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# Development of a highly sensitive and specific enzyme-linked immunosorbent assay (ELISA) for the detection of phenylethanolamine A in tissue and feed samples and confirmed by liquid chromatography tandem mass spectrometry (LC–MS/MS)

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ABSTRACT

Phenylethanolamine A (PA) is a new emerged β-adrenergic agonist illegally used as feed additives for growth promotion. In this study, a highly sensitive and specific indirect competitive enzyme-linked immunosorbent assay (ELISA) for the detection of PA in tissue and feed samples was developed and confirmed by liquid chromatography tandem mass spectrometry (LC–MS/MS). By reduction of nitryl group to amino group, the PA derivative was synthesized and coupled to carrier proteins with diazobenzidine method. The antisera obtained from four immunized rabbits were characterized in terms of sensitivity and specificity. All antisera displayed high sensitivity with $IC_{50}$ values lower than 0.48 ng $mL^{-1}$. The most sensitive ELISA was established with $IC_{50}$ and limit of detection (LOD) values of 0.049 ng $mL^{-1}$ and 0.003 ng $mL^{-1}$, respectively. The cross-reactivity (CR) values of the antisera with three frequently used β-adrenergic agonists (clenbuterol, salbutamol and ractopamine) were lesser than 0.39%; there was no CR of the antisera with other six compounds including two structurally related substances (isoproterenol, phenylephrine). To investigate the accuracy and precision of the assay, swine kidney, liver, meat and feed samples were fortified with PA at different content and analyzed by ELISA. Acceptable recovery rates of 92.2–113.7% and intra-assay coefficients of variation of 3.8–10.9% ($n=3$) were achieved. Seven spiked samples were simultaneously analyzed by ELISA and LC–MS/MS. There was a high correlation coefficient of 0.9956 ($n=7$) between the two methods. The proposed ELISA proven to be a feasible quantitative/screening method for PA analysis in tissue and feed samples with the properties of high sensitivity and specificity, high sample throughput and low expensive.



## 1. Introduction

β-Adrenergic agonists are synthetic phenethanolamine compounds widespread used as bronchodilatory agents for therapeutic purposes. When the drugs were given in doses five to ten times higher than the requirement of therapeutic treatments, they could sharply increase the promotion of muscle growth and the protein-to-fat ratio [1], therefore β-adrenergic agonists were often illicitly abused as growth-promoting agents in animal feeds to enhance the lean meat-to-fat ratio for livestock and as doping drugs to enhance the performance of human athletes [2]. However, their illegal use in livestock production has led to toxic effects after human consumption of meat products. Long-term or high dose use has been shown to illicit deleterious physiological side-effects, and a large enough single dosage may initiate an acute toxic response such as cardiac palpitation, tachycardia, nervousness, muscle tremors and confusion [3]. Poisoning incidents caused by high concentrations of β-agonists residues in edible tissue occurred in many countries such as Spain, France, Italy, and China [4,5]. Therefore, β-agonists are banned as feed additives for growth promotion in animals in many countries. The most commonly abused β-adrenergic agonists are clenbuterol, salbutamol and ractopamine. Unfortunately, with the crackdown of banned β-agonists, some other new β-agonists were emerged. Recently a new alternative of β-adrenergic agonist named phenylethanolamine A [PA, 2-(4-(nitrophenyl) butan-2-ylamino)-1-(4-methoxyphenyl) ethanol, $C_{19}H_{24}N_2O_4$, Fig. 1] has been illegally used in livestock in China [6].

The main analytical methods for the determination of β-adrenergic agonists in biological and feed samples are chromatographic ones including liquid chromatography (LC) [7], liquid

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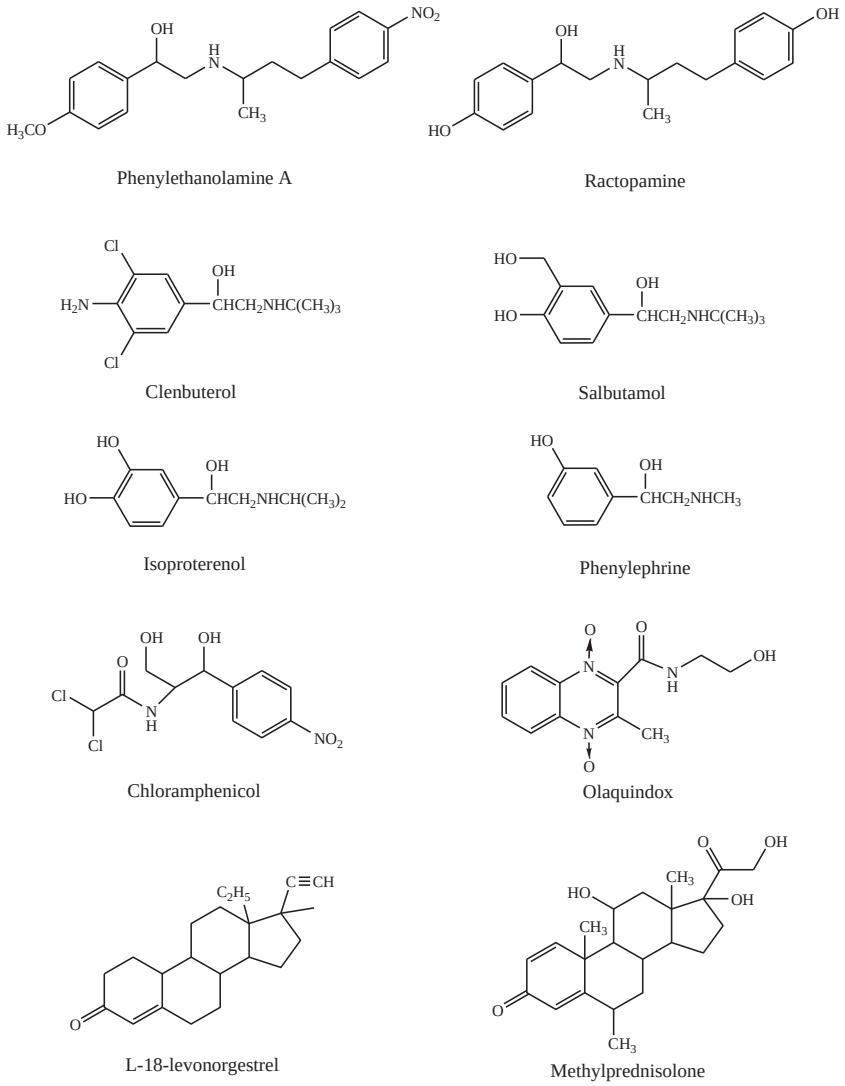


**Fig. 1.** Molecular structures of phenylethanolamine A and other compounds used for cross-reactivity testing.

chromatography/mass spectrometry (LC–MS) [8], liquid chromatography/tandem mass spectrometry (LC–MS/MS) [9,10] and gas chromatography/mass spectrometry (GC–MS) [11]. In 2010, the Ministry of Agriculture of China issued a standard for the detection of PA in feed using high-performance liquid chromatography tandem mass spectrometry (HPLC–MS/MS) [12]. Recently there was a report of the LC–MS/MS for the detection PA in animal hair, tissues and feeds [13]. Although chromatographic methods are accurate, they are expensive and time-consuming. Therefore, there is an urgent need to develop low-cost, sensitive and specific analytical method for the detection of PA in tissue and feed samples.

Immunoassays are analytical methods based on the specific interaction between an antibody and the corresponding antigen. The most used immunoassays were enzyme-linked immunosorbent assays (ELISAs), which have significant advantages over traditional chromatographic methods, such as high sensitivity and specificity, simple sample preparation, high throughput, and, therefore, low cost per sample. ELISAs have been widely applied for the determination of both large and small analytes and even heavy metals in the biological, medical, agricultural, and environmental area [14–21]. In the last decade, ELISAs have also been intensively applied for the detection of β-Adrenergic agonists in different matrix [22–24]. In the last year, a polyclonal antibody based-ELISA for the detection of PA in urine samples was developed with the $IC_{50}$ value of 0.3 ng $mL^{-1}$ [25]. Also, an ELISA test kit for PA detection was commercially available (Shenzhen Lvshiyuan Biotechnology Co., Ltd., Shenzhen, China) [26], unfortunately, no more information including type of sample, sensitivity and specificity was obtained from the web site of that company. Up to date, there are no immunoassays for the detection of PA in animal tissue and feed samples.

The aims of this study are to produce polyclonal antibody against PA and to develop highly sensitive and specific ELISA for the detection of PA in animal tissue and feed samples. The established ELISA was characterized in terms of sensitivity, specificity, accuracy and precision. Under optimal experimental conditions, the proposed ELISA was used for the analysis of fortified samples and was validated by LC–MS/MS.

## 2. Experimental

### 2.1. Chemicals, apparatus, buffers and solutions

Chemicals: Bovine serum albumin (BSA), ovalbumin (OVA), casein, 3,3′,5,5′-tetramethylbenzidine (TMB), Freund's complete and incomplete adjuvants were purchased from Sigma (St. Louis,

MO, USA). Phenylethanolamine A was bought from Toronto Research Chemicals Inc. (Toronto, Ontario, Canada). Hydrazine hydrate, sodium nitrite, carbamide were purchased from Sinopharm Chemical Reagent Co. (Shanghai, China). Horseradish peroxidase labeled goat anti-rabbit IgG conjugate (HRP-GaRIgG) was obtained from Zhong Shan-Golden Bridge Biological Technology CO. (Beijing, China). Ractopamine, salbutamol, clenbuterol, isoproterenol and phenylephrine were purchased from National Institutes for Food and Drug Control. (Beijing, China). HPLC-grade acetonitrile was purchased from Merck (Darmstadt, Germany). HPLC-grade formic acid was purchased from Alladinn (Shanghai, China).

Apparatus: ELISA reader (Sunrise Remote/Touch Screen) was purchased from Columbus plus (Tecan, Grödig, Austria). Model 1575 immunowash Microtiter plate washer was from Bio-Rad (Hercules, California, USA). Spectrophotometer UV-2300 was from Techcomp (Shanghai, China). 96-well polystyrene microtitre plates were from Haimen Plastic (Jiangsu, China). Deionized-RO water machine (Dura 12FV) was purchased from THE LAB Com. (USA).

Buffers and solutions: (1) coating buffer: 0.05 mol $L^{-1}$ carbonate buffer, pH 9.6; (2) coating antigen stock solution: 1 mg $mL^{-1}$ of coating antigen prepared with coating buffer; (3) assay buffer: 0.01 mol $L^{-1}$ phosphate-buffered saline (PBS) pH 7.4, containing 145 mmol $L^{-1}$ NaCl; (4) washing buffer (PBST): assay buffer with 0.1% (v/v) of Tween-20; (5) blocking solution: 1% of casein in assay buffer; (6) acetate buffer: 100 mmol $L^{-1}$ sodium acetate acid buffer, pH 5.8; (7) substrate solution (TMB+$H_2O_2$): 200 μL of 10 mg $mL^{-1}$ TMB dissolved in DMSO, 3.5 μL of 30% $H_2O_2$ and 1 mL of acetate buffer were added to 20 mL of pure water; (8) stop solution: sulfuric acid (5%). (9) phenylethanolamine A standard solutions in the concentration range of 0.01–10 ng $mL^{-1}$ were prepared by diluting the stock solution (1 μg $mL^{-1}$, by dissolving phenylethanolamine A in methanol) with water.

### 2.2. Synthesis of phenylethanolamine A derivative

The PA derivative (PA-$NH_2$) was synthesized as the procedures shown in Fig. 2. Briefly, PA (60 mg, 160 μmol) was dissolved in 15 mL of ethanol with constant stiring, then 28 μL hydrazine hydrate and 10 mg palladium carbon catalyst were added until the temperature rose to 80 °C. The reaction was refluxed for 10 h under nitrogen and the reaction process was monitored by silica gel thin layer chromatography (TLC). After filtration, the solvent was removed under vacuum, then the yellow crude product was obtained. The final product was purified using silica gel chromatography (dichloromethane: methanol:triethylamine, 15:1:0.05%). 400 MHz NMR and HRMS were used to identify the PA derivative

### 2.3. Preparation of immunogens and coating antigens

The PA derivative (PA-$NH_2$) was covalently coupled to carrier proteins (BSA and OVA) by diazobenzidine method (Fig. 2). The synthesis of the diazotized PA-$NH_2$ was performed as described in the literature with a small modification [27]. Briefly, 9.6 mg PA-$NH_2$ were dissolved in 300 μL of water, then 9.3 mg $NaNO_2$ dissolved in 300 μL water were added dropwise. The solution was adjusted to pH 1.5 by adding 0.2 mol $L^{-1}$ HCl and the reaction was carried out in the dark for 7 h at 4 °C. After primary testing with *N,N′*-dimethylaniline, the reaction was stopped by addition of a carbamide (11 mg) solution in water (200 μL). The above diazotized PA-$NH_2$ was added to a solution containing 99 mg of protein (BSA or OVA) dissolved in 1.5 mL of PBS. The pH value of the mixed solution was kept at 7.5 by adding a small amount of 1 mol $L^{-1}$ NaOH. The reaction was continued overnight at 4 °C under slightly stirring. The solution was intensively dialyzed in PBS for 4 days with several changes of the dialyzing buffer solution. The UV–visible spectroscopy was utilized to evaluate whether the coupling was successful. Finally, the PA–protein conjugates were lyophilized and stored at −40 °C until use. Both PA–BSA and PA–OVA were used as immunogens for the production of antibody. In heterogeneous coating antigen/antibody combinations, PA–OVA and PA–BSA were also used as coating antigens to establish indirect competitive ELISAs.

### 2.4. Production of polyclonal antisera

Four adult New Zealand rabbits were immunized with PA–BSA and PA–OVA (two rabbits/one immunogen). Each animal received a dose of 1 mg of immunogen which was dissolved in 0.5 mL physiological saline and emulsified with the same volume of Freund's complete adjuvant. Injections were made intradermally at 10 sites on the back of the animal. For booster immunizations, the same volume of Freund's incomplete adjuvant was used. Four subsequent injections were given at 4-week intervals. Bleeding of animals was performed 10 days after the last immunization. The antisera from four immunized rabbits were designated as I, II (PA–BSA as the immunogen) and III, IV (PA–OVA as the immunogen) and stored at −40 °C until use. After diluted at appropriate times with assay buffer, the antisera were directly used for the development of the ELISA.

### 2.5. Indirect competitive ELISA

An indirect competitive ELISA format was adopted for analyzing PA. The ELISA procedures were as follows. Coating antigen stock solution (1 mg $mL^{-1}$) was diluted with carbonate buffer, pH 9.6, and 200 μL/well added to a 96-well microtiter plate. The plate

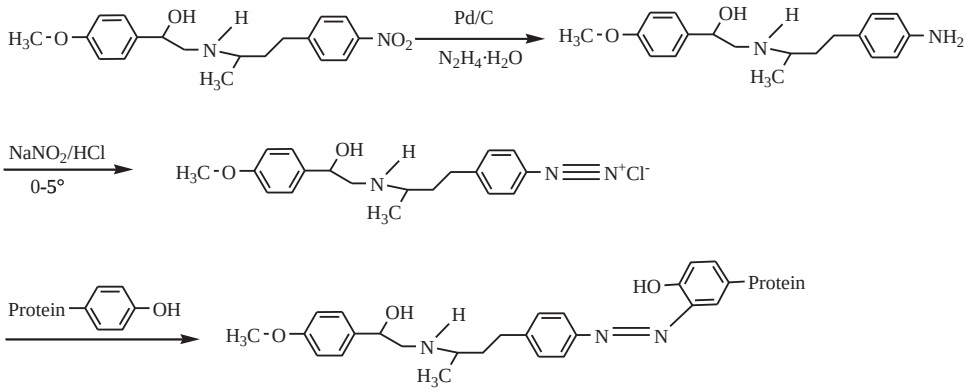


**Fig. 2.** Synthesis of phenylethanolamine A derivative and preparation of PA-protein conjugate.

was incubated overnight at 4 °C and then washed with PBST using an automated plate washer. Some binding sites not occupied by the coating antigen were then blocked by the blocking buffer (280 μL/well) for 1 h at room temperature. After the plate was washed as before, standard solutions or samples in triplicate (100 μL/well) and diluted antiserum (100 μL/well) were added and incubated for 1 h at room temperature. After washing, HRP-GaRIgG was added (200 μL/well) and the plate incubated for 1 h at room temperature. Then, the plate was washed and the substrate solution (200 μL/well) added. After incubation with shaking for about 15 min, sulfuric acid (5%, 80 μL/well) was added and the absorbance measured at 450 nm using a microplate reader. Calibration curves were constructed in the form of $(B/B_0) \times 100\%$ vs. $\log C$, where $B$ and $B_0$ was the absorbance of the analyte at the standard point and at zero concentration of the analyte, respectively.

### 2.6. Cross-reactivity

The specificity of the produced antisera was investigated by cross-reactivity (CR) experiments. Nine compounds including five structurally related compounds (clenbuterol, ractopamine, salbutamol, isoproterenol, and phenylephrine) and four other compounds (chloramphenicol, olaquindox, levonorgestrel, and methylprednisolone) were selected for testing CR. The molecular structures of the CR testing compounds were illustrated in Fig. 1. Standard solutions of testing compounds (0.001–10,000 ng $mL^{-1}$) were prepared and subjected to ELISA procedures. CR was expressed as $IC_{50}$ value based on 100% response of PA, i.e. CR (%)=($IC_{50}$ of PA)/($IC_{50}$ of testing compound) × 100%. The $IC_{50}$ value can be considered as a measure (inverse) of the affinity of an antibody for a given analyte.

### 2.7. Fortification experiment

To obtain the accurate and precise values of the ELISA, four types of samples including swine kidney, liver, meat and feed were collected from local supermarket in Suzhou (China) for fortification experiment. Before fortification, all samples were ground or cut and then intensively homogenized. All collected samples were spiked with PA at different content and detected by ELISA. The fortification experiment and extraction were performed according to reference with small modification [12]. Briefly, 2–5 g of homogenous sample was individually put into a 50 mL plastic centrifuge tube, appropriate amount (2.5–200 μL) of PA stock solution (1 μg $mL^{-1}$) was added into the tubes to prepare a final concentration of 0.5–100 ng $g^{-1}$. Then the fortified samples were vortexed for 1 min and allowed to stand at room temperature for 30 min. Ten milliliter of formic acid/methanol (0.01:100, v/v) was added. The mixture was sonicated for 30 min and centrifuged at 10,000 g for 20 min at 4 °C. Five milliliters of supernatant were taken for clean-up procedure which was carried out on MCX solid phase extraction column (Waters, Japan) to remove the matrix effect arose from animal tissue and feed samples. The MCX SPE column was preconditioned by passing 3 mL of methanol, followed by 3 mL of water through the column. Then, 5 mL of the formic acid/methanol extract was slowly passed through the MCX column at a flow rate of 1 mL $min^{-1}$. After washing with 3 mL of pure water and 3 mL of methanol, the analytes were eluted with 3 mL of ammonia–methanol solution (5%, v/v). The eluent was evaporated to dryness in a 50 °C water bath under nitrogen. Based on individual sample type, the residue was reconstituted with 250 μL to 1000 μL of assay buffer, and then diluted at 1:200–1:1000 with pure water and directly applied to the ELISA procedures [while for LC–MS/MS analysis, the residue was reconstituted with 1 mL of 0.1% formic acid in water/acetonitrile (90:10, v/v) and the extracts were filtered by a 0.45 μm filter cellulose acetate membrane filter (Alltech, Unterhaching, Germany) before injection]. As the final spiked concentration of PA in different samples was in the range of 0.5–100 ng $g^{-1}$, thus after the solid phase extraction, 1:200–1:1000 dilution was required in order to make the PA concentration in the diluted solution fall in the middle part of the standard curve for quantification. For each sample, three separate extractions were performed and each sample was determined in triplicate. Unspiked samples were extracted in the same way and used as blanks. Analytical parameters such as precision and recovery (after subtracting the background concentration) were calculated.

### 2.8. LC–MS/MS analysis

A Shimadzu HPLC system consists of a DGU-20A3 degasser, two LC-20AD pumps, a SIL-20A autosampler, a CTO-20A column temperature oven, a CBM-20A communications bus module (Shimadzu corporation, Kyoto, Japan) for solvent and sample delivery. Separation was carried out on an Agela Venusil $C_{18}$ column (50 mm × 2.1 mm, 3 μm) maintained at 30 °C. The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (acetonitrile). Initial gradient conditions were set to 5% B and held for 0.5 min before incorporating a linear gradient increasing to 80% B at 2.0 min and held for 1.5 min. At 3.6 min the gradient was programmed to initial conditions to re-equilibrate the column for 2.4 min (total run time 6 min). The flow rate was 0.30 mL/min. The injection volume was 10 μL.

Mass spectrometric detection was conducted on an AB SCIEX 40000 QTRAP system (Applied Biosystem Analytical Technologies, Foster City, CA, USA) equipped with an electrospray ionization (ESI) source. The mass spectrometer was operated in positive mode. Quantification was performed by multiple reaction monitoring (MRM). The MS/MS setting parameters were as follows: 20 psi curtain gas; 55 psi nebulizer gas (GS1); 55 psi turbo gas (GS2); 4500 V ion spray voltage; 500 °C ion source temperature; 20 V declustering potential (DP). The data acquisition and processing were performed with Analyst® 1.5.2 software (Applied Biosystems Analytical Technologies, Foster City, CA, USA). The selected MRM transitions for PA were $m/z$ 345.2→327.2 and 345.2→150.1 with a dwell time of 150 ms. The transition chosen for quantification was 345.2→150.1. The optimized collision energies for the transitions of 345.2→327.2 and 345.2→150.1 were 19 eV and 32 eV, respectively. The calibration curve for PA was constructed with standards of 0.5, 1, 5, 10, 50, 100, 200 ng $mL^{-1}$.

## 3. Results and discussion

### 3.1. Synthesis of PA derivative

To prepare highly specific antibody against small molecular compound (e.g. hapten), generally the molecular structure of the hapten should be left unchanged as much as possible. Regarding to the molecular structure of PA, there are two active functional groups: hydroxyl and nitryl. The modification may be conducted at these positions. However, as a bigger space hindrance is existed at the hydroxyl group, it is predicted that the hydroxyl group position is very difficult for molecular modification. Therefore, the nitryl group on the phenyl ring was considered the better position for PA molecule modification. In this study, the nitryl group was reduced to amino group to get the PA derivative bearing an amino group on the phenyl ring. In the synthesized hapten derivative, the amino group is far away from the antigenic determinant, which make the antigenic determinant to be exposed to the immunological system of animal as much as possible. Accordingly, a high sensitive and specific antibody is hopeful.

The structure of PA-$NH_2$ was confirmed by the NMR method. The data from the NMR method are as follow: $^1$H NMR (DMSO-$d_6$, 400 MHz): 1.26 (t, $J$=7.2 Hz, 3H, $CH_3$), 1.66–1.67 (m, 1H, CH–H), 1.98–2.02 (m, 1H, CH–H), 2.32–2.40 (m, 1H, CH–H), 2.50–2.54 (m, 1H, CH–H), 2.94–3.02 (m, 2H, $CH_2$), 3.13–3.14 (m, 1H, CH), 3.75 (s, 3H, $CH_3O$), 4.83–4.89 (m, 2H, $NH_2$), 6.05 (s, 1H, NH), 6.49 (d, $J$=8.0 Hz, 2H, ArH), 6.86 (d, $J$=8.0 Hz, 2H, ArH), 6.94 (d, $J$=7.2 Hz, 2H, ArH), 7.31 (d, $J$=7.31 Hz, 2H, ArH), 8.51 (s, H, OH). HRMS Calcd for $C_{19}H_{26}N_2O_2$, $m/z$: 315.2067 $[M+H]^+$; Found, $m/z$: 315.2060 $[M+H]^+$.

### 3.2. Synthesis of immunogens and coating antigens

PA-$NH_2$ can be coupled to proteins by a diazobenzidine method [27]. The formation of diazotized PA was easily detected by the appearance of a deep yellow color after reaction with *N*,*N*′-dimethylaniline. The UV–visible spectra of BSA, PA-$NH_2$ and PA–BSA conjugate are illustrated in Fig. 3. The absorption peaks of PA-$NH_2$ and BSA were both at 280 nm. However, for PA–BSA conjugate, besides an absorption peak at 280 nm, a shoulder peak at about 330 nm was appeared, displaying a significant red shift as a result of the larger conjugate area formed when PA was coupled to BSA via the diazobenzidine method, which demonstrated the successful linkage between PA and the protein. Similar UV–visible spectra of OVA, PA and the PA–OVA conjugate were also observed, and therefore they are not displayed herein.

### 3.3. Production of polyclonal antibodies

Four rabbits were immunized with PA–BSA and PA–OVA. During the time of immunization, the quality of the polyclonal antisera from four immunized rabbits was controlled. From third immunization, about 0.5 mL of antiserum for each animal was collected via marginal ear vein 10 days after injection. The titer and affinity of the antisera were primarily tested based on an indirect ELISA using checkerboard titration. It was found that with the times of injection increase, the titer and affinity of the antisera were increased. After fifth injection, the titers of four rabbits were more than 1:10,000. Therefore, the rabbits were bled after fifth injections.

### 3.4. Optimization of ELISA conditions

To obtain the highest sensitivity, the assay conditions including type and concentration of the coating antigen, dilution of the antiserum and secondary labeled antibody, etc. should be carefully optimized. In the present investigation, it was performed according to two criteria, i.e. (1) to obtain an $IC_{50}$ value as low as possible; (2) an absorbance limited in the range 0.8–1.5 according to the Lambert–Beer law for the zero standard concentration (blank). On the other hand, to further improve sensitivity, heterogeneous coating antigen/antibody combinations [16] were adopted, e.g. PA–OVA was selected as the coating antigen for the ELISAs using antisera I and II, while PA–BSA was used as the coating antigen for ELISAs based on antisera III and IV. The experimental conditions including coating antigens concentration and dilution of antiserum and secondary antibody were optimized for each combination. The optimized coating antigen concentration and antisera dilutions varied in the range 50–200 ng/well and 1:10,000–1:50,000, respectively, while secondary-labeled antibody was kept at 1:10,000.

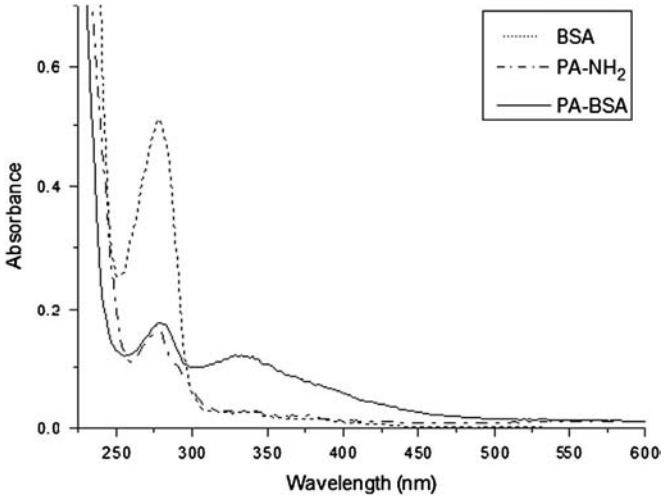


**Fig. 3.** UV–visible spectra of bovine serum albumin (BSA), PA-$NH_2$ and PA–BSA conjugate.

### 3.5. Sensitivity of the assays

The sensitivity of ELISA can be expressed by $IC_{50}$ value. The lower the $IC_{50}$ value, the higher the assay sensitivity. The ELISA standard curves using antisera II, III, IV were constructed in the concentration range of 0.01–5 ng mL$^{-1}$; while for antiserum I, due to its high sensitivity, the standard curve of ELISA using antiserum I was constructed in the concentration range of 0.01–1 ng mL$^{-1}$. Based on four kinds of heterogeneous coating antigen/antibody combinations, four typical ELISA standard curves were established and illustrated in Fig. 4. It was seen that the $IC_{50}$ values of the ELISAs using antisera I, II, III and IV were 0.049, 0.22, 0.21 and 0.48 ng mL$^{-1}$, respectively, indicating high sensitivity of the ELISAs. Apparently, the antiserum I based-ELISA with the $IC_{50}$ value of 0.049 ng mL$^{-1}$ exhibited the superior sensitivity among the four assays and the LOD at a signal-to-noise ratio of 3 ($S/N$=3) was found to be 0.003 ng mL$^{-1}$. It was also noted that the sensitivity achieved in our superior ELISA was six times higher than that in the published paper [25].

### 3.6. Specificity of the assays

The specificity of four antisera was evaluated by cross-reactivity (CR) of the ELISAs with five structurally related compounds (e.g.

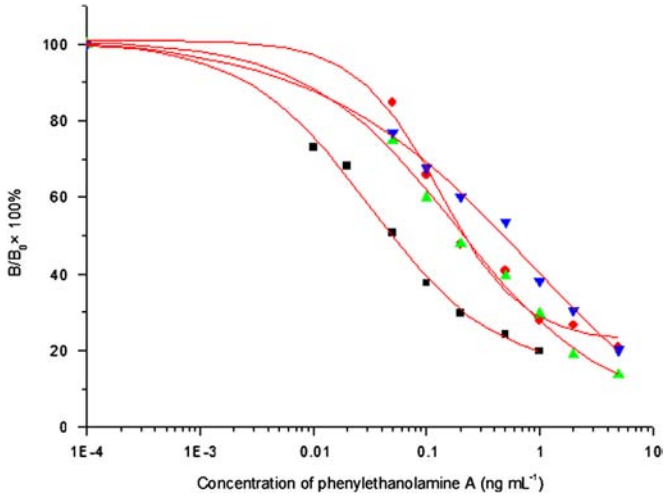


**Fig. 4.** ELISA standard curves for PA with four antisera based on four kinds of heterogeneous coating antigen/antibody combinations: ■ coating antigen PA–OVA, 1:5000 (200 ng/well); antiserum I, 1:50,000; GaRIgG-HRP, 1:10,000; $IC_{50}$ 0.049 ng mL$^{-1}$; ● coating antigen PA–OVA, 1:20,000 (50 ng/well); antiserum II, 1:50,000; GaRIgG-HRP, 1:10,000; $IC_{50}$ 0.22 ng mL$^{-1}$; ▲ coating antigen PA–BSA, 1:5000 (200 ng/well); antiserum III, 1:20,000; GaRIgG-HRP, 1:10,000; $IC_{50}$ 0.21 ng mL$^{-1}$; and ▼ coating antigen PA–BSA, 1:10,000 (100 ng/well); antiserum IV,1:10,000; GaRIgG-HRP, 1:10,000; $IC_{50}$ 0.48 ng mL$^{-1}$.

clenbuterol, salbutamol, ractopamine, isoproterenol, and phenylephrine) and other four compounds (chloramphenicol, olaquindox, levonorgestrel and methylprednisolone). The CR values are summarized in Table 1. It was seen that the CR values of the four antisera with three frequently used β-adrenergic agonist (clenbuterol, salbutamol, and ractopamine) were lower than 0.39%, and there was no CR (<0.01%) of the four antisera with other testing compounds, including two structurally related compounds (isoproterenol, phenylephrine). The CR results indicated high specificity of the produced antisera. Considering its excellent sensitivity and specificity, antiserum I based-ELISA was selected for the further experiment.

### 3.7. Accuracy and precision of the assay

To test accuracy and precision of the assay, four types of samples including swine kidney, liver, meat and feed were collected and fortified with PA at the content of 0.5–100 ng/g of sample and analyzed by ELISA. The results of accuracy and precision of the assay for the detection of PA in fortified samples in triplicate were given in Table 2. It was observed that there was no detectable PA in all unspiked samples. Acceptable recovery rates of 92.2–113.7% and intra-assay coefficients of variation of 3.8–10.9% ($n=3$) were obtained. The inter-assay coefficients of variation were within 5.2–13.4% ($n=3$). These results demonstrated the applicability of the ELISA for detecting PA in different matrices.

**Table 1**
Cross-reactivity of the polyclonal antisera I, II, III, and IV with tested compounds.

| Compound | CR (%) | | | |
|---|---|---|---|---|
| | I | II | III | IV |
| Phenylethanolamine A | 100 | 100 | 100 | 100 |
| Clenbuterol | 0.07 | 0.16 | 0.08 | 0.06 |
| Salbutamol | 0.08 | 0.01 | 0.20 | 0.03 |
| Ractopamine | 0.16 | 0.07 | 0.39 | 0.16 |
| Isoproterenol | <0.01 | <0.01 | <0.01 | <0.01 |
| Phenylephrine | <0.01 | <0.01 | <0.01 | <0.01 |
| Chloramphenicol | <0.01 | <0.01 | <0.01 | <0.01 |
| Olaquindox | <0.01 | <0.01 | <0.01 | <0.01 |
| levonorgestrel | <0.01 | <0.01 | <0.01 | <0.01 |
| Methylprednisolone | <0.01 | <0.01 | <0.01 | <0.01 |

### 3.8. Validation of the assay with LC–MS/MS

The LC–MS/MS calibration curve for PA was constructed in the range of 0.5, 1, 5, 10, 50, 100, 200 ng $mL^{-1}$. The retention time of PA was 3.11 min. Due to different matrix effects, two sets of standard curves of the LC–MS/MS for the detection of PA were established. For meat matrix, the linear equation of the LC–MS/MS standard curve for PA detection was $Y=9413.59x+16{,}175.74$ ($R=0.9971$ $n=7$), while for swine feed matrix, the linear equation of the LC–MS–MS standard curve for PA detection was $Y=6326.78x-5742.61$ ($R=0.9986$ $n=7$).

To validate the applicability of the ELISA, seven spiked samples including three swine meats (spiked at concentration of 1, 5 and 20 ng $g^{-1}$) and four feeds (spiked at concentration of 2, 10, 50 and 100 ng $g^{-1}$) were analyzed by LC–MS/MS and ELISA simultaneously. After sample pretreatment, the extracts were applied to the procedures of the two methods. The comparison of the ELISA with the LC–MS/MS was shown in Fig. 5. A good correlation between LC–MS–MS ($x$) and ELISA ($Y$) was obtained with the linear regression equation of $Y=0.97266x-1.48719$ ($R=0.9956$, $n=7$). These results suggested that PA in tissue and feed samples could be rapidly and accurately detected by the proposed ELISA.

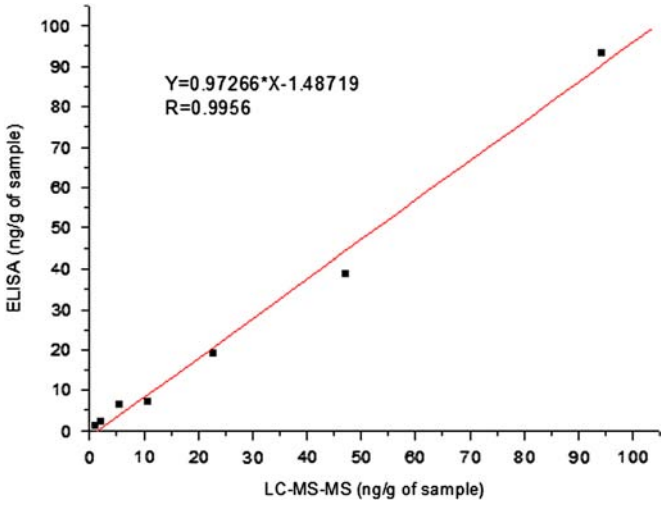


**Fig. 5.** Correlation between the proposed ELISA and LC–MS/MS for the detection of PA in seven spiked samples.

**Table 2**
The recovery and coefficient of variation of the ELISA for the determination of PA in fortified samples.

| Sample | Conc. fortified (ng/g) | Conc. measured ± SD (ng/g) | Recovery (%)[a] | Intra-assay CV (%, $n=3$) | Inter-assay CV (%, $n=3$) |
|---|---|---|---|---|---|
| Swine kidney | 0.5 | 0.48 ± 0.04 | 96.9 | 7.3 | 13.4 |
| | 1 | 1.12 ± 0.07 | 112.4 | 6.1 | 6.6 |
| | 2 | 2.17 ± 0.24 | 108.7 | 10.9 | 5.3 |
| Swine liver | 2 | 2.14 ± 0.08 | 107.2 | 3.8 | 9.4 |
| | 5 | 4.89 ± 0.43 | 97.8 | 8.8 | 7.2 |
| | 10 | 10.4 ± 0.96 | 104.3 | 9.2 | 6.2 |
| Swine feed | 5 | 4.81 ± 0. 38 | 96.3 | 7.9 | 8.1 |
| | 10 | 11.1 ± 0.91 | 110.8 | 8.2 | 11.0 |
| | 20 | 20.7 ± 1.5 | 103.5 | 7.2 | 5.2 |
| Pork | 20 | 18.4 ± 1.5 | 92.2 | 7.9 | 5.4 |
| | 50 | 48.9 ± 3.7 | 97.7 | 7.6 | 8.3 |
| | 100 | 113.7 ± 11.0 | 113.7 | 9.6 | 7.6 |

[a] Recovery (%)=[(Conc. measured−blank)/Conc. fortified] × 100%. No detectable of PA was found in all samples without spiking.

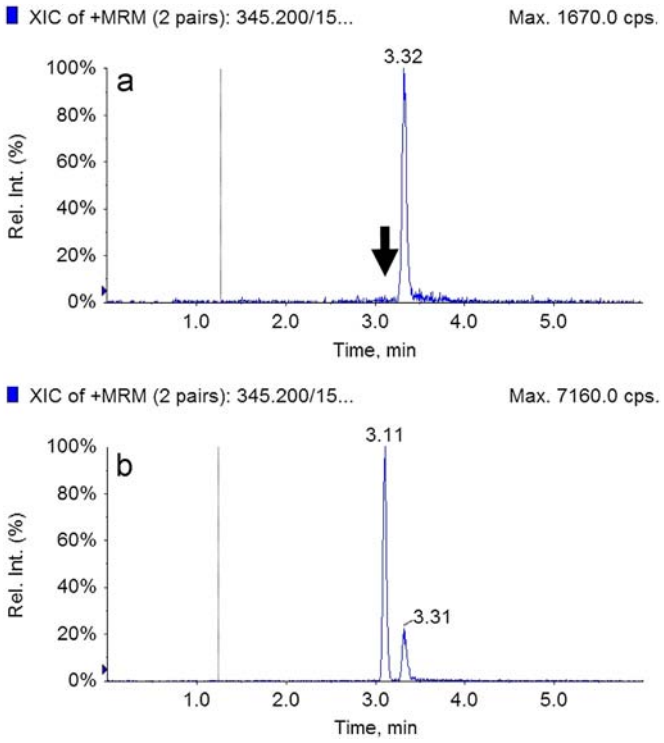


**Fig. 6.** The MRM chromatograms of feed samples, (a) blank sample (the arrow indicates the point PA peak appeared); (b) sample fortified with PA at 2 ng $g^{-1}$ level.

The MRM chromatograms of feed samples without spiking and fortified at the 2 ng $g^{-1}$ level of PA are shown in Fig. 6(a) and (b), respectively. It was seen from Fig. 6(a) that at the retention time of 3.11 min there was no signal intensity, indicating there was no detectable PA in unspiked feed sample; while for the sample fortified with PA at the 2 ng $g^{-1}$ level, as it is seen from Fig. 6(b), the signal intensity at the retention time of 3.11 min was as big as 7160.0 cps., revealing the existence of PA in the sample.

## 4. Conclusions

In this study, the highly sensitive and specific ELISA for the detection of PA was developed and confirmed by LC–MS/MS. The PA derivative was synthesized and coupled to carrier proteins. Polyclonal antibodies against PA were successfully produced. Under optimal experiment conditions, the ELISAs based on heterogeneous coating antigen/antibody combinations were established with $IC_{50}$ values lower than 0.48 ng $mL^{-1}$. The most sensitive ELISA showed $IC_{50}$ value as low as 0.049 ng $mL^{-1}$, which was six times lower than that in the published paper. The cross-reactivity (CR) values of the four ELISAs with nine tested compounds were very small or negligible. The superior ELISA was applied for the detection of PA in spiked tissue and feed samples. Acceptable recovery rates and intra-assay CV for PA detection were achieved with the coefficients of variation of 3.8–10.9%. Seven spiked samples were also simultaneously analyzed by ELISA and LC–MS/MS. There was a high correlation coefficient between the two methods. The proposed ELISA proved to be a sensitive, specific and low cost analytical method for the detection of PA in tissue and feed samples.

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