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## **New steroids on the “supplement” market**

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### *Abstract*

Two products from the “dietary supplement” market, from their labelling already supposed to contain steroidal components, were analysed for their steroid content.

In case of Orastan-A from Gaspari Nutrition the GC-MS analyses of the liberated steroids (after cleavage of the tetrahydropyranyl ether) revealed mass spectra of two components, both inconsistent with the labelling. Thus, the steroids were characterised by different analytical techniques such as mass spectrometry and X-Ray crystal structure analysis. They were identified as 17 $\beta$ -hydroxyandrostano[3,2-c]isoxazole and -[2,3-d]isoxazole. In the product Trenbolox from the company DSP estradienedione was identified by GC-MS comparison with reference material. Following oral administration urinary excretion was studied and metabolites suitable for screening were characterised.

### *Introduction*

Since several years anabolic androgenic steroids are offered on the “dietary supplement” market. Following the classification of so-called prohormones as schedule III controlled substances by the US anabolic steroids control act, more and more products appeared on the market containing steroids that were never approved as therapeutic drugs and mostly without proper labelling of the contents<sup>1-8</sup>. Recently, preparations containing stanozolol analogues, e.g. prostanazol (17 $\beta$ -hydroxy-5 $\alpha$ -androstano[3,2-c]pyrazole), were also advertised on the Internet market for sport supplements<sup>8,9</sup>. In the present study we report the detection of two more unapproved steroids in products purchased recently. After extraction from the matrix the steroids were analysed underivatized and as per-TMS derivatives by gas chromatography-mass spectrometry (GC-MS).

## *Materials and Methods*

### *Supplements, chemicals and reagents*

The product Orastan-A from the company “Gaspari Nutrition” was obtained by Internet order from the sport supplement market, classified as “dietary supplement” according to its labelling. It was labelled to contain “5 $\alpha$ -Androstano[2,3-*c*]furazan-17 $\beta$ -tetrahydropyranol ether”, also called furazadrol-tetrahydropyranyl (THP) ether. The product Trenbolox, labelled to contain estra-4,9-diene-3,17-dione, was ordered by telephone and delivered by mail from a German consignor with cash on delivery. It was labelled to be manufactured for DSP, Wilmington, USA, from the company PharmLabs within their product line “Design Supps”.

Reference material of estra-4,9-diene-3,17-dione and danazol (17 $\alpha$ -ethinyl-17 $\beta$ -hydroxyandrost-4-eno[2,3-*d*]isoxazole) were obtained from Thinker Chemical (Hangzhou, China), and androisoxazol (17 $\beta$ -hydroxy-17 $\alpha$ -methyl-5 $\alpha$ -androstano[3,2-*c*]isoxazole) from Prof. Manfred Donike (Cologne, Germany). Norandrosterone (3 $\alpha$ -hydroxy-5 $\alpha$ -estran-17-one) was purchased from LCG Promochem (Wesel, Germany), 5 $\alpha$ -dihydrotestosterone (17 $\beta$ -hydroxy-5 $\alpha$ -androst-3-one), methyltestosterone (17 $\beta$ -hydroxy-17 $\alpha$ -methylandrost-4-en-3-one), K-selectride (potassium tri-*n*-butylborohydride, 1M in tetrahydrofuran), and hydroxylamine hydrochloride were purchased from Sigma-Aldrich GmbH (Steinheim, Germany).  $\beta$ -Glucuronidase from *E. coli* was obtained from Roche Diagnostics (Mannheim, Germany), N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) from Chem. Fabrik Karl Bucher (Waldstetten, Germany). All other reagents and solvents were of analytical grade and obtained from Merck (Darmstadt, Germany).

### *Supplement analysis*

The homogenised content of one capsule was suspended in 5 mL of methanol. After shaking for 5 min and centrifugation for 5 min at 800 g, the methanolic layer was separated. Aliquots were analysed by GC-MS (underivatized and as TMS derivatives).

For characterisation of the steroid moiety of Orastan-A the extracted compounds were hydrolysed for 1 hour in refluxing aqueous acetic acid (30 %). Aliquots were analysed by GC-MS. The co-crystallizing (from ethyl acetate) steroids were separated by semi-preparative HPLC. After crystallisation from ethyl acetate, the isomers were further characterised by high resolution/high accuracy mass spectrometry and X-Ray crystallography for the identification of the molecular structure.

### *Synthesis of reference material*

#### *17 $\beta$ -Hydroxyandrostanisoxazoles*

For the synthesis of 17 $\beta$ -hydroxyandrostan[3,2-*c*]isoxazole (**1**), 2-hydroxymethylene-5 $\alpha$ -dihydrotestosterone (**4**) was prepared by reaction of 5 $\alpha$ -dihydrotestosterone (**3**) with ethyl formate and sodium methylate in absolute pyridine as described by Schänzer<sup>10</sup>. The [3,2-*c*]isoxazole ring (isomer **1**) was formed by condensation of 33 mg of (**4**) with 27 mg of hydroxylamine hydrochloride using 3.5 mL of pyridine as solvent. Using ethanol as solvent resulted in the formation of the [2,3-*d*]isoxazole (**2**). After refluxing for 3 hours the mixtures were evaporated to dryness, re-dissolved in H<sub>2</sub>O and extracted with *t*-butyl methyl ether (TBME). Following evaporation the 17 $\beta$ -hydroxyandrostanisoxazoles<sup>11-13</sup> were obtained.

#### *Reduction of estra-4,9-diene-3,17-dione with K-Selectride*

Estra-4,9-diene-3,17-dione (**5**, 30 mg) was dissolved in 3 mL of TBME and 140  $\mu$ L of K-selectride solution were added. After 1 h of stirring 3 mL of NH<sub>4</sub>Cl (1 M) solution were added and the mixture was extracted with 20 mL of TBME. The organic layer was evaporated to dryness.

#### *Administration study*

Administration studies using two capsules of the above-mentioned supplements were performed in one healthy male volunteer, each. Urine samples were collected for 24 hours.

#### *Sample pre-treatment*

The samples were prepared according to the routine steroid screening procedure<sup>14</sup>. In brief, after addition of the internal standard methyltestosterone 2 mL of urine were incubated at pH 7 with  $\beta$ -glucuronidase from *E.coli* at 50°C for 1 h. The steroids were extracted with 5 mL of TBME at pH 9.6, the organic layer was evaporated to dryness. For GC-MS analyses the residues were either dissolved in cyclohexane or derivatised with TMIS reagent (MSTFA/ NH<sub>4</sub>I/ ethanethiol, 1000:2:3, v:w:v) by heating for 20 min at 60°C and injected into the GC-MS. Relative retention times (RRT) of the TMS-derivatives were calculated using the internal standard methyltestosterone.

### *Instrumentation*

#### *GC-MS analyses*

The TMS derivatives were analysed on an Agilent 6890 GC coupled to an Agilent 5973 mass selective detector (MSD) using the following parameters: injection volume: 3  $\mu$ L, split 1:16, injection temperature: 300°C, column: Agilent Ultra-1 column (17 m; 0.20 mm inner diameter (i.d.); 0.11  $\mu$ m film thickness), carrier gas: helium, head pressure 1 bar, oven temperature program: 0 min 183°C, +3°C/min, 0 min 232°C, +40°C/min, 2 min 310°C, ionisation: 70 eV, electron ionisation (EI), data acquisition: full scan mode, 40-800 Da.

For the measurement of the underivatized compounds the analyses were carried out on an Agilent 6890 GC coupled to a MSD Agilent 5973, injection volume: 2  $\mu$ L, splitless, injection temperature: 220°C, column: Macherey-Nagel Optima  $\delta$ 3 (20 m, 0.25 mm i.d., 0.25  $\mu$ m film thickness), carrier gas: helium, 1.2 mL/min, oven temperature program: 1.5 min 60°C, +40°C/min, 0 min 240°C, +2°C/min, 0 min 260°C, +40°C/min, 1.5 min 300°C, ionisation: 70 eV, EI, data acquisition: full scan mode, 40-400 Da.

#### *HPLC fractionation*

The HPLC fractionation of the liberated steroids from Orastan-A was performed on an Agilent 1090 equipped with an ODS Hypersil column (Thermo Electron, 10x250 mm, particle size 5  $\mu$ m) using the following parameters: injection volume: 50  $\mu$ L, mobile phase A: H<sub>2</sub>O, B: acetonitrile, gradient: 0-25 min 50 % B to 100 % B, 15-32 min 100 % B, 2 min reequilibration, flow: 3 mL/min, detector wave length: 228 nm. The fractions were collected from 12.9-13.8 min (isomer **1**) and 14.1-15.0 min (isomer **2**). After evaporation of the solvent under reduced pressure, the residues were crystallised from ethyl acetate.

#### *X-Ray crystallography*

Suitable single crystals of isomer **2** (17 $\beta$ -hydroxyandrostand[2,3-*d*]isoxazole, **2**) were obtained from ethyl acetate by evaporation. X-ray data were collected with a Nonius KappaCCD diffractometer equipped with a low temperature device at 100 K by using graphite-monochromated MoK $\alpha$  radiation ( $\lambda = 0.71073$  Å).

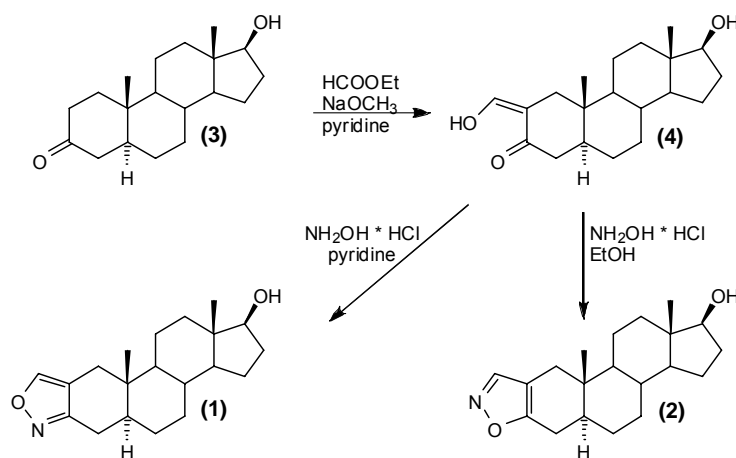
#### *High resolution high accuracy mass spectrometry*

For the verification of the elemental composition high resolution high accuracy mass spectrometry was performed on a Thermo LTQ Orbitrap mass spectrometer.

## Results and Discussion

### Orastan-A

The GC-MS analyses of the underivatised methanolic extract of Orastan-A revealed three pairs of steroidal compounds, which were identified as two isomeric steroid alcohols, their corresponding acetates and THP ethers. As the THP ethers represented the main ingredients, the steroids were released by acidic hydrolysis prior to further characterisation. The mass spectra of the liberated steroids (underivatised and as TMS derivatives) are shown in Fig. 1. In contrast to the labelling of Orastan-A the mass spectra of both compounds did not match furazadrol (17 $\beta$ -hydroxy-5 $\alpha$ -androstano[3,2-*c*]furazan, 20-norfurazabol). High resolution high accuracy mass spectrometry ( $[M+H]^+_{\text{exp.}} = 316.2273$ ,  $[M+H]^+_{\text{theor.}} = 316.2271$ , error 0.56 ppm) deduced the elemental composition C<sub>20</sub>H<sub>29</sub>NO<sub>2</sub> for both isomers. While isomer **1** yielded a mono-TMS derivative, a bis-TMS derivative was obtained from isomer **2** following derivatisation with TMIS reagent. Analogous findings were obtained for androisoxazol ([3,2-*c*-) and danazol ([2,3-*d*]-isoxazole) derivatised with TMIS (mass spectra in Fig. 2). From these findings structures of 17 $\beta$ -hydroxyandrostano[3,2-*c*]isoxazole and -[2,3-*d*]isoxazole were assumed and X-Ray crystal structure analysis of isomer **2** finally confirmed the structure of the assignment 17 $\beta$ -hydroxyandrostano[2,3-*d*]isoxazole for isomer **2**. Both isomers were synthesised as displayed in Scheme 1. The reaction of 2-hydroxymethylene-5 $\alpha$ -dihydrotestosterone (**4**) with hydroxylamine in refluxing pyridine yielded 17 $\beta$ -hydroxyandrostano[3,2-*c*]isoxazole (**1**) as almost single isomer while the reaction carried out in ethanol resulted in a mixture of both isomers (ratio **(1)**:**(2)** ~ 1:10). The synthesised material revealed the same analytical properties as the steroids liberated from the supplement, thus further confirming the identity of the ingredients.



Scheme 1: Synthesis of 17 $\beta$ -hydroxyandrostano[3,2-*c*]- (**1**) and -[2,3-*d*]isoxazole (**2**)

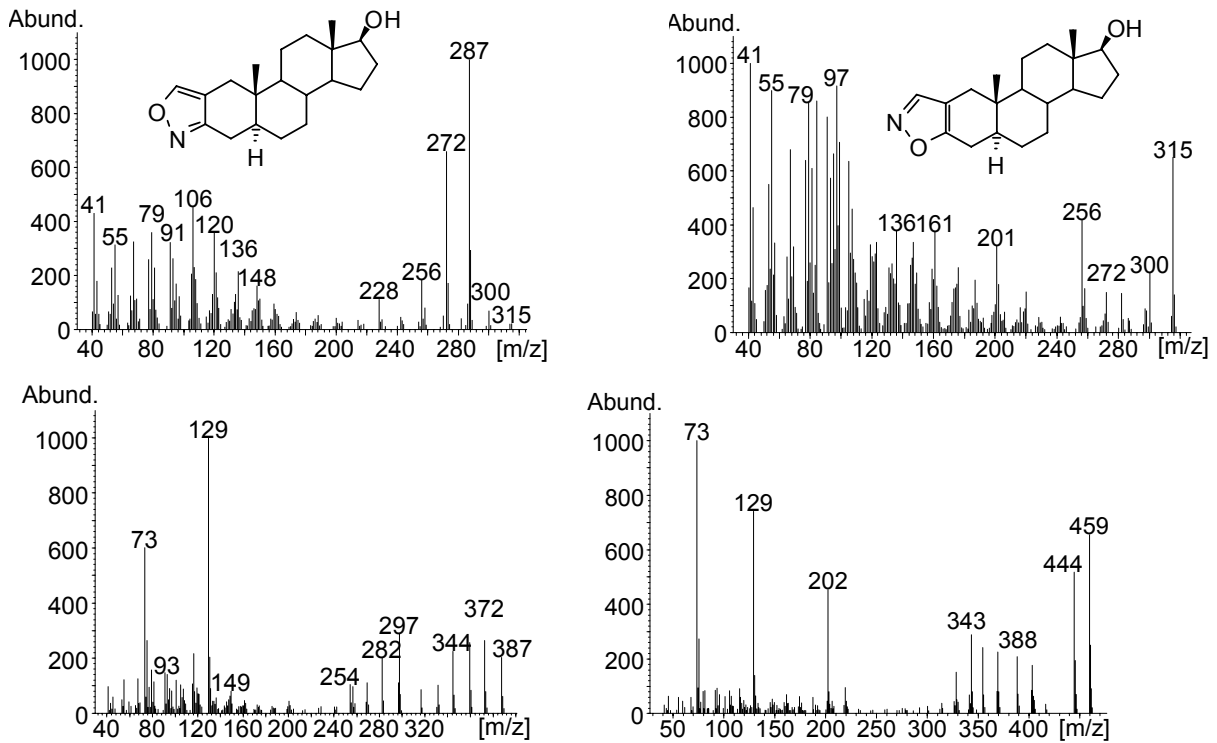


Fig. 1: Mass spectra (EI) of isomer 1 (upper left: underivatized,  $M^+ = 315$ , lower left: TMS derivative,  $M^+ = 387$ ) and isomer 2 (upper right: underivatized,  $M^+ = 315$ , lower right: TMS derivative,  $M^+ = 459$ )

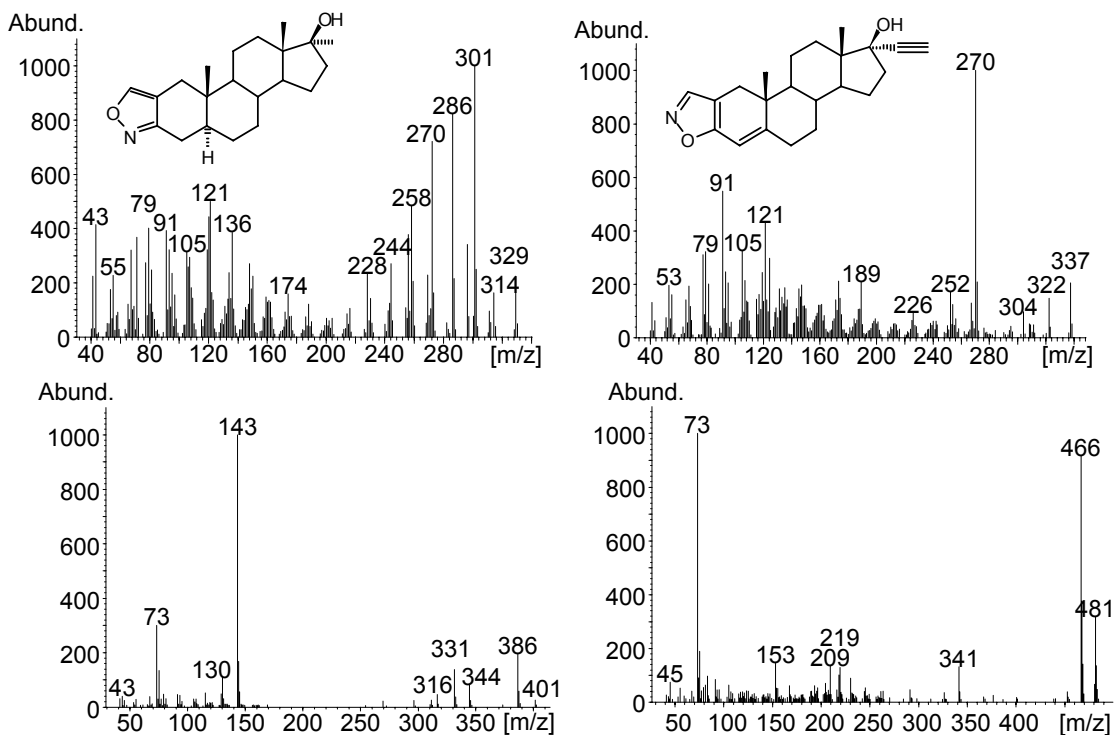


Fig. 2: Mass spectra (EI) of androisoxazol (upper left: underivatized,  $M^+ = 329$ , lower left: TMS derivative,  $M^+ = 401$ ) and danazol (upper right: underivatized,  $M^+ = 337$ , lower right: TMS derivative,  $M^+ = 481$ )

After oral administration of two capsules of Orastan-A only a metabolite in low abundance with  $M^+ = 545$ ,  $RT = 18.81$  min,  $RRT = 1.254$ , presumably the 16-hydroxylated [2,3-*d*]isoxazole, tris-TMS, (mass spectrum in Fig. 3), appearing in the 3'-hydroxystanozolol window in our routine GC-MS screening for anabolic steroids, was detected. To check for a possible loss of the isoxazole ring during metabolism the influence of the administration on the steroid profile and  $\delta^{13}\text{C}$ -values of androsterone (A) and etiocholanolone (E) was determined. No shift in the ratios of A/E and 5 $\alpha$ /5 $\beta$ -androstane-3 $\alpha$ ,17 $\beta$ -diol (Adiol/Bdiol) and the  $\delta^{13}\text{C}$ -values of A and E within the time of urine collection was detected (Fig. 4). Thus, further studies on metabolite identification are required in the future.

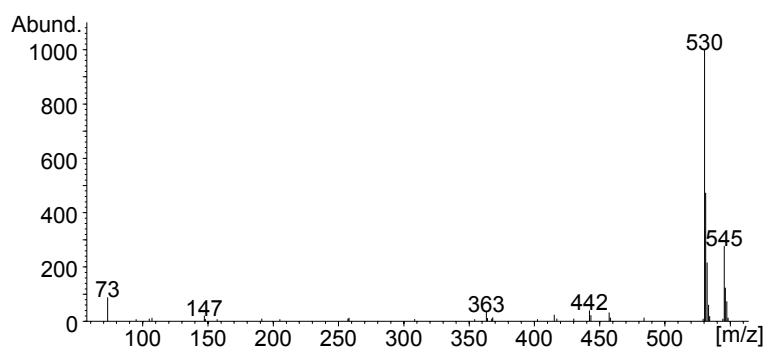


Fig. 3: Mass spectrum (EI) of Orastan-A metabolite, tris-TMS derivative,  $M^+ = 545$

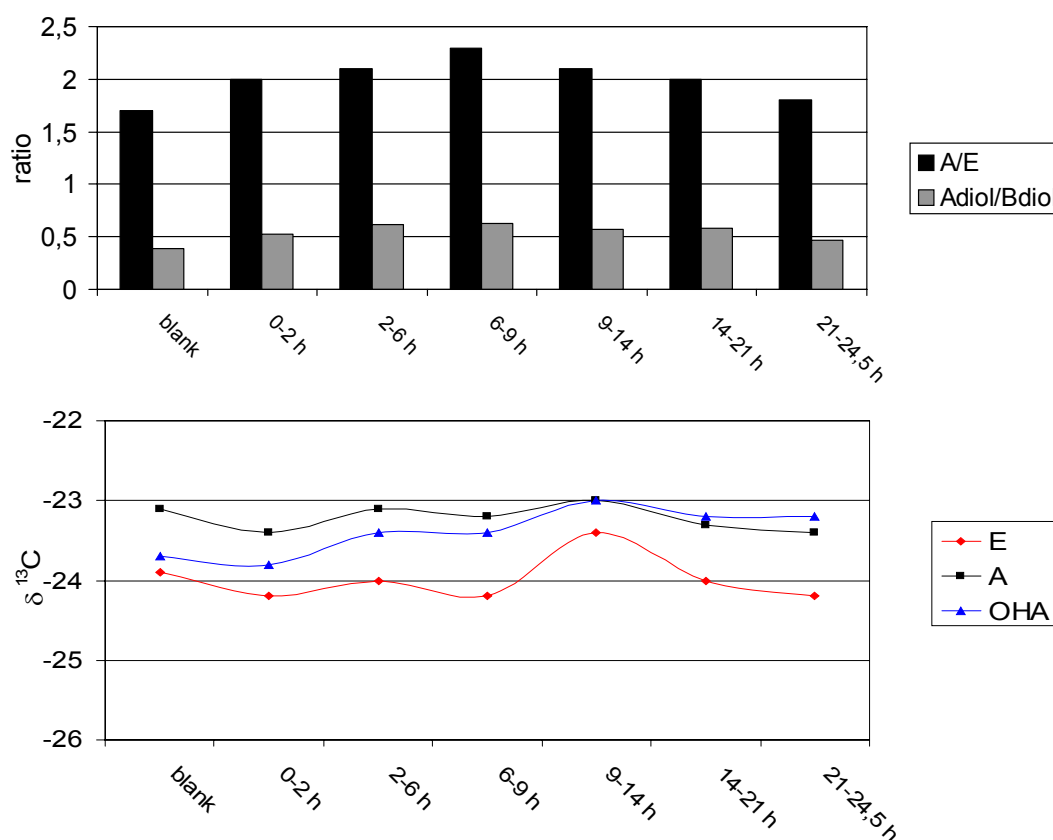


Fig. 4: Time course of urinary steroid profile ratios (upper) and  $\delta^{13}\text{C}$ -values of androsterone (A) and etiocholanolone (E) and the endogenous reference 11-hydroxyandrosterone (OHA),  $\delta^{13}\text{C}$  (Orastan-A) = -32.2

*Trenbolox*

In the product Trenbolox estra-4,9-diene-3,17-dione (**5**) was identified by GC-MS comparison with the reference material. SIM and SCAN analyses revealed no additional steroids present in the product. GC-MS of underivatised estra-4,9-diene-3,17-dione resulted in one single peak. Following derivatisation with TMS reagent two major peaks were obtained, one corresponding to the enol-TMS derivative ( $M^+_{\text{bis-TMS}} = 414$ ,  $RT_{\text{bis-TMS}} = 12.53$  min,  $RRT = 0.833$ ) and the other representing the bis-TMS derivative of an artefact ( $M^+_{\text{bis-TMS}} = 412$ ,  $RT_{\text{bis-TMS}} = 13.14$  min,  $RRT = 0.874$ ). The corresponding mass spectra are shown in Fig. 5.

Reduction of estra-4,9-diene-3,17-dione (**5**) with K-selectride (Scheme 2) yielded three products. Based on their mass spectrometric data they were assigned to  $17\beta$ -hydroxyestra-4,9-diene-3-one (**6**:  $M^+_{\text{bis-TMS}} = 416$ ,  $RT_{\text{bis-TMS}} = 13.10$  min,  $RRT = 0.871$ ) and two  $3\zeta$ -hydroxyestra-4,9-diene-17-ones (**7**:  $M^+_{\text{bis-TMS}} = 416$ ,  $RT_{\text{bis-TMS}} = 11.49$  min,  $RRT = 0.763$ , and **8**:  $M^+_{\text{bis-TMS}} = 416$ ,  $RT_{\text{bis-TMS}} = 11.73$  min,  $RRT = 0.780$ ) (Fig. 6). According to literature<sup>15,16</sup>  $3\beta$ -hydroxy isomers show longer retention times than the  $3\alpha$ -hydroxy isomers.

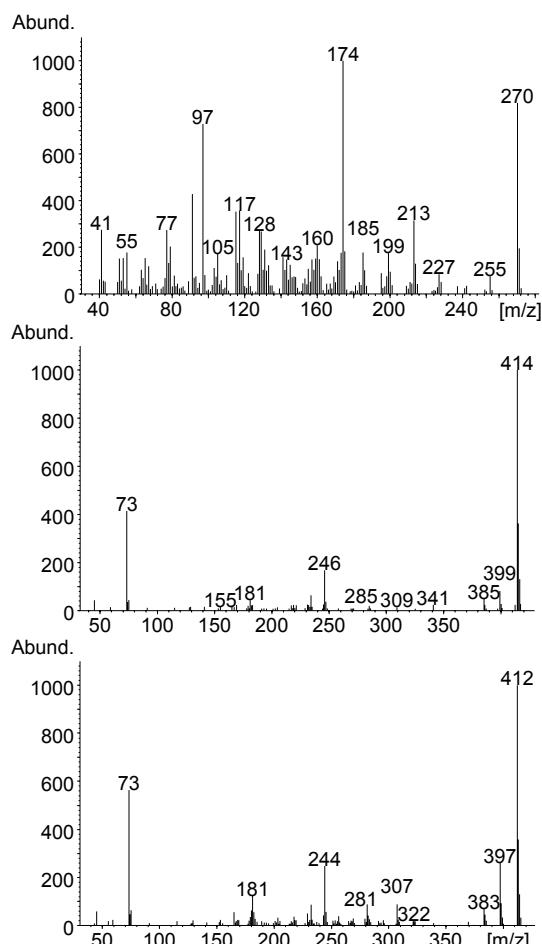
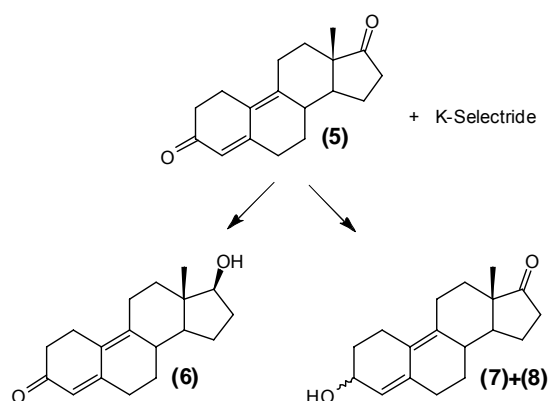


Fig. 5: Mass spectra (EI) of estra-4,9-diene-3,17-dione (**5**) (upper: underivatised,  $M^+ = 270$ , middle: bis-TMS,  $M^+ = 414$ , lower: artefact bis-TMS,  $M^+ = 412$ )



Scheme 2: Reduction of estra-4,9-diene-3,17-dione (**5**) with K-selectride



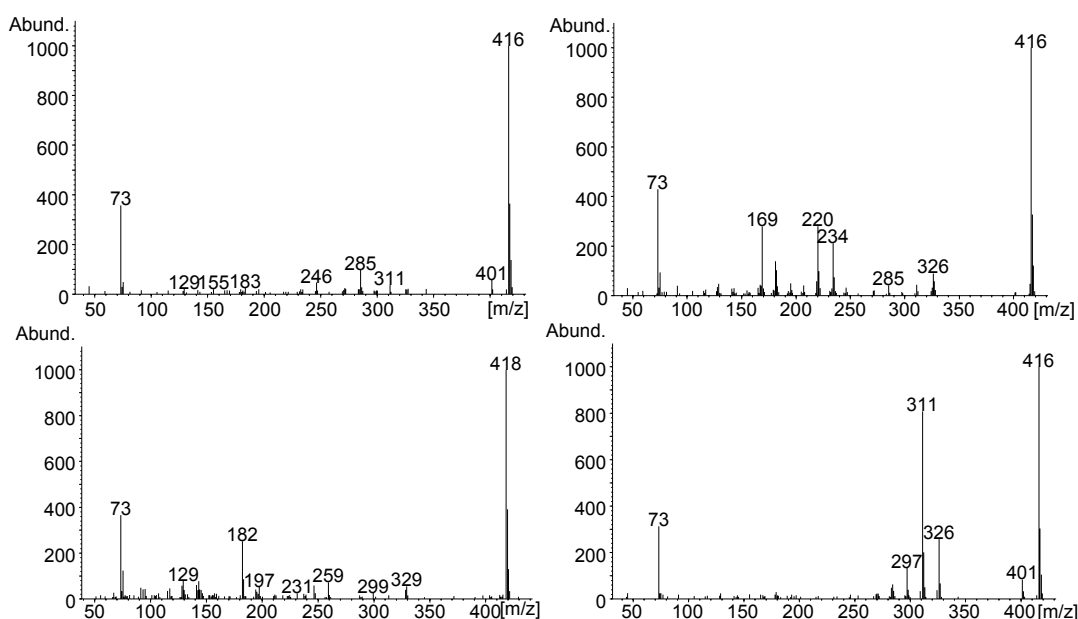


Fig. 6: Mass spectra (EI) of bis-TMS derivatives of urinary metabolites of estra-4,9-diene-3,17-dione (upper left:  $17\beta$ -hydroxyestra-4,9-diene-3-one (**6**),  $M^+ = 416$ , upper right:  $3\alpha$ -hydroxyestra-4,9-diene-17-one (**7**),  $M^+ = 416$ , lower left: estra-4,9-diene-3 $\alpha$ ,17 $\beta$ -ol (**9**),  $M^+ = 418$ , lower right: unidentified metabolite (**11**),  $M^+ = 416$ )

Following the administration of Trenbolox only a small amount of unmetabolised estra-4,9-diene-3,17-dione (**5**), was detected in the post administration urines. Additionally norandrosterone ( $M^+_{\text{bis-TMS}} = 420$ ,  $RT_{\text{bis-TMS}} = 8.99$  min,  $RRT = 0.598$ ) was found to be excreted as metabolite. Its concentration exceeded the WADA threshold limit of 2 ng/mL of urine in the 0:00- 3:40-hours ( $\sim 400$  ng/mL) and the 3:40-10:45-hours urine ( $\sim 20$  ng/mL).  $17\beta$ -Hydroxyestra-4,9-diene-3-one (**6**, main metabolite),  $3\alpha$ -hydroxyestra-4,9-diene-17-one (**7**) and  $3\beta$ -hydroxyestra-4,9-diene-17-one (**8**) were identified by GC-MS comparison with the synthesised references. Furthermore two isomeric steroids (proposed structures estra-4,9-diene-3 $\xi$ ,17 $\beta$ -diol (**9**,  $M^+_{\text{bis-TMS}} = 418$ ,  $RT_{\text{bis-TMS}} = 11.74$  min,  $RRT = 0.781$  and **10**,  $M^+_{\text{bis-TMS}} = 418$ ,  $RT_{\text{bis-TMS}} = 12.03$  min,  $RRT = 0.800$ ) and another metabolite (**11**,  $M^+_{\text{bis-TMS}} = 416$ ,  $RT_{\text{bis-TMS}} = 12.42$  min,  $RRT = 0.826$ ) were detected. The mass spectra of their bis-TMS derivatives are shown in Fig. 6. The main metabolite of estra-4,9-diene-3,17-dione,  $17\beta$ -hydroxyestra-4,9-diene-3-one (**6**) was detectable in all urine samples collected.

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