Simultaneous Determination of Emamectin, its Metabolites, Milbemectin, Ivermectin and Abamectin in Tomato, Japanese Radish and Tea by LC/MS

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We developed a simple and rapid analytical method for determining the residues of emamectin benzoate, milbemectin, abamectin, ivermectin and emamectin metabolites in tomato, Japanese Radish and Japanese tea by liquid chromatography/mass spectrometer (LC/MS) with electrospray ionization (ESI). A sample extracted with acetone was simply cleaned up using only a Sep-Pak C18, and then directly measured by LC/MS (ESI). Several LC/MS measurement conditions were studied that included the mobile phase, solvent for sample solution, range of calibration and standard deviation of the measurement. Detecting target macrocyclic lactone chemicals with methanol as the mobile phase was more sensitive than acetonitrile, especially, milbemectin. The detection limits of these chemicals were 0.1 to 0.5 ng/ml (10 μl injection), and they were similar or more sensitive to the previous fluorescence detection method. For the measurement of these chemicals in tomato, Japanese radish and tea by LC/MS (ESI), ion suppression was always observed. To compensate for such an effect, we used a matrix-matched calibration. This compensation method is effective for obtaining accurate values. The recoveries by the developed method were in an acceptable range for the screening method and 90.1–120.9% except for milbemectin and abamectin from tea leaves that had interfering peaks.

Key words —— emamectin benzoate, milbemectin, ivermectin, abamectin, LC/MS, matrix-matched calibration

INTRODUCTION

Emamectin benzoate and ivermectin are semi-synthetic derivative from abamectin.[1] Milbemectin is produced by Streptomyces hygroscopicus.[2] These four chemicals are used as acaricides or parasiticides for animals or plants. Milbemectin is a mixture of approx. 30% milbemectin A_3 and approx. 70% milbemectin A_4.[3] Emamectin is a mixture of approx. 90% emamectin B_{1a} and approx. 10% emamectin B_{1b}. Emamectin has various metabolites such as the 8,9-Z isomer (defined as 8,9-Z), 4”-epi-amino-4”-deoxy-avermectin B_1 (amino form, defined as Amino), 4”-epi-(*N*-formyl) amino-4”-deoxy-avermectin B_1 (formyl amino form, defined as FA) and 4”-epi-(*N*-formyl-*N*-methyl) amino- 4”-deoxy-avermectin B_1 (methyl formyl amino form, defined as MFA).[4] Avermectin is a natural product in a culture of Streptomyces avermitilis and has the trade name of avermectin B_1.[5] In Japan, emamectin benzoate and milbemectin are registered for use as pesticides by the Ministry of Agriculture, Forestry and Fisheries in 1998 and 1991, respectively. The maximum residue limit (MRL) for emamectin benzoate in crops was officially established by the Ministry of Health and Welfare in November 1999 (The MRLs are 0.1 ppm for radish, cabbage, broccoli, tomato, eggplant and cucumber, and 0.5 ppm for tea.). Abamectin, which is applied to crops in some countries,[3,6,7] has not been allowed as pesticide in Japan, but both abamectin and ivermectin are currently used for parasite of animals.[8,9]

Several methods have been reported for determining the residual macrocyclic lactones in crops.[6,7,10,11] Liquid chromatographic methods using a UV detector was reported for determining abamectin residues in vegetables.[6] This method

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without derivatization is easy and rapid, but UV detection was not sensitive enough for residue determination. As an improved method, derivatization and fluorescence detection for macrocyclic lactone chemicals was developed.[12,13] However, their sample preparation methods are time-consuming, because they have many preparation steps including fluorescence derivatization. The fluorescence derivatization requires anhydrous conditions, because of interfering the derivatization of the target chemicals. In addition, this step causes changing the chemical structure of emamectin and its metabolites.[13]

Recently, liquid chromatography/mass spectrometer (LC/MS) is more useful and effective than conventional LC for pesticide residue analysis, especially for the confirmation of pesticides detected by LC. In the Japanese official method, LC/MS is used only for the confirmation, while the use of LC/MS for determinations is not provided, because LC/MS is not a common detector in Japan. As far as the measurement of emamectin benzoate was concerned, emamectin and its metabolites can be directly detectable by LC/MS without derivatization, and such method is very easy and rapid. Therefore, in this report, we studied the development of a highly sensitive screening method for macrocyclic lactone chemicals by LC/MS.

MATERIALS AND METHOD

Sample —— Tomato, Japanese radish and Japanese powdered tea were purchased from a store in Osaka city.

Reagents and Standard Materials —— Acetone, methanol and ethanol of pesticide grade and acetonitrile and methanol of LC grade were purchased from Wako Pure Chemical Industries Co. Ltd. (Osaka, Japan). Bond Elute C_{18}, Bond Elute NH_2 (each 0.5 g, Varian Co. Ltd., Harbor city, CA, U.S.A.) and Sep-Pak Plus C_{18} (0.5 g, Waters Corporation, Milford, MA, U.S.A.) were used for purification.

Emamectin B_1 (EB_1, including approx. 93% emamectin B_{1a} and approx. 7% emamectin B_{1b}), Amino, FA, MFA, abamectin, milbemectin A_3, milbemectin A_4 and ivermectin standard were purchased from Hayashi Pure Chemical Industries Co. Ltd. (Osaka, Japan). 8,9-Z was provided by Novartis Agro K.K. (Tokyo, Japan). Stock standard solutions contained 100 μg/ml each FA, MFA, Amino, emamectin B_1, 8,9-Z, milbemectin A_3, milbemectin A_4, abamectin and ivermectin in acetonitrile. The working standard solutions were prepared by appropriate dilutions of the stock solutions with acetonitrile (10 μg/ml). Both the stock and working standard solutions were stable for at least 6 months at 4°C.

Table 1. Conditions for Mass Spectrometer during LC/MS Measurement

Ionization mode	ESI(+)
Nebulizing gas	N_2, 4.5 l/min
Probe voltage	+4.5 kV
CDL voltage	5 V
Q-array voltage	38.0 V
Reflector voltage	150 V
Heat-block Temp.	200°C
CDL Temp.	300°C
Analytical mode	Scan and SIM
Target *m/z*	200–1000 (Scan) 872, 886, 922, 936, 895, 897, 551, 565 (SIM)
Acquisition Int.	0.5 sec/scan

Instruments —— The LC/MS system consisted of a Shimadzu (Kyoto, Japan) LCMS-QP2010 LC/MS. The LC separations were carried out with a Shimadzu Shimpak ODS column (2.0 mm i.d. × 150 mm, 5 μm particle size). The solvent was pumped at a flow rate of 0.2 ml/min with a gradient elution. Solvent A was 0.2% ammonium acetate/methanol and solvent B was 0.2% ammonium acetate/water. The gradient elution condition was initially 30% A–70% B, programming to 95% A over 5 min and programming to 100 over 14, then holding 100% A for 5 min (24 min total analysis time). Column equilibration was accomplished using the initial condition for 5 min prior to the next injection. An oven temperature was controlled at 50°C and 5 μl of sample was injected. The MS conditions are summarized in Table 1.

Sample Preparation ——

Extraction:

Tomato and Japanese Radish: The vegetable sample was homogenized using an Ultra turux (IKA-WERKE GMBH & CO., KG., Schtauffen, Germany). The homogenized sample (5.0 g) was placed in a 50 ml centrifuge tube, and 20 ml of acetone was added. The centrifuge tube was capped and shaken for 3 min. After centrifugation (7500 *g*), the supernatant was transferred to a 100 ml round bottomed flask and the sediment was again extracted with 10 ml of acetone. Both supernatants were combined and evaporated to remove the acetone using a rotary

Table 2. Retention Time, Ion Peaks, Calibration Curve and Detection Limits of Target Chemicals

Target compounds	RT (min)	*m/z*	linearity of calibration curve R^2 Value (range, μg/ml)	detection limits[b] (ng/ml) LC/MS	FL
Amino	11.56	872[a] (M) 894 (M−H+Na)	0.9955 (0.05–0.5)	0.2	0.2
Emamectin B_{1a}	11.70	886[a] (M) 908 (M−H+Na)	0.9999 (0.05–1.0)	0.2	0.2
8,9-Z isomer	12.18	886[a] (M) 908 (M−H+Na)	1.000 (0.05–1.0)	0.2	0.2
FA	11.81	922[a] (M−H+Na)	0.9972 (0.05–0.5)	0.2	0.2
MFA	12.63	936[a] (M−H+Na)	0.9980 (0.05–1.0)	0.5	0.5
Abamection	12.45	895[a] (M)	0.9798 (0.05–0.5)	0.2	0.5
Ivermectin	14.38	897[a] (M−H+Na)	0.9821 (0.05–1.0)	0.2	0.5
Milbemectin A_3	12.56	551[a] (M−H+Na)	0.9793 (0.05–1.0)	0.2	0.5
Milbemectin A_4	13.28	565[a] (M−H+Na)	0.9889 (0.05–1.0)	0.1	0.5

a) The values with single asterisk are target ion peaks for LC/MS measurement. *b*) Detection limits are provided as S/N> 3.

evaporator. The aqueous solution was then obtained (Solution A).

Tea: A powdered sample (0.50 g) was placed in a 50 ml centrifuge tube, and 20 ml of acetone was added. The centrifuge tube was capped and shaken for 3 min. After centrifugation (7500 *g*), the supernatant was transferred to a 100 ml round bottomed flask and the sediment was again extracted with 10 ml of acetone. Both supernatants were combined in the round-bottomed flask and evaporated to dryness using a rotary evaporator. The residue was then dissolved with 2.0 ml of methanol (Solution B).

Clean Up and Sample Preparation for LC/MS:

Tomato and Japanese Radish: The Sep-Pak C18 (0.5 g) was conditioned with 5 ml of methanol and 5 ml of water. Solution A was loaded on the column and the aqueous eluent was discarded. The 100 ml-round-bottomed flask was rinsed twice with 2 ml of methanol. The analytes were eluted with the rinse and an additional 10 ml of methanol into another 100 ml-round-bottomed flask. The eluate was evaporated to dryness using a rotary evaporator, dissolved with approx. 2 ml of acetonitrile including 1% acetic acid and determined by LC/MS.

Tea: The Sep-Pak C18 (0.5 g) was conditioned with 5 ml of methanol. Solution B was loaded on the Sep-Pak C18 (0.5 g) and the eluent was collected. The analyte was then prepared in the same manner as tomato and Japanese radish.

RESULT AND DISCUSSION

LC/MS Condition

The mass ion peaks of the target pesticides are shown in Table 2. Most of these spectra had a simple shape and were obtained as the molecular ion peak or their adduct ions. The obtained spectra of milbemectin A_3 and A_4 had some fragment ions. This Shimadzu LC/MS automatically optimizes the voltage condition by itself, but we manually determined the condition for a more sensitive detection. Due to the column contamination and high resolution, the LC separation was carried out with gradient elution. Figure 1 shows the effect of the mobile phase on the peak area. All data was acquired by different mobile phase with same gradient program. Detection of all the macrocyclic lactone chemicals with methanol as the mobile phase was more sensitive than acetonitrile, especially, milbemectin. Concerning the methanol system, detecting with the methanol system including ammonium acetate was similar or more sensitive than with acetic acid. Thus, we selected methanol with ammonium acetate as the mobile phase.

Figure 2 shows a typical mass chromatogram of the standard mixture. Retention times of the target chemicals were between 11.5 min and 14.3 min. The retention times of almost all peaks overlapped but they could be completely separated by each specific *m/z*. For the fluorescence method, the derivatization of emamectin B_{1a} and 8,9-Z isomer produced the same products, accordingly, both chemicals could not be individually measured. However, this developed method could be individually measured with-

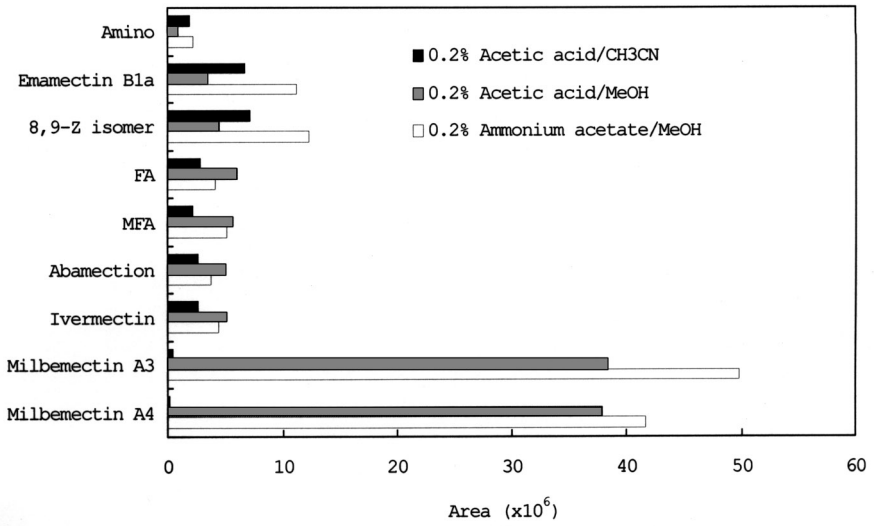

Fig. 1. Comparison of 3 Mobile Phase Conditions on Sensitivity of the Chemicals
Three conditions were carried out with the same gradient program mentioned in LC condition section and different mobile phase.

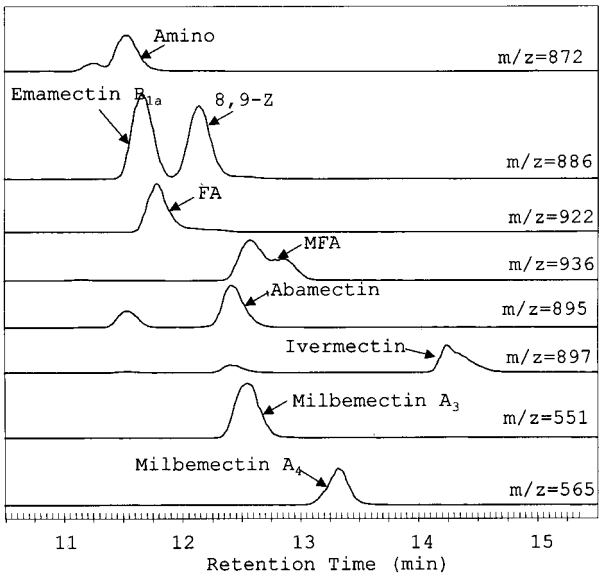

Fig. 2. Typical Mass Chromatogram of Target Macrocyclic Lactone Chemicals

out a derivatization step.

The linearity of the calibration curves and detection limits for the LC/MS are shown in Table 2. The linearities were 0.97–1.0 as the R^2 value between 0.005–0.5 ug/ml. The detection limits were 0.2–0.5 μg/ml and similar or more sensitive compared with fluorescence detection.

Sample Preparation

The stability of emamectin B_{1a} in various solutions are examined. In methanol, 42% emamectin B_{1a} decomposed in 23 hr. Such a phenomenon was also observed only in Amino and 8,9-isomer. Amino is a primary amine, and emamectin B_{1a} and 8,9-Z-isomer are secondary amines. The decomposition was a peculiar phenomenon for the primary and secondary amines in the methanol solution, however, the mechanism is not clear. A sample with acetic acid was more stable than a sample without acetic acid. Therefore, acetonitrile with acetic acid was selected as the final solution of sample.

To reduce the sample preparation time, we developed a simple and easy preparation method. Since the analytical column was a common C18 column, we thought that purification by Sep-pak C18 could result in decreasing contamination of the analytical column and continuous measurements.

Table 3 shows difference in the elution pattern between two types of C18 clean up columns using the standard solution. In our previous report, emamectin B_{1a}, amino and 8,9-Z isomer could be eluted from Bond Elut C18 by methanol.[13] However, in this experiment, they were not eluted from Bond Elut C18 by methanol. Because we could not get same elution pattern from Bond Elut C18 due to the different lots, we selected Sep-Pak C18 for clean up in this experiment.

For the mass spectrometric analysis, ion suppression often occurred due to the sample matrix. Figure 3 shows some raw data of peak areas for the recovery test from tomato. The data are shown in the order of injection to the LC/MS, namely 1st and 8th are the standard solution, 2nd is the blank tomato

Table 3. Comparison of Elution Patterns between Two Types of C18 Preparation Column

	Recovery (%)		
	Bond Elut C18		Sep-pak C18
	10 ml eluent (next additional 10 ml eluent)[a]		10 ml eluent
Eluent	methanol	1% ammonium acetate /methanol	methanol
Amino	0 (6)	94 (0)	97
Emamectin B_{1a}	0 (5)	98 (0)	92
8,9-Z	0 (3)	96 (0)	97
FA	100 (0)	103 (0)	95
MFA	108 (0)	107 (0)	93
Abamectin	106 (0)	96 (0)	94
Ivermectin	100 (0)	102 (0)	94
Milbemectin A_3	96 (0)	103 (0)	91
Milbemectin A_4	90 (0)	98 (0)	96

a) Since Amino, emamectin B_1 and 8,9-Z isomer were not eluted from Bond Elut by 10 ml methanol, an additional 10 ml of eluent was added.

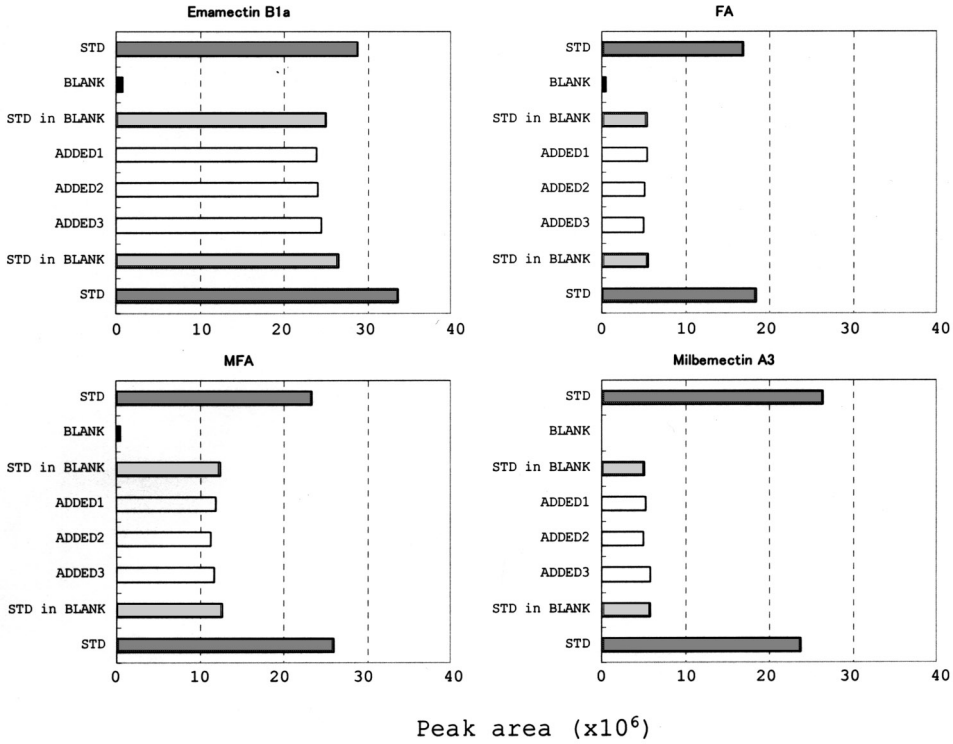

Fig. 3. Effect of Tomato Matorix for the Sensitivity of each Compound on Recovery Test from Tomato

sample, 3rd and 7th are the blank tomato sample added standard solution as 100% recovery, 4th through 6th are three recovery test samples. In all analytes, ion suppression occurred due to the sample matrix, except Emamectin B_{1a}. To avoid the ion suppression due to sample matrix, the determinations of the analytes were carried out using matrix-matched calibration.

Recovery Test

In our previous report with fluorescence detection, FA degraded to emamectin B_{1b}, and a part of emamectin B_{1a} and 8,9-Z isomer degraded to Amino during the fluorescence derivatization. Since such degradations were not observed in this method without fluorescence derivatization, the recovery tests were carried out with a standard mixture including all target macrocyclic lactone chemicals. The fluo-

Table 4. Recoveries of Target Chemicals from Tomato, Japanese Radish and Japanese Powdered Tea[a)]

	Tomato		Japanese Radish		Tea	
	C_{18}	C18 + NH_2[b)]	C_{18}	C18 + NH_2[b)]	C_{18}	C18 + NH_2[b)]
Amino	94.9±3.2	98.1±3.5	95.1±0.7	98.6±1.9	91.4±1.6	82.1±12.3
Emamectin B_{1a}	93.2±0.4	103.1±4.1	100.8±2.3	100.6±2.7	99.7±0.5	91.9± 2.3
8,9-Z isomer	97.3±2.4	101.4±2.9	103.4±1.5	100.9±1.8	115.6±6.9	83.5± 3.6
FA	93.5±3.9	97.4±3.1	103.6±2.8	98.7±2.0	96.9±6.7	89.2± 1.5
MFA	92.0±1.9	102.6±0.3	100.1±2.7	100.6±1.7	95.1±2.4	94.3± 3.1
Abamection	90.1±4.9	94.6±1.4	98.7±1.1	94.6±1.4	—	—
Ivermectin	95.7±4.0	99.2±1.0	105.6±1.8	99.2±1.0	—	—
Milbemectin A_3	98.1±3.9	95.2±2.2	101.3±2.6	95.1±2.3	94.8±4.6	113.3± 3.8
Milbemectin A_4	103.5±3.5	99.3±2.9	103.6±3.8	96.9±2.8	—	—

—: not measured due to interfering peak. *a*) $n = 3$. *b*) C18 + NH_2 means results of clean up with Sep-Pak C18 and Bond Elut NH_2 following the Japanese official method for emamectin benzoate.

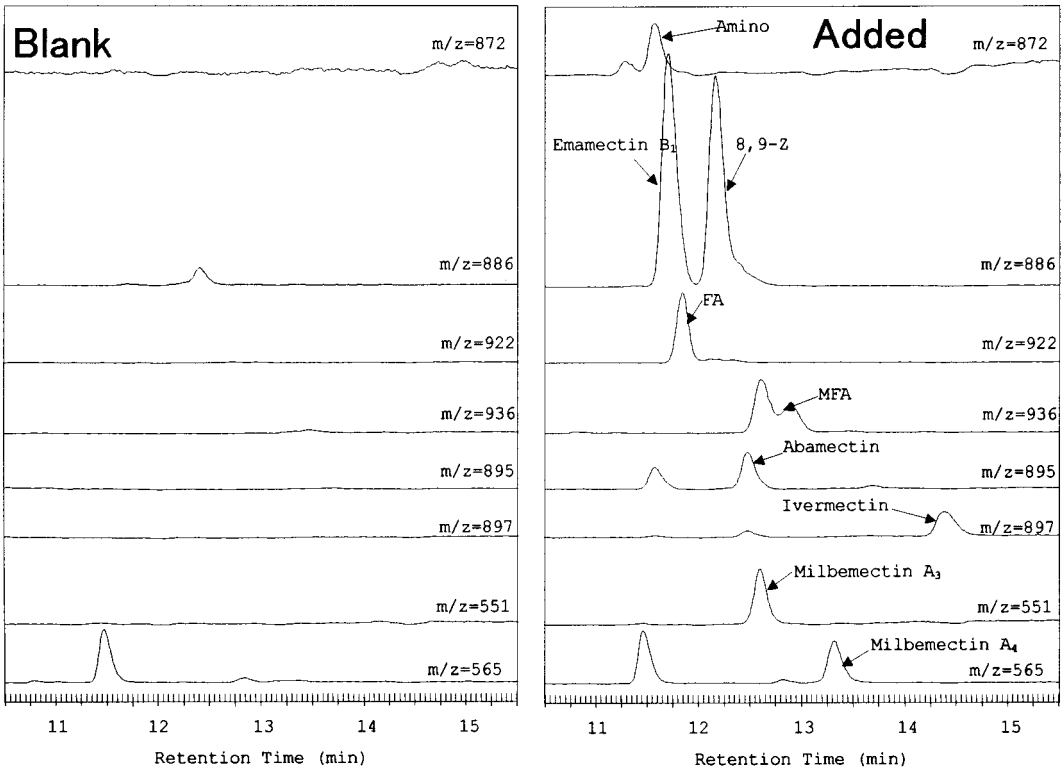

Fig. 4. Typical Mass Chromatograms of Tomato

rescence derivatization was interfered by constituents of radish, therefore, we previously developed an individual method with fluorescence derivatization only for the radish. However, using the LC/MS method without the derivatization, radish and other vegetables could be measured by this method. Table 4 shows the results of the recovery tests from tomato and Japanese radish fortified with 0.1 ppm and Japanese powdered tea fortified with 0.5 ppm . Figures 4–6 show their mass chromatograms using only C18 for clean up. In Figs. 4 and 5, the tomato and Japanese radish had no interfering peaks for any analyte. Recoveries with Sep-Pak C18 from tomato and Japanese radish were 90.1 to 105.6%. In Fig. 6, there are interfering peaks for abamectin and milbemectin A_4 in Japanese powdered tea. The absence of both abamectin and milbemectin A_4 in blank tea sample was confirmed by fluorescence derivatization method following Japanese official method. Recoveries with Sep-Pak C18 from Japa-

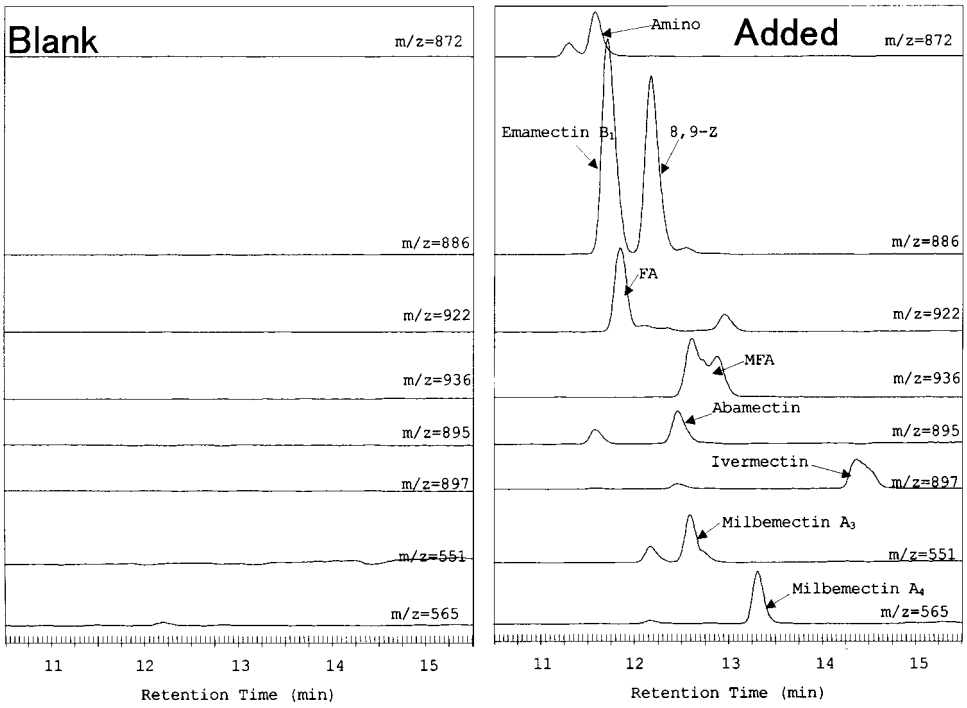

Fig. 5. Typical Mass Chromatograms of Japanese Radish

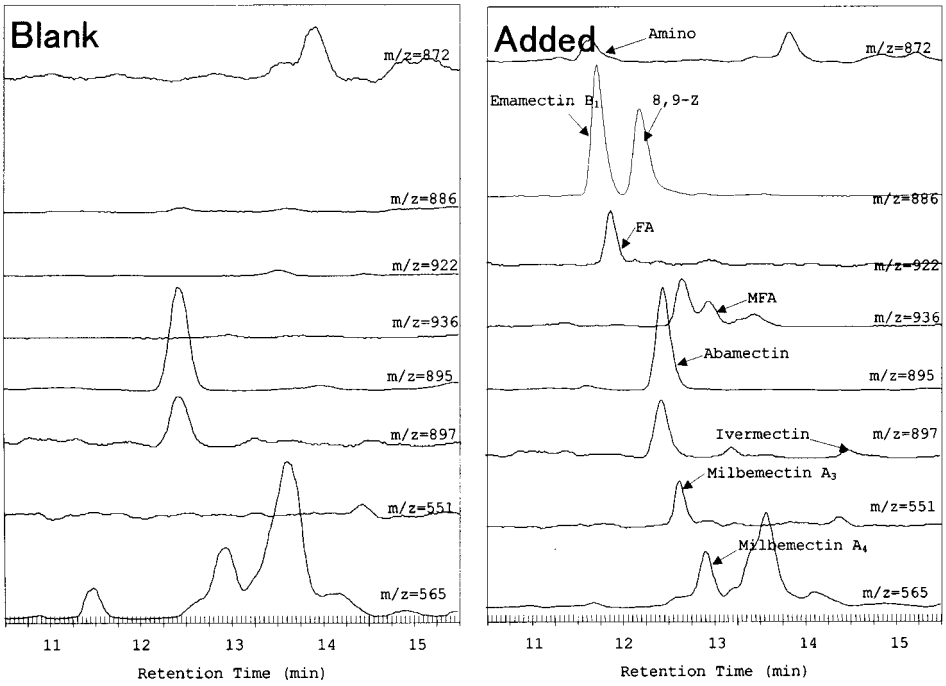

Fig. 6. Typical Mass Chromatograms of Japanese Powdered Tea

nese powdered tea were 91.4 to 120.9% except for abamectin and milbemectin A_4. Abamectin and milbemectin in tea sample could not be measured, because there were interfering peaks overlapping with abamectin and milbemectin. An additional NH_2 column was effective for removing the color component (green color) from the sample, but it was not effective for the mass chromatogram shape and the

recovery value. It may be solved using an additional purification column or additional liquid-liquid partition. In conclusion, the recovery range was acceptable for pesticide analysis except for abamectin, ivermectin, and milbemectin A_4 from Japanese powdered tea.

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