

1 **Determination of selected endogenous anabolic androgenic steroids and ratios in urine by**  
2 **ultra high performance liquid chromatography tandem mass spectrometry and isotope**  
3 **pattern deconvolution**

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7  
8 **ABSTRACT**

9 An isotope dilution mass spectrometry (IDMS) method for the determination of selected  
10 endogenous anabolic androgenic steroids (EAAS) in urine by UHPLC-MS/MS has been  
11 developed using the isotope pattern deconvolution (IPD) mathematical tool. The method has  
12 been successfully validated for testosterone, epitestosterone, androsterone and  
13 etiocholanolone, employing their respective deuterated analogs using two certified reference  
14 materials (CRM). Accuracy was evaluated as recovery of the certified values and ranged from  
15 75% to 108%. Precision was assessed in intraday (n=5) and interday (n=4) experiments, with  
16 RSDs below 5% and 10% respectively. The method was also found suitable for real urine  
17 samples, with limits of detection (LOD) and quantification (LOQ) below the normal urinary  
18 levels. The developed method meets the requirements established by the World Anti-Doping  
19 Agency for the selected steroids for Athlete Biological Passport (ABP) measurements, except in  
20 the case of androsterone, which is currently under study.

21  
22 **INTRODUCTION**

23 Misuse of steroids is nowadays a significant social issue. Apart from doping in sports,  
24 endogenous anabolic androgenic steroids (EAAS) use has become a problem of public health  
25 [1]. Regarding substances prohibited in sports, over the years consensus has been achieved  
26 about which steroidal markers must be controlled as an additional part of the World Anti-  
27 Doping Agency (WADA) Athlete Biological Passport (ABP), the steroidal module [2].  
28 Testosterone (T), epitestosterone (E), androsterone (A), etiocholanolone (Etio), 5 $\alpha$ -androstane-  
29 3 $\alpha$ ,17 $\beta$ -diol (5 $\alpha$ Adiol), 5 $\beta$ -androstane-3 $\alpha$ ,17 $\beta$ -diol (5 $\beta$ Adiol) and the ratios T/E, A/T, A/Etio,  
30 5 $\alpha$ Adiol/5 $\beta$ Adiol, 5 $\beta$ Adiol/E are the parameters of choice. An abnormal steroidal or  
31 longitudinal profiles may constitute a suspicion of doping, thus, reliable analytical methods are  
32 needed to assess the concentration of those EAAS. Moreover, clear verification of exogenous  
33 administration of EAAS is still a challenge. The general workflow includes an initial screening  
34 method followed by a confirmation if adverse results are found. However, in spite of WADA  
35 efforts, a completely standardized methodology has not been established yet, neither by the  
36 mass detector (Q or QqQ), nor by the sample treatment method used in that initial screening  
37 step [3]. Although the accepted WADA quantification method for EAA determination in urine is  
38 GC/MS [2], among current instrumental techniques in drug testing, UHPLC-MS/MS is mainly  
39 used due to its high throughput, chromatographic performance and sensitivity [4-6].  
40 On the other hand, ESI, the most employed ionisation source in LC-MS instrumental  
41 techniques can suffer severe matrix effect problems, mainly related with ion suppression or  
42 enhancement [7-9]. The use of isotope labelled internal standards (ILIS) is widely recognized as  
43 the best way to overcome matrix effect problems. Thus, quantification through isotope  
44 dilution mass spectrometry (IDMS) works out the issues related with signal alteration [10,11].  
45 A recently developed IDMS method of quantification, isotope pattern deconvolution (IPD),

46 does not rely on the construction of any calibration graph. IPD involve the artificial alteration  
47 of the natural isotopomer abundances of a compound in a sample by the addition of a known  
48 amount of a labelled analogue. The isotopic composition of the blend is a linear combination  
49 of two isotope patterns: that of the natural abundance compound and the isotope pattern of  
50 the labelled analogue. The separate contribution of each 'isotope pattern' to the whole mass  
51 spectrum can be calculated by multiple linear regression and provides the molar fractions of  
52 both labelled and unlabelled compound in the sample. [12-14]. This method has been  
53 satisfactorily tested for rapid quantifications in different complex matrices, such as food and  
54 environmental samples [15-18]. IDMS together with IPD can be considered a reliable (precise  
55 and accurate) methodology, free of matrix effect and fast, providing one result per injection.  
56 However, except for a recent paper related with testosterone determination in urine [19], IPD  
57 has never been applied to steroid determination.

58 In this work, an UHPLC-MS/MS method, based in IDMS and IPD quantification approach, is  
59 developed and validated for the determination of selected EAAS in human urine. T, E, A and Etio  
60 were selected among the EAAS included in the ABP, excluding the diols due to the known  
61 ionization difficulties by ESI of hydroxyandrostane compounds [20]. Accuracy and precision has  
62 been checked for the selected compounds, as well as ratios, through the analysis of NMIA  
63 MX002 and MX005 freeze dried human urine CRMs.

64

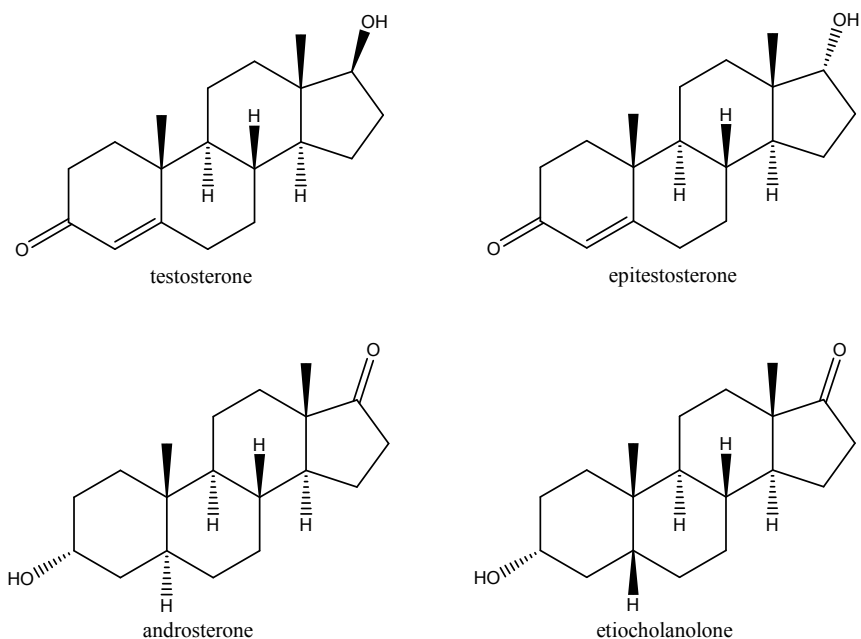
## 65 **EXPERIMENTAL**

### 66 **Reagents and materials**

67 Testosterone (T, purity 99%) and etiocholanolone (Etio, purity 98%) were provided by Sigma-  
68 Aldrich (Saint Louis, MO, USA), epitestosterone (E, purity 96.1%) was provided by LGC  
69 Standards (Luckenwalde, Germany) and androsterone VETRANAL® (A, purity 98.2%) by Sigma-  
70 Aldrich (Seelze, Germany). D3-Testosterone (d3-T, d3≈91%), d3-epitestosterone (d3-E,  
71 d3≈94%), d4-androsterone (d4-A, d4≈81%), d5-etiocholanolone (d5-Etio, d5≈92%) and  
72 certified reference materials (CRMs) NMIA MX002 and MX005 were all purchased to NMI  
73 Australia (North Ryde, NSW, Australia). Molecular structure of the selected EAAS are shown in  
74 Figure 1.

75 Methanol (HPLC quality), acetonitrile (HPLC quality) and methyl tert-butyl ether (MTBE, GC  
76 quality) were provided by Scharlau (Barcelona, Spain). For the sample hydrolysis, β-  
77 glucuronidase from *E. coli* K12 provided by Roche (Indianapolis, IN) was employed. A 1 M  
78 phosphate buffer was prepared by dissolving the proper amount of (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub> (Merck,  
79 Darmstadt, Germany) in Milli-Q water and adjusted to pH=7 with HCl 37% from Scharlau  
80 (Barcelona, Spain). Also, a NaHCO<sub>3</sub>/Na<sub>2</sub>CO<sub>3</sub> (1:2, w/w) (Sigma-Aldrich Co., Madrid, Spain) solid  
81 buffer was prepared. Formic acid (LC additive quality) and a 500 mM solution of NH<sub>4</sub>COOH  
82 (Scharlau, Barcelona, Spain) in methanol HPLC were used as modifiers for mobile phases.

83



84

85

86 Figure 1. Molecular structure of the selected endogenous steroids. Location of D atoms are  
87 displayed for the labeled compounds.

88

89 Individual stock solutions were prepared with 500 µg/mL of T, 200 µg/mL of E, 500 µg/mL of A,  
90 500 µg/mL of Etio and 100 µg/mL of each deuterated analog (d3-T, d3-E, d4-A and d5-Etio) by  
91 dissolving the proper amounts of solid standards in methanol. Then, 10 µg/mL working  
92 solutions of each compounds were prepared by dilution of stock solutions with methanol. A  
93 mix of labelled compounds was prepared in MeOH containing 1 µg/mL of d3-T and d3-E and 25  
94 µg/mL of d4-A and d5-Etio. All standard solutions were stored in amber glass bottles at -20°C.  
95 CRMs were reconstituted following the procedure indicated by the manufacturer and stored in  
96 a refrigerator until use.

97 Ultrapure water was obtained from a Milli-Q gradient A10 from Millipore (Bedford, MA, USA).

98

## 99 Instrumentation

100 Characterization and determination of analytes were performed on an Acquity UPLC system  
101 equipped with binary solvent and sample managers from Waters Corp. (Milford, MA, USA),  
102 coupled to a TQD quadrupole-hexapole-quadrupole tandem mass spectrometer and a Z-spray-  
103 electrospray interface (Waters Corp.). Chromatographic separation was achieved at 55°C on an  
104 Acquity UPLC BEH C18 column (1.7 µm, 2.1 mm x 100 mm, Waters Corp.) at 0.3 mL/min flow  
105 rate and 10 µL injection volume. Mobile phases consisted in H<sub>2</sub>O/ACN (95/5, v/v) as phase A  
106 and H<sub>2</sub>O/ACN (5/95, v/v) as phase B, both containing 0.01% of formic acid and 0.1 mM of  
107 NH<sub>4</sub>COOH as modifiers. The gradient applied was: 10% B (0-1 min), linear increase to 50% B in  
108 4.3 min, 50% B (5.3-9 min), 95% B (9.5-10.5 min), 10% B (11-13 min).

109 Ionization was performed at 120°C desolvation temperature and 350°C source temperature,  
110 while cone gas and desolvation flows were set at 80 and 800 L/h respectively. 3.5 kV capillary  
111 voltage was applied in positive mode. Multiple reaction monitoring (MRM) conditions and  
112 retention times are listed in Table 1.

113 Drying and nebulizing gas was N<sub>2</sub> from a nitrogen generator N<sub>2</sub> LC-MS adapted for LC-MS  
114 analyzers (Claind, Teknokroma, Barcelona, Spain). Collision cell was kept at approximately 5 x  
115 10<sup>-3</sup> mbar of argon 99.995% provided by Praxair (Madrid, Spain). Dwell time was set to 0.1 s

116 per scan for all quantification measurements. Analytical data was processed using Masslynx  
117 v4.1 (Waters) and homemade Excel spreadsheets (Microsoft Office).

118

119

120 Table 1. Experimental conditions of the LC-(ESI)-MS/MS for natural and labeled steroids

Compound	Ret. Time (min)	Precursor ion	Cone voltage (V)	Collision voltage (V)	MRM transitions*
T	6.43	[M+H] <sup>+</sup>	35	25	<b>289.2 &gt; 96.9</b> <b>290.2 &gt; 96.9</b> 289.2 > 109.1
E	7.06	[M+H] <sup>+</sup>	35	25	<b>289.2 &gt; 96.9</b> <b>290.2 &gt; 96.9</b> 289.2 > 109.1
A	8.29	[M+NH <sub>4</sub> ] <sup>+</sup>	35	10	<b>308.3 &gt; 273.1</b> <b>309.3 &gt; 274.1</b> 308.3 > 291.1
Etio	8.05	[M+NH <sub>4</sub> ] <sup>+</sup>	35	10	<b>308.3 &gt; 291.1</b> <b>309.3 &gt; 292.1</b> 308.3 > 273.1
d3-T	6.40	[M+H] <sup>+</sup>	35	25	<b>292.2 &gt; 96.9</b> <b>293.2 &gt; 96.9</b> 292.2 > 109.1
d3-E	7.03	[M+H] <sup>+</sup>	35	25	<b>292.2 &gt; 96.9</b> <b>293.2 &gt; 96.9</b> 292.2 > 109.1
d4-A	8.24	[M+NH <sub>4</sub> ] <sup>+</sup>	35	10	<b>312.3 &gt; 277.1</b> <b>313.3 &gt; 278.1</b> 312.3 > 295.1
d5-Etio	8.00	[M+NH <sub>4</sub> ] <sup>+</sup>	35	10	<b>313.3 &gt; 296.1</b> <b>314.3 &gt; 297.1</b> 313.3 > 278.1

121 \*In bold: MRM transitions employed for IPD quantification.

122

### 123 Sample treatment

124 A previously developed and widely used sample treatment method based on WADA guidelines  
125 [2] has been directly applied. 25 µL of labelled mix was added into 2.5 mL of sample in clean 15  
126 mL-glass tubes followed by 1 mL of 1 M phosphate buffer (pH=7). Hydrolysis was performed by  
127 adding 30 µL of β-glucuronidase solution and incubated in a water bath at 55 ± 2 °C for 1 h.

128 Next, approximately 200 mg of NaHCO<sub>3</sub>/Na<sub>2</sub>CO<sub>3</sub> (1:2, w/w) solid buffer were added to the tube  
129 and stirred until total dissolution of the solid. Liquid-liquid extraction was carried out by adding  
130 6 mL of MTBE, stirring in a vortex for 1 min and centrifuging at 3500 rpm for 5 min. Then, the  
131 upper organic phase was carefully transferred to clean glass tubes using disposable Pasteur  
132 pipettes, avoiding transferring any aqueous phase. MTBE was eliminated by evaporation in a  
133 MiVac at 40°C for 20 min, the residue was redissolved in 300 µL of MeOH/H<sub>2</sub>O (1:1, v/v) and  
134 transferred to LC vials.

135

136

## 137 **Method Validation**

### 138 Accuracy

139 Accuracy was validated by recovery experiments applying the method to two CRMs with  
140 different steroid concentrations and ratios, NMIA MX002 and NMIA MX005 freeze dried  
141 human urine. The method was regarded as accurate if the recovery was between 70% and  
142 110%.

### 143 Precision

144 Using the same CRMs, intraday and interday precisions were validated. Intraday repeatability  
145 was assessed performing the analysis of five replicates. Interday reproducibility was obtained  
146 by the application of the method to four replicates in four consecutive weeks.

147 In order to assess precision in terms of WADA guidelines [2], total combined uncertainty,  $u_c$ ,  
148 was also calculated according to WADA technical document TD2014DL [21] and Nordtest  
149 Guide [22]. A detailed explanation of the measurement uncertainty determination, using T as  
150 model compound in urine, is explained elsewhere [19]. The combined uncertainty for the  
151 ratios was assessed taking into account also the general propagation equation [23] to calculate  
152 the uncertainty associated to the reference material. A detailed explanation is shown in the  
153 supplementary information.

### 154 LOD and LOQ

155 A rough estimation of detection and quantification limits were conducted using the signal to  
156 noise ratio obtained in a real life sample. To this end, 9 urine samples from healthy female  
157 volunteers were collected and analyzed in order to get samples with low concentration of  
158 EAAS. Limits of quantification (LOQ) and detection (LOD) of the method were estimated as S/N  
159 equal to 10 and 3, respectively, in the lowest concentrated sample.

160

## 161 **Quantification by isotope pattern deconvolution (IPD)**

162 Isotope pattern deconvolution is a mathematical tool based on multiple linear regressions that  
163 provides the molar fractions of natural and labelled analytes in the spiked sample. The addition  
164 of the labelled analog alters the natural isotopic distribution of abundances  $A_{nat}^{SRM_i}$  due to the  
165 overlap of the labelled isotopic distribution  $A_{lab}^{SRM_i}$ . Hence, the deconvolution of the measured  
166 distribution in the mix  $A_{mix}^{SRM_i}$  is performed by solving the multiple linear regression in matrix  
167 notation:

$$168 \begin{bmatrix} A_{mix}^{SRM1} \\ A_{mix}^{SRM2} \\ \vdots \\ A_{mix}^{SRMn} \end{bmatrix} = \begin{bmatrix} A_{nat}^{SRM1} & A_{lab}^{SRM1} \\ A_{nat}^{SRM2} & A_{lab}^{SRM2} \\ \vdots & \vdots \\ A_{nat}^{SRMn} & A_{lab}^{SRMn} \end{bmatrix} \begin{bmatrix} X_{nat} \\ X_{lab} \end{bmatrix} + \begin{bmatrix} e^{SRM1} \\ e^{SRM2} \\ \vdots \\ e^{SRMn} \end{bmatrix}$$

169 Where the error vector  $e^{SRM_i}$  is the minimized parameter in the regression to solve the system  
170 and to obtain the molar fractions of natural and labelled analytes ( $x_{nat}$  and  $x_{lab}$  respectively).  
171 This can be easily achieved with the LINEST function in Microsoft Excel or any spreadsheet  
172 software. Then, since the amount of labelled compound  $N_{lab}$  is known, the amount of natural  
173 compound in the sample  $N_{nat}$  is readily calculated as follows:

$$174 N_{nat} = N_{lab} \frac{x_{nat}}{x_{lab}}$$

175 In contrast with commonly used analytical methodologies, IPD does not need methodological  
176 calibration and a concentration value is obtained from a single injection of the spiked sample.  
177 However, an extensive characterization of natural and labelled compounds is required to  
178 construct the calculation matrix, including isotopomer abundance distributions and exact

179 concentration of labelled standard solutions (determined by reverse isotope dilution). If the  
180 individual isotopomer distribution is theoretically calculated, extent of labeling and spectral  
181 purity must be also characterized. Description of the general IPD methodology as well as  
182 examples of characterization of standards can be consulted in the literature [12,24]. In the  
183 present work, isotopomer abundances corresponding to the selected transitions for natural  
184 and labeled compounds have been experimentally obtained according to the method reported  
185 in previous works [19].

186

## 187 **RESULTS AND DISCUSSION**

### 188 **Optimization of LC conditions**

189 In a first approach, gradient conditions using methanol and water both containing 0.01%  
190 HCOOH/1mM NH<sub>4</sub>COOH as mobile phases were tested, as employed for testosterone  
191 determination in previous works [19]. However, due to the similarity between A and Etio  
192 (5 $\alpha$ /5 $\beta$ -position isomers of one H), separation could not be accomplished even with long run  
193 times. Therefore, acetonitrile (ACN) was tested as mobile phase on the basis of the  
194 chromatographic conditions used by Hauser *et al.* [25], which consisted in water/ACN (95/5,  
195 v/v) (Eluent A) and water/ACN (5/95, v/v) (Eluent B), both containing HCOOH and NH<sub>4</sub>COOH.  
196 Different modifier concentrations were tested and 0.01% HCOOH plus 0.1 mM NH<sub>4</sub>COOH  
197 provided the optimal sensitivity and peak shape.

198 Optimization of A/Etio separation was performed starting from isocratic conditions at different  
199 %B (10, 20 and 30%) to ensure that separation was possible with a C<sub>18</sub> column. Once  
200 separation was observed using 30% Eluent B, peak shape and time analysis were tried to be  
201 improved by performing a gradient prior to an isocratic step. Thus, gradients of the same slope  
202 were tested starting from 0% or 10% Eluent B and arriving up to 30, 40 and 50% Eluent B,  
203 followed by the isocratic step. Separation of the A/Etio pair was achieved in all six  
204 experiments, but lower isocratic and initial % of Eluent B produced longer run times and  
205 decreased sensitivity due to peak broadening. Thus, starting conditions were set at 10 %  
206 eluent B, followed by a gradient up to 50% Eluent B in 4.3min and an isocratic step until 9 min.  
207 (Figure 2)

208

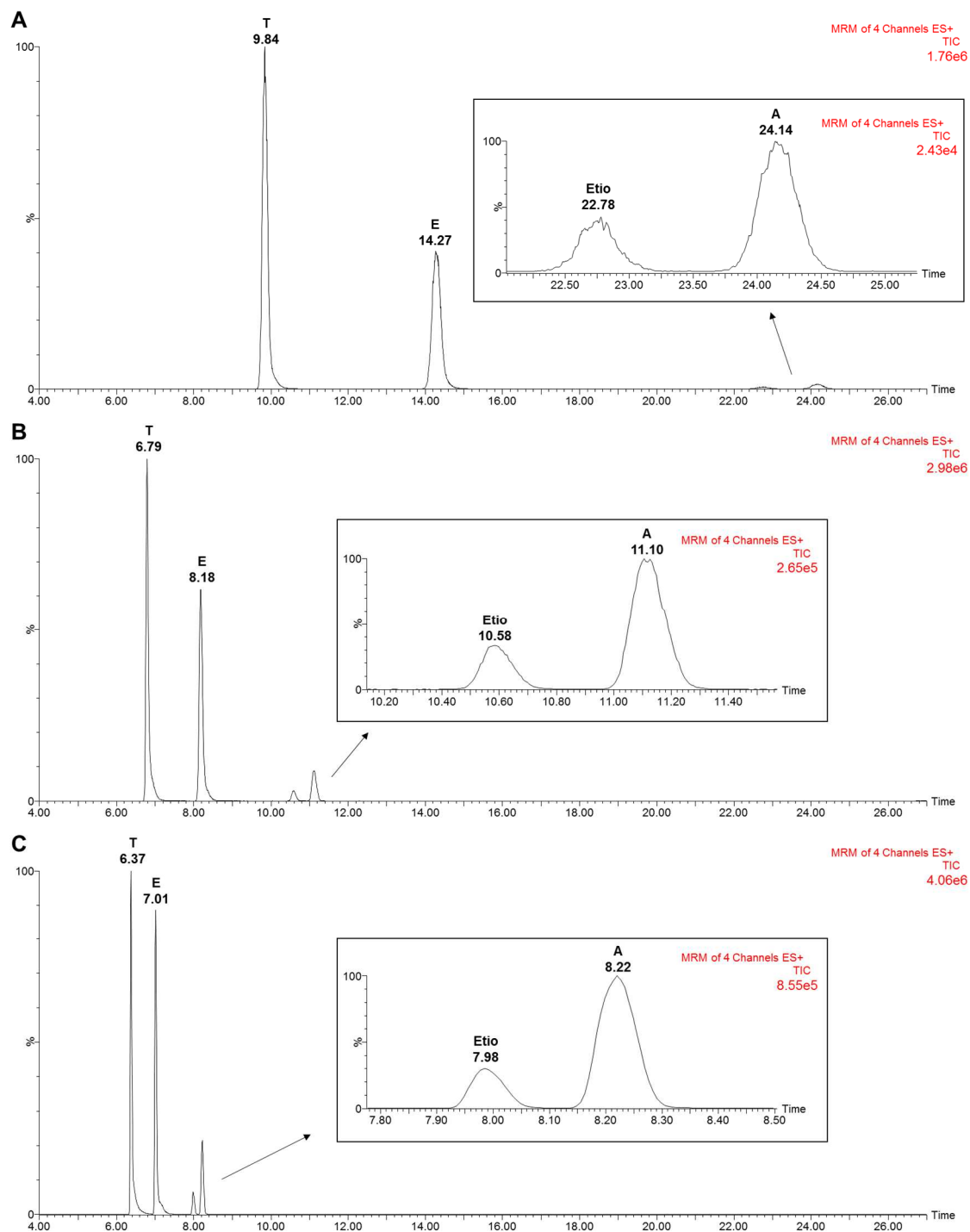
### 209 **Characterization of analytes**

210 Characterization of natural (T, E, A, Etio) and labelled compounds (d3-T, d3-E, d4-A, d5-Etio)  
211 consisted in the determination of the experimental isotopic distribution of abundances and  
212 exact concentration of labelled standard solutions.

213 Experimental abundances were measured by injecting (n=5) individual 500 ng/mL of T, E, d3-T  
214 and d3-E, and 5  $\mu$ g/mL of A, Etio, d4-A and d5-Etio in MeOH/H<sub>2</sub>O (1:1, v/v). MRM transitions  
215 were selected on the basis of theoretical fragmentation calculations by IsoPatrn software [26],  
216 selecting the 10-12 most abundant transitions.

217

218



219

220 Figure 2. Chromatographic optimization using gradients of the same slope from 10% Eluent B  
 221 at 1 min to A) 30% at 3.2 min, B) 40% at 4.25 min and C) 50% at 5.3 min, followed by an  
 222 isocratic step (see text). All three injections correspond to a standard with 500 ng/mL T and E  
 223 and 1.0 µg/mL A and Etio in MeOH/H<sub>2</sub>O (1:1, v/v).

224

225

226 Finally, concentrations of labelled 100 µg/mL standard solutions were checked by reverse  
 227 isotope dilution (RID). That is, quantification of labelled compound spiking the standard  
 228 solution with an accurately prepared natural standard solution using IPD [27]. Results obtained  
 229 by RID were: (100 ± 4) µg/mL for d3-T, (105.8 ± 1.1) µg/mL for d3-E, (98.2 ± 1.1) µg/mL for d4-A  
 230 and (112.7 ± 1.9) µg/mL for d5-Etio.

231

## 232 Method validation

233 Accuracy assessment was carried out by applying the developed IPD methodology to 2  
234 certified reference materials (CRM) from NMI Australia: NMIA MX002 and NMIA MX005.  
235 Recovery, calculated as the percentage ratio between the found and the certified  
236 concentration value, is shown in tables 2 and 3. It is worth noting the high accuracy of the  
237 method for T, E and Etio, with recovery values between 95% and 108% in all experiments.  
238 However, results for A were lower than expected, between 75% and 79%, which may be  
239 caused by insufficient hydrolysis time and/or temperature. Though, interference of matrix  
240 components with d4-A mass spectrum is being also considered since an abnormally high  
241 response in labelled transition measurements produces lower quantifications by IPD. All these  
242 possibilities are currently under study.

243

244

245 Table 2. Validation parameters, accuracy and precision, obtained for NMIA MX002 CRM.

NMIA MX002					
Compound	Certified value <sup>1</sup>	Intra-day repeatability (n=5)		Inter-day reproducibility (n=4)	
		Concentration <sup>1</sup>	Accuracy <sup>2</sup>	Concentration <sup>1</sup>	Accuracy <sup>2</sup>
T	16.6 ± 0.65	16.3 ± 0.3 (1.8%)	98%	15.9 ± 0.4 (2.4%)	96%
E	18.3 ± 1.3	19.7 ± 0.3 (1.8%)	108%	19.0 ± 0.5 (3%)	104%
A	1262 ± 39	963 ± 14 (1.4%)	76%	993 ± 81 (8%)	79%
Etio	814 ± 36	840 ± 15 (1.8%)	103%	804 ± 41 (5%)	99%

246 <sup>1</sup> Expressed as Mean ± SD (%RSD) ng/mL

247 <sup>2</sup> As % recovery respect to the certified value

248

249 Table 3. Validation parameters, accuracy and precision, obtained for NMIA MX005 CRM.

NMIA MX005					
Compound	Certified value <sup>1,3</sup>	Intra-day repeatability (n=5)		Inter-day reproducibility (n=4)	
		Concentration <sup>1</sup>	Accuracy <sup>2</sup>	Concentration <sup>1</sup>	Accuracy <sup>2</sup>
T	40.2 ± 1.8	37.3 ± 0.8 (2.0%)	93%	38.0 ± 0.7 (1.8%)	95%
E	10.74 ± 0.59	11.3 ± 0.3 (3%)	105%	11.0 ± 0.3 (3%)	102%
A	1184 ± 35	886 ± 40 (5%)	75%	890 ± 80 (9%)	75%
Etio	1290 ± 41	1229 ± 30 (2.4%)	95%	1246 ± 55 (4%)	97%

250 <sup>1</sup> Expressed as Mean ± SD (%RSD) ng/mL

251 <sup>2</sup> As % recovery respect to the certified value

252 <sup>3</sup> Corrected as indicated in the manufacturer's instructions (correction factor=0.9977), since  
253 the weighted water after reconstitution was 20.0461g

254

255

256 Intra-day repeatability (n=5) and inter-day reproducibility (n=4) were assessed for both CRMs.  
257 Results in terms of repeatability and reproducibility showed RSD values below 5% and 10%,  
258 respectively, in all cases (Tables 2 and 3). A shows the highest %RSD that should be related  
259 with the poorer recovery. Despite that, the developed method is characterised by a high  
260 precision.



261 In order to assess the method combined uncertainty, Nordtest calculations [22] were applied  
 262 using the available data to obtain combined uncertainty for the determination of the four  
 263 analytes (Table 4), plus the uncertainty of T/E, A/T and A/Etio ratios (Table 5). Total combined  
 264 uncertainty,  $u_c$ , allows the comparison with the WADA requirements for a quantification  
 265 method [2].

266 Since the obtained A concentrations differed from the certified values, its uncertainty derived  
 267 from the bias ( $u(\text{bias})$  around 23%) was found to be higher than the rest ( $u(\text{bias})$  between 3.2%  
 268 and 5.5%). Therefore, combined uncertainties of A, A/T and A/Etio determinations were worse  
 269 than the rest of analytes and ratios. Lower uncertainty values for A and A ratios are expected  
 270 once the aforementioned recovery problems are solved.

271 Regarding T, E and Etio,  $u_c$  was about 6% or lower in all cases. Taking into account that certified  
 272 concentrations in the CRMs (Tables 2 and 3) are  $>5$  ng/mL for T and E, and above five times the  
 273 method LOQ for A and Etio (Table 6), the concentration uncertainty of the method was far  
 274 lower than the limit of 20% set by the WADA for those three analytes [2].

275 Regarding T/E, the method  $u_c$  was 9%, also below the WADA requirements of 15%, and far  
 276 lower than the observed longitudinal individual variation in male urine. In a recent paper [28]  
 277 coefficients of variation of 30% and 46% for longitudinal T/E values were found when one or  
 278 various laboratories were involved respectively. As might be expected, those values contain  
 279 not only the individual variation but the method precision itself. Thus, a method with lower  
 280 measurement uncertainty would help in assessing the actual variability in longitudinal steroidal  
 281 profile for a given individual.

282

283 Table 4. Combined uncertainty,  $u_c$ , for the four selected EAAS.

Compound	$u(\text{bias})$	$R_w$	$u_c^1$
T	5,5%	1,8%	5,8%
E	4,3%	1,8%	4,7%
A	23,2%	10,3%	25,4%
Etio	3,2%	5,2%	6,1%

284 <sup>1</sup>  $u_c$  calculated as the square root of the sum of the squares of interday reproducibility ( $R_w$ ,  
 285 random uncertainty) and  $u(\text{bias})$ , the uncertainty associated to any source of bias including  
 286 that associated to CRMs [19,22]

287

288 Table 5. Combined uncertainty,  $u_c$ , for the selected ratios.

Ratio	$u(\text{bias})$	$R_w$	$u_c^1$
T/E	8,6%	2,7%	9,0%
A/T	19,2%	8,8%	21,1%
A/Etio	21,5%	5,6%	22,2%

289 <sup>1</sup>  $u_c$  calculated as the square root of the sum of the squares of interday reproducibility ( $R_w$ ,  
 290 random uncertainty) and  $u(\text{bias})$ , the uncertainty associated to any source of bias including  
 291 that associated to CRMs [22,23]

292

293 Finally, limits of quantification (LOQ) and detection (LOD) of the method were roughly  
 294 estimated as S/N equal to 10 and 3, respectively. To this end, the lowest concentrated sample  
 295 among 9 healthy female volunteers were selected (see experimental section). Lowest values  
 296 found within the samples for each analyte are shown in Table 6 along with their corresponding  
 297 LOQ and LOD.

298 Table 6. Concentration of steroids in the selected urine sample and calculated LOD and LOQ.

Compound	Sample ID <sup>1</sup>	Mean $\pm$ SD (%RSD) ng/mL	LOD (ng/mL) <sup>2</sup>	LOQ (ng/mL) <sup>3</sup>
T	4	0.520 $\pm$ 0.024 (5%)	0.2	0.7
E	8	1.48 $\pm$ 0.09 (6%)	0.5	1.7
A	8	301 $\pm$ 5 (1.8%)	7.3	24.5
Etio	8	587 $\pm$ 15 (3%)	28.6	95.4

299 <sup>1</sup> Results for all 9 samples can be consulted in the Supplementary Information (Table S.4)

300 <sup>2</sup> S/N = 3

301 <sup>3</sup> S/N = 10

302

303 A brief summary of figures of merit of the here developed method reveals that accuracy (75-  
304 108% recovery) compares well with already published results, while precision shows equal CV  
305 values or better, specifically for T and E and inter-day precision study. On the other hand, as  
306 IPD methodology does not require the use of calibration curves and derivatization steps are  
307 omitted in LC, the application of IPD quantification makes the method fast and reliable.  
308 Table S.5 in supplementary information shows validation results from some selected methods  
309 including those of the present work.

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311

## 312 CONCLUSIONS

313 In this work, an IDMS method for UHPLC-MS/MS has been proved suitable for EAAS  
314 determination in human urine. Isotope pattern deconvolution (IPD) was employed as  
315 mathematical tool to perform the quantification of testosterone, epitestosterone,  
316 androsterone and etiocholanolone, using deuterium-labelled analogs for that purpose.

317 The high similarity of molecular structure between A and Etio required of an extensive  
318 optimization of the chromatographic separation using an acetonitrile gradient.

319 The method was successfully validated with its application to two certified reference materials  
320 in terms of intraday repeatability and interday reproducibility with low relative standard  
321 deviations (%RSD < 10%) in both experiments, as well as in terms of trueness or recovery  
322 respect the certified concentration values (between 75% and 110%).

323 In addition, LODs and LOQs of the method were estimated in real life, low concentrated,  
324 female urine samples. All limits were found suitable for the determination of EAAS since they  
325 fell below the normal range of concentration in adults.

326 Combined standard uncertainty for T, E, Etio and T/E were below the WADA required limits for  
327 a method to be useful in doping suspicion. Moreover,  $u_c(T/E)$  is well below the observed  
328 coefficients of variation for individual longitudinal profiles, thus allowing to improve future  
329 variability assessment studies. Regarding the lower performance of A and their ratios current  
330 studies are being conducted to improve the associated uncertainty.

331 Therefore, the present IPD method by LC-MS/MS is highlighted as a robust, exact and precise,  
332 and constitutes a potential alternative approach for endogenous steroid analysis and a capable  
333 alternative to traditional GC- and calibration-based quantifications. Since the ionization source

334 used in this work, ESI, is not suited for the determination of 5 $\alpha$ Adiol and 5 $\beta$ Adiol, future works  
335 will be focused on their inclusion in the method as alternate forms. In this sense, the direct  
336 determination of glucuronide conjugates seems to be a good alternative to continue  
337 developing IPD as a reliable approach in EAAS determination.

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