methylcarbamate pesticide (aldicarb 98.5%, propoxur 99.0% carbofuran 98.0%, carbaryl 99.5% and methiocarb 98.5% purity) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Florisil (60-100 mesh) and dichloromethane were obtained from J.T. Baker (Deventer, Netherlands). Anhydrous sodium sulfate was from Merck (Darmstadt, Germany). Ultra pure water was supplied by Elga (London, UK).

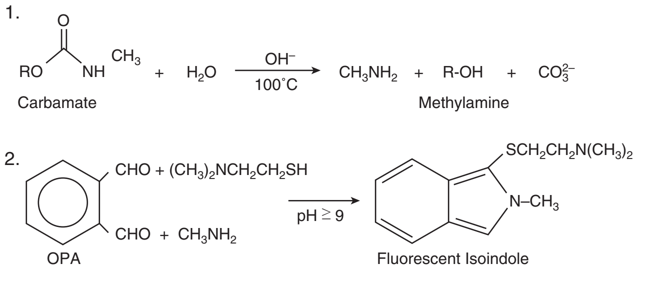

Figure 1. Carbamates hydrolysis and derivatization reaction. The carbamate left from the column was hydrolyzed to methylamine form in the post-column reactor at 100°C and then mixed with OPA and thioflour. At the end of this reaction, the soluction formed fluorescent isoindole that could be read by fluorescence detector(32).

III. *Instruments*

HPLC/PCD–FL was used for analyses. The HPLC system consisted of an Agilent 1100 Series, including gradient pump, fluorescence detection system, autosampler, vacuum membrane degasser, and software Chemstation Rev.b01.03 (2001-2005) (Avondale, PA, USA). The post column system was supplied by Prometheus (Athena, Greece).

Chromatographic separation was achieved on a reversed-phase C18 column, 5 μm, 150 × 4.6 mm I.D obtained from Pickering Laboratories (California, USA). The guard column XDB-C_8 (4.6 × 12.5 mm I.D.) was obtained from Agilent (Avondale, PA, USA).

IV. *Chromatographic Conditions*

The mobile phase consisted of a mixture of water and acetonitrile at a ratio of 90:10 (v/v). The time program of mobile phase system during analysis period is shown in Table 1.

The injection volume was 20 μL. Monitoring wavelength was set at exication 330 nm, and emission 465 nm. Flow-rate of mobile phase was set at 1 mL/min, and the temperature of the column was 42°C. Hydrolysis reaction temperature was set at 100°C and the flow-rate of hydrolysis and OPA reagents were both 0.3 mL/min(45).

(I) *Preparation of the Reagents*

The hydrolysis reagent (950 mL) was prepared in No. 1 bottle.

The OPA diluent (950 mL) was prepared in No. 2 bottle.

(II) *Preparation of the OPA Reagent*

Five milliliter of the OPA diluent was taken from No. 2 bottle and 2 g of thiofluor was dissolved in this OPA diluent. One hundred milligram of OPA was dissolved in 10 mL of HPLC-grade methanol. Both of solutions were added to No. 2 bottle.

V. *Standards and Working Solutions*

Concentration of stock standard solution was 10 ng/

Table 1. Time program of mobile phase system

Step	Time (min)	Interval	Water, %	ACN, %*
0	0	0	90	10
1	0–35	35	90	10
2	35–40	5	10	90
3	40–60	20	90	10

*ACN: Acetonitrile.

μL. Working standard solutions of all carbamate pesticides were prepared at six different concentrations (0.01, 0.05, 0.1, 0.25, 0.5, 1 ng/μL) in methanol. Calibration curve was prepared in the range of 0.01−1 ng/μL for all carbamate pesticides (aldicarb, propoxur, carbofuran, carbaryl, methiocarb) (n = 6).

VI. *Sample Preparation*

Frozen honey samples were thawed at room temperature (25°C) and honey (1 g) sample was weighed in a 25 mL glass tube and added with 2 mL of methanol. The sample was mixed by handling. Florisil was activated at 130°C for at least 48 hr. A little cotton wool was put into a glass column (200 × 20 mm I.D.) Then, 12 g prepared florisil was added with anhydrous sodium sulfate (10 g). After mixing with 20 mL of *n*-hexane/dichloromethane (1/1), the mixture was transferred to the glass column. The column was conditioned. The sample was applied to the glass column and eluted with 15 mL of n-hexane/dichloromethane mixture (1/1, v/v). The eluant was collected in a glass tube and then evaporated (at 40°C, reduced pressure). Residue was dissolved with 1 mL of acetonitrile.

VII. *Validation*

The following parameters were determined for validation of developed method: linearity, precision, accuracy, limit of detection (LOD), limit of quantitation (LOQ), and recovery.

For linearity, six levels (0.01−1 ng/μL) of calibration series with six injections at each concentration level were determined. Calibration curve was calculated automatically using software.

Recoveries were calculated as the percentage using extraction process (with activated florisil) after spiking from 50 to 200 ng/g with three different levels of carbamate pesticides (n = 6).

Precision and accuracy were defined based on spiked pesticide-free honey samples at three concentrations by calibration curve prepared daily (n = 6). Relative standard deviation (RSD) and relative standard error (accuracy) were determined. All samples were freshly prepared from reference standard solutions (10 ng/μL). Accuracy was calculated according to this formula: (Concentration found − concentration known) × 100 / Concentration known.

Considering a signal-to-noise ratio of 3 and 10 for LOD and LOQ, respectively.

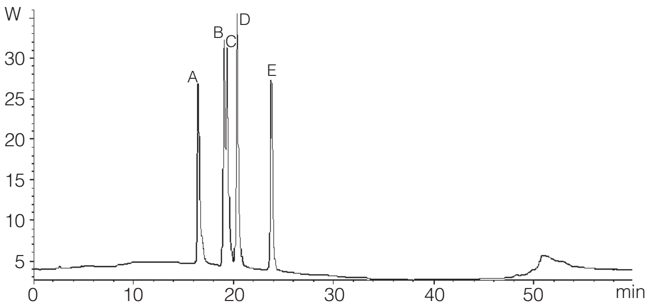

Figure 2. HPLC/PCD-FL chromatogram on C18 column by injecting 20 μL of standard solution (1 ng/μL): A: aldicarb peak, B: Propoxur peak, C: Carbofuran peak, D: Carbaryl peak, E: Methiocarb peak.

Table 2. Limit of detections (LOD, ng/g), limit of quantitations (LOQ, ng/g) and correlation cofficients (R^2) in 0.01-1 ng/μL standard solution range with six levels

Pesticide	LOD (ng/g)	LOQ (ng/g)	Correlation (R^2)
Aldicarb	5	15	0.9998
Propoxur	4	16	0.9999
Carbofuran	4	16	0.9999
Carbaryl	4	16	0.9999
Methiocarb	4	16	0.9999

RESULTS AND DISCUSSION

Chromatogram of carbamate pesticide mix standard (1 ng/μL) is shown in Figure 2. Retention times were 16.528, 18.942, 19.758, 20.437 and 23.972 min for aldicarb, propoxur, carbofuran, carbaryl and methiocarb, respectively (Figure 2).

Calibration graphs were established within the range of 0.01–1 ng/μL with correlation of coefficients from 0.9998 to 0.9999 for all analytes. The correlation of coefficients (R^2), LODs and LOQs are shown in Table 2. LOD of aldicarb was 5 ng/g whereas LODs of others were 4 ng/g. LOQs were found nearly 3.72 times of LODs.

Blasco *et al.*(46) reported that Porteguese honey was more contaminated than Spanish honey. However, little pesticide residues were found in Porteguese honey.

Khan *et al.*(47) reported that among 36 honey samples collected from different bee colonies, carbaryl was detected in one honey sample each of Apis mellifera (901.8 ppb) and Apis florae (800.0 ppb) in India.

Miyata *et al.*(48) reported that the extract was purified on a silica gel column and GC was used for determination of carbamate pesticides. No pesticide residue was found in the 56 samples of polished rice from Thailand. The detection limit of the proposed method was 0.01 ppm for carbamate pesticides. In the present study, LODs were 4-5 ng/g. We used HPLC/PCD/FL because it was more sensitive and selective than GC for carbamate pesticides. Due to their physical chemical properties such as thermal instability and polarity, carbamate pesticides are difficult or even impossible to be analysed using GC techniques without the time-consuming derivatization process. Due to lower sensitivity of LC/UV, HPLC with fluorescence detection by post-column derivatization is the most widely used method for the analysis of carbamate pesticides in foods(49,50). LODs obtained from the present study were consistent with other papers(51,52).

For recovery studies, honey samples were spiked by adding 50, 100, and 200 ng/g standards of *N*-methylcarbamate pesticide. Chromatogram of blank sample and spiked samples (200 ng/g) on C18 column are shown in Figures 3 and 4, respectively.

Precision (RSD) and accuracy ranged 1.77−9.23% and −7.98−27.98%, respectively (n = 6).

Recoveries of the spiked samples, RSD and accuracy are shown in Table 3.

Average recoveries of the spiked samples were 80.09, 77.34, 85.11, 78.02, 79.78% for aldicarb, propoxur, carbofuran, carbaryl and methiocarb, respectively. The recoveries were between 72.02 and 92.02% for varione spiking levels (50, 100, 200 ng/g). Bernal *et al.*(53) and Kawasaki *et al.*(54) reported that recovery of SPE florisil packed-column was higher than liquid-liquid extraction method. Recoveries in the present study were consistent with previous studies(55-57).

Previous studies reported that the extraction of pesticide residues from honey (SPE with florisil packed column) was easier and cheaper than the commonly used procedures(42,44,57). Jimenez *et al.*(42,58) reported that florisil cartridges contributes to reducing the matrix effects. In this study, matrix effect was not observed. This situation resulted from activated florisil and anhydrous sodium sulfate.

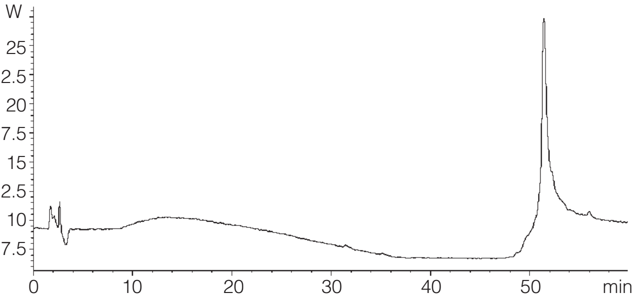

Figure 3. Chromatogram of blank honey sample on C18 column.

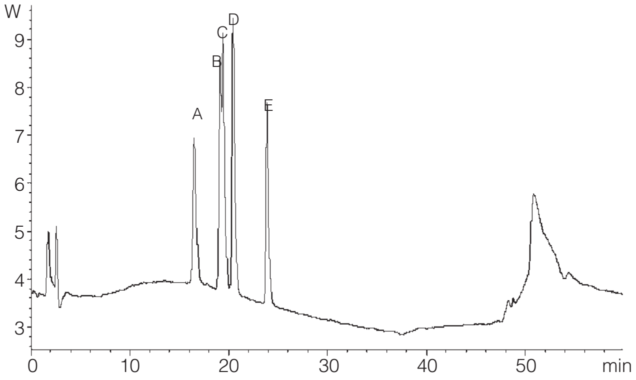

Figure 4. Chromatogram of spiked samples (200 ng/g) on HPLC/PCD–FL. A: aldicarb peak, B: Propoxur peak, C: Carbofuran peak, D: Carbaryl peak, E: Methiocarb peak.

Table 3. Recoveries of spiked samples (n = 6), relative standard deviations (RSD, %) and accuracy in 3 levels

Pesticide	Spiked level (ng/g) (n = 6)	Recovery (%)	RSD (precision, %)	Relative error (accuracy, %)
Aldicarb	50	82.11	5.20	−17.89
	100	83.28	7.36	−16.72
	200	74.89	4.22	−25.11
	average	80.09	8.90	−19.91
Propoxur	50	74.94	1.92	−25.06
	100	80.08	9.23	−19.92
	200	77.01	4.39	−22.99
	average	77.34	6.42	−22.66
Carbofuran	50	78.22	2.76	−21.78
	100	92.02	9.11	−07.98
	200	85.10	4.95	−14.90
	average	85.11	7.28	−14.89
Carbaryl	50	77.09	1.77	−22.91
	100	76.52	8.23	−23.48
	200	80.47	5.19	−19.53
	average	78.02	7.94	−21.98
Methiocarb	50	72.02	3.51	−27.98
	100	86.66	8.40	−13.34
	200	80.68	6.68	−19.32
	Average	79.78	7.55	−20.22

CONCLUSIONS

In the present study, a new method was developed by HPLC with post-column derivatization and fluorescence detection after elution from a florisil column for several carbamate pesticides. A simultaneous extraction/clean-up method was proposed for determination of aldicarb, propoxur, carbofuran, carbaryl and methiocarb residues in honey using a mixture of florisil and anhydrous sodium sulfate in small glass columns. The florisil column is capable of affecting isolation and clean up of these pesticide residues from honey samples. The main advantage of the developed method is that extraction and cleanup are performed in one step with small volume of organic solvent. Additionally, the validation parameters show that the developed method is a sensitive, reproducible and reliable alternative to the normally used methods. Moreover this inexpensive, easy and rapid method can be used in the routine and monitoring studies. The analysis can be confirmed by LC/MS in future studies and the developed method can be used to analyze other carbamate pesticides and their main metabolites.

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