JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Determination of Pyrethroid Residues in Agricultural Products by an Enzyme-Linked Immunosorbent Assay

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To determine cypermethrin and permethrin in agricultural products, a competitive enzyme-linked immunosorbent assay (ELISA) method was employed. The matrix interferences were minimized by direct dilution of the extracts. No further cleanup was needed. A minimum matrix effect with a 1:10 dilution of white wine for cypermethrin and a 1:200 dilution of red and white wines, fruits, and vegetables for permethrin was found when phosphate-buffered saline containing 40% methanol was employed as the diluent. Good recoveries of spiked levels were observed. The mean percentage recoveries of cypermethrin spiked in white wine and permethrin spiked in red and white wines were 99.7, 74, and 78%, respectively. The mean percentage recoveries of permethrin spiked in apple, banana, cucumber, lettuce, onion, and peach were 99.2, 105, 70.2, 97.5, 94.4, and 89.4%, respectively. Validation of the ELISA method with permethrin-spiked lettuce and peach was carried out using gas chromatography with mass spectrometry, resulting in a good recovery and correlation.

KEYWORDS: Pyrethroid; cypermethrin; permethrin; immunoassay; fruits; vegetables; wine; environmental monitoring

INTRODUCTION

In modern agriculture, the pyrethroid class pesticides have been widely used because mammalian toxicity is low. The acute oral LD_{50} values for cypermethrin and permethrin in mice are 138 and 540−2690 mg/kg, respectively (*1*). However, adverse health effects of pyrethroids have been reported including suppression of the immune system after exposure (*2*), lymph node and splenic damage, and carcinogenesis (*3*). Thus, pyrethroid residues on foods are a public health concern.

Traditional analytical methods for cypermethrin and permethrin, mainly GC or high-performance liquid chromatography (HPLC) (*4*−*11*), work well but require costly extraction and cleanup procedures, and the number of samples that can be processed daily is small. In addition, the amounts of chemicals and toxic solvents used are of environmental concern.

Immunoassay has several advantages as compared to the conventional techniques. It has the ability to handle large numbers of samples simultaneously, sample workup is relatively simple, the assay is applicable to many types of analytes and matrices, and there are cost benefits secured from less sample preparation and higher throughput. For this reason, this laboratory has developed immunoassay methods for the determination of cypermethrin (*12*) and permethrin (*7*).

It is known that a major route of exposure to pesticides and other environmental contaminants is through the diet (*13*−*16*). We applied these assays to food and beverage samples on which pyrethroids are applied during production. The present work evaluated the suitability of these immunoassay methods for the analysis of cypermethrin and permethrin residues in agricultural products.

MATERIALS AND METHODS

Chemicals, Immunoreagents, and Solutions. An analytical standard of cypermethrin (mixture of isomers, PS-1068) was purchased from Chem Service (West Chester, PA), and permethrin (mixture of cis- and trans-isomers, Pestanal quality, 45614) was provided by Riedel-de Haën (Seelze, Germany). Stock solutions of the pesticide were prepared in MeOH at 1000 mg/L and stored at −20 °C. GAR-HRP, BSA (fraction V), Tween 20, and 3,3′,5,5′-TMB were purchased from Sigma Chemical Co. (St. Louis, MO). MeOH was HPLC grade.

Sample Preparation. Domestic red and imported white wines (13.5% alcohol, v/v) were purchased from a local market in Davis, CA. The wines were fortified with cypermethrin and permethrin from standard solutions in MeOH to make the appropriate concentrations. The wines spiked with cypermethrin and permethrin were diluted with PBS containing 40% MeOH for analysis. Apple, banana, cucumber, lettuce, onion, and peach were selected as model food samples. They were purchased from a local market in Davis. The samples were rinsed with deionized water. Ten grams of each was chopped and then fortified by adding aliquots of standard solutions of the permethrin in MeOH and allowed to set at room temperature for 30 min prior to extraction. The samples were blended and then placed into a 150 mL flask followed by the addition of 50 mL of MeOH. The flask was shaken for 30 min

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10.1021/jf049438z CCC: $27.50
Published on Web 07/31/2004

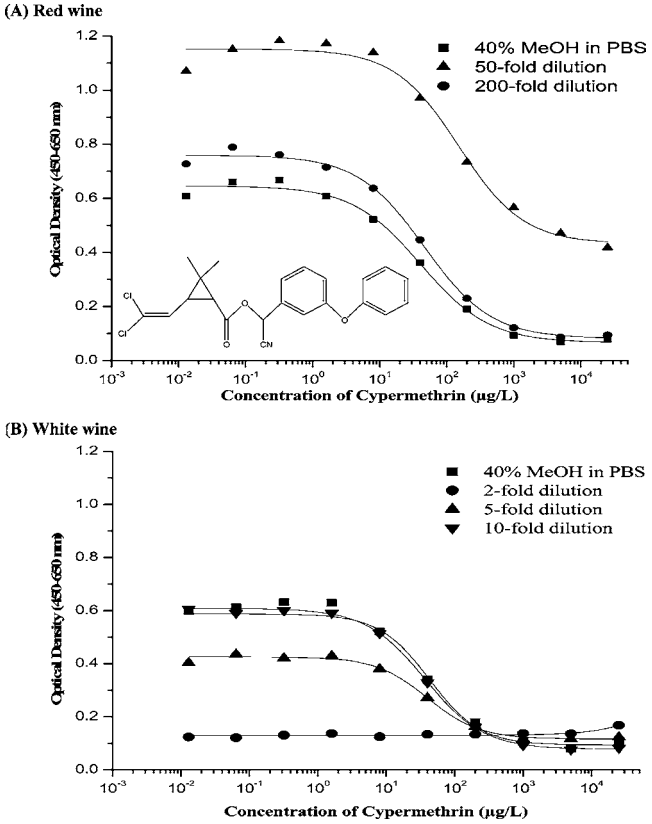

Figure 1. Influence of matrix in (**A**) red wine and (**B**) white wine on the cypermethrin ELISA standard curve. The final dilutions in PBS buffer (40% MeOH) were as follows. In red wine: control (■), 50-fold (▲), and 200-fold (●); in white wine: control (■), 2-fold (●), 5-fold (▲), and 10-fold (▼). Each point represents the average of four well replicates.

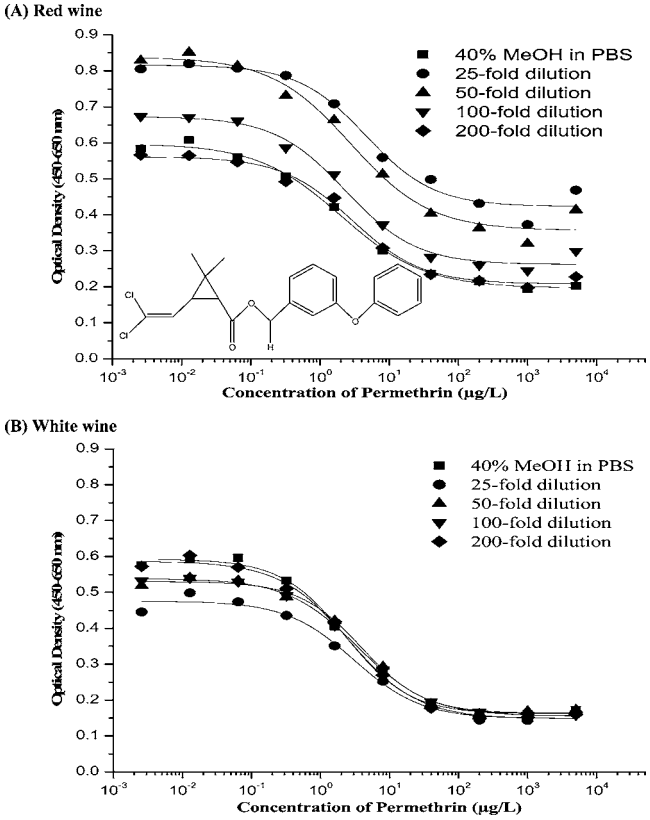

Figure 2. Influence of matrix in (**A**) red and (**B**) white wines on the permethrin ELISA standard curve. The final dilutions in PBS buffer (40% MeOH) were as follows: control (■), 25-fold (●), 50-fold (▲), 100-fold (▼), and 200-fold (◆). Each point represents the average of four well replicates.

at 200 rpm and then filtered through filter paper (no. 1, Whatman) under vacuum. This step was repeated with 40 mL of MeOH. The filtrate was made up to 100 mL with MeOH. This sample was then analyzed by ELISA without a further cleanup procedure.

Instruments for ELISA. The ELISA experiments were performed in 96 well microplates (Nunc, Roskilde, Denmark), and the absorbance was measured with a Versa max microplate reader (Molecular Devices, Menlo Park, CA) in dual-wavelength mode (450−650 nm).

Cypermethrin and Permethrin Immunoassay. A competitive ELISA was used. The methods for both compounds were the same as previously reported (*7*, *12*). For the permethrin assay, antibody (Ab549, 1/2500) and coating antigen (4-BSA, 1/8000) were used, and in the case of cypermethrin assay, antibody (Ab735, 1/7000) and coating antigen (Ag4-BSA, 1/6250) were employed. Briefly, the microplates were coated overnight at 4 °C with 100 μL per well of the appropriate coating antigen concentration in 0.1 M carbonate−bicarbonate buffer (pH 9.6). After the coated plates were washed with the washing solution (0.05% Tween 20 in deionized water), 200 μL of blocking solution (0.5% BSA in PBS) was added and incubated for 30 min at room temperature. After the plates were washed (five times), 50 μL per well of the appropriate antibody concentration in PBS with 0.2% BSA and 50 μL per well of sample or standard were added. The plate was incubated for 60 min and then washed 8−10 times. GAR-HRP (diluted 1:3000 in PBS with 0.05% Tween 20, 100 μL per well) was added and incubated for 60 min at room temperature. Following another washing step (8−10 times), the substrate solution (100 μL per well; 3.3 μL of 30% H_2O_2, 400 μL of 0.6% TMB in DMSO per 25 mL of citrate−acetate buffer, pH 5.5) was added. The blue color development was stopped after 15−20 min with 2 M sulfuric acid (50 μL per well), and the absorbance was measured at 450−650 nm. The standard curves were obtained by plotting absorbance against the logarithm of analyte concentrations, which were fitted to a four-parameter logistic equation: $y = \{(A - D)/[1 + (x/C)^B]\} + D$, where A is the maximum absorbance at no analyte, B is the curve slope at the inflection point, C is the concentration of analyte giving 50% inhibition (IC_{50}), and D is the minimum absorbance at infinite concentration.

Instrument for Chromatographic Analysis. The assay validation was conducted using a Hewlett-Packard 6890 gas chromatograph equipped with an HP 5973 mass selective detector. A 30 m × 0.25 mm capillary column coated with a 0.25 μm thick film of 5% phenylmethylsiloxane (HP-5MS) from Hewlett-Packard was used. An HP model 7683 series autoinjector was used with 1 μL of sample injected. The column temperature was programmed from 200 to 280 °C at 5 °C/min and held for 5 min. Helium was used as a carrier gas (1 mL/min).

Validation Studies. To compare the ELISA and GC-MS methods for permethrin analysis, lettuce and peach were used. The extraction procedure of permethrin for GC-MS was different from that of ELISA. Chopped 10 g samples fortified with permethrin in MeOH to make a final concentration of 2 mg/kg were homogenized with 100 mL of acetone for 3 min, and then, the homogenate was filtered (repeat once). The filtrates were reduced to 30 mL and extracted with 10% NaCl (100 mL) and hexane (100 mL) with shaking for 5 min. After extraction, the hexane layer was transferred and this step was repeated with an additional 50 mL of hexane. Anhydrous Na_2SO_4 was added to the combined hexane and shaken moderately for 15 min. Hexane was then filtered and concentrated for GC-MS measurement.

RESULTS AND DISCUSSION

Analytical Characteristics of the ELISA for Cypermethrin and Permethrin. The immunoassay may be susceptible to food matrix effects. Easy and immediate ways to minimize matrix effects are simple dilution with an appropriate buffer solution. Red and white wines were fortified with cypermethrin and permethrin and directly analyzed by ELISA without any pretreatments other than dilution. **Figures 1** and **2** show the

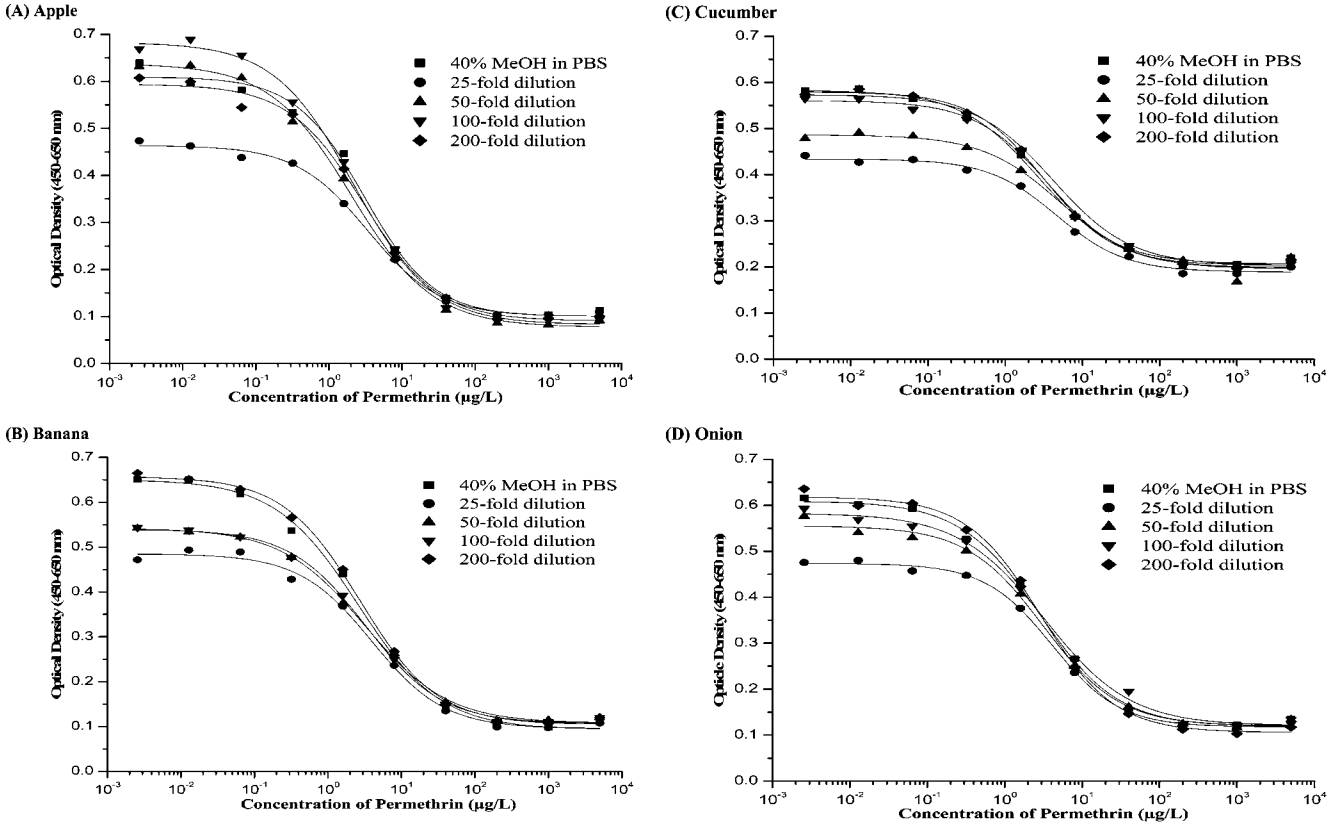

Figure 3. Influence of matrix in (**A**) apple, (**B**) banana, (**C**) cucumber, and (**D**) onion on the permethrin ELISA standard curve. The final dilutions in PBS buffer (40% MeOH) were as follows: control (■), 25-fold (●), 50-fold (▲), 100-fold (▼), and 200-fold (◆). Each point represents the average of four well replicates.

Table 1. Parameters of ELISA Methods for Cypermethrin and Permethrin Analysis in Wines[a] and Agricultural Foods[a]

samples	A_{max} (*A*)	slope (*B*)	IC_{50} (μg/L) (*C*)	A_{min} (*D*)	R^2
			permethrin		
red wine[b]	0.56 ± 0.01	0.86 ± 0.13	2.91 ± 0.59	0.21 ± 0.01	0.993
white wine[b]	0.59 ± 0.01	0.87 ± 0.09	2.56 ± 0.36	0.15 ± 0.01	0.997
apple[b]	0.59 ± 0.01	0.85 ± 0.09	2.92 ± 0.41	0.09 ± 0.01	0.997
banana[b]	0.66 ± 0.01	0.83 ± 0.06	2.74 ± 0.25	0.10 ± 0.01	0.999
cucumber[b]	0.58 ± 0.01	0.94 ± 0.07	3.15 ± 0.28	0.20 ± 0.01	0.998
lettuce[b]	0.57 ± 0.03	0.67 ± 0.12	5.54 ± 0.18	0.06 ± 0.02	0.998
onion[b]	0.62 ± 0.01	0.91 ± 0.08	2.88 ± 0.29	0.11 ± 0.01	0.998
peach[b]	0.53 ± 0.01	0.76 ± 0.16	4.26 ± 0.23	0.04 ± 0.01	0.997
			cypermethrin		
red wine[b]	0.76 ± 0.01	0.87 ± 0.09	46.4 ± 1.60	0.08 ± 0.02	0.997
white wine[c]	0.61 ± 0.01	1.01 ± 0.06	36.8 ± 2.48	0.08 ± 0.01	0.999

[a] Each value was the mean ± SD ($n = 15$). [b] All samples were diluted 200-fold for permethrin analysis and for analysis of cypermethrin in red wine. [c] White wine was diluted 10-fold for analysis of cypermethrin.

Table 2. Recovery of Cypermethrin from Spiked White Wine

spiked concn[a] (μg/L)	theoretical concn in the ELISA (μg/L)	ELISA detected (μg/L)	ELISA mean recovery (%) (SD)
100	10	10.9	110 (10.3)
200	20	21.7	108 (12.1)
400	40	37.5	93.7 (3.3)
600	60	61.4	102 (7.0)
800	80	80.7	101 (4.4)
1000	100	98.2	98.2 (9.5)
1200	120	102.2	85.2 (6.5)

[a] Different amounts of cypermethrin (in MeOH) were added to the white wine. Because of the matrix effect of the white wine, the samples were diluted 10-fold with PBS (40% MeOH). The samples were analyzed blind ($n = 5$).

standard curves for cypermethrin and for permethrin in red and white wines, respectively. The results suggest that both compounds could be analyzed in red and white wines by simply diluting samples in PBS containing 40% MeOH, without further cleanup steps. A minimum matrix effect with a 1:10 dilution of white wine in PBS containing 40% MeOH for cypermethrin was obtained. In contrast, cypermethrin in red wine required a 1:200 dilution, and thus, this assay was not further considered for this matrix. For permethrin in red and white wines, a 1:200 dilution with PBS containing 40% MeOH was satisfactory. **Figure 3** shows the standard curves for permethrin in (**A**) apple, (**B**) banana, (**C**) cucumber, and (**D**) onion, respectively. A minimum matrix effect with a 1:20 dilution of the extracts of fruits and vegetables in PBS containing 40% MeOH (total dilution factor of 1:200 from the original sample) was obtained. **Table 1** summarizes the characteristic parameters for the ELISA assay for cypermethrin and permethrin in diluted wines and the extracts of fruits and vegetables with PBS containing 40% MeOH. The cypermethrin concentrations that reduced absorbance to 50% of the maximum (IC_{50}) were 46.4 ± 1.60 μg/L in 200-fold diluted red wine and 36.8 ± 2.48 μg/L in 10-fold diluted white wine. The IC_{50} values of permethrin in 200-fold diluted red and white wines were 2.91 ± 0.59 and 2.56 ± 0.36 μg/L, respectively. Those of permethrin in 200-fold diluted apple, banana, cucumber, lettuce, onion, and peach were 2.92 ± 0.41, 2.74 ± 0.25, 3.15 ± 0.28, 5.54 ± 0.18, 2.88 ± 0.29, and 4.26 ± 0.23 μg/L, respectively. The limit of detection (LOD) was estimated as the cypermethrin and permethrin concentrations that reduced absorbance to 90% of the maximum (IC_{10}). The LODs of cypermethrin and permethrin in buffer solu-

Table 3. Recovery of Permethrin in Spiked Red and White Wines

			ELISA	
samples[a]	spiked concns in wines (μg/L)	theoretical concn in the ELISA (μg/L)	detected (μg/L)	mean recovery (%) (SD)
red wine	100	0.5	0.44	88 (18.8)
	300	1.5	1.40	93 (0.5)
	600	3	1.86	62 (0.6)
	1000	5	3.25	65 (1.1)
	3000	15	9.15	61 (3.6)
white wine	100	0.5	0.18	36 (1.3)
	300	1.5	1.22	81 (8.1)
	500	2.5	2.38	95 (9.7)
	1000	5	3.95	79 (5.7)
	2500	12.5	12.13	97 (6.5)

[a] Different amounts of permethrin (in MeOH) were added to the wines and then diluted 200-fold with PBS (40% MeOH). The samples were analyzed blind ($n = 3$).

Table 4. Recovery of Permethrin in Spiked Fruits and Vegetables

			ELISA	
sample[a]	spiked concns in samples (μg/kg)	theoretical concn in the ELISA (μg/L)	detected (μg/L)	mean recovery (%) (SD)
apple	100	0.5	0.41	82 (10.4)
	300	1.5	1.53	102 (15.7)
	600	3	2.75	92 (21.1)
	1000	5	5.46	109 (41.5)
	3000	15	16.6	111 (27.6)
banana	100	0.5	0.42	84 (18.3)
	300	1.5	1.57	105 (4.9)
	500	2.5	2.49	100 (2.4)
	1000	5	5.63	113 (17.9)
	3000	15	18.3	122 (27.3)
cucumber	150	0.75	0.5	67 (1.5)
	300	1.5	0.64	43 (0.9)
	600	3	1.92	64 (6.3)
	1000	5	3.9	78 (2.3)
	2700	13.5	13.3	99 (4.8)
lettuce	2000	10	10.0	100 (15.3)
	5000	25	23.7	94.7 (3.5)
onion	100	0.5	0.48	96 (7.2)
	300	1.5	1.39	93 (11.4)
	600	3	2.94	98 (13.8)
	1000	5	4.42	88 (17.7)
	2500	12.5	12.1	97 (10.2)
peach	2000	10	9.71	95.1 (17.6)
	5000	25	20.9	83.6 (6.9)

[a] Different amounts of permethrin (in MeOH) were added to the fruits and the vegetables. The final 200-fold diluted samples with PBS (40% MeOH) were used. The samples were analyzed blind ($n = 3$).

tion were about 3.5 and 0.1 μg/L, respectively. The limit of quantification (LOQ) for cypermethrin and permethrin analyzed by ELISA was determined experimentally. The LOQs of cypermethrin in white wine and permethrin in wines in this experiment were as low as 50 μg/L. The LOQs of permethrin in lettuce and peach were as low as 50 μg/kg. In apple, banana, and onion samples, the LOQ of permethrin was as low as 70 μg/kg, but the LOQ of permethrin in cucumber was as low as 100 μg/kg.

Recovery of Cypermethrin and Permethrin by ELISA. The results of cypermethrin recovery from 10-fold diluted white wine are presented in **Table 2**, indicating that the recoveries were very good (99.7% mean). The permethrin recovery in red and white wines was also satisfactory (74 and 78% mean, respectively) in **Table 3**. However, spike recoveries for the high spike samples (>600 μg/L) in red wine and the low spiked sample (100 μg/L) in white wine were lower in overall means of permethrin recovery. **Table 4** shows that the average of permethrin recovery from apple, banana, cucumber, lettuce, onion, and peach was 99.2, 105, 70.2, 97.5, 94.4, and 89.4%, respectively.

Table 5. Correlation of Permethrin Analysis between ELISA and GC-MS Methods in Lettuce and Peach

	ELISA		GC-MS	
samples[a]	detected (mg/kg)	mean recovery (%) (SD)	detected (mg/kg)	mean recovery (%) (SD)
lettuce	1.69	84.5 (10.6)	2.53	126.3 (11.2)
peach	1.84	92.1 (2.21)	1.45	72.3 (8.31)

[a] Spiked concentration of permethrin (in MeOH) in lettuce and peach was 2 mg/kg. The final 200-fold diluted samples with PBS (40% MeOH) were used for ELISA and with hexane for GC-MS. The samples were analyzed blind ($n = 4$).

Correlation Studies between ELISA and GC-MS Analysis. For permethrin determination, an important peak of fragmentation (m/z 183) was used for quantitation. A comparison of the results obtained by using both ELISA and GC-MS methods for permethrin recovery analysis from lettuce and peach is presented in **Table 5**, indicating that good recovery from both techniques was obtained.

In conclusion, the competitive indirect ELISA presented here successfully determines cypermethrin and permethrin in fruit, vegetable, and wine samples used in this experiment. Both compounds were analyzed by simple dilution without any concentration or cleanup steps. The maximum residue limits (MRLs) for cypermethrin in wine are 0.05 (Australia), 0.5 (Canada), and 2 (Japan) mg/kg. The MRLs for permethrin in wine are 2 (Canada) and 5 (Japan) mg/kg. There are no MRLs for either compound in the United States, meaning that they should not be detected in wines. The agricultural products used in this experiment have different MRLs in each country. In the European Union, the MRLs of all agricultural products used in this experiment are 0.05 mg/kg, but those of apple, onion, peach, and lettuce in the United States are 0.05, 0.1, 5, and 20 mg/kg, respectively. Therefore, the simple dilution with PBS containing 40% MeOH is applicable to determine permethrin residues. The 200-fold dilution factor used in this experiment resulted in no effect from the matrix, but less dilution (100-fold in this experiment) should also be applicable. The comparison between ELISA and GC-MS methods resulted in a good correlation. However, the ELISA method is feasible for determination of pesticide residues on foodstuffs with high throughputs, rapidity, and lower expense. The sensitivity of immunoassays used in this experiment could be easily increased by robotic solid phase extraction methods.

ABBREVIATIONS USED

A_{max}, absorbance in the absence of competing analyte; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; GAR-HRP, goat anti-rabbit immunoglobulin-conjugated horseradish peroxidase; GC-MS, gas chromatography with mass spectrometric detection; IC_{50}, concentration of analyte giving 50% inhibition of the maximum absorbance; MeOH, methanol; PBS, phosphate-buffered saline; SD, standard deviation; TMB, tetramethylbenzidine.

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Received for review April 5, 2004. Revised manuscript received June 15, 2004. Accepted June 16, 2004. This work was supported by NIEHS Superfund Basic Research Program (P42 ES04699) and NIEHS Center for Environmental Health Sciences (P30 ES05707).

JF049438Z