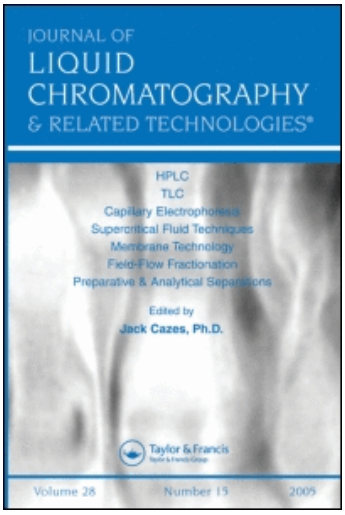

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High Throughput Analysis of N-Methyl Carbamate Pesticides in Cereals and Beans by Dual Countercurrent Chromatography and Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry

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Abstract: We developed a new analytical method for analysis of N-methyl carbamate pesticides in cereals and beans using dual counter-current chromatography (dual CCC) and liquid chromatography-electrospray ionization tandem mass spectrometry (ESI LC/MS/MS). After pesticides were extracted from cereals and beans with ethyl acetate, each extract was cleaned up by dual CCC using a non-aqueous binary solvent system composed of n-hexane-acetonitrile and analyzed by ESI LC/MS/MS with a short column. The average recoveries from cereals and beans fortified at the level of 0.01 ppm ranged from 73.9 to 119.6%, with the coefficients of variation from 0.7 to 6.8%. At the fortified level of 0.5 ppm, the recoveries ranged from 72.1

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to 117.1% with coefficients of variation from 0.4 to 9.3%. The present analytical method of N-methyl carbamate pesticides in cereals and beans is considered to be useful for monitoring the pesticide residues in cereals and beans.

Keywords: High throughput analysis, N-Methyl carbamate pesticides, Beans, Cereals, Dual CCC, ESI LC/MS/MS

INTRODUCTION

N-Methyl carbamate pesticides are used for combatting a variety of pests throughout the world, and have been often found in agricultural products.[1–4] Therefore, it is one of the most important roles for a public health agency to always inspect residues in foods. The analytical method used in the inspection is required to be simple and rapid.

In the past, we reported a simple and rapid method based on ESI LC/MS/MS, without a sample clean-up step, for the determination of nine N-methyl carbamate pesticides, shown in Fig. 1, in juice, wine, fruits, and vegetables.[5,6] However, the samples we examined in our previous reports did not contain an aliphatic sample matrix that interferes with the analysis, and the method could not be applied to cereal and bean samples that contain the aliphatic sample matrix. Therefore, we considered that the clean-up step was needed for determination of these pesticides in these samples. The most widely used sample clean-up methods include liquid-liquid partitioning using a separatory funnel, prepacked cartridge, gel permeation chromatography, etc. However, these methods involve time-consuming processes that require skillful techniques.

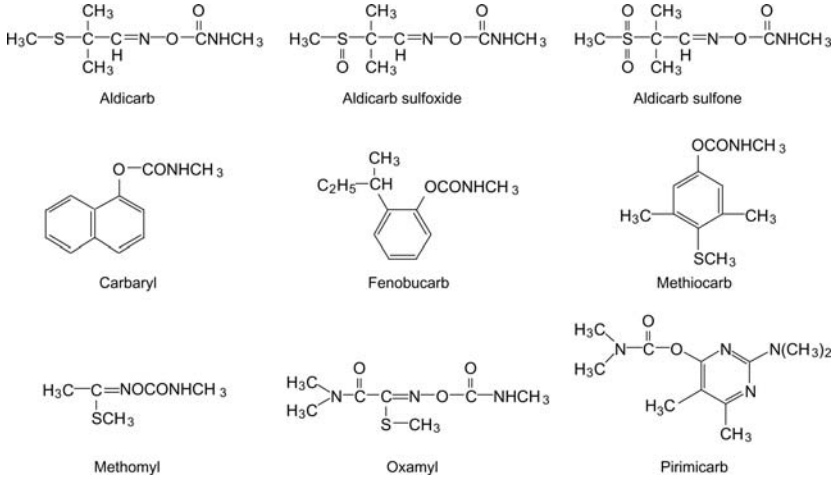

Figure 1. Chemical structures of N-methyl carbamate pesticides.

Dual CCC is a separation method that is based on solute partitioning between lighter and heavier liquid phases moving through a coiled column in the opposite directions (countercurrent) to each other.[7] A liquid-liquid partition clean-up system with n-hexane and acetonitrile, using a separatory funnnel is commonly employed to remove oil and fat from food extracts. In our previous report, we used this two-phase solvent system for dual CCC to effectively remove the aliphatic sample matrix from food extracts. We described the theory of dual CCC separation, and applied it to the dual CCC using this two-phase solvent system for the determination of residual carbaryl, fenobucarb, and methomyl in vegetable oil and fruit.[8] The reported dual CCC method required only 5 min for separation of three pesticides and interfering substances originating from the sample matrix, and successive injection of multiple samples can be performed at suitable intervals without risk of contamination by interfering substances, thus leading to rapid and efficient sample preparation. Therefore, we considered that the previously reported method can be applied to the clean-up of nine carbamate pesticides (Fig. 1) in cereals and beans.

In the present studies, we developed a high-throughput analysis method using dual CCC and ESI LC/MS/MS to determine nine N-methyl carbamate pesticides in cereals and beans.

EXPERIMENTAL

Chemicals and Reagents

All organic solvents were of pesticide-grade, obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Reagent grade formic acid was purchased from Merck (Darmstadt, Germany). The high purity deionized water was obtained from a purification system Auto Pure WT100 (Yamato, Tokyo, Japan). Aldicarb, aldicarb sulfoxide, aldicarb sulfone, methiocarb, methomyl, and oxamyl were obtained from Riedel-de-Haen (Hanover, Germany), carbaryl, fenobucarb and pirimicarb from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and fenobucarb-*d3* (chemical purity $>$99.9%), carbaryl-*d7* (chemical purity $>$99.7%) and methomyl-*d3* (chemical purity $>$99.7%) from Hayashi Pure Chemical Ind., Co., Ltd. (Osaka, Japan).

Each stock standard solution and internal standard was prepared in methanol (1 mg/mL) and the working standard solutions were diluted prior to analysis.

Dual CCC

The dual CCC equipment is a multilayer coil planet centrifuge which was designed and fabricated at the National Institutes of Health, Bethesda, MD,

USA. The coiled column, consisting of a 10 m × 2.6 mm I.D. PTFE tube with a capacity of 50 mL, is connected to five flow channels, the upper phase collection line and the lower phase inlet line at the upper phase inlet line at the tail end, and the sample feed line at the middle portion of the column. Two liquid lines each enter into the coil through a Kel-F (polymonochlorotrifluoroethylene), three-way adaptor at the respective terminus where the tubing extends into the coil for about 50 cm at the head and about 100 cm at the tail. A rheodyne injector is set on the upper phase inlet line, which is joined with the liquid supply pump. A resistance coil, made of PEEK-tube (93 mm long, 0.25 mm I.D.), is placed at the end of the upper phase collection line, which is needed for establishing the hydrodynamic equilibrium to restrain the natural pumping force. The system was rotated at 420 rpm. We reported the details of the design and conditioning in our previous report.[8]

ESI LC/MS/MS

The ESI LC/MS/MS system and the operating conditions were reported in our previous method report.[5,6]

Measurement of Partition Coefficients

In our previous report,[8] we described the measurement of partition coefficients (K) by flow-injection ESI-MS/MS using a simple test tube procedure as follows: A 100 μL aliquot of a mixed standard solution, containing nine carbamate pesticides, and a 20 μL aliquot of a mixture of three internal standards, each 5 mg/L, was pipetted into a test tube, and the solvent was dried gently under a nitrogen gas flow. Then, a 2 mL aliquot of each phase of the equilibrated n-hexane/acetonitrile was added to the above test tube. The contents were thoroughly mixed and allowed to settle at room temperature. After two clear layers were formed, a 1 μL aliquot of each phase was determined by flow-injection ESI-MS/MS. The K was obtained by dividing the concentration of the upper phase by that of the lower phase.

Sample Preparation

The whole sample was ground and homogenized with a mill. A 10 g aliquot of the homogenized sample was weighed into a 250 mL centrifuge tube, and 25 μL of working internal standard solution (40 μg/mL) was added. The mixture was blended with 100 mL of ethyl acetate with a high speed blender. The extracts were centrifuged at 3,100 rpm for 10 min and the upper organic layer was decanted into a flask. Then, the flask was placed in a water bath at 35°C and the solvent was gently evaporated to near dryness.

The remaining solvent was allowed to evaporate in the air. The residue was redissolved in 5 mL of ethyl acetate, and a 100 μL aliquot of the solution was loaded into the dual CCC. The eluate collected between 1 and 4 min was concentrated to dryness. The residue was resolved in 1 mL of ultra pure water by sonication and the precipitate was filtered off by an 0.45 μm syringe driven filter unit (Millipore, Billerica, MA, USA) into the autosampler vial. A 50 μL of the solution was injected into the ESI LC/MS/MS system.

Quantitation

Calibration curves were obtained by the construct which is peak-area ratios of the pesticides to the internal standard. Quantitation of the pesticides in the cereals and beans was achieved from the calibration curves and reported in grams of sample weight (mg/kg). Recoveries were calculated by dividing the peak-area ratio of the analyte to the internal standard from the fortified samples with that of the corresponding standard solutions as follows:

$$\text{Recovery } (\%) = (\text{Aanalyte} \times \text{Sstandard})/(\text{Sanalyte} \times \text{Astandard}) \times 100$$

where Aanalyte is peak area of analyte in the samples; Astandard, peak area of internal standard in the samples; Sanalyte, peak area of analyte in the corresponding standard solutions; and Sstandard, peak area of internal standard in the corresponding standard solutions.

RESULTS AND DISCUSSION

Dual CCC Conditions

We used a non-aqueous binary two-phase solvent system consisting of n-hexane-acetonitrile for dual CCC. When the aliphatic sample matrices are mostly partitioned in the upper n-hexane phase and the target pesticides in the lower acetonitrile phase, dual CCC produces the successful clean up effects. It is important to determine the partition coefficient (K) of the target compounds, from which we can predict their behavior in the two-phase solvent in the running column. In our previous report,[8] we had measured K values of three pesticides (carbaryl, fenobucarb, and methomyl) to investigate their applicability to the dual CCC clean up. In the present study we investigated the K values of nine pesticides in this binary solvent system with the following results: aldicarb, 0.020; aldicarb sulfoxide, 0.002; aldicarb sulfone, 0.032; carbaryl, 0.015; fenobucarb, 0.037; methiocarb, 0.026; methomyl, 0.007; oxamyl, 0.005; and pirimicarb, 0.074. Judging from these K values, we can expect that all nine target pesticides would elute together, immediately, in the acetonitrile phase to facilitate a rapid analysis.

Extraction and Clean Up Procedure

Our previous studies[8] employed the following analytical procedure: Samples were dissolved in n-hexane, the solution was evaporated to dryness, and was then dissolved in 5 mL of n-hexane. This solution was injected into the dual CCC, and the effluent was collected and concentrated to dryness. The residue was redissolved in methanol and injected into the flow-injection ESI/MS/MS. We had targeted only three pesticides using n-hexane as an extraction solvent.[8] However, in the present study, we targeted the nine pesticides and, therefore, it was necessary to investigate a suitable extraction solvent. We examined a variety of solvents, including ethyl acetate, acetonitrile, acetone, cyclohexane, and n-hexane. When acetone, cyclohexane, and n-hexane were used, the recoveries of aldicarb sulfone, aldicarb sulfoxide, and oxamyl were all less than 35% while, if acetonitrile was used, the recoveries of aldicarb sulfone and aldicarb sulfoxide were still less than 70%. Only ethyl acetate was able to extract these pesticides quantitatively, i.e., over 95%. Therefore, we selected ethyl acetate as the extraction solvent in this study.

Next, we evaluated various organic solvents used for sample injection into the dual CCC with the following method: One mL of the mixed standard solution of the N-methyl carbamate pesticides (1 μg/mL, each), dissolved in methanol, was evaporated and the residues were redissolved 1 mL of acetonitrile, n-hexane, n-hexane:ethyl acetate (1 : 1) and ethyl acetate. Then, 200 μL of each sample solution was injected into the dual CCC, while the eluate (acetonitrile phase) was collected for 10 min and concentrated to 0.5 mL. After adding 50 μL of a mixture of three ISs solutions (1 μg/mL), it was made up to 1 mL with acetonitrile. The solution was analyzed by flow-injection ESI MS/MS. At first, n-hexane was examined. The recoveries of aldicarb sulfone, aldicarb sulfoxide, and oxamyl were each less than 35%. These pesticides are more hydrophilic than the others, and we considered that the solubility was the problem, since aldicarb sulfone, aldicarb sulfoxide, and oxamyl were all less soluble in n-hexane than in other pesticides. The resulting recoveries were less than 35%. Then, we tried to use acetonitrile, n-hexane:ethyl acetate (1 : 1), and ethyl acetate. Among these, only ethyl acetate gave a satisfactory result for all pesticides. Therefore, we selected ethyl acetate as the injection solvent in this study.

Next, we investigated the injection volume into the dual CCC. In our previous study,[8] a n-hexane sample solution was injected into the dual CCC column after establishing hydrodynamic equilibrium between n-hexane and acetonitrile. In this case, even an injection volume of 200 μL did not cause any disturbance to the hydrodynamic equilibrium in the column, because the sample solution was prepared from the two-phase solvent system used for separation. However, in this study, we used a relatively large volume of ethyl acetate as the injection solvent, which might alter the hydrodynamic equilibrium and lead to unsatisfactory results. Thus, we compared the effect of

injection volumes of 100 and 200 μL. As shown in Fig. 2, a 100 μL injection gave over 90% recoveries, while a 200 μL showed less than 85%. Therefore, we selected 100 μL as the injection volume in this study.

In order to determine the elution time of the nine carbamate pesticides in the dual CCC, we analyzed each pesticide in the effluent at one minute intervals. All nine pesticides were mostly eluted between 1 and 4 min, i.e., aldicarb, 96%; aldicarb sulfone, 92%; aldicarb sulfoxide, 99%; carbaryl, 96%; fenobucarb, 100%; methiocarb, 99%; methomyl, 98%; oxamyl, 95%; and pirimicarb, 101%. Therefore, we decided to collect the effluent between 1 and 4 min for the analysis of the pesticides.

Validation of Analytical Method with Various Fortified Samples

Based on the above experimental results, we established the analytical method shown in Fig. 3.

Accuracy of the method was determined by calculation from the calibration curves constructed from the peak-area ratios of the pesticides to internal standard; they were found to be linear over the range of 0.005–1.0 μg/mL with correlation coefficients of over 0.999.

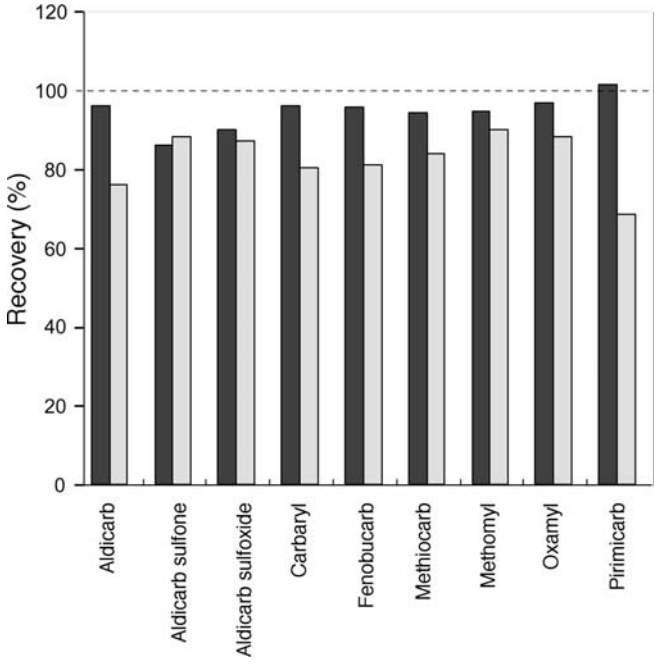

Figure 2. Influence of injection volume on the recoveries of the pesticides from dual CCC: ■ injection volume of 100 μL; ■ injection volume of 200 μL.

The recoveries were measured from various samples, such as brown rice, wheat, soybean, corn, and red bean (adzuki bean), each fortified at levels of 0.01 ppm and 0.5 ppm. They were analyzed according to the sample preparation procedures described above in this paper. The coefficients of variation (C.V.) for precision are listed in Table 1. The average recoveries from fortified brown rice were 78.6 to 117.1%, with C.V. less than 6.3%, from fortified wheat 73.9 to 111.4%, with C.V. less than 11.3%, from fortified soy bean 81.8 to 106.6%, with C.V. less than 5.6%, from fortified brown rice 78.6 to 117.1%, with C.V. less than 6.3%, from fortified corn 76.6 to 113.0%, with C.V. less than 9.1%, and from fortified red bean (adzuki bean), 74.8 to 119.6%, with C.V. less than 6.8%. The limits of detection were 0.005 $\mu g/mL$ $(S/N > 3)$ for each pesticide. Figure 4 shows typical MRM profiles of the fortified brown rice with the pesticides at the a level of 0.01 ppm and the blank brown rice. All of the MRM profiles of the fortified samples were almost the same as the respective standards. The analysis time, including sample preparation and determination, is less than 40 min, which is much shorter than the conventional analytical method.

Overall results indicate that the presented method has satisfactory reproducibility, recovery, and accuracy for the high throughput analysis of nine carbamate pesticides in cereals and beans.

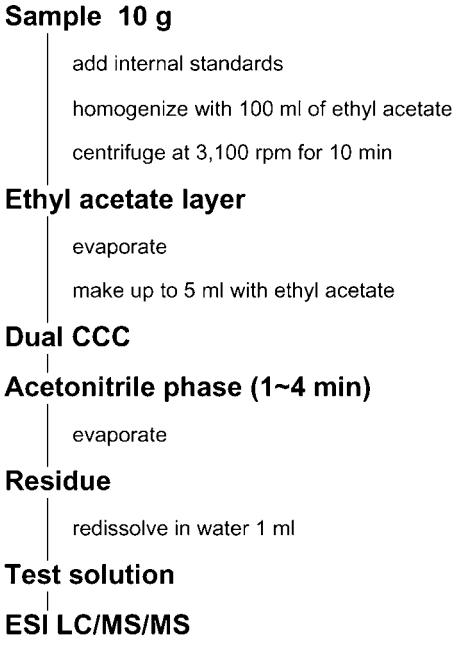

Figure 3. Analytical procedure for the carbamate pesticides.

Table 1. Recoveries of the nine carmbamate pesticides from cereals and beans

		Brown rice		Wheat		Soy bean		Corn		Red bean	
Pesticides	Fortified (ppm)	Recovery[a] (%)	C.V.[b] (%)	Recovery[a] (%)	C.V.[b] (%)	Recovery[a] (%)	C.V.[b] (%)	Recovery[a] (%)	C.V.[b] (%)	Recovery[a] (%)	C.V.[b] (%)
Aldicarb	0.01	88.4	2.4	95.2	5.0	81.8	1.4	88.1	3.6	91.2	4.2
	0.5	92.9	1.1	95.4	2.3	82.1	1.7	102.4	5.1	83.2	1.8
Aldicarb	0.01	95.0	2.3	80.5	1.8	100.2	1.5	94.3	5.4	76.5	5.9
sulfoxide	0.5	95.2	3.9	84.5	5.4	83.6	1.3	79.2	7.5	80.2	1.8
Aldicarb	0.01	87.3	5.4	88.3	11.2	106.6	5.2	109.1	9.1	80.0	4.3
sulfone	0.5	93.5	4.3	89.1	5.3	93.4	1.4	76.6	4.7	89.3	1.2
Carbaryl	0.01	100.9	1.9	103.2	1.1	102.0	1.7	102.5	4.3	100.7	0.9
	0.5	96.0	2.5	96.1	1.5	101.8	0.9	94.8	1.8	100.3	1.6
Fenobucarb	0.01	99.8	1.1	95.4	2.5	99.1	1.9	95.1	1.7	103.0	0.7
	0.5	102.5	1.5	103.4	0.6	100.7	0.6	101.7	2.1	100.6	2.2
Methiocarb	0.01	78.6	3.3	79.7	3.0	90.1	4.3	86.0	3.1	74.8	6.8
	0.5	80.7	6.3	90.0	1.6	90.0	1.6	88.6	3.9	87.6	1.6
Methomyl	0.01	100.2	2.2	107.6	4.2	102.3	2.3	96.6	3.4	98.8	2.7
	0.5	100.1	2.9	99.7	2.2	101.0	0.4	102.4	1.1	100.8	1.6
Oxamyl	0.01	100.5	3.1	73.9	4.1	90.2	5.6	96.1	6.1	98.8	6.0
	0.5	103.8	5.5	94.2	11.3	83.7	3.0	94.3	6.1	85.6	3.5
Pirimicarb	0.01	110.6	3.6	110.2	2.2	106.1	2.0	113.0	3.7	119.6	3.0
	0.5	117.1	1.2	111.4	3.8	104.6	1.9	112.3	4.1	107.5	2.7

[a]Average of five trials.
[b]Coefficient of variation.

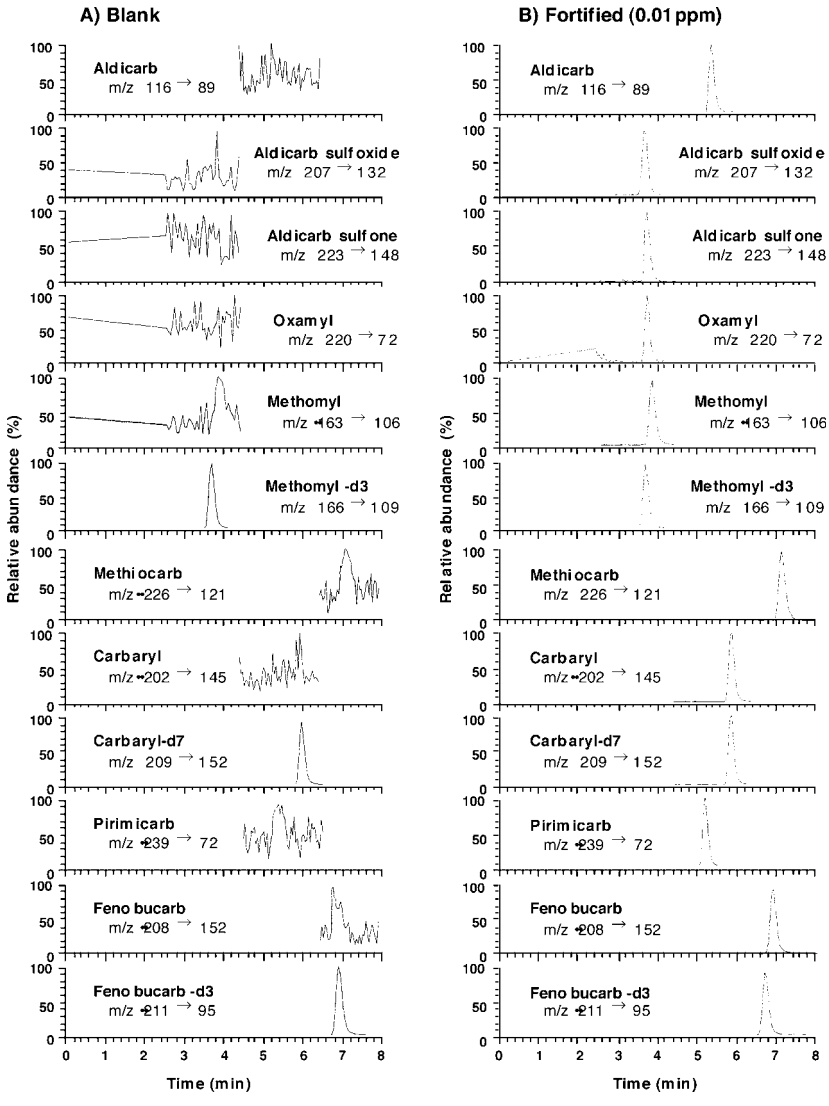

Figure 4. Typical MRM profiles of the fortified and blank brown rice samples.

CONCLUSIONS

We reported, here, a new rapid sample preparation method for the simultaneous analysis of nine carbamate pesticides in cereals and beans using dual CCC. The method was effective for separating the pesticides from the aliphatic matrix in cereals and beans. In addition, this method using LC/MS/MS with isotopically labeled IS enabled us a simple, rapid, and reliable

analysis of N-methyl carbamate in cereals and beans, where the analysis time, including sample preparation and determination is much shorter than that required by the traditional method. Therefore, we strongly recommend the present method for the monitoring of the pesticides in cereals and beans. The present method may also be applicable to other pesticide residues in foods.

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