Simultaneous Determination of Abamectin and Milbemectin Residues in Fruits

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An analytical method was developed to determine abamectin and milbemectin residues in apple, pear, and citrus using HPLC with ultraviolet absorption detection. Abamectin and milbemectin were extracted with methanol from apple, pear, and citrus samples. The extract was diluted with saline water and dichloromethane partition was followed to recover the compounds from the aqueous phase. Florisil column chromatography and aminopropyl solid-phase extraction were employed as cleanup methods to remove most of co-extractives from the sample extract. Reverse-phase HPLC using an octadecylsilyl column was successfully applied to separate and quantitate abamectin and milbemectin residues in sample extracts at the wavelength of 245 nm. Recoveries of abamectin and milbemectin from fortified samples ranged 80.4~90.3% and 90.9~96.8%, respectively. Relative standard deviations of the analytical method were less than 10% for both acaricides. Detection limit of the analytical method was 0.003 mg/kg sample for all the analytes. The proposed method was reproducible and sensitive enough to evaluate terminal residues of abamectin and milbemectin in apple, pear, and citrus.

Key words : *Abamectin, milbemectin, residue analysis, HPLC, apple, pear, citrus.*

Taken in conjunction with the persistence in fruits, use of acaricides inevitably lead to potential occurrence of their terminal residues in the harvest.[1] As fruits are consumed in large quantity, mostly in raw state, residue evaluation of the acaricide is of considerable importance to ensure safety of the harvest. Therefore, development of a highly reliable method is critical for estimation of the acaricide residues in fruits.

Abamectin and milbemectin are commercial acaricides derived from the avermectins, natural macrocyclic lactone compounds produced by the actinomycetes *Streptomyces avermitilis*.[1-3] Abamectin is a mixture of two homologs containing over 80% avermectin B1a and less than 20% avermectin B1b. Milbemectin is also a mixture of two homologs, milbemectin A_3 and A_4 in the ratio of 3 to 7.[2] Acaricidal activity of abamecin and milbemectin is so high that even low application rate of 0.02~0.04 kg a.i./ha, equivalent to one tenth to hundredth dose of conventional acaricides, gives effective control of various mites in fruit trees.[4] Though highly effective they may be, chronic toxicity of avermectins is also known to be high; the acceptable daily intake and maximum residue limits (MRLs) of abamectin are established as 0.002 mg/kg for human and 0.01~0.1 mg/kg for fruits, respectively.[5] Development of a highly sensitive method to analyze the acaricide residues is, therefore, required to meet the MRLs in fruits.

Several studies on the analysis of abamectin residues have been published using HPLC.[6-8] Vuik has analyzed intact

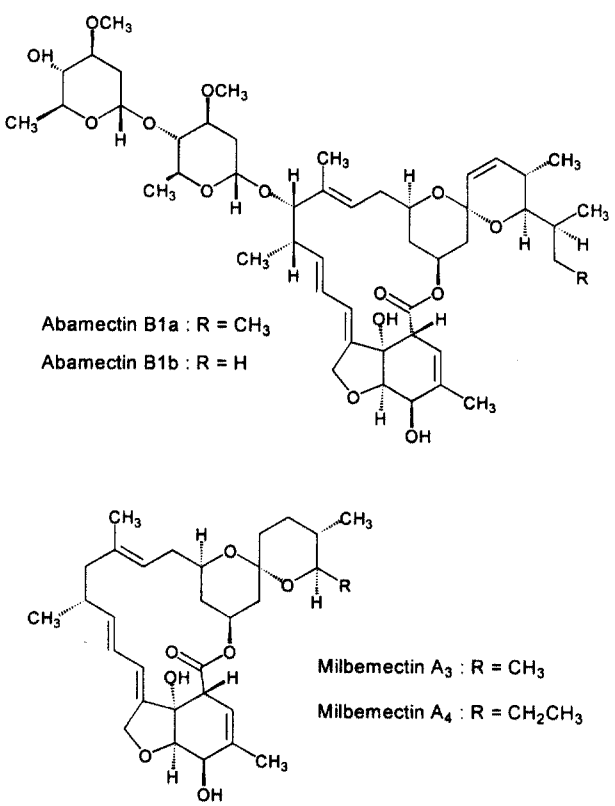

Fig. 1. Molecular structures of abamectin and milbemectin.

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Abbreviations: HPLC, high-performance liquid chromatography; MRLs, maximum residue limits; RSD, relative standard deviation; SPE, solid-phase extraction.

abamectin residues in vegetables using ultraviolet detection, but the sensitivity, 0.05 mg/kg, was not enough to meet current MRLs.[6] Fluorometric method, after derivatization of the ring structure using trifluoroacetic anhydride and N-methylimidazole, was reported to enhance the sensitivity of the compound upto 1~2 μg/kg. However, use of moisture-sensitive reagent and poor reproducibility of derivatizing reaction deterred the method from being routinely applied. The authors themselves had even recommended cautious guidelines for the analyst such as preparing additional samples to confirm the analytical data and careful manipulating of the derivatizing reagent for reaction consistency.[7-8] Accordingly, development of a new method is needed to routinely analyze the abamectin residues. Meanwhile, no appropriate method is yet avaliable for milbemectin.

In the present study, a new analytical method was attempted for the simultaneous determination of abamectin and milbemectin in fruits. The method was developed not only to achieve high sensitivity but also to provide reliability and readiness for the routine analysis of the acaricide residues.

Materials and Methods

Instrumentation. Ultraviolet (UV) absorption spectra were obtained with a Hewlett Packard (HP, USA) Model 8452A photodiode array spectrophotometer. Scanning was conducted at 200~350 nm for 2 s.

HPLC was performed using a Waters (USA) HPLC system equipped with two 510 pumps, 680 gradient controller, 486 tunable UV/VIS absorbance detector, HP (USA) 1100 autosampler, column oven, and 3395 integrator. Nova-Pak C18 (3.9 mm i.d. × 150 mm, 4 μm spherical, Waters Associates, USA) was used as the analytical column. Operating parameters used for the determination of the acaricide residues were as follows: column temperature, 40°C; mobile phase, acetonitrile/methanol/water (52.5/22.5/25, v/v/v), isocratic; flow rate, 1.0 m*l*/min; detection, UV absorption at 245 nm, 0.004 AUFS; sample size, 40 μl; chart speed, 0.5 cm/min.

Chemicals. Analytical standards of abamectin and milbemectin were kindly supplied by Novatis Agro Korea and Aventis Crop Science Korea, respectively. Abamectin standard of 92.8% purity comprised 93.9% B1a and 6.1% B1b. Milbemectin standard of 96.6% purity was consisted of 30.2% A_3 and 69.8% A_4. Milbemectin A_3 standard, 99.1% pure, was separately provided by the manufacturer. Each stock solution of 500 mg/*l* on the basis of total amount of the mixture was prepared in acetonitrile. The stock solution was stable at 4°C for minimum six months. Working solution was prepared in appropriate solvents whenever necessary. Acetonitrile, methanol, and deionized water were HPLC grade. All other solvents were pesticide residue grade or reagent grade freshly redistilled in glass. Florisil (60~100 mesh, pesticide residue grade) and silica gel (70~230 mesh, column chromatography grade) were purchased from Aldrich Chemical (USA) and Merck (Germany), respectively. The adsorbent was activated at 130°C for more than 5 h prior to use. Aminopropyl SPE cartridge (SPE-NH_2, Bond-Elut™, 500 mg packing, 3 m*l* tube) was purchased from Varian Aerograph (USA). All other reagents were reagent grade unless specified.

Fruit samples. At maturity control fruit samples were collected in bulk from orchard fields in Korea, where no analytes had been applied during the whole cultivation period. Varieties and orchard locations were as follows: apple, Fuji from Chilgok, Kyungbuk Province; pear, Singo from Youngchun, Kyungbuk Province; citrus, Koongchun from Seokwipo, Jeju Province. Composite fruit samples were prepared in compliance with the instructions in Korean Test Guidelines for Pesticide Persistence.[9] Apple and pear fruits were minced after removing and discarding the hilum and ovary portions. Whole citrus fruit including peel and flesh was chopped and blended. Each representative sample was stored at -20°C until analysis.

Extraction and partition. A 20-g portion of each fruit sample was weighed into a 500-m*l* homogenizer cup, and 100 m*l* of methanol was added. The mixture was macerated at 10,000 rpm for 2 min in a high-speed homogenizer (Nihonseiki Kaisha AM-8, Japan). The homogenate was suction-filtered through the filter paper (Toyo No. 6, Japan) on porcelain Büchner funnel. The cup and filter cake were washed with fresh 50 m*l* of methanol, and the rinsate was combined with the previous filtrate. The filtrate was quantitatively transferred into a 1000-m*l* separatory funnel, and sequential addition of 150 m*l* of saturated NaCl and 150 m*l* of distilled water was followed. The aqueous phase was then extracted twice each with 50 m*l* portions of dichloromethane. The dichloromethane phase was combined and dried over 20 g of anhydrous sodium sulfate layer. The extract was collected in a 250-m*l* distilling flask and evaporated just to dryness *in vacuo* at 40. The residue was dissolved in 10 m*l* of dichloromethane, ultrasonicated for 30 s, and subjected to Florisil column chromatography.

Florisil column chromatography. A chromatographic column (11 mm i.d. × 40 cm) was dry-packed with 5 g of activated Florisil, and topped with *ca.* 2 cm layer of anhydrous sodium sulfate. The column was pre-washed by passing 25 m*l* of dichloromethane until the solvent level reached the top of the sodium sulfate layer. The dichloromethane extract from the partition step was poured into the column and the column wall was rinsed twice with 2 m*l* portions of dichloromethane. When the liquid was drained to the sodium sulfate layer, the column was eluted with 50 m*l* of ethyl acetate/dichloromethane mixture (10/90, v/v), and the fraction was discarded. The column was then eluted with 50 m*l* of methanol/ethyl acetate mixture (0.5/99.5, v/v), and the fraction was collected. The eluate was concentrated just to dryness, and the residue was reconstituted with 10 m*l* of

n-hexane for solid-phase extraction.

Aminopropyl solid-phase extraction (SPE-NH_2). An SPE-NH_2 cartridge was activated by passing 6 m*l* of *n*-hexane until the solvent level reached the top of the packing. The hexane extract derived from the Florisil column chromatography was poured into the cartridge and eluted at 2 m*l*/min. The tube and wall was rinsed twice with 2 m*l* portions of *n*-hexane. The cartridge was eluted with 3 m*l* of toluene and 10 m*l* of dichloromethane, in turn, and all the eluates were discarded. The cartridge was then eluted with 6 m*l* of acetone/dichloromethane mixture (20/80, v/v), and the fraction was collected. The eluate was evaporated just to dryness under nitrogen stream at 50°C, and the residue was dissolved in 2 m*l* of the mobile phase for HPLC determination.

Validation of the analytical method. Recovery experiments were run on control fruit samples to validate the analytical method developed for acaricide residues. Prior to extraction, series of control samples were fortified with acaricide standard solution in acetonitrile at specified concentrations. After standing for 2 h, analytical procedures mentioned above were carried out to produce quality assurance data.

Results and Discussion

As abamectin and milbemectin have high molecular weights of 528~873 and negligible vapor pressure of 0.013~0.2 μPa,[2] gas chromatography was excluded, and HPLC was chosen as the analytical tool. Milbemectin A_3 and A_4 were reported to be nonpolar with the log P value of 5.3~5.9 and have low water solubility of 0.88~7.2 mg/*l*. Although no log P values were yet available, abamectin B1a and B1b were expected to show the same degree of polarity considering their molecular structures similar to milbemectin and low water solubility of 0.007~0.01 mg/*l*.[2] Therefore, a reverse-phase HPLC, routinely used for nonpolar pesticides, was applied to all analytes.[10-12]

As the present study was focused on the analysis of the acaricides as intact forms and the compounds had neither oxido-reduction characteristics nor intrinsic fluorophores, UV absorption detector was the only choice among the common HPLC detectors. UV absorption spectra were obtained from each 10 mg/*l* solution in acetonitrile/methanol/water mixture (52.5/22.5/25, v/v/v) to find out the optimum wavelength applicable to all the analytes. Abamectin and milbemectin had similar chromophore with the absorption maxima at 238, 246, and 254 nm (Fig. 2). In residue analysis of the acaricides to meet MRLs, analytes should be determined at the longest wavelength as possible with, at least, 10^4 $cm^{-1}M^{-1}$ order of extinction coefficient to avoid interferences by co-extractives as well as to get sensitivity at a nanogram level.[10,11] Estimating the longest wavelength with minimum loss of sensitivity for each compound, the optimum measuring wavelength was set to

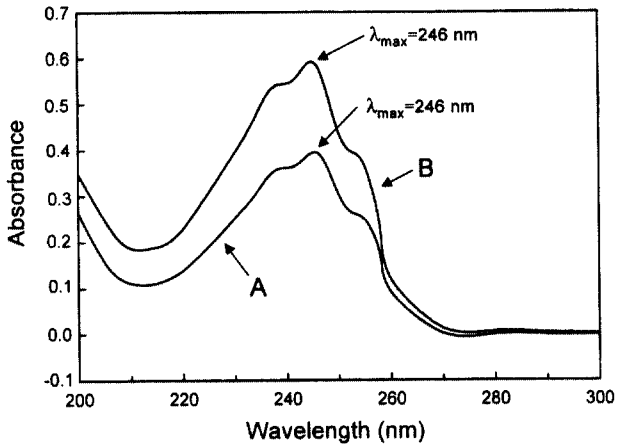

Fig. 2. Ultraviolet absorption spectra of abamectin and milbemectin. A. abamectin (B1a/B1b=93.9/6.1) 10 mg/*l*; B. milbemectin (A_3/A_4= 30.2/69.8) 10 mg/*l*.

Table 1. High-performance liquid chromatographic behavior of abamectin and milbemectin on octadecylsilyl column.

Compound	Capacity factor			MDQ (ng)**
	I*	II	III	
Abamectin B1a	8.1	13.4	10.7	1
Abamectin B1b	5.7	9.8	7.7	1
Milbemectin A_3	8.1	10.6	9.4	1
Milbemectin A_4	11.8	15.0	13.2	1

*Mobile phase I, acetonitrile/water (70/30, v/v), 1.0 m*l*/min; II, methanol/water (80/20, v/v), 1.0 m*l*/min; III, acetonitrile/methanol/water (52.5/22.5/25, v/v/v), 1.0 m*l*/min.
**Minimum detectable quantity at 3% full scale deflection.

245 nm, compensated for UV detector, at which extinction coefficients of abamectin and milbemectin were in the range of 3.1×10^4 ~3.4×10^4 $cm^{-1}M^{-1}$.

When reverse-phase HPLC using an octadecylsilyl column was employed, all analytes showed a sharp symmetrical peak under the mobile phase of acetonitrile/water or methanol/water mixtures. In the range of 50 to 80% acetonitrile or methanol contents in water, their capacity factor increased almost twofold as the contents of organic solvents decreased by 10%. This indicated that all the analytes existed in the neutral form in the mobile phase of acetonitrile/water or methanol/water mixtures.[13] The hydroxyl groups attached to the alkyl chain, two and three groups in milbemectin and abamectin, respectively, were dissociated very weakly in the mobile phase, and little effect was given to the overall polarity of the molecule. Therefore, there was no need for ion-suppression. Chromatographic behaviors of abamectin and milbemectin under different mobile phases are summarized in Table 1. In acetonitrile/water mixture, abamectin B1a and milbemectin A_3 overlapped, while abamectin B1b and milbemectin A_3 were completely resolved. Although B1a and A_3 were well-separated, B1b and A_3 were incompletely resolved in

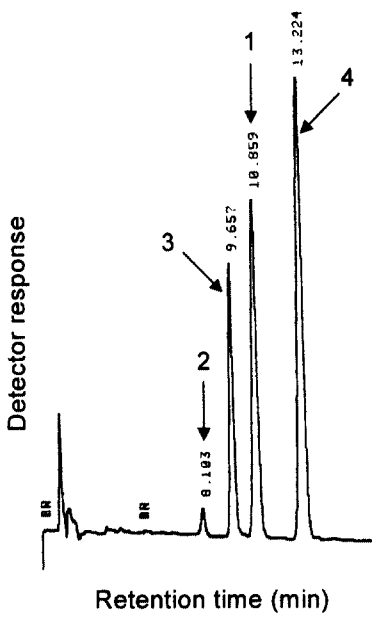

Fig. 3. High-performance liquid chromatogram of the standard mixture. 1, abamectin B1a 18.8 ng; 2, abamectin B1b 1.2 ng; 3, milbemectin A_3 8.5 ng; 4, milbemectin A_4 19.5 ng.

methanol/water mixture. Selectivity of organic solvents affecting the chromatographic behavior of the compounds was hence observed, which might be due to the different solubilization of the compound and/or competition with the hydroxyl groups in the molecule.[13] When the combination of acetonitrile, methanol, and water was optimized, all compounds were completely separated to the baseline within capacity factors of 13.2. A typical chromatogram of the standard mixture is shown in Fig. 3. Minimum detectable quantity (MDQ) of all the compound was calculated to be 1 ng at 3% full scale deflection. The sensitivity appeared *ca.* ten times higher than that of the abamectin method reported by Vuik,[6] as more efficient column and sensitive detector were employed. This sensitivity may be estimated sufficiently high for common residue analysis requiring the detection limit of 0.05 mg/kg.[9] Considering the MRLs of abamectin, 0.01~0.1 mg/kg, however, the developing method should possess a detection limit of 0.005 mg/kg, at least, to surveil the safety of the residues. Consequently, higher concentration of the sample extract should be made to inject the amount of sample size as large as possible. Since concentrating the sample extract frequently led to severe interference by concurrent co-extractives, rigorous purification of the sample extracts might be required. This study was, therefore, mainly focused on the development of efficient but simple cleanup methods.

Regarding the polarity of compounds, an attempt was made at the partition step to remove co-extractives from fruit extracts.[10-12] From the methanol extract diluted with saline water, all compounds were completely recovered into the dichloromethane phase among the three partition methods (Table 2). Hexane partition was favorable in lessening the co-extractives, but failed to recover abamectin.

Table 2. Recovery of abamectin and milbemectin using different partition methods.

Compound	Recovery (%)		
	I*	II	III
Abamectin B1a	10	27	105
Abamectin B1b	19	29	105
Milbemectin A_3	100	102	106
Milbemectin A_4	102	103	104

*I, Single partition with 100 m*l* n-hexane; II, double partition with 50 m*l* portions of *n*-hexane each; III, double partition with 50 m*l* portions of dichloromethane each.

Table 3. Elution profiles of abamectin and milbemectin on Florisil, silica gel, and SPE-NH_2 columns.

Eluting solvent	Recovery (%)			
	Abamectin B1a	Abamectin B1b	Milbemectin A_3	Milbemectin A_4
	Florisil			
I*	NR	NR	NR	NR
II	4	5	88	92
III	87	90	9	6
	Silica gel			
IV**	NR****	NR	NR	NR
V	NR	NR	93	90
VI	87	78	8	6
	SPE-NH_2			
VII***	NR	NR	NR	NR
VIII	NR	NR	NR	1
IX	97	90	100	98

*I, 50 m*l* of ethyl acetate/dichloromethane (10/90, v/v); II, pre-eluted with I and followed by elution with 50 m*l* of ethyl acetate/dichloromethane (45/55, v/v); III, pre-eluted with I, II, and then followed by elution with 50 m*l* of methanol/ethyl acetate (0.5/99.5, v/v).

**IV, 50 m*l* of ethyl acetate/dichloromethane (15/85, v/v); V, 50 m*l* of ethyl acetate/dichloromethane (50/50, v/v); VI, 50 m*l* of methanol/ethyl acetate (0.5/99.5, v/v). Elution sequence was same as 1-III.

***VII, 3 m*l* of toluene; VIII, 12.5 m*l* of dichloromethane; IX, 6 m*l* of acetone/dichloromethane (20/80, v/v). Elution sequence was same as 1-III.

****Not recovered.

Adsorption chromatography and SPE-NH_2 were applied to further purify the extracts. Elution profiles of the acaricides on Florisil, silica gel, and SPE-NH_2 columns are listed in Table 3. Florisil and silica gel exhibited similar elution patterns of abamectin and milbemectin. Milbemectin eluted rather earlier than abamectin but partial overlapping made the fractionation between the two acaricides impossible. As both acaricides had to be collected in a fraction, large difference in polarity between washing and eluting solvent

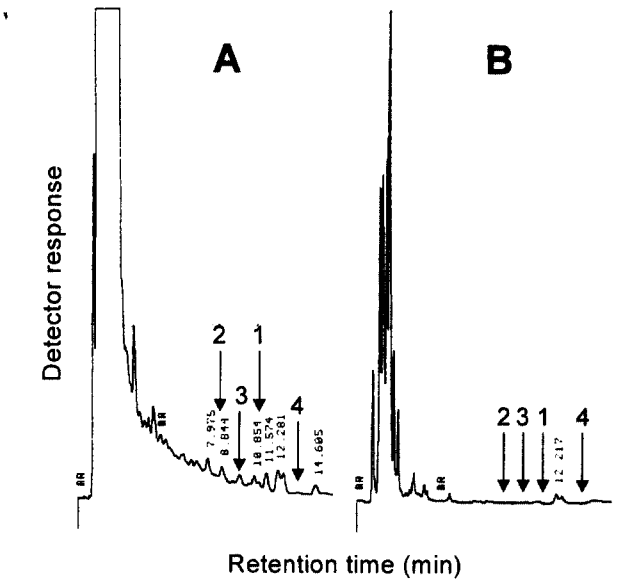

Fig. 4. Effect of SPE-NH_2 on the removal of co-extractives from citrus extract. A, before SPE-NH_2; B, after SPE-NH_2. Each arrow indicates the retention time of abamectin B1a(1), B1b(2), milbemectin A_3(3), and A_4(4), respectively.

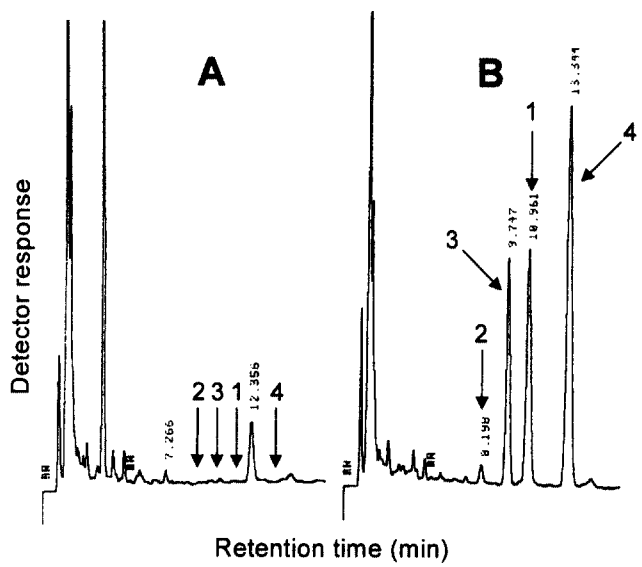

Fig. 5. Typical high-performance liquid chromatograms of extracts from apple samples. A, control; B, fortified with abamectin B1a(1), B1b(2), milbemectin A_3(3), and A_4(4) at 0.047, 0.003, 0.021, and 0.049 mg/kg, respectively.

might cause more chance to co-elute interfering materials from the sample extract. In fact, eluates from silica gel and Florisil columns did contained some co-extractives including yellow pigments. When examined by HPLC, the eluate from Florisil column showed the chromatogram more preferable to that of silica gel column. Florisil was thus chosen as an adsorbent.

As the Florisil eluate still contained some interfering co-extractives, further purification was performed by SPE-NH_2. The aminopropyl group on the SPE surface is known to act

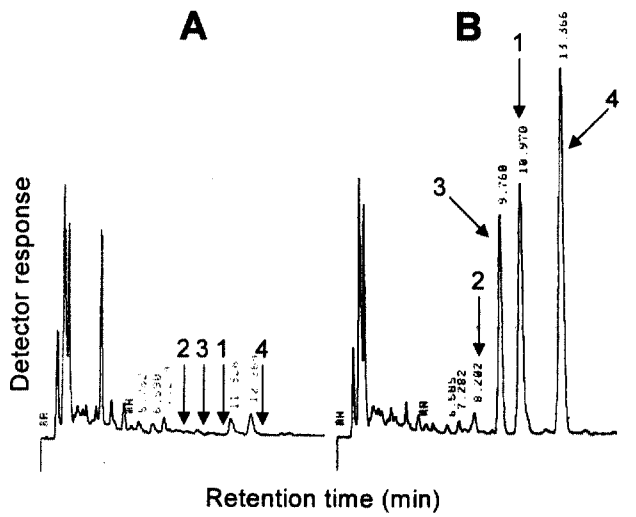

Fig. 6. Typical high-performance liquid chromatograms of extracts from pear samples. A, control; B, fortified with abamectin B1a(1), B1b(2), milbemectin A_3(3), and A_4(4) at 0.047, 0.003, 0.021, and 0.049 mg/kg, respectively.

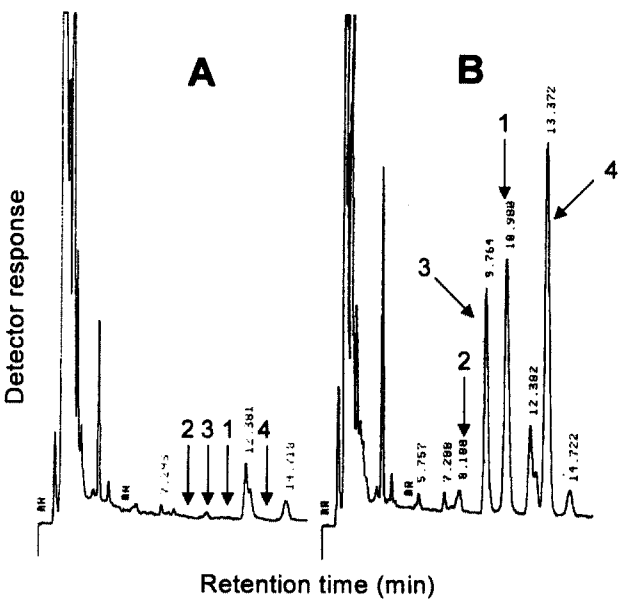

Fig. 7. Typical high-performance liquid chromatograms of extracts from citrus samples. A, control; B, fortified with abamectin B1a(1), B1b(2), milbemectin A_3(3), and A_4(4) at 0.047, 0.003, 0.021, and 0.049 mg/kg, respectively.

as a weak anion exchanger.[14] Even though weakly dissociated it may be, the hydroxyl groups of abamectin and milbemectin would become the counter ions to the amino groups, and, as a result, the analyte would be more retained than the neutral compounds with no anionic or ionizable groups. Applied to the Florisil eluate, abamectin and milbemectin were retained as expected, while much of the co-extractives eluted earlier than the analyte. Most of the interferences from the troublesome citrus extract including yellow pigments were effectively removed, and the colorless eluate ready for HPLC quantitation was successfully obtained (Fig. 4).

Table 4. Recovery and detection limits of abamectin and milbemectin in fruits.

Compound	Fortification (mg/kg)	RecoverySD (%)*			Detection limit (mg/kg)
		Apple	Pear	Citrus	
Abamectin B1a	0.047	80.4±2.6	84.1±2.5	89.9±1.3	0.003
	0.235	86.4±1.2	88.2±1.6	85.3±1.6	
Abamectin B1b	0.003	-	-	-	0.003
	0.015	84.8±3.3	88.4±3.3	90.3±5.7	
Milbemectin A_3	0.021	94.1±1.6	92.6±2.2	96.8±1.3	0.003
	0.085	94.0±0.4	93.2±1.8	92.0±1.2	
Milbemectin A_4	0.049	93.2±1.1	91.7±1.4	93.2±0.3	0.003
	0.195	93.4±0.3	92.2±0.8	90.9±1.3	

*Mean values of triplicate samples with standard deviations.

Coupling with the proposed partition, adsorption chromatography and SPE-NH_2, typical HPLC chromatograms of control and fortified samples of apple, pear, and citrus are shown in Figs. 5, 6, and 7, respectively. The proposed method produced similar, clean HPLC chromatograms, free of interference, for the apple, pear, and citrus samples. In spite that a small peak equivalent to 0.002 mg/kg was found near abamectin B1a in pear sample, no difficulty was found for identification and quantitation of B1a since the retention times were distinctly different each other. Irrespective to sample types, as no late eluting peaks were observed even with 50 consecutive injections of the sample extract for a day, the proposed method would allow a continuous analysis.

Percent recoveries generated during the validation of analytical methods are presented in Table 4. Mean recoveries of abamectin and milbemectin were all over 80%, in the range of 80.4~96.8% with six replicates per sample type. No significant difference in the recovery affecting the analytical precision was found according to the fortification level and fruit type. RSDs over all types of samples were less than 10%, indicating that the method could be reproducibly applied to analyze the acaricide residues in the three fruit samples. As no interference was found in all control samples, detection limits of the method could be directly calculated from minimum detectable quantity of each compound. The detection limit of the proposed method was 0.003 mg/kg sample for each compound based on 3% full scale deflection (S/N>10). These sensitivities were sufficiently high to detect 1/3~1/30 of MRLs (0.01~0.1 mg/kg) established for abamectin in fruits.[5]

The proposed method satisfies the criteria of the analytical method for pesticide residues, with more than 70% recovery and less than 10% RSD, on Test Guidelines for Pesticide Persistence notified by Rural Development Administration.[9] Detection limit of the method is 0.003 mg/kg, which is ten times more sensitive than 0.05 mg/kg of the criteria, and sufficient to cover the lowest MRL of the acaricides. The present method is quite competitive to the current fluorometric method for abamectin residue. Even though the sensitivity is slightly lower, reproducibility is superior to that of the fluorometric method reported as maximum 18% RSD.[7,8] The method has another merit of being able to analyze the compound in an intact form, which enables further confirmatory operation for suspected residues using alternative method such as current fluorometric method. Analytical procedures do not require any special apparatus or instruments except SPE cartridge commercially available, but mostly consist of current techniques familiar to the residue analyst. The method is simple to operate, allowing one experienced person to analyze six samples per day. Therefore, authors suggest that the proposed method could be sufficiently applied to the routine analysis for simultaneous evaluation of abamectin and milbemectin residues in fruit samples.

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