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Application Note

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Life Science

Screening Analysis of Steroid Profiles and Qualitative Doping Substances by GC-MS/MS

Masato Okano*1 , Mitsuhiko Sato*1, Yuma Watanabe*1,
Yuki Nakagawa *2, Yuki Sakamoto *2

Life Science

■ Abstract

WADA-accredited laboratories around the world pursue to fight against doping in sports. Shimadzu GCMS-TQ™ 8050 NX was selected as an instrument of choice for an anti-doping testing at international sports games held in 2021.

The method developed for the games analyzed 180 substances, encompassing both quantitative and qualitative substances with one injection.

The sensitivity, the linearity, and the robustness of the instrument were demonstrated with endogenous steroids quantified for steroid profiling. The sensitivity in qualitative analysis of target substances was also proved to be sufficient, and chromatograms were provided for a dozen of such substances as an illustration in this article.

A document on the rest of the data is available specifically for those working in the WADA-accredited doping control laboratories. The readers interested in the document are encouraged to contact Shimadzu Corporation, or its oversea branches.

1. Introduction

International sporting games are highly anticipated and keenly followed events around the world.

In 1999, the World Anti-Doping Agency (WADA) was established to promote health and well-being of athletes and protect them from harmful substances. To carry out those missions, there are currently 29 operating WADA-accredited laboratories around the world conducting doping analyses (as of Jan 2022 at the time of this writing).

There are two notable trends in the field: the emphasis on screening methods and the reliance on the Athlete Biological Passport (ABP). According to WADA statistics, the importance of screening anti-doping analyses also referred as Initial Testing Procedures (ITPs) has increased in recent years as the number of samples climbed steadily over the years (Fig. 1).The ABP, on the other hand, is a module to monitor selected biomarkers of doping substances (e.g., Steroid Profiles) over an extended period. This module can potentially reveal a covert doping use that would otherwise go undetected with a conventional single-point doping testing scheme. As doping schemes have turned more covert, the importance of the ABP has become more recognized.

In this article, Shimadzu GCMS-TQ8050 NX was employed in an ITP test for simultaneous quantitative and qualitative analyses of 180 substances. The method was developed ensuring the detection of low MRPL compounds while not saturating high concentration quantitative substances for steroid profiles (e.g., androsterone and etiocholanolone).

In addition to the method parameters, the article further introduces steroid profiles analysis (e.g., linearity, carryover, stability) and provides chromatograms for 12 qualitative substances as an example. Carboxy-THC, a major metabolite of cannabis, is also discussed at the end. The robustness of GCMS-TQ8050 NX is also illustrated by plotting a series of detector gains obtained during and after the games.

＊1 LSI Medience Corporation, Anti-Doping Laboratory
＊2 SHIMADZU CORPORATION

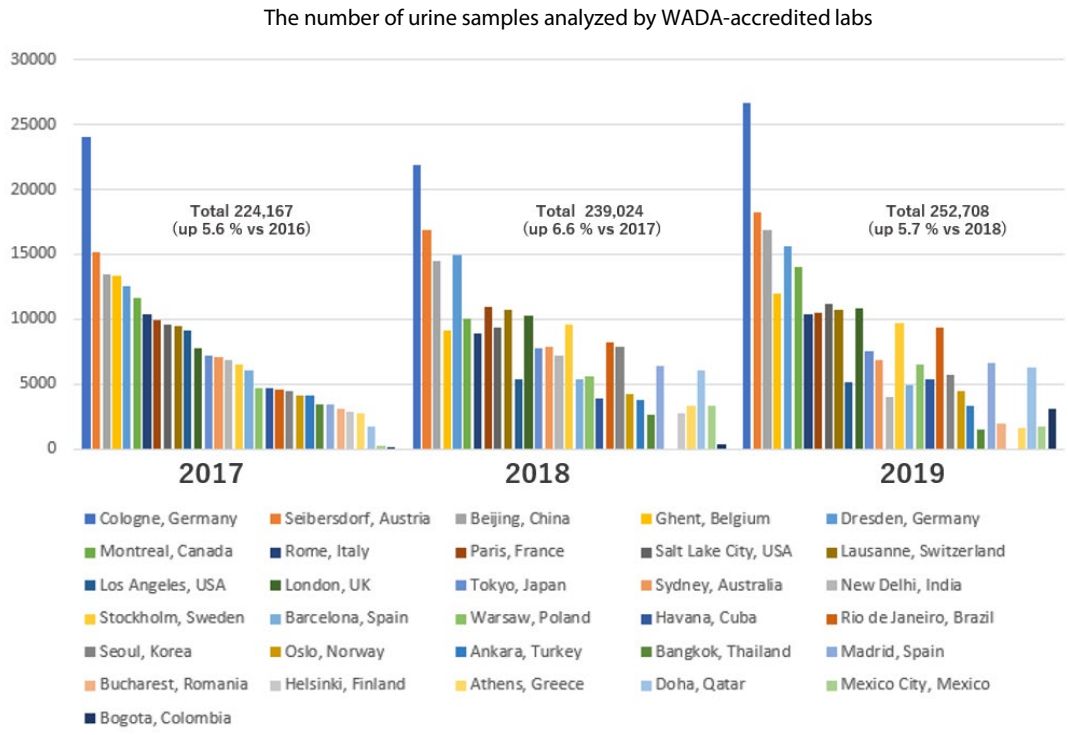


Fig. 1 The number of urine samples analyzed at each laboratory

2. Assay Procedures and Instruments

The sample assay procedure is illustrated in the flow chart below (Fig 2).

2 mL of urine was fortified with an internal standard and passed through a solid phase extraction cartridge. The eluate (i.e., methanol) was then dried down, and solvent-exchanged with a buffer appropriate for the subsequent β-Glucuronidase hydrolysis. The free form substances was then transferred to diethyl ether by liquid-liquid extraction. The solvent was dried down and a TMS-derivatization agent was added as a reconstitution solution (i.e., injection solution) before its injection into the GC/MS.

The assayed sample was subjected to a GC/MS analysis according to the parameters listed in Table 1.

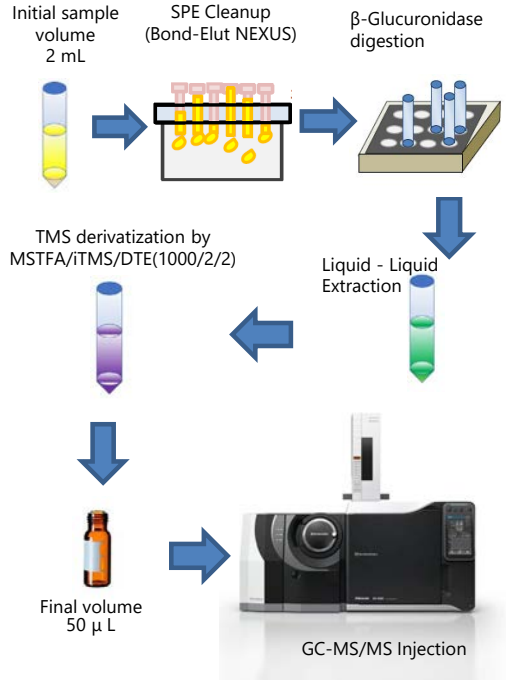


Fig. 2 Flow Chart of Assay Procedure

The split injection was used to take advantage of the highly sensitive GCMS-TQ8050 NX. With the split injection, high concentration substances (e.g., Androsterone and Etiocholanolone) were quantitated. A series of MRM transition channels were created to dynamically capture each substance at a set retention time while minimizing the number of MRM channels within the elution window to increase the sensitivity.

Table 1 System Configurations and Analytical Parameters

GC-MS/MS	: GCMS-TQ8050 NX
Auto Injector	: AOC-20i Plus
Auto Sampler	: AOC-20s Plus
Analytical Column	: HP-ULTRA 1 (17m × 0.20 mm, I.D., df=0.11 µm)

GC	
Inlet temp.	: 280 °C
Injection Mode	: Split
Split Ratio	: 11
Carrier gas	: Helium
Flow Control Mode	: Constant Pressure (ca. 156 kPa)
Column Oven Temp.	: 180 °C (1 min) → (3 °C /min) → 229 °C → (40 °C /min) → 300 °C (3 min) Total 22 mins
Purge flow rate	: 3 mL/min
Sample Inj. volume	: 2 µL
Ion Source Temp.	: 230 °C
Interface temp.	: 300 °C
Detector Voltage	: 0.4 kV Relative to the Tuning Result
Measurement Mode	: Multiple Reaction Monitoring (MRM)

3. Steroid Profiles

Six endogenous steroids (i.e., Androsterone, Etiocholanolone, Testosterone, Epitestosterone, 5α-Androstane-3α,17β-diol, 5β-Androstane-3α,17β-diol) were measured in all the urine samples submitted to the laboratory during the games and the results were reported to Anti-Doping Administration and Management System (ADAMS).

Among the six steroids, androsterone and etiocholanolone are known to exist at high concentrations in some samples. It is therefore important for a laboratory to establish an analytical method capable of detecting those high concentration substances without the detector saturation.

A challenge associated with such substances will be the ITP requirement to detect low concentration qualitative substances (e.g., clenbuterol) in the same injection.

This article showcases the calibration curves of the six steroid profiles, the carryover tests of androsterone and etiocholanolone, and the control chart for the testosterone-to-epitestosterone concentration ratio (hereinafter referred to as T/E ratio).

4. Linearity of endogenous steroids

The calibration curves were drawn at 10 concentrations with each concentration having triplicates, tallying the total to 30 points per curve.

In Fig. 3, two chromatograms are listed for each compound with one at the lowest concentration while the other at the highest.

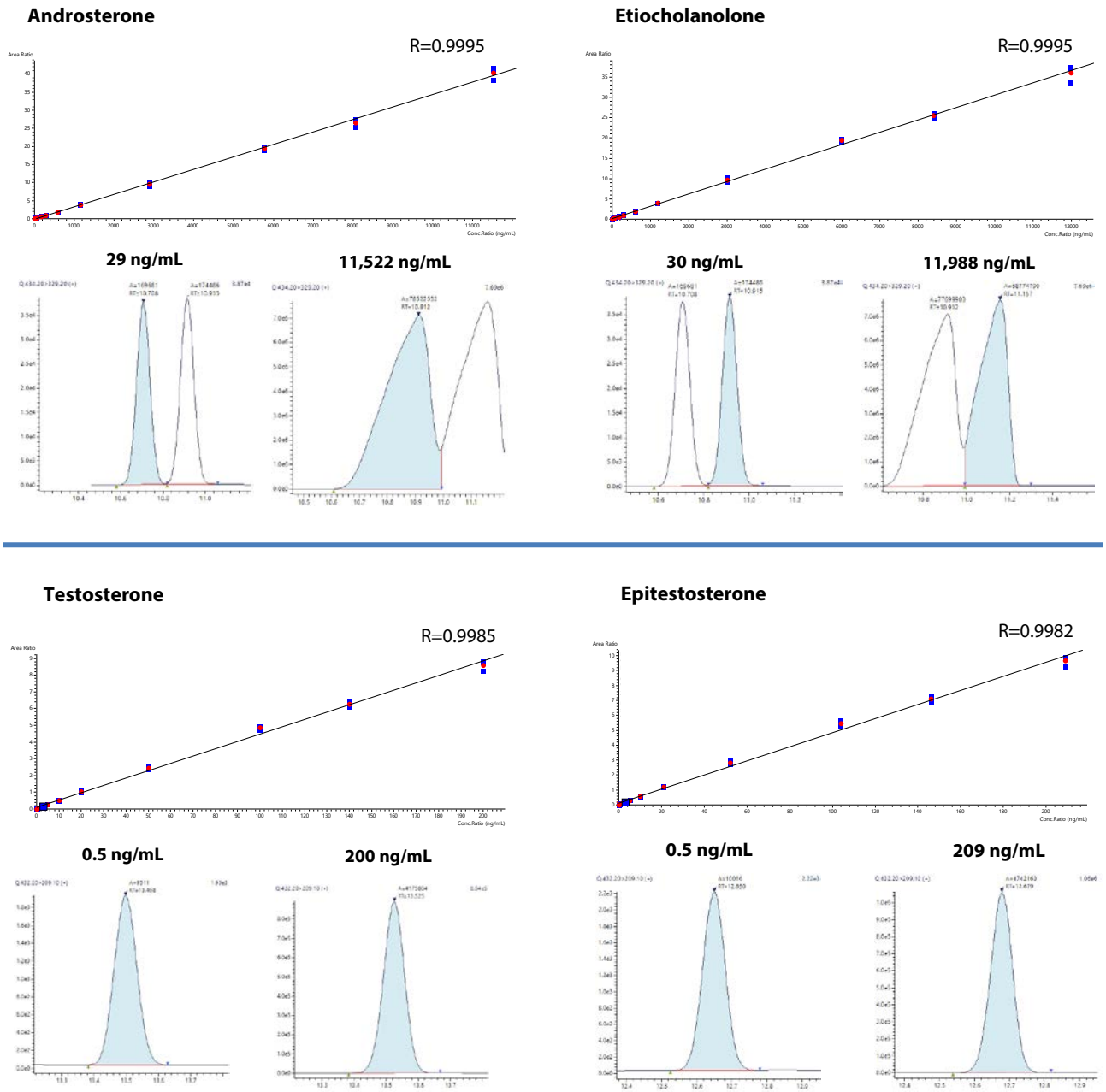


Fig. 3 Calibration Curves and MRM Chromatograms of endogenous steroids (Continue to the next page)

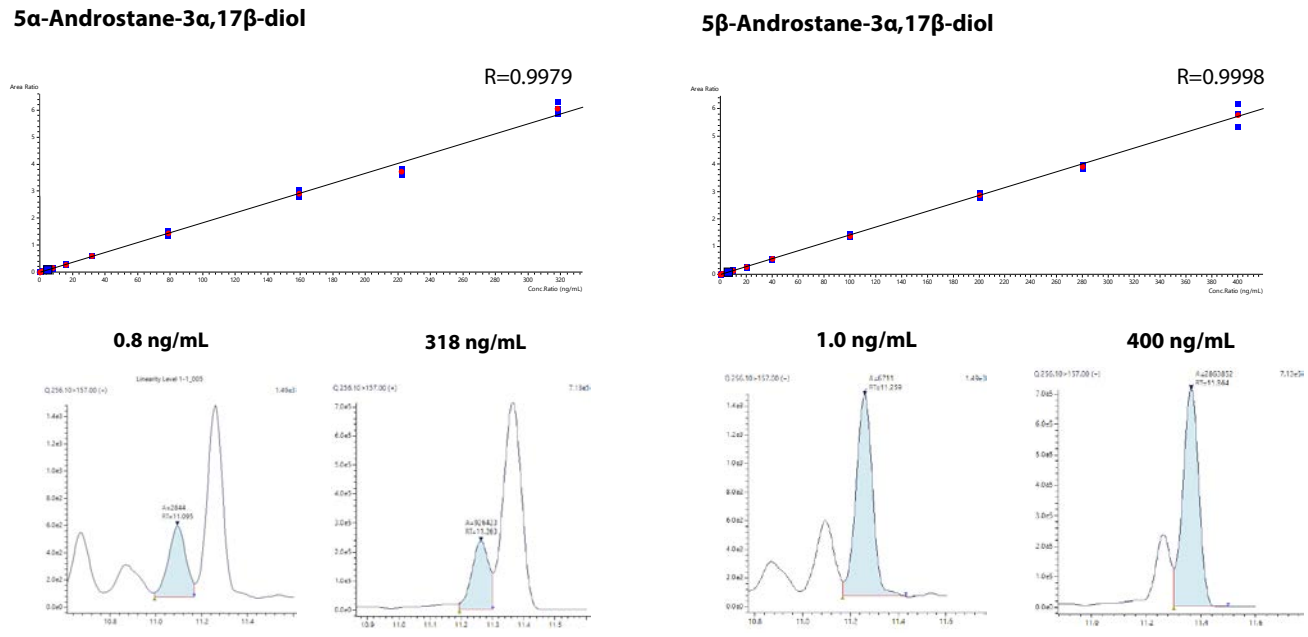


Fig. 3 Calibration Curves and MRM Chromatograms of endogenous steroids (Continuing from the previous page)

5. Carryover Test

In order to ensure the accuracy of quantitative values, the carryover test was performed for androsterone and etiocholanolone. The two substances were injected at 10,000 ng/mL followed immediately by a blank (i.e., Surine) injection (Fig. 4).

The peak top of both substances were not plateaued even at the high concentration. Carryover was not observed in the blank injections immediately following the high concentration injections.

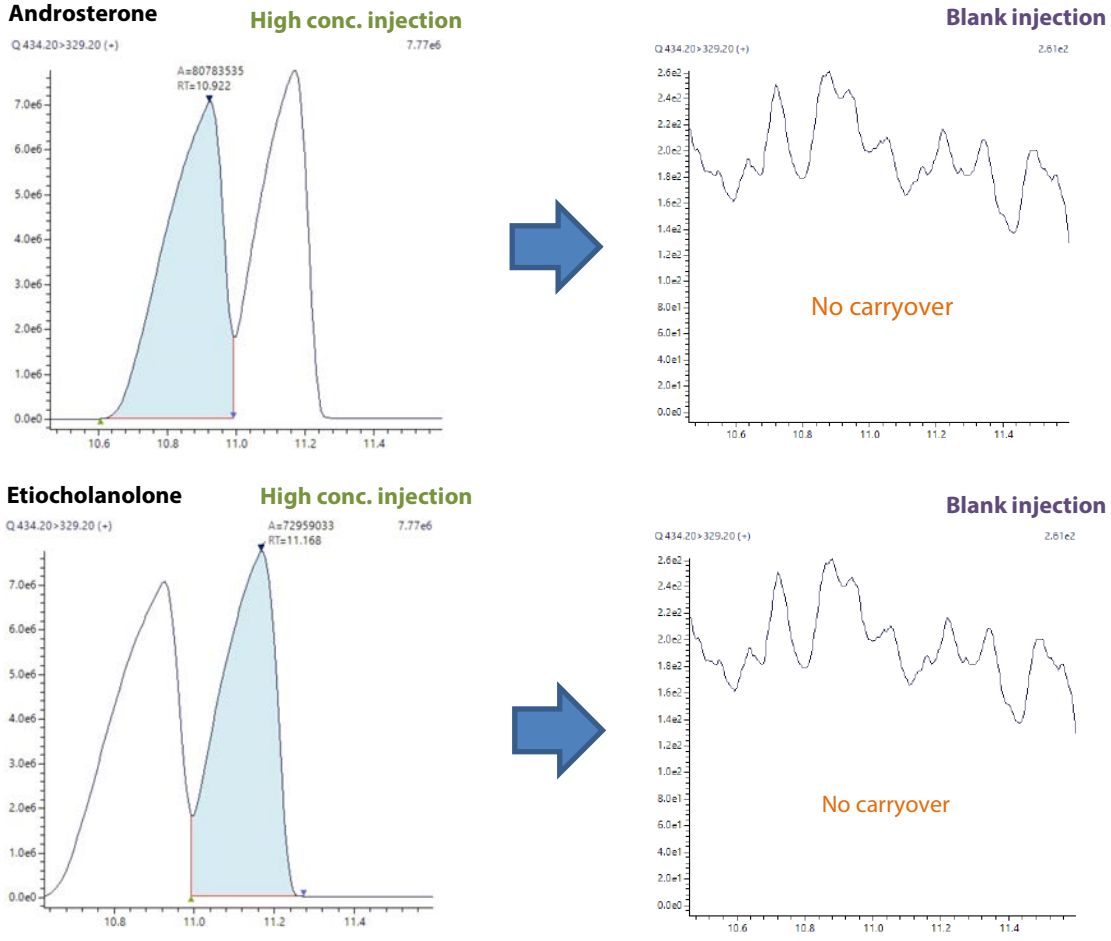


Fig. 4 carryover test after injection of high conc. QC sample

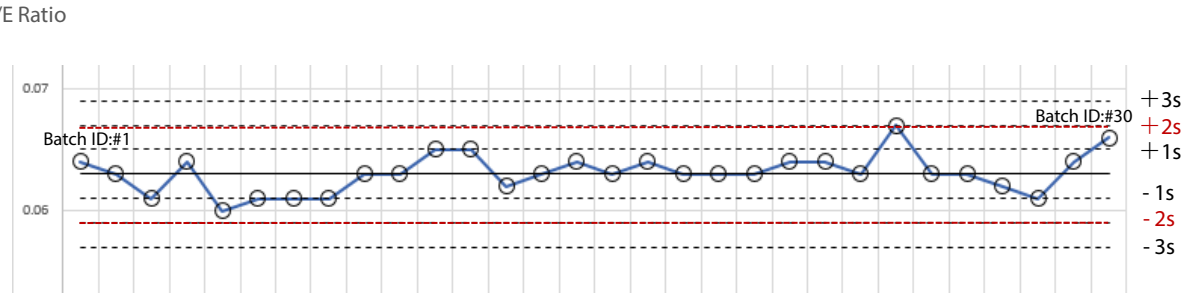


Fig. 5 QC chart plotted for the T/E ratio over the 30 batches run on the same instrument (each batch was assayed separately on a different day)

6. Control Chart for Steroid Profiles

The six endogenous steroids were quantitated, and the results were logged in corresponding control charts. Shown above is the typical control chart for the testosterone-to-epitestosterone ratio (T/E) (Fig. 5) . Each circle in the figure represents a T/E value obtained from a batch that has gone through sample assay pre-treatment. All the batches were run on the same instrument.

The T/E ratios were within ±2s during the measured time period, demonstrating the validity of the data and the robustness of the instrument.

7. Qualitative Analysis

While Steroid Profiles discussed in the previous section are generally found in high concentrations, doping substances are often found in low concentrations and require a high sensitivity analysis.

TD2022MRPL which came into effect in January 2022 lowered MRPLs for a broad range of substances (Table 2). The typical chromatogram is shown below in Fig. 6. Among the 180 quantitative and qualitative substances analyzed, 12 substances were chosen for illustration (Fig. 7). A set of three chromatograms (i.e., blank, 1/4th level of 2019MRPL, and 2019MRPL) are shown for each of the 12 substances in the figure.

The choice of the substances for illustration is based on the retention times (e.g., early, late) and the required sensitivity. For instance, 3'-hydroxy-stanozolol was chosen for illustration as the substance is known to show a poor peak shape in a poorly deactivated instrument. As shown in Figure 7, however, the substance had a symmetrical peak shape in GCMS-TQ8050 NX. Substances with low MRPL such as clenbuterol and DHCMT m2 were also chosen for illustration. Those substances were detected even at four times less than the 2019 MRPL levels, showcasing the sensitivity of the instrument.

Table 2 example substance classes and changes in MRPLs

Prohibited Class *1	Substance Name	TD2019MRPL (ng/mL)	TD2022MRPL (ng/mL)
S1.1 Anabolic Androgenic Steroids (AAS)	3-OH-stonozolol	2	1
S1.1 Anabolic Androgenic Steroids (AAS)	DHCMT metab. 2	2	0.4
S1.1 Anabolic Androgenic Steroids (AAS)	Formebolone metabolite	5	2.5
S1.1 Anabolic Androgenic Steroids (AAS)	Oxymetholone metab. 4	5	2.5
S1.2 Other Anaboic Agents	Clenbuterol	0.2	0.2
S1.2 Other Anaboic Agents	Zeranol metabolite	2	1
S1.2 Other Anaboic Agents	Zilpaterol	2	1
S4.1 Aromatase Inhibitors	Letrozole metabolite	20	20
S4.2 Anti-Estrogenic Substances	Ospemifene	20	20
S7 Narcotics	Morphine	-	-

*1 classification according to WADA Technical Documents

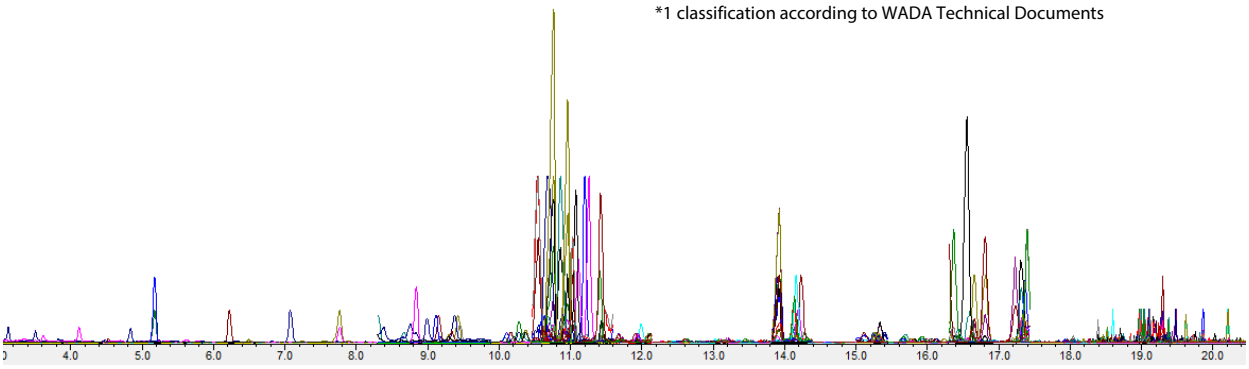
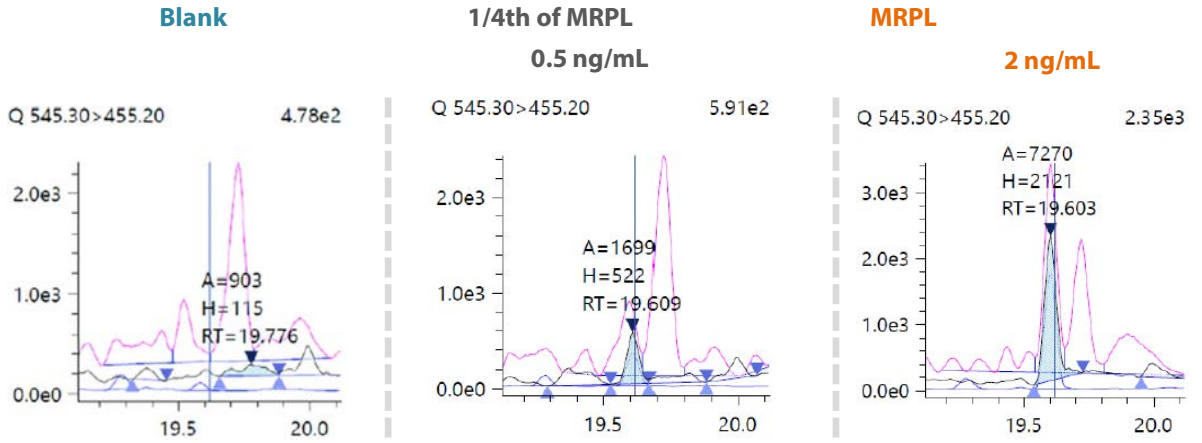
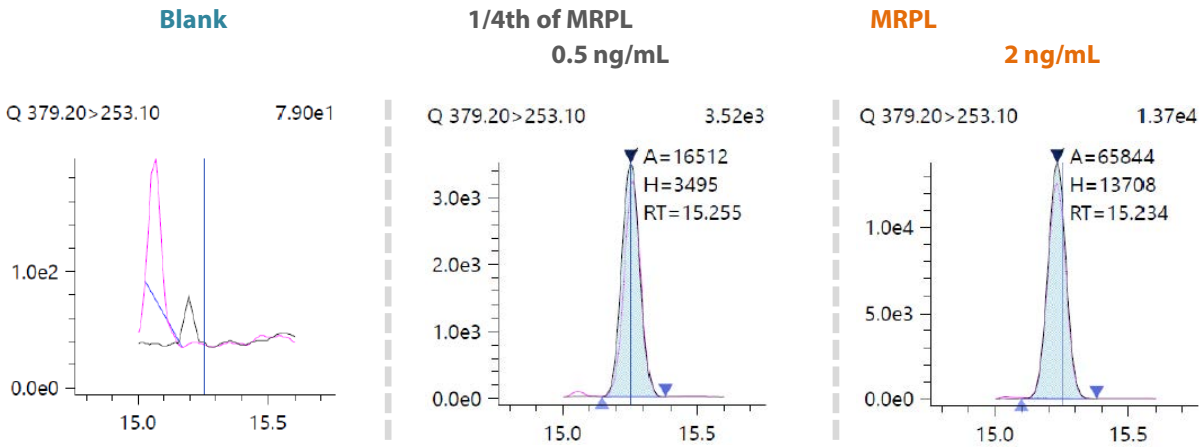


Fig. 6 overlaid MRM chromatograms for 180 substances

3-OH-stanozolol-metabolite and -metabolite 2 (both TMS derivatized)
Synonym: 3-Hydroxystanozolol & 4β-hydroxy-stanozolol



DHCMT metabolite 2 –TMS
Synonym: 4-Chloro-18-nor-17β-hydroxymethyl-17α-methyl-5α-androst-13-en-3α-ol (M3,LTM)



Formebolone metabolite –TMS
Synonym: 4-Androstene-17α-methyl-11α,17β-diol-3-one

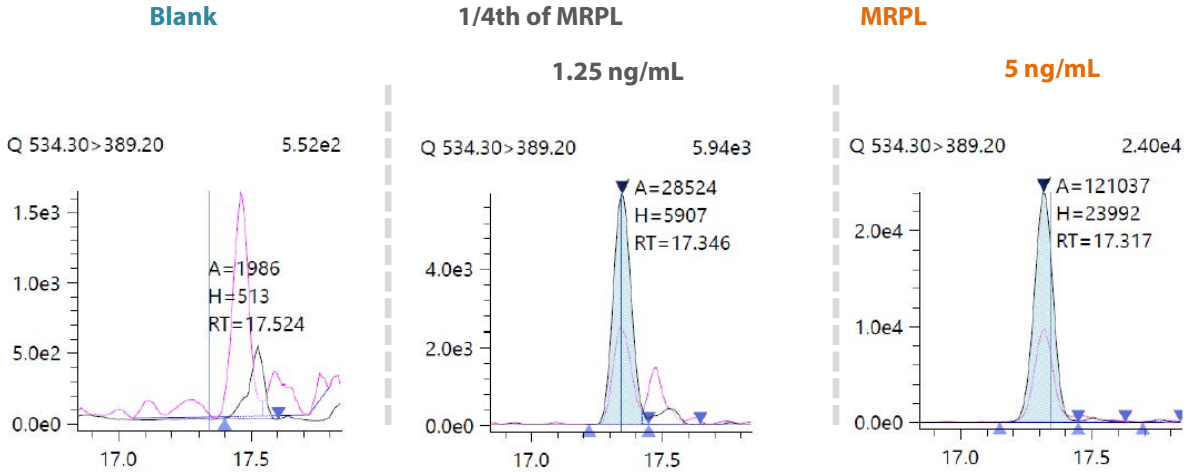
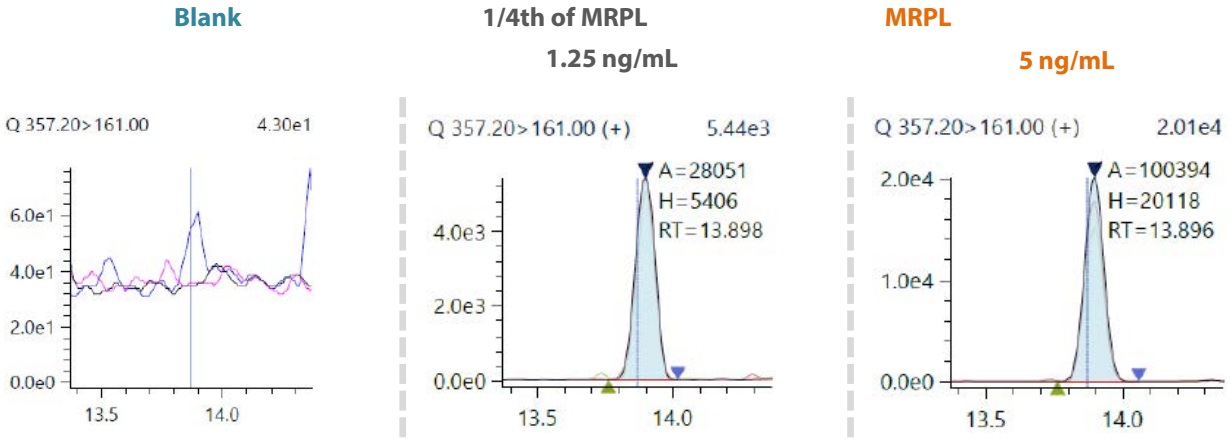


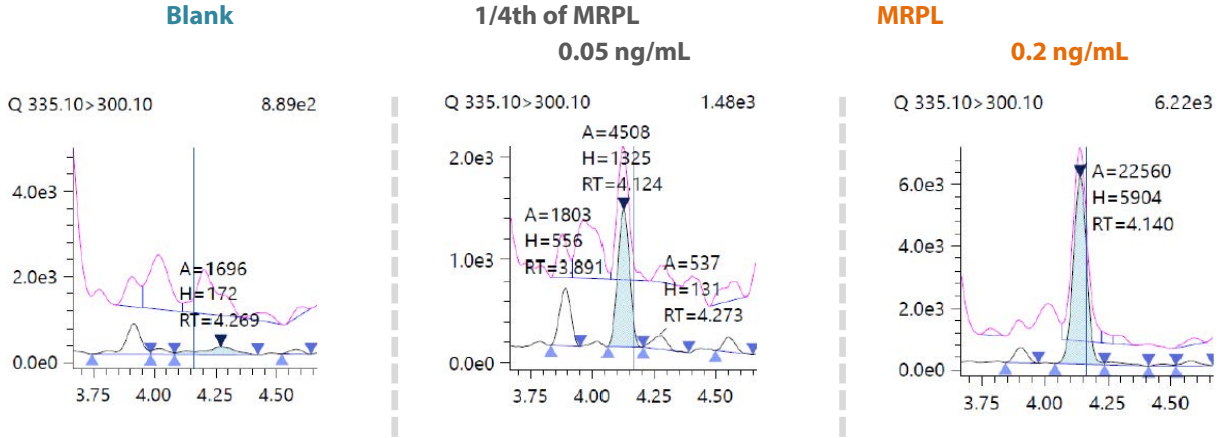
Fig. 7 Examples out of the 180 substances analyzed-part 1

Oxymetholone metabolite 4 –TMS

Synonym: 18-Nor-17β-hydroxymethyl-17α-methyl-2α-methyl-5α-androst-13-en-3-one



Clenbuterol -TMS



Ospemifene -TMS

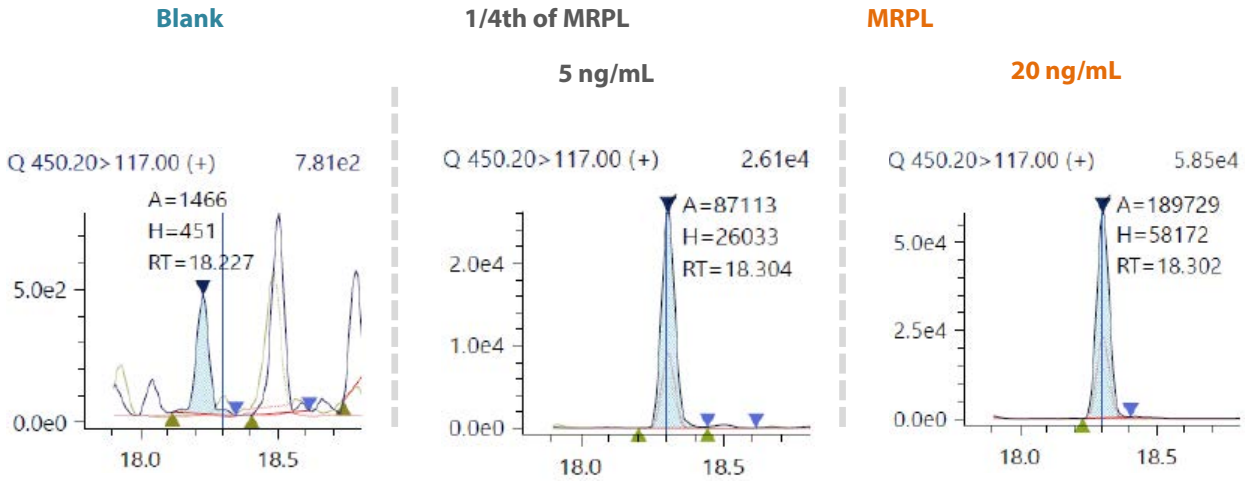
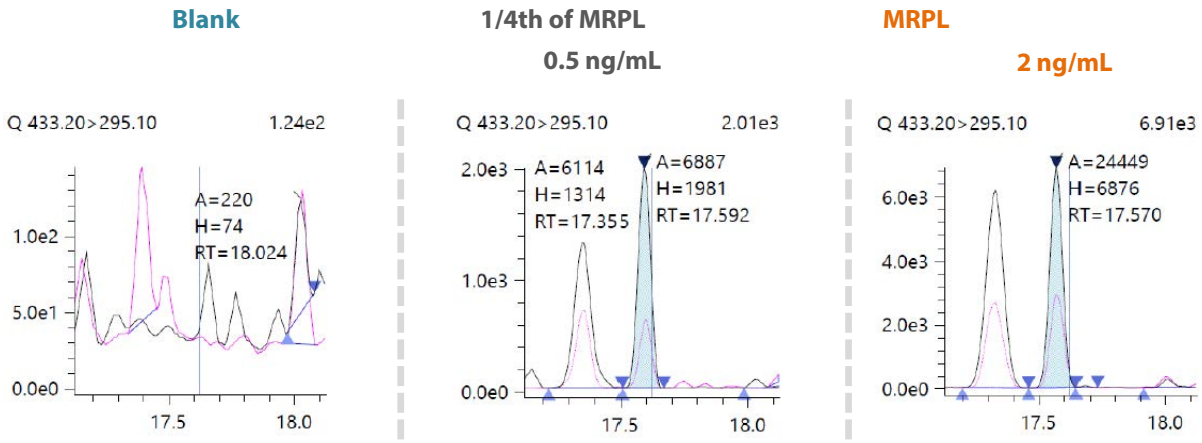
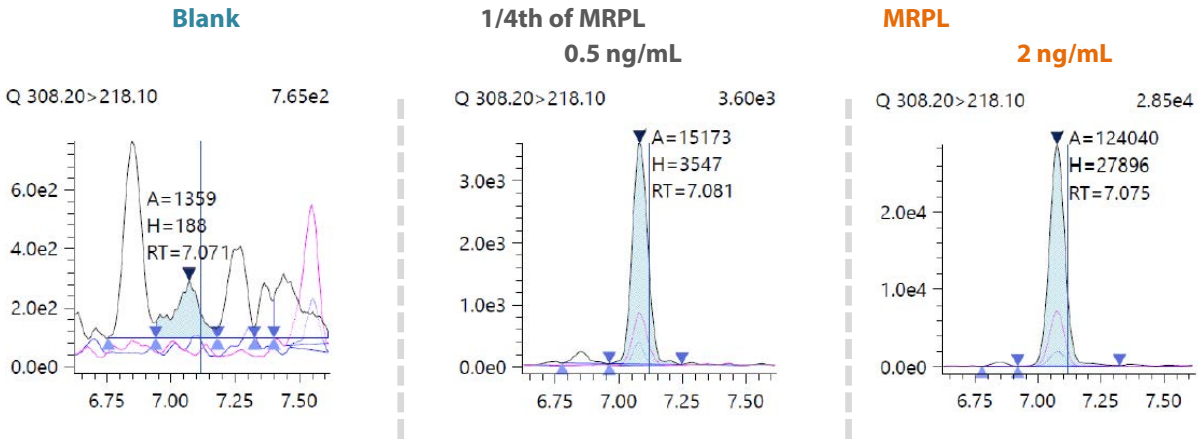


Fig. 7 Examples out of the 180 substances analyzed-part 2

Zeranol metabolite –TMS
Synonym: Taleranol



Zilpaterol -TMS



Letrozole metabolite-TMS
Synonym: Bis(4-Oyanophenyl)methanol

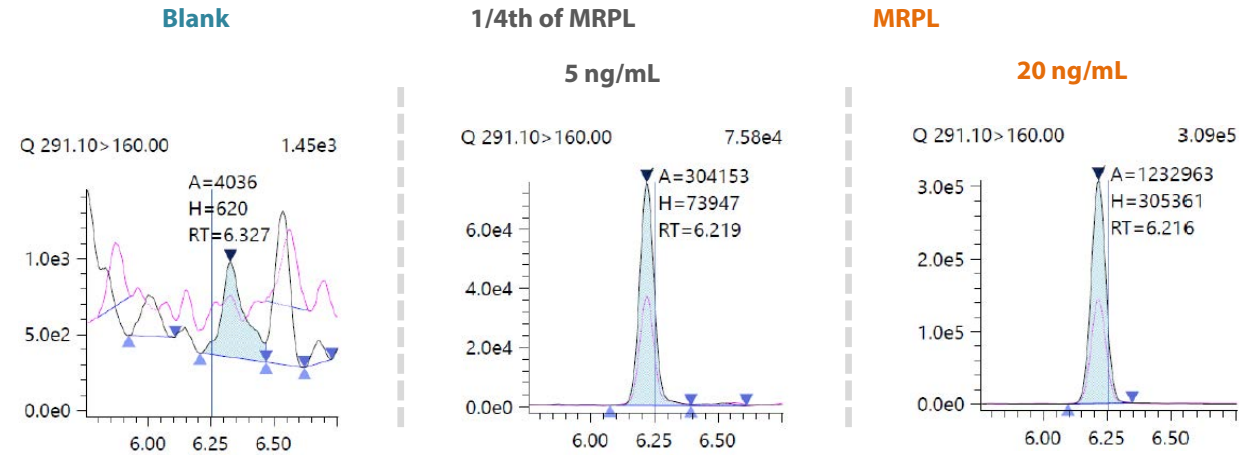
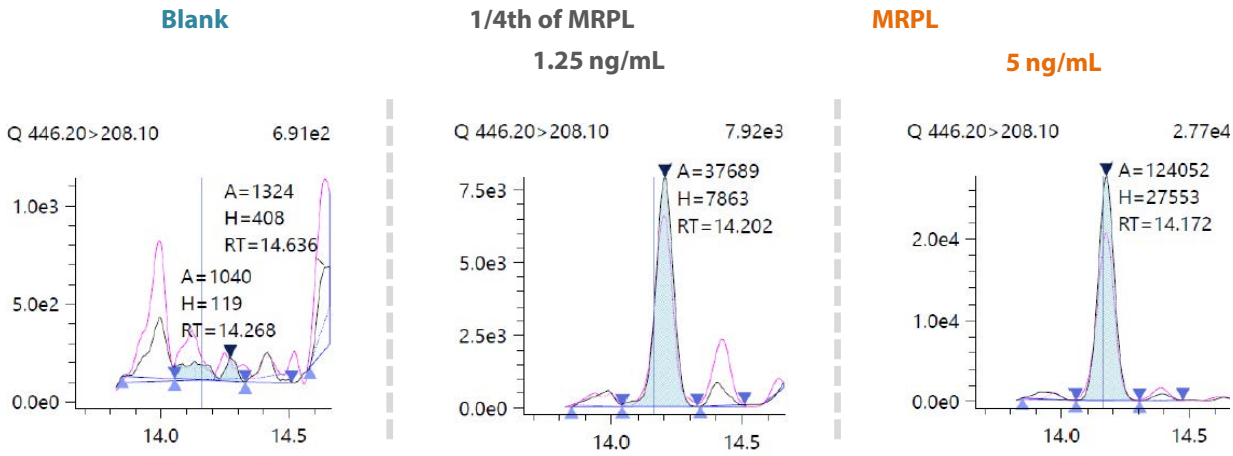
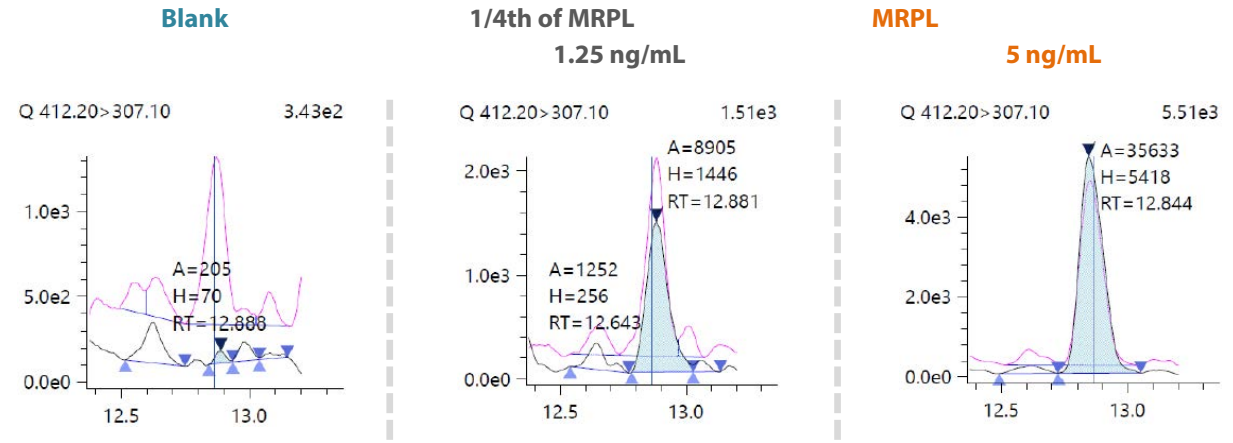


Fig. 7 Examples out of the 180 substances analyzed-part 3

Metenolone-TMS
Synonym: 1-Methylen-5α-androstane-3α-ol-17-one



Trenbolone metabolite –TMS
Synonym: Epitrenbolone [17α-Hydroxytrenbolone]



Norboletone metabolite-TMS
Synonym: 13β,17α-Diethyl-5α-gonane-3α,17β-diol

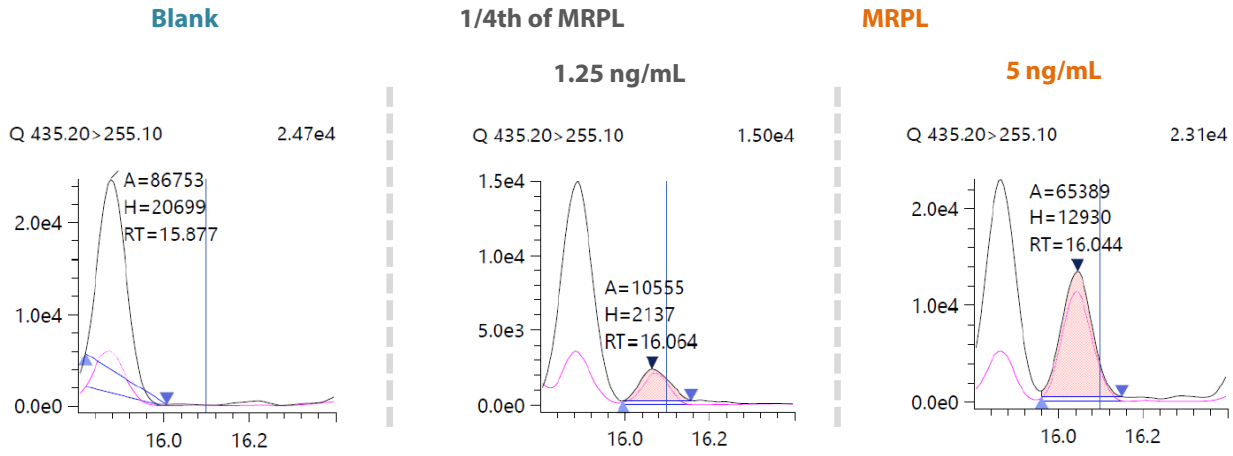


Fig. 7 Examples out of the 180 substances analyzed-part 4

8. Carboxy-THC

Carboxy-THC is of particular interest due to a wide-spread use of cannabis among athletes. Carboxy-THC is the main secondary metabolite of tetrahydrocannabinol which is formed in the body after cannabis is consumed.

The assayed urine fortified with carboxy-THC at the concentration of 150 ng/mL was then injected into the GC-MS, and the chromatograms were obtained as in Fig. 8.

Carboxy-THC at 150 ng/mL
Synonym: Carboxy-tetrahydrocannabinol

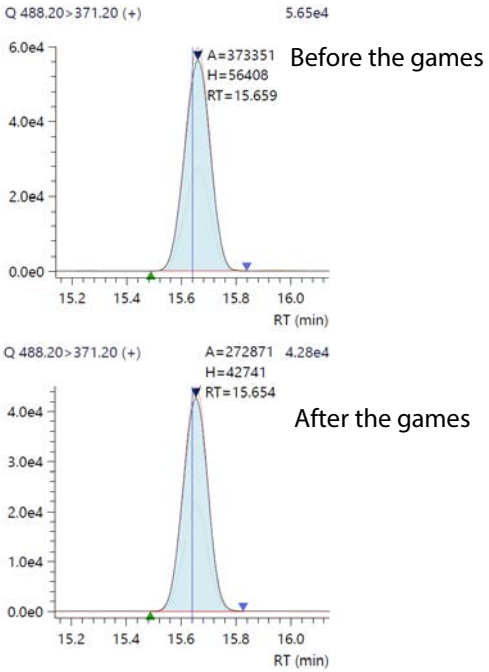


Fig. 8 Carboxy-THC chromatograms(before and after the games)

Though only carboxy-THC is shown in Fig. 8, it should be noted that 180 substances were all analyzed together. The presence of the substance was confirmed with 2 confirmational MRM channels in addition to the quantitative MRM channel.

9. Robustness

During the games, twelve GCMS-TQ8050 NX instruments were employed to process a large number of sample volume. Approximately 6,800 samples came in during the games.

Despite the large number of samples, there was no downtime with any of the GCMS instruments installed at the site. This was due to the Shimadzu patented ion source protecting itself with the shields against contaminations (Fig. 7) . The shields free users from cleaning the ion source every month, and the recommended frequency for cleaning the ion source is only every 6-12 months.

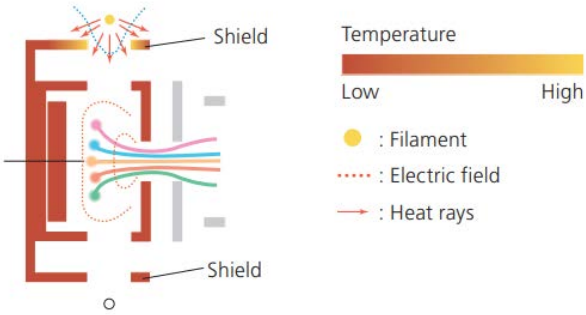


Fig. 9 The patented ion source shields to prevent contamination

The detector gain is an indicator of the contamination level in the mass spectrometer. The detector gain will rise if the contamination level increases (i.e., the detector loses sensitivity). In Fig. 10, the detector gains obtained during and after the games (i.e., from July 15th, 2021 to Dec 16th, 2021) are illustrated with a graph. The orange line indicates the cumulative number of samples, starting the count on July 15th, 2021, while the blue line with dots indicates the detector gains obtained with each batch run on the instrument.

The detector gain was stable during the whole course of testing. About 1,000 samples were injected in the instrument within the tested weeks, and maintenance performed on the instrument were minimal and only on the GC side (e.g., replacing a liner, a septum, etc.).

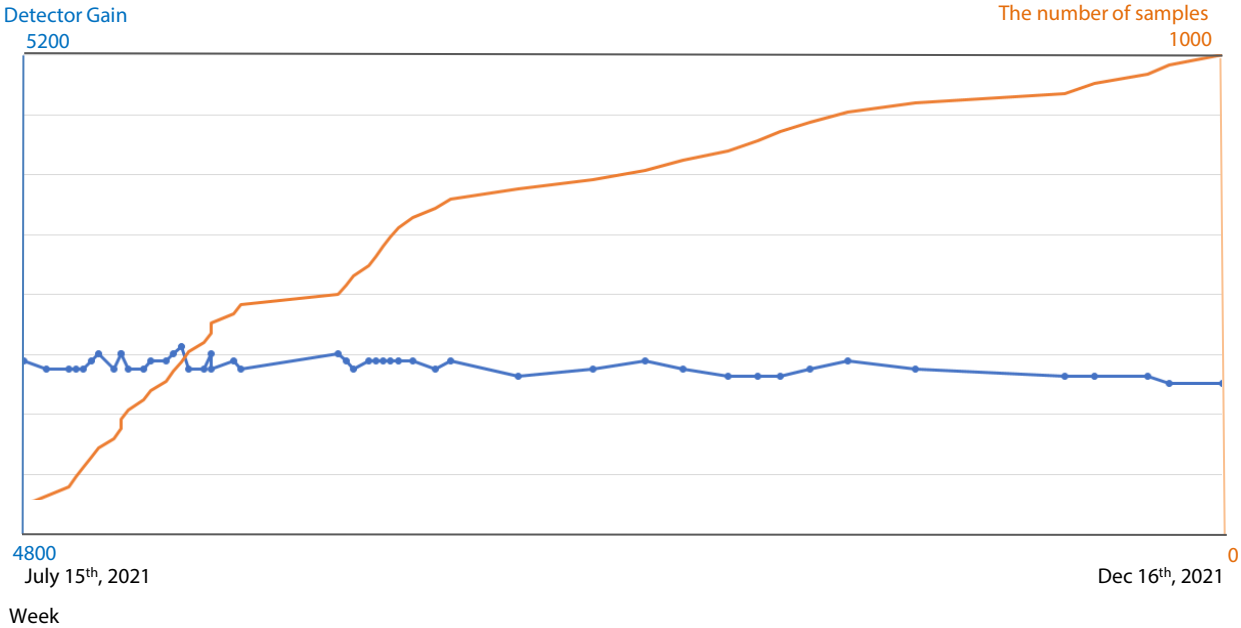
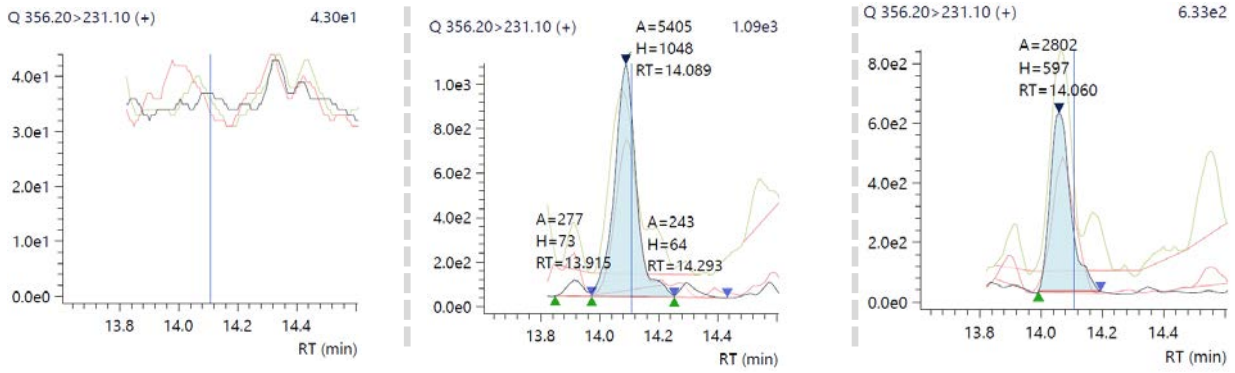


Fig. 10 Detector cleanliness in the course of weeks

Methyl-1-testosterone

Synonym: Methyl-1-testosterone (17β-hydroxy-17α-methyl-5α-andrst-1-ene-3-one)

Blank before the games (July) | Before the games (July) 5 ng/mL | After the games (Dec) 5 ng/mL



Methandriol-m2/Methyltestosterone-m2

Synonym: 17α-Methyl-5β-androstane-3α, 17β-diol

Blank before the games (July) | Before the games (July) 2 ng/mL | After the games (Dec) 2 ng/mL

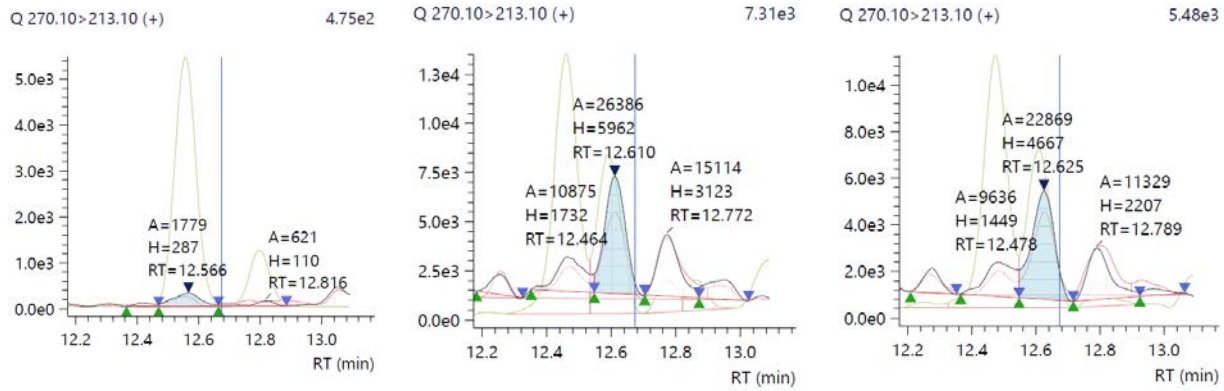


Fig. 11 Chromatograms obtained without any ion source maintenance for 5 high-sample-volume months from July to December 2021

As shown in Fig. 10 on the previous page, the detector gain was consistent, and the mass spectrometer was free of contamination during and after the games. A few substances were chosen to demonstrate how robustly the system performed during the time period. The performance was investigated by comparing the peaks from before and after the games (i.e., July and December 2021).

The substances chosen for illustration are methyl-1-testosterone and methandriol-m2/methyltestosterone-m2 (Fig. 11). These substances were selected based on their propensity to yield poor peak shapes and low sensitivity in a contaminated instrument.

Chromatograms in Fig. 11 show all the substances still being detected with a good sensitivity even in the absence of any ion source cleanings for 5 heavy-sample-volume months that included the games (i.e., from July to December 2021). Methandriol-m2/methyltestosterone-m2 is known to elute close to its interferences but was still detected with ease.

10. Conclusion

WADA accredited laboratories monitor doping substances in athletes' urines. At international sports games held in 2021, GCMS-8050 NX instruments were used to process nearly 6,800 samples in a few weeks.

The method developed here encompassed both the quantitative and qualitative substances with a single injection. The total number of the substances was close to 180, and the run time was approximately 22 mins. There was zero downtime during the games as the patented ion source inside the GCMS-TQ8050 NX prevented contaminations from the urine samples.

Last but not least, a document with further details (i.e., on the data not presented in this article) is available specifically for those working in the WADA-accredited doping control laboratories. The readers interested in the document are encouraged to contact Shimadzu Corporation or its oversea branch.

Acknowledgement

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GCMS-TQ8050 NX

The GCMS-TQ8050 NX features a new highly efficient detector and three noise reduction technologies that enable previously unachievable femtogram-level quantitative analysis of ultra trace quantities. The system also enables quantitative analysis for a variety of new applications, such as utilizing the dramatically high sensitivity for reducing the maintenance frequency and cost of long-term use, for example, or the high mass resolution to achieve even higher separation from contaminants.

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