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# Analytical Challenges in Doping Control: GCxGC: is it an Option?

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## Introduction

Comprehensive bidimensional gas chromatography (GCxGC) is a relatively new technique that has shown remarkable results in different areas (petrochemical [1], forensic [2], essential oils [3], environmental [4] etc.), providing a substantial increase in peak capacity by serially coupling two capillary columns. As a consequence the separation of many unresolved components from the first column is achieved in the second column. A further evolution of GCxGC is represented by its hyphenation to a Time of Flight Mass Spectrometer (TOFMS), so that the system offers not only a superb separation power, but also reliable data for identification, which is obtained from the continuous acquisition of full mass spectra in contrast to the need to scan in other mass filter types in order to acquire total ion current (TIC). The aforementioned hyphenation opens interesting perspectives in antidoping analysis since the enhanced separation power of GCxGC can overcome the problem of sensitivity related to conventional GC-MS analytical methods. Additionally, the TOFMS high data acquisition capability (up to 500 Hz) enables full scan-like acquisition that is not available for steroid analyses with quadrupole mass spectrometers. The availability of full mass-spectral data together with recent developments in software allows an additional dimension in separation derived from deconvolution of mass spectra.

Regarding the doping control field, three direct applications could be envisaged: i) Detection of the World Anti-doping Agency (WADA) target compounds. ii) Metabolic studies. iii) Detection of new drugs and their metabolites such as designer steroids.

In this work, preliminary results in the detection of target compounds for doping control analyses obtained by using a GCxGC–TOFMS system are presented.

**Detection of WADA target compounds**

Aiming at the evaluation of the detection of target compounds, the GCxGC – TOFMS system was optimized for the detection of the five "key" anabolic agents – clenbuterol, norandrosterone, epimetendiol, methyltestosterone metabolite and 3'OH stanozolol – at a concentration level of about 2 ng/ml.

Despite an increasing interest for advanced techniques (GC-MS-MS, HRMS), GC-MS still remains the best choice for the screening of these anabolic steroids in athletes' and animal urines. However, the sensitivity reached by the traditional screening protocol is frequently not enough for some analytes (i.e. stanozolol and its metabolites), mainly due to the poor chromatographic performance of the GC-MS system [5-6].

**Experimental**

*Reagents and chemicals*

3'-hydroxystanozolol (stanozolol metabolite), 5α-androst-1-en-17β-ol-3-one and 5β-androst-1-en-17β-ol-3-one (nandrolone metabolites), 17α-methyl-5α-androstane-3α,17β-diol and 17α-methyl-5β-androstane-3α,17β-diol (methyltestostrone metabolites) were kindly donated by Dr. W. Schänzer and H. Geyer from the Institute of Biochemistry, Germany Sports University, Cologne, Germany. Clenbuterol was bought from Sigma (St. Louis, MO, USA), Epimetendiol from NMI (Sydney, Australia) and Methyltestosterone – used as an internal standard – from Aldrich (Milwaukee, WI, USA). All reagents were analytical grade. MSTFA was purchased from Chem Fabrik (Waldstetten, Germany), $NH_4I$ and ethanethiol from Sigma (St. Louis, MO, USA), tert-butylmethylether (TBME) and methanol from Tedia (Fairfield, USA).

*Sample pre-treatment and purification*

Urine samples were processed as described by Geyer *et al.* [7].

***Apparatus and chromatographic conditions***

The GC×GC–TOFMS system used in this study was a Pegasus 4D (Leco, St. Joseph, MI, U.S.A.). This system is composed of an Agilent 6890 GC (Palo Alto, CA, U.S.A.) equipped with a secondary oven and a non-moving quad-jet dual stage modulator. Data acquisition and processing was performed by ChromaTOF software ver. 2.32 (LECO Corp., St Josephs, MI). The GC column set consisted of a HP-1 (Hewlett Packard, Palo Alto, CA, U.S.A.), 100% methylsiloxane, 17 m, 0.2 mm I.D., film thickness 0.11 μm, as the first dimension (1D) column and an OV-1701 (MEGA, Milan, Italy), poly -14% cyanopropylphenyl -86% dimethylsiloxane, 1 m, 0.1 mm I.D., film thickness 0.1 μm, as

the second dimension (2D) column. The second column was connected to the TOFMS by means of a 0.21 m × 0.25 mm I.D. empty deactivated capillary. The columns and the empty deactivated capillary were connected by SGE unions (P/N 073550) using vespel ferrules for 0.1 – 0.25 mm I.D. columns (P/N 073470). Splitless mode injections of 5 μL at 280 °C were made into the Agilent split/splitless injector with a purge time of 60 s and purge flow of 10 mL/min. Constant carrier gas flow rate of 1 mL/min using helium as carrier gas was maintained throughout the run. Primary oven temperature program was 140 °C for 1 min, ramped at 40 °C/min to 180 °C, then at 3 °C/min to 280 °C, then ramped at 40 °C/min to 320 °C. The secondary oven temperature program had the initial temperature set 20 °C higher than the primary one, although it stopped at the same end temperature (320 ºC). The modulation period was 6 s with 0.8 s hot pulse duration and 45 °C modulator temperature offset versus the primary oven temperature. The MS transfer line was held at 280 °C. The TOFMS used electron ionization mode with collected mass range of m/z 80–750. Ion source temperature was 220 °C, the detector was operated at 2000 V, the applied electron energy was 70 eV and the acquisition rate was 100 spectra/s.

**Results and discussion**

A representative total ion current (TIC) chromatogram of the GCxGC–TOFMS separation of human urine spiked with the 5 key anabolic agents (listed in *Table 1*) at a concentration level of 2 ng/ml is reported in *Figure 1.*

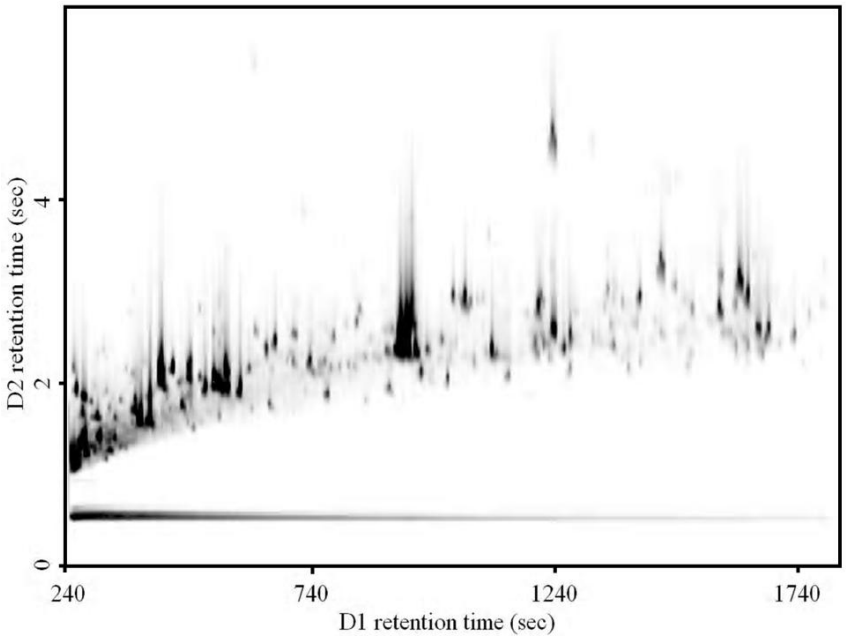


***Figure 1.*** **GCxGC–TOFMS TIC chromatogram of a human urine sample spiked with anabolic agents.**

In the GCxGC separation of the urine sample presented in *Figure 1*, it is possible to distinguish thousands of peaks. Even though the overall separation achieved on the urine sample was good, there were instances where components were not fully resolved by two-dimensional gas chromatography. This is a common occurrence for highly complex samples. For this reason the identification of the five components of interest in the very complex 2D separation was preliminary achieved by extracting from the TIC chromatogram the significant qualifying ions selected from the mass spectrum of each key component (refer to *Table 1*).

The temperature program in the first column was virtually the same as as in routine GC-MS methodology for steroid analysis. However, retention times were larger than those obtained in typical GC-MS methods because the pressure drop throughout the 1D column was smaller, since the same column of GC-MS method was linked to the 2D column instead of linked directly to the mass spectrometer vacuum chamber.

**Table 1. Diagnostic Ions and retention times for the five WADA "key" anabolic agents.**

| | **Anabolic Agent** | **Extracted ions (m/z)** | **1D $t_R$ (s)** | **2D $t_R$ (s)** |
|---|---|---|---|---|
| ***1*** | Clenbuterol | 86, 335, 337 | 456 | 1.78 |
| ***2*** | Norandrosterone M1 | 405, 420 | 816 | 2.22 |
| ***3*** | Epimetendiol (EMD) | 216, 358, 448 | 834 | 2.35 |
| ***4a*** | Methyltestosterone - M1 | 143, 255 | 1032 | 2.41 |
| ***4b*** | Methyltestosterone - M2 | 143, 255 | 1038 | 2.43 |
| ***5*** | 3'-hydroxystanozolol M1 | 254, 545, 560 | 1764 | 2.96 |

In *Figure 2* the regions of the anabolic agents identified by means of GCxGC-TOFMS analysis of the spiked urine are expanded and compared with the same region of the urine sample not containing the key components.

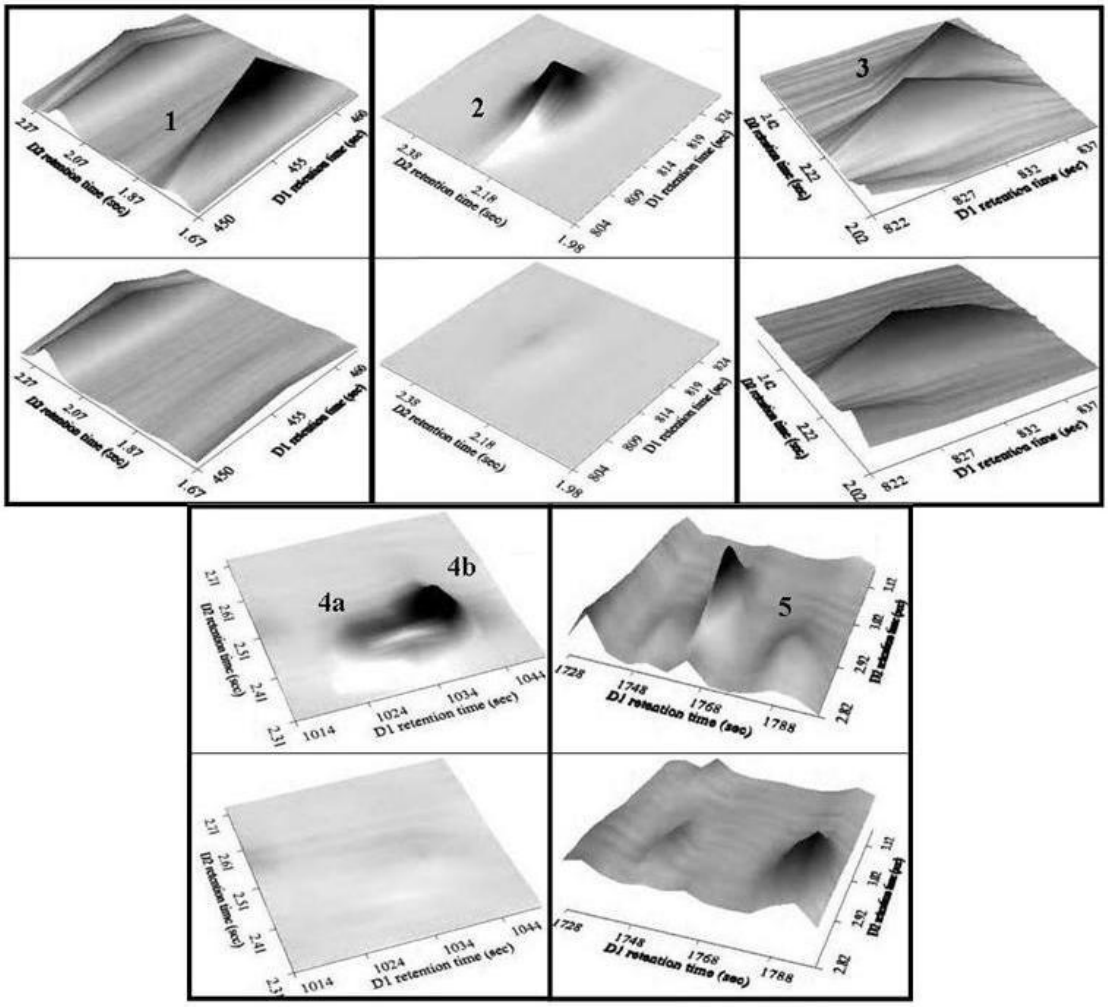


***Figure 2.*** **3D expanded regions of the anabolic agents in the spiked (top) and in the blank (bottom) urine for the key compounds. The expansions were obtained by extracting the qualifying ions reported in *Table 1* from the TIC chromatogram.**

The analytical results obtained are not innovative for sample screening purposes, since they can be already attained for most compounds by routinely applied GC-MS methods. Furthermore, in order to attain the 2 ng/mL level for all the five "key" anabolic agents more sensitive approaches such as GC-HRMS, GC-MS-MS or LC-MS-MS can be used. Nevertheless, none of these latter techniques can provide 2ng/mL **and** full spectra information as demonstrated here.

The most significant improvements in the GCxGC-TOFMS approach for doping analysis are mainly related to higher separation power and complete information on matrix achieved by two-dimensional GCxGC chromatograms. This analytical approach permits detection and identification of key components while simultaneously preserving the matrix information that can be further investigated for research purposes (unknown screening, metabolic studies). The preserved mass spectral information acquired with TOFMS high data acquisition speed can also be used for the mathematical separation

(i.e. deconvolution) of partly co-eluting chromatographic peaks to obtain a "purified spectra". This feature of the GCxGC method can be highlighted through the key anabolic agent, 3'-hydroxystanozolol (*Figure 3)*. The detection of the latter is generally a difficult task in screening doping control analysis.

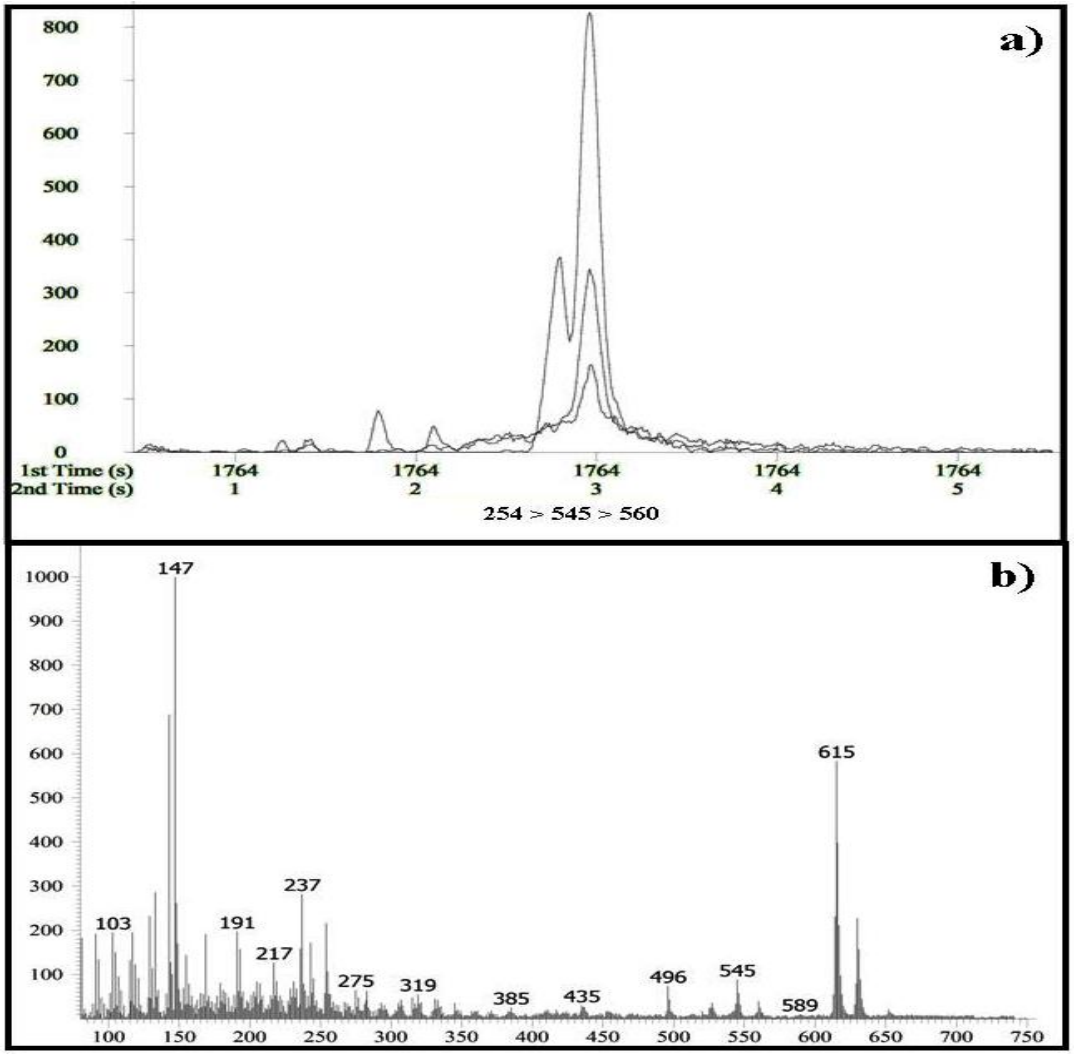


***Figure 3.*** **Expansions of the extracted ion chromatogram of m/z 254, 545 and 560 (a) and the contaminated mass spectrum of 3'-hydroxystanozolol-N-TMS, O-bis-TMS (2 ng/mL) (b) on spiked urine.**

In *Figure 3a* the chromatogram of the qualified ions of 3'-hydroxystanozolol-N-TMS, O-bis-TMS extracted from the TIC GCxGC–TOFMS chromatogram is reported. The chromatogram relative to m/z 254 presents a partial co-elution of the key component with another compound eluting just before in the 2D chromatogram. Further investigation of co-elution can be performed by analyzing the mass spectrum in *Figure 3b*. The main information obtained from mass spectrum profile is the presence of masses higher than the molecular ion of the key component (MW = 560). Moreover, through the observation of the TIC chromatogram represented in *Figure 4*, it was

possible to confirm that the 3'-hydroxystanozolol suffers, indeed, co-elution from a major peak.

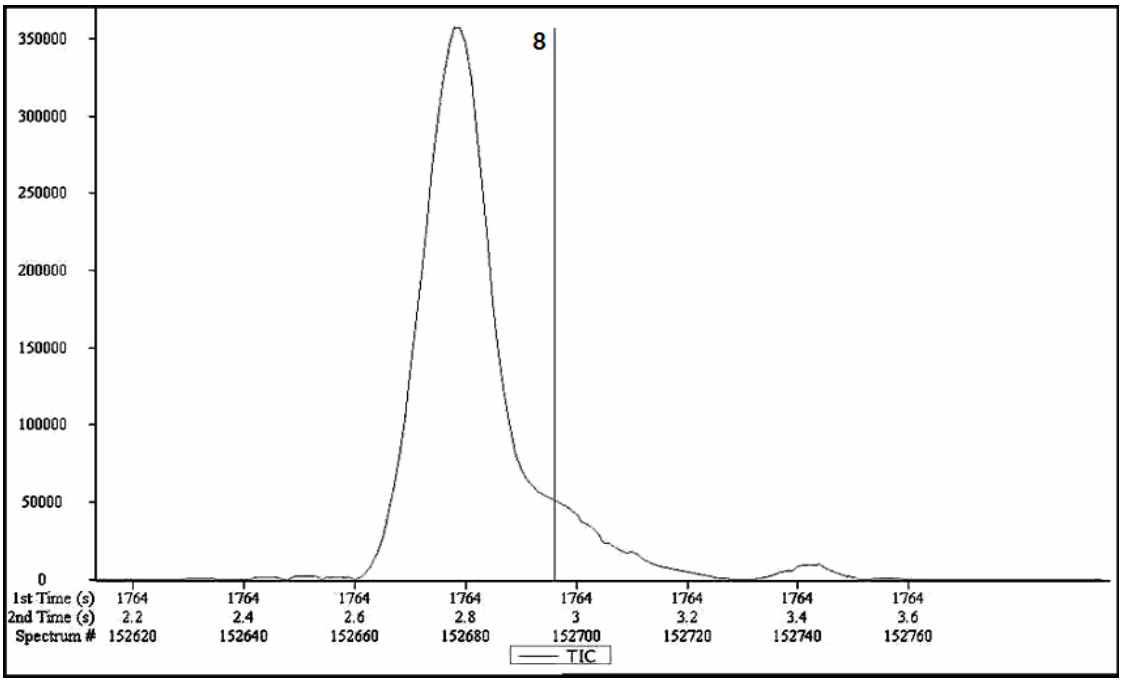


***Figure 4.*** **TIC chromatogram expansion of the region of 3'-hydroxystanozolol-N-TMS, O-bis-TMS. The position of the latter is represented by the vertical line.**

Further investigation in the above led to the identification of the co-elution of 3'-hydroxystanozolol with an endogenous compound because identical peaks were detected in both spiked and blank urines. Since the software used for data mining is able to deconvolute two peaks when their apexes are separated by only a few scans, the mass spectra of key components and endogenous compounds were registered in a laboratory-made library. The latter was developed to smooth the adequate identification of key anabolic agents by means of mass spectra library search and deconvolution.

The higher the purity of the mass spectra registered in the laboratory-made TOFMS library, the better the identification of anabolic agents based on library search matching and deconvolution. In *Figure 5* the deconvolution results for 3'-hydroxystanozolol-N-TMS, O-bis-TMS are reported. The TIC chromatogram in *Figure 4* presented a major component and the 3'-hydroxystanozolol co-eluting in the final part of the peak. After selecting and extracting the fragments with m/z 545 and 615 from the TIC it is possible to distinguish the apexes of the two peaks by the two vertical lines marked as 7 and 8.

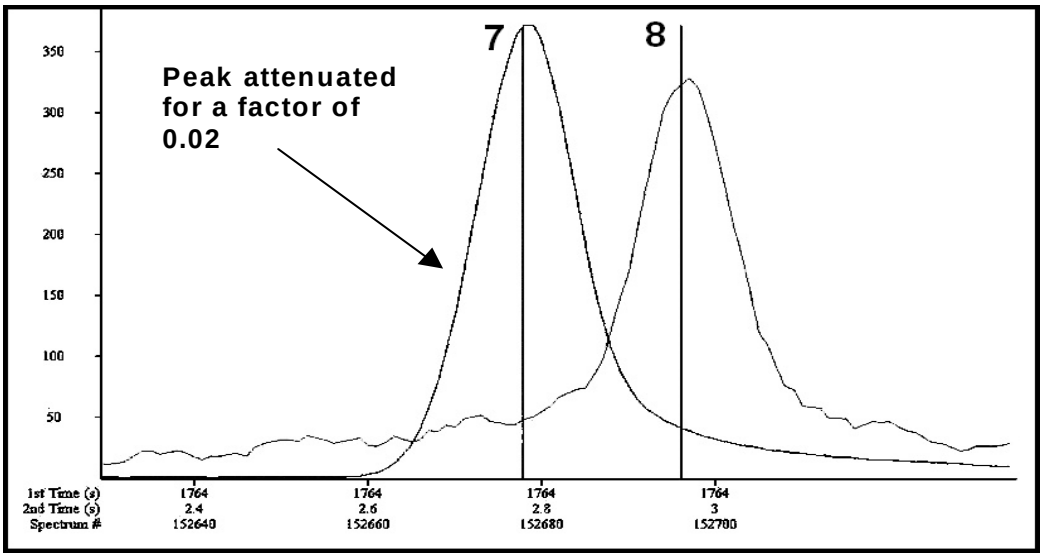


***Figure 5.*** **Software deconvolution of the co-elution of 3'-hydroxystanozolol-N-TMS, O-bis-TMS (#8) and the endogenous compound (#7). (Extracted ions 615x0.02 for the endogenous compound and 545 for the key analyte)**

For better comparison, Figure 6 shows a similar result obtained with conventional GC-MS system in full scan acquisition mode and spiked blank urine, in which 3'OH stanozolol is 10 ng/mL.

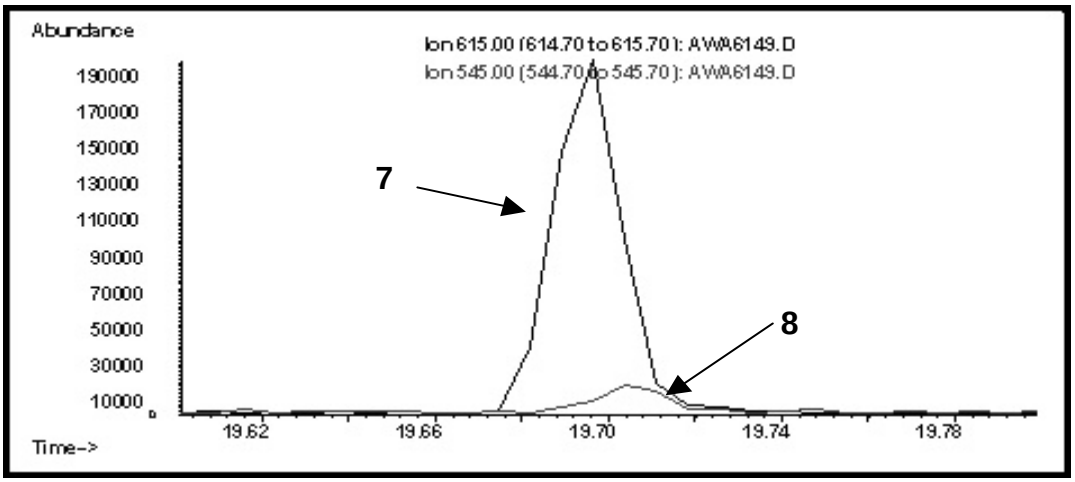


***Figure 6.*** **Demonstration of the co-elution of 3'-hydroxystanozolol-N-TMS, O-bis-TMS (#8) in 10 ng/mL and the endogenous compound (#7) in a conventional GC-MS system. (Extracted ions 615 for the endogenous compound and 545 for the key analyte).**

In *Figure 7* the library search results for the 3'-hydroxystanozolol-N-TMS, O-bis-TMS deconvoluted mass spectrum is shown.

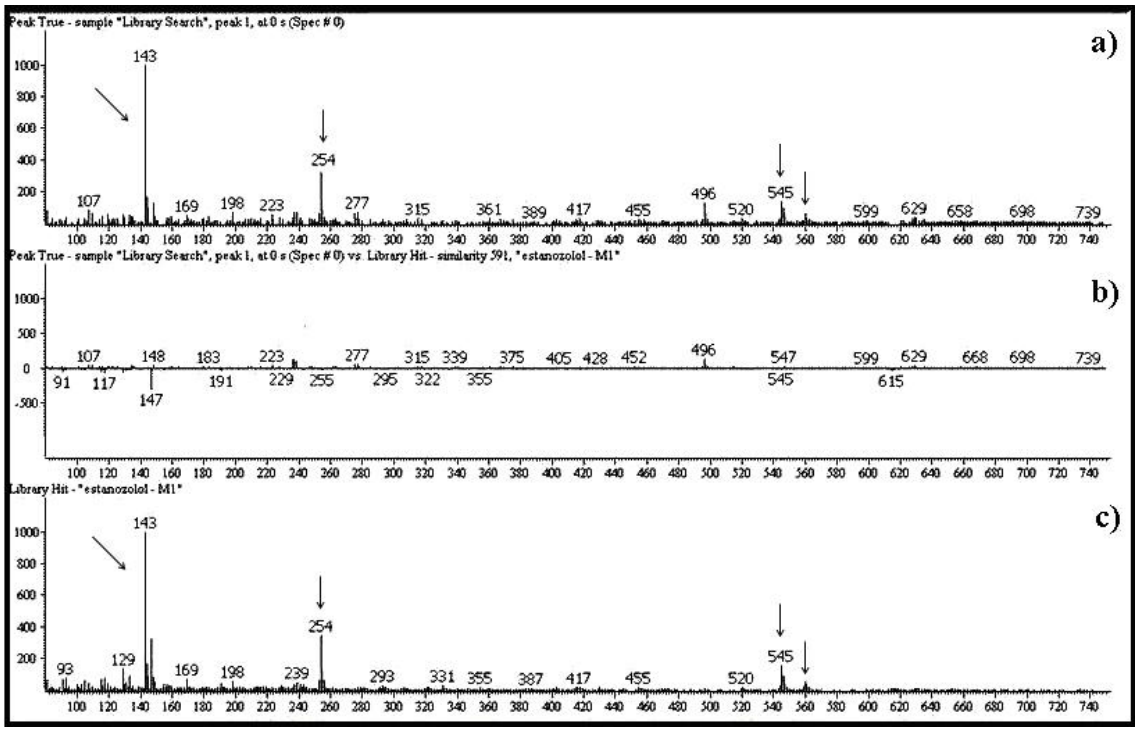


***Figure 7.*** **Library search results for 3'-hydroxystanozolol-N-TMS, O-bis-TMS. a) deconvoluted mass spectrum, b) acquired and library mass spectra differences in fragments, and c) reference mass spectrum contained from the laboratory-made library.**

Further confirmation of the key anabolic agent's identity was obtained by comparing the ratio of the qualifying ions in the spiked urine and in the reference compound. In *Table 2* the relative abundance of these ions are presented.

***Table 2.*** **Qualifying ions ratio for 3'-hydroxystanozolol-N-TMS, O-bis-TMS.**

| Qualifying ion ratios | Spike urine (2 ng/ml) | Spike urine (10 ng/ml) |
|---|---|---|
| m/z 254/545 | 1.9 | 1.8 |
| m/z 545/560 | 4.0 | 3.8 |

The ratios for the qualifying ions of the 3'-hydroxystanozolol-N-TMS, O-bis-TMS present in the spiked urine sample that meet WADA's identification criteria [8] are acceptable when compared to the values attained by injecting the more concentrated standard (10 ng/ml), whose mass spectrum was registered in the laboratory-made library. These results fulfill the expectations regarding the detection of target compounds. The conservation of matrix information as well as the resolution observed strengthen the perspectives of detection of other known and unknown substances.

**Conclusions**

This study has demonstrated the applicability of Comprehensive GC coupled to Time of Flight Mass Spectrometry for the screening of the five key components declared illicit by the World Anti-doping Agency and with the lowest purported concentration levels. The quality of the data obtained will also facilitate meeting the rigorous identification

criteria set up by WADA for analytes in very low concentration. Moreover, the performance of further investigations on the attained separation would surely enable not only the identification of other important exogenous anabolic compounds of doping interest, but also their distinction from the endogenous ones. The ultimate goal will be to provide a tool for tracking designer drugs that may be used by athletes to circumvent present doping control analytical strategies.

**Acknowledgments**

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