Multiresidue Analysis of Eight Acaricides in Fruits

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A multiresidue analytical method was developed for eight acaricides including benzoximate, clofentezine, fenazaquin, fenothiocarb, fenpyroximate, hexythiazox, pyridaben, and tebufenpyrad in four major fruits using high-performance liquid chromatography (HPLC). All the compounds were extracted with acetone from apple, pear, grape, and citrus samples. The extract was diluted with saline water, and *n*-hexane partition was followed to recover the acaricides. Florisil column chromatography was employed to further purify the sample extract. HPLC with ultraviolet absorption detection, using an octadecylsilyl column under the isocratic mobile phase of acetonitrile/water mixture, was successfully applied to separate and quantitate all the compounds in the purified extract. Recoveries of the eight acaricides from fortified samples ranged 86.4~97.0%. Relative standard deviations of the analytical method were all less than 10%. Detection limits of the method were in the range of 0.02~0.05 mg/kg. The proposed method was reproducible and sensitive enough to evaluate the terminal residue of the eight acaricides in the fruit harvest.

Key words: *Acaricide, multiresidue analysis, HPLC, apple, pear, grape, citrus.*

Acaricides have been routinely applied to control various mites in fruit trees. Taken in conjunction with the rapid life cycle of the mites, persistent compounds with ovicidal action have been usually used. Otherwise, application of less persistent acaricides has been timely scheduled to keep the number of mites below the threshold level during cultivation.[1,2] Persistence and use pattern of acaricides inevitably lead to potential occurrence of their terminal residues in the harvest. As fruits are consumed in large quantity and particularly in raw state, evaluation of the residue level of acaricides is of considerable importance to ensure safety of the harvest. For this purpose, development of a highly reliable method is, therefore, required to analyze the acaricide residues in fruits. Although several multiple and individual analytical methods have been published,[3-5] appropriate methods are not yet avaliable for many of compounds, especially for those recently introduced. The present paper describes a new multiresidue analytical method for eight acaricides recently introduced or for which the analytical method has not yet been established. The method was developed not only to achieve high reliability but to fulfill required sensitivity and readiness for analytical operation.

Materials and Methods

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Abbreviations: GLC, gas-liquid chromatography; HPLC, high-performance liquid chromatography; MRLs, maximum residue limits; RSD, relative standard deviation.

Instrumentation. A Hewlett Packard (HP, USA) Model 6890 gas chromatograph, equipped with packed inlet, nitrogen-phosphorus detector (NPD), a capillary column SPB-5, 30 m×0.53 mm i.d., 0.5 μm film (Supelco Inc., USA), and HP 3396 Series II integrator was used for preliminary analysis of acaricides. Operating conditions were as follows: column temperature, 210°C; detector

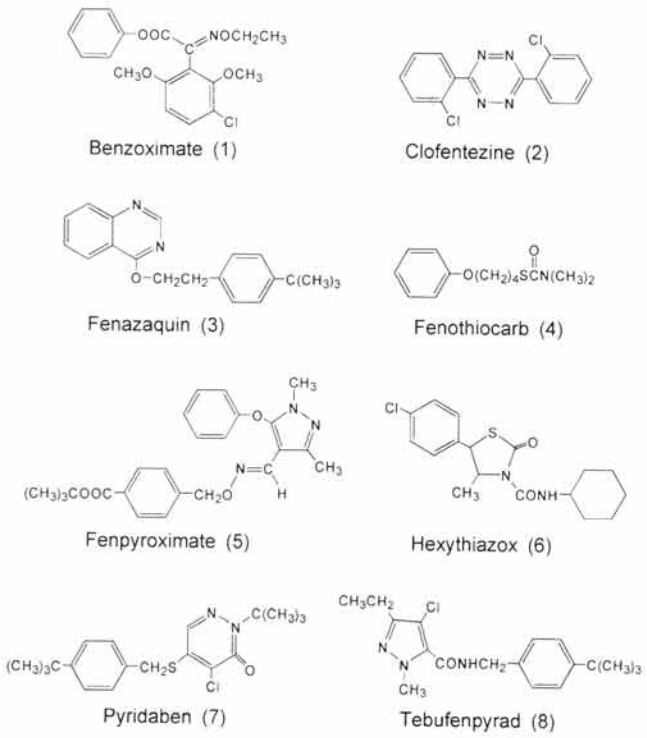

Fig. 1. Chemical structures of acaricides.

temperature, 280°C; inlet temperature, 250°C. Helium was used as a carrier and makeup gase at 20 and 10 ml/min, respectively. Fuel gases for NPD were hydrogen and air at 3.5 and 110 ml/min, respectively.

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

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, USA) was used as the analytical column. Operating parameters used for the determination of eight acaricide residues were as follows: mobile phase, acetonitrile/water (60/40, v/v), isocratic; flow rate, 1.0 ml/min; detection, UV absorption at 228 nm, 0.008 AUFS; sample size, 20 μl; chart speed, 0.5 cm/min.

Chemicals. Analytical standards of eight acaricides were kindly supplied by manufacturers. Stock standard solution of 500 mg/l 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 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) was purchased from Aldrich Chemical (USA) and activated at 130°C for more than 5 h prior to use. 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; grape, Campbell from Chilgok; citrus, Koongchun from Seokwipo, Jeju Province. Composite fruit samples were prepared in compliance with the instructions in Korean Test Guidelines for Pesticide Persistence.[6] Apple or pear fruit was minced after removing and discarding the hilum and ovary portions. Grape fruit was also minced after removing the stem. Whole citrus fruit including peel and flesh was chopped and blended. Each representative sample was stored frozen at -20°C until analysis.

Extraction and partition. A 20-g portion of each fruit sample was weighed into a 500-ml homogenizer cup, and 100 ml of acetone 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 ml of acetone, and the rinsate was combined with the previous filtrate. The filtrate was quantitatively transferred into a 1000-ml separatory funnel, and sequential addition of 100 ml of n-hexane, 50 ml of saturated NaCl, and 450 ml of distilled water was followed. After vigorous shaking for 1 min and standing until two layers clearly separated, the lower aqueous phase was discarded. The hexane phase was dried over 20 g of anhydrous sodium sulfate layer, collected in a 250-ml distilling flask, and evaporated just to dryness *in vacuo* at 40°C. The residue was dissolved in 10 ml of n-hexane and subjected to Florisil column chromatography.

Florisil column chromatography. A chromatographic column (11 mm i.d.×40 cm) was plugged with glass wool, 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 ml of *n*-hexane until the solvent level reached the top of the sodium sulfate layer. The hexane extract from the partition step was poured into the column and the column wall was rinsed twice with 2 ml portions of n-hexane. When the liquid drained to sodium sulfate layer, the column was eluted with 75 ml of dichloromethane, and the fraction was collected (E1 fraction). The column was then eluted with 50 ml of ethyl acetate/dichloromethane mixture (10/90, v/v), and the fraction was also collected (E2 fraction). Each eluate was concentrated just to dryness, and the residue was reconstituted with 5 ml of acetonitrile for HPLC determination.

Validation of the analytical method. Recovery experiments were run on control fruit samples to validate the analytical method proposed 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 all the analytes were known to be nonpolar with the log P value of 2.40~6.37,[7] a preliminary study was conducted to examine the use of GLC in determining the acaricide residue. Fenazaquin, fenothiocarb, pyridaben, and tebufenpyrad each showed a sharp peak with the retention times of 6.8, 2.3, 12.3, and 6.8 min, respectively. However, clofentezine appeared as double peaks with the retention times of 7.9 and 10.0 min suspecting thermal degradation during GLC. Benzoximate, fenpyroximate, and hexythiazox all failed to elute in spite of the variations of all operating parameters. As such, GLC was estimated to be inadequate as an analytical tool, and HPLC was chosen instead for the multiresidue determination of acaricides.

Considering that all the compounds were not readily oxidized nor reduced and had no fluorophore, UV absorption detector was the only choice among common HPLC detectors. UV absorption spectra were obtained from each 10 mg/l solution in acetonitrile/water mixture (60/40, v/v) to find out the optimum wavelength applicable to all the

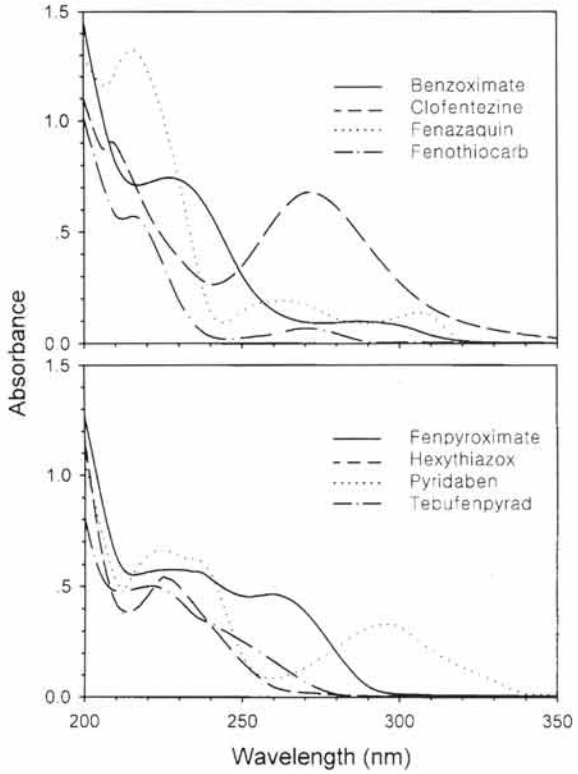

Fig. 2. Ultraviolet absorption spectra of eight acaricides.

analytes. As shown in Fig. 2, each compound has a specific chromophore with different absorption maxima and extinction coefficients. In residue analysis, analytes should be determined at the longest wavelength as possible with 10^4 $cm^{-1}M^{-1}$ order of extinction coefficient to minimize interferences by co-extractives as well as to get sensitivity of nanogram level.[3,8] Benzoximate, fenothiocarb, hexythiazox, and tebufenpyrad had unfortunately no strong absorption at long wavelength. Estimating the longest wavelength with minimum loss of sensitivity for each compound, optimum measuring wavelength was set at 228 nm, even though rather short, at which extinction coefficients of compounds were in the range of 7.4×10^3~2.7×10^4 $cm^{-1}M^{-1}$.

When reverse-phase HPLC using an octadecylsilyl column was employed, every analytes showed a sharp symmetrical peak under the mobile phase of acetonitrile/water mixture. In the range of 50 to 80% acetonitrile contents in water, their capacity factor increased almost twofold as acetonitrile contents decreased by 10%. This indicated that all the analytes existed as neutral form in the mobile phase of acetonitrile/water mixture, thus, there was no need for ion-suppression.[9] Chromatographic behavior of eight acaricides under isocratic and gradient conditions is summarized in Table 1. Under both conditions, every compound was completely separated within capacity factors of 23.7 and 32.8. While gradient condition gave a little higher resolution, isocratic condition was more preferable in view of lower baseline drift and time saving quality. A

Table 1. High-performance liquid chromatographic behavior of eight acaricides on octadecylsilyl column.

Compound	Capacity factor (k')		MDQ (ng)**
	I*	II	
Benzoximate	7.6	15.9	1
Clofentezine	6.4	13.4	2
Fenazaquin	21.1	30.7	2
Fenothiocarb	4.3	8.1	2
Fenpyroximate	16.7	27.3	3
Hexythiazox	13.4	23.6	2
Pyridaben	23.7	32.8	3
Tebufenpyrad	8.7	17.1	2

*Mobile phase I, acetonitrile/water (isocratic, 60/40, v/v), 1.0 ml/min; II, acetonitrile/water (linear gradient from 50/50 to 70/30 for 30 min), 1.0 ml/min.
**Minimum detectable quantity at 3% full scale deflection.

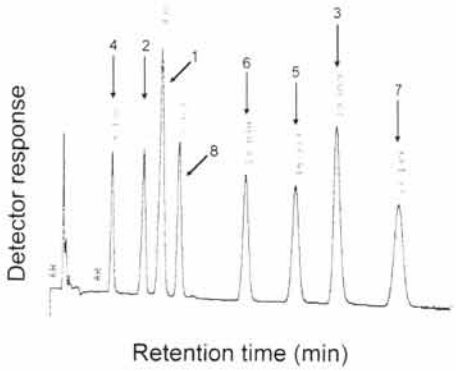

Fig. 3. HPLC chromatogram of the standard solution of eight acaricides. Each 20 ng of the compound was injected.

typical chromatogram of standard mixture is shown in Fig. 3. Minimum detectable quantity of each compound was calculated to be 1 to 3 ng on the basis of 3% full scale deflection. These values were estimated to be sufficiently sensitive for residue analysis. Since measuring at short wavelength frequently led to lower selectivity in the determination step, rigorous purification of 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

Table 2. Recovery of eight acaricides by different partition methods.

Compound	Recovery (%)		
	I*	II	III
Benzoximate	102	101	99
Clofentezine	102	102	95
Fenazaquin	98	99	100
Fenothiocarb	98	103	98
Fenpyroximate	98	98	97
Hexythiazox	102	100	99
Pyridaben	100	102	102
Tebufenpyrad	102	101	99

*I, single partition with 100 ml *n*-hexane; II, double partition with each 60 ml portions of *n*-hexane; III, double partition with each 50 ml portions of dichloromethane.

Table 3. Elution profile of eight acaricides on Florisil column.

Compound	Recovery (%)		
	I*	II	III
Benzoximate	NR**	103	108
Clofentezine	104	E**	E
Fenazaquin	NR	23	97
Fenothiocarb	NR	97	102
Fenpyroximate	NR	95	100
Hexythiazox	20	67	82
Pyridaben	NR	97	100
Tebufenpyrad	NR	97	104

*I, 75 ml of dichloromethane; II~III, pre-eluted with 75 ml of dichloromethane and followed by elution with 50 ml of ethyl acetate/dichloromethane mixture (5/95 or 10/90, v/v).
**NR, not recovered; E, eluted in dichloromethane fraction.

made at partition step to remove co-extractives from fruit extracts. From the acetone extract diluted with saline water,[3,8] all compounds were completely recovered into the organic phase using three partition methods as shown in Table 2. Single hexane partition was quite comparable with other methods even for rather polar compounds, benzoximate (log P=2.40), fenothiocarb (log P=3.28), and hexythiazox (log P=2.40). Thus, single hexane partition was employed as the partition method with merits of low co-extractives and operation readiness.

Adsorption chromatography was applied to further purify the extracts. Elution profiles of eight acaricides on Florisil column are listed in Table 3. Ethyl acetate/dichloromethane mixture among the three eluting solvents exhibited the most favorable pattern for the compounds. When the other mixtures, dichloromethane/acetonitrile/*n*-hexane and ethyl acetate/n-hexane commonly used for current multiresidue analysis,[3,8] were tried, polar combinations were required to elute all the compounds, and, as a result, co-extractives seriously interfering the analysis were eluted together. Though ethyl acetate and dichloromethane were known to have similar degree of solvent strength,[9] elution of acaricides on the Florisil column was largely affected by the ratio of two solvents, and thus solvent selectivity was expected to give more chance to separate the analyte from interferences. Using ethyl acetate/dichloromethane mixtures on Florisil column, acaricides were eluted in earlier order of clofentezine, hexythiazox, benzoximate, pyridaben, fenothiocarb, tebufenpyrad, fenpyroximate, and fenazaquin. As clofentezine was eluted much earlier than other acaricides, interferences might occur if all the eluates covering large range of polarity were combined. Therefore, the eluate was collected in two fractions, E1 for clofentezine and E2 for the rest of acaricides. E1 eluate could also play as a washing fraction for the other compounds. Hexythiazox was variably eluted between E1 and E2, however, separate determination of each fraction was needed for quantification.

Coupling with the proposed partition and adsorption chromatography, typical HPLC chromatograms of E1 and

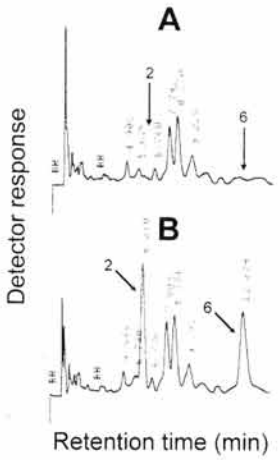

Fig. 4. Typical HPLC chromatograms of E1 fractions from apple samples. A, control; B, fortified with 0.25 mg/kg of each acaricides.

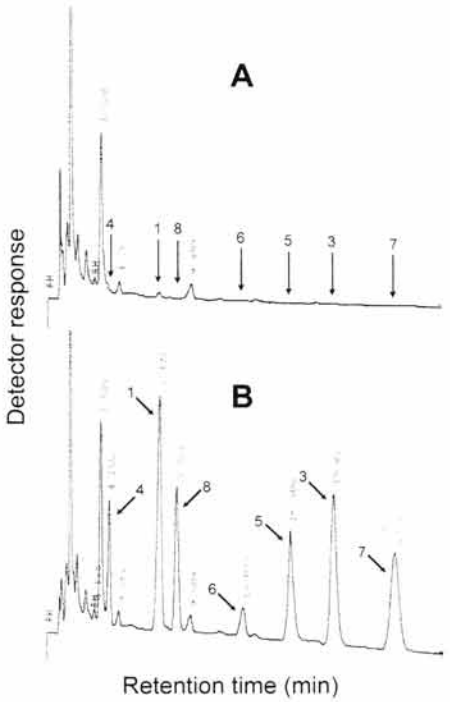

Fig. 5. Typical HPLC chromatograms of E2 fractions from apple samples. A, control; B, fortified with 0.25 mg/kg of each acaricides.

E2 fractions from control and fortified apple samples are shown in Figs. 4 and 5. The proposed method produced simliar clean HPLC chromatograms free of interference for the apple, pear, grape, and citrus samples. Hexythiazox tended to be more eluted in E1 fraction of sample extracts than in that of standard solution. This might be due to slight reduction of Florisil adsorptivity in the presence of co-extractives from the sample. Seven acaricides except hexythiazox showed an identical elution pattern as expected. Although some yellow pigments were also eluted in E2 fraction of citrus samples, no interference was found in chromatograms. Irrespective to sample types, as no late

Table 4. Recovery and detection limit of eight acaricides in fruits.

Compound	Fortification (mg/kg)	Recovery ± SD (%)*				Detection limit (mg/kg)
		Apple	Pear	Grape	Citrus	
Benzoximate	0.25	91.4 ± 2.2	92.3 ± 0.7	87.6 ± 8.6	88.7 ± 2.8	0.02
	1.0	92.4 ± 0.8	93.7 ± 0.7	85.1 ± 1.4	91.3 ± 2.9	
Clofentezine	0.25	93.1 ± 4.5	98.7 ± 0.5	98.9 ± 3.9	95.4 ± 3.7	0.03
	1.0	92.2 ± 3.1	94.7 ± 2.8	94.1 ± 4.2	95.4 ± 0.9	
Fenazaquin	0.25	85.5 ± 2.0	85.9 ± 1.1	92.6 ± 3.1	84.3 ± 1.3	0.03
	1.0	88.7 ± 3.8	92.2 ± 2.5	87.1 ± 4.7	89.9 ± 2.4	
Fenothiocarb	0.25	91.9 ± 1.2	85.9 ± 1.3	82.5 ± 3.6	87.3 ± 1.1	0.03
	1.0	88.6 ± 1.0	90.0 ± 0.4	93.9 ± 1.7	89.7 ± 1.8	
Fenpyroximate	0.25	93.5 ± 2.0	94.7 ± 1.7	95.3 ± 1.7	95.7 ± 1.1	0.04
	1.0	97.0 ± 0.8	97.8 ± 2.0	95.6 ± 2.8	97.6 ± 2.2	
Hexythiazox	0.25	99.6 ± 8.5	92.4 ± 2.5	87.6 ± 3.1	93.6 ± 2.0	0.05
	1.0	94.4 ± 0.7	93.5 ± 1.3	85.2 ± 1.8	94.3 ± 3.2	
Pyridaben	0.25	95.1 ± 4.1	90.5 ± 2.9	89.2 ± 4.5	91.1 ± 1.4	0.04
	1.0	95.6 ± 1.8	94.8 ± 0.3	88.0 ± 4.3	94.0 ± 3.2	
Tebufenpyrad	0.25	92.1 ± 1.8	95.2 ± 1.0	93.5 ± 2.4	94.4 ± 1.3	0.03
	1.0	95.1 ± 1.7	97.7 ± 1.0	95.5 ± 1.0	96.7 ± 1.2	

*Mean values of triplicate samples with standard deviations.

Table 5. Confirmation of eight acaricide residues by monitoring at different wavelength.

Compound	Wavelength (nm)	Ratio of relative peak area*				Confirmation limit (mg/kg)
		Apple	Pear	Grape	Citrus	
Benzoximate	240	0.99	1.00	0.99	1.00	0.03
Clofentezine	266	0.93	0.89	0.92	0.92	0.01
	296	1.01	0.98	0.97	0.99	0.03
Fenazaquin	240	0.99	1.02	1.04	1.01	0.1
	266	1.00	1.07	1.02	0.90	0.1
Fenothiocarb	266	1.18	0.79	0.85	0.92	0.1
Fenpyroximate	240	0.98	0.99	0.96	0.94	0.04
	266	0.95	0.94	0.88	0.92	0.04
Hexythiazox	240	0.90	1.08	1.16	1.11	0.1
Pyridaben	240	1.00	1.02	1.00	1.04	0.05
	296	1.02	1.05	1.08	1.10	0.08
Tebufenpyrad	240	1.03	1.00	0.99	1.03	0.04

*Peak area (PA) of an analyte found in sample extract / PA in standard solution at specified wavelength compared with the ratio at 228 nm.

eluting peaks were found even with 50 consecutive injections of the sample extract for a day, the proposed method would make continuous analysis possible.

Percent recoveries generated during the validation of analytical methods are presented in Table 4. Mean recoveries of eight acaricides were all over 80%, in the range of 86.4~97.0% by six replicates per sample type. No serious difference in the recovery affecting analytical accuracy was found according to 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 eight acaricide residues in four major fruit samples. However variably eluted it was between E1 and E2 fractions, hexythiazox could be analyzed by summing up each residue in two fractions. Overall recovery and RSD of hexythiazox were also acceptable to analytical criteria.[6] 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. Detection limits of the proposed method were 0.02~0.05 mg/kg sample based on 3% full scale deflection (S/N>10). These sensitivities were sufficiently high to detect 1/5~1/50 of MRLs (0.2~2 mg/kg) established for each acaricides in fruits.[10,11]

In this study, a simple method known as peak purity test was applied to confirm the acaricide residues in fruit samples.[12] According to Beer-Lambert Law, absorbances of a compound at two specified wavelengths are solely

dependent upon extinction coefficients while light path and its concentration are constant. Since peak area in a chromatogram is the sum of absorbances measured at unit time, difference in peak areas between two wavelengths is proportional to the difference in extinction coefficients if a peak purely represents an analyte. This is another characteristic inherent to a compound and thus can be used as a confirmatory tool. When determination of a sample is performed at two wavelengths under identical HPLC operating condition, deviations between two data will provide the degree of interference caused by co-extractives. Considering absorption characteristics of each compound, confirmatory wavelengths were set at 240, 266, and 296 nm. Calculated from chromatograms of standard mixture, ratios of difference between peak area and extinction coefficient showed 1.02 ± 0.05 as expected, the purity of each peak being near 100%. Applied to fruit samples fortified with 0.25 mg/kg of each acaricides, the ratios of most compounds ranged 0.90~1.10 showing more than 90% peak purity except more deviations found in fenothiocarb and hexythiazox as shown in Table 5. As a result, most acaricide residues could be confirmed by the peak purity test at limits of 0.01~0.1 mg/kg. Even though this method is qualitatively inferior to using more sophisticated instruments such as liquid chromatography/mass spectrometry, it has merits of simplicity and *in situ* confirmatory operation.

The proposed method satisfies criteria of the analytical method for pesticide residues, with more than 70% recovery, less than 10% RSD, and more sensitive than 0.05 mg/kg detection limit, on Test Guidelines for Pesticide Persistence notified by Rural Development Administration.[6] Analytical procedures do not require any special apparatus or instruments but consist of currently available techniques familiar to the residue analyst as well. The method is so simple to operate that one experienced person can analyze 12 samples per day. Therefore, authors suggest that the proposed method could be sufficiently applied to the routine analysis for evaluation of the eight acaricide residues in fruit samples.

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References

1. Hassall, K. A. (1990) Acaricides. In *The Biochemistry and Uses of Pesticides* (2nd ed.) pp. 224-228, MacMillan Press, London, UK.
2. Agricultural Chemicals Industrial Association (ACIA) (1998) *The Pesticide Use Guidelines*, ACIA, Korea.
3. McMahon, B. M. and Wagner, R. F. (1997) Multiclass multiresidue methods and PESTDATA. In *Pesticide Analytical Manual* Volume I (3rd ed.) Section 301-303, Food and Drug Administration, USA.
4. Cabras, P., Angioni, A., Garau, V. L., Melis, M., Pirisi, F. M., Cabitza, F., Dedola, F. and Navickiene, S. (1998) Determination of buprofezin, pyridaben, and tebufenpyrad residues by gas chromatography-mass-selective detection in clementine citrus. *J. Agric. Food Chem.* **46**, 4255-4259.
5. Cabras, P., M. Melis, C. Tuberoso, D. Falqui, and M. Pala (1992) High-performance liquid chromatographic determination of fenbutatin oxide and its persistence in peaches and nectarines. *J. Agric. Food Chem.* **40**, 901-903.
6. Rural Development Administration (1997) Test guidelines for pesticide persistence. In *Criteria and Guidelines for Pesticide Registration*, Annex 7, Korea.
7. Tomlin, C. (1997) *The Pesticide Manual* (11th ed.) British Crop Protection Council, Surrey, UK.
8. AOAC (1995) Pesticide and industrial chemical residues. In *Official Method of Analysis* (16th ed.) pp. 1-12, AOAC International, Arlington, VA, USA.
9. Snyder, L. R. and Kirkland, J. J. (1979) In *Introduction to Modern Liquid Chromatography* (2nd ed.) pp. 281-288 and 365-374, John Wiley, New York, USA.
10. Ministry of Health and Welfare (1996) In *Maximum Residue Limits for Pesticides in Agricultural Commodities*, Korea.
11. Codex Committee on Pesticide Residues (1999) In *List of Maximum Residue Limits for Pesticides in Food and Animal Feeds*, Codex Alimentarius Commission, FAO/WHO, Rome, Italy.
12. Lawrence, J. F. (1981) Confirmatory tests. In *Pesticide Analysis*, Das, K. G. (ed.) pp. 425-452, Marcel Dekker, NY, USA.