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Development of an indirect competitive immunoassay for parathion in vegetables

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Abstract

An indirect competitive immunoassay for the insecticide parathion has been optimized and characterized. This assay is based on a monoclonal antibody ($2H_9$) produced from an immunogen, a bovine thyroglobulin (BTG) conjugate wherein the reduced form of parathion was multiply bound to the carrier protein via diazo bonds. Assay was performed in the parathion-HSA coated (0.25 μg/ml) ELISA format in which antibody was diluted 1:2000. Several physicochemical factors (pH, ionic strength, BSA concentrations and organic solvent) that influence assay performance were studied and optimized. Finally, the assay was applied to the analysis of parathion in spiked vegetable samples. The sensitivity, estimated as the IC_{50} value, was 360 ng/ml, with a practical working range between 47 and 6000 ng/ml, a limit of detection of 26 ng/ml, and inter-assay and intra-assay variations less than 10%. The average recovery of parathion added to potato, celery and Chinese cabbage were 173 ± 34%, 108 ± 15% and 98 ± 6%, respectively.

Keywords: Parathion; Indirect competitive immunoassay; Recovery

1. Introduction

Synthetic insecticides have been among the most useful weapons for human beings in controlling insect pests. Organophosphates (OP) are a group of highly toxic compounds which are extensively used as agricultural and domestic pesticides (Costa, 1988). Because of similarities in physiological mechanisms across species, non-target organisms are also threatened by insecticides, especially organophosphorus insecticides, such as parathion (*O*,*O*-diethyl-*O*-(4-nitrophenyl) phosphorothioate). OP toxicants generally elicit their effects by inhibition of acetylcholinesterase, which leads to the accumulation of the neurotransmitter acetylcholine (Ach) in synapses, overstimulates the postsynaptic cholinergic receptors with consequent signs of neurotoxicity (Ecobichon, 1996; Gallo & Lawryk, 1991), and produces such symptoms as bradycardia, bronchorrhea, lacrimation, salivation, emesis, diarrhea, diaphoresis, fasciculation, and muscle paralysis. Environmental pollution from organophosphate pesticides is also an important issue that attracts widespread public concern (Fautz & Miltenburger, 1994; Laham, Long, & Broxup, 1986) and the toxicity of parathion for non-target organisms has been the subject of extensive research (Bauer & Römbke, 1997; Chang, Chen, & Yang, 1997). In China, about 1 million tons of various pesticides are used every year, and 25% of them are OPs, such as parathion and methyl-parathion, which has caused serious environmental pollution and human and animal poisoning accidents. Recently, some related policies have been issued and enforced to reduce and limit the employment of some pesticides in vegetables and fruits (since 2004), and highly toxic OPs will be banned absolutely from 2007.

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Current analytical methods involving gas and liquid chromatography for the detection of parathion are sensitive and reliable. However, they require high cost instruments and skilled analysts and involve time-consuming sample preparation steps. Therefore, there is a growing demand for more rapid and economical methods for determining pesticide residues.

Biosensors represent an alternative method for quickly detecting insecticides and this has been an active research area for several years. Most of these biosensors have been based on the inhibition reaction of the enzymes acetylcholinesterase (AchE) or butyrylcholinesterase (BuchE) with electrochemical (amperometric, potentiometric) or optical transducers. Although sensitive, these electrochemical biosensors suffer from several limitations, such as poor selectivity, the required multi-step indirect determination, and irreversible inhibition by many compounds (Schöning et al., 2003). An on-chip enzymatic assay for screening organophosphate nerve agents, based on a pre-column reaction of organophosphorus hydrolase (OPH), electrophoretic separation of the phosphonic acid products, and their contactless-conductivity detection, is described. This OPH-biochip can differentiate between the individual OP substrates and demonstrates, for the first time, the suitability of the contactless-conductivity detection for on-chip monitoring of enzymatic reactions (Wang et al., 2004). A sensitive voltammetric method has also been developed for the determination of parathion (fNO2) using a Na®on1-coated glassy carbon electrode. Using this method, a linear calibration curve for parathion was obtained up to the 15 mM range in pH 1.1 citrate buffer solution with a detection limit ($S/N = 3$) of 50 nM (Zen, Jou, & Kumar, 1999).

Over the past 15 years, the importance and application of immunochemical methods, especially enzyme-linked immunosorbent assay (ELISA), have grown significantly. Immunochemical methods can offer high specificity, sensitivity, simplicity and suitability for the analysis of a large number of samples in a short period. Several groups have developed immunoassays for parathion. The development of an immunoassay requires the production of antibodies for the analyte. Pesticides are small molecules, and pesticide derivatives, namely haptens, must be synthesized and coupled to carrier proteins to induce antibody production. Ercegovich et al. (1981), Vallejo, Bogus, and Mumma (1982) Ibrahim, Morsy, and Hewedi (1994) utilized a hapten prepared by diazotization of the nitro-group on these compounds and conjugated it directly to a carrier protein to develop polyclonal antisera, while Ibrahim et al. (1994) and Garrett et al. (1997) developed monoclonal antibodies (MAbs). In the latter case, a MAb-derived recombinant single chain variable fragment can detect parathion at nanogramme levels. Others (Brimfield, Lenz, & Graham, 1985) have attempted as develop MAbs to organophosphate haptens using diazotized paraoxon rather than parathion as immunogen. The resulting assay did detect parathion, but its lower limit of detection (LLD) was very high (1 mg/l) for both compounds. Subsequently, Vallejo and co-authors (1982) failed to improve the sensitivity of parathion detection by utilizing antibodies prepared (to haptens) by either attaching the carrier protein at a different position (i.e., 2- or 3-phenyl position) or with the use of bridging groups in the spacer arm to extend and expose the hapten structure. In both cases, the resulting antibody was found to preferentially bind to substitutions at the 2- or 3-position or to parathion derivatives that contain the bridging group structures. Final approaches to the development of antibodies to parathion have been through the use of a 'generic' organophosphate hapten, *O*,*O*-diethyl-*O*-4-hydroxybutyl phosphorothioate (Johnson, Van Emon, & Pullman, 1998) or tert-butyl 3-[chloro(methoxy) phosphorthioylamino]-propanoate (Skerritt, Guihot, Asha, Rani, & Karanth, 2003).

Nevertheless, as immunological analysis of pesticides is relatively new in the field, its acceptance by analytical chemists is not yet complete. The key to proper ELISA development is the understanding of the properties of the method itself. Factors affecting assay performance need to be studied and optimized to achieve this knowledge and to produce sensitive assays.

The purpose of this study was to develop, optimize, and characterize an indirect competitive immunoassay based on a monoclonal antibody ($2H_9$) for parathion in the spiked vegetable samples.

2. Materials and methods

2.1. Chemicals and instruments

Unless noted otherwise, biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Bovine thyroglobulin (BTG), human serum albumin (HSA), *O*-phenylenediamine (OPD), fetal calf serum (FCS, Hyclone), polyethylene glycol (PEG, mol. wt. 2000, E. Merck AG, Darmstadt, Germany), penicillin G, streptomycin (Amresco, USA), DMEM, hypoxanthine-aminopterin-thymidine (HAT), hypoxanthine-thymidine (HT), Freund's complete adjuvant (FCA), Freund's incomplete adjuvant (FIA), mineral oil, parathion standard (Riedel-de-Haen AG, Seelze-Hannover, Germany), and peroxidase-conjugated goat anti-mouse IgG (IgG-HRP) (Jackson, West Grove, PA) were used. Mouse anti-parathion MAb ($2H_9$) was prepared in our laboratory. All other reagents used in this study were of analytical grade or better and obtained from standard sources. ELISA plates (96 wells) were purchased from Canada JET Biochemicals Int'l., Inc. (Toronto, Canada). Other cell culture plastic wares were obtained from Costar (Cambridge, MA). Absorbance values were read in dual-wavelength mode (492–630 nm) with a μQuant model microplate reader (Bio-Tek Instruments, Inc., USA).

2.2. Buffers and solutions

Phosphate-buffered saline (PBS) contained 10 mM sodium phosphate buffer, pH 7.4, in 0.14 M sodium chlo-

ride. Coating buffer contained 0.85 mol/l of carbonate buffer, pH 9.6. Blocking solution was prepared with coating buffer containing 1% gelatin. Washing solution was prepared with PBS containing 0.05% Tween-20 (PBST). Dilution solution for antibodies, standards and samples was prepared with PBS containing 0.1% gelatin and 0.01% Tween-20. Substrate buffer was 0.2 mol/l sodium acetate with pH adjusted to 5.0 by addition of 0.1 mol/l of citric acid. Substrate solution for HRP was 2 mg/ml of OPD and 0.012% H_2O_2 in 25 mM citrate/62 mM sodium phosphate buffer, pH 5.0. Stopping solution was 2 M H_2SO_4.

2.3. Preparation of hapten-protein conjugates

Parathion was coupled to carrier protein BTG and HSA through the nitro group using diazotization. Briefly, the nitryl group in phenyl was reduced to amino group, which was used to couple with carrier proteins (Ercegovich et al., 1981).

2.4. Monoclonal antibody production

Animal care was in accordance with institutional guidelines. Five BALB/c female mice, 18–22 g, were immunized subcutaneously with 100 μg of parathion-BTG emulsified in FCA. Four weeks after the initial dose, three other half-monthly boosts were administered, using the half dose in FIA. Ten days after each injection, blood samples were collected from the tail and tested by enzyme-linked immunosorbent assay (ELISA). Four days after the last boost given i.p without adjuvant, spleen cells from one immune mouse were collected and fused with SP2/0 myeloma line, using polyethylene glycol 2000 as a fusing agent. The ratio of spleen cells to myeloma cells in suspension was 5–10:1. Fused cells were resuspended in the selective medium containing hypoxanthine, aminopterin and thymidine (HAT) and distributed into 96-well tissue culture plates on a feeder layer consisting of 10^4 peritoneal cells. Plates were seeded at a density of 10^5 cells/well/200 μl. Supernatants from the cultures showing hybridoma growth were tested for antibody activity on days 7–10. Positive hybridomas were subcloned by the limiting dilution. Ascitic fluids were produced in mineral oil-primed BALB/c mice. MAbs were purified from ascetic fluid by ammonium sulfate precipitation.

2.5. An indirect competitive immunoassay for parathion

An indirect competition ELISA format was utilized to measure parathion binding and cross-reactivity to related compounds. With the checkerboard procedure, the appropriate concentrations of coating antigen were prepared by serial dilutions from 4 to 0.125 μg/ml of parathion-HSA with a dilution factor of 2 in the coating solution and primary antibody (serially diluted purified monoclonal antibody $2H_9$ from 1:500 to 1:12,800 with a dilution factor of 2 in the dilution solution). The optimum reagent concentrations were defined as those, which give maximum absorbance around 1.0 in the absence of analyte with minimum reagent expenses. Usually, several combinations of immunoreagents were evaluated to select the most sensitive assay. A final volume of 100 μl/well was used in all steps, and incubations were performed at 37 °C to avoid well-to-well temperature variability. Plates were washed three times between steps with washing solution and finally, peroxidase activity was revealed with freshly prepared substrate solution. The enzymatic reaction was stopped after 20–30 min by adding 100 μl/well of stopping solution. The absorbance was immediately read at 492 nm with a reference wavelength at 630 nm.

Plates were coated with an appropriate concentration of parathion-HSA in coating buffer by incubation at 4 °C overnight or 37 °C for 1 h. The plates were then washed as described above, and blocked with 120 μl/well of blocking solution at 37 °C for 1 h. 50 μl/well of parathion standard or sample solution, followed by 50 μl/well of $2H_9$ MAb solution at twice the desired assay concentration was added. These solutions were prepared in different buffers, depending on the experiment. Inhibition standard curve was prepared by serial dilutions from 4.7 to 600 ng/well of parathion with a dilution factor of 2. Competitive immunological reaction was allowed to take place for 1 h, and then plates were washed as before. Afterwards, a 1/5000 dilution of IgG-HRP in dilution solution was added, and the reaction lasted for 30 min. After washing, the retained peroxidase activity was determined as indicated above. The resulting curves were fitted with a four-parameter logistic equation to determine the IC_{50}, which was defined as the concentration of inhibitor required to inhibit colour development by 50% compared to control wells containing no competitor.

2.6. Physicochemical parameter influence and optimization

Several physicochemical factors influencing the immunological reaction were studied in this immunoassay. IC_{50} parameters of competitive curves were evaluated under different conditions on three different days.

To evaluate the influence of pH, parathion was diluted in a set of 0.01 M dilution solution buffers with constant ionic strength but different pH values of 5, 6, 7, 7.4, 8, and 9, respectively.

To determine the effect of ionic strength on the assay performance, parathion was added to serial dilutions of a concentrated buffer (0, 0.005, 0.01, 0.05, 0.1, and 1 M, respectively) but with constant pH of 7.4.

Competitive assays were performed in PBS containing different BSA concentrations (0%, 0.5%, 1%, 2.5%, 5% and 10%).

The assay's tolerance to methanol was evaluated between 0% and 50% solvent concentration (v/v). Standard curves of parathion were prepared in dilution solution buffer containing different amounts of methanol (0%, 5%, 10%, 20%, 30% and 50% in PBST).

2.7. Determination of inter- and intra-assay variation

Serial dilutions of parathion (from 600 ng to 18.8 ng/100 μl) were prepared in dilution solution buffer. Parathion concentration in PBST was calculated by using a parathion calibration standard curve of 600, 300, 150, 75, 37.5, 18.8, 9.4 and 4.7 ng/100 μl. Intra-assay (within a day) variation was measured for 12 replicates of each concentration of the parathion, and for inter-assay (between days) variation; each concentration of parathion was determined after 5 days.

2.8. Spiked vegetable samples analysis

Potato, celery and Chinese cabbage, purchased from a local supermarket, were given a good scour and then dried. Parathion, with different concentrations (50, 100 and 200 mg/kg) dissolved in methanol, was spiked on the surface of these vegetables, which were then dried in a fume hood for 30 min and kept in it. To prepare extracts, each vegetable (10 g) was chopped in a vitreous homogenizer and was then transferred to a round-bottom flask. They were shaken (in a shaker) for 4 h at room temperature in 25 ml of methanol and then centrifuged for 20 min at 4000 rpm to remove the solids. The supernatants were treated with excess sodium sulfate overnight to get rid of water from tissue fluid. Blank samples were prepared, as described above, but not spiked with parathion. The extent of matrix interference was determined by extracting a pesticide-free matrix, preparing standards with known concentrations of pesticide, and comparing the standard curve prepared in the extract of the vegetable sample matrix to a similar one produced in dilution solution (Garrett, Appleford, Wyatt, Lee, & Morgan, 1997; Kim, Shelver, & Li, 2004).

3. Results and discussion

3.1. Optimization of competitive ELISA

The synthesis of conjugates between carrier proteins and parathion and the production of high affinity MAbs for parathion were as previously described (Zeng et al., 2005). With MAb $2H_9$, an indirect competitive ELISA, which employed a parathion-HSA coated format, was developed. Optimum reagent concentrations were 0.25 μg/ml of parathion-HSA and 1:1000 dilution of MAb. Fig. 1 shows a representative standard curve for parathion generated by this competitive ELISA. The IC_{50} value was 360 ng/ml, which was similar to results of Garrett et al. (1997) and Ibrahim et al. (1994), 119 ng/ml and 250 ng/ml, respectively. The limit of detection, defined as detectable concentration equivalent to 10% decline of zero binding, was 26 ng/ml, rather below 100 ng/ml which was the result of Ercegovich et al. (1981) and almost the same as 16 ng/ml given by Garrett et al. (1997).

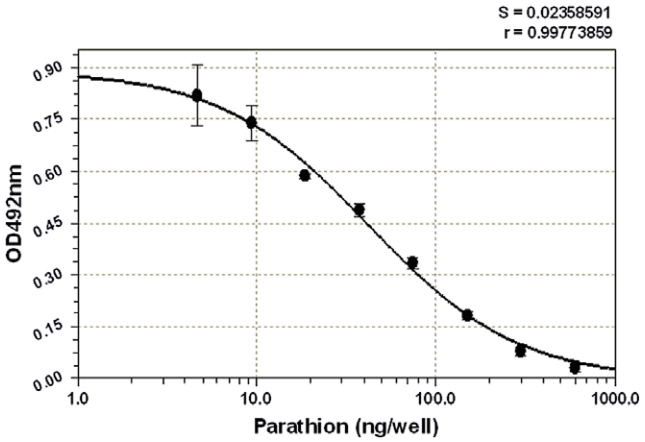

Fig. 1. The standard curve of parathion in indirect competitive ELISA. Values were determined in triplicate and one representative experiment is shown.

3.2. Effects and optimization of physicochemical parameter

To investigate the effect, originating from different physicochemical factors, on ELISA and optimize assay performance, pH, buffer ionic strength and BSA were investigated (Fig. 2).

The value of pH is one of the key factors influencing the characteristics of assay. From Fig. 2a, we can see that IC_{50} and OD_{max} were low at extremely higher pH value, but the former was high with little influence on the latter at lower pH. It seems that the reaction between antibody and analyte occurs more easily in alkali solution, whereas interaction between antibody and coating conjugate may be prone to change in acid solution. The physiological pH (7.4) was selected as the optimum for the assay, based on the favourable IC_{50} value and OD_{max} of the curve.

Due to the non-polar nature of parathion, it seems reasonable to assume that hydrophobic interactions are important in the antibody-parathion reaction as well as antibody–coating antigen interactions. Increasing the concentration of phosphate ions caused an increase in IC_{50} and a reduction in OD_{max}, while low ionic strength led to higher IC_{50}. So it may be speculated that excess high (such as in 0.1 M, 1 M) ionic strength could obstruct the hydrophobic reaction of antibody with parathion or coating conjugate, while the lowest (such as in bidistilled water) may only affect the former (Fig. 2b). Therefore, it is necessary to maintain a certain concentration of buffer to enhance the sensitivity of the assay. Optimum concentration selected was 0.01 M, where the standard curve is sharper.

BSA is the most commonly used additive in ELISA to reduce non-specific interaction. The value of IC_{50} increased as the concentration of BSA was enhanced, that is to say, the lower the BSA concentration, the higher the sensitivity. It was speculated that the reaction of antibody with parathion or coating conjugate could be interfered with by adding protein, which reduced the binding ratio between antigens and antibodies (Fig. 2c). Therefore, it is

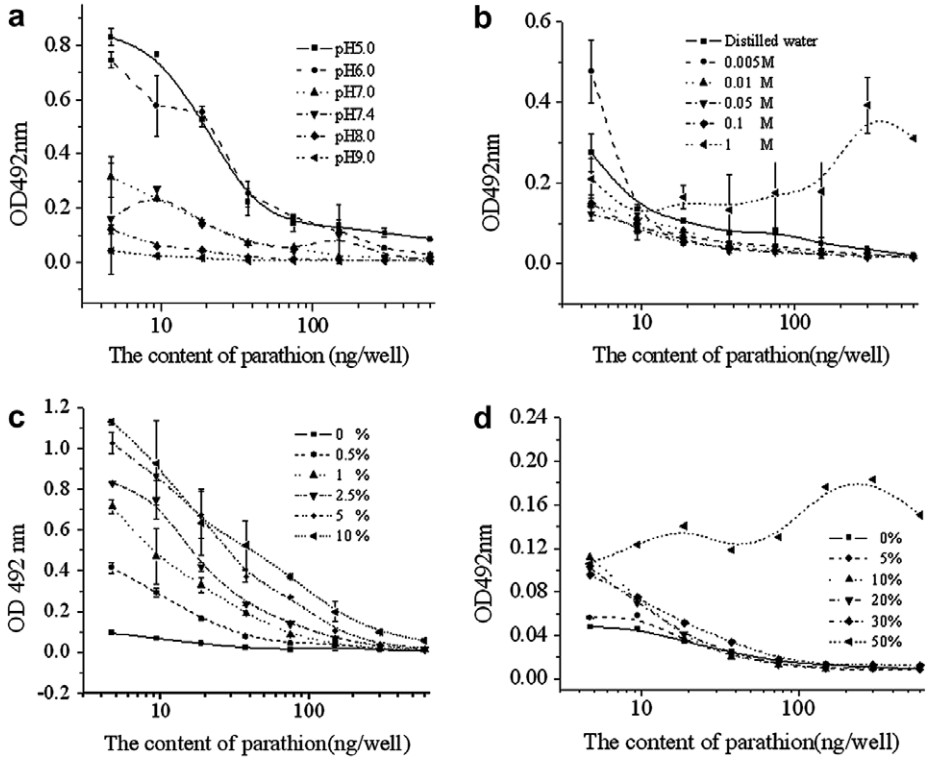

Fig. 2. Influence of different physiochemical factors, pH (a), ionic strength (b), BSA (c), and methanol (d) on the performance of assay. Results are the means of two independent experiments.

noted that high concentrations of BSA (>5%) should be avoided in ELISA.

3.3. Effect of solvent on the assay

Since the use of organic solvents for extraction and/or solid phase clean up is very common in the analysis of non-polar pesticide residues in food and environmental samples, it is desirable to assess the effect of organic solvents on ELISA performance. Parathion dissolves in water slightly but in organic solvent easily, such as methanol, acetone. Stock solutions of parathion were usually prepared in methanol. To evaluate methanol influence on the assay characteristics, various ratios of methanol/assay buffer (0–50%) were used for preparation of working solutions. The final concentration of organic solvent in the reaction mixture given here was obtained by adding analyte and MAb solution in assay buffer to the well (50 μl of analyte and 50 μl of MAb solution). It can be observed that amounts <30%(v/v) of methanol were tolerated, although OD_{max} was slightly reduced and IC_{50} increased little (Fig. 2d). However, when the content of methanol reached 50%, OD_{max} was so greatly reduced that IC_{50} values could not be determined. Several other workers (Kim, Lee, Chung, & Lee, 2003; Mercader & Montoya, 1999) reached

Table 1
Intra-assay, inter-assay variance and recovery of competitive ELISA[a] for $2H_9$

Parathion (ng/well)	Intra-assay (ng/well)	CV (%)	Inter-assay (ng/well)	CV (%)
600	607 ± 1.1	0.18	652 ± 3.3	0.51
300	296 ± 0.2	0.06	272 ± 5.4	1.98
150	153.0 ± 3.1	1.99	154 ± 4.2	2.72
75	74.3 ± 0.3	0.34	72.3 ± 3.4	4.70
37.5	37.3 ± 2.4	6.30	38.9 ± 2.2	5.55
18.75	18.6 ± 4.5	24.24	16.5 ± 5.6	33.9

[a] Representive results are from a series of five experiments. For each experiment $n = 3$.

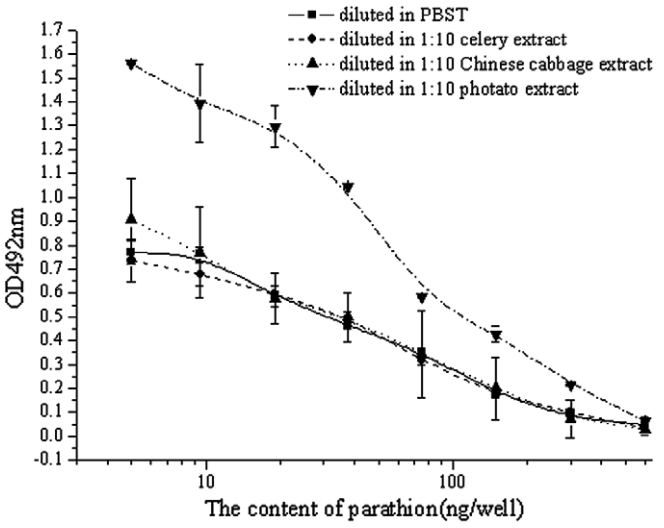

Fig. 3. Standard curves for parathion in spiked vegetable samples diluted in solution buffer.

Table 2
Recovery of parathion from the spiked vegetable samples

	50 mg/kg		100 mg/kg		250 mg/kg	
	Detected (mg/kg)	Recovery (%)	Detected (mg/kg)	Recovery (%)	Detected (mg/kg)	Recovery (%)
Potato	83	166	182	182	354	142
Celery	55	110	103	103	267	107
Chinese cabbage	51	102	98	98	253	101

the same conclusion as ours, and they found that methanol caused the least negative effect on the performance of ELISA, compared to other solvents, such as acetone, acetonitrile.

3.4. Determination of inter- and intra-assay variation

Table 1 summarizes the results of inter- and intra-assay variations. Except that the CV in the lower content was not satisfied, the others were less than 10%. This showed that it was feasible to determine parathion using this indirect competitive immunoassay.

3.5. Analysis of spiked vegetable samples

The immunoassay established above was initially applied to detect the parathion residue. Since potato is a root vegetable, celery contains an abundance chlorophyll, and Chinese cabbage is a common vegetable, they were selected as representative samples to analyze. In order to evaluate and correct the matrix interference caused by a variety of food matrices, blank samples were initially extracted in methanol. Considering the effects of physiochemical factors, the methanol extracts were diluted 1:10 in dilution solution buffer and the plots of absorbance against concentration were obtained for a range of standards (between 4.7 and 600 ng/well) and compared with those of standards prepared in dilution solution. The curves for celery and Chinese cabbage were slightly different from that for standard parathion, while that for potato showed a rightward departure from the standard curve (Fig. 3). This implied that the potato matrix had more influence on the antigen–antibody interaction. Recoveries of spiked celery and Chinese cabbage samples, diluted in 1:10 and analyzed by indirect assay, were quite satisfactory, $108 \pm 15\%$ and $98 \pm 6\%$, respectively. Due to the matrix influence, recoveries of potato were poor ($173 \pm 34\%$) though the sample was dealt with by the same procedure (Table 2, Fig. 3). Overall, the indirect competitive ELISA developed in this study can accurately determine parathion residue in vegetable samples after the simple and rapid extraction procedure.

4. Conclusions

An indirect competitive immunoassay was developed for the detection of parathion by determining the optimal parathion-HSA-coated antigen and MAb (2H9) in a checkerboard fashion, which are defined to be 0.25 μg/ml and dilution of 1:2000, respectively. Considering the effect of factors such as pH, ionic strength, BSA concentration and organic solvent on the performance of ELISA, the sensitivity of 360 ng/ml, with a practical working range between 47 and 6000 ng/ml, a limit of detection of 26 ng/ml, and inter-assay and intra-assay variations of less than 10% were obtained. The average recoveries of parathion in spiked vegetables, such as potato, celery and Chinese cabbage were $173 \pm 34\%$, $108 \pm 15\%$ and $98 \pm 6\%$, respectively, which means that this method provides a tool to monitor parathion in vegetables.

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