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Preparation of an anti-diethylstilbestrol monoclonal antibody and development of an indirect competitive ELISA to detect diethylstilbestrol in biological samples

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Based on the preparation of an anti-diethylstilbestrol (DES) monoclonal antibody, a simple and convenient indirect competitive enzyme-linked immunosorbent assay (ELISA) method for DES detection has been developed. The monoclonal antibody demonstrated high sensitivity to DES with an IC_{50} value of 275 pg mL^{-1} and detection limit (LOD) of 90 pg mL^{-1}. The specificity of the assay was studied by measuring cross-reactivity of the antibody with structurally related compounds of ethinyl estradiol (<7%), estrone (<0.1%), estriol (<0.1%), and diethylstilbestrol benzoate (<0.1%). Chicken, fish, shrimp, urine and bile spiked with different concentration of DES were detected by the developed method, and the recovery rates were greater than 79.5%. Intra- and inter-assay variations were about 6%. This method exhibited high stability with a coefficient of variation less than 10% in buffer and in real samples. The LODs in fish/shrimp, liver, feed and urine spiked with DES were 600, 600, 4800 and 600 pg mL^{-1}, respectively. These results confirmed that the antibody to DES was successfully produced and could be used to establish ELISA methods for DES detection in food producing animals.

diethylstilbestrol, ELISA, monoclonal antibody, drug residue

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Diethylstilbestrol (DES; Figure 1) is an orally active synthetic non-steroid estrogen. Since the 1960s, DES has been used as a growth-promoting agent in livestock [1,2]. Later, DES was found to cause cancer and its use was "phased out in the 1970s" by the FAO/WHO. In 1971, DES was reported to be a teratogen when given to pregnant women and has been associated with a rare vaginal cancer in female offspring [3]. On February 5, 1975, the US Food and Drug Administration (FDA) ordered 25 and 100 mg tablets of DES withdrawn; however, it was still illegally used in the livestock industry. More than 30 years of researches have confirmed that DES is a teratogen, which can cause malformations of an embryo or fetus. Prenatal exposure to DES is associated with an increased incidence of fibroids of adulthood [4,5]. Therefore, the Ministry of Agriculture of the People's Republic of China issued a banned list (Announcement No. 235; http://www.agri.gov.cn/ zcfg/bmgz/ t20060123_540865.htm, accessed on October 24, 2002) of veterinary drugs and announced that the use of DES in food and animal feed was prohibited. The European Union (EU) requires that residual levels of DES in edible animal food should not exceed 2 ng mL^{-1} [6]. Consequently, a rapid screening method with high sensitivity and

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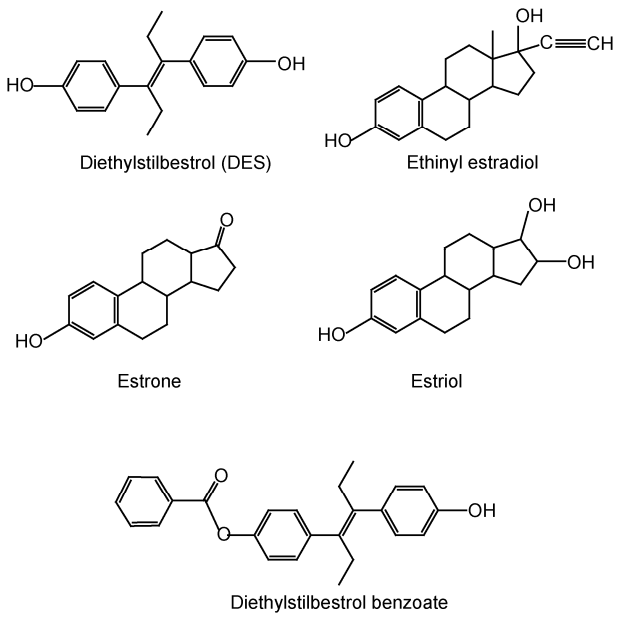

Figure 1 Diethylstilbestrol (DES) and related compounds analyzed in this study.

excellent stability is urgently required.

Determination of DES levels is mainly carried out by high-performance liquid chromatography detection methods [7,8], gas chromatography-mass spectrometry [9], colorimetric assay [10] and chemiluminescence [11]. The chromatography method demonstrated high sensitivity, however the instruments are expensive and sample pretreatment is complicated and time-consuming. Therefore, it is not suitable for rapid screening tests. The enzyme-linked immunosorbent assay (ELISA) technique is sensitive, specific and cost effective, and has been used diagnostically in recent years. Furthermore, ELISA methods have also been used for detection of various drug residues in the real system, such as in food and biological samples [12,13].

The sensitivity of an ELISA test is significantly influenced by the antibody against the compound being detected. Wang et al. [14,15] produced two kinds of similar polyclonal antibodies against DES with IC_{50} of 1.02 and 1.89 μg L^{-1}, respectively. Xu et al. [16,17] also prepared a polyclonal antibody against DES with an average IC_{50} value of 2.4 ng mL^{-1}, a calibration range of 0.2–30.5 ng mL^{-1}, and a detection limit of 0.07 ng mL^{-1}. Compared with the polyclonal antibody, the application of a monoclonal antibody (MAb) was advantageous in terms of purity, sensitivity and specificity. Up to now, there is no research reported on the preparation of monoclonal antibody for DES. The aim of our study is to develop an ELISA method based on the monoclonal antibody toward DES, and detect DES spiked in the animal (chicken, shrimp) tissue, urine and bile.

1 Materials and methods

(i) Chemicals. Bovine serum albumin (BSA), ovalbumin (OVA) and goat anti-mouse IgG-horseradish peroxidase (HRP) conjugated antibody were provided by Beijing Wanger Biotechnology (Beijing, China). 3,3′,5,5′-tetramethylbenzidine (TMB) was purchased from Xinjingke Biotechnology (Beijing, China). *N*-hexane, hydrochloric acid, dimethylsulfoxide (DMSO), sulfuric acid, sodium hydroxide, acetonitrile, hydrogen peroxide (30% H_2O_2) and other reagents were provided by Guangmang Chemical Company (Jinan, China). Chicken samples and pig liver samples were purchased from a supermarket in Jinan (China).

(ii) Instruments and consumables. ELISA was performed in polystyrene 96-well microtitre plates (Bio Basic Inc., Markham, Ontario, Canada) and spectrophotometrically read with a Model 680 microplate reader (Bio-Rad, Hercules, CA, USA). Centrifugation was carried out in a Biofuge Stratos refrigerated centrifuge (Heraeus, Germany). The dialysis bags (MWCO = 8000–14000) were from Aibo Economic & Trade (Jinan, China).

(iii) Buffers. Ultrapure deionized water was used for preparation of all buffers and reagents for the immunoassays, unless otherwise indicated. Phosphate-buffered saline (PBS; pH 7.4) consisted of 138 mmol L^{-1} NaCl, 1.5 mmol L^{-1} KH_2PO_4, 7 mmol L^{-1} Na_2HPO_4 and 2.7 mmol L^{-1} KCl. The wash buffer (PBST) was PBS supplemented with 0.05% (v/v) Tween 20. The 0.05 mol L^{-1} carbonate coating buffer contained 15 mmol L^{-1} Na_2CO_3 and 35 mmol L^{-1} $NaHCO_3$ (pH 9.6). The blocking buffer was a solution of PBS mixed with 1% OVA and 0.05% (v/v) Tween 20. The substrate buffer was 0.1 mol L^{-1} sodium acetate/citrate buffer (pH 5.0). An 80 mL volume of acetonitrile was added to 20 mL of 0.1 mol L^{-1} HCl and used as an extraction solution in the preparation of the biological samples. To prepare the substrate solution, 10 mg of TMB was dissolved in 5 mL of DMSO (substrate solution A) and 5 μL of 30% (w/w) H_2O_2 added to 15 mL of citrate buffer (substrate solution B) was mixed together. A 2 mol L^{-1} H_2SO_4 solution was used to stop the reaction.

(iv) Preparation of the DES hapten. The hapten of DES was synthesized via sodium chloroacetate according to the literature [15] with some modifications (Figure 2). DES (537 mg) was dissolved in 20 mL of dioxane, then 4 mL of sodium chloroacetate solution was slowly added, and 2 mL of sodium carbonate solution was added to the mixture above, then the solution was heated to 70°C and stirred overnight. The mixture was filtered and the filtrate was distilled by rotary evaporators. The residue was dissolved in chloroform and then petroleum ether was added. The precipitated flocculent was collected to obtain the DES hapten.

(v) Preparation of the coating antigen and immunogen. The conjugates of DES coupled with OVA and BSA were used as coating antigen and immunogen, respectively. As shown in Figure 3, the preparation of OVA-DES involved 1.0 mL of hapten as obtained above, 20 mg of dicyclohexylcarbodiimide (DCC) and 12.5 mg of *N*-hydroxysuccinimide (NHS) dissolved in 0.5 mL of dimethylformamide

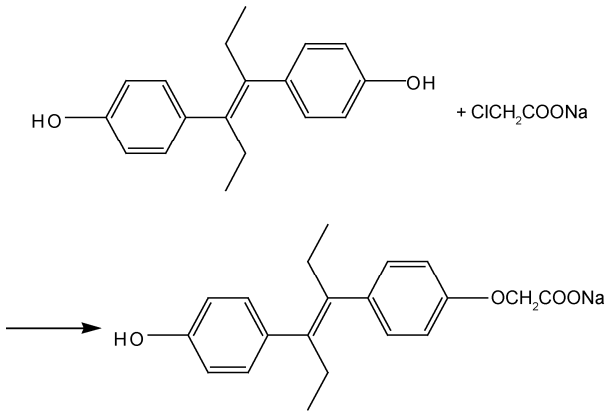

Figure 2 Synthesis of the DES hapten.

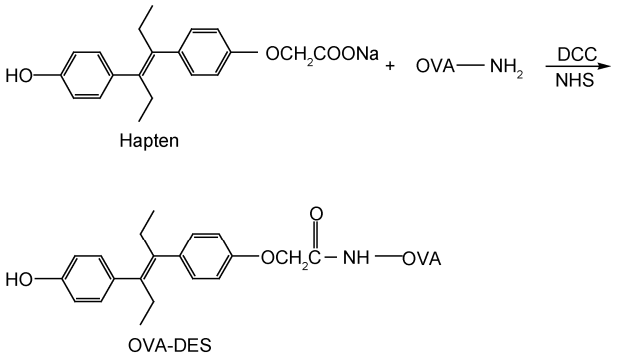

Figure 3 Synthesis of coating antigen OVA-DES.

and mixed for 24 h at room temperature, yielding solution I. OVA (50 mg) was dissolved in 3.5 mL of PBS (0.01 mol L^{-1}, pH 7.2) to obtain solution II. Solution I was added to solution II slowly and the mixture obtained was stirred at room temperature for 3 h to remove small impurities. The mixture was centrifuged at 10000×*g* for 30 min and the supernatant was collected to obtain OVA-DES, which was stored at –20°C for future use. The BSA-DES immunogen was synthesized in the same way.

(vi) Production of anti-DES MAbs. BALB/c mice were immunized by an intraperitoneal injection of 80–100 μg of BSA-DES with complete Freund's adjuvant. Three booster injections were performed using the same amount of BSA-DES with incomplete Freund's adjuvant at an interval of two weeks. Antiserum was collected and their titers were evaluated by an indirect competitive ELISA method. After the immune response was confirmed, the splenocytes from immunized mice were fused with mouse myeloma cells Sp2/0 (7∶1, cell ratio) using the polyethylene glycol (PEG) method [18] and cells were suspended in hypoxanthine-aminopterin-thymidine medium. The hybridoma cells were cultured in medium (pH 7.4) containing 0.2% $NaHCO_3$ and RPMI1640 with the addition of 20% newborn calf serum at 37°C to obtain MAbs for DES. The cells were screened by an ELISA assay and cloned by a limiting dilution method. The purification was performed according to the acid-ammonium sulfate method, and the purified MAbs obtained were stored at –20°C.

(vii) Characteristics of antibody as assessed by an indirect competitive ELISA method. The titer of the antibody was tested by an indirect competitive ELISA method [13]. The procedure was carried out as described below. The microplates were coated with the coating antigen, OVA-DES, at dilutions of 1/500, 1/1000, 1/2000, 1/4000, 1/8000, 1/16000, 1/32000 and 1/64000, and incubated at 37°C for 2 h. Plates were washed three times, blocked with 250 μL/well of blocking buffer, incubated at 4°C overnight. Plates were washed three times again, then the antisera at different dilutions were added and incubated for 1 h at room temperature. After washing, goat anti-mouse IgG-HRP (1∶1000, 100 μL/well) was added and incubated for 30 min at room temperature. Plates were washed three times and TMB substrate solution was added. After that, the plates were incubated for 15 min at room temperature. The color development was inhibited by adding stop solution (100 μL/well) and the absorbance at 450 nm was measured. Absorbance values were corrected by a blank reading. The antibody titer was defined as the reciprocal of the dilution that resulted in an absorbance value twice that of the blank value.

The sensitivity of antibody was determined by the same method as described above, except that different concentrations (0, 50, 150, 450, 1350 and 4050 pg mL^{-1}) of DES (50 μL/well) were mixed with antibody (50 μL/well) that was added to the plates coated with the coating antigen at the optimal concentration. The absorbance values at 450 nm were used to calculate the percent inhibition using the following equation: inhibition% = $B/B_0 \times 100$, where B was the absorbance value for the well containing the competitor and B_0 was the absorbance for the well without competitor.

The specificity of the antibody was evaluated by measuring inhibition curves using four functionally or structurally similar analogues as competitors, including ethinyl estradiol, estrone, estriol and diethylstilbestrol benzoate (Figure 1). The specificity was expressed as the cross-reactivity of each compound, which was determined by comparing the DES concentration required to produce a B/B_0 that was equal to 50% to that of the tested analyte.

(viii) Biological samples used for matrix effect determination. Animal (chicken and shrimp) tissue samples were pretreated by homogenizing for 10–15 min, then 2 g of the homogenized tissue was placed in a polythene tube and 6 mL of extraction solution (acetonitrile/acetone = 4∶1, v/v) was added. The mixture was shaken vigorously for 10 min at 15°C and centrifuged for 5 min at 700×*g*. A 3 mL volume of the supernatant was dried at 55°C. After that, 0.5 mL of chloroform and 2 mL of 2 mol L^{-1} NaOH was added, stirred and shaken for 30 s, then centrifuged at 3000×*g* for 5 min. Approximately 1 mL of the supernatant was mixed with 200 μL of 6 mol L^{-1} H_3PO_4 and 3 mL of acetonitrile. This mix-

ture was centrifuged at 3000×*g* for 10 min. After the organic phase was dried under nitrogen, the residue was mixed with 1 mL of reconstitution solution and used for analysis.

For the treatment of urine and bile, 2 mL of urine (or bile) and glucuronidase was transferred to a polythene tube. The mixture was centrifuged at 3000 r/min for 10 min at room temperature and 1 mL of the supernatant was mixed with 5 mL of chloroform, then centrifuged twice at room temperature for 10 min at 3000 r/min. After the organic phase was dried under nitrogen, the residue was mixed with 1 mL of reconstitution solution and used for analysis.

(ix) Validation of the indirect ELISA method. To detect the precision of the ELISA developed, 0.45 μg L^{-1} DES solution was detected repeatedly. The intra-assay variation was measured by analysis of a 0.45 μg L^{-1} DES standard solution six times on a single day. The inter-assay variation was measured by analysis of a 0.45 μg L^{-1} DES standard solution six times over three different days.

To detect the matrix effect on the repeatability and accuracy of the test, 1.0 and 2.5 μg kg^{-1} L^{-1} DES was added to animal tissues, urine and bile, then analyzed. Samples containing spiked levels were detected five times to ascertain the accuracy of the method. The coefficient of variation (CV) was obtained by the formula: CV% = standard deviation × 100/average.

2 Results and discussion

2.1 Production of immunogen

As a small molecule, DES itself was not immunogenic. A functional group (carboxyl, amino or hydroxyl) in the hapten for coupling the carrier proteins is required for establishing an ELISA method. The structure of DES (Figure 1) showed that the hydroxyl group is an important functional group for hapten synthesis, and likely to be a structural feature for this molecule in immunization.

Monoetherification was carried out during hapten synthesis for preserving a characteristic group and introducing a new functional group. For production of an antibody capable of recognizing an analyte, the haptens should be designed to expose its characteristic group to the largest extent. Spacer arms with different lengths connecting the target analyte to the carrier protein affect antibody formation. The hapten was synthesized by replacing the H residue in the phenolic hydroxyl of DES with a carboxyl group at the end. Haptens were then coupled with the carrier proteins to induce an immune response. The key factor for generating a successful ELISA assay is to produce a sensitive and highly selective antibody to the tested drug; therefore, we mainly focused on the titer and competitive characteristics of the antibody obtained. Consequently, the structure of the hapten and its coupling ratio with a carrier protein was not investigated.

2.2 Antibody evaluation

The titer of the serum from each animal was determined by measuring the binding of serial dilutions of antisera with the coating antigen. The titer of MAbs developed was more than 100000. The optimized concentration of coating and dilution of MAbs used was 1 μg mL^{-1} and 1∶4000, respectively. The representative inhibition and standard curves are shown in Figures 4 and 5, respectively. As can be seen from the Figure 4, the sensitivity of the antibody was satisfactory with an IC_{50} value of 275 pg mL^{-1} and a limit of detection (LOD; IC_{10}) of 90 pg mL^{-1} in buffer, significantly lower than the acceptable level set out by the EU of 2.0 ng mL^{-1}. Comparing the published ELISA methods (IC_{50} = 1.02 μg L^{-1} [14]; IC_{50} = 1.89 μg L^{-1} and LOD = 0.08 μg L^{-1} [15]), the antibody obtained by us demonstrated remarkably higher sensitivity. DES was able to compete with the coating antigen to interact with the antibody in the range of 50–1350 pg mL^{-1}, and in this range the absorbance value exhibited a good linear relationship with a logarithmic DES concentration (R^2 = 0.993; Figure 5). Based on the preparation of the polyclonal antibody against DES, Xu et al. [16,17] established a direct ELISA method for DES detection. A DES-HRP conjugate was required to perform the direct ELISA assay. To avoid synthesis problems in the process of conjugation, commercially available goat anti-mouse IgG-HRP was used to develop an indirect ELISA method in our study. Results show that the assay generated in our research was also more sensitive than the direct ELISA assay (IC_{50} = 2.4 ng mL^{-1}).

The specificity of the antibody was detected with three functionally and one structurally related compounds. As

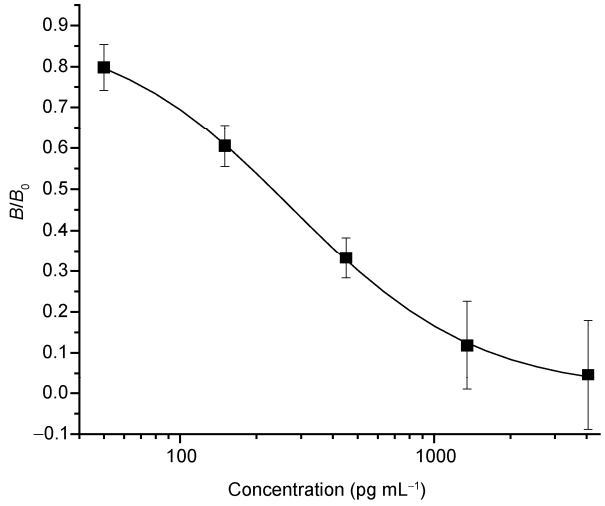

Figure 4 Representative inhibition curve of the anti-DES antibody using DES as the competitor and DES-OVA as the coating antigen in PBS. Each point represents the average of five replicates.

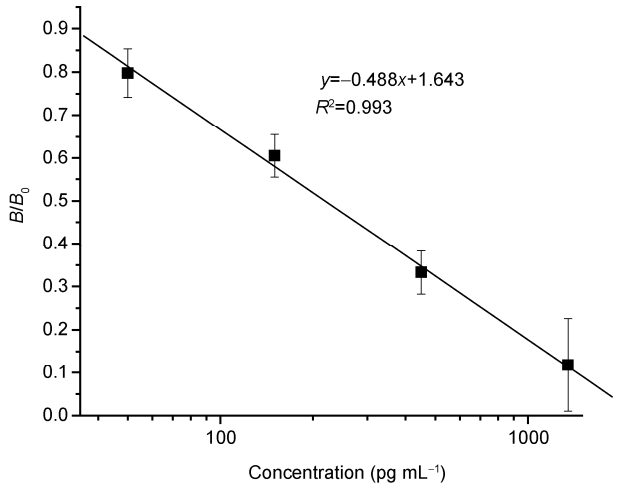

Figure 5 Linear standard curve of the anti-DES monoclonal antibody binding with the coating antigen. Each point represents the average of five replicates.

shown in Table 1, ethinyl estradiol, estrone, estriol and diethylstilbestrol benzoate displayed low cross-reactivity with <7%, <0.1% and <0.1%, respectively, indicating the excellent selectivity of the antibody obtained by us. The high specificity also confirmed our speculation that phenolic hydroxyl is an important structural feature in inducing an immune response against DES.

2.3 Repeatability of the ELISA method

A standard DES solution (0.45 μg L^{-1}) was measured six times on a single day to determine intra-assay variation. This standard solution was assayed over three different days to calculate inter-assay variation. As shown in Table 2, the coefficient of variation was in the range 3.6%–9.4%, intra-assay variation was 5.6%–6.2%, and inter-assay variation was less than 6%. These results demonstrated that the developed ELISA was stable and credible for determining the concentration of DES.

2.4 Matrix effects on the ELISA method

Animal tissue, urine and bile supplemented with 1.0 or 2.5 ug kg^{-1} L^{-1} DES were analyzed by the ELISA method. Each sample was analyzed five times to verify recovery, accuracy and stability of the method in a real system. As shown in Table 3, the coefficient of variation was in the range of 2.9%–6.3% for tissue samples, 5.0%–6.3% for urine samples, and 4.6%–8.5% for bile. The CV% was less than 10% for all samples, showing the acceptable reproducibility of the assay. The results also demonstrated that the recovery rates were greater than 79% for all samples (Table 3), demonstrating accuracy of the ELISA in measuring DES in samples. The recovery for animal tissue samples was mildly higher than that for urine and bile.

The matrix effect on the sensitivity of the test was also evaluated. The real systems (fish/shrimp, liver, feed and urine) spiked with different dilutions of DES were analyzed and the results are given in Table 4. The limit of detection for analyzing DES by the method in fish/shrimp, liver and urine was 0.6 μg L^{-1}, less than the acceptable level of 2 ng mL^{-1}, showing that the method was suitable for a screening analysis of DES in these matrices. However, the sensitivity of the test was extremely influenced by the feed (LOD = 48 μg L^{-1}). Therefore, the method is not fit to be used in DES detection in feed. Also, the recovery and repeatability tests of the method were not determined in the feed samples.

Table 1 Percentage cross-reactivity of the DES analog[a)]

Analogue	Cross-reactivity (%)
DES	100%
Ethinyl estradiol	<7%
Estrone	<0.1
Estriol	<0.1
Diethylstilbestrol benzoate	<0.1

a) Cross-reactivity was determined by comparing the concentration of the analyte required to produce a B/B_0 equal to 50%. Results were expressed as a percentage relative to the value for DES.

Table 2 Coefficient of variation (CV) for detection of the 0.45 μg L^{-1} DES standard solution[a)]

Detection number	CV (%)		
	Day 1	Day 2	Day 3
1	5.2	3.9	5.4
2	4.3	6.4	4.9
3	4.8	5.8	6.2
4	6.1	7.6	5.8
5	5.6	9.4	6.3
6	4.2	4.5	7.4
Intra-assay variation	5.0	6.3	6.0
Inter-assay variation		5.8	

a) Intra-assay variation was determined by measuring 10 replicates on a single day. Inter-assay variation was determined by measuring 10 replicates over three different days.

3 Conclusions

In our research, the immunogen was conjugated via the DCC method and a MAb against DES was obtained from immunized mice. The MAb demonstrated high sensitivity and specificity to DES with an IC_{50} of 275 pg mL^{-1} and LOD of 90 pg mL^{-1} in buffer. The functionally and structurally related compounds exhibited lower levels of cross-reactivity. The ELISA developed was used to detect DES in animal tissue, urine and bile, and the recovery rate was 79.8%–85.6%. The coefficients of variation of DES were both less than 10% in buffer and real systems. The LOD was 0.6 μg L^{-1} in fish/shrimp, liver and urine and 48 μg L^{-1}

Table 3 Detection of DES in animal tissue and urine spiked with DES[a)]

Sample	DES added	Measured concentration (μg kg^{-1}/μg L^{-1})					Recovery (%)	CV (%)
Animal tissue	2.5 μg kg^{-1}	2.0	2.3	2.3	2.1	2.0	85.6	6.3
	1.0 μg kg^{-1}	0.82	0.78	0.83	0.84	0.79	81.2	2.9
Urine	2.5 μg L^{-1}	2.1	2.0	1.9	2.0	2.2	81.6	5.0
	1.0 μg L^{-1}	0.77	0.82	0.73	0.88	0.79	79.8	6.3
Bile	2.5 μg L^{-1}	2.3	2.0	1.8	2.2	2.0	82.4	8.5
	1.0 μg L^{-1}	0.85	0.83	0.83	0.76	0.77	80.8	4.6

a) CV, coefficient of variation.

Table 4 Limit of dentation (LOD) for DES in biological samples with the developed ELISA method

Sample	LOD (μg L^{-1})
Fish/shrimp	0.6
Liver	0.6
Feed	48
Urine	0.6

in feed. In summary, an antibody against DES was obtained and could be used to establish an ELISA method to detect DES in most food-producing animals.

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