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Development of an Enzyme-Linked Immunosorbent Assay To Detect Benzylpenicilloic Acid, a Degradation Product of Penicillin G in Adulterated Milk

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To avoid detection of penicillin G, some producers/merchants illegally add β-lactamase to milk to degrade it into benzylpenicilloic acid (BPA). This degradation product can cause allergic reactions in humans and, therefore, is a potential hazard to human health. To detect BPA in milk, we established a rapid direct competitive enzyme-linked immunosorbent assay (ELISA) with an IC_{50} of 0.32 $\pm$ 0.01 μg L^{-1}, and a detection limit of 0.030 $\pm$ 0.002 μg L^{-1}. Matrix effects in the milk samples were easily eliminated by centrifugation and dilution. Recoveries were 72.75−93.25%. Also heat treatments of raw milk did not affect the detection of the BPA. To validate BPA-ELISA, the spiked milk samples were analyzed by ELISA and LC−MS; the results showed a strong correlation (r^2 = 0.99). Incurred samples obtained from Tianjin Entry−Exit Inspection and Quarantine Bureau (TJCIQ) were tested by BPA-ELISA. The results showed an almost 100% correlation (r^2 = 0.99) with the results supplied by the TJCIQ.

KEYWORDS: Benzylenicilloic acid; degradation product of penicillin; antibody; ELISA

INTRODUCTION

Penicillin G is an inexpensive and highly effective antibiotic that is commonly used in veterinary medicine to prevent various bacterial infections in cows. However, penicillin residues in milk pose a potential threat to public health as they can cause allergic reactions in humans. Maximum residue limits (MRLs) of 4 μg kg^{-1} in milk have been established in China and the European Union (EEC 2377/90) to monitor penicillin residues.

Recently, a new product has appeared on the market which has β-lactamase as its main constituent. The β-lactamase breaks the β-lactam ring and the amide bond of penicillin G, producing penicilloic acid (*1*, *2*). Some producers and/or merchants illegally add β-lactamase to milk to degrade penicillin G, thereby avoiding detection of these residues. However, benzylpenicilloic acid (BPA), which is the main degradation product, can still cause an allergic reaction. Thus, milk containing β-lactamase poses risks to human health.

Detection methods have been developed to screen for penicillin G, including immunological assays (*3*−*6*), physicochemical methods (*7*−*9*), inhibition tests, and receptor assays. However, none of these tests detect BPA. Considering the allergenicity of BPA and the high rates of milk consumption, it is urgent to develop a rapid and efficient method to detect this contaminant.

Compared with physicochemical methods, ELISA can simultaneously detect contaminants in a large number of samples, with only simple pretreatments. The aim of this study was to establish a rapid direct competitive enzyme-linked immunosorbent assay to detect benzylpenicilloic acid in raw milk and milk powder.

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MATERIALS AND METHODS

Chemicals and Equipment. Keyhole limpet hemocyanin (KLH), horseradish peroxidase (HRP), ovalbumin (OVA), penicillin G potassium salt, benzylpenicilloic acid, *N*-hydroxysuccinimide (NHS), *N*,*N*-dicyclohexylcarbodiimide (DCC), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl), 3,3′,5,5′-tetramethylbenzidine (TMB), and hydrogen peroxide were purchased from Sigma-Aldrich (St. Louis, MO). Bovine serum albumin (BSA) was obtained from Merck (Darmstadt, Germany). Protein A-Sepharose 4B was purchased from Amersham Biosciences (Uppsala, Sweden). Purified water was obtained using a Millipore Milli-Q water system (Millipore, USA). Polystyrene 96-well microplates were from Nunc (Rockilde, Denmark). Immunoassay absorbance was read with a Multiskan Spectrum purchased from Thermo (Vantaa, Finland) in dual-wavelength mode (450−650 nm).

Solutions. The solutions used in these experiments were as follows: phosphate-buffered saline (1× PBS, 38.4 mmol L^{-1} $Na_2HPO_4 \cdot H_2O$, 11.5 mmol L^{-1} $NaH_2PO_4 \cdot H_2O$, 154 mmol L^{-1} NaCl, pH 7.5); phosphate-buffered saline (1× PBS, 3.26 mmol L^{-1} $Na_2HPO_4 \cdot H_2O$, 46.7 mmol L^{-1} $NaH_2PO_4 \cdot H_2O$, 154 mmol L^{-1} NaCl, pH 5.7); phosphate-buffered saline with 0.05% Tween 20 (PBST); coating buffer (CB, 50 mmol L^{-1} sodium carbonate buffer, pH 9.6); blocking buffer (1% BSA in 1× PBS); TMB substrate solution (prepared by adding 3.3 mg of TMB in 250 μL of DMSO to 25 mL of phosphate-citrate buffer (0.1 mol L^{-1} citric acid +0.2 mol L^{-1} Na_2HPO_4, pH 4.3) containing 3.25 μL of a 30% H_2O_2 solution); and terminating buffer (2.5 mol L^{-1} H_2SO_4 in DDW).

Immunogen Synthesis. Penicillin G potassium salt (74.4 mg, 0.2 mmol), dissolved in 1 mL of distilled water, was acidified by addition of 2 mol L^{-1} HCl to form penicillin G, which precipitated as a white solid. The penicillin G was washed several times with distilled water to neutralize it, and then freeze-dried. The chemical structures of penicillin G and benzylpenicilloic acid are shown in **Figure 1**.

The active ester was prepared as follows: Penicillin G (0.388 g, 1.16 mmol) and NHS (0.168 g, 1.45 mmol) were dissolved in freshly distilled tetrahydrofuran (THF, 15 mL). Then, *N*,*N*-dicyclohexylcarbodiimide

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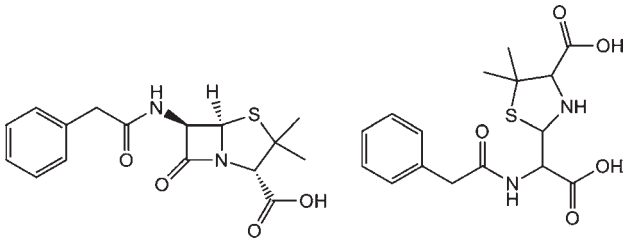

Figure 1. Chemical structure of penicillin G (left) and benzylpenicilloic acid (right).

(DCC, 0.3 g, 1.45 mmol) was added and the mixture was stirred at room temperature for 10 h under nitrogen. The reaction mixtures were filtered to remove the dicyclohexylurea precipitate. The filtered solution was evaporated under reduced pressure, and purified by column chromatography (ethyl acetate/petroleum ether = 3:2).

The active esters dissolved in dried dimethylformamide (DMF) were added dropwise to carrier protein in precooled buffer solution (50 mM K_2HPO_4, pH 9.1). After 8 h of gentle stirring at 4 °C (under the condition, penicillin G was degraded into BPA), the reaction mixture was dialyzed against PBS for 3 days at 4 °C (8000−14000 MWCO). Aliquots of the conjugates of BPA-KLH were stored at −20 °C.

Antibody Production. Antibodies were induced using BPA-KLH as the immunogen. Two New Zealand white rabbits were immunized as described by Wang et al. (*10*). Immunoglobulin G (IgG) in the antisera was purified by Protein A-Sepharose affinity chromatography.

Preparation of Enzyme Conjugate. Penicillin G potassium salt (10 mg, 0.027 mmol) dissolved in 150 μL of 0.1 N NaOH was incubated at 37 °C for 3 h to form benzylpenicilloic acid. Then, the solution was adjusted to pH 6.0 using 2 mol L^{-1} HCl. NHS (5 mg, 0.043 mmol) dissolved in 50 μL of PBS (pH 5.7) and EDC (10 mg, 0.052 mmol) were added to the benzylpenicilloic acid solution, and the mixture was stirred at room temperature for 5 h. The mixture was then centrifuged to remove the precipitate. The supernatant was added dropwise to carrier protein in precooled buffer solution (50 mM K_2HPO_4, pH 9.1). After 8 h of gentle stirring at 4 °C, the reaction mixture was dialyzed against PBS for 3 days at 4 °C. Conjugates of BPA-HRP were stored at −20 °C with glycerol.

Preparation of Standard Solution. To construct the calibration curve, a standard solution of BPA in PBS (pH = 5.7) at concentrations of 0.016−16 μg L^{-1} was freshly prepared by stepwise dilution in glass tubes.

Microwell Assay. Microwell plates were coated with purified anti-BPA IgG at 1 μg per well in 100 μL of coating buffer and incubated overnight at room temperature. Plates were then washed three times with PBST washing solution, and unbound sites were blocked with 200 μL of 1% BSA/PBS per well for 1 h at room temperature. After the plate was blotted dry, 100 μL of 10% methanol in water was added to control and blank wells and 50 μL of standard solution or sample extracts was applied to the allocated wells. Then, 50 μL of enzyme conjugate was immediately added to each well, except for the blank wells, and the mixture was incubated for 1 h. The wells were washed five times, then 100 μL of substrate solution was added to each well and the plate was incubated at room temperature for 30 min. Then, the reaction was stopped by adding 50 μL of termination buffer, and absorbance was determined using a microplate reader.

Optimization of BPA-ELISA. We optimized assay conditions, including concentrations of antibody (0.5, 1.0, 1.5 μg $well^{-1}$) and enzyme conjugate (1:1000, 1:2000, 1:4000, 1:6000), ionic strength (10−50 mmol L^{-1}), and pH of the sample dilution buffer (pH = 5.7, 6.5, 7.5, 8.0).

Cross-Reactivity Determination. To determine the specificity, we investigated cross-reactivity of the anti-BPA antibody with various other penicillins (penicillin G, ampicillin, amoxicillin, cloxacillin, dicloxacillin, oxacillin), and penicilloic acids of penicillins and ceftiofur. Cross-reactivity was determined according to the following equation:

$$CR = 100 \times IC_{50}(BPA)/IC_{50}(\text{cross-reacting compounds})$$

Sample Preparation for BPA-ELISA. We evaluated the performance of BPA-ELISA using raw milk and milk powder samples. All food samples were purchased from local markets. Before conducting spiking and recovery studies, we confirmed that the test samples did not contain BPA by LC−MS analysis. We used various dilution methods, depending on the sample. The concentrations of BPA used to spike the samples were based on the MRL of penicillin G.

Raw Milk. For spiking raw milk, 20 mL of sample was spiked with BPA (4, 12, and 36 μg kg^{-1}). The mixtures were centrifuged for 25 min at 12000*g* at room temperature. The fat layer was discarded, and the upper liquid was diluted 40 times with PBS (pH = 5.7) and then used for analysis.

Milk Powder. For spiking milk powder, 2 g samples were spiked with BPA at three different levels (16, 100, and 200 μg kg^{-1}). The samples were then thoroughly dissolved in 16 mL of distilled water. The reconstituted milk was centrifuged for 25 min at 12000*g* at room temperature. The fat layer was discarded, and the upper liquid was diluted 40 times with PBS (pH = 5.7) and then used for analysis.

Instrumentation for LC−MS Analysis. Liquid chromatography was performed using a HP1200 series instrument (Agilent, CA). Separation was achieved on a Narrow Bore RR C-18 column [2.1 × 150 mm, 3.5 μm]. The mobile phase consisted of 0.1% formic acid in water with 1 mM ammonium acetate (solvent A, pH 2.7) and acetonitrile (solvent B) at a flow rate of 0.3 mL min^{-1}: A/B (10/90, v/v). The injection volume was 10 μL.

We used an Agilent 6410 Triple Quad mass spectrometer fitted with an electrospray ionization (ESI) source in this study. The interfaces were operated in the positive ion mode. Nebulizer gas was nitrogen heated at 350 °C. Ionization collisionally activated dissociation (CAD) MS/MS was performed on the collision cell, and collision energy was optimized. The MRM mode was used for detection, and quanitified by matrix-match standard solution.

Sample Pretreatment for LC−MS Analysis. A 0.5 mL aliquot of sample (raw milk or reconstituted milk) was mixed with 0.5 mL of ethanol, and stirred for 30 s. The mixture was centrifuged in a microcentrifuge (Eppendorf centrifuge 5840R, Germany) at 10000*g* for 5 min. A 0.5 mL aliquot of the supernatant was dried under N_2 gas at 45 °C in a water bath, and the residue was dissolved in 0.5 mL of methanol.

Effects of Food Processing on BPA-ELISA. Raw milk samples spiked with BPA (12 μg kg^{-1}) were subjected to pasteurization and ultrahigh temperature (UHT) treatments. After cooling to room temperature, samples were tested by ELISA. As a control, unheated samples spiked with BPA at the same concentrations were also tested using BPA-ELISA.

Effects of β-Lactamase Addition to Milk on ELISA. Penicillinase (100 μL of a 0.1 U/mL solution) was added to a 20 mL milk sample spiked with penicillin G at three different levels (equivalent to 4, 12, and 36 μg kg^{-1} of BPA). The mixtures were incubated for 1 h at 37 °C, and then for 30 min at 4 °C to inactivate the enzyme (*11*). Samples were pretreated and analyzed according to the method described in sample preparation.

Analysis of Incurred Samples by BPA-ELISA. The Tianjin Entry−Exit Inspection and Quarantine Bureau provided incurred samples, including raw milk and milk powder. These samples were analyzed using BPA-ELISA, and the results were compared with those supplied by the bureau which were analyzed by LC−ESI−MS.

RESULTS AND DISCUSSION

Optimization of BPA-ELISA Method. After the purification of the antibody and the preparation of the enzyme tracer, the direct competitive ELISA was optimized by chessboard titration. The optimal concentration of antibody was 1 μg $well^{-1}$, the optimal dilution of enzyme conjugate was 1:2000, and the optimal ionic strength of the buffer was 10 mmol L^{-1}.

Figure 2 shows the effect of pH of the sample solution buffer on the ELISA. The minimum IC_{50} value was obtained at pH 5.7. Because BPA is a carboxylic acid with two carboxyl groups, antibodies recognize BPA more sensitively in slightly acidic conditions. A six-point calibration curve was constructed using stepwise dilution of BPA standard solution under optimal conditions (**Figure 3**). The average IC_{50} was 0.32 μg L^{-1}, and the average IC_{15} was 0.03 μg L^{-1}.

Cross-Reactivity of Anti-BPA Polyclonal Antibody. The specificity of the BPA-antibody toward its most probable cross-reactants was determined by comparing the dose−response curves of BPA with various penicillins (penicillin G, ampicillin, amoxicillin, cloxacillin, dicloxacillin, oxacillin), and the penicilloic acids of

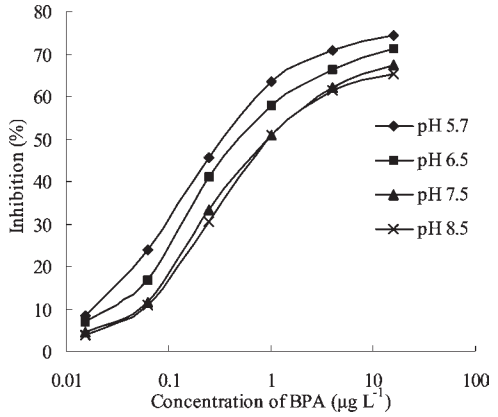

Figure 2. Effects of pH of solution buffer on BPA-ELISA.

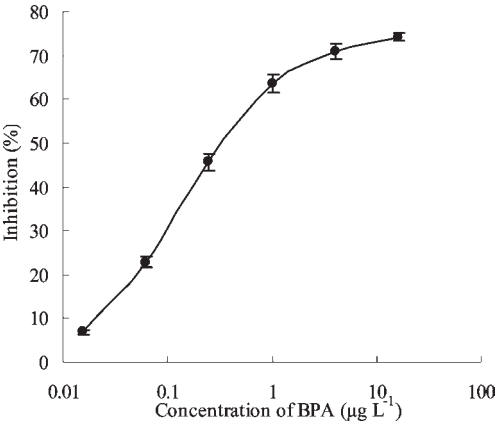

Figure 3. Standard curve for benzylpenicilloic acid.

Table 1. Reproducibility of the BPA-ELISA Method

	coefficient of variation (%)		
BPA (μg L^{-1})	intra-assay	interassay	interday
16.0	0.85	1.06	1.33
4.00	1.21	2.24	3.72
1.00	1.32	4.38	5.23
0.25	2.57	5.57	7.74
0.062	5.06	5.69	8.48
0.016	7.81	8.09	10.10

penicillins and ceftiofur. The antibody was specific to BPA. It exhibited weak cross-reactivity with penicillin G (1.5%) and no cross-reactivity with other compounds (less than 0.01%).

Reproducibility of the BPA-ELISA Method. The precision of the assay was studied by determining intra-assay, interassay, and interday variations. Each result was obtained from 10 replicate experiments (**Table 1**). As the concentration of BPA decreased, the deviation increased.

Stability BPA Antibodies and Enzyme Conjugates. For stability trials, we conducted BPA-ELISA using BPA antibodies and peroxide conjugates stored at 37 °C for 7 days, and compared the results with those obtained from the same assay using antibodies/conjugates stored at 4 °C. The results are shown in **Table 2** (antibody stability) and **Table 3** (enzyme conjugate stability). The assays gave similar IC_{50} values, regardless of whether the antibodies used were stored at 37 °C for 7 days or at 4 °C. In addition, only slight changes were observed in assays conducted using enzyme conjugates stored at 37 °C. Thus, we can conclude that both the antibody and enzyme tracer are sufficiently stable to be used in a BPA-ELISA test kit with a shelf life of 6 months.

Table 2. Stability of BPA Antibody

	IC_{50} (μg L^{-1}, n = 3)	
days of storage	4 °C	37 °C
1	0.32 ± 0.015	0.32 ± 0.015
3	0.32 ± 0.017	0.34 ± 0.018
5	0.33 ± 0.016	0.33 ± 0.016
7	0.32 ± 0.014	0.36 ± 0.014

Table 3. Stability of Enzyme Conjugate

	IC_{50} (μg L^{-1}, n = 3)	
days of storage	4 °C	37 °C
1	0.32 ± 0.012	0.33 ± 0.017
3	0.33 ± 0.016	0.36 ± 0.016
5	0.32 ± 0.014	0.37 ± 0.014
7	0.34 ± 0.017	0.41 ± 0.011

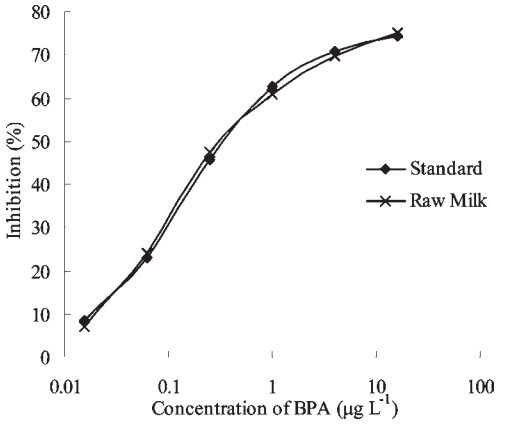

Figure 4. Standard curves for benzylpenicilloic acid in raw milk.

Matrix Effects. Matrix effects can reduce the sensitivity and reliability of the competitive immunoassay. In milk, proteins and fats are the main contributors to matrix effects. These interferences can be eliminated simply by centrifugation and dilution, and no organic solvents are required.

Raw Milk. We tried BSA, Tween 20, and skim milk powder in the standard dilution buffer and the sample dilution buffer to eliminate the matrix effects. After optimization, the standard curve was prepared in PBS (pH = 5.7) containing 0.12% skim milk powder while the milk sample was diluted 40-fold with PBS (pH = 5.7); the matrix effect can be removed (**Figure 4**). Considering the dilution of sample extracts, the detection limit of the assay in raw milk samples was 1.2 μg kg^{-1}. Because there is no MRL established for benzylpenicilloic acid, we used the MRL of penicillin G. The ELISA successfully detected benzylpenicilloic acid residues at concentrations less than the MRL of penicillin G, although dilution lowered the limit of detection.

Milk Powder. Milk powder has a complex composition, due to various additives. We determined whether matrix effects affected the results of BPA-ELISA in milk powder samples. As shown in **Figure 5**, the curve of the sample dilution buffer (320-fold dilution, as used for the milk sample) deviates from the standard curves, indicating that there are much stronger matrix effects in this sample. Thus, we used BSA, Tween 20, and skim milk powder in the dilution buffer to eliminate the matrix effects. After optimization, the matrix effect was eliminated when the standard curve was prepared in PBS (pH = 5.7) containing 0.4% skim milk powder while the sample was diluted 320-fold with PBS (pH = 5.7) (**Figure 6**). The detection limit of the assay in milk powder samples was 9.6 μg kg^{-1}.

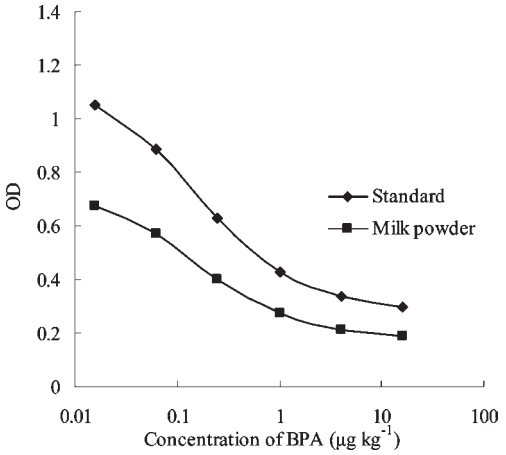

Figure 5. Matrix effects of milk powder.

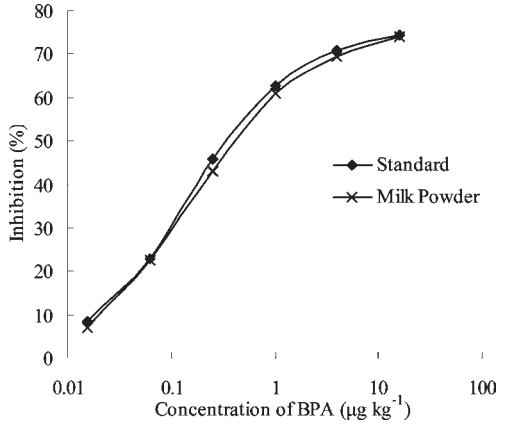

Figure 6. Standard curves for benzylpenicilloic acid in milk powder.

Table 4. Recovery (Two Samples, with Three Levels of BPA) by BPA-ELISA

samples	fortification level (μg kg^{-1})	mean ± SD (μg kg^{-1}) (n = 3)	recovery (%)	CV[a] (%)
raw milk	4	3.73 ± 0.25	93.25	6.70
	12	10.29 ± 0.47	85.76	4.57
	36	32.06 ± 1.19	89.06	2.67
milk powder	16	11.64 ± 1.08	72.75	9.28
	100	79.12 ± 7.25	79.12	9.16
	200	150.38 ± 10.38	75.19	6.90

[a] Coefficient of variation.

Recovery Study. To investigate the accuracy of BPA-ELISA, we conducted a BPA recovery study. Two kinds of samples were fortified with BPA at three different levels, and then BPA concentrations were determined using BPA-ELISA. Each sample was tested at least three times to verify repeatability of the assay. The results are shown in **Table 4**. The recoveries of BPA in these samples were between 72.75 and 93.25%, confirming the accuracy of BPA-ELISA.

Correlations between Data from ELISA and LC−MS Analyses. Blank samples and samples spiked with BPA (4, 12, and 36 μg kg^{-1} in raw milk and 16, 100, and 200 μg kg^{-1} in milk powder) were analyzed by BPA-ELISA and by LC−MS. There was a strong correlation (r^2 = 0.99) between data obtained using the two methods (**Figure 7**), indicating that BPA-ELISA accurately determined the BPA concentrations in milk/milk powder.

Practicality of the BPA-ELISA Method. To ensure that BPA-ELISA will be efficient and reliable in a commercial setting, we investigated whether common food processing techniques affected the results.

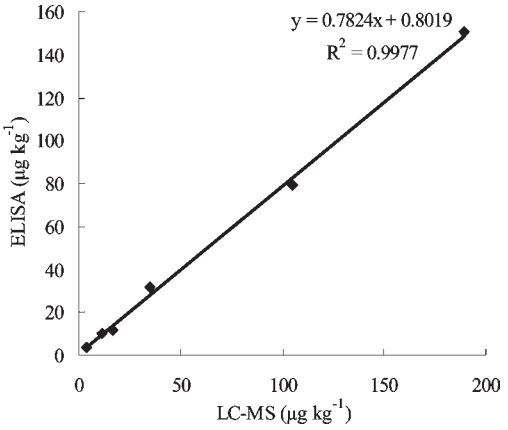

Figure 7. Correlation between ELISA and LC−MS results for milk samples spiked with BPA. $y = 0.7824x + 0.8019$, $R^2 = 0.9977$, $n = 6$.

Table 5. Effects of Processing on BPA-ELISA

milk samples	fortification level (μg kg^{-1})	mean ± SD (μg kg^{-1}) (n = 3)	RSD[a] (%)
unheated	12	10.32 ± 0.42	
heated to 60 °C	12	10.34 ± 0.49	0.14
heated to 100 °C	12	10.23 ± 0.44	0.62

[a] Compared with unheated milk.

Table 6. Ability of BPA-ELISA To Detect BPA with Addition of β-Lactamase to Milk

fortification level (μg kg^{-1})	mean ± SD (μg kg^{-1}) (n = 3)	recovery (%)	CV[a] (%)
4	3.41 ± 0.38	85.25	11.14
12	9.87 ± 0.68	82.25	6.89
36	29.79 ± 1.84	82.75	6.18

[a] Coefficient of variation.

We determined the effects of pasteurization and ultraheat treatment (UHT) on BPA-ELISA (**Table 5**). The relative deviation between BPA concentrations of unheated and heated milk was less than 1%. Thus, we can conclude that pasteurization and UHT do not affect the detection of BPA in milk by BPA-ELISA, and that this method can be used to detect BPA in raw and sterilized milk samples.

Milk spiked with penicillin G was analyzed using BPA-ELISA after addition of β-lactamase. As β-lactamase hydrolyzes 1 mol of penicillin G into 1 mol of BPA, the concentration of BPA should be the same as that of penicillin G in the spiked samples. We investigated recoveries by determining the concentration of newly formed BPA in samples (**Table 6**). The recoveries of BPA residues in these samples were between 82.25 and 85.25%, which indicates that BPA-ELISA can be used to detect BPA that has formed in milk after addition of β-lactamase.

Analysis of Incurred Samples by BPA-ELISA. The interaction between an analyte and the sample matrix in a spiked sample differs from that in incurred samples (*12*). To verify the effectiveness of this ELISA, we analyzed incurred samples obtained from the Tianjin Entry−Exit Inspection and Quarantine Bureau (TJCIQ). Since BPA-ELISA is intended for use in detecting BPA in commercial samples, incurred samples are more appropriate for testing the assay than spiked samples or samples obtained from in vivo experiments. The concentrations were confirmed by TJCIQ in advance of ELISA testing. Samples were randomly numbered before analysis so that the actual concentrations remained unknown.

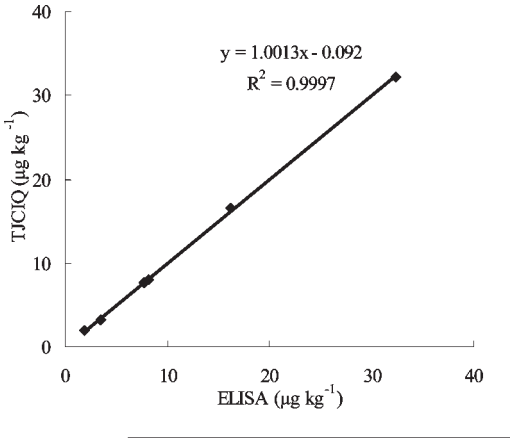

Figure 8. Correlation between ELISA and TJCIQ results for six incurred samples. $y = 1.0013x - 0.092$, $R^2 = 0.9997$, $n = 6$.

We observed an almost 100% correlation ($r^2 = 0.99$) between the results from the ELISA and those supplied by the TJCIQ (**Figure 8**). This confirmed that BPA-ELISA was an effective method for the rapid detection of BPA in incurred samples.

In conclusion, this is the first report of a sensitive and specific ELISA method for the detection of benzylpenicilloic acid in milk and milk powder samples. The method is simple and inexpensive, and thus can be used for routine screening of milk samples. Matrix effects were easily eliminated by centrifugation and dilution, and no cleanup treatments or organic solutions were required. The detection limits of this direct competitive ELISA were 1.2 μg kg^{-1} in raw milk and 9.6 μg kg^{-1} in milk powder. Recoveries were 72.75−93.25%. When the direct competitive ELISA was used to test samples containing known amounts of benzylpenicilloic acid, the results showed an almost 100% correlation ($r^2 = 0.99$) with results from the TJCIQ. Therefore, BPA-ELISA can be used for the rapid and reliable analysis of BPA in milk.

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