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Development of Two Enzyme-Linked Immunosorbent Assays for Detection of Endosulfan Residues in Agricultural Products

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Two competitive immunoassays, a laboratory assay based on microwell plates and a field test based on the use of polystyrene tubes, have been developed for the detection of endosulfan in agricultural products. The limit of detection for the microwell plate format was 0.8 ± 0.1 μg/kg, and the limit of detection for the tube format was 1.6 ± 0.2 μg/kg. A simple, rapid, and efficient extraction method was employed, and 76−112% recoveries of spiked samples were obtained. Methanol extracts of some agricultural product samples such as grape, carrot, spinach, and tobacco could be analyzed directly by immunoassay after dilution in 0.5% fish skin gelatin−phosphate buffered saline. In contrast, extracts of green tea caused significant interference in the assay, and a number of simple cleanup methods were ineffective in removing interference. However, use of the coagulating reagent polyvinyl pyrrolidone removed the matrix effect effectively. For the validation of the enzyme-linked immunosorbent assay (ELISA) tests, samples were analyzed by ELISA and gas chromatography (GC) after solid phase extraction. The relationship between data obtained using the tube assay and microwell assay was good (the lowest r^2 value was 0.94), and also, the immunoassay assay data correlated well with data obtained from GC analysis (the lowest r^2 value was 0.93). The developed immunoassay methods are the suitable methods for the rapid quantitative and reliable determination of endosulfan residues in agricultural products.

KEYWORDS: Immunoassay; endosulfan; agricultural products; matrix effect; validation

INTRODUCTION

Endosulfan is a broad spectrum insecticide and acaricide, which acts as a contact poison to a wide variety of insects and mites (*1*−*5*). It is used primarily on a wide variety of food crops, including tea, coffee, fruit, and vegetables, as well as on cereals such as rice, maize, sorghum, or other grains (*6*−*9*).

Currently, organochlorine residue analysis such as endosulfan in most developed and developing countries is carried out using gas chromatography (GC) with electron capture detection (ECD) (*10*−*12*). The equipment required is comparatively expensive, and the analysis consumes large amounts of solvent and requires a stable and continuous electricity supply and high-purity gases. Also, samples need to be carefully cleaned up prior to analysis (*13*). Therefore, although sensitive and well-established, the method is not very well-suited for the analysis of the large number of samples required in comprehensive monitoring programs.

China is a large exporter of agricultural products. In recent years, the problem of pesticide residues in agricultural products has become more serious. Monitoring of pesticide residues in agricultural products is thus an important means of detecting unacceptable practices or violations and implementing strategies to limit the possibility of reoccurrence (*14*−*16*). Failure to adequately screen and control residues in food has limited the ability of China to more effectively enter the export market for a range of agricultural commodities (*17*). With the increasing demand for pesticide residue analysis certification in export, there is a need to develop simple, cost effective, and sensitive assays to detect pesticide residues. Immunoassays have the benefits of being inexpensive and rapid and can be performed on simple, inexpensive equipment (*18*, *19*). In addition, immunoassays are able to simultaneously analyze a large number of samples with minimal sample cleanup and with accuracy and precision comparable to those reached by chromatographic methods (*20*, *21*).

Enzyme-linked immunosorbent assay (ELISA) was previously developed and employed for detecting endosulfan in water and soil (*22*). In the present study, we have utilized these haptens to develop two sensitive, competitive immunoassays based on polyclonal antibodies for the detection of endosulfan in agricultural products. These two enzyme immunoassays, a laboratory assay using microwell plates and a tube-based assay using polystyrene tubes, were validated with real samples. The tube assay was mainly suitable for field use as a qualitative or semiquantitative test, which requires only 15 min, using two short incubation steps, and the resulting color was read by a portable photometer or visually. The microwell plate assay was

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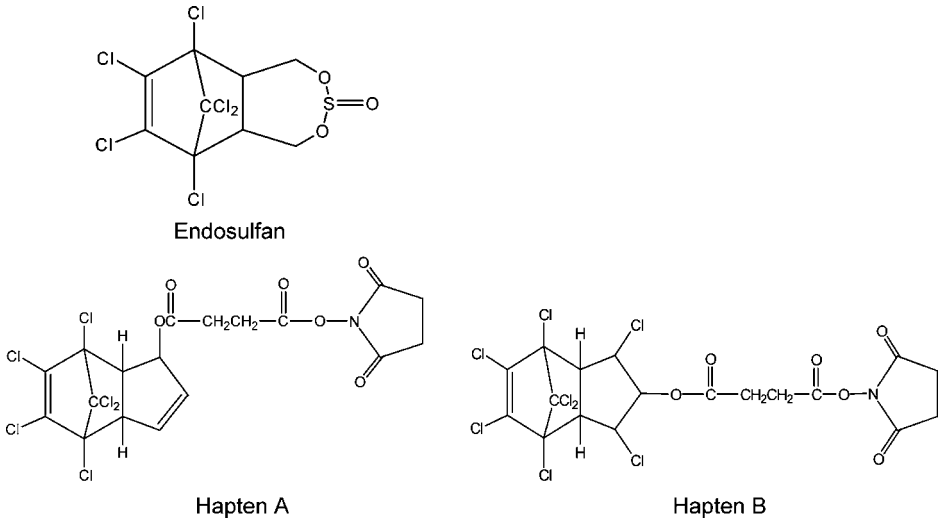

Figure 1. Chemical structures of endosulfan and haptens.

mainly used under laboratory conditions. It required 90 min to perform and utilized a microwell reader but had the advantage of allowing a higher number of samples.

Before assays can be applied to the analysis of a particular agricultural product sample, sample matrix interference must be evaluated and methods for the removal of any interference must be developed (*23*, *24*). Although the reliability of ELISA has already been demonstrated, that reliability needs to be assessed for a given sample type subjected to a particular preparation or extraction procedure. Also, the extraction efficiency of the extraction method needs to be evaluated (*25*).

The objectives of this study were (i) to develop the immunoassays for endosulfan residue analysis; (ii) to determine the precision, accuracy, sensitivity, matrix effects, and utility of ELISA for detecting endosulfan in different agricultural products; (iii) to assess the possibility of eliminating the sample cleanup step for ELISA determination; and (iv) to compare the quality of ELISA results with those obtained by a traditional GC methodology.

MATERIALS AND METHODS

Chemicals and Materials. Horseradish peroxidase (HRP), keyhole limpet hemocyanin (KLH), fish skin gelation (FG), and Freund's complete and incomplete adjutants were purchased from Sigma (St. Louis, MO). Bovine serum albumin (BSA) was obtained from Merck (Darmstadt, Germany). Protein A-Sepharose 4B was purchased from Amersham Biosciences (Uppsala, Sweden). Pesticide grade *n*-hexane for GC was obtained from Dima (Richmond Hill, Canada). Reagent grade 3,3′,5,5′-tetramethyl-benzidine and hydrogen peroxide were from Sigma. Endosulfan was obtained from Chem Service (West Chester, PA). Polystyrene 96 well microwell plates were from Nunc (Rockilde, Denmark), and the microplate washer was from Bio-Rad (Hercules, CA). Immunoassay absorbance was read with a Multiskan Spectrum purchased from Thermo (Labsystems, Vantaa, Finland) in dual-wavelength mode (450−650 nm).

Preparation of the Immunogens. The synthesis of endosulfan haptens reported by Lee et al. was accomplished according to their procedures (*22*). Haptens A and B consist of a five-membered ring next to a norbornane structure and thus retained the basic ring structure of endosulfan. While the two haptens contained the same hemisuccinate linker, they were different in the total number of chlorine atoms (i.e., haptens A and B consisted of six and eight chlorine atoms, respectively) and the presence of a double bond in the five-membered ring (hapten A). Chemical structures for endosulfan and these haptens are shown in **Figure 1**.

Haptens were attached to protein and enzyme using the active ester method (*26*). The haptens were coupled to KLH for use as immunogens or coupled to HRP for use as enzyme tracers.

Antibody Production. New Zealand white rabbits were used for the production of antibody according to the method described by Wang et al. (*27*). Briefly, the primary immunogens containing the hapten A and hapten B conjugated to KLH were injected by the modes of intradermal and intramuscular injections. The sera that exhibited strong positive responses were purified using a Protein A-Sepharose 4B affinity column. The IgG fraction was dialyzed, and the antibodies were concentrated and then used for the competitive ELISA format described below.

Microwell Assay. In this study, we employed direct competitive ELISA for endosulfan analysis. The optimum concentrations of antibody and HRP required to get the most sensitive assay were determined using checkerboard analysis. Microwell plates were coated with 100 μL of the purified polyclonal antibody (10 μg/mL) diluted in 50 mM carbonate buffer, pH 9.6, per well, and incubated overnight at room temperature. Then, coated plates were washed three times with PBS/T washing solution [phosphate-buffered saline, pH 7.2, with 0.05% (v/v) Tween 20], and then, unbound sites were blocked with 200 μL of 1% BSA/PBS per well for 1 h. After the plates were washed three times with PBS/T, the assay was performed by the addition of 100 μL of pesticide standard in 0.5% (w/v) FG in PBS solution and 100 μL of enzyme conjugate solution (HRP-hapten conjugate) diluted in the PBS to each well and was incubated for 1 h. After it was washed with washing solution, 150 μL of 3,3′,5,5′-tetramethylbenzidine-peroxide-based substrate solution was added to each well. Color development was stopped after 30 min by adding 50 μL of 1.25 M H_2SO_4, and absorbances were read in the microplate reader in dual-wavelength mode (450−650 nm).

Tube Assay. Antibodies were diluted in 50 mM carbonate buffer (pH 9.6) to 15 μg/mL and immobilized at 500 μL per tube (Nunc) overnight at room temperature. After the tube was washed three times with PBS/T, the unbound sites were blocked by adding 600 μL per tube of 1% BSA−PBS for 1 h. The reagents were prepared in the different dropper bottles, and the samples and standards were added into the tube using the plastic droppers of different sizes. Four drops (about 450 μL) of sample and four drops (about 50 μL) of HRP conjugate were added to a tube and incubated for 10 min. The tube was washed with distilled water five times. Then, six drops (about 750 μL) of hydrogen peroxide substrate/chromogen was added for color development and incubated for 5 min. Finally, four drops (about 250 μL) of stopping solution (1.25 M sulfuric acid) was added to stop the color development and read with a portable photometer at 450 nm.

GC Analysis of Endosulfan. A Shimadzu GC-2010 (Tokyo, Japan) gas chromatograph equipped with an ECD detector was used for endosulfan instrumental analysis with external calibration. Chromatographic separation was performed using a low polarity fused silica

capillary column DB-5 (30 m × 0.25 mm i.d. with 0.25 μm film thickness) supplied by Agilent and with high-purity (over 99.999%) nitrogen as a carrier gas at 1.2 mL/min. The column temperature was programmed as follows: 130 °C maintained for 1 min, then programmed at 8 °C/min to 220 °C, held 1 min, and programmed at 5 °C/min to 260 °C, held 4 min. The injector port was maintained at 280 °C, and the detector temperature was 300 °C. One microliter of sample was injected in the splitless mode.

For GC analysis, 10 g of samples was vigorously shaken with 50 mL of methanol for 60 min in a rotary shaker. After it stood for 5 min, the supernatant was cleaned up with solid phase extraction (SPE) or Florisil columns, and then, the elution was analyzed by GC-ECD.

Sample Extraction. Fruits, vegetables, teas, and tobacco were bought from local markets. Once confirmed by GC analysis that the samples contained residues of endosulfan at less than 0.1 μg/kg, they were used for recovery studies.

The extent of matrix interference was initially determined by extracting a pesticide-free matrix on a rotary shaker for 60 min, preparing standards with known concentrations of pesticide, and comparing the standard curve prepared in the extract of the food sample matrix to the ones prepared in diluted solvent used in the extraction. These experiments were followed by spiked and recovery studies. Initially, samples were extracted with neat methanol, and the details of extraction methods are specified in next section.

For a spiking study, 10 g of sample in a glass jar was spiked with 200 μL of solution of endosulfan dissolved in methanol at different levels; the samples were thoroughly mixed and then allowed to stand at room temperature overnight.

For ELISA determinations, 10 g of chopped sample was weighed into conical bottles, and 50 mL of methanol was added. Different extraction methods were compared as follows: (i) Ten grams of sample and 50 mL of methanol were homogenized at 4000 rpm for 2 min using a Waring blender obtained from Omini (Warrenton, VA). (ii) Ten grams of sample and 50 mL of methanol were shaken by hand for 5 min. (iii) Ten grams of sample and 50 mL of methanol were shaken at 200 rpm on the rotary shaker for 60 min. The sample extracts were diluted in appropriate folds (based on the samples matrix) before ELISA analysis, and these appropriate folds are specified in the Results and Discussion section.

Sample Cleanup Procedures. *Solid Phase Extraction (SPE).* The supernatant was cleaned up with a SPE cartridge (StrataC18-E, 50 μm, 70 Å, 500 mg/3 mL). The cartridge was previously conditioned with 5 mL of methanol followed by 10 mL of purified water without allowing the cartridge to dry out. Then, the samples were passed though the cartridge at a rate of 4 mL/min, followed by washing with 4 mL of 40% methanol in water. The cartridge was dried by blowing nitrogen. Adsorbed pesticides were eluted by 5 mL of hexane; hexane was then evaporated to dryness under a gentle stream of nitrogen, and the residue was redissolved with 1 mL of hexane.

Cleanup Using Florisil Columns. Florisil was dried by heating at 600 °C for 4 h and stored in a desiccator. Purified water was added dropwise to dried Florisil while shaking to a final 5% water (v/w) content and rotated on the vertical rotor overnight to mix well. A glass column plugged with cotton wool was packed with 2 g of Florisil and topped with 1 cm height anhydrous sodium sulfate. One milliliter of the sample extract was added to the top of the column. The column was then eluted with 6 mL of hexane followed by 12 mL of acetone/hexane (1:3). The first 2 mL of elute was discarded, and then, the following 10 mL was collected and concentrated to about 1 mL. Finally, it was made up to 5 mL with hexane in a volumetric flask.

RESULTS AND DISCUSSION

Analytical Characteristics of ELISA. In preliminary work, a range of immobilized antibody and conjugate concentration was assessed for the polyclonal Ab-based assay. Absorbance of each well was read at 450 nm with reference at 650 nm, and the condition chosen in this paper gave color development of absorbance values of 0.7−1.2 for pesticide-free control.

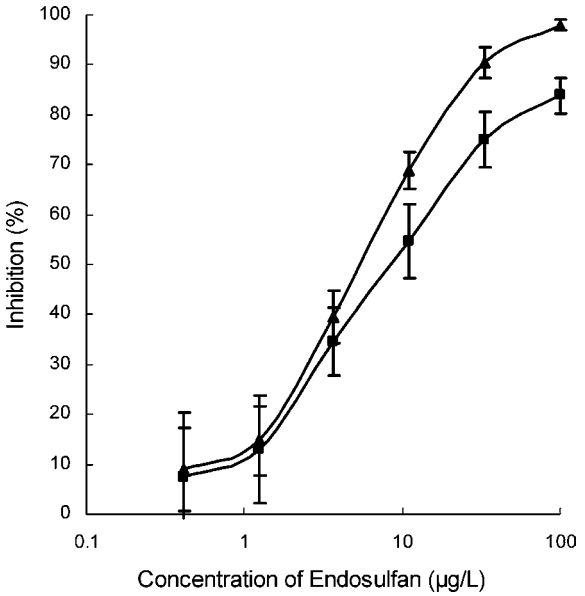

Figure 2. Endosulfan standard curves for plate ELISA (▲) and tube ELISA (■). Each point represents the average of 10 well replicates.

We also checked titers of two antibodies raised from KLH conjugates of hapten A and hapten B with two enzyme conjugates, and optimized conditions for all of the combinations were established by titration of serum dilutions against various concentrations of the immunizing hapten coupled to ovalbumin. It was found that the antibody raised from the hapten B−KLH conjugate was not suitable for the current assay test. The color development was very low, and the sensitivity of this antibody for the target compound was also low (data not shown). Therefore, the antibody raised from hapten A−KLH conjugate was chosen and used in the subsequent work, because it gave better assay sensitivity and color development.

In the course of endosulfan analysis, a six-point standard curve was included in each ELISA plate assay to estimate analyte concentrations. The same ELISA procedure was repeated 10 times in 5 days. The standard curves for endosulfan are shown in **Figure 2**. The sensitivity of the plate ELISA for endosulfan (IC_{50}, calculated as the concentration of endosulfan giving 50% inhibition of color development) was 5.3 ± 0.4 μg/kg. The limit of detection (LOD) was calculated as the IC_{15} value, where it is an endosulfan's concentration at which the analyte gives 15% inhibition of color development and it is approximately the lowest part of the linear portion of the standard curve. According to this criterion, the LOD for endosulfan was 0.8 ± 0.1 μg/kg.

The standard curve from the tube assay of the same antibodies was also shown in **Figure 2**. It displayed that the detection limit of the tube assay was 1.6 ± 0.2 μg/kg. The slope of the standard curve for the tube assay was less steep, and its IC_{50} value was 8.4 ± 0.5 μg/kg.

The intraassay reproducibility and interassay reproducibility were determined to study the assay precision. The variations of percent inhibition for 100, 33.3, 11.1, 3.7, 1.4, and 0.4 μg/kg of endosulfan tested four times on the same day using the microwell assay were 1.07, 3.15, 6.96, 8.24, 15.72, and 28.79%, respectively. The data using the tube assay were 8.69, 12.54, 10.59, 18.84, 25.83, and 38.67%, respectively. The microwell assay of the same material run over 5 days gave a deviation from the mean values of 2.03, 2.83, 8.11, 11.09, 17.70, and 32.44% for each of the respective concentrations and of 7.54, 15.38, 21.52, 26.75, 32.92, and 42.56%, respectively, using the

tube assay. The tendency for increased deviation with low concentration was likely due to the serial dilution. This suggests that extra caution should be taken in the standard curve preparation, as the precision of the standard curve will influence the precision and accuracy of the analysis. The relatively higher deviation of the tube assay in each point of standard curve would suggest that this assay was less suitable for quantitative analysis than the microwell assay for the determination of endosulfan, and it was mainly used as a semiquantitative tool in field tests.

Cross-reactions can affect analytical results by either giving a false positive or by elevating the predicted concentration of the target compound when both the target and one or more structurally similar compounds are present. Therefore, the specificity of the antibody toward compound and its most probable cross-reactants should be determined. Among the cyclodienes tested, aldrin and heptachlor showed cross-reactivity less than 5% (data not shown). Dieldrin and endrin have been found to exhibit significant cross-reactivities (22.6 and 46.4%, respectively). Pesticides other than cyclodienes such as DDT did not show any cross-reaction at 1000 ppb. The results are consistent with the previous studies by Lee et al. (*22*). Because dieldrin and endrin have been strictly prohibited in China, they should not appear in foodstuffs and not a concern for the immunoassay in these matrices and samples. Therefore, the developed immunoassay could be used to specifically detect endosulfan in agricultural products.

Matrix Effect Studies. Immunoassays are rapid and convenient for food sample analysis primarily because they usually do not require sample preconcentration and cleanup steps. However, ELISA methods often have a high potential for nonspecific binding between nontarget analytes and antibodies and are consequently prone to matrix interferences. Chemical compounds present in samples or sample extracts, such as pigment, solvents, and others, might affect the binding of antibody and analytes, and they also might affect other aspects of the assay. This "matrix effect" is a common problem for the immunoassay, which could reduce the sensitivity and reliability of the competitive immunoassay and cause false positives by lowering the color development. There are several methods available for the quantitative evaluation the matrix effect. Typically, interferences are quantified by comparing a standard curve produced in a control such as diluted methanol with a calibration curve generated in the sample matrix (*28*). If the two curves are superposable, the effect of the matrix is not significant, and then, the samples can be analyzed using the standard curve prepared in the control solution.

Grape, carrot, spinach, teas, and tobacco were chosen as test samples to study the matrix effect. Dilution is a commonly used procedure to reduce the interferences, but this procedure would also reduce the sensitivity. The approach works well with immunoassays exhibiting very high sensitivity able to accommodate the dilution factors and still maintain the detection limit at legal requirements. In our study, dilution of methanol extracts in PBS alone could not reduce matrix interfence from extract even diluting 1/200. The addition of Teleostean FG, BSA, and Tween 20 to diluent of methanol extract was examined to reduce nonspecific interactions, and finally, 0.5% FG-PBS was found to be good as a diluent because matrix interference can be overcome after appropriate dilution with this diluent. The FG in the diluent seemed to act like a stabilizer to protect the enzyme from the interfering materials or to stabilize the antibody−antigen interaction.

Grapes and Nongreen Vegetables, i.e., Carrot. The standard curves for endosulfan standards prepared in methanol were not significantly different from those obtained when standards were prepared in methanol extracts of either grapes or carrots diluted 20-fold times in PBS containing 0.5% FG/PBS buffer. The matrix effect was fairly diminished. **Figure 3** shows the standard curves of endosulfan in different sample extracts after appropriate dilutions.

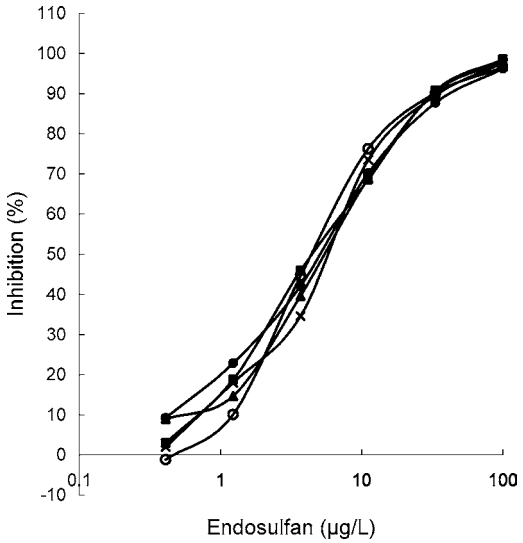

Figure 3. Standard curves of endosulfan in methanol diluted in 0.5% FG/PBS (▲), grape extract (■), carrot extract (●), spinach extract (×), and tobacco extract (○). Each point represents the average of six well replicates.

Colored Samples. There were some troubles in dealing with those colored samples such as spinach and green tea. Spinach was used as an example of a foodstuff with high chlorophyll content, and it gave a highly pigmented extract when either methanol or acetonitrile was selected as extractants. A significant effect of this matrix was seen for endosulfan standards in each of methanol, acetonitrile, and acetone extracts, with depression of absorbency by about 40%. At least 1:100 dilution in PBS/T was needed to remove the matrix effect from spinach. The removal of matrix effect of tobacco extract is similar to spinach with the 200-fold dilution. **Figure 4** showing the standard curves of various dilutions with 0.5% FG-PBS for tobacco demonstrated that the matrix effect disappeared when it was diluted 200-fold times. The maximum residue limits (MRL) for endosulfan formulated by the Food and Agriculture Organization (FAO) in grape, carrot, spinach, tea, and tobacco are 1, 0.2, 2, 30, and 3.5 mg/kg, respectively. Therefore, the simple dilution with 0.5% FG/PBS still maintains the detection limit at legal requirements and is applicable to determine endosulfan residues with the currently developed ELISA.

It was shown above that in many instances, removing the matrix effect could be simply achieved by selecting a diluent and an appropriate dilution range. However, fewer methods were successful with all commodities. For the ELISA analysis of green tea with high chlorophyll and polyphenol contents, there were significant difficulties, presumably arising from coextractant interfering in the antigen−antibody binding or direct deleterious effects on the conjugated enzyme, and the assay color was reduced by half. Some cleanup methods including SPE and Florisil column could remove chlorophyll effectively, but they led to very poor recoveries of endosulfan from matrix and could not sustain the advantage of immunoassay as ease of use. However, the addition of the coagulating reagent, polyvinyl pyrrolidone (PVP, molecular weight 10000) to the tea−methanol extracts before diluting 200-fold times in 0.5% FG/PBS, can

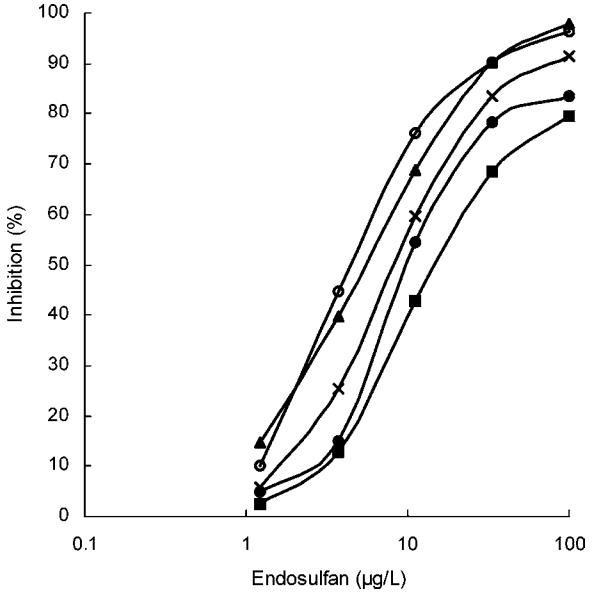

Figure 4. Standard curves of various dilutions with 0.5% FG/PBS for tobacco. Methanol diluted in 0.5% FG/PBS (▲), 20-fold dilution (■), 50-fold dilution (●), 100-fold dilution (×), and 200-fold dilution (○). Each point represents the average of six well replicates.

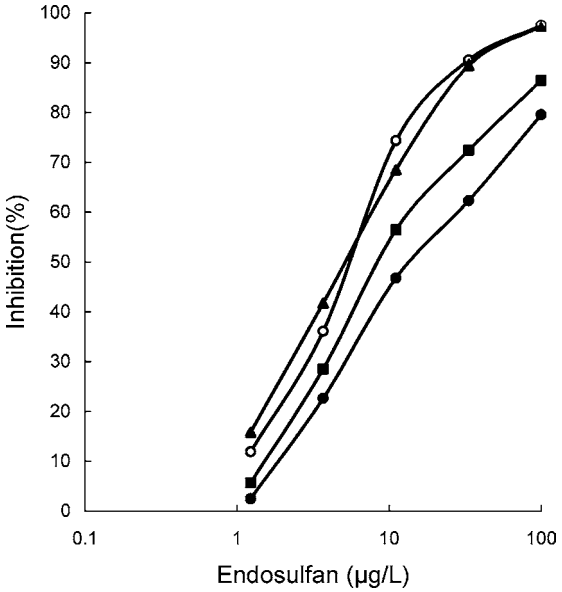

Figure 5. Standard curves of endosulfan in methanol diluted in 0.5% FG/PBS (▲), green tea extract without cleaning up (■), green tea extract cleaned up by SPE (●), and green tea extract treated by PVP (○). Each point represents the average of six well replicates.

make the endosulfan standard curve prepared in the extract similar to the standard curve as shown in **Figure 5**.

Extraction of Residues. Three kinds of organic solvents, methanol, acetonitrile, and acetone, were tested as extractants. The study showed that acetonitrile and acetone were not effective to remove the endosulfan from samples and their recoveries are lower than methanol (data not shown). So, as a result, the methanol was chosen for extracting endosulfan.

We tested three different extraction methods and investigated the efficiency of each method. Each kind of sample was fortified with several concentration levels and then analyzed by ELISA. Each sample was evaluated with four replicates to verify repeatability. The results of recovery studies are shown in **Table 1**. Shaking by a rotary shaker for 1 h could give more than

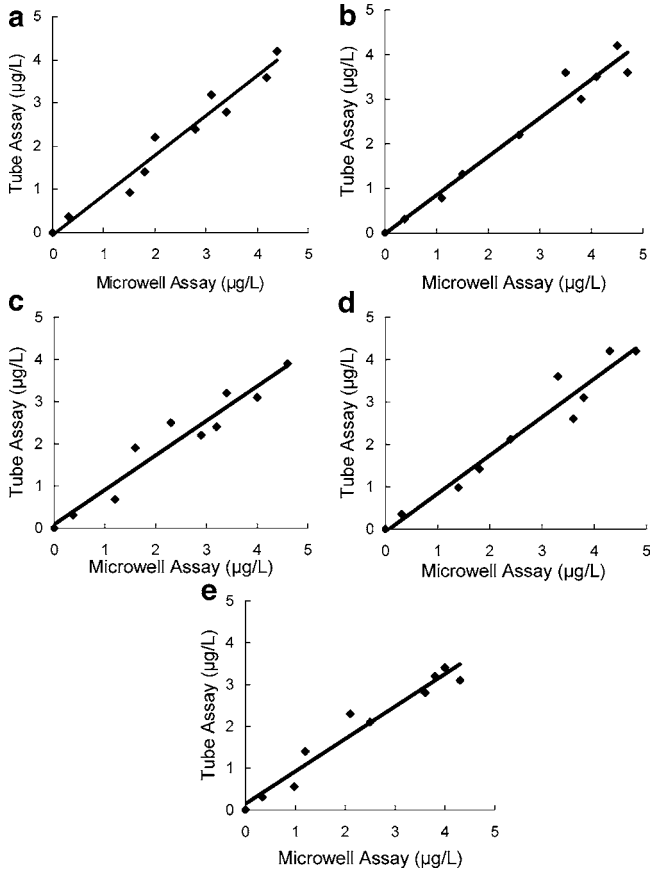

Figure 6. Relationship between the microwell assay and the tube assay for endosulfan in different samples. (**a**) Grape: $y = 0.9159x - 0.0443$, $r^2 = 0.96$. (**b**) Carrot: $y = 0.8644x - 0.0121$, $r^2 = 0.96$. (**c**) Spinach: $y = 0.8197x + 0.0869$, $r^2 = 0.94$. (**d**) Green tea: $y = 0.9016x - 0.0609$, $r^2 = 0.95$. (**e**) Tobacco: $y = 0.7759x + 0.1464$, $r^2 = 0.96$.

90% recovery in every sample, but the procedure was time-consuming. A more rapid extraction method was often required for use with a rapid test. Two minutes of blending could conform to the requirement, since it was efficient, extracting over 95% of the residue, and fast enough to suit the rapid test. However, this extraction method is unsuitable for a field test because it needs equipment and an electricity supply. Although the recovery values obtained from the method by hand shaking for 5 min were somewhat lower than those obtained from the other two methods, this method was also reasonably efficient, extracting over 76% of the residue in every sample. Moreover, by reason that this extraction method did not require the instruments for extraction, such as a shaker, Waring blender, or ultrasonic bath, it was the simplest extraction method of the tested methods and reproducible enough to be used for extraction for on-site residue analysis.

Correlation Studies between Microwell Assay and Tube Assay. All of the samples were analyzed by microwell assays and tube immunoassays. Regression equations determined for two immunoassays are shown in **Figure 6**. The regression indicated that the analysis of the tube assay correlated well with the analysis of microwell assay.

Pesticide-free samples were spiked with endosulfan concentrations at 0.5 MRL, MRL, and 2 MRL and then analyzed by the tube assay. It indicated that each concentration level had an obviously different color development. The higher the concentration was, the lighter the color was. In the field, analysis of the five food samples using the rapid tube assay resulted in all five samples being negative (negative means that the endosulfan

Table 1. Recovery Studies from Agricultural Products Spiked with Endosulfan at Several Levels by ELISA ($n = 4$ Replicates)

matrix	fortification level (mg/kg)	theoretical concn in the ELISA (μg/L)	extration method	mean ± SD (μg/L)	recovery (%)	CV (%)
grape	0.2	2	2 min blend	1.82 ± 0.04	91.0	2.20
	0.5	5		4.51 ± 0.15	90.2	3.33
	1	10		9.35 ± 0.22	93.5	2.35
				mean	91.6	
	0.2	2	5 min shaking	1.65 ± 0.03	82.5	1.82
	0.5	5	by hand	4.31 ± 0.15	86.2	3.48
	1	10		8.87 ± 0.20	88.7	2.25
				mean	85.8	
	0.2	2	60 min shaking	1.78 ± 0.05	89.1	2.8
	0.5	5	by rotary shaker	4.82 ± 0.18	96.4	3.73
	1	10		9.70 ± 0.32	97.2	3.3
				mean	94.1	
carrot	0.1	0.4	2 min blend	0.37 ± 0.03	92.5	8.11
	0.2	0.8		0.71 ± 0.08	88.8	11.27
				mean	90.7	
	0.1	0.4	5 min shaking	0.32 ± 0.05	80.0	15.63
	0.2	0.8	by hand	0.61 ± 0.12	76.3	19.67
				mean	78.1	
	0.1	0.4	60 min shaking	0.35 ± 0.05	87.5	14.28
	0.2	0.8	by rotary shaker	0.74 ± 0.08	92.5	10.81
				mean	90.0	
spinach	0.5	1	2 min blend	0.93 ± 0.06	93.1	6.45
	1	2		1.81 ± 0.08	90.5	4.42
	1.5	3		2.85 ± 0.12	95.0	4.21
	2	4		3.67 ± 0.10	91.8	2.72
				mean	92.6	
	0.5	1	5 min shaking	0.83 ± 0.04	83.0	4.8
	1	2	by hand	1.78 ± 0.08	89.2	4.5
	1.5	3		2.72 ± 0.06	90.7	2.2
	2	4		3.41 ± 0.12	85.2	3.5
				mean	87.0	
	0.5	1	60 min shaking	0.89 ± 0.08	89.1	9.0
	1	2	by rotary shaker	1.83 ± 0.08	91.5	4.37
	1.5	3		2.76 ± 0.14	92.1	5.07
	2	4		3.51 ± 0.16	87.8	4.56
				mean	90.1	
green tea	0.5	1	2 min blend	0.98 ± 0.02	98.0	2.04
	1	2		2.01 ± 0.05	100.5	2.49
	1.5	3		2.98 ± 0.10	99.3	3.36
	2	4		3.91 ± 0.15	97.8	3.84
				mean	98.9	
	0.5	1	5 min shaking	0.96 ± 0.08	96.0	8.3
	1	2	by hand	1.85 ± 0.13	92.5	7.0
	1.5	3		2.78 ± 0.10	92.7	3.5
	2	4		3.49 ± 0.20	87.2	5.7
				mean	92.1	
	0.5	1	60 min shaking	1.12 ± 0.04	112.0	3.57
	1	2	by rotary shaker	2.12 ± 0.08	106.0	3.77
	1.5	3		2.96 ± 0.12	98.7	4.05
	2	4		3.87 ± 0.10	96.8	2.6
				mean	103.4	
tobacco	0.5	0.5	2 min blend	0.46 ± 0.02	91.6	4.35
	1	1		0.89 ± 0.04	88.7	4.49
	2	2		1.86 ± 0.10	92.8	5.38
	3	3		2.72 ± 0.12	90.7	4.41
				mean	90.9	
	0.5	0.5	5 min shaking	0.41 ± 0.04	82.0	9.7
	1	1	by hand	0.81 ± 0.10	81.2	12.4
	2	2		1.79 ± 0.10	89.5	5.57
	3	3		2.52 ± 0.14	84.1	5.56
				mean	84.1	
	0.5	0.5	60 min shaking	0.51 ± 0.04	102.3	7.8
	1	1	by rotary shaker	0.92 ± 0.06	92.1	6.5
	2	2		1.95 ± 0.16	97.5	8.2
	3	3		2.64 ± 0.15	88.2	5.68
				mean	94.9	

concentration was less than MRL). The confirmation step with the microwell assay and GC method also resulted in negative samples. This means that no false negative or false positive results were obtained. The interpretation of the results was visual. Therefore, the tube assays were highly sensitive and can be used as convenient qualitative tools, particularly interesting for on-site semiquantitative detection of endosulfan in food samples.

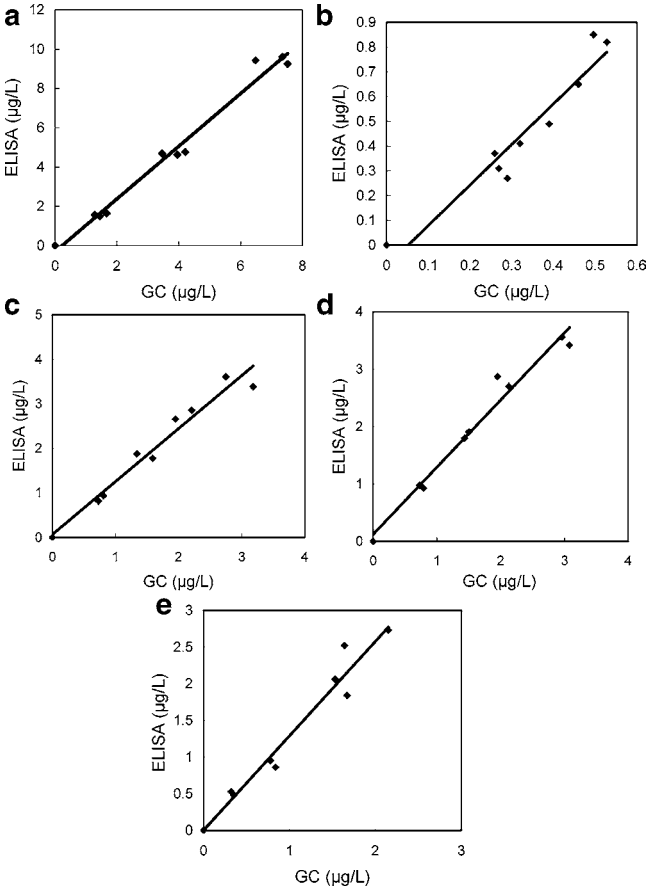

Figure 7. Correlation between ELISA and GC results for each nonpurified agricultural product spiked with endosulfan. (**a**) Grape: $y = 1.3671x - 0.4467$, $r^2 = 0.98$. (**b**) Carrot: $y = 1.6376x - 0.0851$, $r^2 = 0.93$. (**c**) Spinach: $y = 1.1902x + 0.0678$, $r^2 = 0.96$. (**d**) Green tea: $y = 1.1705x + 0.1234$, $r^2 = 0.98$. (**e**) Tobacco: $y = 1.2886x + 0.0013$, $r^2 = 0.96$.

Correlation Studies between ELISA and GC Analysis. Column-purified sample extracts were analyzed by GC, and crude extracts were analyzed by plate ELISA without cleanup. In general, the ELISA data were in good agreement with the GC data for all of the samples. The correlation coefficients (r^2) of the ELISA and GC data are 0.98 in grapes, 0.93 in carrots, 0.96 in spinach, 0.98 in green tea, and 0.96 in tobacco. **Figure 7** displayed the relationship of the ELISA and GC data and the linear regression lined for these data. In summary, there was a good correlation between the ELISA and the GC data for these samples. The slopes were all greater than 1.0 and indicate a high positive bias for the ELISA method relative to the GC method. This was possibly due to the loss of endosulfan incurred as a consequence of sample cleanup and evaporation steps for GC analysis. The accuracy of results obtained using the ELISA method was investigated by comparison of results obtained with GC method. Therefore, the proven ability of this immunoassay to analyze endosulfan rapidly in contaminated samples entails an undoubted practical advantage over methods requiring a tedious sample cleanup procedure.

In conclusion, this article mainly describes the development of two immunoassays including a microwell assay and a tube-based assay. These assays were sufficiently sensitive to detect endosulfan in agricultural products. The developed ELISAs have an IC_{50} value of 5.3 ± 0.4 μg/kg and a LOD of 0.8 ± 0.1 μg/kg for the laboratory test and an IC_{50} value of 1.6 ± 0.2 μg/kg and a LOD of 8.4 ± 0.5 μg/kg for the tube-based method. The specific antibody employed allows for the detection of endosulfan in the presence of other structurally similar pesticides, with the exception of dieldrin and endrin, which have been found to exhibit cross-reactivities (22.6 and 46.4%, respectively). A simple, rapid, and efficient extraction method has been developed for endosulfan. Methanol extracts of some agricultural product samples such as grape, carrot, spinach, and tobacco could be analyzed directly by immunoassay after dilution in 0.5% FG-PBS. Some simple cleanup methods were ineffective in removing interference from green tea. However, use of the coagulating reagent PVP could remove matrix interference effectively. The spike and recovery studies gave about 76−112% recovery with a maximum variation coefficient of ±19.67%. Correlation of ELISA and GC results was good ($r^2 \geq 0.93$). It indicated that these immunoassays could be just as reliable as GC methods for analyzing endosulfan in food samples. Therefore, these ELISA methods could be applied to future large-scale site detection of endosulfan in foodstuffs with high throughputs, rapidity, and lower expense.

ABBREVIATIONS USED

ELISA, enzyme-linked immunosorbent assay; 0.5% FG/PBS, phosphate-buffered saline containing 0.5% fish skin gelation; HRP, horseradish peroxidase; IC_{50}, concentration of analyte giving 50% inhibition of color development; IC_{15}, concentration of analyte giving 15% inhibition of color development; KLH, keyhole limpet hemocyanin; LOD, limit of detection; GC, gas chromatography; ECD, electron capture detector; SPE, solid phase extraction; PVP, polyvinyl pyrrolidone; PBS, phosphate-buffered saline; PBS/T, phosphate-buffered saline with 0.05% Tween 20; BSA, bovine serum albumin; FAO, Food and Agriculture Organization (of the United Nations); CV, coefficient of variation.

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