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Synthesis of three haptens for the class-specific immunoassay of O,O-dimethyl organophosphorus pesticides and effect of hapten heterology on immunoassay sensitivity

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ABSTRACT

A general and broad class-specific enzyme-linked immunosorbent assay was developed for the *O,O*-dimethyl organophosphorus pesticides, including malathion, dimethoate, phenthoate, phosmet, methidathion, fenitrothion, methyl parathion and fenthion. Three haptens with different spacer-arms were synthesized. The haptens were conjugated to bovine serum albumin (BSA) for immunogens and to ovalbumin (OVA) for coating antigens. Rabbits were immunized with the immunogens and six polyclonal antisera were produced and screened against each of the coating antigens using competitive indirect enzyme-linked immunosorbent assay for selecting the proper antiserum. The effect of hapten heterology on immunoassay sensitivity was also studied. The antibody–antigen combination with the most selectivity for malathion was further optimized and tested for tolerance to co-solvent, pH and ionic strength changes. The IC_{50} values, under optimum conditions, were estimated to be 30.1 $\mu g L^{-1}$for malathion, 28.9 $\mu g L^{-1}$ for dimethoate, 88.3 $\mu g L^{-1}$ for phenthoate, 159.7 $\mu g L^{-1}$ for phosmet, 191.7 $\mu g L^{-1}$ for methidathion, 324.0 $\mu g L^{-1}$ for fenitrothion, 483.9 $\mu g L^{-1}$ for methyl parathion, and 788.9 $\mu g L^{-1}$ for fenthion. Recoveries of malathion, dimethoate, phenthoate, phosmet and methidathion from fortified Chinese cabbage samples ranged between 77.1% and 104.7%. This assay can be used in monitoring studies for the multi-residue determination of *O,O*-dimethyl organophosphorus pesticides.

1. Introduction

In recent years, OP pesticides have been widely used in agriculture, forestry and horticulture all around the world, and they made a major contribution to improvements in agricultural output and the control of disease vectors [1]. Although OP pesticides are known to degrade relatively rapidly, their high acute toxicity necessitates more prudent monitoring of their residues in crops and the environment [2,3].

Many methods for the determination of OP pesticides have been reported, such as gas chromatography [4,5] and high-performance liquid chromatography [6]. In spite of their good sensitivity and reliability, the procedure for sample preparation in such methods is relatively complicated, expensive and not suitable for high throughput screening. The ELISA, which is known as a simple, rapid and cost-effective method for monitoring pesticide residue, especially for its capability of high throughput screening analysis, have being demonstrated as

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an alternative to the traditional instrument analysis methods [7,8]. Most of the immunochemical assays developed were aimed at detecting individual pesticides [9–12]. The demand of cost-effective fast screening of multi-pesticide residues in the agro-products and environment has called for the development of class-specific immunoassays.

Several attempts have been made to develop class-specific immunoassays for OP pesticides. Johnson et al. [13] described the class-specific immunoassay for determination of the OP pesticides using *O*,*O*-diethyl thiophosphate as generic hapten, the IC_{50} values ranged from 4.8 $\mu g L^{-1}$ to 28000 $\mu g L^{-1}$ for *O*,*O*-diethyl phosphorothionates and 140 $\mu g L^{-1}$ to 6600 $\mu g L^{-1}$ for *O*,*O*-dimethyl phosphorothionates; Alcocer et al. [14] used phosphonic acid to develop the class-specific immunoassay for OP pesticides including chlorfenvinphos, ethyl parathion, fenamiphos and tetrachlorvinphos with IC_{50} ranged from 24 $\mu g L^{-1}$ to 600 $\mu g L^{-1}$; Jang et al. [15] used *O*,*O*-diethyl *O*-(5-carboxy-2-fluorophenyl) phosphorothioate as a generic hapten to generate monoclonal antibodies, the IC_{50} values of four *O*,*O*-diethyl OP pesticides including bromophos-ethyl, chlorpyrifos-ethyl, dichlofenthion and parathion-ethyl were fairly uniform ranging from 100 $\mu g L^{-1}$ to 300 $\mu g L^{-1}$. Banks et al. [16] described the class-specific immunoassay using thiophosphate as generic hapten with IC_{50}s for fenitrothion, methacrifos, propetamphos and dichlorvos ranged from 480 $\mu g L^{-1}$ to 911 $\mu g L^{-1}$. All of the class-specific immunoassays developed for OP pesticides with low or no sensitivity to *O*,*O*-dimethyl OP pesticides.

For developing class-specific immunoassay, the generic hapten should preserve the common structure as much as possible. Beside this, it is generally accepted that the structure of the hapten spacer arm is an important factor in the production of antibodies with high affinity to the analyte and the hapten heterology influences the sensitivity of immunoassay [7,17], but there has been limited experimental data supportive of this notion [18–20].

The primary goal of our study was to develop a class-specific immunoassay for *O*,*O*-dimethyl OP pesticides with the sensitivity higher than the maximum residue limits (MRL) of pesticides in vegetable, for example, the MRL of malathion, dimethoate, phosmet and fenitrothion is 0.5 $\mu g g^{-1}$ and is 0.05 $\mu g g^{-1}$ for fenthion. Another objective was to investigate the effect of hapten heterology on the sensitivity of ELISA. The most suitable combination of coating antigen and antiserum was selected for further optimization and its applicability for the determination of *O*,*O*-dimethyl OP pesticides in Chinese vegetable samples was also studied.

2. Experimental

2.1. Reagents and materials

Malathion, dimethoate, phenthoate, phosmet, methidathion, fenitrothion, methyl parathion, fenthion, omethoate, methamidophos, parathion and chlorpyrifos were obtained from National Standards Company (Tianjin, China). Forty-five percent sodium *O*,*O*-dimethyl phosphorodithioate water solution was kindly provided by Dafeng pesticide factory (Dongtai, Jiangsu, China). Chloroacetic acid was obtained from Green Apple Chemical Co., Ltd. (Wuxi, Jiangsu, China). 732-strong acid cation resin was obtained from Sanyou Chemical Reagent Factory (Shanghai, China), *p*-nitrophenol, sodium nitrite and glutaraldehyde were from Sinopharm Group Chemical Reagent Co., Ltd. (Shanghai, China). *N*-Hydroxysuccinimide (NHS), *N*,*N*-dicyclohexylcarbodiimide (DCC), bovine serum albumin (BSA, MW 67,000), ovalbumin (OVA, MW 45,000), complete and incomplete Freund's adjuvants were purchased from Sigma (Saint Louis, Missouri, USA). Enzyme immunoassay-grade HRP-labeled goat anti-rabbit immunoglobulin was supplied by KPL, Inc. (Gaithersburg, MD, USA). Water used in experiments was purified with a Water Pro Plus water system. (LABCONCO, USA). All other reagents were of analytical grades.

Thin-layer chromatography (TLC) was performed on 0.2 mm precoated silica gel F_{254} (200 mesh) on glass sheets. Column chromatographic purifications were carried out using silica gel (200–300 mesh) from Qingdao Haiyang Chemical Co., Ltd. (Qingdao, Shandong, China). The dialysis membrane (MW cutoff: 8000) was obtained from Huamei Biotechnology Co. (Luoyang, Henan, China). Polystyrene 96-well microtiter plates were from Costar (Corning, Massachusetts, USA).

2.2. Instruments

^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were obtained with a Bruker ARX spectrometer (300 MHz, Rheinstetten, Germany). Chemical shift values are given in parts per million (ppm) downfield from internal standard tetramethylsilane (TMS). Coupling constants are expressed in Hz and the abbreviations s, d, t, q, m and ar represent singlet, doublet, triplet, quartet, multiplet, and aromatic, respectively. Mass spectra were measured using an Agilent 1100 mass spectrometer (Agilent, Foster City, CA, USA). High resolution mass spectra were obtained on a Bruker FT-ICR Mass Spectrometer (Bruker, Germany). UV–vis spectra were recorded on a DU-640 spectrophotometer (Beckman, USA). ELISA plates were washed with Thermo wellwash plus plate washer (Thermo, USA). Absorbance was measured at 450 nm of wavelength using an automated microtiter plate reader (Labsystem, Helsinki, Finland).

2.3. Hapten synthesis and verification

Three haptens with different spacer arms were designed and synthesized. The synthetic routes for these haptens are illustrated in Fig. 1. NMR spectral data and mass spectra supported all structures of target molecules.

2.3.1. *S*-Carboxymethyl *O*,*O*-dimethyl phosphorodithioate (hapten I)

S-Carboxymethyl *O*,*O*-dimethyl phosphorodithioate was prepared as described by G. Berkelhammer et al. [21]. A solution of 45% sodium *O*,*O*-dimethyl phosphorodithioate (4 g) was added dropwise over a 45-min period, with efficient stirring, to a refluxing solution of chloroacetic acid (0.95 g) in a mixture of chloroform (10 mL) and water (1.5 mL). Heating under refluxing and rapid stirring were continued for an additional 30 min. The reaction mixture was cooled to room temperature and the layers separated. The water layer was extracted with chloroform

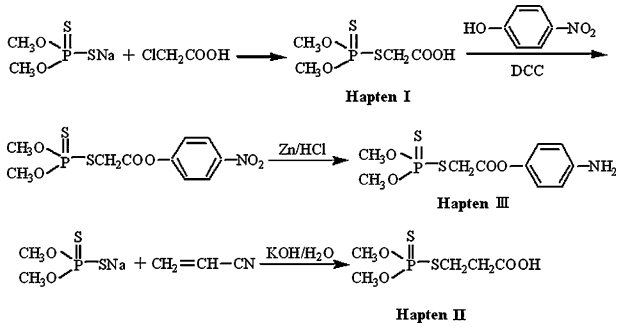

Fig. 1 – Synthetic route for Hapten I, II and III.

(3 × 4 mL), the combined extracts and original chloroform layer was washed with water (2 mL) and dried over anhydrous magnesium sulfate. The solvent removed under vacuum to give orange-brown oil which crystallized on cooling. Three recrystallizations from mixtures of approximately equal volumes of carbon tetrachloride and hexane gave white crystalline solid (0.91 g, Yield 51%). TLC Rf 0.48 (ethyl acetate: petroleum ether, 1: 2). m.p 41–43.5 °C.

^{1}H NMR ($CDCl_3$) δ: 3.69 (d, J = 17.8Hz, 2H, SCH_2CO), 3.81 (d, J = 15.2 Hz, 6H, CH_3O), 10.99 (s, 1H, COOH); ^{13}C NMR ($CDCl_3$) δ: 34.85 (d, J = 3.6 Hz, SCH_2), 54.32 (d, J = 5.5 Hz, CH_3O), 174.77 (d, J = 3.9 Hz, COOH); MS (ESI) m/z: 216[M], 199[M-OH], 185[M-CH_3O], 171[M-COOH], 125[M-SCH_2COOH].

2.3.2. *S-Carboxyethyl O,O-dimethyl phosphorodithioate (hapten II)*

A mixture of 45% Sodium *O,O*-dimethyl phosphorodithioate (4 g), acrylonitrile (0.58 g) and 6% KOH (18 mL) was refluxed for 4 h in oil bath. The reaction mixture was cooled to room temperature and acidified with 732-strong acid cation resin. The solvent was evaporated and the residue was subjected to column chromatography to give the yellow oil (0.42 g, yield 23%). TLC Rf: 0.31 (ethyl acetate:petroleum ether, 1:2).

^{1}H NMR (dimethylsulphoxide-d_6, DMSO-d_6) δ: 2.18 (t, J = 6.3 Hz, 2H, CH_2CO), 2.31 (t, J = 6.6Hz, 2H, SCH_2), 3.35 (d, J = 14.1 Hz, 6H, CH_3O); ^{13}C NMR (DMSO-d_6) δ: 37.99 (CH_2CO), 51.19 (d, J = 2.7 Hz, SCH_2), 57.45 (d, J = 30.9, CH_3O), 173.55 (COOH); MS m/z: 230[M], 213[M–OH], 182[M–OH–OCH_3], 125[M–SCH_2CH_2COOH].

2.3.3. *S-Acetyl 4-aminophenyl O,O-dimethyl phosphorodithioate (hapten III)*

1.03 g DCC was added into a solution of 1.08 g S-carboxymethyl *O,O*-dimethyl phosphorodithioate (Hapten I) and 1.39 g *p*-nitrophenol in 10 mL dried dichloromethane cooled in ice-water bath while stirring, the ice-water bath was removed after stirring for 4 h, the reaction mixture was continuously stirred for 12 h at room temperature and filtered to remove the dicyclohexylurea (DCU), and then washed with 5% $NaHCO_3$ (2 × 5 mL). The solvent was dried over anhydrous magnesium sulfate and concentrated under vacuum to give *S*-acetyl 4-nitrophenyl *O,O*-dimethyl phosphorodithioate as fawn oil (0.51 g, yield 47%).

^{1}H NMR ($CDCl_3$) δ: 3.83 (d, J = 15.3 Hz, 6H, CH_3O), 3.92 (d, J = 18.9 Hz, 2H, SCH_2), 7.33 (m, 2H, ar), 8.33(m, 2H, ar).

Four millilitre of 9:1 acetic:HCl and 1.0 g zinc particles were added into a solution of 0.5 g S-acetyl 4-nitrophenyl *O,O*-dimethyl phosphorodithioate in 8 mL ethyl ether, the reaction mixture was stirred for 30 min at room temperature and then refluxing for 30 min. The mixture was decanted from the reaction flask and the zinc was washed with ethyl ether. The combined organic phase was then washed with water (2 mL × 2 mL) and dried over magnesium sulfate. The solvent was evaporated and the residue was subjected to column chromatography to give the *S*-acetyl 4-aminophenyl *O,O*-dimethyl phosphorodithioate as brown syrup (48mg, yield 10%). TLC Rf: 0.16 (ethyl acetate: petroleum ether, 1:2).

^{1}H NMR ($CDCl_3$) δ: 3.66 (d, J = 21.3Hz, 2H, SCH_2), 3.78 (d, J = 15.3 Hz, 6H, CH_3O), 6.76 (m, 2H, ar), 7.27 (m, 2H, ar), 8.32(s, 2H, NH_2). ^{13}C NMR ($CDCl_3$) δ: 37.00 (SCH_2), 54.71(CH_3O), 115.76 (ar), 122.32 (ar), 129.63 (ar), 153.53 (ar). High resolution FT-ICR MS ESI (+) formula: $C_{10}H_{14}NPS_2O_4$ m/z calculated: 308.0175[M+H], found: 308.0174[M+H], error: 0.1 ppm.

2.4. *Preparation of hapten–protein conjugates*

For immunization purpose, hapten I and II were covalently attached to BSA by the active ester method [22], hapten III with amino group was attached to BSA by diazotization [23]. The active ester method and the mixed anhydride method [24] were used to synthesize Hapten I-OVA and hapten II-OVA, respectively which were used as coating antigens. The diazotization method and the Schiff base formation and reductive amination method [25] were used to synthesize Hapten III-OVA which were also used as coating antigens. The immunogens and coating antigens were purified by dialysis for three days in phosphate buffer (PBS: 0.01 mol L^{-1}, pH 7.4). The conjugates were stored at −20 °C until use. UV–vis spectral data were used to confirm the structures of the final conjugates. Assuming that the molar absorptivity of haptens was the same for the free and conjugated forms, the hapten densities (the number of hapten molecules per molecule of protein) of the conjugates were estimated directly by the mole absorbance ε [26]:

$$\text{Hapten density} = \frac{(\varepsilon_{\text{conjugation}} - \varepsilon_{\text{protein}})}{\varepsilon_{\text{hapten}}}$$

2.5. *Immunization and antiserum preparation*

New Zealand white rabbits weighing 1.5–2 kg were used to obtain polyclonal antibodies to the immunogens hapten I-BSA (rabbits #3024, and #3025), hapten II-BSA (rabbits #3055, and #3056) and hapten III-BSA (rabbits #3128, and #3129). 800 μg immunogen for each animal dissolved in 0.5 mL PBS was emulsified with Freund's complete adjuvant (1:1, v:v) and injected intradermally at multiple sites (10–15 sites) on the back. Further injections of the immunogens (800 μg for each animal) emulsified with Freund's incomplete adjuvant were given (1:1, v:v) after 2 weeks. Booster injections were given at 2-week intervals. The rabbits were bled 7 days after each boost. Serum was isolated by centrifugation (10,000 rpm, 10 min) at 4 °C and stored at −20 °C. The results of antibody characterization were obtained from sera of terminal bleeds after five boosters.

2.6. Competitive indirect ELISA

The procedure of the competitive assay was described similarly by Shan et al. [27]. All incubations except the antigen coating were carried out at 37 °C and after each incubation the plates were washed three times with PBST (10 mM PBS containing 0.05% Tween 20, pH 7.4). Microplates were coated overnight at 4 °C with 100 μL per well of the appropriate coating antigen concentration in 0.1 M carbonate-bicarbonate buffer (pH 9.6). After the plates were washed with PBST, the surface of the wells were blocked with 200 μL of 1% OVA in PBS by incubation for 1 h to minimize the non-specific binding in the plate. After another washing step, 100 μL per well of antiserum diluted in PBS per well (for titration experiment) or 50 μL well^{-1} of antiserum diluted in PBS and 50 μL well^{-1} of standard analyte (for inhibition assay) or sample solution were added and incubated for 2 h. The standard analyte concentrations ranged from 0.05 μg L^{-1} to 10 mg L^{-1}. Following a washing step, the goat anti-rabbit IgG–HRP conjugate (diluted 1:5000 in PBS with 1%OVA, 100 μL well^{-1}) was added and incubated for 1 h. The plates were washed again, and 100 μL per well of substrate solution (70 μL of 0.65% H_2O_2, 250 μL of 10 mg mL^{-1} 3,3′5,5′-tetramethylbenzidine (TMB) in dimethylsulfoxide (DMSO) per 25 mL of phosphate–citrate buffer, pH 5.4) was added. After incubation for 15 min, the reaction was stopped by adding 50 μL of 2 M H_2SO_4 per well. The absorbance was measured at 450 nm and recorded. Standard curves were obtained by plotting absorbance against the logarithm of analyte concentration, which were fitted to a four-parameter logistic equation:

$$y = \left\{ \frac{(A - D)}{[1 + (x/C)^B]} \right\} + D$$

where A is the maximum absorbance at no analyte present, B is the curve slope at the inflection point, C is the concentration of analyte giving 50% inhibition (IC_{50}), and D is the minimum absorbance at infinite concentration [28].

2.7. Antibody characterization and assay optimization

The polyclonal antibodies raised against each of the three immunogens (Hapten I–III conjugated to BSA) were screened against each of the six coating antigens in a two-dimensional titration for the best dilution of coating antigen and antiserum. The detailed procedure is described in the Section 2.6.

The effect of spacer arm heterology and conjugation method heterology on binding of antibodies to the coating antigens was evaluated by comparing the affinity of antibodies to each coating antigen, then the effect on the sensitivity of ELISA was also evaluated by measuring competitive inhibition curves (the detailed procedure is described in the Section 2.6) for different antibody and coating antigen combinations. The one with the lowest IC_{50} to malathion (a OP pesticide most similar to the haptens in structure) was selected for further assay development.

The influences of organic solvents, pH and ionic strength on the performance of immunoassay were further optimized. The organic solvents selected, including methanol, acetone and dimethyl sulfoxide (DMSO), were added to PBS at different rates (5, 10, 25 and 50%), respectively. The mixture of organic solvents and PBS was used as solvents to dissolve pesticide in the ELISA assay. The influence of the solution pH on ELISA performance was studied by preparing analytes in PBS buffer at pH values of 5, 6, 7.4 and 8. The effect of ionic strength was also studied by preparing analyte standard solutions in 0.01, 0.05, 0.1 and 0.2 M PBS.

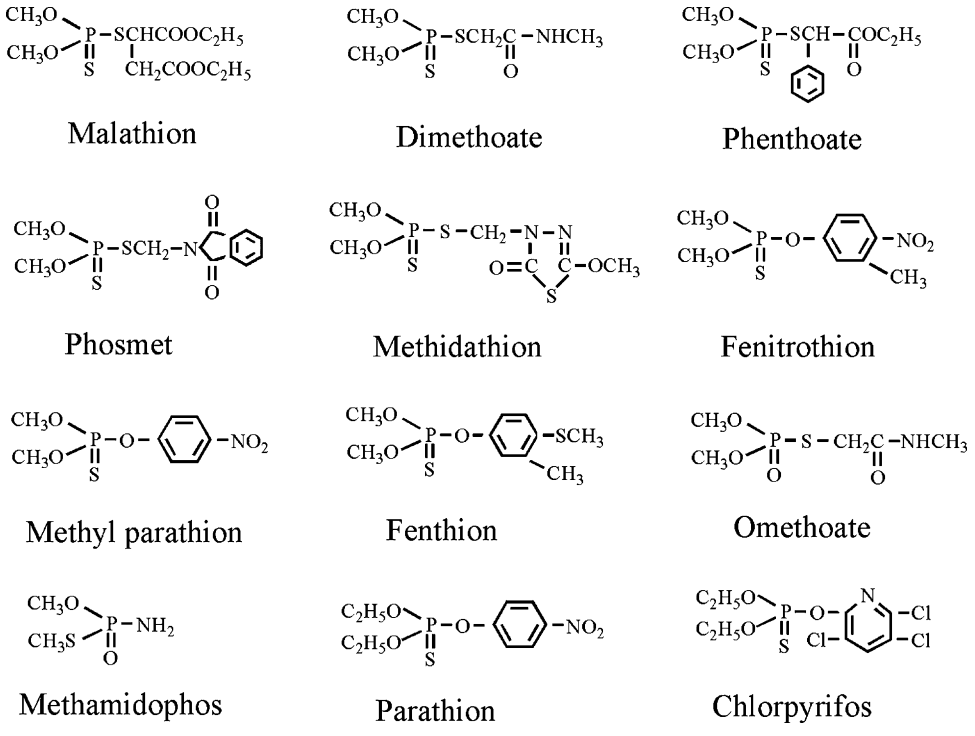

Fig. 2 – Structures of organophosphorus pesticides.

2.8. Determination of cross-reactivity (CR)

The optimized assay was applied to cross-reactivity studies by using the standard solution of the analyte and other structurally related compounds (Fig. 2). The cross-reactivity values were calculated by dividing the IC_{50} of malathion assigned to be 100% by the IC_{50} of another compound and multiplying by 100.

2.9. Analysis of spiked samples

Chinese cabbages from a local supermarket were finely chopped. Malathion was spiked on vegetables with three final concentrations (20, 100, 200 ng g^{-1}). After standing in a hood in the dark for 6 h at room temperature, 1 g spiked vegetables were added with 2 mL methanol and the mixture was shaken for 2 h at room temperature. The mixtures were centrifuged for 10 min at 5000 rpm. The supernatant was evaporated to dryness under vacuum and the residue was then dissolved in 1 mL 5% methanol-PBS and analyzed by the ELISA without any other purification procedure. The cabbages with no pesticides spiked were used as control. In the quantification, the result of the sample spiked minus the result of the control was used to eliminate or reduce the influence of the intrinsic pesticides in the cabbage. In addition, the recoveries of other dimethyl OP pesticides such as dimethoate, phenthoate, phosmet and methidathion were also tested.

3. Results and discussion

3.1. Hapten design and conjugate verification

The initial and critical step in the development of effective class-specific immunoassay for low molecular weight chemicals lies in the selection of haptens which preserve the structure of the target compounds as much as possible [18]. The majority of organophosphorus pesticides can be derived from two general structural formulas $(MeO)_2$ P(X)Y and $(EtO)_2$ P(X)Y where X represents S or O and Y represents a range of chemical structures. Based on the purpose of this study, the haptens were thus designed to contain the *O*,*O*-dimethyl phosphorodithioate group.

Sodium *O*,*O*-dimethyl phosphorodithioate was used as material to react with chloroacetic acid and acrylonitrile, respectively to synthesize hapten I and hapten II. Hapten I was used as material to react with *p*-nitrophenol and then deoxidized by zinc powder to make hapten III. These methods were successfully applied to the synthesis of the three haptens. The hapten I has been used successfully for malathion immunoassay by Brun et al. [29]. Hapten II and hapten III differed from previous reports, in which thiophosphate [13,16] or phosphonic acid [14] was used as a generic hapten. The haptens differed in the structure of spacer arm, this offered options for selecting the most sensitive antiserum and for investigating the effect of hapten heterology on the sensitivity of the method. From the data of ^{1}H NMR, ^{13}C NMR and MS, the products were as expected.

Verification of conjugate synthesis and estimation of the hapten/protein ratio were performed at the same time. Scanning the UV–vis spectrum is the simplest and effective method for these analyses. A scan of 200–400 nm was used. Three haptens, two carrier proteins, and nine conjugates were scanned. There were obvious differences in the absorbance patterns of the conjugates compared to those of the corresponding carriers, especially at around 251 nm. Hapten/protein ratios were calculated by measuring the absorbance of the hapten, the protein, and the hapten–protein conjugate at the same wavelength, and the results are shown in Table 1.

3.2. Effect of hapten heterology on the affinity of antisera to coating antigens

The antisera of six rabbits were screened against each of the six coating antigens using two-dimensional titration method to examine the effect of hapten heterology on the affinity of antisera to coating antigens. It could be difficult to draw solid conclusions from the results of this type of experiments, since the immune responses to interindividual vary among animals

Table 1 – Ratios of hapten/protein conjugates

Hapten	Conjugates	Binding ratio to carrier protein	Wavelength for calculation (nm)[e]
Hapten I	I-BSA[a]	57	256
	I-OVA[a]	43	
	I-OVA[b]	24	
Hapten II	II-BSA[a]	49	243
	II-OVA[a]	45	
	II-OVA[b]	30	
Hapten III	III-BSA[c]	18	268
	III-OVA[c]	10	
	III-OVA[d]	11	

[a] The conjugate was synthesized using the active ester method.
[b] The conjugate was synthesized using the mixed anhydride method.
[c] The conjugate was synthesized using the diazotization method.
[d] The conjugate was synthesized using Schiff base formation and reductive amination method.
[e] A characteristic wavelength for a corresponding hapten was generally selected to detect absorbance of hapten, carrier protein and conjugate at some known concentration, and then values of molar absorbance coefficients were calculated.

Table 2 – Titration results for antisera using different coating antigens

Ab/immunogen	Coating antigen[e]					
	I-OVA[a]	I-OVA[b]	II-OVA[a]	II-OVA[b]	III-OVA[c]	III-OVA[d]
3024/Hapten I-BSA[a]	H	L	L	L	L	–
3025/Hapten I-BSA[a]	H	L	L	–	L	–
3055/Hapten II-BSA[a]	M	M	H	L	M	L
3056/Hapten II-BSA[a]	L	L	H	L	L	L
3128/Hapten III-BSA[c]	H	M	M	L	M	M
3129/Hapten III-BSA[c]	M	L	M	–	L	L

[a] The conjugate was synthesized using the active ester method.
[b] The conjugate was synthesized using the mixed anhydride method.
[c] The conjugate was synthesized using the diazotization method.
[d] The conjugate was synthesized using Schiff base formation and reductive amination method.
[e] H, M and L indicate the serum dilution factor range that produced absorbances of 0.5 after 15 min of color development. H: more than 1:12800; M: between 1:12800 and 1:1600; L: less than 1:1600; "–" indicates not tested. The data shown are at coating antigen concentration of 2 $\mu g\,mL^{-1}$.

and the number of haptens designed with different spacer arm is small. However, the results of the experiment showed that there are certain trends in the behavior of the antibodies. The results of the titration experiments using the final bleeds are shown in Table 2.

The titer difference between homologous and heterologous was great. The homologous assay had higher titer than the heterologous assay for antibodies generated from immunogen I-BSA[a] and II-BSA[a]. The reverse trend was observed for the antibodies generated from the immunogen III-BSA[c] which has the most complicated spacer arm. Low titers for hapten III with antibodies generated from haptens I and II suggest that the more complex spacer arm in hapten III may not be recognized by antibodies generated from simpler haptens. The titer of antibodies generated from the immunogen I-BSA[a] and II-OVA[a] were much higher with coating antigen I-OVA[a] and II-OVA[a] than that of I-OVA[b] and II-OVA[b], respectively. Titer difference may be due to the different coupling methods resulting in different hapten loads.

3.3. *Selection of antisera and relationship between hapten heterology and ELISA sensitivity*

In order to select the most proper combination of antiserum and coating antigen and examine the effect of hapten heterology on the sensitivity of immunoassay, the combinations of the antiserum and coating antigen were further tested with competitive inhibition experiments to malathion and dimethoate, IC_{50} values of each assay are given in Table 3.

Hapten heterology may improve ELISA sensitivity [10]. For the heterologous combinations in Table 3, the IC_{50} values of antisera 3128 versus heterologous coating antigens I-OVA[a],

Table 3 – Combination data for screening with competitive ELISA[e] ($n = 3$)

Immunogen	Ab/cAg	Malathion				Dimethoate			
		A_{max} (A)	Slope (B)	A_{min} (D)	IC_{50} ($\mu g\,L^{-1}$) (C)	A_{max} (A)	Slope (B)	A_{min} (D)	IC_{50} ($\mu g\,L^{-1}$) (C)
I-BSA[a]	#3024/I-OVA[a]	0.861	0.601	0.177	132.7	0.892	0.589	0.326	59.8
	#3025/I-OVA[a]	0.688	0.654	0.159	76.7	0.898	0.456	0.015	122.8
II-BSA[a]	#3055/I-OVA[b]	1.059	0.552	0.171	456.5	1.174	0.396	0.217	491.5
	#3055/II-OVA[a]	0.948	0.760	0.429	45.9	1.002	0.536	0.304	53.5
	#3055/III-OVA[c]				>1000	0.970	1.459	0.089	520.3
	#3056/I-OVA[a]	0.775	0.410	0.092	734.9	0.986	0.367	0.178	799.3
	#3056/II-OVA[a]	1.132	0.323	0.117	52.3	0.956	0.575	0.307	96.5
III-BSA[c]	#3128/I-OVA[a]	0.866	0.582	0.016	215.4	0.971	0.510	0.025	190.3
	#3128/I-OVA[b]	1.148	0.730	0.131	397.9	1.037	1.030	0.222	437.2
	#3128//II-OVA[a]	0.544	1.026	0.100	505.5	0.683	0.394	0.085	438.6
	#3128/III-OVA[c]	0.674	0.340	0.091	649.1	0.650	0.953	0.236	450.9
	#3128/III-OVA[d]				>1000	0.758	0.565	0.063	843.8
	#3129/I-OVA[a]	1.187	0.758	0.505	540.8	1.203	0.614	0.187	405.6
	#3129/II-OVA[a]	0.425	0.660	0.241	978.3	0.469	0.419	0.171	568.8

[a] The conjugate was synthesized using the active ester method.
[b] The conjugate was synthesized using the mixed anhydride method.
[c] The conjugate was synthesized using the diazotization method.
[d] The conjugate was synthesized using Schiff base formation and reductive amination method.
[e] The results were the means of three experiments on the different days.

Table 4 – Effect of organic solvents on assay parameters[a,b] ($n = 3$)

Organic solvent (%)	A_{max} (A)	Slope (B)	A_{min} (D)	IC_{50} (C) ± S.D. ($\mu g L^{-1}$)	R^2 of linear equation
Methanol					
5	0.859	0.794	0.122	38.5 ± 1.45	0.998
10	0.944	0.886	0.390	44.6 ± 2.06	0.997
25	0.897	0.861	0.131	45.2 ± 1.99	0.997
50	0.964	0.658	0.085	81.9 ± 5.23	0.989
Acetone					
5	0.923	0.803	0.226	52.3 ± 3.18	0.999
10	0.849	1.162	0.081	51.5 ± 2.67	0.999
25	0.841	0.899	0.100	96.0 ± 5.09	0.991
50	0.812	0.745	0.022	129.0 ± 7.43	0.997
DMSO					
5	0.776	0.739	0.030	182.1 ± 29.59	0.988
10	0.592	1.174	0.184	491.5 ± 25.94	0.990
25	0.547	0.342	0.083	931.7 ± 50.83	0.936
50	0.312	0.733	0.028	1110.7 ± 97.77	0.941

[a] ELISA conditions: coating antigen II-OVA[a]; antiserum 3055 (1:6400); goat anti-rabbit IgG–HRP (1:5000).
[b] The results were the means and standard deviations of three experiments on the different days.

I-OVA[b], II-OVA[a] and III-OVA[c] were lower than homologous coating antigen III-OVA[c]. However, this situation was not observed for antisera 3055 and 3056. Although the differences are not great, it appears that hapten heterology is more important for the antiserum derived from the hapten with the complicated spacer arm than the simple spacer arm to improve the sensitivity of the immunoassay.

The IC_{50} values of the homologous assay for antibodies generated from I-BSA[a] and II-BSA[a] were almost the same and much lower than the 3128/III-OVA[c], suggesting simple spacer arm of hapten may be more suitable than complicated spacer arm to the sensitivity of immunoassay. After preliminary screening, the combination of antiserum 3055 with coating antigen II-OVA[a] gave the lowest IC_{50} values for malathion. It may be that the hapten I linked to a carrier protein suffer a little amount of masking in the region of the hapten near the site of linkage, antisera produced would suffer a little lack of specificity for those determinant groups that have been masked. Hapten II extended the determinant group out in space via a spacer arm to avoid this problem [7,17]. Although Shan et al. described an acceptable assay using immunizing haptens with zero spacer arm length [30]. The combination of antiserum 3055 and coating antigen II-OVA[a] with the lowest IC_{50} values to malathion was chosen for further assay optimization and cross-reactivity studies.

3.4. *Assay optimization*

Although most of OP pesticides are hydrophilic, a few are hydrophobic such as methyl parathion and parathion, so organic solvent is needed for this assay. Table 4 shows the effect of methanol, acetone and DMSO concentration on sensitivity. The results indicated that the DMSO concentration strongly interferes with the assay sensitivity and maximum absorbance. The maximum absorbance was significantly decreased with an increase in DMSO concentration. Following the increase in acetone concentration, the maximum absorbance and sensitivity was decreased. Methanol concentration does not affect absorbance remarkably, the sensitivity decreased with an increase in methanol concentration. Based on the IC_{50} value obtained from standard curve, 5% methanol concentration ($IC_{50} = 38.5\ \mu g L^{-1}$) was selected for subsequent experiments.

To identify potential interferences from vegetable samples, the effects of pH and ionic strength on ELISA performance were also evaluated in this study. Fig. 3 illustrates the effect of pH value on the assay sensitivity. No significant effect on IC_{50} was detected when analyte was dissolved in buffer at different pH values ranging from 5.0 to 8.0. However, acid matrix resulted in little lower sensitivity and the ratio of maximum absorbance to minimum absorbance for malathion standard curve at pH 8.0 was much lower than others, indicating that the assay could effectively detect malathion at pH values ranging from 6 to 7.4. The effect of ionic strength was shown in Fig. 4. Ionic strength strongly influenced ELISA performance, a higher salt concentration in the assay system resulted in

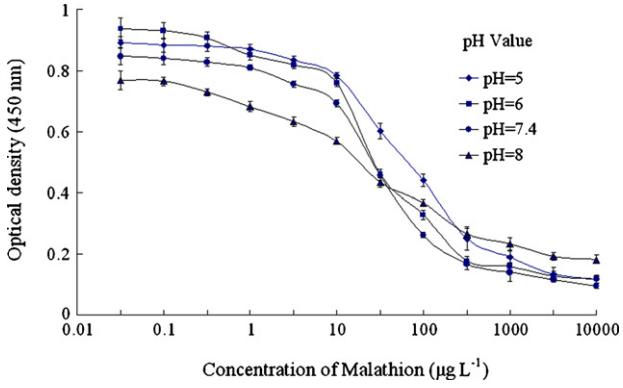

Fig. 3 – ELISA competitive curves of malathion prepared in PBS buffer containing various pH values. Data represent mean ± S.D. of three experiments on the different days. Reagent concentrations: coating antigen II-OVA[a] ($2\ \mu g mL^{-1}$); antiserum 3055 (1:6400); goat anti-rabbit IgG–HRP (1:5000).

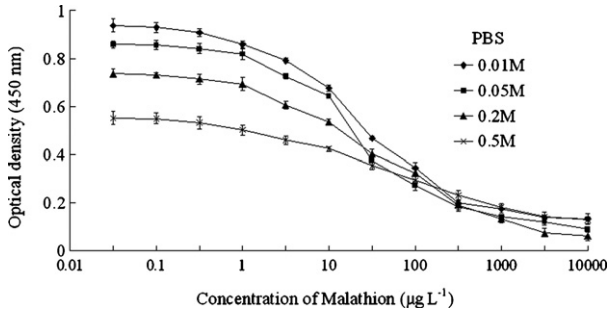

Fig. 4 – ELISA competitive curves of malathion prepared in PBS buffer containing various concentrations of ionic strength. Data represent mean ± S.D. of three replications on the different day. Reagent concentrations: coating antigen II-OVA[a] (2 μg mL^{-1}); antiserum 3055 (1:6400); goat anti-rabbit IgG–HRP (1:5000).

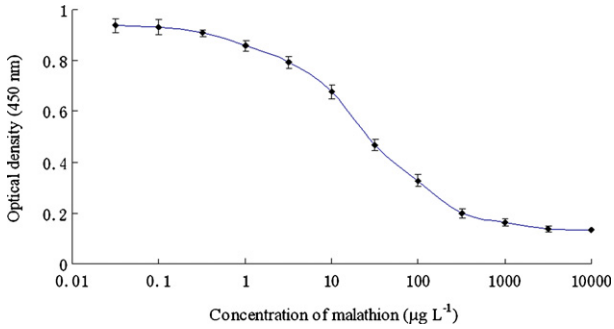

Fig. 5 – ELISA inhibition curve of malathion. Data represent mean ± S.D. of three experiments on the different days. Reagent concentrations: coating antigen II-OVA[a] (2 μg mL^{-1}); antiserum 3055 (1:6400); goat anti-rabbit IgG–HRP (1:5000).

lower absorbance and higher IC_{50} values. According to data from Fig. 3, 0.01 M of salt concentration was chosen in ELISA system.

The optimized ELISA used coating antigen II-OVA[a] at 2 μg mL^{-1}, antibody 3055 at a dilution of 1:6400, and analyte in 5% methanol–0.01 M PBS buffer (pH 7.4).

Fig. 5 illustrates the typical standard curve for malathion under the optimum conditions. The detection limit (0.5 μg L^{-1}) was estimated as the concentration that corresponded to the absorbance of the control (zero concentration of analyte) minus three times the standard deviation of the control [31]. The sensitivity for detection of malathion and other specific pesticides in this paper was lower than other published assays which were aimed at detecting individual pesticides [11,20,29], but it was much higher than those which were aimed at detecting multi-pesticides[13,16].

3.5. *Cross-reactivity*

Table 5 demonstrates the cross-reactivity of the ELISA system 3055/II-OVA[a] with different OP pesticides. This system had the highest cross-reactivity with the dimethoate (104.2%), followed by phenthoate (34.1%), phosmet (18.8%), methidathion (15.7%), fenitrothion (9.3%), methyl parathion (6.2%) and fenthion (3.8%). The results demonstrate that the assay was more sensitive for *O*,*O*-dimethyl OP pesticides than *O*,*O*-diethyl OP pesticides.

3.6. *Recovery*

To estimate reliability, recovery tests were performed using Chinese cabbage as samples. Table 6 shows the recovery of malathion from Chinese cabbages. The recoveries of malathion (>85.5%) were satisfactory. Furthermore, the recoveries of other *O*,*O*-dimethyl organophosphorus pesticides using a malathion standard curve were also tested. Chinese cabbages were spiked with appropriate concentrations of dimethoate, phenthoate, phosmet and methidathion standard, respectively. When recoveries of each sample were calculated, cross-reactivity factors shown in Table 5 were used to correct the amount of OP pesticides, which was also described by Mak et al. [32] and Watanabe et al. [33]. The results were shown in Table 7. The recoveries ranged from 77.1% to 104.7% with the CV from 2.18% to 13.81%. The results show

Table 5 – Cross-reactivities (CR) and limit of determination (LOD) of organophosphorus pesticides[a,b]

Compounds	A_{max} (A)	Slope (B)	A_{min} (D)	IC_{50} (C) ± S.D. (μg L^{-1})	CR (%)	LOD (μg L^{-1})
Malathion	0.900	0.738	0.123	30.1 ± 1.05	100.0	0.5
Dimethoate	1.029	0.567	0.124	28.9 ± 1.22	104.2	0.3
Phenthoate	0.908	0.820	0.093	88.3 ± 3.62	34.1	0.6
Phosmet	0.892	0.641	0.038	159.7 ± 3.09	18.8	1.9
Methidathion	0.940	0.748	0.045	191.7 ± 13.96	15.7	1.1
Fenitrothion	0.973	0.426	0.011	324.0 ± 45.32	9.3	10.6
Methyl parathion	0.934	1.028	0.221	483.9 ± 39.65	6.2	14.5
Fenthion	0.876	0.762	0.170	788.9 ± 100.48	3.8	39.8
Omethoate				>1000		
Methamidophos				>1000		
Parathion				>1000		
Chlorpyrifos				>1000		

[a] ELISA system 3055/II-OVA[a] was used in the cross-reactivity studies. Malathion was assigned as 100%.
[b] The results were the means and standard deviations of three experiments on the different days.

Table 6 – Recovery test of malathion in Chinese cabbage[a] ($n = 3$)

Analyte	Spiked (ng g^{-1})	Measured (ng g^{-1})	Recovery (%)	CV (%)	
				Intra-assay[b]	Inter-assay[c]
Malathion	20	17.8	89.0	3.72	4.24
	100	88.4	88.4	1.77	2.18
	200	171.0	85.5	1.50	3.53

[a] Percent recovery was calculated as the measured spiked concentration of malathion divided by the theoretical spiked concentration of malathion × 100.
[b] The data was the results of three experiments on the same day.
[c] The data was the results of three experiments on the different days.

Table 7 – Recovery test of the OP pesticides in Chinese cabbage ($n = 3$)

Analyte	Spiked (ng g^{-1})	Malathion equivalent (ng g^{-1})	Relative recovery (%)	Actual recovery (%)	CV (%)	
					Intra-assay[a]	Inter-assay[b]
Dimethoate	100	102.4	102.4	98.3	2.77	4.69
	200	218.2	109.1	104.7	3.04	3.73
Phenthoate	100	28.1	28.1	82.4	8.94	11.99
	200	60.4	30.2	88.6	6.39	7.02
Phosmet	100	17.7	17.7	94.1	5.48	9.61
	200	32.6	16.3	86.7	9.25	13.81
Methidathion	100	12.1	12.1	77.1	7.35	12.88
	200	26.0	13.0	82.8	10.62	13.54

[a] The data was the results of three experiments on the same day.
[b] The data was the results of three experiments on the different days.

that the assay matches the requirements of residue analysis and would be a useful screening method for *O*,*O*-dimethyl OP pesticides.

4. Conclusions

A general and broad selective immunoassay for the *O*,*O*-dimethyl OP pesticides has been developed by using II-BSA[a] as the immunogen and II-OVA[a] as the coating antigen. The ELISA system of 3055/II-OVA[a] had a relatively lower IC_{50} for *O*,*O*-dimethyl OP pesticides and demonstrated little inhibition to the diethyl OP pesticides. This assay is useful for screening *O*,*O*-dimethyl OP pesticides and the use of such a general assay has several advantages. One such advantage is to screen out samples containing low level residues thus enhancing efficiency of further instrumental analysis such as GC–MS. It also can be used in a variety of ways in environmental chemistry. Beside these, it can be used as a highly sensitive and specific detector following chromatographic separation [33].

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REFERENCES

[1] K.D. Racke, Rev. Environ. Contam. Toxicol 131 (1991) 1.
[2] Y. Picó, G. Font, J.C. Moltó, J. Maòes, in: L.M.L. Nollet (Ed.), Food Analysis by HPLC, Marcel Dekker, New York, 1996, p. 717 (Chapter 17).
[3] K.A. Hassal, The Biochemistry and Uses of Pesticides, Macmillan, Toronto, 1990, pp. 142.
[4] A. Hercegova, M. Domotorova, E. Matisova, M. Kirchner, R. Otrekal, V. Stefuca, J. Chromatogr. A 1084 (2005) 46.
[5] Q. Xiao, B. Hu, C.H. Yu, L.B. Xia, Z.C. Jiang, Talanta 69 (2006) 848.
[6] S. Inoue, T. Saito, H. Mase, Y. Suzuki, K. Takazawa, I. Yamamoto, S. Inokuchi, J. Pharm. Biomed. Anal. 44 (2007) 258.
[7] F. Szurdoki, H.K.M. Bekheit, M.P. Marco, M.H. Goodrow, B.D. Hammock, in: D.A. Kurtz, J.H. Skerritt, L. Stanker (Eds.), New Frontiers of Agrochemical Immunoassay, AOAC International, Arlington, VA, 1995, p. 39.
[8] M.C. Hennion, D. Barcelo, Anal. Chim. Acta 362 (1998) 3.
[9] Y.H. Liu, M.J. Jin, W.J. Gui, J.L. Cheng, Y.R. Guo, G.N. Zhu, Anal. Chim. Acta 591 (2007) 173.
[10] Q. Zhang, Q. Sun, B.S. Hu, Q. Shen, G. Yang, X. Liang, X. Sun, F.Q. Liu, Food Chem. 106 (2008) 1278.
[11] Y.A. Cho, J.A. Seok, H.S. Lee, Y.J. Kim, Y.C. Park, Y.T. Lee, Anal. Chim. Acta 522 (2004) 215.
[12] A.Y. Kolosova, J.H. Park, S.A. Eremin, S.J. Park, S.J. Kang, W.B. Shim, H.S. Lee, D.H. Chung, Anal. Chim. Acta 511 (2004) 323.
[13] J.C. Johnson, J.M.V. Emon, D.R. Pullman, K.R. Keeper, J. Agric. Food Chem. 46 (1998) 3116.
[14] M.J.C. Alcocer, P.P. Dillon, B.M. Manning, C. Doyen, H.A. Lee, S.J. Daly, R.O. Kennedy, M.R.A. Morgan, J. Agric Food Chem. 48 (2000) 2228.

[15] M.S. Jang, S.J. Lee, X. Xue, H.M. Kwon, C.S. Ra, Y.T. Lee, T. Chung, Korean Chem. Soc. 23 (2002) 1116.
[16] J.N. Banks, M.Q. Chaudhry, W.A. Matthews, M. Haverly, T. Watkins, B.J. Northway, Food. Agric. Immun. 10 (1998) 349.
[17] M.P. Marco, S.J. Gee, B.D. Hammock, Trends Anal. Chem. 14 (1995) 415.
[18] Y.J. Kim, Y.A. Cho, H.S. Lee, Y.T. Lee, S.J. Gee, B.D. Hammock, Anal. Chim. Acta 475 (2003) 85.
[19] Y.J. Kim, Y.A. Cho, H.S. Lee, Y.T. Lee, Anal. Chim. Acta 494 (2003) 29.
[20] Q. Zhang, L.B. Wang, K.C. Ahn, Q. Sun, B.S. Hu, J. Wang, F.Q. Liu, Anal. Chim. Acta 596 (2007) 303.
[21] G. Berkelhammer, S. Dubreuil, R.W. Young, J. Org. Chem. 26 (1961) 2281.
[22] D.P. McAdam, A.S. Hill, H.L. Beasley, J.H. Skerritt, J. Agric. Food. Chem. 40 (1992) 1466.
[23] C.D. Ercegovich, R.P. Vallejo, R.R. Getting, L. Woods, E.D. Bogus, R.O. Mumma, J. Agric. Food. Chem. 29 (1981) 559.
[24] B. Riggle, J. Agric. Food. Chem. 38 (1990) 1922.
[25] G.T. Hermanson, Preparation of Colloidal-Gold-Labeled Proteins, in: Bioconjugate Techniques, Academic Press, New York, NY, USA, 1996, pp. 593.
[26] M.J. Moreno, A. Abad, A. Montoya, J. Agric. Food Chem. 49 (2001) 72.
[27] G. Shan, D.W. Stoutamire, I. Wengatz, S.J. Gee, B.D. Hammock, J. Agric. Food. Chem. 47 (1999) 2145.
[28] G.M. Raab, Clin. Chem. 29 (1983) 1757.
[29] E.M. Brun, M. Garces-Garcia, M.J. Banuls, J.A. Gabaldon, R. Puchades, A. Maquieira, Environ. Sci. Technol. 39 (2005) 2786.
[30] G. Shan, R.L. Whitney, W.S. Donald, S.J. Gee, D.P.Y. Chang, B.D. Hammock, J. Agric. Food. Chem. 48 (2000) 4032.
[31] H.E. Grotjan, B.A. Keel, Data interpretation and quality control, in: E. Diamandis, T. Christopoulos (Eds.), Immunoassay, Academic Press, San Diego, CA, 1996.
[32] S.K. Mak, G.M. Shan, H.J. Lee, T. Watanabe, D.W. Stoutamire, S.J. Gee, B.D. Hammock, Anal. Chim. Acta 534 (2005) 109.
[33] T. Watanabe, G.M. Shan, D.W. Stoutamire, S.J. Gee, B.D. Hammock, Anal. Chim. Acta 444 (2001) 119.