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APPLICATION NOTE 30405

GC-MS-IRMS: Undisputable results by coupling of GC-IRMS with high-resolution mass spectrometry for final confirmation in sports drug testing

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Keywords

Sports doping, testosterone, Q Exactive GC, Delta V, GC-MS-IRMS

Goal

To demonstrate that hyphenation of the Thermo Scientific™ Delta V™ IRMS or the Thermo Scientific™ 253 Plus™ IRMS systems with the Thermo Scientific™ Q Exactive GC™ connected to a single Thermo Scientific™ Trace™ 1310 GC provides complementary information. Isotopic composition and structural information with high resolution can be simultaneously determined in a single run.

Introduction

The administration of performance-enhancing anabolic substances has been banned since the 1970s, first by the International Olympic Committee (IOC) and subsequently by the World Anti-Doping Agency (WADA). Nowadays, endogenous steroids are still among the most frequently detected class of substances misused in sports.

Gas chromatography coupled with isotope ratio mass spectrometry is routinely applied in doping controls. It allows distinguishing between endogenous steroids from their synthetic analogs in urine by the determination of $^{13}C/^{12}C$ isotope ratios.

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Pharmaceutically produced anabolic-androgenic steroids are predominantly derived from C3-plant material. Natural isotopic Fractionation in plants results in depletion of the $^{13}C/^{12}C$ isotope ratios of derived compounds in comparison with endogenously produced steroids. The administration of those synthetic steroid copies reveals depleted $^{13}C/^{12}C$ isotope ratios of the excreted compound and its metabolites. Endogenous reference compounds are not affected and are selected for an individual internal standardization of the isotope ratios.

The Thermo Scientific™ GC Isolink II™ conversion unit combines the high performance of the Trace 1310 GC with the market-leading Delta V or 253 Plus mass spectrometer, providing a seamless solution for GC-IRMS. The GC IsoLink II conversion unit continuously and quantitatively converts substances into simple gases, such as CO_2 and N_2 by combustion, and H_2 and CO by high temperature conversion.

Doping control laboratories accredited by WADA obligatorily use methods for the determination of the $^{13}C/^{12}C$ ratios of steroids in urine.[1] Prior to GC-IRMS, analytes must be efficiently isolated and purified. Sample preparation of the urine specimen includes several steps, such as solid phase extraction, liquid-liquid extraction, and high-performance liquid chromatography (HPLC) with Fraction collection.[2] Finally, depending on the overall sample preparation and analytical strategy, specimens are acetylated before analysis by GC-Combustion IRMS. Precision of the analysis of acetates is increased due to enhanced chromatographic properties, resulting in narrower peaks with less tailing.

The isotopic determination of steroids requires proper separation of compounds and clean peak integration. Despite comprehensive sample cleanup, satellite peaks and matrix compounds may interfere with compounds of interest. Any doubts of peak purity or integrity must be eliminated to avoid the possibility of false positives or negatives. The $^{13}C/^{12}C$ isotope ratios of the target compounds and endogenous reference compounds must be unambiguously determined.

However, GC-IRMS solely supplies information on isotopic composition of a compound of interest. Identification of compounds is based only on comparison of retention times of steroid standards previously injected. Additionally, samples can be repeatedly analyzed by conventional GC-MS to confirm compound identities. This approach requires applying identical chromatographic conditions in both analytical systems and using the same GC column is essential. Batch-to-batch differences and inhomogeneity of GC columns by the manufacturing process might result in analytical uncertainties.

With the introduction of the GC IsoLink II, the IRMS system has been routinely coupled with the Thermo Scientific™ ISQ™ single quadrupole system connected to a single Trace 1310 GC. Concomitant data are critical to qualify the true identity of a compound. This has become mandatory in confirmation of so-called adverse analytical findings.

The Q Exactive GC Orbitrap™ GC-MS/MS is a new class of GC-MS system with high mass resolution and exceptional mass accuracy for the detection and identification of potentially co-eluting matrix compounds. By coupling GC-IRMS with the Q Exactive GC system, the isotopic compositions and the comprehensive qualitative and quantitative sample information with high levels of selectivity, sensitivity, and confidence are accessible simultaneously.

The Q Exactive GC Orbitrap system can provide the highest confidence in assessing peak purity and identity, making this system a valuable contribution due to its unmatched selectivity. Identification of minor compounds masked by dominant co-eluting compounds can be accomplished due to superior sensitivity.

Instrument and method setup

Excretion study after testosterone administration

One male volunteer was administered 30 mg of testosterone orally. Urine samples were collected before and up to 24 hours after administration (1.5 h, 3 h, 6 h, 8.5 h and 24 h).

Sample preparation

Sample preparation was performed according to the routine methodology for doping analysis. The detailed description of sample preparation was published before.[2,3]

Two consecutive HPLC Fractionation steps are included, resulting in seven Fractions after the first HPLC cleanup (see Table 1). All Fractions were acetylated to improve

Table 1. List of different Fractions collected during HPLC cleanup and analyzed by QE GC-IRMS.
Steroids are excreted into urine as glucuronide conjugates.

Fraction	Abbreviation	Compound Name
I	11-KETO	3a-hydroxy-5b-androstane-11,17-dione
II	TESTO	17b-hydroxy-androst-4-en-3-one
III	5b DIOL, (DHEA,EPIT)	5b-androstane-3a,17b-diol, (3b-hydroxy-androst-5-en-17-one) (17a-hydroxy-androst-4-en-3-one)
IV	5a DIOL, (ETIO)	5a-androstane-3a,17b-diol, (3a-hydroxy-5b-androstane-17-one)
V	ANDRO	3a-hydroxy-5a-androstane-17-one
VI	PD	5b-pregnane-3a,20a-diol
VII	16EN	5a-androst-16-en-3a-ol

chromatographic separation and peak shapes of steroids on both the HPLC and GC column. Derivatization was accomplished by adding 50 µL of pyridine and 50 µL of acetic anhydride. The mixture was incubated for 60 min at 70 °C and evaporated to dryness under a stream of air. The dried residue was transferred to either GC or LC autosampler vials. Fractions II (containing TESTO), III (EPIT, DHEA and 5bDIOL), and IV (ETIO, 5aDIOL) were further purified by an additional HPLC Fractionation.

Q Exactive GC-IRMS analysis

All experiments used a Trace 1310 GC with a Q Exactive GC hybrid quadrupole-Orbitrap mass spectrometer coupled with a GC-IRMS system consisting of a GC IsoLink II preparation unit, ConFlo IV interface, and a Delta V Plus mass spectrometer. The GC oven incorporates a micro channel device (MCD) for splitting the effluent of the GC column. The split of the GC effluent was set to 5:1 (IRMS / Q Exactive GC) and was adjusted by the length of transfer fused silica capillary from the MCD to the ion source of the Q Exactive GC.

Sample introduction was performed using a TriPlus RSH autosampler, and chromatographic separation was obtained using a Thermo Scientific™ TraceGold™ TG-5MS 30 m × 0.25 mm I.D. × 0.25 µm film capillary column (P/N: 26098-1420). Additional details of instrument parameters are shown in Tables 2a and 2b.

Table 2a. GC conditions.

Trace 1310 GC inlet parameters	
Injection volume	1.0 µL
Liner	Single taper (P/N453A1345)
Injector	280 °C
Injector module and mode	SSL, splitless
Splitless time and purge	1 min, 100 mL/min
Carrier gas	Helium, 1.5 mL/min
Oven temperature program	
Initial temperature	100 °C
Hold time	2 min
Rate 1	25 °C/min
Temperature 1	270 °C
Hold time 1	0 min
Rate 2	2 °C/min
Temperature 2	290 °C
Hold time 2	0 min
Rate 3	30 °C/min
Temperature 3	300 °C
Hold time 3	2 min

Table 2b. Mass spectrometer conditions.

GC-IRMS parameters	
IRMS	Delta V Plus
Amplifications	*m/z* 44: 3 * 10^8 *m/z* 45: 3 * 10^{10} *m/z* 46: 10^{11}
Conversion unit	GC IsoLink II
Mode	Combustion
Oxidation reactor	Ceramic tube, ID 0.5 mm with NiO, CuO, and Pt wiring
Reactor temperature	950 °C
Reactor conditioning	1.5 h with gentle flow of oxygen (0.7 mL/min)
Interface	ConFlo IV
Q Exactive GC-MS parameters	
Transfer line	280 °C
Ionization type	EI
Ion source	250 °C
Electron energy	70 eV
Acquisition mode	Full Scan
Mass range	50–600 *m/z*
AGC target	1e6
Maximum injection time	auto
Mass resolution (FWHM at *m/z* 200)	120,000
Filament on delay	10 min
Method duration	21 min

Table 3. Retention times.

Steroid	RT(s) = [RT (min)] IRMS	RT (min) QE GC
11-KETO	873 [14.6]	14.3
TESTO	922 [15.4]	15.1
5b DIOL	893 [14.9]	14.6
5a DIOL	898 [15.0]	14.7
ANDRO	847 [14.1]	13.8
PD	1032 [17.2]	16.9
16EN	689 [11.5]	11.2

Correction for the acetate moieties

In the acetylation step, additional carbon atoms from the acetate moiety are incorporated into the steroidal backbone. Based on an adapted mass balance formula the measured δ^{13}CSA values have to be corrected using the following equation:[4]

$$\delta^{13}C_{Steroid} = \frac{(n_{SA} * \delta^{13}C_{SA}) - (n_A * \delta^{13}C_{A\,corr})}{n_{Steroid}}$$

n = number of moles of carbon
SA = steroid acetate
A = acetate moiety (derivative group)

The $^{13}C_{A\,corr}$ is empirically determined by consecutive measurements of both the native and the derivatized steroid reference material.

Why high resolution, accurate mass?

- Confidence in confirmation
- High resolution, accurate mass for non-target screening
- Retrospective search of new compounds
- Sensitive, simultaneous non-targeted and targeted quantitation in a non-regulated environment

Figure 1. Q Exactive GC.

Results and discussion

An excretion study was performed after testosterone administration by a volunteer. Urine samples collected were prepared according to the standard protocol of the doping control routine and analyzed by a GC-IRMS system online coupled with a Q Exactive GC.

Figure 2a shows an exemplary IRMS chromatogram of the KETO Fraction, and Figure 2b shows the QE GC total ion chromatogram of the same injection. This urine sample was collected 8.5 hours post-administration of testosterone. Peaks in both chromatograms show the same peak resolution. Small differences in signal intensities can be found due to the different mass spectrometric approaches. Sample compounds eluting from the GC column were split using micro channel devices. For Q Exactive GC analysis, compounds are directly introduced into the EI ion source by a transfer line; whereas, the portion transferred to the IRMS system was firstly combusted into CO_2 and H_2O. After online purification of the sample gas and removal of water by the GC IsoLink II, CO_2 is admitted into the isotope ratio mass spectrometer. The GC IsoLink preparation device maintains peak separation and resolution.

The $\delta^{13}C$ values of the excretion study are presented in Tables 4a and 4b. The IRMS methodology for doping control comprises the comparison of the $\delta^{13}C$ values of target compounds (TCs) and endogenous reference compounds (ERCs). TCs are the group of exogenous anabolic steroids or metabolites exhibiting depleted $\delta^{13}C$ values, while the ERCs are not affected and typically enriched in their corresponding 13C/12C ratio. WADA has established in general a minimum of 3% difference between any ERC and TC to constitute an adverse analytical finding.

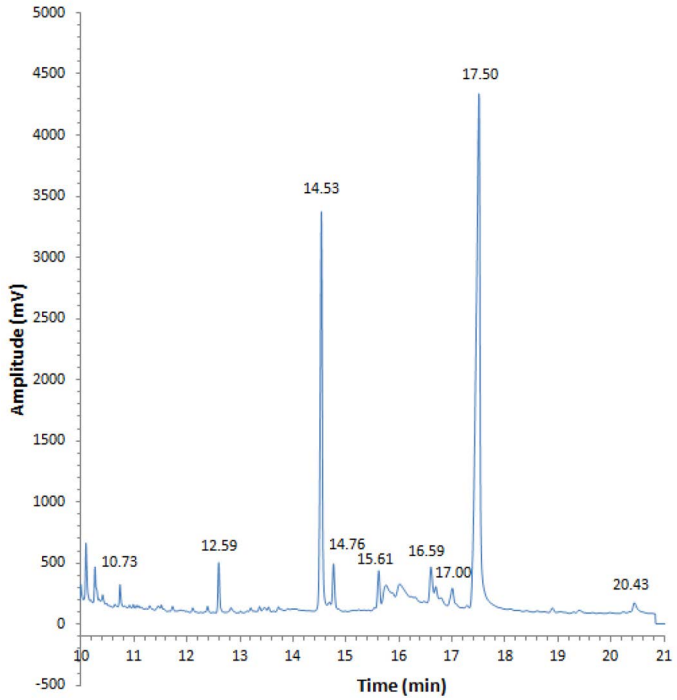


Figure 2a. IRMS chromatogram (*m/z* 44 trace) of 11-KETO Fraction (8.5 hours post-administration).

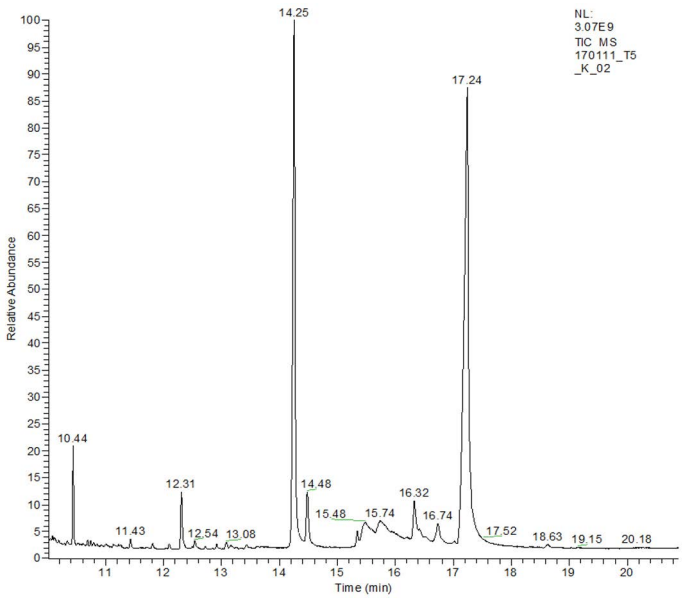


Figure 2b. QE GC chromatogram of 11-KETO Fraction (8.5 hours post-administration).

The results of the target compounds testosterone (TESTO) and its main metabolites 5α-androstane-3a,17b-diol (5a DIOL) and 5β-androstane-3a,17b-diol (5b DIOL), are shown in Table 4a. In doping control, pregnanediol (PD), 5a-androst-16-en-3b-ol (16EN), or 11-ketoetiocholanolone (KETO) is commonly selected as the ERC. The corresponding results are shown in Table 4b. All samples were analyzed in duplicate; the tables show the average and the precision (SD) achievable by this method of analysis. The measured $\delta^{13}C$ values and the corrected values considering the added carbon atoms by derivatization can be found in the corresponding columns.

Table 4a. $\delta^{13}C_{VPDB}$ of target compounds, mean values (n=2), and standard deviations (SD) of acetylated (measured) and corrected values.

	Measured Values		Corrected Values	
	Mean [‰]	SD [‰]	Mean [‰]	SD [‰]
TESTO				
T1	-26.43	0.03	-22.90	0.04
T2	-33.00	0.04	-30.15	0.04
T3	-32.88	0.33	-30.02	0.37
T4	-32.86	0.08	-30.00	0.09
T5	-31.70	0.25	-28.72	0.27
T6	-28.96	0.08	-25.69	0.09
5a DIOL				
T1	-31.09	0.03	-25.01	0.04
T2	-33.43	0.16	-27.84	0.19
T3	-35.27	0.02	-30.07	0.03
T4	-34.80	0.20	-29.49	0.24
T5	-34.25	0.01	-28.83	0.02
T6	-33.32	0.10	-27.70	0.12
5b DIOL				
T1	-31.27	0.13	-25.22	0.16
T2	-35.10	0.17	-29.86	0.21
T3	-35.56	0.21	-30.41	0.26
T4	-34.84	0.02	-29.54	0.03
T5	-34.85	0.01	-29.55	0.01
T6	-34.06	0.13	-28.60	0.15

Table 4b. $\delta^{13}C_{VPDB}$ of endogenous reference compounds, mean values (n=2), and standard deviations (SD) of acetylated (measured) and corrected values.

	Measured Values		Corrected Values	
	Mean [‰]	SD [‰]	Mean [‰]	SD [‰]
PD				
T1	-29.35	0.07	-23.51	0.08
T2	-29.56	0.23	-23.76	0.28
T3	-29.76	0.10	-24.00	0.12
T4	-29.63	0.30	-23.84	0.35
T5	-29.48	0.03	-23.67	0.03
T6	-29.58	0.02	-23.79	0.02
Mean [‰]	**-29.56**		**-23.76**	
SD [‰]	**0.14**		**0.16**	
11-KETO				
T1	-26.70	0.20	-23.19	0.22
T2	-26.96	0.15	-23.48	0.16
T3	-26.85	0.03	-23.36	0.03
T4	-27.15	0.01	-23.69	0.02
T5	-26.97	0.04	-23.49	0.05
T6	-27.14	0.02	-23.68	0.02
Mean [‰]	**-26.96**		**-23.48**	
SD [‰]	**0.17**		**0.19**	
16EN				
T1	-27.83	0.05	-24.44	0.06
T2	-26.85	0.23	-23.36	0.26
T3	-27.05	0.10	-23.58	0.11
T4	-27.50	0.09	-24.08	0.10
T5	-27.36	0.01	-23.92	0.01
T6	-27.12	0.29	-23.66	0.33
Mean [‰]	**-27.29**		**-23.84**	
SD [‰]	**0.35**		**0.39**	

The temporal progress of the $\delta^{13}C$ values are shown in Figure 3. The ERCs exhibit nearly constant $\delta^{13}C$ values with small variation. The plot reveals the largest deviation of the $\delta^{13}C$ values of the TCs from ERCs between 2 and 6 hours. However, the data demonstrates that testosterone doping can be still approved after 24 hours by utilizing the IRMS results of TESTO and its metabolites 5a DIOL and 5b DIOL. The administration of 30 mg of testosterone for the purposes of this study can be considered to be a low and realistic dose typically applied in sports.

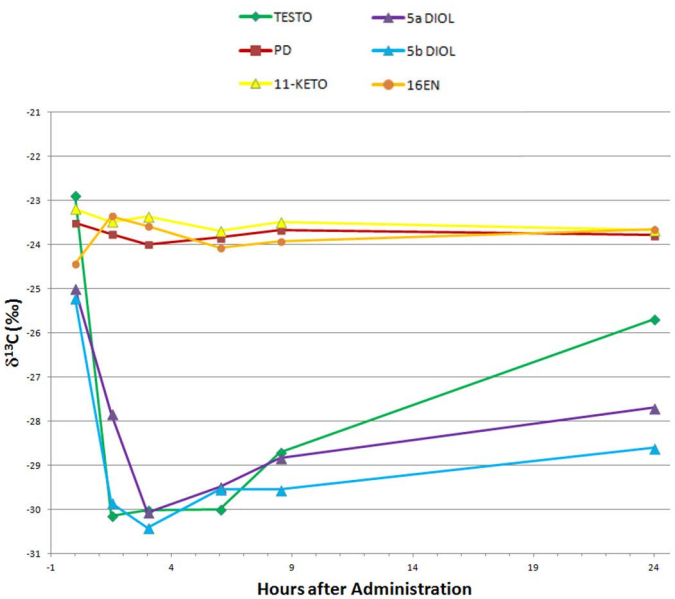


Figure 3. Plot of the $\delta^{13}C$ (%) values of target compounds and endogenous reference compounds.

In GC-IRMS, compounds are quantitatively converted into simple gases, such as CO_2, by combustion, and the corresponding *masses 44, 45,* and *46* are simultaneously measured on separated Faraday collectors with different amplification. Therefore, the structural information and compound identity is not directly accessible by IRMS. The specificity of the methodology is ensured by sample preparation and HPLC Fractionation and can be confirmed by GC/MS. TCs and ERCs are identified by direct comparison of mass spectral data and retention times of steroid standards. However, satellite peaks and matrix compounds may interfere with compounds of interest. Any doubts of peak purity or integrity must be eliminated avoiding the possibility of false positives or negatives.

IRMS software shows the *45/44* ion current ratio (isotope ratio trace). The *45/44* ion current ratio varies strongly across each peak as a result of the slightly shorter retention time for molecules containing one ^{13}C atom. This phenomenon has been observed on most of the gas chromatographic systems.

The *45/44* isotope ratio trace can be considered as a helpful tool for assessing the peak purity. Baseline separated compounds show a clean isotopic swing on the isotope ratio trace. Any interference or co-elution results in a disturbed isotopic swing.

Figure 4a shows the partial chromatogram of the TESTO Fraction. It shows the *mass 44* ($^{12}CO_2^+$) ion current (bottom) and the instantaneous *45/44* ion-current ratio (top) from GC-IRMS analysis. Based on the retention time, the large peak is supposed to be testosterone while the small peak in front had to be identified.

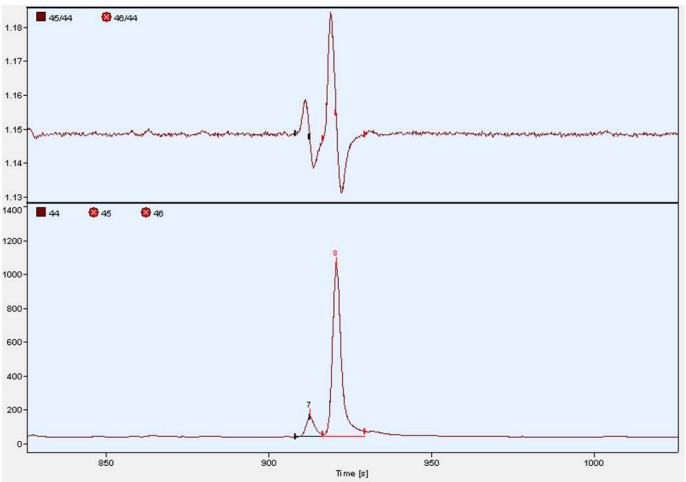


Figure 4a. IRMS chromatogram (*m/z 44* trace) and ratio trace *45/44* of TESTO Fraction (24 hours post-administration).

The compound identity and purity was confirmed by the Q Exactive GC. Figure 4b shows a section of the Q Exactive GC total ion chromatogram of the TESTO Fraction. The peak with RT 15.08 min could be identified as testosterone acetate by a NIST® library search. Figure 4c displays the HRAM mass spectra and Figure 4d shows the comparison with the NIST library.

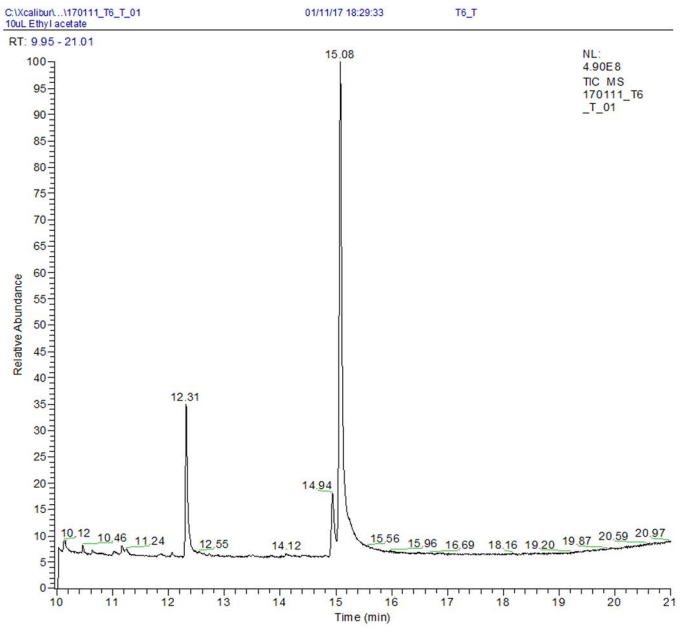


Figure 4b. Q Exactive GC total ion chromatogram of TESTO Fraction (24 hours post-administration).

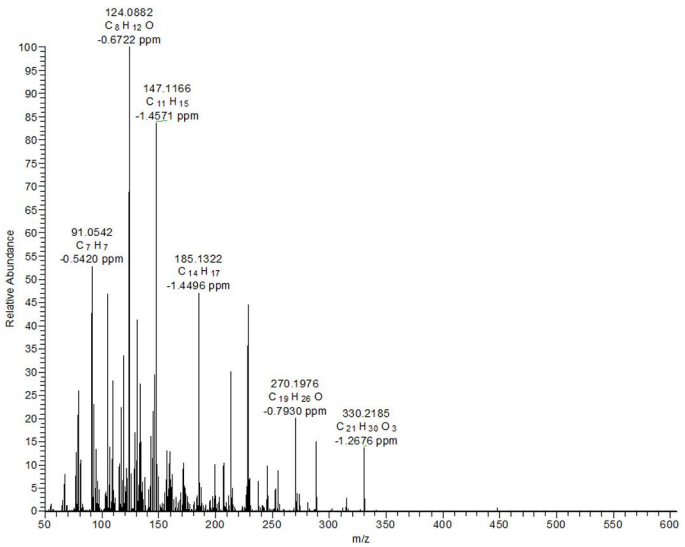


Figure 4c. Q Exactive GC MS spectrum of the peak at 15.08 min (TESTO Fraction, 24 hours post-administration), identified as testosterone.

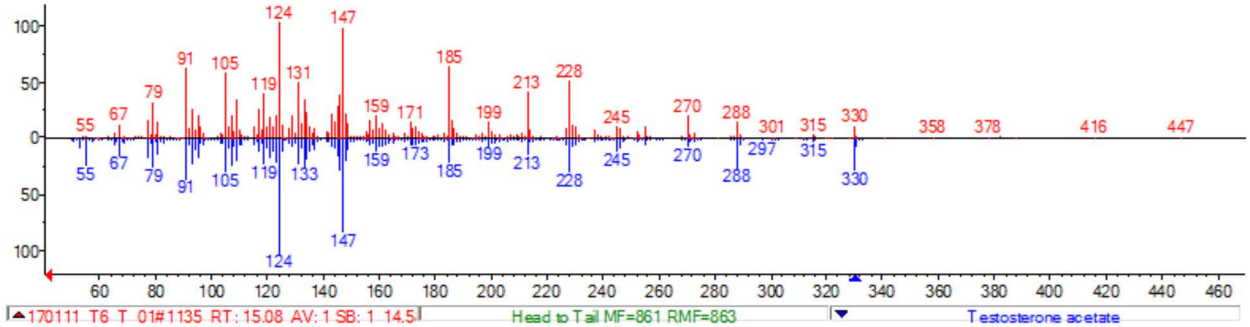
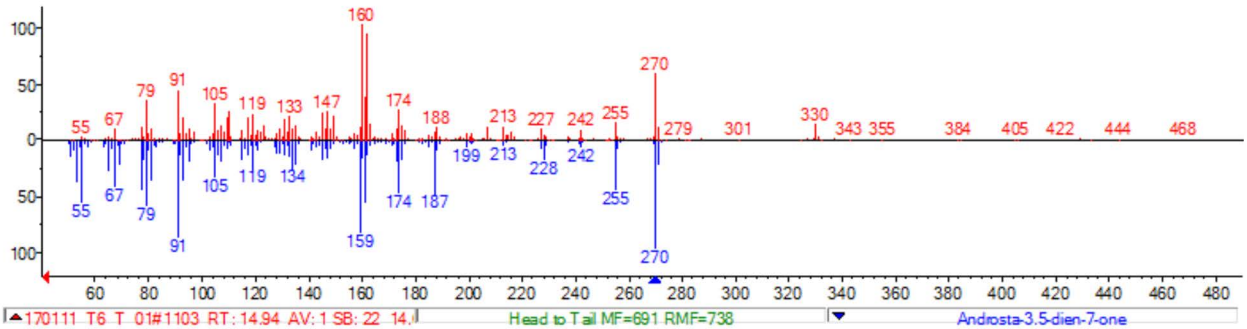


Figure 4d. Comparison of Q Exactive GC MS spectrum of peak at 15.08 min (TESTO Fraction, 24 hours post-administration) with a NIST spectrum of testosterone, Full MS Mode (*m/z* range: 50–600 amu).

The elemental composition of the masses and mass accuracy is automatically calculated and displayed in the mass spectra. The Q Exactive GC shows excellent mass accuracy below 2 ppm.

The peak eluting from the GC column before testosterone acetate exhibits a different mass spectrum. The first hit of the NIST library search of this peak shows androsta-3,5-dien-7-one with only small differences in spectra (Figure 4e). The analysis of the corresponding standard compound can help to unambiguously identify the compound structure.



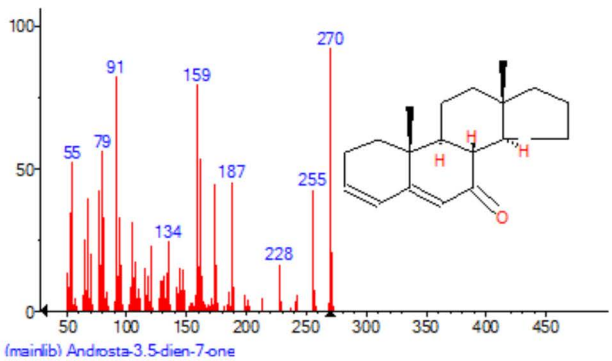


Figure 4e. Comparison of Q Exactive GC MS spectra of peak at 14.94 min (TESTO Fraction, 24 hours post-administration) with first hit of NIST database.

Conclusion

The GC-IRMS methodology and corresponding sample preparation procedures have been continuously improved and intensively investigated since their introduction to sports drug testing in 1994. In the last years, the online coupling of GC-IRMS with quadrupole GC/MS (Thermo Scientific ISQ Series) has been established for confirmatory analysis. The GC-MS-IRMS system provides vital complementary information. However, a quadrupole GC/MS only provides mass spectra based on the nominal mass.

The Thermo Scientific Q Exactive GC Orbitrap GC-MS/MS system fulfills the demand for high resolution and accurate mass (HRAM) determination. It can focus on analyzing the samples using full-scan non-targeted acquisition and using high mass resolving power to obtain accurate mass measurements. This resolving power is important to enable confident elemental composition proposals, structural elucidation, and discrimination of co-eluting and isobaric compounds. Fast acquisition speeds, in combination with a high in-scan dynamic range and high sensitivity, facilitate the detection of new metabolites and minor constituents.

Combining the Thermo Scientific Delta V or the Thermo Scientific 253 Plus IRMS system with Thermo Scientific Q Exactive GC connected to a single Thermo Scientific Trace 1310 GC gas chromatograph provides a refined methodology for doping control. Simultaneous analysis by an HRAM GC/MS system enables confirming the peak identity and purity with highest accuracy and confidence.

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