Rapid Determination of Carbendazim Residues in Mushrooms by Immunosorbent Assay[1]

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Abstract. In this study, the testing time was reduced to 1 h through optimizing, and an LOD of 0.3±0.15 μg·L^{-1} and an IC_{50} of 2.7±0.3 μg·L^{-1} were achieved by the optimized CD-ELISA. The sample extraction of 1-fold acetonitrile followed by a 20-fold dilution in assay diluted buffer has proven to be sufficient to eliminate the influence of matrix. The CD-ELISA was validated further by comparing with the standard HPLC. The average recovery ratio of 89.7%～112.7% was obtained, and the coefficients of variation were less than 11.0%. In actual samples detection, except for Pleurotus eryngii, carbendazim was not detected, and other mushrooms were all detected, and only carbendazim in Volvariella volvacea exceeded the standard, and the over-standard rate was 16.7 %. In a word, the rapid, sensitive and efficient CD-ELISA for quantifing carbendazim in mushrooms was established in the study.

1 INTRODUCTION

As a broad spectrum benzimidazole fungicide, carbendazim is widely used in agricultural production with the characteristics of high efficiency, low toxicity and the inner attraction [1]. In addition to being applied to a wide range of cereals, fruits, vegetables, field crops etc, the carbendazim also was, until recently, routinely recommended for the control of cobweb disease of mushrooms [2]. The study found carbendazim had certain teratogenic effect, and was very stable in soil and water and difficult to decompose [3]. In 2011, the United States environmental protection agency (EPA) had limited the amount of carbendazim in orange juice to 100 μg·L^{-1}, 200 μg·L^{-1} was allowed in the EU, and the amount was not more than 3 mg·L^{-1} for Japan[4]. The EU allowed the MRLS of carbendazim in fresh mushrooms to be 0.1 mg·L^{-1}, the MRLS in the edible fungus is 1 mg·L^{-1} in Korea, Britain and China, and 3 mg·L^{-1} is for Japan[5].

In recent years, some immunochemical methods [6], such as enzyme linked immunosorbent assay (ELISA)[7-8] or Surface Plasmon Resonance(SPR) were widely used. Some sensitive and selective ELISAs for quantifing carbendazim in fruits and vegetables had been set up and applied[9-10]. In 2018, Guo, Linglinget al[11] had proposed a method based on the inhibition of plant esterase from pesticides and validated for the determination of carbendazim residues in aqueous samples. After optimization in pH, temperature, and detectiontime, a lower detection limit of 0.105 mu M was obtained in the linear range from 0.105 to 41.84 mu M. In 2015, Ana Ucl´es et al[12] had established an ELISA for detecting carbendazim, imazalil and thiabendazole residues in vegetable samples, and through 2h' testing, the 0.5 μg·kg^{-1}～13.8 μg·kg^{-1} of IC_{50} value of the calibration curve was obtained, after simple pretreatment, the method has a linearity range of 10 μg·kg^{-1} ～80 μg·kg. Above these researches, complex pretreatment method can't meet the needs of the rapid detection.

In the study, to develop a repid and sensitive ELISA for the carbendazim in mushrooms, some research carried out were as follows: the direct competitive ELISA(CD-ELISA) for detecting carbendazim was established with simple sample extraction and fast determination only costing 1h after optimizing experiments. The CD-ELISA could be applied to the rapid quantitative determination of carbendazim in food, especially in mushrooms.

2 EXPERIMENTAL

2.1 Chemical and Reagents

Carbendazim, benomyl, thiophanate, thiophanatem -ethyl, thiabendazole, 2-aminobenzimidazole, metat -olyl-N-methylcarbamate were obtained from Toronto Research Chemicals INC(TRC, Canada). 3,3′,5,5′-tetramethyl-

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benzidine (TMB) and hydrogen peroxide were obtained from Sigma Chemical Co. (St. Louis, MO). Bovine serum albumin (BSA) was obtained from Merck (Darmstadt, Germany). Protein A-Sepharose 4B was purchased from GE. Carbendazim antibody was from the laboratory.

2.2 Buffers and Solutions

Phosphate-buffered saline (PBS), phosphate buffered saline with 0.05% Tween-20 (PBST), assay diluted buffer(PBSB, PBS+0.1%BSA), coating buffer, blocking buffer, TMB substra -te solution, and stopping solution were used.

Stock standard solutions (1mg·mL^{-1}) of all pesticides were prepared by dissolving in methanol. The individual stock solutions were stored away from light at -20℃ in brown glass bottles.

2.3 Instrumentation

ELISA experiments were performed in 96-well microplates (Nunc, Roskilde, Denmark), a microplate washer was from Bio-Rad (Hercules, CA) and the absorbances were read with a Multiskan Spectrum from Thermo (Labsystems, Vantaa, Finland) in dual-wavelength mode (450 nm and 650 nm).

2.4 Preparation of Protein Conjugates

The immunogen, coating antigen and enzyme conjugate were prepared by conjugating the SBI to OVA and POD respectively by the active ester method described by Wang et al [13].

2.5 Direct ELISA

Polystyrene ELISA plates were coated with purified antibodies in 100 μL of CB and incubated for 3 h at 37℃. Plates were then washed three times with 10 mmol·L^{-1} of PBST, and unbound active sites were blocked with 200 μL of 0.5% skim milk power per well for 1 h. After the plate had been washed four times, competitive assays were performed by adding respectively 50 μL of standard or samples and 50 μL of SBI-POD conjugate diluted in PBS to each well and incubating for 1 h at room temperature. After washings of five times, 150 μL of TMB substrate solution was added to each well. And the enzymatic reaction was stopped after 15 min by adding 50 μL of 1.25 mol·L^{-1} H_2SO_4 per well, and the absorbance was read in the micro-plate reader in dual wavelength mode (450 nm as test and 650 nm as reference).

2.6 Assay Specificity

The carbendazim and its structural analogs were detected by an optimized ELISA method, and the inhibition curve was drawn to obtain the corresponding IC_{50} value, and the cross reaction was calculated. Cross-reactivity (CR) was calculated as follows:

$$CR = [IC_{50}(\text{carbendazim})/IC_{50}(\text{compound})] \times 100 \cdots\cdots\cdots\cdots\cdots\cdots I$$

2.7 Sample Preparation

Five different mushroom matrices: selection of shiitake, Agaricus bisporus, Pleurotus eryngii, Pleurotus and Volvariella volvacea (Bull.:Fr.) Sing to assess the accuracy of the CD-ELISA method, all samples from different markets. Each sample tested was determined to contain no carbendazim by HPLC prior to the spike recovery experiment. Firstly, the edible raw portion from the individual samples was minced to fine pieces by the food processor, then which were instantly freezed and stored at -20℃ until used.

2.8 Sample Extraction

For ELISA: 1 g of samples were spiked with carbendazim (except blank samples), and standed 30 min at room temperature, then 1 mL of acetic acid/acetonitrile (1:99, v/v) plus 0.4 g anhydrous Na_2SO_4 and 0.05 g NaAc were added acetonitrile was added and vortexed for 2 min. The mixture was centrifuged at 6000 rpm for 3 min, and the clear supernatant was collected and stored at -20℃, and diluted appropriately with buffer before ELISA analysis.

For HPLC: To validate the ELISA method, samples were analyzed by HPLC for comparison. The way of sample extraction was same as the report by Guo B et al[14].

2.9 HPLC Determination

HPLC determination was performed by Agilent 1200 series.Operating conditions were as follows: injection volume, 20 uL; flow-rate, 1.0 ml/min; test wavelength, 286 nm; the mobile phase, methanol/water (1:1, v/v).

3. RESULTS AND DISCUSSION

3.1 Optimization of working conditions

To establish working curve of CD-ELISA, some relevant reaction conditions such as ionic strength and organic solvent were optimized. A checkerboard method was used for optimization to get the lowest IC_{50} and maximum absorbance value ranging from 0.8 to 1.2 [15]. The results show that A^0 / IC_{50} at pH 7.4 is good.

The effect of acetonitrile concentration from 2.5% to 15% (v / v) on immunoassay performance was investigated. Figure 1 showed that acetonitrile could change the sensitivity of the assay. When the acetonitrile concentration was more than 5%, the detection sensitivity was lowered.

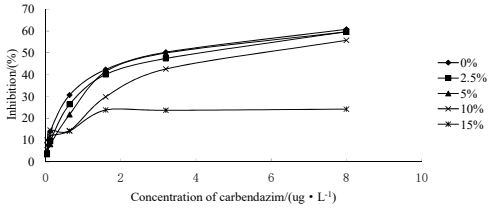

Fig.1. The effect of acetonitrile content in ELISA

The blocked and reaction time were optimized. No antibody was coated on plate to evaluate the effect. As

shown in table 1, the blocked assay had no influence on IC_{50} or absorbance value. So the blocked assay can be omitted in the study. Secondly, when the time of the competitive assays reached 45 min the absorbance value raised to about 0.85 A and kept stand, however, the IC_{50} also enlarged to about 2.8 μg·L-1. Thus, to obtain less IC_{50}, 30 min of the competitive assays were appropriate.

Tab.1. The optimization of time of competitive assays and blocked

Competitive Assays (min)	0 min		15 min	
	A^0(A)	IC_{50} (μg·L^{-1})	A^0(A)	IC_{50} (μg·L^{-1})
15	0.52	1.7	0.50	1.5
30	0.73	1.8	0.80	1.7
45	0.85	2.8	0.84	2.7
60	0.84	2.9	0.86	3.0

Note: The time of blocked were 0 min and 15 min; the time of competitive assays were 15, 30, 45, 60 min.

3.2 LOD and working range

The working curves of carbendazim were set up by optimizing the conditions related to CD-ELISA, as shown in Figure 2. The detailed conditions were as follows: coating antibody dose and enzyme conjugate dilution factors were 5 μg·mL^{-1} and 150-fold (diluted with PBS); the blocked was left out, adding 45 min of competitive assays, so only 1 h of the total reaction time was needed; Working standard solution of carbendazim was prepared by diluting the stock standard solution in Buffer (PBS + 0.5% Tween-20) and was stored at 4℃. The linear working range was 0.4 μg·L^{-1}～58.0 μg·L^{-1}, which is determined as the concentration causing 20–80% inhibition of color development [8], and IC_{15} and IC_{50} were 0.3±0.15 μg·L^{-1} and 2.7±0.3 μg·L^{-1}, respectively.

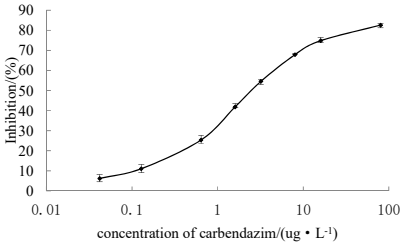

Fig.2. The standard curves of carbendazim in CD-ELISA

3.3 Assay Specificity

The cross reactivity of a set of analogs was determined using the optimized CD-ELISA system. (Tab.2.). From Table 2, we found that the antibody was specific to carbendazim highly. And because the active components of benomyl just was carbendazim.

Tab.2. The IC_{50} and CR of compounds

compounds	IC_{50}(μg·L^{-1})	CR(%)	compounds	IC_{50}(μg·L^{-1})	CR(%)
Carbendazim	2.5	100	2-Aminobenzimidazole	1200	0.2
Benomyl	4.0	62.5	Thiabendazole	1800	0.1
Thiophanate	71	3.5	Thiophanate methyl	150	1.7
Metolcarb	n	0			

3.4 Matrix effects and their removal

Matrix effects can induce some influence on the antigen–antibody interaction and enzymatic activity, and also lead to false positives. Bigger dilution ratio means smaller the influence of the matrix, but in consideration of LOD(limit of detection) of samples and sensitivity of the ELISA, smaller dilution ratio was needed. Some masking agent (BSA) or surfactant (Tween-20) was used to increase sensitivity.

As shown in Table 3 and 4, when the percentage of BSA raised to 0.1% the absorbance value(0.73A) and the IC_{50}(2.1 μg·L^{-1}) would not reduce and remain, 0.1% of BSA could eliminate the impact of non-specific binding. Thus, if the matrix was diluted with PBSB, the diluted ratio of matrix would be reduced from 40-fold to 20-fold. And the IC_{15} and IC_{50} of the standard curves(PBS+0.5% Tween-20) were 0.3 μg·L^{-1} and 3.0 μg·L^{-1}, so the limit of detection and sensitivity of the way was 6 μg·L^{-1} and 60 μg·L^{-1}.

Tab.3. The influence of content of BSA and Tween-20 on standard curves (PBS)

Buffer	PBS	BSA			Tween-20		
		0.01%	0.1%	1%	0.005%	0.05%	0.5%
IC_{50}(μg·L^{-1})	2.5	2.4	2.1	1.9	2.5	2.6	3.0
A_0(A)	0.86	0.80	0.73	0.73	0.71	0.6	0.51

Tab.4. The comparation of different sample pretreatment

way	IC_{15}(μg·L^{-1})	IC_{50}(μg·L^{-1})	LOD(μg·L^{-1})	Sensitivity(μg·L^{-1})
A	0.4	2.6	16	104
B	0.3	3.0	6	60

Note: A: standard curves(PBST), matrix curves (40-fold, with PBS); B: standard curves(PBS+0.5% Tween-20), matrix curves(20-fold, with PBS+0.1%BSA).

In the study, standard curve was prepared in PBS with different ratios of the Shiitake extract. As shown in Figure 3, the different percentage of the matrix also had different levels of impact on the sensitivity (5% and 10%). When the percentage was 2.5% the similar IC_{15} and IC_{50} were showed in matrix calibration curves and the standard curves, thus, extracts should be diluted at least 40-fold before analyzed accurately.

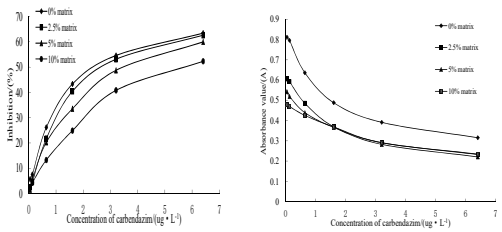

Fig. 3. The effect of matrix on inhibition curve and Absorbance curve

At the same time, a significant effect on the absorbance value could be observed when the matrix was added to the

buffer (2.5%, 5% and 10%), and even the matrix was diluted 40-fold, its absorbance value still was much less than the standard curves'(PBS). From Figure 4 and Table 3, only the percentage of Tween-20 was less than 0.005%, the Tween-20 could reduce the non-specific binding without affecting the sensitivity, and when the content of Tween-20 between 0.005% and 0.05% it had small impact on the sensitivity. As shown in Figure 4, the absorbance value decreased with the Tween-20 increasing. Thus, when the standard curves was prepared with PBST the absorbance value and inhibition of standard curves and matrix curves(40-fold,with PBS) were almost the same.

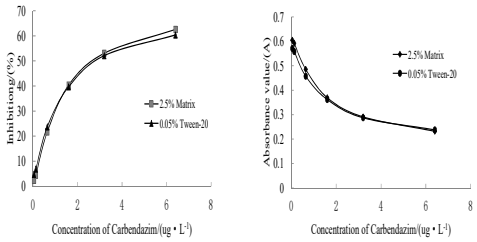

Fig.4. The comparation between the standard curves(0.05%Tween-20) and the matrixcurves (2.5%Matrix).

3.5 Recovery studies

In order to demonstrate accuracy and efficiency of the CD-ELISA, the recovery experiment by adding three-level standard samples, was carried out, as shown in Table 5, the average recovery ratio were 89.7%～112.7%, despite this slight overestimation or underestimation, which is found within the range accepted in an analytical methodology, and all of coefficients of variation (CV) were less than 11.0%, which indicated the developed sample extraction and the CD-ELISA of detecting carbendazim in food samples could be applied.

Tab.5. The recovery ratio of five mushrooms at three levels by CD-ELISA(n=3)

sample(n=3)	Spiked level（μg·L^{-1}）	Mean±SD（μg·L^{-1}）	Recovery(%)	CV (%)
Shiitake	10	9.7±1.1	97.0	11.0
	20	18.1±1.7	89.7	9.1
	40	41.3±2.1	103.2	4.2
Pleurotus eryngii	10	11.3±0.9	112.7	6.4
	20	20.8±1.5	104.0	5.3
	40	38.2±3.6	95.5	8.8
Agaricus bisporus	10	9.3±0.6	93.0	4.3
	20	22.3±2.0	111.5	9.3
	40	38.3±3.2	95.8	7.4
Pleurotus	10	9.2±0.7	92.0	4.5
	20	19.3±2.6	96.5	3.2
	40	40.2±0.5	100.5	6.3
Volvariella volvacea (Bull.:Fr.) Sing.	10	9.5±1.4	95.0	10.2
	20	19.8±1.3	99.0	5.5
	40	39.7±2.2	99.3	7.1

Note: All of the sample extraction were diluted 20-fold with the solution(PBS + 0.1% BSA) before analyzed.

Then the CD-ELISA was validated further by comparing with the standard HPLC, two different samples that did't contain carbendazim and benomyl were added respectively standard at three levels(10 μg•kg^{-1}, 20 μg•kg^{-1}, 40 μg•kg^{-1}), then extracted by their method, and analyzed simultaneously through the HPLC and the CD-ELISA. The monitoring results of same matrix of two methods of were linear. And the correlation coefficient (R^2) of HPLC and CD-ELISA was 0.9822, indicating two methods had high consistency.

3.6 Actual sample test results

Five mushrooms were purchased from six different markets and carbendazim was detected by established CD-ELISA and HPLC methods, respectively. As shown in Table 6 except for the carbendazim that was not detected by Pleurotus eryngii, other mushrooms were all detected, and only the carbendazim in Volvariella volvacea exceeded the standard, and the over-standard rate was 16.7%. At the same time, the detection result of CD-ELISA is consistent with the detection result of HPLC.

Tab.6. detection of carbendazim in actual samples (n=3)

Sample (n=3)	number	CD-ELISA			HPLC		
		Carbendazim residue (μg/kg)	detection rate (%)	excess rate (%)	Carbendazim residue (μg/kg)	detection rate (%)	excess rate (%)
Shiitake	1	32.3±1.4	66.7	0	31.4±1.2	66.7	0
	2	21.2±3.1			20.3±2.8		
	3	18.3±0.3			18.1±0.3		
	4	41.3±0.6			40.3±0.7		
	5-6	-			-		
Pleurotus eryngii	1-6	-	0	0	-	0	0
Agaricus bisporus	1	53.2±0.6	83.3	0	51.4±0.8	83.3	0
	2	51.3±2.1			50.8±2.3		
	3	61.3±1.8			63.2±1.3		
	4	50.8±0.7			52.1±1.2		
	5	72.4±0.9			71.8±2.6		
	6	-			-		
Pleurotus	1	69.2±2.7	33.3	0	68.2±2.1	33.3	0
	2	73.2±1.8			72.9±1.4		
	3-6	-			-		
Volvariella volvacea	1-3	109.5±1.4	50.0	16.7	108.3±1.6	50.0	16.7
	4	-			-		
	5	72.3±2.4			71.8±3.0		
	6	61.4±1.4			60.9±1.1		

Note:(-) means not detected.

4 CONCLUSIONS

In the study, a rapid and highly sensitive direct competitive ELISA immunoassay has been developed for detecting carbendazim in mushroom samples within 1 h. The standard working curves of CD-ELISA was set up after lots of optimization with the linear working range of 0.4 $\mu g \cdot L^{-1}$ ~58.0 $\mu g \cdot L^{-1}$, with the LOD of 0.3±0.15 $\mu g \bullet L^{-1}$ and the IC_{50} of 2.7±0.3 $\mu g \bullet L^{-1}$. The mushrooms were extracted with acetonitrile followed by dilution in buffer PBSB, which has been proven to be sufficient to eliminate the influence of matrix, and 89.7%~112.7% of the average recovery ratio was gained. The new CD-ELISA was further verified by comparing with conventional HPLC, and the correlation coefficient (R_2) of 0.9822 showed high accuracy and efficiency of the CD-ELISA, coupled with the detection limit of 6 $\mu g \bullet L^{-1}$, indicating the new CD-ELISA for rapid screening carbendazim in food could be applied widely.

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