

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

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## ABSTRACT

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  $^{13}\text{C}/^{12}\text{C}$  isotope ratios.

By coupling GC-IRMS with the Thermo Scientific™ Q Exactive™ GC Orbitrap™ GC-MS/MS the isotopic compositions and the comprehensive qualitative and quantitative sample information with high levels of selectivity, sensitivity and confidence are accessible simultaneously. From a single injection, the structure and isotope ratio of each compound can be determined. This has become mandatory in confirmation of so-called adverse analytical findings.

Here we present the results of an excretion study after testosterone administration. The data demonstrates that testosterone doping can be still approved after 24 hours by utilizing the IRMS results of testosterone and its main metabolites.

## INTRODUCTION

Endogenous steroids are the most frequently detected class of substances misused in sports. The misuse of endogenous anabolic steroids can be detected by measuring the  $\delta^{13}\text{C}$  values of the steroid itself or its metabolites.

Samples showing suspicious steroid profile parameters are isotopically characterized in order to prove the origin of steroids. This is possible since pharmaceutically produced anabolic-androgenic steroids are predominantly derived from C3-plant material. Natural isotopic fractionation in plants results in depletion of the  $^{13}\text{C}/^{12}\text{C}$  isotope ratios of derived compounds in comparison with endogenously produced steroids. The administration of those synthetic steroid copies reveals depleted  $^{13}\text{C}/^{12}\text{C}$  isotope ratios of the excreted compound and its metabolites. Endogenous reference compounds are not affected and selected for an individual internal standardization of the isotope ratios.

However, GC-IRMS solely supplies information on isotopic composition of a compound of interest. The Q Exactive GC Orbitrap GC-MS/MS can provide highest confidence in assessing peak purity and identity making this system a valuable contribution due to today unmatched selectivity. Identification of minor compounds masked by dominant co-eluting compounds can be accomplished due to superior sensitivity.

Figure 1. Thermo Scientific Q Exactive GC Orbitrap GC-MS/MS coupled with a GC IsoLink™ IRMS System.



## MATERIALS AND METHODS

All experiments used a Thermo Scientific™ TRACE™ 1310 GC with a Thermo Scientific™ Q Exactive GC™ Orbitrap Mass Spectrometer coupled with the Thermo Scientific™ GC IsoLink™ IRMS System consisting of a Thermo Scientific™ GC IsoLink II™ Preparation Unit, Thermo Scientific™ ConFlo IV™ Interface and a Thermo Scientific™ DELTA V™ Isotope Ratio Mass Spectrometer (Figure 1). The GC oven incorporates a micro channel device (MCD) for splitting the effluent of the GC column.

Sample introduction was performed using a Thermo Scientific™ 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.

### Sample Preparation

Sample preparation was performed according to the routine methodology for doping analysis.<sup>1,2</sup> One male volunteer administered 30 mg of testosterone orally. Urine samples were collected before and up to 24 hours after administration (1.5h, 3h, 6h, 8.5h and 24 h).

Two consecutive HPLC fractionation steps are included resulting in seven fractions after the first HPLC clean up (see Table 1). All fractions were acetylated in order to improve 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 and the dried residue was transferred to either GC or LC auto-sampler vials. Fractions II (containing TESTO), III (EPIT, DHEA and 5bDIOL) and IV (ETIO, 5aDIOL) were further purified by an additional HPLC fractionation.

| 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   |

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

After sample injection in the TRACE 1310 GC, the effluent was split between the IRMS and the Q Exactive GC Orbitrap Mass Spectrometer via a micro channel device (MCD). The split ratio can be adjusted by the length of transfer fused silica capillary from the MCD to the ion source of the Q Exactive GC Orbitrap Mass Spectrometer.

For Q Exactive GC Orbitrap Mass Spectrometer analysis compounds are directly introduced into the EI ion source by a transfer line whereas the portion transferred to the IRMS was firstly combusted into CO<sub>2</sub> and H<sub>2</sub>O. After online purification of the sample gas and removal of water by the GC IsoLink Preparation Unit, CO<sub>2</sub> is admitted into the IRMS.

### Measurement principles

The IRMS methodology for doping control comprises the comparison of the  $\delta^{13}\text{C}$  values of target compounds (TC) and endogenous reference compounds (ERC's). TC are the group of exogenous anabolic steroid or its metabolites exhibiting depleted  $\delta^{13}\text{C}$  values while the ERC's are not affected and typically enriched in their corresponding  $^{13}\text{C}/^{12}\text{C}$  ratio. WADA has established in general a minimum of 3 ‰ difference between any ERC and TC to constitute an adverse analytical finding.



## RESULTS

The temporal progress of the  $\delta^{13}\text{C}$  values are shown in Figure 2. The ERCs exhibit nearly constant  $\delta^{13}\text{C}$  values with small variation. The plot reveals the largest deviation of the  $\delta^{13}\text{C}$  values of the TCs from ERCs between 2 and 6 hours.

The data demonstrate 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 2. The  $\delta^{13}\text{C}$  (‰) values of the target compounds testosterone (TESTO) and its main metabolites 5a-androstane-3a,17b-diol (5a DIOL) and 5b-androstane-3a,17b-diol (5b DIOL). In doping control, pregnenediol (PD), 5a-androst-16-en-3b-ol (16EN) or 11-ketoetiocholanolone (KETO) are commonly selected as endogenous reference compounds (ERC).

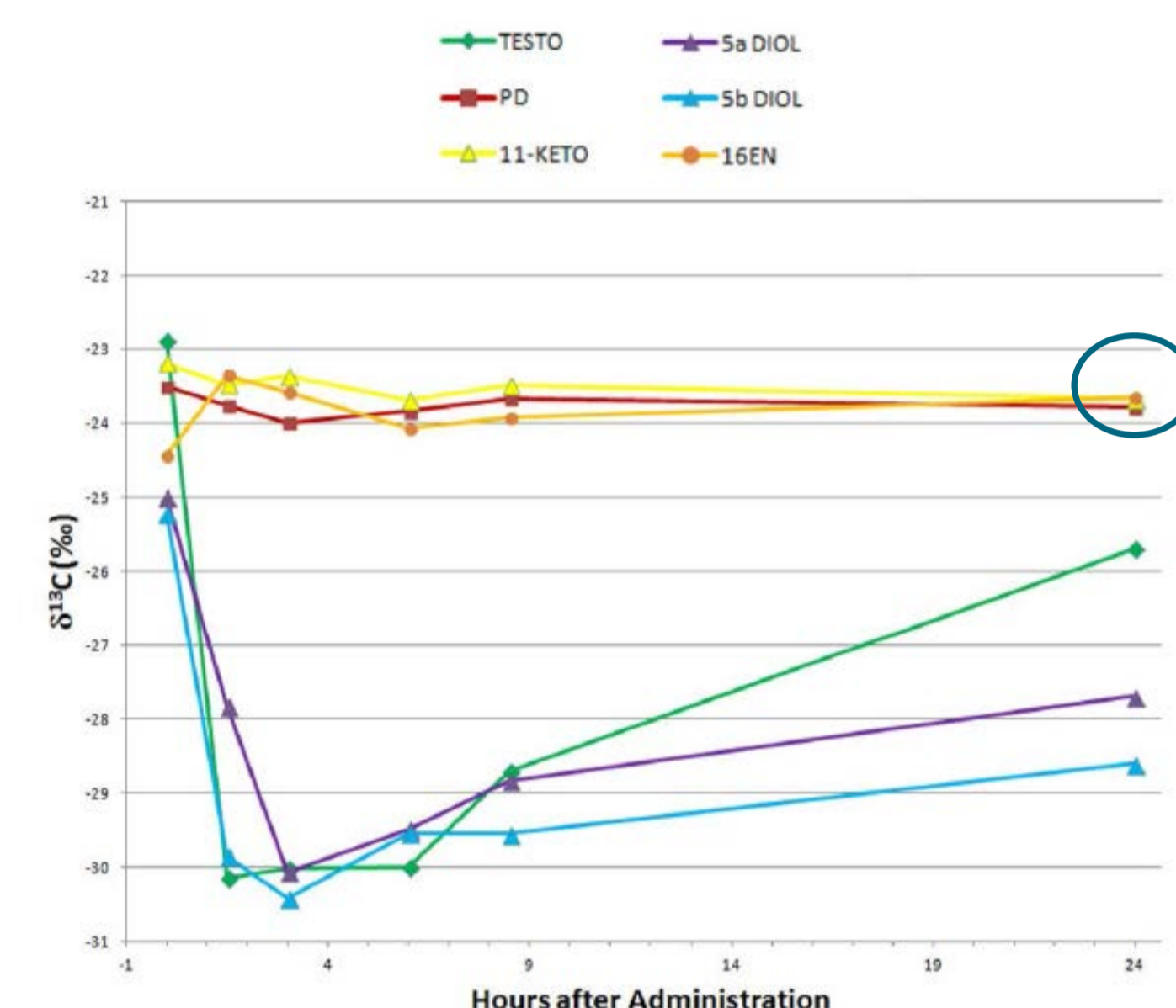


Figure 3a. IRMS Chromatogram (m/z 44 Trace) and Ratio Trace 45/44 of TESTO Fraction (24 hours post-administration).

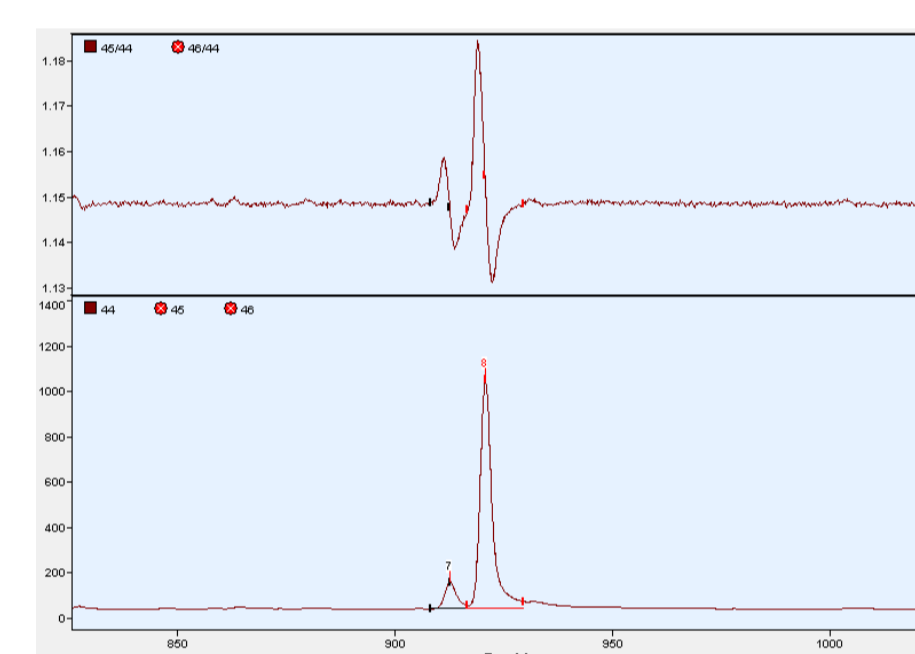


Figure 3c. Comparison of Q Exactive GC Orbitrap GC-MS/MS spectra of peak at 14.94 min (TESTO Fraction, 24 hours post-administration) with first hit of NIST database.

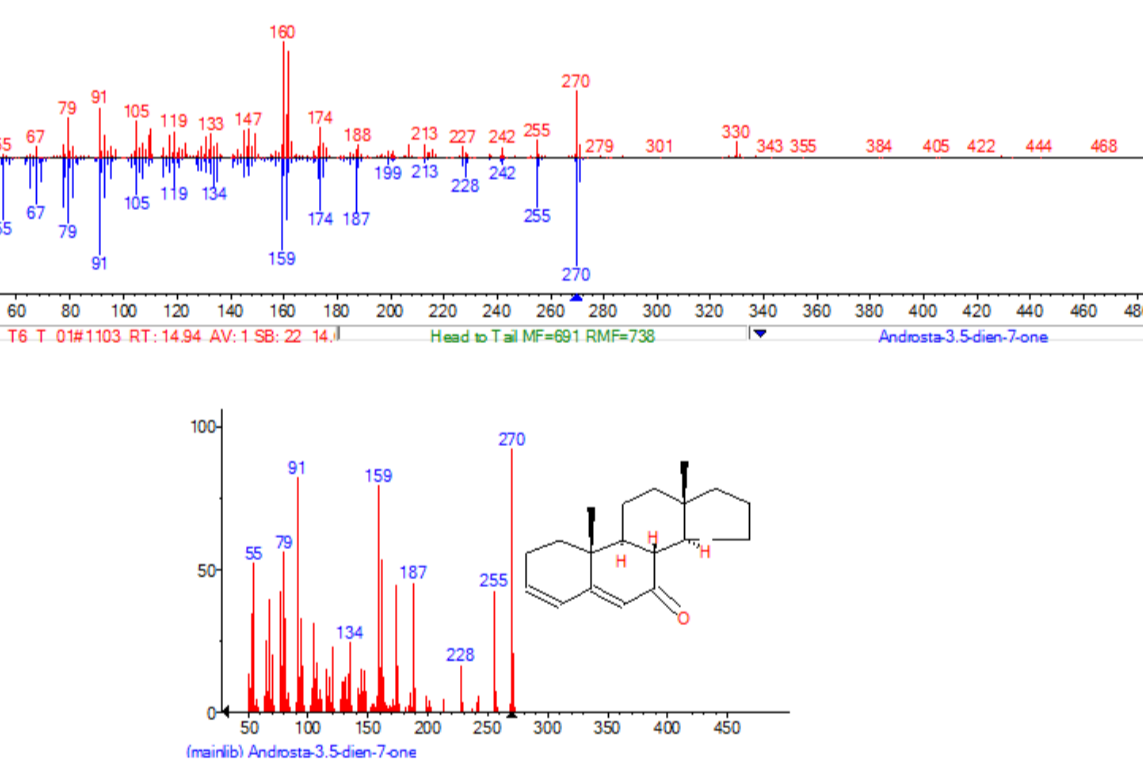


Figure 3b. Q Exactive GC Orbitrap GC-MS/MS Total ion chromatogram of TESTO Fraction (24 hours post-administration).

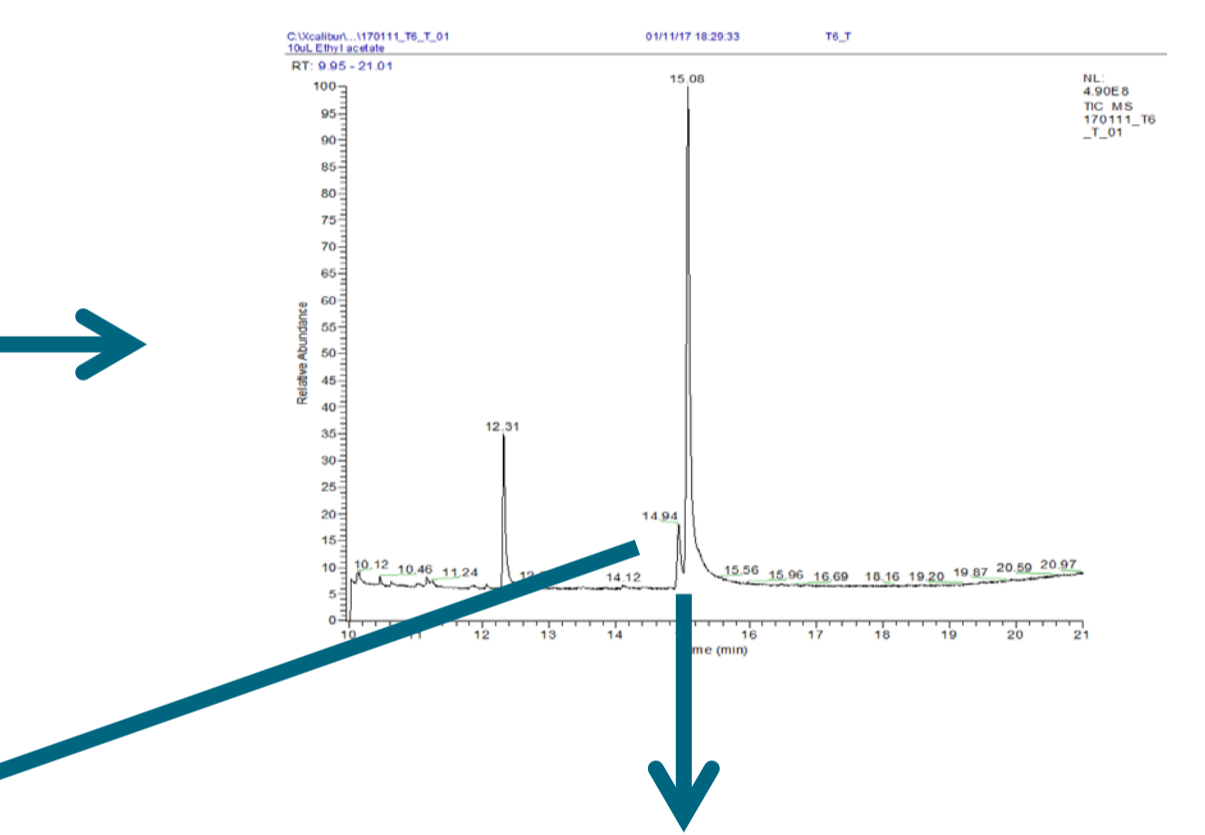
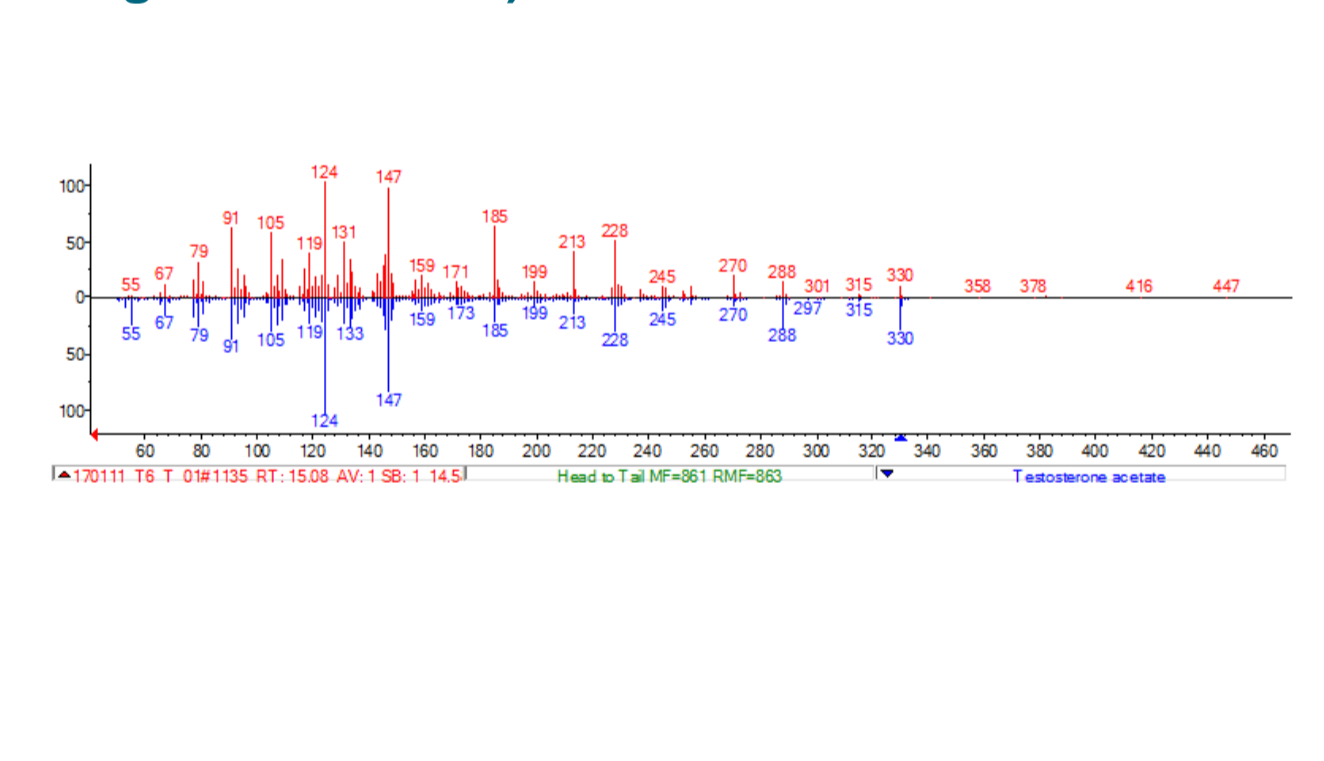


Figure 3d. Comparison of Q Exactive GC Orbitrap GC-MS/MS spectrum of peak at 15.08 min (TESTO Fraction, 24 hours post-administration) with a NIST spectrum of testosterone, Full MS Mode (m/z range: 50-600 amu).



## CONCLUSIONS

Combining the Thermo Scientific™ GC IsoLink™ IRMS System with the Q Exactive GC Orbitrap GC-MS/MS provides a refined methodology for doping control. Simultaneous analysis by a HRAM GC/MS system fulfills the demand for high resolution and accurate mass 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.

## REFERENCES

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## TRADEMARKS/LICENSING

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