Application Note

Doping Control

Agilent

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Extend the time window for the detection of low level anabolic-androgenic steroids and their metabolites

Steroid profiling, screening, and confirmatory analysis using the Agilent 7010 GC/MS mass spectrometer

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Introduction

Doping laboratories want to have lower detection limits to be able to identify and quantitate illegal drugs for a longer time window between doping and urine sample collection. This Application Note describes how the doping control lab in Kreischa, Germany used the Agilent 7010 Triple Quadrupole GC/MS System (TQ) for enhanced targeted detection of anabolic-androgenic steroids in urine samples. The 7010 TQ enabled higher sensitivity, longer maintenance-free intervals, and more stable operation than earlier models, and satisfied all measurement specifications required by the World Anti-Doping Agency (WADA). The 7010 TQ GC/MS ion source produces more ions, and provides exceptional sensitivity for the detection of low intensity metabolites weeks after drug ingestion. However, this higher sensitivity may lead to saturation of endogenous components naturally present at higher concentrations. This method provides a sensitive and robust operation over a wide concentration range, permitting the detection of low-concentration exogenous species in the presence of high-concentration endogenous species, which can vary with ethnicity.

Experimental

Instrument configuration

Figure 1. The Agilent 7010 GC/TQ system consists of an Agilent 7890 GC with multimode inlet and backflush, an Agilent 7993A, and the Agilent 7010 TQ mass spectrometer.

Columns and backflush configuration

The optimum chromatographic resolution and robustness of the system was obtained using a 1 m precolumn and a 10 m × 0.18 mm, 0.18 µm analytical column with dimethyl polysiloxane phase. Backflush occurs at the end of the 1 m precolumn. The backflush system enables the removal of residual matrix components by reversing the column flow and backflushing the heavy contaminants from the system. The removal of high-boiling matrix components reduces contamination build-up in the column and in the ion source. This results in longer maintenance-free intervals and fewer column changes. The lab in Kreischa still uses the first column and, after 6,000 sample injections, has not been required to clean the ion source.

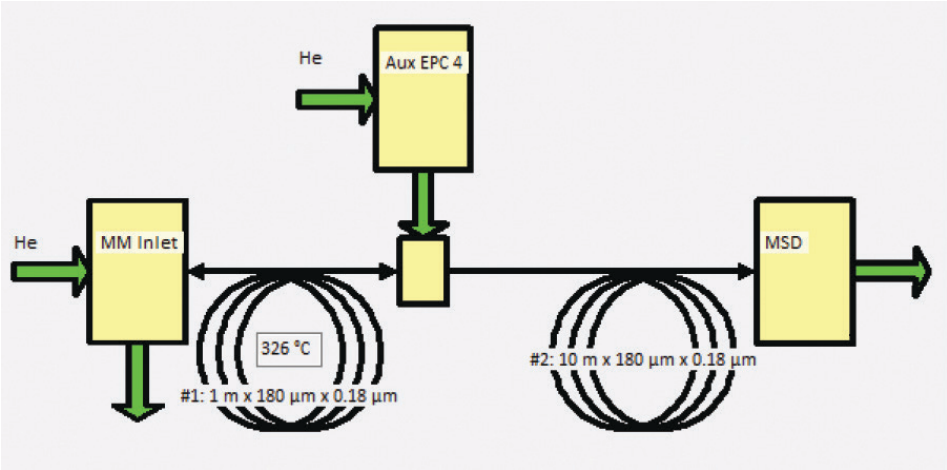


Figure 2. Backflush setup.

Table 1. Instrument parameters.

Parameter	Value
Injection volume	0.4 µL
Inlet: MMI	260 °C
Injection mode	Split, 15:1
Column	Col 1: 1m Col 2: 10 m Both 0.18 mm id, 0.18 µm film thickness, dimethyl polysiloxane
Carrier gas	Col 1: 0.885 mL/min He Col 2: 1.083 mL/min He
Backflush starts	16.5 minutes
Oven temperature	130 °C, hold for 0 minutes
	56°C/min to 186 °C, 2 °C/min to 206 °C 5 °C/min to 221 °C, 35 °C/min to 326 °C, hold for 1.5 minutes
Transfer line temperature	280 °C
Ionization mode	EI
Source temperature	230 °C
Electron energy	Variable by time segment, 70 eV or 130 eV
Detector gain	1.0
Quadrupole temperature	150 °C

MS Method—extending the dynamic range

With the increased sensitivity of the 7010 MS, lower injected amounts (0.4 µL in this study) can be used. The gain factor controlling the electron multiplier voltage should be set at 1.0 instead of 10, or the higher values common for an Agilent 7000B.

Several optimization steps were performed to benefit from the higher sensitivity for low-abundance compounds while not saturating the signals of high-abundance compounds. These steps allowed detection of long-term excreted metabolites of exogenous anabolics and endogenous markers androsterone and etiocholanolone. The high-abundance and low-abundance species were segregated into different chromatographic time windows. To control the signal intensity of the high-abundance compounds and maintain dynamic range, the electron energy was increased from 70 eV to 130 eV. This increase reduced the signal intensity to 10–20 % of the original response (Figure 3). When switching eV, the filament is instantaneously stabilized, without impacting data quality or filament lifetime.

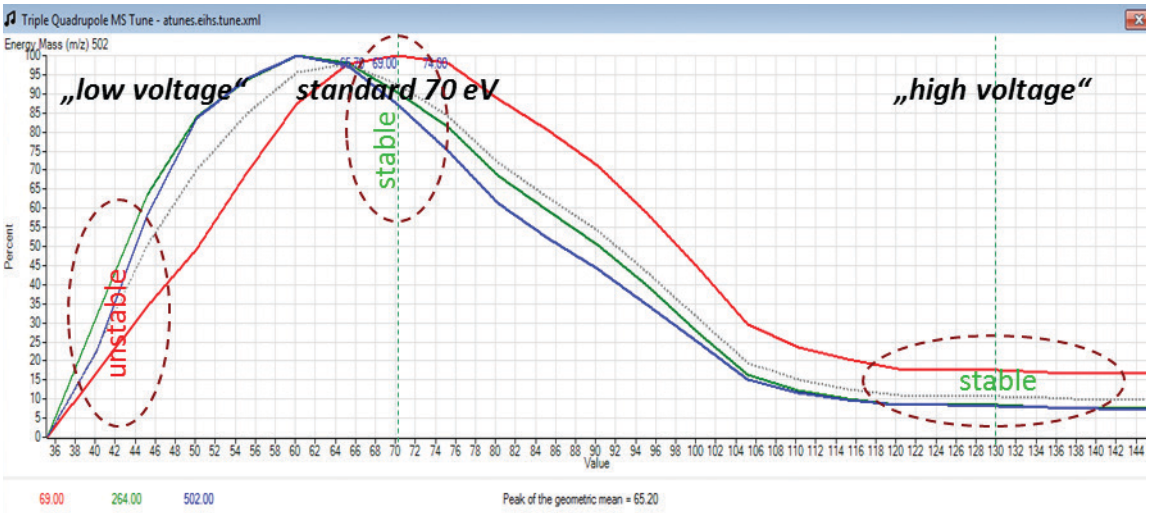


Figure 3. As the result of varying electron energy from 35 eV to 145 eV, signal intensity and stability changes. At 130 eV, the signal was stable, and produced approximately 10–20 % of the response obtained at the standard 70 eV operation, eliminating the saturation of the high-concentration species. Note that at 35 eV, the signal is significantly reduced, but the response is not stable. This is characteristic for most filaments.

The electron energy was increased between 10.2–11.7 minutes, where the high-intensity species eluted (Figure 4). This eliminated signal saturation, and expanded the linear dynamic range while yielding reproducible results. Using this special feature of the Agilent MassHunter acquisition software, enhanced and suppressed detection can be combined in one run (Figures 5A and 5B).

To gain the most accurate results, suitable stable isotope-labeled internal standards such as deuterated etiocholanolone, androstanediol, epitestosterone, and testosterone were used for the most critical analytes to efficiently compensate for undesired matrix effects.

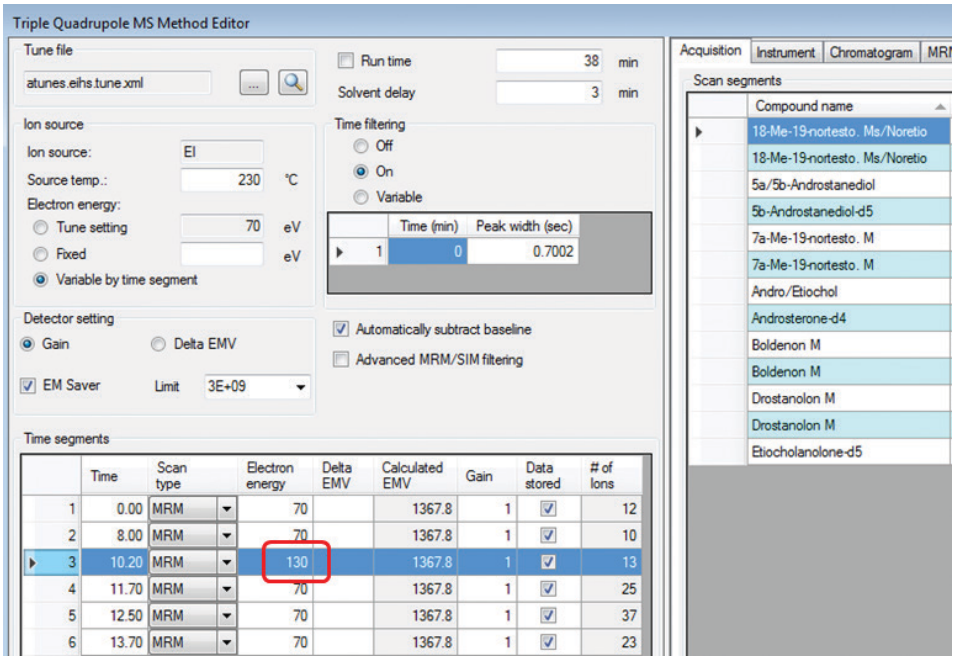


Figure 4. The electron energy was increased only in the chromatographic time window from 10.2–11.7 minutes, where the high-intensity species eluted.

Results and discussion

Meeting WADA requirements

Using the high, 130 eV approach, the linear dynamic range for androsterone and etiocholanolone could be extended by a factor of approximately five compared to the default 70 eV operation (Figure 6). Despite the lower on-column sample amount (only 0.4 µL of sample at a split rate of 15:1 was injected) the performance targets required by WADA can be fulfilled[2].

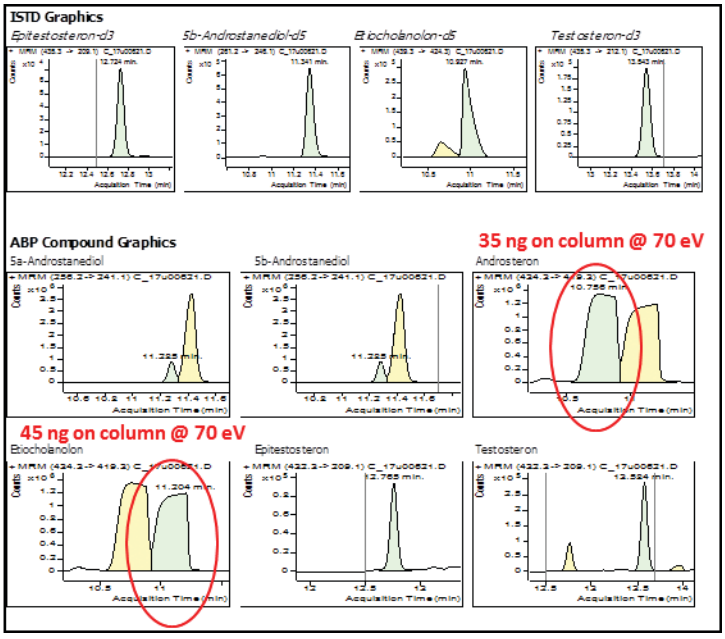


Figure 5A. The endogenous steroids androsterone and etiocholanolone may be present in urine at high concentrations, resulting in chromatographically overloaded, saturated peaks, hindering the initial testing procedure (screening).

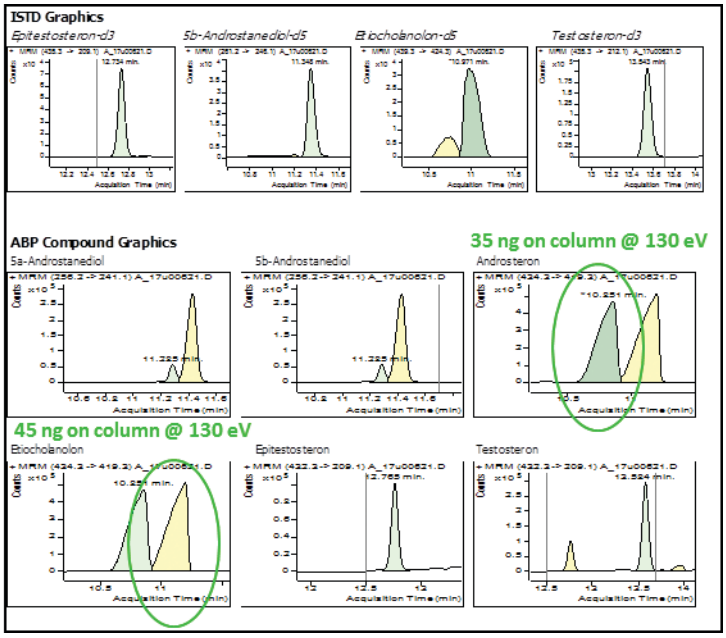


Figure5B. Suppressed response at 130 eV electron energy prevents saturation, and gives the expected look for chromatographically overloaded peaks.

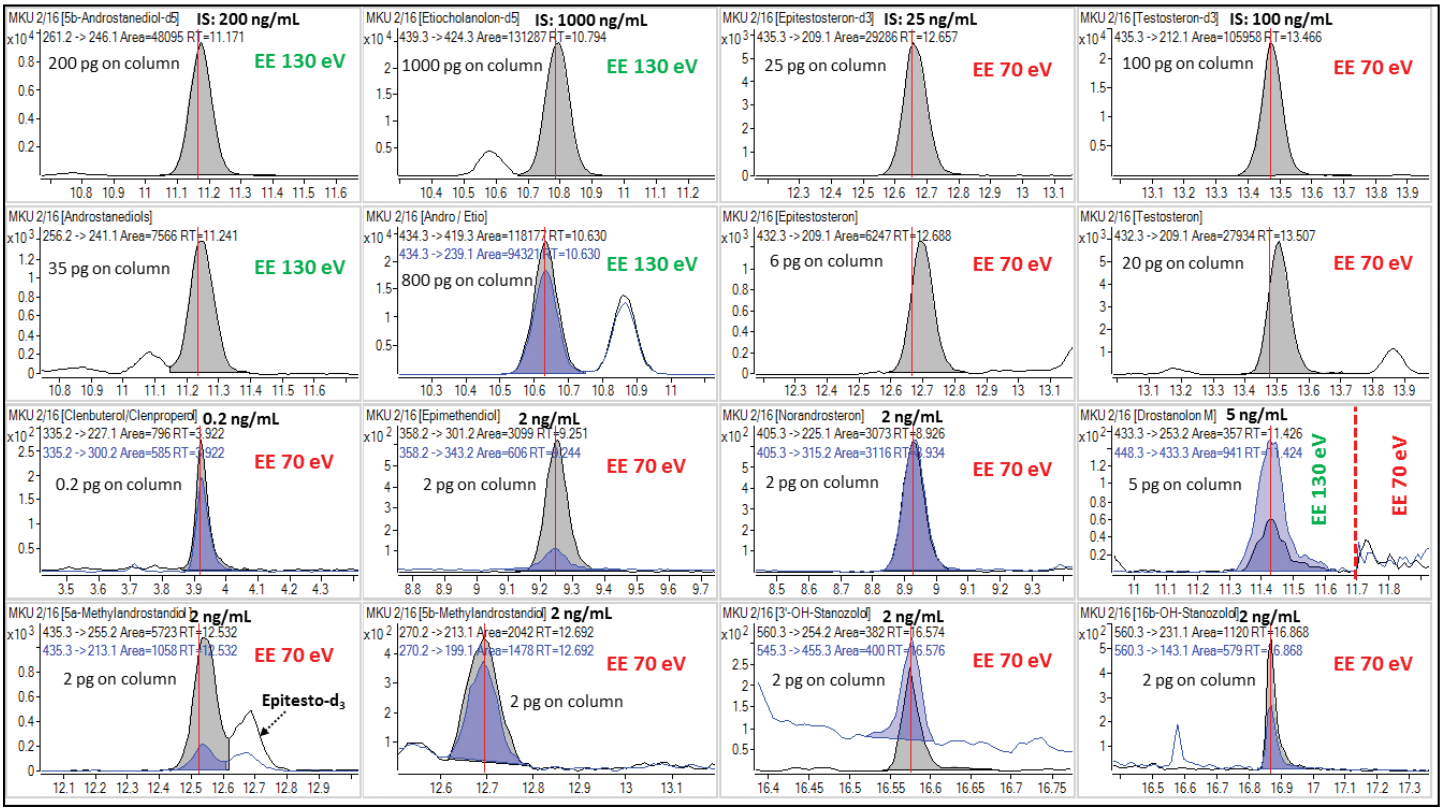
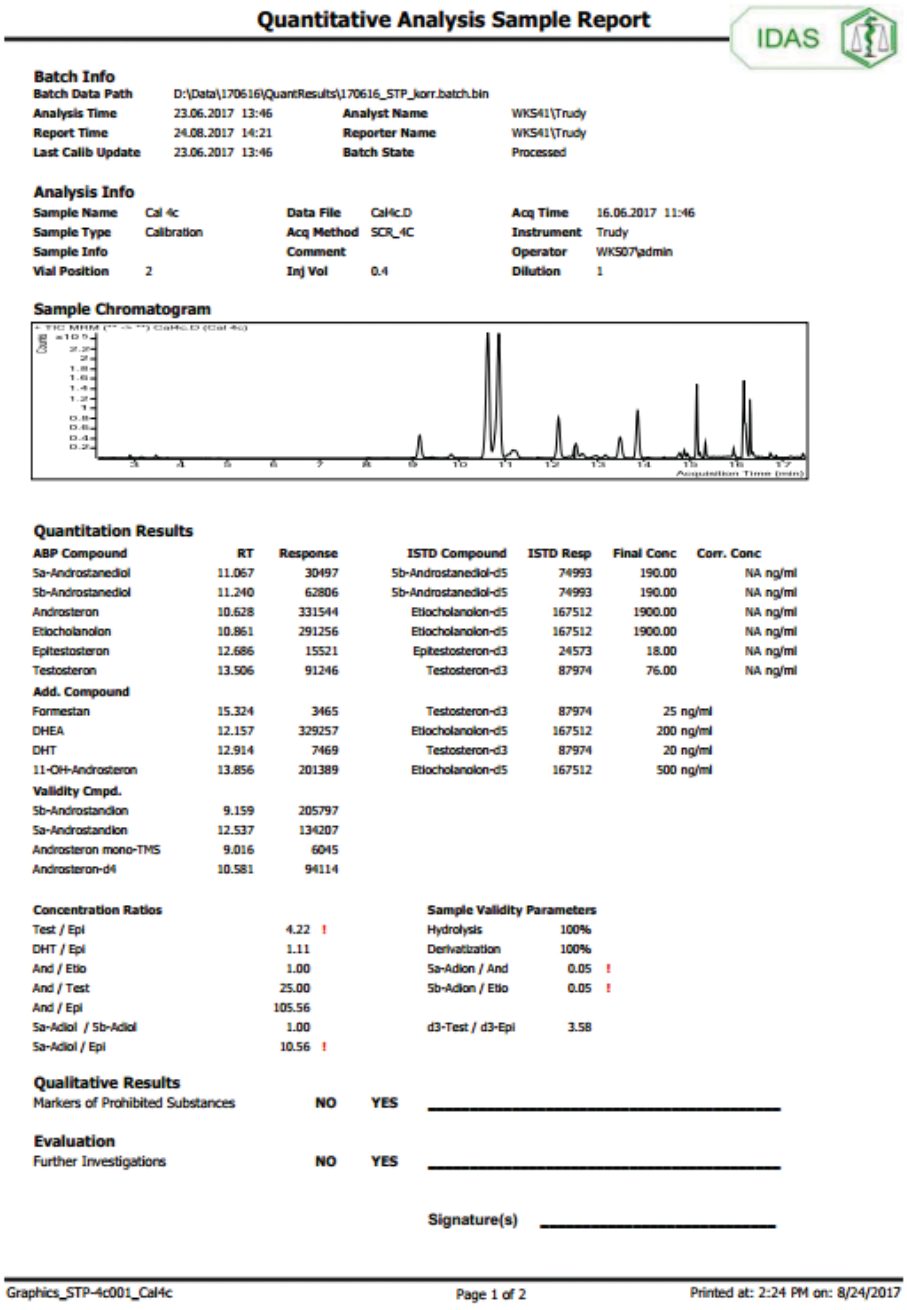


Figure 6. This is a positive control sample showing that the developed screening method is fit-for-purpose, fulfilling the WADA Minimum Required Performance Limits (MRPLs) at 0.2–2 ng/mL for the exogenous and 200–1,000 ng/mL for the endogenous species in the same run.

Data review and reporting: initial testing procedure

The screening results evaluation was performed in two steps using different features of the Agilent MassHunter Quantitative Analysis software:

1. All endogenous markers to be quantified are analyzed using a single-point-calibration method. The integration quality of all relevant peaks is checked on-screen before the sample-template-based report is created and automatically uploaded into the LIMS system (Figure 7A).
2. All qualitative markers are reviewed using the Compounds-at-a-Glance feature of MassHunter fast on-screen recognition (Figure 7B). This is followed by printing and report archival using the Sample report template.



Quantitative Analysis Sample Report

IDAS

Batch Info

Batch Data Path	D:\Data\170616\QuantResults\170616_STP_korr.batch.bin		
Analysis Time	23.06.2017 13:46	**Analyst Name**	WKS41\Trudy
Report Time	24.08.2017 14:21	**Reporter Name**	WKS41\Trudy
Last Calib Update	23.06.2017 13:46	**Batch State**	Processed

Analysis Info

Sample Name	Cal 4c	**Data File**	Cal4c.D	**Acq Time**	16.06.2017 11:46
Sample Type	Calibration	**Acq Method**	SCR_4C	**Instrument**	Trudy
Sample Info		**Comment**		**Operator**	WKS07\admin
Vial Position	2	**Inj Vol**	0.4	**Dilution**	1

Sample Chromatogram

Quantitation Results

ABP Compound	RT	Response	ISTD Compound	ISTD Resp	Final Conc	Corr. Conc
5a-Androstanediol	11.067	30497	5b-Androstanediol-d5	74993	190.00	NA ng/ml
5b-Androstanediol	11.240	62806	5b-Androstanediol-d5	74993	190.00	NA ng/ml
Androsteron	10.628	331544	Etiocholanolon-d5	167512	1900.00	NA ng/ml
Etiocholanolon	10.861	291256	Etiocholanolon-d5	167512	1900.00	NA ng/ml
Epitestosteron	12.686	15521	Epitestosteron-d3	24573	18.00	NA ng/ml
Testosteron	13.506	91246	Testosteron-d3	87974	76.00	NA ng/ml
Add. Compound						
Formestan	15.324	3465	Testosteron-d3	87974	25 ng/ml	
DHEA	12.157	329257	Etiocholanolon-d5	167512	200 ng/ml	
DHT	12.914	7469	Testosteron-d3	87974	20 ng/ml	
11-OH-Androsteron	13.856	201389	Etiocholanolon-d5	167512	500 ng/ml	
Validity Cmpd.						
5b-Androstandion	9.159	205797				
5a-Androstandion	12.537	134207				
Androsteron mono-TMS	9.016	6045				
Androsteron-d4	10.581	94114				

Concentration Ratios		Sample Validity Parameters	
Test / Epi	4.22 !	Hydrolysis	100%
DHT / Epi	1.11	Derivatization	100%
And / Etio	1.00	5a-Adion / And	0.05 !
And / Test	25.00	5b-Adion / Etio	0.05 !
And / Epi	105.56		
5a-Adiol / 5b-Adiol	1.00	d3-Test / d3-Epi	3.58
5a-Adiol / Epi	10.56 !		

Qualitative Results

Markers of Prohibited Substances **NO** **YES** _____

Evaluation

Further Investigations **NO** **YES** _____

Signature(s) _____

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Figure 7A. Example screening report showing the relevant markers of the endogenous steroid profile.

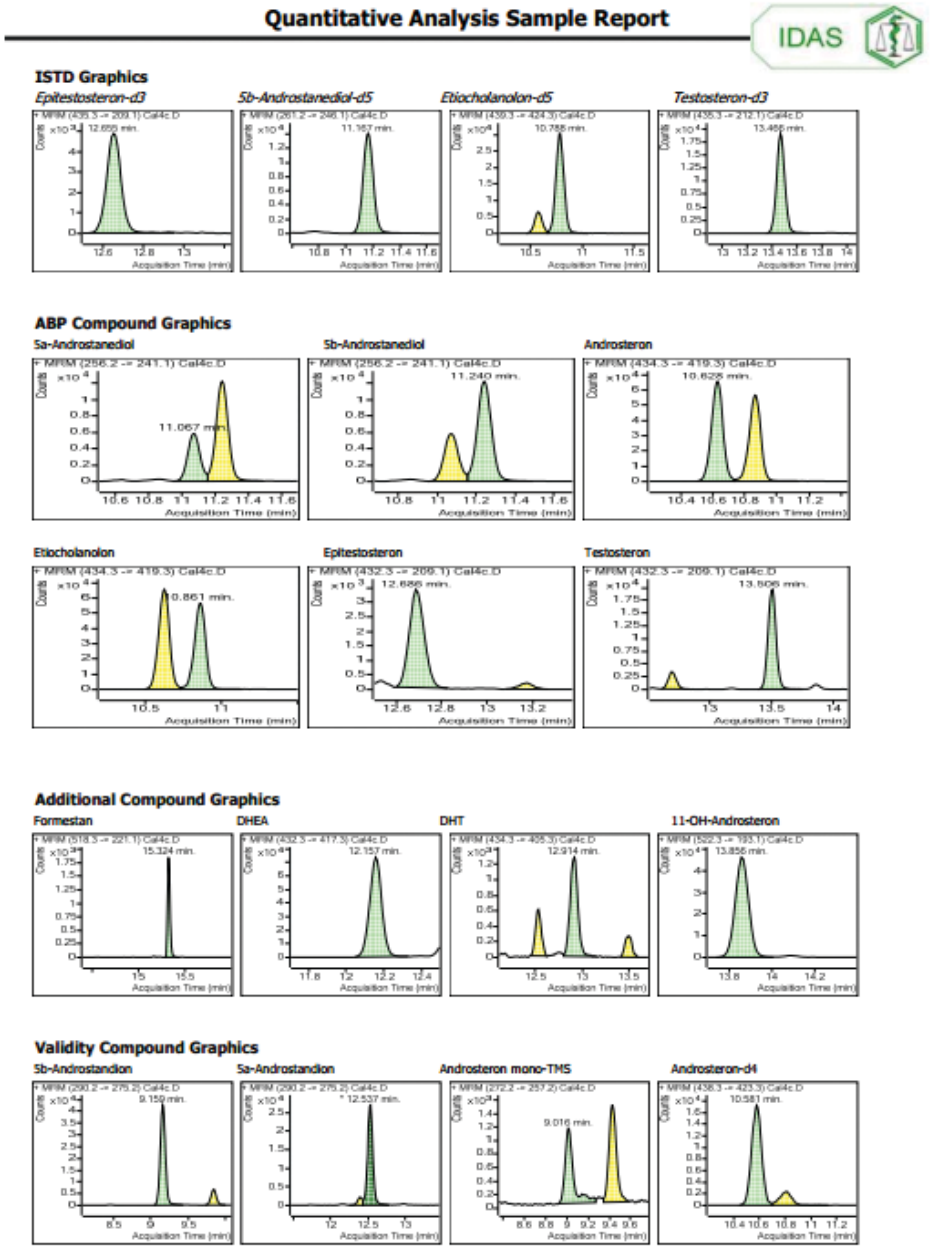

Figure 7B. Example screening report showing the relevant markers of the endogenous steroid profile.

Steroid profile confirmation procedure

The final test report of a sample on the WADA platform Anti-Doping Administration and Management System (ADAMS) includes the steroid profile variables. It requires the evaluation of the steroidal module of the Athlete Biological Passport program for consistency with respect to population-based or individual reference ranges. In case of deviation, the system automatically triggers a Confirmation Procedure Request[3]. For this purpose, a four-level calibration is used. It is necessary to determine the nonconjugated moiety of testosterone as an additional marker of a microbial activity present in the urine sample. The following example demonstrates the usability of the method even for low-testosterone excreters, typical for Asians and approximately 10 % of Caucasians[4]. The combined steroid fraction is obtained after enzymatic hydrolysis, and covers the glucuronides and the nonconjugated (free) moiety (Figures 8 and 9). To isolate the free steroid fraction, no hydrolysis was applied.

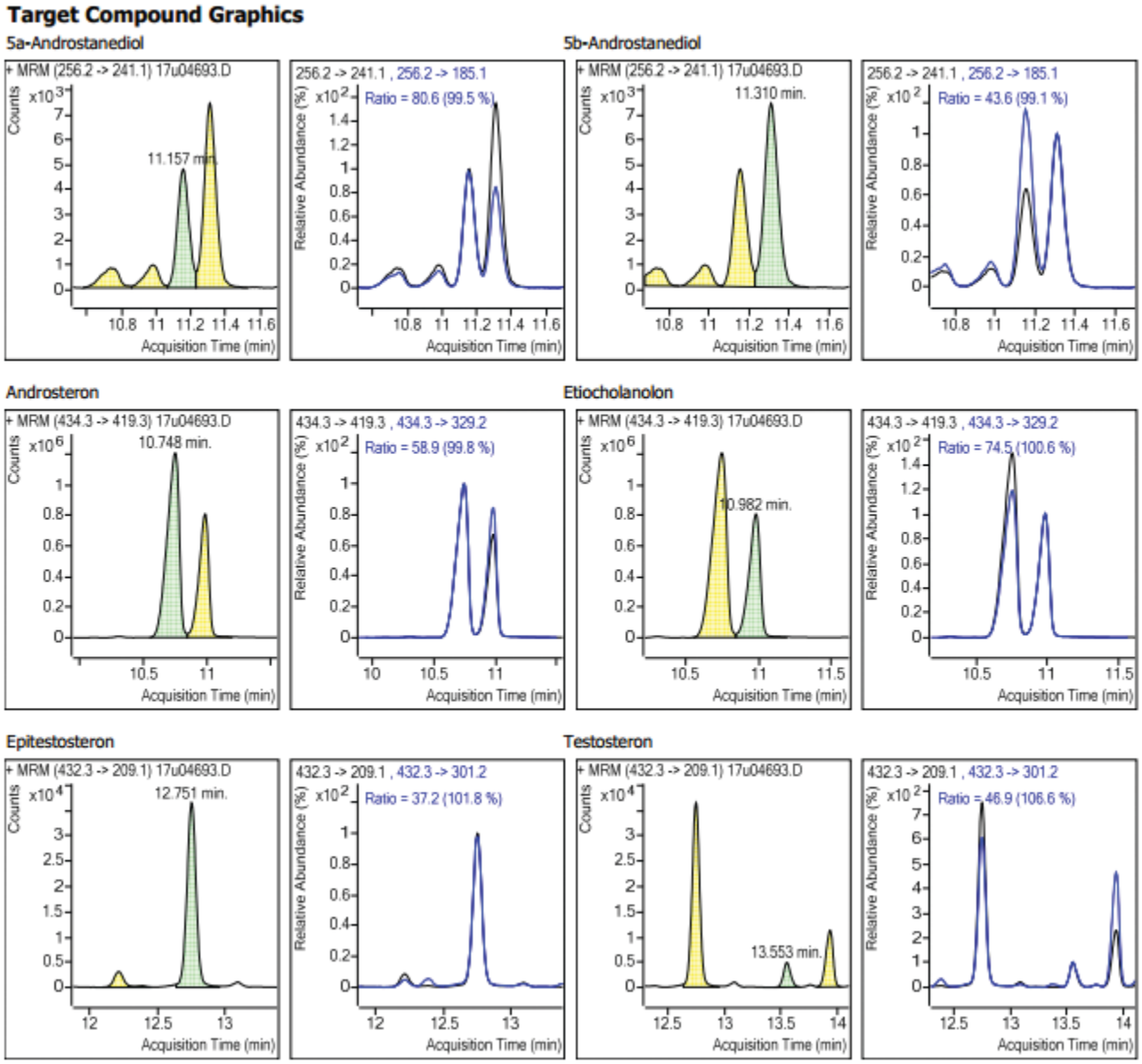

Figure 8. A sample's combined steroid fraction containing 6.95 ng/mL testosterone (RT 13.553 minutes) and 44.79 ng/mL epitestosterone (RT 12.751 minutes).

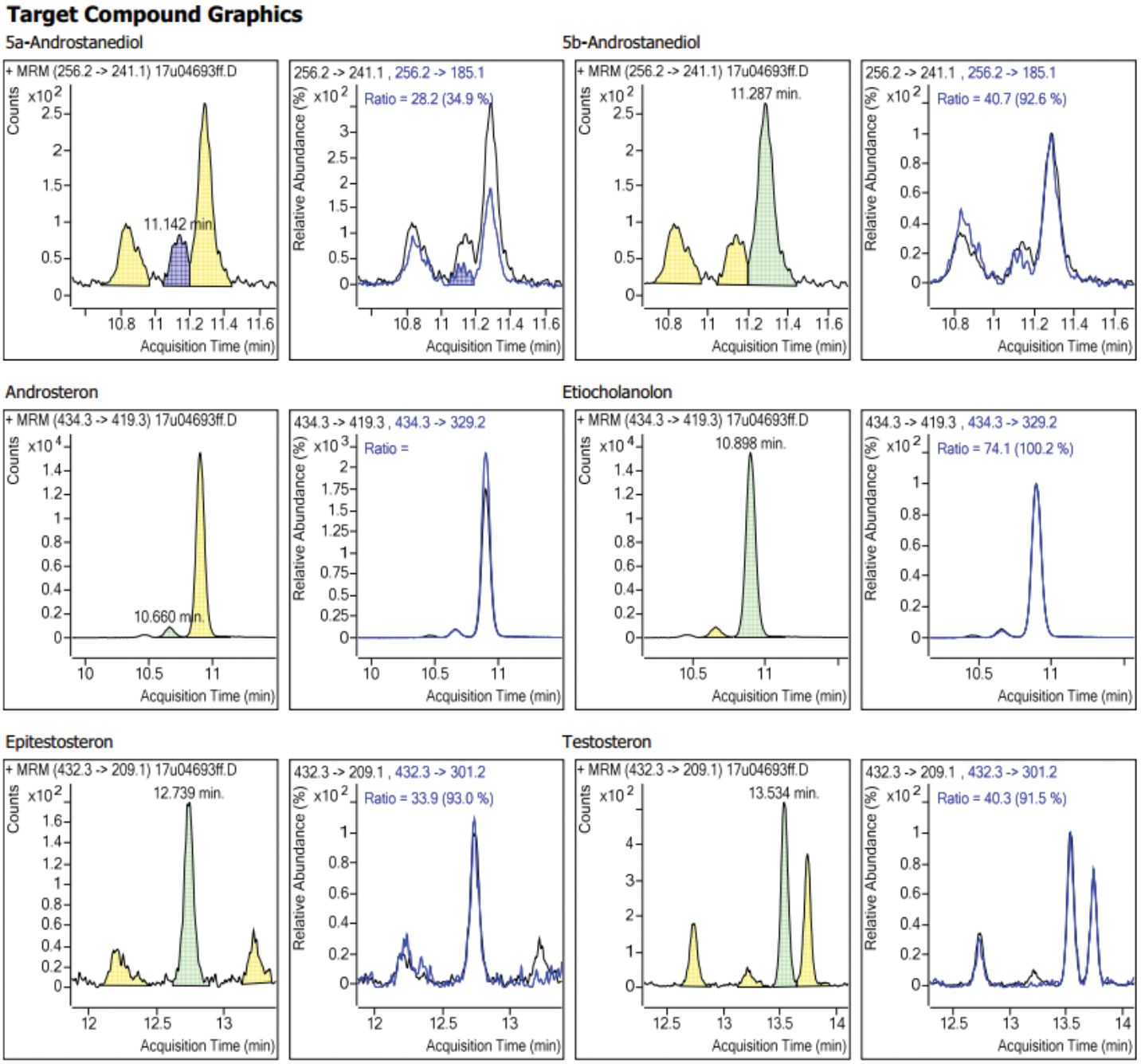

Figure 9. The same sample's free steroid fraction containing 0.70 ng/mL testosterone (RT 13.534 minutes) and 0.31 ng/mL epitestosterone (RT 12.739 minutes).

Conclusions

Based on the method optimization and thorough testing at the Kreischa lab, an Agilent 7010 GC/MS-based protocol was developed. This protocol fulfills the screening and confirmation requirements of steroid measurements in doping control labs. With this method, it is possible to detect traces of exogenous anabolic steroid metabolites while quantifying small concentrations of testosterone and epitestosterone typical in Asian populations. It permits the measurement of high-abundance endogenous steroids such as androsterone and etiocholanolone in the same injection, while analyzing testosterone/epitestosterone and androsterone/etiocholanolone ratios without suppression.

The backflush capability of the system protects the analytical column, and consequently the mass spectrometer, by removing heavy matrix components. This allowed more than 6,000 samples to be analyzed on the same column without requiring ion source cleaning or filament change. It also demonstrated that the higher, 130 eV electron energy did not sacrifice the filament lifetime. It performed reliable measurements of both trace (0.2 pg on-column) and high (10,000 pg on-column) concentration analytes in the same run.

The method validation revealed that the exceptional sensitivity offered by the 7010 GC/MS can be used to:

- Improve the trace detection ability of the method
- Reduce the amount of sample injected
- Extend maintenance-free intervals

References

1. D. Thieme, C. Rautenberg, J. Grosse, M. Schoenfelder. Significant increase of salivary testosterone levels after single therapeutic transdermal administration of testosterone: suitability as a potential screening parameter in doping control. *Drug Test. Anal*. **2013**, *5*, 819.
2. WADA. Technical Document TD2017MRPL, Available at: https://www.wada-ama.org/en/resources/science-medicine/td2017-mrpl-0 (17 October **2017**).
3. WADA. Technical Document TD2016EAAS, Available at: https://www.wada-ama.org/en/resources/science-medicine/td2016-eaas (17 October **2017**).
4. J. Jakobsson, L. Ekström, N. Inotsume, M. Garle, M. Lorentzon, C. Ohlsson, H-K. Roh, K. Carlström, A. Rane. Large differences in testosterone excretion in Korean and Swedish men is strongly associated with an UDP-glucuronosyl transferase 2B17 polymorphism. *J. Clin. Endocrinol. Metab*. **2006**, *91*, 687.

Complete analytical methodology including GC and MS configuration and methods with an analyte list and MS/MS conditions is available from the Doping Control Segment Marketing Manager, at doping_control@agilent.com.

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