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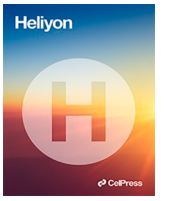

Research article

Development of a monoclonal antibody-based indirect competitive enzyme-linked immunosorbent assay for screening of diethylstilbestrol in animal-derived foods

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ABSTRACT

Diethylstilbestrol (DES), a synthetic non-steroid estrogen, it has been prohibited from being added to animal feed for any purposes. Herein, an indirect competitive enzyme-linked immunosorbent assay (ic-ELISA) based on a specific monoclonal antibody (mAb) was developed for the rapid screening of DES. Primarily, conjugates of mono-O-3-carboxypropyl diethylstilbestrol with keyhole limpet hemocyanin were used to raise a specific mAb, 1B7, which had IC_{50} value for DES of 213.0 ng L^{-1}. The limits of detection and limits of quantification value for DES in animal-derived foods ranged from 68.1 to 103.1 ng L^{-1} and 100.8–192.7 ng L^{-1}, respectively. The DES recovery ranged from 70.1 % to 103.1 %, with coefficients of variation below 13.9 %. A positive correlation ($R^2 = 0.997$) was observed between the results of ic-ELISA and HPLC-MS/MS for milk. In order to inspect its detection effect, milk and animal-derived foods were chosen as the testing object. The results showed that this ic-ELISA method (specific mAb, 1B7) can effectively examine for DES residues.

1. Introduction

Diethylstilbestrol (DES), an orally active synthetic non-steroid estrogen, it has been extensively served as a feed additives in raising, fattening and increasing feed conversion efficiency of aquaculture and stockbreeding [1,2]. Later, the application of DES was found that brought about unscheduled DNA synthesis, exchanging sister-chromatid, and disruptive mitotic and meiotic spindles, etc [3]. In addition, the prevalence of vaginal and cervical cancers increased in fetuses when exposed to DES [4]. Moreover, DES as a synthetic estrogen, it was also added to cosmetics [5].

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However, the carcinogenic and toxicological properties of DES to animal and human appeared extra consciousness. DES has been forbidden in most areas since 1970s, which was "phased out in the 1970s" by the FAO/WHO. Europe has banned adding DES to feed and has restricted the import of food animals with remaining DES from other countries in 1986 [6]. In 2002, the Ministry of Agriculture and Rural Affairs of the People's Republic of China issued a banned list of veterinary drugs and declared that DES was prohibited the employment in food and animal feed [7]. However, DES was still unlawful utilized extensively for huge profitable interests, which implied that the remainders of DES in animal foods grievously damage to human health, causing a worriment for food security.

During last twenty years, several techniques have been applied to quantitative monitoring anabolic hormone remnants in animal-derived foods such as fish, milk, meat, liver, etc [8,9].In order to protect the humans healthy, there has been developed several analytical methods for monitoring DES, such as high performance liquid chromatography (HPLC) [10], HPLC equipped with diode array detection (HPLC-DAD) [11], HPLC-mass spectrometry (HPLC-MS) [12], liquid chromatography-tandem mass spectrometry (LC-MS/MS) [13] and gas chromatography tandem mass spectrometry (GC-MS/MS) [14]. However, these above instrumental methods are exorbitant, require professional technicians and complex sample pretreatment before chromatographic examination. So, it is urgent problem to develop method with the low cost, high efficiency and easy operate instead of expensive testing means.

The exemplary analysis procedure towards screening of DES possess the qualities of high efficiency, manageability and profitability, which capacitate measured in miniature volume of biological fluid [15]. Immunoassay methods are constructed by molecular identification that connected antigens with antibodies to configurative a steady compound. Moreover, it is an extremely sensitive and available method owing to the high affinity antibody and convenient operation. Immunoassay methods, including radioimmunoassay (RIA) [16], fluorescence immunoassay (FIA) [17], radio receptor assay (RRA) [18] and enzyme linked immunosorbent assay (ELISA) [19], were successfully prepared for evaluating DES remnants. Distinctive with FIA, RIA and RRA methods, ELISA possesses additional predominance of harmlessness, environment-friendly, further speediness, more reactivity and low-budget [20,21]. However, most developed ELISA methods just monitor DES in limited animal tissues, such as milk [22], chicken muscle and liver [8], and shrimp [23], simultaneously, there are absence of studies about indirect competitive enzyme-linked immunosorbent assay (ic-ELISA) procedures that surrounded with the objective residual foods of pig and cattle for DES.

In this study, we established an ic-ELISA method that possesses a highly sensitive to determine DES with specific monoclonal antibody (mAb). Meanwhile, the simple preparation procedure was applied in animal-derived foods, incorporating milk, fish, and the muscle, kidney and liver of chickens, cattle and pigs.

2. Materials and methods

2.1. Chemicals

Bovine serum albumin (BSA), N′, N′-dicyclohexyl carbodiimide (DCC), dimethyl sulfoxide (DMSO), culture media RPMI-1640, hypoxanthine-aminopterin-thymidine (HAT), peroxidase-labelled goat anti-mouse immunoglobulins (HRP-IgG), hypoxanthine-thymidine (HT) medium supplements, incomplete and complete Freund's adjuvants, keyhole limpet haemocyanin (KLH), mono-O-3-carboxypropyl (MCPE), N-hydroxysuccinimide (NHS),mono-O-3-carboxymethyl (MCME), ovalbumin (OVA), polyethylene glycol 4000 (PEG 4000, 50 %) and N-acetylsulfanilyl chloride, 3,3′,5,5′-tetramethylbenzidine (TMB), which were obtained from Sigma-Aldrich (St. Louis, MO, USA). Fetal calf serum was purchased from Beijing Thermo Fisher Scientific Biological Engineering Materials Co., Ltd. (Beijing, China). The standard analytes were purchased from Dr. Ehrenstorfer. GmbH (Augsburg, Germany),including diethylstilbestrol (DES), dienestrol (DIEN), estrone (E), estradiol (E2), estriol (E3) and hexestrol (HEX).

2.2. Cells and animals

SP2/0 mouse plasmacytoma line was conserved in Liquid nitrogen in our laboratory.

Female Balb/c mice (NO.42816300004043, 5–8 weeks old) were obtained in the Animal Experiment Center of Huazhong Agricultural University (Wuhan, China), which was growth by surroundings with humidity conditions and specific temperature. The totals of experiments related animal in this subject, which were performed in acceptance of the Animal Experiment Centerwith guiding principle and were accepted though the Animal Ethics Committee of Huazhong Agricultural University (Wuhan, China).

2.3. Synthesis of haptens

The design and synthesis of hapten DES-MCPE were shown in Fig. 1. To prepare hapten mono-O-3-carboxypropyl diethylstilbestrol (DES-MCPE, $C_{22}H_{26}O_4$) and mono-O-3-carboxymethyl diethylstilbestrol (DES-MCME, $C_{20}H_{22}O_4$), DES (5.3 g) was dissolved in N,N-dimethylformamide (DMF, 50 mL) while mixed with Na_2CO_3 (9 g), which were reacted in an oil-bath (60 °C) on a magnetic stirrer. Later, 4-bromo-acetic acid ester (5 mL) was added dropwise to the mixture and reacted overnight, which thin layer chromatography was used to monitor the reaction process. After that, the organic layer was accumulated by adding ethyl acetate and water, and dried it within rotary evaporation. Finally, the product was obtained from the reaction result mixture (Intermediate A) by silica gel column chromatography, and was eluted by ethylacetate: petroleum ether (1:6). The elution was collected and dried by rotary evaporation. And then, the powder was dissolved in water: methanol (1:1), and the KOH (1 g) was added, which were reacted in an oil-bath for 12 h to obtain the final product DES-MCPE. The m/z calculated for $C_{22}H_{26}O_4$ was M = 354.1831.

The design and synthesis of hapten DES-MCME were shown in Fig. 2. To prepare hapten mono-O-3-carboxymethyl diethylstilbestrol (DES-MCME, $C_{20}H_{22}O_4$), the NaH (0.6 g) and DES (5.3 g) were blended in N,N-dimethylformamide (DMF, 50 mL), which were

reacted in an ice-bath on a magnetic stirrer. Later, the ethyl bromoacetate (3.2 g) was added to the mixture after 20 min. It was reacted overnight and monitored with thinlayer chromatography. After that, the ethyl acetate and water were added and gathered the organic layer, and dried it later by rotary evaporation. Finally, it was dissolved in water: methanol (1:1), and the KOH (0.7 g) was added, which were reacted in an oil-bath for 12 h to obtain the final product DES-MCME. The *m/z* calculated for $C_{20}H_{22}O_4$ was M = 326.1445.

2.4. Synthesis of antigens

The immunogens DES-MCPE-KLH and DES-MCME-KLH were composited according to a modified procedure (Figs. 1 and 2) utilizing the active ester method [24]. Concisely, the hapten (DES-MCPE or DES-MCME, 17 mg) was dissolved in DMF (0.3 mL) with NHS (12 mg) and DCC (18 mg), which were stirred for 12 h at room temperature. Next, the KLH (10 mg) was dissolved in phosphate buffer (PBS, 0.1 M, pH 8.0, 10 mL), which was added dropwise into the system and stirred overnight in 4 °C. After that, it was centrifuged for 10 min at 5000 g to obtained the supernatant. At last, the solute was depurated in exhaustive dialysis with PBS (0.01 M, pH 7.4) to gain the purified liquid. It was verified by UV–Visible spectrophotometry (Aglient 8453) and preserved at −20 °C until used.

The synthesis of DES-MCPE-BSA and DES-MCME-BSA coating antigens were the same as DES-MCPE-KLH and DES-MCME-KLH immunogens, but the KLH was replaced with BSA.

2.5. Animal immunization

Antigens (DES-MCPE-KLH or DES-MCME-KLH) with 50 μg and 100 μg were used to immunize female healthy Balb/c mice (aged 6–8 weeks). It was diluted with PBS (500 μL) and emulsified by Freund's adjuvant with an equal volume. The complete adjuvant containing immunogens was used to each mouse for primary immunization by subcutaneous injection of multiple sites. Subsequently, two subsequent booster immunizations about 2-week intervals were carried by the incomplete adjuvant. Collected the serum samples from the mice at intervals starting 7 days after the last booster immunization, and assessed the titre and specificity by ic-ELISA.

2.6. Preparation of monoclonal antibodies

Three days before cell fusion, the mice were injected with the final dose of enhancer, which consisted of double the dose of antigen in sterile isotonic saline without adjuvant. Mouse spleen cells that produce the best titer and specificity of the serum will be fused with Sp2/0 myeloma cells in a ratio of 10:1. After propagation in HAT medium, the supernatant of the hybridoma was measured using ic-ELISA. Subcloned and amplified the hybridoma from the well with the strongest positive reaction three times using limited dilution method. In addition, the cultured hybridoma was suspended in 0.5 mL 1640 medium and intraperitoneally injected into mice to produce ascites. Purification of ascites using octanoic acid and ammonium sulfate precipitation method, which stored at −20 °C and 50 % glycerol. The mouse mAb isotype assay kit (Proteintech Group, Inc., Chicago, IL, USA) was used to determine the class and subclass of secreted antibody isotypes [22].

2.7. Standard curve and specificity determination

The conditions, including the dilution of antibody, coating conjugate and the incubation time of antibody, were optimized to screening DES. Therefore, the DES standard solution was used to establish the standard curve (ELISA Calc software), which also calculated the half maximal inhibition (IC_{50} value) and across-reactivity (CR), including DES, dienestrol (DIEN), estradiol (E2), estriol (E3), estrone (E), HES and progesterone (P) The CR values were calculated as followed: CR= (IC_{50} of DES/IC_{50} of competitor) × 100 %.

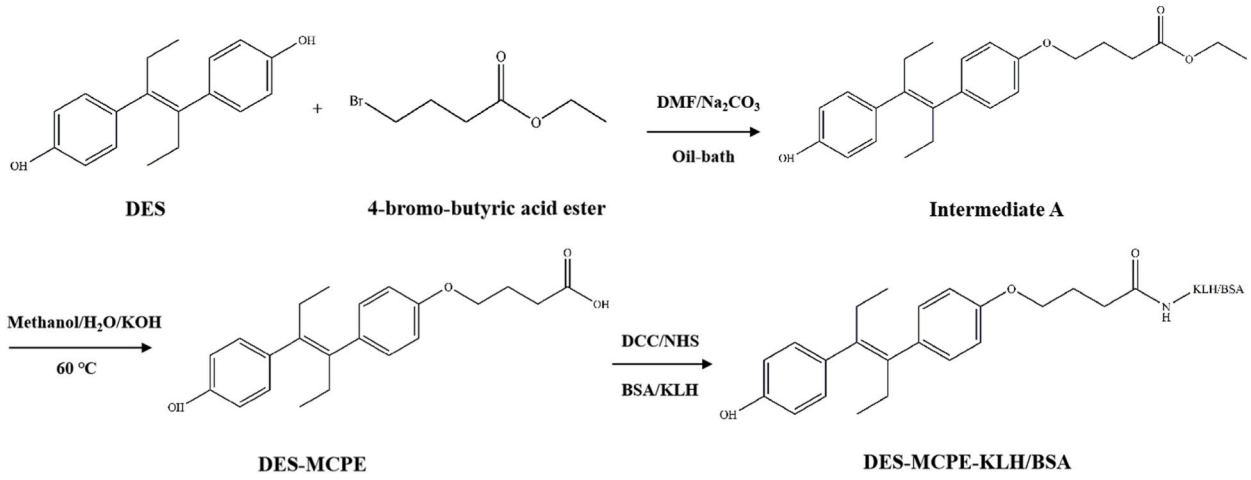


Fig. 1. Design and synthesis of the DES-MCPE, DES-MCPE-BSA/KLH.

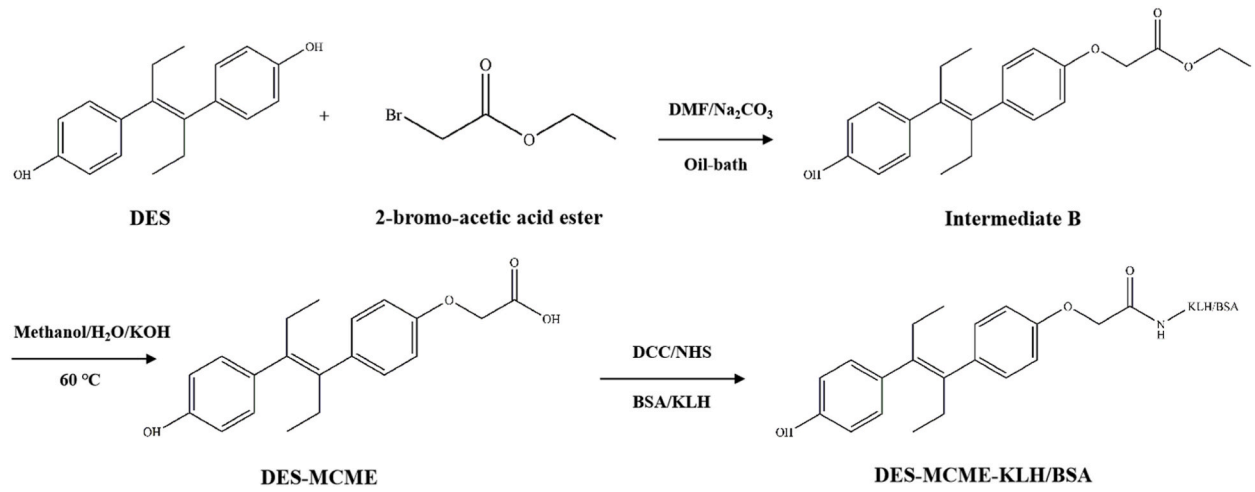


Fig. 2. Design and synthesis of the DES-MCME, DES-MCME-BSA/KLH.

2.8. Sample preparation

The samples, including milk, fish, and the muscle, kidney and liver of chicken, cattle and pig, were purchased from local markets. Firstly, milk sample (10 mL) was centrifuged at 5000 g for 10 min at 4 °C, and the middle layer of the milk was diluted 10 times with 20 % methanol PBS (0.01 M, pH 7.4) for use in ic-ELISA. Secondly, the animal tissue samples were minced and homogenized. Each sample (2 g) was placed in 30 % methanol-PBS (20 mL), vortexed for 5 min and centrifuged at 6000 g for 10 min. Obtained the supernatant and centrifuge again. Collected the supernatant for the ic-ELISA [22].

2.9. Validation of the ic-ELISA

All samples had been declared to be free of DES, which related verification by HPLC-MS/MS, according to a modified procedure [25]. Referring to the article of Gao et al., 2021 [22], the HPLC-MS/MS analytical separation was performed in a negative ion mode (ESI^-).

The ic-ELISA validation was putted into practice through 20 different blank samples, which was investigated to determine the LOD and LOQ value. The LOD values were calculated with the mean value of 20 blank samples plus 3*SD, but the LOQ values were plus 10*SD. Three different analyses at levels of 1-, 2- and 4-times the LOQ of DES were established by spiked blanks for five repeated trials.

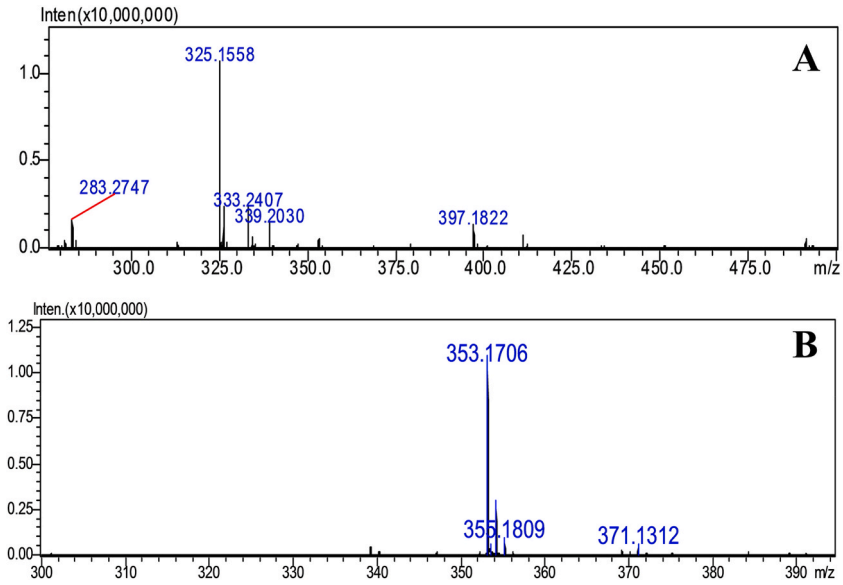


Fig. 3. MS spectrum of hapten DES-MCPE (A), calculated for $C_{22}H_{26}O_4$ $[M-H]^-$ 353.1831, found 353.1706; DES-MCME (B), calculated for $C_{22}H_{26}O_4$ $[M-H]^-$ 325.1445, found 325.1558.

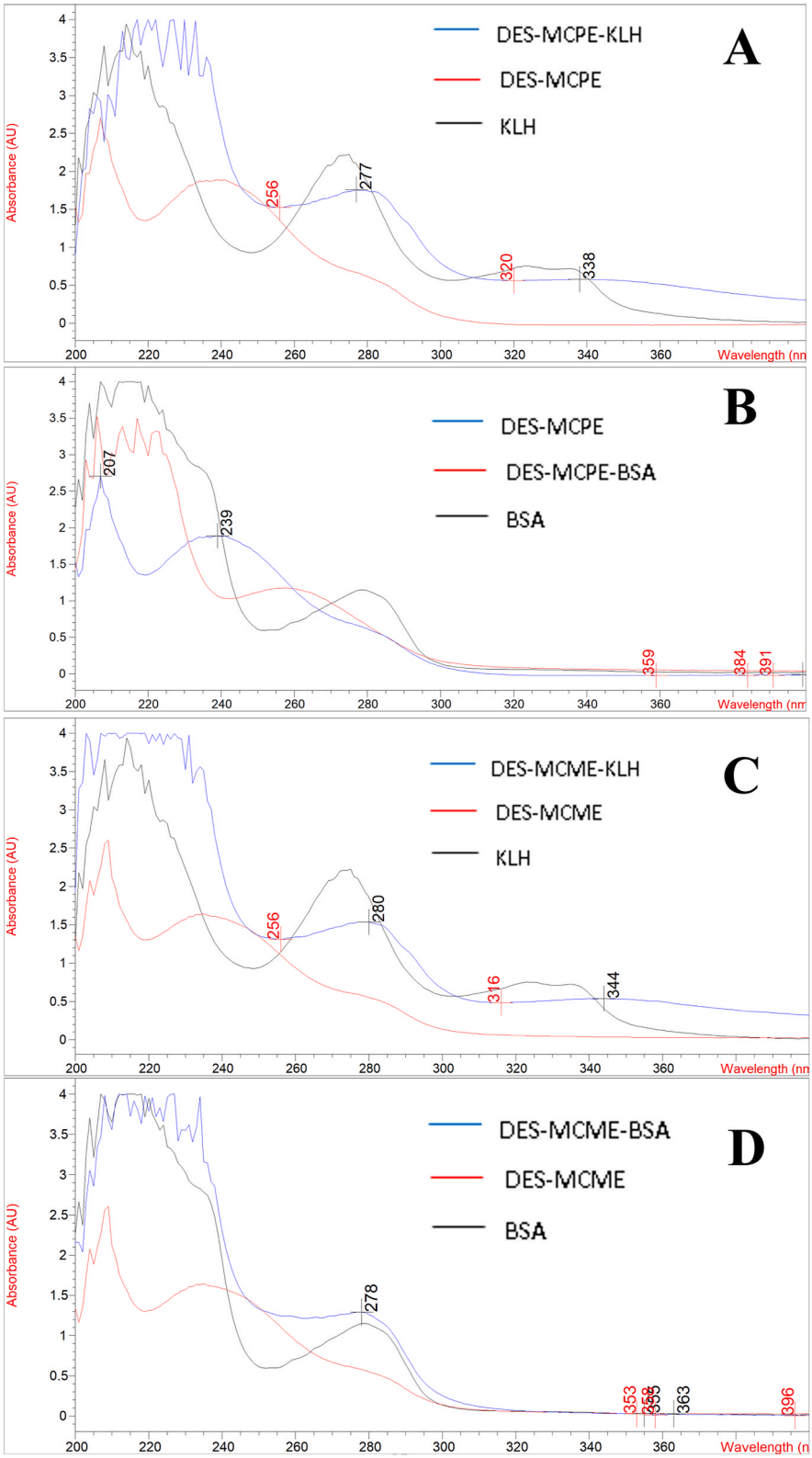


Fig. 4. The haptens (DES-MCPE and DES-MCME), carrier proteins (KLH and BSA) and synthetic antigens (DES-MCPE-KLH, DES-MCPE-BSA, DES-MCME-KLH and DES-MCME-BSA) were identified using UV–Visible spectrophotometry (Aglient 8453).

The mean recovery was determined as following: (conc. measured/conc. spiked) × 100 %. The coefficient of variation (CV) was determined with the same way and spiked with DES for five duplicated.

Simultaneously, the HPLC-MS/MS was performed to compare the reliability of the ic-ELISA, and the correlation of two methods was calculated. One half of the milk sample was use to the ic-ELSIA, and the other milk with equal volume was subjected to the HPLC-MS/MS.

2.10. Statistical analysis

All the experiments were repeated three times, and the data were statistically sorted out by Excel 2019 and analyzed by SPSS 23.0. Oneway ANOVA Duncan test was used for data difference analysis and normal distribution test.

Measurement data in accordance with the normal distribution law were expressed as x±SD.

3. Results and discussion

3.1. Characterization of haptens

Haptens DES-MCPE and DES-MCME were recognized by full scan mass spectra (Fig. 3). The MS *m/z* of hapten DES-MCPE and hapten DES-MCME were found in $C_{22}H_{26}O_4$ $[M-H]^-$ M = 353.1706 and $C_{20}H_{22}O_4$ $[M-H]^-$ 325.1558, respectively.

Anabolic estrogens are characterized by a non-steroidal parent ring and two benzene rings that connected with single or double bond in the carbon chain and resulted in diversely distorted levels in spatial structure. To obtain specific antibody that capable of recognizing analyte, the hapten ought to be formulated to perfectly mimic the structure of target molecule, and expose its characteristic group in accordance with electronic and hydrophobic properties. Therefore, the macro-molecular DES was introduced with connecting 4-bromo-acetic acid ester that characterized by 4 carbon chain length, which leaving neither too short because of the epitope cannot be fully exposed, but will not be too long to cause the folding connecting arms [26]. Tochi et al. (2016) and Mukunzi et al. (2016) both reported that the carbon lengths, between haptens and carrier proteins, can provide antibodies with higher affinity by longer spacer arms [19,27]. Moreover, because of the rotatability of the single bond on the alkane, the hapten structure get more flexible by the chain-like spacer arm, which result the sensitivity of antibodies differed greatly from each others [23].

3.2. Characterization of antigens

Sensitivity and specificity of antibody were largely determined by the conjugation ratio of hapten-protein and construction of hapten. In this study, we similarly synthesized antigens DES-MCPE conjugated carrier proteins KLH/BSA with the modified functional hydroxyl group [8,20]. An 8453 UV–Visible spectrophotometer was utilized to recognize the synthetic antigens (Fig. 4). The antigens were successfully synthesized, which could be identified the difference between the ultraviolet absorbance spectra of DES-MCPE-BSA (λ_{max}, 280), DES-MCPE-KLH (λ_{max}, 283), DES-MCME-BSA (λ_{max}, 278) and DES-MCME-KLH (λ_{max}, 282) and the BSA (λ_{max}, 279), KLH (λ_{max}, 279), DES-MCPE (λ_{max}, 239) and DES-MCME (λ_{max}, 235). The estimated conjugation ratios of DES-MCPE-BSA, DES-MCPE-KLH, DES-MCME-BSA and DES-MCME-KLH were 10.6, 23.6, 5.2 and 11.8 respectively. Compared with other researches, the detection capability of the present study was more specific and sensitive. The immunogen was well immunogenic, which the conjugation proportion of DES-MCPE-KLH was 23.6 that belonging the substitution degree range from 20 to 30 [28,29], which certificated that KLH possess the characteristic of strengthened immunogenicity and multiple coupling groups [30].

3.3. Characterization of the monoclonal antibody and the antisera

Two antigens, DES-MCPE-KLH and DES-MCME-KLH, were selected as an immunogen to produce mAb. Following the third injected, the indirect ELISA and indirect competitive ELISA were applied to take the measurements of the titres and the specificity of the antisera from the immunized mice, respectively. Compared with DES-MCPE-KLH (50 μg), the antisera with immunized in 100 μg group

Table 1
Titre and specificity of the antiserum and specificity of the hybridomas.

Immunogen		DES-MCPE-KLH		DES-MCME-KLH	
Dose (μg)		50	100	50	100
Titres (1:X)	Mouse 1	4000	4000	2000	3000
	Mouse 2	8000	10000	3000	4000
	Mouse 3	4000	4000	4000	4000
	Mouse 4	16000	16000	3000	4000
B/B0 values	Mouse 1	0.59	0.45	0.55	0.58
	Mouse 2	0.41	0.27	0.69	0.77
	Mouse 3	0.70	0.53	0.62	0.70
	Mouse 4	0.55	0.43	0.62	0.68
Hybridomas			1B7	2G2	
B/B0 values	DES(10 μg L^{-1})		0.33	0.61	

exhibited a vertical extent titre and more excellent inhibition ratio (B/B0<50 %). Differently, the antisera withimmunized in DES-MCME-KLH (50 μg) exhibited a higher titre than 100 μg group. Consequently, the fusion experiment would employ with DES-MCME-KLH (50 μg) and DES-MCPE-KLH (100 μg) to immunize spleen cells from the mice. Through the cell rescreening and subcloning three times, the hybridoma cells 1B7 and 2G2 were obtained, which the broad sensitivity was investigated by experiment with B/B0 against DES. As is shown in Table 1, the 1B7 demonstrated the more senior sensitivity against DES. Consequently, the hybridoma cell 1B7 was adopted to secrete the mAb, which observed by 8453 UV–Visible spectrophotometer (Fig. S1).

3.4. Optimization and standard curve for the ic-ELISA

The diluted mAb (from 1/8000 to 1/512000) and the coated concentrations of DES-MCPE-BSA (from 4 to 0.0625 μg mL^{-1}) were assessed by indirect ELISA (Table S1). Accordingly, the optimal concentration of the coating antigen (DES-MCPE-BSA) was determined to 0.5 μg mL^{-1}, which was obviously lower than Li et al. (2011) (1 μg mL^{-1}) and Wang et al. (2007) (1.5 μg mL^{-1}). In addition, the cell line 1B7 was capable of firmly secreting highly specific antibodies, which were diluted 72000 times and was sensitive to the concentration of DES. The inhibition rate in the DES concentration range of 0.8 μg L^{-1} to 0.2 μg L^{-1} increased rapidly from 20 % to 80 %. At the same time, mAb and second antibody reaction time were verified and optimized, as show in Table S2. It was represented the best reaction time of monoclonal antibody maintains 40 min, which keeping the lower IC_{50} values and the appropriate OD_{450} of 2.0 in the "0″ well. The incubation time of the secondary antibody was kept at 30–40 min, and the IC_{50} increased significantly over 40 min.

As shown in Table 2, the standard curve based on DES solvent calibration ranged from 64 to 2500 ng L^{-1} was established. The IC_{50} values for DES and HEX were 213.0 and 6200 ng L^{-1} exhibiting CR towards DES (100 %) and HEX (3.2 %), respectively.

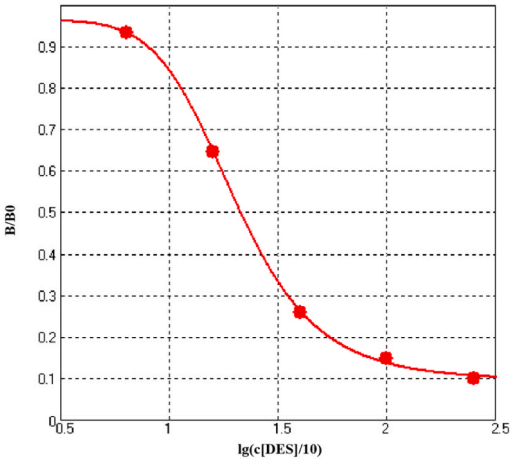
3.5. Sample preparation

Sample preparation definitely affects the reliability of the ELISA, thus it is a crucial step in the analysis process. Sex hormones are a categorization of water-insoluble medicine, which frequently dissolved in methanol to organize immunoassay [31]. According to the chemical properties of DES, it is easily soluble in organic solvents such as methanol, acetonitrile, etc.

In this study, we detected samples, which include milk, fish, and the muscle, kidney and liver of kidney, cattle and pig. Firstly, the milk was centrifuged and diluted 10 times accompanied by 20 % methanol-PBS, which was the same with previous researches [19]. Secondly, the preparation procedure of fish sample had improved enormously in accordance with the method reported by Mi et al. (2019). In addition, the sample solution was centrifuged twice at 6000 r/min for 10 min, and was guaranteed to be clear. The rotation rate of centrifuge was increased and the rotation time of centrifuge was reduced, which compared with Xu et al. (2006) (centrifuged at 2000 r/min for 10 min) and Mi et al. (2019) (centrifuged at 5000 r/min for 30 min). Thirdly, 30 % methanol-PBS (0.01M, pH 7.4) was prepared as a solvent extraction for animal-derived foods preparation procedure, which was a substitute for acetonitrile and ethyl acetate [32,33]. Li determined a complex method of biological samples pretreatment, which the sample was pretreated by centrifuged and diluted third times using acetonitrile/acetone, NaOH (2 M) and H_3PO_4 (0.6 M) [8].

Through the uncomplicated sample preparation, the general recovery rates of upon foods were 70.1 %–103.1 %, within coefficient of variation lower 13.9 % (shown in Table 3). Supplementary, the average recovery rates were distinct (fish < kidney < liver < muscle) within the modified procedure from samples, which signified that the recovery efficiency for liver and muscle was higher than fish and kidney.

Table 2
Standard curve for the ic-ELISA and cross-reactivity of the mAb DES/1B7.

The standard curve of ic-ELISA	Estrogen	IC_{50} (ng L^{-1})	Cross-reactivity (%)
	DES	213.0	100.0
	HEX	6200.0	3.4
	Dienestrol	>21300.0	<1.0
	Estradiol	>21300.0	<1.0
	Estriol	>21300.0	<1.0
	Estrone	>21300.0	<1.0
	Progesterione	>21300.0	<1.0

The calibration range from 64 to 2500 ng L^{-1} y=(A-D)/[1+(1000x/C)^B]+D, A = 0.96272,B = 6.90278, C = 1.30317, D = 0.09481, R^2 = 0.99959.

3.6. Validation of the ic-ELSIA method

The LODs and LOQs for DES in number of matrix samples as shown in Table 3, inclusive of milk, fish muscle, and the muscle, kidney and liver of chicken, cattle and pig, with ranging from 68.1 to 103.1 ng L^{-1} and 100.8–192.7 ng L^{-1}, respectively. By the above described sample preparation programs, negative sample increased of DES in several concentrations ((1*LOQ, 2*LOQ, 4*LOQ) that detected by optimized ic-ELISA measure. Moreover, the LOD and LOQ of muscle were lower than other foods, which meant the muscle qualified higher extraction efficiency. The accuracy and precision of the method were represented by the recovery and coefficient of variation (CV), respectively.

For the purpose of verifying the dependable detection of current ic-ELISA, it was frequently applying the HPLC-MS/MS to identify the drug residue. Consequently, the ic-ELISA and HPLC-MS/MS was performed and compared and to screening DES residue in milk. As shown in Fig. S2, a fine correlation (R2 = 0.997) among the two techniques was discovered, which suggested the present ic-ELISA with a reliable detection DES residue in samples.

3.7. Assessment of the ELISA methods for DES

Over the last few years, there have been developed competitive ELISA techniques to detect DES and its analogues, as shown in Table 4. Firstly, the mAb 1B7 possesses a lower IC_{50} value compared with other mAbs, and is able to recognize DES. Secondly, previous studies prepared the immunogen though the activated ester method, such as Li et al. (2011), Wang et al. (2007) and Mi et al. (2019), while Mukunzi et al. (2016) employed the EDC method. However, there were significantly different in the sensitivities between of these mAbs and polyclonal antibodies by using the same hapten of DES-MCPE. On the other hand, the sensitivities of polyclonal antibodies established by Mi et al. (2019) represented less different compared with the mAbs obtained in this study even they synthesized different hapten of DES-Bn. Furthermore, the biological samples pretreatment method for ic-ELISA should convenient, rapid and inexpensive. Nevertheless, the established sample preparation methods in the published studies detected deficient animal foods, including chicken liver and muscle, fish, shrimp and milk, while we prepared the milk and 10 kinds of animal-derived foods in this study. Meanwhile, we also summarized other detection methods for steroid hormones residues, as shown in Table S3, which showed significant differences in test values with different methods mentioned in this article.

Table 3
LODs, LOQs, recoveries, and CVs for the DES in 11 kinds of animal-derived foods.

Samples	LOD (ng L^{-1})	LOQ (ng L^{-1})	Spiked level (ng L^{-1})	Recovery ± SD (%, n = 15)	CV (%)
Milk	84.5	165.1	170	85.3 ± 9.8	11.5
			340	75.9 ± 5.9	7.8
			680	79.9 ± 14.6	13.9
Fish	87.2	159.2	160	77.8 ± 8.9	11.5
			320	78.7 ± 7.1	9.1
			640	71.6 ± 7.4	10.3
Beef	69.9	105.4	110	103.1 ± 6.5	6.3
			220	88.9 ± 3.7	4.2
			440	78.3 ± 5.6	7.2
Bovine liver	91.8	184.3	190	89.6 ± 4.9	5.5
			380	75.9 ± 7.9	10.4
			760	71.6 ± 9.1	12.7
Bovine kidney	81.4	170.6	180	91.0 ± 4.9	5.3
			360	81.9 ± 5.6	6.7
			720	76.7 ± 9.4	12.3
Chicken muscle	75.7	134.4	140	102.2 ± 4.6	4.5
			280	82.9 ± 7.9	9.5
			560	92.7 ± 11.7	12.6
Chicken liver	72.7	100.8	110	101.8 ± 4.7	4.6
			220	87.2 ± 6.8	7.8
			440	76.5 ± 5.7	7.5
Chicken kidney	103.1	192.7	200	88.1 ± 7.0	8.0
			400	75.2 ± 7.9	10.5
			800	74.6 ± 9.1	10.8
Porcine muscle	68.1	121.4	130	101.0 ± 8.3	8.2
			260	92.1 ± 7.1	7.7
			520	77.9 ± 7.7	9.9
Porcine liver	98.8	183.3	190	86.9 ± 5.3	6.1
			380	78.1 ± 7.8	10.0
			760	72.2 ± 6.9	9.6
Porcine kidney	87.6	168.8	170	89.9 ± 5.8	6.4
			340	83.5 ± 6.4	7.6
			680	70.1 ± 8.1	11.6

Table 4
Comparison between different antibody based ELISA methods for DES detection.

Antibody	Hapten	Coupling method	IC_{50} (μg L^{-1})	CR (%)		Samples	Reference
				DES	HEX		
Monoclonal	DES-MCPE	DCC	0.21	100	3.39	Milk, Fish, Bovine, Chicken, Porcine (muscle, liver, kidney)	This study
Polyclonal	DES-MCPE	DCC	1.02	100	6.1	Water	[20]
Monoclonal	DES-Sodium chloroacetate	DCC	0.275	100	–	Chicken (liver and muscle) Fish, Shrimp, urine	[8]
Monoclonal	DES-MCPE	EDC	0.23	100	–	Milk	[19]
Monoclonal	–	–	3.33	100	–	Urine	[21]
Polyclonal	DES-Bn	DCC	0.14	100	4.1	Shrimp	[23]
Polyclonal	DES-MCPE	–	2.4	100	–	chicken meat and liver tissue samples	[24]

4. Conclusion

In this study, to raise a high specific mAb against DES, we synthesized two distinct haptens and developed an ic-ELISA method for multi-resides recognition in milk and animal-derived foods. It was show that the mAb 1B7 demonstrated a high sensitivity for DES with the IC_{50} and the LOD are 213.0 ng L^{-1} and 74.0 ng L^{-1}. Furthermore, the established ic-ELISA method simplified the sample pre-treatment, including milk, fish, chicken (muscle, liver and kidney), cattle (muscle, liver and kidney) and pigs (muscle, liver and kidney), which is advantageously realizing beneficial throughput examination for DES residues.

CRediT authorship contribution statement

Bofei Fu: Formal analysis, Data curation. **Haijiao Gao:** Formal analysis, Data curation, Conceptualization. **Cuilan Fang:** Investigation, Funding acquisition. **Guyue Cheng:** Methodology, Investigation. **Hui Wang:** Software, Resources. **Yulian Wang:** Validation, Supervision. **Haihong Hao:** Visualization, Validation. **Xu Wang:** Writing – review & editing. **Lingli Huang:** Writing – review & editing. **Dapeng Peng:** Supervision, Funding acquisition.

Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

Ethics approval and consent to participate

This study was reviewed and approved by the Science Ethics Committee of Huazhong Agricultural University with the approval number: HZAUMO-2019-110, dated May 26, 2019.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations

BSA	bovine serum albumin
CBS	carbonate-buffered saline
CV	coefficient of variation
DCC	N,N′-dicyclohexylcarbodiimide
DES	Diethylstilbestrol
DMF	N,N′-Dimethylformamide
Hex	hexestrol
HRP	horseradish peroxidase
IC_{50}	50 % Inhibition concentration

Ic-ELISA	indirect competitive enzyme-linked immunosorbent assay
KLH	keyhole limpet haemocyanin
LOD	limit of detection
LOQ	limit of quantity
mAb	monoclonal antibody
MCPE	mono-O-3-carboxypropyl
MEME	mono-O-3-carboxymethyl
NHS	N-hydroxysuccinimide
OVA	ovalbumin
PBS	phosphate-buffered saline
SD	standard deviation
TMB	3,3′,5,5′-Tetramethyl benzidine dihydrochloride.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e39769.

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