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Analysis of 19-nortestosterone residue in animal tissues by ion-trap gas chromatography-tandem mass spectrometry*

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Abstract: A rapid sample treatment procedure for the gas chromatography-tandem mass spectrometry (GC-MS) determination of 19-nortestosterone (19-NT) in animal tissues has been developed. In our optimized procedures, enzymatic hydrolysis with β-glucuronidase from *Escherichia coli* was performed in an acetate buffer (pH 5.2, 0.2 mol/L). Next, the homogenate was mixed with methanol and heated at 60 °C for 15 min, then placed in an ice-bath at −18 °C for 2 h. After liquid-liquid extraction with *n*-hexane, the analytes were subjected to a normal-phase solid phase extraction (SPE) C_{18} cartridge for clean-up. The dried organic extracts were derivatized with heptafluorobutyric anhydride (HFBA), and then the products were injected into GC-MS. Using electron impact mass spectrometry (EI-MS) with positive chemical ionization (PCI), four diagnostic ions (*m*/*z* 666, 453, 318, and 306) were determined. A standard calibration curve over the concentration range of 1–20 ng/g was reached, with Y=467084X−68354 (R^2=0.9997) for 19-NT, and the detection limit was 0.3 ng. When applied to spiked samples collected from bovine and ovine, the recoveries ranged from 63% to 101% with relative standard deviation (RSD) between 2.7% and 8.9%. The procedure is a highly efficient, sensitive, and more economical method which offers considerable potential to resolve cases of suspected nandrolone doping in husbandry animals.

Key words: 19-Nortestosterone (19-NT), Gas chromatography-tandem mass spectrometry (GC-MS), Animal tissues
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1 Introduction

19-Nortestosterone (19-NT) was synthesized for the first time by Birch in 1950 and by Wilds and Nelson in 1953 (Le Bizec *et al*., 2002; Kohler and Lambert, 2002), which is designed for treatments of a wide span of haematological, post surgical conditions, cachexia, and substitutive progestin supplementation, and is also used in veterinary as well as human medicine in case of protein deficiency, breast cancer, osteoporosis, or burns (Delbeke *et al*., 1995; Sauer *et al*., 1998; Bricout and Wright, 2004; Chen *et al*., 2004). In animal husbandry, 19-NT can enhance lean tissue growth, reduce fat deposition, increase the weight gain, and feed conversion efficiency (Roda *et al*., 2003; Conneely *et al*., 2007). Administration is usually through intramuscular injection of the esterified drug, or through insertion into the fleshy part of the animal's ears. The hormones are gradually released over a period of 50 d to ensure a relatively constant level in the animal's blood (Conneely *et al*., 2007). 19-NT has also been employed as a doping agent to increase muscle mass and boost physical

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performance of athletes in sports and horse racing (Delbeke *et al*., 1995; Yamada *et al*., 2008).

Previous studies showed that 19-NT and its metabolite residues in meat produce a series of adverse effects, such as peliosis hepatitis, nephromegaly, adrenal atrophy, hypoproteinemia, semen scarcity, testicle shrink, menstrual cycle disorder, cerebral dysfunction, dyssecretosis, emotional instability, and masculinization in children and women (Heitzman, 1976; Bagatell and Bremner, 1996; Bahrke and Yesalis, 2004; Brännvall *et al*., 2005). Therefore, the use of natural and synthetic hormones for growth promotion purpose in meat-producing animals has been prohibited in the European Community since 1986 (Council Directive 86/469/EEC, Document No. L 275:36), and 19-NT or its esters are also included in the banned veterinary drug list in China (Notice No. 193 of the Ministry of Agriculture in April 2002).

This has resulted in the development of a broad range of methods to detect this drug and its metabolites in biological matrices. Traditionally, the gas chromatography coupled to mass spectrometry (GC-MS) method is accurate and suitable for detecting steroid hormones and their metabolites simultaneously, and is used as the confirmatory method. In the veterinary field, methods based on GC-MS have been reported to confirm the administration of 19-NT to horse (Houghton *et al*., 1978; Yamada *et al*., 2008), to swine (Bagnati and Fanelli, 1991; Petersen *et al*., 1994; Ventura *et al*., 2008), and to bovine (Dubois *et al*., 1998), through identification of 19-NT or its main metabolites. However, no reports were detailed to directly identify 19-NT residue in meat from bovine or ovine.

The aim of the present study was to optimize a GC-MS procedure for the quantification of 19-NT in muscle tissue, to develop a reliable sample preparation in which amounts of chemicals and time were to be minimized, and to aid in the selection of the best target ions to detect the administration of 19-NT esters.

2 Materials and methods

2.1 Chemicals and reagents

19-NT (17β-hydroxyestr-4-en-3-one) was purchased from Dr. Oetker Company (Germany). β-Glucuronidase from *Escherichia coli* was obtained from Sigma-Aldrich (USA). Solid phase extraction (SPE) C_{18} cartridges were purchased from Dalian Sipore Co., Ltd. (China). Heptafluorobutyric anhydride (HFBA) from Alfa Aesar (USA) was used for derivatization. All other solvents, reagents, and chemicals were of analytical grade or high-performance liquid chromatography (HPLC) grade, unless otherwise stated.

2.2 Preparation of samples

A total of 10 g fresh meat samples (muscle tissue, liver, kidney) of ovine or bovine, determined to be free of 19-NT by HPLC, were homogenized with a high speed triturator, and collected in a 50-ml round-bottomed plastic flask. For recovery investigations, standard solutions of 19-NT in 1 ml of methanol-water (60:40, v/v), corresponding to 2, 5, and 10 ng/g, respectively, were added at this step. The spiked sample was mixed thoroughly and allowed to stand at room temperature for 2 h, and then a volume of 10 ml acetate buffer (pH 5.2, 0.2 mol/L) was added. The mixture was subjected to enzymatic hydrolysis with 50 μl of β-glucuronidase from *E. coli* and incubation on an oscillator at 37 °C for 6 h. Next the homogenate was mixed with 30 ml of methanol, heated in a water-bath at 60 °C for 15 min, and then placed in an ice-bath at −18 °C for 2 h. The mixture was centrifuged at 3500 r/min for 5 min and this procedure was repeated one more time. The supernatant was extracted twice with 20 ml *n*-hexane to remove fat, and the aqueous methanol layer was transferred into a calibrated flask. Methanol was evaporated by a vacuum evaporator at 40 °C. The remaining aqueous phase was cooled to room temperature and afterwards submitted to solid-phase for clean-up process.

2.3 Separation and clean-up of crude extract

The SPE C_{18} cartridges were consecutively conditioned with 5 ml of methanol and then 5 ml of deionized water at a flow rate of 0.3 ml/min. After drying for 5 min under vacuum, the cartridges were loaded with the aqueous extract solution (weak vacuum applied, approx. 7×10^4 Pa). The cartridge was then washed with 20 ml of the elution solution (*n*-hexane-ether (70:30, v/v)) at a flow rate of less than 0.5 ml/min. Next, the eluate was washed with 2 ml of 1.0 mol/L sodium hydroxide. After centrifugation (3500 r/min, 5 min), the organic layers were

collected in a tapered glass tube, and the solvents were removed under a stream of nitrogen in a water bath at 45 °C. The extracts were kept in a desiccator containing P_2O_5 and maintained under vacuum for at least 30 min before derivatization.

2.4 Derivatization steps

The dry extracts were dissolved in 100 μl of isooctane and 50 μl of HFBA (2 g/ml) was mixed thoroughly. The derivatization process was performed on an oscillator at 80 °C for 30 min. The derivatization pathway is presented in Fig. 1. After cooling to room temperature, the mixture was evaporated to dryness under a stream of nitrogen. Then, the derivatization mixture was redissolved in 200 μl of isooctane. Finally, the derivatized products were transferred to injection vials, and 20 μl of this solution was injected directly into GC-MS (injection volume: 1 μl).

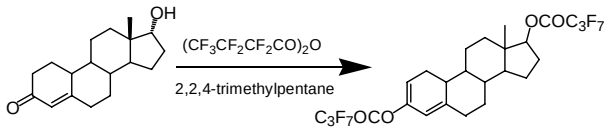

Fig. 1 Derivatization procedure of 19-NT through HFBA

2.5 GC-MS analysis

The GC-MS analyses were performed on a DSQ II mass spectrometer (Thermo Scientific Company, USA) linked to a TRACE GC Ultra gas chromatograph (Thermo Scientific Company, USA), equipped with a TR-5MS (Thermo-Fisher, USA) fused-silica capillary column (30 m×0.25 mm i.d., 0.25 μm film thickness). Helium (purity: 5.0) was used as the carrier gas at a flow rate of 1 ml/min (measured at 180 °C). The oven temperature was programmed as follows: initial temperature 80 °C (1 min); increased at 10 °C/min to 170 °C, 2 °C/min to 220 °C (hold 12 min); and then at 20 °C/min to a final temperature of 260 °C, at which temperature it was maintained for 5 min. The injections were made in the splitless mode (1.0 μl) using a TRIPLUS AS autosampler, and the injection temperature was 260 °C.

The MS was operated in the electron impact (EI) mode at electron ionization energy of 70 eV. Selected ion monitoring (SIM) with four target ions was used for screening of individual steroid tested in this study and full scan mode was followed for confirmation. The mass spectrum was obtained by scanning from *m*/*z* 50 to 700. The ionization mode was positive ion chemical ionization (PCI) and the ion source temperature was maintained at 260 °C.

2.6 Validation study

The validation protocol was performed and the following parameters were evaluated: the calibration linear range, limit of detection (LOD), limit of quantification (LOQ), and intra- and inter-assay precisions and accuracies.

Quantitative analysis of 19-NT was performed using the external standard method. A 6-point calibration curve was calculated and fitted by a linear regression of the peak area ratios versus 19-NT concentrations, using an equal weighting factor. The calibration was established over the range of 0–20 ng/ml (corresponding to 0, 1, 2, 4, 8, and 16 ng/g) with standards spiked in the blank samples. The calibration linear curves were prepared in quadruplicate. The LOD and LOQ were defined as three and ten times the value of noise, respectively (Roig *et al*., 2007). The estimated concentration value for the lowest calibration point of each sample was used as a measure of the noise.

Precision and accuracy detections of the overall analytical procedures were determined by spiking a pool of negative meat samples with three different 19-NT concentrations (low 2 ng/g, medium 5 ng/g, high 10 ng/g) based on the calibration curves. To determine the intra-assay, triplicate spiked samples within a single batch were analyzed on six different days, while the inter-assays were determined from triplicate runs of six separate analytical samples on a single day. Precision was expressed as the relative standard deviation (RSD) of the sample concentration calculated and accuracy was expressed as the recovery (%) of the estimated concentration.

3 Results and discussion

3.1 Extraction and purification of steroids

The purities of the isolated target analytes are of utmost importance to guarantee capillary column overloading and loss of resolution is avoided. This is of particular importance in order to achieve gas

chromatographic baseline resolution of 19-NT. Sample preparation for steroids in biological specimen was developed quickly in 1990s, in which the traditional and well-accepted method is the one used in thin layer chromatography (TLC) (Hartmann and Steinhart, 1997). In spite of the excellence in applying all kinds of steroids, this method requires a lengthy procedure (2.5 d) and plenty of poisonous chemical reagents (900 ml per sample). To overcome the shortcomings, some authors used simple SPE methods to take the place of column chromatographic method (Draisci *et al*., 2003), coupled SPE to HPLC (van Poucke and van Peteghem, 2002), and employed the liquid-liquid extraction step (Gasparini *et al*., 2007), immunoaffinity columns (Barrón *et al*., 1996), or solid-phase microextraction (SPME) (Saito *et al*., 2010). These observations led us to consider an alternative extraction procedure which would be less time-consuming and more suitable for routine analysis.

In this study, we followed the method by Hartmann and Steinhart (1997), with significant modifications. In our optimization procedure, the defeating step had to be carried out with liquid-liquid extraction by *n*-hexane, and an additional heating for 15 min at 60 °C resulted in cleaner extracts. Because the presence of lipophilic compounds in the crude extracts caused foaming during vacuum evaporation, we put the crude products in a refrigerator at −20 °C for 2–3 h to achieve an efficient purification process. In these cases, however, considerable interferences are also extracted from the meat matrix and upset the GC-MS analysis; we therefore applied a reversed-phase (C_{18}) SPE cartridge to purify the analytes. To avoid hazardous aromatic solvents, mixtures of *n*-hexane and ether were chosen for rinsing and eluting. In order to eliminate the time-consuming purification steps with Sephadex ion exchangers, the organic phase was washed with aqueous NaOH to remove organic acids.

3.2 Derivatization of steroids

Because of the retention character of hydroxyl group, 19-NT displays a poor chromatographic peak when the GC-MS analysis was performed directly. This compound, therefore, should be derivatized before injection in order to protect the hydroxyl group and to enhance chromatographic performance. The traditional derivatization methods include acylation, oximation, silylation, bis(trimethylsilyl) trifluoroacetamide (BSTFA) or HFBA reaction, according to individual characteristics of the steroids. In these reactions, BSTFA reaction obtains complicated products which hinder the analysis, and silylation produces low molecular derivatives (Daeseleire *et al*., 1998). In the mass chromatogram of 19-NT derivatives, the silylation reaction produces a base peak of *m*/*z* 73, whereas the relative abundance of *m*/*z* 418 (molecular ion peak) is little. HFBA reaction is a universally-applicable derivatization method which is especially suitable for 19-NT GC-MS analysis. The HFBA is useful because it is more volatile than BSTFA and, therefore, chromatographic separation may be carried out more rapidly. The advantages of using HFBA also include the ease of reagent removal by simple evaporation and formation of intense molecular ions under GC-MS conditions, so that interferences are less likely to be encountered (Choi *et al*., 1999). In comparison to the sole silylation, the proposed derivatization blocks the hydroxyl group, producing stable 19-NT derivatives. Furthermore, in the EI^+ mass chromatogram, HFBA produced abundant ion fragments and a base peak of *m*/*z* 666 (molecular ion peak), which contributed to qualitative and quantitative analyses. Fig. 2 shows exemplarily the MS spectra of 19-NT derivative. One of the drawbacks of HFBA is that it belongs to acid anhydride and dissolves quickly in aqueous solution. To overcome the disadvantage, analytes should be kept in a desiccator for 30 min before derivatization.

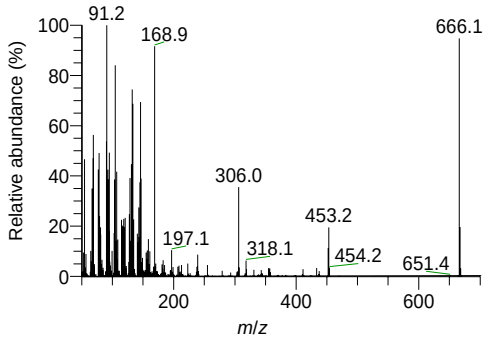

Fig. 2 Full scan mass spectrum of HFBA derivatives of 19-NT

3.3 Quantitative ion selection

Because most of the steroids have hydroxyl groups, dehydrated fragment ions could be observed when GC-MS procedure was performed, which would

be used for qualitative and quantitative analyses. After HFBA derivatization, SIM with four target ions was used for 19-NT quantitative analysis. The mass spectral properties of 19-NT derivatives in PCI are displayed in Fig. 2. Fig. 3 presents the example of chromatograms obtained from 19-NT derivatives, in which retention time of the target substance was 38.81 min. Based on the mass spectrum data in Fig. 2, four diagnostic ions (*m*/*z* 666, 453, 318, and 306) were determined, and their structures are listed in Fig. 4. These results are consistent with that of Petersen *et al*. (1994), and *m*/*z* 666 (molecular ion peak) is chosen as the base peak and the quantization ion of the analyte. The *m*/*z* 453 is M-OCOC$_3$F$_7$, derived from the precursor ion, and *m*/*z* 318 and 306 are fragment ions produced by rearrangement in B ring. The rearrangement ways are also common in other steroids (de Boer *et al*., 1995).

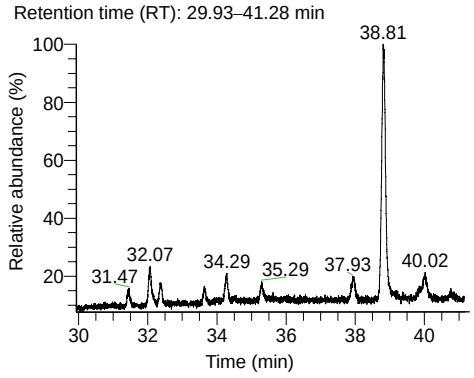

Fig. 3 Chromatogram of HFBA derivatives of 19-NT

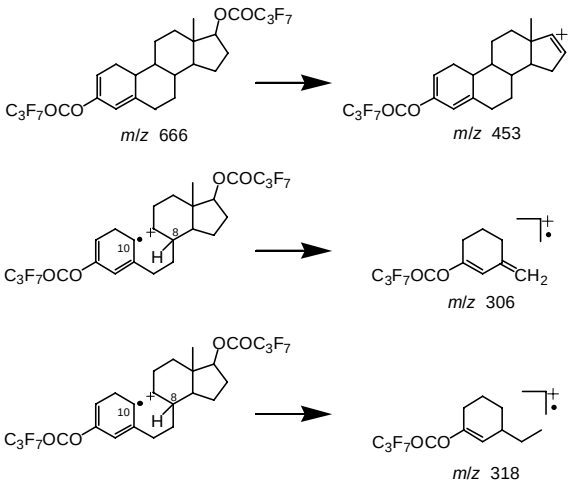

Fig. 4 Structures of characteristic ions *m*/*z* 666, 453, 318, and 306

3.4 Evaluation of the method

It is generally agreed that the control of the abuse of anabolic steroids in husbandry animals is effective only if sensitive tests are used. As seen in Fig. 5, our methods showed the good linearity of a standard calibration curve over the concentration range of 1–20 ng/g, with Y=467084X−68354 (R^2=0.9997) for 19-NT. The LOD and LOQ values for this steroid by the procedure were 0.3 and 1.0 ng/g, respectively.

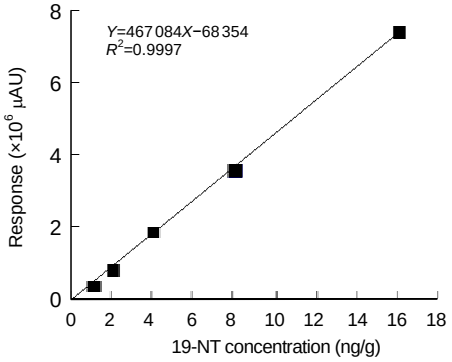

Fig. 5 A standard calibration curve of 19-NT

Six spiked specimens collected from bovine or ovine (muscle tissue, liver, and kidney) were employed to validate the methods. SIM chromatographic spectra of blank and spiked samples are shown in Fig. 6. It is noteworthy that the retention time obtained for 19-NT was 38.81 min, while the total cycle time was about 60 min per analysis, and the standard deviation of the retention time of the 19-NT derivative was ±0.05 min. Despite testing having been performed on six different tissue samples, in general terms, no matrix interfering signals were observed at the retention time of the analytes.

Results of the precision and accuracy values for the different animal tissues from ovine or bovine are presented in Table 1. These values were quantitated by the ratio of the peak area from the spiked samples to that from the corresponding external standard (19-NT), and absolute values were calculated. For the three target concentrations (2, 5, 10 ng/g), the recoveries in bovine muscle ranged from 71% to 86% with precision (RSD) ranging from 3.4% to 7.7%; the recoveries in bovine liver ranged from 65% to 95% with RSD from 2.9% to 8.9%; as for the bovine kidney, the recoveries were all higher than 81% with RSD between 3.3% and 7.4%. Regarding the liver

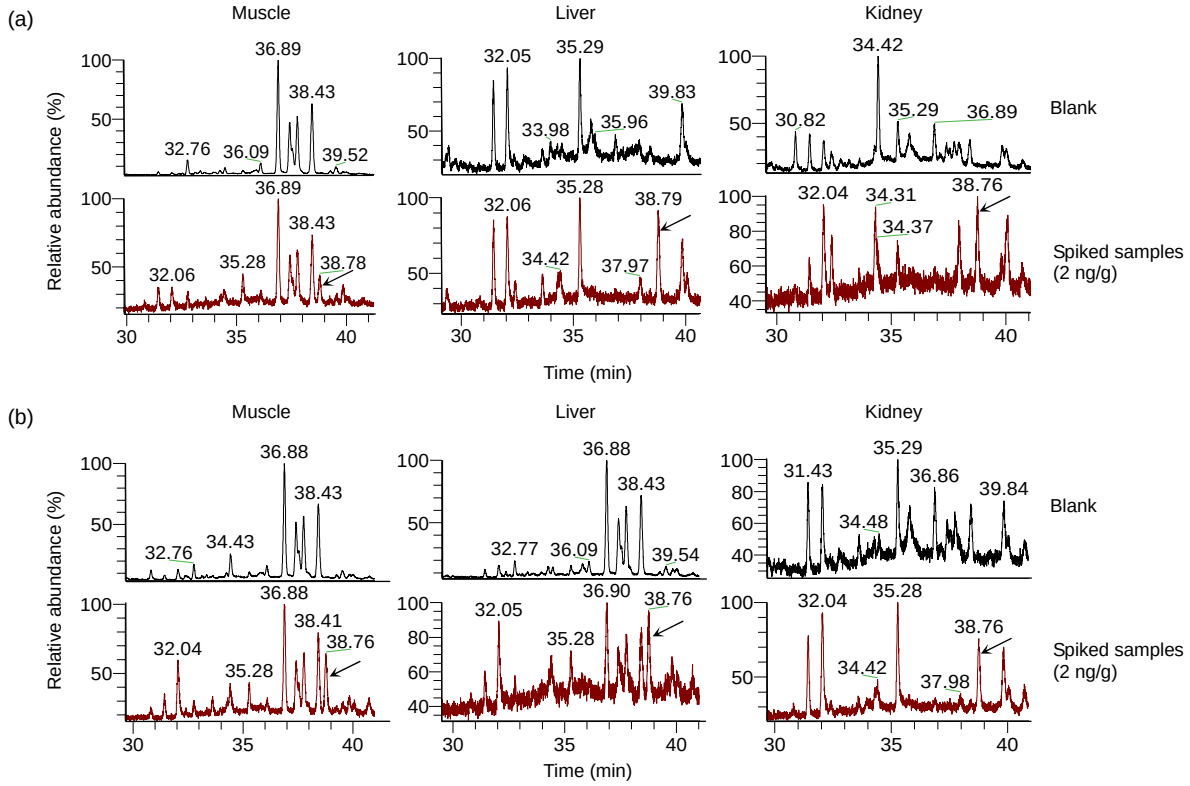

Fig. 6 SIM total ion chromatograms of blank and spiked samples (2 ng/g)
(a) Bovine; (b) Ovine. Arrow means the retention time of 19-NT spiked

Table 1 Intra- and inter-assay precisions and accuracies for animal tissues tested

Samples	c_{NT} (ng/g)	Intra-assay			Inter-assay		
		c_{found} (ng/g)	Precision (%)	Recovery (%)	c_{found} (ng/g)	Precision (%)	Recovery (%)
Bovine muscle	2	1.42±0.11	7.7	71	1.53±0.10	6.5	77
	5	3.93±0.21	5.3	79	3.92±0.19	4.8	78
	10	8.56±0.36	4.2	86	8.48±0.29	3.4	85
Bovine liver	2	1.35±0.12	8.9	67	1.30±0.11	8.5	65
	5	4.35±0.26	6.0	87	4.33±0.25	5.8	87
	10	9.53±0.39	4.1	95	9.44±0.27	2.9	94
Bovine kidney	2	1.62±0.12	7.4	81	1.63±0.11	6.7	82
	5	4.29±0.25	5.8	86	4.31±0.27	6.3	86
	10	9.88±0.42	4.3	99	10.06±0.33	3.3	101
Ovine muscle	2	1.33±0.11	8.3	66	1.40±0.11	7.9	70
	5	4.12±0.22	5.3	82	4.09±0.18	4.4	82
	10	8.53±0.34	4.0	85	8.61±0.33	3.8	86
Ovine liver	2	1.30±0.11	8.5	65	1.25±0.10	8.0	63
	5	4.38±0.25	5.7	88	4.32±0.26	6.0	86
	10	9.38±0.41	4.4	94	9.29±0.25	2.7	93
Ovine kidney	2	1.58±0.12	7.6	79	1.62±0.12	7.4	81
	5	4.27±0.21	4.9	85	4.34±0.27	6.2	87
	10	9.82±0.37	3.8	98	9.86±0.33	3.3	99

c_{NT}: 19-NT concentration; c_{found}: found concentration

and kidney samples from ovine (Table 1), the intra- and inter-assay accuracy values were all better than 63%, with RSD between 2.7% and 8.5%. As can be observed, the precision in ovine muscles ranged from 3.8% to 8.3%, while the recoveries were in the range of 70% to 86% except for 2 ng/ml in intra-assay. These results are in agreement with the internationally-accepted ranges for these parameters, and the standard deviations indicate that the methods are sufficiently precise.

4 Conclusions

In summary, a modified GC-MS method to quantify 19-NT in meat has been developed and validated, and this method has proven to be suitable for the quantitative determination of 19-NT after administration of 19-NT esters to bovine and ovine. To ensure rapid, effective, and reliable results in relation to meat control for the residue program in connection with the European Commission (EC) directive (86/469/EEC), the proposed method employed an alternative extraction, isolation, and purification scheme, in which the use of aromatic solvents (like benzene or toluene) could be avoided, the organic solvents reduced to a minimum of about 100 ml, and the sample preparation time (approx. 1.5 d per analysis) was reduced to 60% of the reference method (Hartmann and Steinhart, 1997). Using EI-MS with PCI, the present study produces highly-informative fragment ions and allows definitive identification and quantization of 19-NT. The method described seems to have merits with respect to accuracy and sensitivity as compared to previous studies, and offers considerable potential to resolve cases of suspected nandrolone doping in husbandry animals.

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