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Liquid chromatographic determination of emamectin, milbemectin, ivermectin and abamectin in crops and confirmation by liquid chromatography–mass spectrometry

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Abstract

Emamectin, milbemectin, ivermectin and abamectin are similar macrocyclic lactone chemicals used as an acaricides or parasiticides. We developed a simultaneous analytical method for determining the residual amounts of these compounds and emamectin metabolites in crops. A sample extracted with acetone was cleaned up with Bond Elut C_{18} and NH_2. The sample was then fluorescence-derivatized with trifluoroacetic anhydride and 1-methylimidazole in acetonitrile. The analyte was measured by HPLC with fluorescence detection using an octadecylsilyl column with 3 μm particle size and gradient elution. In most crops, their recoveries by the developed method were ca. 80–110%. The detection limits of the analytes in vegetables were 0.1–0.3 ppt. Using the developed method, we surveyed the residues of these compounds in 20 commercial crops in Osaka, Japan. The result of the surveillance was that emamectin benzoate of 0.2–6.7 ppb was detected in nine cases and milbemectin of 16.7–279.3 ppb was detected in four cases. The detected samples were confirmed by LC–electrospray ionization (ESI) MS. The limit of detection by LC–ESI-MS was similar to the fluorescence detection level of 0.1–0.3 ppt in vegetables except for milbemectin.

Keywords: Food analysis; Emamectin; Milbemectin; Ivermectin; Abamectin; Pesticides

1. Introduction

Emamectin, milbemectin, ivermectin and abamectin are macrocyclic lactone chemicals used as acaricides or parasiticides for animals or plants [1–5]. Emamectin has various metabolites such as the 8,9-Z isomer, *N*-demethylated, *N*-formylated and *N*-methylformylated emamectins [5]. In Japan emamectin benzoate and milbemectin were recently permitted use by the Environmental Agency [6], and the maximum residue limit (MRL) for emamectin benzoate in crops was officially determined by the Ministry of Health and Welfare [7]. Abamectin applied to crops in some countries [4,8,9] still has not been allowed for use in Japan, but both abamectin and ivermectin are currently used against parasites in animals [10,11].

Several methods have been described for determining the residual macrocyclic lactones in crops [8,9,12,13]. Liquid chromatographic methods using a UV detector were reported to determine abamectin residues in vegetables [8]. However, UV detection was not sensitive enough for residue determination. As an improved method, fluorescence detection of a derivative was developed [14]. Most of the derivatizations are carried out with trifluoroacetic anhydride and 1-methylimidazole, and these methods are very sensitive. Only a few attempts have so far

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been made for the simultaneous determination of macrocyclic lactones in crops [15]. Most of reported chromatographic techniques were isocratic elution, which is not good enough to separate various macrocyclic lactones including metabolites. We proposed our developed method as the official method for emamectin in crops, and the residue level of emamectin benzoate in crops was surveyed using the method. In addition, other macrocyclic lactone chemicals were studied for the simultaneous residue determination by the developed method.

2. Experimental

2.1. Chemicals

Pesticide-grade acetone, ethyl acetate, methanol, ethanol and HPLC-grade acetonitrile were purchased from Wako (Osaka, Japan). Standard materials included emamectin B_1 (hereafter defined as EB_1, including approx. 92% emamectin B_{1a} and approx. 8% emamectin B_{1b}), abamectin (hereafter defined as Aba), milbemectin A_3 (hereafter defined as MA_3), milbemectin A_4 (hereafter defined as MA_4) and ivermectin (hereafter defined as Ivr) were purchased from Hayashi (Osaka, Japan). Emamectin metabolites, 4-epi-amino avermectin B_1 (hereafter defined as AMI), 4-epi-(*N*-formyl) amino-4-deoxy avermectin B_1 (hereafter defined as FA), 4-epi-(*N*-formyl-*N*-methyl)amino-4-deoxy avermectin B_1 (hereafter defined as MFA) and 4′-deoxy-4′-epi-methylamino-4-deoxy avermectin B_1 $\Delta^{8,9}$ isomer (hereafter defined as 8,9-Z) were provided by Novartis Agro (Tokyo, Japan). Other reagents such as trifluoroacetic anhydride, ammonium acetate and molecular sieves were from Wako. 1-Methylimidazole (99%) was from Aldrich (Milwaukee, WI, USA).

Standard stock solutions contained 100 μg/ml each of FA, MFA, AMI, B_1, 8,9-Z, MA_3, MA_4, Aba and Ivr in acetonitrile. Working standard solutions were prepared by appropriate dilutions of the stock solutions with acetonitrile. Bond Elut C_{18}, PRS and NH_2 (1 g each, Varian) were used for purification. Sep-Pak Plus C_{18} (360 mg, Waters, Milford, MA, USA) was used for removal of the ammonium acetate.

2.2. Chromatographic conditions

2.2.1. High-performance liquid chromatography (HPLC)

The LC system included a Hewlett-Packard 1100 liquid chromatograph with fluorescence detection operated at an excitation wavelength of 365 nm and emission wavelength of 470 nm. LC separations were performed with a 150 mm×4.6 mm I.D. Wakosil-II 3C18HG column with a 3 μm particle size (Wako). The flow-rate was 1.0 ml/min with an oven temperature of 50°C and an injection volume of 5 μl. Solvent A was acetonitrile and solvent B was water. The gradient elution conditions were initially A–B (80:20), programming to A–B (90:10) over 5 min, programming to A–B (93:7) over 20 min, and programming to 100% A over 2 min (27 min total analysis time). Column equilibration was accomplished by using the initial conditions for 5 min prior to the next injection.

2.2.2. LC–MS

The LC–MS system included a Shimadzu (Kyoto, Japan) LCMS-QP8000 liquid chromatography–mass spectrometer. LC separations were performed with a 150 mm×2.0 mm I.D. Wakosil-II 3C18HG column with a 3 μm particle size. The flow-rate was 0.2 ml/min with an oven temperature of 50°C and an injection volume of 5 μl. Solvent A was acetonitrile with 0.2% acetic acid and solvent B was 0.2% acetic acid in water. The gradient elution conditions were initially A–B (30:70), programming to 100% A over 20 min, an holding 100% A for 2 min (22 min total analysis time). Column equilibration was accomplished by using the initial condition for 5 min prior to the next injection. The other operational parameters of the LC–MS system used for the confirmation are summarized in Table 1.

Table 1
Operational parameters for LC–MS

Operational parameters	
Analytical mode	ESI (positive)
Drying gas (N_2) flow	4.5 l/min
Probe voltage	−65 V
Deflector voltage	+65 V
Detector gain	−2.0 kV

2.3. Sample preparation

2.3.1. Extraction

2.3.1.1. Tea

A powdered sample (1 g) was placed in a 25-ml centrifuge tube, and 20 ml 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 an additional 10 ml of acetone. Both supernatants were mixed in a round bottomed flask and evaporated to dryness by a rotary evaporator. The extract was diluted with 2 ml of methanol (solution A).

2.3.1.2. Vegetables

A chopped sample (20 g) was placed in a 200 ml homogenizer cup, and 100 ml of acetone was added. After homogenizing, the extract was filtered by a filter paper, and the extraction was repeated with an additional 50 ml of acetone. All extracts were combined in a 300 ml round bottomed flask and evaporated to completely remove the acetone by a rotary evaporator. An aqueous solution was obtained (solution B).

2.3.2. Clean-up

2.3.2.1. Tea

Bond Elut NH_2 (1 g) was attached to the end of the Bond Elut C_{18} (Fig. 1). Solution A was loaded to the Bond Elut C_{18}. The centrifuge tube was rinsed with 2 ml of methanol. The analytes were eluted with the rinse and an additional 10 ml of methanol, and eluate was collected in the 100 ml round bottomed flask. The Bond Elut NH_2 was then detached from the Bond Elut C_{18}, and eluted with 10 ml of ethanol into the same flask. The eluate was evaporated to dryness by a rotary evaporator, and dissolved with approx. 4 ml of methanol–ethyl acetate (1:1).

2.3.2.2. Vegetables except for radish

(i) Trapping analytes with Bond Elut C_{18}: Bond Elut C_{18} (1 g) was conditioned with 5 ml of ethanol followed by 10 ml of water. Solution B was loaded to the column. The 300-ml round bottomed flask was rinsed with 10 ml of 5% methanol in water, and the rinse was used to rinse the column. The end of the column was aspirated to remove the water.

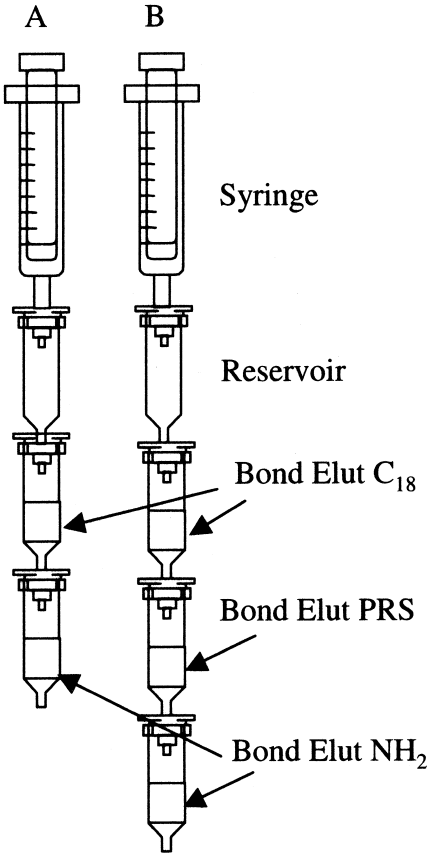

Fig. 1. Attachment of preparation column. Combination A was for the preparation of tea and vegetables except for radish. Combination B was for the preparation of radish.

(ii) Elution of analytes from column: Bond Elut NH_2 (1 g) already conditioned with 5 ml of methanol was attached to the end of Bond Elut C_{18} holding the analytes (Fig. 1). The 300-ml round bottomed flask was rinsed with 10 ml of methanol. The analytes were eluted with the rinse and an additional 10 ml of methanol, and eluate was collected in the 100-ml round bottomed flask. The Bond Elut NH_2 was then detached from the Bond Elut C_{18}, and eluted with 10 ml of ethanol into the same flask. After detachment of the Bond Elut C_{18} from the NH_2, the sample preparation was completed the same as for tea samples.

2.3.2.3. Radishes

(i) Trapping analytes with Bond Elut C_{18}: trapping analytes with C_{18} was done in a way similar to that described in Section 2.3.2.2.

(ii) Elution of analytes from column: Bond Elut PRS (1 g) already conditioned with 5 ml of methanol was attached the end of the Bond Elut C_{18} holding the analytes. Bond Elut NH_2 (1 g) already conditioned with 5 ml of ethanol was attached to the end of Bond Elut PRS (Fig. 1). The 300-ml round bottomed flask was rinsed with 10 ml of methanol and the rinse was poured into the column. The analytes were eluted with 10 ml of methanol into the 100-ml round bottomed flask. Bond Elut PRS-NH_2 was detached from the Bond Elut C_{18} and eluted with 10 ml 1% of acetic acid in methanol, and then the Bond Elut NH_2 was detached from the Bond Elut PRS and then eluted with 10 ml of ethanol.

(iii) Removal of ammonium acetate: the eluate was evaporated using a rotary evaporator, 10 ml of water added, loaded onto Sep-Pak C_{18} (360 mg) conditioned with 5 ml of ethanol and 5 ml of water. The cartridge column was rinsed with 10 ml of water, which was then discarded. The round bottomed flask was rinsed with 4 ml of methanol. The rinse was poured into the cartridge column and the eluate was collected into a 50 ml round bottomed flask. Analytes were eluted with 10 ml of methanol into the round bottomed flask. The eluate was evaporated to dryness by a rotary evaporator, and dissolved with approx. 4 ml methanol–ethyl acetate (1:1).

2.3.3. Derivatization and determination

Fluorescence derivatization was modified by the Method of Standard to Withhold Registration of emamectin benzoate samples [6]. The samples and standards removed in 15 ml silylated tubes from the 50-ml round bottomed flask were evaporated to dryness by a rotary evaporator and completely dried under a nitrogen purge. The sample and standards were reconstituted in 0.2 ml ethyl acetate and 1 ml acetonitrile. To the sample and standard was added 0.1 ml of 1-methylimidazole (99%). The tubes were capped and vortex-mixed, and all samples, standards, and the freshly prepared trifluoroacetic anhydride–acetonitrile (1:3) were placed in a cooling box at ice temperature for 10 min. After cooling, 0.3 ml of the trifluoroacetic anhydride–acetonitrile (1:3) was added to each sample and the standard tube. The tube was capped and allows to stand for 10 min. The sample and standards were diluted to 2 ml with acetonitrile in a volumetric flask and determined by liquid chromatography.

2.3.4. Quantitation

Each residue of emamectin benzoate, AMI, FA, MFA and 8,9-Z, was quantified by peak area on a chromatogram. Each amount was estimated as emamectin benzoate, and the total emamectin was shown as their sum. Namely, the total emamectin means an emamectin benzoate equivalent. Since EB_{1a} and 8,9-Z were derivatized into the same fluorescence derivative [13], the amount of EB_{1a} included 8,9-Z. The equation is shown as follows:

$$\text{Total emamectin (ppm)} = A + 1.16B + 1.12C + 1.10D$$

where *A*: amount of emamectin benzoate (ppm), *B*: amount of AMI (ppm), *C*: amount of FA (ppm), *D*: amount of MFA (ppm).

The molecular masses of emamectin benzoate divided by one of the metabolite give constants of 1.16, 1.12 and 1.10 for conversion from emamectin metabolites to emamectin benzoate. Residues of MA_3, MA_4, Aba and Ivr were determined by each calibration curve.

3. Results and discussion

3.1. LC measurements

The MRL of emamectin benzoate was 0.1 ppm for vegetables and 0.5 ppm for tea. On the other hand, fluorescence detection was sensitive enough to determine the emamectin benzoate. In order to reduce the run time with good resolution, a gradient elution with the 3 μm particle size octadecylsilyl column was used. Fig. 2 shows the LC chromatograms of crop samples fortified with the analytes. The major peak of each emamectin, emamectin metabolites and four other macrocyclic lactone compounds could be separated except for EB_{1a} and 8,9-Z. For reasons already stated, the derivatization of EB_{1a} and 8,9-Z

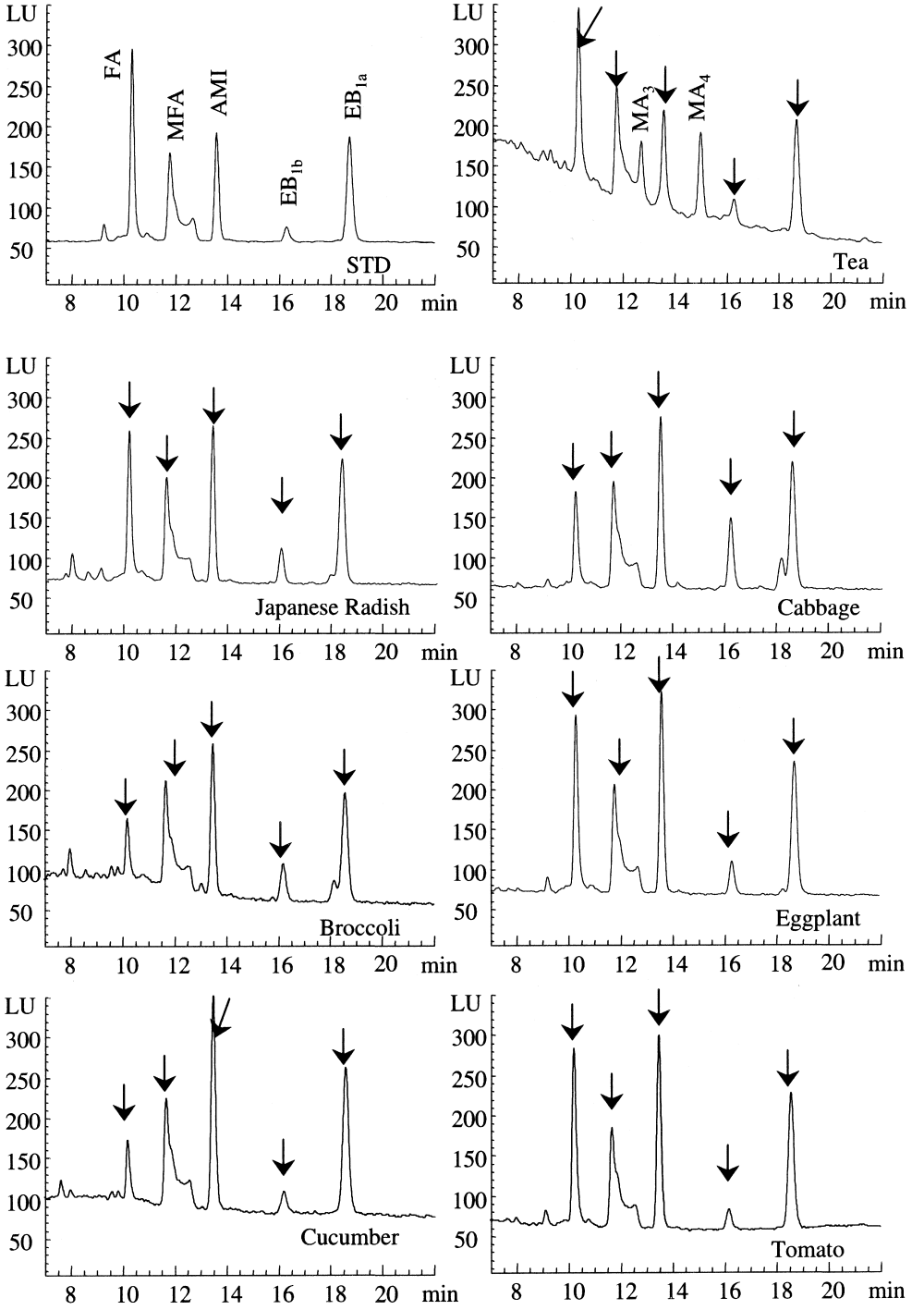

Fig. 2. LC chromatograms of crops fortified with emamectin and its metabolites. Vegetables and tea were fortified with target compounds at 0.005 ppm and 0.1 ppm, respectively. LU = luminescence unit.

resulted in the same derivative. Chromatograms of some fortified samples had unknown peaks near EB_{1a}. Since chromatograms of the blank sample did not have the peak, we suppose that the unknown peaks originated from emamectin benzoate. Fig. 3 shows chromatograms of crops fortified with other

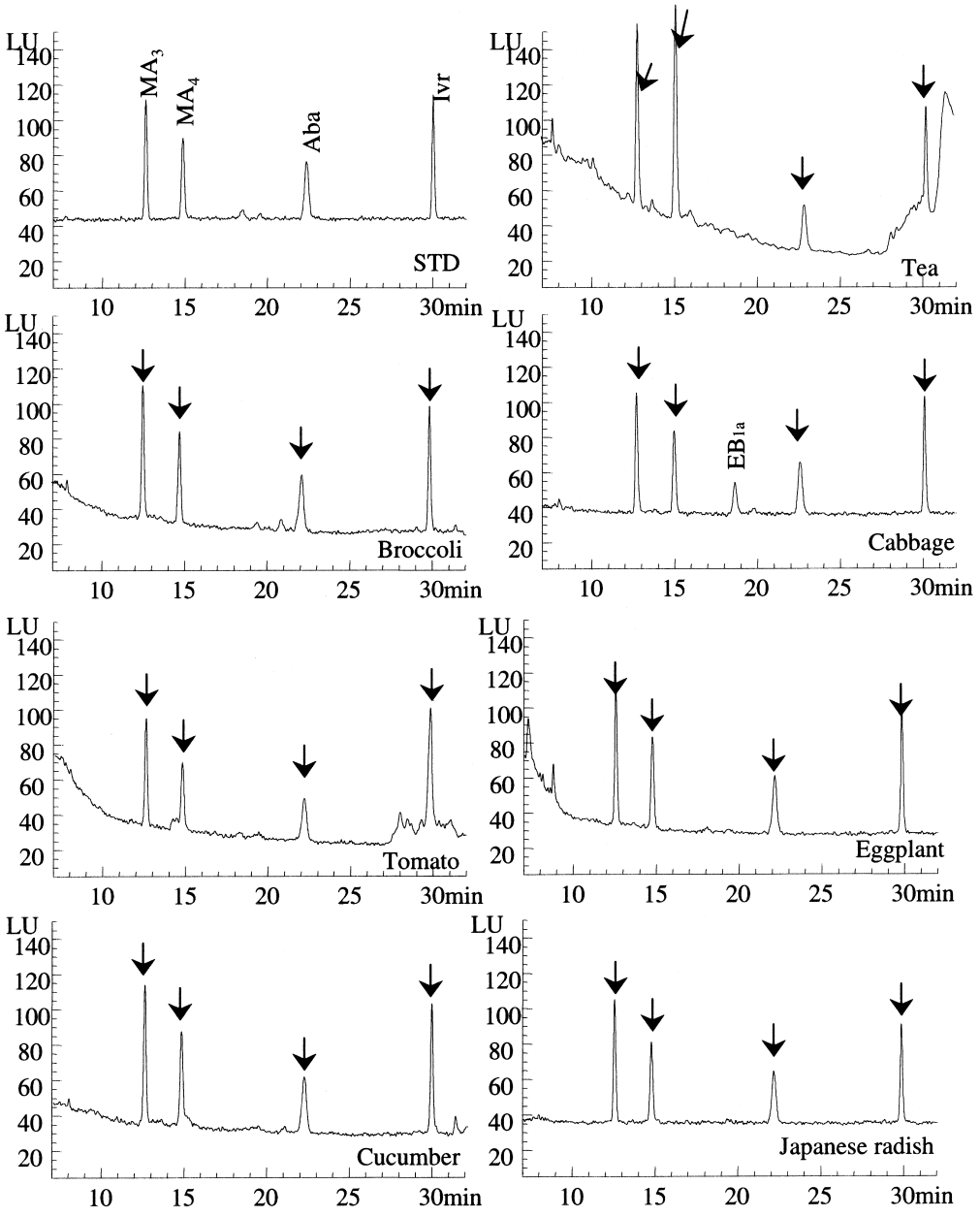

Fig. 3. LC chromatograms of crops fortified with milbemectin, abamectin and ivermectin. Vegetables and tea were fortified with target compounds at 0.005 ppm and 0.1 ppm, respectively.

Table 2
Limit of detection (LOD) by LC with fluorescence detection (LC–FL) and (SIM) LC–ESI-MS

Compound	LC–FL, LOD (ng)	(SIM) LC–ESI-MS	
		m/z	LOD (ng)
FA	0.002	908	0.005
MFA	0.005	936	0.005
AMI	0.002	872	0.002
EB_{1a}	0.002	886	0.002
8,9-Z	0.002	886	0.002
MA_3	0.005	511	0.05
MA_4	0.005	525	0.05
Aba B_{1a}	0.005	895	0.005
Ivr B_{1a}	0.005	897	0.005

macrocyclic lactone compounds. The limits of detection (LODs) were between 0.1 ppt for EB_{1a} and through 0.3 ppt for MFA in vegetables (Table 2).

3.2. Sample preparation

To reduce the frequency of evaporation, we used two or three cartridge columns connected to each other. This technique enabled the reduction of the preparation time and decomposition during evaporation. Since acetone in the extract for loading onto the column caused a low absorption rate in the C_{18} column, acetone in the extract must be completely removed.

A radish extract contained an inhibitor for fluorescence derivatization. EB_{1a} and AMI were influenced very strongly by the inhibitor and hardly recovered. To remove the inhibitor, the PRS column eluting with 1% ammonium acetate in methanol was effective. However, residual water contained ammonium acetate was hardly evaporated by a rotary evaporator, and the residue of water inhibited the fluorescence derivatization. Moreover, dry nitrogen gas flowing for 30 min was not enough for dryness, so more nitrogen flowing was needed.

3.3. Recovery test

Figs. 4 and 5 show the recoveries of each analyte fortified in various crops. Results of the various crops showed the fortified compound as the *x*-axis and the recovered compounds as bars, respectively. During sample preparation, FA degraded to EB_{1b}. Emamectin is a mixture of EB_{1a} as the main component and B_{1b} as minor component, and a part of EB_{1a} degraded to AMI during sample preparation. In addition, 8,9-Z degraded to AMI. The rate of degradation to AMI was not constant, however, the total emamectin recovered was relatively constant. A part of AMI fortified in eggplant was recovered as EB_{1b}. Accordingly, it is likely that AMI and EB_{1a} are converted into each other. As mentioned above, emamectin, FA, 8,9-Z and AMI degraded to other metabolites. For this reason, the content of emamectin and its metabolites in crops was calculated as total emamectin benzoate. Both abamectin and ivermectin mainly consist of B_{1a}, therefore, the contents in crops were calculated only using B_{1a}. Residual milbemectin in commercial powdered tea actually consisted of A_3 and A_4 (Fig. 6), therefore, the content of milbemectin in crops was calculated from both MA_3 and MA_4. In Japan, the common acceptable range of recovery was 70–120%. For most crops, compound recoveries were ca. 80–110% and within the acceptable range.

3.4. Surveillance of emamectin benzoate and other macrocyclic lactones in commercial crops

Using the developed method, we surveyed the residues of target compounds in commercial crops in Osaka, Japan. Table 3 shows the results of 20 surveyed cases. Most of detected cases were in May through June. In Japan, the temperature gets warmer in May, while in June starts the rainy season, and mites grow. Therefore, acaricide can be reasonably used in this season. All detected compounds were confirmed by LC–electrospray ionization (ESI) MS. The fluorescence-derivatized standard solution could be detected by LC–MS, however, in case of the fluorescence-derivatized sample, target compounds could not be detected. It was likely that a derivatized contaminant from sample might interfere with the ionization of the target compound in the mass spectrometer. Therefore, a sample without fluorescence derivatization was measured by LC–ESI-MS. Fig. 7 shows a detected case of emamectin and

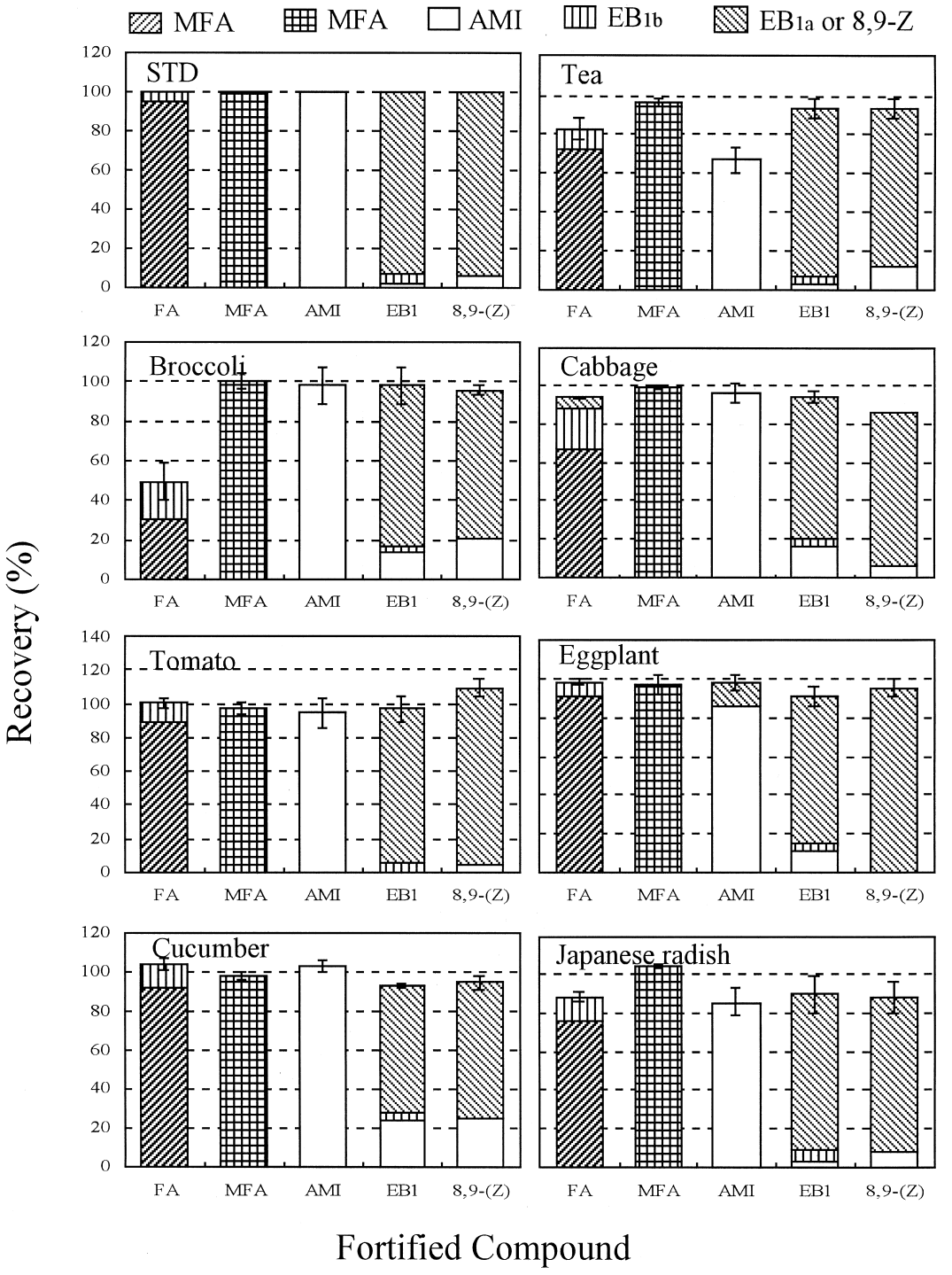

Fig. 4. Recoveries of emamectin benzoate and its metabolites from various crops. Vegetables and tea were fortified with target compounds at 0.005 ppm and 0.1 ppm, respectively. A bar chart of STD shows constituents in each emamectin and the metabolite standard. Other charts show the recoveries of fortified compound shown on the *x* axis. Values are average and standard deviation of three replicate determinations.

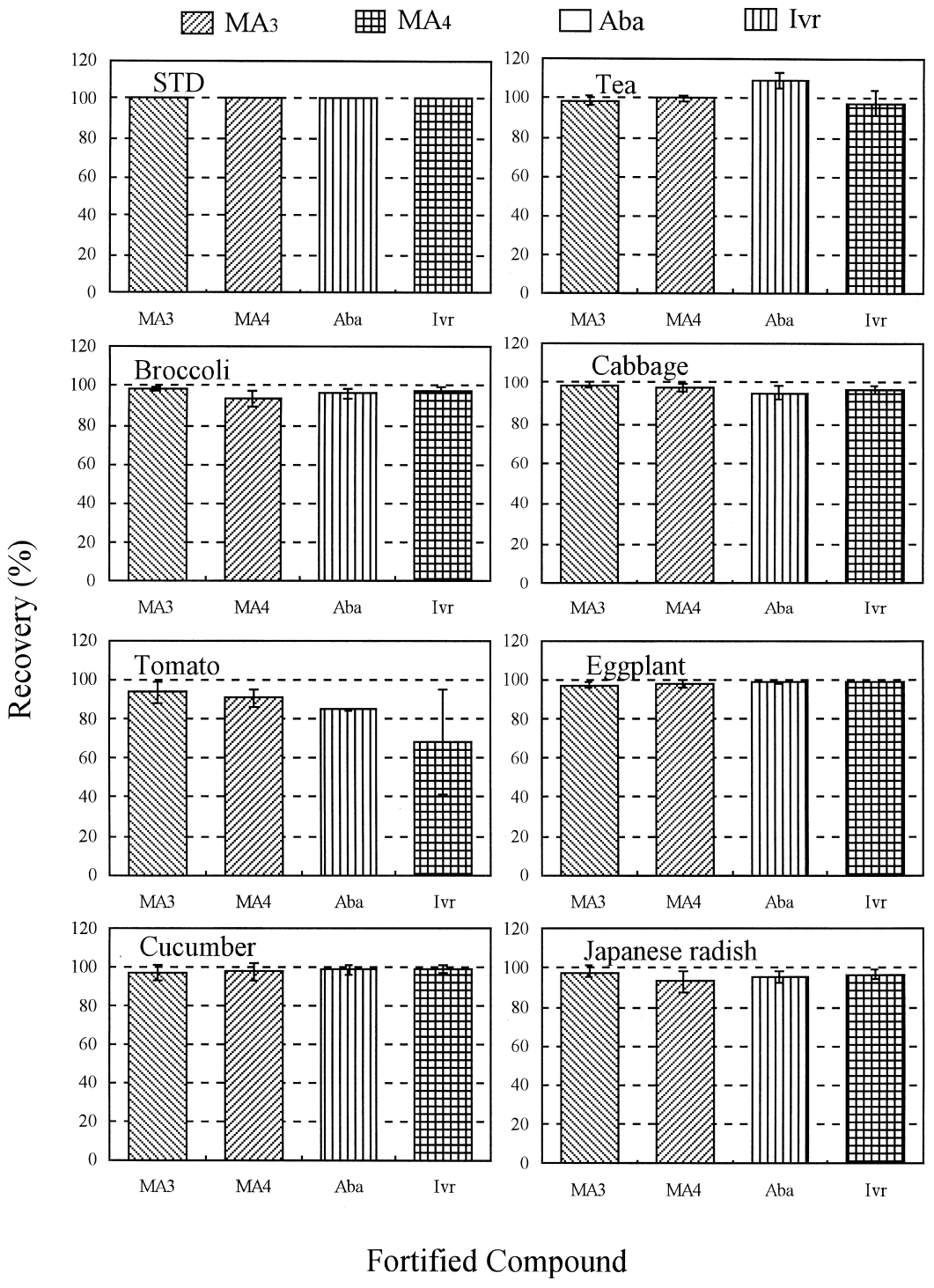

Fig. 5. Recoveries of other macrocyclic lactone compounds from various crops. Vegetables and tea were fortified with target compounds at 0.005 ppm and 0.1 ppm, respectively. A bar chart of STD shows constituents in each macrocyclic lactone standard. Other charts show the recoveries of fortified compound shown on the *x* axis. Values are average and standard deviation of three replicate determinations.

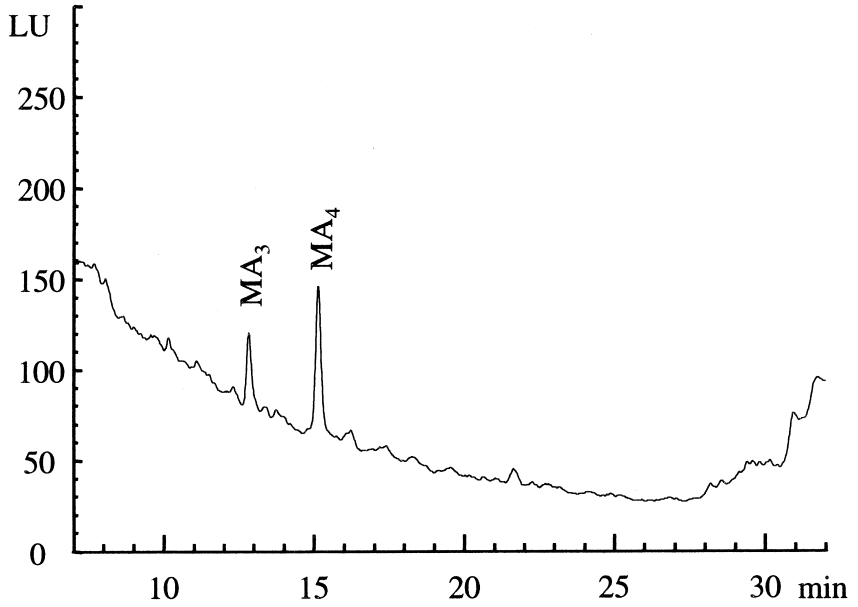

Fig. 6. Fluorescence chromatogram of commercial tea sample.

milbemectin in a cabbage sample. The LOD by LC–ESI-MS was similar to the fluorescence detection except for milbemectin (Table 2).

4. Conclusions

Four macrocyclic lactones and emamectin metabolites in crops were simultaneously extracted with acetone, cleaned up by soild-phase extraction (SPE) cartridge columns, and determined by LC with a fluorescence detector. The method is rapid and easy to perform without liquid–liquid partition, and the sensitivity is very high. In most crops, recoveries by the developed method were ca. 80–110% and within the acceptable range for the Japanese official method.

Table 3
Residual emamectins and milbemectin from commercial crops[a]

	Emamectins (ppb)						Milbemectin (ppb)	
	FA	MFA	AMI	EB_{1b}	EB_{1a}	Total	MA_3	MA_4
Powdered tea	ND	ND	ND	ND	ND	ND	55.5	186.4
Powdered tea	ND	ND	ND	ND	ND	ND	70.8	208.5
Japanese radish	ND	ND	ND	1.0	2.7	3.7	ND	ND
Japanese radish	ND	ND	ND	ND	0.3	0.3	ND	ND
Cabbage	ND	ND	ND	ND	0.2	0.2	ND	ND
Cabbage	ND	ND	0.3	ND	1.4	1.7	ND	ND
Cabbage	ND	ND	ND	ND	4.0	4.0	1.6	15.1
Tomato	ND	ND	0.1	0.7	2.1	2.9	18.9	58.0
Tomato	ND	ND	ND	ND	1.0	1.0	ND	ND
Cucumber	ND	ND	0.4	ND	0.6	1.0	ND	ND
Cucumber	ND	ND	0.5	1.7	4.5	6.7	ND	ND

[a] Results were obtained from 20 samples (powdered tea: 2, Japanese radish: 4, cabbage: 4, broccoli: 2, tomato: 3, cucumber: 4). ND means not detected.

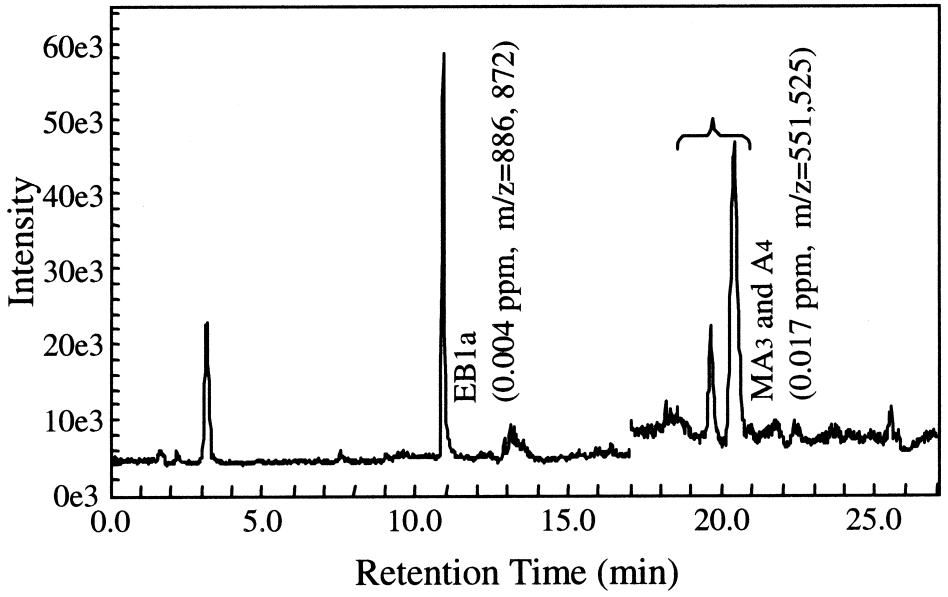

Fig. 7. Selected-ion monitoring chromatogram obtained by LC–ESI-MS of commercial cabbage.

A fluorescence-derivatized sample could not be confirmed by LC–ESI-MS. Therefore, confirmation by LC–MS was carried out using a sample without fluorescence derivatization. We surveyed the target compounds in 20 commercial crops using the developed method. As a result, emamectin was detected in nine crops and milbemectin was detected in four crops. Both of them were below the maximum residue limit.

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