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Ultra-high performance liquid chromatography–tandem mass spectrometry in high-throughput confirmation and quantification of 34 anabolic steroids in bovine muscle

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ABSTRACT

An ultra-high performance liquid chromatography tandem mass spectrometry multi-residue method for the determination of 34 anabolic steroids (10 estrogens including stilbenes, 14 androgens and 10 gestagens) in meat of bovine origin is reported. The extraction and clean-up procedure involved homogenization with methanol, defatting with hexane, liquid/liquid extraction with diethylether and finally SPE clean-up with coupled Si and NH_2 cartridges. The analytes were separated on a 1.9 μm Hypersil Gold column (100 × 2.1 mm) and quantified on a triple quadrupole mass spectrometer (TSQ Vantage) operating simultaneously in both positive and negative atmospheric pressure chemical ionisation (APCI) modes. This analytical procedure was subsequently validated according to EU criteria (CD 2002/657/EC), resulting in decision limits and detection capabilities ranging between 0.04 and 0.88 μg kg^{-1} and 0.12 and 1.9 μg kg^{-1}, respectively. The method obtained for all, natural and synthetic steroids, adequate precisions and intra-laboratory reproducibilities (relative standard deviation below 20%), and the linearity ranged between 0.991 and 0.999. The performance characteristics fulfill the recommended concentrations fixed by the Community Reference Laboratories. The developed analysis is sensitive, and robust and therefore useful for confirmation and quantification of anabolic steroids for research purposes and residue control programs.

1. Introduction

The use of anabolic steroids for growth promotion purposes in meat producing animals results in an improvement of muscle growth, more lean meat and a higher feed conversion efficiency [1,2]. However, toxicological/epidemiological studies show that these compounds pose a risk for public health. As a consequence, the use of anabolic steroids for fattening purposes has been banned in the European Union since 1986 [3]. Therefore, national plans of the individual Member States were developed to monitor the abuse of anabolic steroids. In this context, the development of sensitive, specific and multi-residue analytical methods, allowing an adequate control of the possible illegal use of growth promotors in meat production, is requisite. These analytical methods must be in compliance with the criteria of Commission Decision 2002/657/EC [4].

Until recently, the standard technique for steroid analysis has been gas chromatography coupled to mass spectrometry (GC–MS) [5]. GC–MS is a sensitive, robust and therefore suitable technique for the assay of hormones, but requires derivatization to reduce the analytes polarity and thermal instability, using silylation, acylation or oxime/silylation reactions [6–9]. The combination of liquid chromatography with mass spectrometry (LC–MS) on the other hand offers a rapid, simplified, specific and sensitive alternative to GC–MS methods involving simpler extraction procedures and removing the need for derivatization reactions. During recent years many LC–MS/MS applications have been described with respect to the analysis of anabolic steroids in various biological samples including urine or serum from bovine and equine origin, bovine hair and kidney fat [10–15], since these tend to be the matrices of choice when monitoring for anabolic steroids. For controls at retail level and for products imported in the EU, it is however necessary to have analytical methods applicable to meat samples. The difficulty in monitoring for these compounds in tissue samples is caused by the complexity of the matrix and the low minimum required performance limits (MRPLs), recommended concentrations (RCs) and action limits (ALs) established by respectively CD 96/23/EC [16], the Community Reference Laboratories (CRLs) [17] and the National Reference Laboratory (WIV) in Belgium. Indeed, the levels that accumulate in tissue are lower than those found in

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other matrices. Several methods on the determination of anabolic steroids in muscular tissue of animal origin have been reported [18–22], monitoring a selection of the existing anabolic steroids (10–20), sometimes in combination with corticosteroids.

As the number of growth promotors likely to be abused in cattle fattening increases and encompasses both the natural as the synthetic steroids, the use of multi-residue methods that cover different classes of compounds becomes increasingly important. Besides this increasing number of analytes, current routine LC–MS/MS methods for detection, quantification and confirmation of residues also require a high-throughput and concomitant short analysis time [23,24]. The use of U-HPLC coupled to modern fast-switching triple quadrupole mass spectrometers provides a rapid separation and universal detection and confirmation of multiple analytes. U-HPLC significantly increases sample throughput by using sub-2-micron particle sized columns with low dead volume and able to withstand high-pressure LC equipment, thus drastically shortening analysis time without loss of separation efficiency while increasing sensitivity [24].

The current study presents evidence of an efficiently selective U-HPLC–MS/MS method with atmospheric pressure chemical ionization (APCI) for the high-throughput detection, confirmation and quantification of 34 anabolic steroids in muscle meat, covering the classes of gestagens, estrogens (including stilbenes) and androgens, both the synthetic as well as the natural derivates. Five deuterated and one structurally related internal standards were used for the quantification of these anabolic steroids. Two ion transitions per compound were monitored in a total U-HPLC–MS/MS analysis time of 8 min. As a whole the method proved to be simple, reliable and reached the required sensitivity, accuracy and precision. Hence, it provides a suitable means for residue control programs.

2. Materials and methods

2.1. Reagents and chemicals

Standards of 17β-nortestosterone, fluoxymesterone, progesterone, estrone, estriol, 17α-estradiol, 17β-estradiol, 17α-hydroxyprogesterone, 17β-ethinylestradiol, diethylstilbestrol, dienestrol, β-zearalanol, α-zearalanol, 17β-testosterone, 17α-testosterone, hexoestrol, norgestrel, acetoxyprogesterone, medroxyprogesteron acetate, methyltestosterone and the internal standard androstadienedione were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methylboldenone (or methandienone), norethandrolone, α-nortestosterone, methandriol, β-boldenone, 4-androstenedione and caproxyprogesterone were provided by Steraloids Inc. (Newport, RI, USA). α-Boldenone and the internal standards 17β-estradiol-d3, medroxyprogesteron acetate-d3, methyltestosterone-d3, 17β-testosterone-d2 and hexoestrol-d4 were obtained from RIKILT (Wageningen, The Netherlands). β-trenbolone, flugestone acetate, trenbolone acetate, megestrol acetate, chlormadinone acetate and melengestrol acetate were kindly provided by WIV (Brussels, Belgium). Solvents were of analytical grade when used for extraction and purification steps, and of LC–MS Optima grade for U-HPLC–MS/MS application. They were obtained for VWR International (Merck, Darmstadt, Germany) and Fisher Scientific UK (Loughborough, UK), respectively.

Primary stock solutions were prepared in methanol at a concentration of 1000 ng μL^{-1}. Working solutions were prepared by 100× and 1000× dilution in methanol/water (50/50). When necessary, sonication was applied to ensure the complete dissolution of the substances. Solutions were stored in dark glass bottles at −20 °C.

Sodium acetate buffer 0.2 M (pH 5.2) was prepared by dissolving 54.5 g sodium acetate in 600 mL 0.2 M acetic acid and diluted to 2 L with ultrapure water. Ultrapure water was produced with an Arium 611 UV system (Sartorius Stedim Biotech, Aubagne, France). Isolute Si (500 mg, 10 mL) and NH_2 (100 mg, 1 mL) cartridges were purchased from Biotage (Uppsala, Sweden).

2.2. Instrumentation

The LC system consisted of a Thermo Fisher Scientific (San José, USA) Accela U-HPLC pumping system, coupled with an Accela Autosampler and Degasser. Chromatographic separation was achieved by reversed phase chromatography and gradient elution. Separation of the anabolic steroids was carried out on a Hypersil Gold C18 column (1.9 μm, 100 mm × 2.1 mm, Thermo Fisher Scientific), kept at 30 °C. The mobile phase constituting of water and methanol, was pumped at a flow rate of 0.3 mL min^{-1}. Optimized separation of all analytes was obtained using a linear gradient starting with a mixture of 50% water and methanol. In 0.5 min the amount of methanol was increased to 65% and kept there for 2.75 min. Next, the amount of methanol was increased to 100% in 0.5 min and kept there for 2 min. Finally, the column was allowed to re-equilibrate for 2.25 min at initial conditions, this before each run. All analytes could be separated in a total runtime of 8 min. Analysis was performed on a triple quadrupole mass analyzer (TSQ Vantage, Thermo Fisher Scientific, San José, USA), fitted with an atmospheric pressure chemical ionization source operating simultaneously in positive and negative ion mode. The following working conditions were applied: spray voltage at 4 (+) and 6 (−) kV; vaporizer and capillary temperature at 450 and 360 °C, respectively; sheath and auxiliary gas at 35 and 10 arbitrary units (a.u.), respectively; cycle time of 0.8 s. Argon pressure in the collision cell (Q2) was set at 1.5 mTorr and the mass resolution at the first (Q1) and third (Q3) quadrupole was set at 0.7 Da at full width at half maximum (FWHM). Precursor ion, S-lens RF amplitude, and collision energy (CE) in Q2 were optimized individually per compound or transition (Table 1). Quantification and confirmation data were acquired in selected reaction monitoring (SRM) mode, the transitions followed are displayed in Table 1. Instrument control and data processing were carried out by means of Xcalibur Software 2.0.7 SP1 (Thermo Electron, San José, USA).

2.3. Muscle samples

Bovine muscle tissue was obtained from three different local supermarkets (different meat brands), cut into pieces of ca. 100 g and stored at −20 °C in polypropylene vessels until use.

2.4. Sample extraction and clean-up

Sample extraction and clean-up was adopted and downscaled from Impens et al. [25], with omission of the derivatization step, resulting in the following procedure. Muscular beef tissue samples (5.0 ± 0.1 g wet weight) were weighed into 100 mL Sovirel glass flasks. Samples were fortified with mixed internal standard at a level corresponding to 5 μg kg^{-1} by adding 50 μL of 500 ng mL^{-1} internal standard mix solution. Depending on the fortification level required (0.25, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0 or 10.0 times the compound-specific RC or AL) samples were fortified with 12.5, 25, 50, 100, 200, 300, 400 or 500 μL of a mix solution, containing 50, 100, 200, 300, 500 or 1000 ng mL^{-1} respectively for those compounds with an RC/AL of 0.5, 1, 2, 3, 5 or 10 μg kg^{-1}. After fortification, samples were held for 15 min, then 2 mL of sodium acetate buffer and 8 mL of ultra pure water were added to each flask. Next, samples were subjected to a microwave (Zanker, The Netherlands) treatment for 72 s at 100 W, contents transferred to 50 mL polypropylene tubes and homogenized using an Ultra-turrax instrument for 1 min. Methanol (10 mL) was added to the tubes, which were subsequently vortexed for 1 min and centrifuged (9000 × *g*, 10 min, 4 °C). Any meat

Table 1
Collected SRM transitions and compound specific MS parameters.

Analyte	tR (min)	Precursor ion (*m/z*) (polarity)	Product ions (*m/z*)	S-lens (RF amplitude) (V)	Collission energy (eV)
β-Zearalanol	2.81	305.1 (+)	161.1, 189.1	85	24, 18
Androstadienedione	2.95	285.2 (+)	91.1, 121.1	77	41, 24
Flugestone acetate	3.49	407.1 (+)	225.2, 267.2	103	28, 23
α-zearalanol	3.68	305.1 (+)	161.1, 189.1	85	24, 18
Estriol	3.68	271.2 (+)	159.2, 253.3	74	21, 12
Fluoxymesterone	3.70	337.2 (+)	241.2, 281.2	110	24, 20
β-Trenbolone	3.71	271.1 (+)	165.1, 253.2	110	59, 20
β-Boldenone	3.71	287.1 (+)	121.1, 135.1	77	25, 15
17β-Ethinylestradiol	3.85	279.1 (+)	133.1, 159.1	77	17, 20
17β-Estradiol-d3	3.85	258.2 (+)	133.1, 159.1	67	20, 18
4-Androstenedione	3.85	287.1 (+)	97.1, 109.1	93	21, 25
17β-Estradiol	3.89	255.1 (+)	133.1, 159.2	70	19, 18
Estrone	4.03	271.2 (+)	159.2, 253.3	74	21, 12
17α-Estradiol	4.12	255.1 (+)	133.1, 159.2	70	19, 18
17β-Nortestosterone	4.14	275.1 (+)	109.1, 257.2	85	30, 14
Diethylstilbestrol	4.21	269.1 (+)	107.1, 135.1	71	31, 13
α-Boldenone	4.21	287.1 (+)	121.1, 135.1	77	25, 15
Methylboldenone	4.44	301.2 (+)	121.1, 149.2	71	28, 15
17α-Nortestosterone	4.53	275.1 (+)	109.1, 257.2	85	30, 14
Dienestrol	4.55	267.1 (+)	107.1, 121.1	80	30, 19
Hexoestrol-d4	4.61	273.1 (-)	121.1, 136.1	90	43, 18
Hexoestrol	4.62	269.2 (-)	119.2, 134.1	68	46, 17
17α-Hydroxyprogesterone	4.73	331.2 (+)	97.1, 109.1	91	29, 30
17β-Testosterone	4.76	289.2 (+)	97.1, 109.1	85	23, 26
17β-Testosterone-d2	4.77	291.2 (+)	99.1, 111.1	79	22, 25
Acetoxyprogesterone	4.93	373.2 (+)	271.2, 313. 3	84	17, 13
Norgestrel	4.94	313.1 (+)	91.1, 245.2	85	43, 18
17α-Testosterone	4.95	289.2 (+)	97.1, 109.1	85	23, 26
Methyltestosterone	5.03	303.2 (+)	97.1, 109.1	96	31, 29
Methyltestosterone-d3	5.03	306.2 (+)	97.1, 109.1	82	30, 29
Methandriol	5.04	269.2 (+)	91.1, 213.2	82	43, 19
Chlormadinone acetate	5.12	405.1 (+)	309.2, 345.2	92	15, 12
Megestrol acetate	5.13	385.2 (+)	267.2, 325.3	88	18, 14
Medroxyprogesteron acetate	5.14	387.2 (+)	123.1, 327.3	94	32, 14
Medroxyprogesteron etate-d3	5.14	390.2 (+)	126.1, 330.3	87	31, 13
Melengestrol acetate	5.18	397.2 (+)	279.2, 337.3	93	20, 14
Trenbolone acetate	5.19	313.1 (+)	165.1, 253.2	105	60, 20
Norethandrolone	5.20	303.2 (+)	109.1, 285.3	96	31, 15
Progesterone	5.23	315.2 (+)	97.1, 109.1	89	22, 28
Caproxyprogesterone	5.69	429.3 (+)	271.2, 313.2	106	17, 13

residues were removed from the supernatant by filtration over a cotton plug. Addition of 5 mL hexane, followed by rigorously shaking for 2 min and centrifugation (9000 × *g*, 10 min, 4 °C) allowed elimination of the more lipophilic matrix fraction by discarding the hexane fraction.

Extraction of the analytes was performed by adding 20 mL diethylether to the water–methanol mixture and rigorously shaking for 2 min. The samples were allowed to settle and the ether layer transferred to a 15 mL polypropylene flask and evaporated to dryness at 60 °C under nitrogen. The residue was reconstituted in 0.5 mL of chloroform, vortexed for 1 min and diluted with 5 mL of hexane just prior to the SPE. This residue was applied onto Si cartridges (500 mg), which were preconditioned with 2 times 2.5 mL of hexane. Next, the tubes containing the extract were rinsed with 5 mL of hexane, which was applied onto the Si cartridges. NH_2 cartridges (100 mg) were placed underneath the Si cartridges and the whole rinsed with 5 mL of hexane and allowed to run dry. Finally, the cartridges were eluted with 5 mL chloroform:acetone (4:1, v/v) and the eluates reduced to dryness under nitrogen at 45 °C before reconstituting in 125 μL of methanol:water (50:50, v/v). An aliquot (10 μL) was injected onto the U-HPLC column.

2.5. Quality assurance

Prior to the sample analysis, a standard mixture of the targeted compounds was injected to check the operational conditions of the U-HPLC–MS/MS device. To every sample, a mixture of procedure internal standards (ISTDs) was added at a concentration of 5 μg kg^{-1}, prior to the extraction. The identification of the anabolic steroids was based on their retention time relative to the internal standard of choice and on the ion ratios of the product ions, carried out according to the criteria described in CD 2002/657/EC [4]. After identification, the analytes concentration was calculated by fitting its area ratio in a eight-point calibration curve, established by blank meat samples spiked with the ISTDs at 5 μg kg^{-1} and 34 anabolic steroids in the range of 0.25–10 times the ALs or RCs, which implies for most of the compounds 0.25–10 μg kg^{-1}. Area ratios were determined by integration of the area of an analyte under the specific SRM chromatograms in reference to the integrated area of the internal standard.

3. Results and discussion

3.1. U-HPLC and MS conditions

An U-HPLC–MS/MS method was developed to provide efficient, rapid and sensitive confirmatory quantification of 34 different anabolic steroids, both natural as synthetic from the different classes of androgens, gestagens and estrogens in bovine muscle. Acquisition parameters of the mass spectrometer were optimized by direct continuous pump infusion of standard working solutions of each individual analyte (10 ng μL^{-1}). Data acquisition was performed initially in full scan to determine an abundant precursor ion. During this tuning step acetic acid and formic acid were evaluated as candidate mobile phase additives, to enhance ionization. Addition of acidic mobile additives did however not significantly

Table 2
Decision limits (CC_α), detection capabilities (CC_β), mean recovery and precision of the developed method for 34 anabolic steroids in meat.

Analyte	Internal standard used	RC/AL (μg kg^{-1})	CC_α (μg kg^{-1})	CC_β (μg kg^{-1})	Recovery[a]	Repeatability[a]	Within-lab reproducibility[b]
					Mean ± SD (%)	RSD (%)	RSD (%)
β-Zearalanol	17β-Estradiol-d3	1	0.34	0.85	81.6 ± 11	18.5	17.1
Flugestone acetate	Medroxyprogesteron ac.-d3	1	0.07	0.20	97.5 ± 15	11.2	14.0
α-Zearalanol	17β-Estradiol-d3	5	0.88	1.77	81.4 ± 11	18.3	16.8
Estriol	17β-Estradiol-d3	1	0.29	0.71	119.2 ± 1.0	15.7	15.6
Fluoxymesterone	Androstadienedione	5	0.46	1.20	100.2 ± 2.0	17.6	17.7
β-Trenbolone	Androstadienedione	1	0.45	0.79	91.0 ± 16	13.1	12.1
β-Boldenone	Androstadienedione	1	0.21	0.35	91.6 ± 8.0	7.4	8.2
17β-Ethinylestradiol	17β-Estradiol-d3	1	0.21	0.33	93.5 ± 12	13.0	12.1
4-Androstenedione	Androstadienedione	1	0.13	0.17	91.9 ± 7.0	6.5	7.0
17β-Estradiol	17β-Estradiol-d3	1	0.25	0.46	101.4 ± 1.0	10.2	9.9
Estrone	17β-Estradiol-d3	1	0.29	0.55	110.0 ± 1.0	8.4	9.6
17α-Estradiol	17β-Estradiol-d3	1	0.14	0.27	93.4 ± 7.0	7.3	8.2
17β-Nortestosterone	17β-Testosterone-d2	1	0.56	0.87	93.0 ± 11	6.2	6.8
Diethylstilbestrol	17β-Estradiol-d3	1	0.47	0.88	85.4 ± 16	17.5	19.6
α-Boldenone	Androstadienedione	1	0.49	0.94	99.5 ± 11	12.6	13.6
Methylboldenone	Androstadienedione	3	0.46	0.65	92.4 ± 6.0	6.3	7.0
17α-Nortestosterone	17β-Testosterone-d2	1	0.38	0.68	98.5 ± 7.0	7.6	7.6
Dienestrol	17β-Estradiol-d3	1	0.13	0.25	97.4 ± 14	17.0	18.5
Hexoestrol	Hexoestrol-d4	1	0.32	0.39	99.5 ± 7.0	9.9	10.4
17α-Hydroxyprogesterone	Medroxyprogesteron ac.-d3	1	0.14	0.22	94.2 ± 11	12.4	12.2
17β-Testosterone	17β-Testosterone-d2	1	0.16	0.35	80.7 ± 10	9.0	9.1
Acetoxyprogesterone	Medroxyprogesteron ac.-d3	10	0.15	0.22	96.4 ± 10	8.0	8.0
Norgestrel	Medroxyprogesteron ac.-d3	5	0.33	0.48	97.2 ± 10	9.4	8.8
17α-Testosterone	Methyltestosterone-d3	1	0.09	0.19	93.4 ± 9.0	6.9	6.6
Methyltestosterone	Methyltestosterone-d3	1	0.19	0.23	98.2 ± 3.0	4.1	4.0
Methandriol	17β-Testosterone-d2	5	0.46	0.92	93.1 ± 5.0	6.1	7.2
Chlormadinone ac.	Medroxyprogesteron ac.-d3	0.5	0.13	0.23	96.4 ± 10	12.2	12.9
Megestrol ac.	Medroxyprogesteron ac.-d3	1	0.04	0.12	94.1 ± 6.0	6.5	6.5
Medroxyprogesteron ac.	Medroxyprogesteron ac.-d3	1	0.08	0.17	96.8 ± 6.0	6.4	5.8
Melengestrol ac.	Medroxyprogesteron ac.-d3	1	0.11	0.15	94.8 ± 5.0	4.8	5.9
Trenbolone ac.	Methyltestosterone-d3	2	0.12	0.21	91.9 ± 15	14.1	14.5
Norethandrolone	Methyltestosterone-d3	2	0.59	0.75	96.2 ± 5.0	4.2	4.3
Progesterone	Medroxyprogesteron ac.-d3	1	0.06	0.12	88.2 ± 9.0	9.9	10.3
Caproxyprogesterone	Medroxyprogesteron ac.-d3	10	0.5	1.34	97.1 ± 15	18.1	19.7

[a] Three series of six replicates of fortified samples of an identical matrix at each 1.0, 1.5 and 2.0 times the RC or AL, under identical conditions.
[b] Four series of six replicates of fortified samples of an identical matrix at each 1.0, 1.5 and 2.0 times the RC or AL, analyzed by two different operators.

improve and for some compounds, in particular the estrogens, even decreased ionization. APCI was selected as ionization source since it provided higher detection sensitivities for the majority of steroidal compounds as compared to ESI. Indeed, it has been reported in literature that APCI results in higher signals for multiresidue analysis of hormones [18]. Next, the MS/MS fragmentation conditions were investigated and collision energies and S-lens voltages were optimized for each individual compound and/or transition (Table 1). According to CD 2002/657/EC [4] a confirmatory method requires 4 identification points. Therefore, for each analyte a precursor ion (parent mass) and two product ions were monitored (Table 1). Since this yields 4 identification points (1 for the parent ion and 1.5 for each product ion), this method complies with the requirements of a confirmatory method.

The gradient U-HPLC program was optimized to facilitate separation of matrix constituents from the hormone molecules and at the same time allow separation between isobaric analytes i.e. the β and α forms of molecules such as nortestosterone, boldenone, testosterone, estradiol and zearalanol. Several U-(H)PLC columns: Nucleodur Sphinx (Machery-Nagel; 1.8 μm, 100 mm × 2.1 mm), Aqcuity HSS C18 (Waters; 1.8 μm, 100 mm × 2.1 mm), and Hypersil Gold (Thermo Scientific; 1.9 μm, 100 mm × 2.1 mm) were evaluated for their performances. The Hypersil Gold provided not only the highest signal to noise ratio (S/N) and peak intensity for the different analytes, but most important the best separation of the β and α forms. Additionally, retention times, optimal separation, and good resolution were ameliorated by careful selection of the gradient program. To this end, the organic solvent methanol was preferred, as the higher elution strength of acetonitrile proved to be disadvantageous for separation and resolution of certain steroids. The selected gradient accomplished adequate separation of isobaric analytes (see Table 1 and Fig. 1). This gradient allowed quantitative determination of a much higher number of steroids in meat than so far reported in literature [18–22].

3.2. Validation study

The newly developed analytical method was validated according to the criteria specified in CD 2002/657/EC for quantitative confirmation [4]. For each compound 2 transitions were monitored (Table 1). Both product ions were used for quantification purposes. Firstly, appropriate internal standards were chosen, capable of anticipating fluctuations in the signal intensity upon extraction of anabolic steroids from muscle tissue. The use of isotopically labeled internal standards in MS-based chemical analysis has always been recommendable, as well as compounds that are structurally related to the analyte (basic structure identical) [4]. Therefore, several deuterated compounds (10 in total) were evaluated as internal standards, resulting in the final selection of 17β-estradiol-d3, medroxyprogesteron acetate-d3, methyltestosterone-d3, 17β-testosterone-d2 and hexoestrol-d4. The deuterated compounds estrone-d4, 19-nortestosterone-d2, 17β-trenbolone-d2, megestrol acetate-d3 and melengestrol acetate-d3 did not provide better results for any of the compounds and were not retained in the method. In addition, one structural similar analogue, namely androstadienedione was selected as internal standard as well, as a result of its superior corrective activities for some of the androgens as opposed to the deuterated analogues. Finally, for

each compound, the most reliable internal standard was chosen (Table 2).

Validation of the method occurred by adopting the protocol proposed by Antignac et al. [26]. This protocol was tailored for validating analytical methods based on MS detection and offers a compromise between CD 2002/657/EC [4] and practical aspects and limitations related to laboratory work. The validation protocol was designed as follows. Analysis of 20 blank muscle samples was performed to check the ruggedness of the method. This permitted to determine the specificity by calculating the average (μ_N) and standard deviation (σ_N) of the noise amplitude, expressed relative to the selected internal standard signal amplitude. The calibration curve consisted of eight fortification levels together with the previously estimated noise average (μ_N) as forced intercept. The linearity of

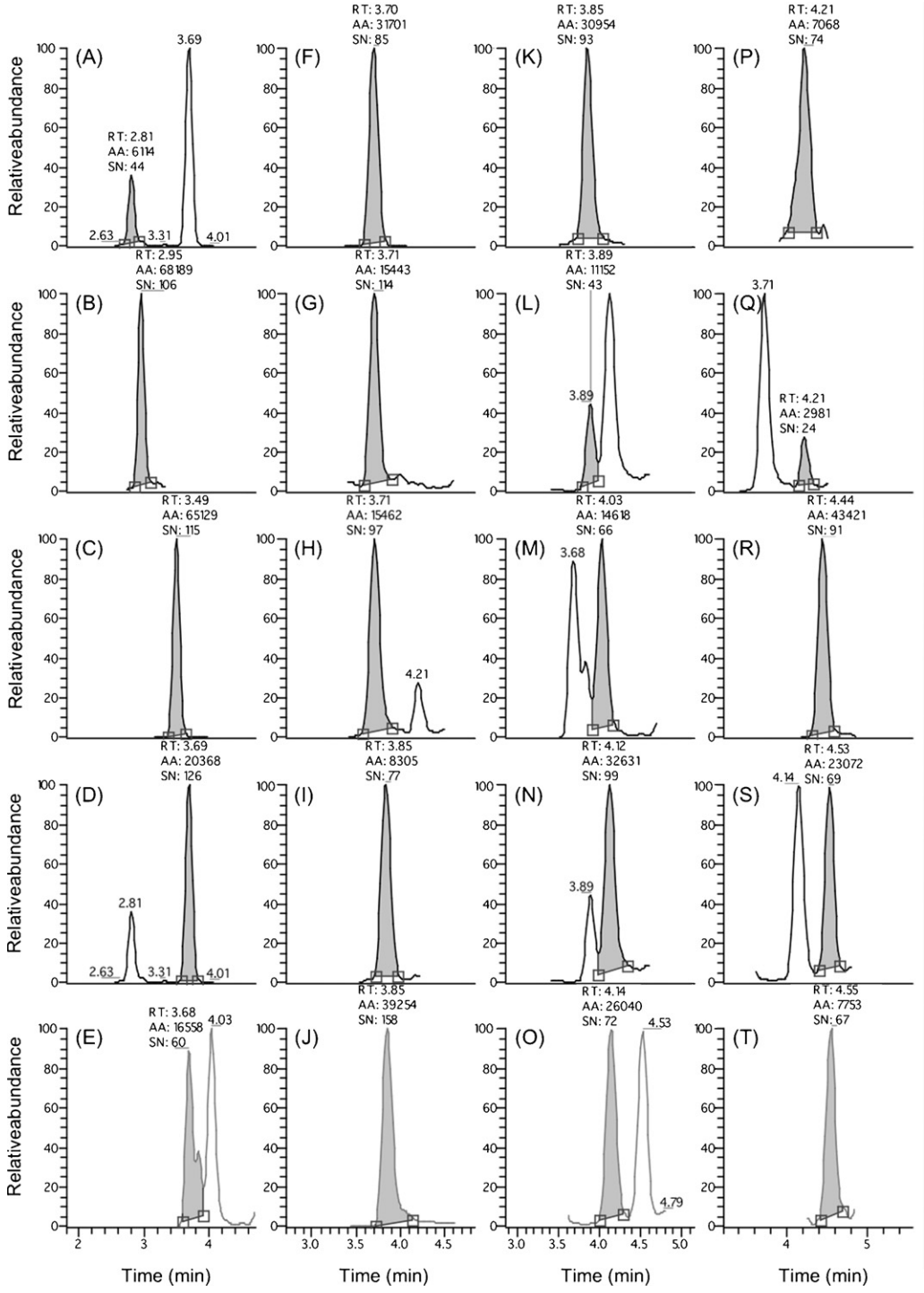

Fig. 1. SRM chromatogram (MS/MS) in APCI mode of a meat sample fortified with (A) β-zearalanol; (B) androstadienedione; (C) flugestone acetate; (D) α-zearalanol; (E) estriol; (F) fluoxymesterone; (G) β-trenbolone; (H) β-boldenone; (I) 17β-ethinylestradiol; (J) 17β-estradiol-d3; (K) 4-androstenedione; (L) 17β-estradiol; (M) estriol; (N) 17α-estradiol; (O) 17β-nortestosterone; (P) diethylstilbestrol; (Q) α-boldenone; (R) methylboldenone; (S) 17α-nortestosterone; (T) dienestrol; (U) hexoestrol-d4; (V) hexoestrol; (W) 17α-hydroxyprogesterone; (X) 17β-testosterone; (Y) 17β-testosterone-d2; (Z) acetoxyprogesterone; (A′) norgestrel; (B′) 17α-testosterone; (C′) methyltestosterone; (D′) methyltestosterone-d3; (E′) methandriol; (F′) chlormadinone acetate; (G′) megestrol acetate; (H′) medroxyprogesteron acetate; (I′) medroxyprogesteron acetate-d3; (J′) melengestrol acetate; (K′) trenbolone acetate; (L′) norethandrolone; (M′) progesterone and (N′) caproxyprogesterone at their respective RC or AL levels.

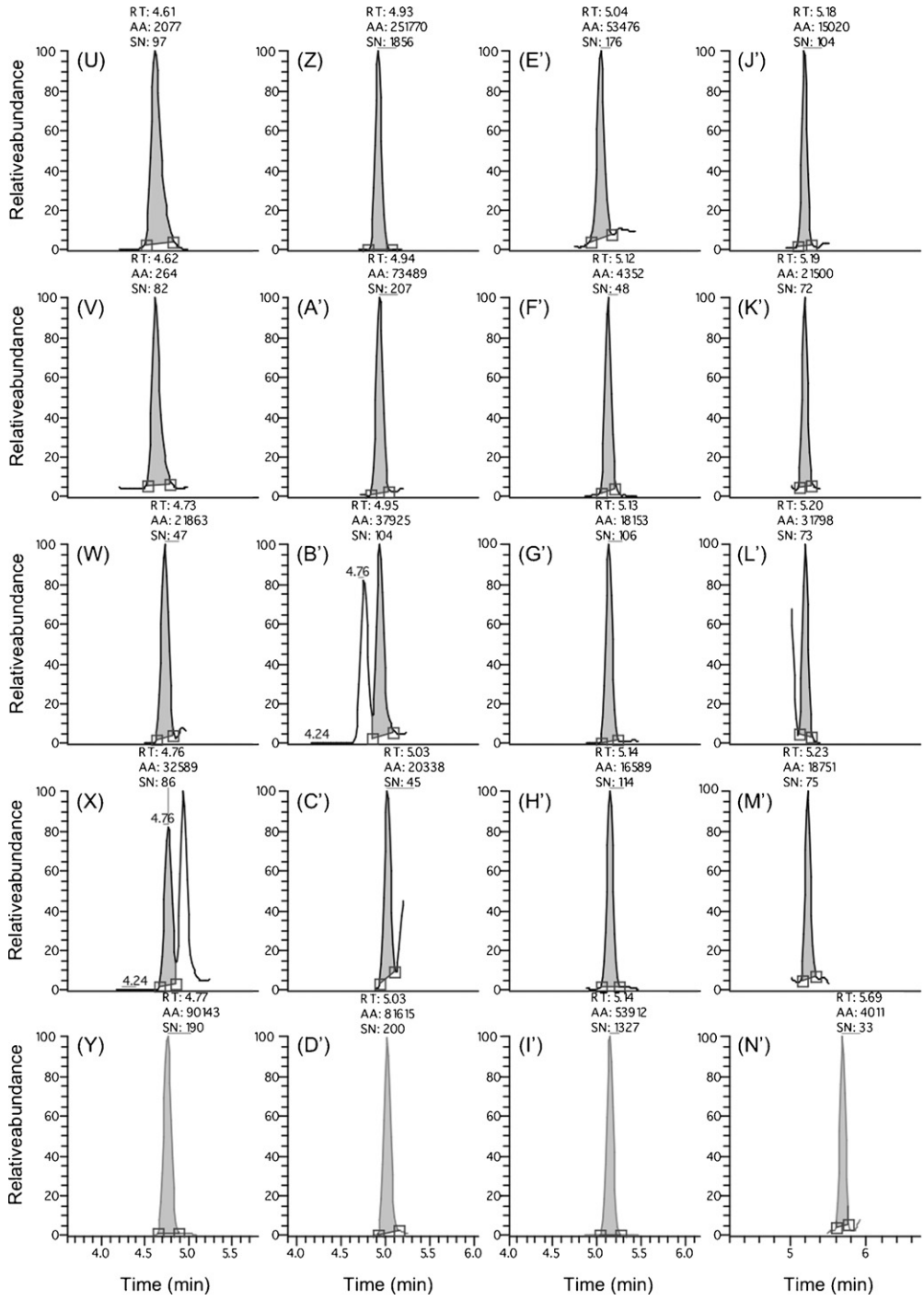

Fig. 1. (*Continued*)

this calibration graph was evaluated by calculation of the correlation coefficient (R^2) and the sensitivity i.e. the slope of the fitted curve (a). Based on these data, the decision limit (CC_α) was calculated as 2.33 times the standard deviation of the noise amplitude divided by the slope of the fitted curve. For calculating CC_β, 20 blank samples were spiked at the determined CC_α level. To this end, a spike mixture containing the different analytes at a concentration, resulting in the CC_α in meat upon spiking with 100 μL of this mixture, was prepared. This permitted to estimate the repeatability through the standard deviation of the signal amplitude (σ_S). In order to minimize the estimation error, the signal relative standard deviation ($(R.S.D.)_S$) was preferred above the standard deviation (σ). Finally with σ_N, a, and $(R.S.D.)_S$, the detection capability (CC_β) could be calculated, according to the following equation:

$$CC_\beta = \frac{[2.33\sigma_N + 1.64\mu_N(R.S.D.)_S]}{a[1 - 1.64(R.S.D.)_S]}$$

To determine the recovery (trueness) and precision, 18 identical blanks, were amended with the target compounds at 1.0, 1.5 and 2.0 times their RC or AL (Table 2) and the internal standards, divided over four sets ($n = 6$). This procedure was repeated by two different operators on four separate occasions.

3.2.1. Specificity

To establish the specificity of the method blank muscle samples were fortified with the 34 analytes and internal standards separately at their RC or AL. In addition, blank muscle samples were analyzed as well. To this end, meat samples from different origins (super markets) were examined. Besides, standard solutions of the individual compounds were injected as well, to rule out potential interferences between the target compounds. For each analyte spiked or standard solution injected, chromatograms showed a significant increase in peak area and intensity at the compound-specific retention time compared to the blanks, taking a signal to noise ratio of at least 15 into account (Fig. 1). No other matrix substances interfered at the compound-specific retention time window and no interferences between the individual compounds could be noticed. In some cases minor matrix components could be observed but were chromatographically resolved from the compounds of interest and did not hinder the quantification or identification. Therefore, the newly developed method was found to be specific for all 34 anabolic steroids and its internal standards in the presence of matrix components.

3.2.2. Selectivity

Tandem mass spectrometry itself as a detection technique offers a high degree of selectivity. In accordance with CD 2002/657/EC, analytes were identified on the basis of their relative retention time, i.e. the ratio of the chromatographic retention time of the analyte to that of the internal standard [4]. In addition, a system of identification points was used to interpret the data, based on the ion ratios of the precursor and product ions in the acquired spectrum. For the confirmation of anabolic steroids, listed in Group A of Annex I of Directive 96/23/EC [19], a minimum of 4 identification points (IPs) is required [4]. Precursor (1 IP) and product ions (1.5 IP/ion) of each analyte are presented in Table 1. The individual relative retention time ($n = 6$) of the extracted hormones showed in every case a standard deviation lower than 0.006, with a coefficient of variation smaller than 0.56%. This falls well within the stated tolerance level of 2.5% for liquid chromatography. As a result, the identification of the 34 steroidal compounds, extracted from muscle meat was unambiguously. As for the identification points, the minimum required amount of IPs, set at 4, was achieved for every compound at the respective RC or AL levels.

3.2.3. Calibration curves

The linearity of the chromatographic response was evaluated with matrix-matched calibration curves in blank muscle meat using 8 calibration points, i.e. 0.25, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0 times the RCs or ALs, which implies for the compounds with a recommended concentration (1 $\mu g\,kg^{-1}$) a range of 0.25–10 $\mu g\,kg^{-1}$. These were the same calibration curves as acquired to calculate the CC_α and CC_β levels of the analytes. Linear regression analysis was carried out by plotting the peak area ratios of the analyte against the I.S. versus the analyte concentrations. The estimated noise average of the pool of blanks ($n = 20$) was used as a forced intercept [20]. Good linearity was obtained; all correlation coefficients (R^2) were ≥ 0.990.

3.2.4. Trueness

Since no certified reference material was available, the trueness of the method was assessed by fortifying blank muscle samples at 1.0, 1.5 and 2.0 times the RC or AL for each analyte. Mean corrected recovery ($n = 18$) of the analytes, determined in three separate assays, as presented in Table 2, was between 80.7% and 119.2%. All calculated mean recoveries fulfill the criteria put forward in CD 2002/657/EC stating that a mass fraction below 1 $\mu g\,kg^{-1}$ should comply with a mean recovery range of 50–120%, while a mass fraction between 1 and 10 $\mu g\,kg^{-1}$ should obtain a mean recovery range of 70–110%, whereas a mean recovery of 80–110% should be required for a mass fraction of, or greater than 10 $\mu g\,kg^{-1}$ [4].

3.2.5. Precision

To evaluate the precision of the method, repeatability and within-laboratory reproducibility were determined. Repeatability was evaluated by calculating the coefficients of variation (CV). To this purpose, data from three series of six replicates of samples of an identical origin fortified at 1.0, 1.5, and 2.0 times the compound-specific RC or AL were used. These analyses were carried out on three different occasions by the same analyst under repeatable conditions. For all anabolic steroids considered, good repeatability was obtained, since the individual overall calculated CVs for each compound were mostly below 15% (Table 2). Low values could be achieved for a number of compounds (<10%), the main reason for this can be attributed to the availability of deuterated structural or structural analogues of the compounds being examined. For those compounds without a deuterated structural or structural analogue the internal standard providing the best correlation coefficient upon linear regression of the calibration line was used. These non-identical, to a lesser extent structurally similar internal standards, however resulted in some cases in apparent lower correction factors for losses or matrix suppression and ultimately higher CVs.

For evaluation of the reproducibility only the within-laboratory reproducibility was considered. Four series of six replicates of fortified samples at 1.0, 1.5, and 2.0 times the compound-specific RC or AL were analyzed by two different operators on three different days with different spiking solutions and using two different blank meat matrices. The results, summarized in Table 2 indicate the good precision of the method. The obtained CVs were in accordance with CD 2002/657/EC [4] stating that in case of repeated analysis of a sample carried out under within-laboratory reproducibility conditions, the intra-laboratory coefficient of variation of the mean should not exceed 20% in case of a mass fraction smaller than 100 $\mu g\,kg^{-1}$. The modified function suggested by Thomson [27] stating that the reproducibility CV should be lower than 22% for mass fractions smaller than 120 $\mu g\,kg^{-1}$ is also fulfilled by these data.

3.2.6. Decision limit (CC_α) and detection capability (CC_β)

In accordance with CD 2002/657/EC the decision limit (CC_α) was used instead of the detection limit and the detection capability (CC_β) was used instead of the limit of quantification. The decision limit (CC_α) is defined as the limit above which it can be concluded with an error probability of α, that a sample contains the analyte. For prohibited substances an α value equal to 1% is applied. The detection capability (CC_β) is the smallest content of the substance that may be detected, identified and quantified in a sample, with a statistical certainty of $1 - \beta$, with $\beta = 5\%$. During this study, the CC_α and CC_β were determined by analysis of 20 blank muscle samples respectively, non-fortified and fortified at CC_α level as already widely described under Section 3.2. The signal associated with CC_α corresponded to the maximal noise amplitude. Table 2 summarizes the calculated CC_α and CC_β values for the different anabolic steroids. Decision limits and detection capabilities ranged respectively, between 0.04 and 0.88 $\mu g\,kg^{-1}$ and between 0.12 and 1.9 $\mu g\,kg^{-1}$. All analytes were clearly detected with a S/N ratio higher than 10 upon analysis of samples fortified at the CC_α level and were successfully confirmed using the ion ratios of the two product ions. These high S/N values provide evidence that detection and confirmation is surely possible at or below the CC_β values. Compared to values reported in literature for a smaller number of analytes [18–22], the calculated CC_α and CC_β values from this study are equal (in most cases) indicating high sensitivity of the reported methodology. In some cases higher values are reported but this may be explained by the higher ALs applied for these compounds, in line with the Belgian National Reference Laboratory's

recommendations and the fact that the signal relative standard deviation ($(R.S.D.)_S$) was preferred above the standard deviation (σ_S) for calculation of the CC_β as proposed by Antignac et al. [26].

4. Conclusions

A specific, sensitive and reliable multi-residue U-HPLC-MS/MS method has been developed that simultaneously identifies and quantifies 34 natural and synthetic anabolic steroids of various chemical sub-categories in bovine muscle. The method can be considered as rapid, since it utilizes ultra high performance fast chromatography with all analytes eluting within 4 min with a total run time of only 8 min and still achieving sufficient resolution of the different β and α isomers. Data obtained showed satisfactory precision and trueness and results were validated and confirmed, according to the criteria laid down by the European CD. This study shows that the developed method meets the required sensitivity of 1–10 $\mu g\,kg^{-1}$, which has been set forward by the Community's and National Reference Laboratories. All calculated CC_α and CC_β values are considerably lower than these levels. Hence, the method is a useful tool for official residue control analyses and is used as such in our laboratory.

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