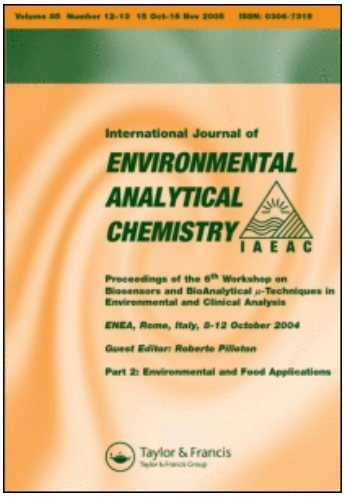

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Determination of 19-nortestosterone residues in aquaculture tissues by enzyme-linked immunosorbent assay and comparison with liquid chromatography and tandem mass spectrometry

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Determination of 19-nortestosterone residues in aquaculture tissues by enzyme-linked immunosorbent assay and comparison with liquid chromatography and tandem mass spectrometry

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19-Nortestosterone (17β-NT) was oximated by carboxymethoxylamine and then coupled with bovine serum albumin (BSA) in a mixed-anhydride reaction in order to produce an antibody. The conjugate rate of 17β-NT and BSA was estimated to be 24 by ultraviolet spectrophotometry. Polyclonal antibody of 17β-NT was acquired from the animal immunized with the conjugate. Through an indirect enzyme-linked immunosorbent assay (ELISA), which demonstrated that the synthesis of immunogen was successful, the titre of antiserum was found to be 6.4×10^5. Based on the purified antibody, a competitive indirect ELISA was developed. ELISA revealed that the limit of detection (LOD) was $0.07\,ng\,g^{-1}$, the recovery (in edible tissues) was 71–89%, and the working range was $0.05–31.25\,ng\,g^{-1}$. The preliminary evaluation of assay performance through specificity, sensitivity, precision, and accuracy revealed that this ELISA method could be used in the practical detection of 17β-NT in tissue samples. Moreover, this method was compared with high-performance liquid chromatography tandem mass spectrometry, for which the transition for quantification of 17β-NT was 275.4/109.1.

Keywords: Determination; 19-Nortestosterone; Residues; Aquaculture; Enzyme-linked immunosorbent assay (ELISA); Liquid chromatography and tandem mass spectrometry

1. Introduction

19-Nortestosterone (17β-NT) is one kind of anabolic steroids (ASs). Anabolic steroids have been extensively used in stock raising with beneficial effects on animal growth promotion and feed efficiency. Application of ASs as growth promoters has a history going back over 50 years [1]. 19-Nortestosterone (17β-NT) has been banned in livestock breeding as growth promoters in China since 2002 [2]. Until recently, the standard technique for ASs analysis has been gas chromatography mass spectrometry (GC/MS). This required the derivatization of the ASs using silylation, acylation,

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or oxime/silylation reactions, according to the individual characteristics of the ASs [3–5]. The lack of a universal derivatization reagent, together with the chemical rearrangement of others, hinders the availability of this method [6, 7].

Liquid chromatography tandem mass spectrometry (LC/MS/MS) provides a universal detector, since ASs may be analysed without derivatization. Recently, LC/MS/MS has been successfully applied to the analysis of ASs in various biological samples including urine from bovine and horse, bovine hair, and kidney fat [8–16], but few applications of the methods on muscular tissue of aquaculture have been reported. The limits of detection (LOD) of LC/MS/MS were above $0.1\,\mu g\,kg^{-1}$ [17–24], and the sample preparation was trivial [25–27].

Recently, several studies in China have been carried out on steroid residues, endogenous or exogenous, in domestic animal food. Surprising results were obtained; for example, some meat samples have above $0.1\,mg\,kg^{-1}$ of testosterone 17-propionate residue [28] or about $1.0\,mg\,kg^{-1}$ progesterone in some areas [29]. Some steroids, whether endogenous or exogenous, are used illegally or improperly in some areas now, where veterinary drugs have not been administered or under control. Studies were carried out on natural steroid hormone residues in animal food, such as estradiol, estriol, or estrone, but the methods are not sensitive enough [30, 31]. Therefore, it is worth establishing more sensitive methods suitable for inspection of steroid residues in animal food in China.

The objective of the present study is to develop a method for the determination 17β-NT residues in aquaculture tissues by using enzyme-linked immunosorbent assay (ELISA), and to compare it with high-performance liquid chromatography and tandem mass spectrometry. The present article describes an inexpensive, simple, rapid, and reliable ELISA method for detecting residues in 17β-NT aquaculture tissues, which is potentially applicable to other androgens or androgen-metabolite determinations.

2. Experimental

2.1 *Reagents*

19-Nortestosterone (17β-NT), 17β-hydroxyestra-4,9,11-trien-3-one (trenbolone), 17α-hydroxyestra-4,9,11-trien-3-one (17-α-trenbolone), and 17β-hydroxyandrost-4-ene-3-one (testosterone) were kindly donated by the Chinese Academy of Inspection and Quarantine. 17α-Hydroxyandrost-4-ene-3-one (epitestosterone) standard sample was purchased from Sigma-Aldrich (St. Louis, MO), and all other steroids used were supplied by Riedel-deHaën (Seelze, Germany). BSA, OVA (ovalbumin), and a protein-A-agarose affinity chromatography column (Sino-American Biotechnology Company, imported in bulk); Coomassie Brilliant Blue G_{250} (Shanghai Boao Biotech Co., Ltd); hydroxylamine HCl (Shanghai Chemical Agent Company, AR); carboxymethoxylamine hemihydrochloride and *N*,*N*-dimethylformamide (DMF) (Wuxi Chinese Traditional and Western Medicine Group Co., Ltd, AR); triethylamine (Shanghai Chemical Agent Company, AR); tributylamine (Shanghai Chemical Agent Company, CP); and isobutyl chlorocarbonate (Shanghai Feixiang Chemical Factory, CP) were used. Methanol, ethyl acetate, isopropanol, perchloric acid, and sodium borate were from Sigma (analytical- or HPLC-grade). β-Glucuronidase/arylsulphatase was from Helix Pomatia (Roche Diagnostics GmbH, Mannhein, Germany). Ammonium sulphate

and phosphate-buffered saline (PBS) were from Shanghai Chemical Reagent Station (AR). Isobutyl chloroformate was obtained from Feixiang Chemical Plant (Shanghai, China). Acetonitrile, *n*-hexane, chloroform, and 1-propanol for HPLC were all commercially available from Scharlau Chemic SA (Barcelona). Ultrapure water was made using the Milli-Q Ultra pure System (Millipore, Bedford, MA). All standards were stored at −20°C. Sep-Pak silica and amino-propyl solid-phase extraction cartridges containing 500 mg materials (3 mL) were purchased from Waters Co. (Milford, MA). To avoid contamination, all the glassware was baked for 4 h at 400°C prior to use. In addition, procedural blanks were conducted for each batch of samples to ensure minimal contamination.

2.2 *Apparatus*

The following equipments were used: electronic balance AB104-N (Shanghai Mettler Toledo Group); electro-thermostatic blast oven (Shanghai Yuejin Medical Instruments Factory); PHS-3TC acidimeter (Shanghai Tianda Apparatus Co., Ltd); auto-double-layer quartz distillatory (Ronghua Apparatus Manufacturing Co., Ltd, Jintan, Jiangsu); U-3000 UV scanner (Shimadzu, Japan); 722-style UV-Vis-NIR spectrophotometer (Shanghai Analytical Instrument Overall Factory); Bio-Rad mini gelatin cataphoresis apparatus (American Bio-Rad Company); FTS EI585-Q freeze dryer (American Stone Ridge); and a Multiscan MK3 Luminometer, transfer pipette, tips, and 8 × 12 Microlon ELISA plates (Thermo Labsystems).

An Agilent 1100 series LC system (Delaware, USA) including a G1313A quaternary pump, a G1313A autosampler, and a G1316A column oven were used for all analyses. All analytes were separated using a 150 × 2.1 mm SUPELCO Discovery® C_{18} column (Supelco, Bellefonte, PA) with a 5 μm particle size. A binary gradient consisting of acetonitrile (A) and purified water (B) at a flow rate of 0.3 mL min^{-1} was used. An injection volume of 10 μL was used for all analyses (Fluid Management System Inc., Minneapolis, MN). The gradient was as follows: for androgens and progestogens, the linear gradient protocol was 50% A to 100% A within 15.0 min. Mass spectrometry was performed using an API3000 tandem triple-quadrupole mass spectrometer equipped with a TurboIonSpray ESI source (HP Lab, Palo Alto, CA). The ion-spray voltage was 3000 V. High-purity nitrogen was used as nebulizer, heater, curtain, and collision gases. The heater gas was set at 7.5 L min^{-1}, and the TurboIonSpray probe temperature was maintained at 550°C. The nebulizer and curtain gases were, respectively, at 12 and 8 L min^{-1}, while the gas pressure in the collision cell was set at 3.4×10^{-5} Torr. Multiple reaction monitoring (MRM) was used for the multiple product ions of each analyte. Precursor/daughter ions were set to a unit resolution, and the dwell time was 150 ms.

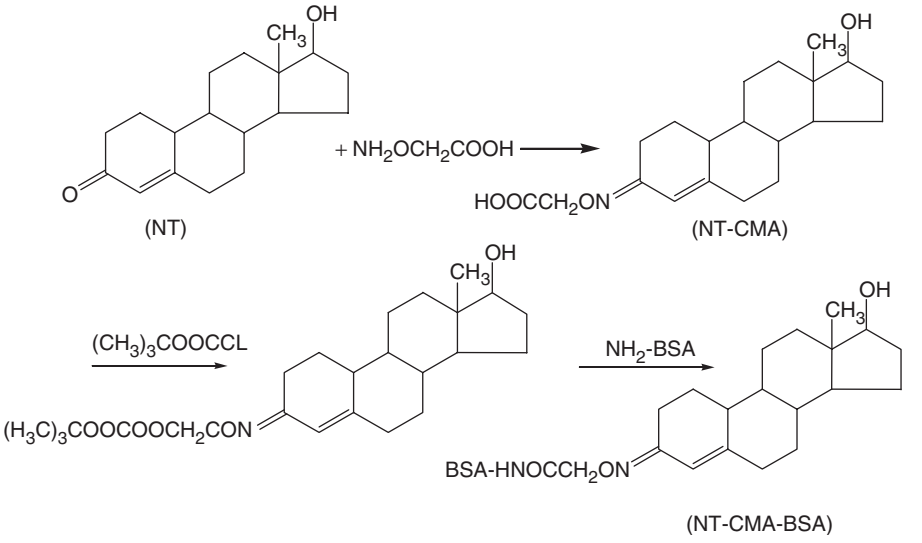
The conditions of total ion chromatography of standard androgens and progestogens in LC-MS-MS analysis were as follows: TEM: 550°C; IS: 3000 V; CAD: 7.0; mobile phase: acetonitrile (A) and water (B), gradient used for elution: 0.01 min A 50% (φ), 15.0 min A 100% (φ), 10 μL injection volume (1 μg mL^{-1}). The conditions of LC/MS/MS chromatography of androgens and progestogens in spiked fish meat tissues at 1.0 μg kg^{-1} were set as follows: TEM 550°C; IS 3000 V; CAD 7.0; mobile phase acetonitrile (A) and water (B); linear gradient used for elution: 0.01 min A 50%, 15.0 min A 100%; 10 μL injection volume.

2.3 *Preparation of protein–hapten conjugate*

2.3.1 Synthesis and characterization of 19-nortestosterone derivative. One millimole of 17β-NT (275 mg) was dissolved in anhydrous pyridine (10 mL), and then carboxymethoxylamine hemihydrochloride (219 mg, 2 mmol) was added. The mixture was stirred at 50°C for 30 min, and the pyridine was removed by distillation under reduced pressure. The residue was dissolved in ethyl acetate (100 mL). The organic layer was washed four times with distilled water (60 mL), dried over anhydrous Na_2SO_4, and removed by distillation under reduced pressure. The resulting crude product was purified by crystallization with diethyl ether to yield a white powder and then characterized by mass spectrometry.

2.3.2 Synthesis of 19-nortestosterone-BSA conjugate (17β-NT-BSA). The hapten of 17β-NT (100 mg) was dissolved in DMF (1 mL) and triethylamine (15 μL), and then isobutyl chlorocarbonate (45 μL) was dripped gradually to the solution at 4°C. The mixture was maintained at 4°C for 1 h and at room temperature for 20 min. The mixture product was added, by dripping gradually to a solution of a carrier protein (218 mg of BSA in 2 mL water; the pH was adjusted to 8.5 with 1 mol L^{-1} NaOH, then 1 mL DMF was added at 4°C). The reaction mixtures were stirred at 4°C for 4 h, and then the resulting conjugates were purified by exhaustive dialysis in PBS. Finally, the conjugates were dialysed in water and kept after lyophilization.

17β-NT-OVA (ovalbumin) conjugation was performed in the same way as detailed above for 19-NT-BSA conjugation. The protein conjugation of the 19-nortestosterone derivative is shown in scheme 1.



Scheme 1. The conjugation of protein and 19-nortestosterone derivative.

2.4 *Hapten density analysis*

The hapten density of 19-nortestosterone (17β-NT) was determined spectrophotometrically by measuring the ratios of absorbance at 250 and 280 nm of 100 μg mL^{-1} solutions according to the following equation: $n = (\varepsilon_{P,280}/\varepsilon_{H,250}) \times [(A_{250}/A_{280}) - (\varepsilon_{P,250}/\varepsilon_{P,280})]$. Where ε_H is the specific absorbance of the hapten and ε_P, the specific absorbance of the protein. The parameters $\varepsilon_{P,250}$, $\varepsilon_{H,250}$, and $\varepsilon_{P,280}$ were calculated by measuring the absorbance at 250 nm for the solutions of the free haptens (50 μg mL^{-1}) and proteins (100 μg mL^{-1}) at 250 and 280 nm [32].

2.5 *Immunization of animals*

Three New Zealand female white rabbits were immunized with 19-nortestosterone (17β-NT)-BSA conjugate synthesized by the methods of the mixed anhydride using the intradermal–intravenous route [33, 34]. 19-Nortestosterone (17β-NT)-BSA lyophilized powder was dissolved with physiological saline solution to make a solution of 1 mg mL^{-1}. The titres of antiserum were determined by ELISA. Antibodies were purified by ammonium sulphate precipitation followed by further purification using a protein-A-agarose affinity chromatography column. Antibody activity in the fractions was verified by ELISA. Pool fractions that showed maximum activity were stored at −80°C in small portions (0.5 mL) [35].

The determination procedures of the titre of antiserum by ELISA were carried out as follows:

1. Microtitre plates (96 wells) were coated with the solution of 19-nortestosterone (17β-NT)-OVA (Ovalbumin) (0.2 g L^{-1}), 100 μL for each well, and placed in the refrigerator overnight at 4°C.
2. Microtitre plates were then blocked with the solution of OVA (20 g L^{-1}), 200 μL for each well, and kept for 2 h at 37°C.
3. Four microtitre strips were prepared. The dilution of antibody was added in the first row as control, the second row was for the negative serum ($\varphi_r = 1:1000$), and the next six rows were separate for the positive serum ($\varphi_r = 1:250$, 1 : 1000, and 1 : 256,000), 100 μL for each well, and the strips were then kept for 1 h at a room temperature.
4. Goat anti-rabbit IgG marked with horseradish peroxidase (HRP) was added, 100 μL for each well, and kept for 1 h at a room temperature.
5. TMB, a colour-development reagent, was added, and the solution was kept for 15 min at room temperature.
6. H_2SO_4 (2 mol L^{-1}) was added to stop the reaction, and the absorbance at $\lambda = 450$ nm (OD_{450}) was then determined.

At each step above, the solution was washed with PBST solution three times, each time for 3 min. A diagram was drawn with OD_{450} as ordinate and the negative logarithm of dilution times of antibody as abscissa. With P/N (positive/negative) ≥ 2.1, the titre of the antibody could then be measured.

2.6 *ic-ELISA procedure*

After blocking the plates with 19-nortestosterone (17β-NT)-OVA, the sample (50 μL) or standard solution was dispensed into the appropriate well using a positive

displacement pipette. Rabbit anti-19-nortestosterone (17β-NT) antibody (50 μL) was added to each well, and plates were incubated for at least 2 h. Each plate was washed prior to the addition of goat anti-rabbit HRP-IgG (100 μL per well). After another 2 h of incubation, followed by washing, substrate solution (100 μL per well) was added. The colour development proceeded in the dark for about 20 min. The assay was stopped by the addition of 100 μL of 1.25 M H_2SO_4 per well, and the absorbance read at 450 nm on a Multiscan MK_3 Luminometer. The zero standard was assayed in quadruplicate, and all other standards and samples were assayed in duplicate. Using the Sigmaplot software package, sigmoidal competitive curves were fitted to a four-parameter logistic equation:

$$B/B_0 = \frac{A - D}{[1 + (\chi/C)^B]} + D,$$

where A is the asymptotic maximum (maximum absorbance in absence of analyte, A_{max}), B the curve slope at the inflexion point, C the χ value at the inflexion point (corresponding to analyte concentration giving 50% inhibition of A_{max}, IC_{50}), and D the asymptotic minimum (background signal). The four-parameter sigmoidal standard curve could be produced, based on the data above using the Softmax software package.

2.7 *Assay of cross-reactivity (CR)*

There were several structural analogues of 19-nortestosterone (17β-NT): 4-estren-3,17-dione (19-norandrostendione), 17α-19-nortestosterone, 5-estrarie-3,17-dione (19-norandrostandione), 17α-estradiol,17β-hydroxyestra-4,9,11-trien-3-one (trenbolone), 17β-estradiol, estrone, estriol, 17α-hydroxyestra-4,9,11-trien-3-one (17α-trenbolone), 5β-androstan-17β-ol-3-one (dihydrotestosterone), 4-androsten-17α-methyl-17β-ol-3-one, 5α-androstan-3-ol-17-one (androsterone), 17α-hydroxyandrost-4-ene-3-one (epitestosterone), pregn-4-en-3,20-dione (progesterone), diethylstilbestrol, 17β-hydroxyandrost-4-ene-3-one (testosterone), and ethinylestradiol. These standard compounds were prepared with the same buffer as for 19-nortestosterone (17β-NT) but in different concentrations, which acted as competitive substances to the antibody. All operations were carried out according to the ic-ELISA process, and IC_{50} values were calculated based on the sigmoidal curves, respectively: CR (%) = (IC_{50} of 19-nortestosterone)/(IC_{50} of anabolic steroid analogue) × 100%.

2.8 *Sample-pretreatment protocol*

Healthy fish were produced with feeds without using any anabolic steroids on the laboratory farm of our school. After they were slaughtered, their meat tissues were removed separately, and the fat, skin, and bones discarded. Each fish (100–200 g) was cut into pieces, homogenized with a household mixer, and then preserved in airtight conditions at −20°C.

Ten grams of each sample was weighed, and each sample was put into a 100 mL glass conical flask. Ten millilitres of 0.2 mol L^{-1} acetate buffer (pH 5.2) was added, and the samples were homogenized with an ultra-turrax machine for about 1 min. The pH of each mixture was readjusted to 5.2, 100 μL of β-glucuronidase/arylsulphatase from

Helix Pomatia (Roche Diagnostics GmbH, Mannhein, Germany) was added, and the mixture was incubated overnight at 54°C. Then, each sample was cooled to room temperature, and 35 mL methanol was added to homogenize the sample. Each mixture was centrifuged at 2000*g* for 10 min at 0°C. The supernatant was decanted into a separatory funnel and extracted with 20 mL *n*-hexane twice to remove any fat. The upper layer was discarded (*n*-hexane), and 5 mL of 1-propanol was added to prevent foaming during evaporation. Methanol was evaporated at 50°C with a rotary evaporator. One hundred millilitres of water was added, and the aqueous solution was subjected to solid-phase extraction (SPE).

An HLB cartridge was conditioned sequentially with 6 mL of methanol containing 50 mmol L^{-1} of triethylamine, 6 mL of methanol, and 6 mL of water. The aqueous extract was applied to the cartridge at a flow rate of 3–4 mL min^{-1}. The glass reservoir and cartridge were rinsed with 2×4 mL of water. The cartridge was dried with high-purity nitrogen. The crude analytes were eluted with 10 mL of methanol containing 50 mmol L^{-1} of triethylamine. The eluate was dried under a gentle nitrogen stream. The residue was dissolved by ultrasonication for 30 s with 0.5 mL of chloroform, and 5 mL of *n*-hexane was added. The solution was then passed through a Sep-Pak Silica solid-phase extraction cartridge and conditioned with 6 mL of *n*-hexane without any pressure. Five millilitres of *n*-hexane was used to wash the interference. The analytes were eluted sequentially with 6 mL of water-saturated ethyl acetate. The eluate was dried under a gentle nitrogen stream, and the residue was redissolved with 2 mL of methanol–ethyl acetate (40 : 60, v/v). The methanol–ethyl acetate solution was applied to amino-propyl solid-phase extraction cartridges conditioned with 4 mL of methanol–ethyl acetate (40 : 60, v/v) and 4 mL of water-saturated ethyl acetate. The eluate was collected, and 2 mL of methanol-ethyl acetate (40 : 60, v/v) was used to rinse the analytes. The eluate was dried under a gentle nitrogen stream and then the residue was reconstituted with 0.5 mL of mobile phase and mixed in a vortex stirrer.

2.9 *Recovery*

Recoveries of the ELISA assay were calculated based on optical density (OD) values of samples with different additional concentrations, respectively. OD values were interpolated from the standard curve using a computerized technique for automating calculation.

3. Results and discussion

3.1 *Calculation coupling rates of conjugate*

The conjugate band of 19-nortestosterone (17β-NT)-BSA showed the tailing phenomenon, which was lower than that of BSA. According to the scanning illustration, the maximum wavelength of 19-nortestosterone (17β-NT) was 250 nm.

Under two different concentrations, the absorbances of BSA and 19-nortestosterone-BSA at $\lambda = 250$ nm could be obtained, and the coupling rates were 24 using the following equation: $n = (\varepsilon_{P,280}/\varepsilon_{H,250}) \times [(A_{250}/A_{280}) - (\varepsilon_{P,250}/\varepsilon_{P,280})]$.

3.2 *Titre of antibodies*

The titre of 19-nortestosterone (17β-NT)-BSA was evaluated by indirect competitive ELISA. As shown in figure 1, the titre value for R1, R2, and R3 was 1 : 640,000, 1 : 160,000, and 1 : 320,000, respectively, which indicated that the 19-nortestosterone (17β-NT)-BSA conjugate was highly antigenic. The antiserum titre value, by definition, corresponds to the antiserum dilution resulting in an uninhibited assay signal three times the background signal under the specific assay conditions [36]. Since R1 displayed the highest titre value, this was used in our subsequent experiments.

3.3 *ic-ELISA procedure*

The optimized ic-ELISA standard curve is shown in figure 2. The IC_{50} value represents the concentration of the analyte resulting in a 50% decrease in the maximal corrected assay signal in the competitive ELISA system, and IC_{50} was 2.4 ng g^{-1}. The LOD value is defined as the analyte concentration reducing the mean blank assay signal by three standard deviations of the blank reading, and the limit of detection was 0.07 ng g^{-1}. The assay range was 0.05–31.25 ng g^{-1}.

3.4 *Assay of cross-reactivity (CR)*

The cross-reactivity of 19-nortestosterone antisera with anabolic steroid analogues was carried out by ic-ELISA. CR of 4-estren-3,17-dione (19-norandrostendione) was more than 20%. The CRs of 5-estrarie-3,17-dione (19-norandrostandione) and 17β-hydroxyestra-4,9,11-trien-3-one (trenbolone) were more than 10%. The CRs of 17α-19-nortestosterone and 17α-hydroxyestra-4,9,11-trien-3-one (17α-trenbolone) were more than 2%. The CRs of 5β-androstan-17β-ol-3-one (dihydrotestosterone), 4-androsten-17α-methyl-17β-ol-3-one, 5α-androstan-3-ol-17-one (androsterone), 17α-hydroxyandrost-4-ene-3-one (epitestosterone), pregn-4-en-3,20-dione (progesterone), 17α-estradiol, 17β-estradiol, estrone, estriol, diethylstilbestrol, 17β-hydroxyandrost-4-ene-3-one

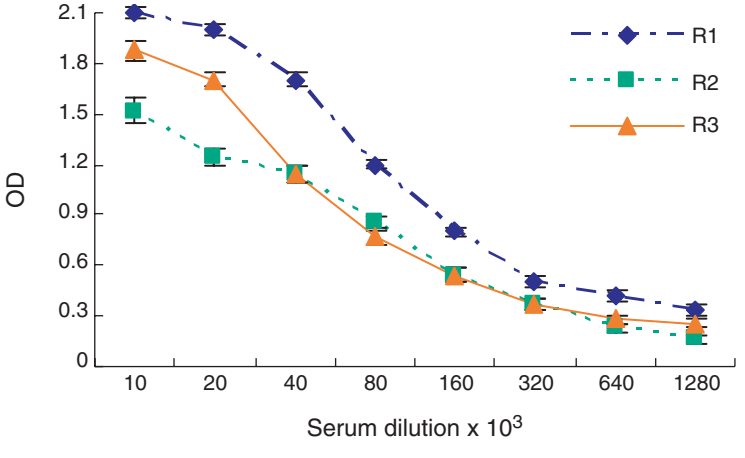


Figure 1. Determination of the antiserum titre by indirect ELISA.

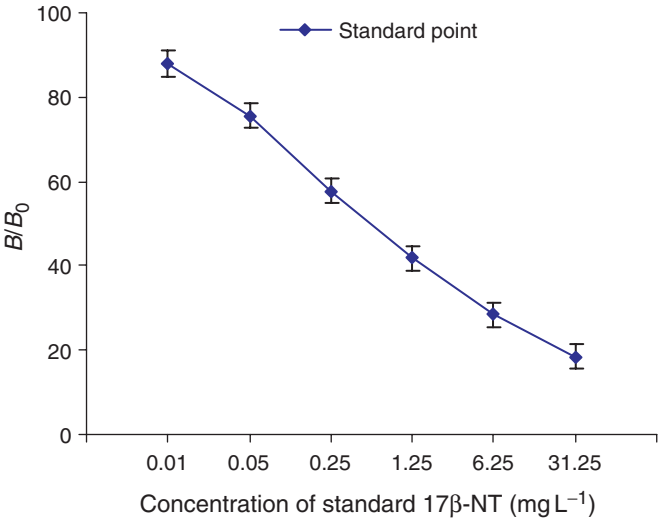


Figure 2. Calibration curve of ic-ELISA for 19-nortestosterone.

Table 1. Cross-reactivity of 19-nortestosterone antisera with anabolic steroids.

Anabolic steroid analogue	Cross-reactivity (%)
17β-19-Nortestosterone	100
17α-19-Nortestosterone	2.5
4-Estren-3,17-dione (19-norandrostendione)	32.1
5-Estrarie-3,17-dione (19-norandrostandione)	15.2
17β-Hydroxyestra-4,9,11-trien-3-one (trenbolone)	11.6
17α-Hydroxyestra-4,9,11-trien-3-one (17α-trenbolone)	3.27
5β-Androstan-17β-ol-3-one (dihydrotestosterone)	< 0.1
4-Androsten-17α-methyl-17β-ol-3-one	< 0.1
5α-Androstan-3-ol-17-one (androsterone)	< 0.1
17α-Hydroxyandrost-4-ene-3-one (epitestosterone)	< 0.1
Pregn-4-en-3,20-dione (progesterone)	< 0.1
17α-Estradiol	< 0.1
17β-Estradiol	< 0.1
Estrone	< 0.1
Estriol	< 0.1
Diethylstilbestrol	< 0.1
17β-Hydroxyandrost-4-ene-3-one (testosterone)	< 0.1
Ethinylestradiol	< 0.1

(testosterone), and ethinylestradiol were less than 0.1%. The results are presented in table 1. The data show that the antisera were highly selective. The cross-reactivity of the antiserum with metabolites of the drug was thus a useful characteristic of this ELISA, and it not only measured the parent drug but also the main metabolites, albeit with less sensitivity, and our antiserum did not cross-react with endogenous hormones. This analytical control of aquaculture in farms may be used to detect the precursor drug or its main metabolites.

Table 2. Coefficient of variation between microplates, batches, and in batches ($n = 6$).

	Standard concentration of 17β-NT ($ng\,g^{-1}$)					
	Blank	0.05	0.25	1.25	6.25	31.25
C.V.% (between microplates, $n = 6$)	7.8	5.8	6.3	5.5	6.7	5.8
C.V.% (between batches, $n = 6$)	8.5	7.9	7.2	6.9	7.5	6.7
C.V.% (in batches, $n = 6$)	11.2	12.7	10.3	11.3	8.6	8.9

3.5 *Precision and accuracy*

To assess the precision and accuracy of the assay, samples of the spiked 19-nortestosterone in fish meat tissues at six concentrations, corresponding to 0, 0.05, 0.25, 1.25, 6.25, and 31.25 $ng\,g^{-1}$, were studied, as shown in table 2. The coefficient of variation between microplates was 5.5–7.8%, the coefficient of variation between batches was 6.7–8.5%, and the coefficient of variation in the batches was 8.6–12.7%. All coefficients of variation were less than 15%.

3.6 *Recovery*

Recovery of the spiked 19-nortestosterone in the fish muscle tissues was 71–89% for concentrations within the range of 1–3 $ng\,g^{-1}$ of ELISA, as shown in table 3.

To evaluate the performance of the LC-MS-MS method, blank fish samples were fortified at 1.0 and 2.0 $\mu g\,kg^{-1}$. Table 4 lists the results of the fortification tests of each steroid in fish tissues. At a fortification concentration of 1.0 $\mu g\,kg^{-1}$, the average recovery of the 19-nortestosterone was 72%. The coefficient of variation (CV) was 18.1% at the 1.0 $\mu g\,kg^{-1}$ level. At a fortification concentration of 2.0 $\mu g\,kg^{-1}$, the average recovery of 19-nortestosterone was 83.5%, and CV% was 10.7%.

3.7 *Comparison with LC/MS/MS*

To evaluate the performance of the methods, fish blank samples were fortified at 1.0 and 2.0 $\mu g\,kg^{-1}$ level. Six replicates for each concentration were determined by ELISA and LC/MS/MS. The results are shown in table 5 and figures 3–7. In order to develop a method with the desired limit of detection ($< 0.1\,mg\,kg^{-1}$), it was necessary to use MS-MS detection, as MS-MS methods provide improved limits of detection for trace-mixture analyses. The inherent selectivity of MS-MS detection was also expected to be beneficial in developing a selective and sensitive method. Electrospray ionization (ESI) is selected in this experiment because of the differences in molecular structures of the ASs. The retention times of 17β-NT, MTS, ETS, MED, PG, and PTS was 2.88, 3.80, 4.14, 5.42, 7.27, and 11.32 min, respectively. The most sensitive mass transition was from m/z 275.4 to 109.1 for 17β-NT, from m/z 303 to 97.3 for MTS, from m/z 289.3 to 97.3 for MED, from m/z 345.2 to 97.3 for PTS, from m/z 315.3 to 97.3 for PG, from m/z 287.1 to 171.2 for EST, from m/z 271.1 to 145.2 for 17β-ES, from m/z 295.1 to 145.2 for EES and from m/z 269 to 145.2 for ESN. The MRM technique was chosen for the

Table 3. Average recoveries of fish meat samples as analysed by ic-ELISA.

Fish meat samples ($n=6$)	Added concentration of 17β-NT ($ng\,g^{-1}$)	Average value	Coefficient of variation (C.V.%)	Rate of recovery (%)
1	1	0.74	11.5	74
	3	2.61	8.7	87
2	1	0.71	11.3	71
	3	2.52	8.7	84
3	1	0.81	11.4	81
	3	2.67	8.5	89

Table 4. Recoveries and precision of the LC/MS/MS method ($n=6$).

Spiked sample ($n=6$) ($\mu g\,kg^{-1}$)		17β-NT	MTS	ETS	MPA	PG	PTS
1.0	Mean ± S.D. ($\mu g\,kg^{-1}$)	0.72 ± 0.13	0.71 ± 0.21	0.67 ± 0.23	0.66 ± 0.17	0.64 ± 0.21	0.69 ± 0.17
	Recovery (%)	72	71	67	66	64	69
	C.V. (%)	18.1	20.3	17.4	14.1	17.2	16.7
2.0	Mean ± S.D. ($\mu g\,kg^{-1}$)	1.67 ± 0.23	1.44 ± 0.21	1.72 ± 0.13	1.56 ± 0.12	1.34 ± 0.15	1.41 ± 0.13
	Recovery (%)	83.5	72	86	78	67	70.5
	C.V. (%)	10.7	11.3	10.6	8.4	10.4	9.7

Table 5. Comparative results for ELISA and LC/MS/MS for 19-nortestosterone in fish meat tissues.

Method	ELISA		LC/MS/MS	
Added ($ng\,g^{-1}$)	1.0	2.0	1.0	2.0
Determined ($ng\,g^{-1}$)	0.72 ± 0.03	1.47 ± 0.11	0.74 ± 0.04	1.45 ± 0.13
C.V. (%)	8.9 ± 0.26	10.1 ± 0.36	7.5 ± 0.19	12.9 ± 0.43
No. of determined samples	6	6	6	6

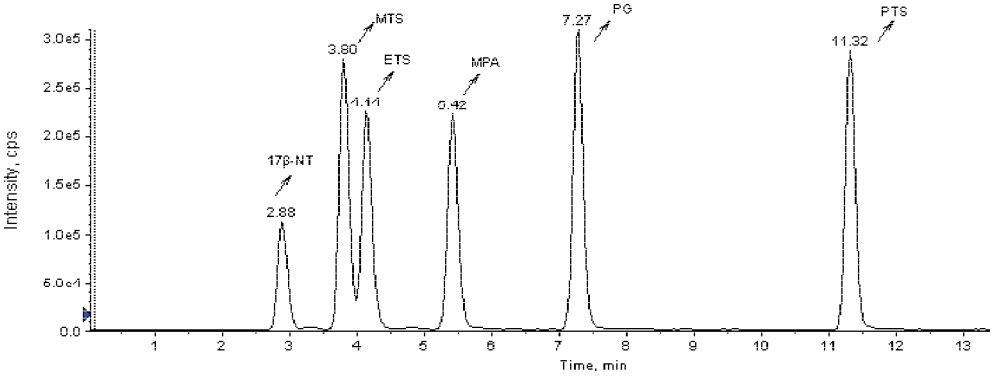


Figure 3. Total ion chromatography of standard androgens and progestogens in LC-MS-MS analysis.

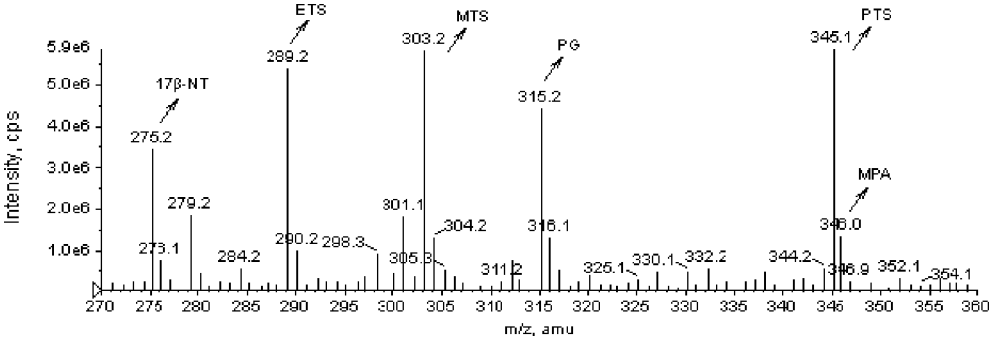


Figure 4. Full scan positive ESI Q1 scan of 17β-NT (19-Nortestosterone), MTS (17α-methyltestosterone), ETS (epitestosterone), MPA (Medroxyprogesterone acetate), PG (Prostaglandin), and PTS (testosterone 17-propionate).

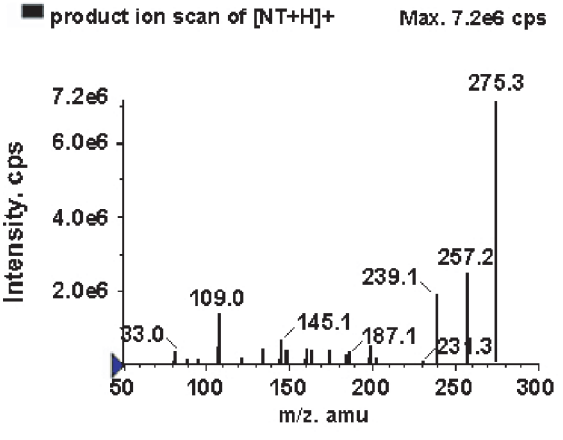


Figure 5. Product ion scan of 19-Nortestosterone parent ion $[M^{+}H]^{+}$.

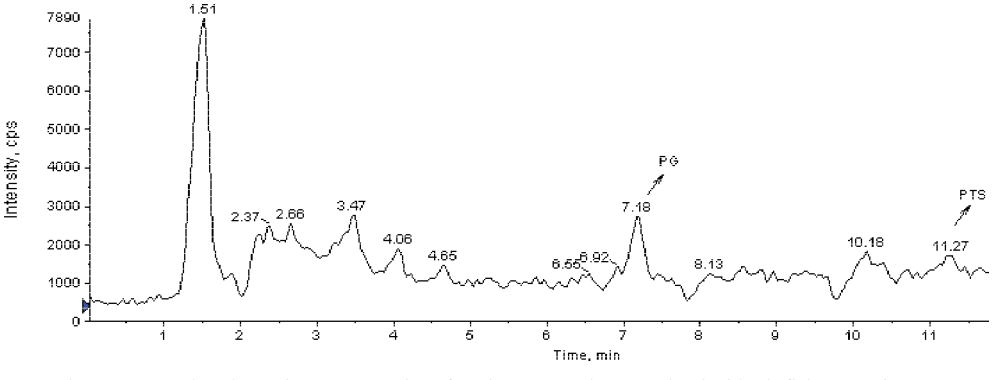


Figure 6. LC/MS/MS chromatography of androgens and progestins in blank fish meat tissues.

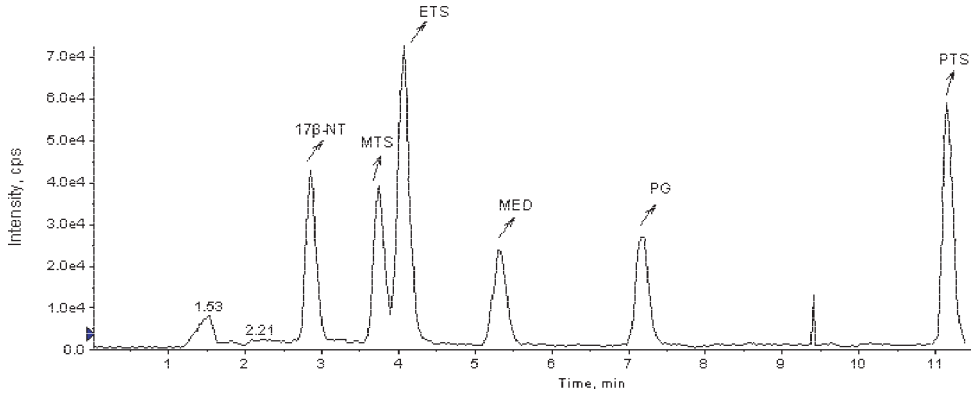


Figure 7. LC/MS/MS chromatography of 17β-NT (19-Nortestosterone), MTS (17α-methyltestosterone), ETS (epitestosterone), MPA (Medroxyprogesterone acetate), PG (Prostaglandin), and PTS (testosterone 17-propionate) in spiked fish meat tissues at $1.0\,\mu g\,kg^{-1}$.

assay development. Separate MRMs were preferred for ASs to provide the most sensitive and selective method. The MRM state file parameters were optimized to maximize the response for the analytes. The transition for quantification of 19-nortestosterone was 275.4/109.1, and the linear equation of 19-nortestosterone of LC/MS/MS: $y = 3.38 \times 10^3 x + 1.35 \times 10^3$, $r = 0.9997$. The linear equation of 19-nortestosterone in ELISA: $y = 6.09 \times 10^3 x + 9.12 \times 10^2$, $r = 0.994$.

3.8 *LOD and LOQ*

By determining the standard errors of blank samples, the limits of detection (LOD), which were defined as three times the standard errors of blank samples, were obtained. The LOD of the method for the 19-nortestosterone was $0.064\,\mu g\,kg^{-1}$. The limits of quantification (LOQ) of the method were determined according to the formula $S/N = 10$ and found to be $0.12\,\mu g\,kg^{-1}$.

4. Conclusion

This ELISA method has a high specificity and no cross-reaction with natural hormones. The detection limit is $0.07\,ng\,g^{-1}$ in aquaculture tissue residues and considerably lower than the temporary MRL in China. Therefore, this ensures the minimum occurrence of false-negative results for the detection of 17β-NT treatment. The ELISA methods presented here are suitable as screening methods for the detection of 17β-NT treatment in aquaculture tissue samples. For confirmatory or forensic purposes, the necessary MS-based methods should be employed.

The ic-ELISA method was developed and validated for the determination of 19-nortestosterone concentrations in aquaculture tissues. On the basis of the characteristics of the antiserum and assay described here, it can be concluded that this ELISA is appropriate for screening the illegal use of nortestosterone. This method was

verified by LC/MS/MS. The two analytical methods can be used to measure 19-nortestosterone at subnanogram levels and are suitable for monitoring 19-nortestosterone residues in edible foods.

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