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Article

Determination of Seven Free Anabolic Steroid Residues in Eggs by High-Performance Liquid Chromatography–Tandem Mass Spectrometry

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A cheap, reliable and practical high-performance liquid chromatography–tandem mass spectrometric method was developed for the simultaneous determination of seven anabolic steroids in eggs, including trenbolone, boldenone, nandrolone, stanozolol, methandienone, testosterone and methyl testosterone. The analytes were extracted from the egg samples using methanol. The extracts were subjected to the removal of fat by freezing-lipid filtration and then further purified by liquid–liquid extraction using *tert*-butyl methyl ether. The analytes were separated on a Luna C18 column by a gradient elution program with 0.1% formic acid and acetonitrile. This method was validated over 1.00–100 ng/g for all steroids of interest. The correlation coefficients (*r*) for each calibration curve are higher than 0.99 within the experimental concentration range. The decision limits of the steroids in eggs ranged from 0.20 to 0.44 ng/g, and the detection capabilities were below 1.03 ng/g. The average recoveries were between 66.3 and 82.8% in eggs at three spiked levels of 1.00, 1.50 and 2.00 ng/g for each analyte. The between-day and within-day relative standard deviations were in the range of 2.4–11%. High matrix suppression effects were observed for all compounds of interest.

Introduction

Anabolic androgenic steroids (AASs) refer to substances that can be used to promote muscle growth. In animal husbandry, AASs are used in increasing the weight of meat-producing animals, enhancing nitrogen retention and building up proteins, which in turn results in an improvement of muscle growth and higher carcass quality (1, 2); AASs are banned from use. Evidence of the illegal use of AASs for growth-promoting purposes has been presented. Residues of these compounds in edible animal products are a potential risk for the consumers. Therefore, monitoring of AAS residues in animal-derived food is very important.

Over the years, many analytical methods have been developed to determine AAS in biological samples. Gas chromatography–mass spectrometry (GC–MS and tandem MS) methods have been used to determine and confirm AAS residues (3–7), and currently, liquid chromatography–mass spectrometry (LC–MS and tandem MS) methods are preferred because they provide easier sample preparation without a derivatization step, and because the detection of substances (i.e., stanozolol and trenbolone) by GC–MS is difficult or impossible. For example, Van Poucke *et al.* determined 21 AAS residues in bovine urine by LC–MS-MS and GC–MS, respectively (8). Xu *et al.* developed an LC–MS-MS method for the determination of 10 AAS residues in animal muscle tissues (9). Yang *et al.* developed an ultra-performance liquid chromatography (UPLC)–MS-MS method for the simultaneous determination of 50 steroid hormones (including 18 AASs and their metabolites) with a wide range of polarity and classification in samples of pork, beef, milk and shrimp (10). Currently, only a few papers have been reported to analyze steroid hormones in eggs. Yang *et al.* developed a UPLC–MS-MS method for the simultaneous screening and determination of eight free progestogens in eggs (11). A sensitive UPLC–MS-MS method was developed for the simultaneous determination of 17 sex hormones in egg products by Wang *et al.* (12), and the limits of detection of the method for the three AASs such as testosterone propionate, methyltestosterone and nandrolone were in the range of 0.035–0.071 μg/kg. The authors' previous publication (13) described a simple and fast UPLC–MS-MS method for the analysis of 11 AAS residues in animal muscles and eggs. However, a problem was encountered in the interlaboratory study with generating three layers from egg sample solutions in the final centrifugation at 36,000 × g. In addition, there are quantities of endogenous progesterone in eggs, so the method is unfit for the determination of progesterone. The aim of this work is to develop and validate a practical, cheap and reliable LC–MS-MS method for the simultaneous screening and quantification of seven anabolic androgenic steroid compounds (including trenbolone, boldenone, nandrolone, stanozolol, methandienone, testosterone and methyl testosterone) in eggs by removing fat with a freezing technique and liquid–liquid extraction protocol. The method was successfully applied to the determination of seven AASs in eggs from local supermarkets. Figure 1 shows the chemical structures of the studied compounds.

Experimental

Materials and chemicals

Trenbolone (TBL), boldenone (BDN), nandrolone (NLE), stanozolol (SNZ), methandienone (MDN), testosterone (TSN), and methyltestosterone (MTS), all (analytic grade >98% purity) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Formic acid, acetonitrile and methanol (all LC grade) were purchased from Merck (Darmstadt, Germany). *Tert*-butyl methyl ether (TBME) was purchased from Sigma-Aldrich (St. Louis, MO). Hexane, sodium acetate and sodium carbonate (all analytical reagents) were purchased from Guangzhou Chemical Reagent Factory (Guangzhou, China).

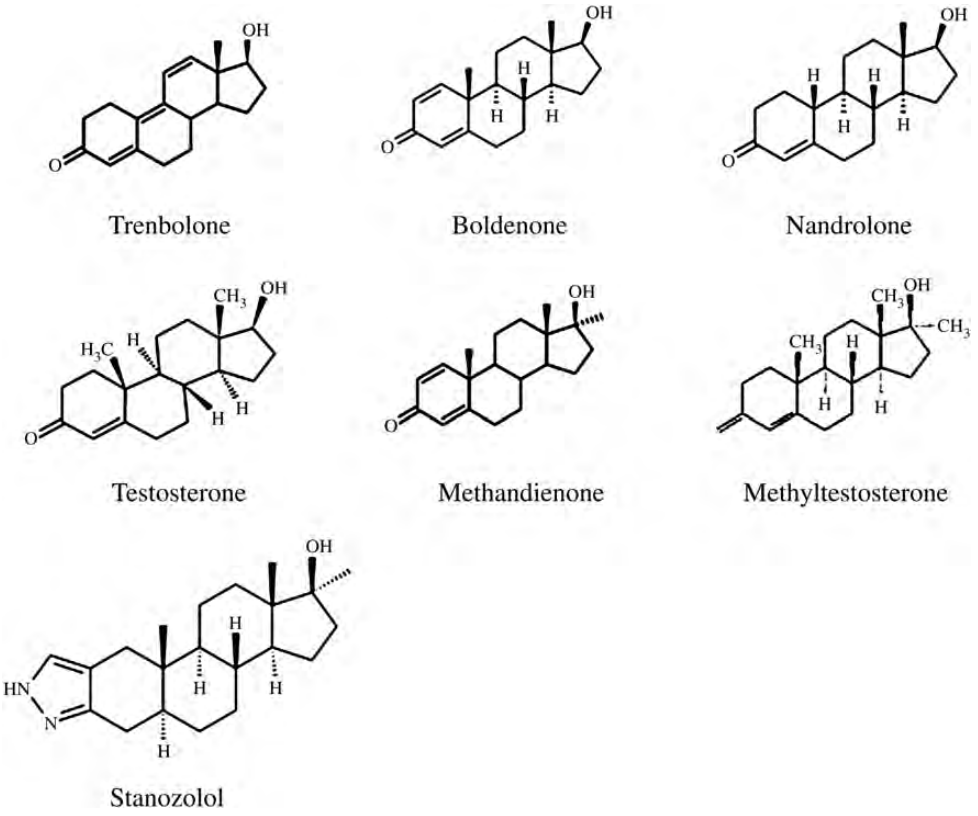

Figure 1. Chemical structures of seven anabolic androgenic steroids.

Ultra-pure water was purchased from Watsons Company (Hong Kong, China)

Preparation of stock and working solutions

Individual steroid standard stock solutions were prepared by weighing approximately 10 mg each of TBL, BDN, NLE, SNZ, MDN, TSN and MTS into separate 10 mL volumetric flasks in methanol (1.00 mg/mL) and storing at −20°C.

Intermediate mixed standard solution was prepared by transferring 100 μL of every standard stock solution to a single 10 mL volumetric flask and diluting to volume with methanol (10 μg/mL) and storing at −20°C.

Working standards at concentrations of 0.25–200 ng/mL were freshly prepared daily by a series of dilutions of the intermediate mixed standard solution with mobile phase (0.1% formic acid aqueous solution–acetonitrile, 50/50, v/v).

Instrumentation and analytical conditions

The chromatographic system was composed of an Agilent 1200 series high-performance liquid chromatography system, including quaternary pump and autosampler (Milford, MA). The mass system included an Applied Biosystems API 4000 triple quadrupole mass spectrometer with electrospray ionization interface and Analyst 1.5 software (Foster City, CA). Chromatographic separation was performed using a Luna C18 column (150 × 2.0 mm i.d., 5 μm), which was purchased from Phenomenex (Torrance, CA). The mobile phase consisted of a gradient of 0.1% formic acid solution (solvent A) and acetonitrile (solvent B) at a flow rate of 0.25 mL/min. The linear elution gradient profile was as follows: 0–2.0 min, 20–90% B; 2.0–8.0 min, 90% B; 8.0–10 min, 90–20% B; 10–16 min, 20% B. Injection volumes of 10 μL were used in LC.

The mass analyses were performed using an electrospray source in positive ionization mode. The ionspray voltage was 5.0 kV. The source temperature was set at 600°C. Curtain gas and ion source gas 1 and gas 2 were set at 20, 55 and 50 psi, respectively. Multiple reaction monitoring (MRM) experiments were conducted. The optimization of the primary mass spectrometry parameters was performed by flow injection analysis for each compound. Table I shows the optimized parameter values and the MRM transitions used for the confirmation and quantification of the seven AAS residues.

Sample preparation

Whole eggs were homogenized in a blender. A 2 g portion of the sample was weighed into a 50 mL polypropylene centrifuge tube with a screw cap. Two milliliters of sodium acetate buffer (0.4 mol/L, pH 5.2) and 10 mL methanol were added to the centrifuge tube and vortexed for 10 s. The mixture was sonicated for 20 min and centrifuged at 6,000 × g for 10 min at 4°C. The supernatant was left overnight in a refrigerator at

Table I
MRM Settings and Analytical Data for Confirmation of Seven Anabolic Androgenic Steroids in Eggs by LC–MS-MS*

Compound	t_R(min)	Transitions (amu)	DP (V)	CE (eV)	MRM ratio	
					Solvent (%)†	Egg (%)†
Trenbolone	7.84	271/**199**	44	26	74.8 (14)	77.5 (17)
		271/253		24		
Boldenone	7.98	287/**121**	36	25	51.6 (10)	50.4 (13)
		287/135		20		
Nandrolone	8.21	275/**109**	45	35	57.2 (15)	58.3 (18)
		275/257		20		
Stanozolol	8.34	329/**81**	90	75	29.0 (11)	25.7 (14)
		329/121		55		
Methandienone	8.53	301/**121**	40	26	55.3 (7.0)	57.6 (8.8)
		301/283		20		
Testosaterone	8.83	289/**97**	60	35	79.1 (5.9)	77.5 (9.0)
		289/109		34		
Methyl testosterone	9.11	303/**97**	58	32	84.6 (6.5)	90.8 (8.7)
		303/109		35		

*Note: Quantitative ions are in bold; precursor ions are [M + H] + ; t_R = retention time; DP = declustering potential; CE = collision energy; MRM ratio = average ion ratio of the qualifier ion to the corresponding quantitation ion at three spike levels ($n = 3$).

−18°C and subsequently centrifuged at 5,000 × g for 2 min at 4°C. The suspended solid lipids were removed from the tube, the solution was transgerred to a 50 mL pear-shaped flask and evaporated to approximately 2 mL at 55°C under vacuum. Then, 2 mL sodium carbonate solution (10%, w/v) and 10 mL TBME were added to the flask. The mixture was sonicated for 1 min in an ultrasonic bath, and the solution was decanted to a new 50 mL centrifuge tube. The solution was shaken for 10 min and then centrifuged at 3,500 × g for 5 min at 4°C. The upper ether layer was collected and transferred to another centrifuge tube and re-extracted once under aqueous layer with 5 mL of TBME. The TBME layers were combined, evaporated to dryness at 50°C under a stream of nitrogen, and the residues were dissolved in 1.0 mL 0.1% formic acid aqueous solution–acetonitrile (90/10, v/v). The solutions were filtered through a 0.22 μm syringe filter before LC–MS-MS analysis.

Method validation

Validation of the method complied with the 2002/657/EC guideline for confirmation analysis procedures (14). According to the criteria, the performance characteristics of a conventional method include calibration curves, specificity, recovery, repeatability, reproducibility, decision limit, detection capability and stability. Egg was used for full validation purposes and validation data were obtained for egg matrix.

Matrix-matched calibration curves

Negative eggs obtained from local layer farms were used as blank matrix samples. Blank egg was prepared as described previously. The extract residues were used to prepare concentrations of 1.00, 2.00, 5.00, 10.0, 20.0 and 100 ng/g. The matrix-matched calibration standard curves were made by plotting the response of the respective AAS versus its concentration in the matrix solution. Five analyses were performed and repeated on three days. The acceptance criterion was that the coefficient of correlation (r) must be more than 0.99.

Recovery and precision

Intermediate mixed standard solution was diluted with methanol to obtain concentrations of 20, 30 and 40 ng/mL for each of analyte. An aliquot of 0.1 mL of the diluted standard solutions was added into 2 g blank egg composites, corresponding to final concentrations of 1, 1.5 and 2 ng/g, respectively. Five replicates for each concentration level were performed and repeated on three consecutive days. The spiked samples were allowed to stand for 30 min before extraction and then prepared and analyzed as described previously. The extraction recoveries of AAS at the spiked samples were determined by measuring the peak area response from samples spiked with a particular standard solution of AAS before extraction with those from blank egg samples extracted and spiked with same concentration of analytes after extraction. The precision was expressed as relative standard deviation (RSD).

Decision limit and detection capability

The decision limit (CCα) is the lowest concentration at which a method can discriminate with a statistical certainty of $1 - \alpha$ that the analyte is present. In the case of AAS (prohibited substances), CCα was established by the following (14): 20 blank matrix samples of egg were analyzed and mean signal (noise height, N) was calculated at the time window in which the analyte is expected. CCα was then defined as the concentration that yields a signal-to-noise (S/N) ratio of 3.

The detection capability (CCβ) is the concentration at which the method is able to detect truly contaminated samples with a statistical certainty of $1 - \beta$. CCβ was calculated by analyzing 20 egg spiked with the analytes at CCα, and then the CCα value plus 1.64 times the corresponding standard derivation was equal to CCβ ($\beta = 5\%$).

Specificity

The analysis of more than 100 egg samples collected from different local markets was performed to check the selectivity of the proposed method, and then a spike with a concentration of 1.0 μg/kg in egg was investigated. The results were evaluated by the presence of interfering substances around the AAS retention time.

Stability

Stability must be taken into account during the validation of residue analysis. The stability of the neat standard solution (10 ng/mL) and the matrix-matched standard solution (5 ng/g) at −20, 4 and 20°C was evaluated within four weeks. The measured values were compared to those of freshly prepared standard solutions.

Statistical analysis

All calculations and data analysis were made with Origin 6.0 software (Originlab Co., Northampton, MA).

Results and Discussion

Optimization of instrumental conditions

Because TBL, BDN, NLE, MDN, TSN and MTS are neutral compounds, their retention times are not very relevant with the value of pH of the mobile phase. However, SNZ is a basic molecule containing imine nitrogen group in its structure (Figure 1), and its retention time is greatly affected by the pH value of the mobile phase. The simple water–acetonitrile mobile phase has a pH of approximately 7, which should dissociate any residual silanols to weakly anionic species that can strongly retain the basic SNZ. Therefore, these compounds are expected to be separated well, and the pH value of mobile phase should be adjusted to be acidic. Based on these trials, a water–acetonitrile mobile phase containing 0.1% formic acid was an adequate condition of pH, which allowed good chromatographic resolution and peak shape of the seven anabolic steroids. A Luna C18 column was enough to separate the compounds of interest.

MS parameters for the steroids were optimized in positive electrospray ionization full scan mode. In the second phase, the MS-MS conditions were adjusted in collision-induced dissociation (CID) mode under various collision energies. Steroids with a 3-one-4-ene functional group (all the steroids except SNZ) are preferably ionized in the positive mode, and form a stable $[M + H]^+$ ion. Stanozolol has no 3-one-4-ene functional group, but has a conjugated imine nitrogen group, which is also easily protonated (15). Therefore, $[M + H]^+$ molecular

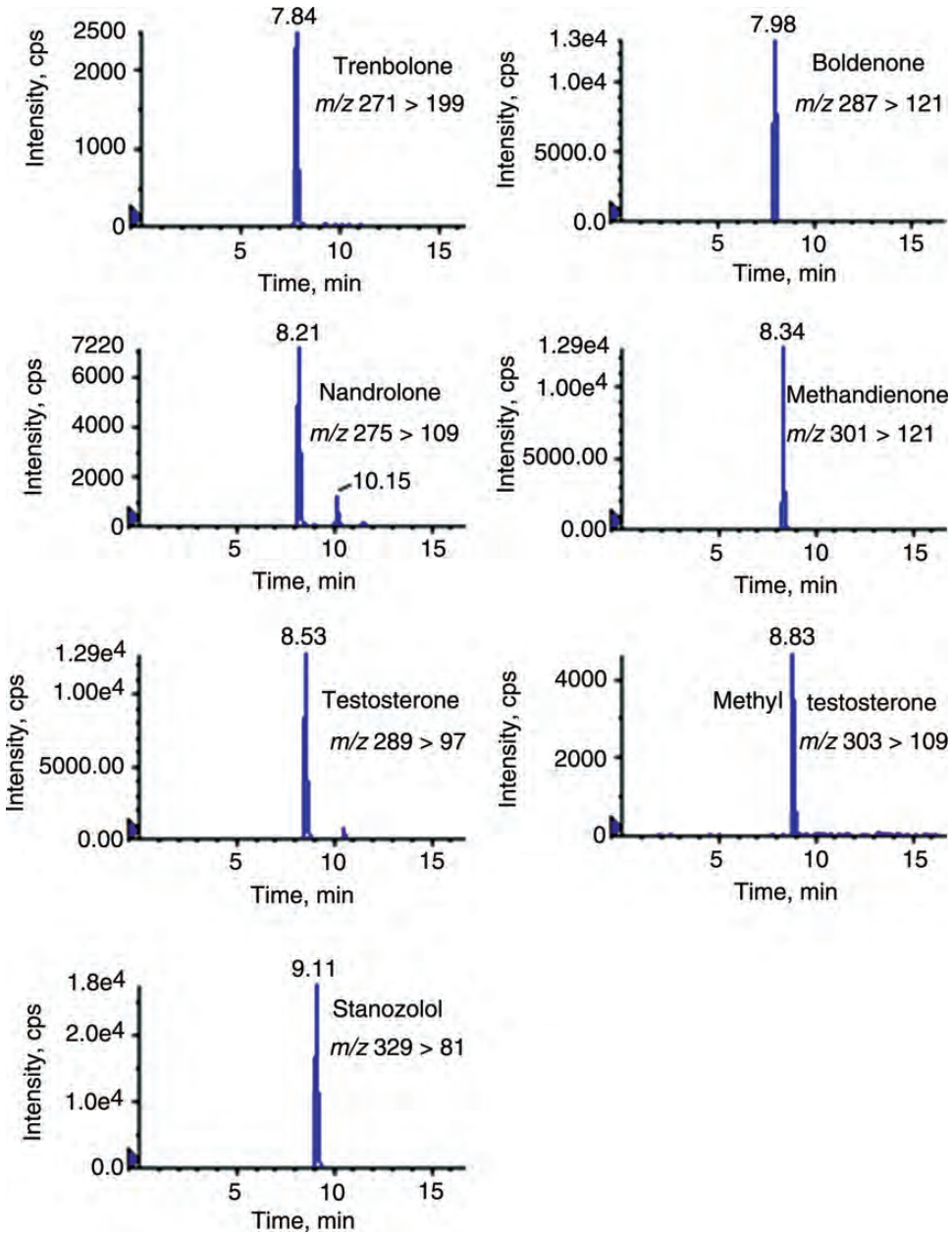

Figure 2. Typical MRM chromatograms of spiked blank egg sample, 1.0 ng/g for all target analytes except for methyl testosterone (0.5 ng/g).

ions, which were predominant for TBL, BDN, NLE, SNZ, MDN, TSN and MTS, were selected as the precursor ions.

Optimization of sample preparation

Because the anabolic steroids are insoluble in water, methanol and acetonitrile are used to precipitate protein and extract these compounds from complex matrices. Egg matrices are rich in protein and lipid components, which are known to cause significant ion suppression effects in positive ion electrospray mode (16). Thus, it is vital to remove proteins and lipids from egg samples for LC–MS-MS analysis. Because the steroids were considered to be prone to conjugate with proteins, the whole egg sample was hydrolyzed by β-glucuronide sulfatase. However, the sample solution became very complex after hydrolysis, and poor recoveries were obtained. Therefore, a fast and practical method was established for the determination of unconjugated steroid fractions in eggs. A higher recovery was obtained when extracting the target analytes from egg samples with methanol than with acetonitrile when the freezing-lipid filtration step was performed. This defatted method could save organic solvent and achieve higher recoveries than the conventional defatted protocol with *n*-hexane (liquid–liquid distribution). For further purification and liquid–liquid extraction with TBME was conducted according to the previous study (13).

The developed sample preparation procedure not only obtained high recoveries, but was also cheap. In comparison with the authors' previous study (13), the current sample preparation procedure avoided emulsification in the final centrifuge. Although some literature reported the use of solid-phase extraction (SPE) cartridges (including C18, –NH2, Silica and Oasis HLB) for cleanup of steroids in certain types of biomatrices (10, 12, 17, 18), in this study low recoveries (less than 50% for TBL, NLE and TSN) were obtained when using C18 or amine group SPE cartridge for cleanup. Moreover, the cost of sample preparation using SPE cleanup is more expensive, so the developed method is practical. The proposed sample preparation procedure is also satisfactory for extracting progesterone from egg, however, progesterone is difficult to quantify due to its high endogenous amounts in eggs (13).

Method validation

Specificity

Compared with the background noise in egg matrix, the results demonstrated that no interfering peaks could be detected within the 2.5% margin of the retention time of these target analytes. Typical chromatograms of spiked egg sample in the MRM mode are shown in Figure 2, which is cleaner than that found by He *et al.* (13). The chromatographic resolution and peak performance were good. Therefore, the specificity of method was satisfying for the compounds under investigation.

Table II
Matrix-Matched Calibration Curves for Seven Anabolic Androgenic Steroids ($n = 6$)

Compound	Regression equation y = (slope ± SD) x + (intercept ± SD)*	Correlation coefficient (r)
Trenbolone	$y = (1.11 \pm 0.02) \times 10^5 x - (1.88 \pm 1.59) \times 10^4$	0.9994
Boldenone	$y = (1.27 \pm 0.03) \times 10^5 x - (1.38 \pm 2.25) \times 10^4$	0.9991
Nandrolone	$y = (1.70 \pm 0.04) \times 10^4 x - (3.09 \pm 3.16) \times 10^3$	0.9986
Stanozolol	$y = (3.14 \pm 0.05) \times 10^5 x - (9.49 \pm 4.88) \times 10^4$	0.9993
Methandienone	$y = (2.47 \pm 0.04) \times 10^4 x - (5.13 \pm 3.49) \times 10^3$	0.9994
Testosaterone	$y = (3.33 \pm 0.05) \times 10^5 x - (9.01 \pm 4.72) \times 10^4$	0.9994
Methyl testosterone	$y = (2.23 \pm 0.03) \times 10^5 x - (4.91 \pm 2.82) \times 10^4$	0.9995

*SD = standard deviation; x in the range from 1.00 to 100 ng/g.

Table III
Recoveries of Seven Anabolic Androgenic Steroids from Spiked Egg and CCαs and CCβs ($n = 5$)

Compound	Added	Recovery (RSD) (%)		CCα	CCβ
	ng/g	Intra-day	Inter-day	ng/g	ng/g
Trenbolone	1.00	70.5 (10)	69.0 (11)	0.44	1.03
	1.50	75.0 (7.0)	73.7 (8.5)		
	2.00	72.6 (6.8)	74.3 (2.8)		
Boldenone	1.00	70.6 (9.7)	72.1 (10)	0.28	0.98
	1.50	72.2 (6.0)	73.6 (7.4)		
	2.00	70.3 (7.2)	71.3 (5.9)		
Nandrolone	1.00	71.2 (7.5)	70.3 (8.2)	0.40	1.00
	1.50	75.2 (9.0)	73.5 (9.2)		
	2.00	81.7 (9.2)	82.8 (10)		
Stanozolol	1.00	74.0 (9.6)	73.7 (11)	0.22	0.87
	1.50	80.9 (5.5)	78.6 (9.4)		
	2.00	79.6 (4.7)	80.0 (8.0)		
Methandienone	1.00	77.6 (3.9)	75.5 (4.4)	0.20	0.53
	1.50	75.0 (3.0)	77.3 (3.5)		
	2.00	75.4 (2.4)	74.8 (3.8)		
Testosaterone	1.00	70.5 (6.9)	66.3 (8.3)	0.30	0.98
	1.50	73.9 (7.0)	71.7 (6.3)		
	2.00	77.6 (5.1)	78.2 (7.0)		
Methyl testosterone	1.00	75.6 (7.3)	77.4 (6.0)	0.22	0.55
	1.50	77.0 (6.2)	78.5 (7.2)		
	2.00	78.2 (6.7)	80.5 (5.1)		

Table IV
Stability of Anabolic Androgenic Steroids Under Different Storage Conditions ($n = 3$)

Compound	Storage temperature and day	Mean ± SD (ng/mL)
Trenbolone	−20°C for 28 days (10 ng/mL pure standard solution)	9.8 ± 3.8
Boldenone		10.2 ± 1.4
Nandrolone		9.7 ± 2.5
Stanozolol		10.1 ± 1.6
Methandienone		10.0 ± 2.2
Testosaterone		9.9 ± 5.0
Methyl testosterone		10.1 ± 3.7
Trenbolone	4°C for seven days (10 ng/mL pure standard solution)	9.7 ± 3.3
Boldenone		9.8 ± 2.6
Nandrolone		9.6 ± 3.8
Stanozolol		9.9 ± 2.4
Methandienone		9.8 ± 4.1
Testosaterone		9.7 ± 4.4
Methyl testosterone		9.7 ± 2.0
Trenbolone	4°C for five days (5.0 ng/g matrix-matched standard solution)	9.7 ± 4.9
Boldenone		9.8 ± 2.6
Nandrolone		9.5 ± 4.3
Stanozolol		9.9 ± 1.9
Methandienone		9.8 ± 3.5
Testosaterone		9.6 ± 4.8
Methyl testosterone		10.0 ± 2.2
Trenbolone	20°C for three days (5.0 ng/g matrix-matched standard solution)	9.7 ± 2.5
Boldenone		9.8 ± 3.3
Nandrolone		9.6 ± 5.0
Stanozolol		9.9 ± 2.4
Methandienone		9.8 ± 3.2
Testosaterone		9.6 ± 4.1
Methyl testosterone		9.8 ± 2.3

Confirmation

For the confirmation of seven AAS residues in egg and on the basis of European Union (EU) guidelines, the concept of identification points (IPs) was applied for confirmatory purposes (14). Four IPs by the selection of two MRM transitions in LC–MS-MS system were acquired. Table I shows the MRM transitions used for confirmation and quantification of seven AAS residues and retention times. Confirmation of trace residues in complex matrices adopted in the EU guidelines also requires the determination of relative abundance criteria. In the samples of egg under analysis, the relative intensities of the MRM transitions met those of the standards in solvent (mobile phase). In all cases for the 1.0, 1.5 and 2.0 ng/g spikes, no matter how high the relative abundance of the target analyte, the RSDs of the ion ratios in samples were below 20%, the RSDs of the ion ratios of all analytes in solvent were below 15%, and the RSDs of the ion ratios of the analytes in the samples to that of the analytes in the standards were all less than 10%. That is, the ion ratios of the qualifier ion to the corresponding quantitation ion for each compound investigated were within the acceptability criteria set by EU guidelines.

Linearity

The matrix-matched calibration standard curves for detection of the steroid residues were obtained by performing a linear regression analysis in the range of 1.00–100 ng/g. Good linearity was obtained for each analyte. The correlation coefficients (r) for the calibration curves are higher than 0.99. Detailed results

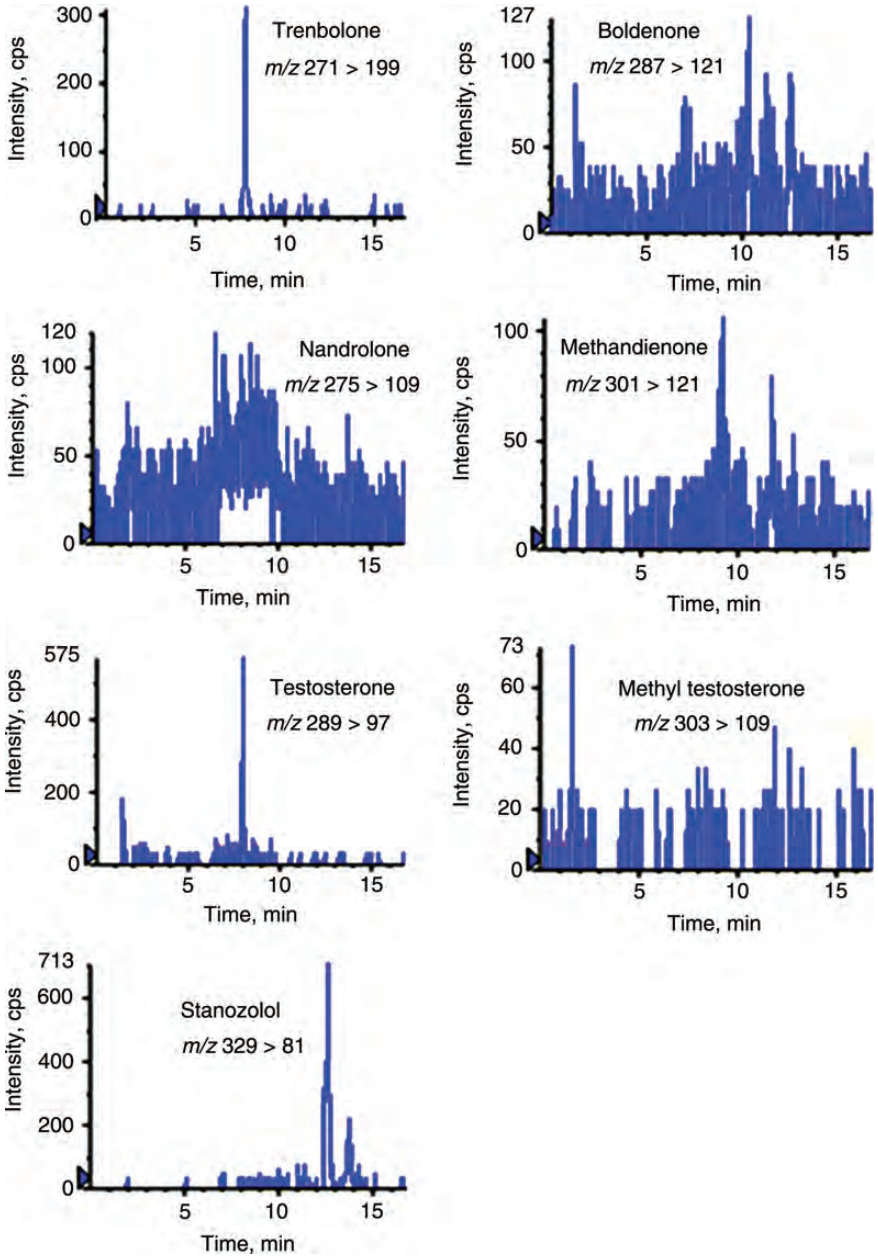

Figure 3. Typical MRM chromatograms of egg sample (trenbolone more than 0.3 ng/g).

are presented in Table II. The data of the pure solvent calibration standard curves are omitted.

Accuracy and precision

Recoveries for the AASs were estimated with the matrix-matched standard solutions. Data are presented in Table III. At the three spiked levels of 1.0, 1.5 and 2 ng/g, recoveries of TBL, BDN, NLE, SNZ, MDN, TSN and MTS were greater than 65%. The intra-day repeatability was between 2.4 and 13%, and the inter-day reproducibility was below 11% for all analytes of interest. The recovery rate and the precision of the developed method were acceptable for the residue analysis.

CCα and CCβ

Under the conditions specified in the method, the CCα values were in the range of 0.20–0.44 ng/g and the CCβ values were in the range of 0.50–1.03 ng/g for all analytes, as shown in Table III.

Stability

The experiments revealed that the response factors of the studied compounds remained almost constant in the pure solvent (10 ng/mL) for 28 days (−20°C) and 7 days (4°C), and in matrix solution (5 ng/g) for five days (4°C) and three days (20°C), respectively (shown in Table IV). Therefore, the prepared sample solutions should be injected within two days for good quantification.

Matrix effect

Matrix effects were investigated during the determination of AASs by using the established LC–MS-MS method. The areas of the seven compounds in solvent (free matrix) were compared with those obtained from the corresponding blank extracts spiked with the same amount of the steroids after extraction (19). The experiment showed that the area ratios (matrix to solvent) for seven AAS drugs were between 0.57 and 0.66. High matrix suppression effects were observed. Therefore, for evaluation of the proposed method and analysis of real samples, the matrix-matched standard solutions are suitable and scientific.

Application to egg samples

The method developed was used to determine the seven AAS drug residues in over 400 egg samples collected from local wholesale markets, farmer's markets and supermarkets. All of the studied compounds were less than 1.0 ng/g in the analytical eggs. Typical chromatograms of egg samples in the MRM mode are shown in Figure 3.

Conclusions

A multi-residue, cheap LC–MS-MS method was established that was capable of simultaneously determining seven anabolic androgenic steroids (TBL, BDN, NLE, SNZ, MDN, TSN and MTS) in eggs. Good performance data in terms of the quality of the results (linearity, specificity, recovery, repeatability and reproducibility) and the practical aspects (low cost, operability and high sample throughput) were obtained for all analytes. CCαs of the method developed were all below 0.44 ng/g for the steroids in eggs. The proposed method may be applied in the routine analysis and monitoring of trenbolone, boldenone, nandrolone, stanozolol, methandienone, testosterone and methyl testosterone in eggs.

Acknowledgments

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