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Analytical Methods

Development of a direct competitive enzyme-linked immunoassay for carbofuran in vegetables

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Abstract

Three ELISA formats, antigen coated, antibody coated and the second antibody coated for the determination of carbofuran were investigated with conjugations including hapten–BSA, hapten–OVA, hapten–HRP and anticarbofuran IgG–HRP. Results showed that the second antibody-coated method of ELISA had a better performance in the establishment of standard curves and detection of carbofuran residue in vegetables samples. The sensitivity for detection, the I_{50} value was 36.1 ng/ml at a practical working concentration range from 3.44 to 380.1 ng/ml and the limit of detection for carbofuran was 3.44 ng/ml. The average recoveries of determination for carbofuran spiked in cabbage, lettuce, carrot, winter fragrant-flowered garlic, bamboo shoot and green soy bean were 85.24%, 101.8%, 103.6%, 90.52%, 106.9% and 94.08%, respectively. Additional analyses confirmed that the results given by the ELISA method was in agreement with those of the gas chromatography (GC) method.

Keywords: Carbofuran; Residue; Vegetable; Enzyme-linked immunoassay (ELISA)

1. Introduction

Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofurany1 methylcarbamate) is a widely used systemic and contact *N*-methylcarbamate pesticide applied to control insect and nematode pests on a variety of agricultural crops. It is a cholinesterase inhibitor with a high toxicity to human and wildlife through the oral and nasal routes of exposure (Liu & Zhu, 1999). Current analytical methods for detection of carbofuran involving gas chromatography (GC) and high performance liquid chromatography (HPLC) with post-column derivatization are sensitive and reliable (Fu et al., 2005; Li, Lu, Yu, & Yu, 2005). However, these methods require expensive instruments, 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.

A monoclonal antibody-based ELISA method for carbofuran was developed by Montoya and coworkers (Abad, Moreno, & Montoya, 1997). However, further study is necessary for the determination of carbofuran in vegetables. In this study, an ELISA method for detection of carbofuran residues in vegetable samples was developed. The background value, limit of detection (LOD) and the range of recovery of each samples was developed. For cabbage, lettuce, carrot, winter fragrant-flowered garlic, bamboo shoot and green soy bean, the carbofuran residue could be detected by the developed method directly. To investigate whether the matrix in vegetable samples affected the antigen–antibody reaction, direct competitive time resolved fluorescence immunoassay (TRFIA) for carbofuran was also evaluated in analysis of blank samples. An additional experiment was conducted to investigate whether the matrix would inhibit the color development of Horseradish Peroxidase (HRP).

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2. Experimental

2.1. Chemicals and instruments of immunoassay

Pesticide standards were obtained from Hangzhou Geling Scientific Instrument (Hangzhou, China). Bovine serum albumin (BSA), ovalbumin (OVA), Tween 20, *o*-phenylenediamine (OPD), Horseradish Peroxidase (HRP), goat anti-rat immunoglobulins, Freund's complete and incomplete adjuvants were purchased from Sigma–Aldrich (Madrid, Spain). *N*′-[*p*-Isothiocyanatobenzyl]-diethylenetriamine-N^1,N^2,N^3,N^3-tetraacetate-Eu^{3+} (DTTA-Eu) was obtained from Tianjin Radio-Medical Institute (Tianjin, China). Enhancement solution of TRFIA was purchased from Wallac/Pharmacia (Turku, Finland). All other chemicals and organic solvents were of analytical grade or better.

The ELISA was carried out in 96-well polystyrene microplates (Corning, New York, USA). Polystyrene TRFIA plates were purchased from Thermo Scientific (Waltham, USA). Micro-plates were washed with a Dynex Plus microplate washer (Dynex Technologies, Chantilly, USA). Absorbance and fluorescence intensity were measured with a multifunctional plate reader (Tecan Group, Mannedorf, Switzerland).

2.2. Preparation of hapten–protein conjugate

The synthesis of hapten (BFNP) and the preparation of monoclonal antibody followed the method as described in Abad et al. (1997). BFNP–OVA conjugates and BFNP–HRP were prepared by the isobutyl chloroformate method, and IgG–HRP was prepared by the $NaIO_4$ method (Jung, Gee, & Harrison, 1989; Zhu, Wu, & Wu, 2003). Conjugate formations were confirmed spectrophotometrically. Molar ratios of approximately 4.3, 3.5, 8.3 and 3.1 were estimated for anti-BFNP IgG–HRP, BFNP–HRP, BFNP–OVA and BFNP–BSA conjugates, respectively (Jin, Wu, Zhu, Chen, & Shou, 2006).

2.3. Selection of the ELISA formats

Three ELISA formats including antigen-coated format, anti-BFNP IgG-coated format and the second antibody (goat anti-rat IgG)-coated format were compared in this study. Antigen-coated format was first considered, where BFNP–OVA was coated in the 96-well microtiter plate. After incubation, the carbofuran standard and anti-BFNP IgG–HRP were added. Anti-BFNP IgG in liquid phase was competed by BFNP–OVA which was coated in the plate and the pesticide in the liquid phase. The standard curve was generated (Jung et al., 1989). The second format was the anti-BFNP IgG-coated method. Anti-BFNP IgG was coated in the microtiter plate, and after incubation, the carbofuran standard and BFNP–HRP were added. In the reaction period the IgG coated in the plate was competed by the carbofuran standard and BFNP–HRP in the liquid phase (Gui, Jin, Chen, Cheng, & Zhu, 2006; Skerritt, Guihot, & Asha, 2003). The third format was the secondary antibody-coated method. Briefly, the second antibody was coated in the microtiter plate. After incubation, carbofuran standard, BFNP–HRP and anti-BFNP IgG were added. The pesticide, BFNP–HRP and IgG were all in the liquid phase. The antibody was competed by the pesticide and BFNP–HRP thoroughly.

For the purpose of sensitivity and reproducibility evaluation, the second antibody-coated method was further applied to the analysis of carbofuran in spiked vegetable samples. Immunoassay was performed using the following procedure. Microtiter plates were coated with the second antibody (goat anti-mouse immunoglobulin, 10 μg/ml, 200 μl/well) by incubating overnight in 10 mM phosphate-buffered saline (PBS, pH 7.4) at 4 °C. Plates were washed four times with PBST (PBS containing 0.05% Tween 20) and then blocked by incubation with 2% skimmed milk in PBS (300 μl/well) for 30 min at 37 °C. After another wash step, 50 μl/well of standard or sample solutions were added, followed by 50 μl/well of BFNP–HRP (0.143 μg/ml) in PBS containing 4% skimmed milk and 100 μl/well of anti-BFNP IgG (4 μg/ml) in PBS containing 2% skimmed milk. After incubation at 37 °C for 1 h, the plates were washed as before, and 100 μl/well of OPD solution (0.4 g/L OPD, 25 mM citrate, 62 mM sodium phosphate, pH 5.4, containing 0.012% H_2O_2) was added. After another incubation for 15 min at 37 °C, the enzymic reaction was stopped by adding 50 μl/well of 2.5 M sulfuric acid, and the absorbance was read immediately at 492 nm with a reference wavelength at 630 nm (Jung et al., 1989; Meyer & Guven, 1986).

2.4. Development of TRFIA for carbofuran

Microtiter plates were coated with the BFNP–OVA (10 μg/ml, 100 μl/well) in 50 mM carbonate-buffered saline (CBS, pH 9.6) at 37 °C for 2 h. Plates were washed four times with PBST and were then blocked by incubation with 2% skimmed milk in 50 mM Tris–HCl buffer, pH 7.2 (300 μl/well) for 30 min at 37 °C. After another wash step, 50 μl/well of standards or samples were added, followed by adding 50 μl/well of anti-BFNP IgG–Eu (1:16.7 dilutions) in Tris–HCl buffer containing 4% skimmed milk. After incubation at 37 °C for 1 h, the plates were washed as before, and 200 μl enhanced solution was then added. The microtiter plate was oscillated for 5 min at room temperature, and then the fluorescence intensity was read in the Tecan Genios multifunctional machine at excitation and emission wavelengths of 340 and 613 nm, respectively (Bush, Vanden, Sherman, & Peterson, 1991; Yu, Reed, & Golden, 2002).

2.5. Chromatographic analysis

Analysis of GC was performed with a Hewlett–Packard 6890 (Agilent technologies, Palo Alto, USA) system

equipped with a nitrogen–phosphorus detector and a split/spiltless injector. Samples were pretreated with Method NY/T 761.3-2004 (Ministry of Agriculture of China, 2004). The capillary column was a DB-17 (30 m × 0.32 mm I.D.) (Hewlett–Packard). Injector and detector were operated at 260 °C and 280 °C, respectively. Nitrogen was used as the carrier and make-up gas at 4.8 ml/min and 5.0 ml/min, respectively. Hydrogen and air were used as the detector gases at 2.5 ml/min and 60 ml/min, respectively. The oven temperature was initially kept at 100 °C for 1 min. It was then increased to 160 °C at 30 °C/min, and held at 160 °C for 1 min. The temperature was finally increased to 230 °C at 10 °C/min and held at 230 °C for 1 min.The total run time was 31.0 min. The retention time of carbofuran was 11.75 min. The injection volume was 1 μl. The samples were spiked with carbofuran standard at 0, 10, 50 and 250 ng/ml.

2.6. Preparation of carbofuran standards and samples

From a 3.41 μg/ml stock solution of carbofuran in methanol, standards in range of 2.67–341 ng/ml were prepared daily by serial dilution in 2 × PBS/methanol solution (9/1, v/v) (Zeng et al., 2007). Agricultural products (cabbage, lettuce, carrot, winter fragrant-flowered garlic, bamboo shoot and green soy bean) were purchased from a local market. The extraction and purification of carbofuran in winter fragrant-flowered garlic, green soy bean, carrot, lettuce, cabbage and bamboo shoot were performed by the following procedures. To 10 g of pre-dollied sample was added 1 ml of carbofuran standard in acetonitrile, followed by the addition of 9 ml of acetonitrile. After oscillating for 1 min on a vortex mixer, the mixture was added 1 g of sodium chloride and 4 g of magnesium sulphate and oscillated for an additional 1 min. After centrifugation (5 min, 3000 rpm), 1 ml of the supernatant was transferred and evaporated at 40 °C under a nitrogen stream. The dry residue was diluted with the mix solution of PBS/methanol (9/1, v/v) and oscillated on a vortex mixer for 1 min. At last 50 μl portions were used for the test.

2.7. Evaluation of matrix effect

Some ingredients of the vegetable samples could affect the ELISA reaction. Diluting the sample was previously found effective to reduce the matrix interfere (Abad, Moreno, & Pelegr, 1999). The following experiment was conducted to determine the optimal dilution ratio. Blank samples were prepared with the same procedure as described for sample preparation. Eight glass tubes of dry residue were dissolved in 1 ml of a series of standard solutions of eight different concentrations, or diluted to 1:2, 1:3, 1:4, and 1:5 (v/v) with the series of standard solutions, respectively. A series of standard solutions containing matrix of different fold of dilution were prepared. After the preparation, several standard curves were generated by the selected method to investigate the matrix interference to the standard curve.

The matrix effect may interfere the reaction of antigen and antibody, or the activity of the HRP. Experiments were carried out to investigate the potential interference. TRFIA for carbofuran was developed, and the blank samples of winter fragrant-flowered garlic were analyzed. The results showed that the reaction of antigen and antibody was inhibited by the matrix of winter fragrant-flowered garlic. Another experiment was performed by the following procedure. Microtiter plates were coated with anticarbofuran IgG–HRP (0.8 μg/ml, 100 μl/well) in the coating buffer (10 mM phosphate-buffered saline, pH 7.4) for 2 h at 37 °C. Plates were washed four times with PBST and then blocked by incubation with 2% skimmed milk in PBS (300 μl/well) for 30 min at 37 °C. After another wash step, 100 μl/well of pre-treated solution of blank sample or PBS/methanol solution (9:1, v/v) were added directly. After incubation at 37 °C for 1 h, the plates were washed as before, and then 100 μl of a 0.4 mg/ml OPD solution in reaction buffer (25 mM citrate, 62 mM sodium phosphate, pH 5.4, containing 0.012% H_2O_2) was added. After 15 min at 37 °C, the enzymic reaction was stopped by adding 50 μl of 2.5 M sulfuric acid, and the absorbance was immediately read at 492 nm with a reference wavelength at 630 nm.

3. Results and discussion

3.1. Comparison of ELISA formats

Three types of standard curves were established for carbofuran by ELISA with the antigen-coated, the anti-BFNP IgG-coated and the second antibody-coated format, respectively. The typical standard curves of the three ELISA formats are given in Fig. 1 and the comparative results are shown in Table 1. From standard curves generated by the three ELISA formats, it is clear that the anti-BFNP IgG-coated format had the best performance in sensitivity and the second antibody coated format had the smallest variation coefficient. For immunoassays, the difference between the anti-BFNP IgG-coated format and the second antibody-coated format in sensitivity was not significant. However, the second antibody-coated format had a better performance in the variation coefficient. The possible reason was that all the reactants of the competitive reaction were in the liquid phase for the second antibody coated format, and the antibody was competed by the pesticide and BFNP–HRP thoroughly, while the antigen was in the solid phase for the antigen-coated format. And for the anti-BFNP IgG-coated format, the anti-BFNP IgG was in the solid phase but the other reactants were in the liquid phase.

The phenomenon known as the time-dependent drift was caused by different incubation times within the plate due to the time required for pipetting different samples or standards into 96 wells (Meyer & Guven, 1986; Munro & Tabenfeldt, 1984; Skerritt et al., 2003). The present study indicated that under the same conditions, the drift phenomenon was more obvious for the anti-BFNP MAb

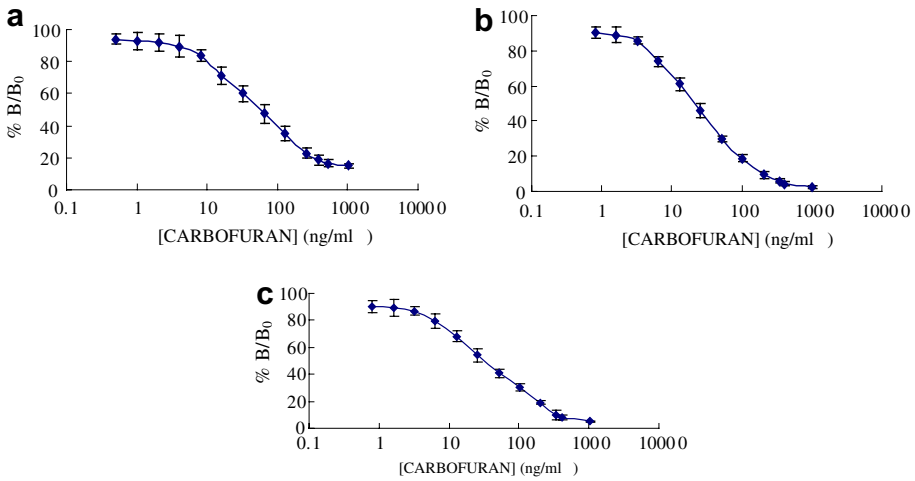

Fig. 1. Typical standard curves of three ELISA formats for carbofuran: (a) antigen-coated format, (b) anti-BFNP IgG-coated format, (c) the second antibody-coated format. Data represent the means of 10 determinations.

Table 1
The comparative result of three ELISA formats

Three ELISA formats	Regression equation	I_{10} (ng/ml)	I_{50} (ng/ml)	Rang of CV (%)	Linear range[a] (ng/ml)
Antigen-coated format	$y = -16.584\,\mathrm{Ln}(x) + 116.29$	4.88	54.45	4.04–14.88	4.88–607.4
Anticarbofuran IgG-coated format	$y = -18.967\,\mathrm{Ln}(x) + 108.01$	2.58	21.29	2.33–20.85	2.58–175.4
The second antibody coated format	$y = -17\,\mathrm{Ln}(x) + 110.99$	3.44	36.15	3.44–7.48	3.44–380.1

[a] The linear range was from I_{10} to I_{90} of the regression equation.

coated format. Several options were considered to prevent the drift. Oscillating the microtiter plates for 5 min prior to the competitive reaction proved to be effective for reducing the drift. The possible reason was that oscillating the microtiter plates could asunder the carbofuran-IgG and BFNP-IgG combination. As a result, the reaction solution in each well of the plate was given the same time to finish the competitive reaction.

In view of sensitivity and reproducibility of the standard curves and the contribution of the time-dependent drift, the second antibody coated format was chosen to develop the immunoassay in this study.

3.2. Matrix effect

The effect of matrix in extract solutions from carrot, green soy bean, bamboo shoot, cabbage, lettuce and winter fragrant-flowered garlic is summarized in Table 2. Results showed that the matrix of carrot and green soy bean had a negligible effect to the standard curves. However, the matrix of bamboo shoot and cabbage affected the standard curves when the sample extracts were not diluted. When they were diluted to 1:2 (v/v) and 1:4 (v/v), the interference of matrix to the standard curve became insignificant. The matrix of lettuce showed negligible interference to the standard curve when it was diluted to 1:2 (v/v) or 1:4 (v/v). As for the winter fragrant-flowered garlic the matrix affected the standard curve when it was not diluted or diluted to 1:4 (v/v). When the extract was diluted to 1:2 (v/v), the interference of matrix to the standard curve became negligible.

Table 2
I_{10} and I_{50} of stand curves in different matrix dilution

Samples	No matrix	1:1 (v/v)	1:2 (v/v)	1:4 (v/v)
Cabbage	3.114/27.07[a]	3.279/34.20	2.984/25.84	3.160/26.03
Lettuce	2.724/25.30	2.518/24.82	3.676/28.37	4.044/31.46
Carrot	3.005/29.55	2.822/28.60	3.354/31.14	3.524/30.64
Winter fragrant-flowered garlic	2.726/24.79	4.201/40.48	3.366/30.96	3.870/34.55
Bamboo shoot	3.017/27.61	5.038/44.68	2.635/25.73	2.748/26.35
Green soy bean	2.554/24.08	2.503/24.52	2.622/24.38	2.817/24.43

[a] 3.114/21.07 was the value of I_{10}/I_{50}, the others was same to it.

The blank samples of winter fragrant-flowered garlic were prepared, and the dry residues were dissolved in 2 ml of 9:1 (v/v) Tris–HCl buffer/methanol. The solution was then tested by the TRFIA method directly. The average value of eight blank samples was 48.96 ng/ml. And the variation coefficient was 24.55%. Due to the unique fluorescent properties of the lanthanide chelates and the time-resolved measurement mode, the specific fluorescence was measured after the background fluorescence had already declined. This indicates that the antigen–antibody reaction was interfered by the matrix.

Eight blank samples of winter fragrant-flowered garlic were prepared with the method described above. Each residue was dissolved in 2 ml of mix solution (PBS/methanol = 9/1, v/v). Then a 100-μl subsample was added to

Table 3
Reproducibility and recovery of carbofuran from spiked vegetable samples

Samples	Mean background value ± SD (ng/mg, $n = 96$)	Spiked level (ng/mg)	Mean ± SD (ng/mg)	Mean recovery (%, $n = 96$)	CV (%)
Cabbage	19.51 ± 5.516	100	94.06 ± 17.32	74.55	18.41
		500	499.2 ± 88.29	95.94	17.69
Lettuce	10.64 ± 4.159	50	50.94 ± 10.36	80.61	20.34
		250	318.1 ± 36.52	123.0	11.48
Carrot	8.364 ± 2.816	50	48.44 ± 9.791	80.15	20.21
		250	326.2 ± 36.60	127.1	11.83
Winter fragrant- flowered garlic	16.95 ± 4.848	50	50.87 ± 10.30	67.84	20.25
		250	299.9 ± 18.17	113.2	12.12
Bamboo shoot	6.208 ± 1.766	50	58.31 ± 9.984	104.2	17.12
		250	280.6 ± 40.78	109.7	14.53
Green soy bean	4.922 ± 1.371	50	45.50 ± 6.984	81.16	15.35
		250	272.4 ± 31.88	107.0	11.70

the ELISA plate which was pre-coated with 0.8 μg/ml of anti-BFNP IgG–HRP. A 100-μl aliquot of the mix solution (PBS/methanol = 9/1, v/v) was then added to the same ELISA plate for check. The average OD value of the solution without matrix of winter fragrant-flowered garlic was 1.412. The average OD value of the solution with the matrix was 1.076. This result suggests that the color development of HRP was inhibited by the matrix. The possible reason was that the activity of HRP was affected by the matrix.

The experiment for determining the optimal dilution ratio showed that the interference of matrix to the standard curve was negligible when the sample was properly diluted. However, too much dilution would eventually reduce the LOD of the sample. The experiments of TRFIA and the inhibition of HRP showed that the competitive reaction and color development of HRP could both be interfered for specific samples. Changing the tracer should be another option for controlling the matrix interference.

3.3. Analysis of spiked vegetable samples

The immunoassay established above was applied to the detection of carbofuran residue. In order to evaluate and correct the matrix interference caused by sample matrices, blank samples were initially extracted in mix solution (PBS/methanol = 9/1, v/v). The cabbage was spiked with carbofuran at 100 and 500 ng/ml. The residue was dissolved in 4 ml of mix solution (PBS/methanol = 9/1, v/v). Lettuce, carrot, winter fragrant-flowered garlic, bamboo shoot, green and soy bean were spiked with carbofuran at 50 and 250 ng/ml, respectively. The residue of bamboo shoot and green soy bean were dissolved in 1 ml of mix solution (PBS/methanol = 9/1, v/v) and the residue of lettuce, carrot and winter fragrant-flowered garlic were dissolved in 2 ml of mix solution (PBS/methanol = 9/1, v/v), respectively. The final solution was tested by the immunoassay directly. From Table 3, the mean recoveries were 85.24%, 101.8%, 103.6%, 90.52%, 106.9% and 94.08% for cabbage, lettuce, carrot, winter fragrant-flowered garlic, bamboo shoot and green soy bean, respectively. The samples free with carbofuran (i.e., non-spiked samples) were also included in the analysis as negative controls. Carbofuran concentrations below the detection limit of the assay were found in all the cases, and no false positive results were obtained. Since most coefficients of variation were around or below 20%, precision obtained for all samples meets the demand for a residue method.

3.4. Comparison with GC analysist

Forty eight lettuce samples were spiked with carbofuran at 0, 10, 50 and 250 ng/ml. Half of the samples were analyzed

Table 4
Analysis by ELISA and GC of lettuce spiked with carbofuran at 0, 10, 50 and 200 mg/kg

No.	Spiked concentration (mg/kg)	Detection of GC method (mg/kg)	Detection of ELISA method (mg/kg)
1	0	N.D.	N.D.
2	0	N.D.	N.D.
3	0	N.D.	N.D.
4	0	N.D.	N.D.
5	0	N.D.	N.D.
6	0	N.D.	N.D.
7	0.01	0.012	N.D.
8	0.01	0.015	N.D.
9	0.01	0.011	N.D.
10	0.01	0.016	0.003
11	0.01	0.015	0.006
12	0.01	0.019	0.002
13	0.05	0.056	0.038
14	0.05	0.062	0.058
15	0.05	0.067	0.048
16	0.05	0.061	0.044
17	0.05	0.056	0.087
18	0.05	0.043	0.035
19	0.25	0.286	0.319
20	0.25	0.272	0.294
21	0.25	0.239	0.270
22	0.25	0.311	0.319
23	0.25	0.229	0.365
24	0.25	0.274	0.393

by GC, while the remainder half were analyzed with the ELISA method. Results are given in Table 4. At low concentrations (0.01 and 0.05 mg/kg), the results of ELISA showed a tendency for slight underestimation. At high concentrations (0.25 mg/kg), it had a tendency of overestimation. Correlation between the GC results and the ELISA results showed a good linear relationship, with R^2 values 0.92.

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