

WADA Technical Document – TD2021EAAS

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Written by:	WADA Science/EAAS Working Group	Approved by:	WADA Executive Committee
Reviewed by:	WADA <u>Laboratory Expert Group</u>		
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Measurement and Reporting of Endogenous Anabolic Androgenic Steroid (EAAS) Markers of the Urinary Steroid Profile

1.0 Introduction

The purpose of this *Technical Document (TD)* is to harmonize the measurement and reporting of the “steroid profile” of urine *Samples* in support of the steroidal module of the *Athlete Biological Passport (ABP)* (the steroidal Passport).

1.1 The Steroid Profile

The measurement of steroidal *Markers* [concentrations and ratios of defined Endogenous Anabolic Androgenic Steroids (EAAS)] in a urine *Sample* form the steroid profile for that *Sample* (see Table 1).

The steroid profiles of a series of urine *Samples* collected from an *Athlete* over a period of time constitute the steroidal Passport of that *Athlete*.

The administration of synthetic forms of EAAS can alter one or more of the *Markers* of the urinary steroid profile, resulting in increased or decreased concentrations and/or ratios of specific pairs of steroid *Markers* ^[1-3]. This effect forms the basis for the use of the steroidal Passport as a tool for the detection of doping with EAAS, in particular testosterone (T), its precursors (for example, 4-androstenediol, androstenedione and prasterone), its active *Metabolite* [dihydrotestosterone (DHT)], or its epimer epitestosterone (E).

The steroidal module of the *ABP* utilizes the Adaptive Model in *ADAMS* to trigger *Atypical Passport Findings (ATPFs)*, which can lead to the performance of Confirmation Procedures (CP), *Target Testing* of an *Athlete*, or to establish *Use of a Prohibited Substance* and/or *Prohibited Method* as per Code Article 2.2 (see *International Standard for Results Management*, Annex C ^[4]).

1.2 Procedure for Determination of the Steroid Profile

Each urine *Sample* shall be analyzed to determine its steroid profile. The determination and reporting of a *Sample*'s steroid profile follows a two-step procedure:

- i. An Initial Testing Procedure (ITP) is conducted to estimate the steroid profile of the *Sample*, and
- ii. A subsequent CP is performed when the reported steroid profile constitutes an *ATPF*, as determined by the Adaptive Model, or upon request from the Athlete Passport Management Unit (APMU), the Testing Authority or *WADA*.

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Table 1. Markers of the Urinary Steroid Profile.

Type of Marker	Steroid Profile Markers	Determination
Concentrations of Steroids	<ul style="list-style-type: none"> - Androsterone (A); - Etiocholanolone (Etio); - 5α-Androstane-3α,17β-diol (5αAdiol); - 5β-Androstane-3α,17β-diol (5βAdiol); - Testosterone (T); and - Epitestosterone (E). 	Determined by the <u>Laboratory</u> by GC-MS ⁿ from the combination of the free steroid fraction and the conjugated fraction released after hydrolysis with β -glucuronidase from <i>E. coli</i> .
Ratios of Steroids	- T/E	As reported by the <u>Laboratory</u> in ADAMS.
	<ul style="list-style-type: none"> - A/T; - A/Etio; - 5αAdiol/5βAdiol; and - 5αAdiol/E 	Automatically computed in ADAMS from respective steroid concentrations after the reporting of the steroid profile by the <u>Laboratory</u> .

1.3 Factors Impacting the Steroid Profile

In addition to the effects mediated by the administration of EAAS, alteration of the urinary steroid profile can occur for a number of other reasons including, but not limited to, the following factors ^[1-3]:

- Intake of alcohol (ethanol);
- The administration of other anabolic androgenic steroids (e.g. stanozolol);
- The administration of human chorionic gonadotrophin (hCG) in males;
- The administration of aromatase inhibitors and anti-estrogenic substances;
- The administration of inhibitors of 5 α -reductase (e.g. finasteride, dutasteride);
- The administration of ketoconazole or other similar compounds (e.g. fluconazole, miconazole);
- The use of masking agents (e.g. probenecid) and diuretics;
- Microbial activity;
- *Sample* manipulation.

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2.0 Initial Testing Procedure (ITP)

2.1 ITP Method Requirements

The quantification of the *Markers* of the steroid profile shall be based on gas chromatography combined with mass spectrometry (GC-MSⁿ; $n \geq 1$).

Table 2. Requirements of the ITP for Quantification of the *Markers* of the Steroid Profile.

2.1.1 <u>ITP</u> Validation Requirements								
Range of the Method	Shall cover the ranges of <i>Marker</i> concentrations normally found in males and females.							
Enzymatic Hydrolysis	Assess the efficiency of the enzymatic hydrolysis using β -glucuronidase from <i>E. coli</i>							
Derivatization	Assess the efficiency of the trimethylsilyl (TMS) derivatization							
Limits of Quantification (LOQ)	<p>The <u>LOQ</u> shall be determined during method validation as the lowest concentration that can be measured with an u_c (%) not greater than (\leq) 30% and shall meet the following criteria:</p> <ul style="list-style-type: none"> • T, E \leq 1 ng/mL; • 5αAdiol, 5βAdiol \leq 10 ng/mL; • A, Etio \leq 500 ng/mL 							
Measurement Uncertainty, u_c (%)	Level	A	Etio	T	E	Adiols (5α-, 5β-)	T/E	
	The estimated u_c (%) shall be not greater than (\leq) the u_{c_Max} (%) value given below							
	at <u>LOQ</u>	\leq 30%						
	at 5 x <u>LOQ</u>	\leq 20%				\leq 25%		
	(T, E) > 5 ng/mL							\leq 15%
(T, E) \leq 5 ng/mL							\leq 30%	
2.1.2 <u>ITP</u> Analysis Requirements								
Sample	The <u>ITP</u> for the quantification of the <i>Markers</i> of the steroid profile shall be conducted on a single <u>Aliquot</u> . When needed, the volume of the <u>Aliquot</u> may be adjusted as a function of its specific gravity (SG) and of the sex of the <i>Athlete</i> .							

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Calibration	Calibration standard(s) or a calibration curve shall be included in each sequence of analysis.
Quality Control	At least two (2) quality control (QC) urine samples containing representative low and high concentrations of the <i>Markers</i> of the steroid profile shall be included in each sequence of analysis.
Enzymatic Hydrolysis	Purified β -glucuronidase from <i>E. coli</i> shall be used for the hydrolysis of the glucuroconjugated urinary steroids, and the completeness of hydrolysis shall be monitored in each <u>Aliquot</u> with isotopically labeled A-glucuronide (or an equivalent scientifically recognized alternative). <i>H. pomatia</i> mixtures shall not be used.
Derivatization	The <i>Markers</i> of steroid profile shall be analyzed as TMS derivatives (TMS enol ethers and/or TMS ethers). Completeness of the derivatization shall be controlled in each <u>Aliquot</u> through the monitoring of mono-O-TMS vs. di-O-TMS derivative of A.
T/E Ratio	The T/E ratios shall be determined from the ratios of chromatographic peak areas or peak heights after correction against a calibrator or a calibration curve.
Factors Impacting the Steroid Profile	The <u>Laboratory</u> shall: <ul style="list-style-type: none"> • Monitor for signs of microbial activity [e.g. presence of indicators of 3α-hydroxysteroid dehydrogenase (HSD) activity]; <i>[Comment: The direct enzymatic hydrolysis of urine Samples may increase the effects of microbial contamination.]</i> • Test for the presence of conjugated <i>Metabolite(s)</i> of ethanol [e.g. ethanol glucuronide (EtG)], 5α-reductase inhibitors (e.g. finasteride, dutasteride) and ketoconazole (and similar substances).

2.2 Reporting the *Sample's* Steroid Profile from the ITP

Following the performance of the ITP, the Laboratory shall report in ADAMS the steroid profile for each *Sample* analyzed.

The Laboratory shall report in ADAMS:

- i. The SG of the *Sample*, as determined by the Laboratory (see TD DL ^[5]);
- ii. The uncorrected concentrations of T, E, A, Etio, 5 α Adiol and 5 β Adiol, and the T/E ratio;

*[Comment: When the ITP measurement of a steroid profile Marker is not possible due to, for example, dilution, unusual matrix interferences, inhibition of the enzymatic hydrolysis or incomplete derivatization, the Laboratory should repeat the analysis with an alternative *Sample* preparation procedure (e.g. changing Aliquot volumes, application of solid phase extraction, or extraction with a different solvent).*

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If, however, a Marker of the steroid profile cannot be quantified, the concentration of the affected Marker shall be reported as “-1”. The Laboratory shall make a comment in the Test Report on why this Marker could not be quantified (e.g. < LOQ, incomplete derivatization).

When the chromatographic peak signal for a Marker cannot be detected (i.e. is below the detection capability of the assay), the concentration of the Marker shall be reported as “-2” (See Table 3 for reporting of specific situations for [T], [E], and T/E).

The Laboratory may also provide information on other steroidal parameters such as prasterone (DHEA), dihydrotestosterone (DHT) and 6 α -hydroxy-androstenedione (6 α -OH-AD) at the request of the Testing Authority, Results Management Authority or the APMU.]

- iii. Any signs of microbial activity in the *Sample*, e.g. ratios of 5 α -androstenedione (5 α AND) to A and 5 β -androstenedione (5 β AND) to Etio, as determined from the respective steroid concentrations;
- iv. The presence or absence in the *Sample* of substance(s) that may alter the steroid profile (see Article 1.3). The Laboratory shall report the estimated levels of:
 - EtG if $\geq 5 \mu\text{g/mL}$;
 - Carboxy-finasteride if $\geq 5 \text{ ng/mL}$;
 - 4-hydroxy- and/or 6-hydroxy-dutasteride if $\geq 5 \text{ ng/mL}$;
 - Ketoconazole if $\geq 100 \text{ ng/mL}$;
 - Fluconazole if $\geq 500 \text{ ng/mL}$;
 - Miconazole if $\geq 1,000 \text{ ng/mL}$.

2.2.1 Validity of the *Sample* Steroid Profile

The validity of the *Sample* will be determined automatically upon reporting of the steroid profile in ADAMS. A *Sample* will be invalid only when the *Sample* shows signs of extensive degradation, as determined by:

- 5 α AND/A ≥ 0.1 , and/or
- 5 β AND/Etio ≥ 0.1

*[Comment: In addition, following the reporting of the steroid profile in ADAMS by the Laboratory, the *Sample* may be evaluated as “invalid” by the APMU upon review of the steroid profile data, for example, by considering the presence of substances that may alter the steroid profile in the *Sample*.]*

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Table 3. Summary of conditions for reporting T and E concentrations and T/E ratio.

Concentration of T	Concentration of E	T/E ratio
Chromatographic peak signal of T measured at or above (\geq) the <u>LOQ</u> . $[T] \geq \text{LOQ}_{(T)}$ Report T as measured	Chromatographic peak signal of E measured at or above (\geq) <u>LOQ</u> . $[E] \geq \text{LOQ}_{(E)}$ Report E as measured.	Report T/E (as determined by the <u>Laboratory</u> from corrected peak heights/areas)
	Chromatographic peak signal of E detected, but below ($<$) <u>LOQ</u> . $\text{LOD}_{(E)} \leq [E] < \text{LOQ}_{(E)}$ Report E as “-1”	
	Chromatographic peak signal of E not detected. $[E] < \text{LOD}_{(E)}$ Report E as “-2”	Report T/E as “-1” Report the <u>LOD</u> _(E) <i>Comment in ADAMS: T/E ratio could not be measured accurately because E could not be detected.</i>
Chromatographic peak signal of T detected, but below ($<$) the <u>LOQ</u> . $\text{LOD}_{(T)} \leq [T] < \text{LOQ}_{(T)}$ Report T as “-1”	Chromatographic peak signal of E measured at or above (\geq) <u>LOQ</u> . $[E] \geq \text{LOQ}_{(E)}$ Report E as measured	Report T/E (as determined by the <u>Laboratory</u> from corrected peak heights/areas)
	Chromatographic peak signal of E detected, but below ($<$) <u>LOQ</u> . $\text{LOD}_{(E)} \leq [E] < \text{LOQ}_{(E)}$ Report E as “-1”	
	Chromatographic peak signal of E not detected. $[E] < \text{LOD}_{(E)}$ Report E as “-2”	Report T/E as “-1” <i>Comment in ADAMS: T/E ratio could not be measured accurately because the concentration of T could not be measured, and E could not be detected</i>
Chromatographic peak signal of T not detected. $[T] < \text{LOD}_{(T)}$ Report T as “-2”	Chromatographic peak signal of E measured at or above (\geq) <u>LOQ</u> . $[E] \geq \text{LOQ}_{(E)}$ Report E as measured	Report T/E as “-1” Report the <u>LOD</u> _(T) <i>Comment in ADAMS: T/E ratio could not be measured accurately because T could not be detected</i>
	Chromatographic peak signal of E detected but below ($<$) <u>LOQ</u> . $\text{LOD}_{(E)} \leq [E] < \text{LOQ}_{(E)}$ Report E as “-1”	Report T/E as “-1” Report the <u>LOD</u> _(T) <i>Comment in ADAMS: T/E ratio could not be measured because T could not be detected, and E could not be measured.</i>
	Chromatographic peak signal of E not detected. $[E] < \text{LOD}_{(E)}$ Report E as “-2”	Report T/E as “-2” Report the <u>LOD</u> _(E) and <u>LOD</u> _(T) <i>Comment in ADAMS: T/E ratio could not be measured because T and E could not be detected.</i>

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3.0 Confirmation Procedures (CP)

The CP for the EAAS *Markers* include the GC-MSⁿ ($n \geq 1$) identification (in compliance with the TD IDCR ^[6]) and quantification, as well as the GC/C/IRMS analysis ^[7] of the *Marker(s)* of the steroid profile.

In addition, the Laboratory shall confirm the presence or absence of factors impacting the steroid profile (see Article 1.3).

3.1 CP Requests (CPRs)

3.1.1 CPRs triggered by *Atypical Passport Findings (ATPF)* through ADAMS

Once the *Sample's* steroid profile data are entered in ADAMS and matched with an *Athlete*, the Adaptive Model automatically updates the steroidal Passport. If an *ATPF* is identified based on an abnormally high T/E value, a CP request (*ATPF-CPR*) is triggered and sent automatically to Laboratories through ADAMS.

Upon receipt of an *ATPF-CPR*, the Laboratory shall proceed with the CP of the steroid profile as soon as possible, unless the presence of ethanol or other factors impacting the steroid profile has been detected in the *Sample*. In such cases, the Laboratory shall receive, within fifteen (15) days from the *ATPF-CPR* notification, an advice from the Passport Custodian or the Testing Authority (or Results Management Authority, if different) on whether to proceed or not with the CP of the *Sample's* steroid profile.

*[Comment: In the absence of communication from the Passport Custodian or the Testing Authority (or Results Management Authority) within fifteen (15) days from the *ATPF-CPR* notification, the Laboratory shall proceed with the CP of the steroid profile (see Article 3.2)].*

Any justification from the Passport Custodian or the Testing Authority (or Results Management Authority) not to proceed with the CP shall be provided in writing and in compliance with the TD APMU ^[8].

*[Comment: In cases when the Laboratory is instructed by the Passport Custodian or the Testing Authority (or Results Management Authority) not to perform the CP, the Laboratory shall update the ADAMS Test Report for the *Sample* with a comment stating that the Passport Custodian, Testing Authority (or Results Management Authority) requested not to perform the CP, and the reasons given.]*

When the Laboratory receives an *ATPF-CPR* for a *Sample* for which *Adverse Analytical Finding(s) (AAF)* have been reported for other *Prohibited Substance(s)* or *Method(s)*, the Laboratory shall consult the Testing Authority (or Results Management Authority, if different) about the need to conduct the CP for the *Markers* of the steroid profile.

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3.1.2 CPRs from the APMU, the Testing Authority (or Results Management Authority, as applicable) or WADA.

The Adaptive Model will also determine abnormal values or sequences of the other ratios of the “steroid profile” (A/T, A/Etio, 5 α Adiol/5 β Adiol, 5 α Adiol/E). However, in such cases the Laboratory will not receive an automatic “ATPF-CPR” notification through ADAMS. Instead, the APMU will advise the Testing Authority (or Results Management Authority, if different) on whether the Sample shall be subjected to CP. Therefore, in these cases the Laboratory shall receive a written request from the Testing Authority (or Results Management Authority, if different) before proceeding with the CP.

In the absence of an ATPF-CPR, requests for CP can be made also by the Testing Authority (or Results Management Authority, if different), the APMU *, or WADA.

* where the respective client of the APMU has agreed to bestow such authority to the APMU.

3.2 CP Test Methods

3.2.1 CP of Steroid Profile *Markers* by GC-MSⁿ

The Laboratory shall quantify all the *Markers* of the steroid profile in one Aliquot by a validated Fit-for-Purpose GC-MSⁿ ($n \geq 1$) quantification method. Identification (in compliance with the TD IDCR ^[6]) of the *Markers* that triggered the CP shall be performed as well.

- In every case, the Laboratory shall confirm quantitatively all the *Markers* of the steroid profile before proceeding with the GC/C/IRMS analysis;

*[Comment: This requirement does not apply if the Testing Authority (or Results Management Authority, as applicable) has authorized the Laboratory to proceed directly to GC/C/IRMS analysis without a need for a quantitative confirmation of the steroid *Markers* (for example, in cases of limited Sample volume).*

For T/E values, only T needs to be confirmed if E is not detected or the volume of the Sample is not sufficient.]

- In the case of an ATPF-CPR for an abnormally high T/E ratio, GC/C/IRMS analysis is not mandatory when the confirmed T/E value is below the confirmation T/E cut-off calculated by the Adaptive Model and provided within the ATPF-CPR notification received from ADAMS;
- For other CP requests, when the steroid profile CP does not confirm the ITP values that triggered the CP (e.g. 5 α Adiol/E value), taking into consideration the expanded uncertainty of the measurement ($U_{95\%}$, $k = 2$), the Laboratory shall consult the Testing Authority to determine if the GC/C/IRMS analysis is necessary. In the event that GC/C/IRMS analysis is deemed unnecessary, the Laboratory shall update the ADAMS report for the Sample with the

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confirmed values of all the *Markers* of the steroid profile and include a comment that GC/C/IRMS analysis was not necessary.

[Comment: for ratios other than the T/E, the u_c (%) of the ratio shall be calculated by propagation of uncertainties of the corresponding Marker concentrations.]

The same analytical requirements presented in Table 2 for the ITP shall apply for the GC-MSⁿ CP, with the following modifications:

- GC-MSⁿ CP Validation Requirements
 - For determinations of A, Etio, 5 α Adiol and 5 β Adiol, the u_c (%) shall be not greater than (\leq) 15% when the concentrations are five times (5x) the respective LOQ;
 - For determinations of T, E and T/E ratios, the u_c (%) shall be not greater than (\leq) 15% when the concentrations of T and E are greater than (>) 5 ng/mL.
- GC-MSⁿ CP Analysis Requirements
 - A Solid Phase Extraction (SPE) shall be performed prior to the enzymatic hydrolysis of the *Sample*;
 - Calibration standard(s) and at least two (2) QC urine samples containing representative low and high levels of the *Markers* of the steroid profile shall be included.

3.2.2 GC/C/IRMS CP

Technical and reporting requirements for the GC/C/IRMS CP are specified in the TD IRMS ^[7].

When an *AAF* is reported for the *Marker(s)* of the steroid profile based on the results of a GC/C/IRMS analysis performed on the “A” *Sample*, only the GC/C/IRMS analysis, including the identification of the relevant *Markers* (target compounds and endogenous reference compounds) shall be repeated during the “B” *Sample CP*.

3.3 Reporting Results from the CP

3.3.1 “A” *Sample*

Following the CP performed for the steroid profile on the “A” *Sample*, the Laboratory shall report in ADAMS:

- i. The SG of the *Sample* (determined from a new Aliquot of the “A” *Sample*);
- ii. The confirmed value of the *Markers* of the steroid profile (concentrations, T/E value), without adjustment for the SG of the *Sample*;

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- iii. The associated u_c (expressed in units);
- iv. The GC/C/IRMS confirmation results, if performed (see TD IRMS ^[7]). The Laboratory shall update the Test Report for the *Sample* in ADAMS (as AAF, Atypical Finding (ATF), or Negative Finding) based on the results of the GC/C/IRMS CP;
- v. The confirmed results (presence/absence) for signs of microbial activity: 5 α AND/A, 5 β AND/Etio, and T_{free}/T_{total} ; based on concentrations;

*[Comment: In addition to the determination of the 5 α AND/A and 5 β AND/Etio ratios as signs of microbial contamination, the determination during the CP of an elevated ratio of free Testosterone to total Testosterone ($T_{free} / T_{total} > 0.05$) will also invalidate (the steroid profile of) the *Sample*. However, this shall not preclude the performance of the GC/C/IRMS CP or invalidate its results.]*

- vi. The presence or absence in the *Sample* of substance(s) that do not constitute an AAF but may alter the steroid profile (see Article 1.3): if detected in the *Sample*, the Laboratory shall report the confirmed estimated levels of EtG, 5 α -reductase inhibitors and -azoles as specified in Article 2.2 (without the need to report the u_c for these determinations).

3.3.2 “B” Sample

Following the performance of the GC/C/IRMS CP for the steroid profile on the “B” *Sample*, the Laboratory shall report the GC/C/IRMS confirmation results (see TD IRMS ^[7]) in ADAMS.

*[Comment: If the *Sample* has not been reported as an AAF for the Marker(s) of the steroid profile based on the results of the GC/C/IRMS analysis, but the steroid profile CP by GC-MSⁿ has been requested for the “B” *Sample*, then the Laboratory shall report in ADAMS the results of the “B” confirmation of the steroid profile as described for the “A” *Sample* in Article 3.3.1.]*

4.0 Reporting Sample Manipulation (Tampering or Attempted Tampering)

Tampering or Attempted Tampering aims to alter the integrity and validity of *Samples* collected during *Doping Control*, including, but not limited to *Sample* substitution with another fluid and urine exchange and/or adulteration (e.g. addition of proteases to *Sample*).

*[Comment: the substitution of an Athlete’s urine *Sample* with the urine of another individual (urine exchange) can be uncovered using the steroidal Passport and confirmed by DNA analysis across multiple *Samples*, as described in the TD APMU ^[8].]*

In cases when a *Sample* is not consistent with human urine (e.g. SG ≤ 1.001 , creatinine ≤ 5 mg/dL ^[9], non-physiological salt concentration, abnormal pH values, absence or abnormally low levels of endogenous steroids, corticosteroids, proteins, etc.), the Laboratory shall:

- i. Report the finding as an AAF for *Tampering* or *Attempted Tampering* (class M2.1 of the *Prohibited List*) if the Laboratory can determine the general nature/type of the adulterated *Sample*, which is not consistent with human urine (e.g. water, liquor, synthetic urine);

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- ii. Report the finding as an *ATF* for *Tampering* or *Attempted Tampering* and include a comment in *ADAMS* advising the Testing Authority to perform further investigations (e.g. additional analyses on the *Sample*, *Target Testing the Athlete*).

5.0 References

- [1] Mareck U *et al.* Factors influencing the steroid profile in doping control analysis. *J Mass Spectrom.* **43**(7):877-91, 2008.
- [2] Ayotte C. Detecting the administration of endogenous anabolic androgenic steroids. *Handb Exp Pharmacol.* **195**:77-98, 2010.
- [3] Kuuranne T, Saugy M, Baume N. Confounding factors and genetic polymorphism in the evaluation of individual steroid profiling. *Br J Sports Med.* **48**(10): 848-55, 2014.
- [4] The World Anti-Doping Code International Standard for Results Management.
- [5] WADA Technical Document TD DL: *Decision Limits* for the Confirmatory Quantification of Exogenous Threshold Substances by Chromatography-based Analytical Methods.
- [6] WADA Technical Document TD IDCR: Minimum Criteria for Chromatographic-Mass Spectrometric Confirmation of the Identity of Analytes for *Doping Control* Purposes.
- [7] WADA Technical Document TD IRMS: Detection of Synthetic Forms of *Prohibited Substances* by GC/C/IRMS.
- [8] WADA Technical Document TD APMU: Athlete Passport Management Unit – Requirements and Procedures.
- [9] Cook J D *et al.* The Characterization of Human Urine for Specimen Validity Determination in Workplace Drug Testing: A Review. *J Anal Toxicol* **24**: 579-588, 2000

[Comment: Current versions of WADA Technical Documents may be found at <https://www.wada-ama.org/en/what-we-do/science-medical/laboratories>]