Simultaneous Determination of Amitraz, Bromopropylate, Coumaphos, Cymiazole and 2,4-Dimethylaniline in Korean Honey Samples by High-Performance Liquid Chromatography

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Simultaneous determination of amitraz, bromopropylate, coumaphos, cymiazole and 2,4-dimethylaniline in 200 honey samples purchased in Korea was performed by reversed-phase high-performance liquid chromatography with multiple UV detection. 2% Acetone in hexane was used for a liquid-liquid extraction and 20-40% water in acetonitrile solutions were used as mobile phases. The LOD for the analytes varied between 0.4 and 1.5 μg/L and the recoveries were yielded between 64 and 94%. Relative standard deviation of the repeatability of the method is less than 15%. Amitraz was not present in amount above 10 μg/L and one for coumaphos and cymiazole and two for bromopropylate, and three for 2,4-dimethylanilne were detected in amount above 10 μg/L. Levels of the acaricide residues found were less than 50 μg/L.

Key Words : Simultaneous analysis, Honey, Acaricide, HPLC-UV

Introduction

Amitraz, bromopropylate, coumaphos and cymiazole are frequently used as acaricides by beekeepers.[1] Even though very small amounts of the acaricides are used, a certain amount of the active ingredient can remain in the hive, resulting in the contamination of honey.[2] Many countries have established Maximum Residue Limit (MRL) for these four acaricides.[3] Korea has also established MRL. Many methods for extraction, purification, and quantitative analysis of acaricides in honey have been introduced very recently.[3,4] According to the recent review paper,[3] about 65% of them are based on GC or GC/MS, and others, based on HPLC. Honey is freely soluble in water. When it is analyzed by reversed phase HPLC, additional purification is not necessary after extraction. This is a distinct merit of the RP-HPLC method for analysis of honey samples. Various kinds of mobile phases and extraction solutions used for analysis of acaricides resulted in different results.[2,3,5-7]

In this study, an additional effective extraction and simultaneous analysis method for amitraz, bromopropylate, coumaphos, cymiazole, and 2,4-dimethylaniline in honey were developed. This method involves simple sampling procedures followed by liquid-liquid extraction without purification of the samples and quantification of acaricide residues by HPLC with multiple UV detection. The recovery yields, limits of detection (LOD), and linearity of calibration curves were examined, and quantitative analyses of amitraz, bromopropylate, coumaphos, cymiazole and 2,4-dimethylaniline in 200 honey samples purchased in Korea were performed.

Experimental

Reagents and apparatus. Amitraz (98%), bromopropylate (99%), and coumaphos (99%) were purchased from Chem Service Inc. (West Chester, PA, USA). Pure cymiazole (more than 99%) was obtained from Barokil, a commercial name of cymiazole, produced by Iljin F&B (Seoul, Korea) by several extractions and column chromatography. Benzoinmethylether (internal standard, IS) and 2,4-dimethylaniline were purchased from Sigma-Aldrich Korea (Seoul, Korea). HPLC grade acetone, methanol, hexane and acetonitrile were used for HPLC analysis and extraction. All of the HPLC and extraction solvents were supplied by Merck Korea (Seoul, Korea). Ultra-pure water was obtained from Millipore Milli-Q plus apparatus (Bedford, MA, USA). HPLC was performed on a JASCO HPLC system, consisting a JASCO PU-2080 Plus Intelligent Pump, a Rheodyne Model 7125 injector with a 20 μL sample loop, and a JASCO UV-2075 Plus Intelligent UV/VIS detector from JASCO (Tokyo, Japan). The liquid chromatographic column was an Intersil ODS-3 (5 μm particles, 250 mm × 4.6 mm i.d.) from G.L. Science INC. (Seoul, Korea). All honey samples used in this study were purchased from various places including traditional markets, internet on-line markets, department stores, and post offices in Korea from Dec. 2006 to Feb. 2007.

Preparation of standard solution. A 100.0 mg/L (each component) mixed stock standard solution of these compounds was prepared by dissolving 50.00 mg of each compound, amitraz (abbreviate to A), 2,4-dimethylaniline (ab-

breviate to A'), bromopropylate (abbreviate to B), coumaphos (abbreviate to C), and cymiazole (abbreviate to Cy), into 500 mL 98:2 hexane-acetone containing 1.00 ppm (1000 μg/L) benzoinmethylether (IS), and stored at −20 °C in the dark.[8] The stock solution was diluted to 0.5, 1, 2, 3, 5, 10, 12.5, 25, 50, 100, 200, 500, and 1000 μg/L (each component) mixed standard solutions with the same solvent.

Preparation of sample solution. Each honey sample (5.00 ± 0.01 g) was weighed in a 300 mL Erlenmeyer flask. 70 mL of water with 10 μL NH_4OH solution was added and stirred for 5 min at room temperature. 70 mL of 2% acetone in hexane solution was added and stirred for 25 min. The stirred solution was poured into a 250 mL separating funnel. The Erlenmeyer flask was rinsed with 20 mL 2% acetone in hexane solution and the solution was added to the separating funnel. The aqueous phase was moved into other separating funnel for additional extraction with the organic phase after vigorous shaking and the organic phase was washed with 50 mL of 0.25% ammonia water twice. The combined organic phase was dried with magnesium sulfate and the solution filtered. The filtrate was concentrated to dryness by evaporation under reduced pressure at 30 °C. The recovery flask was flushed with nitrogen gas to remove all solvent. The residue was dissolved with 2.00 mL of internal standard (1000 μg/L) containing 96:4 (v:v %) hexane-acetone.

Chromatographic conditions. The mobile phase was 70:30 (v/v) acetonitrile-water and the flow rate was 1.2 mL/min. However, when complex samples were analyzed, the composition (60:40 or 65:35) of mobile phase and the flow rate (1.0 or 0.8 mL/min) were changed for exact determination. The compounds were analyzed under different detection wavelength for each analyte: 289 nm, 250 nm, 233 nm, 313 nm, and 264 nm, respectively, for amitraz(A), 2,4-dimethylaniline(A'), bromopropylate(B), coumaphos(C), and cymiazole(Cy). Sample injection volume was adjusted to 20 μL by using a sample loop. The average value from more than five separate analyses was taken for the quantification data of each compound. An external calibration was employed.

Results and Discussion

Optimum detection wavelength. To find the optimum detection wavelength of our HPLC system for simultaneous analysis of the five acaricides and internal standard, the UV spectra of each chemical were obtained.

As shown in Figure 1, the optimum wavelengths between 210 nm and 780 nm (detection range for HPLC) for each analyte were determined as 289 nm, 240 nm, 233 nm, 313 nm, and 264 nm, respectively, for amitraz(A), 2,4-dimethylaniline(A'), bromopropylate(B), coumaphos(C), and cymiazole(Cy). These results are similar to previous results.[2,9] From these results and retention times of individual analytes, the conditions necessary for multiple UV detection were established as follows: 250 nm (0-5.5 min), 264 nm (5.5-7.0 min), 313 nm (7.0-10.5 min), 233 nm (10.5-16.5 min), and 289 nm (16.6-30.0 min). These conditions were applied to the HPLC analysis of the 1000 μg/L (each component) standard and the chromatogram is shown in Figure 2. The six peaks were clearly separated under the outlined conditions, therefore, the conditions were applied to analyze the 200 honey samples.

Selection of extraction solvent. Four generally used extraction solutions including previous used solution were tested to select a good extraction solvent.[3] As shown in Figure 3, the smallest impurity peaks were found in the 2% acetone containing hexane solution, therefore, this solution was selected as an extraction solvent for this study. A further study about this is in progress.

Limit of detection (LOD). → Limits of detection (LODs) were calculated at a signal-to-noise ratio of 3:1 based on chromatographic data obtained with various concentrations (each component) of the mixed standard as follows: 4 μg/L, 10 μg/L, 20 μg/L, and 50 μg/L.

As shown in Figure 4, all the five peaks were easily found on the chromatogram of the 4 μg/L standard mixture. After

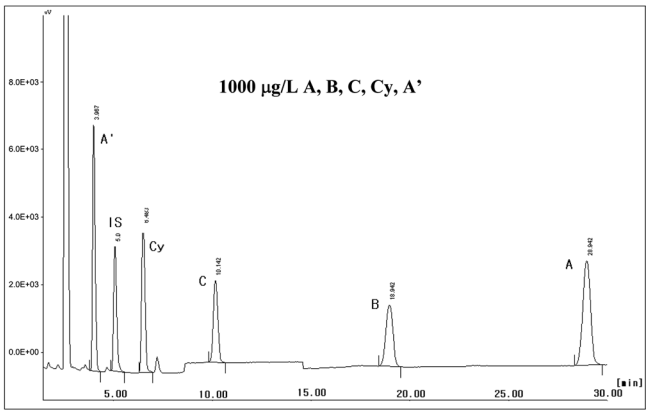

Figure 2. Chromatogram of a 1.0 ppm standard mixture of amitraz (A), bromopropylate (B), coumaphos (C), cymiazole (Cy), and 2,4-dimethylaniline (A') on ODS column. Flow rate: 1.2 mL/min, detection; multi-UV (0.0-8.0 min; 233 nm, 8.0-14.5 min; 313 nm, 14.5-23.5 min; 233 nm, 23.5-30.0 min; 289nm.), Peak 1: A' (3.99 min), Peak 2: Cy (6.48 min), Peak 3: C (10.1 min), Peak 4: B (18.9 min), Peak 5: A (28.9 min).

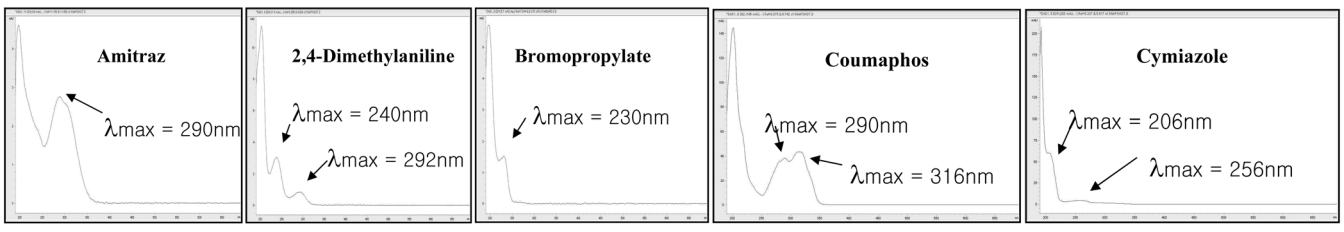

Figure 1. UV spectra of amitraz (A), 2,4-dimethylaniline (A'), bromopropylate (B), coumaphos (C), and cymiazole (Cy).

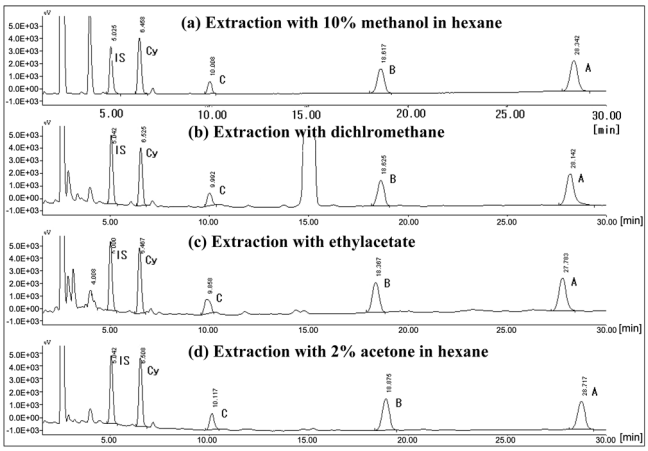

Figure 3. Chromatograms of four acaricides mixtures obtained from four different extraction solutions on ODS column. Flow rate: 1.2 mL/min, detection; multi-UV (0.0-8.0 min; 233 nm, 8.0-14.5 min; 313 nm, 14.5-23.5 min; 233 nm, 23.5-30.0 min; 289 nm.)

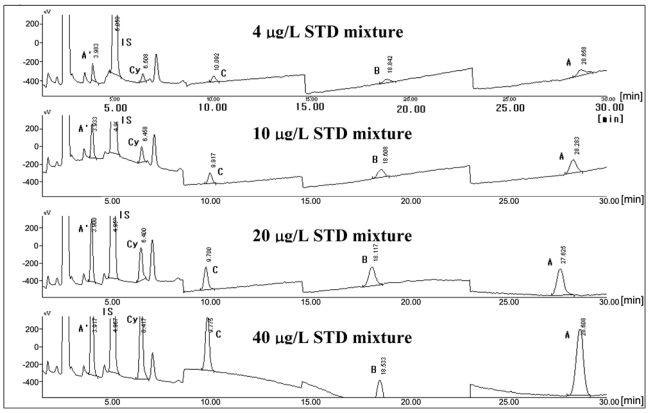

Figure 4. Chromatograms of 4, 10, 20, and 40 μg/L A, A', B, C, and C' standard mixtures on ODS column. Flow rate: 1.2 mL/min, detection; multi-UV 264, 313, 233, 289 nm.

further HPLC experiments at different concentration, 0.5, 1, 1.5, and 2 μg/L, the LOD and LOQ (s/n = 10) values for each analyte were determined and shown in Table 1. These results are lower than the previous results.[5,10-12] Because the concentration of final honey sample was 2.5 times larger than that of the original honey sample (amount of starting sample: 5.00 g $\rightarrow$ amount of analyzing sample: 2.00 mL), LODs of this analysis method are 2.5 times larger than the absolute detection limits of each compound.

Calibration curves. Calibration curves were constructed from peak areas versus acaricide concentrations. Good linearity was observed (r^2 = 0.995, at least) for all molecules (Figure 5).

Therefore, these calibration curves could be used for quantification of each acaricide in honey samples.

Recovery test. The recovery test of acaricides was carried out by separate addition of a series (4 different levels) of 2.0 mL of mixed standard solutions to a honey sample. The blank sample from the same honey without spiking was treated and analysed at the same time. Honey samples were spiked just before analysis. The acaricides were extracted as described before and the sample solutions were analysed by HPLC. In the course of extraction procedure, many organic solvents (hexane, methanol, dichloromethane, acetone, ethylacetate, and their mixtures) and many bases (ammonia water, sodium hydroxide, triethylamine, and pyridine) were used to get high recovery yields. (More advanced extraction

Table 1. Analytical parameters for four acaricides and amitraz degradation product residue[a]

Compounds	Concentration	R^2 value	LOD[c]	LOQ[d]
Amitraz	50, 100, 200, 500, 1000	0.996	0.4	1.5
2,4-DMA[b]		0.998	1.5	5
Bromopropylate		0.999	1.5	5
Coumaphos		1.000	0.5	1.5
Cymiazole		1.000	1.0	3

[a]Analytical parameters (linearity: R^2 value, limit of detection: LOD, limit of quantitation: LOQ), Values in μg/L. [b]2,4-dimethylaniline. [c]Limit of detection as the calculated concentration at the signal/noise = 3. [d]Limit of quantitation as the calculated concentration at the signal/noise = 10.

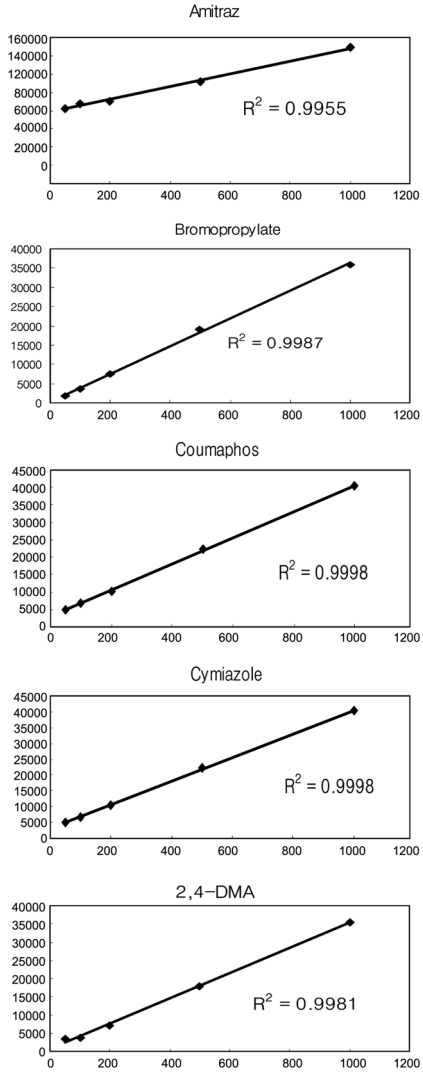

Figure 5. Calibration graphs with 50, 100, 200, 500, 1000 μg/L standard solution of amitraz, bromopropylate, coumaphos, cymiazole, and 2,4-dimethylaniline.

Table 2. Recoveries obtained for four acaricides and amitraz degradation product residue in honey at four different fortification levels[a]

	Tentative MRL[b]	Fortification level[c]	Recovery (%)[d]	RSD (%)[d]
A	100	50	89.7	4.7
		100	86.5	4.9
		200	89.5	7.9
		400	89.2	4.6
A'	100	50	70.1	10.5
		100	68.1	8.4
		200	86.8	14.8
		400	63.9	8.6
B	100	50	90.9	3.1
		100	85.4	7.1
		200	82.8	9.8
		400	90.1	6.6
C	100	50	84.9	2.0
		100	88.8	5.3
		200	93.7	12.7
		400	86.8	9.5
Cy	100	50	90.1	3.5
		100	87.0	8.4
		200	87.8	6.7
		400	87.8	5.8

[a]Amitraz (A), 2,4-dimethylaniline (A', Amitraz metabolite), Bromopropylate (B), Coumaphos (C), and Cymiazole (Cy), Values in μg/L. [b]MRL of coumaphos (the highest toxic compound among the four acaricides). [c]Fortification level; 0.5, 1.0, 2.0, 4.0 times of MRL of each compound. [d]Calculated from 7 replicated experimental data.

and chromatographic conditions for quantification of acaricides in honey samples are still being studied.) At present, a very small amount of ammonia water was found helpful for good extraction. Each solute was spiked at four different concentrations and seven repetitions were carried out for each fortification level. The recoveries ranged from 63.9 to 93.7% and the relative standard deviations (RSDs) varied from 3.1 to 14.8% (Table 2).

The reason for the low recoveries of 2,4-dimethylaniline (A') was assumed that the compound could easily be removed by the acidic polysaccharides in honey by acid-base salt formation. To improve the recovery of the A', a small amount of ammonia water was used in the course of extraction.

Analysis of acaricides in 200 Korean honey samples. The highest toxic compound among the five chemicals (A, A', B, C, Cy) is coumaphos. The MRL of coumaphos in US and EU is 100 μg/L. One tenth of the MRL was set as the quantitation limit of our experiments. Therefore, if there was less than 10 μg/L of an acaricide in a sample, the component was reported as ND (not detected). In addition, the final honey sample was concentrated 2.5 times compared to the original honey sample (5.00 g $\rightarrow$ 2.00 mL), the amounts (more than 10 μg/L) of the five acaricides could be detected more easily and correctly.

70:30 (v/v %) acetonitrile–water was used as the main mobile phase and the operating flow rate was set at 1.2 mL/min. However, the mobile phase composition and/or the flow rate were varied when very complex samples were analyzed. Simultaneous detection of the acaricides was performed at multiple UV wavelengths: 250 nm [0-5.5 min; 2,4-dimethylanilne (A') and benzoinmethylether (IS)], 264 nm [5.5-7.0 min, cymiazole (Cy)], 313 nm [7.0-10.5 min, coumaphos (C)], 233 nm [10.5-16.5 min, bromopropylate (B)], and 289 nm [16.6-30.0 min, amitraz (A)].

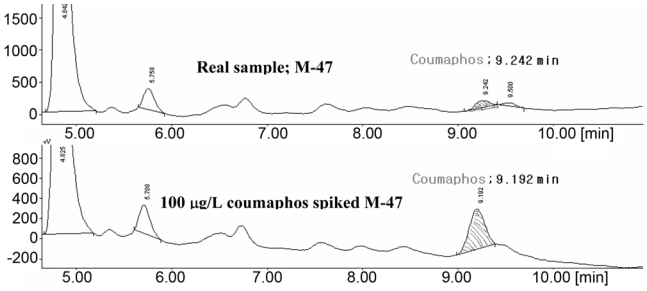

Figure 6. Chromatograms of a honeybee sample, M-47 and 100 μg/L coumaphos spiked M-47 on ODS column. Flow rate: 1.2 mL/min, detection; multi-UV (0.0-8.0 min; 233 nm, 8.0-14.5 min; 313 nm, 14.5-23.5 min; 233 nm, 23.5-30.0 min; 289 nm.)

Table 3. Number and amount of acaricides found in 200 honey samples

Sample	Number of detected one[a]	Detection range (μg/L)	Detection ratio (%)
Amitraz	0	–	0
2,4-Dimethylaniline	3	25-40	1.5
Bromopropylate	2	12-50	1
Coumaphos	1	22	0.5
Cymiazole	1	21	0.5

[a]More than 10 μg/L.

For rapid monitoring of the 200 honey samples, all of the samples were extracted twice, and 800 chromatograms (2 runs for each extracted sample) were obtained. After comparing the peak areas of the five acaricides on each chromatogram with those of the 25 μg/L (equal to 10 μg/L of real sample) standard mixture, the samples having similar or larger peak areas to those of the 25 μg/L standard were repeatedly (3 times) extracted and measuered. When similar peaks appeared near the acaricides peaks, the 50 or 100 μg/L standard was spiked to find the right components. Figure 6 shows an example of such work.

From the comparison between the spiked and the real samples, the amount of coumaphos in the real sample, M-47, was determined as 22 μg/L by internal and external calibration.

In the course of HPLC analysis, more than 1,500 HPLC runs were performed, but an additional column was needed only near the end of this study. The summarized results from the analysis of the 200 Korean honey samples are assembled in Table 3.

As shown in Table 3, amitraz was not present above the

level of 10 μg/L, and one occurrence of coumaphos and cymiazole, two occurrences of bromopropylate, and three occurrences of 2,4-dimethylanilne, were found above the level of 10 μg/L. The levels of the acaricide residues were found less than 50 μg/L.

Conclusion

A simple liquid-liquid extraction without additional purification was found useful in the analysis by reversed-phase HPLC in this study. 98:2 hexane-acetone was useful for extraction of acaricides, and acetonitrile solutions with 20-40% water proved to be good mobile phases. The LODs for the analytes varied between 0.4 and 1.5 μg/L and the recoveries were found between 64 and 94%. The relative standard deviation of the repeatability of the method is lower than 15%. Simultaneous analysis of amitraz, bromopropylate, coumaphos, cymiazole, and 2,4-dimethylaniline in 200 honey samples purchased in Korea was concisely performed by reversed-phase high-performance liquid chromatography with multiple UV detection. Amitraz was not present in the level above 10 μg/L, and one case for coumaphos and cymiazole, two cases for bromopropylate, and three cases for 2,4-dimethylanilne, were found above 10 μg/L. The levels of the acaricide residues were found less than 50 μg/L.

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