Development of an Enzyme-Linked Immunosorbent Assay for the Fungicide Imazalil in Citrus Fruits

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Imazalil has been widely used in citrus fruits such as lemons, oranges, and grapefruits. A competitive enzyme-linked immunosorbent assay (ELISA) was developed for the detection of residual imazalil in citrus fruits. A monoclonal antibody (MoAb) generated to the synthetic imazalil hapten (EIT-0073)−protein conjugate was used. This assay was applied to lemon, orange, and grapefruit matrices for an imazalil analysis. The acceptable residue level for lemons, oranges, and grapefruits in Japan is 5 ppm. The matrix interference was minimized by direct dilution of the sample homogenate. No further cleanup was needed. The detection limit for imazalil in these citrus fruits was 0.1 ng/mL. The recovery of each fortified citrus fruit sample was >81.0%. The imazalil recovery measured by the proposed ELISA was compared to the recovery determined by a conventional HPLC. A good correlation was observed between the proposed ELISA and the HPLC. This proposed ELISA would be useful for monitoring for residual imazalil.

Keywords: *ELISA; imazalil; monoclonal antibody; citrus fruit; HPLC*

INTRODUCTION

Imazalil [1-(β-allyloxy-2,4-dichlorophenethyl)imidazole], which is one of the azole fungicides, was produced by Laville and was introduced by Janssen (Laville, 1973). It is a highly effective systemic fungicide with a protective and curative action (Tomlin, 1994). It can control a wide range of fungal diseases on fruits, vegetables, and ornamentals, for example, powdery mildews on cucurbits and ornamentals; powdery mildew on roses; and storage diseases (particularly *Penicillium*, *Gloeosporium*, etc.) of citrus fruits, pome fruits, and bananas based on the inhibition of ergosterol biosynthesis (Tomlin, 1994; Takeda et al., 1995a). It is also used for the control of diseases (particularly *Fusarium*) of cereals (Tomlin, 1994; Takeda et al., 1995a). In Japan, the tolerance levels for imazalil are regulated to be 5 ppm for citrus fruits, for example, lemons, oranges, and grapefruits, and range from 0.01 to 0.05 ppm for cereals, for example, wheat, rice, and corn. On the other hand, the U.S. Environmental Protection Agency (EPA) regulates the tolerance levels for imazalil to be 10 ppm for citrus fruits and 3 ppm for bananas. These tolerance levels are the total values of imazalil and its metabolite [1-(2,4-dichlorophenyl)-2-(1*H*-imidazole-1-yl)-1-ethanol] (Takeda et al., 1995a). Particularly, imazalil is frequently detected in citrus fruits imported from the United States and also in food processed from citrus fruits such as orange marmalade and concentrated lemon juice (Nagayama et al., 1995a, 1996; Hori, 1997; Akiyama et al., 1997). In most reports on surveys of residual imazalil in citrus fruits and their processed foods, the residual concentrations of imazalil were <5 ppm. Imazalil in citrus fruits has been mainly analyzed by instrumental analysis using HPLC (Tonogai et al., 1992a, 1998; Nagayama et al., 1995b; Takeda et al., 1995b; Nakazato et al., 1995; Kawahara et al., 1995; Chatani et al., 1996; Ito et al., 1998) or gas chromatography (GC) (Tonogai et al., 1992b; Nagayama et al., 1995b; Akiyama et al., 1996; Yamazaki and Ninomiya, 1996; Garrido et al., 1997; Torres et al., 1997). Both methods require a complicated pretreatment for the determination and are expensive and laborious. On the other hand, an ELISA is rapid, sensitive, and selective and is generally cost-effective for large sample loads (Harris et al., 1995; Ellis et al., 1996; Yuasa, 1998). Recently, there have been a large number of reports regarding residual analyses for pesticides using an ELISA (Bushway et al., 1995; Szekacs and Hammock, 1995; Cairoli et al., 1996; Mercader and Montaya, 1999; Abad et al., 1999). In this paper, we describe the development of a sensitive ELISA for the detection of imazalil and its application to citrus fruit samples such as lemons, oranges, and grapefruits.

MATERIALS AND METHODS

Reagents and Apparatus. Imazalil was purchased from Kanto Chemicals Ltd. (Tokyo, Japan). The purity of the imazalil was >97.0% as analyzed by GC. The other azole

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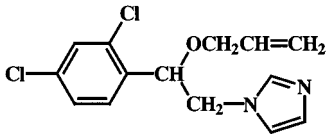
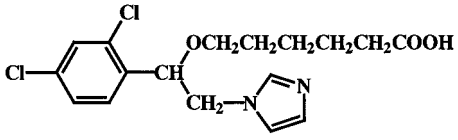


Imazalil



Imazalil hapten (EIT-0073)

Figure 1. Chemical structures of imazalil and imazalil hapten (EIT-0073) used for preparing the protein conjugates.

fungicide standards were obtained from Wako Pure Chemicals Industry Co. Ltd., (Osaka, Japan). Bovine serum albumin (BSA) fraction V, rabbit serum albumin (RSA) fraction V, and *o*-phenylenediamine (OPD) were from Sigma Chemical Co. (St. Louis, MO). Horseradish peroxidase (HRP), *N*-hydroxysuccinimide (NHS), dicyclohexylcarbodiimide (DCC), PEG 1500, Freund's complete and incomplete adjuvants, and organic solvents for pesticide residue analysis were from Wako Pure Chemicals Industry Co. Ltd. Block Ace was from Dainippon Chemical Industries (Osaka, Japan). Peroxidase-labeled goat anti-mouse immunoglobulins (Igs) were from Biosource International (Camarillo, CA). A Chem Elut disposable cartridge for solid phase extraction (SPE) was from Varian (Harbor City, CA). A mouse typer EIA grade isotyping kit was from Bio-Rad (Richmond, CA). An Sp2/0 Ag14 mouse plasmacytoma cell line was from American Type Culture Collection (Rockville, MD). Cell culture media, fetal bovine serum, and supplements were purchased from Gibco BRL (Grand Island, NY), Nissui Pharmaceutical Co. Ltd., (Tokyo, Japan), and Intergen (New York), respectively. Culture plasticware was from Corning Inc. (Corning, NY) or Sumitomo Bakelite Co. Ltd. (Tokyo, Japan). Water used in all reactions and ELISA tests was purified using a Milli-Q system (Millipore Corp., Milford, MA). All other chemicals used were of analytical reagent grade. Flat-bottom polystyrene 96-well plates (Sumitomo Bakelite Co. Ltd.) were washed with a Nunc-Immuno Wash 8 microplate washer (Roskilde, Denmark), and absorbances were read with a Bio-Rad model 550 microplate reader (Hercules, CA). The HPLC system consisted of a Nanospace SI-1 pump, a UV−visible detector, and a column oven from Shiseido (Tokyo, Japan). The detection wavelength was at 230 or 202 nm. The column used was a J'sphere ODS-H80 (4.6 × 150 mm, 4 μm) (YMC, Kyoto, Japan). The mobile phase used was methanol/water (80:20 v/v) delivered at a flow rate of 1.0 mL/min.

Imazalil-free citrus fruit samples were obtained from the Federation of Japanese Consumer Cooperatives (Kanagawa, Japan). Commercially available samples were purchased from a local supermarket.

Imazalil Hapten (EIT-0073) Synthesis. Imazalil hapten (EIT-0073) (Figure 1) was gift from Environmental Immunochemical Technology Co., Ltd. (Tokyo, Japan).

Preparation of EIT-0073−Protein Conjugate. *(1) Immunizing Conjugate and Coating Conjugate.* The EIT-0073 was covalently linked to BSA or RSA according to the active ester method described by Karu et al. (1994). EIT-0073 (74.2 mg, 0.2 mmol), NHS (23.0 mg, 0.2 mmol), and DCC (41.3 mg, 0.2 mmol) were dissolved in 1 mL of dry dimethylformamide (DMF). The reaction mixture was stirred for 4 h at room temperature. The precipitate was removed by centrifugation, and 250 μL of supernatant was added dropwise to 50 mg of BSA or RSA solution. After reaction overnight at 4 °C, the solution was dialyzed against 10 mM phosphate-buffered saline [PBS; 1.1 g/L Na_2HPO_4, 0.306 g/L KH_2PO_4, 0.9% (w/v) NaCl, pH 7.2].

(2) Enzyme Conjugate. The EIT-0073 was conjugated with HRP according to the active ester method described by Karu et al. (1994). EIT-0073 (74.2 mg, 0.2 mmol) was dissolved in 1 mL of dry DMF. NHS (23.0 mg, 0.2 mmol) and DCC (41.3 mg, 0.2 mmol) were dissolved in the DMF solution, and then the mixed solution was reacted for 4 h at room temperature. After reaction, the solution was centrifuged at 10000 rpm for 15 min (supernatant, liquid A). After 25 mg of HRP was dissolved in 2.5 mL of PBS, 525 μL of DMF was slowly added to this HRP solution (liquid B). Liquid A (125 μL) was added dropwise to liquid B, and the mixture was reacted for 16 h at 4 °C. After reaction, this solution was dialyzed against PBS. The concentration of the EIT-0073−HRP conjugate in the PBS solution was determined by Lambert−Beer's law using an extinction coefficient of 2.275 for 1 mg/mL solution.

Immunization and Fusion Protocol. Following the previous practice (Schlaeppi et al., 1989), BALB/c female mice (4−6 weeks) were immunized with EIT-0073−BSA conjugate by subcutaneous injection. The EIT-0073−BSA conjugate (50 or 100 μg) was dissolved in PBS and emulsified with an equal volume of Freund's complete adjuvant before immunization. A month after the first immunization, the second immunization was performed with 50 μg of EIT-0073−BSA conjugate emulsified with Freund's incomplete adjuvant. One week after the last injection, blood was collected and the antisera obtained from BALB/c mice were tested for the antibody titer and recognition properties for imazalil. The mice that gave a strong response to imazalil were given a booster injection in the tail vein. Four days after the booster injection, cell fusion was performed with the polyethylene glycol (PEG) method. Spleen cells from immunized mice (2×10^8) and myeloma cells (4×10^7) were mixed in the ratio of 5:1 and fused with PEG 1500. The fused cells were cultured with a HAT selection medium. Cells were grown in the HAT medium for 2−4 weeks. Culture supernatants were screened for the presence of antibodies that recognize imazalil. Selected hybridoma cells in ELISA positive wells were cloned with the limiting dilution method. Stable antibody-producing clones were expanded and cryopreserved under liquid nitrogen. Ascites obtained from BALB/c mice were subjected to salting out with saturated ammonium sulfate. Two milliliters of the saturated ammonium sulfate was slowly added to stirred 4 mL of ascite fluid, and then the mixture was centrifuged at 10000 rpm for 20 min at 20 °C. After centrifugation, the precipitate deposit was collected and dissolved in 1 mL of PBS. The PBS solution was dialyzed against PBS overnight at 4 °C. The concentration of MoAbs was determined by Lambert−Beer's law using an extinction coefficient of 1.4 for 1 mg/mL solution. The purified MoAbs were stored at −20 °C in PBS. The isotype of the purified MoAbs was determined using a commercial kit.

ELISAs. *Noncompetitive Conjugate-Coated ELISA Format.* The titer of mice sera, the antisera dilution, and the MoAbs concentration to be used in the competitive assays were determined according to this ELISA format. Ninety-six-well polystyrene ELISA plates were coated with the EIT-0073−RSA conjugate (0.1 μg/mL, 50 μL/well) in coating buffer (10 mM PBS) overnight at 4 °C. After the coated plates were washed five times with 10 mM PBS, 300 μL of blocking solution [25% (v/v) Block Ace in distilled water containing 0.1% (w/v) sodium azide] was added and incubated at 4 °C. The coated plates received 50 μL of IgG solutions (sera, culture supernatants, or MoAb) diluted in PBS. An immunological reaction took place for 1 h at 25 °C, and the plates were washed five times according to the above-mentioned method. Next, 50 μL of a 1/1000 dilution of HRP-labeled goat anti-mouse IgGs in PBS containing 10% (v/v) Block Ace was added to each well, and the plates were incubated for 1 h at 25 °C. After washing, the bound peroxidase activity was determined by adding 50 μL of 2 mg/mL OPD and 0.02% (v/v) H_2O_2 in phosphate−citrate buffer, pH 5.2 (substrate−chromogen). The enzymatic reaction was stopped after 10 min at room temperature by adding 50 μL of 1.0 M sulfuric acid. The absorbance was immediately read at 490 nm.

Competitive Conjugate-Coated ELISA Format. Microtiter plates were coated as described above with the appropriate concentration of EIT-0073−RSA conjugate. The washed plates then received 50 mL of standard solution of imazalil or related compounds in methanol plus IgG solution diluted with PBS. A competition was established between the immobilized conjugate and the free analytes for the antibody binding sites. The plates were incubated at 25 °C for 1 h and washed five times according to the above-mentioned method. Next, 50 μL of a 1/1000 dilution of HRP-labeled goat anti-mouse IgGs in PBS was added to each well, and the plates were incubated for 1 h at 25 °C. The enzymatic activity was determined as described for the noncompetitive ELISA format. The sera, culture supernatants, and MoAb affinity were estimated by IC_{50}.

Direct Antibody-Coated ELISA Format. Microtiter plates were coated with the MoAb (10 μg/mL, 50 μL/well) in coating buffer overnight at 4 °C. After washing, 300 μL of blocking solution was added, and the resultant mixture was incubated overnight at 4 °C. The competition step for the standard curves was performed as follows: 450 μL of PBS, 50 μL of an imazalil standard solution of a concentration ranging from 0.2 ng/mL to 20 μg/mL, and 500 μL of EIT-0073−HRP conjugate (1:16000 in PBS) were mixed and added to each well (50 μL/well). Then, an immunoreaction was performed for 1 h at 25 °C, and subsequently, after washing, the HRP activity bound to the well was read as previously described for the conjugate-coated ELISA format.

Stocks and Standard Curves. Analyte was prepared at 1 mg/mL in methanol and kept at 4 °C in glass vials. For standard curves, a 1:10 dilution was prepared in methanol from stock solution.

Sample Preparation. *ELISA Analysis.* Citrus fruit samples were prepared as follows: 10 g of each homogenate was shaken with 20 mL of methanol for 1 h. After filtration, each sample was made up to 50 mL with methanol. The extract solution was diluted 10 times with PBS before an ELISA analysis.

HPLC Analysis. Citrus fruit samples were prepared as follows: 20 g of each homogenate was shaken with 100 mL of acetone and 5 mL of 1.0 M sodium hydroxide for 30 min. After the sample mixture was filtered, the solution was concentrated to ∼30 mL with a rotary evaporator and then was applied to a Chem Elut SPE cartridge. The target compound was eluted with 100 mL of ethyl acetate and concentrated to ∼30 mL. Next, 30 mL of 0.05 M sulfuric acid was added to this extract residue and was vigorously shaken with a separatory funnel. The aqueous phase was washed with 30 mL of *n*-hexane and then re-extracted with 50 mL of ethyl acetate containing 1.0 M sodium hydroxide. The organic phase was evaporated to dryness with a rotary evaporator and was made up to 5 mL with acetonitrile for an HPLC analysis.

RESULTS AND DISCUSSION

Evaluation of Polyclonal Mice Antisera. Preliminary studies of mice antisera may provide some information on the immunogenicity of synthetic antigens and on further work performance such as the screening of fusion cultures. Following a booster injection, the resulting antisera were subjected to a competitive inhibition study to imazalil by the indirect competitive ELISA. The antisera from mice immunized with each dose of EIT-0073−BSA conjugate showed that the IC_{50} value for imazalil was 1−10 μg/mL. From mice with the lowest IC_{50} values, four mice immunized with 50 μg and three mice immunized with 100 μg were used for cell fusion. The supernatants from 2688 wells were screened for antibody activity to EIT-0073−RSA by the indirect ELISA, and 12 cell lines were ultimately established. The subclass of all 12 cell lines was all of the IgG_1 (κ) isotype. Furthermore, the reactivity to imazalil was estimated with culture supernatants containing MoAbs from each cell line. The IC_{50} values for imazalil were in the range of 3−60 ng/mL (Table 1).

Table 1. Characterization of Anti-imazalil MoAbs

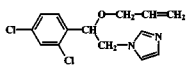
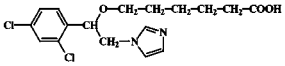
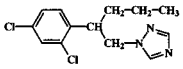
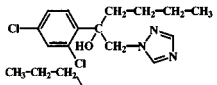
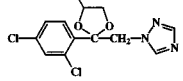
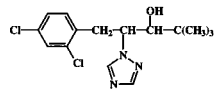
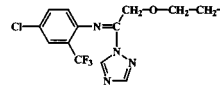
MoAb	IC_{50} (ng/mL)	MoAb	IC_{50} (ng/mL)
9C1-1-1	18	9C14-1-1	18
9C5-1-1	6	9C16-1-1	9
9C6-1-1	18	9C18-1-1	9
9C8-1-1	4	9C19-1-1	39
9C9-1-1	3	9E1-1	60
9C12-1-1	7	9G2-1	60

Specificity of the Imazalil ELISA. The cross-reactivity (CR) of each anti-imazalil MoAb was evaluated by using five kinds of azole fungicides: penconazole, hexaconazole, propiconazole, diclobutrazol, and triflumizole. As shown in Table 2, the specificity patterns were almost the same except 9C14-1-1 MoAb; that is, although 9C14-1-1 MoAb showed 26.1% CR (IC_{50} = 230 pmol/mL) to penconazole, other MoAbs showed CR < 2.0% to five kinds of related azole fungicides. In this study, based on the specificity and the sensitivity to imazalil, MoAbs for further study were selected. Furthermore, in consideration of the growth condition of each cell line, the productive condition of ascite, and so on, four MoAbs (9C5-1-1, 9C9-1-1, 9C14-1-1, and 9C18-1-1 MoAbs) shown in Table 3 were selected for further study. The CR of these MoAbs was further examined with five kinds of imazalil analogues. The reactivity of the four kinds of MoAbs to EIT-0111 was higher than that to propiconazole. The CR of 9C14-1-1 MoAb against EIT-0111 was 15.0%. In the case of EIT-0158, all of the MoAbs showed CR values of <2.0%. The reactivity of all MoAbs to K-240 was higher than that to EIT-0158. From these results, it was suggested that all MoAbs distinguished imidazole rings from triazole rings because the reactivity to EIT-0111 was higher than that to propiconazole. The length of an alkyl chain may have affected reactivity, but it was difficult to estimate whether the alkyl chain was part of the epitope of these MoAbs corresponding to the length of the alkyl chain. These findings indicated that the length of the alkyl chain bound to the end of oxygen was important for the immunological interaction. From the above-mentioned results, 9C9-1-1 MoAb, which had the highest sensitivity (IC_{50} = 9 pmol/mL) and the highest specificity to imazalil, was finally selected and examined for application to actual samples.

Effect of Methanol. An ELISA usually requires a process of extraction from citrus fruits. An organic solvent, such as methanol or acetone, is needed for such an extraction procedure because imazalil is a hydrophobic compound. However, it is well-known that organic solvents affect the reactivity and the sensitivity of an antibody. Therefore, we investigated the effect of organic solvents on one of the MoAbs. In this study, we selected methanol as the solvent for extraction.

The extraction efficiency from each citrus fruit was evaluated. In the case of the instrumental analysis, acetone and acetonitrile are generally used as the solvents for extraction. Furthermore, the official procedure for extraction of imazalil was carried out with acetone under alkaline condition (Ministry of Health and Welfare of Japan, 1993). Akiyama et al. (1996) and Tonogai et al. (1998) reported the extraction with acetonitrile under alkaline conditions using sodium acetate. On the other hand, in the case of the ELISA, methanol was usually used as the solvent for extraction of a hydrophobic compound from a citrus fruit. First, to

Table 2. Cross-Reactivity of Various Related Compounds with Anti-imazalil MoAbs

compound	structure	IC_{50} (pmol/mL) (Cross-reactivity : % [a])										
		9C1-1-1	9C5-1-1	9C6-1-1	9C8-1-1	9C9-1-1	9C12-1-1	9C14-1-1	9C16-1-1	9C18-1-1	9C19-1-1	9E1-1	9G2-1
Imazalil		60 100	20 100	60 100	13 100	9 100	22 100	60 100	30 100	30 100	130 100	200 100	200 100
EIT-0073		2 3000	2 1000	3 2000	1.8 722	1.3 692	1.4 1571	20 300	0.6 5000	1.3 2308	4 3250	60 333	17 1176
Penconazole		27000 0.2	6000 0.3	12000 0.5	4200 0.3	4800 0.2	4300 0.5	230 26.1	7200 0.4	6000 0.5	40000 0.3	>1000 <20	>1000 <20
Hexaconazole		>100000 <0.1	13000 0.2	40000 0.2	12000 0.1	22000 0.04	20000 0.1	>100000 <0.1	23000 0.1	20000 0.2	>100000 <0.13	>1000 <20	>1000 <20
Propiconazole		>100000 <0.1	13000 0.2	40000 0.2	12000 0.1	22000 0.04	20000 0.1	>100000 <0.1	23000 0.1	20000 0.2	>100000 <0.13	N.T. ------	N.T. ------
Diclobutrazol		>100000 <0.1	>100000 <0.1	>100000 <0.1	>100000 <0.1	>100000 <0.1	>100000 <0.1	>100000 <0.1	>100000 <0.1	>100000 <0.1	>100000 <0.1	N.T. ------	N.T. ------
Triflumizole		N.T. ------	>1000 <2.0	N.T. ------	>1000 <2.0	>1000 <2.0	>1000 <2.0	N.T. ------	>1000 <2.0	>1000 <2.0	N.T. ------	N.T. ------	N.T. ------

[a] Cross-reactivity is defined as (imazalil concentration for 50% inhibition)/(related compound concentration for 50% inhibition) × 100. N.T., not tested.

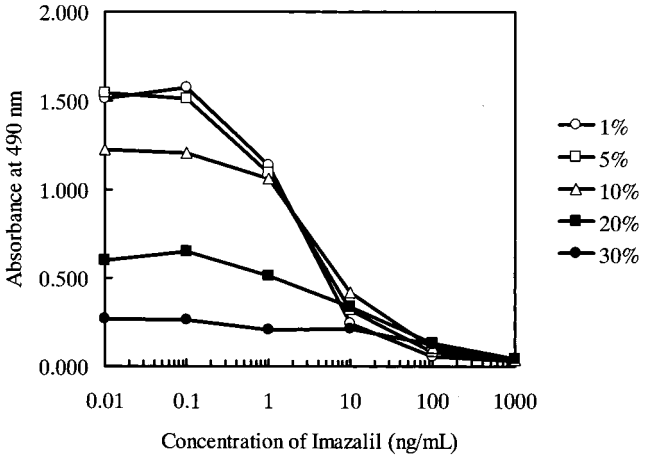


Figure 2. Effect of methanol on reactivity and sensitivity of 9C9-1-1 MoAb. The data are corrected for background and are the average of two replicates. IC_{50} values were as follows: 1%, 2 ng/mL; 5%, 3.5 ng/mL; 10%, 5.5 ng/mL; 20%, 15 ng/mL; 30%, 75 ng/mL. Conditions for assay were as follows: immobilized concentration of 9C9-1-1 MoAb, 10 μg/mL; assay buffer, PBS; final concentration of EIT-0073−HRP conjugate, 1/16000 in PBS; incubation time and temperature, 1 h at 25 °C.

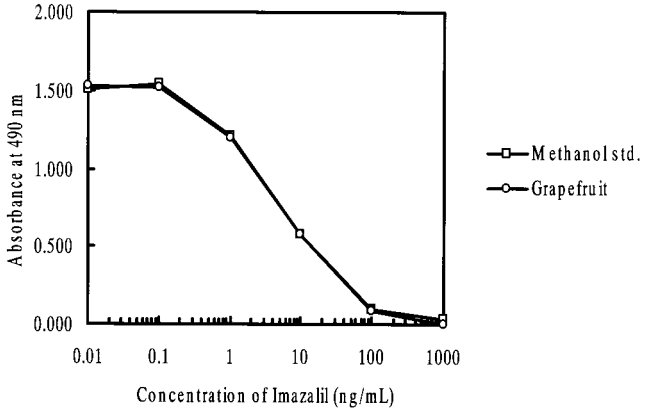
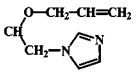
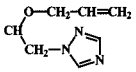
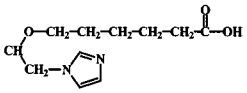
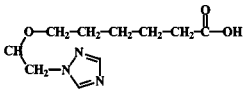
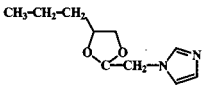
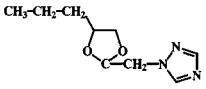
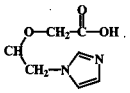
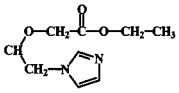


Figure 3. Influence of grapefruit matrix on the standard curve. The data are corrected for background and are the average of four replicates with coefficient of variation below 17%. Conditions for assay were as follows: immobilized concentration of 9C9-1-1 MoAb, 10 μg/mL; assay buffer, PBS; final concentration of grapefruit matrix, 5% (v/v); final concentration of EIT-0073−HRP conjugate, 1/16000 in PBS; incubation time and temperature, 1 h at 25 °C.

evaluate the extraction efficiency, the extraction with acetone under alkaline conditions and the extraction with methanol under neutral conditions were compared. The extraction efficiencies were measured according to the above-mentioned HPLC method. Because the extraction efficiencies were almost the same as recovery data at each citrus fruit sample fortified at 1 ppm (extraction with acetone, ≥91%; extraction with methanol, ≥93%), the methanol extraction under neutral conditions was comparable to conventional procedures. Therefore, we confirmed that it was possible to extract imazalil from citrus fruit samples by using methanol as the solvent for extraction. The effect of methanol on the 9C9-1-1 MoAb was evaluated by preparing imazalil in PBS containing 1−30% (v/v) methanol (Figure 2). Color development was significantly decreased with an increase in the concentration of methanol. As shown in Figure 2, methanol addition influenced the sensitivity and absorbance for the ELISA, and 10% (v/v) methanol was selected for the ELISA analysis.

Effects of Citrus Fruit Matrices. An ELISA has a potential advantage of requiring no cleanup procedures. However, citrus fruits matrices may contain interference. Therefore, we estimated the matrix effect of citrus fruits on the 9C9-1-1 MoAb. Imazalil standards were added to lemon, orange, and grapefruit extracts, and

Table 3. Cross-Reactivity of Related Compounds with Selected Anti-imazalil MoAbs

compound	structure	IC_{50} (pmol/mL) (Cross-reactivity : % [a])			
		9C5-1-1	9C9-1-1	9C14-1-1	9C18-1-1
Imazalil		20	9	60	30
		100	100	100	100
EIT-0183		14000	9000	600	17000
		0.1	0.1	10	0.2
EIT-0073		2	1.3	20	1.3
		1000	692	300	2308
EIT-0180		1300	1000	15	1500
		1.5	0.9	400	2.0
EIT-0111		500	300	400	700
		4.0	3.0	15.0	4.3
Propiconazole		13000	22000	>100000	20000
		0.2	0.04	<0.1	0.2
EIT-0158		>6000	>3000	3600	>9000
		<0.3	<0.3	1.7	<1.0
K-240		520	180	3.6	510
		3.8	5.0	1667	5.9

[a] Cross-reactivity is defined as (imazalil concentration for 50% inhibition)/(related compound concentration for 50% inhibition) × 100.

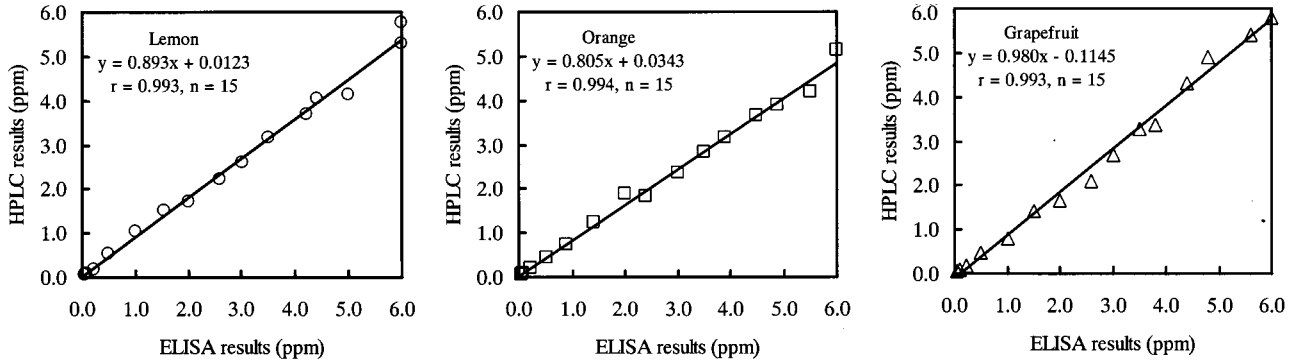


Figure 4. Correlation between the proposed ELISA and HPLC for imazalil in citrus fruits. The data are the average of five replicates with coefficient of variation below 15% for ELISA and of three replicates with coefficient of variation below 19% for HPLC.

the standard curves were compared. Figure 3 shows the result obtained from the grapefruit extract. There was no significant matrix interference from the grapefruit extract. Also, there was no significant difference between the lemon or orange extract and the control (data not shown). Therefore, these extracts required no cleanup procedure and the method could be applied to actual samples.

Analyses of Spiked Citrus Fruit Samples. Citrus fruit samples were fortified with imazalil at 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, and 5.0 ppm and analyzed by using the optimized ELISA. Determinations were made in quadruplicate, and the mean absorbance was used to estimate the imazalil concentration by interpolation of the standard curve obtained with the same plate. Each spiked sample was analyzed five times. The detection limit of the ELISA, calculated as the imazalil concentration that reduces the absorbance to 90% of the maximum, was 0.1 ng/mL for lemons, oranges, and grapefruits. Amounts of imazalil were accurately recovered at all concentration levels with an average recovery in the range of 81.0−107.0% (Table 4). This optimized ELISA was precise with CV ranging from 1.7 to 14.6%. These results show that the established analytical

Table 4. Percentage Recovery of Imazalil from Citrus Fruit Samples[a]

sample	imazalil spike level (ppm, μg/g)	mean ± SD (ppm)	CV (%)	recovery (%)
lemon	0.05	0.05 ± 0.01	14.6	104.0
	0.1	0.09 ± 0.01	5.6	90.8
	0.2	0.20 ± 0.02	9.4	100.0
	0.5	0.51 ± 0.03	6.5	102.4
	1.0	0.93 ± 0.07	7.0	93.4
	2.0	1.90 ± 0.11	5.7	96.0
	5.0	4.90 ± 0.08	1.7	97.6
orange	0.05	0.05 ± 0.01	11.0	97.6
	0.1	0.10 ± 0.01	13.0	99.0
	0.2	0.20 ± 0.01	7.1	100.0
	0.5	0.49 ± 0.04	8.0	97.6
	1.0	0.81 ± 0.07	8.2	81.0
	2.0	2.00 ± 0.12	6.1	100.0
	5.0	5.00 ± 0.28	5.6	99.6
grapefruit	0.05	0.05 ± 0.01	11.5	94.4
	0.1	0.10 ± 0.01	10.2	96.4
	0.2	0.21 ± 0.03	13.5	107.0
	0.5	0.49 ± 0.03	6.0	97.6
	1.0	1.00 ± 0.09	9.0	100.8
	2.0	2.00 ± 0.14	7.1	100.0
	5.0	5.00 ± 0.15	3.0	99.6

[a] $n = 5$ replicates.

Table 5. Application to Actual Samples Using the Proposed ELISA

sample	imazalil[a] (ppm) ELISA	imazalil[a] (ppm) HPLC
lemon (California)	1.10	1.04
orange (Florida)	1.70	1.42
grapefruit (California)	0.60	0.55

[a] Values were based on a single analysis.

conditions of this study were the same as those of the conventional methods conducted in Japan in terms of the detection limits. The results of the recovery test also show that the ELISA is able to analyze imazalil in a concentration of ~5 ppm, which is the tolerance level for imazalil in Japan.

Correlation between the Proposed ELISA and HPLC. The sensitivity of the ELISA for imazalil was compared with that of an HPLC analysis. As shown in Figure 4, good correlations were obtained between the proposed ELISA and the HPLC data from lemons (slope = 0.893, $r = 0.993$), oranges (slope = 0.805, $r = 0.994$), and grapefruits (slope = 0.980, $r = 0.993$). These results show that imazalil in citrus fruits could be simply and rapidly analyzed using the ELISA without any cleanup procedures. Furthermore, these results also indicated the lower recovery by the HPLC analysis because the pretreatment for HPLC required a very complicated and lengthy procedure, whereas the proposed ELISA can analyze imazalil in citrus fruit samples without any cleanup procedure. Although it takes 4−5 h to analyze imazalil in citrus fruit samples with the conventional methods, the proposed ELISA is able to analyze each sample within 2 h and permits high-throughput screening.

Application to Actual Samples. To evaluate the proposed ELISA, we analyzed imazalil residues in commercially available citrus fruits imported from California and Florida. The residue level for imazalil had been previously measured by HPLC. As shown in Table 5, the amount of residue measured by the proposed ELISA corresponded to that measured by the HPLC. These findings suggested that it is possible to apply the proposed ELISA to the analysis of residue imazalil in actual citrus fruit samples.

Conclusion. The anti-imazalil MoAbs were generated by immunization with EIT-0073 conjugated to BSA. No cross-reactivities were measured to other azole fungicides such as penconazole, hexaconazole, propiconazole, diclobutrazol, and triflumizole, and we obtained satisfactory sensitivity by using an ELISA. Furthermore, no significant matrix effects were observed with direct dilution by using a buffer solution. This ELISA was able to determine residual imazalil in citrus fruit samples without any cleanup procedures. Therefore, the proposed ELISA would be useful in coping with the increasing amount of citrus fruits imported to Japan.

LITERATURE CITED

Abad, A.; Moreno, M. J.; Pelegri, R.; Martinez, M. I.; Saez, A.; Gamon, M.; Montaya, A. Determination of carbaryl, carbofuran and methiocarb in cucumbers and strawberries by monoclonal enzyme immunoassays and high-performance liquid chromatography with fluorescence detection. An analytical comparison. *J. Chromatogr. A* **1999**, *833*, 3−12.

Akiyama, Y.; Yano, M.; Mitsuhashi, T.; Takeda, N.; Tsuji, M. Simultaneous determination of pesticides in agricultural products by solid-phase extraction and gas chromatography−mass spectrometry. *J. Food Hyg. Soc. Jpn.* **1996**, *37*, 351−362.

Akiyama, Y.; Yoshioka, N.; Yano, M.; Mitsuhashi, T.; Takeda, N.; Tsuji, M.; Matsushita, S. Pesticide residues in agricultural products (F. Y. 1994−1996). *J. Food Hyg. Soc. Jpn.* **1997**, *38*, 381−389.

Bushway, R. J.; Brandon, D. L.; Bates, A. H.; Li, L.; Larkin, K. A.; Young, B. S. Quantitative determination of thiabendazole in fruit juices and bulk juice concentrates using a thiabendazole monoclonal antibody. *J. Agric. Food Chem.* **1995**, *43*, 1407−1412.

Cairoli, S.; Arnoldi, A.; Pagani, S. Enzyme-linked immunosorbent assay for the quantitation of the fungicide tetraconazole in fruits and fruit juices. *J. Agric. Food Chem.* **1996**, *44*, 3849−3854.

Chatani, Y.; Chikamoto, T.; Munehisa, M.; Adachi, T.; Komatsu, M. Systematic determination of 6 postharvest pesticides in imported citrus fruits. *J. Food Hyg. Soc. Jpn.* **1996**, *37*, 187−194.

Ellis, R. L. Rapid test methods for regulatory programs. In *Immunoassays for Residue Analysis. Food Safty*; Beier, R. C., Stanker, L. H., Eds.; ACS Symposium Series 621; American Chemical Society: Washington, DC, 1996; pp 44−58.

Garrido, J.; de Alba, M.; Jimenez, I.; Casado, E.; Folgueiras, M. L. Chromatographic analysis of imazalil and carbendazim in fruits. Method validation and residue monitoring program 1995. *J. Chromatogr. A* **1997**, *765*, 91−97.

Harris, A. S.; Lucas, A. D.; Kramer, P. M.; Marco, M.-P.; Gee, S. J.; Hammock, B. D. Use of immunoassay for the detection of urinary biomarkers of exposure. In *New Frontiers in Agrochemical Immunoassay*; Kurtz, D. A., Skerritt, J. H., Stanker, L., Eds.; AOAC International: Arlington, VA, 1995; pp 217−235.

Hori, S. Profile of the pesticides residues in foods. *J. Food Hyg. Soc. Jpn.* **1997**, *38*, J-175−J-176.

Ito, Y.; Ikai, Y.; Oka, H.; Hayakawa, J.; Kagami, T. Application of ion-exchange cartridge clean-up in food analysis I. Simultaneous determination of thiabendazole and imazalil in citrus fruit and banana using high-performance liquid chromatography with ultraviolet detection. *J. Chromatogr. A* **1998**, *810*, 81−87.

Karu, A. E.; Goodrow, M. H.; Schmidt, D. J.; Hammock, B. D.; Bigelow, M. W. Synthesis of haptens and derivation of monoclonal antibodies for immunoassay of the phenylurea herbicide diuron. *J. Agric. Food Chem.* **1994**, *42*, 301−309.

Kawahara, A.; Yamada, M.; Kato, Y.; Nakamura, M.; Nishijima, M.; Ito, Y. Systematic determination of diphenyl, *o*-phenylphenol, thiabendazole and imazalil in citrus fruits. *Food Food Ingredients J. Jpn.* **1995**, *163*, 104−110.

Laville, E. Etudes des activites du R 23979 et de ses sels sur les pourritures a *Penicillium* (*P. digitatum* et *P. italicum*) des oranges. *Fruits* **1973**, *28*, 545−547.

Mercader, J. V.; Montaya, A. Development of monoclonal ELISAs for azinphos-methyl. 2. Assay optimization and water sample analysis. *J. Agric. Food Chem.* **1999**, *47*, 1285−1293.

Ministry of Health and Welfare of Japan. Bulletin 159, Sept 14, 1993.

Nagayama, T.; Kobayashi, M.; Shioda, H.; Tamura, Y. Pesticide residues in imported raw agricultural commodity. *J. Food Hyg. Soc. Jpn.* **1995a**, *36*, 643−655.

Nagayama, T.; Kobayashi, M.; Shioda, H.; Ito, M.; Tamura, Y. Relationship between pesticide residues in fruit peel and flesh. *J. Food Hyg. Soc. Jpn.* **1995b**, *36*, 383−392.

Nagayama, T.; Kobayashi, M.; Ito, M.; Shioda, H.; Tomomatsu, T. Pesticide residues in imported fruit products. *J. Food Hyg. Soc. Jpn.* **1996**, *37*, 127−134.

Nakazato, M.; Tadano, K.; Ogawa, H.; Ushiyama, H.; Kawai, Y.; Kobayashi, C.; Tateishi, Y.; Tamura, Y. Determination of imazalil, diphenyl, thiabendazole and *o*-phenylphenol in citrus fruits, and of imazalil and thiabendazole in banana, by high performance liquid chromatography. *Jpn. J. Toxicol. Environ. Health* **1995**, *41*, 392−397.

Schlaeppi, J. M.; Fory, W.; Ramsteiner, K. Hydroxyatrazine and atrazine determination in soil and water by enzyme-linked immunosorbent assay using specific monoclonal antibodies. *J. Agric. Food Chem.* **1989**, *37*, 1532−1538.

Szekacs, A.; Hammock, B. D. Development of an enzyme-linked immunosorbent assay for the detection of the triazole fungicide myclobutanil. *J. Agric. Food Chem.* **1995**, *43*, 2083−2091.

Takeda, M.; Ito, Y.; Odanaka, Y.; Komatsu, K.; Maekawa, Y.; Matano, O. *Saishin Noyaku No Zanryu Bunsekiho* (in Japanese); Noyaku Zanryu Bunsekiho Kenkyuhan, Eds.; Chuo Hoki Publishing: Tokyo, Japan, 1995a; pp 349−351.

Takeda, N.; Tsuji, M.; Akiyama, Y. HPLC analysis of pesticides in brown rice using UV absorption and postcolumn photolysis-fluorescence detection. *J. Food Hyg. Soc. Jpn.* **1995b**, *36*, 601−606.

Tomlin, C. *A World Compendium, The Pesticide Manual Incorporating the Agrichemical Handbook*, 10th ed.; Crop Protection Publications: Thornton Health, England, 1994; pp 580−581.

Tonogai, Y.; Tsumura, Y.; Nakamura, Y.; Ito, Y. Analysis of post-harvest application pesticides in citrus fruits. *J. Food Hyg. Soc. Jpn.* **1992a**, *33*, 23−30.

Tonogai, Y.; Tsumura, Y.; Nakamura, Y.; Matsuki, H.; Ito, Y. Studies on simultaneous determination of 12 kinds of fungicides containing unstable captan, captafol *etc.* in preparation of test solution. *Jpn. J. Toxicol. Environ. Health* **1992b**, *38*, 270−281.

Tonogai, Y.; Tsumura, Y.; Nakamura, Y.; Shibata, T. Simultaneous determination of pesticides and their metabolites in fresh fruits and vegetables by HPLC. *J. Food Hyg. Soc. Jpn.* **1998**, *39*, 13−25.

Torres, C. M.; Pico, Y.; Manes, J. Comparison of octadecylsilica and graphitized carbon black as materials for solid-phase extraction of fungicide and insecticide residues from fruit and vegetables. *J. Chromatogr. A* **1997**, *778*, 127−137.

Yamazaki, Y.; Ninomiya, T. Determination of imazalil residues in lemons by gas chromatography with nitrogen−phosphorus detection. *J. AOAC Int.* **1996**, *79*, 787−790.

Yuasa, Y. Analysis of pesticide residues by immunoassay. *J. Food Hyg. Soc. Jpn.* **1998**, *39*, 61−66.

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