Production of antibodies and development of an enzyme-linked immunosorbent assay for 17β-estradiol in milk

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ABSTRACT
The residue of 17β-estradiol (E2) in milk could potentially lead to the occurrence of various reproductive diseases; therefore, a rapid and sensitive method for monitoring E2 residues in milk was highly necessary. In this study, we produced new polyclonal and monoclonal antibodies using E2-3-O-carboxymethyl ether as a hapten and developed an indirect competitive enzyme-linked immunosorbent assay (icELISA) for the detection of E2 in milk. The results showed that the sensitivity of polyclonal antibody was higher than that of monoclonal antibody, providing a half maximum inhibition concentration (IC50) against E2 of 0.17 ng/mL, high cross-reactivity (CR) to E2 benzoate (150%) and oestriol (18.02%), and negligible CR with other oestrogen compounds. Under optimized conditions, the developed icELISA based on the polyclonal antibody had a limit of detection values of 0.093 μg/L, which was enough sensitive to detect E2 in milk. In spiked samples (0.5, 1, and 2 μg/L), the recoveries ranged from 83.12% to 94.58% with coefficients of variation <12.8%. These results indicated that the icELISA method we developed was suitable for screening of E2 residue in milk.

1. Introduction
17β-Estradiol (E2) is the most active endogenous oestrogen derived from cholesterol and employed for promoting the propagation and breeding of animals (Chambers, Casey, Hakk, DeSutter, & Shappell, 2014). However, there are many adverse effects, including naupathia and inappetence, for consumers when animal-of-origin food contains E2 residue (Xiao, Zhang, Wu, Han, & Zhang, 2017). Therefore, many countries have banned E2 use in the livestock and poultry industries. Milk is considered a perfect natural food for people; however, the illegal and uncontrolled use of E2 in dairy cattle could cause a wide range of health problems (Chen, Mi, et al., 2014; Yang et al., 2015). Therefore, it is of great significance to establish a rapid and sensitive method for monitoring E2 residue in milk.
Currently, high-performance liquid chromatography (HPLC), liquid chromatography–tandem mass spectrometry (LC–MS/MS), and gas chromatography–mass spectrometry (GC–MS) represent the primary analytical methods used for E2 detection (Afifi et al., 2016; Choi, Kim, & Chung, 2000; Torres et al., 2015; Wang, Yan, Yang, Li, & Qiao, 2016). Although these methods are highly sensitive and specific, conventional oestrogen-residue analyses comprise complex, time-consuming, and high-cost techniques required for sample extraction and cleaning, making them unsuitable for screening high-throughput samples. An effective alternative for screening large numbers of samples involves immunoassays, such as indirect competitive enzyme-linked immunosorbent assays (icELISAs), using highly sensitive antibodies. This technique provides many advantages, including rapid, simple operation, and high-throughput capabilities (Li et al., 2014). Antibody represents the key reagent in immunoassays, and some E2-specific antibodies have been reported (Caron, Sheedy, & Farenhorst, 2010; Li & Jiang, 2012; Yang et al., 2015). However, the sensitivities of some of these antibodies are too low [half maximum inhibition concentration (IC50) = 243 ng/mL (Caron et al., 2010) or 3.76 ng/mL (Li & Jiang, 2012)] to be used for establishing a highly sensitive icELISA to detect traces of E2 residue in milk. Therefore, this study prepared polyclonal and monoclonal antibodies against E2 and established a sensitive icELISA using the antibodies exhibiting the highest sensitivities for detecting E2 in milk.

2. Experimental

2.1. Materials and equipment

E2, oestriol, E2 valerate, and E2 benzoate were obtained from the China Pharmaceutical Biological Products Analysis Institute (Beijing, P.R. China). Oestrone, quinestrol, and diethylstilbestrol were obtained from the China Food and Drug Verification Research Institute (Beijing, P.R. China). Ethinyloestradiol and nonylphenol were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Bovine serum albumin (BSA), ovalbumin (OVA), N-hydroxysuccinimide, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC), polyethylene glycol 1450, incomplete Freund’s adjuvant, complete Freund’s adjuvant, hypoxanthine-thymidine, and hypoxanthine-aminopterin-thymidine media supplement (50x; Hybrid-Max) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Peroxidase-conjugated goat anti-rabbit IgG and anti-mouse IgG were obtained from Bethyl Laboratories, Inc. (Montgomery, TX, USA). 3,3′,5,5′-Tetramethylbenzidine (TMB) was obtained from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, P.R. China). N, N-Dimethylformamide (DMF) was obtained from Sinopharm Chemical Reagent Co., Ltd. (Beijing, P.R. China). Reagent-grade solvents and salts were supplied by Beijing Chemical Reagent Co. (Beijing, P.R. China). Dulbecco’s modified Eagle medium-high glucose (4500 mg/L) and foetal calf serum were obtained from Gibco BRL (Carlsbad, CA, USA). Deionized water was prepared using a Milli-Q water purification system (Millipore, Bedford, MA, USA). Polystyrene ELISA plates were procured from Costar, Inc. (Cambridge, MA, USA). The microplate absorbance reader Multiscan GO 1510 was obtained from Thermo Fisher Scientific (Shanghai, China). E2-free skimmed milk was supplied by the Analytical and Testing Center of Wenzhou Agricultural Sciences Academy, Agricultural Products Quality Safety Risk Assessment Laboratory of Chinese
Ministry of Agriculture (Wenzhou, P.R. China). The other reagents and solvents were of analytical grade or higher.

Four-month-old male rabbits and 8-week-old female BALB/c mice were obtained from Wenzhou Medical University and raised under strict controlled conditions. The experimental procedures involving animals in this study were approved by the Animal Care Center of Wenzhou Medical University (Wenzhou, China).

2.2. Buffers and solutions

Stock standard solutions (5 mg/mL) of all oestrogens were prepared by dissolving an appropriate amount of each standard in DMF. The individual stock solutions were stored at −20°C in amber glass bottles. Working standards of each oestrogen were prepared by diluting the stock standard solution in assay buffer and stored at 4°C.

The following buffers were used for ELISA: (1) coating buffer consisted of 0.05 M carbonate buffer (pH 9.6); (2) blocking buffer consisted of 0.01 M phosphate-buffered saline (PBS; pH 7.4), 10% foetal bovine serum, and 0.01% Tween 20; (3) washing buffer consisted of 0.01 M PBS with 0.05% Tween 20; (4) antibody dilution buffer consisted of 0.01 M PBS containing 0.2% albumin; (5) enzyme-labelled secondary antibody dilution buffer comprised the antibody dilution buffer containing 5% fetal bovine serum; (6) the substrate consisted of 0.1% TMB and 50% H2O2 in 0.05 M citrate buffer (pH 4.5); and (7) the stopping reagent was 2 M H2SO4.

2.3. Preparation of immunogen and coating antigen

2.3.1. Synthesis of the E2-3-O-carboxymethyl hapten

The E2-3-O-carboxymethyl (E2-CME) hapten was synthesized using the C3–OH group of E2 according to the method reported by Dhar, Samanta, and Ali (1988) (shown in Figure 1). Briefly, E2 (100 mg) and sodium hydroxide (265 mg) were both dissolved in dimethyl sulphoxide (DMSO) (10 mL) at 30°C, followed by dropwise addition of bromoacetic acid (100 mg) to the E2 solution. After reacting for at least 10 h, the mixture was dropped into ice-cold water (10 mL), and the pH was adjusted to 6.0 using 1 M HCl solution. The mixture was extracted by ethyl acetate (10 mL) three times and washed with saturated salt water (5 mL), followed by addition of anhydrous sodium sulphate to vapourize the liquid. After further purification by column chromatography (dichloromethane:methanol ratio = 20:1). The hapten E2-CME was confirmed by HPLC–MS/MS and NMR. ESI+ (M + Na)+ 353.2659, 1H NMR (400 MHz, d6-DMSO) δ 12.88 (s, 1H), 7.16 (d, 1H), 6.64 (d, 1H), 6.57 (s, 1H), 4.58 (s, 2H), 4.45 (s, 1H), 3.52 (s, 1H), 2.75 (s, 2H), 2.25 (d, 1H), 2.08 (t, 1H), 1.78–1.89 (m, 3H), 1.58 (s, 1H), 1.11–1.39 (m, 8H), 0.87 (s, 3H).

2.3.2. Antigen preparation

The E2-CME hapten was conjugated to BSA and OVA to prepare the immunizing and coating conjugates using an active ester method (Wang et al., 2015; Zhang et al., 2016). Briefly, 33 mg E2-CME (0.1 mM), 95 mg EDC (0.5 mM), and 58 mg N-hydroxysuccinimide (0.5 mM) were dissolved in 2 mL of DMF and stirred at room temperature for 6 h. After centrifugation at 8000g for 10 min, the clear solution was added dropwise to 66 mg BSA (0.001 mM) or 45 mg OVA (0.001 mM) in 10 mL 0.01 M PBS at 4°C with
gentle stirring overnight, followed by dialysis against PBS for 3 days and characterization by matrix-assisted laser desorption/ionization time-of-flight (MALDI–TOF)–MS. Hapten-to-protein molar ratios were calculated as follows (Peng et al., 2016):

\[
\text{hapten-to-protein molar ratios} = \frac{M_{\text{conjugation}} - M_{\text{carrier protein}}}{M_{\text{hapten}}}.
\]

### 2.4. Antibody production

Six Japanese white rabbits (each weighing 2–3 kg) were used for immunization to produce polyclonal antibodies as previously described (Chen, Huang, Li, Kong, & Huai, 2014; Gandhi, Sharma, Capalash, Verma, & Suri, 2008; Wang, Liu, Hsu, & Yu, 2011). Rabbits were injected intradermally at multiple sites with 0.5 mL E2-CME-BSA (2 mg/mL) thoroughly emulsified with an equal volume of Freund’s complete adjuvant for the first injection, with Freund’s incomplete adjuvant used for subsequent booster injections at 3-week intervals for 24 weeks. At 7 days after the eighth injection, sera were collected from ear marginal veins and characterized by indirect non-competitive ELISA and icELISA as described below. Rabbits showing the highest antibody titre and sensitivity were sacrificed, and the collected antiserum was stored at −20°C until use.

Seven female BALB/c mice were immunized with E2-CME-BSA to produce monoclonal antibodies as previously described (Guan et al., 2015; Sheng et al., 2012; Zhang et al., 2015). The immunization was performed as described above, but with 50 μL E2-CME-BSA (1 mg/mL) mixed with Freund’s complete adjuvant for the first injection. At 7 days after the fourth immunization, the titre and selectivity of antisera against free E2 were tested by icELISA. Mice showing the highest inhibitory rates associated with free E2 were sacrificed to extract the monoclonal antibody.
2.5. Indirect ELISA and icELISA

Indirect ELISA was performed as follows (Isanga et al., 2016). High-binding microtiter plates (96 wells) were coated with 100 μL/well coating antigen E2-CME-OVA (0.1 μg/mL) and incubated at 4°C overnight. The plates were washed three times with washing buffer and then blocked with 150 μL/well blocking buffer at 37°C for 2 h, followed by addition of 50 μL/well 0.01 M PBS and 50 μL/well diluted antibodies and incubation for 30 min at 37°C. Unbound antibodies were removed by washing steps, and 100 μL/well goat anti-rabbit IgG–horseradish peroxidase or goat anti-mouse IgG–horseradish peroxidase (1:5000 dilution using 0.01 M PBS) was added and incubated for 30 min at 37°C and then washed, followed by addition of 100 μL/well substrate solution, incubation at 37°C for 15 min, and reaction termination by adding 50 μL/well 2 M H2SO4. Optical density (OD) values were measured at 450 nm (OD450). The icELISA procedure was similar to that described for indirect ELISA, except that 50 μL of oestrogen-standard solution (or samples) and 50 μL of antibody were added to the wells instead of 50 μL PBS.

Standard curves were developed as OD450 versus the logarithm of analyte concentration (Log C) and fitted with a four-parameter logistic equation. The IC50 was calculated to evaluate assay sensitivity.

2.6. Cross-reactivity

To determine polyclonal and monoclonal antibody specificities, antibody cross-reactivity (CR) with other oestrogen analogues, including E2 benzoate, oestrone, oestriol, diethylstilbestrol, quinestrol, ethinyloestradiol, E2 valerate, and nonylphenol, were determined. CR values were calculated according to the following equation (Chen, Xu, et al., 2014; Mukunzi, Isanga, Suryoprabowo, Liu, & Kuang, 2017):

\[
CR(\%) = \left(\frac{\text{IC50}_{E2}}{\text{IC50}_{\text{analogue compounds}}}\right) \times 100\%.
\]

2.7. Sample preparation

Negative milk samples were spiked with a 1 mg/L solution of E2 to obtain milk containing 0.5, 1, and 2 μg/L E2, followed by subsequent dilutions using 0.01 M PBS for icELISA. Accuracy and precision were evaluated by performing recovery and calculating coefficients of variation (CVs), respectively. A total of 20 blank milk samples were used to calculate limit of detection (LOD) using the following equation (Zhang et al., 2017):

\[
\text{LOD} = \bar{X} + 3\text{SD}.
\]

3. Results and discussion

3.1. Preparation of haptens and conjugates

E2 is a small molecule requiring conjugation with a carrier protein, such as BSA and OVA, to elicit immune response. Normally, two strategies are employed to conjugate E2 to protein carriers. One uses hydroxyl groups at the 3-position of E2, and the another uses hydroxyl groups at the 17-position (Figure 1). Since the hydroxyl groups at position 17 of the E2 is the characteristic structure and the aim of the study is to produce high specifically antibody to E2, the derivative site should be at the 3-position. Actually, there were reports showing that the antibody obtained from at 3-position were more sensitive than
that obtained from 17-position of E2. Watanabe, Kubo, Kanzaki, and Nakazawa (2010) previously prepared β-estradiol 17-hemisuccinate as a hapten for producing polyclonal antibodies with an IC$_{50}$ values of 6.9 ng/mL, and Yang et al. (2015) synthesized E$_2$-CME as a hapten for conjugating carrier proteins, obtaining a monoclonal antibody specific for E$_2$ with an IC$_{50}$ value of 0.18 ng/mL using a lateral-flow colloidal gold immunnoassay strip. Thus, we synthesized the E$_2$-CME used as hapten to produce antibodies to E$_2$ in this study. The synthesized hapten E$_2$-CME was identified by HPLC–MS/MS and NMR. The molecular ions (m/z, M + Na) of E$_2$-CME was 353.2659, indicating that CME was successfully conjugated with E$_2$. The E$_2$-CME hapten was subsequently conjugated with the carrier protein and characterized by MALDI–TOF (Figure 1), which showed a shift in the molecular weight relative to the control protein (the molecular weights of BSA and E$_2$-CME-BSA were 65,886 and 69,709 Da, respectively), suggesting that the antigen was successfully synthesized and the hapten-to-protein molar ratio was 11.6, which was suitable for producing antibody according to a previous report (Wang et al., 2015).

### 3.2. Antibody production

Six rabbits were immunized with E$_2$-CME-BSA to produce polyclonal antibodies against E$_2$. Seven days after the eighth boost immunization, antiserum was collected and characterized by indirect non-competitive ELISA and icELISA (Table 1). Our results indicated that rabbit no. 2 provided the best antibody (evaluated by titre and IC$_{50}$); therefore, the polyclonal antibody derived from this rabbit was selected for subsequent study. Additionally, seven mice were also immunized with E$_2$-CME-BSA to produce monoclonal antibodies, and antiserum analysed following the fourth immunization was collected and characterized by indirect ELISA and icELISA (Wang et al., 2016) (Table 2). Mouse no. 6 was sacrificed for cell fusion based on its best inhibition ratios using free E$_2$. One cell line, 3H3, was ultimately obtained, and the monoclonal antibody 3H3 was used for subsequent study.

### 3.3. Development of icELISA in milk

After optimization of the working dilutions for the polyclonal antibody (1:100,000), monoclonal antibody (1:80,000), and coating antigen (0.1 μg/mL), standard solutions of E$_2$ were diluted in PBS at concentrations of 0, 0.033, 0.1, 0.3, 0.9, 2.7, 8.1, and 24.3 ng/mL, standard curves for icELISA were determined (Figure 2). The IC$_{50}$ value of the

| Rabbit number | OD$_{450}$ | IC$_{50}$ (ng/mL) |
|---------------|------------|------------------|
| 1             | 1.463      | 2.55             |
| 2             | 2.174      | 0.22             |
| 3             | 1.986      | 0.37             |
| 4             | 1.685      | 3.46             |
| 5             | —          | —                |
| 6             | 1.235      | 5.87             |

Note: “—” indicates that the rabbit died.

*aAntisera were evaluated by icELISA for primary screening. The antiserum dilution was 1:80,000 and coating-conjugate dilution was 1:5000.
monoclonal antibody was 2.18 ng/mL, whereas that for the polyclonal antibody was 0.17 ng/mL. Therefore, the polyclonal antibody was selected to develop the icELISA to detect E2 in milk samples in term of lower IC50 values. Our results indicated that the sensitivity of the polyclonal antibody produced in this study was among the best reported (Yang et al., 2015). The specificity of the polyclonal antibody was evaluated by comparing the IC50 values of other structurally related compounds to that of E2 as expressed by CR values (Table 3). The polyclonal antibody exhibited high CR to E2 benzoate (150%), oestriol (18.02%), oestrone (3.11%), and ethinyloestradiol (1.52%), with negligible CRs with other oestrogen compounds, including quinestrol, E2 valerate, diethylstilbestrol, and nonylphenol.

As a screening method to detect E2 in milk, sample preparation should be as simple as possible. Dilution is a common method used to reduce the matrix effect of milk in immunoassays; therefore, the milk-matrix effect was evaluated here by preparing E2 standard curves in milk diluted 2-, 4-, 6-, 8-, and 10-fold with 0.01 M PBS and comparing the data against E2 standard curves prepared in 0.01 M PBS. We observed that increases in the dilution factor resulted in increases in the maximum OD values along with relatively stable and improved IC50 values (Figure 3). Additionally, inhibition curves without

### Table 2. The OD450 and IC50 of immunized mice antisera.\(^a\)

| Mouse number | OD450 (0 ng/mL) | IC50 (ng/mL) |
|--------------|----------------|--------------|
| 1            | 1.6471         | 135.40       |
| 2            | 1.3349         | 117.60       |
| 3            | 1.1834         | 88.35        |
| 4            | 1.9516         | 106.77       |
| 5            | 1.1981         | 75.46        |
| 6            | 1.8499         | 36.47        |
| 7            | 1.0795         | 60.89        |

\(^a\)Antisera were evaluated by icELISA for primary screening. Antiserum dilution was 1:10,000, and coating-conjugate dilution was 1:5000.

![Figure 2. Standard curves of icELISA for E2 in buffer based on the polyclonal antibody no. 2 and monoclonal antibodies 3H3.](image-url)
interference were obtained after a six-fold dilution, indicating that skimmed milk could be measured following dilution. Furthermore, the LOD of the icELISA was 0.093 ng/mL, which was much lower than that reported previously (Caron et al., 2010; Li & Jiang, 2012). The accuracy of the ELISA developed for E2 detection in milk was then assessed by measuring spiked raw milk samples and estimating the overall recovery. Our results showed recoveries ranging from 83.2% to 94.5%, with CVs <12.8% (Table 4). These findings suggested that the icELISA based on the polyclonal antibody developed in this study was capable of detecting of E2 in milk samples.

Table 3. The IC$_{50}$ and CR values of the polyclonal antibody.

| Estrogens          | Structure | IC$_{50}$ (ng/mL) | CR (%) |
|--------------------|-----------|-------------------|--------|
| Oestradiol         | ![Image](image1.png) | 0.170 ng/mL       | 100    |
| Oestradiol benzoate| ![Image](image2.png) | 0.113 ng/mL       | 150    |
| Oestriol           | ![Image](image3.png) | 0.943 ng/mL       | 18.02  |
| Oestrone           | ![Image](image4.png) | 5.466 ng/mL       | 3.11   |
| Ethinyloestradiol  | ![Image](image5.png) | 11.184 ng/mL      | <1.52  |
| Oestradiol valerate| ![Image](image6.png) | >50 μg/mL         | <0.1   |
| Quinestrol         | ![Image](image7.png) | >50 μg/mL         | <0.1   |
| Diethylstilbestrol | ![Image](image8.png) | >50 μg/mL         | <0.1   |
| Nonylphenol        | ![Image](image9.png) | >50 μg/mL         | <0.1   |
4. Conclusions

In this study, an E2-CME hapten was synthesized and used to produce polyclonal and monoclonal antibodies against E2. The sensitivity of the polyclonal antibody exceeded that of the monoclonal antibody, and a more sensitive icELISA based on using the polyclonal antibody was established, resulting in an IC50 value of 0.17 ng/mL. Additionally, we were able to completely remove the matrix effect of milk following a six-fold dilution, ultimately resulting in recoveries by the icELISA ranging from 83.2% to 94.5%, with CVs <12.8%. These results indicated that the icELISA developed here was suitable as a rapid screening method for detection of E2 residue in milk samples.

Disclosure statement

No potential conflict of interest was reported by the authors.

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