Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Pharmaceutical and Biomedical Analysis 41 (2006) 616–621
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Short communication

Development of a faster determination of 10 anabolic steroids residues in animal muscle tissues by liquid chromatography tandem mass spectrometry

Chuan Lai Xu *, Xiao Gang Chu, Chi Fang Peng, Zheng Yu Jin, Li Ying Wang

School of Food Science and Technology, Southern Yangtaze University, 214036 WuXi, JiangSu Province, China

Received 6 October 2005; received in revised form 25 November 2005; accepted 29 November 2005
Available online 6 January 2006

Abstract

A method had been developed for determination of residues of 10 anabolic steroids (ASs) in animal muscle tissues by liquid chromatography tandem mass spectrometry (LC/MS/MS). After enzymolysis, the sample was extracted with *tert*-butyl methyl ether, cleaned up through reverse solid-phase extraction and further determined by LC/MS/MS under multiple reaction monitoring (MRM) mode. The limits of detection (LOD) of LC/MS/MS method used for testing epitestosterone (ETS), nandrolone (17 β-NT), 17 α-methyl-testosterone (MTS), testosterone 17-propionate (PTS), medroxyprogesterone (MED), progesterone (PG), estrone (ESN), 17 β-estradiol (17 β-ES), 17α-ethynylestradiol (EES) and estriol (EST) in animal muscle ranged from 0.06 to 0.22 μg/kg, and the limits of quantification (LOQ) were from 0.12 to 0.54 μg/kg. Experiments on spiked samples of pork, beef, chicken and fish showed that at addition level of 1.0 μg/kg, the average recoveries of the ASs ranged from 64% to 77%, and coefficients of variation from 7.1% to 20.3%, while at addition level of 2.0 μg/kg, the average recoveries ranged from 70% to 89%, and coefficient of variation from 7.1% to 19.1%.

Keywords: Determination; Anabolic steroids; Residues; Animal muscle tissues; Liquid chromatography tandem mass spectrometry

1. Introduction

Anabolic steroids (ASs) have been extensively used in husbandry practice with beneficial effects such as animal growth promotion and feed efficiency. Application of ASs as growth promoters has a history over 50 years [1]. They have been banned in food producing livestock as growth promoters in china since 2002 [2]. Until recently, the standard technique for steroid analysis has been gas chromatography (GC)–MS. This required the derivatisation of the steroids using silylation [3], acylation [4] or oxime/silylation [5] reactions, depending on the individual properties of the steroid. The lack of a universal derivatisation reagent [6], together with the chemical rearrangement of others [7], hinders the availability of a method to statutory testing laboratories.

LC/MS/MS provides a universal detector, since steroids may be analyzed without derivatisation. During recent years, LC/MS/MS has been successfully applied to the analysis of anabolic steroids in various biological samples including urine from bovine and horse, bovine hair and kidney fat [8–15], but few applications of the methods on muscular tissue of the animal have been reported. In china, inspection on muscular tissue of the animal plays an important role. But it has not been powerful because of the low level in muscular tissues and complex matrix.

Recently, some studies in china were carried out on the steroid residues, endogenous or exogenous, in domestic animal food. And some surprising results were obtained, for example, some pork samples have above 0.1 mg/kg 17-testosterone propionate residue [16], or about 1.0 mg/kg progesterone in some areas [17]. It is worrying that some steroids are used illegally in some areas, where ASs residues have not been controlled. Some studies were carried out on natural steroid hormones residus in animal food, such as estradiol, estriol or estrone, but the method is not sensitive enough [18,19]. So it is valuable to establish a more sensitive method against the steroids applying to inspect their residues in animal food in China.

This study describes a relatively simple sample preparation procedure for detecting 10 anabolic steroids using enzyme hydrolysis and solid-phase extraction, and subsequent detection by LC/MS/MS, which is more sensitive than before.

* Corresponding author. Tel.: +86 510 5881769; fax: +86 510 5881769.
E-mail address: xcl@sytu.edu.cn (C.L. Xu).

0731-7085/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2005.11.033

2. Experimental

2.1. Standards and reagents

Hormone standards were purchased from Riedel-de Haen (Seelze, Germany) but epitestosterone provided by Sigma–Aldrich (St. Louis, MO, USA). β-Glucosidase/aryl sulfatase solution (EC3.2.1.31 + EC3.1.6.1, Helix pomatia), methanol, methyl *tert*-butyl ether, acetonitrile, toluene, and all other reagents were purchased from Merck (HPLC grade, Darmstadt, Germany). Acetic acid buffer (0.04 mol/L, pH 5.0) was prepared by dissolving $NaAc{\cdot}3H_2O$ (43.0 g) together with glacial acetic acid (25.2 g) in water and diluting it to 1000 ml. Standard solutions of the mixtures of ten ASs in methanol are prepared with concentration of 100 mg/L and stored at −18 °C as stock solutions in the darkness. Work solutions were prepared into 1.0 mg/L by diluting 1 ml stock solution to 100 ml with methanol. The further dilutions were prepared by diluting with acetonitrile–water (50:50 v/v).

2.2. Instruments and apparatus

An Agilent 1100 series LC system (Palo Alto, CA, USA) including G1313A quaternary pump, G1367A autosampler, G1316A column oven were used for all analysis. All analytes were separated using a 150 mm × 2.1 mm SUPELCO Discovery®C_{18} column (MA, USA) 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 was used. Injection volume of 10 μl was used for all analyses (fluid Management System Inc. USA). The gradient was as follows: for androgens and progestogens the linear gradient protocol was 50%–100% A within 15.0 min, while for the determination of estrogen was 50%–60% A within 15.0 min.

Mass spectrometry was performed using an API3000 tandem triple-quadrupole mass spectrometer equipped with a TurboIonSpray ESI source (Applied Biosystems/MDS-SCIEX, Palo Alto, CA, USA) using MRM. Tandem mass spectrometer main working parameters were set as follows: the ion spray voltage was 3000 V. High-purity nitrogen gas was used as nebulizer, heater, curtain and collision gases. Heater gas was set at 7.5 L/min and the TurboIonSpray probe temperature was maintained at 550 °C. The nebulizer and curtain gases were, respectively, 12 and 8 L/min, while the gas pressure in the collision cell was set at 6.6×10^{-4} psi. MRM was used for the multiple product ions of each analyte. Precursor/product ions were set to unit resolution and dwell time was 150 ms. For the androgens and progesterones, a turbo-electrospray interface in positive ionization mode was used and entrance potential (EP) was 10 V, declustering potential (DP) was 40 V, collision cell exit potential was 15 V, focusing potential (FP) was 210 V and collision energy (CE) was shown in Table 1. For the estrogens, negative ionization mode was adopted and EP was 10 V, DP was −90 V, collision cell exit potential was 10 V, and FP was −380 V and CE was displayed in Table 2.

2.3. Sample pretreatment protocol

2.3.1. Sample preparation

The healthy swine, cattle, chicken and fish were bred with feedstuff without using any ASs on our school laboratory farm. When they were slaughtered, their muscle tissues of them were taken out separately, and fat, skin and bones tissues were eliminated. Every animal muscle tissues (100–200 g) were cut into pieces and treated with household stirring machine and then were preserved by sealing it airtight at −20 °C.

2.3.2. Homogenization and enzymolysis

Muscular tissues (5.0 ± 0.1 g) were transferred to centrifuge tube (50 ml), and then acetic acid buffer (10 ml) was added. The solution was homogenized at 10,000 rpm twice, each time 20 s.

Table 1
The diagnostic ions of androgens and progestogens acquired in MRM mode

Compounds	Precursor ion Q1 *m/z* $[M+H]^+$	Rt (min)	Diagnostic ions Q1/Q3 (optimised collision energy, CE, unit: eV)
17 β-NT	275.4	2.88	109.1(40), 257.2(25), 239.3(25)
MTS	303.0	3.80	97.3(40), 109.1(40), 285.4(25)
ETS	289.3	4.14	97.3(38), 109.1(38), 253.3(28)
MED	345.2	5.42	97.3(40), 123.1(40), 327.4(25)
PG	315.3	7.27	97.3(39), 109.1(39), 297.4(25)
PTS	345.2	11.32	97.3(40), 109.1(40), 253.3(27)

Table 2
The diagnostic ion of estrogens acquired in MRM mode

Compound	Precursor ion Q1 *m/z* $[M-H]^-$	Rt (min)	Diagnostic ions Q1/Q3 (optimised collision energy, CE, unit: eV)
EST	287.1	2.06	171.2(50), 145.2(55), 183.1(55)
17 β-ES	271.1	4.82	145.2(55), 183.1(55), 169.0(60)
EES	295.1	6.30	145.2(58), 159.0(47), 183.1(58), 199.0(55)
ESN	269.0	6.82	145.2(55), 159.0(50), 183.1(50)

After the enzyme solution (100 μl of β-glucosidase/aryl sulfatase) was added, which was used to release the steroids from both steroid glucuronide and sulphate conjugates, the mixture was left at room temperature for 3–4 h.

In preparation of spiked samples, homogenized standard solution was added into a centrifugal tube and enzyme solution was added after 10–15 min. Then it was hydrolyzed by the same way mentioned above.

2.3.3. Extraction and purification

Methanol (10 ml) was added into the solution after enzymolysis. It was mixed by a vortex oscillator for 1 min, extracted by immersing the tube into an ultrasonic bath for 5 min at room temperature and centrifuged at 2500 × *g* for 10 min. The supernatant was moved to another tube, mixed with *tert*-butyl methyl ether (15 ml) by oscillator and then centrifuged at 2000 × *g* for 3 min. The underlayer was reextracted with *tert*-butyl methyl ether (20 and 15 ml) twice. The ether layers were collect, transferred into a rotary evaporate flask and dried at 40 °C under vacuum. The residue was dissolved in 0.5 ml methanol, and 5 ml water was added.

The solution in the flask was loaded onto a C_{18} solid-phase extraction cartridge (500 mg/3 ml), which had previously been primed with methanol (3 ml) and water (3 ml). The flask was washed with 5 ml methanol (10%, v/v) and the wash solution was transferred onto the cartridge. The cartridge was rinsed with 5 ml methanol (10%, v/v) and 5 ml water, dried under vacuum for at least 1 min and finally eluted with 6 ml methanol under 1 ml/min flow rate. The eluates were dried at 40 °C under a stream nitrogen.

The residues were dissolved in 0.5 ml acetonitrile-water (50%, v/v). The suspended sample was analyzed by LC/MS/MS.

2.4. Data evaluation

2.4.1. Confirmatory analysis

If the LC/MS data of the sample was in accordance with the follows, it can be determined that there are some hormone residues:

a. Under the same trial conditions, retention time of the target substances in the sample is the same with that of standard solution, within deviation of 2.5%.
b. Signal to noise ratio of monitoring ion (precursor/daughter ions) is higher than 3.
c. The relative intensity of the monitoring ions of the sample to be tested should be the same as that of corresponding compounds in the standard solution, within deviation of 20%.

The optimal MS/MS parameters for each compound were determined by flow injection. In Tables 1 and 2, an overview is given of the precursor and diagnostic ions and also the retention time of each compound.

2.4.2. Quantitative analysis

Calibration curve were constructed, ranging from 0.1 to 20 μg/kg.

3. Results and discussion

3.1. Mass spectrometer detection

3.1.1. Selection of characteristic diagnostic ions for MS/MS

In order to develop a method with the desired limit of detection (less than 0.1 mg/kg), it was necessary to use MS/MS

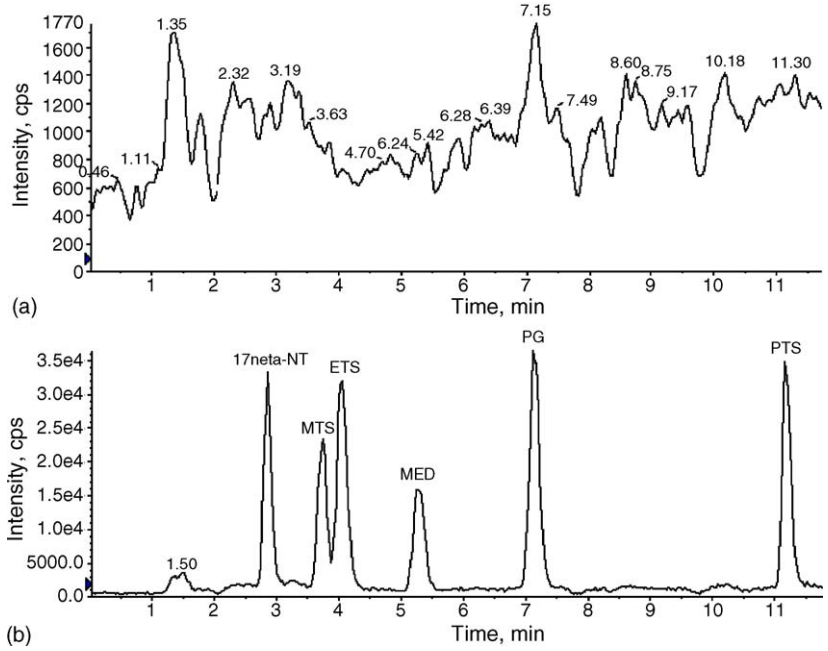

Fig. 1. (a) The LC/MS/MS chromatography of androgens and progestogens in blank pork. (b) The LC/MS/MS chromatography of androgens and progestogens in spiked pork at 0.001 mg/kg. Conditions for androgens and progestogens specify positive mode: TEM, 550 °C; IS, 3000 V; CAD, 7.0; mobile phase, acetonitrile (A) and water (B); linear gradient used for elution: 0.01 min A 50%, 15.0 min A 100%. Ten microliters injection.

detection, as MS/MS methods provide improved limit of detection for trace-mixture analysis [20]. 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 product ion mass spectrum of the androgens and progesterones are shown in Table 1, and of estrogens are in Table 2. $[M+H]^+$ and $[M-H]^-$ were the predominant ion in the Q1 spectrum and was used as the precursor ion to obtain the product ion spectra. Even though, the estrogens containing phenolic hydroxyl groups, the sensitivity of electrospray in negative ion mode was the same as the positive mode. The most sensitive mass transition was from *m/z* 275.4 to 109.1 for 17 β-NT, from *m/z* 303 to 97.3 for MTS, from *m/z* 289.3 to 97.3 for MEP, from *m/z* 345.2 to 97.3 for PTS, from *m/z* 315.3 to 97.3 for PQ from *m/z* 287.1 to 171.2 for EST, from *m/z* 271.1 to 145.2 for 17 β-ES, form *m/z* 295.1 to 145.2 for EES and from *m/z* 269 to 145.2 for ESN. Tables 1 and 2 show the precursor ion, product ions and optimized collision energy for each compound. Monitoring multiple ions is necessary for the unambiguous identification of the analytes according to the criteria laid down in the Commission Decision 2002/657/EC [21]. From Tables 1 and 2, the results were shown that the fragments contained co-precursor-ring ions and specific ions after collision, and this was the reason that various chemical bond energy led to the different optimum collision energy. LC-MRM is a very powerful technique for determine ASs since it provides sensitivity and selectivity requirements for analytical methods. Thus, the MRM technique was chosen for the assay development. Separate MRMs were preferred for ASs to provide a most sensitive and selective method. The MRM state file parameters were optimized to maximize the response for the analytes.

Table 3
The LODs and LOQs of ASs of LC/MS/MS

ASs	LOD (μg/kg)	LOQ (μg/kg)
17 β-NT	0.16	0.35
MTS	0.06	0.16
ETS	0.10	0.24
MED	0.06	0.16
PG	0.06	0.12
PTS	0.08	0.19
ESN	0.12	0.32
17 β-ES	0.22	0.54
EES	0.20	0.50
EST	0.19	0.46

3.1.2. The analysis of LC/MS/MS

Ten ASs were analyzed under optimized LC/MS/MS conditions. Several different matrixes were chosen, which were lean meats from pork, beef, chicken and fish. Figs. 1 and 2 show the typical total ion chromatograms which was obtained from pork meat. Good separation was carried out between ASs with corresponding gradient procedures. Retention time (Rt) of each hormone was listed in Tables 1 and 2, the CV% of Rt ($n=5$) was less than 1.5%.

3.1.3. LOD and LOQ

The LODs were estimated by calculating three times the blank signal at the expected retention time and considering the analyte losses of the method, which are concluded in Table 3.

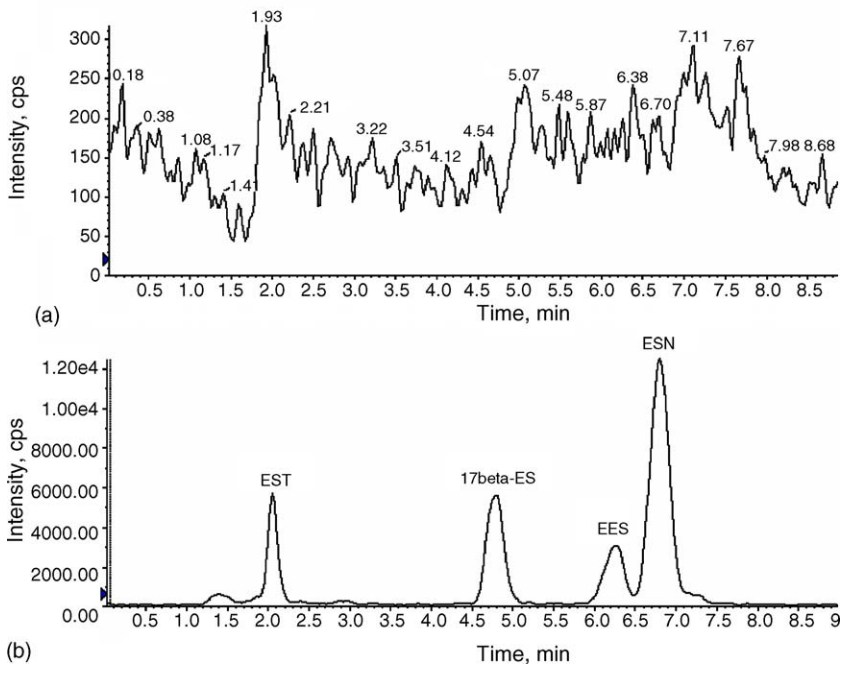

Fig. 2. (a) The LC/MS/MS chromatography of estrogens in blank pork. (b) The LC/MS/MS chromatography of estrogens in spiked pork at 0.001 mg/kg. Conditions for estrogens specify negative mode: TEM, 550 °C; IS, −4000 V; CAD, 7.0; mobile phase, acetonitrile (A) and water (B); linear gradient used for elution: 0.01 min A 60%, 15.0 min A 50%. Ten microliters injection.

Table 4
The recoveries and precision of LC/MS/MS method ($n = 5$)

Spiked samples	17 β-NT	MTS	ETS	MED	PG	PTS	EST	17 β-ES	EES	ESN
Pork										
1.0 level (μg/kg)										
Mean ± S.D. (μg/kg)	0.71 ± 0.15	0.74 ± 0.25	0.69 ± 0.24	0.64 ± 0.16	0.65 ± 0.21	0.67 ± 0.18	0.71 ± 0.23	0.69 ± 0.15	0.66 ± 0.18	0.70 ± 0.14
Recovery (%)	71	74	69	64	65	67	71	69	66	70
CV (%)	19.6	20.2	19.2	15	19.0	17.7	15.1	14.9	13.8	14.3
2.0 level (μg/kg)										
Mean ± S.D. (μg/kg)	1.69 ± 0.28	1.54 ± 0.24	1.70 ± 0.23	1.52 ± 0.14	1.44 ± 0.17	1.43 ± 0.23	1.73 ± 0.24	1.66 ± 0.27	1.55 ± 0.17	1.71 ± 0.25
Recovery (%)	84.5	77	85	76	72	71.5	86.5	83	77.5	85.5
CV (%)	11.2	9.9	12.7	7.9	12.9	11.8	11.1	10.9	12.6	10.8
Beef										
1.0 level (μg/kg)										
Mean ± S.D. (μg/kg)	0.67 ± 0.20	0.65 ± 0.17	0.70 ± 0.23	0.66 ± 0.14	0.68 ± 0.20	0.67 ± 0.14	0.67 ± 0.17	0.70 ± 0.22	0.64 ± 0.11	0.72 ± 0.15
Recovery (%)	67	65	70	66	68	67	67	70	64	72
CV (%)	17.7	20.3	14.6	14.2	15.8	20.0	17.9	18.6	11.9	12.4
2.0 level (μg/kg)										
Mean ± S.D. (μg/kg)	1.72 ± 0.23	1.78 ± 0.22	1.54 ± 0.19	1.47 ± 0.23	1.40 ± 0.23	1.41 ± 0.19	1.55 ± 0.16	1.43 ± 0.19	1.49 ± 0.22	1.52 ± 0.18
Recovery (%)	86	89	77	73.5	70	70.5	77.5	71.5	74.5	76
CV (%)	9.0	14.3	14.7	15.0	16.7	13.4	10.7	9.7	10.7	12.3
Chicken										
1.0 level (μg/kg)										
Mean ± S.D. (μg/kg)	0.71 ± 0.14	0.70 ± 0.24	0.74 ± 0.22	0.66 ± 0.20	0.64 ± 0.17	0.67 ± 0.18	0.65 ± 0.14	0.74 ± 0.26	0.72 ± 0.25	0.77 ± 0.24
Recovery (%)	71	70	74	66	64	67	65	74	72	77
CV (%)	20.3	15.8	11.7	16.8	7.1	15.6	17.1	16.6	15.8	13.2
2.0 level (μg/kg)										
Mean ± S.D. (μg/kg)	1.67 ± 0.27	1.59 ± 0.25	1.41 ± 0.27	1.46 ± 0.19	1.49 ± 0.26	1.50 ± 0.14	1.41 ± 0.22	1.45 ± 0.20	1.47 ± 0.16	1.45 ± 0.24
Recovery (%)	83.5	79.5	70.5	73	74.5	75	70.5	72.5	73.5	72.5
CV (%)	16.0	15.6	13.1	14.3	14.7	11.1	14.7	13.4	14.8	12.8
Fish										
1.0 level (μg/kg)										
Mean ± S.D. (μg/kg}	0.72 ± 0.23	0.75 ± 0.26	0.64 ± 0.13	0.67 ± 0.18	0.65 ± 0.17	0.64 ± 0.21	0.67 ± 0.22	0.69 ± 0.20	0.71 ± 0.20	0.75 ± 0.23
Recovery (%)	72	75	64	67	65	64	67	69	71	75
CV (%)	18.4	14.3	14.2	15.4	14.1	13.6	15.0	12.4	17.7	13.9
2.0 level (μg/kg)										
Mean ± S.D. (μg/kg}	1.65 ± 0.19	1.51 ± 0.14	1.55 ± 0.11	1.44 ± 0.12	1.43 ± 0.21	1.45 ± 0.18	1.49 ± 0.25	1.50 ± 0.16	1.47 ± 0.14	1.49 ± 0.11
Recovery (%)	82.5	75.5	77.5	72	71.5	72.5	74.5	75	73.5	74.5
CV (%)	16.4	19.1	16.7	14.1	12.3	10.6	11.8	7.1	7.4	7.8

The LODs of the method for the four androgens, two progestogens and four estrogens was from 0.06 to 0.22 μg/kg.

The LOQs of the method were defined as 10 times the standard errors of blank samples and the results were from 0.12 to 0.54 μg/kg.

3.1.4. Recovery and precision

To evaluate the performance of this method, pork, beef, chicken and fish blank samples were fortified at 1.0 and 2.0 μg/kg level (five samples were prepared for each species). Table 4 shows the results of fortification tests of each hormone in different animal muscle tissues. At fortification concentration of 1.0 μg/kg, the average recovery of the ASs ranged from 64% to 77%. Since, no modifications were carried out with isotopic internal standards in sample pretreatment, the coefficient of variation (CV) was high and the highest was 20.3% at 1.0 μg/kg level. At fortification concentration of 2.0 μg/kg, the average recoveries ranged from 70% to 89% and CV% ranged from 7.1%–19.1%. It was found that different matrixes have not significant effect on the recovery of each analyte at 1.0 μg/kg or 2.0 μg/kg level.

3.1.5. Linearity

Considering the actual application, linearity was studied in the range 0.1–20 μg/kg. It can be concluded that the linearity is satisfactory for quantitative analysis ($r > 0.99$) after subtracting the blank level.

The method established can be used as a first step method for study some steroids residues in animal foods, although some steroids such as testosterone have not been discussed. Moreover, it is urgent to establish some methods analyzing more steroids simultaneously against animal samples, which will need other studies in the future.

4. Conclusion

A rapid analytical confirmation method of ASs multiresidues in various animal muscle tissues has been developed. Samples are pretreated by enzymatic digestion, tissue homogenization and sonication, then cleaned up through SPE. LOD and LOQ ranged between 0.06–0.22 and 0.12–0.54 μg/kg, respectively, as determined by LC/MS/MS under negative or positive mode.

Acknowledgement

We greatly appreciate the financial support from NSFC on research project No. 20475022 and SFC of Jiangsu province on research project No. BK2004023.

References

[1] R. Verbeke, J. Chromatogr. 177 (1979) 69–84.
[2] Y.F. Zhou, Chin. J. Veterinary Drug 12 (2003) 19–21.
[3] A. Geier, D. Bergemann, L. von Meyer, Int. J. Legal Med. 109 (1996) 80–83.
[4] G. Casademont, B. Perez, J.A. Garcia Regueiro, J. Chromatogr. B 686 (1996) 189–198.
[5] P. Teale, E. Houghton, Biol. Mass Specrom. 20 (1991) 109–114.
[6] D. De Boer, M.E. Gainza Bernal, RD. van Ooyen, RA.A. Maes, Biol. Mass Spectrom. 20 (1991) 459–466.
[7] D. Stockl, R. de Sagher, L.M. Thienpont, G. Debruyckere, C.H. van Peteghem, The Analyst 119 (1994) 2587–2590.
[8] S.A. Hewitt, M. Kearney, J.W. Currie, P.B. Young, D.G. Kennedy, Anal. Chim. Acta 473 (2002) 99–109.
[9] N.H. Yu, E.N. Ho, D.K. Leung, T.S. Wan, J. Pharm. Biomed. Anal. 37 (2005) 1031–1038.
[10] L. Rambaud, E. Bichon, N. Cesbron, F. André, B.L. Bizec, Anal. Chim. Acta 532 (2005) 165–176.
[11] Y. Gaillard, F. Vayssette, Pépin, Forensic Sci. Int. 107 (2000) 361–379.
[12] C. Van Poucke, C. Van Peteghem, J. Chromatogr. B 772 (2002) 211–217.
[13] H. Hooijerink, E.O. van Bennekom, MW.F. Nielen, Anal. Chim. Acta 483 (2003) 51–59.
[14] A. Leinonen, T. Kuuranne, T. Kotiaho, R. Kostiainen, Steriods 69 (2004) 101–109.
[15] C. Van Poucke, M. Van De Velde, C.J. Van Peteghem, Mass Spectrom. 40 (2005) 731–738.
[16] X.Y. Luo, Y. Lin, L.Z. Liu, H.W. Zhou, Chin. J. Health Lab. Tech. 15 (2005) 387–389.
[17] Q. Li, J. Liu, Z.Q. Li, JY. Feng, Lit. Inf. Prev. Med. 8 (2002) 651–652.
[18] C.Q. Fang, Q. Li, A. Wang, Chin. J. Publ. Health Eng. 2 (2003) 166–168.
[19] Q. Li, D.X. Zhang, C.G. Fang, W.S. Liu, Chin. J. Publ. Health 20 (2004) 1500–1510.
[20] M. Jemal, Biomed. Chromatogr. 14 (2000) 422–429.
[21] Commission Decision 2002/657/EC.