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Review

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Review of Enzyme-Linked Immunosorbent Assays (ELISAs) for Analyses of Neonicotinoid Insecticides in Agro-environments

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ABSTRACT: Immunoassay is a promising method that is suitable for rapid and simple analyses of pesticides, which are likely to persist at a trace level in agro-environments, including agricultural products, soil, and water. Particularly, enzyme-linked immunosorbent assay (ELISA) has wide application to development of analytical methods for pesticide residues because it can very sensitively and very accurately determine them in samples. This paper presents a review of the fundamental analytical performance, a device for the sample pretreatment methods before determination, and cases of applications to various samples on ELISA methods that have been developed for detection of neonicotinoid insecticides in food or environmental matrices. The reviewed ELISAs can be ranked as quantitative, rapid and simple analytical methods for single analytes. The recognition of ELISA as an analytical methodology for pesticide residues is expected to advance rapidly in the future.

KEYWORDS: *immunoassay, ELISA, neonicotinoid insecticides, pesticide residue, matrix effect*

INTRODUCTION

Pesticides are vital agricultural materials necessary for the stable production of agricultural products. They show various physiological activities such as insecticidal, fungicidal, or herbicidal action, necessitating that their actual behavior and that of their residues be supervised in terms of food safety and environmental preservation. Conventionally, gas chromatography (GC) with element-selective detectors or high-performance liquid chromatography (HPLC) with a UV detector has been used for pesticide residue analyses. Because of drastic technical innovation in recent years, highly accurate and sensitive analytical instruments such as GC or HPLC with mass spectrometry (MS) or tandem MS (MS/MS) has familiarized pesticide residue analyses.[1−4] They can provide both qualitative and quantitative information simultaneously, and can simultaneously analyze numerous pesticides.

The immunoassays selected in this review have been widely used in clinical tests for disease diagnosis or in the field of biochemistry. Reports of the world's first development of immunoassay described the use of radioimmunoassay (RIA) for detection of insulin by Yalow and Berson.[5,6] Using immunoassay for determination of trace amounts of pesticide or environmental pollutant by Hammock and Mumma in 1980,[7] Western countries such as the United States of America, Spain, and Australia have advanced energetically to take the lead in the research and development of immunoassays for various pesticides.[8−13] Because immunoassay is an analytical technique based on the specific reactivity of an antibody against an antigen, i.e., antigen−antibody interaction, it can theoretically detect a target analyte accurately in a sample containing a complicated matrix component. Immunoassay techniques possess a predominant benefit that they obviate troublesome sample pretreatment procedures, especially cleanup and concentration stages, which are indispensable for pesticide residue analyses using chromatographic techniques.[11,12] Immunoassays have various modifications such as RIA and fluorescent immunoassay (FIA). Those methods notwithstanding, enzyme-linked immunosorbent assays (ELISAs) have remained the most versatile methods among immunoassays for pesticide residue analyses.

Imidacloprid, which is the pioneer among neonicotinoid insecticides, acts as agonist on the insect postsynaptic nicotinic acetylcholine receptors. Developed as a systemic pesticide showing prominent insecticidal effects at low doses, it has been used globally as a next-generation insecticide, replacing classical insecticides such as organophosphorus, carbamate, and synthetic pyrethroid insecticides. Neonicotinoid insecticides such as acetamiprid, thiamethoxam, and thiacloprid have been marketed one after another. The active ingredients of seven kinds are used at present. Because the thermolability and high polarity of the neonicotinoid insecticides generally make them difficult to analyze using GC, HPLC with UV, MS or MS/MS is currently preferred for determination in various matrices.[14] In 2000, an ELISA based on polyclonal antibody (PoAb) against imidacloprid was reported for the first time by Li and Li[15] Shortly thereafter, ELISAs for detection of other neonicotinoid insecticides such as acetamiprid and thiamethoxam,[14] and even ELISA kits have appeared on the market.[16,17]

This review is intended to summarize the reports in the literature published on the development of the ELISAs and other immunoassays for detection of neonicotinoid insecticides and ELISA application to agro-environmental samples such as agricultural products, soil, and water.

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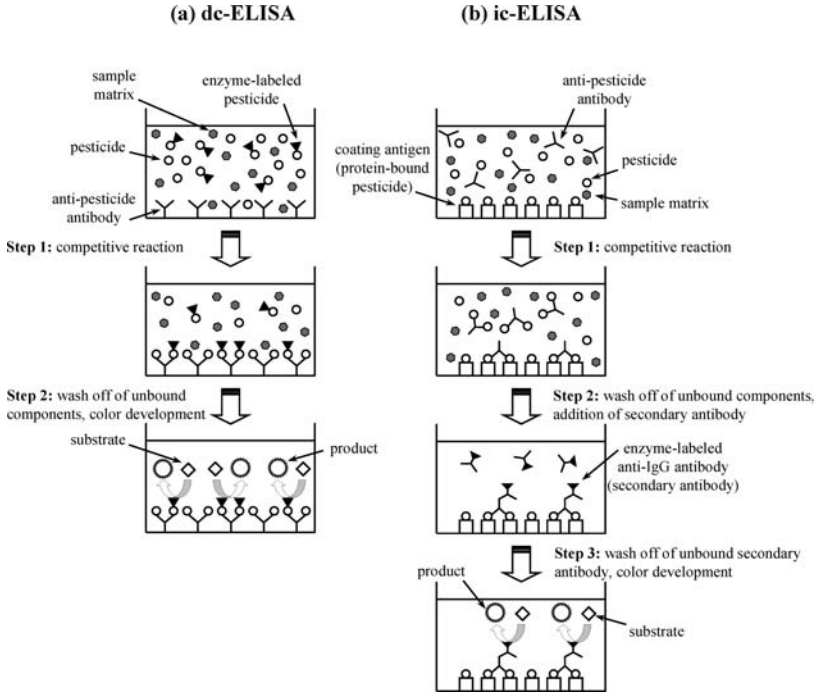


Figure 1. Principles of competitive ELISAs.

PRINCIPLE OF ELISA FOR NEONICOTINOID INSECTICIDES

ELISA is an analytical technique by which a complex yielded by antigen−antibody interaction is detected secondarily through an enzyme with which the antigen or the antibody is labeled. Color development of the substrate by catalytic action of the enzyme is effective for intensifying and visualizing antigen−antibody interaction. Therefore, ELISA is a highly sensitive and accurate method. It remains the most versatile method among various immunoassays. Formats of two kinds, direct competitive ELISA (dc-ELISA) and indirect competitive ELISA (ic-ELISA), are generally used for ELISAs intended for the evaluation of neonicotinoid insecticides.

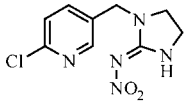
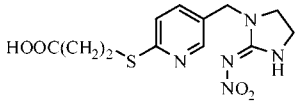
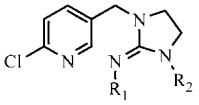
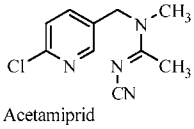
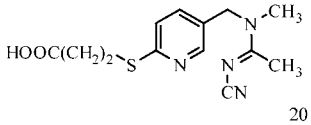
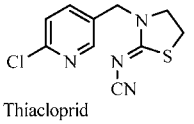
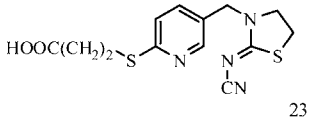
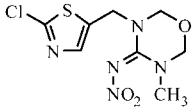
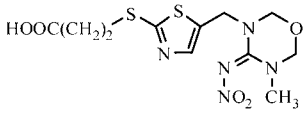
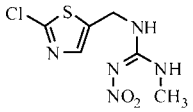
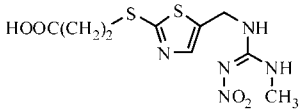
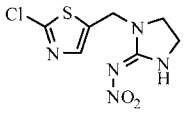
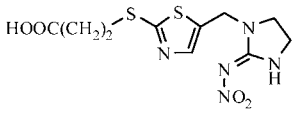
dc-ELISA Format. The dc-ELISA derives from the fact that the format detects a pesticide in a sample directly based on competitive antigen−antibody interaction between a target pesticide (antigen) and a derivative of the target pesticide covalently conjugated with an enzyme (enzyme-labeled pesticide) beforehand. As shown in Figure 1a, dc-ELISA uses a format that is immobilized with antibody of a fixed quantity on the surface of each well of a microtiter plate. Pesticide and enzyme-labeled pesticides are combined competitively with the immobilized antibody in each well (Step 1 in Figure 1a). Because the quantities of the immobilized antibody and the enzyme-labeled pesticide are constant, the competitive reaction varies depending on the amount of a target pesticide in a sample. Here, the concentration range of pesticide showing competitive reaction corresponds to the dynamic (or working) range of the dc-ELISA format. After the competitive reaction for constant time, the unbound components are washed off; then the absorbance is measured by adding a substrate of the labeled enzyme and developing color (Step 2 in Figure 1a). The pesticide concentration in samples is estimated based on the calibration curve produced by plotting the known concentrations of pesticide in the x-axis and the yielded absorbance in the y-axis. All commercially available ELISA kits for neonicotinoid insecticides use this direct competitive format.[16,17]

ic-ELISA Format. In fact, ic-ELISA derives from indirect detection of a pesticide by capturing a complex of protein-bound pesticide (coating antigen) and antibody generated by interaction with enzyme-labeled secondary antibody recognized as an antipesticide antibody. As shown in Figure 1b, ic-ELISA uses the format, which is immobilized (coated) with coating antigen of the fixed quantity on the surface of each well of microtiter plate. The pesticide and coating antigen are combined competitively with the antibody in each well (Step 1 in Figure 1b). Because the quantity of the antibody and the coating antigen are constant, the competitive reaction varies depending on the amount of a target pesticide in a sample. Like the dc-ELISA format, the concentration range of pesticide showing competitive reaction corresponds to the dynamic range of the ic-ELISA format. After the competitive reaction for some time, the unbound components are washed off; then secondary antibody is added (Step 2 in Figure 1b). After washing off of the unbound secondary antibody, the absorbance is measured by adding a substrate and developing color (Step 3 in Figure 1b). The pesticide concentration in samples is estimated based on the calibration curve, which is produced similarly to the dc-ELISA method.

DESIGN STRATERGY OF HAPTEN MOLECULE

Although low-molecular compounds such as pesticides generally do not show immunogenicity, these compounds acquire it by combination with high-molecular compounds such as proteins. Such compounds are called hapten molecules. However, the combination of a pesticide and a protein (a carrier protein) often must newly introduce a functional group into the target pesticide's molecule. A carboxyl group is often used as the functional group for covalent conjugation with the free amino group in the protein molecule. It is also necessary to maintain a certain distance between a pesticide's and a carrier

Table 1. Chemical Structures of Neonicotinoid Insecticides, Of Which Antibody Had Developed, and Hapten Molecules for Immunization and for Assay Development

Insecticide	Hapten, Ref.	
Type 1 (chloropyridine ring)		
 Imidacloprid	 15,20−22	 For immunization: $R_1 = NO_2$, $R_2 = CO(CH_2)_2COOH$ For assay: $R_1 = NHCO(CH_2)_2COOH$, $R_2 = H$ 19
 Acetamiprid	 20	
 Thiacloprid	 23	
Type 2 (chlorothiazole ring)		
 Thiamethoxam	 24,25	
 Clothianidin	 26,27	
 Imidaclothiz	 28	

protein's molecules by introducing a linkage group comprising some methylene ($-CH_2-$) chains. Furthermore, the position to introduce the carboxyl group and the linkage group into the target pesticide's molecule is also important to acquire an antibody showing the desired reactive property (sensitivity and specificity). Therefore, the molecular design of the carboxylic derivative (that is, hapten molecules) for preparation of a complex with carrier protein strongly affects the fundamental property of antibodies. From a series of previous research efforts at developing ELISAs for various pesticides, the fundamental strategy for design of the hapten molecule for a pesticide is thought to be desirable to introduce the carboxyl group and the linkage group into the position as far as possible away from the structural characteristics of a target pesticide moiety.[11,18]

An enzyme-labeled or a protein-bound pesticide is used in each ELISA format, as described in a previous section (Figure 1). Therefore, it is necessary to design and synthesize hapten molecules for assay development. The same hapten molecule used as an immunogen for antibody production can be appropriated for assay development as it is (homologous ELISA). In addition, using other hapten molecules with different chemical structure of hapten molecule for immunogens, the analytical sensitivity or specificity to a target pesticide might be improved (heterologous ELISA).[11,18]

Table 1 presents chemical structures of neonicotinoid insecticides for which ELISA has been developed and designed, with hapten molecules used for immunogen and assay development. Neonicotinoid insecticides are classifiable into two types, as possessing a chloropyridine ring (e.g., imidacloprid, acetamiprid, and thiacloprid; Type 1 in Table 1) and with a chlorothiazole ring (e.g., thiamethoxam, clothianidin, and imidaclothiz; Type 2 in Table 1) based on their chemical structure characteristics. It is clear that the fundamental design of hapten molecules in neonicotinoid insecticides is common for the following points (1) replacing a chlorine atom in each heterocyclic ring with a sulfur atom, (2) extending the linkage group with two methylene chains from the sulfur atom, and (3) introducing a carboxyl group on the end finally. The design of hapten molecules for neonicotinoid insecticides agrees well with the concept of the general strategy described above.[11,18]

The ELISAs developed for neonicotinoid insecticides have made use of homologous format with same hapten molecules in immunogen and assay development (Table 1). However, related to imidacloprid, one report describes a hapten molecule

Table 2. Cross-Reactivities of Developed ELISAs and Assessed Commercial ELISA Kits Against Structurally Related Neonicotinoid Analogues

	imidacloprid									acetamiprid	
	PoAb				MoAb			NR[a]	MoAb	MoAb	
	ic-ELISA	ic-ELISA	ic-ELISA	ic-CL-ELISA	dc-ELISA	ic-ELISA	ic-ELISA	dc-ELISA kit	dc-ELISA kit	dc-ELISA	dc-ELISA kit
imidacloprid	**100** (35)[b]	**100** (17.3)	**100** (2.7)	**100** (15)	**100** (6.4)	**100** (1.6)	**100** (6.2)	**100** (1.05)	**100** (5)	0.3	0.62
acetamiprid	–[c]	7.1	0.3	***19***[d]	0.3	0.6	0.6	***24***	0.21	**100** (1.3)	**100** (0.8)
thiacloprid		1.5						***81***	0.83		***40***
thiamethoxam						NC[e]	<0.1	0.05	<0.05		<0.02
clothianidin			<0.01			3.6	3.6	0.07	***11.9***		0.1
dinotefuran						NC	<0.1		<0.05		0.031
nitenpyram			<0.01		0.3				<0.05	<0.1	0.025
imidaclothiz											
ref	15	19	22	29	20	21	30	17	31	20	32

	thiacloprid	thiamethoxam				clothianidin			dinotefuran	imidaclothiz
	PoAb	PoAb	MoAb			MoAb	PoAb		MoAb	MoAb
	ic-ELISA	dc-ELISA	FIA	ic-ELISA	dc-ELISA kit	dc-ELISA	ic-ELISA	ic-CL-ELISA	dc-ELISA kit	ic-ELISA
imidacloprid	0.23	0.8	NC	<0.01	0.095	<0.1	0.8	0.4	<0.1	***91.7***
acetamiprid	0.72	<0.4	0.01	0.01	0.084	<0.1	<0.05	<0.02	<0.1	<0.05
thiacloprid	**100** (10)				1.8	<0.1	<0.05	<0.02	<0.1	<0.05
thiamethoxam	<0.01	**100** (9.0)	**100** (0.03)	**100** (0.5)	**100** (0.76)	<0.1	<0.05	<0.02	<0.1	<0.05
clothianidin	0.12	1.8	0.06	0.06	0.45	**100** (4.4)	**100** (46)	**100** (15)	***184***	
dinotefuran	0.02	<0.25	NC	<0.01	<0.01	***64***	***11.8***	***9.4***	**100** (7)	
nitenpyram	<0.01				<0.01	<0.1	<0.05	<0.02	<0.1	<0.05
imidaclothiz	<0.01						0.6	0.4		**100** (87.5)
ref	23	24	25	30	33	26	27	27	34	28

[a]Not reported. [b]Units of IC_{50} value were unified in nanograms per milliliter. [c]Not reported. [d]Bold and italic figures denote insecticides that showed significant cross-reactivity. [e]No competition.

for an immunogen by introducing a linkage group into a nitrogen atom at the 3-position of the imidazolidine ring, which is the characteristic structure that was designed.[19] Furthermore, other hapten molecules for assay development were designed as different from the hapten molecules for immunogen in the chemical structure (Table 1). A highly sensitive heterologous ELISA by comparison with the previous homologous ELISA described by Li and Li[15] was developed. This was the sole ELISA based on a heterologous format for neonicotinoid insecticides.

FUNDAMENTAL CHARACTERISTICS OF ELISA FOR NEONICOTINOID INSECTICIDES

ELISAs developed for determination of pesticides must be studied for their reactivity to a target pesticide and for their cross-reactivity with chemical structure analogues as fundamental analytical parameters. Cross-reactivity of an antibody is a common problem for immunoassays. Cross-reactivities of the ELISAs developed for neonicotinoid insecticides are shown in Table 2. Although most ELISAs showed high specificity only to a target insecticide, some of them highly cross-reacted with other neonicotinoid insecticides.[17,26−29,31,32,34] Because ELISA methods have no capability to identify an unknown compound, they cannot discriminate a target compound from potential cross-reactants if a sample contains a compound showing cross-reactivity. Therefore, ELISA should be used for pesticide residue analysis after due consideration about its inherent problems.

However, the dynamic range to a target compound is an important parameter. As clarified from Table 3 showing the dynamic range of the reported ELISAs, the range greatly varies

Table 3. Dynamic Range of Developed ELISAs and Assessed Commercial ELISA Kits

insecticide	assay type	dynamic range (ng/mL)[a]	ref
imidacloprid	ic-ELISA	5−125[b]	19
		0.1−4.0	21
	dc-ELISA	1.3−50	20
	dc-ELISA kit	0.2−6	17
		1−39[b]	31
acetamiprid	dc-ELISA	0.3−12.5	20
	dc-ELISA kit	0.18−3[b]	32
thiamethoxam	dc-ELISA kit	0.21−4.1[b]	33
clothianidin	dc-ELISA	1.5−15[b]	26
	ic-ELISA	2.8−770[b]	27
	ic-CL-ELISA	1.4−150[b]	27
dinotefuran	dc-ELISA kit	1.0−30[b]	34
imidaclothiz	ic-ELISA	17.8−745	28

[a]Units of dynamic range were unified in nanograms per milliliter. [b]IC_{20}-IC_{80}.

because of the reactivity of the antibody used in an ELISA, even if the same pesticide was determined. Furthermore, because of the narrow range from 10 times to 40 times, when estimating the concentration of a pesticide by application to actual samples, dilution magnification of a sample should be adjusted so that the final concentration of a target pesticide at the time of the determination goes into the dynamic range.

Imidacloprid. Numerous examples exist of development of ELISAs for imidacloprid: specifically, four kinds of PoAb-based ic-ELISAs,[15,19,22,29] and three kinds of monoclonal antibody (MoAb)-based dc-ELISA[20] and ic-ELISAs[21,30] have been

reported. Moreover, easy-to-use kits based on the dc-ELISA format are commercially available from Horiba, Ltd.[16] and EnviroLogix Inc.[17] The cross-reactivities of these ELISAs against various structurally related neonicotinoid analogues have been assessed (Table 2).

Although PoAb-based ic-ELISA developed by Li and Li[15] was no examination of the cross-reactivity against other neonicotinoid insecticides, it showed somewhat high cross-reactivity against major metabolites of imidacloprid (imidacloprid olefin, 16%; 5-hydroxy imidacloprid, 11%). However, ic-chemiluminescent-ELISA (ic-CL-ELISA) developed by Girotti et al.,[29] who used a PoAb prepared by Li and Li[15] showed high cross-reactivity against acetamiprid (19%). A heterologous ic-ELISA with PoAb developed by Lee et al.[19] showed minor cross-reactivity with acetamiprid (7.1%) and remarkable one cross-reactivity with ketonic metabolite (1-[(6-chloro-3-pyridinyl)-methyl]imidazolidin-2-one, 152%). A PoAb-based ic-ELISA developed by Wang et al.[22] is reported as highly specific to imidacloprid because of its slight cross-reactivity with acetamiprid and nitenpyram, which belong to the same category, with a chloropyridine ring (Table 1).

Watanabe et al.[20] made a point of the specificity of the prepared MoAbs rather than reactivity to imidacloprid in development of dc-ELISA: the reactivity of the selected MoAb to the insecticide was an eighth of that of the most sensitive MoAb. However, because the selected MoAb showed little cross-reactivity to acetamiprid and nitenpyram, they established a dc-ELISA based on it. The MoAb-based ic-ELISAs developed by Kim et al.[21] and assessed by Xu et al.[30] also showed high specificity to imidacloprid.

The ELISA kit marketed by EnviroLogix Inc. cross-reacted strongly with acetamiprid (24%), thiacloprid (81%), and metabolites of imidacloprid of three kinds (32−60%).[17] Watanabe et al.[31] reported that the ELISA kit developed by Horiba, Ltd. showed somewhat high cross-reactivity against clothianidin (11.9%).

Other Neonicotinoid Insecticides. Some reports describe the development of ELISAs for neonicotinoid insecticides of six kinds,[20,23−28,30] except imidacloprid. Others present assessments of the analytical performance of the commercial ELISA kits respectively designed for acetamiprid,[32] thiamethoxam,[33] and dinotefuran[34] (Table 2). Related to acetamiprid, MoAb-based dc-ELISA developed by Watanabe et al.[20] showed high specificity only to the insecticide. The ELISA kit strongly cross-reacted with thiacloprid (40%).[32] In addition, PoAb-based ic-ELISA for thiacloprid showed high specificity only to itself.[23]

PoAb-based dc-ELISA,[24] MoAb-based FIA,[25] and ic-ELISA developed by Xu et al.[30] and the commercial ELISA kit[33] were assessed for the determination of thiamethoxam. Consequently, all of these ELISAs and FIA reacted specifically only to the insecticide.

Each ELISA for clothianidin, dinotefuran, and imidaclothiz showed remarkable cross-reaction with other neonicotinoid insecticides. Specifically, dc-ELISA[26] and ic-ELISA[27] for clothianidin, ELISA kit for dinotefuran,[34] and ic-ELISA for imidaclothiz[28] cross-reacted respectively with dinotefuran (9.4−64%), clothianidin (184%), and imidacloprid (91.7%) (Table 2). Particularly, although Li et al.[27] used the hapten molecule for immunogens used by Uchigashima et al.,[26] who developed a MoAb-based dc-ELISA for clothianidin (Table 1), it is interesting that cross-reactivity to dinotefuran decreased approximately 85% in comparison with the dc-ELISA (Table 2).

ELISAs developed for neonicotinoid insecticides, except ELISAs for thiamethoxam and thiacloprid, and a part of ELISAs for imidacloprid and acetamiprid, showed cross-reactivity with any analogue with sufficiently similar chemical structure (Table 2). ELISA methods are based on the specific antigen−antibody interaction. Therefore, they often appear to cross-react with analogues. However, no response to their analogues is ideal for ELISA methods. Actually, although hapten molecules for immunization are designed while considering specificity of antibody sufficient, cross-reaction often occurs in ELISA methods.

For ELISA methods, it is thought that they should be used practically for pesticide residue analysis in agricultural samples by considering the following matters. (1) It is unusual that the same classes of insecticides are applied to crops during the cultivation period at once. (2) It should be determined in consideration of the residue concentrations of any potential metabolite of a target analyte that might show a cross-reaction. However, it cannot be denied that it is possible to have multiple residues of neonicotinoid insecticides in environmental samples such as soil or water. Therefore, when applying ELISA methods to such samples, false-positive results caused by cross-reaction should always be kept in mind.

INFLUECE OF ORGANIC SOLVENT ON ELISA PERFORMANCE

Usually, water-miscible methanol (MeOH), acetonitrile (MeCN), or acetone is used as an extractant for neonicotinoid insecticides from solid samples such as agricultural or soil samples.[14] However, the analytical performance (e.g., sensitivity or color development) of ELISA is susceptible to these organic solvents because the method relies upon functional proteins such as antibody and enzyme molecules. For that reason, it is necessary to consider in advance how they might affect the performance of ELISA. This section presents a summary of the results on the influence of organic solvents on analytical performance. The degree of the influence of organic solvents varieds among every ELISA that has been developed. However, the influence on analytical performance of ELISAs with MeOH tended to be small in comparison with those of other organic solvents. Because MeCN or acetone is a more common extractant for pesticide residue analyses with chromatographic techniques than MeOH,[14] the equivalence of the extraction efficiency of MeOH for a target pesticide to other solvents should be verified.[32−36]

Imidacloprid. Li and Li[15] investigated the influence of organic solvents of four kinds (MeOH, MeCN, acetone and DMSO) on the analytical performance of developed PoAb-based ic-ELISA. Although the influence gradually increased along with increasing concentration of every organic solvent, they concluded that no influence would occur if each concentration was less than 2%. Lee et al.[19] reported that the influence on the analytical sensitivity indexing of IC_{50} values of their developed PoAb-based ic-ELISA was elucidated using phosphate-buffered saline (PBS) containing 10% organic solvents (MeOH, MeCN, DMSO, and DMF). The results show that the sensitivity decreased considerably in the presence of DMSO and DMF. Declines in the IC_{50} values of 3.5 and 7.5 times were also observed with MeOH and MeCN, respectively, compared with the control (PBS containing no organic solvent). They indicated that it is important to use the running assay buffer containing very little or no organic solvents in the ELISA because their presence suppresses antigen−antibody

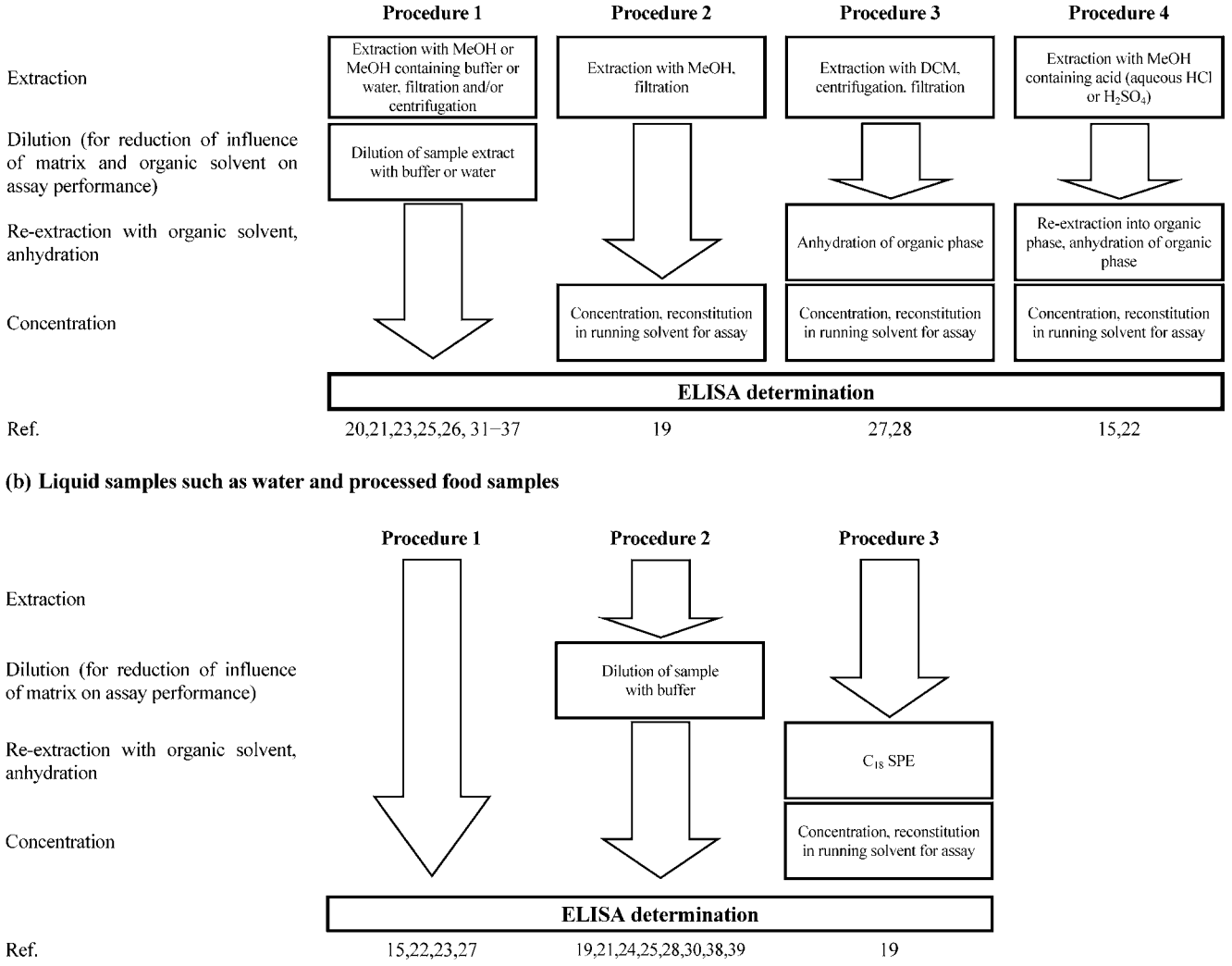


Figure 2. Schematic diagram of sample pretreatments before determination with ELISA.

interaction. However, Kim et al.[21] investigated the influence of organic solvents of four kinds (MeOH, MeCN, acetone, and DMSO) on the analytical performance (both IC_{50} and color development indexing of A_{max} values) sensitivity of developed MoAb-based ic-ELISA. Consequently, although both parameters affected with increasing concentration of MeCN and DMSO, there was little influence of MeOH and acetone, even if their concentrations were increased up to 20%, especially in cases of MeOH. Results indicate that the prepared MoAb is tolerant of organic solvents. Similar investigations related to the influence of organic solvents on PoAb-based ic-CL-ELISA[29] and commercial ELISA kit[31] have also been reported. In both results, because the influence of each solvent tested on analytical performance clearly existed depending on the kind and concentration of solvent used, MeOH that the influence was the smallest was selected as a best cosolvent for each ELISA. The final concentration in each well was adjusted to less than 5%.

Thiamethoxam. Kim et al.[24,25] reported the influence of four kinds of organic solvents (MeOH, MeCN, acetone, and DMSO) on analytical performance of the PoAb-based dc-ELISA and MoAb-based FIA using kinetic exclusion assay. Little change in IC_{50} values of the dc-ELISA was observed up to 5% of MeCN and acetone. However, it increased gradually as concentrations of MeOH and DMSO increased. The high susceptibility of FIA to low solvent concentrations appears to be somewhat unusual when compared to other ELISA methods. Although only slight influence on IC_{50} value with PBS (containing Tween 20) containing 5% of MeOH, other solvents affected it remarkably. For a commercial ELISA kit assessed by Watanabe and Miyake,[33] MeOH was the best organic solvent for a maximum tolerable concentration in 10%.

Other Neonicotinoid Insecticides. The influences of MeOH on analytical sensitivities (IC_{50} values) of ic-ELISAs for thiacloprid,[23] clothianidin,[27] and imidaclothiz[28] were also investigated, with the results showing a marked decline of sensitivity observed as more than 10% or 20%. Watanabe et al.[32,34] assessed the influence of MeOH, MeCN, and acetone using commercial ELISA kits for acetamiprid and dinotefuran, and concluded that MeOH should be selected as the best solvent for the assessed kits and that the final concentration in each well should be adjusted to 5%.

Table 4. Overview of Application of ELISAs for Neonicotinoid Insecticides to Agricultural Samples

insecticide	sample matrix		assay type	spiked levels (ng/mL or ng/g)[a]	recovery (%)	correlation coefficient[b]	ref
imidacloprid	fruiting vegetables	cucumber	ic-ELISA	10−500	70−107 (n = 4)	NR[c]	21
				1−10	85−90 (n = 3)	0.99[d]	22
			dc-ELISA	150−1500	90−95 (n = 5)	1.00[d]	20
			dc-ELISA kit	500−1500	86−**130**[e] (n = 3) (hand-shaking extraction)	0.98[d]	37
					88−98 (n = 3) (ultrasonic extraction)		
		eggplant	dc-ELISA kit	500−1500	91−117 (n = 3) (hand-shaking extraction)	1.00[d]	
					89−108 (n = 3) (ultrasonic extraction)		
		green pepper	dc-ELISA	150−1500	99−119 (n = 5)	NR	20
			dc-ELISA kit	4000−6000	91−100 (n = 3) (hand-shaking extraction)	0.91[d]	37
					87−111 (n = 3) (ultrasonic extraction)		
		tomato	dc-ELISA	150−1500	80−120 (n = 5)	NR	20
	leafy vegetables	cabbage	ic-ELISA	1−10	83−94 (n = 3)	0.99[d]	22
		lettuce	dc-ELISA kit	1000−3000	80−93 (n = 3) (hand-shaking extraction)	0.98[d]	37
					83−96 (n = 3) (ultrasonic extraction)		
	legume vegetables	bean	ic-ELISA	−500	94	NR	15
	pome fruits	apple	ic-ELISA	16−2000	94−**213** (n = 3)	NR	19
				1−10	80−94 (n = 3)	0.99[d]	22
			dc-ELISA	150−1500	89−120 (n = 5)	NR	20
			dc-ELISA kit	100−2000	88−112 (n = 3)	1.00[d]	31
	others	coffee cherry	ic-ELISA	−500	108	NR	15
acetamiprid	fruiting vegetables	cucumber	dc-ELISA	50−1000	88−94 (n = 5)	NR	20
			dc-ELISA kit	50−1000	113−**146** (n = 3)	1.00[d]	32
		eggplant	dc-ELISA kit	100−1500	104−**123** (n = 3)	1.00[d]	
		green pepper	dc-ELISA	50−1000	82−90 (n = 5)	NR	20
		strawberry	dc-ELISA kit	100−5000	89−101 (n = 3)	1.00[d]	32
		tomato	dc-ELISA	50−1000	90−100 (n = 5)	NR	20
			dc-ELISA kit	50−1000	113−**123** (n = 3)	1.00[d]	32
	stone fruits	peach	dc-ELISA kit	100−5000	82−**130** (n = 3)	1.00[d]	
	pome fruits	apple	dc-ELISA	50−1000	80−93 (n = 5)	NR	20
			dc-ELISA kit	100−5000	92−113 (n = 3)	1.00[d]	32
thiacloprid	fruiting vegetables	tomato	ic-ELISA	50−1000	80−119 (n = 3)	0.99[d]	23
	pome fruits	pear	ic-ELISA	25−500	91−101 (n = 3)	0.99[d]	
thiamethoxam	fruiting vegetables	cucumber	FIA	10−500	90−116 (n = 4)	NR	24
			dc-ELISA kit	50−500	91−110 (n = 3)	HPLC[f]	33
		eggplant	dc-ELISA kit	50−500	100−104 (n = 4) (hand-shaking extraction)	1.00[d]	36
					99−105 (n = 4) (mechanical extraction)		
		green pepper	dc-ELISA kit	100−1000	101−105 (n = 4) (hand-shaking extraction)	1.00[d]	
					98−109 (n = 4) (mechanical extraction)		
		tomato	dc-ELISA kit	200−2000	88−96 (n = 3)	HPLC[f]	33
	leafy vegetables	spinach	dc-ELISA kit	500−10000	99−103 (n = 4) (hand-shaking extraction)	1.00[d]	36
					99−107 (n = 4) (mechanical extraction)		
	root vegetables	potato	FIA	10−500	86−112 (n = 4)	NR	25
	pome fruits	apple	FIA	10−500	77−**125** (n = 4)	NR	

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Table 4. continued

insecticide	sample matrix		assay type	spiked levels (ng/mL or ng/g)[a]	recovery (%)	correlation coefficient[b]	ref
clothianidin	grain	rice	ic-ELISA	10−500	100−106 (n = 3)	1.00[g]	27
			ic-CL-ELISA	5−500	96−107 (n = 3)	0.99[g]	
	fruiting vegetables	cucumber	dc-ELISA	100−600	104−110 (n = 3)	HPLC[f]	26
		tomato	ic-ELISA	10−500	89−103 (n = 3)	1.00[g]	27
			ic-CL-ELISA	5−500	91−104 (n = 3)	0.99[g]	
			dc-ELISA	100−600	109−**124** (n = 3)	HPLC[f]	26
	leafy vegetables	cabbage	ic-ELISA	10−500	90−116 (n = 3)	1.00[g]	27
			ic-CL-ELISA	5−500	76−92 (n = 3)	0.99[g]	
	pome fruits	apple	dc-ELISA	100−600	105−106 (n = 3)	HPLC[f]	26
dinotefuran	grain	rice	dc-ELISA kit	100−2500	93−113 (n = 3)	0.99[d]	34
	fruiting vegetables	green pepper	dc-ELISA kit	300−3000	103−118 (n = 3)	1.00[d]	35
	leafy vegetables	cabbage	dc-ELISA kit	200−2000	104−107 (n = 3)	1.00[d]	
		komatsuna	dc-ELISA kit	500−5000	107−**130** (n = 3)	1.00[d]	
		spinach	dc-ELISA kit	1500−15000	101−118 (n = 3)	1.00[d]	
	bulb vegetables	leek	dc-ELISA kit	500−5000	105−118 (n = 3)	1.00[d]	
	root vegetables	carrot	dc-ELISA kit	200−700	100−101 (n = 3)	1.00[d]	
imidaclothiz	leafy vegetables	cabbage	ic-ELISA	50−500	81−95 (n = 3)	NR	28

[a]The units of spiked levels were unified, respectively, in nanograms per milliliter or nanograms per gram. [b]Correlation coefficient between ELISA and chromatographic methods was rounded off to three decimal places. [c]Not reported. [d]Correlation coefficient (r or r^2) between ELISA and HPLC methods. [e]Bold figures present results that exceeded the acceptable range of recovery: 70−120%. [f]Data on method comparison between ELISA and HPLC methods are available, but no report describes the correlation coefficient. [g]Coefficient of determination (r^2) between ELISA and GC methods.

SAMPLE PRETREATMENT PROCEDURES FOR NEONICOTINOID INSECTICIDE DETERMINATION BY ELISA

When ELISA methods are applied to analysis for pesticide residues in food samples such as agricultural samples or environmental samples such as soil and water samples, it is possible to omit complicated cleanup and concentration procedures of sample extracts. Consequently, greater facility and acceleration of sample pretreatment procedures can be accomplished. However, ELISA methods also require sample pretreatment procedures aside from cleanup and concentration. Therefore, some methods for sample pretreatments being suitable for ELISA have been devised. Several examples of sample pretreatment procedures used in the analysis of neonicotinoid insecticides in various samples with ELISA methods are presented in Figure 2.

Solid Samples. For solid samples such as agricultural or soil samples, thorough homogenization and extraction are crucial procedures that are independent of analytical methods. Accordingly, it is important to examine how to simplify the procedures after extraction to rank ELISA methods with rapid and simple analytical methods for pesticide residues. The reported sample pretreatments that are conducted before determination of neonicotinoid insecticides with ELISA methods can be categorized as four procedures (Figure 2a). Of these, the simplest procedure is Procedure 1, in which, after extraction of a target pesticide with MeOH or mixture of MeOH and buffer (or water), filtration and/or centrifugation of sample extracts, they are properly diluted with buffer or water. Then, determined with ELISA, the procedure is adopted most.[20,21,23,25,26,31−37] In addition, three examples shown below are reported. The first is an example by which a target pesticide was determined after extraction with MeOH, concentration of sample extract and reconstitution of concentrate in running solvent, PBS (Procedure 2 in Figure 2a).[19] The second is that a target pesticide was determined after ultrasonic extraction with MeOH or dichloromethane (DCM), anhydration, and concentration of the organic phase, and reconstitution of concentrate in mixture of MeOH and buffer (Procedure 3 in Figure 2a).[27,28] The final examples are as follows: after a target pesticide was extracted with a mixture of MeOH-diluted acid, it was re-extracted with organic solvent; then, the organic phase was concentrated. Finally, a pesticide dissolved in running solvent was determined using ELISA (Procedure 4 in Figure 2a).[15,22] Although Procedures 2, 3, and 4 are aimed at determining each pesticide in a lower concentration levels, the advantage of ELISA methods as a rapid and simple analytical technique for pesticide residues might be spoiled. Fundamentally, the authors hold that the Procedure 1 should be applied to a sample pretreatment method for ELISA methods. However, it can present the possibility of becoming effective to use Procedures 2, 3, and 4 if analytical performance, such as sensitivity, is inadequate.

Liquid Samples. As presented in Figure 2b, pesticide in liquid samples such as water or fruit juice samples can be determined directly or only given dilution with buffer because ELISA methods were developed at first for detection of a compound in aqueous samples, for example, biological samples such as serum or saliva. Direct determination is applicable to ELISA simply by filtering a sample that contains few matrix components such as lake or paddy water samples (Procedure 1

in Figure 2b).[22,23] Some examples show that even filtration can be skipped for tap or river water samples (Procedure 1 in Figure 2b).[15,27] Dilution with buffer shown in the Procedure 2 requires that concentration of a target pesticide in a sample be higher than the dynamic range of ELISA. Furthermore, it is used to eliminate the influence of ionic strength or pH of a sample on analytical performance.[19,21,24,25,28] Because dilution of a sample is a part of pipet work, which is a basic operation for ELISA method, an operation that increases a procedure is easy to deal with and is therefore used effectively for determination of fruit juice or honey samples.[30,38,39] However, a sample pretreatment procedure has also been reported by which pond and groundwater samples showing a significant matrix effect were cleaned up with a C_{18} solid-phase extraction (SPE) cartridge (Procedure 3 in Figure 2b).[19]

If some highly sensitive ELISA with antibody showing superior reactivity to a target pesticide is developed, then the matrix effect derived from any coexisting components should be reduced by dilution of the sample before determination with ELISA. If the effect cannot be reduced, then cleanup procedures using SPE method should be exploited as a last resort.

APPLICATION OF ELISA TO AGRICULTURAL SAMPLES

Pesticide residue analysis in agricultural products is a main objective of ELISA methods. Especially, neonicotinoid insecticides are frequently applied to various crops and can be used during long periods of cultivation from dissemination to harvest. Therefore, it is necessary to analyze the insecticides in agricultural products. Actually, many examples on application of ELISAs developed and commercial ELISA kits for detecting neonicotinoid insecticides to various agricultural samples have been reported, as Table 4 shows.

Imidacloprid. Five kinds of originally developed ELISAs and a commercial ELISA kit were applied mainly to vegetable samples such as fruiting or leafy vegetables (Table 4). The recoveries from artificially spiked samples were within the permissible range (70−120%),[40,41] and it turns out in almost all reports that each proposed ELISA can determine imidacloprid accurately and quantitatively in complicated agricultural samples without troublesome matrix effects.[15,20−22,31,37] However, some cases showing excess of the permissible recovery range were also found. One such case is described in a report by Lee et al.,[19] who applied PoAb-based ic-ELISA to detection for imidacloprid in apple samples. In this example, the recoveries of samples spiked at lower levels (16 and 80 ng/mL) and were, respectively, 213 and 149% (Table 4). Furthermore, the recovery of a cucumber sample spiked at the lowest level, 500 ng/g, which also varied from the valid recovery range, as described by Watanabe et al.,[37] who evaluated a commercial ELISA kit (Table 4). Overall, the ELISA kits tended to estimate recoveries at the range of lower detectable concentration levels excessively in comparison with theoretical levels. Although a major factor causing the results might be the matrix effect derived from agricultural samples, the recovery of the cucumber sample that spiked at 500 ng/g was improved by changing hand-shaking extraction into ultrasonic extraction (Table 4).[37] This point is interesting for consideration of properties of sample matrices. Furthermore, because the analytical results obtained from the proposed ELISAs agreed well with those of the reference HPLC method ($r > 0.9$),[20,22,31,37] these ELISA methods might be suitable as quantitative analytical methods for imidacloprid.

Imidacloprid in samples was determined accurately solely by extraction with MeOH and dilution of methanolic sample extract (Procedure 1 in Figure 2a) in four of seven reports that were studied for application of each ELISA method to agricultural samples.[20,21,31,37] Therefore, it might be concluded that the ELISAs for detecting imidacloprid are used as a rapid and simple analytical method from these findings.

Acetamiprid. A dc-ELISA and a commercial ELISA kit were applied to several fruiting vegetable and fruit samples (Table 4). Watanabe et al.[20] evaluated the accuracy of developed MoAb-based dc-ELISA using three kinds of fruiting vegetable and apple samples spiked at three different concentration levels (50, 300, and 1000 ng/g). Consequently, all recoveries were within the permissible recovery range. Therefore they reported that it is possible to determine acetamiprid with the ELISA quantitatively, largely without a matrix effect. A commercial ELISA kit assessed by Watanabe et al.[32] showed tendencies to recover over 120% in many examined agricultural samples attributable to matrix effect. Good recoveries were obtained by compensating for the matrix effect with a calibrator prepared by methanolic extract of an acetamiprid-free strawberry sample as a remedy for the negative effect (Table 4). However, because excellent correlation was found between the ELISA kit results and the reference HPLC in all examined agricultural samples ($r \approx 1.00$), it was regarded as useful for determination with consideration of its tendency toward overestimation.

Thiamethoxam. An FIA and a commercial ELISA kit were applied mainly to vegetable samples (Table 4). Kim et al.[25] developed a highly sensitive FIA method based on kinetic exclusion assay, and applied the method to cucumber, potato, and apple samples. The FIA method had restriction of concentration of organic solvents in the final solution for the determination as described in previous section because it was susceptible to the influence on the analytical performance because of them. Therefore, it was necessary to zero the concentration limitlessly for the method. However, because of the high sensitivity (IC_{50} = 30 pg/mL), the influence of the organic solvent (MeOH) on the sensitivity was minimized by diluting the sample extract. The recoveries from the spiked agricultural samples were good, except that the apple sample spiked at 100 ng/g (Table 4). Watanabe et al.[33,36] applied a commercial ELISA kit to fruiting vegetable and spinach samples of four kinds. As shown in Table 4, good recoveries from all examined agricultural samples were provided solely by dilution of sample extracts following extraction with MeOH. They were able to determine thiamethoxam accurately even in spinach samples containing pigments such as chlorophyll, which is a main cause of the potential matrix effect in ELISA methods. The results were thought to support the robustness of the ELISA kit that was evaluated in that study.[36] Furthermore, the analytical results determined using the proposed ELISA kit after rapid and simple hand-shaking extraction also agreed with those of the reference HPLC ($r \approx 1.00$).

In summary, because both methods described herein can determine thiamethoxam in agricultural samples only giving the simple dilution of sample extracts after extraction with MeOH or aqueous MeOH (Procedure 1 in Figure 2a), they would be suitable methods for detecting the insecticide rapidly and simply in complicated agricultural samples. Because the FIA method was highly sensitive, it was necessary to dilute more

Table 5. Overview of Application of ELISAs for Neonicotinoid Insecticides to Liquid Food Samples

insecticide	sample matrix		assay type	spiked levels (ng/mL or ng/g)[a]	recovery (%)	correlation coefficient[b]	ref
imidacloprid	fruit juices	apple juice	ic-ELISA	50−1000	86−102 (n = 4)	0.91[c]	30
			dc-ELISA kit	10−100	82−100 (n = 3)	0.99[d]	39
		grape juice	ic-ELISA	50−1000	90−104 (n = 4)	0.93[c]	30
			dc-ELISA kit	10−100	95−**129**[e] (n = 3)	0.99[d]	39
		orange juice	ic-ELISA	100−2000	95−108 (n = 4)	0.9[c]	30
			dc-ELISA kit	50−400	96−113 (n = 3)	0.99[d]	39
		peach juice	ic-ELISA	100−2000	108−**129** (n = 4)	0.89[c]	30
	others	honey	ic-ELISA	50−2000	90−114 (n = 3)	0.96[c]	38
thiamethoxam	fruit juices	apple juice	ic-ELISA	20−200	75−95 (n = 4)	0.91[c]	30
		grape juice	ic-ELISA	20−200	87−110 (n = 4)	0.93[c]	
		orange juice	ic-ELISA	20−200	91−118 (n = 4)	0.9[c]	
		peach juice	ic-ELISA	20−200	112−**125** (n = 4)	0.89[c]	
	others	honey	ic-ELISA	10−1000	96−**122** (n = 3)	0.96[c]	38

[a]The units of spiked levels were unified, respectively, in nanograms per milliliter or nanograms per gram. [b]Correlation coefficient between ELISA and chromatographic methods was rounded off to three decimal places. [c]Coefficient of determination (r^2) between ELISA and LC−MS methods. [d]Correlation coefficient (r) between ELISA and HPLC methods. [e]Bold figures present results that exceeded the acceptable range of recovery: 70−120%.

sample extracts, which made the operation complicated. It might be said that this is an example indicating the necessity for compatibility between sensitivity requiring for a particular target pesticide in a sample and robustness for an appropriate analytical method.

Thiacloprid and Imidaclothiz. A PoAb-based ic-ELISA for detection of thiacloprid developed by Liu et al.[23] was applied to tomato and pear samples. The insecticide was determined using ELISA by diluting the sample extract with PBS after extraction with a mixture of MeOH-PBS (3:1) according to Procedure 1 presented in Figure 2a. It was possible to determine the insecticide accurately without a matrix effect by diluting the tomato extract 10-fold and the pear extract at 5-fold. The recoveries and the correlation between the proposed ELISA and the reference HPLC were also excellent (Table 4). Fang et al.[28] applied MoAb-based ic-ELISA developed for detection of imidaclothiz to cabbage samples, with the result that the recoveries from those spiked at 50, 100, and 500 ng/g were also excellent (Table 4). However, the sample pretreatment procedures for the insecticide were complicated because they required concentration of DCM that was used as an extractant for cabbage samples (Procedure 3 in Figure 2a).

Clothianidin. A MoAb-based dc-ELISA for detection of clothianidin developed by Uchigashima et al.[26] was applied to cucumber, tomato, and apple samples. Among the examined samples, the matrix effect was observed in the side of lower concentration only slightly in the tomato sample. Consequently, the recovery from the tomato sample spiked at the lowest level (100 ng/g) was slightly higher than at the theoretical level (Table 4), which suggested that the result reflected some matrix effect. However, the recoveries of other samples aside from the tomato sample described above were excellent (Table 4). Actually, a PoAb-based ic-ELISA and ic-CL-ELISA was applied to rice, cabbage, and tomato samples for detecting clothianidin.[27] Although the sample pretreatment procedures were complicated because methanolic extract from examined agricultural samples was concentrated (Procedure 3 in Figure 2a), matrix effects were evaded respectively by diluting concentrated sample extract with mixture of MeOH and PBS at 8-fold for rice samples and at 4-fold for cabbage and tomato samples. Good recoveries from all examined agricultural samples are shown in both ELISAs (Table 4).

Dinotefuran. Watanabe et al.[34,35] assessed the potential application of the commercial ELISA kit for detection of dinotefuran using agricultural samples of seven kinds including rice, green pepper, komatsuna (Japanese mustard spinach), and others. The recoveries except for those of the komatsuna samples, which spiked at 500 and 1000 ng/g, were excellent. Good agreement between the proposed ELISA and the reference HPLC were also observed in all examined agricultural samples (Table 4). It was thought that determination in the neighborhood of the maximum residue limit (MRL) level with the assessed ELISA kit might not give any trouble; it therefore might be a useful method for rapid and simple residue analysis for dinotefuran.

As described above, most ELISAs developed to date can determine a target insecticide in various agricultural samples quantitatively and accurately using simple sample pretreatment procedures: extraction and dilution of sample extracts. Several investigations have revealed the possibility that ELISA methods can contribute greatly to future residue analyses undertaken to investigate neonicotinoid insecticides in agricultural products.

APPLICATION OF ELISA TO LIQUID FOOD SAMPLES

Procedures for extraction of a target pesticide are unnecessary for liquid food samples such as fruit juice and honey samples. Therefore, generally, a target pesticide can be determined using ELISA only by diluting samples to avoid matrix effects (Procedure 2 in Figure 2b). Table 5 presents some examples of application of ELISAs for imidacloprid and thiamethoxam to liquid food samples.

Matrix effects can be reduced efficiently by raising dilution magnification, but the analytical sensitivity of ELISA comes off properly. It is therefore necessary to set an appropriate dilution magnification without affecting the determination with ELISA by a matrix effect while considering the balance of the sensitivity and the concentration levels that are necessary for food safety, for example, concentration levels of MRLs neighborhood.[30] Applications of ELISAs for neonicotinoid insecticides to liquid food samples were some cases. However, it was concluded that quantitative determination of the target

Table 6. Overview of Application of ELISAs for Neonicotinoid Insecticides to Environmental, Wood, and Animal Samples

insecticide	sample matrix		assay type	spiked levels (ng/mL or ng/g)[a]	recovery (%)	correlation coefficient[b]	ref
imidacloprid	water	canal water	ic-ELISA	1−10	80−87 (n = 3)	NR[c]	22
		ground water	ic-ELISA	16−2000	92−**156**[d] (n = 3)	1.00[e]	19
				16−400 (C_{18} SPE)	**125−152** (n = 3)		
		lake water	ic-ELISA	1−10	78−83 (n = 3)	NR	22
		paddy water	ic-ELISA	1−10	79−89 (n = 3)	NR	
		pond water	ic-ELISA	16−2000	108−**213** (n = 3)	1.00[e]	19
				16−400 (C_{18} SPE)	92−107 (n = 3)		
				1−10	83−93 (n = 3)	NR	22
				1−10 (fishpond)	78−82 (n = 3)	NR	
		stream water	ic-ELISA	2−200	101−120 (n = 4)	NR	21
		tap water	ic-ELISA	−3000	good	NR	15
				2−200	86−104 (n = 4)	NR	21
	soil		ic-ELISA	10−1000	73−92 (n = 3) (soils of three kinds)	0.99[e]	22
	others	honeybee	ic-CL-ELISA	100−1000	73−76	LC−MS[f]	29
		hemlock	dc-ELISA kit	0.2−5	91−**150** (n = 3) (1% wood tissue extract)	NR	43
					101−**127** (n = 3) (1% needle tissue extract)		
		wiliwili leaf	ic-ELISA	100−10000	78−100 (n = 3)	0.98[e]	44
thiacloprid	water	paddy water	ic-ELISA	5−100	98−108 (n = 3)	NR	23
		tap water	ic-ELISA	5−100	105−109 (n = 3)	NR	
	soil		ic-ELISA	100−1000	82−113 (n = 3)	0.99[e]	
thiamethoxam	water	stream water	dc-ELISA	−100	good	NR	24
			FIA	0.5−10	93−106 (n = 4)	NR	25
		tap water	dc-ELISA	−100	good	NR	24
			FIA	0.5−10	88−96 (n = 4)	NR	25
clothianidin	water	river water	ic-ELISA	10−500	89−102 (n = 3)	1.00[g]	27
			ic-CL-ELISA	5−500	83−99 (n = 3)	0.99[g]	
	soil		ic-ELISA	10−500	95−108 (n = 3)	1.00[g]	
			ic-CL-ELISA	5−500	90−101 (n = 3)	0.99[g]	
imidaclothiz	water	river water	ic-ELISA	50−500	93−106 (n = 3)	NR	28
		tap water	ic-ELISA	50−500	93−107 (n = 3)	NR	
	soil		ic-ELISA	50−500	80−114 (n = 3)	NR	

[a]The units of spiked levels were unified, respectively, in nanograms per milliliter or nanograms per gram. [b]Correlation coefficient between ELISA and chromatographic methods was rounded off to three decimal places. [c]Not reported. [d]Bold figures present results that exceeded the acceptable range of recovery: 70−120%. [e]Correlation coefficient (r or r^2) between ELISA and HPLC methods. [f]Data on method comparison between ELISA and LC−MS methods are available, but no report describes the correlation coefficient. [g]Coefficient of determination (r^2) between ELISA and GC methods.

insecticide with each ELISA was possible. It is thought that a target pesticide can be determined accurately only by simple dilution for reducing matrix effect in ELISA method because of the lack of a requirement of extraction procedures from liquid food samples. Furthermore, it might be a great benefit that no organic solvent is necessary in these samples.

Imidacloprid. Xu et al.[30] tried to reduce matrix effects by diluting apple and grape juice samples 50-fold and orange and peach juice samples 100-fold with PBS when applying ic-ELISA to those fruit juice samples. Consequently, good recoveries, except for the peach juice sample, spiked at 2000 ng/g, for which recovery exceeding 120% was shown. The correlation coefficient (r^2) with the reference LC−MS was higher than 0.89 (Table 5). Ma et al.[38] also applied the ic-ELISA described above to honey samples containing waxes or pigments and sought to reduce matrix effects by diluting them with PBS-like application to fruit juice samples.[30] Recoveries showed strong agreement with the spiked concentrations. The analytical results obtained using the proposed ELISA were comparable to those obtained using the reference LC−MS with a reasonably high coefficient of determination (r^2 = 0.96) (Table 5). Furthermore, Watanabe et al.[39] applied a commercial ELISA kit to fruit juice samples of three kinds, finding that that matrix effect was avoided by diluting samples with PBS, and obtaining good recoveries overall, except for the grape juice sample spiked at 100 ng/mL, in which recovery exceeded 120%. High correlation with the reference HPLC (r = 0.99) was shown (Table 5).

Thiamethoxam. Xu et al.[30] and Ma et al.[38] respectively applied ic-ELISA to four kinds of fruit juice and honey samples. Although some samples such as the peach juice sample spiked at 500 ng/mL and the honey sample spiked at 10 ng/g led to results exceeding 120% a little, good recoveries were obtained overall. In addition, a fair correlation is found between the ELISA and the reference LC−MS (Table 5).

APPLICATION OF ELISA TO ENVIRONMENTAL SAMPLES

Table 6 presents application of the developed ELISAs to environmental samples. Because of the lack of requirement of extraction procedures in water samples composed of a simple matrix in comparison with food samples generally, it is possible to determine a target pesticide directly merely by giving simple filtration or dilution of sample with buffer for adjustment of ionic strength or pH before analysis (Figure 2b).[19,21−25,28]

However, because the concentration levels of overall pesticides including neonicotinoid insecticides in water samples are lower than those in food samples, it is necessary to develop more highly sensitive ELISAs for the samples. Nevertheless, soil samples require some extraction procedure, as do solid food samples (Figure 2a). Actually, there are yet some examples of ELISAs for neonicotinoid insecticides being applied to soil samples. Much the same sample pretreatment procedures were adopted for them as for food samples.[22,23,27,28]

Imidacloprid. ic-ELISAs of four kinds were applied to environmental and tap water samples. Accurate recoveries were obtained given only filtration or dilution with buffer at the most (Procedures 1 and 2 in Figure 2b).[15,21,22] However, a report by Lee et al.[19] specifies that, although pond and groundwater samples were diluted with PBS to reduce the potential matrix effect, recoveries in low spiked concentration levels (16 and 80 ng/mL) greatly exceeded 120% (pond water, 204−213%; groundwater, 133−156%) (Table 6). The recoveries in pond water samples were improved significantly by cleaning up each water sample with C_{18} SPE cartridges (92−107%). However, the additional procedure had practically no effect on groundwater samples (125−152%) (Table 6).

Wang et al.[22] applied PoAb-based ic-ELISA to soil samples of three kinds and reported good recoveries and excellent correlation (r = 0.99) with the reference HPLC. The proposed sample pretreatment procedures that are necessary to re-extract imidacloprid with DCM after extraction from soil samples are complicated (Procedure 4 in Figure 2a).

Thiacloprid, Thiamethoxam, Clothianidin, and Imidaclothiz. Liu et al.[23] recovered thiacloprid from paddy and tap water samples simply by filtering with PoAb-based ic-ELISA (Procedure 1 in Figure 2b and Table 6). Furthermore, they found that the insecticide accurately recovered from spiked soil samples after ultrasonic extraction with mixture of MeOH-PBS (1:3), centrifugation and 10-fold dilution with PBS (Procedure 1 in Figure 2a and Table 6). Good correlation was obtained between the proposed ELISA and the reference HPLC (r^2 = 0.99). Kim et al. applied PoAb-based dc-ELISA[24] and MoAb-based FIA[25] to detection of thiamethoxam in stream and tap water samples, obtaining good recoveries from mere dilution with buffer in all samples (Procedure 2 in Figure 2b and Table 6).

Li et al.[27] applied PoAb-based ic-ELISA and ic-CL-ELISA directly to river water samples without dilution of the sample (Procedure 1 in Figure 2b). On application of both ic-ELISAs to soil samples, although it was necessary to concentrate methanolic sample extracts, good recovery was obtained (Table 6). Furthermore, the analytical results of clothianidin-positive paddy water and soil samples with both ic-ELISAs were approximately equal to those obtained using GC method.

On imidaclothiz, MoAb-based ic-ELISA was applied to river and tap water samples. Consequently, recoveries of the insecticide were excellent by dilution with a mixture of PBS-MeOH (8:2) (Procedure 2 in Figure 2b and Table 6).[28] However, recoveries from soil samples that spiked between 50 ng/g and 500 ng/g were generally good (Table 6). It was necessary to extract DCM and concentrate them before determination (Procedure 3 in Figure 2a).[28]

APPLICATION OF IMIDACLOPRID ELISA TO HONEYBEE AND WOOD SAMPLES

ELISAs for neonicotinoid insecticides have also been applied to honeybee and wood samples (Table 6). It is thought to be premature to discuss the potential matrix effect in these samples described in this section. Nevertheless, development of a method for determination to be able to draw the characteristic of ELISA method is expected by the adoption of a simple sample pretreatment method in the same way as other solid samples such as agricultural products (Procedure 1 in Figure 2a).

Honeybee Samples. Girotti et al.[29] developed PoAb-based ic-CL-ELISA for the monitoring of honeybees polluted with the insecticide. After imidacloprid was extracted with acetone from lyophilized honeybee samples, and after the sample extracts were coagulated with mixtures of ammonium chloride and phosphoric acid solutions, it was re-extracted using DCM. Then the DCM was concentrated.[42] Good recoveries of imidacloprid with the ic-CL-ELISA were obtained by diluting the concentrated samples 100-fold with water (Table 6). Furthermore, the analytical results of 27 real honeybee samples in which five samples were positive with the proposed ic-CL-ELISA agreed well with those of LC−MS analyses. They reported that the developed ic-CL-ELISA might allow rapid screening to detect imidacloprid in honeybee samples.

Wood Samples. In the eastern United States of America, imidacloprid is often used as the primary insecticide to prevent the exotic invasive insect hemlock woolly adelgid (*Adelges tsugae* Annand), a pest of eastern hemlock trees. Eisenback et al.[43] evaluated a commercial ELISA kit for studies of the distribution, the metabolic pathways, and the decision on threshold concentration for providing control of the insect infestation. When applying the ELISA kit to the studies, imidacloprid in wood and needle tissue samples of hemlock tree was ascertained by extraction with water and dilution of sample extracts with water at 100-fold before determination (Table 6). They reported the ELISA kit as a valuable tool for a semiquantitative screening method of imidacloprid in hemlock tree samples and alerted future kit users that it has some limitations in terms of its performance and that it shows a tendency to analytical error, probably because of the matrix effect.

In contrast, Xu et al.[44] investigated the applicability of ic-ELISA developed by Kim et al.[21] for detection of imidacloprid applied to endemic wiliwili trees to control the erythrina gall wasp (*Quadrastichus erythrinae* Kim). Imidacloprid in lyophilized leaf samples was extracted ultrasonically with a mixture of MeOH-diluted sulfuric acid (4:1). Then the concentrated sample extract after evaporation of MeOH was re-extracted with DCM. After evaporation of DCM, the residue was dissolved in mixture of MeOH-water (1:1) then further diluted with water prior to determination. When diluting 10-fold by water, good recoveries were obtained and an excellent coefficient of determination (r^2 = 0.98) between the proposed ELISA and the reference HPLC analyzing several leaf samples contained different levels of the insecticide (Table 6). By way of conclusion they proposed that ic-ELISA can quantitatively determine imidacloprid in leaf samples of wiliwili trees and that it might be effective for the management and control of the wasps.

OVERVIEW AND FUTURE PROSPECT OF ELISA FOR PESTICIDE RESIDUES

To date, numerous ELISAs have been developed and assessed with the aim of assessing the presence and concentrations of neonicotinoid insecticides rapidly and simply. The present review summarized a series of flows from design of hapten

molecules for production of antibody and assay development to application to various sample matrices on an immunoassay and especially addressed the potential applicability of ELISAs as a rapid and simple analytical technique. Because ELISA methods can obviate laborious and onerous sample pretreatment procedures that are necessary to conduct determination using chromatographic methods, they are characterized not only as a rapid analytical technique but also as a straightforward analytical technique requiring no sophisticated skills. Furthermore, ELISAs are generally inexpensive because they entail no requirement of costly equipment. It might be advantageous to use them outside of a laboratory. Nevertheless, ELISA methods have no capability of qualifying unknown components, which GC−MS and LC−MS/MS can achieve, and are analytical techniques fundamentally intended to assess a single component. Consequently, they are inadequate for application to samples in which the used pesticide is unknown, and are therefore restricted to determination for samples that clearly contain the target pesticide.

The matrix effect is a common problem in both ELISA and chromatographic methods. Sample pretreatment procedures can overcome the effect. Above all, because ELISA methods make use of antibody's specific reactivity to a target pesticide, it is possible to analyze it theoretically in a complicated sample even if matrix components coexist in some quantity. However, false-positive results are well-known to occur because of matrix effects when determining pesticides in samples using an ELISA method. Means of avoiding awkward effects have been devised in various ways. The present review describes the tricky means of minimizing matrix effects and the analytical results when determining target pesticides in actual samples with ELISAs under respectively optimized conditions. Almost all reports have presented the conclusion that a target pesticide can be determined quantitatively with ELISA by reducing the matrix effect after extraction. However, an assessment using real samples, not artificially spiked samples, is necessary for the confirmation of the veritable validity of ELISA method as an analytical technique for pesticide residues.[27,29,35,36,44] From this perspective, it is the present conditions to remain in confirmation of the validity of using spiked samples in most cases.

Established ELISA methods are convenient to determine a pesticide rapidly and simply because of the lack of a requirement of sophisticated skills and expensive experimental equipment. However, establishment of an ELISA for the determination of pesticides demands extensive experimental techniques such as design and synthesis of hapten molecules, production of antibodies, and design of ELISA including optimization of assay conditions, fundamental analytical characteristics, and application to real samples as described above. The use of ELISAs to evaluate pesticide residues has promoted research and development in earnest since the 1980s, and nowadays, it amounts to several hundred varieties. Nevertheless, not just anyone can obtain these developed ELISAs easily; it, therefore, is hoped that more ELISAs will come to be marketed as kits.

Considering both the benefits and the shortcomings of ELISA methods in pesticide residues, they seem to be fit for a preliminary screening method that complements determination using chromatographic methods. Moreover, reports clarify the respective results thoroughly. ELISA methods can be useful as an important analytical method for quantifying pesticide residues. As the assessment of ELISA methods as an analytical method for pesticide residue advances, it is hoped that ELISA kits that analysts can use easily for various pesticides will be made available on the market.

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Notes

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