Multiresidue Analysis of Pesticides in Fresh Fruits and Vegetables by Supercritical Fluid Extraction and HPLC

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A screening method was established for the determination of 27 pesticides in fresh fruits and vegetables by a super critical fluid extraction (SFE), cleaned up with cartridge columns and HPLC. The multiresidue and semiautomatic analysis was useful for a screening examination, because the determination methods for pesticides under the Japanese Food Sanitation Law are mostly individual determinations. Reported methodologies for multiresidue analysis by HPLC were not adequate to regulated pesticides in Japan. In this report, multiresidue determination of pesticides and their metabolites are discussed using SFE and HPLC. Details of the proposed method are as follows: Wet samples such as fruits and vegetables were not suitable for the SFE instrument, so the water in the samples was removed with an absorptive polymer (Arasorb® S-310) prior to SFE. The pesticides were extracted by SFE, the extracts trapped with Extrelut® NT + Bond Elut® C_{18} and then eluted with acetonitrile. The eluate was cleaned up with Sep-Pak® Florisil+Bond Elut® PSA cartridges. After washing with *n*-hexane, the pesticides were eluted from the cartridges with 15% ether/*n*-hexane, 15 and 50% acetone/*n*-hexane. These three fractions were individually determined by HPLC with a photodiode-array detector. The pesticides spiked in samples at 0.5 ppm showed satisfactory recoveries except for thiabendazole, imazalil and clofentezine. Detection limits were 0.005–0.01 ppm for the 27 pesticides.

Key words —— pesticide, super critical fluid extraction, HPLC, polymer, Sep-Pak® Florisil, Bond Elut® PSA

INTRODUCTION

Extraction of pesticides in food is commonly performed using an organic solvent, but it is time-consuming, laborious, requires much space and glassware as well as generates a large amount of hazardous waste. Therefore, SFE has recently been noted as a new method of extraction in the laboratory.[1] Because SFE uses liquid carbon dioxide (CO_2) for extraction, the technique poses little threat to humans and the environment, saves laboratory space and analytical time, and solves the rest of the above problems. To reduce hazards to human health and the environment, many hydrophilic pesticides have been developed and registered in Japan. Some of these pesticides can be measured by GC with derivatization[2, 3] or by HPLC. Especially, the pesticides measured by HPLC[4–7] have been increasing. Many studies have demonstrated the capability of SFE for pesticide residues in matrices such as soil, grain and wet samples such as vegetables and fruits.[8–10] The excess water in a sample tends to reduce the extractability by SFE; therefore, several approaches for dehydration from green-grocery items have been reported.[11–13] For some of them, an absorptive polymer that has high absorptivity serves as a water sorbent. The aim of this study was to establish a systematic analytical method for the rapid and sensitive monitoring of pesticide residues in green-grocery items and to reduce the consumption of solvent and analytical time by semi-micronized HPLC with photodiode array detection and SFE.

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MATERIALS AND METHODS

Apparatus ——

SFE: Super-201 (JASCO, Hachioji, Japan). Extraction conditions: extraction fluid, CO_2; pressure, 300 kg/cm^2; extraction temperature, 40°C; flow rate of CO_2, 4.9 ml/min; flow rate of acetone modifying

CO_2, 0.1 ml/min; extraction time, 40 min; extraction vessel volume,10 ml. Trapping conditions of extract: nozzle temperature, 75°C; trap packing of column, layered Extrelut® NT 1 g over BondElut® C_{18} 1 g into glass tube (150 × 14 mm i.d.).

HPLC analysis: The HPLC system used in this work consisted of a Hewlett-Packard 1100 series (Waldbronn, Germany) with a photodiode array detector setting at 230 nm, a gradient pump, which delivered an acetonitrile–water mobile phase to a Wakosil II – 3C18RS (150 × 2 mm i.d.; 3.5 μm particles, Wako Pure Chem, Osaka, Japan) at a flow rate of 0.2 ml/min at 50°C. A linear gradient from 20 to 60% of acetonitrile, for 10 min, next from 60 to 70% for 20 min was used. Hereafter, the mobile phase composition was maintained at 100% acetonitrile at 22 to 30 min. The injection volume was 10 μl.

Reagents ——

Reagent: Acetonitrile, acetone, ether, *n*-hexane, diatomaceous earth Celite 545 were pesticide residue analysis grade (Wako Pure Chem. Industries, Co., Ltd., Osaka, Japan); Liquid CO_2 was 99.99% pure. (Kinki Sanso Co., Ltd., Sasayama, Japan); Extrelut® NT (Merck KgaA, Darmstadt, Germany); Bond Elut® C_{18} (1 g); PSA (500 mg: Varian, Harbor City, CA); Sep-Pak® Florisil (690 mg: Waters, Massachusetts, U.S.A.); water-absorbent polymer Arasorb S-310 (Arakawa Chemical Industries, Ltd., Osaka, Japan). Other reagents used were of the highest grade commercially available.

Pesticide Standards: Pesticides standards were obtained from Wako Pure Chem. The 33 pesticides are listed in Table 1. Cyromazine, flusulfamide, chlorimuronethyl, and metsulfuronmethyl were also purchased from Wako. Each stock solution was prepared at 1000 μg/ml in acetonitrile. Working standard mixtures (A–E groups) and other pesticides in acetonitrile, containing 10 μg/ml of each pesticide, were used for spiking the samples and preparing calibration standards.

Sample Preparation and Analysis ——

Extraction: Commercially purchased cucumber, potato, apple, radish and banana served as the blank or spiked sample. One piece of each sample was thoroughly shredded and homogenized. Then 5 g of the sample, 1 g of Celite and 1 g of Arasorb® S-310 polymer were mixed well with a glass rod. To granulate them completely, a bit of Celite was added to the sample and mixed well. Thereafter, the mixture was packed into the extraction vessels, and for the fortified samples, 0.5 μg/g of each pesticide was added to the sample in the vessel. The pesticides were trapped with Extrelut® NT and the Bond Elut® C_{18} column.

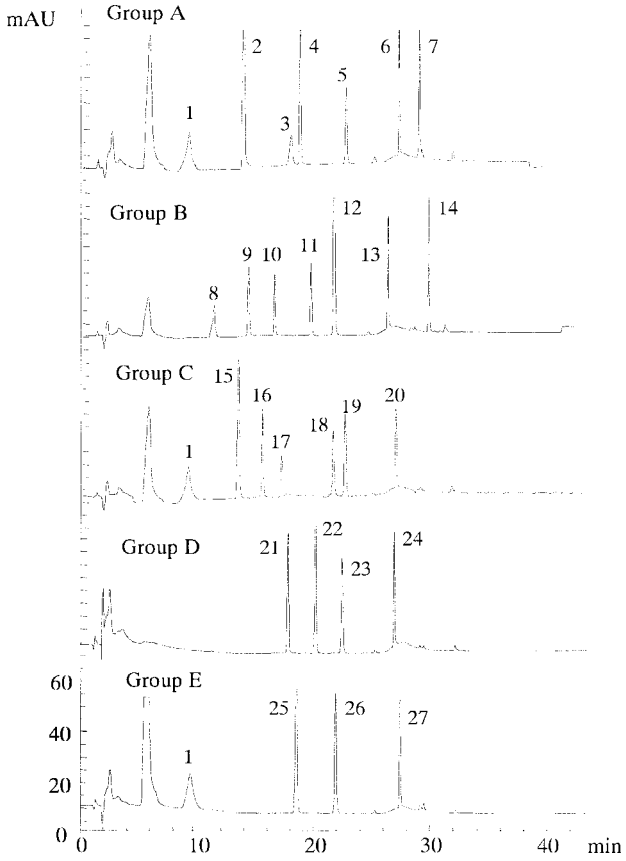

Fig. 1. HPLC Chromatograms of Standard Mixture
Peak numbers are indicated in Table 1. 0.025 ng of each pesticide was injected.

Purification: The column trapping the pesticides was removed from the SFE instrument, the pesticides were eluted with 40 ml of acetonitrile, and the eluate was concentrated to dryness. The residue was dissolved in 2 ml of *n*-hexane, and then applied to Bond Elut® PSA over Sep-Pak® Florisil columns connected in tandem. After washing with *n*-hexane, the pesticides were eluted from these cartridges sequentially with 15% ether/*n*-hexane, 15 and 50% acetone/*n*-hexane 20 ml for each solvent mixture. Each fraction was evaporated to dryness. The residue was filled to exactly 1 ml with acetonitrile.

Determination: Recording of chromatograms and quantitative measurements of peak areas were performed with a Hewlett-Packard HPLC Chemstation.

Confirmation: The absorption spectrum of the detected peaks were confirmed by comparison with a standard of each pesticide.

Table 1. Recoveries of 33 Pesticides from 5 Cartridge Columns

Group	No.	Pesticide	Recovery (%)[a]				
			PSA	Florisil	Silica	SAX	NH_2
A	1	Thiabendazole	91.2	78.5	101.5	69.4	31.0[b]
	2	Carbaryl	93.8	94.6	99.9	95.9	63.0
	3	Imazalil	87.4	86.5	64.9	71.0	60.0
	4	Iprodione	107.1	92.6	98.7	56.7	54.8
	5	Hexaflumuron	105.6	95.5	100.3	94.5	96.1
	6	Chlorfluazuon	93.4	89.6	100.7	101.7	103.0
	7	Ethofenprox	94.3	94.8	98.8	92.5	92.8
B	8	Imibenconazole metabolite	107.4	90.9	92.5	103.3	99.0
	9	Furametpyr	94.2	97.1	96.6	99.3	89.3
	10	Benfuresate	91.6	91.2	98.1	97.5	98.1
	11	Pyrazoxyfen	89.1	92.1	95.7	98.8	82.8
	12	Iprodione metabolite	95.4	97.4	100.6	100.3	19.3
	13	Hexythiazox	82.1	88.1	72.1	89.0	88.3
	14	Silafluofen	91.2	96.9	95.8	94.7	89.9
C	15	Methabenzthiazuron	98.7	96.3	17.6	102.1	98.8
	16	Inabenfide	102.4	86.7	91.7	46.9	82.3
	17	Myclobutanil	104.1	71.0	48.5	0.0	82.1
	18	Ethobenzanid	100.8	102.6	99.1	119.9	101.5
	19	Pencycuron	91.8	93.6	98.9	98.3	90.7
	20	Buprofezin	93.2	88.3	90.7	94.6	97.3
D	21	Dymron	93.0	94.7	94.8	89.8	96.9
	22	Tebufenozide	92.2	96.7	97.7	97.3	93.7
	23	Clofentezine	89.3	102.7	94.4	85.5	90.0
	24	Flufenoxuron	91.7	95.7	102.4	99.4	96.8
E	25	Diflubenzuron	101.7	87.9	100.3	98.8	96.3
	26	Triflumizole	83.8	93.3	90.5	100.6	90.0
	27	Fenpyroximate	99.0	100.3	102.4	100.7	23.9
F		Pyridate	42.1	90.8	89.4	64.0	7.1
		Tecloftalam	95.6	36.7	0.0	23.4	0.0
		Tecloftalam metabolite	20.8	94.8	95.5	97.3	10.0
		Trichlamide	90.8	43.7	89.1	94.4	92.8
		Cymoxanil	89.5	31.1	0.0	32.4	38.9
		Diclomezine	91.5	39.8	92.1	91.5	89.4

a) Recovery was total amount from following 20 ml of each fraction, 15% ether/*n*-hexane,15% acetone/*n*-hexane and 50% acetone/*n*-hexane 20 ml, respectively. *b*) Recovery less than 50% is covered by mesh.

RESULTS AND DISCUSSION

HPLC Conditions

In this study, a reversed-phase semi-micro column was used for simultaneous determination, reducing the solvent volume and the measurement time and obtaining high separativity. These pesticides, capable of determination by HPLC, were mainly selected among pesticides to set a regulated value in Japan. Recovery tests from samples were ultimately performed for 27 pesticides divided into 5 groups, because the retention times of some pesticides were very close. The HPLC chromatograms of standard mixtures are shown in Fig. 1. Concerning the possibility of confirmation by liquid chromatograph mass spectrometer (LC/MS), acetonitrile-water without a precipitable salt as the mobile phase was selected. Under this condition, however, efficient separation and peak shape could not be obtained for flusulfamide, chlorimuronethyl, and metsulfuron-methyl. The cyromazine peak overlapped the solvent peak. Various approaches by adding acetic acid, sodium acetate or an ion pair reagent to the mobile phase did not improve the situation (data not shown). Consequently, these 4 pesticides were excluded from this work as references. Detection at 254 nm results in decreasing the interfering peaks and horizontal baseline; however, it caused lower sensitivity for

Table 2. Recoveries of 27 Pesticides from Sep-Pak® Florisil + Bond Elut® PSA

Group	Pesticide	Recovery (%)[a)]			
		1 fr.	2 fr.	3 fr.	total
A	Thiabendazole			71.7	71.7
	Carbaryl		89.8		89.8
	Imazalil			87.4	87.4
	Iprodione		91.6		91.6
	Hexaflumuron			96.8	96.8
	Chlorfluazuon			91.5	91.5
	Ethofenprox	94.5			94.5
B	Imibenconazole metabolite			103.7	103.7
	Furametpyr		93.5		93.5
	Benfuresate		90.4		90.4
	Pyrazoxyfen		90.8		90.8
	Iprodione metabolite		96.9		96.9
	Hexythiazox		85.5		85.5
	Silafluofen	94.0			94.0
C	Methabenzthiazuron		97.5		97.5
	Inabenfide			98.7	98.7
	Myclobutanil			87.9	87.9
	Ethobenzanid		100.8		100.8
	Pencycuron		91.8		91.8
	Buprofezin	35.8	56.1		91.9
D	Dymron		93.0		93.0
	Tebufenozide		94.5		94.5
	Clofentezine	89.3			89.3
	Flufenoxuron		14.3	75.1	89.4
E	Diflubenzuron		67.8	20.9	88.7
	Triflumizole		32.8	61.3	94.1
	Fenpyroximate		90.5		90.5

a) 1 μg of each pesticide was used. *b*) elute conditions 1 fr.; 20 ml of 15% ether/*n*-hexane, 2 fr.; 20 ml of 15% acetone/*n*-hexane, 3 fr.; 20 ml of 50% acetone/*n*-hexane.

hexythiazox, buprofezin, silafluofen and ethofenprox than detection at 250 nm, and the sensitivities were not sufficient for regulation values in Japan. Ultimately, the wavelength was set at 230 nm for greater sensitivity for pesticides. The peaks of thiabendazole and imazalil were still broad and unstable under these conditions.

Extraction with SFE

Generally speaking, SFE is not adequate for extraction from wet samples but is suitable from relatively dry ones. To subject wet samples to SFE, freeze drying or drying agents are used. In this study, we used a water-absorbent polymer for vegetables and fruits, and SFE conditions were in accordance with previous reports.[8–10] In our experiments, it was found that the manner of mixing the sample, Celite and dry agent had a significant effect on the extractability of the pesticides by SFE. The wet sample was mixed with Celite 545 and Arasorb® S-310 in turn, and a granular sample was obtained. To avoid conjugation of the granular sample, a small amount of additional Celite was added. Consequently, the mixture became completely granular, easy to pack and the reproducibility of the recoveries increased. Acetone was used as a modifier for CO_2 to prevent clogging of the SFE instrument by repeated extraction. To avoid breakage of the trapping column by high pressure and freezing extract, Bond Elut® C_{18} was packed into a glass tube and layered with Extrelut® NT as a trapping material over it.

Cleanup with Cartridge Columns

The extract from the trapping column of SFE was directly injected to HPLC. There were too many interfering peaks to measure on the chromatogram. In this paper, several cartridge columns (Sep-Pak® Florisil, Silica and Bond Elut® PSA, SAX, NH_2) were tested, and the recoveries were compared for cleaning up the extract. The recoveries of about 33 pesti-

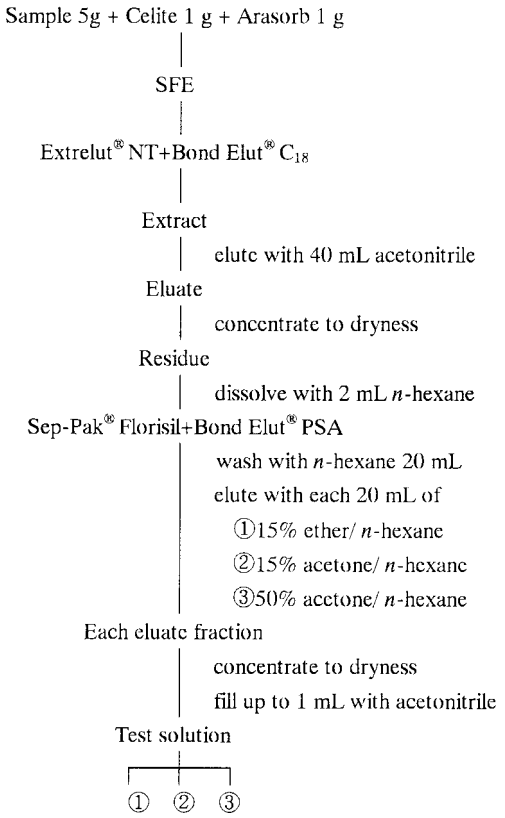

Chart 1. Procedure for the Preparation of Test Solution

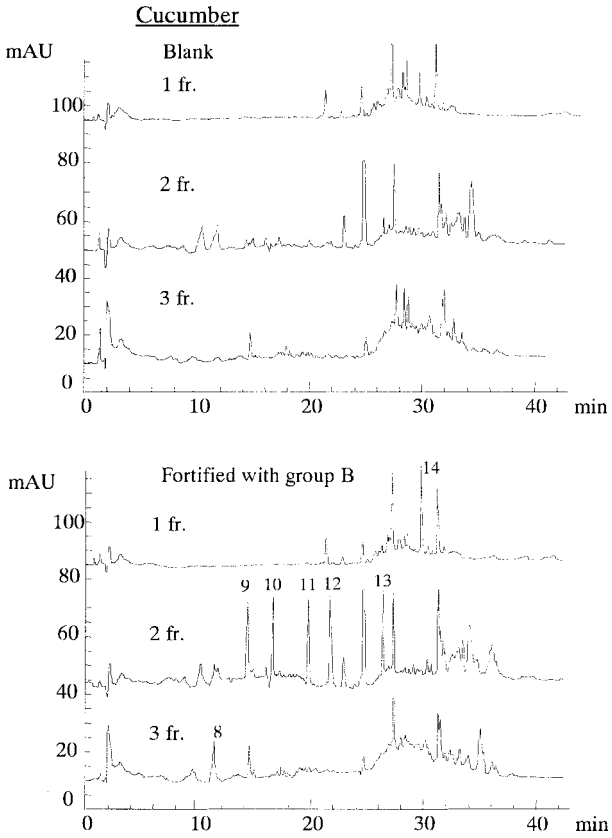

Fig. 2. HPLC Chromatograms of Cucumber Blank and Sample Fortified with Group B
Each pesticide was spiked at 0.5 ppm.

cides from 5 cartridges are indicated in Table 1. The recoveries of 6 pesticides (group F) among them were inadequate for all of the cartridges; therefore, these pesticides were eliminated from this study. Other pesticides gave satisfactory recoveries with Sep-Pak® Florisil and Bond Elut® PSA. To reduce cleanup time, both of these cartridges were adopted with attachment in tandem. Purification of the sample eluted from these cartridges was not sufficient; therefore, the eluted material from these cartridges was divided into 3 fractions, 15% ether/*n*-hexane, 15 and 50% acetone/*n*-hexane. This allowed dispersion and reduction of the interference peaks as well as quantitative analysis. The interfering peaks from Arasorb® S-310 were not detected. The recoveries of 27 pesticides by the respective fractions from Sep-Pak® Florisil and Bond Elut® PSA are indicated in Table 2.

For Florisil, it was necessary to observe the preservation conditions and the difference in activity among lots, because deficient conditions may cause inadequate reproducibility.

The procedure for preparation of the test solution is indicated in Chart 1.

Recovery Test from Agricultural Products

In this work, focused on green-grocery items, the critical problem mentioned above was the method of removing water from the sample. Therefore, besides the conventional method, Celite and the Arasorb® S-310 polymer were added to the samples and simply mixed, ultimately adding a small amount of Celite to the sample again.

Recoveries of 27 pesticides added to green-groceries are shown in Table 3. HPLC chromatograms of a cucumber blank and a cucumber fortified with B group are shown in Fig. 2. The present method was capable of analyzing quantitatively by dividing into 3 fractions the eluates from Sep-Pak® Florisil and Bond Elut® PSA for most of the 27 pesticides. However, thiabendazole and imazalil showed low recoveries; thiabendazole, in particular, showed considerable variation, which may be caused by high solubility in water or diffusion due to too much injection volume or insufficient recoveries from the cartridges.

The recoveries at a level of 0.5 ppm were 61.3–103.6%, except for thiabendazole in 5 agricultural products, imazalil in 3 agricultural products, clofentezine and diflubenzuron in 2 agricultural products and triflumizole in apple. Concerning a

Table 3. Recoveries of 27 Pesticides Added to Agricultural Products by the Proposed Method

Group	Pesticide	Recovery (%, mean ± S.D.)[a]				
		Cucumber	Potato	Apple	Radish	Banana
A	Thiabendazole	75.3 ± 6.7	71.1 ± 0.8	54.9 ± 10.1	63.3 ± 15.5	94.7 ± 33.1
	Carbalil	83.7 ± 6.1	89.4 ± 7.2	97.0 ± 0.9	87.0 ± 0.2	90.9 ± 0.6
	Imazalil	58.9 ± 10.5	64.0 ± 15.4	58.1 ± 7.9	49.3 ± 9.9	61.3 ± 2.7
	Iprodione	88.4 ± 6.0	80.0 ± 6.3	82.3 ± 3.3	75.9 ± 5.5	86.0 ± 3.4
	Hexaflumuron	73.9 ± 5.6	77.1 ± 9.4	71.4 ± 5.4	74.0 ± 4.0	83.1 ± 6.4
	Chlorfluazuon	72.5 ± 1.2	73.5 ± 3.3	74.8 ± 7.8	68.9 ± 4.5	84.1 ± 5.9
	Ethofenprox	81.4 ± 4.6	72.0 ± 4.3	81.9 ± 0.2	72.9 ± 8.7	80.1 ± 3.5
B	Imibenconazole metabolite	103.6 ± 4.6	89.1 ± 5.1	91.0 ± 5.8	76.8 ± 10.0	85.3 ± 3.3
	Furametpyr	103.3 ± 10.5	92.6 ± 6.4	94.4 ± 1.1	95.6 ± 5.0	99.2 ± 1.0
	Benfuresate	78.8 ± 15.1	90.7 ± 7.7	75.6 ± 0.5	89.1 ± 5.4	73.9 ± 10.1
	Pyrazoxyfen	95.9 ± 0.8	98.3 ± 3.9	82.2 ± 0.5	96.8 ± 2.5	85.8 ± 8.5
	Iprodione metabolite	90.6 ± 3.8	94.7 ± 4.8	79.0 ± 4.1	85.1 ± 4.7	79.7 ± 4.8
	Hexythiazox	72.2 ± 1.4	80.6 ± 8.3	74.0 ± 6.9	77.4 ± 4.7	73.7 ± 6.8
	Silafluofen	73.5 ± 1.5	85.8 ± 3.7	65.9 ± 8.7	75.6 ± 4.7	67.7 ± 9.4
C	Thiabendazole	68.8 ± 16.5	73.0 ± 10.2	56.4 ± 3.9	59.9 ± 16.6	38.2 ± 5.0
	Methabenzthiazuron	88.5 ± 5.1	88.3 ± 0.8	85.3 ± 0.9	88.7 ± 4.4	87.2 ± 5.1
	Inabenfide	77.3 ± 5.8	84.4 ± 2.6	78.5 ± 2.1	87.4 ± 3.1	67.3 ± 12.6
	Myclobutanil	85.3 ± 0.4	93.5 ± 3.0	93.4 ± 0.6	88.9 ± 2.9	92.1 ± 7.1
	Ethobenzanid	87.0 ± 0.9	93.8 ± 2.1	87.5 ± 2.1	95.8 ± 0.4	87.3 ± 13.8
	Pencycuron	83.3 ± 3.6	89.4 ± 0.4	88.7 ± 1.3	90.2 ± 1.9	87.2 ± 8.5
	Buprofezin	64.0 ± 7.2	82.6 ± 10.2	72.1 ± 11.2	68.1 ± 8.9	65.9 ± 0.3
D	Dymron	87.5 ± 4.4	89.0 ± 5.1	85.9 ± 4.6	85.0 ± 7.3	94.2 ± 5.5
	Tebufenozide	88.0 ± 2.9	93.6 ± 4.5	93.1 ± 1.5	85.5 ± 8.8	96.0 ± 4.1
	Clofentezine	66.3 ± 7.6	25.1 ± 11.6	58.4 ± 9.9	—[b]	79.3 ± 8.8
	Flufenoxuron	78.3 ± 0.7	78.0 ± 7.4	75.8 ± 3.4	81.2 ± 5.8	79.0 ± 6.3
E	Thiabendazole	49.3 ± 30.5	47.6 ± 33.6	56.7 ± 19.8	79.4 ± 6.6	0.0
	Diflubenzuron	69.2 ± 0.6	56.3 ± 3.0	70.4 ± 7.6	57.7 ± 0.2	76.4 ± 13.1
	Triflumizole	71.6 ± 7.0	74.1 ± 0.9	55.4 ± 3.3	65.4 ± 0.7	61.3 ± 6.7
	Fenpyroximate	75.0 ± 6.7	77.3 ± 5.1	81.9 ± 4.3	72.2 ± 2.2	75.3 ± 16.3

a) Each pesticide was spiked at 0.5 ppm to the samples. Recovery was an average of triplicate determinations. *b)* Bar means unmeasurable due to interfering peak.

widely applicable method and the limit of quantitation, the added level was determined to be 0.5 ppm.

Besides the above agricultural products, the proposed method was applied to spinach, tea, orange, eggplant, onion and carrot. On the chromatograms, there are many interfering peaks in the 15% acetone/*n*-hexane fraction except for eggplant. In the case of onion, it was impossible to analyze the pesticides due to interfering peaks resulting from the sample. However, these analyses are capable of improvement by introducing LC/MS. It is suggested that this method is sufficiently applicable to eggplant (shown in Fig. 3). In this figure, it was confirmed that the peak near 10 min was not thiabendazole by comparison with the UV spectrum of a standard using photodiode array detection.

In conclusions, in this study, a screening method using SFE and HPLC-photodiode array detection for the simultaneous determination of 27 pesticides, especially in vegetables and fruits, has been developed. This method permitted qualitative and quantitative determination by SFE for extraction and Sep-Pak® Florisil and Bond Elut® PSA for purification. The recoveries at a level of 0.5 ppm were 61.3–103.6%, except for thiabendazole in 5 agricultural products, imazalil in 3 agricultural products, clofentezine and diflubenzuron in 2 agricultural products and triflumizole in apple. Detection limits were 0.05–0.1 ng for the 27 pesticides. This proposed method could generally avoid interfering peaks as a result of separating the eluate from the cartridge into 3 fractions. The results clarified that this method is highly suitable for monitoring purposes. Hereafter, we are planning to introduce LC/MS in the interest of further confirmation of the peaks ascertained.

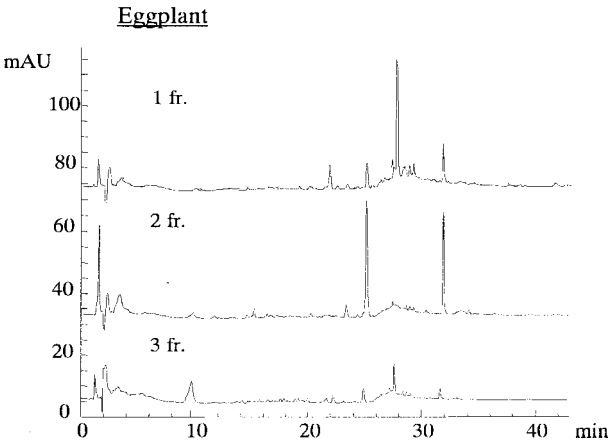

Fig. 3. HPLC Chromatograms of Eggplant Blank Sample
1 fr., 15% ether/*n*-hexane. 2 fr., 15% acetone/*n*-hexane. 3 fr., 50% acetone/*n*-hexane.

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