Technical note | 001896

Thermo Fisher SCIENTIFIC

Anti-doping

Staying ahead of regulations for the analysis of steroids in urine with GC-MS/MS

Authors

Łukasz Rajski[1], Adam Ladak[2] and Daniel Kutscher[1]

[1]Thermo Fisher Scientific, Bremen, Germany

[2]Thermo Fisher Scientific, Hemel Hempstead, United Kingdom

Keywords

Anti-doping controls, TSQ 9610 GC-MS, helium, HeSaver-H_2Safer carrier gas saving technology

Goal

The goal of this technical note is to demonstrate the utility of the Thermo Scientific™ TRACE™ 1610 GC system and Thermo Scientific™ TSQ™ 9610 triple quadrupole GC-MS system for the anti-doping controls.

Introduction

Anti-doping controls are an important part of modern professional sport to avoid the misuse of pharmacological treatments and assure fair play. The World Anti-Doping Agency (WADA) was established in 1999 and is responsible for the harmonization and coordination of the anti-doping regulations across all sports and countries.[1] WADA publishes an annually updated document called the List of Prohibited Substances and Prohibited Methods. The substances in the document are grouped into ten different categories according to their pharmacological actions (anabolic agents, beta-2 agonists, peptide hormones, growth factors, etc.).[2] WADA also publishes technical documents that must be followed by the accredited anti-doping laboratories. The documents specify the detection and identification criteria for LC-MS and GC-MS methods[3] as well as the analytical parameters of the method.

Minimum Required Performance Level (MRPL) is the minimum concentration of a prohibited substance, or a metabolite or marker of a prohibited substance, that a laboratory shall be able to detect and identify in routine operations. The Minimum Reporting Level (MRL) defines a cut-off level, below which laboratories should not report an adverse analytical finding (a positive result) for certain classes of specific prohibited substances. The MRL is established to ensure harmonization of reporting and should be equal to or greater (≥) than the MRPL. The estimated Limit of Detection (LOD) of

thermo scientific

the Initial Testing Procedure (ITP) shall be less than or equal to 50% of the corresponding MRPL or less than or equal to the corresponding MRL. It is not necessary to estimate the LOD for all potential metabolites, marker(s), or degradation products of a given non-threshold substance. However, the laboratory shall estimate, during method validation, the Limit of Identification (LOI) of the Confirmation Procedure. At the LOI for a given target analyte, for which a Reference Material is available, the false negative identification rate cannot be higher than 5%.[4]

Anti-doping laboratories apply two strategies. One is limited to a single sample analysis, which occurs mostly at competitive events; whereas the other involves a longitudinal testing where results are included in a larger framework of test for performance profiling. While the first strategy aims to detect a prohibited substance, the latter strategy is the base for the so-called Athlete Biological Passport (ABP). This approach is focused on monitoring of the impact of doping on the organism. Urine is the predominant matrix analyzed in anti-doping laboratories, as it can be easily obtained close to competition, and because of the wide range of prohibited substances that can be identified through its analysis. However, blood is also analyzed to a much smaller extent.

Liquid chromatography (LC) and gas chromatography (GC) coupled to mass spectrometry (MS) are the typical analytical techniques applied in the single sample testing because of their selectivity and sensitivity. They cover all low to medium molecular weight drugs of abuse that need to be detected according to the WADA regulations. Additionally, GC-MS/MS is applied in the longitudinal testing (ABP) where the target compounds are pseudo-endogenous steroids.[5] Although HRMS seems to be an interesting alternative,[6] triple quadrupole mass spectrometers remain the predominant instrument type in anti-doping laboratories. This application is focused on the use of gas chromatography coupled to triple quadrupole mass spectrometry for the analysis of steroids in urine. This note addresses the most relevant aspects of the steroids analysis, namely detection and quantitation capabilities, as well as robustness. The sensitivity was checked at and below the new MRPLs that were introduced in 2022, a two-point calibration curve was employed to evaluate the quantitation, and moreover the presence of carry-over was thoroughly checked. Finally, the robustness during long sample sequences was tested. For the experiments described here, a subset of the most challenging steroids was selected.

Experimental

The analyses were performed using a TRACE 1610 GC system coupled to a TSQ 9610 triple quadrupole GC-MS/MS equipped with a Thermo Scientific™ Advance Electron Ionization (AEI) source. The samples were injected using a Thermo Scientific™ AI/AS 1610 liquid autosampler. Table 1 contains the operational parameters of the TRACE 1610 GC and TSQ 9610 MS/MS, whereas Table 2 shows the list of transitions applied to identify and quantify the targeted analytes.

Table 1. GC/MS parameters

TRACE 1610 GC parameters	
Injector	
Injector type	Thermo Scientific™ iConnect Split/Splitless (SSL) Injector
Liner	Thermo Scientific™ LinerGOLD™ Precision (P/N 453A1255-UI)
Operating mode	Split
Split flow [mL/min]	51
Split ratio	30
Purge flow [mL/min]	5
Vacuum compensation	On
Temperature [°C]	220
Oven	
Analytical column	Thermo Scientific™ TraceGOLD™ TG-5SilMS (30 m × 0.25 mm × 0.25 µm) (P/N 26096-1420)
Carrier gas	He
Carrier gas flow [mL/min]	1.7
Oven temperature program	
Temperature 1 [°C]	160
Hold [min]	0.3
Rate [°C/min]	60
Temperature 2 [°C]	210
Hold [min]	0
Rate [°C/min]	4
Temperature 3 [°C]	270
Hold [min]	0
Rate [°C/min]	70
Temperature 4 [°C]	320
Hold [min]	1.5
TSQ 9610 triple quadrupole GC-MS/MS parameters	
Ion source	Advanced Electron Ionization (AEI)
Electron energy [eV]	*35*
Transfer line temperature [°C]	300
Ion source temperature [°C]	250
Acquisition threshold [counts]	1,000

Table 2. List of transitions

Name	RT (min)	Precursor (*m/z*)	Product (*m/z*)	Collision energy (eV)
Clenbuterol	5.08	335.1	227.1	10
Clenbuterol	5.08	335.1	300.1	14
Clenbuterol	5.08	337.1	302.1	8
Zilpaterol	7.71	308.1	218.1	14
Zilpaterol	7.71	405.2	308.1	10
Androsterone	10.23	434.3	329.1	14
Androsterone	10.23	434.3	419.4	8
Androsterone	10.23	436.4	240.5	5
Androsterone	10.23	436.4	322.6	5
Androsterone	10.23	436.4	422.7	5
Androsterone	10.23	437.4	239.3	5
Androsterone	10.23	437.4	241.7	20
Androsterone	10.23	437.4	329.4	25
Androsterone	10.23	437.4	332.5	5
Androsterone	10.23	437.4	420.4	10
Androsterone	10.23	437.4	423.6	20
Ethiocholanolone-d_5	10.32	439.4	334.3	10
Ethiocholanolone-d_5	10.32	439.4	424.4	10
Etiocholanolone	10.38	434.3	329.1	14
Etiocholanolone	10.38	434.3	419.4	8
Etiocholanolone	10.38	436.4	240.5	5
Etiocholanolone	10.38	436.4	322.6	5
Etiocholanolone	10.38	436.4	422.7	5
Etiocholanolone	10.38	437.4	239.3	5
Etiocholanolone	10.38	437.4	241.7	20
Etiocholanolone	10.38	437.4	329.4	25
Etiocholanolone	10.38	437.4	332.5	5
Etiocholanolone	10.38	437.4	420.4	10
Etiocholanolone	10.38	437.4	423.6	20
Etiocholanolone (5A-diol)	10.54	256.2	185.2	10
Etiocholanolone (5A-diol)	10.54	256.2	241.2	10
Etiocholanolone (5A-diol)	10.54	421.3	255.2	12
D5-5B-Androstane-3a,17B-diol	10.61	261.2	185.1	15
D5-5B-Androstane-3a,17B-diol	10.61	261.2	199.1	15
Etiocholanolone (5B-diol)	10.66	256.2	185.2	10
Etiocholanolone (5B-diol)	10.66	256.2	241.2	10
Etiocholanolone (5B-diol)	10.66	421.3	255.2	12
Methyltestosterone-M1	11.67	270.2	199.1	18
Methyltestosterone-M1	11.67	270.2	213.2	10
Epitestosterone-d_3	11.69	435.4	196.1	20
Epitestosterone-d_3	11.69	435.4	196.1	20
Epitestosterone-d_3	11.69	435.4	209.2	10
Epitestosterone-d_3	11.69	435.4	209.2	10
Epitestosterone	11.72	327.2	297.1	22
Epitestosterone	11.72	432.3	209.1	16
Methyltestosterone-M2	11.74	270.2	199.1	18
Methyltestosterone-M2	11.74	270.2	213.2	10
Testosterone-d_3	12.33	435.4	196.1	20
Testosterone-d_3	12.33	435.4	209.2	10
Testosterone	12.34	432.3	209.2	14
Testosterone	12.34	432.3	417.3	12
Testosterone	12.34	432.3	432.3	3
Testosterone	12.34	433.3	418.2	12
Testosterone	12.34	433.3	433.3	3
Calusterone-M	12.46	284.2	227.2	10
Calusterone-M	12.46	374.3	269.2	10
Calusterone-M	12.46	449.3	282.9	8
Methylstenbolone	13.39	460.4	192.9	24
Methylstenbolone	13.39	460.4	207.8	12
Methylstenbolone	13.39	460.4	220	10
Methylstenbolone	13.39	460.4	244.9	16
Norbolethone-M1	14.39	144.1	73.2	12
Norbolethone-M1	14.39	144.1	75.1	14
Norbolethone-M1	14.39	144.1	144.1	3
Furazabol-M	18.25	490.3	143.1	24
Furazabol-M	18.25	490.3	231.1	12
Furazabol-M	18.25	491.3	143.1	26

Data acquisition, processing, and reporting

Data were acquired, processed, and reported using the Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software, version 7.3.1. Integrated instrument control ensures full automation from instrument set up to data processing, reporting, and storage. Simplified e-Workflows™ deliver effective data management ensuring ease of use, sample integrity, and traceability. Chromeleon CDS software also offers the option to scale up the entire data handling from a single workstation to an enterprise environment.

Results and discussion

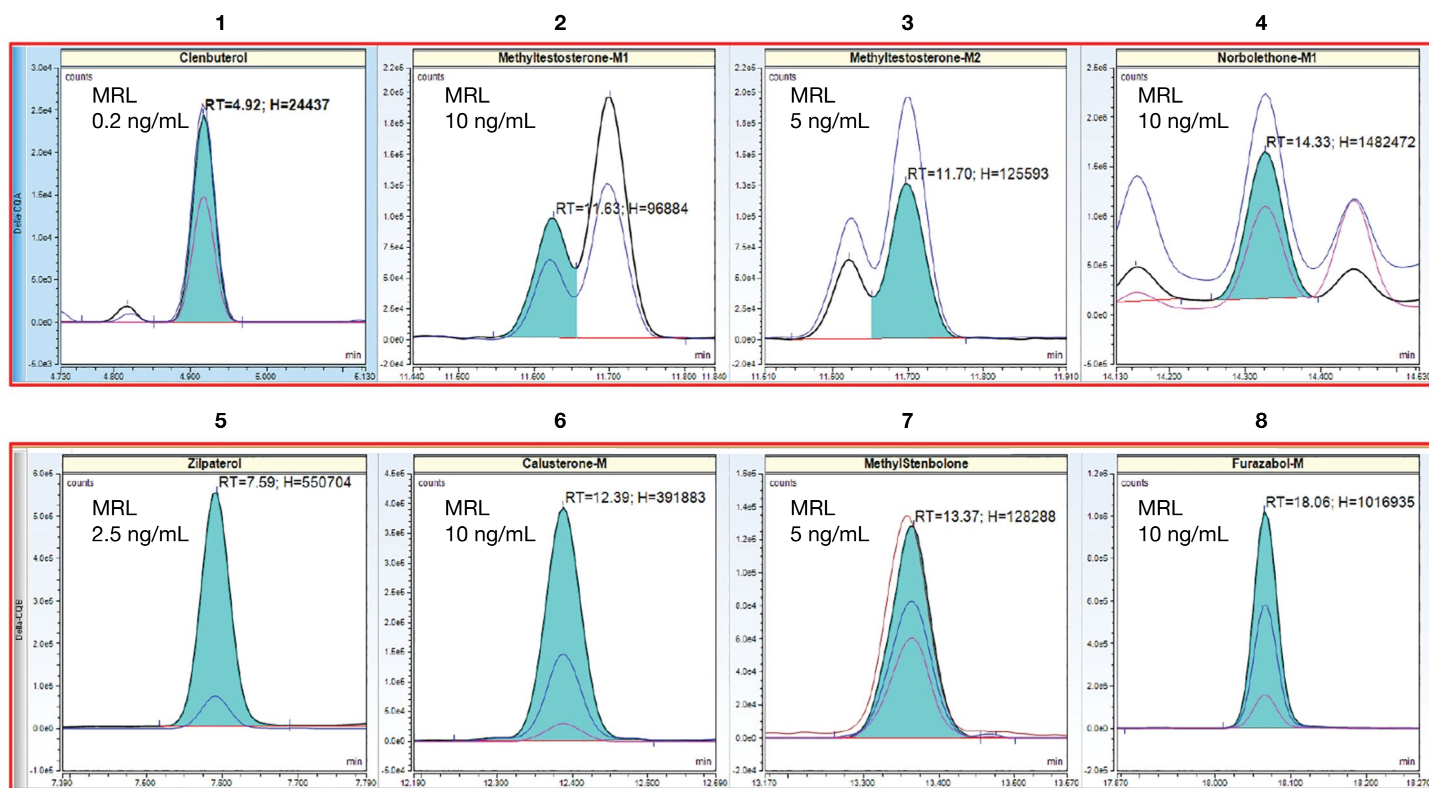
Sensitivity

In 2022, WADA decreased the MRPL for some steroids, for example zilpaterol (from 0.5 to 0.25 ng/mL), calusterone-M (from 2.5 to 1.25 ng/mL), methylstenbolone (from 2.5 to 1.25 ng/mL), norbolethone (from 2 to 1.25 ng/mL), and 16b OH furazabol (from 2.5 to 1.25 ng/mL), which means that testing laboratories need to make sure they can detect these compounds with a sufficient signal to noise ratio at the new limits. Table 3 presents a comparison of the new MRPLs with the results obtained on the TSQ 9610 GC-MS/MS system. In many of the shown cases, obtained sensitivity is considerably higher than the sensitivity necessary to fulfil the WADA requirements, as a signal-to-noise ratio of higher than 3 was achieved for all compounds.

As it was mentioned in the introduction, the MRL should be equal to or higher (≥) than the MRPL. Figure 1 shows examples of compounds at their MRLs. All analytes investigated in this study show a very high S/N and consistent ion ratio.

Table 3. Peak height and signal-to-noise ratio of selected analytes at or below their MRPL

Name	MRPL (ng/mL)	Injected concentration (ng/mL)	Peak height	S/N
Clenbuterol	0.1	0.1	12846	16
Methyltestosterone M2	1	1	23315	4
Methylstenbolone	1.25	1	11979	5
Zilpaterol	0.25	0.25	24294	21
Methyltestosterone M1	1	1	11912	7
Calusterone-M	1.25	1	15027	7
Norbolethone M1	1.25	1	106549	16
Furazabol	1.25	1	42005	215



1. Clenbuterol
2. Methyltestosterone M1
3. Methyltestosterone M2
4. Norbolethone M1
5. Zilpaterol
6. Calusterone-M
7. Methylstenbolone
8. Furazabol-M

Figure 1. Steroids injected at their MRLs

Quantitation

To evaluate the quantitation accuracy, three QC samples containing different concentration levels of the targeted analytes were prepared. A synthetic urine served as a surrogate matrix. A two-point calibration curve, built with a standard and the calibration plot origin, was used to quantify the QCs. The results were evaluated using an acceptance criterion of ±30%. As can be seen in Table 4, all the results were within the acceptable range, except for testosterone and etiocholanolone (5B-diol) in the low QC. These two compounds were overestimated because of the presence of some matrix interferences.

Carry-over

One of the most relevant characteristics of a good GC-MS method is the absence of carry-over. Carry-over can lead to an overestimation of the concentration of a given compound or even to a false positive result. When a method is free from carry-over, it allows highly concentrated samples to be analyzed without affecting subsequent samples. To evaluate the presence of carry-over, blank samples were injected directly after highly concentrated standards. None of the steroids under investigation were found to be present in the blank (Figure 2). This means that the method was free of carry-over.

Table 4. Quantitation accuracy (the deviation should not be greater than ±30%)

Compound	Low QC	Mid QC	High QC
Androsterone	4%	15%	2%
Etiocholanolone	10%	3%	-18%
Etiocholanolone (5A-diol)	26%	25%	4%
Etiocholanolone (5B-diol)	49%	8%	-7%
Epitestosterone	29%	-2%	-8%
Testosterone	74%	-7%	-11%

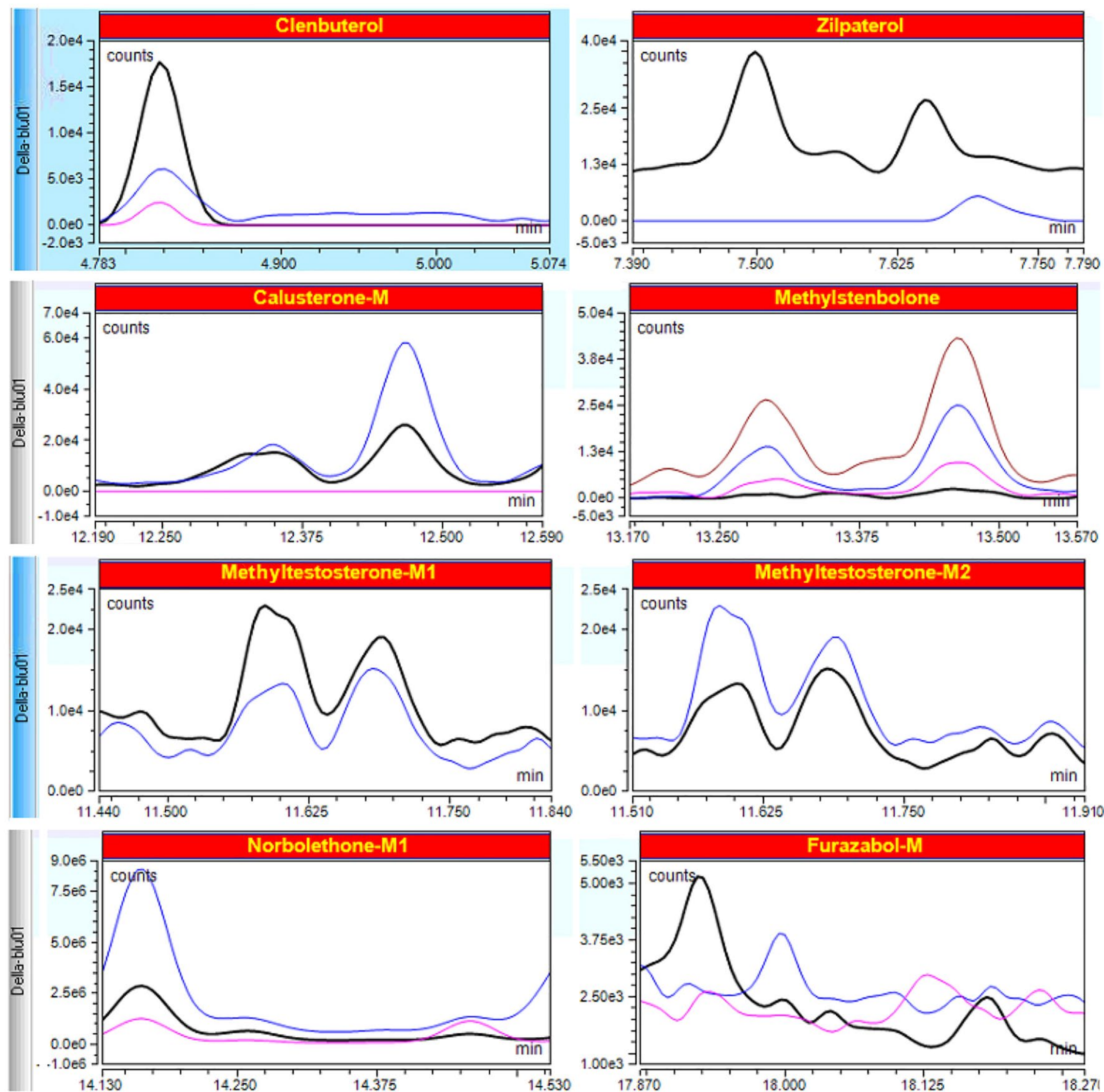


Figure 2. Carry-over test. In a blank matrix injected directly after a high concentrated standard, no analyte peak was present.

Robustness

Analytical testing laboratories need to be able to produce consistent results daily. A gradual decrease in sensitivity is not acceptable as it could lead to missing low level positives. When the sensitivity falls below the acceptable level, the instrument must be stopped for maintenance (i.e., ion source cleaning, liner replacement, column trimming, etc.). In a robust GC-MS system, the sensitivity decrease is considerably slower and thus helps to maintain sample throughput over an extended period. To test the robustness, a long sequence of matrix samples was injected. After every 30 injections, a QC sample that contained steroids at their MRLs was analyzed and no relevant sensitivity lost was observed. Figure 3 shows four consecutives QC samples, i.e., samples analyzed after 30, 60, 90, and 120 injections of urine.

Helium conservation

Helium is the most commonly used carrier gas for gas chromatography thanks to its high chromatographic efficiency and inertness. Recent price rises in helium and supply issues caused by shortages have led GC manufacturers, researchers, and analysts to investigate possible mitigation options that entail either switching to alternative carrier gases or reducing the helium consumption. The Thermo Scientific™ HeSaver-H_2Safer™ carrier gas saving technology[7] offers significant gas savings not only when the GC is idle, but mainly during sample injection and analysis. It decouples the gas flow for inlet pressurization, analyte vaporization, and transfer to the analytical column from the main carrier gas flow. This way, an inexpensive gas (e.g., nitrogen or argon) can be used while the consumption of helium is greatly decreased. At the same time, an established method does not require any changes or modifications. In this case, the employment of the helium saver would decrease the helium consumption 4.5 times in comparison to a standard SSL. The exact cost reduction can be calculated using the Thermo Scientific™ Gas Saver Calculator tool[8], an easy-to-use and intuitive interface to estimate the helium consumption and cost impact. The user is only required to insert the column geometry, the carrier and split flow settings, and helium and nitrogen costs. The tool provides an estimation of both the helium cylinder lifetime and the cost savings.

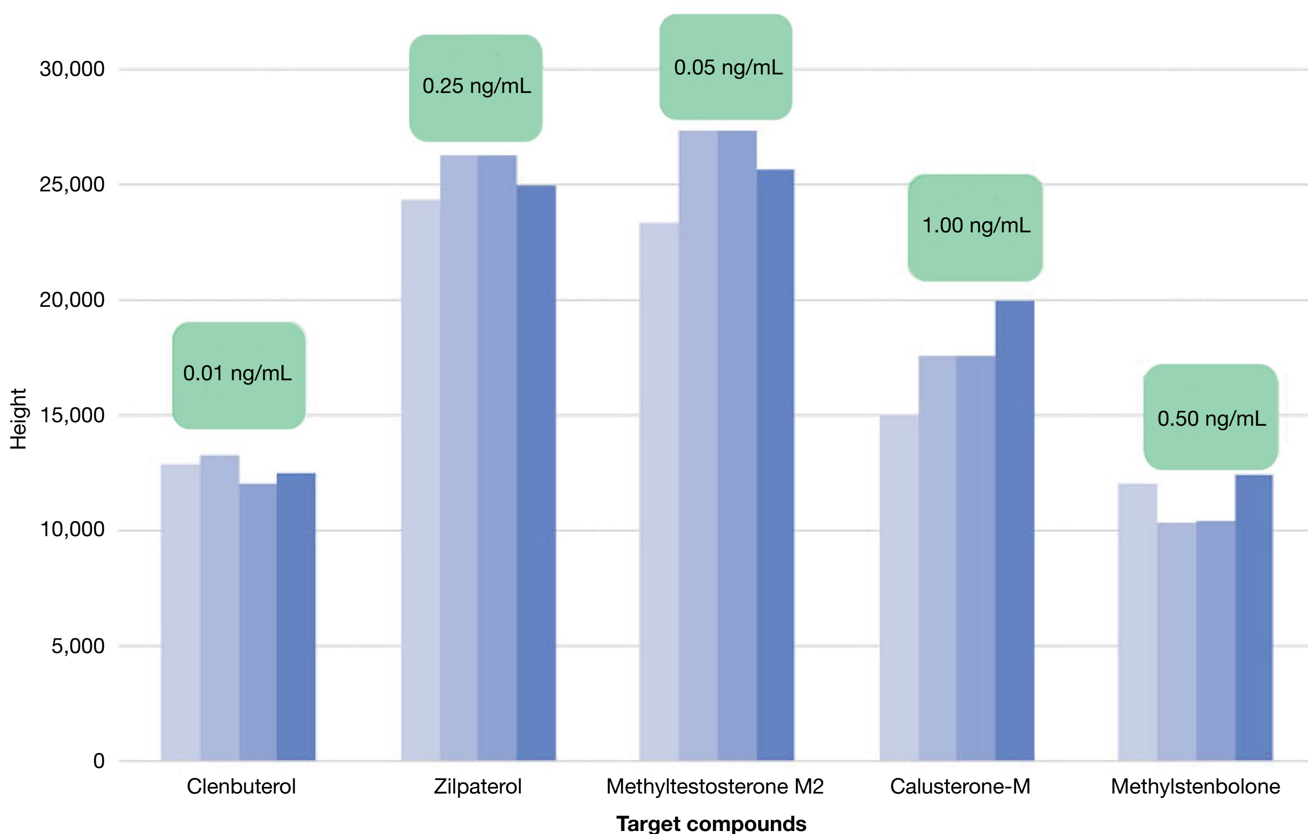


Figure 3. Robustness test. A QC sample (MRPL) was analyzed after 30, 60, 90, and 120 urine injections.

Conclusions

The combination of the TRACE 1610 GC and the TSQ 9610 GC-MS/MS system showed to be an excellent tool the steroids analysis. It should be remarked that:

- High sensitivity of the TSQ 9610 GC-MS/MS system allowed the analysis of steroids at the recently updated MRPLs.
- In the majority of cases, the quantitation accuracy was better than 30%.
- It was demonstrated that the method was not subject to carry-over after high concentrations were injected.
- Excellent robustness over 100 injections was demonstrated to give consistent instrument response.
- Helium usage can be decreased by 4.5 time using the HeSaver-H_2Safer injector.

References

1. www.wada-ama.org
2. The 2023 List of Prohibited Substances and Methods (https://www.wada-ama.org/sites/default/files/2022-09/2023list_en_final_9_september_2022.pdf)
3. WADA Technical Document – TD2023IDCR (https://www.wada-ama.org/sites/default/files/2022-09/td2023idcr_eng_final2.pdf)
4. The International Standard for Laboratories (ISL) (https://www.wada-ama.org/sites/default/files/resources/files/isl_2021.pdf)
5. Parr, M.K.; Botrè, F. Supercritical fluid chromatography mass spectrometry as an emerging technique in doping control analysis, *TrAC Trends in Analytical Chemistry* **2022,** *147,* 116517. https://doi.org/10.1016/j.trac.2021.116517
6. de Albuquerque, C.G.; Rodrigues, L.M.; Dos Santos, L. et al. Non-targeted acquisition strategy for screening doping compounds based on GC-EI-hybrid quadrupole-Orbitrap mass spectrometry: a focus on exogenous anabolic steroids. *Drug Test Anal.* **2018,** *10,* 507–517. https://doi.org/10.1002/dta.2227
7. Thermo Fisher Scientific, Technical Note 001218: Addressing gas conservation challenges when using helium or hydrogen as GC carrier gas. https://assets.thermofisher.com/TFS-Assets/CMD/Technical-Notes/tn-001218-gc-hesaver-h2safer-trace1600-tn001218-na-en.pdf
8. Thermo Fisher Scientific Helium Saver Calculator. https://www.thermofisher.com/us/en/home/industrial/chromatography/chromatography-learning-center/chromatography-consumables-resources/chromatography-tools-calculators/helium-saver-calculator.html

General Laboratory Equipment – Not For Diagnostic Procedures. ©2023 Thermo Fisher Scientific Inc. All rights reserved. All trademarks are the property of Thermo Fisher Scientific and its subsidiaries. This information is presented as an example of the capabilities of Thermo Fisher Scientific products. It is not intended to encourage use of these products in any manners that might infringe the intellectual property rights of others. Specifications, terms, and pricing are subject to change. Not all products are available in all countries. Please consult your local sales representative for details. **TN001796-EN 0323S**

thermo scientific