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REVIEW
The Present State and Perspective on Simple and Rapid Immunochemical Detection for Pesticide Residues in Crops

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Abstract
The presence of pesticide residues in crops is a matter of global public concern, and simple, rapid, and reliable methods for pesticide residue analysis are essential to ensure food safety. In this review, I evaluate a commercially available kit-based enzyme-linked immunosorbent assay (ELISA), which uses specific or selective antigen-antibody interactions, for simple and rapid pesticide residue analysis in crops, particularly before shipment of the crops. The evaluated ELISAs were found to be sufficiently sensitive to detect three pesticides (imidacloprid, fenitrothion, and chlorothalonil) at levels close to the maximum residue limits. Simple dilution of sample extracts alone was sufficient to surmount the problem of matrix interference, which can be troublesome with ELISA. The average recovery rates of the three pesticides exceeded 84%, and the average coefficients of variation were less than 13% for all tested crop samples. The results obtained with the ELISAs correlated well with those obtained by reference chromatographic methods for all three pesticides (r > 0.96). These findings strongly suggest that ELISA is a suitable method for quantitative and reliable screening analysis of these pesticides in crops without the need for sample pretreatment. Elimination of this need can be expected to save time and money and considerably increase sample throughput.

Discipline: Agricultural chemicals
Additional key words: chromatography, ELISA, matrix interference, screening method

Introduction

The poisoning of Chinese frozen dumplings (*gyoza* in Japanese) with the organophosphorus insecticide methamidophos, which is not registered for use to cultivate crops in Japan[6], remains fresh in public memory and sparked a sudden increase in public concern about food safety. Farmers or distributors of crops have tried to provide access to records dealing with crop production processes (for example, the moment of dissemination and the types and frequency of dispersion of agricultural materials such as fertilizer and pesticides) and address public concerns concerning the health risks of pesticide residues. To determine whether pesticide residue levels exceed the prescribed maximum residue limits (MRLs), residue analysis in crops before shipment to market is an important means of ensuring consumer safety. However, any analytical method adopted for such purposes must be simple, rapid and have a high sample throughput.

Gas chromatography (GC) with element-selective detection[15, 22] and high-performance liquid chromatography (HPLC) with UV or fluorescence detection[5, 26, 33] have been used to analyze pesticide residues in crops. More recently, highly sensitive and accurate chromatographic techniques involving mass spectrometric (MS) detection (single MS or tandem MS) have also been used [2, 5, 12, 21]. For accurate determination, chromatographic techniques require multistage sample pretreatment procedures before the analysis. In contrast, the enzyme-linked immunosorbent assay (ELISA), which is based on highly specific or selective antigen-antibody interactions, gives a sensitive response against only one or a few trace level pesticides in various sample matrices, and is therefore a promising method for pesticide residue analysis [9, 11, 20, 27].

Herein, I review the potential utility of ELISA for simple and rapid pesticide residue analysis in crops on

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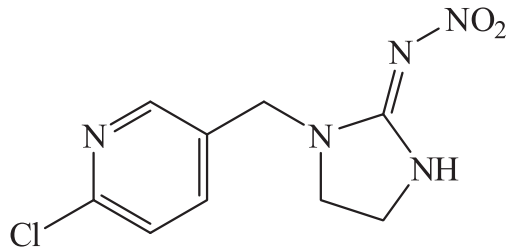
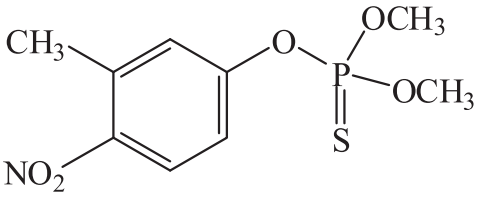
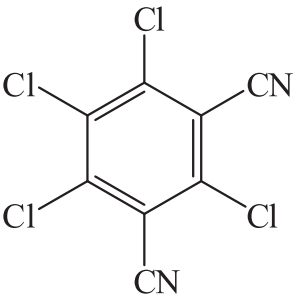


(a) Imidacloprid



(b) Fenitrothion



(c) Chlorothalonil

Fig. 1. Chemical structures of (a) imidacloprid, (b) fenitrothion, and (c) chlorothalonil

the basis of previous research carried out in my laboratory to evaluate kit-based ELISAs for the neonicotinoid insecticide imidacloprid[29, 30], the organophosphorus insecticide fenitrothion[31], and the fungicide chlorothalonil[32] (Fig. 1) developed by Horiba Ltd. (Kyoto, Japan). I also briefly mention some problems regarding the use of ELISA for pesticide residue analysis, and I discuss the future prospects for the method.

Fundamental characteristics of ELISAs

In this section, I evaluated the fundamental characteristics of kit-based ELISAs developed to analyze imidacloprid, fenitrothion, and chlorothalonil residues in crop samples. The evaluated ELISA using a direct competitive method can be used to directly determine the concentration of a pesticide in a sample[9, 23, 27] (Fig. 2). In this method, because a pesticide (antigen) and a given amount of enzyme-labeled pesticide (labeled antigen) competitively bind to the binding site of the antibody, there is no competitive reaction between the two antigens when either is in excess. Accordingly, the range of concentrations formed by a sigmoidal curve dropping to the right (i.e. the ELISA standard curve) corresponds to the dynamic range, and is calculated as the concentration of analyte providing 20-80% inhibition (I_{20-80}) of the maximum signal[18] (Fig. 3). Conversely, I_{50} showing assay sensitivity and I_{10} showing the detection limit can be also calculated as the concentration of analyte at which 50 or 10% of the maximum antibody binding is inhibited[18] (Fig. 3).

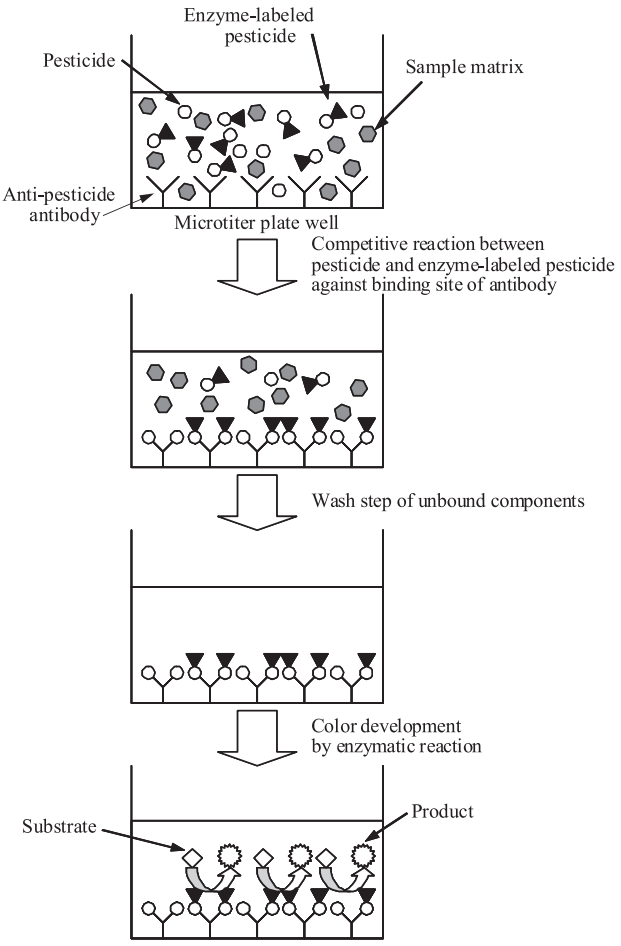


Fig. 2. Principle of direct competitive ELISA

To estimate the above-mentioned analytical parameters of the evaluated ELISAs, standard curves for each pesticide were prepared by using standard solu-

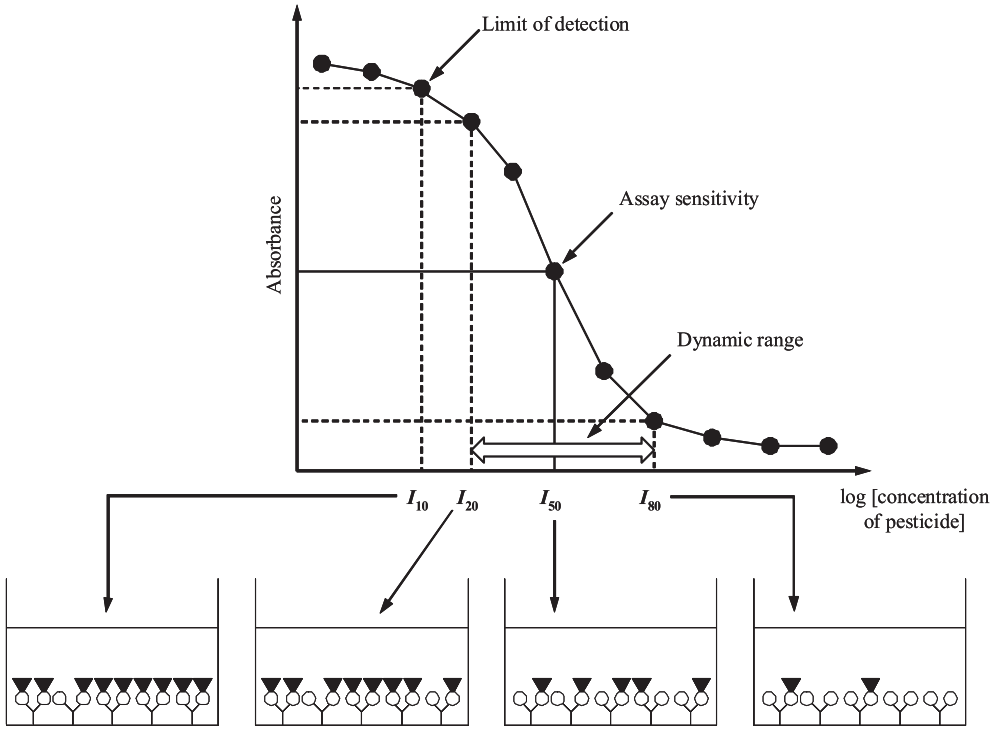


Fig. 3. Typical sigmoidal curve. Absorbance is plotted against log (concentration of pesticide)

tions prepared in the laboratory in water/methanol (9:1) (final methanol concentration in each well, 5%; Fig. 4). The sensitivities (I_{50} values) of the ELISAs for imidacloprid[29], fenitrothion[31], and chlorothalonil[32] exceeded those of previously developed ELISAs (Table 1)[3, 10, 13, 14, 16, 17]. When applying ELISA to pesticide residue analysis in crop samples, the pesticide concentration is diluted by sample pretreatments (extraction, dilution of extracts to adjust the concentration of organic solvents and elimination of matrix interference, and mix with enzyme-labeled pesticide solution) prior to ELISA analysis (Fig. 5). When ELISA is used to confirm whether pesticide residue levels exceed the MRLs for each crop, the dilution factor of sample pretreatments and interpolation of concentration of the MRL after dilution into the dynamic range must be considered. From these perspectives, any evaluated ELISAs can adequately determine the concentration levels close to the MRLs.

Influence of organic solvents on ELISA sensitivity

Water-miscible organic solvents such as acetone[33], acetonitrile[2, 5, 12, 15], and methanol[21, 26] are usually used to efficiently and quantitatively extract pesticides from crop samples. However, the sensitivity of ELISA is known to be affected by the presence of organic solvents, depending on the concentration and type of sol-

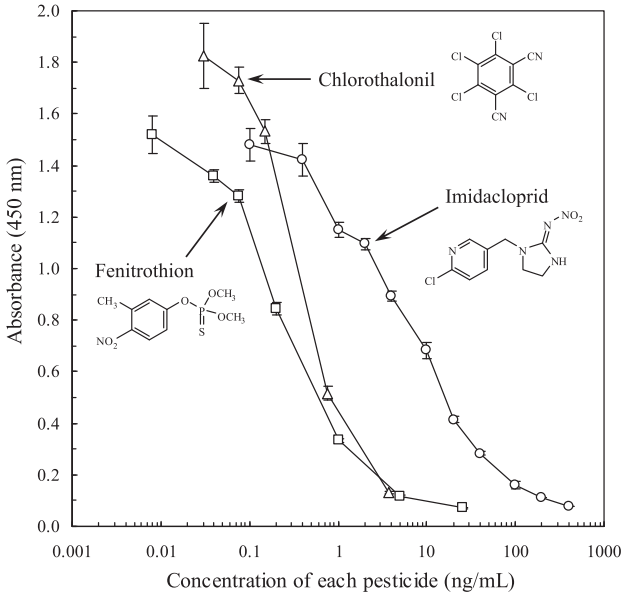


Fig. 4. Standard curves for imidacloprid, fenitrothion, and chlorothalonil produced with standard solutions[29, 31, 32]

The final methanol concentration (in each well) is 5%. Each point is the average of three or four replicate determinations. Error bars indicate ± standard deviations from the average absorbance.

Table 1. Comparison of analytical characteristics of the evaluated ELISAs and previously developed ELISAs

	I_{50} (ng/mL)	Dynamic range (ng/mL)	Detection limit (ng/mL)	Reference
Imidacloprid				
Evaluated ELISA	8	1-39	0.5	29
Polyclonal antibody-based ELISA	17.3	5-125	Data not shown	14
Polyclonal antibody-based ELISA	35	Data not shown	Data not shown	16
Fenitrothion				
Evaluated ELISA	0.23	0.087-2	0.033	31
Polyclonal antibody-based ELISA	3.7	Data not shown	0.5	3
Monoclonal antibody-based ELISA	23	Data not shown	2	17
Chlorothalonil				
Evaluated ELISA	0.34	0.13-1.2	0.052	32
Magnetic particle-based ELISA	1.12	0.1-5.0	0.07	13
Monoclonal antibody-based ELISA	2.7	0.7-11.0	Data not shown	10

vent[11, 20, 23]. Therefore, before ELISA can be used for crop samples, the most suitable organic solvent and the tolerable concentration for each ELISA should be determined as described in several reports[3, 8, 14, 16, 28]. The influences of the three above-mentioned organic solvents were evaluated via standard curves prepared using water containing 5% (final concentration) of each solvent in the well. The evaluated ELISAs showed the weakest influence on the sensitivity (I_{50} value) and the maximum signal (A_{max} value) reached at a zero dose of analyte against methanol (Fig. 6). Acetone and acetonitrile substantially decreased the sensitivity at the same final concentrations as methanol, meaning the latter was the most suitable organic solvent for the evaluated ELISAs[29, 31, 32].

Cross-reactivity of ELISAs

Cross-reactivity between antibodies and compounds that are structurally similar to the target compound is an inherent problem with ELISA. Cross-reactions can affect the analytical result either by suggesting that the target compound is present when it is not (false positive) or by overestimating the concentration of the target compound when both the target and one or more structurally similar compounds are present, hence the cross-reactivity of an ELISA toward the target pesticide and its most probable cross-reactants must be determined. The cross-reactivities of the ELISAs for the three pesticides were evaluated using analogues structurally related to the pesticides (Table 2). The evaluated ELISAs were highly selective for each of the pesticides, but several other pesticides or analogues show significant cross-

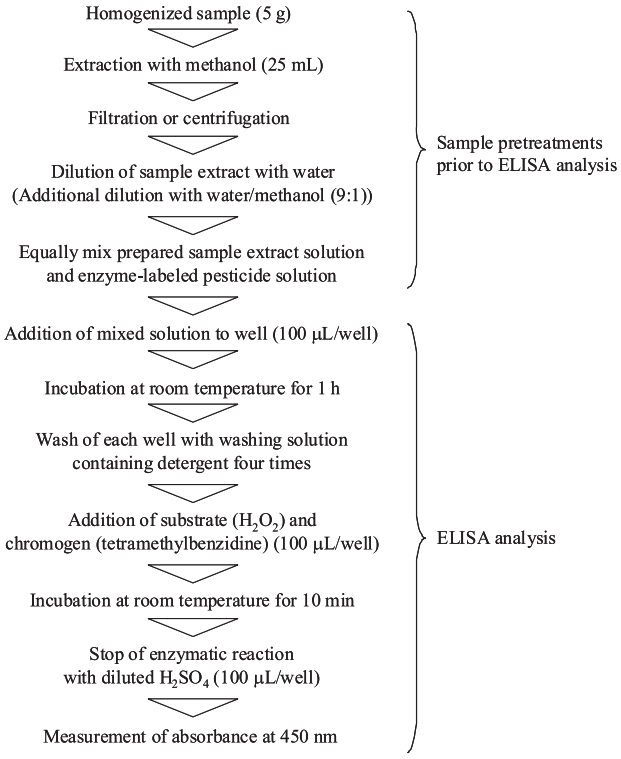
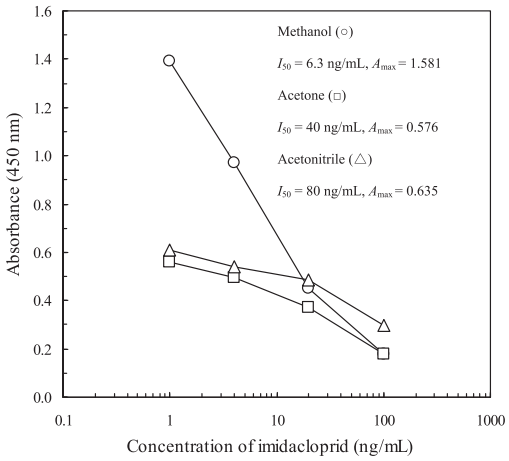


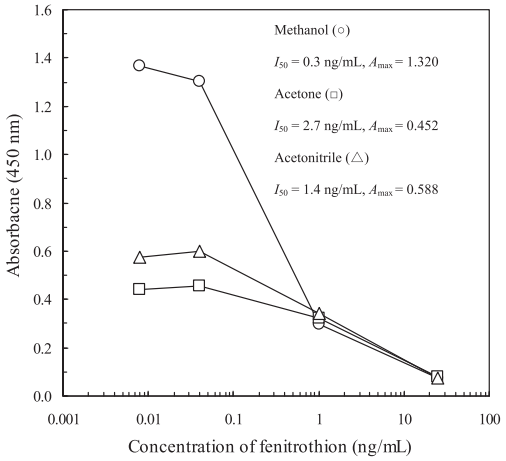
Fig. 5. Common procedures of kit-based ELISA for pesticide residue analysis in crop samples

reactivity: for example, clothianidin (12%) shows cross-reactivity for imidacloprid[29]; EPN (42%), parathion-methyl (13%), and parathion (12%) show cross-reactivity for fenitrothion[31]; and fthalide (59%), pentachloronitrobenzene (quintozene, 20%), and some non-agrochemical compounds show cross-reactivity for chlo-

(a) Imidacloprid



(b) Fenitrothion



(c) Chlorothalonil

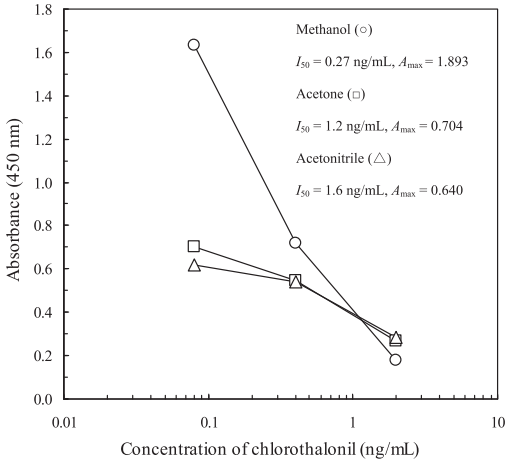


Fig. 6. Influence of commonly used organic extractants, methanol, acetone, and acetonitrile for pesticide residues in crop samples on ELISA performance[29, 31, 32]

The data are the average of two replicates. The final concentration of each solvent (in each well) is 5%.

rothalonil[32]. Such cross-reactivities cannot be ignored, and when the ELISA results are unusual or doubtful or the assay shows false positives for certain samples, the samples in question should be subjected to chromatographic analyses to confirm the ELISA results. Note however that because the use of parathion-methyl and parathion has been banned in Japan due to their high toxicity, no domestic crops should contain residues of these compounds. However, because they may be used abroad, especially in developing countries, the potential for cross-reactivity must be considered when dealing with imported crops.

Elimination or minimization of interference from the sample matrix

Matrix interference is a common problem in pesticide residue analysis, both when using ELISA[9, 20, 24] and chromatographic methods[2, 12, 15]. For both methods however, the problem can generally be resolved via appropriate sample pretreatment procedures such as solid-phase extraction (SPE), classical column chromatography, and so on (Fig. 7). However, the advantages of the ELISA method (as described above) are negated by these complicated pretreatment procedures. As described in several previously published reports[8, 25, 28–32], the simplest way to avoid matrix interference is to dilute the sample extract with water or a buffer (Fig. 7).

To evaluate matrix interference on assay performance, pesticide-free methanol extracts were prepared, and then properly diluted either with water or water/methanol (9:1), and serial standard solutions prepared with each diluted extract. Conversely, similar solutions were prepared with water/methanol alone (9:1) as a control. The matrix interference in the analysis of crop samples was quantified by comparing control curves with those generated in the diluted extract solution. As shown in Fig. 8, since all standard curves in diluted apple extracts substantially agreed with the control curves, the results suggest that any pesticides evaluated can be directly analyzed in the tested samples only by simple dilution of the extracts with water or water/methanol (9:1)[29–32]. However, when the ELISA for imidacloprid was used to analyze spinach samples, avoiding matrix interference was difficult, even with additional dilution (data not shown)[30]. For such samples, a minimal sample pretreatment procedure such as SPE may be necessary to prevent matrix interference.

Table 2. Cross-reactivity of the ELISAs toward analogues structurally related to the target pesticide[29, 31, 32]

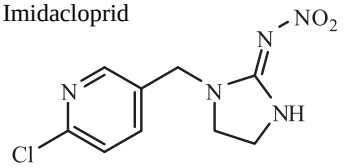
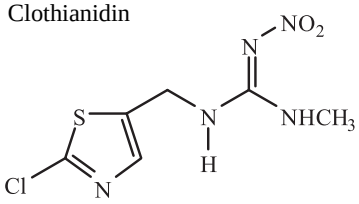
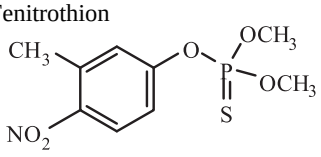
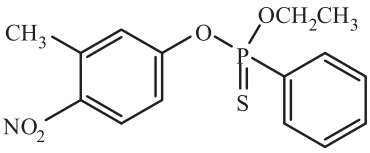
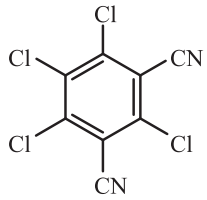
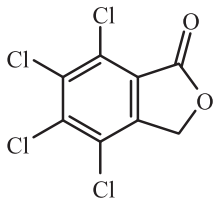
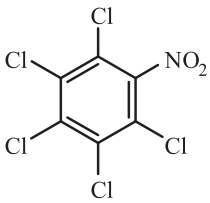
Analogue	I_{50} (ng/mL)	Cross-reactivity (%) [a]
ELISA for Imidacloprid		
Imidacloprid 	5	100
Clothianidin 	42	12
Thiacloprid	600	0.8
Acetamiprid	2,400	0.2
Thiacloprid-amide [b]	8,000	0.06
Nitenpyram	> 10,000	< 0.05
Dinotefuran		
Thiamethoxam		
6-Chloronicotinic acid [b]		
ELISA for Fenitrothion		
Fenitrothion 	0.22	100
EPN 	0.52	42
Parathion-methyl	1.7	13
Parathion	1.8	12
Chlorthion	6	4
Fenitrooxon [b]	18	1
Dicapthon	20	1
EPN-oxon [b]	270	0.08
Paraoxon-methyl [b]	400	0.06
Paraoxon [b]	710	0.03
Fenthion	900	0.02
Mesulfenfos	1,000	0.02
Fenthion sulfone [b]	>1,000	<0.02
Dichlofenthion		
Prothiofos		

Table 2. Continued.

Analogue	I_{50} (ng/mL)	Cross-reactivity (%) [a]
Chlorpyrifos-methyl		
Chlorpyrifos		
Diazinon		
Pirimiphos-methyl		
Pirimiphos-ethyl		
Pyridafenthion	>1,000	<0.02
Phenthoate		
Dimethoate		
Trichlorfon		
Acephate		
Methamidophos		
ELISA for Chlorothalonil		
Chlorothalonil 	0.28	100
Tetrachloroterephthalonitrile [b]	0.31	97
Tetrachlorophthalonitrile [b]	0.41	68
Fthalide 	0.51	59
Pentachloronitrobenzene (Quintozene) 	1.5	20
Pentachloroaniline [b]	2.2	14
Tetrachlorophthalimide [b]	3.5	8
Pentachloroanisole [b]	6	5
Pentachlorophenol	8	4
2, 3, 4, 6-Tetrachlorophenol [b]	51	0.6
Tetrachlorophthalic anhydride [b]	60	0.5
Isophthalonitrile [b]	> 10,000	< 0.01

[a] : Cross-reactivity(%)=(I_{50} of target pesticide / I_{50} of other chemicals)×100.
[b] : These compounds are metabolites of the parent pesticide or non-agrochemicals.

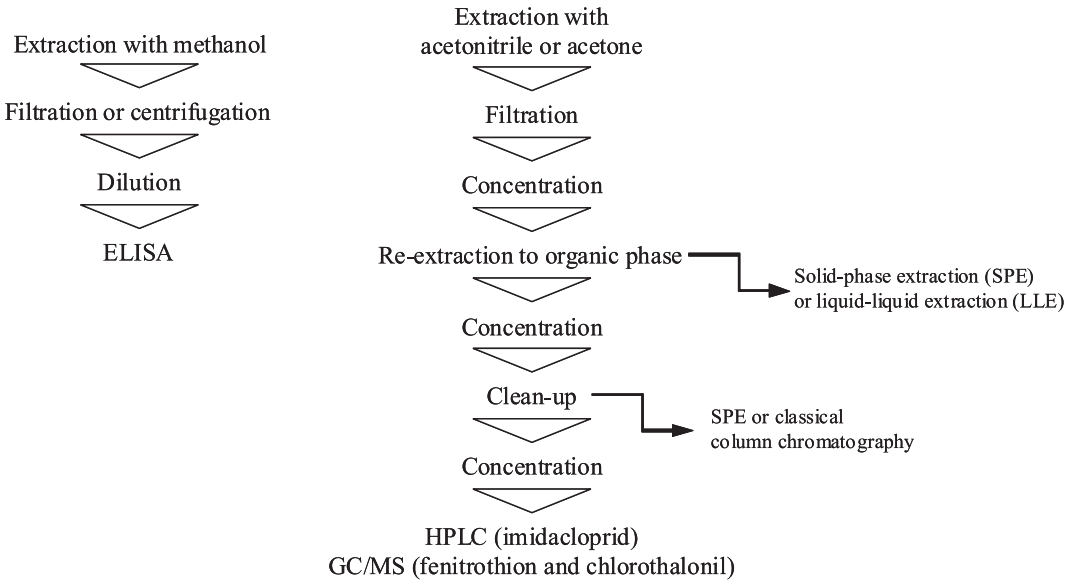


Fig. 7. Sample pretreatment procedures to eliminate or minimize matrix interference for the ELISA and the chromatographic methods[29–32]

Reliability of ELISA and comparison between ELISA and conventional chromatographic techniques

To investigate the reliability of ELISAs, several kinds of crop samples artificially spiked with imidacloprid, fenitrothion, or chlorothalonil at various concentrations were analyzed (Table 3). Average recoveries ranged from 85 to 105% for imidacloprid[29, 30], 111 to 112% for fenitrothion[31], and 102 to 114% for chlorothalonil[32]; with average coefficients of variation less than 13% for the three pesticides. The high recovery rates for the spiked crop samples suggest that the ELISAs are a suitable method for the simple and rapid analysis of residues of these pesticides.

The correlation coefficients (r) of the ELISA and the reference chromatographic results were 0.9938 in imidacloprid, 0.9648 in fenitrothion, and 0.9860 in chlorothalonil respectively[29–32] (Fig. 9). The ELISA for imidacloprid showed the highest correlation with the HPLC method in the evaluated ELISAs. Although the HPLC method for imidacloprid showed a tendency to estimate the concentration to a relatively higher extent than the ELISA results for some spiked crop samples (Fig. 9-(a)), the difference between both results was slight and may be within the permissible range considering that ELISA is generally a suitable analytical technique as a screening method. Conversely, it was presumed that for the following two results, namely, the ELISA for fenitrothion showed a tendency toward overestimation due to matrix interference likely coming from apple and peach samples (Fig. 9-(b)), and the recovery rates depending on the crop varieties were low in the GC/MS method for chlorothalonil (Fig. 9-(c)), which may account for the dispersion between ELISA and the chromatographic methods.

Altogether, the correlation between ELISAs and the chromatographic methods differed depending on the types of pesticides used. Although fundamentally, the ELISA results were nearly equal to the chromatographic results, or tended to somewhat overestimate theoretical values except for some imidacloprid results, when considering average recovery rates as a whole (Fig. 9), the evaluated ELISAs showed tolerable agreement with the chromatographic methods for the three pesticides, and it can be concluded that all evaluated ELISAs are practical analytical techniques that can be used as quantitative screening methods to detect pesticides in crop samples, as described in several related reports[1, 8, 19, 28].

Potential ranking of ELISAs in pesticide residue analysis and prospective outlook

Due to the important role of crops and foods in human health, maintaining their quality is essential. In pesticide residue analysis, which contributes significantly to ensuring food safety, simple, rapid, sensitive and reliable analytical methods are needed. ELISA, one of the immunochemical methods, provides a screening method to fulfill the above-described four requirements because they have several advantages over conventional chromatographic techniques: eliminating the need for

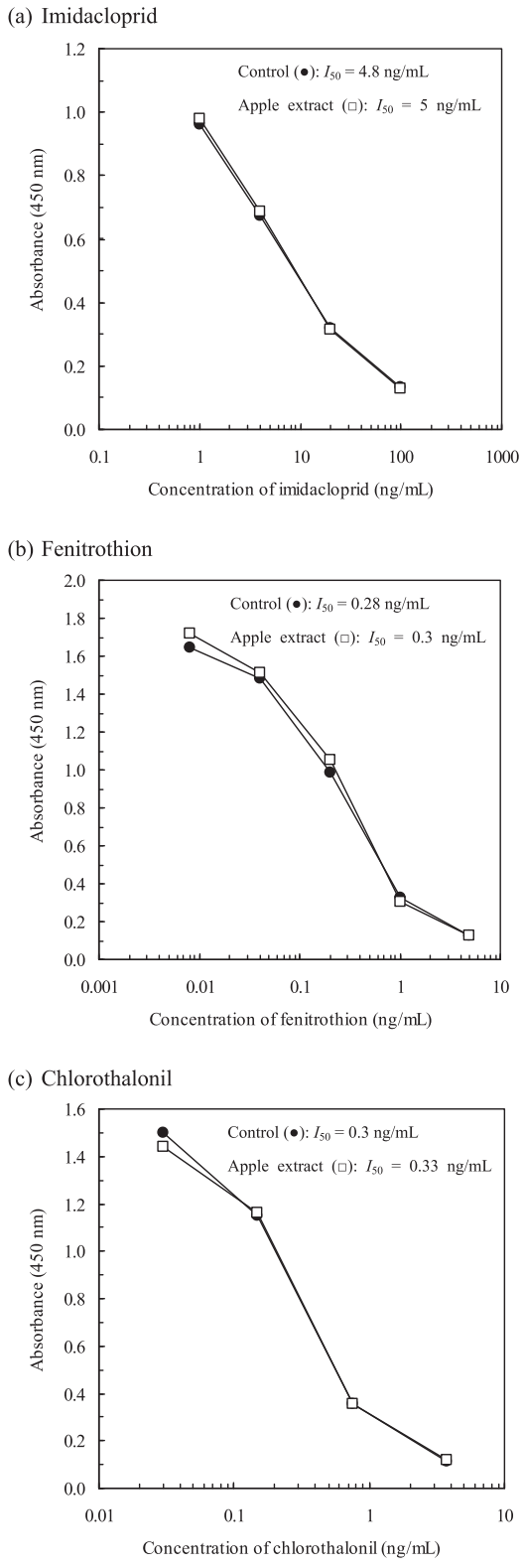


Fig. 8. Influence of matrix interference on ELISA standard curves in apple extracts[29, 31, 32]
The data are the average of two replicates.

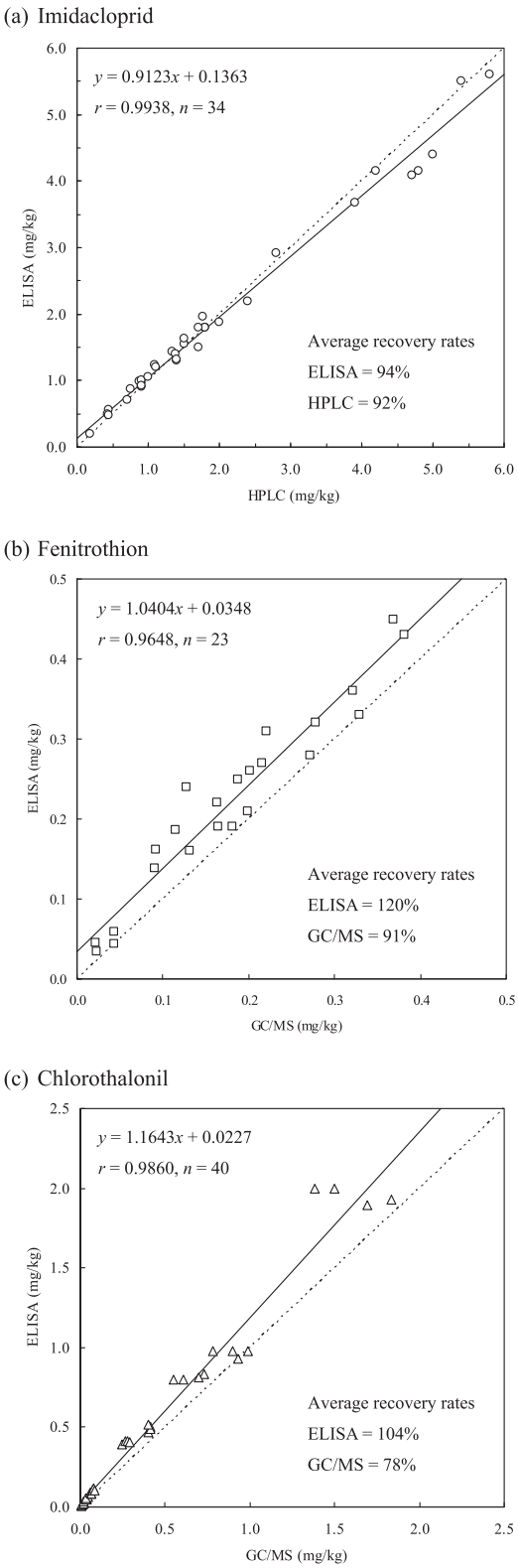


Fig. 9. Correlations between estimated concentrations of three pesticides in spiked crop samples determined by ELISAs and reference chromatographic methods[29–32]
The dotted line corresponds to a perfect correlation ($y = x$).

Table 3. Accuracy of the ELISAs on crop samples spiked at a wide range of concentrations[29–32]

Pesticide	Crop sample	Spiked concentration range (mg/kg)	MRLs in Japan (mg/kg)	Average recovery [a] (%, n = 3 [b])	Average CV [a] (%)
Imidacloprid	Apple	0.1 − 2	0.5	99	7
	Cucumber	0.5 − 1.5	1	105	9
	Eggplant	0.5 − 1.5	1 [c]	104	7
	Lettuce	1−3	2 [c]	85	9
	Green pepper	4−6	5 [c]	96	7
Fenitrothion	Apple	0.1 − 0.4	0.2	112	10
	Peach	0.1 − 0.4	0.2	111	12
Chlorothalonil	Apple	0.01 − 1.0	2	102	6
	Peach	0.01 − 1.0	2	109	3
	Cucumber	0.01 − 1.0	5	114	3
	Tomato	0.01 − 1.0	5	112	5

[a]: Each value is an average of all spiked concentrations.
[b]: Each spiked concentration was evaluated three times to verify repeatability.
[c]: The values are as of May 2004, and the MLRs prescribed for these crops have been revised.

both time-consuming sample pretreatment procedures, and sophisticated skills for operation and maintenance of analytical instruments. Taking the opportunity for development of an immunochemical method based on radioimmunoassay (RIA) for organophosphorus parathion by Ercegovich et al.[4], the methods, mainly ELISAs supplanted with RIAs, have been aggressively developed and applied as simple and rapid analytical means for wide-ranging pesticides in various samples as shown in numerous reviews[9, 20, 23, 27]. However, it is unfortunate that most are restricted to in-house use, and in relative terms, the number of universally accessible commercial kit-based ELISAs remains small[7].

In Japan, kit-based ELISAs for about 20 kinds of pesticides have been released on the market to date. Although kit-based ELISAs are ready-to-use, the supposed users - academic and industrial researchers, and end-users - should not only be aware of the above-mentioned advantages of ELISA but also its analytical characteristics, such as the influences of the organic solvent for the extraction procedure on assay sensitivity, cross-reactivity against structurally-related analogues, matrix interferences having originated from real sample matrices, and so on. Therefore, I have evaluated the analytical performance of three kinds of kit-based ELISAs for their practical application in pesticide residue analysis. All the evaluated ELISAs have sufficient sensitivity to detect target pesticides at MRL levels for the tested crop samples. Furthermore, it has been suggested that they can simply, rapidly and reliably determine each pesticide in crop samples following minimization of matrix interference merely by simple dilution of sample extracts, substantially excellent recovery rates, and good correlation between the ELISAs and chromatographic methods.

Finally, I concluded that the kit-based ELISAs evaluated to date surely provide not only simplicity and rapidity but also reliability in pesticide residue analysis through these findings, and that they are particularly well-suited for use as a preliminary screening method for pesticide residues in crops before shipment, making a significant contribution toward ensuring food safety.

Moreover, I am constantly aware of the vital need to select analytical methods carefully according to circumstance, and ELISAs should be ranked as methods that complement chromatographic methods for pesticide residue analyses.

Acknowledgments

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