Development of the Assay Methods for Pesticides by Immunological Technique

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List of Abbreviations

ELISAs	enzyme-linked immunosorbent assays
dc-ELISA	direct competitive enzyme-linked immunosorbent assay
ic-ELISA	indirect competitive enzyme-linked immunosorbent assay
MoAb	monoclonal antibody
QuEChERS	the quick, cheap, effective, rugged and safe
PoAb	polyclonal antibody
BSA	bovine serum albumin
RSA	rabbit serum albumin
HRP	horseradish peroxidase
FBS	fetal bovine serum
TLC	thin layer chromatography
PBS	phosphate buffered saline
PBS-BSA	PBS modified with 0.2% BSA
ADI	acceptable daily intake
MRLs	maximum residue limits

GC	gas chromatography
HPLC	high performance liquid chromatography
QCM	quartz crystal microbalance
SPR	surface plasmon resonance
2,4-D	2,4-dichlorophenoxyacetic acid
2,4,5-T	2,4,5-trichlorophenoxyacetic acid
NHS	*N*-hydroxysuccinimide
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, hydrochloride
SDS	sodium dodecyl sulfate
GdnHCl	guanidine hydrochloride
TPN-BSA	*N*-(pentachlorophenoxyacetyl)glycine and BSA
RU	resonance unit
KLH	keyhole limpet hemocyanin
HAT	hypoxanthine-aminopterin-thymidine
CH_2Cl_2	dichloromethan
SPR-sensor	immunosensor based on surface plasmon resonance

Introduction

In recent years, it is rise of national consciousness to pesticide residues in a food. Based on the Food Hygiene Law, the positive list system was enacted, and established the maximum residue limits (MRLs) of each food about all pesticides. A simultaneous analysis by high-performance liquid chromatography (HPLC) - a mass spectrograph (MS) and gas liquid chromatography (GC) - MS was introduced into actual determination. But it was difficult for a food enterpriser to use because expensive apparatus and high technique were necessary. On the other hand, immunoassay (directly competing ELISA) for the pesticide determination was developed and was marketed. This determination method is used in the production and distribution site of food, because of its quickness, easiness and cheapness.

Therefore, in this study, I aimed at the development of the direct competitive ELISA for fungicide chlorothalonil and boscalid which were detected frequency. In addition, ELISA accuracy varies depending on the skill level of the user. Therefore, immunosensor was developed based on surface plasmon resonance (SPR) which is intermolecular interaction measuring equipment (Biacore T200 : GE healthcare Inc.), and tried automation of the immunoassay.

Chlorothalonil is one of fungicides which was developed in 1964. Chlorothalonil residues have been often detected in crops, because it is widely used even now. Already development of ELISA for determination of chlorothalonil was reported, and commercially ELISA kits are available. However, the specificity of the reaction was low because

chlorothalonil structure was simple, and there were many similar pesticides. Therefore a carboxylic acid derivative of chlorothalonil was prepared as reported previously. And it was used to prepare an anti-chlorothalonil monoclonal antibody (MoAb). By repeating of screening, specific MoAb TPN9A was obtained. Working range was 0.10-6.0 ng/mL in the established direct competitive ELISA. It had sufficient sensitivity to determine chlorotharonil at the MRLs in crops (0.5-50 ng/g). In addition, it showed the specificity that was higher than existing ELISA although it showed cross-reactivity with phthalide (22 %) and Quintozene (17 %). The recovery of chlorotharonil spiked in vegetables was 97.1-125 %. The results correlated well with those obtained by HPLC analysis (R^2=0.98-0.99). The established direct competitive ELISA was useful to determination of chlorotharonil in vegetables.

An immunosensor based on SPR was developed. The sensitivity was 10 times lower compared to the indirect competitive ELISA. However, the working range of the sensor was 8.0-44 ng/mL and it was enough for determinate at the MRLs. The sensor showed sufficient accuracy and it needs only 3 minutes to determinate. Chlorotharonil spiked in vegetables was recovered at 90-118 %. The results correlated well with those obtained by HPLC results (R^2=1.00). Developed immunosensor is the first example of success as pesticide residue determination in vegetables, using SPR.

Boscalid is one of fungicides which is effective to plant diseases caused by fungi such as *Botrytis* spp. and *Sclerotini*a spp. In Japan boscalid was registered in 2005. Boscalid is one of the most generally used fungicides, consequently it is detected frequently. For that

reason determination of boscaid is important. Already, ELISA for boscalid, using polyclonal antibodies to boscalid, have been developed.

But its sensitivity is too high to determinate at the MRLs in vegetables. Therefore, newly immunoassay was developed for suitable working range. A carboxylic acid derivative of boscalid was prepared, and it was used to prepare an anti-boscalid monoclonal antibody (MoAb). Direct competivive ELISA and SPR-immunosensor were developed using obtained MoAb BSC7. Boscalid spiked in vegetables was recovered at 100-124 % by measurement of direct competitive ELISA, 85-109 % by SPR-immunosensor. The results correlated well with those obtained by HPLC results (R^2=0.97 and 0.95). These methods showed good performance and these are useful for determination of boscalid.

Clothianidin and nitenpyram are belong to neonicotinoid insecticide which is the most widely used currently. They are sometimes applied in a simultaneous period with fungicide boscalid. SPR-immunosensor has 3 channels are measured in parallel, thus simultaneous analysis was developed. MoAbs to clothianidin and nitenpyarm were offered from Horiba, Ltd. Carboxylic acid derivatives of clothianidin and nitenpyram were prepared as described previously. The recovery of boscalid, clothianidin and nitenpyram spiked in vegetables were 72-105 %. The results correlated well with those obtained by direct conpetitive ELISA (R^2=0.98-1.00). Achievement of this study suggests that it's possible to develop SPR-immunosensor for simultaneous multicomponent measurement.

In this study, immunoassays were developed for pesticide residues determination. Developed direct competitive ELISAs are able to use for pre-shipment inspection as

commercially available kits. And it was indicated that developed SPR-immunosensor are high practicality. It is expected that the new sensor system corresponding to this concept will be developed.

Chapter 1

Development of Direct Competitive ELISA for Residue Analysis of Fungicide Chlorothalonil in Vegetables

Abstract. With the aim of developing rapid and simple determination method for chlorothalonil residue in vegetables, a direct competitive (dc)-ELISA was developed. A carboxylic acid derivative of pentachlorophenol was used for preparation of anti-chlorothalonil monoclonal antibody (MoAb). MoAb TPN9A was adequate reactivity for the dc-ELISA constitution. Chlorothalonil in vegetables is rapidly decomposed by the enzyme contained in the vegetables after homogenization. For the prevention, phosphoric acid is added on the homogenization (vegetable/10% phosphoric acid (2: 1, w/v)). The addition gave influence to reaction of the dc-ELISA, but the influence could be neglected by using phosphate buffer (100 mmol/L, pH 7.0) on competitive reaction in the dc-ELISA. Working range was 0.10 to 6.0 ng/mL in optimized dc-ELISA. The recovery results of chlorothalonil spiked in cucumber and eggplant were 97.1 to 125% by the dc-ELISA. The results were sufficient although the rate was slightly over 100%, and correlated well with those by HPLC analysis. The dc-ELISA could rapidly determine the chlorothalonil only with simple sample preparation.

INTRODUCTION

Chlorothalonil, tetrachloroisophthalonitrile, is a non-systemic organochlorine fungicide which is effective against various fungal diseases in wide range of vegetables. Since chlorothalonil was developed on 1964, it has been frequently used for pathogen control in farms because of its high effectivity.[1] About 4700 ton of chlorothalonil, which is the largest amount as a fungicide next to thiophanate-methyl, was actually produced in 2011 in Japan.[2] It was also reported that chlorothalonil had been frequently detected in environment samples and in fruit and vegetable samples from Ontario, Canada.[3,4] Furthermore, chlorothalonil has been categorized in Group 2B as a possibly carcinogenic substance by International Agency for Research on Cancer, while acute oral LD_{50} value for rats was relatively large (>5000 mg/kg).[1] Residue monitoring of chlorothalonil is therefore highly important for public health and safety.

Chlorothalonil is generally analyzed by GC-ECD or GC-MS, or by HPLC-UV or LC-MS.[5-8] Different from the typical cases of other pesticides, chlorothalonil is extracted from vegetable samples under acidic condition for GC/HPLC analysis, by adding an adequate acid, e.g. phosphoric acid, sulfuric acid or formic acid.[5,8,*1] It was exactly reported that when acetonitrile extraction with gel permeation chromatography or the quick, cheap, effective, rugged and safe (QuEChERS) method was used for simultaneous determination of various pesticides in vegetables, the analysis result of residual chlorothalonil was problematic. [9, 10] It was found that the sensitivity of QuEChERS method for chlorothalonil could be improved by adding acetic acid to the vegetable samples. [10] This means acidic

[*1]Ministry of Health, Labour and Welfare in Japan, http://www.mhlw.go.jp/topics/bukyoku/iyaku/syoku-anzen/zanryu3/siken.htm [11 September 2013]. *in Japanese*

condition is required for ensuring high yield at chlorothalonil extraction from the vegetables.

Although instrumental analyses described above are sufficiently sensitive and accurate for laboratory testing, they are not applicable for the on-site monitoring of vegetables because they need expensive instruments and the complicated, time-consuming sample preparation. As the other method which can be widely used for the on-site monitoring, various ELISAs have been developed for determining many kinds of pesticides. [11] They are not only simple, rapid and cost-effective methods compared to the instrumental analyses, but are also practically sensitive and accurate for the on-site monitoring of the vegetables. Authors have already developed several kinds of direct competitive (dc)-ELISAs for analyzing residual pesticides in vegetables. [12-14] The dc-ELISAs could correctly determine the target pesticides only with simple sample preparation consisting of extraction with 5 folds volume of methanol, filtration and dilution of methanolic sample extract with 7.5 folds volume of water (final 10% methanol).

A magnetic particle-based ELISA was developed for chlorothalonil analysis in water and vegetable samples. [15] This study however used polyclonal antibody (PoAb) which generally shows different reactivity by immunizing individual animals. On the other hand, analysis results with commercially available kits were also reported on vegetable samples from some research groups. [16-18] They did not define the hapten structure and the antibody characteristics. One of the kit reported by our group showed high sensitivity (IC_{50} value: 0.34 ng/mL) but low specificity (cross-reactivity with phthalide: 58.8%).[18]

In this study, ELISA development was newly examined to establish adequate reactive and specific determination method for chlorothalonil residue analysis in vegetables, with the anti-chlorothalonil specific MoAb.

MATERIALS AND METHODS

Materials. The following standard pesticides (analytical grade for pesticide residue analysis), chlorothalonil, phthalide, pentachlorophenol, quintozene, 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid, were purchased from Wako Pure Chemical Industries (Osaka, Japan). Bovine serum albumin (BSA, Pro. No. A7030), rabbit serum albumin (RSA, Pro. No. A0764), HAT media supplement (Pro. No. H0262-10VL), HT media supplement (Pro. No. H0137-10VL) and polyethylene glycol (molecular weight 1450, Pro. No. 7181-5X5ML) solution were from Sigma-Aldrich (St. Louis, MO, USA), and horseradish peroxidase (HRP, Pro. No. PEO-131) was from Toyobo (Osaka, Japan). Freund's complete adjuvant and incomplete adjuvant were from Difco Laboratories (Detroit, MI). Seven-week-old female Balb/c mice were from Nippon SLC (Shizuoka, Japan) for immunization. RPMI 1640 medium was purchased from Nacalai Tesque (Kyoto, Japan). Fetal bovine serum (FBS) was purchased from PAA Laboratories (Pasching, Austria). Microtiterplate with 96-wells for ELISA (Nunc MaxiSorp) was purchased from Thermo Fisher Scientific (New York, USA). Gel for ion-exchange chromatography (abbreviated to DEAE-gel, TOYOPEARL DEAE-650M) was from Tosoh (Tokyo, Japan). All other chemicals and reagents were analytical grade purchased from Wako Pure Chemical Industries or Nacalai Tesque.

Hapten Synthesis. Carboxylic acid derivative of chlorothalonil (hereinafter called hapten) as N-(pentachlorophenoxyacetyl)glycine was newly synthesized through the flow (compound 1 - 4) as shown in Fig. 1. Lawruk *et al.* only described its chemical name without the method of the synthesis and the results of the structure determination. [15]

General methods: all reactions were performed under nitrogen or an atmosphere of argon in oven-dried glassware unless otherwise noted. All reactions were monitored by thin layer chromatography (TLC), TLC were performed on glass plates and aluminum sheets pre-coated with silica gel Merck KGaA 60 F254, layer thickness 0.2 mm. All the starting materials are commercially available and were used without any purification. The products were visualized by irradiation with UV light or using I_2 or *p*-anisaldehyde. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectrum were recorded on JEOL 400 spectrometer. Chemical shifts are reported as values (ppm) relative to internal tetramethylsilane (0.00 ppm) in $CDCl_3$. IR spectra were recorded by using JASCO FT/IR-230 spectrometer and are reported in reciprocal centimeter (cm^{-1}). Elemental analyses were measured on a Yanaco CHNCORDER MT-6.

A mixture of ethyl 2-(2-chloroacetamido)acetate (compound 1; 89.8 mg, 0.56 mmol) and NaI (8.0 mg, 1.7 mmol) in dry acetone (3 mL) was heated to mild reflux (oil bath temp = 56˚C) for 16.5 hr. Ethyl 2-(2-iodoacetamido)acetate (compound 2) was obtained in quantitative yield. Structural information of compound 2: ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.50 (br s, 1H, -CON*H*-), 4.25 (q, 2H, *J* = 7.02 Hz, -$CO_2C\mathit{H}_2CH_3$), 4.05 (d, 2H, *J* = 5.19 Hz, -NHCH_2CO-), 3.75 (s, 2H, ICH_2CO-), 1.31 (t, 3H, *J* = 7.02 Hz, -$CO_2CH_2C\mathit{H}_3$) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.6(*C*=O, ester), 167.9(*C*=O, amide), 61.8(-$CO_2\mathit{C}H_2CH_3$), 42.2(-NH$\mathit{C}H_2$CO-), 14.2(-$CO_2CH_2\mathit{C}H_3$), -1.3(I$\mathit{C}H_2$CO-) ppm.

Ethyl 2-(2-iodoacetamido)acetate (compound 2) was used for next step without any purification. To this mixture was added K_2CO_3 235.0 mg, 1.7 mmol, (3 equiv.) and 2,3,4,5,6-pentachlorophenol (149.1 mg, 0.56 mmol) at room temperature. The resulting mixture was again heated to mild reflux (oil bath temp = 56˚C) for 4.5 hr and then stirred for 17 hr at room temperature. At the end of this period, the solvent was removed off and water was added. The organic product was extracted with CH_2Cl_2 and dried with Na_2SO_4. The solvent was removed under reduced pressure and the residue was purified by flash column chromatography on silica gel (Merck, Art. No. 7734) using hexane/ethyl acetate (4:1, v/v) as the mobile phase to give ethyl ester of the desired product (compound 3) in 53% yield (two steps). Structural information of compound 3: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36 (br s, 1H, -CON*H*-), 4.57 (s, 2H, -OC*H*$_2$CO-), 4.27 (q, 2H, *J* = 7.02 Hz, -CO_2C*H*$_2CH_3$), 4.19 (d, 2H, *J* = 5.19 Hz, -NHC*H*$_2$CO-), 1.32 (t, 3H, *J* = 7.02 Hz, -CO_2CH_2C*H*$_3$) ppm. ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.4(*C*=O, ester), 167.1(*C*=O, amide), 150.1(-O*C*$_6Cl_5$), 132.3(*o*-*C*$_6Cl_5$), 129.2(*m*-*C*$_6Cl_5$), 128.0(*p*-*C*$_6Cl_5$), 71.3(-O*C*H_2CO-), 61.9 (-CO_2*C*H_2CH_3), 41.1(-NH*C*H_2CO-), 14.3(-CO_2CH_2*C*H_3) ppm. Anal. $C_{12}H_{10}Cl_5NO_4$ · 0.6

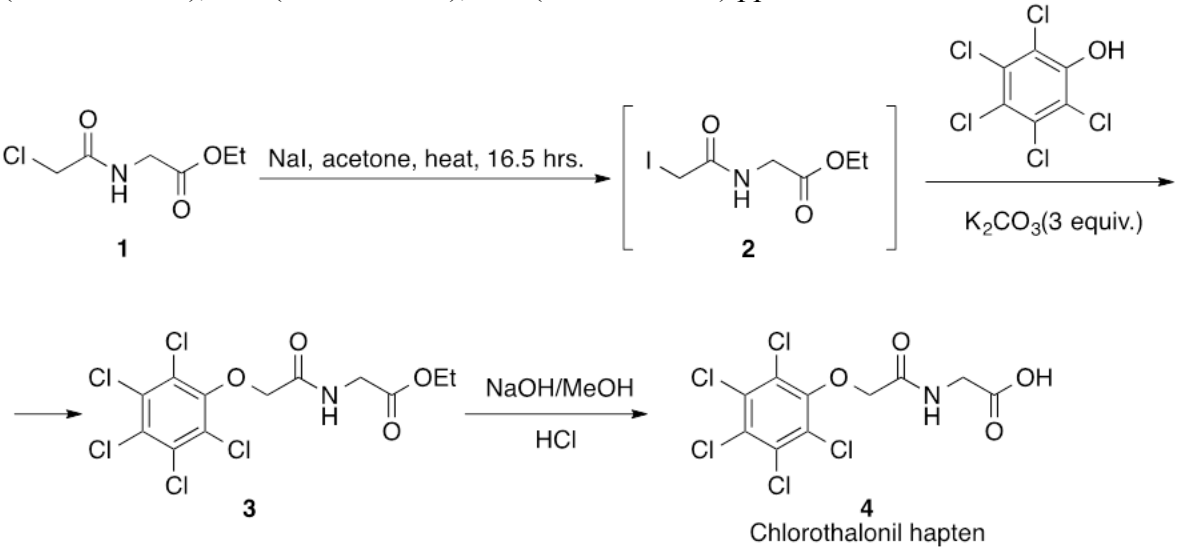


Figure 1. Scheme of the hapten synthesis

H_2O Calcd: C 34.29, H 2.69, N 3.33 %; Found: C 33.94, H 2.29, N 3.33 %. IR (neat) 3413, 3336, 2982, 2933, 1756, 1679 cm^{-1}.

The ester compound 3 (233 mg, 0.57 mmol) was treated with 8 mL of NaOH/methanol solution (1 mol/L) and neutralized with 10% HCl solution. After the removal of the solvent, the product was extracted with ethyl acetate and dried with Na_2SO_4 to give the carboxylic acid (compound 4) in 64% yield. Structural information of compound 4: ^1H NMR (400 MHz, DMSO-d6) δ 12.64 (br s, -COO*H*), 5.10 (s, 2H, -OC*H*$_2$CO-), 4.63 (s, 2H, -NHC*H*$_2$COOH) ppm. N-(pentachlorophenoxyacetyl)glycine (compound 4) was used as chlorothalonil hapten for next step without any further purification.

Hapten-Protein Conjugate Preparation. The synthesized hapten was conjugated with proteins (BSA, RSA and HRP) by the activated ester method as previously described. [13] One mL of the hapten (20 μmol) in dried dimethyl sulfoxide was mixed with 225 μL of N-hydroxysuccinimide (40 μmol) and 1-ethyl-3-(3-dimethylamiopropyl) carbodiimide hydrochloride (40 μmol). Each of 1 mL of BSA, RSA and HRP (10 mg) was used for conjugation with 400 μL of the activated ester of hapten. The hapten-BSA conjugate was used for immunization. The hapten-RSA conjugate was used for constitution of a direct-bind (db)-ELISA and an indirect competitive (ic)-ELISA. The hapten-HRP conjugate was used for constitution of a dc-ELISA.

MoAbs Preparation. MoAbs-secreted-cells were prepared based on the previously described procedure. [13] Five numbers of mice were intraperitoneally immunized with 100 μL of the hapten-BSA conjugate (100 μg per a mouse) after it had been emulsified with an

equal volume of Freund's complete adjuvant. Booster immunization (25 μg per a mouse) was performed 2 times using the emulsion with Freund's incomplete adjuvant at intervals of 2 weeks. On the 3rd days after the last immunization, spleen cells from the mice (5×10^8 cells) were fused with P3U1 myeloma cells (5×10^7 cells) by using polyethylene glycol solution. The fused cells were suspended at 2.5×10^6 cells/mL as spleen cells in RPMI1640 medium modified with 10% FBS and with HT media supplement, and each 100 μL was transferred to the wells of a 96-well microplate. The microplate was incubated at 37°C for 24 h in 5% CO_2, and then 40 μL of RPMI 1640 modified with 10% FBS and with HAT media supplement was added to each well. After confirming that the growing hybridoma had formed a colony, each of secreted antibodies in the cultured fluids was screened on the basis of reactivity with the hapten–RSA conjugate by the db-ELISA. Fluids in the positive wells were secondary screened on the basis of reactivity with chlorothalonil by the ic-ELISA. Each hybridoma grown in the positive wells was cloned by limiting dilution technique (2 times), and the representative cell clone was incubated in RPMI1640 modified with 2% FBS at 37°C in 5% CO_2. The cultured fluid was used for preparation of the MoAb. MoAbs were purified with ammonium sulfate fractionation and with open column chromatography on DEAE-gel. Two L of cultured fluid of the hybridoma was continuously stirred, and saturated ammonium sulfate was gradually added to the fluid up to 50% of saturation to co-precipitate IgG with BSA contained in the fluids. After stirred for 30 min, the solution was centrifuged at 8000 rpm at 4°C for 30 min. The precipitate was dissolved with minimum volume of phosphate buffer (10 mmol/L, pH 7.0) modified with NaCl (150 mmol/L), phosphate buffered saline (PBS), and was dialyzed with PBS. The solution was re-precipitated with saturated ammonium sulfate up to 35% of saturation to prepare MoAb

fraction, and was dialyzed with Tris-HCl buffer (20 mmol/L, pH 8.0). Ten mL of DEAE-gel in column was equilibrated with the same buffer and 4 mL of the above fractionated MoAb solution was applied to the column. After washing with 30 mL of the Tris-HCl buffer modified with NaCl (40 mmol/L), MoAb was eluted with 30 mL of the Tris-HCl buffer modified with NaCl (50 mmol/L). The MoAb fraction of which purity was checked with SDS-PAGE was assembled. The MoAb concentration was determined from the extinction coefficient (1.4 for 1.0 mg/mL of IgG).

db-ELISA and ic-ELISA. A db-ELISA and an ic-ELISA were constituted on microtiterplate with 96-wells by the previously described procedure. [13] For both of the ELISA constitutions, 50 μL of the prepared hapten-RSA (5.0 μg/mL) dissolved in PBS was added to each well of microtiterplate with 96-wells. For ic-ELISA, chlorothalonil (1 μg/mL) was dissolved in methanol/PBS (1:9, v/v).

dc-ELISA. A dc-ELISA was constituted on microtiterplate with 96-wells by the previously described procedure. [13] Chlorothalonil was dissolved and serially diluted with methanol. The diluents were further diluted to 2-100 folds with deionized water, respectively: the prepared chlorothalonil concentrations were 50 pg/mL-50 ng/mL and methanol concentration was 1-50%. A hapten-HRP conjugate was diluted to the adequate concentration (50 ng/mL for TPN9A and TPN11D) with PBS modified with 0.2% BSA (PBS-BSA), so that the maximum absorbance showed 0.5-1 at 450 nm on this dc-ELISA. On the other hand, the absorbance for TPN12E was almost not developed on the same condition. TPN12E was necessary to use 500 ng/mL of the hapten-HRP conjugate.

However, the condition increased the absorbance base line; The absorbance was 0.15 for TPN12E, and 0.08 for TPN9A and TPN11D. Phosphate buffer concentrations of the PBS-BSA were varied from 10 mmol/L to 200 mmol/L to optimize the assay condition. Each of the chlorothalonil solutions or measurement solutions prepared from vegetables was mixed with an equal volume of the hapten-HRP conjugate solution. The mixture was added to the MoAb coated well, and the dc-ELISA proceeded.

Vegetable Samples. Cucumber and eggplant samples were collected from market. The samples were initially chopped, respectively. After the chopped sample was gently mixed maintaining the piece form, 0.1 mL of chlorothalonil standard solution (0.5, 2.0, 5.0 and 10 mg/kg for cucumber, 0.2, 1.0, 2.0 and 4.0 mg/kg for eggplant) in methanol was added to each sample (100 g), and was stand for 30 min. Each sample was mixed with 50 mL of 10% phosphoric acid and was homogenized by blender.

Treatment for dc-ELISA: 25 mL of methanol was added to the homogenized sample (7.5 g) in each tube (50 mL), and the tube was hermetically sealed with the screw cap. It was vigorously shaken for 30 min to extract chlorothalonil. The extract was centrifuged with 3000 rpm at 4 °C for 15 min. The supernatant was diluted to 8.5-folds with deionized water. The diluted sample was 10% methanol equivalent. Further dilutions of the sample were carried out with water/methanol (9:1, v/v; 10% methanol).

Treatment for HPLC analysis: A vegetable sample was prepared by the procedure of Ministry of Health, Labour and Welfare in Japan, 2005.[*1] The homogenized sample (20 g) was extracted with 100 mL of acetone for 3 min using a homogenizer (Polytron PT2100; Kinematica, Lucerne, Switzerland). After the extraction, the mixture was filtered through a

funnel with diatomaceous earth by suction. The residue was then re-treated with 50 mL of acetone. All filtrate was concentrated to about 30 mL with a rotary evaporator. The concentrated extract was transferred to separation funnel with 100 mL of 10% (w/v) sodium chloride solution. The above flask was washed with 100 mL of n-hexane, and the washed solution was also transferred to the funnel. After vigorously shaking for 5 min, the n-hexane phase was separated. The liquid-liquid partition was 2 times repeated. The separated n-hexane phase was anhydrated with anhydrous sodium sulfate. After filtration, the anhydrated n-hexane phase was concentrated, and the residue was dissolved in 5 mL of n-hexane. Next, the resulting solution was loaded to a Florisil column, which was packed with Florisil (5 g) and anhydrous sodium sulfate (5 g) suspended in adequate amounts of n-hexane, and then the column was washed with 100 mL of n-hexane. After chlorothalonil was eluted with 150 mL of ethyl acetate/ n-hexane (1:9, v/v), the eluate was concentrated, and then the dried residue was dissolved in 2 mL of n-hexane. A portion of the solution (0.5 mL) was dried by evaporation and re-dissolved with 1.0 mL of methanol. The methanol solution was used for HPLC analysis.

HPLC Analysis. HPLC system was consisted on an Agilent 1100 series equipped with a quaternary pump, an auto-sampler, a column oven and a diode array detector. The wavelength for chlorothalonil detection was 246 nm. The column was a SunFire C18 reversed-phase column (i. d. 4.6 mm × 250 mm, 5 μm particle size) (Waters, Milford, MA, USA) with a guard column (i. d. 4.6 mm × 20 mm, 5 μm particle size). The column oven temperature was maintained at 40°C. A volume of 20 μL was injected. The mobile phase was methanol/water (70:30, v/v) at flow rate of 0.8 mL/min. The linearity range was 0.02

μg/mL – 2.0 μg/mL (Y = 83.0X + 0.0202, r = 0.9999) and the LOD was 0.01 μg/mL (S/N = 3).

RESULTS AND DISCUSSION

Preparation of Anti-Chlorothalonil MoAbs. Anti-chlorothalonil MoAbs were prepared using N-(pentachlorophenoxyacetyl)glycine which was synthesized as a hapten with a process shown in Fig. 1. The hapten has 5 chloro-groups as well as pentachlorophenol and quintozene, while chlorothalonil had 4 chloro-groups and 2 cyano-groups which have smaller molecular size than chloro-group. Because chlorothalonil does not have characteristic antigen structure except for such difference of the function groups, it was supposed that the majority of prepared MoAbs was not able to recognize the difference of these 3 fungicides, or that the MoAbs would react more strongly with pentachlorophenol and/or quintozene than chlorothalonil. Here, we made a working hypothesis that at least one hybridoma which can produce chlorothalonil-specific MoAb would be contained among prepared hybridoma population producing MoAbs highly reactive to chlorothalonil. Hybridoma cells were therefore screened by db-ELISA and ic-ELISA to find the cells producing the highly reactive MoAbs and to select chlorothalonil-specific MoAbs.
Fused cells were incubated in 12 pieces of microplates with 96 wells, and cell colonies were formed in almost all wells on the microplates. Cultured fluids in 68 wells were positive for reaction with the hapten-RSA in db-ELISA. Because the immunogen was the hapten-BSA conjugate, the positive wells contained the hapten but not BSA reactive MoAb. Fifteen wells among them were positive with chlorothalonil (1 μg/mL) in ic-ELISA. The reactivity of only 4 wells among them was higher than the target, 10 ng/mL as 50% of inhibition value. This target reactivity was required to constitute dc-ELISA for residual

analysis of chlorothalonil in vegetables, for which the maximum residue levels (MRLs) are 0.5 to 10 mg/kg, and to determine chlorothalonil in the samples after diluting to 10 - 200 ng/mL through the sample preparations. Three kinds of MoAbs, TPN9A, TPN11D and TPN12E, were finally prepared. These 3 MoAbs were expected to be highly reactive and specific to chlorothalonil, because they were selected from numbers of MoAbs contained in the wells through 2 times of screenings. Subclass of the MoAbs was IgG_1. Each of the MoAb was purified with ammonium sulfate fractionation and ion-exchange chromatography.

Comparison of MoAb reactivity by dc-ELISA. A dc-ELISA has less assay steps than an ic-ELISA and has been usually used for pesticide determinations in vegetables. [18-20] The dc-ELISAs were therefore constituted to develop chlorothalonil determination method using each of the prepared MoAbs. When chlorothalonil standard prepared using PBS was examined by the constituted dc-ELISAs, it was found that reactivity was unstable and fluctuated in the range of more than 10 folds and that the quantitative determination of chlorothalonil was impossible (data not shown). This phenomenon was considered to occur due to solubilization of chlorothalonil in PBS at the determination concentration range. Actually, the problem of instability was solved as expected, by substituting deionized water for PBS (data not shown). Reactivity of the MoAbs was therefore compared using chlorothalonil standards with deionized water with 10% methanol, as shown in Fig. 2. The concentration range of TPN9A was between 0.10 ng/mL (IC_{20} value) and 6.0 ng/mL (IC_{80} value), and the IC_{50} value was 1.1 ng/mL. The concentration range and IC_{50} value for TPN11D were similar with TPN9A: between 0.23 ng/mL and 7.0 ng/mL, and 1.4 ng/mL.

TPN12E showed slightly higher reactivity with chlorothalonil than the other MoAbs; the concentration range was between 0.092 ng/mL and 2.1 ng/mL, and the IC_{50} value was 0.40 ng/mL. However, the actual concentration of hapten-HRP conjugate for TPN12E was 500 ng/mL as described in the method, which was 10 folds higher than that for TPN9A and TPN11D (50 ng/mL). Higher reactivity of TPN12E with chlorothalonil would be caused

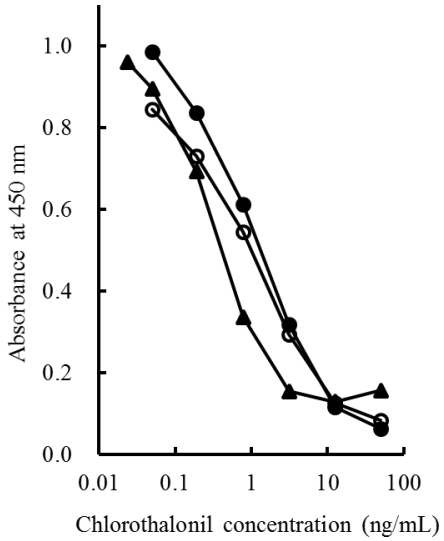


Figure 2. Reactivity of prepared antibodies with chlorothalonil in the dc-ELISA
(○) shows MoAb TPN9A, (●) shows MoAb TPN11D, and (▲) shows MoAb TPN12E. Each data point is the mean of 3 replicates for independent examinations.

from the relative affinity ratio of chlorothalonil and the hapten-HRP conjugate rather than the absolute high affinity to chlorothalonil because concentration of the hapten-HRP conjugate used was 10 folds higher for TPN12E than for the other MoAbs. This condition produced higher baseline absorbance which would increase the instability of standard curve and be easily influenced by matrix from vegetable samples. It was therefore considered that TPN12E is hardly applied to the dc-ELISA for chlorothalonil residue in vegetables.

Optimization of dc-ELISA. It is known that chlorothalonil is rapidly decomposed in homogenized agricultural products. In Japan, 10% phosphoric acid is generally added to the samples before homogenization to avoid such decomposition. [*1] At GC or HPLC analysis, added phosphoric acid is removed from the measurement samples through preparation process and does not influence on measurement results. On the other hand, dc-ELISA is highly influenced by phosphoric acid because the acid cannot be removed from the samples. The pH value of PBS (10 mmol/L), which is used as hapten-HRP conjugate solution, exactly shifted to strong acidic condition (pH 3) after mixing with the measurement sample. Therefore, a neutralization process is necessary during the sample preparation. It should be noted that the increases of salt concentration in solution by neutralization may cause insolubilization of chlorothalonil. Buffer condition dissolving the hapten-HRP conjugate was therefore examined for optimization because the neutralization on this step was carried out immediately before the competitive reaction of the dc-ELISA.

The hapten-HRP conjugate was dissolved in phosphate buffer (10 to 200 mmol/L, pH 7.0) modified with NaCl (150 mmol/L): hereinafter abbreviated to PBS (10 to 200 mmol/L). The reactivity after mixing the hapten-HRP solutions with chlorothalonil standard solution with 10% methanol was compared in the dc-ELISA with TPN9A as shown in Fig. 3A. The reactivity trend was almost equal between PBS (10, 40 and 100 mmol/L), while it shifted to slightly lower side with PBS (200 mmol/L). The pH value of mixture of PBS and measurement sample prepared from cucumber and eggplant showed the concentration dependence, as shown in Fig. 3B. There was no difference between both of the vegetables. It was pH 6.8 for PBS (100 mmol/L) and pH 7.0 for PBS (200 mmol/L) where the influence of sample pH can be practically neglected. From these results, PBS (100 mmol/L)

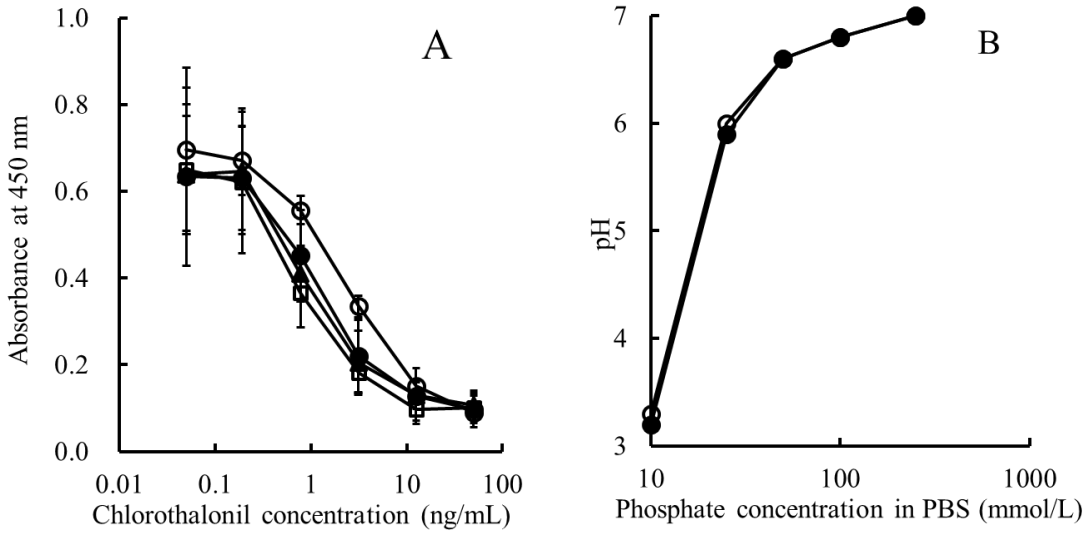


Figure 3. Influence of PBS in the dc-ELISA with MoAb TPN9A and its neutralization effect to the determination sample

(A) shows the reactivity for the hapten-HRP conjugate solution dissolved in PBS (10 mmol/L: □, 40 mmol/L: ▲, 100 mmol/L: ●, 200 mmol/L: ○; pH 7.0). Each data point of (A) is the mean of 3 replicates for independent ± SD.

(B) shows pH for the mixture of PBS (10 - 200 mmol/L, pH 7.0) and the determination samples prepared from cucumber (○) and eggplant (●).

was chosen as the buffer condition dissolving the hapten-HRP conjugate. A typical reaction curve on this condition was shown in Fig. 4.

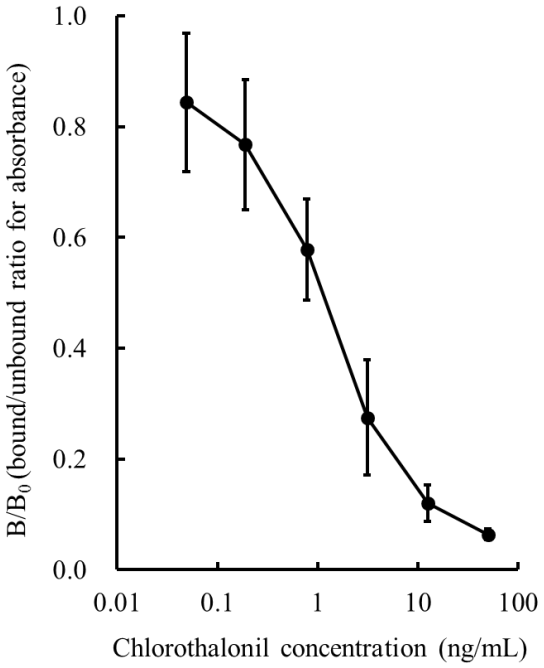
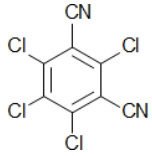
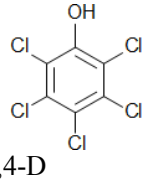
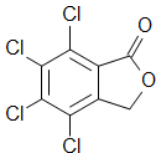
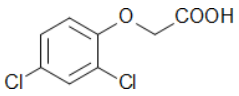
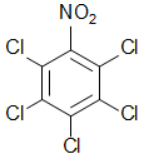
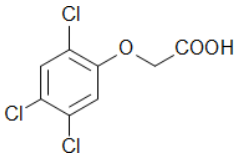


Figure 4. Typical standard curve for chlorothalonil in the dc-ELISA with MoAb TPN9A
Chlorothalonil was dissolved in 10% methanol with deionized water, and the hapten-HRP conjugate was dissolved in PBS (100 mmol/L, pH 7.0).
Each data point is the mean of 5 replicates for independent examinations ± SD.

Cross-reactivity of prepared MoAbs. Cross-reactivity was examined for TPN9A, TPN11D and TPN12E to compare their specificity as summarized in Table 1. TPN9A was the most specific to chlorothalonil among the prepared MoAbs; the cross-reactivity of TPN9A was 22% for phthalide and 17% for quintozene, TPN11D was 210% for phthalide and 490% for quintozene, and TPN12E was 40% for phthalide and 33% for quintozene. The specificity is important factor to choose the representative MoAb to the dc-ELISA constitution for chlorothalonil residue analysis in vegetables. TPN9A also showed lower baseline absorbance compared to TPN12E as described the above. Further, TPN12E was

Table 1. Cross-reactivity of prepared MoAbs with chlorothalonil and its structurally related pesticides

Pesticide	CR (%)[a]			Pesticide	CR (%)		
	9A	11D	12E		9A	11D	12E
chlorothalonil 	100[b]	100	100	pentachlorophenol 	<0.7	<2.1	<0.3
phthalide 	22	210	40	2,4-D 	<0.7	<2.1	<0.3
quintozene 	17	490	33	2,4,5-T 	<0.7	<2.1	<0.3

[a]CR (%) shows cross-reactivity (%) compared to chlorothalonil.

[b]Each data point is the mean of three replicates for independent examinations.

PBS (100 mmol/L, pH 7.0) was used for the HRP-hapten solution.

too sensitive to determine chlorothalonil residue in vegetables around the MRLs because over 1000 folds dilutions would be necessary to the sample preparations. TPN9A was therefore chosen to constitute the dc-ELISA.

TPN9A and TPN12E highly reacted with chlorothalonil having nitrile-group compared to quintozene having nitro-group and phthalide having furanone-group. The cross-reactivity result suggested that, compared to TPN11D, TPN9A and TPN12E would have smaller apertural area in their paratope, which was bound with the anchor part of the hapten. On the other hand, TPN11D would have deep space in the paratope which more specifically bound with the chloride-group of quintozene and phthalide than nitrile-group of chlorothalonil.

Pentachlorophenol, which has the most similar structure to the immunized hapten, showed no reaction in the dc-ELISA with all of MoAbs. Lawruk *et al.* also reported that the prepared polyclonal antibody reacted with quintozene and chlorothalonil but not pentachlorophenol. [15] It might be caused by change of binding ability due to acidity of hydroxy-group because the paratope space of the MoAbs is expected also to fit hydroxy-group from the viewpoint of molecular size.

Recovery of Chlorothalonil Spiked in Vegetables by dc-ELISA. Recovery examination was carried out to confirm applicability of the dc-ELISA to vegetables. Chlorothalonil was spiked to each chopped cucumber at 0.5, 2.0, 5.0 and 10 mg/kg and eggplant at 0.2, 1.0, 2.0 and 4.0 mg/kg. After preparation of the samples, the chlorothalonil concentrations were determined by the dc-ELISA with TPN9A. Each of the examination was repeated 6 times. As described in Table 2, all of the recovery results for the dc-ELISA agreed well with the spiking concentration; the recovery rates were 97.1 to 117% for cucumber and 110 to 125% for eggplant, excepting 172% for cucumber at 0.5 mg/kg: 1/10 of the MRL. The low concentration of chlorothalonil for cucumber was not quantitatively determined by the dc-ELISA although eggplant at 0.2 mg/kg could be determined. The reason might be derived from interference to the competitive reaction or pipetting work by cucumber matrix as reported by Amano *et al*. [21] Determined chlorothalonil around this concentration for cucumber should be re-examined by the other method to take the precise value. It is also important to check the matrix effect by such a recovery examination when the dc-ELISA is applied to the other vegetables.

Table 2. Recovery concentration of chlorothalonil spiked in vegetable by dc-ELISA

cucumber			eggplant		
Spiked (mg/kg)	Recovery (%)	RSD (%)	Spiked (mg/kg)	Recovery (%)	RSD (%)
0.5[a]	172	18.8	0.2	117	11.3
2.0	105	8.93	1.0	110	14.4
5.0[b]	97.1	14.6	2.0[b]	116	5.95
10.0	117	15.3	4.0	125	9.24

[a]Each data point is the mean of 6 replicates for independent examinations.

[b] shows the maximum residue limit.

PBS (100 mmol/L, pH 7.0) was used for the HRP-hapten solution.

Further, the recovery results basically showed slightly over 100% that the dc-ELISA has potential tendency over estimation. The reason might be derived from difference of physical property of solutions between standard and sample. The composition of standard solution must be improved to take precise results in future.

Next, chlorothalonil was spiked to each chopped cucumber at 0.5, 1.0, 2.0, 5.0 and 10 mg/kg, and to eggplant at 0.2, 0.4, 1.0, 2.0 and 4.0 mg/kg, independently from the above recovery examinations. The chlorothalonil concentrations were determined by the dc-ELISA with TPN9A and by HPLC, with each sample preparation method. Each of the examination was repeated 4 times. Fig. 5 shows the correlation results by plotting each data obtained. Regression line for cucumber was $y = 0.99x + 0.2$, correlation coefficient was $R^2 = 0.98$. The result was affected by dispersion of the dc-ELISA results on the spiked concentration at 10 mg/kg. On the other hand, the results of eggplant highly agreed; regression line was $y = 1.08x$, correlation coefficient was $R^2 = 0.99$. These results suggested

that the developed dc-ELISA can be basically applied to the residue analysis of chlorothalonil in these vegetables as well as HPLC. However, it was found that y-intercept for cucumber was 0.2 and slight bias for eggplant was 1.08. Especially, cucumber must be taken care because the y-intercept means strong overestimation of the chlorothalonil concentration around 0.2 mg/kg which exists false in cucumber. The reason might be derived from interference on the competitive reaction or pipetting work as well as the above discussion for the recovery examination. It is important to check the matrix effect when the dc-ELISA is applied to the other vegetables not only cucumber.

In conclusion, a set of results suggested that the dc-ELISA will be useful for on-site screening analysis by production site of vegetables and quality control laboratory of their foods, however decision of good safety based on food hygiene law must be naturally warranted by instrumental analysis for which validity has been inspected. The dc-ELISA is simple, rapid, cost-effective and high throughput, compared to the conventional

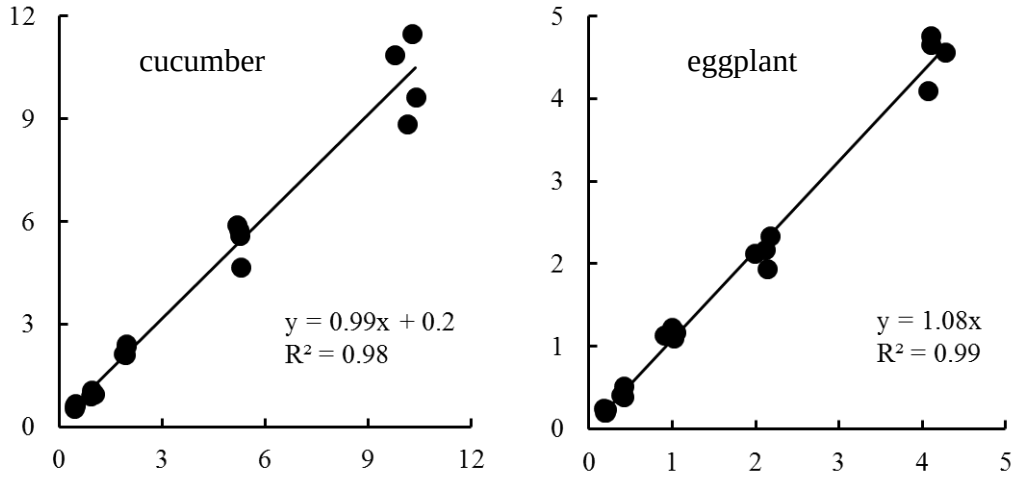


Figure 5. Correlation results for the dc-ELISA with TPN9A and HPLC
Cucumber and eggplant were used. Each data point is the mean of duplicates.

instrumental analysis, in obvious from comparison of their methods. It is expected that the dc-ELISA is widely used for on-site residual chlorothalonil analysis in agricultural products and foods.

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Chapter 2

Development of an Immunosensor for Determination of the Fungicide Chlorothalonil in Vegetables, using Surface Plasmon Resonance

Abstract. An immunosensor based on surface plasmon resonance (SPR-sensor) was developed to analyze chlorothalonil residues and maximum residue limits (MRLs; 0.5–50 mg/kg) in vegetables in Japan. Conjugates of *N*-(pentachlorophenoxyacetyl)glycine and BSA were covalently coated on the sensor chip. The SPR-sensor quantitatively determined chlorothalonil at concentrations ranging from 8.0 to 44 ng/mL, using TPN9A, a monoclonal chlorothalonil antibody. The 50% inhibition concentration was 25 ng/mL. The reactivity was 10-fold lower than that of indirect competitive enzyme-linked immunosorbent assay (ic-ELISA). However, the SPR-sensor could determine chlorothalonil residues in vegetables at the concentrations around the above MRLs. Chlorothalonil spiked in vegetables was recovered at 90–118% within 1 day and 90–115% across 3 days, correlating with HPLC results. The sensor showed good performance for chlorothalonil residue analysis in vegetables with rapid determination although the sensitivity and the cross-reactivity were less effective than the ic-ELISA.

INTRODUCTION

Chlorothalonil, 2,4,5,6-tetrachloroisophthalonitrile, is widely used as an organochlorine fungicide that acts on various pathogenic fungi infecting a wide range of vegetables.[1] Chlorothalonil residues have often been detected in vegetables, as reported in Japan and Canada.[2-4] The acceptable daily intake (ADI) of chlorothalonil is set at 0.018 mg/kg/day in Japan, and the maximum residue limits (MRLs) are set at 0.5–50 mg/kg based on the ADI, depending on the vegetables. It is important that chlorothalonil residues are monitored for public health and food safety because of their wide application range, the distribution volume, and the detection frequency.

Chlorothalonil residues in vegetables are generally determined using gas chromatography (GC) or high performance liquid chromatography (HPLC).[5-8] Such instrumental analyses are sufficiently sensitive and accurate, but complicated and time-consuming pre-treatments are necessary. Acid solutions are added to prevent the degradation of chlorothalonil before the vegetables are homogenized. The homogenized samples are extracted with organic solvents, and chlorothalonil is cleaned up from the extracts through a liquid-liquid partition and/or column chromatography.

Several types of enzyme-linked immunosorbent assays (ELISAs), for which the above clean-up steps are not necessary, have been developed for the determination of chlorothalonil.[9-12] The assay methods are simple and rapid, but complicated pipetting procedures are necessary at each reaction step. Immunosensors based on electrochemistry (electrochemical-sensors) were developed for determination of pesticides as automatic assay methods for which the pipetting procedures in the ELISAs were not necessary.[13,14] Biosensors were also examined by using enzymes which was group specific to some

pesticides.[15,16] Further, immunosensors based on quartz crystal microbalance (QCM-sensor) and surface plasmon resonance (SPR-sensors) were developed for pesticide determinations.[17-26] The QCM-sensor and the SPR-sensors enabled the direct detection of target pesticide in real time without bound/free separation and any labeling techniques. They were more rapid and simpler methods compared with the ELISAs and the electrochemical-sensors. The SPR-sensors were especially examined to determine pesticides in water, fruit juices or milk, but extracts from vegetables with organic solvents have been not examined although they were examined for the electrochemical-sensors.

In this study, an SPR-sensor was developed and examined for the determination of chlorothalonil residue analysis in vegetables.

MATERIALS AND METHODS

Materials. Chlorothalonil, phthalide, quintozene, pentachlorophenol, 2,4-dichlorophenoxyacetic acid (2,4-D), and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) were purchased from Wako Pure Chemical Industries (Osaka, Japan). The pesticides used for residue analysis were of analytical grade. *N*-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, hydrochloride (EDC) were purchased from Wako Pure Chemical Industries and Dojindo Laboratories (Kumamoto, Japan), respectively. Bovine serum albumin (BSA, Pro. No. A7030) was purchased from Sigma-Aldrich (St. Louis, MO). Sodium dodecyl sulfate (SDS) and guanidine hydrochloride (GdnHCl) were purchased from Nacalai Tesque (Kyoto, Japan). Horseradish peroxidase-labeled anti-mouse IgG (H+L) antibody from rabbit (Invitrogen) and 96-well micro-titer plates (Nunc MaxiSorp) were purchased from Thermo Fisher Scientific (New York, NY). All other chemicals and

reagents were of analytical grade and purchased from Wako Pure Chemical Industries or Nacalai Tesque. Anti-chlorothalonil mouse monoclonal antibody (MoAb TPN9A; IgG_1) and the conjugate of *N*-(pentachlorophenoxyacetyl)glycine and BSA (TPN-BSA) were prepared as described previously.[12]

SPR-sensor. SPR-sensors consisted of commercially available instruments of micro-flow type (Biacore T200: GE Healthcare Europe, Munich, Germany) and the sensor chip which was coated with carboxymethyldextran (CM5: GE Healthcare Europe). They were functionalized via the following steps based on the instruction manual.

1) Immobilization of TPN-BSA. TPN-BSA was covalently bound to the chip surface by the active ester method. The sensor chip was put to the instrument, and was initially rinsed with 10 mmol/L PBS (10 mmol/L phosphate buffer adjusted to pH 7.0, 150 mmol/L NaCl) modified with 0.005% Tween 20 at 5 μL/min for 600 s. A mixture of 80 μL of EDC (400 mmol/L) and 80 μL of NHS (100 mmol/L) in distilled water was made to flow to the channel for 350 s to esterify the carboxyl group of the sensor chip with NHS. The TPN-BSA solution (17 or 44 μg/mL) was dissolved in acetic acid buffer (10 mmol/L; adjusted to pH 5.0) and flown for 45, 60, 90, or 120 s to bind the activated carboxyl groups to the amino-group residues of TPN-BSA. Ethanolamine (1 mol/L; adjusted to pH 8.5) was made to flow for 350 s to block the residual carboxyl groups. After rinsing with running buffer (50 mmol/L phosphate buffer adjusted to pH 7.0, 75 mmol/L NaCl, 5.0% methanol, and 0.1% BSA), the sensor chip was used for chlorothalonil determination.

2) Pre-mixture of MoAb TPN9A and chlorothalonil. On the other hand, chlorothalonil was dissolved in methanol and serially diluted to concentrations from 7.8 to 1000 ng/mL in

tubes. The diluents were further diluted 10 folds with distilled water, resulting in concentrations from 0.78 to 100 ng/mL in 10% methanol. MoAb TPN9A was also diluted to concentrations from 1.56 to 60 μg/mL with 100 mmol/L PBS (100 mmol/L phosphate buffer adjusted to pH 7.0, 150 mmol/L NaCl) modified with 0.2% BSA, as described previously. [12] The prepared chlorothalonil solution (75 μL) was mixed with the MoAb TPN9A solution (75 μL) in tubes. In this phase, the complex between the MoAb TPN9A and chlorothalonil was formed.

3) Reaction of MoAb TPN9A not bound to chlorothalonil with the immobilized TPN-BSA. The tubes were put to the instrument. Each mixture was made to flow to the sensor at 20 μL/min for 180 s to react the free MoAb TPN9A, which was not bound to chlorothalonil, with TPN-BSA on the chip. Immediately after the reaction, the flowing solution was replaced with running buffer at 20 μL/min for 180 s.

4) Dissociation of the bound MoAb TPN9A from the immobilized TPN-BSA. The sensor chip must be regenerated by remove the bound antibody from the immobilized TPN-BSA. As the condition, 2 kinds of solutions were used for the dissociation of the bound MoAb TPN9A after examinations had been carried out to optimize the conditions as described in the results. The dissociation solution A was GdnHCl (3.0 mol/L) in acetic acid (1.0 mol/L; pH 1.9), and the dissociation solution B was 0.2% SDS. The sensor chip was initially washed with solution A at 20 μL/min for 60 s. After rinsing with distilled water at 20 μL/min for 60 s, it was washed again with solution B at 20 μL/min for 120 s. The sensor chip on which the MoAb TPN9A had been dissociated was used again by repeating the above each of steps between 2) pre-mixture of MoAb TPN9A and chlorothalonil and 4) dissociation of the bound MoAb TPN9A.

5) Data processing. Raw data was output as a resonance unit (RU), where 1000 RU was defined as 0.1 degrees of the refractive index shift by the SPR phenomenon. The actual response curve was drawn; the x-axis represented chlorothalonil concentration and the y-axis represented the delta RUs between the starting point of the reaction and a time point of 180 s.

ic-ELISA. Indirect competitive (ic)-ELISA was developed as described previously. [12] TPN-BSA (1 μg/mL) was dissolved in 10 mmol/L PBS and added to each well of a 96-well micro-titer plate at 100 μL/well. The plate was incubated overnight at 4 °C. Blocking buffer (10 mmol/L PBS modified with 0.4% BSA) was added at 300 μL/well after the wells were washed once with washing buffer (10 mmol/L PBS modified with 0.02% Tween 20). The plate was incubated at 25 °C for 1 h, and the solution was removed from the wells. Chlorothalonil standards were prepared at concentrations from 0.05 to 200 ng/mL in 10% methanol, and the solutions were added at 50 μL/well. The MoAb TPN9A was dissolved in 100 mmol/L PBS with 0.2% BSA and was prepared to a concentration (100 ng/mL) showing an absorbance between 1.0 and 2.0 at 450 nm in this ic-ELISA. MoAb TPN9A was added at 50 μL/well to each well immediately after the standard was added. The plate was incubated at 25 °C for 1 h. After washing three times with the washing buffer, horseradish peroxidase-labeled anti-mouse IgG (H+L) antibody, which was diluted 2000 folds in 10 mmol/L PBS with 0.2% BSA, was added at 100 μL/well. The plate was incubated at 25 °C for 1 h. After washing three times with the washing buffer, the color development solution (100 μg/mL 3,3',5,5'-tetramethylbenzidine, 0.006% H_2O_2, 100 mmol/L acetic acid; adjusted to pH 5.5) was added at 100 μL/well. After 10 min at 25 °C,

0.5 mol/L sulfuric acid was added at 100 μL/well to stop the color development reaction. The absorbance was measured at 450 nm by the microplate reader, xMark (Bio-RAD Laboratories, CA).

Treatment of vegetable samples. Lettuce, cabbage, and long green onion (200 g of each), purchased from a market in Kyoto city, were used for recovery examinations. Cucumber and eggplant (1.0 kg of each), harvested from the experiment farm in the National Institute for Agro-Environmental Sciences, were used for correlation examinations between the SPR-sensor and HPLC. Phosphoric acid solution (phosphoric acid:water = 1:9 v/v) was added at a ratio of 1:2 (w/w) to the vegetable samples and homogenized by a blender. Chlorothalonil was added to the homogenized samples. The final concentrations ranged from 1.0 to 10 mg/kg for the recovery examinations, and from 0.2 to 5.5 mg/kg for the correlation examinations.

For the SPR-sensor, pre-treatment of the homogenized samples was carried out with the same procedure as that of direct competitive (dc)-ELISA, as described previously. [12] Methanol (25 mL) was added to the samples (7.5 g) in a 50-mL tube hermetically sealed with a screw cap. The tube was vigorously shaken for 30 min to extract chlorothalonil. The mixture was centrifuged at 3,000 rpm at room temperature for 10 min. The supernatant was diluted 8.5 folds with distilled water to become 10% methanol equivalent. The diluted sample was applied to the SPR-sensor without other pre-treatments.

For HPLC, acetone was added at 100 mL/sample to the homogenized samples (20 g) and extracted for 3 min, using a homogenizer (Polytron PT2100; Kinematica, Lucerne, Switzerland). The mixture was filtered through a funnel with diatomite by suction. The

residue was then re-treated with 50 mL of acetone. Both filtered extracts were mixed and concentrated to 30 mL by evaporation at a temperature lower than 40 °C, in a flask. The sample was transferred to a separating funnel with 100 mL of 10% sodium chloride solution. The above flask was washed with 100 mL of hexane and the washed solution was also transferred to the funnel. After vigorous shaking for 5 min, a hexane layer was recovered. The liquid-liquid partition was repeated twice. The recovered hexane layer was mixed with appropriate quantities of anhydrous sodium sulfate. After the filtration, the solution was transferred to a flask and evaporated to dryness at a temperature lower than 40 °C. The dried residue was dissolved with 5 mL of hexane. The dissolved solution was applied to a column in which Florisil (5 g) and anhydrous sodium sulfate (5 g) were filled up with hexane. After the column was washed with hexane (100 mL), chlorothalonil was eluted with a mixture of ethyl acetate and hexane (1:9, v:v; 150 mL). The eluate was dried by evaporation and re-dissolved with hexane (2.0 mL). A portion of the solution (0.5 mL) was dried by evaporation and re-dissolved with methanol (1.0 mL). The methanol solution was applied to HPLC.

HPLC analysis. The HPLC system consisted of an Agilent 1100 series equipped with a quaternary pump, an autosampler, a column oven, and a diode array detector. The wavelength for chlorothalonil detection was 246 nm. The column was a SunFire C18 reversed-phase column (5 μm, 4.6 i.d. × 250 mm; Waters, MA) with a guard column (5 μm, 4.6 i.d. × 20 mm). The mobile phase was methanol/water (70/30, v/v). The flow rate was 1.0 mL/min, the column oven temperature was 40 °C, and the injection volume was 20 μL.

RESULTS AND DISCUSSION

Development of the SPR-sensor. An SPR-sensor was developed for chlorothalonil determination, using MoAb TPN9A and antigen TPN-BSA. The TPN part of the TPN-BSA has a chlorothalonil-like structure as shown in Figure 1, and reacts with MoAb TPN9A as described previously. [12] Because the structure is much more chemically stable than that of the binding site in the MoAb TPN9A, TPN-BSA was chosen for immobilization on the sensor chip surface, as it was expected to tolerate the dissociation condition of the TPN-BSA and TPN9A complex.

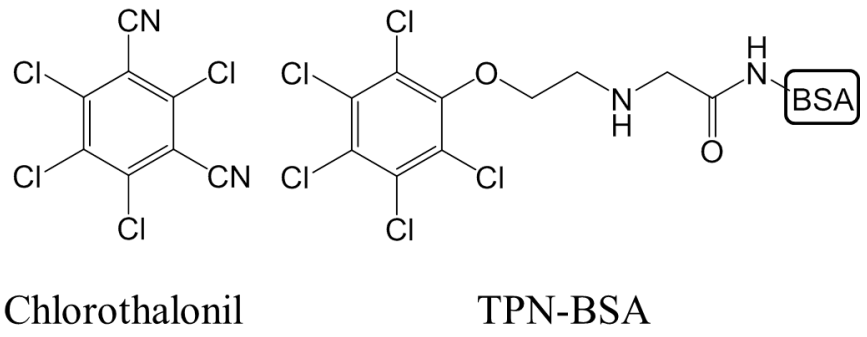


Figure 1. Structures of chlorothalonil and TPN-BSA

The TPN-BSA was immobilized at several concentrations and reaction times to determine the optimum condition: 17 μg/mL and 90 s, 44 μg/mL and 45 s, 44 μg/mL and 60 s, and 44 μg/mL and 120 s. The RU values changed depending on the concentration and the reaction time as shown in Figure 2. Sufficient signal was acquired at a condition of 44 μg/mL and 120 s. These conditions were the minimum concentration of TPN-BSA and time required to obtain a sufficient signal.

The sensor chip should be repeatedly regenerated by dissociation of MoAb TPN9A from the TPN-BSA immobilized on the chip surface. The recommended condition in the

instruction manual was SDS (up to 0.5%) or GdnHCl (up to 5.0 mol/L). Only 10% of MoAb TPN9A was dissociated from TPN-BSA by 0.2% SDS, as shown in Figure 3, while 84% of MoAb TPN9A was dissociated by 3.0 mol/L GdnHCl, but the dissociation was still incomplete. The k_d value, which was determined after the condition was optimized, was 3×10^{-5} mol/L/s. The result means that the complex is difficult to dissociate at these condition. Such a phenomenon has already been reported by Svitel *et al.* for the complex of 2,4-D-BSA and anti-2,4-D antibody. [19] Anti-2,4-D antibody was successfully dissociated from the sensor chip surface by using a non-covalently bound 2,4-D-concanavalin A conjugate instead of 2,4-D-BSA conjugate, with which sugar chains were covalently immobilized on the sensor chip surface. This idea was useful for the regeneration of the sensor chip, but re-immobilization of 2,4-D-concanavalin A was necessary for each test. Improvement of the dissociation condition was therefore examined using a combination of GdnHCl and SDS. The dissociation condition was consequently established such that GdnHCl (3.0 mol/L) in acetic acid (1.0 mol/L) was initially made to flow for 60 s, followed by flowing distilled water for 60 s to remove the GdnHCl residue, and finally, 0.2% SDS was made to flow for 120 s. The base line returned completely to the initial level at this condition, as shown in Figure 3. The established condition made it possible for the sensor chip to be used continuously for 200 cycles, after which the micro-channel was physically destructed by delamination of the thin gold film. It suggests that salting out by GdnHCl is an effective way to dissociate the complex, and the residual part was dissociated with the surfactant potency of SDS. The MoAb TPN9A and the TPN-BSA complex may have heterogeneous binding forces.

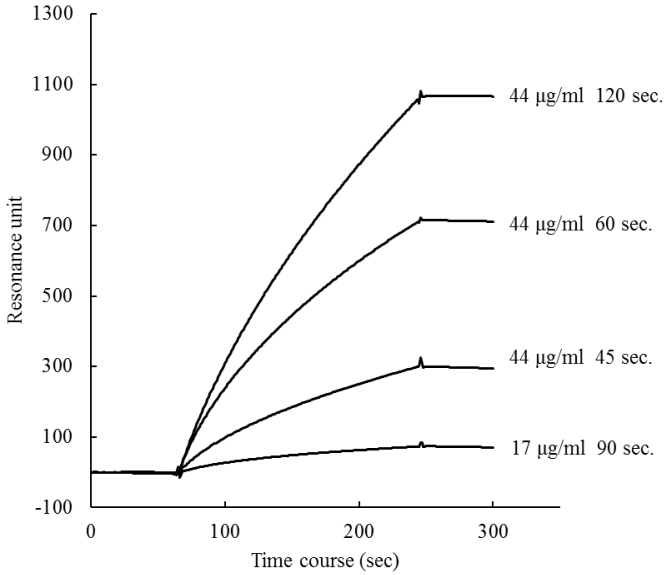


Figure 2. Typical time course of the MoAb TPN9A (7.5 µg/mL) reaction with the immobilized TPN-BSA in the SPR-sensor: The numerals described on the right show TPN-BSA concentrations and the reaction times for the immobilization.

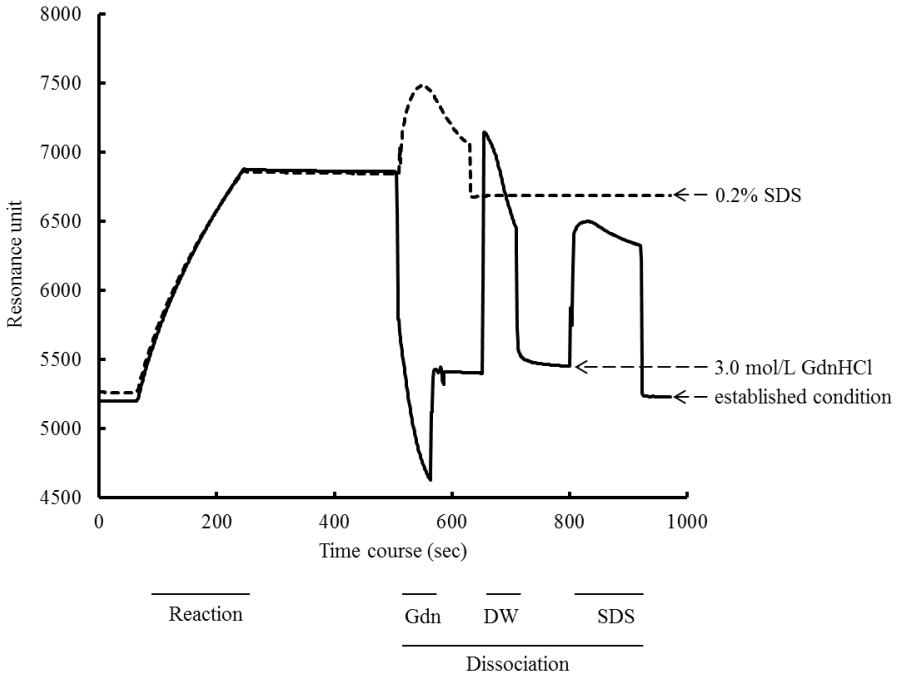


Figure 3. Dissociation of the MoAb TPN9A bound to the immobilized TPN-BSA on the sensor chip: Solid line shows time course of the signal for the established condition. Dashed line shows time course of the signal for the dissociation by 0.2% SDS.

Reactivity in the SPR-sensor. Various concentrations of the MoAb TPN9A solution (1.88–60 μg/mL) were initially applied to the sensor chip, on which TPN-BSA (44 μg/mL) was immobilized, to determine the application dose of MoAb. The minimum required concentration of MoAb TPN9A was 7.5 μg/mL to achieve a ΔRU value of greater than 400 at a time point of 180 s after injection. In turn, this concentration was set as the application dose.

Chlorothalonil solutions were mixed with equal volumes of the MoAb TPN9A solution, and then applied to the SPR-sensor. The ΔRU value decreased with an increase in the chlorothalonil concentration (0.78 ng/mL-100 ng/mL), as shown in Figure 4. The *Ka* value was calculated as 1×10^{10} $(mol/L)^{-1}$ from the results of the SPR-sensor. The inhibition curve 180 s after sample injection for the SPR-sensor was compared with that for

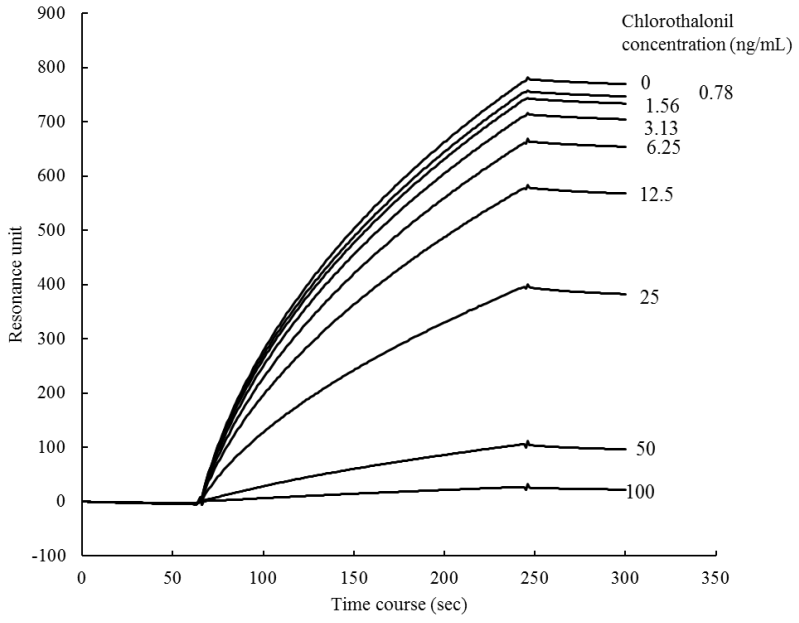


Figure 4. Typical time course of the MoAb TPN9A reaction not bound to chlorothalonil with the immobilized TPN-BSA in the SPR-sensor

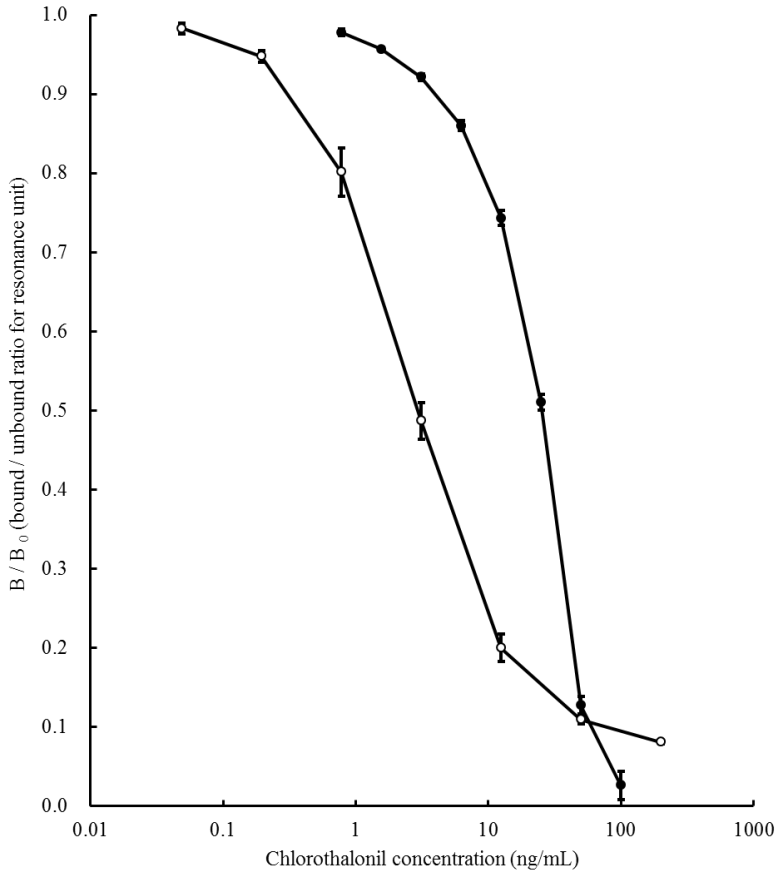


Figure 5. Inhibition curves by chlorothalonil in the ic-ELISA and in the SPR-sensor: (○) represents ic-ELISA, and (●) represents SPR-sensor. Each data point is the mean of three replicates from independent examinations. Error bars indicate standard deviations.

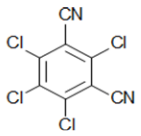
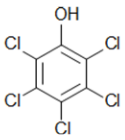
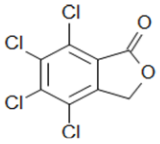
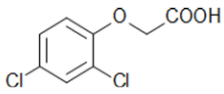
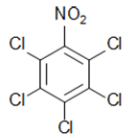
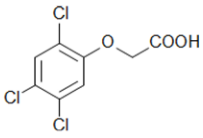
ic-ELISA, as shown in Figure 5. The values of 20%, 50%, and 80% inhibition concentration (IC_{20}, IC_{50}, and IC_{80}) were 8.0, 25, and 44 ng/mL, respectively, for the SPR-sensor. The reactivity at IC_{50} was 10-folds lower than that acquired by ic-ELISA, of which the IC_{20}, IC_{50} and IC_{80} values were 0.44, 2.5, and 12 ng/mL, respectively. The SPR-sensor required a 44-fold higher concentration of TPN-BSA for immobilization compared with ic-ELISA, which was 44 μg/mL in the SPR-sensor as shown in Figure 2 whereas 1 μg/mL in the ic-ELISA. The high concentration for the SPR-sensor was derived from the mechanism

to monitor the direct signal of the antigen-antibody interaction, differed from signal amplification by horseradish peroxidase in the ic-ELISA. It would be the reason that the sensitivity between them was differed as shown in figure 5 although the same MoAb was used to their constitutions. On the other hand, the SPR-sensor required only 15 min to examine chlorothalonil and showed high reproducibility. The relative standard deviations (RSDs) of SPR were 0.76% (6.25 ng/mL), 2.0% (25 ng/mL), and 8.5% (44 ng/mL) for chlorothalonil while the RSDs of ic-ELISA were 3.8% (0.44 ng/mL), 4.8% (3.1 ng/mL), and 8.6% (13 ng/mL). Thus, the SPR-sensor would be a useful immunoassay method for simple, quick, and accurate determination, although it had lower sensitivity than ic-ELISA.

Cross-reactivity was examined by the SPR-sensor for structurally related pesticides. The results were slightly less specificity compared to those acquired by ic-ELISA, as described in Table 1. Compared with the results of ic-ELISA, phthalide examined by the SPR-sensor had a slightly lower cross-reactivity, but quintozene, pentachlorophenol, and 2,4,5-T had slightly higher cross-reactivities. The SPR-sensor monitors the early response of the antigen-antibody interactions while ic-ELISA monitors the saturated interactions after 1 h. Because both assays showed only minor differences, it was confirmed that the specificity was a particular characteristic of the MoAb TPN9A.

The MRLs of chlorothalonil are set from 0.5 to 50 mg/kg in vegetables in Japan. On the other hand, the SPR-sensor showed a working range from 6.5 to 42 ng/mL. The MRL values were included in concentrations that were 70 folds higher than the working range, for which the dilution rate was usually used for the pre-treatment of vegetables in dc-ELISAs.[12] The developed SPR-sensor showed adequate sensitivity to determine chlorothalonil residues in vegetables.

Table 1. Cross-reactivity of MoAb TPN9A with chlorothalonil and the other structurally related pesticides

Pesticide	CR (%)[a]		Pesticide	CR (%)	
	ELISA	SPR		ELISA	SPR
chlorothalonil			pentachlorophenol		
	100	100		<0.7[b]	1.2
phthalide			2,4-D		
	22	9.1		<0.7	<2.0
quintozene			2,4,5-T		
	17	22.6		<0.7	6.5

[a] CR (%) shows cross-reactivity (%) compared to chlorothalonil.

[b] < 0.7 shows less than 0.7%.

Recovery of chlorothalonil spiked into vegetables. Recovery examination was carried out to confirm applicability of the SPR-sensor in determining the chlorothalonil residue in vegetables. Lettuce in the Asteraceae family, cabbage in the Brassicaceae family, and Welsh onion in the Alliaceae family, which are typical leaf vegetables, were chosen as representative species. The vegetable samples before chlorothalonil was spiked had not contained any detectable chlorothalonil in the SPR-sensor (the data not shown). The recovery results within the day are shown in Table 2, and the results across several days are shown in Table 3. The SPR-sensor showed good recovery results for both examinations. The recovery ranged from 90.2 to 118% within the day, and from 90.2 to 115% across several days. The RSD ranged from 2.71 to 13.5% within the day, and from 6.79 to 12.6%

across several days. These results showed that the developed SPR-sensor has adequate accuracy for the determination of chlorothalonil residues in the vegetables. It was suggested that the SPR-sensor would be applicable to a wide range of vegetables.

Table 2. Intraday validation of chlorothalonil recovered from vegetables by SPR-sensor

Spiked (mg/kg)	lettuce[a]		cabbage		long green onion	
	Recovery	RSD	Recovery	RSD	Recovery	RSD
1	96	3.3	103	9.9	NT	NT
2	90	7.1	90	3.5	118	2.7
4	NT[b]	NT	103	13.5	NT	NT
5	NT	NT	NT	NT	103	3.1
10	NT	NT	NT	NT	114	7.4

[a] The values show recovery (%) and RSD (%).

[b] NT shows not tested.

Each date point is the mean of 3 replicates for independent examination.

Table 3. Interday validation of chlorothalonil recovered from vegetables by SPR-sensor

Spiked (mg/kg)	lettuce[a]		cabbage		long green onion	
	Recovery	RSD	Recovery	RSD	Recovery	RSD
1	101	12.6	105	10.4	NT	NT
2	90	7.1	92	9.2	115	6.8
4	NT[b]	NT	107	7.9	NT	NT
5	NT	NT	NT	NT	112	7.5
10	NT	NT	NT	NT	111	8.7

[a] The values show recovery (%) and RSD (%).

[b] NT shows not tested.

Each date point is the mean of 3 replicates for independent examination.

Examination of the correlation between HPLC and SPR-sensor. The results of recovery were confirmed by HPLC analysis, using eggplants and cucumbers, which are typical fruit vegetables. As shown in Figure 6, although the analytical values generally have a bias toward the proposed method (slope = 1.2) and have y-intercept (-0.22), no significant difference was found in the values obtained from both methods (R^2 = 1.00 and p = 0.23).

The results showed that the SPR-sensor developed could determine the chlorothalonil residues in vegetables with adequate quantitative performance in addition to HPLC analysis.

In conclusion**,** this study showed that the SPR-sensor developed was applicable for the rapid determination of chlorothalonil residues in vegetables around the MRL although the sensitivity and the cross-reactivity were less effective than the ic-ELISA. The principle of SPR is quite simple, making the real time determination of pesticides possible. It would be useful for rapid and accurate pesticide residue analysis in garden crops.

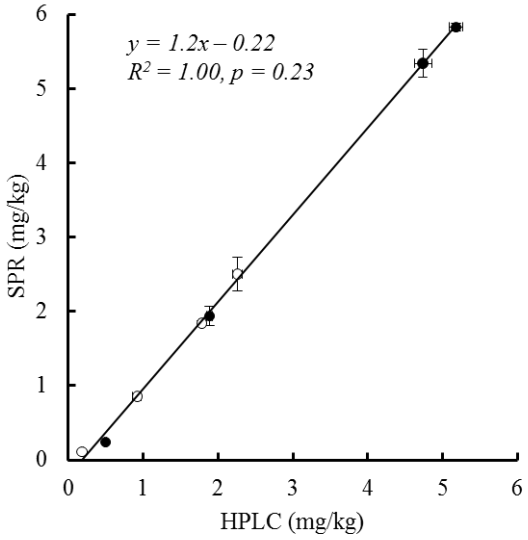


Figure 6. Correlation between the results of the SPR-sensor and the results of HPLC: (○) represents eggplant, and (●) represents cucumber. Each data point is the mean of three replicates from independent examinations. Error bars indicate standard deviations.

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Chapter 3

Analysis of the Fungicide Boscalid in Horticultural Crops, Using an Enzyme-Linked Immunosorbent Assay and an Immunosensor Based on Surface Plasmon Resonance

ABSTRACT: A direct competitive enzyme-linked immunosorbent assay (dc-ELISA) and an immunosensor based on surface plasmon resonance (SPR-sensor) were developed for fungicide boscalid determination in horticultural crops. To produce anti-boscalid monoclonal antibodies (MoAb BSC7 and MoAb BSC72) for these assays, a hapten of boscalid was synthesized and conjugated to keyhole limpet hemocyanin for Balb/c mice immunization. The working range of the dc-ELISA was 0.8–16 ng/mL with MoAb BSC7 and 2.5–120 ng/mL with MoAb BSC72, and that of the SPR-sensor was 17–80 ng/mL with MoAb BSC7. The dc-ELISA and SPR-sensor were compared for their sensitivity in determining boscalid residues at the maximum residue limit of 1–40 mg/kg for horticultural crops in Japan. Recovery of the spiked boscalid was 85%–109% by the SPR-sensor and 100%–124 % by the dc-ELISA. On real tomato samples, the results obtained by both these immunoassays correlated well with results obtained by high-performance liquid chromatography.

INTRODUCTION

Boscalid, 2-chlor-*N*-(4′-chlorobiphenyl-2-yl)nicotinamide, one of the fungicides that have a carboxamide structure as the functional group, specifically sterilizes pathogenic fungi by inhibiting succinate ubiquinone reductase in the fungal mitochondrial electron transport chain.[1] Introduced to agricultural fields in the USA in 2003 and in Japan in 2005, boscalid is applied to plant diseases caused by fungi such as *Alternaria* spp., *Botrytis* spp., *Sclerotinia* spp., *Mycosphaerella* spp. and *Monilinia* spp. Surveys of boscalid's behavior in the agricultural fields for various horticultural crops, such as green beans, spring onions, strawberries, grapes, blueberries, tomatoes, and raspberries, have demonstrated that the fungicide residues disappear within a few weeks.[2–8] Nevertheless, boscalid is one of the most frequently detectable pesticides in the field, because it is one of the most generally used fungicides.[9,10] Furthermore, the pesticide often drifts to other locations such as rivers, lakes, and ground water, and has been detected in bed sediments, fish, invertebrates, surface water, and ground water.[11–14] It is therefore important to monitor the boscalid concentration in horticultural crops and the environment.

Boscalid is generally determined by gas or liquid chromatography with mass spectrometry.[14–18] Although such chromatographic analytic methods are sensitive and accurate, they require a lot of time to prepare samples for measurement, rendering them impractical for the rapid and convenient monitoring of boscalid in many samples. As an alternative method, enzyme-linked immunosorbent assays (ELISAs) have been developed for the monitoring of many pesticides in agricultural products.[19–23] ELISAs are more simple, rapid, and cost-effective than the above-mentioned instrumental methods.[24–26]

Immunosensors based on electrochemistry (electrochemical sensors) have also been developed for the determination of pesticides.[27,28] These sensors are automated methods, unlike ELISAs in which manual pipetting work is necessary. Immunosensors based on surface plasmon resonance (SPR-sensor) have achieved real-time monitoring of pesticides without any bound/free separation and labeling techniques that are necessary in the ELISA and the electrochemical sensors.[29–34] The SPR-sensor has successfully been used to determine pesticides in water[35,36], fruit juices[37], milk[38], and vegetables and fruits.[39]

ELISAs for boscalid, using anti-boscalid polyclonal antibodies (PoAbs), have been developed[40,41] and found to be highly sensitive and useful for the determination of boscalid in environmental samples. However, the 50% of inhibition concentration (IC_{50}) values of 0.4–1.6 μg/L were too sensitive to determine boscalid at the maximum residue limits (MRLs) of 1–40 mg/kg in horticultural crops in Japan. Thus, a new strategy was examined to develop an adequate sensitive immunoassay for the analysis of boscalid residues in horticultural crops.

In this study, the synthesis of a new hapten, preparation of monoclonal antibodies (MoAbs) to boscalid, methodologies of the direct competitive enzyme-linked immunosorbent assay (dc-ELISA) and SPR-sensor, and comparison of the dc-ELISA and SPR-sensor (using the same MoAb) for determination of boscalid in horticultural crops are described.

MATERIALS AND METHODS

Materials. Boscalid, benalaxyl, fenhexamid, pencycuron, and tecloftalam, which were of analytical grade for pesticide residue analysis, and *N*-hydroxysuccinimide and keyhole limpet hemocyanin (KLH) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Bovine serum albumin (BSA; Prod. No. A7888), and hypoxanthine-aminopterin-thymidine (HAT) medium supplement and polyethylene glycol (molecular weight 1450) solution were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). Dichloromethane (CH_2Cl_2) was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Sodium dodecyl sulfate (SDS), guanidine hydrochloride (GdnHCl), and RPMI 1640 medium were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Fetal bovine serum (FBS) was purchased from PAA Laboratories, GmbH. (Pasching, Austria). Ninety-six-well microplates for cell culture (Nunc), 96-well microtiterplates for ELISA (Nunc MaxiSorp), and horseradish-peroxidase (HRP)-labeled anti-mouse IgG (H+L) antibody from rabbit (Invitrogen) and anti-mouse IgG (H+L) antibody from goat (Pierce) were purchased from Thermo Fisher Scientific K. K. (New York, NY, USA). HRP was purchased from Toyobo Co. Ltd. (Osaka, Japan). Freund's complete adjuvant and incomplete adjuvant (Difco) were purchased from Becton, Dickinson and Company (Franklin Lakes, NJ, USA). All other chemicals and reagents used were of analytical grade and purchased from Wako Pure Chemical Industries, Ltd. or Nacalai Tesque, Inc.

Hapten Synthesis.

General. Unless otherwise noted, all reactions were performed under an atmosphere of argon. All reactions were monitored by thin-layer chromatography (TLC) on glass plates precoated with silica gel of a layer thickness of 0.2 mm (60 F_{254}) from Merck KGaA (Darmstadt, Deutschland). The products were visualized by irradiation with UV light, or by treatment with a solution of phosphomolybdic acid, or by treatment with a solution of *p*-anisaldehyde. Flash column chromatography was performed using silica gel (Art. No. 7734) from Merck KGaA. ^{1}H NMR (500 and 400 MHz) and ^{13}C NMR (125 and 100 MHz) spectra were recorded on JEOL JNM-ECX500 and JEOL JNM-ECS400 spectrometers. Chemical shifts are reported as δ values (ppm) relative to $CDCl_3$ (7.26 ppm). Elemental analyses were measured on a Yanaco CHN CORDER MT-6 analyzer. Optical rotations were performed with a JASCO P-1030 polarimeter at the sodium D line (1.0 mL sample cell). DART mass (positive mode) analyses were performed on an LC-TOF JMS-T100LP spectrometer.

Synthesis of 2-chloro-*N*-(2-iodophenyl)pyridine-3-carboxyamide (3). A solution of chloronicotinoyl chloride (**1**) (704.0 mg, 4.0 mmol) in CH_2Cl_2 (20 mL) was stirred into a mixture of 2-iodoaniline (**2**) (876.1 mg, 4.0 mmol) and Et_3N (836 µL, 6.0 mmol, d = 0.73) in CH_2Cl_2 (4 mL) for over 15 min at 0 °C. The resulting mixture was stirred at room temperature for an additional 2 h. Then, the solvent was removed under reduced pressure. The residue was purified by column chromatography on a silica gel that was eluted with a hexane and ethyl acetate mixture (2:1, v:v) to give the desired product **3** (603.4 mg) in 42% yield. **3**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.56 (dd, 1H, J = 4.88, 1.83 Hz, H-6 Py), 8.43 (br s,

1H, NH), 8.38 (d, 1H, J = 8.24 Hz, H-4 Py), 8.23 (dd, 1H, J = 7.63, 1.83 Hz, H-6 Ph), 7.86 (dd, 1H, J = 8.10, 1.34 Hz, H-3 Ph), 7.41–7.45 (m, 2H, H-5 Py, H-5 Ph), and 6.94 (dt, 1H, J = 7.63, 7.63, 1.53 Hz, H-4 Ph) ppm.

Synthesis of boscalid hapten precursor, methyl 2'-(2-chloronicotinamido)-biphenyl-4-carboxylate, (5). A mixture of product (**3)** (179.3 mg, 0.5 mmol), 4-(methoxycarbonyl)phenylboronic acid (**4**) (90.0 mg, 0.5 mmol), $(Ph_3P)_4Pd$ (28.9 mg, 0.0025 mmol), and K_2CO_3 (207.3 mg, 1.5 mmol) in anhydrous *i*-PrOH (6.3 mL) was degassed and exchanged with Ar.[42] The resulting mixture was heated to 50 °C for 2 h and 100 °C for 21 h with stirring. The suspension was then cooled to room temperature and filtered. The solvent was removed under reduced pressure and the residue was purified by column chromatography on silica gel that was eluted with a hexane and ethyl acetate mixture (2:1–1:1, v:v). At this stage, the *i*-propyl ester produced as a side reaction product was a contaminant, and, therefore, the mixture of methyl ester and *i*-propyl ester was re-purified and eluted with a chloroform and acetone solution (30:1, v:v) to give the desired precursor **5** (27.5 mg) in 15% yield. **5**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.41 (dd, 1H, J = 4.59, 1.91 Hz, H-6 Py), 8.38 (d, 1H, J = 8.03, H-3′ Ph), 8.19 (br s, 1H, NH), 8.09–8.11 (m, 3H, H-4 Py, H-3 and H-5 Ph), 7.47–7.51, 7.29–7.34 (m, 6H, H-5 Py, H-2 and H-6 Ph, H-4′ and H-5′ and H-6′ Ph), and 3.93 (s, 3H) ppm. ^{13}C NMR (125 MHz, $CDCl_3$) δ 166.5, 162.5, 151.2, 146.6, 142.6, 140.0, 134.1, 132.5, 131.0, 130.2, 130.0, 129.8, 129.5, 129.1, 125.4, 122.8, 122.3, and 52.3 ppm. IR (neat) υ 3279, 3045, 1714, and 1676 cm^{-1}. HRMS (DART) *m/z*: Anal. Calcd for $C_{20}H_{16}ClN_2O_3$ ($M+1^+$) 367.0849; Found 367.0850.

Boscalid hapten, 2'-(2-chloronicotinamido)-biphenyl-4-carboxylic acid, (6). To a solution of boscalid hapten precursor **5** (36.8 mg, 0.1 mmol) in CH_3OH (1.3 mL) was added a solution of NaOH (40.0 mg, 1.0 mmol) in H_2O (0.5 mL) at 0 °C. The mixture was stirred for 3 h at room temperature and then acidified with 1N HCl solution to pH 2.0. The solvent was removed and the residue was washed with tetrahydrofuran. The tetrahydrofuran mixture was concentrated to give the desired hapten **6** (23.7 mg) in 67% yield. **6**: ^{1}H NMR (500 MHz, CD_3OD) δ 8.41 (dd, 1H, *J* = 4.97, 1.91 Hz, H-6 Py), 8.07–8.10 (m, 2H, H-3 and H-5 Ph), 7.77 (dd, 1H, *J* = 7.64, 1.91 Hz, H-4 Py), 7.64 (d, 1H, *J* = 7.64 Hz, H-6′ Ph), 7.55–7.57 (m, 2H, H-2 and H-6 Ph), 7.47–7.51 (m, 1H, H-5′ Ph), and 7.42–7.44 (m, 3H, H-5 Py, H-3′ and H-4′ Ph) ppm. ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.0, 167.1, 151.5, 148.4, 143.0, 139.5, 139.2, 136.5, 134.6, 133.9, 131.5, 130.5, 129.8, 129.5, 128.6, 128.4, and 124.1 ppm. HRMS (DART) *m/z*: Calcd for $C_{19}H_{14}ClN_2O_3$ ($M+1^+$) 353.0693; Found 353.0657. IR (neat) υ 3417, 1644–1660 cm^{-1}.

Preparation of Hapten–Protein Conjugate. The synthesized hapten was conjugated with proteins (KLH, BSA, or HRP) by the activated ester method as described previously.[23] In brief, 100 μL of the hapten (5 μmol) in anhydrous dimethyl sulfoxide was mixed with 5 μL of *N*-hydroxysuccinimide (6 μmol) and 10 μL of EDC (6 μmol) in anhydrous dimethyl sulfoxide, and the mixture was allowed to stand at room temperature for 1.5 h. Then, 1 mL of each of the proteins (10 mg) dissolved in 10 mmol/L PBS (10 mmol/L phosphate buffer and 150 mmol/L NaCl; pH 7.0) was added to the mixture, which was then gently stirred for 1.5 h. The hapten–KLH conjugate and the hapten–BSA conjugate were respectively

dialyzed in 10 mmol/L PBS, whereas the hapten–HRP conjugate was purified through gel filtration chromatography. The hapten–KLH conjugate was emulsified with an equal volume of Freund's complete adjuvant or incomplete adjuvant for use in mice immunization and booster immunization, respectively. The hapten–BSA conjugate was used for the indirect ELISA (i-ELISA), indirect competitive ELISA (ic-ELISA), and SPR-sensor experiments. The hapten–HRP conjugate was used for the dc-ELISA.

Preparation of Monoclonal Antibodies. MoAbs were prepared as described previously.[23] In brief, Balb/c mice (7-week-old females) purchased from Japan SLC, Inc. (Shizuoka, Japan) were immunized with 100 μL of the hapten–KLH conjugate (100 μg/mouse) in Freund's complete adjuvant. After 1 month, the booster immunization was performed twice at 2-week intervals, using 100 μL of the hapten–KLH conjugate (25 μg/mouse) in Freund's incomplete adjuvant. After 3 days from the last immunization, spleen cells of the mice were collected and prepared for cell fusion with P3-X63-AG8.653 myeloma cells (5.5×10^7 cells), using polyethylene glycol solution. The fused cells were suspended at 8×10^5 cells/mL in HAT medium (RPMI 1640 medium, 10% FBS, and HAT medium supplement) and transferred to 96-well microplates. After incubation at 37 °C for 7–10 days, colonies secreting anti-boscalid MoAbs were identified by i-ELISA and ic-ELISA and picked up. The selected hybridoma cells were cloned by the limiting dilution method. Two MoAbs, BSC7 and BSC72, were purified from the ascite fluid by ammonium sulfate fractionation and anion-exchange chromatography. BSC7 showed the highest reactivity upon the ELISA

screenings, whereas BSC72 was the representative MoAbs among various MoAbs showing similar reactivity.

The mouse experiments were performed under the guidelines of the Animal Experiment Committee of Kyoto Women's University following the bulletin (No. 71, 2006) of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

i-ELISA and ic-ELISA. The i-ELISA and ic-ELISA methods were conducted as described previously.[23] The specific conditions were that the hapten–BSA concentration was 1.0 μg/mL, and boscalid standard solution was prepared at concentrations from 0.6 to 2500 ng/mL in 10% methanol.

dc-ELISA. The dc-ELISA method was conducted as described previously.[23] Each well of a 96-well microtiter plate was coated overnight with 100 μL of anti-mouse IgG goat antibody (5 μg/mL) in 10 mmol/L PBS at 4 °C and then blocked for 1 h with 300 μL of 10 mmol/L PBS containing 0.4% BSA at 25 °C. One hundred microliters of purified MoAb BSC7 (500 ng/mL) or culture fluid containing MoAb BSC72 in 10 mmol/L PBS containing 0.2% BSA was added to the appropriate wells and incubation was carried out at 25 °C for 1 h. The plate was washed once with washing buffer (10 mmol/L PBS containing 0.02% Tween 20). Each solution (150 μL) of boscalid (0.038–156 ng/mL), boscalid-related pesticides (10 μg/mL) used in a cross-reactivity test, and vegetable samples prepared in 10% methanol were mixed with an equal volume of the hapten–HRP conjugate (250 ng/mL) in 10 mmol/L

PBS containing 0.2% BSA. The mixture (100 μL) was added to the wells of the plate for competitive reaction and incubated at 25 °C for 1 h. After washing 3 times with the washing buffer, 100 μL of the color development solution (100 μg/mL 3,3′,5,5′-tetramethylbenzidine, 0.006% H_2O_2, and 0.1 mol/L acetate buffer; pH 5.5) was added. After 10 min at 25 °C, 100 μL of 0.5 mol/L sulfuric acid was added to stop the color development reaction. The absorbance was measured at 450 nm with an xMark microplate reader (Bio-Rad Laboratories, CA, USA), so that the maximum absorbance reading was around 1.3.

SPR-Sensor. The SPR-sensors consisted of commercially available instruments of microflow type (Biacore T200; GE Healthcare Europe, Munich, Germany), and the sensor chips were coated with carboxymethyl dextran (CM5; GE Healthcare Europe). The sensor chips were functionalized via the following steps, based on the instruction manual.

Step 1: Preparation of the sensor chip for boscalid. The sensor chip was placed in the instrument, and the surface was initially rinsed with 10 mmol/L PBS containing 0.005% Tween 20 at a rate of 5 μL/min for 600 s. It was activated by flushing with a mixture of 80 μL of EDC (400 mmol/L) and 80 μL of *N*-hydroxysuccinimide (100 mmol/L) in distilled water, for 350 s. The hapten–BSA (40 μg/mL) solution dissolved in acetic acid buffer (10 mmol/L; pH 5.0) was then flowed through the system for 120 s to bind the chip's activated carboxyl groups to the amino group residues of hapten–BSA. This was followed by flowing ethanolamine (1 mol/L; pH 8.5) through the system for 350 s, to block residual carboxy groups. After rinsing with the running buffer (50 mmol/L phosphate buffer and 75 mmol/L

NaCl; pH 7.0 containing 5% methanol and 0.1% BSA), the sensor chip was ready to be used.

Step 2: Preparation of samples. The boscalid solutions were serially diluted at final concentrations in the range of 2.4–3100 ng/mL in 10% methanol with distilled water. The other structurally related pesticides were prepared at 10 μg/mL. MoAb BSC7 was diluted to 15 μg/mL with 100 mmol/L PBS (100 mmol/L phosphate buffer, and 150 mmol/L NaCl; pH 7.0) containing 0.2% BSA. The boscalid or other pesticide solution (75μL) was mixed with an equal volume of MoAb BSC7 solution (75 μL) to form the pesticide–MoAb BSC7 complex. This mixture (final 5% methanol solution) was used for pesticide determination by the SPR-sensor.

Step 3: Determination of pesticides. The mixed solutions were flushed through the sensor system at 20 μL/min for 180 s, to allow free MoAb BSC7 to bind with the hapten–BSA immobilized on the sensor surface. The solution was then changed to the running buffer, which was allowed to flow continuously at 20 μL/min for 180 s.

Step 4: Regeneration of the sensor surface. After the pesticide determination had been completed, the MoAb BSC7 was removed from the immobilized hapten–BSA in the following way. The sensor surface was initially washed with GdnHCl (3.0 mol/L) in acetic acid (1.0 mol/L; pH 1.9) at 20 μL/min for 60 s. After rinsing with distilled water at 20 μL/min for 60 s, the sensor surface was washed with 0.2% SDS at 20 μL/min for 120 s, as described previously.[39] After the regeneration had been completed, the sensor chip was used again, repeating steps 2 to 4 described above.

Step5: Data processing. Raw data were output as a resonance unit (RU), where 1000 RU was defined as a 0.1-degree refractive index shift by the SPR phenomenon. The actual response curve was drawn, where the x-axis represented the boscalid concentration and the y-axis represented the delta RUs between the starting point of the reaction and a time point of 180 s.

Treatment of Vegetable Samples. Cucumber, tomato, green sweet pepper, cabbage, spinach, and orange, purchased from a market in Kyoto city, were used at 100 g each for the recovery examinations. Boscalid-treated tomato (200 g) was harvested from an experimental farm at the Aichi Agricultural Research Center. Real tomato samples were used to examine for correlations between the dc-ELISA or the SPR-sensor and high-performance liquid chromatography (HPLC). The vegetable samples were homogenized in a blender. For the recovery examinations, boscalid was added to the homogenized samples at final concentrations in the range from 1 to 15 mg/kg.

For the dc-ELISA and the SPR-sensor, 25 mL of methanol was added to the homogenized samples (5.0 g) in a 50 mL screw-cap tube. The mixture was first vigorously shaken (Shaker SA320; Yamato Scientific Co., Ltd, Tokyo, Japan) for 30 min to extract the boscalid and then was centrifuged at 3000 rpm at room temperature for 10 min. The supernatant was diluted to 8.5-folds with distilled water, to prepare a 10% methanol equivalent solution. The diluted sample was applied to the dc-ELISA and the SPR-sensor. Further dilution using 10% methanol with distilled water was carried out to adjust the

concentrations of boscalid for most of the samples in the dc-ELISA and a part of the samples in the SPR-sensor.

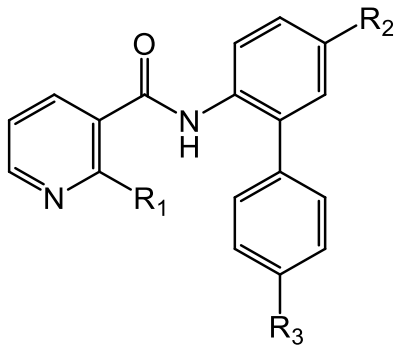
For the HPLC, 100 mL of acetone was added to the homogenized sample (20 g), and the boscalid was extracted for 30 min using a shaker (Recipro Shaker SR-II; Taiyo Chemical Industry, Gunma, Japan). The mixture was filtered through a filter-paper-lined funnel by suction. The residue was then re-treated with 60 mL of acetone. Both filtered extracts were mixed, and the acetone was distilled away by evaporation at <40 °C in a flask. The concentrated solution was applied to 20 mL of a porous kieselguhr column (Inert Sep k-solute; GL Science, Tokyo, Japan) and held at room temperature for 10 min. The fraction containing boscalid was eluted with 100 mL of hexane. The eluted sample was evaporated to dryness by evaporation and N_2 purge. The dried sample was dissolved in 5 mL of acetonitrile and then applied to 6 mL of a bilayer column with activated charcoal and aminopropyl silica gel (GL-Pak GC/NH2; GL Sciences). The column was washed 3 times with 5 mL of acetonitrile, and the boscalid was then eluted with a mixture of acetonitrile and toluene (3:1, v:v; 15 mL). The eluted sample was dried by evaporation and N_2 purge and re-dissolved with a mixture of acetonitrile and distilled water (50:50, v:v; 2 mL). A portion of the solution (4 μL) was applied to HPLC.

HPLC Analysis. The HPLC system consisted of the Waters Acquity UPLC-PDA detector, equipped with a Waters BEH C18 reversed-phase column (1.7 μm, 2.1 i.d. ×50 mm; Waters, MA, USA). The mobile phase was a methanol and water mixture (45:55, v:v), running at a

flow rate of 0.5 mL/min. The column oven temperature was 40°C, and the injection volume of the sample was 4 μL. Boscalid was detected at the wavelength of 230 nm.

RESULTS AND DISCUSSION

Hapten Design. Boscalid is one of the carboxamide fungicides that have two chlorine atoms, as shown in Figure 1. In designing the hapten, the chlorine residues are potent candidates for introducing the hapten linker. Abad-Fuentes *et al*. actually used the chlorine (R_1) for the linker site, and prepared a highly reactive PoAb to boscalid from hapten 1.[40] Esteve-Turrillas *et al.* (of the same research group) subsequently synthesized the other haptens by introducing alkyl-carboxylic acid to the position of R_2 (hapten 2), as well as introducing the carboxy group to the position of R_3 (hapten 3) using a thiol group.[41] In the dc-ELISAs, the IC_{50} values of the prepared PoAbs were 0.4–1.6 ng/mL for hapten 1, 0.6–1.2 ng/mL for hapten 2, and 0.8–1.6 ng/mL for hapten 3. The reactivity values, however, seemed too sensitive to determine boscalid at the MRLs for horticultural crops (1–40 mg/kg), because the concentration was more than 1000-folds. It seemed that the PoAb with hapten 3 had the lowest reactivity. In our study, therefore, the R_3 position was chosen as the linker site of the newly synthesized hapten. Furthermore, the linker was directly introduced without a thiol group, because the structure is more stable in the mouse abdominal cavity. The key step of the synthesis of the boscalid hapten was achieved by using the Suzuki–Miyaura coupling reaction in the presence of a Pd catalyst, as shown in Figure 2.[42]



compound	R_1	R_2	R_3	reference
boscalid	Cl	H	Cl	1
hapten 1	$S(CH_2)_4COOH$	H	Cl	40
hapten 2	Cl	$(CH_2)_5COOH$	Cl	41
hapten 3	Cl	H	$S(CH_2)_4COOH$	41
new hapten	Cl	H	COOH	

Figure 1. Structure of boscalid and the haptens

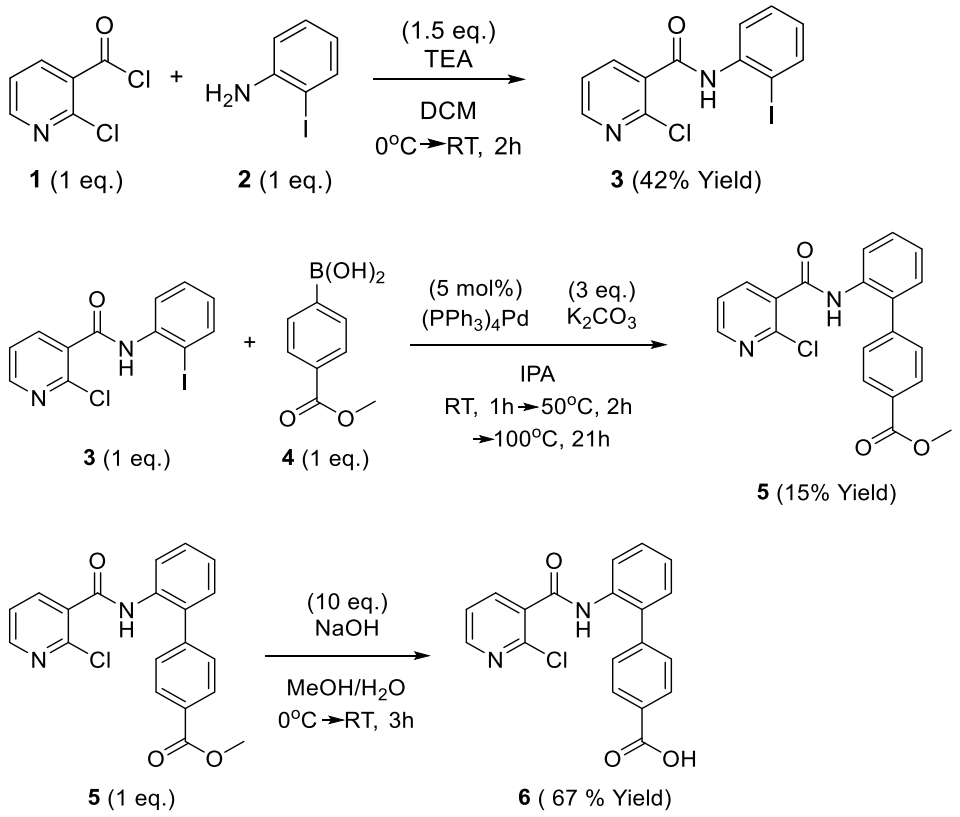


Figure 2. Scheme of the hapten synthesis

Preparation of MoAbs Reacting to Boscalid. The MoAb preparation was carried out by using the conjugate of the new hapten and KLH as the immunogen. MoAbs reacting to the boscalid hapten conjugate were screened, via i-ELISA and ic-ELISA, from 2000 wells in which the colony had formed, and the reactivity of each MoAb was compared with that of the PoAb prepared from the same mouse. Of the 2 MoAbs finally prepared, MoAb BSC7 had the highest reactivity of all the MoAbs upon screening, whereas MoAb BSC72 was the representative one among various MoAbs showing similar reactivity. As shown in Figure 3, with the ic-ELISA, the IC_{50} values of the PoAb, MoAb BSC7, and MoAb BSC72 were 90, 3.3, and 43 ng/mL, respectively, indicating that the reactivity of MoAb BSC72 was much closer to that of the PoAb. The reactivity of the ic-ELISA seemed adequate when compared

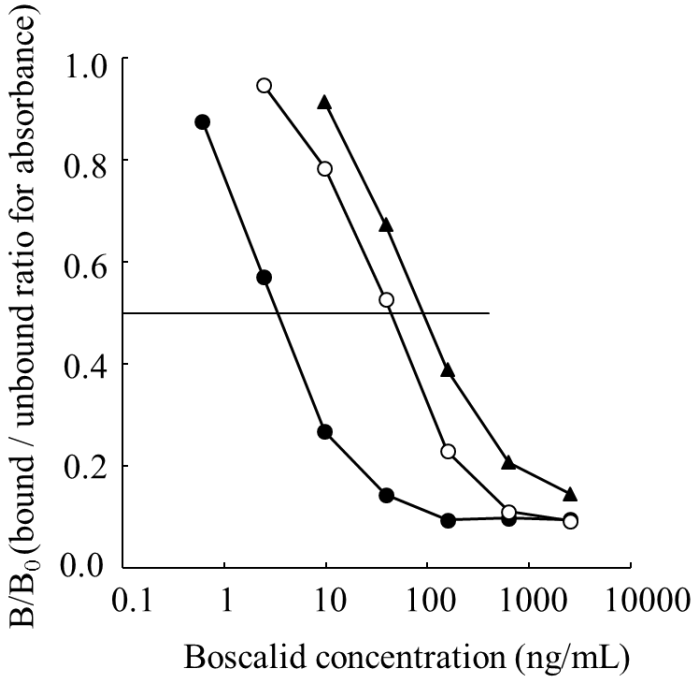


Figure 3. Reactivity of the prepared antibodies with boscalid in ic-ELISA : (●) MoAb BSC7; (○) MoAb BSC72; (▲) PoAb. Each data point is the mean of duplicates in independent

with the boscalid MRLs. On the other hand, MoAb BSC7 had a 30 times higher reactivity than PoAb, which was too high for determining the boscalid residue by ic-ELISA. Hence, the test samples would have to be diluted to the adequate boscalid concentrations to adjust to the working MRL range.

Reactivity of MoAbs with Boscalid in the dc-ELISA. The dc-ELISA is the most widely used method for pesticide residue analysis among the various immunoassays, and many of the test kits based on the dc-ELISA are commercially available for pesticide determination. The kits have been validated for agricultural products.[24–26] The effectiveness of the dc-ELISA for determining boscalid using MoAb BSC7 and MoAb BSC72 was therefore examined, and the results are shown in Figure 4.

The working range was defined as concentrations between the IC_{20} and IC_{80} values. The IC_{20}, IC_{50}, and IC_{80} values with MoAb BSC7 were 0.8, 3.3, and 16 ng/mL, respectively, similar to the results obtained with the ic-ELISA. These results suggest that it would be possible to use the dc-ELISA with MoAb BSC7 for boscalid residue analysis in horticultural crops, although most of the test samples would have to be diluted to adequate concentrations prior to analysis. On the other hand, the IC_{20}, IC_{50}, and IC_{80} values with MoAb BSC72 were 2.5, 15, and 120 ng/mL, respectively, in the dc-ELISA. The degree of slope on the standard curve for dc-ELISA was more gradual than that for the ic-ELISA, indicating dc-ELISA's higher sensitivity and hence adequacy for residue analysis around the MRLs of horticultural crops.

Reactivity of MoAb BSC7 in the SPR-Sensor. SPR-sensors are rarely used for pesticide residue analysis in horticultural crops, although various electrochemical sensors have been applied.[28,39] There may be several reasons for this. First, the organic solvent containing high concentrations of the sample matrices may gum the microflow channel in the sensor chip by precipitation. To resolve the precipitation problem, the test samples were solubilized in 5% of methanol as well as the condition of competitive reaction for the above-described dc-ELISA, and the running buffer was also mixed with 5% of methanol to maintain the solubility of the sample. Second, the antibody bound to the immobilized antigen may be unable to dissociate from the sensor chip. A combination of GdnHCl (3.0 mol/L) in acetic acid (1.0 mol/L; pH 1.9) and 0.2% SDS was therefore recently applied to dissociate the bound antibody, as described previously.[39]

The reactivity of MoAb BSC7 with boscalid was examined in the SPR-sensor, as shown in Figure 4. The working range was defined as concentrations between the IC_{20} and IC_{80} values as well as the dc-ELISA. The IC_{20}, IC_{50}, and IC_{80} values were 17, 42, and 80 ng/mL, respectively. MoAb BSC7 showed a 15-fold lower reactivity in the SPR-sensor than in the dc-ELISA. The reactivity of MoAb BSC72 in the SPR-sensor was too low to determine boscalid residues in horticultural crops (data not shown). The lower reactivity may be because much more MoAb and hapten–BSA were necessary for the SPR-sensor compared with the dc-ELISA.

The SPR-sensor with MoAb BSC7 seemed to show adequate sensitivity for determination of boscalid residues in horticultural crops, with higher reproducibility for the standard curve than the dc-ELISA, as shown in Figure 4. The advantages of the SPR-sensor

with MoAb BSC7 are its ability to make both quantitative and rapid determinations of boscalid residues in horticultural crops. Further comparisons of the SPR-sensor and the dc-ELISA, using MoAb BSC7 only, were therefore carried out.

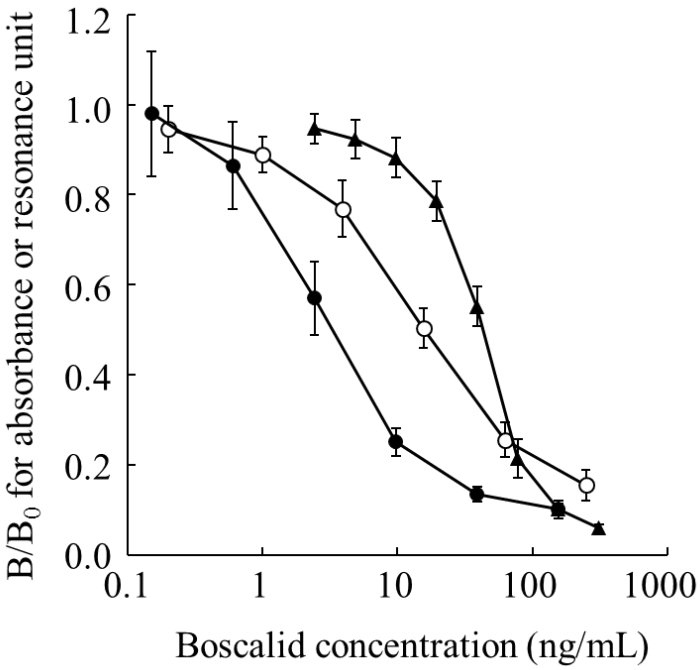
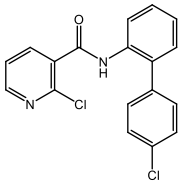
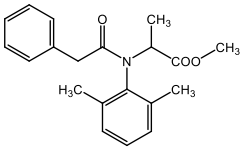
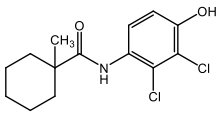
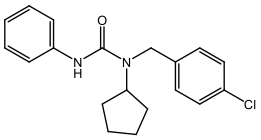
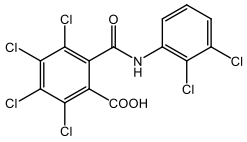


Figure 4. Reactivity of MoAbs with boscalid in dc-ELISA and in SPR-sensor: (●) MoAb BSC7 in dc-ELISA; (○) MoAb BSC72 in dc-ELISA; (▲) MoAb BSC7 in SPR-sensor. Each data point is the mean of 4 replicates in independent examinations; error bars indicate ± SD.

Cross-reactivity of MoAb BSC7. Cross-reactivity of MoAb BSC7 in the dc-ELISA and the SPR-sensor was examined using other structurally related pesticides: benalaxyl, fenhexamid, pencycuron, and tecloftalam. MoAb BSC7 was highly specific to boscalid, as shown in Table 1. MoAbs generally recognize slight differences in the immunogen, such as the position and molecular size of the functional group, and even the optical isomerism of

the molecules. Since the boscalid structure has many differences from the other carboxamide fungicides of the same group, the high assay specificity would be due to its structural uniqueness.

Table 1. Cross-reactivity of MoAb BSC7 with boscalid and the other structurally related pesticides in dc-ELISA and SPR-sensor[b]

Pesticide	CR (%)[a]		Pesticide	CR (%)	
	ELISA	SPR		ELISA	SPR
boscalid	100	100	benalaxyl	<0.03	<0.4
					
fenhexamid	<0.03	<0.4	pencycuron	<0.03	<0.4
					
tecloftalam	<0.03	<0.4			
					

[a] CR (%) shows cross-reactivity (%) compared to IC_{50} value of boscalid.

[b] Each data point is the mean of 3 replicates in independent examinations.

Boscalid Recovery in Pesticide-spiked Horticultural Crops. The applicability of the dc-ELISA and the SPR-sensor, using MoAb BSC7, to boscalid residue analysis in horticultural crops was examined by recovery examinations. The limits of quantity value of boscalid for

horticultural crops were 0.04 mg/kg in the dc-ELISA and 0.87 mg/kg in the SPR-sensor. The fruit vegetables cucumber (*Cucumis sativus* L.), tomato (*Solanum lycopersicum* L.), and green sweet pepper (*Capsicum annuum* L. var. *grossum*); the leaf vegetables cabbage (*Brassica oleracea* L. var. *capitata*) and spinach (*Spinacia oleracea* L.); and the fruit orange (*Citrus unshiu*) were chosen as the representative horticultural crops. As shown in Table 2, the recovery values were 100%–124% in the dc-ELISA and 85.0%–109% in the SPR-sensor, indicating that both immunoassays could be applied to boscalid residue analysis in horticultural crops. The SPR-sensor especially showed a higher quantitative performance than the dc-ELISA, since each of the recovery results was closer to the trueness value (100%), except for the results of spinach. The RSD values for the SPR-sensor were also lower than those for the dc-ELISA, although some exceptions were found for green sweet pepper, cabbage and orange.

Table 2. Recovery of boscalid spiked in horticultural crops by dc-ELISA and SPR-sensor[a]

Cucumber					Tomato				
spiked	ELISA		SPR		spiked	ELISA		SPR	
concn[b]	Rec.[c]	RSD[d]	Rec.	RSD	concn	Rec.	RSD	Rec.	RSD
3	116	5.6	101	4.3	3	109	13.3	104	5.5
5	114	12.4	85	3.1	5	122	16.1	90	7.4
7	124	12.1	94	8.8	7	124	8.9	93	5.8
Green sweet pepper					Cabbage				
spiked	ELISA		SPR		spiked	ELISA		SPR	
concn	Rec.	RSD	Rec.	RSD	concn	Rec.	RSD	Rec.	RSD
5	120	13.2	89	6.3	1	109	9.8	104	10.6
10	114	2.6	95	9.0	3	116	9.6	109	5.2
15	122	7.4	94	14.5	5	113	7.4	105	9.2
Spinach					Orange				
spiked	ELISA		SPR		spiked	ELISA		SPR	
concn	Rec.	RSD	Rec.	RSD	concn	Rec.	RSD	Rec.	RSD
1	100	9.7	95	2.9	1	103	5.4	102	10.2
3	104	13.4	107	3.5	2	120	4.2	97	11.1

[a] Each data point is the mean of three replicates in independent examinations.

[b] The unit of the concentration are milligrams per kilogram.

[c] The abbreviation “rec” is short for recovery (%).

[d] RSD = relative standard deviation (%).

The dc-ELISA performance for determination of boscalid residues was as good as previously reported dc-ELISAs for other pesticides.[24-26] The SPR-sensor also showed performance equal to or more than the dc-ELISA except for the sensitivity. The reasons would be that for the SPR-sensor, 1) sample dilution was not necessary for most of the samples, 2) bound/free separation of the MoAb was not necessary, and 3) labeling of the MoAb, such as with HRP in the dc-ELISA, was not necessary.

Correlation between HPLC and dc-ELISA or SPR-Sensor. The applicability of the dc-ELISA and the SPR-sensor was also examined by comparing them with HPLC, using real tomato samples to which a boscalid formulation had been applied before harvesting. As shown in Figure 5, the correlation values for HPLC with both immunoassays were high (R^2 = 0.97 with dc-ELISA; R^2 = 0.95 with SPR-sensor), suggesting that both of the newly developed immunoassays had high applicability to boscalid residue analysis in horticultural crops. The SPR-sensor had a lower bias (slope = 1.14) than the dc-ELISA (slope = 1. 26), and did not show a visible y-intercept difference, whereas the y-intercept difference from the dc-ELISA was considerable (0.53 mg/kg). The result suggested that the SPR-sensor has high accuracy even near the lowest determination limit. Therefore, this SPR-sensor would be useful as a rapid, simple, and accurate method for the determination of boscalid residues in horticultural crops. On the other hand, the dc-ELISA has sufficient applicability for on-site analysis. In conclusion, each of the two immunoassays developed for boscalid determination should be used according to the required purpose.

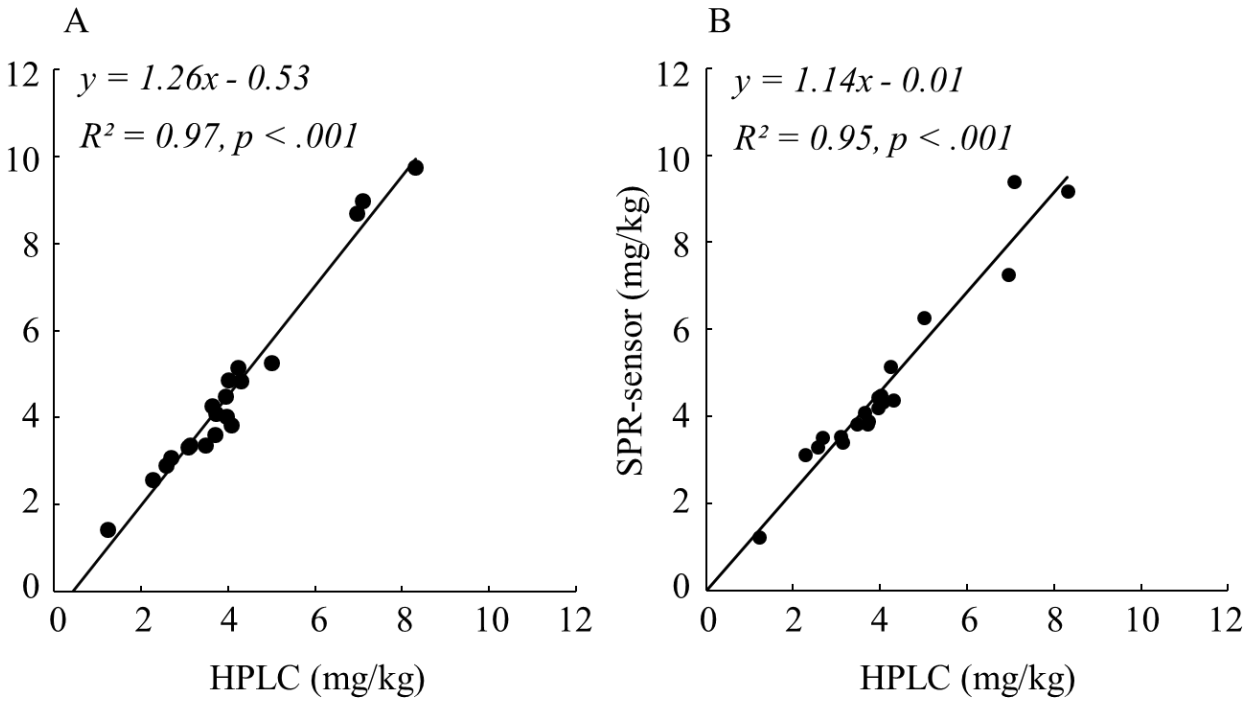


Figure 5. Correlation of determined boscalid residue in real tomato samples between (A) dc-ELISA and HPLC or (B) SPR-sensor and HPLC: Each data point is the mean of duplicates in independent examinations.

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Chapter 4

Simultaneous Analysis of Three Pesticides, Boscalid, Clothianidin and Nitenpyram in Vegetables, Using an Immunosensor Based on Surface Plasmon Resonance

ABSTRACT: An immunosensor based on surface plasmon resonance (SPR-sensor) were developed for simultaneous analysis of fungicide boscalid, and insecticide clothianidin and nitenpyram in vegetables. The SPR-sensor was constituted on 3 parallel channels immobilized with each of the conjugates of the pesticide haptens and bovine serum albumin. Mixture of 3 kinds of anti-pesticide monoclonal antibodies (MoAb) for boscalid, clothianidin and nitenpyram were used to the SPR-sensor. The mixed MoAbs solution was further mixed with various dispensed standard pesticides or samples, and flowed to the SPR-sensor. Each working range was 14–77 ng/mL for boscalid, 8.5–34 ng/mL for clothianidin, and 8.2–62 ng/mL for nitenpyram with almost no cross-reaction each other. Recovery of the spiked pesticide mixture in vegetables was 75%–90% for boscalid, 88%-104% for clothianidin and 72%-105% for nitenpyram, respectively. Results for the developed simultaneous analysis were correlated well with the results for each of the conventional dc-ELISA.

INTRODUCTION

Boscalid, 2-chloro-*N*-(4′-chlorobiphenyl-2-yl)nicotinamide, is one of the fungicides. It is widely applied in vegetable fields.[1-3] Neonicotinoid insecticides, clothianidin, (*E*)-1-(2-chloro-1,3-thiazol-5-ylmethyl)-3-methyl-2-nitroguanidine, and nitenpyram, (*E*)-*N*-(6-chloro-3-pyridylmethyl)- *N*-ethyl-*N*'-methyl-2-nitrovinylidenediamine, are also applied widely to the fields. Clothianidin was developed by modification of nitenpyram although they are different type neonicotinoid insecticides that clothianidin belongs chlorothiazol ring type and nitenpyram belongs chloropyridine ring type. Boscalid and their insecticides are often applied in combination to prevent both of fungi disease and insect pests in the same season.

Boscalid, clothianidin and/or nitenpyram are generally determined by liquid chromatography with mass spectrometry (LC-MS).[4–7] The chromatographic technique can simultaneously determine their 3 pesticides as constituents in a multi-residue analysis not only it is sensitive and accurate. It however requires a lot of time to prepare measurement samples and labor intensive. Enzyme-linked immunosorbent assays (ELISAs) have been developed for the monitoring of their pesticide residues as more simple, rapid, and cost-effective method than the LC-MS. [8-11] The ELISAs however are individual analysis but not multi-residue analysis.

Immunosensor using surface plasmon resonance (SPR-immunosensor) was recently developed for determinations of fungicide chlorothalonil and boscalid residues, respectively.[12,13] It is an automatic analyzer as well as the above LC-MS, and the maximum 3 pesticides can be determined at the same time by its parallel 3 channels in the sensor chip.

In this study, the SPR-immunosensor was developed for simultaneous analysis of boscalid, clothianidin and nitenpyram. Applicability of the SPR-sensor was compared to each of the dc-ELISAs developed for boscalid, clothianidin and nitenpyram.[13,14]

MATERIALS AND METHODS

Materials. Analytical grade of boscalid, benalaxyl, fenhexamid, tecloftalam, clothianidin, nitenpyram, imidacloprid, dinotefuran, thiacloprid and thiamethoxam for pesticide residue analysis and *N*-hydroxysuccinimide (NHS) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Analytical grade of acetamiprid was from Hayashi Pure Chemical Ind., Ltd. (Osaka, Japan). Dichloromethane (CH_2Cl_2) was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Glass plates pre-coated with silica gel (60 F_{254}, layer thickness 0.2 mm) for thin layer chromatography (TLC), and silica gel (Art. No. 7734) were purchased from Merck KGaA (Darmstadt, Deutschland). Bovine serum albumin (BSA; Prod. No. A7888) was purchased from Sigma-Aldrich Co. (St. Louis, MO). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). Guanidine hydrochloride (GdnHCl) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Ninety-six-wells of microtiterplate for ELISA (Nunc MaxiSorp) and anti-mouse IgG (H+L) antibody from goat (Pierce) were purchased from Thermo Fisher Scientific K. K. (New York, NY, USA). Horse radish peroxidase (HRP) was purchased from Toyobo Co. Ltd. (Osaka, Japan). Anti-boscalid MoAb was prepared as described previously, [13] anti-clothianidin MoAb and anti-nitenpyram MoAb were provided from Horiba Ltd (Kyoto, Japan). Boscalid hapten and

clothianidin hapten were prepared as described previously. [13,14] All other chemicals and reagents used were of analytical grade, and purchased from Wako Pure Chemical Industries, Ltd., Nacalai Tesque, Inc, or Kanto Chemical Co., Inc..

Synthesis of Nitenpyram Hapten.

General: All reactions were performed under an atmosphere of argon unless otherwise noted. All reactions were monitored by TLC. The products were visualized by irradiation with UV light or by treatment with a solution of phosphomolybdic acid or by treatment with a solution of *p*-anisaldehyde. Flash column chromatography was performed using silica gel. IR spectra were measured on a JASCO FT/IR-4600 spectrometer. ^{1}H NMR (500 MHz, 400 MHz) and ^{13}C NMR (125 MHz, 100 MHz) spectra were recorded on JEOL JNM-ECX500, JEOL JNM-ECS400 spectrometer. Chemical shifts are reported as δ values (ppm) relative to TMS (0 ppm) and DMSO-d_6 (2.50 ppm). DART mass (positive mode) analyses were performed on a LC-TOF JMS-T100LP.

Synthesis of *N*-((6-chloropyridin-3-yl)methyl)ethanamine (2) [15]: To a solution of ethanamine (3.865 g, 60 mmol, 70 wt% aqua.) in CH_3CN (3.0 mL) was slowly added a solution of 2-chloro-5-(chloromethyl)pyridine (**1**) (972.1 mg, 6.0 mmol) in CH_3CN (1.5 mL) over 25 min. at 0 °C (ice-bath). The resulting mixture was stirred for 4hr. at 0 °C (ice-bath) followed by addition of H_2O (10 mL). The product was extracted with CH_2Cl_2 and dried with Na_2SO_4. The solvent was removed to give crude *N*-((6-chloropyridin-3-yl)methyl)ethanamine (**2**) (1.007 g) in 98% yield. **2** was used for next step without any

further purification. **2**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.33 (d, 1H, *J* = 2.29 Hz, H-2 Py), 7.67 (dd, 1H, *J* = 2.29, 8.03 Hz, H-4 Py), 7.29 (d, 1H, *J* = 8.03 Hz, H-5 Py), 3.79 (s, 2H, -PyC*H*$_2$NH-), 2.67 (q, 2H, *J* = 7.26 Hz, -NHC*H*$_2$CH$_3$), 1.13 (t, 3H, *J* = 7.26 Hz, -NHCH$_2$C*H*$_3$) ppm. ^{13}C NMR (125 MHz, $CDCl_3$) δ 149.6, 149.0, 138.5, 134.7, 123.7, 50.0, 43.4, 14.9 ppm. HRMS(DART) *m/z* : Calcd for $C_8H_{12}ClN_2$ (M+1$^+$) 171.0689, Found 171.0682. IR (neat) 3418, 3087, 2968, 1458, 1104 cm^{-1}.

Synthesis of *N*-((6-chloropyridin-3-yl)methyl)-*N*-ethyl-1-(methylthio)-2-nitroethen-1-amine (3)[15]: *N*-((6-chloropyridin-3-yl)methyl)ethanamine (**2**) (1.00 g, 5.9 mmol) was dissolved in anh. EtOH (3.0 mL) followed by addition of (2-nitroethene-1,1-diyl)bis(methylsulfane) (886.4 mg, 5.4 mmol). The resulting mixture was heated to reflux for 6 hr. Then the stirring was continued for 17.5 hr. at room temperature. At this stage, the reaction was not completed. The mixture was refluxed again for 4.5 hr. The mixture was cooled to room temperature and the solvent was removed. The residue was purified by column chromatography on silica gel (hexane/ethyl acetate = 3/1→1/1→1/3 v/v) to give *N*-((6-chloropyridin-3-yl)methyl)-*N*-ethyl-1-(methylthio)-2-nitroethen-1-amine (**3**) (616.3 mg) in 40% yield. **3**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.27 (d, 1H, *J* = 2.44 Hz, H-2 Py), 7.55 (dd, 1H, *J* = 2.44, 8.24 Hz, H-4 Py), 7.35 (d, 1H, *J* = 8.24 Hz, H-5 Py), 6.80 (s, 1H, -C*H*NO_2), 4.69 (s, 2H, -PyC*H*$_2$N-), 3.49 (q, 2H, *J* = 7.02 Hz, -NC*H*$_2$CH$_3$), 2.47 (s, 3H, -SC*H*3), 1.25 (t, 3H, *J* = 7.02 Hz, -NCH$_2$C*H*$_3$) ppm. ^{13}C NMR (125 MHz, $CDCl_3$) δ 165.7, 151.2, 148.6, 137.9, 130.1, 124.5, 113.8, 53.1, 48.5, 17.7, 13.1 ppm. HRMS(DART) *m/z*: Calcd for $C_{11}H_{15}ClN_3O_2S$ (M+1$^+$) 288.0574, Found 288.0549. IR (neat) 2976, 1519, 1388, 1258, 1106 cm^{-1}.

Synthesis of *tert*-butyl 4-((1-(((6-chloropyridin-3-yl)methyl)(ethyl)amino)-2-nitrovinyl)amino)butanoate (4)[16]: To a mixture of *N*-((6-chloropyridin-3-yl)methyl)-*N*-ethyl-1-(methylthio)-2- nitroethen-1-amine (28.8 mg, 0.1 mmol) and K_2CO_3(41.5 mg, 0.3 mmol) in CH_3CN (0.6 mL) was added dropwise a solution of *tert*-butyl 4-aminobutanoate hydrochloride (19.6 mg, 0.1 mmol) in CH_3CN (0.1 mL) at room temperature. The resulting mixture was stirred for 23 hr. at room temperature. At the end of this period, the solvent was removed and the residue was purified by column chromatography on silica gel (hexane/ethyl acetate = 2/1→1/1→1/0→ethyl acetate/methanol = 1/1 v/v) to give *tert*-butyl 4-((1-(((6-chloro pyridin-3-yl)methyl)(ethyl)amino)-2-nitrovinyl)amino) butanoate (**4**) (21.6 mg) in 54% yield. **4**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.56 (bs, 1H, -N*H*), 8.29 (d, 1H, *J* = 2.29 Hz, H-2 Py), 7.56 (dd, 1H, *J* = 2.68, 8.03 Hz, H-4 Py), 7.36 (d, 1H, *J* = 8.03 Hz, H-5 Py), 6.52 (s, 1H, -C*H*NO_2), 4.35 (s, 2H, -PyCH_2N-), 3.40 (q, 2H, *J* = 6.88 Hz, -NC$H_2$$CH_3$), 3.12 (q, 2H, *J* = 7.26 Hz, -NC$H_2$$CH_2CH_2$C=OOtBu), 2.36 (t, 2H, *J* = 6.88 Hz, -NCH_2CH_2CH_2COO*t*-Bu), 1.93-1.99 (m, 2H, -NCH_2C$H_2$$CH_2$COO*t*-Bu), 1.44 (s, 9H, *t*-Bu), 1.18 (t, 3H, *J* = 6.88 Hz, -NCH_2CH_3) ppm. ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.8, 162.1, 151.1, 148.6, 138.0, 130.2, 124.5, 103.8, 80.7, 49.8, 44.9, 44.6, 31.9, 27.9, 25.2, 12.0 ppm. HRMS(DART) *m/z*: Calcd for $C_{18}H_{28}ClN_4O_4$ ($M+1^+$) 399.1799, Found 399.1799. IR (neat) 3452, 3131, 2977, 2934, 1724, 1517, 1366 cm^{-1}.

Synthesis of nitenpyram hapten, 4-((1-(((6-chloropyridin-3-yl)methyl)(ethyl) amino)-2-nitrovinyl)amino) butanoic acid (5)[16]: A solution of 4-((1-(((6-chloro pyridin-3-yl)methyl)(ethyl)amino)-2-nitrovinyl) amino) butanoate (**4**) (17.2 mg, 0.043 mmol) in CH_2Cl_2 (0.43 mL) was treated with CF_3CO_2H (46 μL). The mixture was stirred for 23 hr. at room temperature. Then the solvent was removed and the residue was purified by column

chromatography on silica gel (ethyl acetate/methanol = 15/1→8/1 v/v) to give the desired nitenpyram hapten, 4-((1-(((6-chloropyridin-3-yl)methyl)(ethyl) amino)-2-nitrovinyl)amino)butanoic acid (**5**) (13.4 mg) in 91% yield. **5**: ^{1}H NMR (500 MHz, DMSO-d$_6$) δ 12.17 (bs, 1H, -COO*H*), 8.67 (bs, 1H, -N*H*), 8.34 (d, 1H, *J* = 2.29 Hz, H-2 Py), 7.34 (dd, 1H, *J* = 2.29, 8.03 Hz, H-4 Py), 7.51 (d, 1H, *J* = 8.03 Hz, H-5 Py), 6.43 (s, 1H, -C*H*NO$_2$), 4.51 (s, 2H, -PyC*H*$_2$N-), 3.22-3.27 (m, 4H, -NC*H*$_2$CH$_3$, -NC*H*$_2$CH$_2$CH$_2$C=OOH), 2.23 (t, 2H, *J* = 7.26 Hz, -NCH$_2$CH$_2$C*H*$_2$COOH), 1.70-1.77 (m, 2H, -NCH$_2$C*H*$_2$CH$_2$COOH), 1.11 (t, 3H, *J* = 7.26 Hz, -NCH$_2$C*H*$_3$) ppm.

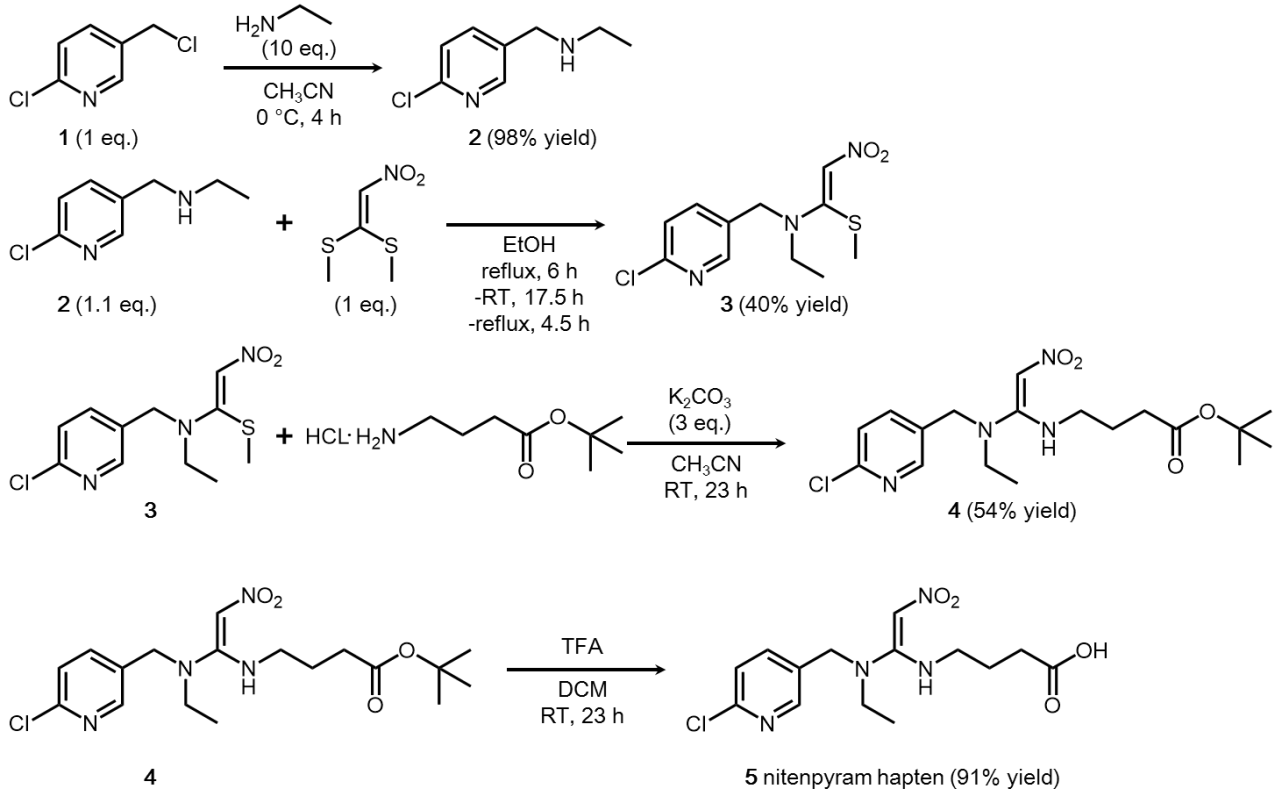

Figure 1. Scheme of the nitenpyram hapten synthesis

Preparation of Hapten–Protein Conjugate. The boscalid hapten, the clothianidin hapten and the nitenpyram hapten were respectively conjugated with BSA and HRP by the activated ester method as described previously.[17] In brief, 100 μL of each of the haptens (5 μmol) was dissolved to anhydrous dimethyl sulfoxide, and was mixed with NHS (6 μmol) and EDC (6 μmol). The mixture was stood for 1.5 hr at room temperature. Then, 1 mL of BSA or HRP (10 mg) was dissolved in phosphate buffer (10 mmol/L; pH 7.0), and was added to the mixture, followed by gently stirring for 1.5 hr. The hapten-BSA conjugates prepared were dialyzed in phosphate buffer modified with 150 mmol/L NaCl (phosphate buffered saline; PBS), and the hapten-HRP conjugates were purified through gel filtration chromatography.

dc-ELISA. The dc-ELISAs were constituted as described previously.[17] The specific conditions were as described below. The anti-pesticide MoAbs were prepared for coating on the wells of microtiterplate at each of concentrations: boscalid (500 ng/mL), clothianidin (250 ng/mL) and nitenpyram (125 ng/mL). The hapten-HRP conjugates were prepared for competitive reaction at each of concentrations: boscalid (250 ng/mL), clothianidin (50 ng/mL) and nitenpyram (1000 ng/mL) in PBS modified with 0.2% BSA. The standard solutions were prepared at each of concentrations: boscalid (0.038–156 ng/mL), clothianidin and dinotefuran (0.024–100ng/mL), nitenpyram (0.24–1000 ng/mL), thiacloprid (24-100,000 ng/mL) and the other pesticides (10 μg/mL) in 10% methanol.

SPR-Sensor. The SPR-sensors consisted of commercially available instruments of microflow type (Biacore T200; GE Healthcare Europe, Munich, Germany), and the sensor chips were coated with carboxymethyl dextran (CM5; GE Healthcare Europe), as described previously.[12] In brief, the conditions for the sensor constitution were described below.

Step 1: Preparation of the sensor chip for 3 pesticides. The sensor chip was initially rinsed with PBS containing 0.005% tween 20 for 600 s at a rate of 5 μL/min. Carboxy group on the chip was activated with a mixture of 80 μL of EDC (400 mmol/L) and 80 μL of NHS (100 mmol/L) dissolved in distilled water, for 350 s. The hapten–BSA conjugate (each 40 μg/mL) dissolved in acetic acid buffer (10 mmol/L; pH 5.0) was flowed to each of the channels for 360 s to immobilize it on the sensor surface. It was followed by flowing ethanolamine (1 mol/L; pH 8.5) for 350 s. The sensor constituted was rinsed with the running buffer (50 mmol/L phosphate buffer and 75 mmol/L NaCl; pH 7.0 containing 5% methanol and 0.1% BSA), and was ready to be used.

Step 2: Preparation of pesticide samples. The pesticide solutions were prepared at each of the concentrations or at the mixture: boscalid (3.1–200 ng/mL), nitenpyram (3.1–200 ng/mL), clothianidin (1.6–100 ng/mL), dinotefuran (1.6–100 ng/mL), thiacloprid (1.6–100 μg/mL) and the other pesticides (10 μg/mL) in 10% methanol. Anti-pesticide MoAbs were prepared at each of the concentrations or at the mixtures: boscalid (15 μg/mL), clothianidin (15 μg/mL), nitenpyram (15 μg/mL) in 100 mmol/L PBS modified with 0.2% BSA. The pesticide solutions (75μL) were mixed with an equal volume of the MoAb solution (75 μL). The mixtures were applied to the SPR-sensor.

Step 3: Determination of pesticides. The mixed solutions were flowed to each of the channels, to which the hapten-BSAs were respectively immobilized, in the sensor chip for 180 s at 20 μL/min. The solution was continuously changed to the running buffer, and was flowed for 180 s at 20 μL/min.

Step 4: Regeneration of the sensor surface. After the reactions had been finished, the sensor-chip was regenerated by removal of the bound MoAbs as described previously.[12] The channels were firstly washed with GdnHCl (3.0 mol/L) in acetic acid (1.0 mol/L; pH 1.9) for 60 s at 20 μL/min. After rinsing with distilled water for 60 s at 20 μL/min, it was further washed with 0.2% SDS for 120 s at 20 μL/min to complete the regeneration. The sensor chip was re-used by repeating the steps among 2 and 4.

Step 5: Data processing. Raw data were output as a resonance unit (RU). A 0.1-degree refractive index shift by the SPR phenomenon was defined as 1000 RU. The actual reaction signal was represented as the delta RUs from the starting point to a time point of 180 s on the reaction.

Treatment of Vegetable Samples. Broccoli (*Brassica oleracea* var. *italica*), cucumber (*Cucumis sativus* L.), lettuce (*Lactuca sativa* L.), spinach (*Spinacia oleracea* L.), tomato (*Solanum lycopersicum* L.) and Welsh onion (*Allium fistulosum* L.) were purchased from a market in Kyoto city. They did not contain boscalid, chlorothalonil or nitenpyram residue when the residue analysis was carried out by the above dc-ELISAs. The vegetable samples (100 g) were homogenized in a blender. The pesticide mixture dissolved in methanol were

spiked to the homogenized samples at the concentrations of boscalid, clothianidin and nitenpyram (A: 2μg/g, 0.75μg/g, 1.5 μg/g; B: 4μg/g, 1.5μg/g, 3 μg/g; C: 8μg/g, 3μg/g, 6 μg/g), and were stood for 30 min at room temperature.

Twenty five mL of methanol was added to the homogenized samples (5.0 g) in a 50 mL screw-cap tube. It was vigorously shaken by the reciprocal shaker (Shaker SA320; Yamato Scientific Co., Ltd, Tokyo, Japan) for 30 min to extract the pesticides. The extract was centrifuged at 3000 rpm for 10 min at room temperature. The supernatant was diluted to 8.5-folds with distilled water to prepare a 10% methanol equivalent solution. The diluted sample was applied to the SPR-sensor and the dc-ELISA as well as standard pesticides. The sample was adjusted to the concentrations of the working range in the SPR-sensor and the dc-ELISA by dilution with 10% methanol.

RESULTS AND DISCUSSION

Hapten Design of Nitenpyram. Hapten design is important to constitute immunosensor for simultaneous analysis of 3 pesticides, removing the cross-reaction with each other. Nitenpyram is not similar structure to boscalid except for 2-chloropyridine group but it is considerably similar to clothianidin as shown in Figure 2. The boscalid hapten and the clothianidin hapten as described previously were used for this study because there were no similar structures. [13,18] On the other hand, Nitenpyram hapten was examined to synthesize. It is necessary that the hapten compound does not cross-react with anti-clothianidin MoAb not only anti-boscalid MoAb. Nitenpyram has a 2-chloropyridine group in the structure as

well as the boscalid, but the chlorine atom of the nitenpyram is para position differed from it of the boscalid which is ortho position. Biphenyl group of the boscalid has a chlorine atom at the para position but it is 2 folds larger than a 2-chloropyridine group. The anti-boscalid Moab would not react with the nitenpyram and the hapten. On the other hand, clothianidin, which was derived from nitenpyram, has a guanidine structure which is similar to nitrovinylidenediamine structure of the nitenpyram. But the nitenpyram includes bulky ethyl group. Linker of the nitenpyram hapten was therefore extended from aminomethyl group of the structure, considering that there would not be cross-reacted by steric hindrance of the ethyl group as shown in Figure 2. The 3 haptens were almost not cross-reacted with each other, as described in Table 1.

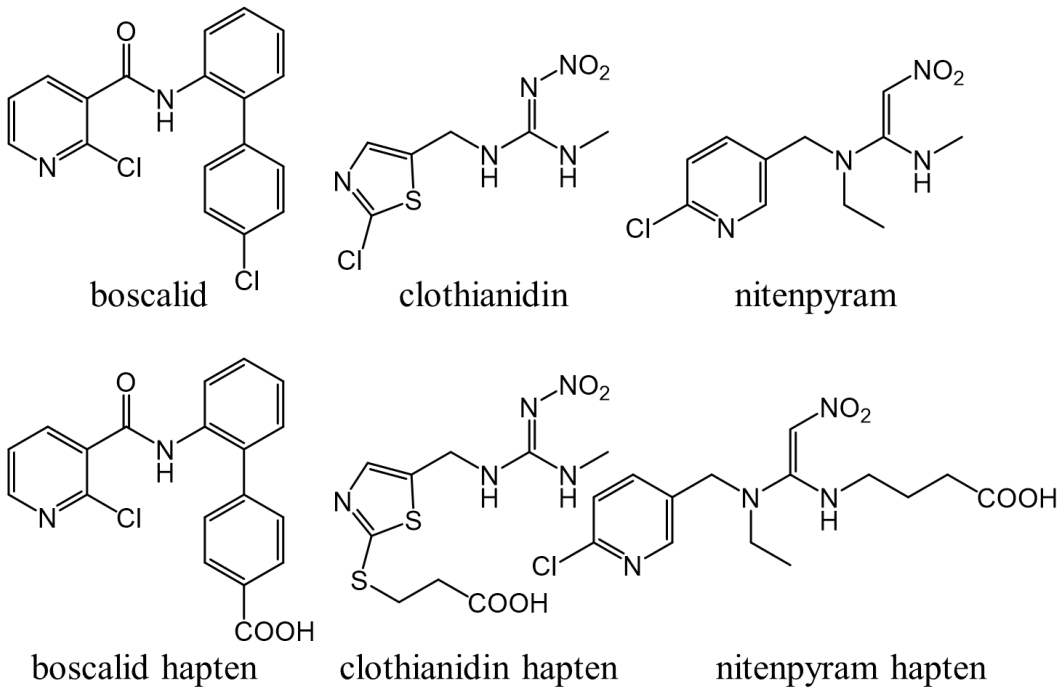

Figure 2. Structure of boscalid, clothianidin, nitenpyram and their haptens

Table 1. Typical signal of immobilized antigens with anti-pesticide MoAbs in each of the SPR-sensors

MoAb	boscalid-BSA		clothinidin-BSA		nitenpyram-BSA	
	*RU	%	*RU	%	*RU	%
anti boscalid MoAb	771	100	0	0	0	0
anti clothianidin MoAb	3	0.2	1524	100	12	0.8
anti nitenpyram MoAb	0	0	2	0.5	426	100

*RU means resonance unit

Competitive reaction of pesticides in the SPR-Sensor. Each of boscalid, clothianidin and nitenpyram was determined as described previously.[13] Boscalid, clothianidin or nitenpyram solution was initially mixed with the corresponding MoAb solution, and the mixture was applied to the SPR-sensor. The RU value for all of pesticides was increased time course dependently as shown in Figure 3, and the signal strength was 450 RU for boscalid, 1500 RU f clothianidin or and 310 RU for nitenpyram on 180 s from the starting point of the reaction. The signals were pesticide concentration dependently decreased on the range between 3.1 ng/mL and 200 ng/mL in boscalid, 1.6 ng/mL and 100 ng/mL in clothianidin and 3.1 ng/mL and 200 ng/mL in nitenpyram. The Kd value was calculated as 2.85×10^{-12} (mol/L) for the boscalid, 8.14×10^{-12} (mol/L) for the clothianidin, and 7.74×10^{-12} (mol/L) for the nitenpyram.

The inhibition curves processed from the time course curve on 180 s were shown in Figure 4. The 20%, 50%, and 80% inhibition concentration (IC_{20}, IC_{50}, and IC_{80}) values were 15,

41, and 93 ng/mL for the boscalid, 6.7, 15, and 27 ng/mL for the clothianidin, 7.3, 24, and 62 ng/mL for the nitenpyram. Next, mixture of 3 MoAbs was mixed with each of the corresponding pesticides, and the mixture was applied to the SPR-sensor. As shown in Figure 4, the inhibition curves were almost completely identical with the curves of the above individual determinations. Mixture of 3 MoAbs was further mixed with mixture of the 3 pesticides, and the mixture was applied to the SPR-sensor. As shown in Figure 4, the inhibition curves were also identical with the curves of individual determinations as well as the mixture of 3 MoAbs and each of pesticides. The results indicated that the simultaneous determination method was actually constituted for the 3 pesticides using the SPR-immunosensor.

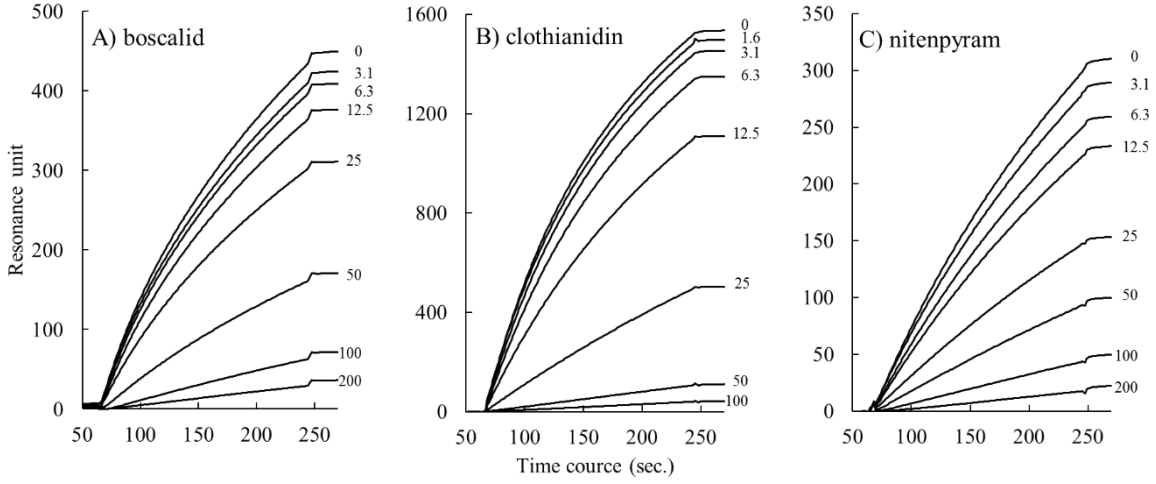

Figure 3. Typical time course of reaction for each of pesticide specific MoAbs with the corresponding pesticides by SPR-sensor: (A) boscalid; (B) clothianidin; (C) nitenpyram.
The numerals described on the right on each graph show the pesticide concentrations (ng/mL).

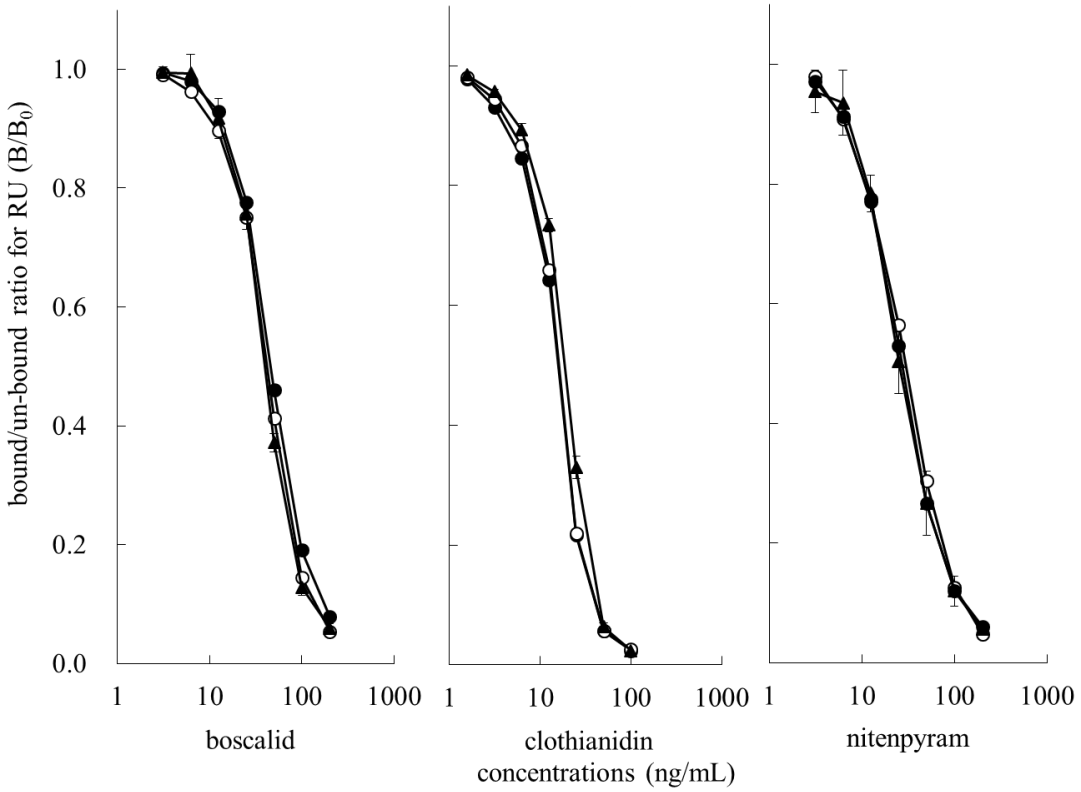

Figure 4. Reactivity of MoAbs with the corresponding pesticides on sensor-chips immobilized with each of the hapten-BSA conjugates (A: boscalid hapten, B: clothianidin hapten, C: nitenpyram hapten) in SPR-sensor: (●) reactivity of the corresponding MoAb and pesticide; (○) reactivity of the mixture of 3 MoAbs and the corresponding pesticide; (▲) reactivity of the mixture of 3 MoAbs and 3 pesticides. Each data point is the mean of 3 replicates in independent examinations; error bars indicate ± SD.

Cross-reactivity. The cross-reactivity was examined using fenhexamid, tecloftalam and benalaxyl that are structurally related fungicides of boscalid, and acetamiprid, imidacloprid, dinotefuran, thiacloprid and thiamethoxam that are structurally related insecticides of clothianidin and nitenpyram. Three of the MoAbs were mixed, and the mixture was further mixed with each of pesticides. After injection, the signal in the SPR- immunosensor was

Table 2. Cross-reactivity of the structurally related pesticides in each of the SPR-sensors and the conventional dc-ELISAs

pesticides examined	cross-reactivity (%)					
	boscalid-BSA		clothianidin-BSA		nitenpyram-BSA	
	SPR	ELISA	SPR	ELISA	SPR	ELISA
boscalid	100	100	<0.18	<0.02	<0.22	<0.23
clothianidin	<0.40	<0.03	100	100	<0.22	<0.23
nitenpyram	<0.40	<0.03	<0.18	<0.02	100	100
fenhexamid	<0.40	<0.03	NT*	NT	NT	NT
tecloftalam	<0.40	<0.03	NT	NT	NT	NT
benalaxyl	<0.40	<0.03	NT	NT	NT	NT
acetamiprid	NT	NT	<0.18	<0.02	<0.22	<0.23
imidacloprid	NT	NT	<0.18	<0.02	<0.22	<0.23
dinotefuran	NT	NT	119	79.3	<0.22	<0.23
thiacloprid	NT	NT	0.21	0.03	<0.22	<0.23
thiamethoxam	NT	NT	<0.18	<0.02	<0.22	<0.23

*NT means not tested.
Each data is the mean of duplicates in independent examinations.

shown as the cross-reactivity (%) compared to the original pesticides. The dc-ELISA was also used as the control. The boscalid and the nitenpyram were highly specific as expected, as described in Table 2. On the other hand, anti-clothianidin MoAb cross-reacted with dinotefuran: the cross-reactivity was 119% in SPR-immunosensor. The clothianidin has a chlorothiazol ring and the dinotefuran has a tetrahydrofuran ring. The compositions were different but their 3 dimensions were quite similar except for the clothianidin has chlorine atom substituent. Anti-clothianidin MoAb would have sufficient space in which the dinotefuran fits.

The SPR-immunosensor showed similar cross-reactivity as the corresponding dc-ELISAs, but the dinotefuran in the clothianidin SPR-immunosensor showed a little higher reactivity than it in the dc-ELISA. The SPR-immunosensor determines the pesticide concentration at the kinetics of the initial antigen antibody reaction but dc-ELISA determines the reaction after it was equilibrated. The difference might be the reason that the cross-reactivity was slightly different.

The SPR-immunosensor was almost not cross-react with each other pesticides. It was considered that the sensor can be used for simultaneous determination of the 3 pesticides, boscalid, clothianidin and nitenpyram.

Recovery Examination of Pesticides Spiked in Vegetables. The applicability of the SPR-immunosensor to simultaneous determinations of boscalid, clothianidin and nitenpyram residues in vegetables was examined by recovery examinations. The lower limits of quantity value of boscalid, clothianidin and nitenpyram for vegetables were 0.7 mg/kg, 0.43 mg/kg and 0.41 mg/kg in the SPR-immunosensor. The Welsh onion, lettuce, cucumber, tomato, broccoli and spinach were chosen as the representative vegetables. The 3 pesticides were mixed and were spiked to the vegetable homogenates at the following concentrations: boscalid, clothianidin and nitenpyram were A) 2 μg/g, 0.75 μg/g and 1.5 μg/g; B) 4 μg/g, 1.5 μg/g and 3 μg/g; C) 8 μg/g, 3 μg/g and 6 μg/g, respectively. As shown in Table 3, the recovery values were 74.7%–89.6% for boscalid, 87.9%–104% for clothianidin, and 71.9%–105% for nitenpyram in the SPR-sensor. The results indicated that the simultaneous SPR-immunosensor could be applied to determination of 3 pesticide residues in vegetables.

Table 3. Recovery examination of pesticide mixture spiked in vegetables

spiked pesticide mixture			Welsh onion		lettuce		cucumber	
		(μg/g)	Rec	RSD	Rec	RSD	Rec	RSD
A	boscalid	2	79.7	2.49	76.4	1.44	77.2	0.00
	clothinidin	0.75	92.3	1.15	100	0.77	99.8	0.38
	nitenpyram	1.5	78.0	8.63	80.8	5.07	84.1	6.91
B	boscalid	4	87.2	2.49	78.9	1.44	89.6	2.49
	clothinidin	1.5	96.3	3.32	98.5	1.01	96.9	2.39
	nitenpyram	3	84.1	18.3	98.5	5.07	95.2	6.91
C	boscalid	8	83.8	3.80	85.5	1.44	88.8	1.44
	clothinidin	3	98.7	1.01	87.9	0.38	99.8	1.38
	nitenpyram	6	90.7	1.91	94.1	1.92	101	5.07
spiked pesticide mixture			tomato		broccoli		spinach	
		(μg/g)	Rec	RSD	Rec	RSD	Rec	RSD
A	boscalid	2	75.5	3.80	78.9	1.44	74.7	2.49
	clothinidin	0.75	96.7	3.66	97.4	1.92	95.6	2.39
	nitenpyram	1.5	85.2	5.07	74.1	1.92	71.9	5.07
B	boscalid	4	82.2	4.98	82.2	2.49	81.3	7.61
	clothinidin	1.5	94.5	2.99	99.6	1.33	98.7	4.32
	nitenpyram	3	89.6	3.32	94.1	8.36	94.1	15.0
C	boscalid	8	78.0	10.1	83.0	3.80	77.2	2.49
	clothinidin	3	100	2.39	104	1.15	95.8	1.53
	nitenpyram	6	98.5	8.36	101	8.36	105	18.9

Rec shows recovery (%) and RSD shows relative standard deviation (%).

The SPR-immunosensor also showed a higher quantitative performance that the RSD values were 0.00%-8.63% except that the RSD for Welsh onion (3 μg/g) was 18.3%, for spinach was 15.0% at 3μg/g and 18.9% at 6 μg/g in nitenpyram. Thus, the boscalid and the clothianidin showed high accuracy but the nitenpyram showed sometimes outlier value. It was indicated that the SPR-immunosensor constituted could be used for quantitative analysis of boscalid and clothianidin, and be also used for semi-quantitative analysis of nitenpyram.

Correlation between dc-ELISA and SPR-immunosensor. The applicability of the SPR-immunosensor was further examined by comparing them with the dc-ELISA, using cucumber and tomato samples spiked with mixture of boscalid, clothianidin and nitenpyram. As shown in Figure 5, the SPR-immunosensor was highly correlated with the corresponding dc-ELISA: $R^2 = 0.98$, 1.00 and 0.98, respectively. It was suggested that the SPR-immunosensor had high applicability for simultaneous residue analysis of the 3 pesticides in vegetables.

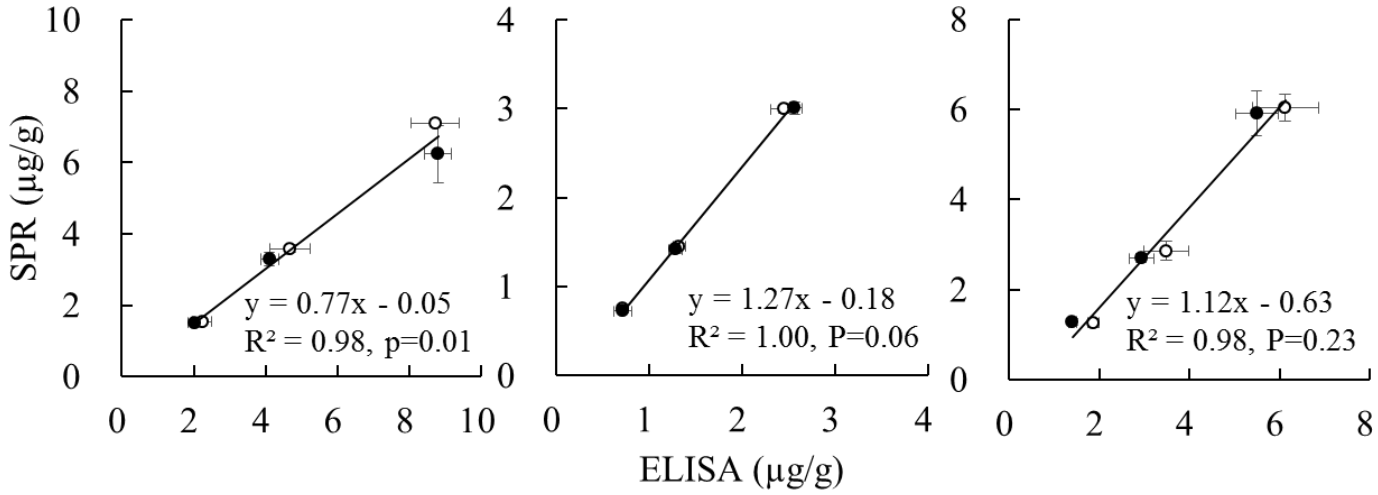

Figure 5. Correlation of pesticide concentrations determined in cucumber (○) and tomato (●) samples spiked with 3 pesticides, between dc-ELISA and SPR-sensor: (A) boscalid hapten immobilized, (B) clothianidin hapten immobilized, (C) nitenpyram hapten immobilized.
Each data point is the mean of 3 replicates in independent examinations ; error bars indicate ± SD.

The SPR-immunosensor had slight bias: slope = 0.77 for boscalid, 1.27 for clothianidin and 1.12 for nitenpyram, and showed a visible y-intercept (0.63) for nitenpyram. It must be careful to determine low concentration of nitenpyram especially.

In conclusion, it was indicated that the developed SPR-immunosensor would be useful for their simultaneous residue analysis in vegetables. The instrument used is maximum 3 channels in the sensor chip, however the results in this study showed that immunosensor by SPR would have useful potential for multi residue analysis over 3 pesticides. It is expected that the new sensor system corresponding to this concept will be developed.

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List of Publications

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