Available online at www.sciencedirect.com
ScienceDirect
Analytica Chimica Acta 586 (2007) 57–72
ANALYTICA CHIMICA ACTA
www.elsevier.com/locate/aca

Mass spectrometric detection of and similarities between 1-androgens

K. Verheyden [a], B. Le Bizec [b], D. Courtheyn [c], V. Mortier [a], M. Vandewiele [c], W. Gillis [d], P. Vanthemsche [d], H.F. De Brabander [a,*], H. Noppe [a]

[a] *Ghent University, Faculty of Veterinary Medicine, Department of Veterinary Public Health and Food Safety, Laboratory of Chemical Analysis, Salisburylaan 133, B-9820 Merelbeke, Belgium*
[b] *LABERCA, Ecole Nationale Vétérinaire de Nantes, BP 50707, F-44087 Nantes Cedex 03, France*
[c] *Federal Feed & Food Laboratory, Braemkasteelstraat 59, B-9050 Ghentbruges, Belgium*
[d] *Federal Agency for the Safety of the Food Chain, WTC III 16nd Floor, Simon Bolivarlaan 30, B-1000 Brussels, Belgium*

Received 19 June 2006; received in revised form 9 October 2006; accepted 12 October 2006
Available online 11 November 2006

Author's personal copy

Abstract

Regularly new anabolic steroids appear on the black market. In most cases these substances are marketed on websites or are confiscated during inspections. 1,(5α)-Androstene-17β-ol-3-one, also known as 1-testosterone, is one of these substances presented to body-builders as a nutritional supplement or a pro-hormone. 1-Testosterone closely resembles the natural hormone testosterone except for a 1,2-double bound instead of a 4,5-double bound. 1-Androstene-3β,17β-diol is transformed into 1-testosterone after oral administration.

1-Testosterone, 1-androstene-3β,17β-diol and some other related 'new' anabolic steroids were studied with gas chromatography coupled to mass spectrometry (GC–MS) and Liquid chromatography coupled to tandem mass spectrometry (LC–MS^2) methods. Similarities in spectra to known analytes, which may lead to pitfalls in the interpretation of the derivatised analytes, are discussed.
© 2006 Elsevier B.V. All rights reserved.

Keywords: 1-Testosterone; 1-Androstenediol; Multiple mass spectrometry

1. Introduction

The use of anabolic steroids, indicated as estrogens, gestagens and androgens (EGAs), in products of animal origin has been forbidden in the European Union since 1981, due to possible toxicological effects on consumer's health [1]. Within the scope of steroid residue control, the Federal Agency for the Safety of the Food Chain (FAVV–AFSCA) performs inspection controls on the abuse of EGAs both at the farm and the slaughterhouse. According to the national residue plan a number of EGAs must be monitored by analysis of different matrices. However, regularly new EGAs appear on the black market [2–4]. In most cases these substances are marketed on websites for body-building or are confiscated during inspections.

1-Testosterone (1,(5α)-androstene-17β-ol-3-one) is one of these substances presented to body-builders as a nutritional supplement or a pro-hormone. 1-Testosterone closely resembles the natural hormone testosterone except that it has a 1,2-double bound instead of a 4,5-double bound (Fig. 1). 1-Testosterone can also be seen as a 5α-reduced version of the hormone boldenone, a molecule studied extensively [5]. Being a 5α-reduced androgen it cannot aromatise to estrogens and therefore it is claimed to be much more anabolic than testosterone. Since estrogen treatment is accompanied by water and fat retention, unwanted side effects are said to be non-existent after administration of this substance. 1-Androstene-3β,17β-diol is transformed into 1-testosterone after oral administration and hence has a similar action [6].

1-Testosterone, 1-androstene-3β,17β-diol and some other related 'new' anabolic steroids (Fig. 1) were studied with gas

* Corresponding author. Tel.: +32 9 264 74 60; fax: +32 9 264 74 92.
E-mail address: Hubert.DeBrabander@UGent.be (H.F. De Brabander).

0003-2670/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.aca.2006.10.058

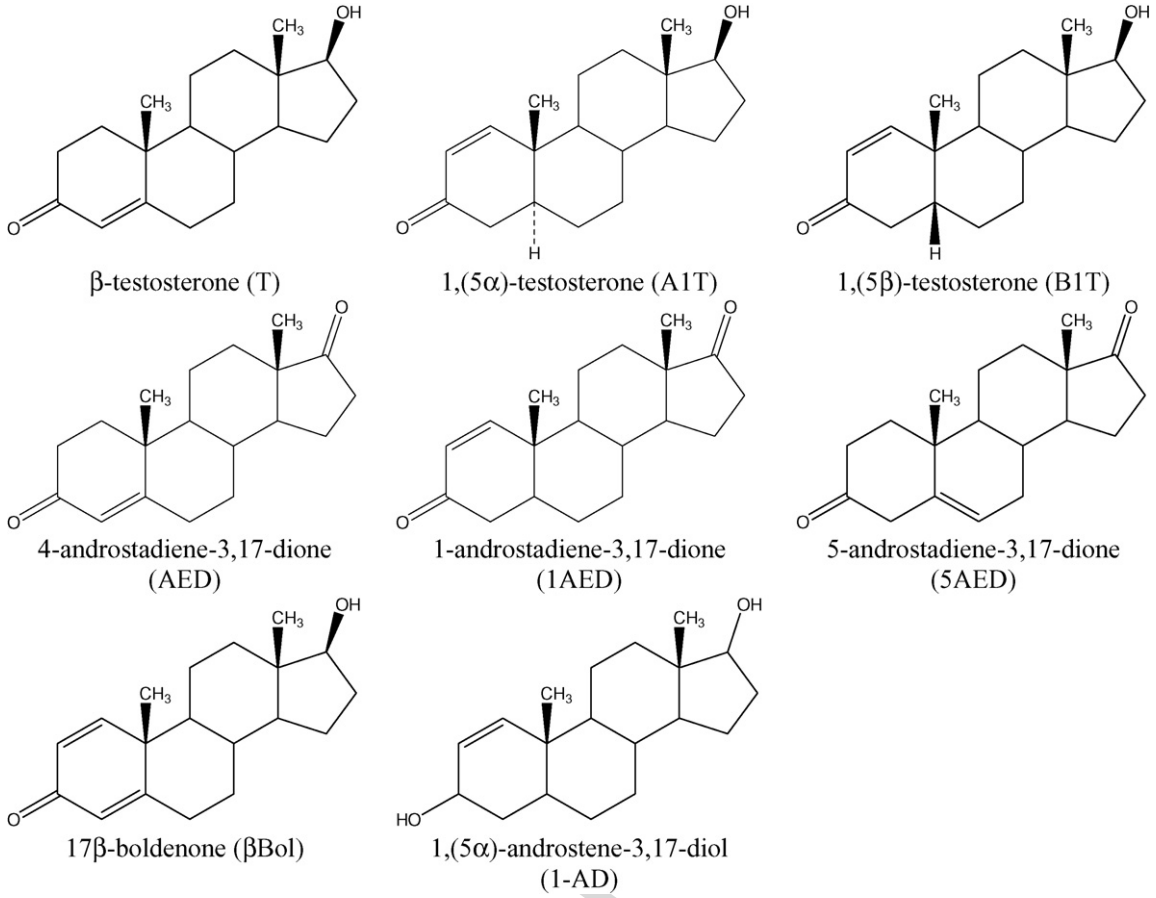

Fig. 1. Chemical structure of the different analytes.

chromatography coupled to mass spectrometry (GC–MS) and liquid chromatography coupled to tandem mass spectrometry (LC–MS^2) methods. Similarities in spectra to known analytes, which may lead to pitfalls in the interpretation, are discussed.

2. Experimental

2.1. Reagents and chemicals

Standards of 17β-boldenone (βBol), 1,(5α)-androstene-3,17-dione (1AED), 4-androstene-3,17-dione (AED), 5-androstene-3,17-dione (5AED), 17β-testosterone (βT), 17α-testosterone (αT) and 1-testosterone (1,(5α)-androstene-17β-ol-3-one) (A1T) were obtained from steraloids (Wilton, NY, USA), while 17α-boldenone (αBol) was purchased from the Community Reference Laboratory (Bilthoven, The Netherlands). 1,(5α)-Androstene-3β,17β-diol (body-builder product 1-AD) was a generous gift from the Belgian Doping Lab and 1,(5β)-androsten-17β-ol-3-one (B1T) was obtained from the National Measurements Institute (Pymble, Australia) (Table 1). Standard stock solutions of 200 ng μL^{-1} were prepared in ethanol (EtOH). All chemicals used were of analytical grade from Merck (Darmstadt, Germany) and Acros (Geel, Belgium).

The derivatisation reagent $MSTFA^{++}$ for GC–MS^n was prepared by dissolving 100 mg ammonium iodide (NH_4I) (Sigma, St. Louis, MO, USA) and 0.2 mL ethanethiol (Acros, Geel, Belgium) in 5 mL *N*-methyl-*N*-(TMS)-trifluoroacetamide (MSTFA) (Macherey-Nagel, Düren, Germany), followed by dilution of 1.5 mL of this solution with 10 mL MSTFA. The final derivatisation reagent is MSTFA/TMS-I.

Prior to GC–MS analysis, the analytes were fractionated by preparative high performance liquid chromatography (HPLC)

Table 1
Overview of the different analytes sorted by mass, code and product number

Analyte	Code	Mass	Product number
17α-Boldenone	αBol	286.193	CRL
17β-Boldenone	βBol	286.193	ST-A0200
1-Androstene-3,17-dione	1AED	286.193	ST-A6030
4-Androstene-3,17-dione	AED	286.193	ST-A4400
5-Androstene-3,17-dione	5AED	286.193	ST-A8020
17α-Testosterone	αT	288.209	ST-A6900
17β-Testosterone	βT	288.209	ST-A6950
1,(5α)-Testosterone	A1T	288.209	ST-A4600
1,(5β)-Testosterone	B1T	288.209	D564
1,(5α)-Androstene-3,17-diol	1-AD	290.225	BDL-gift

ST = steraloids, USA; CRL = Community Reference Laboratory, The Netherlands; D = National Measurements Institute, Australia; BDL = Belgian Doping Lab.

Author's per

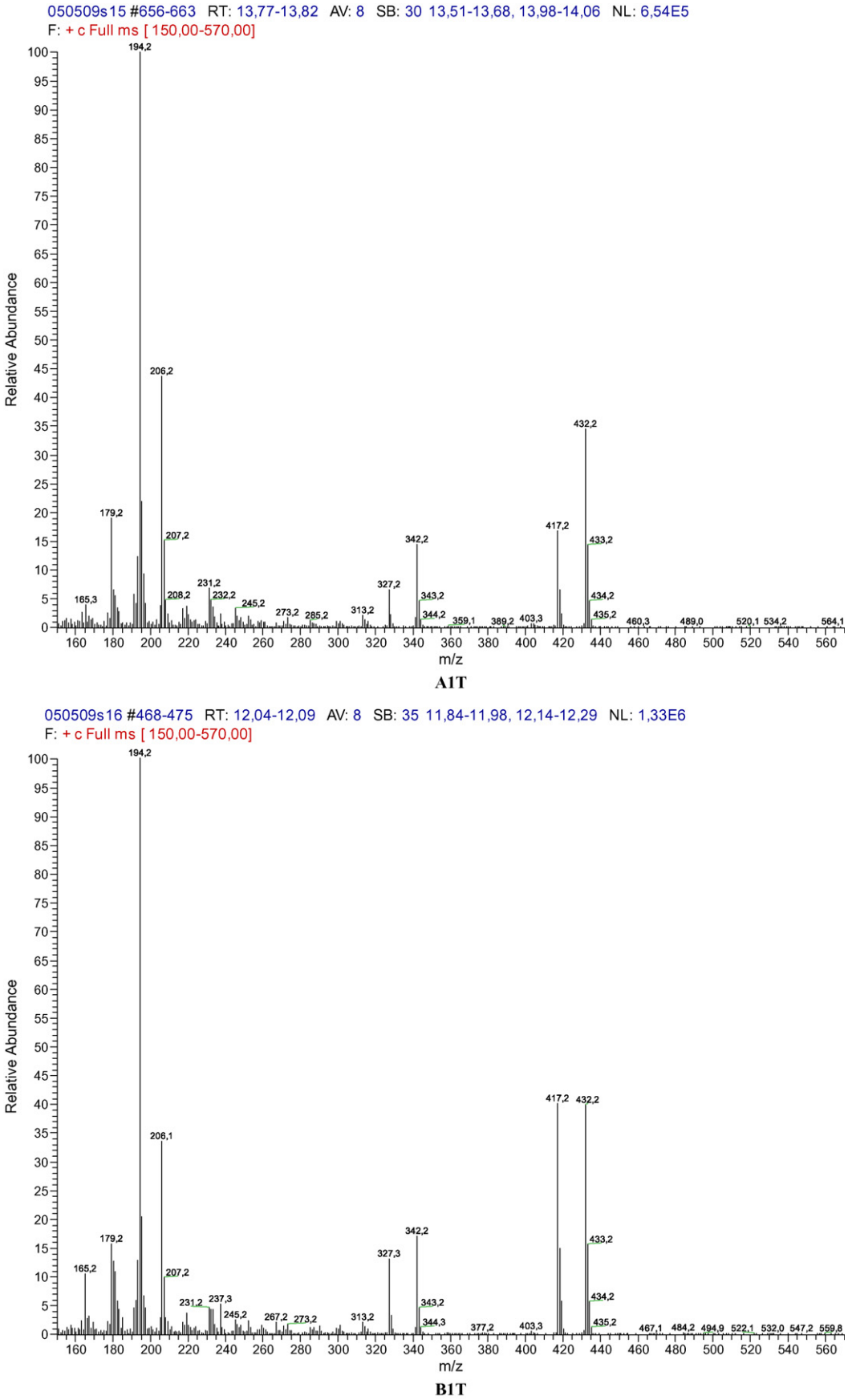

Fig. 2. GC–MS full scan of 1,(5α)-testosterone (A1T) and 1,(5β)-testosterone (B1T).

(HPLC Intelligent pump, Merck, Darmstadt, Germany) on a C_{18}-reversed phase column [7].

2.2. GC–MS analysis

Chromatographic separation was achieved using a ThermoQuest CE Trace GC gas chromatograph (Thermo Finnigan, Austin, TX, USA) with split/splitless injector (splitless injection). Analysis was performed using a non-polar 5% phenyl-polysilphenylene-siloxane SGE BPX-5 GC-column (25 m × 0.22 mm i.d. 0.25 μm) (SGE Incorporated, Austin, TX, USA). Helium gas was used as GC carrier gas at a flow rate of 1 mL min^{-1}. A Carlo Erba autosampler AS2000 (Thermo Finnigan, Austin, TX, USA) was used to inject the samples. Detection was done using a Polaris ion trap mass spectrometer (Thermo Finnigan, Austin, TX, USA) with electron ionisation in positive ion mode (ion source: 200 °C; transfer line: 275 °C). A temperature gradient was used starting at 100 °C, increasing to 250 °C at a rate of 17 °C min^{-1}. In a second step the temperature was increased from 250 to 300 °C at a rate of 2 °C min^{-1}.

After HPLC fractionation, the mobile phase was evaporated and afterwards, the analytes were derivatised with $MSTFA^{++}$. A 25 μL aliquot of $MSTFA^{++}$ was added to each vial and incubated for 60 min at 60 ± 2 °C. Then, 1 μL was injected into the gas chromatograph [8]. Starting from a 50 ng μL^{-1} working solution, an amount of 2 ng of each analyte was obtained after derivatisation and 1 μL injection. Androsterone was added prior to the derivatisation reaction as an internal standard to control the derivatisation. The mass spectrometer experiments are performed in GC–MS full scan.

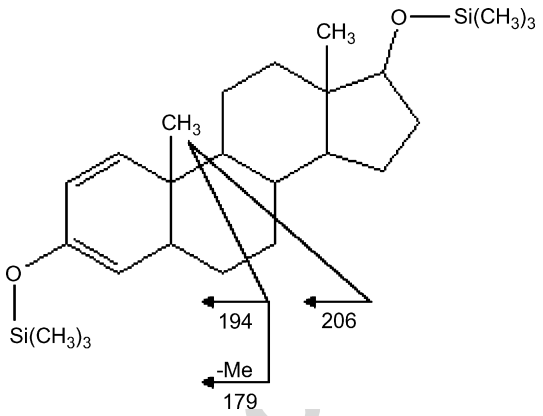

Fig. 3. Fragmentation of the TMS enol TMS ether derivative of 1-testosterone in GC–MS full scan.

2.3. LC–MS analysis

The LC apparatus consisted of an 1100 series quaternary gradient pump and autosampler of Agilent (Palo Alto, CA, USA). Chromatographic separation was achieved using a Symmetry C_{18} column (5 μm, 150 mm × 2.1 mm, Waters, Milford, USA). The mobile phase consisted of a mixture of 0.02 M formic acid

Table 2
The relative retention time (t_R) and the relative intensities of the MS ions using GC–MS and the HPLC fraction in which each analyte elutes

	B1T	1AED	A1T	αBol	αT	AED	5AED	βBol	βT
t_R[a]	0.956	1.068	1.086	1.089	1.102	1.135	1.135	1.136	1.153
MS ions									
432	40		34		**100**				**100**
430		43		53		**100**	**100**	56	
428									
417	40		17		11				36
415		**100**		9		13	13	26	
413									
342	17		14		7				
340		14							
327	13		6		14				
325		15		49		6	6	39	
323									
260						5	5		
237									
235									
234						9	9		
222									
221		29							
209					10	8	8		10
206	34		44	**100**				**100**	
196					7				7
194	**100**	24	**100**						
191				16				17	
179	16		19						
HPLC									
Fraction	3	3	3	2	3	2	3	2	3

[a] Relative to androsterone.

Author's personal copy

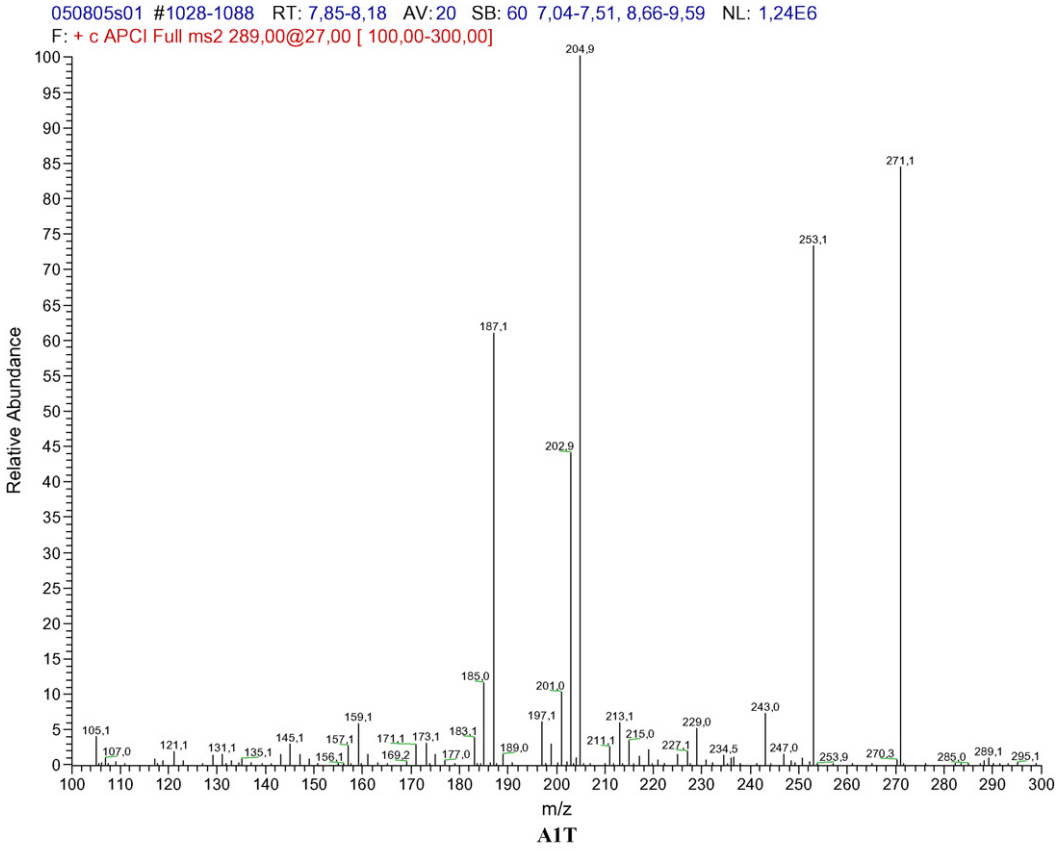

Fig. 4. LC–MS^2 scan of 1,(5α)-testosterone (A1T) and 1,(5β)-testosterone (B1T), precursor ion *m/z* 289.

in water (A) and methanol (B) (65% of B was increased to 80% in 25 min). The flow rate was 0.3 mL min^{-1}. MS^n detection was carried out with a ThermoFinnigan LCQ Deca ion trap with atmospheric pressure chemical ionisation (APCI) interface in positive ion mode (San José, CA, USA). With a capillary temperature of 200 °C, a vaporizer temperature of 450 °C, a sheath gas flow of 80.00 and an auxiliary gas flow of 3.00. The stock solutions were diluted in 0.02 M formic acid prior to injection. An amount of 5 ng of each analyte was brought onto the LC column by injecting 10 μL of a 0.5 ng μL^{-1} working solution.

3. Results

First, the mass spectrometric behaviour of the new anabolic steroid 1-testosterone was investigated. Subsequently, some related anabolic steroids were studied and similarities in mass spectra were highlighted. Finally, a mixture of all the studied anabolic steroids was injected both in GC– and LC–MS to investigate possible pitfalls.

3.1. Mass spectrometric behaviour of 1-testosterone

1-Testosterone, 1,(5α)-testosterone (A1T) as well as 1,(5β)-testosterone (B1T) (Fig. 1), was derivatised and injected into the GC–MS apparatus in order to acquire data for its identification. The molecular mass of 1-testosterone is 288 (Table 1), so after derivatisation to a trimethylsilyl enol trimethylsilyl ether (TMS enol TMS ether) derivative, a molecular ion of *m/z* 432 can be expected. Fig. 2 shows the GC–MS spectrum of 1-testosterone. As could be expected, 1-testosterone had a TMS molecular ion of *m/z* 432 (M^+). The fragment ions present in MS full scan were 417 $(M - Me)^+$, 342 $(M - TMSOH)^+$, 327 $(M - TMSOH - Me)^+$ and the ions 179, 194 and 206 which are due to fragmentation of the B-ring (Fig. 3). Both 1,(5α)-testosterone (A1T) and 1,(5β)-testosterone (B1T) had the same ions in MS full scan. However, the relative intensities of these ions were, in general, different in both MS spectra. Concerning, the relative retention time (versus androsterone), B1T (0.956) elutes before A1T (1.089).

In the case of unknown samples, after extraction and clean-up, extracts are fractionated into four different parts by preparative HPLC on a C_{18}-reversed phase column to further reduce interfering matrix compounds. To become extra information, it was also investigated in which HPLC fraction A1T and B1T elute. Both analytes elute in fraction three.

In Table 2 an overview is given of the GC–MS ions in MS-full scan and their relative intensities, as well as their relative retention time (versus androsterone) and the HPLC fraction in which the analyte elutes.

Next to GC–MS, 1-testosterone was also investigated using LC–MS. Therefore, no derivatisation was used. As mentioned previously, the molecular mass of 1-testosterone is 288, so the pseudo-molecular ion in MS full scan in positive ion mode is *m/z* 289. Fig. 4 shows the MS^2 fragmentation of *m/z* 289. The product ions are *m/z* 187, 205, 253 and 271. The relative intensities of the product ions *m/z* 187 and 205 were far lower in the MS^2 spectrum of B1T compared to the mass spectrum of A1T.

The precursor ions and the relative intensities of their MS^2 product ions, as well as their elution time, are summarised in Table 3.

3.2. Mass spectrometric behaviour of related anabolic steroids

The analytes 17α-boldenone (αBol), 17β-boldenone (βBol), 1,(5α)-androstene-3,17-dione (1AED), 4-androstene-3,17-dione (AED), 5-androstene-3,17-dione (5AED), 17β-testosterone (βT) and 17α-testosterone (αT) (Fig. 1) were injected individually in order to acquire data for their identification.

3.2.1. GC–MS analysis

The molecular mass of boldenone, 1AED, AED and 5AED is 286 (Table 1), so after derivatisation to TMS enol TMS ether

Table 3
The elution time, precursor ion and the relative intensities of the product ions using LC–MS^2

	βBol	AED	αBol	1AED	5AED	βT	αT	A1T	B1T
Elution time	4.28	4.63	5.32	5.87	6.57	6.07	7.67	7.67	7, 67
Precursor ion	287	287	287	287	287	289	289	289	289
Product ions									
271						98	**100**	**100**	**100**
269	**100**	**100**	**100**	88	**100**				
253						**100**	82	82	82
251		49		21	41				
229					44				
211		24							
205								66	
203				**100**				32	
187								47	
185				91					
147	15		6						
135	65		12						
121	20		6						
109		28			20				

Author's personal copy

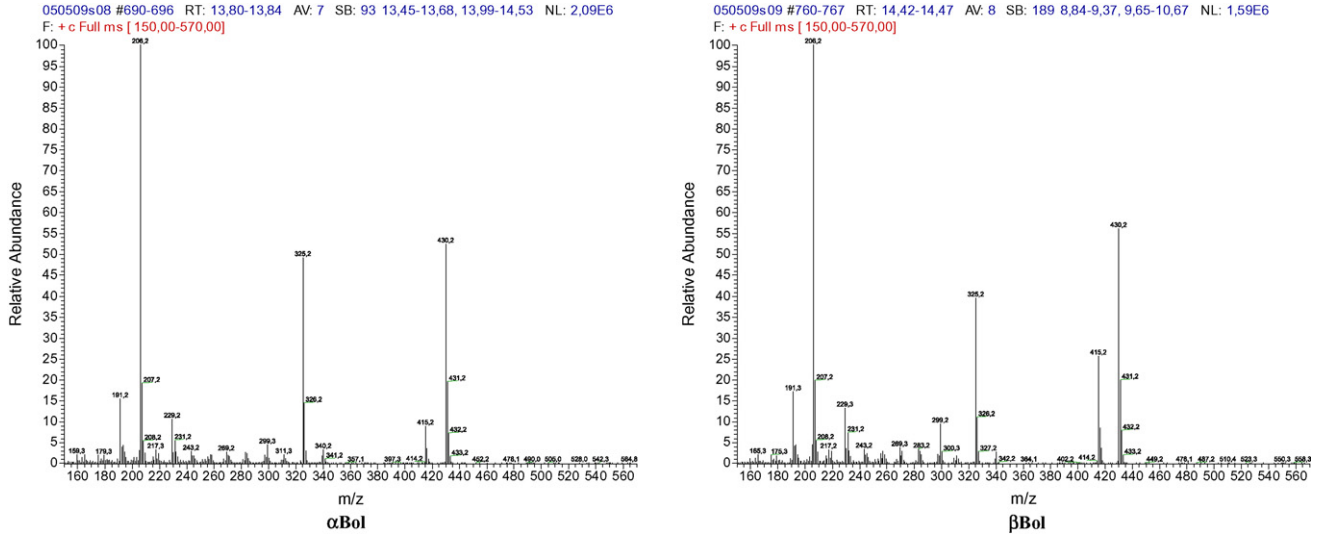

Fig. 5. GC–MS full scan of 17α-boldenone (αBol) and 17β-boldenone (βBol).

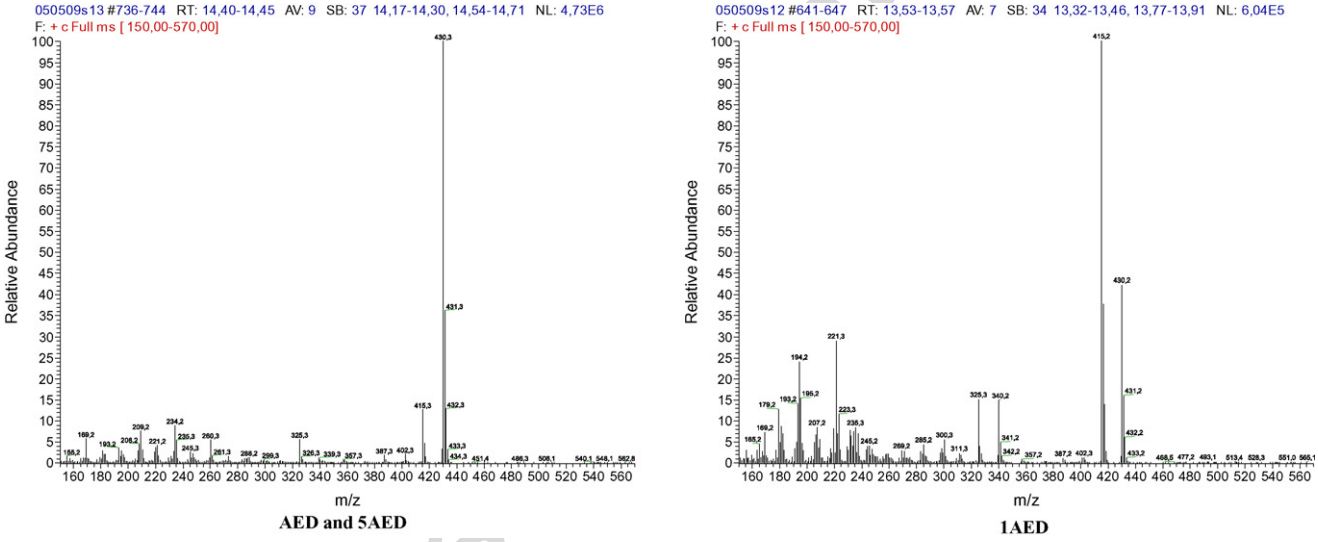

Fig. 6. GC–MS full scan of 4-androstene-3,17-dione (AED), 5-androstene-3,17-dione (5AED) and 1-androstene-3,17-dione (1AED).

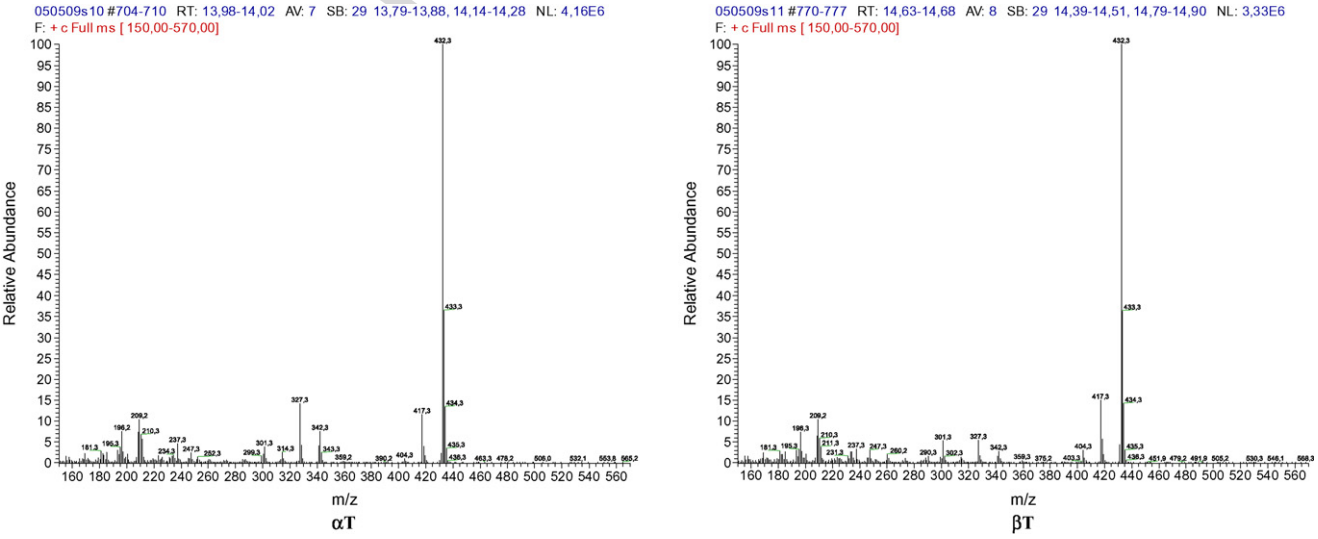

Fig. 7. GC–MS full scan of α-testosterone (αT) and β-testosterone (βT).

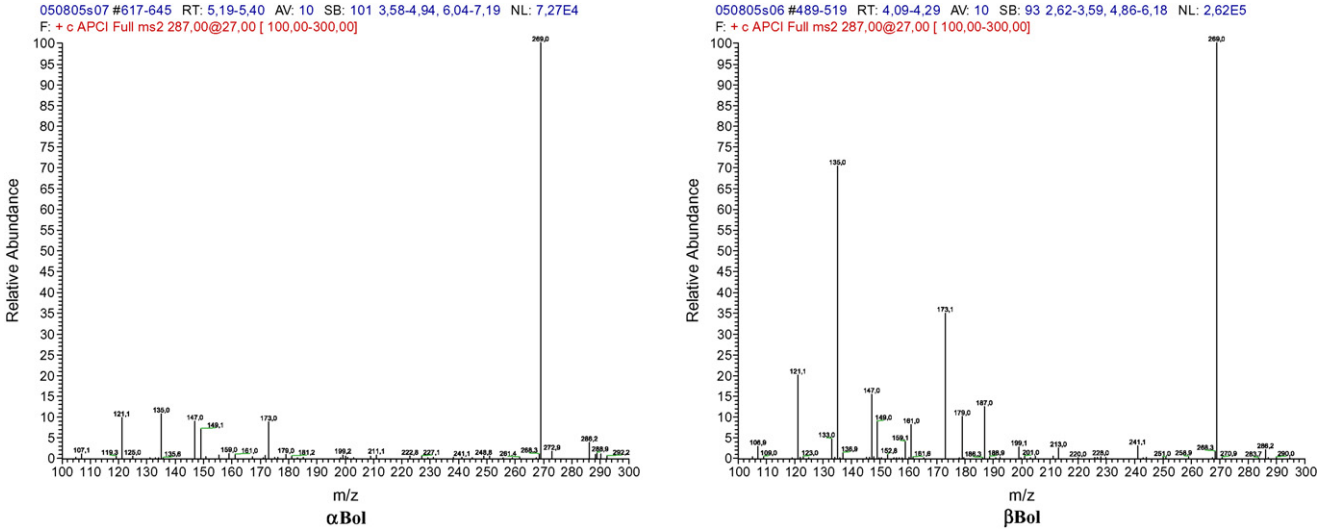

Fig. 8. LC–MS^2 scan of 17α-boldenone (αBol) and 17β-boldenone (βBol).

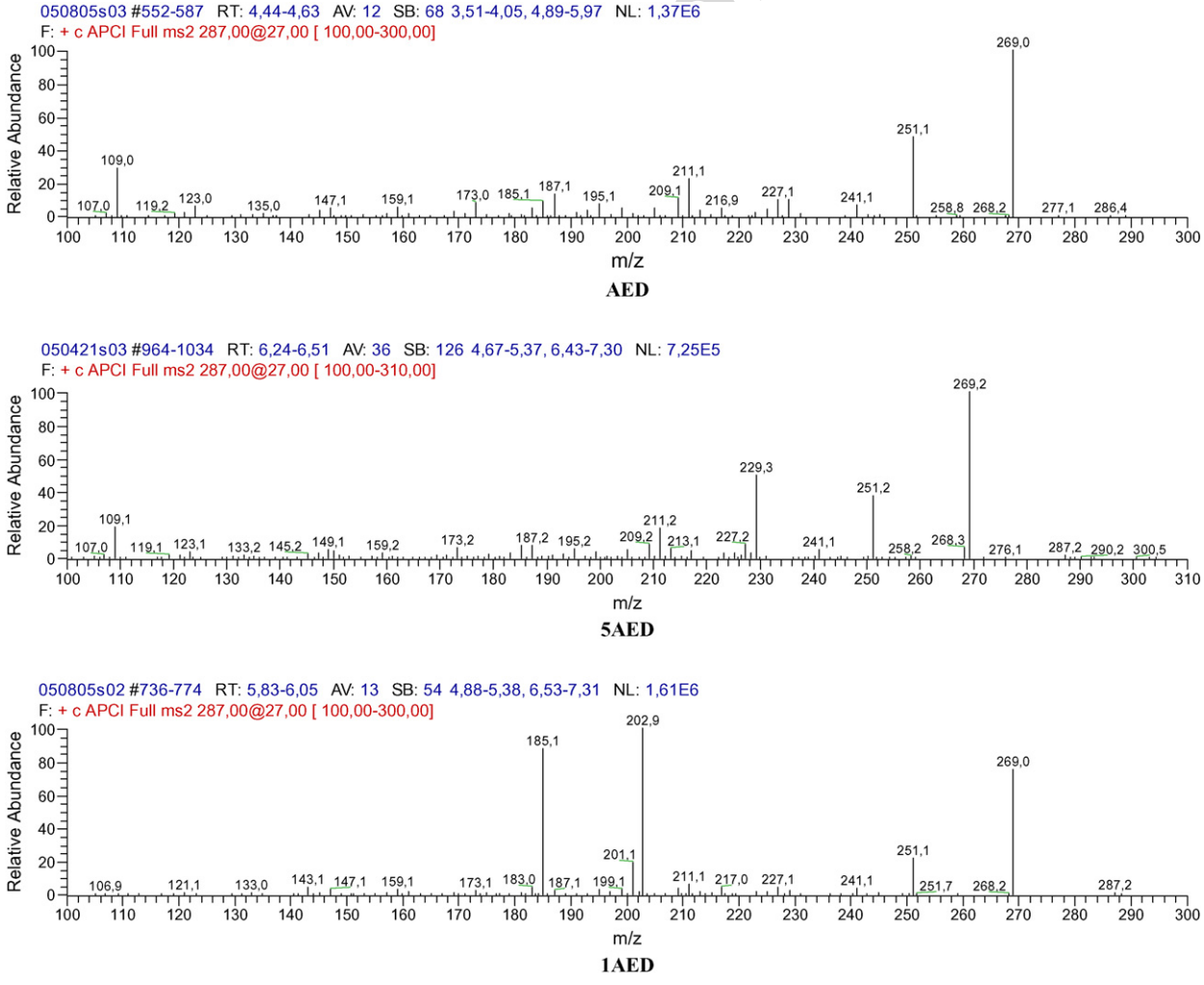

Fig. 9. LC–MS^2 scan of 4-androstene-3,17-dione (AED), 5-androstene-3,17-dione (5AED) and 1-androstene-3,17-dione (1AED).

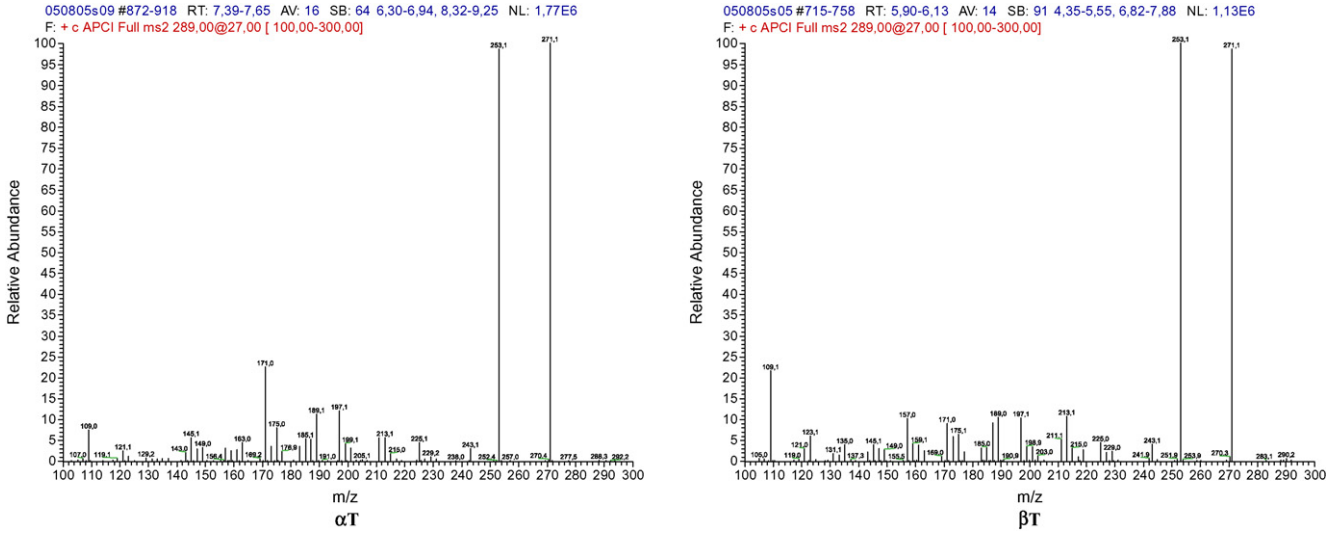

Fig. 10. LC–MS^2 scan of α-testosterone (αT) and β-testosterone (βT).

derivatives, a molecular ion of *m*/*z* 430 can be expected. The molecular mass of testosterone is 288 (Table 1), so after derivatisation, a molecular ion of *m*/*z* 432 can be expected.

The GC–MS spectrum of boldenone (Fig. 5) had a TMS molecular ion of *m*/*z* 430 (M^+). The fragment ions present in MS full scan were 415 $(M - \text{Me})^+$, 340 $(M - \text{TMSOH})^+$, 325 $(M - \text{TMSOH–Me})^+$ and the ion 206 which is due to fragmentation of the B-ring. Both αBol and βBol have the same ions in MS full scan, but their relative intensities differ.

4-Androstene-3,17-dione (AED) and 5-androstene-3,17-dione (5AED) have the same TMS enol TMS ether derivative. They had a TMS molecular ion of *m*/*z* 430 (M^+). The fragment ions present in MS full scan were 415 $(M - \text{Me})^+$ and some other ions with a relative intensity of less than 15% (Fig. 6).

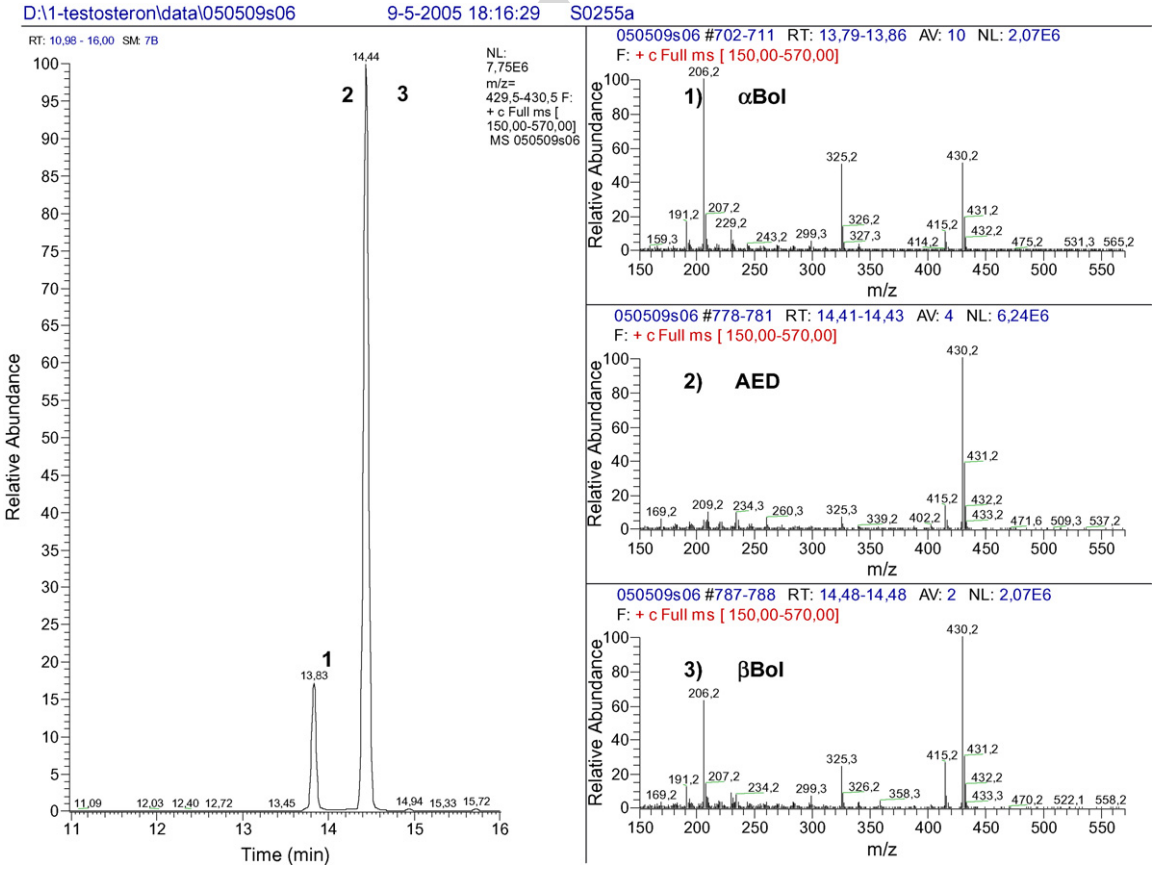

Fig. 11. (A) Combined GC chromatogram of the analytes αBol (1), AED (2) and βBol (3) present in HPLC fraction 2 and their MS spectra. (B) Combined GC chromatogram of the analytes B1T (1), 1AED (2), A1T (3), αT (4), 5AED (5) and βT (6) present in HPLC fraction 3 and their MS spectra.

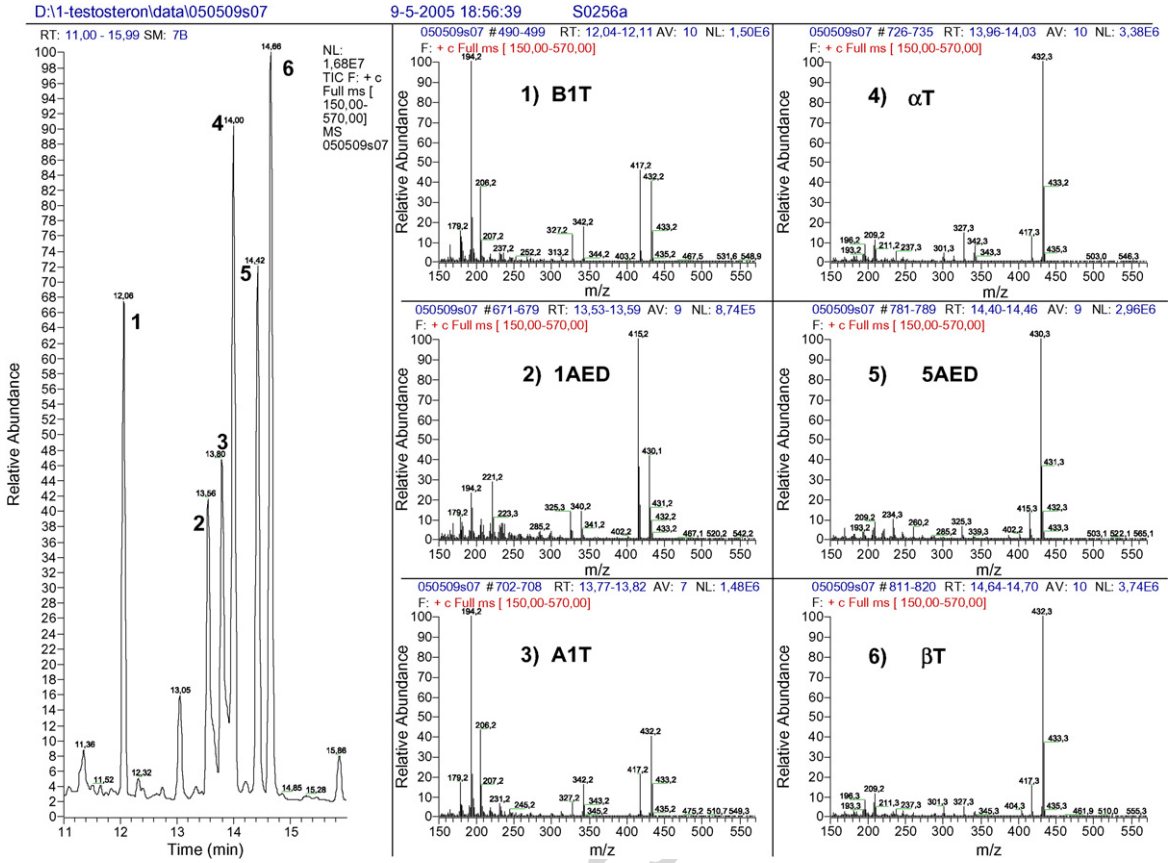

Fig. 11. (*Continued*).

1-Androstene-3,17-dione (1AED) had a TMS molecular ion of *m/z* 430 (M^+). The fragment ions present in MS full scan were 415 $(M-\text{Me})^+$, 340 $(M-\text{TMSOH})^+$, 325 $(M-\text{TMSOH}-\text{Me})^+$ and the ions 194 and 221 which are due to fragmentation of the B-ring. The ion fragment at *m/z* 169 indicated the presence of a keto-group at position 17 ($C_9H_{17}OSi$) (Fig. 6).

The GC–MS spectrum of testosterone had a TMS molecular ion of *m/z* 432 (M^+). The fragment ions present in MS full scan were 417 $(M-\text{Me})^+$, 342 $(M-\text{TMSOH})^+$, 327 $(M-\text{TMSOH}-\text{Me})^+$ and the ions 196 and 209 which are due to fragmentation of the B-ring. Both αT as βT have the same ions in MS full scan, but the relative intensities of the ions with *m/z* 327 and 342 is below 5% in the mass spectrum of βT (Fig. 7).

In Table 2 an overview is given of the GC–MS ions in MS full scan and their relative intensities, as well as their relative retention time (versus androsterone) and the HPLC fraction in which the analyte elutes.

3.2.2. LC–MS^2 analysis

Apart from GC–MS, the analytes were also investigated using LC–MS. The pseudo molecular ion of boldenone, 1AED, AED and 5AED in MS full scan in positive ion mode was *m/z* 287, for testosterone it was *m/z* 289. The analytes were further studied by MS^2 fragmentation of these pseudo molecular ions.

Fig. 8 shows the MS^2 spectrum of boldenone. The product ions are *m/z* 121, 135, 147 and 269. The relative intensities of these product ions were much lower in the MS^2 spectrum of αBol compared to the mass spectrum of βBol.

Using LC–MS as detection technique, a differentiation between the analytes AED and 5AED could be made. The MS^2 spectra of 1AED, AED and 5AED all showed the product ions with *m/z* 269 and 251. Differentiation between these analytes was possible considering product ions with a lower *m/z*. The MS^2 spectrum of 1AED showed the intense product ions with *m/z* 185 and 203. In the mass spectrum of AED the ions with *m/z* 109 and 211 were present. And the mass spectrum of 5AED showed the product ions with *m/z* 109 and 229 (Fig. 9).

Fig. 10 shows the MS^2 spectrum of testosterone. The product ions are *m/z* 253 and 271. The relative intensities of these product ions are different for αT and βT.

The precursor ions and the relative intensities of the MS^2 product ions of these analytes, as well as their elution time, are summarised in Table 3.

3.3. Chromatographic and mass spectrometric behaviour of a mixture of 1-testosterone and related anabolic steroids

3.3.1. GC–MS analysis

A mixture of A1T, B1T, αBol, βBol, 1AED, AED, 5AED, βT and αT was derivatised and injected in the GC–MS apparatus. Fig. 11A and B show a combined chromatogram of the analytes present in fraction 2 and fraction 3, respectively. The different

Author's per-

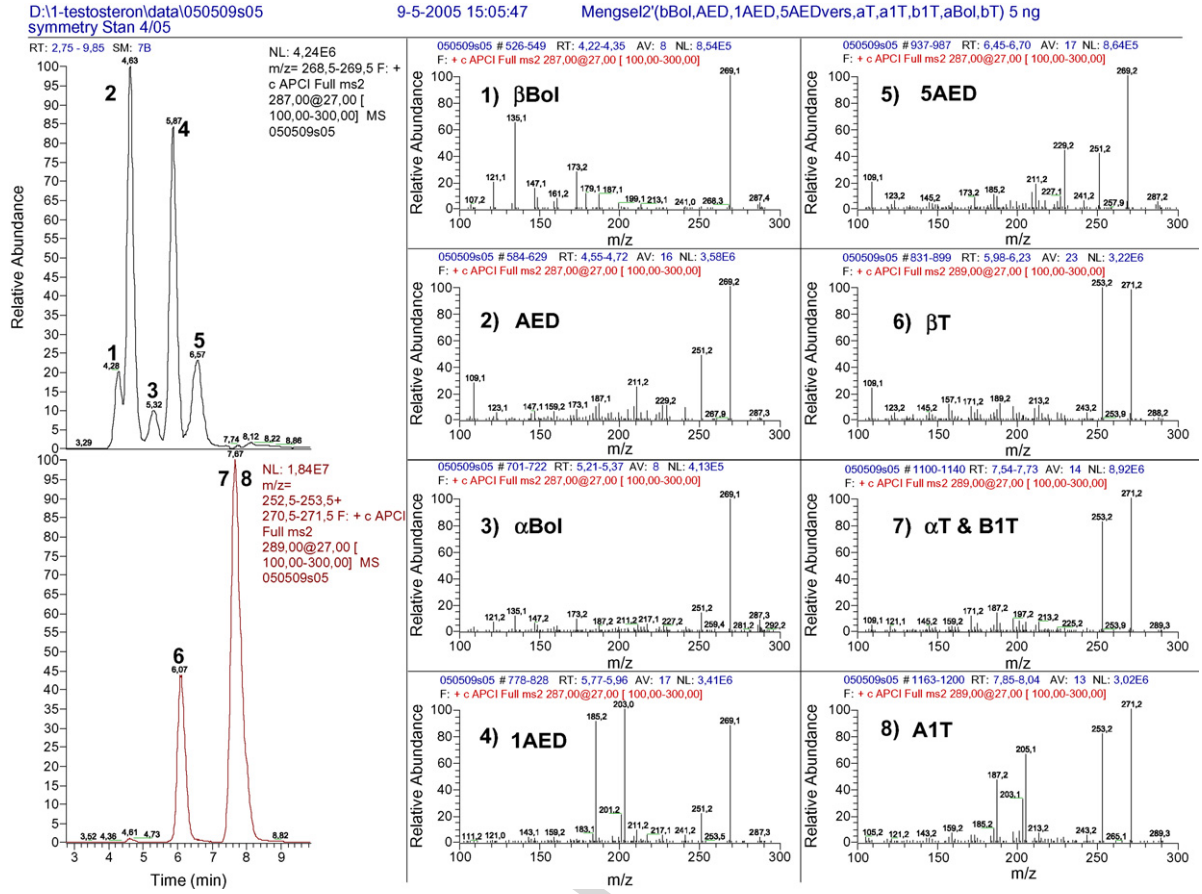

Fig. 12. Combined LC chromatogram of the analytes βBol (1), AED (2), αBol (3), 1AED (4), 5AED (5), βT (6), αT (7), A1T (8) and B1T (7) and their MS^2 spectra.

analytes could be separated chromatographically, based on their MS spectra and by HPLC fractionation.

The analytes βBol and AED were not separated chromatographically and elute in the same HPLC fraction. However, their MS spectra differ (Figs. 5 and 6), but the interpretation can be difficult when both analytes are present in a mixture. When a large amount of AED is present (endogenously), the presence of a small amount of βBol could be masked.

The analytes AED and 5AED elute at the same retention time and have the same ions in MS full scan, because they have the same TMS enol TMS ether derivative, as mentioned before. However, both analytes can be distinguished because they are present in different HPLC fractions.

Co-elution also occurs for A1T and αBol, but both analytes are present in different HPLC fractions.

3.3.2. LC–MS^2 analysis

The same mixture of A1T, B1T, αBol, βBol, 1AED, AED, 5AED, βT and αT was injected in the LC–MS apparatus. Fig. 12 shows a combined chromatogram of all analytes. Not all analytes were separated chromatographically. However, a mass spectrometric differentiation could be obtained for most of those analytes that were not baseline separated.

For the analytes with precursor ion *m/z* 287 for example, βBol and AED were not baseline separated, but their MS^2 spectra differ.

The analytes αT, A1T and B1T, with precursor ion *m/z* 289, were not separated chromatographically. The MS^2 spectrum of A1T had two distinct product ions, which are only weakly present in the other MS^2 spectra. The analytes αT and B1T, however, could not be separated, nor chromatographically, nor based on their MS^2 spectra.

The injection of the standard of 5AED resulted in two chromatographic peaks (Fig. 13). The first peak co-elutes with AED and has a MS^2 spectrum equal to AED (Fig. 9). The MS^2 spectrum of the second peak is similar to the one of AED, but the product ion *m/z* 229 is more abundant (Fig. 9). It was observed that 5AED was transformed into AED after a couple of injections. This transformation also occurred in the stock solution, but at a slower rate. Most probably the transformation is catalysed by the formic acid used in the working solution. Therefore, the time prior to injection of a sample will determine the result of an analysis for AED or 5AED and may lead to a contradiction between LC–MS^n and GC–MS^n results.

4. Discussion

In this work the new anabolic steroid 1-testosterone and a number of related compounds were studied. These analytes, with closely related structures to the natural hormone testosterone, are formed by changing the position of the double

Author's personal copy

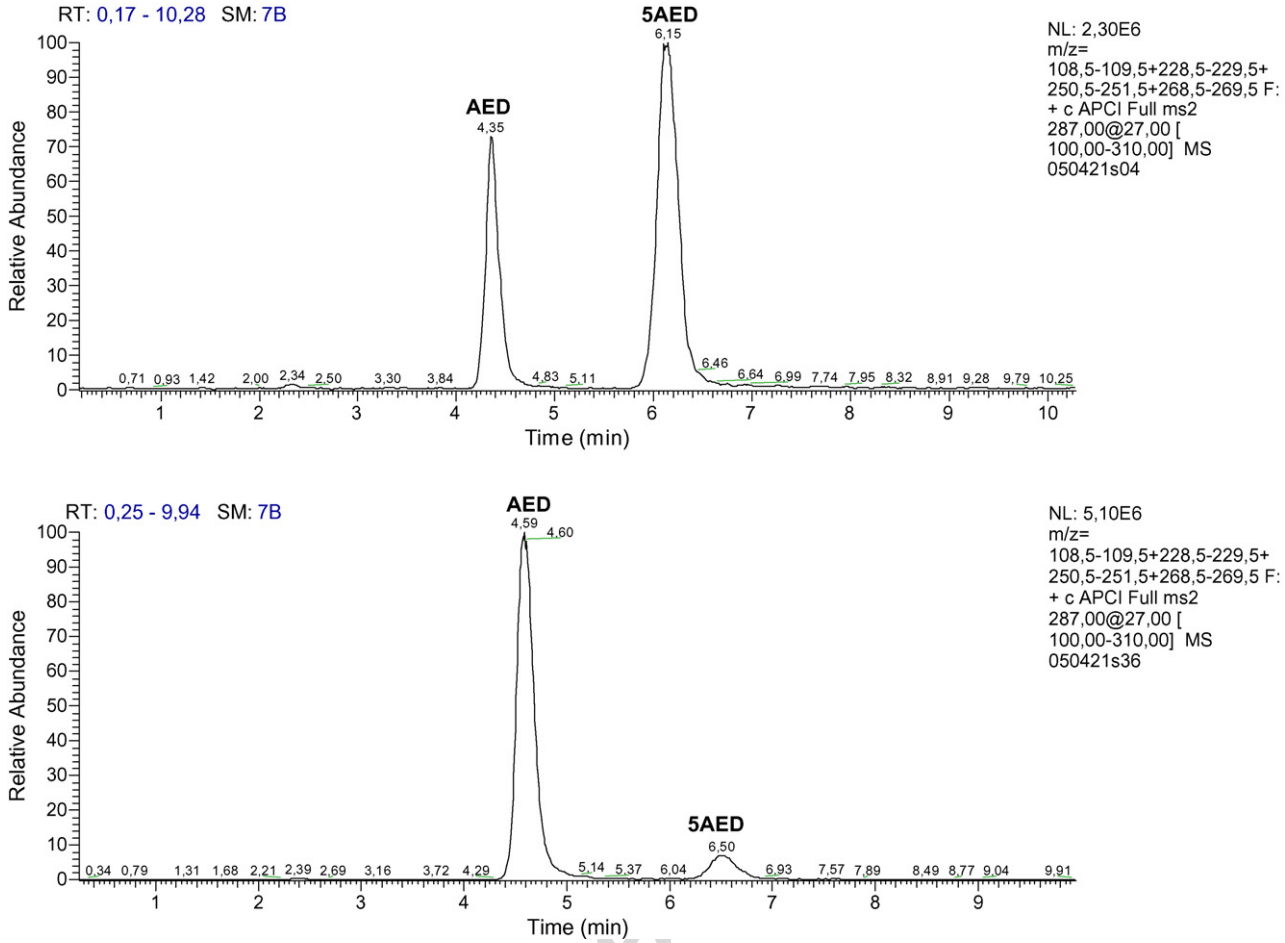

Fig. 13. Ion chromatogram (LC) of the standard 5AED; first injection (upper trace) and after 32 injections (lower trace).

bound, by epimerisation of the 5-hydrogen or the hydroxy-group, by reduction of the keto-group or by oxidation of the hydroxy-group. It concerns the analytes, 17α-boldenone (αBol), 17β-boldenone (βBol), 1,(5α)-androstene-3,17-dione (1AED), 4-androstene-3,17-dione (AED), 5-androstene-3,17-dione (5AED), 17β-testosterone (βT), 17α-testosterone (αT), 1,(5α)-testosterone (A1T), 1,(5β)-testosterone (B1T) and 1,(5α)-androsten-3,17-diol (1-AD) (Fig. 1). In Table 1 these analytes are summarised and their code and molecular mass is mentioned.

In residue analysis the identification of a substance is based on the comparison of the relative retention time and the mass spectrum of the unknown with these of an analytical standard [9]. Therefore, the availability and purity of analytical standards is very important. The standards A1T and B1T contain traces of boldenone and 17β-estradiol. Fig. 14 shows the ion chromatogram of A1T. The chromatographic peak of A1T has a shoulder with the same MS ions, but with different relative intensities. Also, the standard of B1T contains traces of this unknown compound. Knowing that these analytical standards already contain unknowns, serious questions can be asked about the purity of commercial products, sold to body-builders, for example through the internet. By-products can be harmful for the consumer (man or animal) and they can also interfere with residue analysis [10].

Prior to GC–MS^n analysis, the analytes were derivatised. Derivatisation leads to structural changes and therefore, the interpretation of a mass spectrum is not always unambiguous. In the case of indistinctness about the identity of an analyte, it is advisable to use or develop complementary methods, such as LC–MS^n. Misinterpretations in GC–MS^n due to co-elution can sometimes be anticipated by HPLC fractionation.

The different analytes, αBol, βBol, 1AED, AED, 5AED, βT, αT, A1T, B1T, were examined with both LC–MS^2 and GC–MS to be aware of possible pitfalls in the interpretation of their mass spectra. These pitfalls may be due to the presence of unknown anabolic steroids, such as 1-testosterone, or due to the selection of a non-specific precursor ion. Samples, to be analysed for the presence of anabolic steroids, will routinely be analysed using GC–MS. Therefore, the major analytical problems encountered in GC–MS, which can lead to pitfalls in the interpretation, are discussed below.

The first analytical problem is the co-elution of AED and βBol in GC–MS. Both analytes belong to the same HPLC fraction, but their mass spectra are different. However, the presence of large amounts of AED can mask the presence of small amounts of βBol in MS full scan. MS^2 fragmentation can be

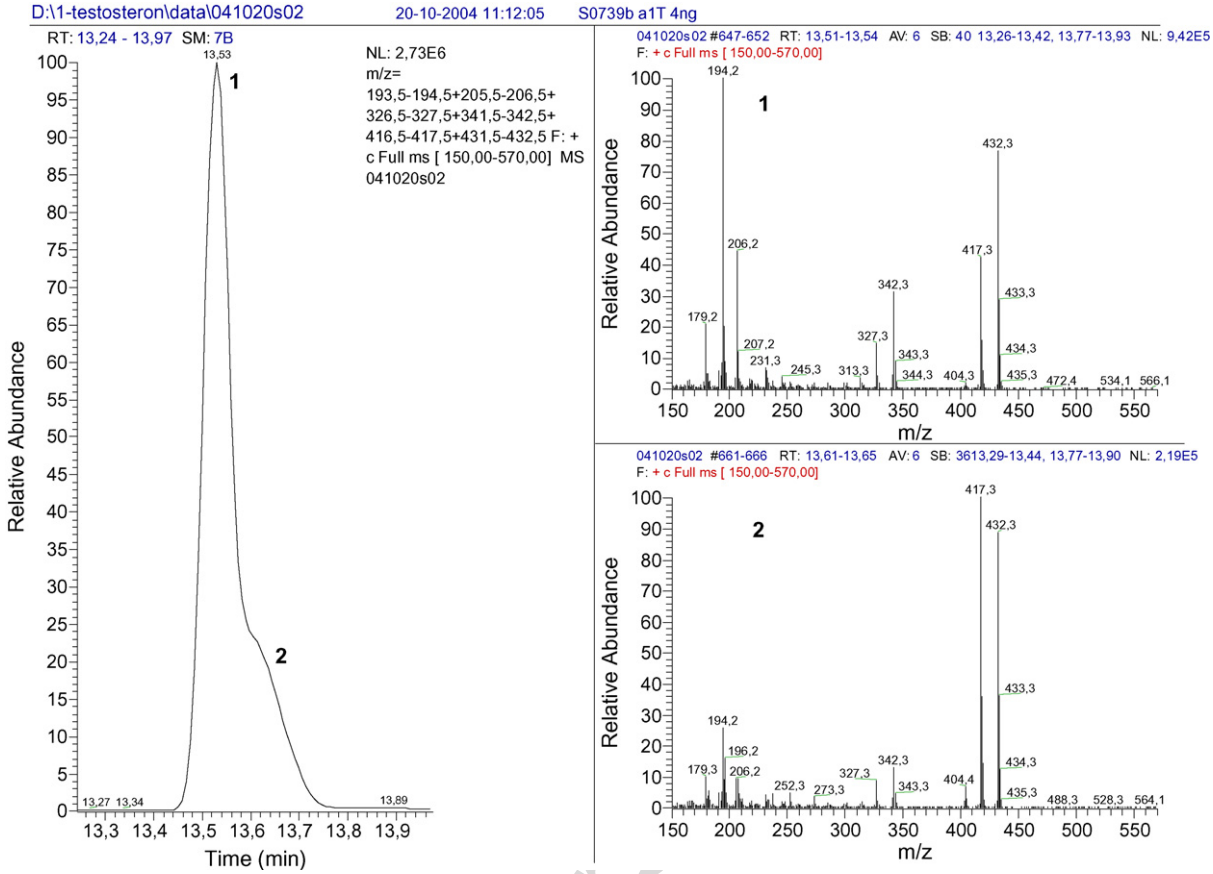

Fig. 14. Ion chromatogram and MS spectrum of the standard A1T.

a solution, but it can also lead to pitfalls in the interpretation. The most abundant ion of βBol, the ion with *m/z* 206, can be chosen as precursor ion for MS^2 fragmentation. The product ions present in MS^2 scan are *m/z* 163, 175 and 191. When large amounts of AED are present, the MS^2 spectrum of βBol is produced as well, although βBol is not present. This is due to the presence of the ion with *m/z* 209 in MS full scan. Because of large amounts of AED in a sample, the C-isotope ion cluster around *m/z* 209 will contain the ion with *m/z* 206, and this ion will lead to the MS^2 spectrum of βBol. A solution to this problem could be to use a more selective HPLC fractionation with a narrow retention time window, which can separate both analytes, or to choose a more suitable precursor ion for MS^2 fragmentation.

A second analytical problem is the possible confusion between A1T and αBol when no HPLC fractionation is performed prior to GC–MS analysis. In this case, these analytes are not separated chromatographically and therefore αBol can be mistaken for A1T, or vice versa. Fig. 15 shows the MS^2 spectra of both analytes when the ion *m/z* 206 is chosen as precursor ion. The ion with *m/z* 206 was chosen as precursor ion because it is the most abundant ion in MS full scan of αBol. Both mass spectra contain the same product ions and have the same ion ratios. Analysts, unaware of the existence of the new anabolic steroid A1T and of the co-elution of αBol with A1T, will only detect αBol. This is of high importance because αBol has already been detected many times in samples of animal origin. However, distinction between both analytes is possible, in the case when the MS full scan mass spectrum is used for interpretation (Figs. 2 and 5) or when a suitable precursor ion, preferably the molecular ion M^+, is chosen instead of the most abundant ion *m/z* 206.

Recently, Le Bizec et al. [11] demonstrated the presence of B1T (referred to as metabolite M4 in their article) in urine of cattle treated with βBol. Since most transformations in the body are equilibrium reactions it could also be possible that βBol is a metabolite of B1T or A1T. Therefore some faeces samples with substantial amounts of αBol were reanalysed with GC–MS in our laboratory. In Fig. 16 the presence of A1T with the unknown shoulder is shown in a faeces sample containing substantial amounts of αBol. The question arises whether A1T has a possible natural source, analogous to βBol, or whether the presence of A1T is the result of a treatment with βBol.

Another analytical challenge was the analysis of 1-AD. For 1-AD or 1,(5α)-androstene-3,17-diol no analytical standard is available. Only the product that circulates on the illegal prohormone market could be used. Van Eenoo et al. [12] derived the identity of 1-AD from a comparison with 4-androstene-3,17-diol and 5-androstene-3,17-diol. 1-AD was also analysed in our lab with $GC–MS^n$ and $LC–MS^n$. The GC–MS full scan mass spectrum contained an abundant ion with *m/z* 434, corresponding to

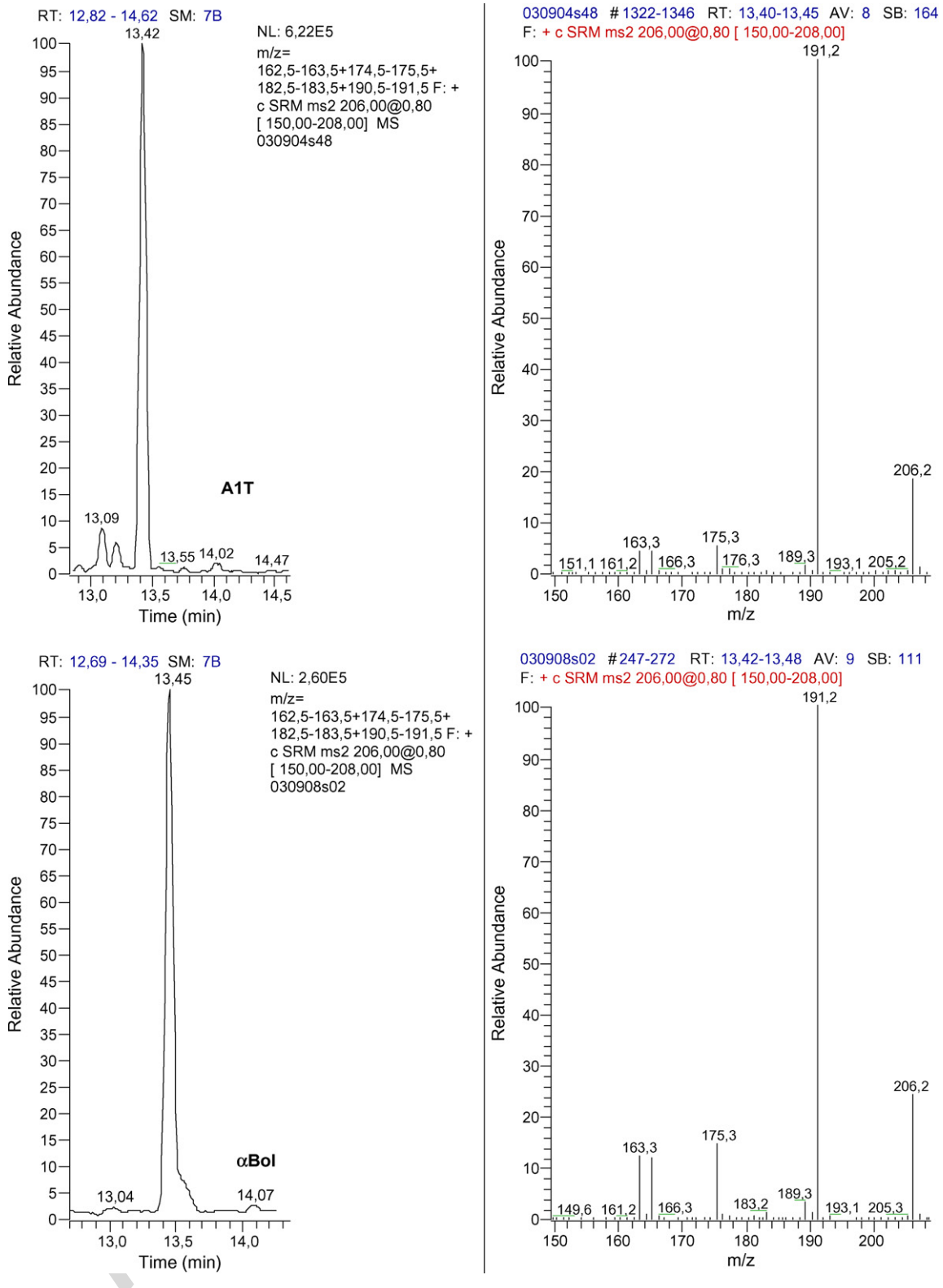

Fig. 15. Ion chromatograms and MS^2 spectra after fragmentation of the ion *m/z* 206, of A1T (upper trace) and αBol (lower trace).

1-AD. Next to this major ion peak, a number of smaller peaks were observed. One ion peak was identified as βBol ($M^+ = 430$), another is possibly the nor-equivalent of 1-AD ($M^+ = 420$). Low flow infusion of the product 1-AD into the LC–MS system resulted in identification of the ion with *m/z* 291 as 1-AD. Also an ion peak with *m/z* 289 (corresponding to αT, A1T or B1T) and an unknown ion peak with *m/z* 306 were observed. After injection onto an analytical column and acquisition in MS^2 scan mode, also βBol, traces of A1T and an unidentified peak with a similar spectrum as A1T were identified.

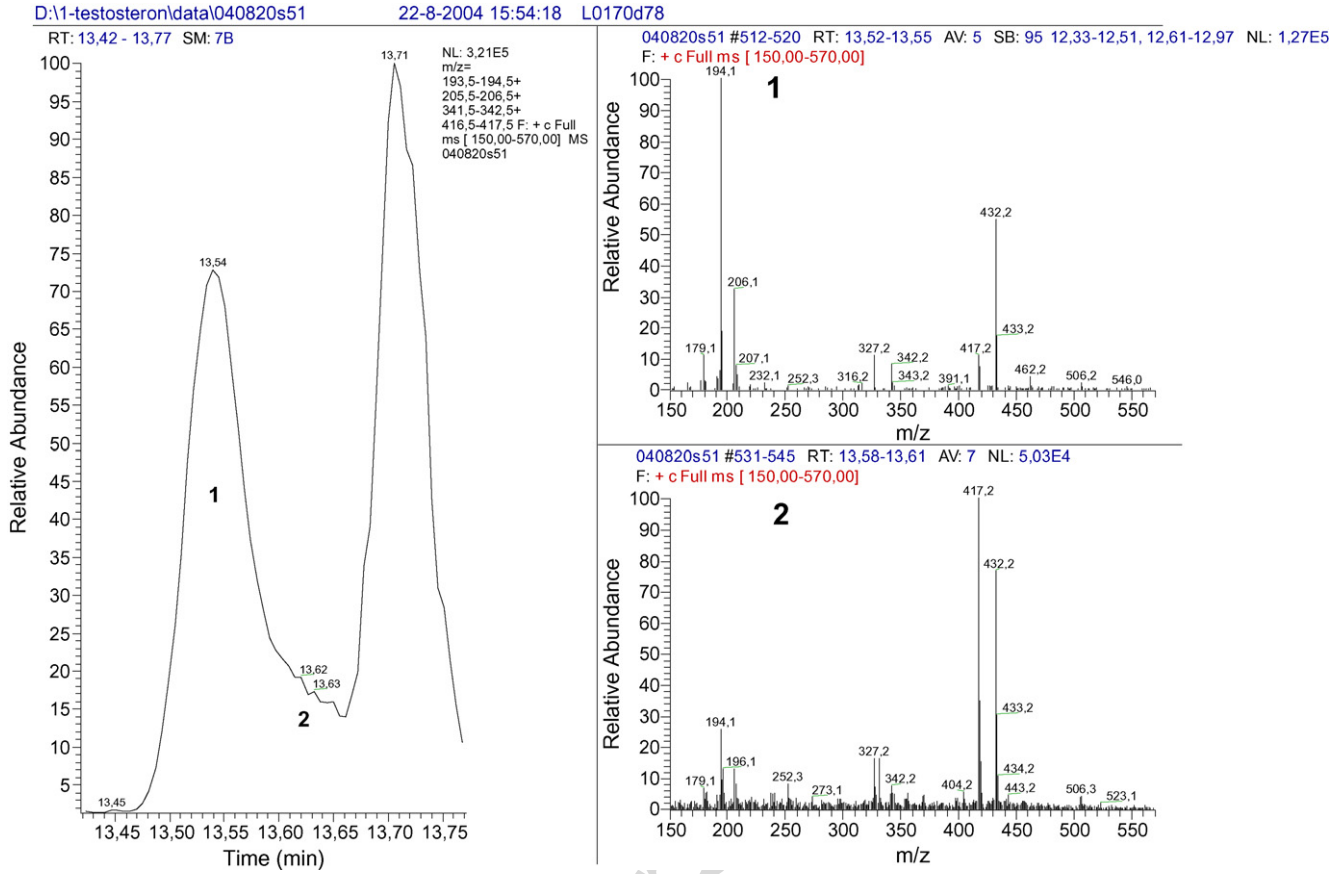

Fig. 16. Presence of a trace of A1T and unknown shoulder in a faeces sample containing αBol analysed by GC–MS.

5. Conclusion

Because of the increase in availability of 'semi-natural' hormones and pro-hormones on the black market (and the internet), laboratories analysing animal matrices have to be alert for the possible presence of residues of these substances. In particular 1-androgens, having a 1,2-double bound instead of a 4,5-double bound, deserve the necessary attention.

GC–MS^n data have to be interpreted with caution because of the similarity between the analytes and because some analytes are not separated chromatographically. Moreover, certain analytes may occur naturally in some samples. Furthermore, the selection of a non-specific precursor ion can give the same MS^2 fragmentation for different analytes. A more selective clean-up, using HPLC fractionation, can be a solution to differentiate between co-eluting analytes. Furthermore, LC–MS^n can function as a complementary tool since all analytes are separated. If not chromatographically, a mass spectrometric differentiation was obtained. However, the method of choice remains GC–MS^n because of the large number of anabolic steroids that can be screened in one acquisition and because the chromatographic resolution using GC is still better than using LC. A combination of GC–MS^n and LC–MS^n is preferred for the analysis of the different pro-hormones and hormones.

In our laboratory the investigated analytes were incorporated into the multi-residue screening method for anabolic steroids in different matrices. Consequently, future research should focus on the complete validation of this method in different matrices such as urine, faeces and kidney fat. Considering all the information collected in this study, the presence of 1-androgens can also be monitored in other residue laboratories.

Author's personal copy

Acknowledgements

The authors are grateful to Mieke Naessens and Dirk Stockx for the assistance in experimental work and the skillful operation of the LC–MS^n and GC–MS^n apparatus. The authors wish to thank the Belgian Federal Ministry of Public Health and the Federal Agency for the Safety of the Food Chain for the grant financing this research (S6150).

References

[1] Council Directive 96/23/EC of 29 April 1996, Off. J. Eur. Commun., L 125 (1996) 10.
[2] D.H. Catlin, M.H. Sekera, B.D. Ahrens, B. Starcevic, Y.C. Chang, C.K. Hatton, Rapid Commun. Mass Spectr. 18 (2004) 1245–1249.
[3] M.H. Sekera, B.D. Ahrens, Y-Ch. Chang, B. Starcevic, C. Georgakopoulos, D.H. Catlin, Rapid Commun. Mass Spectr. 19 (2005) 781–784.
[4] N. Van Hoof, K. De Wasch, S. Poelmans, D. Bruneel, S. Spruyt, H. Noppe, C. Janssen, D. Courtheyn, H. De Brabander, J. Chromatogr. 59 (2004) S85–S93.
[5] H.F. De Brabander, S. Poelmans, R. Schilt, R.W. Stephany, B. Le Bizec, R. Draisci, S.S. Sterk, L.A. van Ginkel, D. Courtheyn, N. Van Hoof, A. Macrì, K. De Wasch, Food Addit. Contam. 21 (2004) 515–525.

[6] V.P. Uralets, P.A. Gilette, Cologne: Sport und Buch Strauβ, in: Proceedings of the Manfred Donike 20th Cologne Workshop on Dope Analysis, 2002, pp. 73–83.
[7] F. Smets, H.F. De Brabander, G. Pottie, Arch. Lebensmittelhyg 48 (1997) 30–34.
[8] S. Impens, K. De Wasch, M. Cornelis, H.F. De Brabander, J. Chromatogr. A 970 (2002) 235–247.
[9] F. André, K.K.G. De Wasch, H.F. De Brabander, S.R. Impens, L.A.M. Stolker, L. van Ginkel, R.W. Stephany, R. Schilt, D. Courtheyn, Y. Bonnaire, P. Furst, P. Gowik, G. Kennedy, T. Kuhn, J.-P. Moretain, M. Sauer, Trends Anal. Chem. 20 (2001) 435–445.
[10] F. Smets, G. Pottie, H.F. De Brabander, P. Batjoens, L. Hendriks, D. Courtheyn, B. Lancival, Ph. Delahaut, Analyst 119 (1994) 2571–2576.
[11] B. Le Bizec, F. Courant, I. Gaudin, R. Schilt, R. Draisci, F. Monteau, F. André, Steroids, in press.
[12] P. Van Eenoo, F.T. Delbeke, N. Desmet, Cologne: Sport und Buch Strauβ, in: Proceedings of the Manfred Donike 14th Cologne Workshop on Dope Analysis, 1997, pp. 185–201.

Author's personal copy