Immunoassay for Cadmium Detection and Quantification[1]

GONG-LIANG LIU [*], JU-FANG WANG[*,2], ZHI-YONG LI [#], SHI-ZHONG LIANG [*], AND XIAO-NING WANG [*]

[*]*School of Biosciences & Bioengineering, South China University of Technology, Guangzhou 510006, Guangdong, China;* [#]*Guangdong Inspection and Quarantine Technical Center, Guangzhou 510623, Guangdong, China*

Objective To detect cadmium in environmental and food samples by graphite furnace atomic absorption spectroscopy (GFAAS) and inductively coupled plasma atomic emission spectroscopy (ICPAES). **Methods** An indirect competitive enzyme-linked immunosorbent assay (IC-ELISA) was developed based on a cadmium-specific monoclonal antibody. IC-ELISA for cadmium in environmental and food samples was evaluated. **Results** IC-ELISA showed an IC_{50} of 45.6 μg/L with a detection limit of 1.95 μg/L for cadmium, and showed a mean recovery ranging 97.67%-107.08%. The coefficient of variations for intra- and interassay was 3.41%-6.61% and 4.70%-9.21%, respectively. The correlation coefficient between IC-ELISA and GFAAS was 0.998. **Conclusion** IC-ELISA can detect and quantify cadmium residue in environmental or food samples.

Key words: Cadmium; ITCBE; Monoclonal antibody; ELISA; Food; Environmental samples

INTRODUCTION

Some environmental toxic substances, such as cadmium, have a biological half-life of more than 10 years once accumulated in human body. Depending upon the route of exposure, high cadmium level can damage the kidney or lung[1-2]. Thus, rapid detection of environmental cadmium ion is critical for farm industry and human health. Current methods for detecting cadmium in water, vegetables and many other farm products include graphite furnace atomic absorption spectroscopy (GFAAS) and inductively coupled plasma atomic emission spectroscopy (ICPAES). Although they are sensitive and reliable, they are labor-intensive, time-consuming and expensive[3-4]. Immunoassays such as enzyme-linked immunosorbent assay (ELISA) have recently emerged as an alternative to the traditional methods, because they are usually less time-consuming, inexpensive, simple, specific and reasonably portable[5-6]. Moreover, immunoassays are able to analyze many samples simultaneously[7-8]. In this study, we generated a highly specific monoclonal antibody against cadmium ion. Based on this monoclonal antibody, we developed an indirect competitive enzyme-linked immunosorbent assay (IC-ELISA), which is sensitive and specific, and therefore can be used in detecting cadmium residues in environmental and food samples.

MATERIALS AND METHODS

Reagent

All metal ions were under atomic absorption standards. Cd^{2+}, Pb^{2+}, Hg^{2+}, Zn^{2+}, Na^{+}, Ca^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, Cu^{2+}, and Mg^{2+} (1000 μg/mL in 2% HNO_3) were purchased from National Research Center of Standard Materials (Beijing, China). 1-(4-isothiocyanobenzyl) ethylenediamine-N, N, N′, N′-tetraacetic acid (ITCBE) was purchased from Dojindo Laboratories (Shanghai, China). Ultrafiltration centrifuge tubes were products of Millipore Co (Bedford, MA, USA). Keyhole limpet

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[2]Correspondence should be addressed to Ju-Fang WANG. Tel: 86-20-20-39380626. Fax: 86-20-20-39380626. E-mail: jufwang@scut.edu.cn

Biographical note of the first author: Gong-Liang LIU, male, born in 1980, a candidate for doctor's degree at School of Bioscience and Bioengineering, South China University of Technology, majoring in fermentation engineering.

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hemocyanin (KLH), bovine serum albumin (BSA), primary antibodies against mouse IgG1, IgG2a, IgG2b, IgG3, IgM, IgA, secondary antibody goat anti-mouse IgG/IgM coupled to horseradish peroxidase, 3, 3′, 5, 5′-tetramethylbenzidine (TMB), Freund's complete and incomplete adjuvants, ethylenediamine tetraacetic acid (EDTA) and antibody isotyping kit were purchased from Sigma Chemical Co (St. Louis, MO, USA). HiTrap IgM purification column was purchased from GE Co (Rockford, IL, USA). BCA protein assay kit and TNBS were purchased from Pierce Co (Rockford, IL, USA). Wheat flour, rice flour, tea, spinach, and bush branches and leaves were obtained from Guangdong Inspection and Quarantine Technical Center. Electroplating waste water was collected from a polishing factory in Guangzhou, Guangdong. Apple juice was purchased from a supermarket in Guangzhou.

Preparation of Cadmium-protein Conjugates

Cadmium and ITCBE in PBS (0.1 mol/L，pH 10.0) were mixed at an equal molar ratio. The pH of solution was adjusted to 7.4 with ammonia and the solution was incubated for 24 h at 25 ℃[9]. An equal amount of BSA or KLH was then added to Cd-ITCBE solution and the pH of solution was adjusted to 9.0 with ammonia before an additional 24-hour incubation at 25 ℃[10]. Small unconjugated molecules were removed by buffer exchange with centricon-30 micro-concentrators. The conjugates were washed with HEPES (0.1 mol/L, pH 7.4)[11]. ITCBE-BSA was also prepared following the similar procedure as previously described[12]. The concentration of protein conjugate was determined by Pierce BCA protein assay.

Monoclonal Antibody Production

Eight 6-week old BALB/c mice were immunized with 50 μL of Cd-ITCBE-KLH (1 mg/mL, in pH 7.4 HEPES) on days 0, 21, and 42. Blood samples were collected from mouse tails on days 21, 42, and 49. Freund's complete adjuvant and incomplete adjuvant were used for primary and secondary injections, respectively. In the third injection, immunogen was mixed with an equal volume of HEPES. The former two injections were intraperitoneal and the later one was subcutaneous. Mice with the highest antibody response were given a final intraperitoneal boost with 50 μg of Cd-ITCBE-KLH for 3 days prior to fusion. Mouse spleen cells were harvested, washed in RPMI 1640 medium, and fused with SP2/0 myeloma cells. Hybridomas that produce antibodies against cadmium-chelate were subcloned twice by limiting dilution until stable clones were established. About 10-15 days after hybridoma cell (1.27×10^6) injection, ascites fluid was obtained from the intraperitoneal cavity of inoculated mice. Monoclonal antibody (MAb) in ascites was isolated by affinity chromatography using HiTrap HP IgM purification column. Protein concentration of the purified antibody was determined with a BCA protein assay kit.

Optimal Concentrations of ELISA Coating Antigen and Antibody

Cd-ITCBE-BSA was diluted into HBS (137 mmol/L NaCl, 3 mmol/L KCl, and 10 mmol/L HEPES, pH 7.4) at concentrations of 1, 2, 4, 8, and 16 μg/mL and coated onto microwell plates by incubation at 4 ℃ overnight. After it was washed and blocked with 1% BSA, MAb 3A9D9G7 was serially diluted in HBS through the wells of plates and incubated in the microwells. After 1 h at 37 ℃, the plates were washed again and incubated with goat anti-mouse IgM horseradish peroxidase conjugate and TMB. The enzymatic reaction was stopped by 2 mol/L H_2SO_4 and absorbance was measured at 450 nm.

Indirect Competitive Enzyme-linked Immunosorbent Assay (IC-ELISA)

Microwell plates were coated with Cd-ITCBE-BSA at 4 ℃ overnight, washed three times with HBS-Tween (274 mmol/L NaCl, 6 mmol/L KCl, 20 mmol/L HEPES and 0.05% Tween-20, pH 7.4), and then blocked with 1% BSA in HBS. MAb 3A9D9G7 was mixed with an equivalent volume of HBS or HBS containing 10^5, 10^4, 5×10^3, 10^3, 200, 100, 80, 40, 20, 10, 1, 0.1 μg/L of cadmium at 37 ℃ for 1 h, and the mixed solution was added to each well. After incubation at 37 ℃ for 1 h, the plates were washed three times, goat anti-mouse IgM horseradish peroxidase conjugate was added to each well and then incubated at 37 ℃ for 45 min. After incubation, the plates were washed three times. TMB was then added to each well and the plates were incubated at 37 ℃ for 30 min. The enzymatic reaction was stopped by adding 2 mol/L H_2SO_4 and absorbance was measured at 450 nm.

Determination of Cross-reactivity

Cross-reactivity for several metal-chelates including Cd-EDTA, Pb-EDTA, Hg-EDTA, Zn-EDTA, Na-EDTA, Ca-EDTA, Fe-EDTA, Mg-EDTA, Mn-EDTA, Cu-EDTA, or K-EDTA was tested by IC-ELISA[13]. Values for IC_{50} were those that gave the best fit to the following equation: $A=A_0-[Cd]\ (A_0-A_1)/\{IC_{50}+[Cd]\}$[14], where A is the signal

at a definite known concentration of cadmium, A_0 is the signal in the absence of cadmium, A_1 is the signal at the saturating concentration of cadmium, and IC_{50} is the cadmium concentration that produces a 50% inhibition of the signal. The cross-reactivity values were calculated as % CR =100× [IC_{50} (Cd-EDTA) / IC_{50} (other metal-chelate)][15].

Analysis of Spiked Wheat Sample

Wheat flour (5 g) was added to 20 mL ultrapure nitric acid overnight. The mixture was heated to boiling until it was solved completely. The liquid was centrifuged for 10 min at 8 000 rpm. The supernatant was then mixed (9:1) with 10×HBS. The pH of solution was adjusted to 7.4 with saturated KOH. With the titration of GFAAS, varying concentrations of atomic absorption grade cadmium were added into the wheat solution to give final cadmium concentrations of 5, 10, 20, 40, and 60 μg/L. The wheat solution with spiked cadmium was analyzed with IC-ELISA.

Analysis of Environmental and Food Samples

A solid sample was added to ultrapure nitric acid overnight. The mixture was heated to boiling until solved completely. The liquid was centrifuged for 10 min at 8 000 rpm. The supernatant was then mixed (9:1) with 10×HBS. The pH of solution was adjusted to 7.4 with saturated 10 mol/L KOH. The solution after pretreatment was then spiked with different concentrations of cadmium and analyzed by IC-ELISA. A liquid sample was centrifuged for 10 min at 8 000 rpm. The supernatant was then mixed (9:1) with 10×HBS and the pH of solution was adjusted to 7.4. Both the solid and liquid samples were analyzed by IC-ELISA and GFAAS. The coefficient correlation of results between IC-ELISA and GFAAS was calculated with excel.

RESULTS

Preparation of Antibodies against Cadmium-chelate

One mice with the highest serum antibody titer was selected from those immunized with Cd-ITCBE-KLH for cell fusion. After fusion and cloning, the potential positive clones were initially screened. As shown in Fig. 1, the supernatant of seven positive clones showed a strong response to Cd-ITCBE-BSA with a minimal response to ITCBE-BSA.

To determine whether the antibodies in the supernatant recognize Cd-EDTA, antigen inhibition ELISA was performed. As shown in Fig. 2, Cd-EDTA

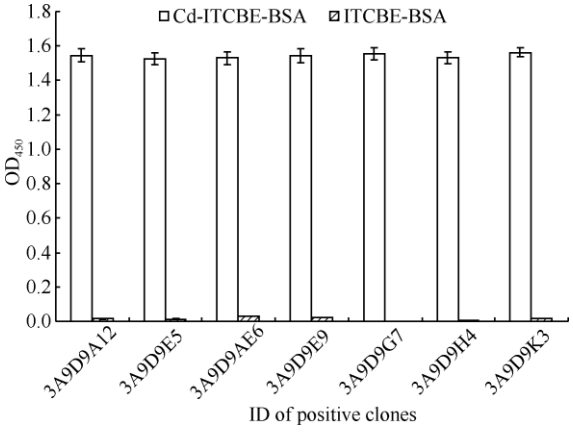

FIG. 1. Supernatant reactivity of subcloned hybridoma. Hybridomas that produced antibodies against cadmium-chelate were subcloned twice by limiting dilution. Antibody response was determined by indirect ELISA as described in the section "Materials and Methods". Each point represents the mean of triplicate ± SD.

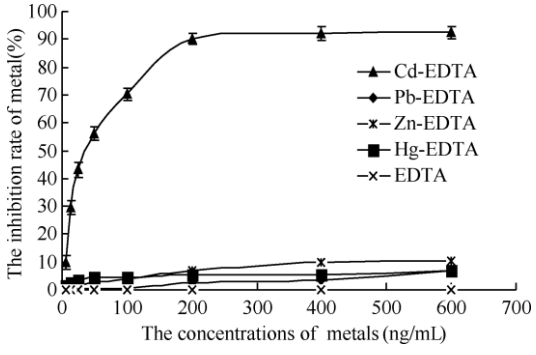

FIG. 2. Inhibitory effect of different metal-chelates on the binding of mAb 3A9D9G7 to immobilized Cd-ITCBE-BSA. The equation used to calculate the inhibition ratio (IR) is IR = (A-B) / A × 100%, where A is OD450 of the microwell without any metal-chelate, B is OD450 of the microwell with one of the following metal-chelates: Cd-EDTA, Pb-EDTA, Hg-EDTA and Zn-EDTA in competitive inhibition ELISA. Each point represents the mean of triplicate ± SD.

exhibited the highest inhibition (91%) with the concentration of 200 ng/mL cadmium ion, while other metal-chelates, including Pb-EDTA, Zn-EDTA. and Hg-EDTA, had scarcely any inhibitory effects in this assay. All these metal chelates had an inhibition rate of less than 3% at the concentration of 200 ng/mL, and less than 6% even at the highest tested concentration of 600 ng/mL. The inhibition rate with EDTA (20 mmol/L) alone was below 0.1% (detail not showed). These results demonstrate that mAb

3A9D9G7 could recognize Cd-EDTA with a high specificity. Therefore, this clone was selected for further experiments. MAb 3A9D9G7 was an IgM with kappa light chain as determined by isotype typing.

Optimum Concentrations of Coating Antigen and Antibody for IC-ELISA

To establish IC-ELISA, the optimum concentrations of MAb 3A9D9G7 and coating antigen Cd-ITCBE-BSA were determined by checkerboard titration. As shown in Fig. 3, at 4 μg/mL of Cd-ITCBE-BSA in HBS, increasing the concentration of MAb 3A9D9G7 from 40 to 200 ng/mL did not significantly elevate the absorbance ranging 0.950-1.015. In addition, at a fixed concentration of 50 ng/mL for MAb 3A9D9G7, dilution of Cd-ITCBE-BSA from 4 μg/mL to 1 μg/mL resulted in a absorbance decrease from 0.950 to 0.315. These results thus indicated that the most optimal concentrations for MAb 3A9D9G7 and Cd-ITCBE-BSA in IC-ELISA were 40 ng/mL and 4 μg/mL, respectively.

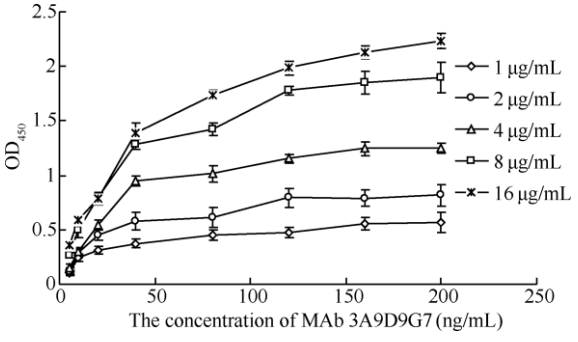

FIG. 3. Optimal concentrations of antibody and coating antigens for IC-ELISA as determined by a checkerboard assay. Cd-ITCBE-BSA was coated onto the microwells at the concentrations of 1, 2, 4, 8, and 16 μg/mL. Serial dilutions of MAb 3A9D9G7 were allowed to bind to the antibody-coated plate. The assay was performed as described in the section "Materials and Methods". Each point represents the mean of triplicate ± SD.

Sensitivity of IC-ELISA

The sensitivity of IC-ELISA to cadmium-chelate detection was determined. The assay's sensitivity determined by identifying the limit of detection was 1.953 μg/L. As shown in Fig. 4, quantification of cadmium-chelate was carried out by IC-ELISA with MAb 3A9D9G7. These results indicated that MAb 3A9D9G7-based IC-ELISA for cadmium-chelate detection was highly sensitive and could be used in detecting the concentration of cadmium in water or other samples.

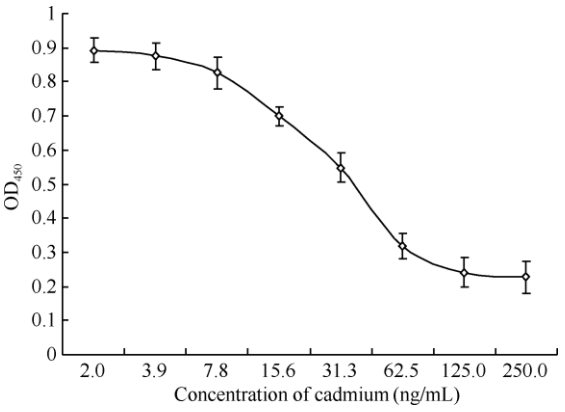

FIG. 4. Standard curve of IC-ELISA for cadmium-chelate detection. An IC-ELISA for detecting cadmium-chelate was established based on MAb 3A9D9G7, and the standard curve was developed by a serial diluted cadmium-chelate. The IC50 value was 45.6 μg/L, and the detection limit was 1.953 μg/L. Each point represents the mean of three replicates ± SD.

Recovery and Cross-reactivity of IC-ELISA

As shown in Table 1, recoveries of cadmium from the wheat spiked with different concentrations of cadmium were determined by IC-ELISA based on the values obtained from the standard curves. The mean recovery rate was 97.67%-107.08%, and the coefficient of variability ranged from 4.19% to 8.37%, suggesting that IC-ELISA could accurately detect cadmium in spiked wheat samples.

TABLE 1

Recovery of Cadmium from Spiked Wheat Sample*

Wheat Sample with Fortified Cadmium (μg/L)	Cadmium Detected (μg/L)	Mean Recovery (%, *n*=3)	CV (%)
5	5.33 ± 0.40	106.67	7.58
10	9.77 ± 0.76	97.67	7.83
20	20.10 ± 1.68	100.50	8.37
40	42.83 ± 1.79	107.08	4.19
60	61.90 ± 2.95	103.17	4.78

Note. *Microwells were coated with 4 μg/mL of Cd-ITCBE-BSA. Varying concentrations of atomic absorption grade cadmium were added into the wheat pretreated as described in the Materials and Methods to give final cadmium concentrations of 5, 10, 20, 40, and 60 μg/L by titration of GFAAS. IC-ELISA was performed as described in the section "Materials and Methods". Each value represents the mean of three replicates ± SD.

The specificity of assay for cadmium was determined in a mixed sample in the presence of different metal ions (Cd^{2+}, Pb^{2+}, Hg^{2+}, Zn^{2+}, Na^{+}, Ca^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, Cu^{2+}, or Mg^{2+}) at the concentration of 10^{-1}-10^{5} μg/L. With the chelating of EDTA, all metal ions were converted to metal-chelate complexes. As shown in Table 2, the cross reactivity exhibited by any of the tested metal-chelates was <0.9%. Furthermore, Pb-EDTA and Hg-EDTA showed a cross-reactivity of 0.80% and 0.73%, respectively, in our study, which was lower than the reported data[16].

TABLE 2

Cross-reactivity of MAb 3A9D9G7 to Metal-chelate*

Metal-chelates	IC_{50} (μg/L)	Cross-reactivity (%)
Cd-EDTA	45.6	100
Pb-EDTA	5712.14	0.80
Hg-EDTA	6230.39	0.73
Zn-EDTA	6573.88	0.69
Mg-EDTA	5246.84	0.87
Ca-EDTA	5260.11	0.87
Mn-EDTA	15478.92	0.29
Cu-EDTA	19267.69	0.24
Fe-EDTA	7944.6	0.58
K-EDTA	7047.83	0.65
Na-EDTA	5851.17	0.78

Note. *Microwells were coated with 4 μg/mL of Cd-ITCBE-BSA. The specificity of assay for cadmium was determined in samples in the presence of different metal-chelates by IC-ELISA. IC_{50} and CR were calculated as described in the section "Materials and Methods".

Determination of IC-ELISA Reliability by Intra- and Interassay

To determine the reproducibility of results, intra- and inter-assay of IC-ELISA in spiked wheat samples was performed. The inter-assay was assessed by analyzing 6 replicates of samples in a single run, and the intraassay was assessed by analyzing the same sample, as a triplicate, in two separate runs. As shown in Table 3, the coefficients of variations for intra- and inter-assay were 3.41%-6.61% and 4.70%-9.21%, respectively, suggesting that IC-ELISA could reliably detect cadmium.

Comparison of IC-ELISA with GFASS

Because GFASS is a conventional method used to detect cadmium in environmental and food samples, we compared IC-ELISA with GFASS in testing a variety of samples. As shown in Table 4, in experiments detecting cadmium residues in environmental and food samples, including electroplating waste water, bush branches and leaves, apple juice, rice flour, wheat flour, tea, and spinach, the overall coefficient of variations of IC-ELISA was 3.54%-9.63%. The coefficient of correlation of results obtained by IC-ELISA and GFAAS was 0.998.

TABLE 3

Reliability of IC-ELISA for Detecting Cadmium Determined by Inter- and Intra-assay

Wheat Sample with Fortified Cadmium (μg/L)	Inter-assay, n=6		Intra-assay, n=6	
	Cadmium Concentration (μg/L)	CV (%)	Cadmium Concentration (μg/L)	CV (%)
5	5.38±0.50	9.21	4.91±0.32	6.61
10	9.93±0.66	6.67	10.07±0.56	5.61
20	20.35±1.17	5.77	19.85±1.22	6.17
40	42.54±2.00	4.70	41.00±1.40	3.41
60	62.97±3.22	5.11	61.00±2.40	3.93

Note. *The concentrations of cadmium in fortified wheat samples were determined by IC-ELISA. The inter-assay was assessed by analyzing six replicates of each sample in a single run, and the intra-assay was assessed by analyzing the same sample, as triplicates, in two separate run.

TABLE 4

Comparison of IC-ELISA with GFAAS for Analysis of Cadmium Residues in Environmental and Food Samples

Samples	CI-ELISA	CV (%)	GFAAS
Electroplating Waste Water	601.42±21.28 μg/L	3.54	655.44±14.74 μg/L
Bush Branches and Leaves	344.69±23.92 μg/kg	6.94	320.72±40.84 μg/kg
Apple Juice	3.37±0.32 μg/L	9.50	3.59±0.09 μg/L
Rice Flour	96.38±7.78 μg/kg	8.07	87.78±5.04 μg/kg
Wheat Flour	21.80±2.10 μg/kg	9.63	18.48±4.21 μg/kg
Tea	56.87±3.91 μg/kg	6.88	62.22±4.28 μg/kg
Spinach	169.62±12.78 μg/kg	7.53	150.24±25.08 μg/kg

Note. *All the samples were pretreated as described in the section "Materials and Methods". Each value represents the mean of three replicates ± SD.

DISCUSSION

Cadmium, like most of other small and simple inorganic molecules, is non-immunogenic by itself and lacks a functional group for protein coupling. Therefore, steps to conjugate it to carrier proteins are critical in the preparation of antigens for immunization and microplate coating. In this study, ITCBE as a bifuncional chelator was used for conjugating carried protein and chelating cadmium ion. Therefore, hapten Cd-ITCBE was conjugated to KLH as an immunogen for mouse immunization, and Cd-ITCBE was conjugated with BSA as a coating antigen. ITCBE-BSA was used for screening non-specific clones. Successful preparation of these conjugates is the first critical step for generating antibodies against cadmium-chelates.

MAb 3A9D9G7 we generated was highly specific with little or no cross-reactivity to other metal-chelates, including Pb-EDTA, Zn-EDTA, and Hg-EDTA. Furthermore, MAb 3A9D9G7 showed no cross-reactivity to EDTA (20 mmol/L). With addition of enough EDTA, the metals were chelated and the concentration of metal chelates could then be analyzed by IC-ELISA. The concentration of metal chelates depending on the concentration of metals and MAb 3A9D9G7 could not recognize metal-free chelator, thus allowing the use of antibody in immunoassay of environmental cadmium. In addition, MAb 3A9D9G7-based IC-ELISA was capable of detecting cadmium ion as low as 1.953 μg/L. The recovery rate of cadmium from wheat samples was 97.67%-107.08%, which was comparable to the reported data[16]. Moreover, the coefficients of intra- and inter-assay were 3.41%-6.61% and 4.70%-9.21%, respectively, indicating that IC-ELISA based on MAb3A9D9G7 was a reliable assay for cadmium residue in food samples.

Antigen-antibody interaction in immunoassays can be affected by various substances in solid samples with complex matrices, which is a great challenge to immunoassays for cadmium residues in solid samples. In this study, solid samples were ground into flour and dissolved in ultrapure nitric acid in order to reduce matrix interferences, thus allowing all solid samples to be converted into a soluble form of nitrate. With addition of EDTA, the metals were chelated and the concentration of metal chelates could then be analyzed by IC-ELISA. We, therefore, were able to use IC-ELISA to detect cadmium residues not only in water samples, but also in a wide variety of solid samples (Table 4). Furthermore, we compared IC-ELISA with conventional GFASS for detecting cadmium residues in environmental and food samples. The coefficient of IC-ELISA was 3.54%-9.63% and the overall coefficient correlation of results obtained by IC-ELISA and GFAAS was 0.998. In conclusion, IC-ELISA based on MAb3A9D9G7 can be used in detecting and quantifying cadmium residues in environmental and food samples.

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