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Reduction of Hazardous Organic Solvent in Sample Preparation for Hydrophilic Pesticide Residues in Agricultural Products with Conventional Liquid Chromatography

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ABSTRACT: An original extraction method using water as an extractant has been established for environmentally friendly sample preparation procedures for hydrophilic pesticides (acetamiprid, clothianidin, dinotefuran, flonicamid, imidacloprid, methomyl, pymetrozine, thiacloprid, and thiamethoxam) in agricultural samples with conventional HPLC. Water-based extraction and cleanup with two solid-phase extraction cartridges can recover target hydrophilic pesticides quantitatively. The matrix effects of tested samples on the proposed method developed herein were negligibly small. Under the optimized conditions, the recoveries of almost all tested pesticides were 70−120% with satisfactory precision (%CV < 20%). The analytical data are in good accordance with Japanese or European Union guidelines for pesticide residue analysis. The reduction rate of hazardous organic solvents used for the proposed method and by reducing the sample size for extraction was about 70% compared with the Japanese authorized reference method used in this work. The results demonstrate the feasibility of the proposed sample preparation procedures for hydrophilic pesticides.

KEYWORDS: *water extraction, hydrophilic pesticides, conventional HPLC, residue analysis, matrix effect, vegetables*

INTRODUCTION

Pesticide residue analysis is a tool used to ensure food safety by confirming that produce is in compliance with maximum residue limits (MRLs). Therefore, it has been recognized worldwide that important developments for pesticide residue analyses based on mainly chromatographic techniques combined with conventional detectors such UV, diode array (DAD), and element-selective detectors have taken place to date. In the past 20 years, GC-MS, LC-MS, and/or LC-MS/MS have become the predominant methods to analyze pesticide residues in complex food samples because they can provide both qualitative and quantitative information simultaneously.[1−3] General methods for pesticide residue analysis comprise (1) sample preparations consisting of extraction and cleanup procedures and (2) chromatographic determination. Although sample preparation procedures are necessary to analyze pesticide residues accurately in samples, they are rate-limiting in the analysis. In 2003, a very rapid and easy sample preparation procedure well-known as the quick, easy, cheap, effective, rugged, and safe (QuEChERS) method was developed.[4] This method has quickly become a preferred method by laboratories for pesticide residue analysis and was recently adopted as official multiresidue methods, for example, AOAC Official Method 2007.01[5] or European Committee for Standardization (CEN) Standard Method EN 15662 (foods of plant origin − determination of pesticide residues using GC-MS and/or LC-MS/MS following acetonitrile extraction/partitioning and cleanup by dispersive SPE − QuEChERS-method). Precisely, the sample preparation procedure is adequate for highly sensitive analytical instruments such as GC-MS[4−8] and/or LC-MS/MS.[5−10] The reasons are as follows: (1) the method does not need any troublesome concentration procedure that can increase analytical sensitivity, and (2) because only a part of the sample extract is cleaned up to avoid matrix effects as much as possible, concentrations of pesticides in a final solution prepared for determination are lower than those in samples rightly. It may be said that such procedures have superior sensitivity. On the other hand, in such context, it cannot be denied that pesticide residue analytical methods using chromatographic techniques combined with conventional UV, DAD, and/or element-selective detectors are becoming outdated. However, in pesticide residue analysis to secure food safety by (1) testing of "foods" on the market and (2) testing of "agricultural products" before shipment, it is questionable to completely depend only on GC-MS and LC-MS/MS for pesticide residue analysis. The following points can be given as reasons. In the former situation, the history of pesticides that have been used is often unknown. Therefore, multiresidue analysis using GC-MS or LC-MS/MS is suitable, in which as many pesticides as possible can be tested and unknown ingredients can be identified. On the other hand, in the latter situation, the subject pesticide can be selected on the basis of the history of use. Furthermore, only a few pesticides may be used during the cultivation period of a crop and, therefore, a multiresidue analytical method that can determine simultaneously several hundred kinds of pesticides is too excessive in the latter situation, and even chromatographic methods with conventional detectors should be able to support enough. Above all, because conventional HPLC-UV and/or HPLC-DAD methods are clearly inferior to LC-MS/MS at some points including analytical sensitivity and selectivity, thorough sample preparation is necesssary, requiring a large amount of hazardous organic solvents.

First of all, nine hydrophilic pesticides most commonly used in crop protection including neonicotinoid insecticides, which have attracted attention due to the possibility of the ecological effect on the bee nowadays (Table 1), were selected, and the study was conducted by advocating the following aim: (1) development of an original environmentally friendly sample preparation procedure

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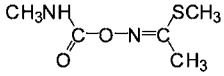
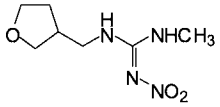
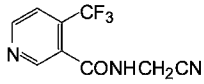
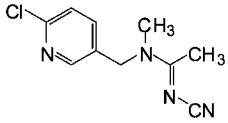
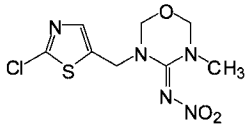
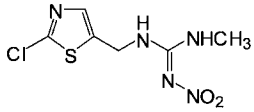
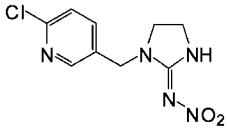
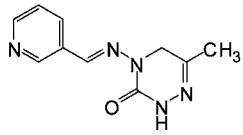
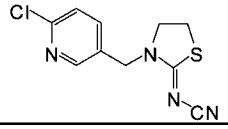
Table 1. Chemical Structures and Physicochemical Properties of the Hydrophilic Pesticides Selected in the Current Work[a]

pesticide		water solubility (g/L)	log *P*
methomyl		57.9 (25°C)	0.093
dinotefuran		54.3 ± 1.3 (20°C)	-0.644 (pH 7)
flonicamid		5.2 (20°C)	0.30
acetamiprid		4.25 (25°C)	0.80 (25°C)
thiamethoxam		4.1 (25°C)	-0.13 (25°C)
clothianidin		0.340 (pH 10, 20°C)	0.7 (25°C)
imidacloprid		0.61 (20°C)	0.57 (21°C)
pymetrozine		0.29 (pH 6.5, 25°C)	-0.18
thiacloprid		0.185 (20°C)	0.73 (pH 7)

[a]Each value was referred to *The Pesticide Manual*.[22]

comprising water extraction of the smallest feasible sample scale, and the following cleanup with two types of commercially available solid-phase extraction (SPE) cartridges; and (2) validation in development of a new HPLC-DAD pesticide residue analytical method that is versatile even today by applying the established sample preparation procedures to some complicated agricultural samples.

MATERIALS AND METHODS

Chemicals and Reagents. Certified standards of pesticides were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan), Hayashi Pure Chemical Ind. Ltd. (Osaka, Japan), and Kanto Chemical Co. Inc. (Tokyo, Japan). Pesticide analysis grade and HPLC grade organic solvents were purchased from Wako Pure Chemical Industries Ltd. Water used for HPLC was prepared directly in the laboratory using a Milli-Q water purification system (Millipore Corp., Bedford, MA, USA). Cartridges used for SPE were Oasis HLB (225 mg; Waters, Milford, MA, USA) and Envi-Carb/LC-NH_2 (500 mg + 500 mg/6 mL; Supelco, Bellefonte, PA, USA).

Standard and Working Solutions. Individual standard stock solutions (1000 μg/mL) were prepared by dissolving 10 mg of each analyte in 10 mL of HPLC grade acetonitrile (MeCN). A working standard multicomponent solution (10 μg/mL) was prepared daily, diluting each primary stock solution with mobile phase (MeCN/water (25:75, v/v)). It was used for spiking agricultural matrices and for preparing calibration standards. The stock solutions were stored under refrigerated conditions (4 °C) and were protected from light. Under these conditions, the stock solutions were stable for at least 6 months.

Samples. Tomatoes, green peppers, and spinaches were used in this work. For the preparation of real samples, agricultural samples of three kinds were grown in a plastic greenhouse on arable land of the National Institute for Agro-Environmental Sciences. Each sample at the harvesting stage was sprayed with a mixture of some pesticide formulations diluted according to the manufacturer's labels using a handy sprayer. Then vegetables were harvested at 1, 3, 7, and 14 days after spraying. After harvesting, the residue samples were placed in a food cutter and chopped until homogeneous. The chopped samples were placed in 500 mL glass jars and frozen at −20 °C until extraction.

Extraction Experiments. In all cases the samples were spiked with a mixed solution of the test analytes in mobile phase such that the concentrations in the sample were 0.1, 0.5, and 1.0 mg/kg. The spiked samples were allowed to stand for 30 min before extraction.[11]

Proposed Water Extraction Method. Five grams of sample was weighed into a 50 mL of conical centrifuge tube made of polypropylene with a screw cap. Water (15 mL) was added to the sample and extracted for 3 min using a high-speed homogenizer (Polytron PT2100; Kinematica, Lucerne, Switzerland). The mixture was centrifuged for 10 min at 16200*g* (high-speed refrigerated centrifuge, himac CR22G, Hitachi Koki Co., Ltd., Tokyo, Japan). Then the supernatant was filtered on a Büchner funnel with suction. The solid residue in the tube was extracted again with 10 mL of water. Then the mixture was centrifuged

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Table 2. Analytical Data of Pesticides Selected in the Current Work Using HPLC-DAD

pesticide	detection wavelength (nm)	equation of calibration curve	linearity (μg/mL)	r	LOD (ng/mL)
acetamiprid	246	$y = 81.3x + 1.37$	0.01−2	1.0000	5
clothianidin	270	$y = 66.8x + 2.74$	0.01−2	1.0000	5
dinotefuran	270	$y = 61.7x + 1.32$	0.01−2	0.9998	5
flonicamid	270	$y = 10.4x - 0.04$	0.03−2	0.9999	15
imidacloprid	270	$y = 90.7x + 2.35$	0.01−2	1.0000	5
methomyl	230	$y = 42.5x + 1.31$	0.02−2	1.0000	10
pymetrozine	298	$y = 67.2x + 2.41$	0.04−2	0.9999	20
thiacloprid	246	$y = 69.5x + 2.25$	0.01−2	0.9999	5
thiamethoxam	254	$y = 35.2x + 1.16$	0.02−2	0.9999	10

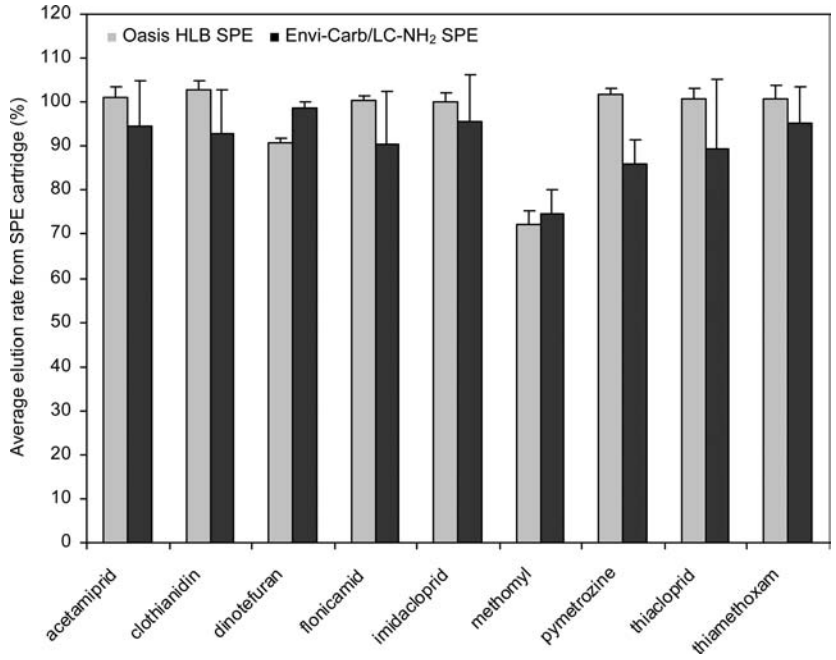

Figure 1. Elution profiles of hydrophilic pesticides from two SPE cartridges used in this study (n = 3 replicates).

and filtered. The water extract was percolated through an Oasis HLB SPE cartridge preconditioned with 6 mL of methanol (MeOH) and 6 mL of ultrapure water. The cartridge was rinsed with 5 mL of ultrapure water and vacuum-dried for 10 min to remove excess water. Finally, the retained pesticides were eluted with 10 mL of MeOH, and the eluate was concentrated to a final volume of about 1 mL under reduced pressure. The residue was reconstituted in 2 mL of MeCN/toluene (3:1, v/v) and the solution was applied to an Envi-Carb/LC-NH_2 cartridge that had been preconditioned with 10 mL of MeCN/toluene (3:1, v/v). The retained pesticides were eluted with 20 mL of MeCN/toluene (3:1, v/v). The eluate was concentrated under reduced pressure and evaporated under a gentle nitrogen stream at 50 °C. The residue was reconstituted in 1 mL of mobile phase and syringe-filtered using a 0.45 μm PTFE filter (Millipore Corp., Billerica, MA, USA) into an autosampler vial.

Japanese Authorized Reference Multiresidue Pesticide Analytical Method. To verify the proposed sample preparation procedures, the authorized official method[12] was selected as a reference method in this work.

To 20 g of sample was added 50 mL of MeCN, and the mixture was extracted for 3 min with a high-speed homogenizer. The mixture was filtered with suction, and the solid residue on the funnel was extracted again with 20 mL of MeCN. Both extracts were accurately made up to 100 mL with MeCN in a volumetric flask, and then 20 mL aliquots of the extract, equivalent to 4 g of sample, was mixed with 10 g of sodium chloride and 20 mL of 0.5 M phosphate buffer (pH 7.0). The mixture was vigorously shaken for 5 min and allowed to stand for about 10 min. After the aqueous phase was discarded, the MeCN phase was anhydrated, filered, and then concentrated. After the residue was treated similarly as described above, the eluate from an Envi-Carb/LC-NH_2 cartridge was concentrated. Then the residue was reconstituted in 10 mL of acetone. The solution was concentrated. Then to the residue was added 5 mL of acetone. The acetone was evaporated under a gentle nitrogen stream as described above. The residue was reconstituted in 1 mL of mobile phase and syringe-filtered using a 0.45 μm PTFE filter into an autosampler vial.

HPLC Analysis. The HPLC system consisted of an Agilent 1100 HPLC equipped with a quaternary pump, an autosampler, a column oven, and a DAD. The detection wavelengths were 230, 246, 254, 270, and 298 nm. A reversed phase column (250 mm × 4.6 mm, 5 μm particle size; SunFire C18, Waters, Milford, MA, USA) fitted with a guard column (20 mm × 4.6 mm, 5 μm particle size; Waters) was used. The column oven temperature was maintained at 40 °C. A volume of 20 μL was injected. The mobile phase was MeCN/water (25:75, v/v) at flow rate of 0.85 mL/min.

Fundamental Analytical Performance of HPLC-DAD Method. The external standard procedure was used and calibration curves were constructed by plotting concentration against peak area using several concentration levels and following linear regression analysis. The linearity range was checked from 0.005 to 2 μg/mL. Excellent linearity and coefficient of regression (r) were achieved for the nine pesticides as given in Table 2. The limit of detection (LOD) for each pesticide was determined as the lowest concentration of each pesticide that gave a signal-to-noise ratio of 3.[13] This was as low as 5 ng/mL for acetamiprid, clothianidin, dinotefuran, imidacloprid, and thiacloprid, approximately 10 ng/mL for methomyl and thiamethoxam, 15 ng/mL for flonicamid, and as high as 20 ng/mL for pymetrozine, respectively (Table 2).

Elution Profiles of SPE. *Oasis HLB SPE Cartridge.* As an alternative sample, 5 μg of each pesticide dissolved with 5 mL of water

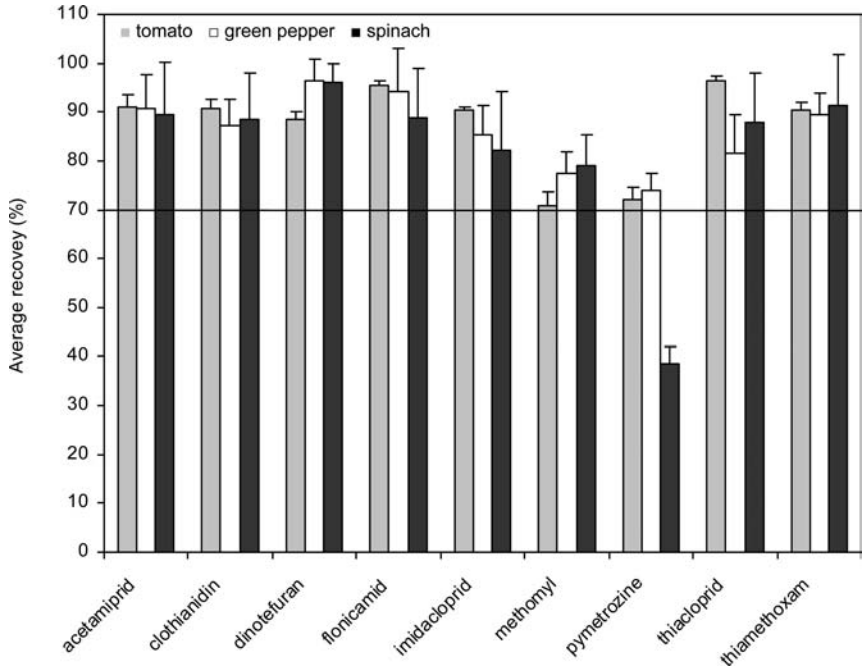

Figure 2. Average recoveries of hydrophilic pesticides in tomato, green pepper, and spinach samples spiked at the level of 1 mg/kg using water extraction and SPE cleanup (n = 3 replicates).

Table 3. Average Recoveries of Hydrophilic Pesticides from Artificially Spiked Tomato, Green Pepper, and Spinach Samples Using the Proposed Sample Preparation Based on Water Extraction and SPE Cleanup

	average recovery[a] (%) (%CV) (n = 5 replicates)								
spike level (mg/kg)	acetamiprid	clothianidin	dinotefuran	flonicamid	imidacloprid	methomyl	pymetrozine	thiacloprid	thiamethoxam
				Tomato					
0.1	99 (10)	93 (6)	94 (5)	110 (10)	93 (5)	74 (10)	77 (4)	94 (1)	85 (8)
0.5	94 (3)	95 (3)	94 (2)	98 (5)	91 (3)	85 (10)	75 (3)	93 (4)	89 (2)
1.0	91 (3)	92 (3)	90 (3)	95 (5)	91 (3)	79 (4)	73 (5)	93 (4)	91 (2)
				Green Pepper					
0.1	93 (6)	83 (4)	87 (14)	101 (14)	91 (11)	89 (6)	82 (6)	93 (6)	99 (3)
0.5	87 (9)	87 (8)	89 (5)	103 (13)	102 (5)	88 (6)	76 (7)	82 (9)	89 (8)
1.0	88 (11)	82 (10)	91 (5)	80 (8)	82 (8)	74 (8)	77 (6)	76 (13)	86 (11)
				Spinach					
0.1	87 (11)	87 (9)	90 (2)	90 (14)	89 (14)	94 (10)	**47** (6)	85 (8)	95 (9)
0.5	104 (3)	90 (3)	92 (8)	92 (4)	93 (4)	89 (9)	**44** (8)	87 (2)	98 (10)
1.0	87 (8)	84 (8)	84 (5)	88 (13)	85 (9)	71 (6)	**32** (9)	82 (10)	88 (7)

[a]Bold figures show recoveries outside 70−120%.

was used. The spiked water sample was diluted with 25 mL of water. Then the diluted water sample was applied to the cartridge.

Envi-Carb/LC-NH$_2$ SPE Cartridge. Two milliliters of MeCN/toluene (3:1, v/v) dissolved with 5 μg of each pesticide as an alternative sample was applied to the cartridge.

The preconditioning and washing of the cartridge and the elution of each pesticide for both SPE cartridges were performed according to the aforesaid method.

Evaluation of Matrix Effects. Matrix effects, expressed as a signal from the pesticide in matrix compared to the signal in pure solvent (mobile phase), were tested in all matrices. To an aliquot of blank extract in mobile phase was added a mixture of pesticides, producing a final concentration of 0.1 mg/kg of each agricultural sample. The effect was evaluated according to a method described in an earlier paper.[9]

$$\text{matrix effect (\%)} = [(\text{peak area of standards in cleaned up extract}/\text{peak area of standards in pure solvent}) - 1] \times 100$$

RESULTS AND DISCUSSION

Applicability of Water Extraction. This study was conducted to reduce organic solvent consumption in all sample preparation procedures (1) by using water as an extractant and two types of SPE cartridges (Oasis HLB SPE and Envi-Carb/LC-NH$_2$ SPE) and (2) by reducing the sample scale. Initially, the elution profiles of two SPE cartridges of each pesticide were studied. In Figure 1, although the elution rate of methomyl from both cartridges was somewhat low (72−75%), other pesticides were recovered from them quantitatively. It might therefore be inferred that the SPE processes have practically no contribution to the low recovery rate in the whole procedures. The suitability of procedures consisting of water extraction and SPE cleanup was verified using artificially spiked samples at 1 mg/kg of each pesticide, and the applicability was evaluated according to the authorized criteria in which recovery rates were considered to be acceptable between 70 and 120% and

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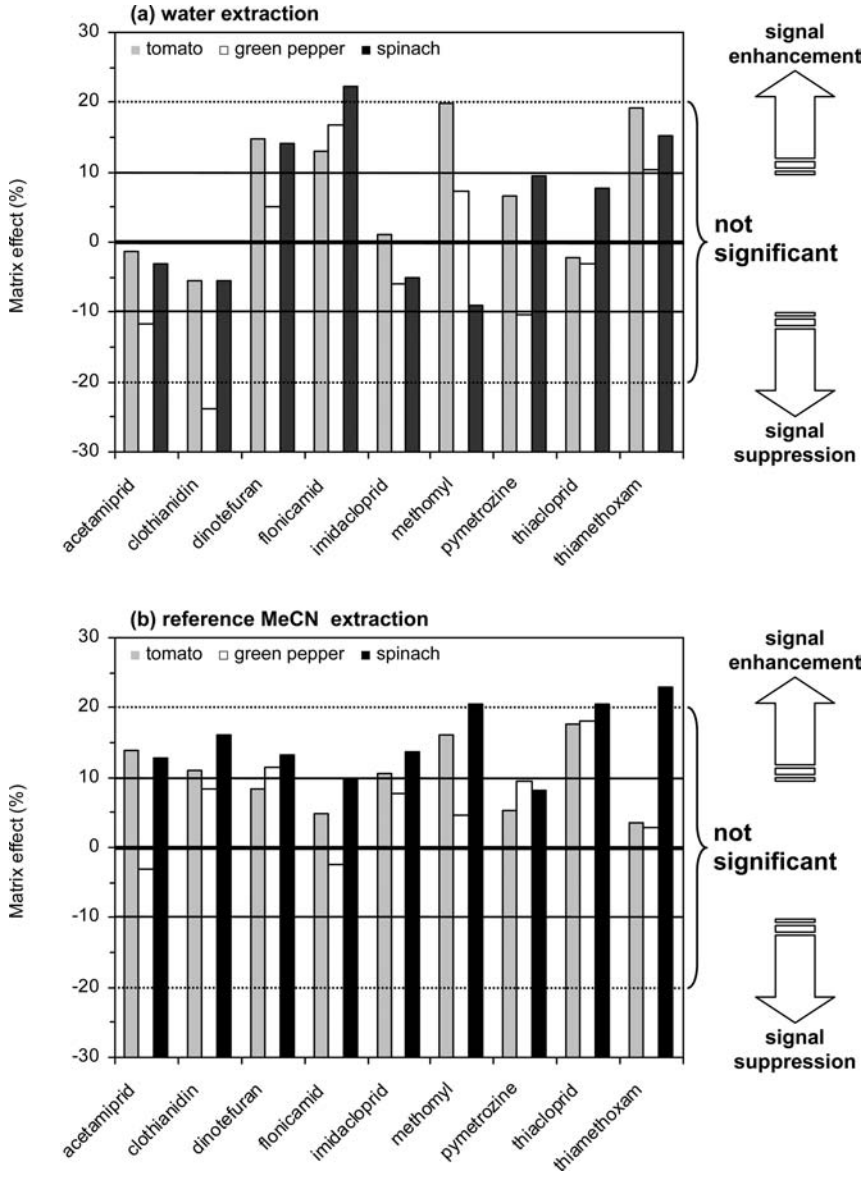

Figure 3. Matrix effects in the proposed sample preparation procedures based on water extraction and the authorized reference method.

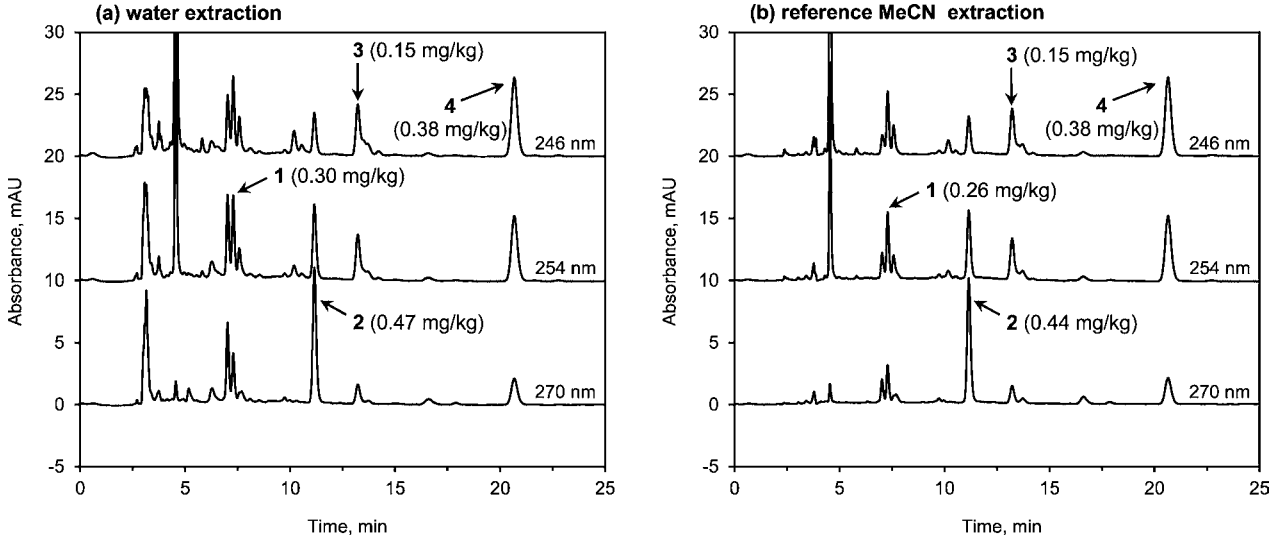

Figure 4. Representative HPLC chromatograms of real green pepper samples harvested at 7 days after spraying. Peaks: 1, thiamethoxam; 2, imidacloprid; 3, acetamiprid; 4, thiacloprid.

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repeatability (%CV) of ≤20%.[14,15] From Figure 2, it is apparent that the studied sample preparation procedure is applicable to hydrophilic pesticides except pymetrozine in spinach sample. Because >85% of the pesticide was eluted from two SPE cartridges, the inadequate extraction efficiency of water considerably contributes to the loss during the sample preparation procedures in spinach sample only. From these perspectives, we can ascertain the suitability of the proposed sample preparation procedure for hydrophilic pesticides using environmentally friendly water as an extractant.

Matrix Effect. The influence that a matrix effect gives in terms of the reliability of the analytical results may be immeasurable. Therefore, an important issue in the method development of quantitative pesticide residue analysis using chromatographic techniques is the possible occurrence of matrix effects. Figure 3 shows the degree of matrix effects of pesticides selected in the current study in the proposed sample preparation procedures and the authorized reference method of every agricultural sample. Most combinations of tested pesticides and agricultural samples showed no considerable signal suppression or enhancement (matrix effect within ±20%), which it is likely to be an obstacle to accurate determination according to the criteria explained by Mol et al.[16] The degree to which each pesticide catches the matrix effect seems to vary slightly according to agricultural sample. In the proposed sample preparation procedures, the combinations of pesticides that were analyzed without receiving substantial matrix effects (matrix effect within ±10%, Figure 3) and tested samples were nearly equal with that of the authorized reference method; that is to say, it might be inferred that the cleanup efficiencies of both sample preparation procedures were equal.

The representative HPLC chromatograms of real-world green pepper samples treated with four kinds of neonicotinoid insecticides and extracted with water or MeCN are shown in Figure 4. By both sample preparation procedures, the peaks of thiamethoxam and acetamiprid and matrix components were not completely separated. Nevertheless, the trouble attributable to the matrix effect does not arise in their determinations because the matrix effects of these pesticides in green pepper samples were slight (between nonsignificant level (±10%) and minor level (±20%), Figure 3).

Validation. The accuracy of the established sample preparation procedures was estimated using recovery experiments conducted at three concentration levels, 0.1, 0.5, and 1.0 mg/kg (Table 3). For all matrices, the results obtained for most of the analytes were satisfactory, with recovery rates of 70−120% and %CV values <20%.[14,15] Only pymetrozine in tested pesticides presented a lower recovery rate in spinach samples at all spiked levels. This pesticide is well-known as a problematic pesticide for development of simultaneous multiresidue methods described in several papers,[6−8,10,17] showing an especially low recovery rate in acidic sample matrices such as citrus samples.[6,17] The poor recovery rate for some analytes such as pymetrozine also underscores the difficulties of developing an original multiresidue method for the determination of a number of pesticides with widely diverse physicochemical properties.

For the evaluation of analytical methods under development, it has been acknowledged that recoveries of field-incurred analytes from environmental matrices are far more realistic than recoveries based on laboratory spiking into the sample matrices.[18] To accomplish the objective of the work, which is reduction of organic solvent consumption during sample preparation procedures, miniaturization of sample sizes for extraction can be one effective means.[4,19] Typically, 20−100 g for extraction is subsampled from a

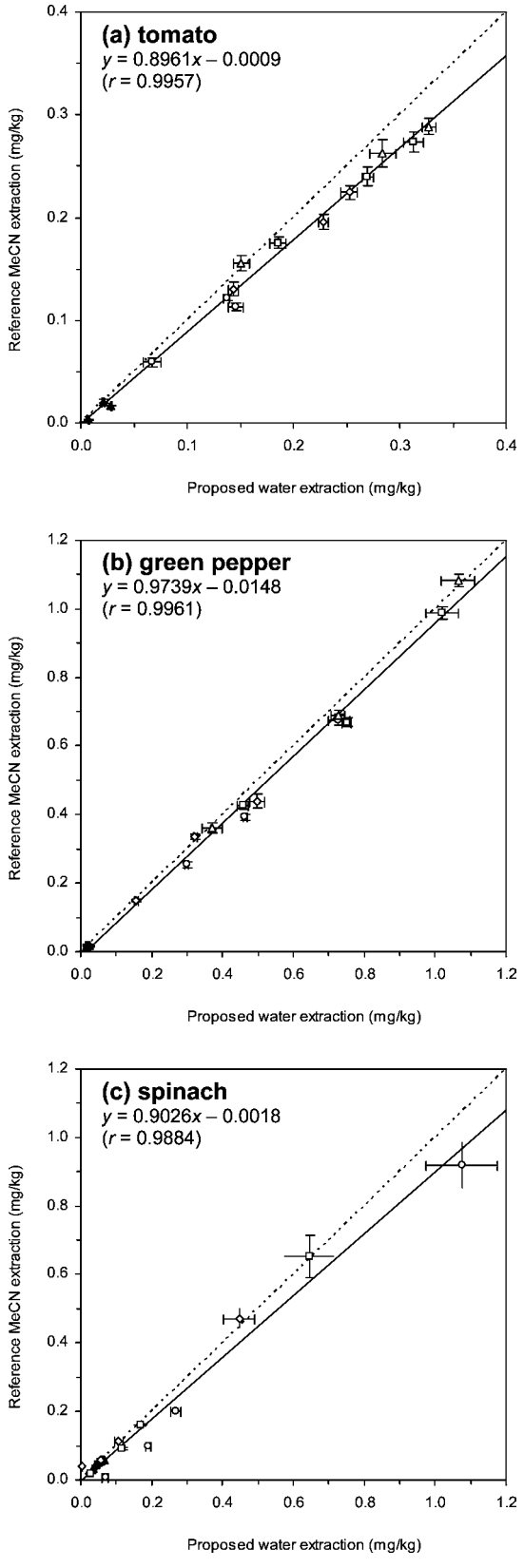

Figure 5. Validity of the proposed sample preparation procedures related to the accuracy and the sample size for extraction by comparison with the authorized reference method: (◇) acetamiprid; (▲) clothianidin; (□) imidacloprid; (△) thiacloprid; (○) thiamethoxam. Each point is the average of individual quintuplicate determinations.

larger quantity of homogenized produce to ensure reproducible results. Therefore, an extraction stage in many multiresidue methods uses about 100−200 mL of organic solvent as extractant. A much smaller sample size in our work, with 5 g of well-homogenized sample extracted, was incorporated into the proposed sample preparation procedures. Therefore, the validity of small sample size for extraction was assessed herein using real agricultural samples. The analytical results obtained using the proposed method were compared with those obtained using an authorized official analytical method by which the customary sample size (20 g) was used for extraction.[12] As shown in Figure 5, the detected concentrations of pesticides in samples prepared according to the proposed method were equivalent with those detected using an official method ($r > 0.98$). These results strongly indicate that the reduction in sample size for extraction does not affect the reproducibility of the sample preparation procedure. Moreover, they suggest the possibility of considerably reducing organic solvent consumption by using water as an extractant and by reducing the sample size. The proposed method, consuming about 50 mL of organic solvents per sample, accomplished up to 70% of organic solvent consumption as the reduction rate in comparison with the authorized method used in this work (about 150 mL per sample)[12] and some previously developed multiresidue methods.[20,21]

Our results suggest that the proposed organic solvent saving sample preparation procedures based on water extraction of downsized samples allow quantitative recovery of hydrophilic pesticides and, furthermore, that the proposed method is applicable as an analytical method that could satisfy the requirements of, for example, quickness or simplicity in screening method development for agricultural products before shipment by combination with SPE methods. It may be concluded that the conventional HPLC-DAD method can conjugate as a quantitative screening method from the viewpoint of the accuracy and sensitivity of the analytical results of the examined spiked and real agricultural samples.

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Notes

The authors declare no competing financial interest.

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