Application Note

Forensics and Toxicology

Introducing Semi-Automated GC/Q-TOF Screening with the AssayMAP Bravo Sample Prep Platform for Antidoping Control

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Abstract

In doping control, there is a continuously growing demand for high sensitivity of the analytical method as well as for screening approaches with necessary throughput, that would provide the possibility for retrospective analysis. Apart from the choice of highly sensitive analytical instrumentation, the limits of detection (LOD) can be further decreased by subsequent improvements to the method, including automated solid phase extraction (SPE). This application note describes a workflow combining automated sample preparation using the Agilent AssayMAP Bravo sample prep platform with a GC quadrupole time-of-flight (GC/Q-TOF) mass spectrometer (MS) to complement traditionally used GC triple quadrupole (GC/TQ) MS systems. This combination provides doping control laboratory analysts with superior efficiency and confidence in their results, along with the possibility to perform retrospective analysis.

Introduction

To maintain World Anti-Doping Agency (WADA) accreditation, doping control laboratories strive to achieve high sensitivity, selectivity and reproducibility, as well as quantitative capability of the analytical method. There is additionally an ever-increasing demand for reliable screening approaches that readily enable retrospective analysis.[1]

The WADA List of Prohibited Substances and Methods includes both endogenous and exogenous substances. Endogenous substances (mostly endogenous androgenic anabolic steroids (EAAS)) must be quantitated from very low (<1 ppb) to very high (>10 ppm) concentrations. The exogenous substances represent a wide variety of compound classes, such as (exogenous) androgenic anabolic steroids (AAS), anabolic agents, peptides, growth factors, beta-2 agonists, hormones and metabolic modulators, diuretics and other masking agents, stimulants, narcotics, cannabinoids, and beta-blockers, among others. This prohibited substance list becomes longer every year, and therefore analytical methods should always be updated to be able to screen for more compounds.

For every compound class (and sometimes an individual substance) WADA has set a Minimum Required Performance Level (MRPL). A screening method or Initial Testing Procedure (ITP) should be able to detect these exogenous substances at a half of the MRPL, which typically go down year after year.

GC/MS is an essential tool in doping control that is complimentary to the LC/MS technique, and can cover up to over 400 compounds out of approximately 600 screened for in urine.

The LC/MS technique suffers from a lack of ionization efficiency for many AAS metabolites, and does not offer sufficient chromatographic resolution to separate many stereoisomers of doping agents. Therefore, for the quantification of the endogenous steroid profile, WADA now relies exclusively on GC/MS, where GC ITP methods should be able to accurately quantify EAAS at concentrations over 10 ppm, as well as in the low ng/ml range. GC triple quadrupole systems are extensively used in doping analysis applications, and are highly valued for their unsurpassed sensitivity and wide dynamic range, making them an instrument of choice for targeted analysis and quantitation.[2] However, even with modern GC/TQ instrumentation, there is a limit to the number of compounds that can practically be analyzed in a method. The increased number of samples puts pressure on scientists to develop faster chromatographic methodologies, which also puts pressure on the number of compounds that can be analyzed. Moreover, many of the AAS metabolites undergo a very strong fragmentation in an EI source, and thus are not very suitable for MS/MS. In contrast, high resolution accurate mass time-of-flight (TOF) instruments do not have a restriction on the number of compounds monitored in a single run, and are capable of high sensitivity, wide dynamic range in complex matrices, as well as retrospective analysis.

Hence, a combination of two GC ITP methods is suggested: one that uses the GC/TQ equipped with PTV injector for large volume injection (for the most demanding AAS, and a few other compounds with very low MRPLs), and a second one for a broader range of compounds with higher MRPLs (such as stimulants, narcotics, beta-agonists, hormone modulators, and diuretics) as well as some AAS with highly fragmented spectra, taking advantage of the high-resolution GC/Q-TOF.

WADA statistics show that AAS have remained among the most-detected doping substances over past decades (approximately 50% of all findings). Over the last few years, several "long-term metabolites" of AAS have been discovered. These metabolites are excreted from the body over a much longer time, and often present at lower concentrations than classic metabolites.

Sulfation plays an important role in the formation of these long-term metabolites, and until recently, LC/MS/MS was assumed to be the only technique to detect the sulfated metabolites of AAS. Recently, the GC/MS detection of these sulfates has also been demonstrated, made possible by cleaving off the sulfate group in a hot injector.[4,5] This provides higher resolving power and additional structural information compared to LC/MS/MS, which can only detect the loss of the sulfate group.

The challenge with the GC/MS-based approach is sample preparation, which can either be performed using a liquid-liquid extraction (LLE) with ethyl acetate, or with a solid phase extraction (SPE). SPE has a higher extraction recovery, and, with an adequate rinsing procedure, also leads to cleaner extracts. In addition, SPE is more amenable to automation. One powerful platform for automated SPE-based sample preparation is the Agilent AssayMAP Bravo system. The AssayMAP Bravo is a micro chromatography-based system that can process 1 to 96 samples in parallel, using packed resin bed cartridges that are available with a variety of surface chemistries. While the AssayMAP Bravo system is typically used as a protein sample preparation platform, this study shows that not only sulfated metabolites – but virtually all polar compounds – are extracted with significantly higher recovery, compared to the LLE method. With the miniaturization of the process, the amount of waste is reduced, and so is the environmental impact.

A unified approach using GC/TQ, GC/Q-TOF, and the AssayMAP Bravo sample prep platform is suggested to help achieve reliable screening results in a time-efficient manner for doping applications. The detection of nonhydrolyzed sulfate metabolites of steroids that can serve as long-term markers is also described.

Experimental

Samples

The samples included six calibrators (calibration curves for the endogenous steroid profile) in steroid-stripped urine, four Quality Control (QC) samples at four different levels in steroid-stripped urine, one blank water sample, one negative control urine, and 84 authentic urine samples.

Hydrolysis

Sample preparation was performed in 96-well plate format. 0.5 mL of urine was spiked with deuterated Internal Standard (IS) mixture and incubated with *E. coli* β-glucuronidase in phosphate buffer at pH 7 for at least one hour at 56 °C.

Sample extraction using automated SPE with the AssayMAP Bravo sample prep platform

Automated SPE was performed with Agilent AssayMAP 25 µL Reversed Phase (RP-S) cartridges (G5496-60023) using a modified version of the Peptide Cleanup application on the AssayMAP Bravo (Figure 1). The RPS cartridges were primed with 250 µL MeOH at 300 µL/min, then equilibrated with 100 µL 20% MeOH at 25 µL/min. The 1 mL samples were loaded onto the cartridges at 25 µL/min. The cartridges were then washed with 250 µL 20% MeOH at 25 µL/min. Two sequential eluates (first with 75 µL MeOH at 7.5 µL/min, and the second one with 75 µL ACN at 7.5 µL/min) were collected and combined.

Sample extraction using liquid-liquid extraction with ethyl acetate

Hydrolysis was performed as described in the "Hydrolysis" section, except using individual glass tubes with screw caps. The samples were extracted with ethyl acetate in alkaline conditions (pH 9.5) for 20 minutes, and the organic layer was collected.

The extracts were dried under a nitrogen stream at 40 °C, derivatized using 50 µL of a mixture of MSTFA:NH_4I:ethanethiol at 80 °C for 30 minutes.

Data acquisition and data processing

GC/MS analysis was performed using two GC/MS systems, the Agilent 7250 GC/Q-TOF and the Agilent 7000C GC/TQ. Instrument parameters are listed in Table 1.

An accurate mass Personal Compound Database and Library (PCDL) containing 320 WADA-prohibited exogenous compounds and their metabolites was created to facilitate the accurate mass GC/Q-TOF screening approach. Accurate mass EI fragments were converted to the theoretical *m/z* using Agilent MassHunter Qualitative Analysis software (version 10). The spectra were then imported into the accurate mass PCDL, using Agilent PCDL Manager software (version 8.0). Further data processing was performed using MassHunter Quantitative Analysis software (version 10.2).

Table 1. Data acquisition parameters.

MS	Agilent 7250 Q-TOF	Agilent 7000C TQ
GC	Agilent 7890 GC	
Inlet and Liner	SSL, 4 mm UI liner single taper with wool	PTV, multibaffled PTV liner
Inlet Temperature	275 °C	120 °C for 0.05 min 12 °C/sec to 360 °C
Injection Volume	1.4 µL	7 µL
Injection Mode	Splitless	Solvent vent
Columns	Agilent J&W HP-1 ms Ultra Inert, (2 m + 10 m) × 0.25 mm, 0.25 µm	Agilent J&W DB-35MS Ultra Inert, 15 m × 0.25 mm, 0.25 µm
Oven Temperature Program	110 °C for 0.1 min; 70 °C/min to 125 °C for 0.15 min, 35 °C/min to 186°C for 0.15 min, 2.2 °C/min to 204 °C, 20 °C/min to 245 °C, 50 °C/min to 270 °C, 75 °C/min to 320 °C, 1.1 min hold	110 °C for 0.25 min; 60 °C/min to 185 °C, 15 °C/min to 220 °C, 5 °C/min to 250 °C for 0.25 min hold, 55 °C/min to 330 °C, 1.4 min hold
Run Time	14.85 min	12.94 min
Carrier Gas	Helium	Helium
Column Flow	Column 1: 1 mL/min, Column 2: 1.2 mL/min	1 mL/min
Backflushing Conditions	2 minutes (post run), 320 °C (oven), 10 psi (AUX EPC pressure), 2 psi (inlet pressure)	–
Transfer Line Temperature	310 °C	310 °C
Quadrupole Temperature	150 °C	150 °C
Source Temperature	230 °C	280 °C
Ionization Mode	EI	EI
Electron Energy	70 eV	70 eV
Emission Current	5 µA	35 µA
Collision Cell Gas Flow	Nitrogen 1 mL/min, Helium 4 mL/min	Nitrogen 1.5 mL/min, Helium 2.25 mL/min
Mass Range	50 to 750 *m/z*	dMRM

Results and discussion

Advantages of automated sample preparation in doping control using the AssayMAP Bravo sample prep platform

Manual sample extraction with ethyl acetate is significantly more time-consuming and less efficient compared to automated sample preparation using the AssayMAP Bravo system. It takes about 45 minutes to evaporate 5 mL of ethyl acetate, while for 150 µL MeOH:ACN, it only takes 10 minutes. In addition, LLE involves an extra sample transfer that takes another 30 minutes. Automation also significantly decreases the hands-on time, as one can walk away from the AssayMAP Bravo during the sample preparation, whereas the manual method requires nearly continuous attention.

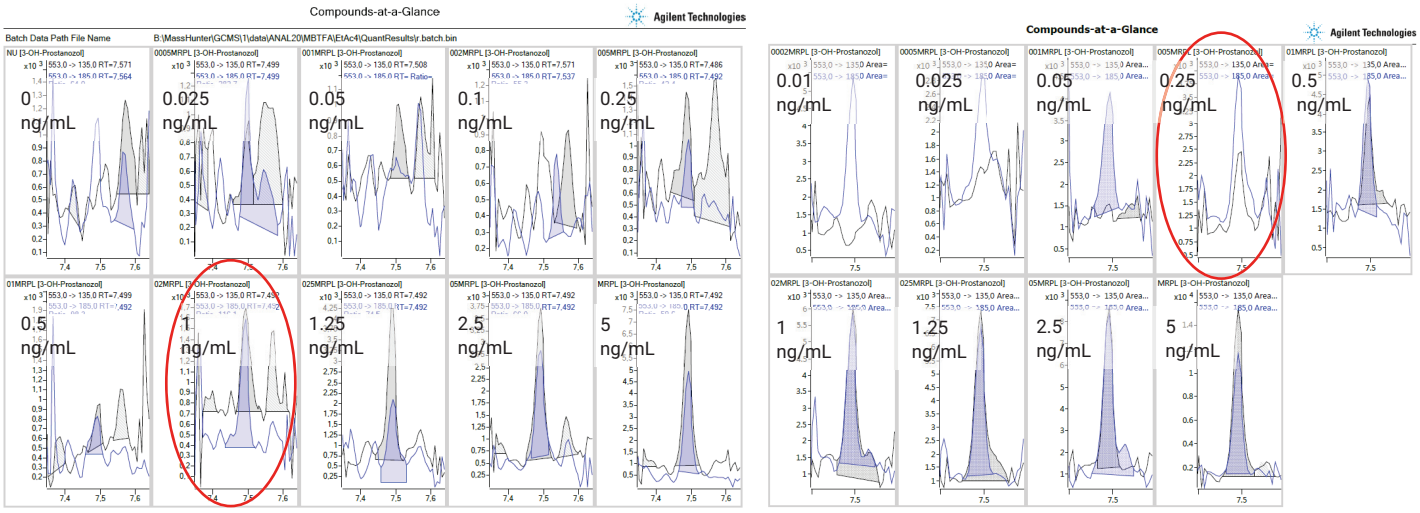
The risk of error is also significantly reduced when using automated sample preparation. Additionally, many volatile compounds (mostly stimulants) evaporate with the ethyl acetate, and have very poor extraction recoveries compared to automated SPE. Furthermore, an additional sample transfer step after adding the derivatization reagent leads to further losses.

A comparison of the detection limits of the LLE procedure (using ethyl acetate) with the AssayMAP Bravo solid phase extraction protocol was performed using both GC/Q-TOF and GC/TQ instruments, and is illustrated in Figure 2. For nonpolar compounds (such as steroids), LOD improvement when using AssayMAP Bravo sample prep platform was not very significant, from only a few percent and up to approximately four-fold. The four-fold LOD improvements were due to substantially lower background with AssayMAP extraction.

For more polar compounds (such as furosemide, for which the new LOD is less than 0.4 ng/mL) the AssayMAP Bravo extraction method helped to improve the sensitivity over 200-fold compared to the previous LLE extraction method and achieve detection of the new WADA MRPL (Figure 3).

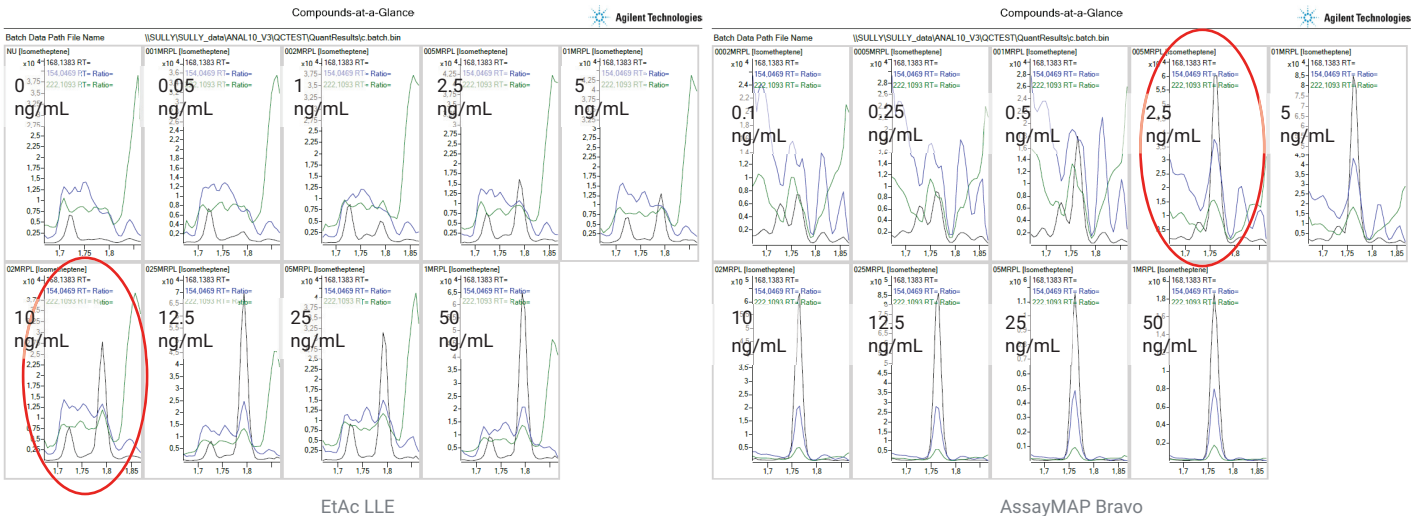
Figure 1. AssayMAP Bravo sample prep platform.

A 3-OH-prostanozol: minimum LOD 1.25 ng/mL



EtAc LLE AssayMAP Bravo

B Isometheptene: minimum LOD 50 ng/mL



EtAc LLE AssayMAP Bravo

Figure 2. Comparison of LOD when using LLE with ethyl acetate (left) and AssayMAP Bravo (right) extractions (A) for 3-OH-prostanozol analyzed by the GC/TQ, and (B) for isometheptene analyzed by GC/Q-TOF. LOD for each extraction technique is outlined in red circle.

Furosemide: minimum LOD 20 ng/mL

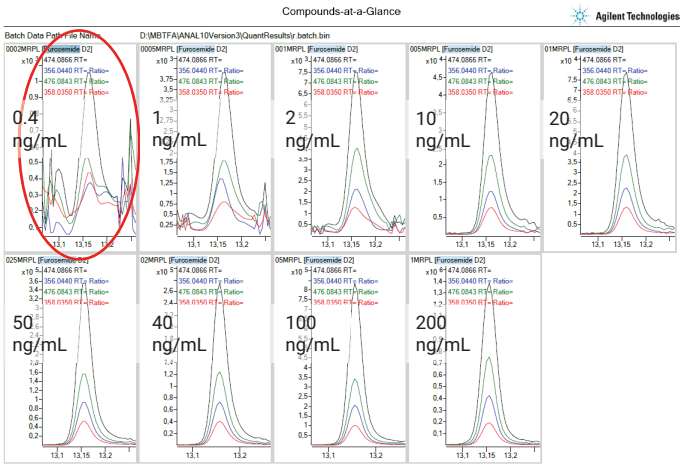


Figure 3. GC/Q-TOF LOD when using extractions with AssayMAP Bravo for furosemide. LOD is outlined in red circle.

New semi-automated GC/Q-TOF screening approach for routine antidoping analysis

For GC/MS analysis, most doping control labs use triple quadrupole instruments. When operated in optimal conditions in multiple reaction monitoring (MRM) mode, the GC/TQ systems can deliver exceptionally high sensitivity for target compounds. Fundamental differences in time-of-flight measurement enables GC/Q-TOF-based approaches the ability to extend what is possible compared to GC/TQ. Since data are collected in an untargeted fashion, method development is greatly simplified, as optimization is not required for every target compound. The untargeted data collection also enables retrospective data analysis, opening the door to looking for additional targets, even if they are not known at time of sample analysis. The full-scan collection also avoids any duty-cycle related concerns that can be present in MRM methods[3] in portions of chromatograms where many compounds may be coeluting.

In order to incorporate GC/Q-TOF into the screening approach, the quantitative range of the GC/Q-TOF was first evaluated and compared to that of the GC/TQ (Figure 4). Anabolic steroids are some of the most analytically challenging compounds due to their low MRPL and endogenous interferences. Thus, a total of five batches of 20 urine samples each were assessed with a focus on the steroids that require quantitation according to WADA. Satisfactory correlation of linearity between the two systems was observed.

Further examples of the calibrations, showing extensive quantitative range for steroids using the GC/Q-TOF are shown in Figure 5. Correlation coefficients (R^2) for calibration curves of all evaluated steroids exceeded 0.997 and are summarized in Table 2.

Table 2. Correlation coefficients for calibration curves of steroids that require quantitation.

Substance	Calibration Levels in Urine (ng/mL)	Coefficient of Determination (R^2)
Testosterone	1-3-10-30-100-400	0.9995
Epitestosterone	1-3-10-30-100-400	0.9997
Androsterone	24-72-240-720-2,400-9,600	0.9993
Etiocholanolone	24-72-240-720-2,400-9,600	0.9987
Dihydrotestosterone	0.5-1.5-5-15-50-200	0.9986
Dehydroepiandrosterone	2-6-20-60-200-800	0.9993
4-Androstene-3,17-dione	0.5-1.5-5-15-50-200	0.9988
5α-Androstane-3α,17β-diol	2-6-20-60-200-800	0.9997
5β-Androstane-3α,17β-diol	2-6-20-60-200-800	0.9995
5α-Androstane-3,17-dione	0.5-1.5-5-15-50-200	0.9987
5β-Androstane-3,17-dione	0.5-1.5-5-15-50-200	0.9986
6αOH-androstenedione	0.25-0.75-2.5-7.5-25-100	0.9988
4OH-androstenedione	0.25-0.75-2.5-7.5-25-100	0.9986
5β-Pregnanediol	2-6-20-60-200-800	0.9971

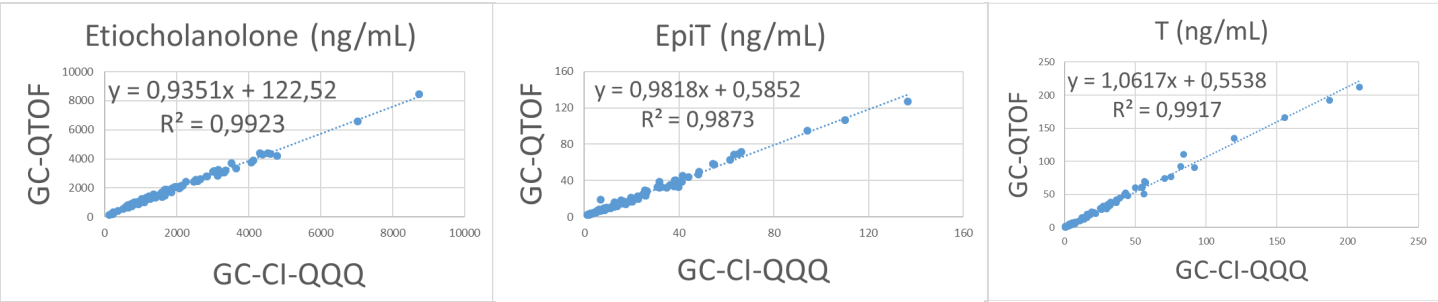


Figure 4. Steroid profiling of GC/TQ versus GC/Q-TOF.

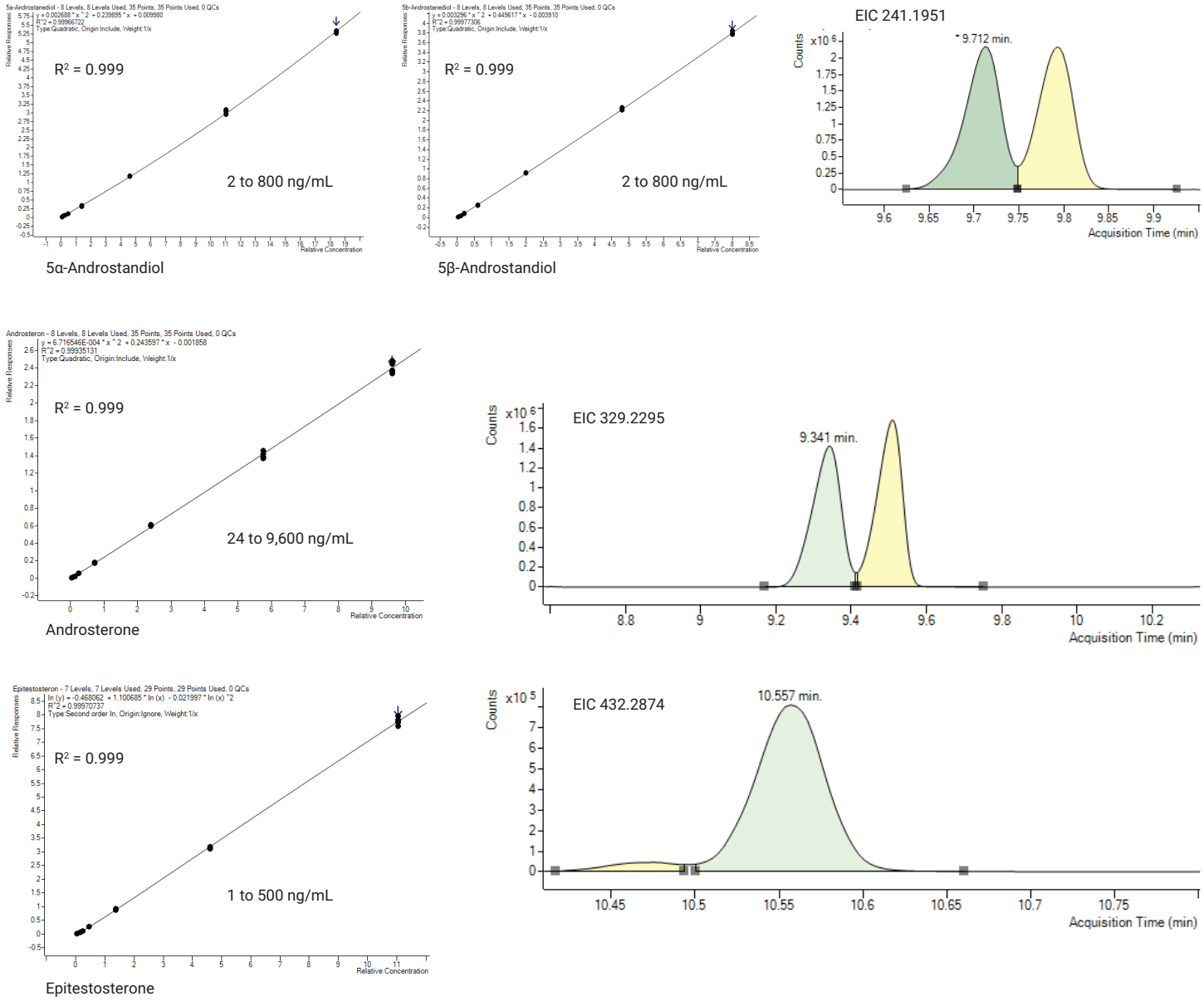


Figure 5. Examples of GC/Q-TOF calibration curves for steroids and accurate mass extracted ion chromatograms (EICs) for target ions (at the highest point of the calibration range).

The mass axis stability of the GC/Q-TOF has been evaluated in a course of 200 injections, where no mass calibration was performed during the first 100 injections (or approximately 24 hours). For the subsequent 100 injections, the automatic calibration of the system, enabled in the sequence, was performed every five samples (Figure 6). It worth mentioning that when automated calibration is performed, no additional time is spent for the calibration, since it is performed during the oven cooling part of the GC cycle. The *m/z* of four target ions have been monitored to detect mass axis drift. A small positive bias was observed during the first 100 injections that was largely corrected for during the subsequent 100 injections. The amplitude of the mass shift was less than ±1.5 ppm, with the absolute drift up to about 4 ppm without mass calibration.

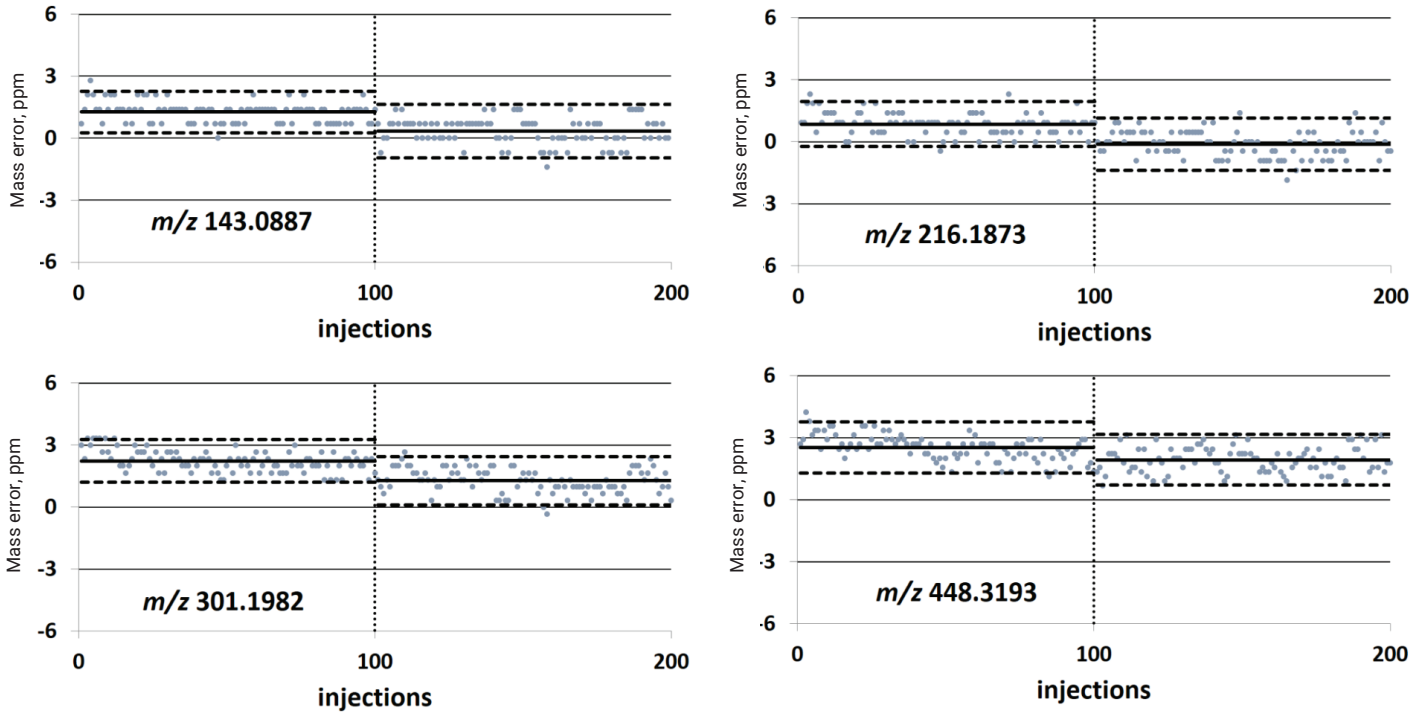


Figure 6. Mass axis stability of the 7250 GC/Q-TOF. Injections 0 to 100 were performed without calibration. For the injections 100 to 200, the system was automatically calibrated every five runs.

Thus, the 7250 GC/Q-TOF system can deliver high-quality data with respect to both mass accuracy and the quantitative capability required for doping control analysis. Currently, a typical approach for data processing includes data analysis and report generation, followed by a time-consuming manual review by two independent scientists. Automation of the data review process in a reliable way would provide a number of important advantages, including improving time-efficiency, consistency, and unbiasedness due to the drastically decreased need for human involvement.

The new GC/Q-TOF suspect screening workflow, which also includes target quantitation, is performed entirely in MassHunter Quantitative Analysis software and is based on an accurate mass library. It has been widely used for pesticide and environmental contaminant screening[6], but in principle can also be applied to other applications, such as doping control. As outlined in Figure 7, after automatic creation of a screener method from accurate mass library spectra, the GC/Q-TOF screener's two-stage algorithm validates each quantifier and qualifier ion based on outliers (such as mass accuracy, signal-to-noise ratio, etc.) at the first stage. At the second stage, it decides whether a compound is "confirmed" (present), "rejected" (not present), or needs an additional review (could be present). At this second step, various criteria are assessed on a compound level, such as library match score and number of qualified ions.

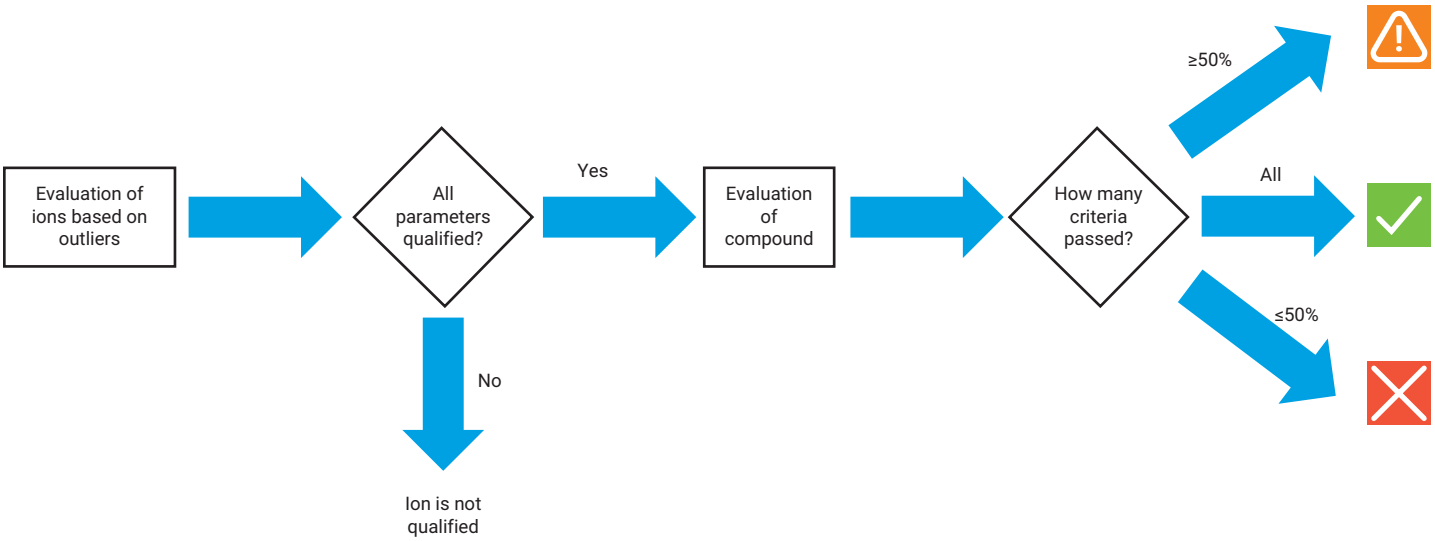
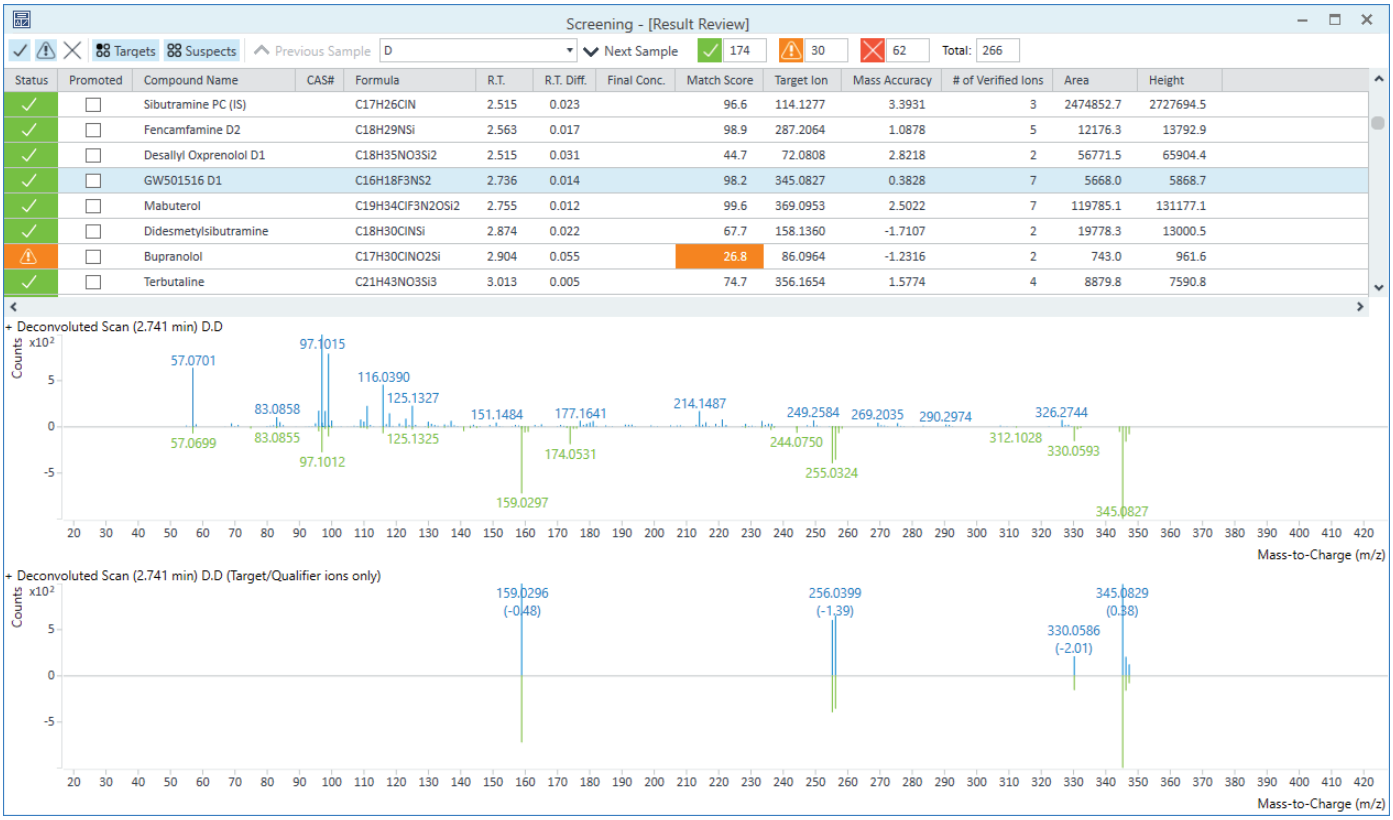


Figure 7. Automated compound verification in the GC/Q-TOF screener. Depending on the specific compound criteria that do not pass, a compound is either rejected or tentatively confirmed.

The screener setup provides a high degree of user flexibility to customize the criteria individually for each compound, if needed. The screener results summary window is displayed in Figure 8.

When using the new GC/Q-TOF screener, the need to review only questionable compounds is much more time-efficient compared to conventional targeted approaches.

A



B

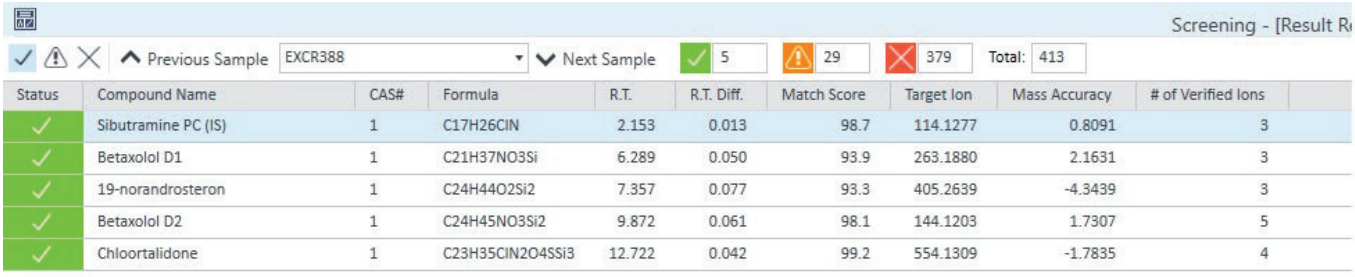


Figure 8. Screener results. (A) The summary window allows the user to quickly review the questionable hits that require additional attention. (B) Summary report for an EQAS sample containing betaxolol (beta blocker), chlorthalidone (diuretic), and 19-norandrosteron. Sibutramine is an internal standard.

Detection of hydrolyzed glucuronidated and nonhydrolyzed sulfated metabolites of steroids

Anabolic androgenic steroids (AAS) have been the most frequently detected compounds in sports doping testing.[4, 5] Identification of the long-term sulfated metabolites of AAS plays an important role in doping control, since these metabolites can be used as alternative markers and potentially extend the detection time of some AAS.[7–9] In order to identify nonhydrolyzed sulfates, excretion studies of mesterolone and metenolone have been performed.[10, 11] The characteristic ions of the hydrolyzed glucuronidated metabolites and the sulfated metabolites of these steroids are listed in Table 3.

An example of a chromatogram containing metenolone sulfate metabolite S1 (S-Met-1) from the same individual is shown in Figure 9.

At day 2, the metabolite is still clearly visible, while at day 10, it has disappeared.

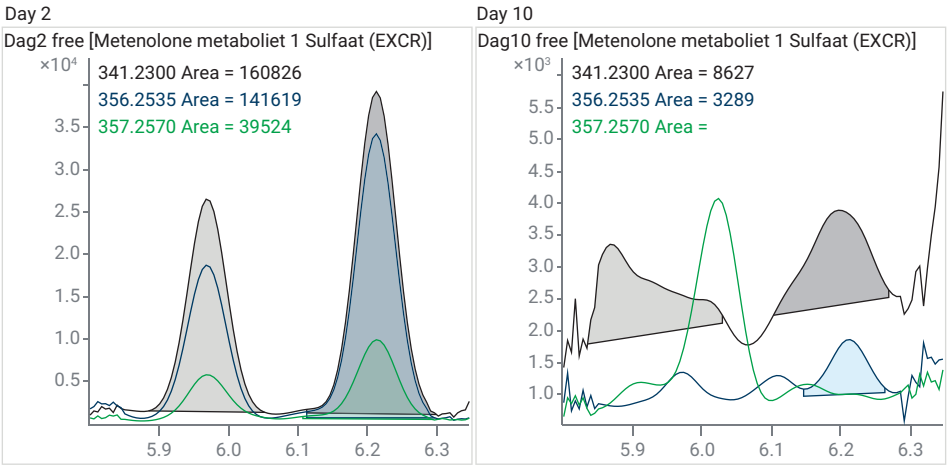


Figure 9. Metenolone metabolite S-Met-1 in different urine samples.

Table 3. List of identified ions for sulfated metabolites of metenolone and mesterolone.

Label of Marker	Name of Marker	GC-EI-QTOF-MS			
		RT (min)	Ion 1 (*m/z*)	Ion 2 (*m/z*)	Ion 3 (*m/z*)
Metenolone					
Met-PC	Metenolone (17β-hydroxy-1-methyl-5α-androst-1-en-3-one)	11.75	446.3031	208.1278	–
Met-1	3α-Hydroxy-1-methylene-5α-androstan-17-one	10.19	446.3031	447.3050	–
Met-2	16ζ-Hydroxy-1-methyl-5α-androst-1-ene-3,17-dione	12.83	532.3219	517.2975	–
S-Met-PC	Monosulfated metenolone	7.52	356.253	266.2029	–
S-Met-1	1-Methylene-5α-androstan-17-one-3α-sulfate	6.2	356.2535	357.2571	341.2300
S-Met-2	1-Methyl-5α-androst-1-ene-3,17-dione-16ζ-sulfate	11.54	444.2874	445.2913	429.264
S-Met-3	1β-Methyl-5α-androstan-17-one-3ζ-sulfate	6.68	358.2686	359.2726	
Mesterolone					
Mest-1	3α-Hydroxy-1α-methyl-5α-androstan-17-one	10.34	448.3187	449.3900	–
Mest-2	3,6,16-Trihydroxy-1α-methyl-5α-androstan-17-one	12.98	624.3880	610.3684	609.3650
S-Mest-4	1α-Methyl-5α-androstan-17-one-3α-sulfate and 1α-Methyl-5α-androstan-17-one-3β-sulfate	5.81	358.2696	359.2726	–
S-Mest-5	Mono-sulfate form of 3β,16ζ -dihydroxy-1α-methyl-5α-androstan-17-one	9.64	446.3031	447.3045	–
S-Mest-6	Mono-sulfate form of 17ζ,4ζ -dihydroxy-1α-methyl-5α-androstan-3-one	10.21	446.3031	447.3045	–

An example of the accurate mass GC/Q-TOF spectrum of one of the nonhydrolyzed sulfated metabolites of mesterolone is shown in Figure 10. Due to high sensitivity in full data acquisition mode, as well as the benefits of accurate mass and high resolution, the GC/Q-TOF is an ideal tool to search for these metabolites. All metabolites can be easily tracked in a single run.

An additional benefit of the GC/Q-TOF-based approach is that with the discovery of new metabolites, they can always be searched for retrospectively, since all data have been collected in full spectrum acquisition mode.

Excretion studies of mesterolone indicated that its nonhydrolyzed sulfated metabolites, S-Mest-4 and 5, showed a significant improvement in detection time compared to conventional hydrolyzed markers (Figure 11). This strongly suggests the benefits of adding non-hydrolyzed sulfated metabolites to the doping control routine methods, including those based on GC/TQ, for ultimate sensitivity when necessary.

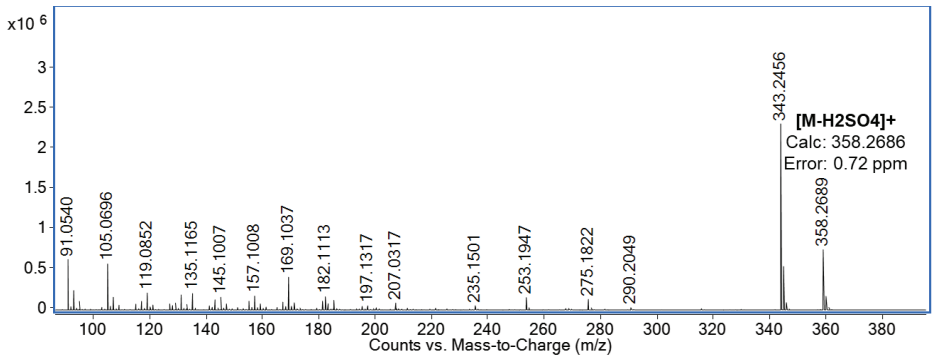


Figure 10. Accurate mass high resolution spectrum of the nonhydrolyzed sulfated metabolite of mesterolone (S-Mest-4).

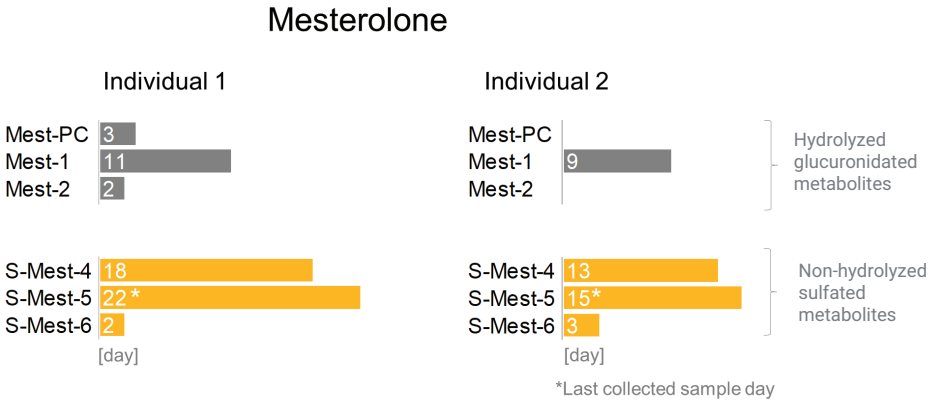


Figure 11. Results of two excretion studies of mesterolone analyzed with GC/Q-TOF.

Conclusion

With a continuously growing list of WADA-prohibited substances and new WADA regulations, there is a need for novel workflow solutions in doping control to address the updated WADA requirements. This application note describes a new workflow that can bring the productivity and efficiency of doping control laboratories to a new level. The complete solution suggested here is focused on highly sensitive quantitative and qualitative analysis with retrospective potential, and involves GC/Q-TOF and GC/TQ instruments combined with sample preparation using the Agilent AssayMAP Bravo system. This solution can significantly improve throughput (achieving 20,000 samples per year) and is equipped with automation of the time-consuming data review process enabled by the GC screener capability of Agilent MassHunter software. In addition, identification and the benefits of including the sulfated metabolites of anabolic steroids into the routine doping screening methods have also been discussed here.

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