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A novel ultra-high-performance supercritical fluid chromatography hyphenated to tandem mass spectrometry method for the analysis of urinary endogenous steroids in the anti-doping context

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ABSTRACT

The first step in the detection of testosterone (T) doping is to measure the urinary steroid profile for the athlete biological passport (ABP). To harmonise the analysis between anti-doping laboratories, urinary steroid profiling is parametrised in deep detail and shall be performed by gas chromatography hyphenated to mass spectrometry (GC–MS). However, due to its requirement for extensive sample preparation, alternatives to GC–MS are being actively pursued. The aim of this study was the evaluation of Ultra-High-Performance Supercritical Fluid Chromatography hyphenated to tandem Mass Spectrometry (UHPSFC-MS/MS) as an alternative for the quantification of endogenous urinary steroids. In this context, we developed a high throughput sample extraction method, followed by a novel UHPSFC-MS/MS method for the analysis of 10 endogenous urinary steroids which are relevant for doping control analysis. Depending on the steroid, the herein presented method is capable of quantification from 0.5 ng/mL up to 10 μg/mL. After validation, the applicability of the method was evaluated by analysing 132 authentic urine samples, which demonstrated results similar to classical GC–MS analysis. Steroid concentrations determined by UHPSFC-MS/MS were slightly overestimated in comparison with GC–MS, but the ratios had <10 % difference between the two methods. As the ABP considers the steroid ratios for passport evaluation, the herein presented method could be used for steroid profiling without reducing the sensitivity of the ABP. Thus, we would propose to consider UHPSFC-MS/MS as an alternative to GC–MS after more tests would have been performed to support our findings. Furthermore, we have also investigated the potential of this technology for sample purification prior to Isotope Ratio Mass Spectrometry (IRMS) for the differentiation between exogenous and endogenous origin of T and its metabolites. While the achieved separation was sufficient to purify urine samples for IRMS analysis in our proof-of-concept study, the instrumental parameters should be further refined for future use.

1. Introduction

Doping with endogenous steroids like testosterone (T) is highly prevalent and challenging to uncover, since they are produced naturally and alterations in urinary concentrations could also be due to medical conditions. To prove the exogenous origin, the carbon isotope ratio (CIR) of the steroid(s) of interest in urine shall be compared with the CIRs of endogenous reference compounds (ERC) in the suspect sample [1,2]. CIRs are expressed as δ^{13}C-values versus the international primary reference material Vienna PeeDee Belemnite (VPDB). To achieve harmonised interpretation of the results, the technical document of WADA defines the differences in delta values between the steroid of interest and the ERC that imply a synthetic nature and would lead to an adverse analytical finding [3]. Since the CIR determination by Gas Chromatography Combustion Isotope Mass spectrometry (GC/C/IRMS) is a lengthy procedure with multiple manual steps and relatively low throughput, only targeted urine samples are analysed with this method. This selection is based on the urinary steroid profile which is determined

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by quantitative Gas Chromatography (tandem)-Mass Spectrometry (GC–MS(/MS)) measurements of T, its inactive isomer epitestosterone (E) and the metabolites androsterone (A), etiocholanolone (Etio), 5α-androstanediol (5αAdiol) and 5β-androstanediol (5βAdiol).To account for circadian variations in urinary excretion and different dilution of urine samples, five ratios of the steroid concentrations are then calculated (T/E, A/T, A/Etio, 5αAdiol/5βAdiol, 5αAdiol/E) [4]. The most sensitive marker for T administration is the T/E ratio, followed by 5αAdiol/E, especially for individuals with naturally low T/E values [5–7]. Steroid profiles are monitored to establish an individual and longitudinal Athlete Biological Passport (ABP). Based on the data of the ABP, individual thresholds are calculated using Bayesian statistics [8] and samples which exceed these thresholds for one or several steroidal markers are then analysed by GC/C/IRMS to determine the origin of T and its metabolites.

Since the data for urinary steroid profiling originates from different accredited antidoping laboratories, the methods must be standardised and similar, otherwise the inter-laboratory variation would exceed the alterations due to potential steroid use, and lead to compromised sensitivity of the individual thresholds. The routine analysis is therefore well parametrised in the corresponding technical document (TDEAAS) [4] of the World Anti-Doping Agency (WADA). This technical document dictates the use of GC–MS to identify and quantify the steroids after trimethylsilyl (TMS) derivatisation. Not only have anti-doping laboratories historical expertise with TMS derivatisation [9], but the resulting electron ionisation (EI) spectra are highly informative and robust. Furthermore, GC–MS analyses exhibit high resolving power, capable of distinguishing between most steroid isomers and the negligible matrix effects make it an excellent choice for quantitative analysis. On the other hand, steroids are excreted in urine as phase II metabolites, which require hydrolysis, purification by Solid Phase Extraction (SPE) or Liquid-Liquid Extraction (LLE) and derivatisation to make them volatile enough for GC–MS analysis.

With increasing numbers of doping control samples, fast and automatable procedures are needed, especially during major sports competitions [10]. Due to instrumental improvements and development of new stationary phases with smaller particles, modern Ultra-High-Performance Liquid Chromatography coupled to Mass Spectrometry (UHPLC-MS) became capable of efficiently and rapidly separating endogenous steroids in reversed phase mode and was proposed for urinary steroid profiling in antidoping as well [11–13]. Another separation technique that has gained a lot of interest over the last few years for the analysis of endogenous steroids [14–18], is Ultra-High Performance Supercritical Fluid Chromatography coupled to tandem Mass Spectrometry (UHPSFC-MS/MS). In the past, SFC had a negative reputation and was considered irreproducible and non-robust, but with the metamorphosis to UHPSFC thanks to more robust instrumentation and the use of columns packed with sub-2 μm particles [19,20], it became a faster orthogonal alternative to the widely used reversed phase UHPLC separations [21]. Whereas UHPSFC-MS/MS has not been used in an anti-doping context for the analysis of endogenous steroids so far, the capability of analysing exogenous steroids [22,23] and other classes of prohibited substances was already demonstrated [24–26]. In various designs hyphenating UHPSFC and MS, the flow is split and not entirely transferred to the MS [27], a part is diverted to the waste through the Automatic Back-Pressure Regulator (ABPR) [28]. Interestingly, if the split-flow configuration is used, this flow to waste could be collected to perform semi-preparative chromatography, while verifying the content of the collected fractions at the same time. Indeed, semi-preparative SFC was even proposed as a mean to purify urine samples for further analysis of endogenous steroids [29]. Despite its wide applicability, UHPSFC-MS/MS has not yet gained full acceptance in routine analysis but demonstrated its potential only in research projects.

The aim of this study was to develop and validate an UHPSFC-MS/MS method for the determination of the urinary steroid profile for anti-doping purposes and compare the results of 132 urine samples with those obtained using the classical GC–MS analysis. Furthermore, the possibility of semi-preparative SFC for the sample purification prior to GC/C/IRMS was also explored as proof-of-concept.

2. Material & methods

2.1. Reagents and solutions

Methanol (MeOH) and ammonium formate (both ULC/MS - CC/SFC grade) were supplied by Biosolve BV (Valkenswaard, Netherlands); acetic anhydride (puriss. >99 %) and pyridine (anhydrous >99.8 %) were supplied by Sigma Aldrich (Buchs, Switzerland); dichloromethane (DCM) and acetonitrile (ACN) were supplied by VWR International (Fantanay-sous-Bois, France); tertiary butyl-methylether (TBME) was supplied by Thermo Scientific AG (Reinach, Switzerland); He (>99.999 %) and CO_2 (99.99 %) were supplied by Carbagas (Gümlingen, Switzerland); β-glucuronidase from *E. Coli* was supplied by Roche (Basel, Switzerland), *N*-methyl-*N*-(trimethylsilyl)-trifluoracetamide (MSTFA), ammoniumiodide and ethanethiol were supplied by Macherery-Nagel (Oensingen, Switzerland). The steroid standards T, D_3-T, E, D_3-E, A, D_4-A-glucuronide, Etio, D_5-Etio, 5βAdiol, D_5-5βAdiol, 5αAdiol, D_3-5αAdiol, 17-methyl-testosterone (MeT), dehydroepiandrosterone (DHEA), 5β-pregnane-3α,20α-diol (PD), androst-16en-ol, (16EN) and 11-oxo-etiocholanolone (11-O-Etio) were obtained from Steraloids (Newport, RI, USA). All stock solutions were prepared at concentrations of 1 mg/mL in MeOH and further diluted in MeOH to obtain the required solutions. All solutions were stored frozen in glass tubes at −20 °C.

Synthetic urine was prepared by dissolving urea, ammonium bisphosphate, creatinine, bovine albumin, glucose (all supplied by Sigma, Buchs, Switzerland), glycin, l-alanine, oxalic acid (all supplied by Fluka, Buchs, Switzerland), and sodium chloride (Acros Organics, Geel, Belgium) in water and pH was adjusted to 6.0 with either NaOH (12 mol/L) or HCl (3 mol/L). Calibrators were prepared by spiking methanolic standards in synthetic urine with serial dilutions, according to the concentrations in supplementary data S1. The internal standard (IS) was prepared as methanolic solution and contained D_3-T (2 μg/mL), D_3-E (0.5 μg/mL), D_5-Etio (20 μg/mL), D_4-A-glucuronide (12.5 μg/mL), D_5–5βAdiol (5 μg/mL) D_3–5αAdiol (5 μg/mL) and MeT (5 μg/mL).

2.2. Samples

Authentic urine samples from athletes ($n = 132$; 87 male, 45 female) for the comparison of UHPSFC-MS/MS and GC–MS were obtained from the routine activities of the Swiss Laboratory for Doping Analyses. The samples were collected during routine doping controls and were anonymised and stored at 4 °C before the analysis.

For the proof-of-concept sample purification prior to IRMS analysis quality control samples of the Swiss Laboratory for Doping Analyses were used. The positive quality control sample (PQC) used for these preliminary experiments were collected from a male volunteer (30 years) that had orally administered testosterone undecanoate, whereas the negative quality control sample (NQC) was donated by a male volunteer (27 years) that did not take any medications Both subjects gave their written consent for their samples to be used for research purposes.

2.3. UHPSFC-MS/MS analysis

2.3.1. Sample preparation

In a 96-well plate, 300 μL of each urine sample or calibrator were fortified with 20 μL of IS and diluted with 150 μL of acetate buffer (0.5 M ammonium acetate at pH 6.3) containing 10 % β-glucuronidase from *E. coli*. The plate was covered and incubated for 1 h at 50 °C, while shaking gently for enzymatic hydrolysis. Then, 400 μL of each hydrolysate were loaded onto the SLE+ plates (Biotage, Uppsala, Sweden) before applying 3 bar of positive pressure for 30 s. After an equilibration phase of 5 min, 700 μL of DCM were added to each well and the steroids were eluted into

Table 1
MS parameters for the steroids and the internal standards. The transitions that were used for quantification are highlighted in bold.

Substance	RT [min]	Precursor ion [*m/z*]	Product ion [*m/z*]	Cone voltage [V]	Collision energy [eV]
16EN	2.32	257	**147**	20	20
			161	20	20
5βAdiol	4.01	257	**147**	20	20
			161	20	20
5αAdiol	4.19	257	**147**	20	20
			161	20	20
PD	4.40	285	109	20	10
			149	10	10
Etio	4.66	315	**147**	30	20
			161	30	20
A	4.87	315	**147**	30	20
			161	30	20
DHEA	5.30	271	**97**	10	30
			253	20	10
11-O-Etio	5.70	287	229	10	35
			269	20	35
E	6.04	331	**97**	40	50
			109*	30	50
T	6.77	331	97	40	50
			109	30	50
D_5–5βAdiol	4.00	262	147	20	20
D_3–5αAdiol	4.18	260	147	20	20
D_5-Etio	4.62	260	145	20	30
D_4-Andro	4.84	259	145	10	20
D_3-E	6.02	334	97	30	20
D_3-T	6.76	334	97	30	20
MeT	7.15	285	109	25	25

* only informative, see chapter 3.2 Method validation.

glass micro-vials by applying 3 bar of positive pressure for 1 min. The solvent was evaporated under a stream of nitrogen at 40 °C during approximately 30 min. To acetylate the steroids, 20 µL pyridine and 20 µL acetic anhydride were added, the plate closed and incubated for 1 hour at 70 °C while shaking gently. Excess derivatisation reagents were evaporated under a stream of nitrogen at 40 °C for 10 min.

2.3.2. Analysis method

The final extract was reconstituted in 100 µL of ACN:H_2O (2:1, v:v) and 1 µL was injected into the Acquity UPC^2 coupled to a Xevo TQ-S mass spectrometer (Waters, Milford, USA). The UHPSFC system consisted of a binary pump, a fixed-loop autosampler, a column oven and an ABPR, and was coupled with a quaternary pump (Acquity H-class) to deliver the make-up solvent via the SFC-MS splitter device to the triple quadrupole MS. Chromatographic separation was performed on a Torus 1-AA column (3.0 × 100 mm, 1.8 µm) from Waters at 60 °C with the ABPR set to 150 bar. Mobile phase A was CO_2 and mobile phase B was 20 mM ammonium formate in MeOH:H_2O (98:2, v:v). Pure MeOH was used as make-up solvent at a constant flow rate of 0.1 mL/min. From the initial conditions of 1 % B, the amount of modifier was increased to 2 % over 2 min, followed by an increase to 10 % B over 4 min. Afterwards the column was flushed for 2 min at 20 % B, followed by a 2-minutes re-equilibration phase at initial conditions.

All compounds were analysed in positive ESI ionisation mode using 2.6 kV capillary voltage and a source temperature of 150 °C. The desolvation temperature was set to 250 °C, the desolvation flow to 700 L/hr, the cone gas flow to 150 L/hr and the nebuliser gas was set to 7 bar. MS analysis was performed in Multiple Reaction Monitoring (MRM) mode with two transitions per analyte, their fragmentation parameters are shown in Table 1. MassLynx (version 4.2) was used for data acquisition and TargetLynx XS (version 4.2) for data processing.

2.4. UHPSFC-MS/MS method validation

Method validation was performed in accordance with ISO 17,025, the International Standard for Laboratories (ISL) and the publication by Matuszewski et al. [30] on three distinct days by two operators.

2.4.1. Matrix effects, extraction recovery and memory effects

Matrix effects (ME) and extraction recoveries (ER) were determined by spiking six urine samples (3 male, 3 female) in duplicates with isotope labelled standards before and after the extraction and comparing the peak areas with the corresponding injections of pure isotope labelled standards. Memory effects were assessed by injecting a urine sample spiked at the highest calibration level and three blank urine samples afterwards.

2.4.2. Selectivity

The selectivity of the method was established by comparing the transitions of the substances in 12 authentic urine samples in duplicates (6 male, 6 female) before and after spiking with a standard. Furthermore, the ratios of the transitions were compared with a standard injected as triplicate.

2.4.3. Calibration range

To establish the range of calibration, eight calibrators were prepared as described in supplementary data S1 and injected as duplicates each per day of validation. Quadratic regression was performed with a 1/x weighting function. From the 10 endogenous steroids analysed with this method, the six steroids of the urinary steroid profile (i.e. T, E, Andro, Etio, 5αAdiol and 5βAdiol) were considered for quantification in both GC–MS and UHPSFC-MS/MS.

2.4.4. Accuracy, precision, and measurement uncertainty

Quality control samples at four concentration levels (LOQ, QC-low, QC-mid and QC-high) were extracted as triplicates per day of validation to calculate inter-, intra-day precision and accuracy. From these results, the measurement uncertainties were calculated in percentage and the LOQs were set as the concentrations where the measurement uncertainty did not exceed the WADA criteria of 30 % [4]. The concentrations of the remaining four steroids (i.e. DHEA, 16EN, PD and 11-OH-Etio) were estimated using the calibrators, as no specific isotope labelled internal standards were used or available for them. Method validation results were calculated with Excel (Office365, Microsoft).

2.5. Comparison with GC–MS analysis

The validated GC–MS method that was employed as reference method to determine the urinary steroid profiles according to the appropriate technical document [4] has recently been described elsewhere [31]. Briefly, a urine aliquot of 2.5 mL per sample was spiked with 20 μL internal standard and hydrolysed with β-glucuronidase from *E. Coli*. Subsequently, the steroids were extracted at basic pH with TBME, and after removal of the solvent, they were derivatised with MSTFA, ammonium iodide and ethanethiol. From the final extract, 1 μL was injected into the GC–MS for the analysis of endogenous steroids. The injector of the GC–MS (Agilent 7890B/5977B, Waldbronn, Germany) was operated at 300 °C in split-mode (1:10). Separation was performed on an Agilent HP-1 column (17 m x 0.2 mm, 0.11 μm) with He as carrier gas. The oven was kept initially at 181 °C and the temperature increased with 4 °C/min to 230 °C followed by a second ramp of 120 °C/min up to 310 °C. The final temperature was maintained for 4 min before returning to the initial conditions. The ion source of the single quadrupole was operated in positive EI mode with an ionisation energy of 70 eV, generated ions were analysed in Selected Ion Monitoring (SIM). Instrument control, data acquisition and analysis were performed on the MassHunter software from Agilent. Samples were quantified based on a single-point calibration using a quality control sample (QC-mid). The limits of quantification (LOQ) for each marker of the urinary steroid profile (i.e. T: 0.5 ng/mL, E: 1 ng/mL, 5αAdiol: 2 ng/mL, 5βAdiol: 2 ng/mL, A: 50 ng/mL, Etio: 50 ng/mL) were compliant with the WADA requirements [4]. Method comparison was performed with Excel for the creation of Bland-Altman plots and with RStudio (R, version 2022.12.0 Build 353) for the calculation and visualisation of Passing-Bablok regressions.

2.6. Semi-preparative UHPSFC of endogenous steroids: a proof of concept

The possibility of using UHPSFC for sample fractionation and purification prior to IRMS was investigated. Fractions were manually collected from the ABPR-capillary and the CIRs of the containing steroids were determined by GC/C/IRMS.

As a relatively high sample volume was needed for GC/C/IRMS analysis, an established sample preparation protocol was used and modified for SFC purification [31]. In brief, 10 mL of urine samples were extracted on C18 SPE cartridges (previously conditioned with MeOH and water, elution of steroids with MeOH). After removal of the solvent, the extract was hydrolysed with β-glucuronidase from *E. coli* at pH 6.8 for one hour at 50 °C. Free steroids were extracted by double LLE with TBME and after evaporation, the extract was acetylated with pyridine and acetic anhydride for one hour at 70 °C. Excess reagents were evaporated, and the final extract was reconstituted in cyclohexane before injecting 10 μL into the SFC.

To further improve the separation, the gradient was geometrically scaled to a longer Torus 1-AA column (consisting of two columns in series 3.0 × 100 mm and 3.0 × 150 mm, both 1.8 μm) [32]. Pure MeOH was used as modifier and make-up solvent at 0.4 mL/min, all other conditions were kept similar as for the quantification method. The amount of modifier increased from the initial conditions at 1 % to 2 % within 5.5 min. After an increase to 10 % over the next 10 min, a washing phase at 20 % B was performed for 4 min. In the end, the column was re-equilibrated at the initial conditions for a total gradient time of 30 min. Fractions were collected manually by submerging the end of the ABPR capillary into a glass tube containing 1 mL of MeOH. The entirety of the steroid peaks was collected with an additional margin of 10 s before and after each peak. After the solvent evaporation, the extracts were transferred to autosampler vials and reconstituted with

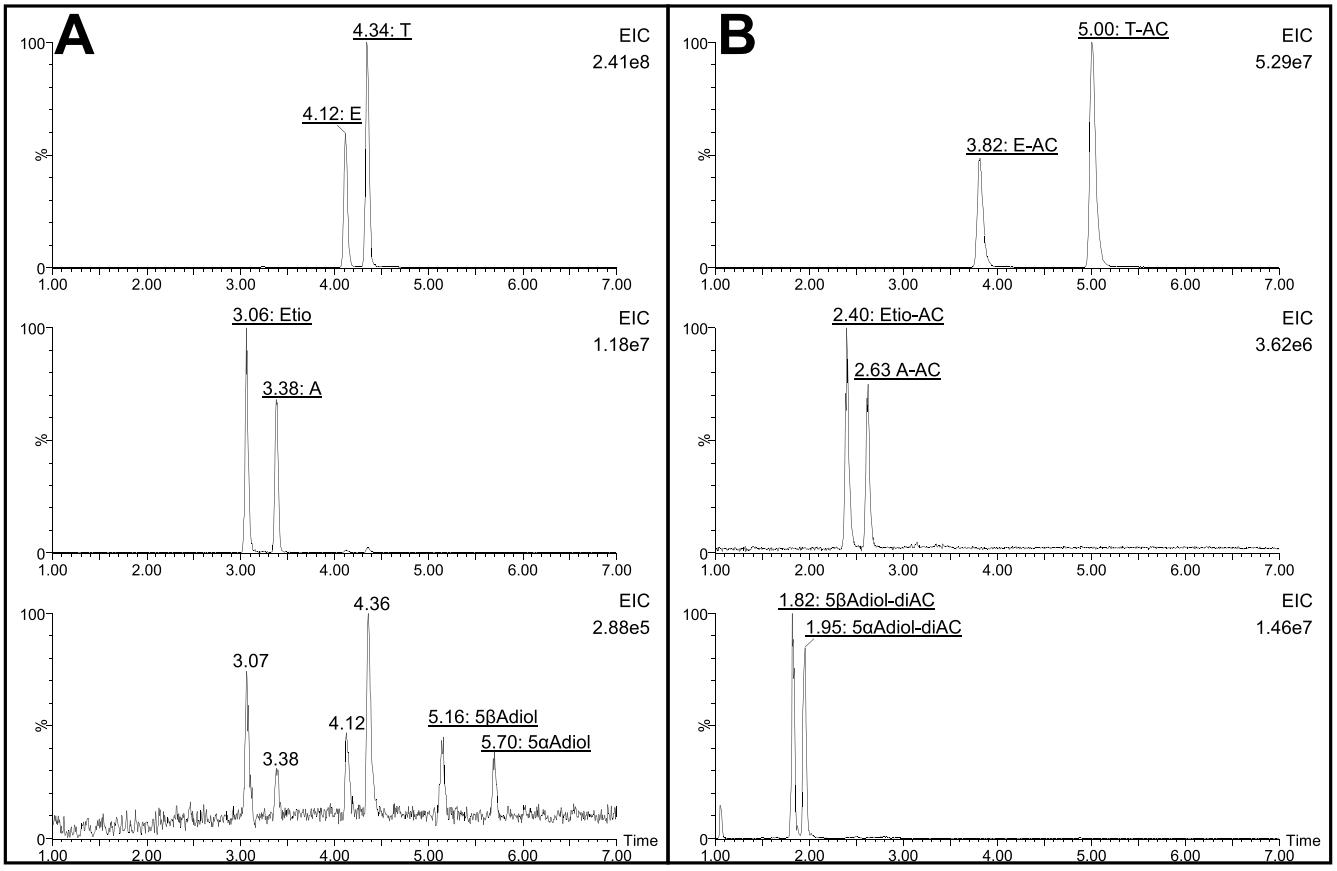

Fig. 1. Summed Extracted Ion Chromatograms of a standard mix at 100 ng/mL of free steroids (A) or steroid acetates (B). The peaks of the steroids of interest are labelled and their retention times are underlined.

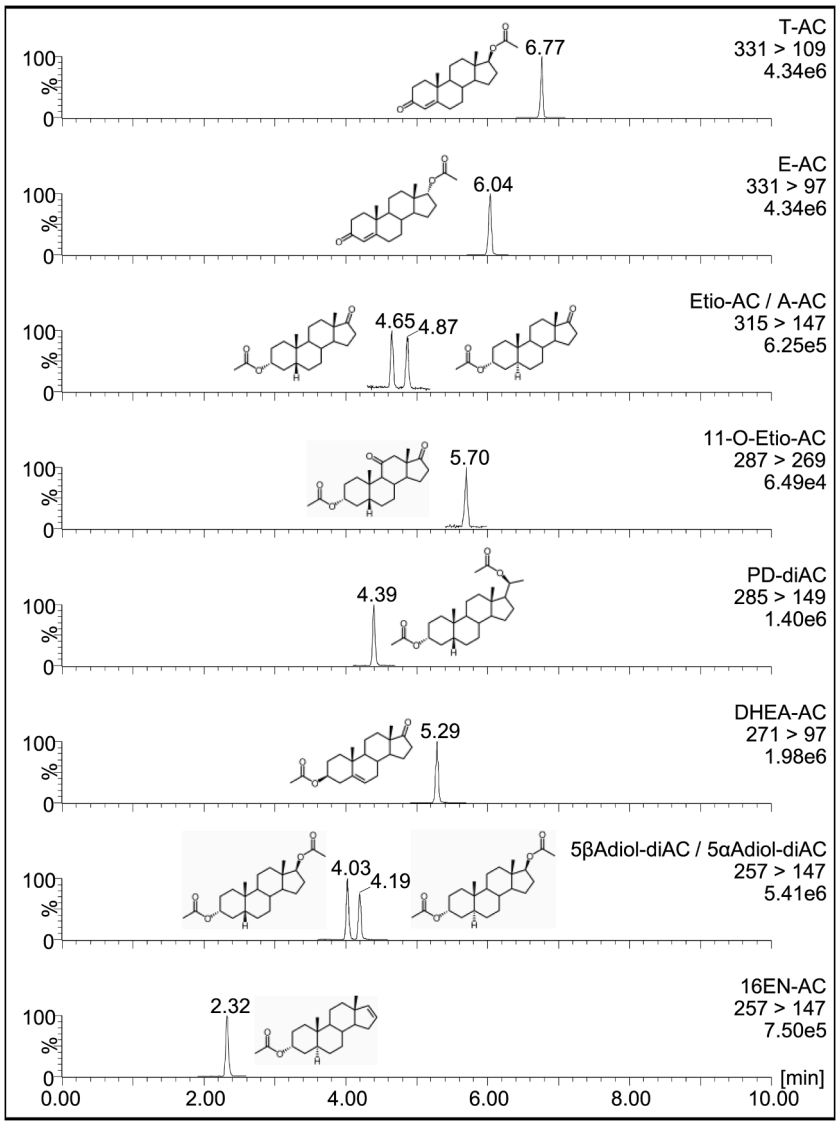

Fig. 2. Examples of chromatograms of the acetylated steroids in a standard mix at 100 ng/mL with the corresponding structural formulas over the 10 min separation on a Torus 1-AA column (3.0 × 100 mm, 1.8 μm) kept at 60 °C using CO_2 and MeOH containing 2 % water and 20 mM ammonium formate as mobile phases. For further details please refer to chapter 2.2 UHPSFC-MS/MS analysis. The intensities of the transition used for quantitation are given for each substance on the right side of the chromatogram.

cyclohexane according to the urinary concentrations. Of each fraction, 1 μL was injected into the Trace 1310 GC coupled to an IsoLink II combustion interface, a ConFlo IV continuous flow interface and a Delta V IRMS (Thermo Fisher Scientific, Bremen, Germany). Chromatographic separation was performed on an Agilent J&W DB-17 MS fused silica capillary column (30 m x 0.25 mm, 0.25 μm) using He as mobile phase at a constant flow of 1.4 mL/min. Samples were injected in splitless mode with 1.5 min of purge into the liner that was kept at 280 °C. The oven was kept at 70 °C for 2 min followed by a ramp of 30 °C/min until 270 °C and a second ramp of 2 °C/min up to 300 °C. The final conditions were held for 5 min before returning to the initial temperature of 70 °C, resulting in a total runtime of about 21 min. A small portion of the gas flow was diverted through a restriction capillary to an ISQ 7000 single quadrupole mass spectrometer (Thermo Fisher Scientific) operated in positive EI mode. IRMS results were obtained by analysing the raw data with the IsoDat software and single quadrupole MS-spectra were analysed with Xcalibur. The quality of the purification was assessed by comparing IRMS chromatograms as well as the peak spectra of the steroids of interest with the corresponding chromatograms and spectra from a routinely performed semi-preparative HPLC purification [31]. Furthermore, the completeness of the fraction collection was checked, as this parameter is important to prevent isotopic fractionation. The CIRs of the steroids of interest in quality control samples were compared with results obtained from routinely performed analysis to verify the correct fraction collection.

3. Results and discussion

The aim of this project was to present an alternative method for quantitative analysis of urinary steroids in the ABP context. Due to harmonisation in the anti-doping community and mandatory specifications set by WADA, this analysis shall be currently performed by GC–MS (/MS) in the antidoping routine, which limits the use of alternative chromatographic technologies [4]. Nonetheless, with this study, we would like to demonstrate the capabilities of this so far underrepresented technique in doping-analyses and to propose an approach to urinary steroid profiling by UHPSFC-MS/MS.

Table 2
Method validation parameters and results: extraction recoveries (ER), matrix effects (ME) and range of the calibrators with classical quadratic regression parameters are shown for the 6 quantified steroids. Inter- and intra-day variability, accuracy and measurement uncertainty (MU) were determined at 4 different levels for the 6 steroids and the T/E ratio as well.

Substance	ER [%]	ME [%]	range [ng/mL]	regression $Y=ax^2+bx+c$	R^2	LOQ			QC-low			QC-mid			QC-high		
						conc. [ng/mL]	intra- / inter-dayvariability (n = 4 / 8)[%]	accuracy / MU[%]	conc. [ng/mL]	intra- / inter-day variability (n = 3 / 6) [%]	accuracy / MU [%]	conc. [ng/mL]	intra- / inter-day variability (n = 3 / 6) [%]	accuracy / MU [%]	ng/mL	intra- / inter-day variability (n = 3 / 6) [%]	accuracy / MU [%]
T	96.5	100.6	0.5–500	$-1.81e^{-6}$ x^2+0.0534 x + 0.0807	0.9993	1	2.4 / 3.0	123.9 / 24.6	2	3.1 / 2.6	116.2 / 16.8	40	1.0 / 2.8	110.5 / 11.4	150	3.0 / 4.9	111.2 / 13.8
E	89.3	101.8	0.5–500	$5.73e^{-6}$ x^2 +0.0675 x + 0.01117	0.9998	1	1.5 / 3.7	109.1 / 10.9	2	0.6 / 3.8	108.2 / 10.2	10	2.0 / 4.8	99.9 / 7.2	100	1.6 / 3.4	101.4 / 5.3
A	95.4	113.9	50–10,000	$-3.19e^{-6}$ x^2 +0.272 x −1.313	0.9990	50	2.8 / 5.2	123.1 / 24.9	100	0.5 / 1.5	109.8 / 10.1	2000	3.1 / 5.3	101.1 / 8.0	6000	1.0 / 1.8	102.3 / 3.6
Etio	135.8	93.7	50–10,000	$-4.41e^{-6}$ x^2 +0.128 x + 0.357	0.9992	50	6.3 / 12.2	91.7 / 18.4	100	2.9 / 2.9	101.4 / 4.5	2000	1.4 / 1.5	98.8 / 2.6	5000	1.2 / 2.4	97.2 / 4.4
5αAdiol	93.8	116.6	2.5–500	$1.51e^{-5}$ x^2 +0.739 x + 0.821	0.9995	2.5	10.1 / 11.1	105.72 / 18.2	5	4.8 / 5.4	112.0 / 15.0	50	1.0 / 5.0	99.6 / 7.3	200	0.8 / 4.9	103.6 / 8.3
5βAdiol	90.8	126.3	2.5–500	$5.70e^{-7}$ x^2 +0.583 x + 0.0271	0.9998	5	0.7 / 3.5	107.6 / 9.3	10	3.5 / 3.5	105.5 / 8.4	100	1.1 / 2.9	96.5 / 5.4	500	2.3 / 4.9	103.3 / 8.3
T/E							2.9 / 5.9	113.8 / 16.9		2.8 / 4.3	107.6 / 10.2		2.6 / 2.9	110.7 /11.7		2.8 / 2.5	109.6 / 10.5

3.1. Method development

While the sample preparation could be adapted from already established methods, the chromatographic separation and MS-detection had to be newly developed. Especially the MS-detection was less straightforward and required derivatisation, so the chromatographic separation had to be adjusted for this necessity as well.

3.1.1. Sample preparation

At the same time as suggesting an alternative separation method, we aimed at modernising the sample preparation process as well. The extensive sample preparation for analysis of urinary steroids by GC–MS included optional preliminary SPE, followed by enzymatic hydrolysis, LLE/SPE of the hydrolysed steroids, evaporation to dryness, TMS derivatisation and finally GC–MS(/MS) analysis. While a few steps (e.g. SPE) in this procedure could be automated, there were still many manual steps that included a transfer from one vial to another and therefore, risks for errors which should be reduced to minimum. Furthermore, these manual steps would be lengthy and therefore limiting the potential for high throughput analysis. Due to better sensitivity of modern MS instrumentation, lower sample volumes could be used for extraction, allowing the use of SLE 96-well plates instead of single cartridges, as already well established in our laboratory [33–35]. Different sample volumes (2.5 mL, 1 mL, 300 μL) were therefore tested in this study, starting with the conventional volume that was used for the GC–MS analysis (2.5 mL). Using only 300 μL of urine, LOQs in the low ng/mL range were still reached using 400 μL SLE plates and dichloromethane as extraction solvent. Different amounts of β-glucuronidase were tested for the enzymatic hydrolysis of the phase II steroid metabolites. While adding a high amount of enzyme would ensure efficient hydrolysis, glycerol from the enzyme-mixture could influence the hydrolysis and the subsequent extraction. Therefore, experiments were carried out to minimize the amount of added enzyme, also for cost efficiency reasons. The efficiency of hydrolysis was monitored through the deuterated internal standard ratio of D_4-A/D_5-Etio, as D_4-A was added in glucuronidated form and therefore was undergoing the hydrolysis as well [36]. For this, samples with different steroid concentrations were hydrolysed with different enzyme amounts (150 μL, 100 μL, 50 μL, 15 μL), and the resulting peak areas were compared. It was found that 15 μL of enzyme solution *per* 300 μl sample were sufficient to obtain complete hydrolysis of the steroid glucuronides. Finally, the derivatisation with acetic anhydride was based on a previously published method [31,37] but the added amounts were scaled down by testing different urine samples with varying concentrations.

3.1.2. UHPSFC-MS/MS conditions

Regarding the UHPSFC-MS/MS method, the steroids were initially injected in their free form without derivatisation. Satisfying chromatographic separation was well achieved on a Viridis BEH column with a simple linear gradient using the same mobile phases as used for the final method described in the materials and methods section. However, important differences in MS/MS signal intensities were observed between the steroids, as demonstrated in Fig. 1A, despite thorough optimisation of source parameters, modifier composition and make-up solvent. Signals from heavy isotopologues of T, E, A and Etio showed significantly higher peaks than 5αAdiol and 5βAdiol. Since the only structural difference between A and 5αAdiol was the change of the 17-oxo group to a 17β-hydroxy group and since PD also yielded low signal intensities, we suspect, that the signal intensities negatively correlate with the number of hydroxy groups in the steroid structure. To mitigate the negative impact of hydroxy groups, a specific derivatisation with methoxyamine was investigated, as this approach has been already used for steroid analysis in UHPSFC-MS/MS [17]. TMS derivatisation was also tested. However, these two derivatization approaches were ruled out as they would either only target oxo groups or both oxo and hydroxy groups. Another common derivatisation for steroids to increase

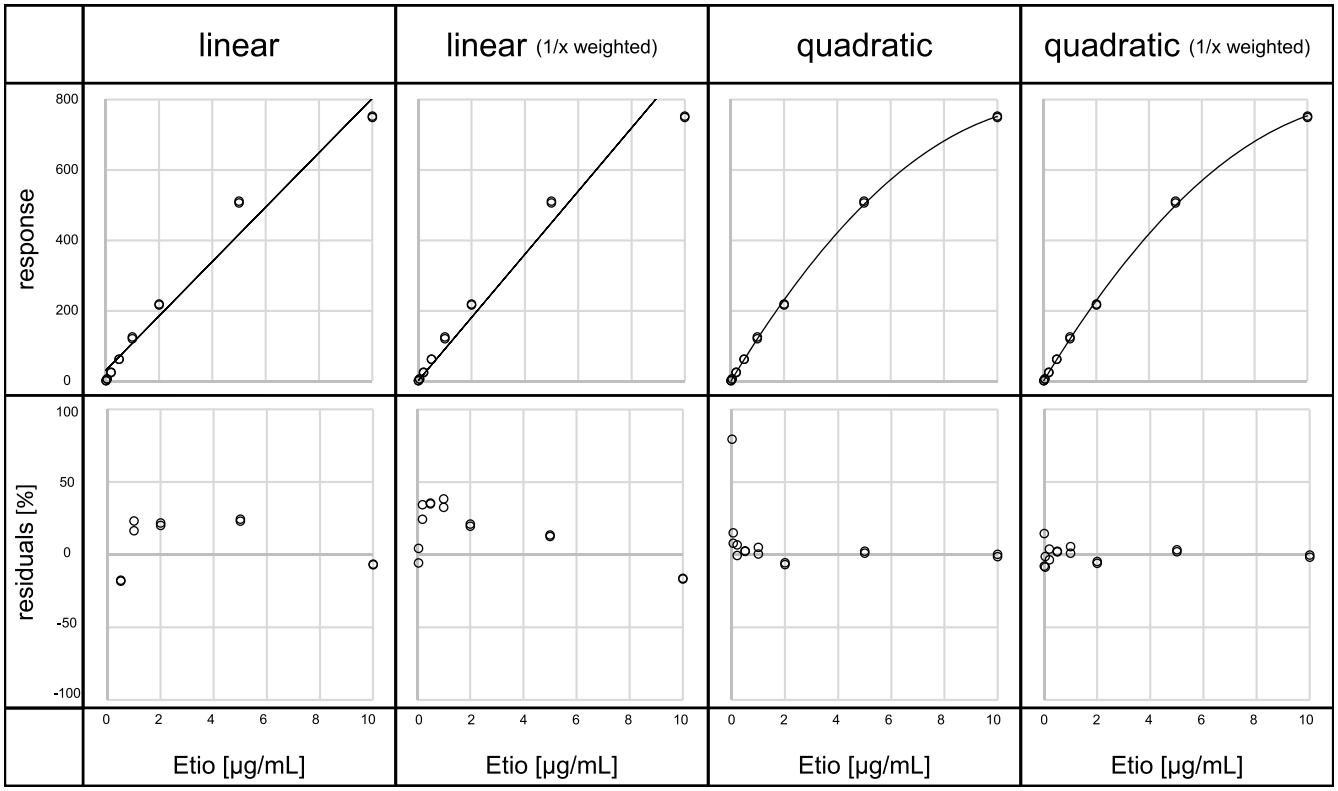

Fig. 3. Different regression models for the quantification of steroids and the resulting relative residuals, with etiocholanolone as example. Please note that the relative residuals of the linear regression for low concentrations were not depicted, as their values were too far off.

their volatility and make them more suitable for GC separations was acetylation, which is already used for GC/C/IRMS analysis [31,37]. In UHPSFC-MS/MS, the acetylation resulted in satisfying signals for all investigated substances as demonstrated for six steroids in Fig. 1B. This chromatogram was obtained on a Torus 1-AA column, using the same gradient as the separation of the free steroids. This stationary phase was selected after having tested multiple columns with different chemistries, according to classifications by West *et al.* [38]: Viridis BEH, Torus Diol, HSS C18 SB, Torus 2-PIC, BEH 2-EP, Torus 1-AA. All columns were provided by Waters and had identical dimensions (3.0 × 100 mm, 1.7 µm, 130 Å), except the HSS C18 SB, which contained 1.8 µm particles with 100 Å pores. The columns were evaluated based on their performance to separate endogenous steroids based on chromatographic resolution between isobaric compounds, peak shapes, and retention times. Most columns were ruled out, because the earliest eluting compound, 16EN-acetate showed no retention and was eluted within the system's dead volume. Finally, Torus 1-AA was chosen as a suitable stationary phase chemistry, allowing an efficient separation between all isomeric species with acceptable peak shapes. As all tests were performed with a common modifier composition, MeOH:H_2O (98:2, v:v) + 20 mM ammonium formate, and already yielded acceptable results, the mobile phase composition was not further changed, only the gradient was optimised to improve separation. With the final conditions, it was possible to separate all steroids, even the structural isomers, within 10 min, with a baseline resolution as shown in a chromatogram in Fig. 2.

As the volatility and ionisation efficiency of the steroids were altered due to the acetylation, the source parameters were also further optimised to improve sensitivity. In comparison with published UHPSFC-MS/MS methods for the analysis of free steroids [14,15], lower desolvation temperature and gas flow were used for the analysis of acetylated steroids. Interestingly, the investigated steroids that have their ionisation efficiency enhanced by the acetylation, lose the acetate moiety during ionisation, which is reflected by the detection of their precursor ions in the free form (non-acetylated), as demonstrated before in Table 1. For the acetylated 3-oxo-4-ene steroids (T and E), the quasi-molecular ions $[M+H]^+$ were detected with very high intensities in full scan mode. The most intense precursor ions of the acetylated 17-oxo steroids (A and Etio) resulted from an in source loss of water [M - H_2O + $H]^+$, while other steroid acetates lost up to two times acetic acid [M - AcOH + $H]^+$ and [M - 2AcOH + $H]^+$ during the ionisation. A similar behaviour was already described for the non-acetylated steroids [39]. Regarding the selection of product ions, the most intense fragments of the acetylated steroids originated from B-ring fragmentations, with the charge remaining on either side.

3.2. Method validation

The method was successfully validated for the quantification of the six steroids that comprise urinary steroid profile in the ABP to comply with the criteria set by WADA as summarised in Table 2.

3.2.1. Matrix effects and extraction recovery

ME and ER were determined as described by Matuszewski [30] using six real urine samples. As the endogenous steroids were present in all urine samples, the ME had to be determined with isotope labelled standards. The use of blank synthetic urine would not be applicable as different authentic samples should be used and due to limited availability of isotope labelled standards, the ER and ME were assessed only for the six steroids of the urinary steroid profile. We achieved satisfactory ERs, that were similar to another method utilizing SLE for extracting endogenous steroids [33]. For the other four steroids, ERs and MEs were estimated by spiking aliquots of the six urine samples before and after extraction at the highest point of the calibration curve to minimise the impact of the endogenous concentrations. They were then

compared with each other or a corresponding standard. These additional steroids showed no carry-over (<0.5 %), low ion enhancement (+8.5 % on average) and excellent ER (88.9 % on average) except 16EN, which had an ER of 23 %. However, this could be due to the volatility of 16EN which is increased by acetylation, therefore losses during the sample preparation can be expected.

3.2.2. Selectivity

As these endogenous steroids are present in all urine samples, the selectivity of the monitored transitions had to be demonstrated differently than for exogenous substances. Therefore, the ratios of the transitions in authentic and spiked urine samples were compared with neat standards. The ratios of transitions observed in urine samples corresponded with those found in steroid standards, apart from E. The second transition of E was influenced by an endogenous interference especially at low E concentrations, as a slight shift in retention time and an asymmetric peak shape were observed. Moreover, the ratio of transitions differed from that of a pure standard, rendering the second transition unsuitable for quantification purposes.

3.2.3. Calibration range

Regarding the calibration, different regression models were evaluated. From the calibration curves as exemplarily shown for Etio in Fig. 3, it is clearly visible that the relation between the concentration and signal response is not simply linear and even with a 1/x weighting function, the correlation did not improve. As neither the most concentrated calibrator nor a more concentrated standard was saturating the detector (data not shown), a non-linear relation was assumed. Although a linear regression would be easier to compute and would require less calibrators in the sequence, previous demonstrations have shown that quadratic regression is better suited for MS quantification [40]. With a quadratic regression, the correlation coefficients improved, but the residuals of the first points were still too high, so a 1/x weighting function was included to achieve the best possible correlation.

3.2.4. Accuracy, precision, and measurement uncertainty

Inter- and intra-day variability below 15 % at four different concentration levels were found to be acceptable, as reported in Table 2. Calculated measurement uncertainties and LOQs were compliant with the necessary requirements for urinary steroid profiling by WADA [4] and the methods range of calibration encompassed the reference values for urinary steroid concentrations of athletes [41].

3.3. Comparing UHPSFC-MS/MS and GC–MS

Concerning the sample preparation, both methods contained variations of the same elements: enzymatic hydrolysis under buffered conditions, followed by liquid extraction with a non-polar solvent. While the relative volume of buffer to sample was similar in both methods, the buffer capacity used for the GC–MS extraction was higher than for the UHPSFC-MS/MS method. For specific samples that have extraordinary pH-values or are heavily charged with matrix components, the higher buffer capacity could support the enzymatic hydrolysis better. Differences regarding the extraction solvent were less pronounced, TBME and DCM have similar polarities and both methods used a similar ratio of sample to extraction solvent.

To demonstrate the applicability for the analysis of authentic doping control samples, 132 urine samples from athletes were analysed by the herein presented UHPSFC-MS/MS method as well as with the routinely performed GC–MS method. From the steroids that were analysed by UHPSFC-MS/MS, only the six steroids that were quantified were compared with the GC–MS results, as the concentration of the others were only estimated with both methods. The concentrations that were below the LOQs of any of the two methods were excluded from the comparison, as it was the case for the T concentrations in eight samples. Four samples were completely excluded from the comparison due to complications during the extraction for UHPSFC-MS/MS analysis. Either

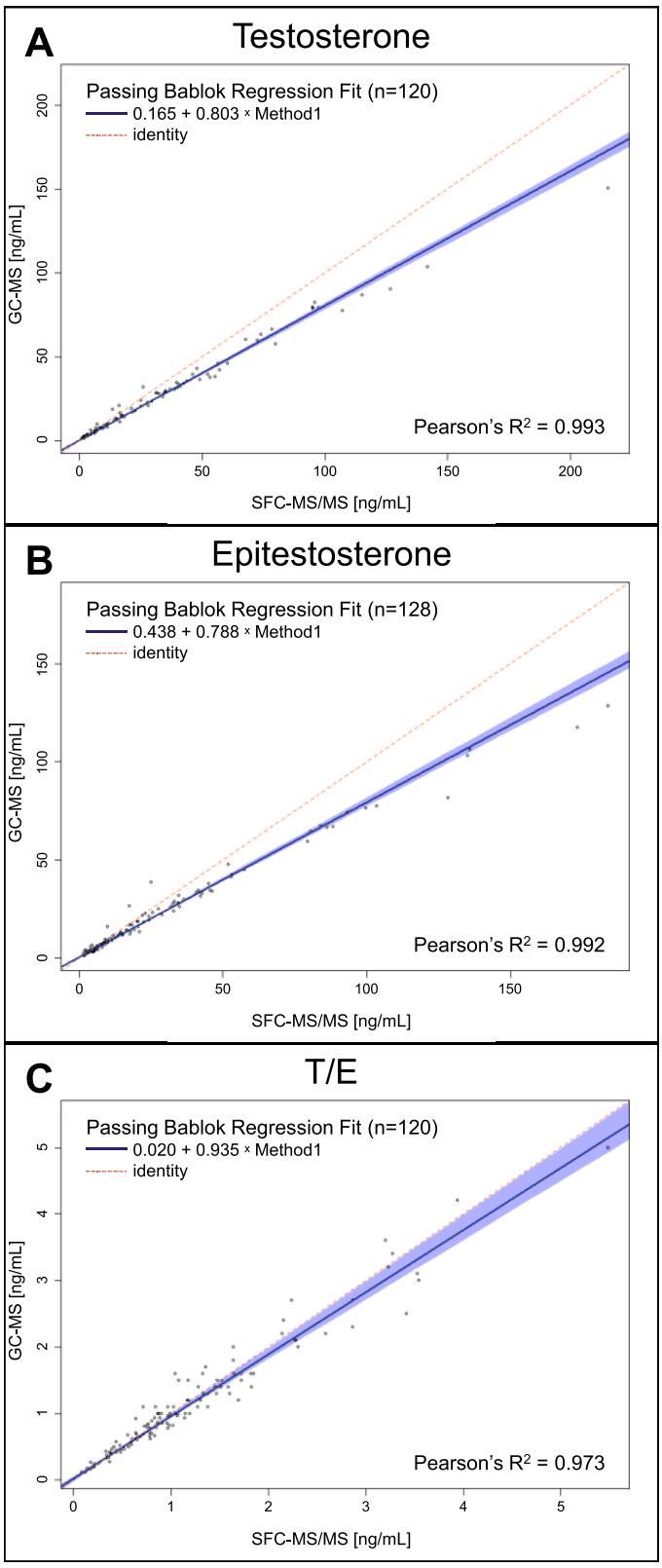

Fig. 4. Passing-Bablok regression of T (A), E (B) and the T/E-ratio (C) comparing the analysis by SFC-MS/MS with GC–MS.

they exhibited a significantly different ratio between D_4-A and D_5-Etio, lower signals of the internal standard, or both, suggesting incomplete hydrolysis or extraction. The objective of this method was to be used as an initial testing procedure (ITP), a re-extraction or confirmation of the

steroid profile could be done by GC–MS with an additional SPE to facilitate the enzymatic hydrolysis.

For the remaining 128 samples, the results from both methods were found to be similar, with the steroid ratios aligning more closely between the two methods in comparison to the steroid concentrations, as exemplarily demonstrated in Fig. 4 for T and E concentrations and the T/E ratio. Passing-Bablok regression [42] revealed a proportional bias when comparing the steroid concentrations analysed by the two methods. Generally, the concentrations were overestimated in UHPSFC-MS/MS in comparison with GC–MS, as demonstrated by the slope of Passing-Bablok regression, suggesting a proportional bias. As the intercept of the Passing-Bablok regressions were close to 0 and the confidence intervals included the origin, no significant systematic bias was observed for any of the steroids. All Passing-Bablok regressions and Bland-Altman plots for all steroids and their ratios were provided in the supplementary data S2. The use of different reference materials and the creation of calibrators could be a plausible explanation for this proportional bias. For highly sensitive longitudinal monitoring of urinary steroid profiles, the individual points should be accurate, therefore the routinely quantification by GC–MS was performed with quality control samples (LOQ, QC-low, QC-mid and QC-high) created from certified reference solutions with specified concentrations. On the other hand, the standards that were used for the creation of calibrators for the UHPSFC-MS/MS analysis were produced by weighing certified reference materials in powdered form and diluting them accordingly. During the method validation the calibrators and quality control samples were compared and gave equivalent results, however, the use of powdered standards is associated with higher risks of variation. Therefore, if the UHPSFC-MS/MS methodology would be applied for longitudinal monitoring of urinary steroids in an anti-doping context, it is recommended to use reference solutions with certified concentrations to maximise the accuracy.

Another contributing factor to the discrepancies between the two methods could be the employed calibration type. While the UHPSFC-MS/MS method relied on weighted quadratic regression with multiple calibrators, quantification for GC–MS was performed by cross-multiplication with a single point calibrator (QC-mid) assuming a linear correlation. This explanation seemed more plausible, as the quadratic influence became more noticeable at high signal intensities. Concentrations of T and E, which exhibited the highest responses in UHPSFC-MS/MS, as well as concentrations of A and Etio, which had the highest urinary concentrations, were overestimated on average by 20 % in comparison with GC–MS analysis. In contrast, the concentrations of 5αAdiol and 5βAdiol were only overestimated by 5 % on average.

Since authentic urine samples of athletes were used for the comparison, the true steroid concentrations of the samples used for method comparison were unknown. However, urinary steroid profiling by GC–MS is subject to multiple interlaboratory comparisons per year, organised by WADA for their External Quality Assessment Scheme (EQAS). As the used GC–MS method has passed the criteria of these interlaboratory comparisons, it is deemed as the gold-standard. Nevertheless, to put the method comparison into perspective, it should be highlighted at this point that the technical document for endogenous steroid profiling for the ABP [4] allowed for a maximal measurement uncertainty of 20 % or larger at lower concentrations or for certain analytes. Considering the proposed application of the method as an ITP, the quantification results in UHPSFC-MS/MS were found to be acceptable.

Moreover, it should be mentioned that the urinary steroidal module in the ABP is comprised of ITP data from GC–MS analysis. If the herein presented UHPSFC-MS/MS method was used as an ITP for the ABP, slightly overestimated steroid concentrations could be introduced. In extreme cases with large differences in comparison to previous points, the responsible Athlete Passport Management Unit (APMU) would request a confirmation of the steroid profile, which should then be performed by GC–MS to either correct or confirm the values of the ITP. However, the ABP considers the urinary steroid ratios more than the individual concentrations to account for different renal dilutions. The ratios determined by UHPSFC-MS/MS showed values much closer to the GC/MS measurements than the individual concentrations. The proportional bias for the ratios was <10 % except for A/Etio, which showed a larger bias due to differences in the overestimation of A and Etio with UHPSFC-MS/MS in comparison with GC–MS. The most sensitive urinary markers for the detection of T doping, if used for longitudinal monitoring in the ABP, are T/E and 5αAdiol/E [7,31,36]. As these two ratios had almost no bias between the two methods, the presented UHPSFC-MS/MS method could be used for urinary steroid profiling without compromising the sensitivity of the ABP.

Considering all these results, the herein presented UHPSFC-MS/MS method could be used as an ITP for urinary steroid profiling as an alternative to GC–MS. If a confirmation of the urinary steroid profile would be requested later, we would propose to perform this confirmation by GC–MS. Thus, the majority of the samples would only be analysed by the faster UHPSFC-MS/MS method and only special cases would require GC–MS to ensure orthogonality of the methods, especially for samples that show difficulties during the extraction process. Finally, to further assure the similarity of the results of the two methods, more tests should be performed in the future.

3.5. Proof-of-concept: semi-preparative UHPSFC prior to IRMS analysis

Currently, IRMS is needed to distinguish between endogenous and exogenous origin of urinary testosterone and its metabolites. As all organic matter is oxidised in the combustion oven of the GC/C/IRMS, co-eluting substances would invalidate any determined CIR, therefore the sample extracts needed to be accordingly purified beforehand. Routinely, this is achieved either by a single [43], double [31,37] or multidimensional [44,45] semi-preparative HPLC purification. In this context, due to the splitter configuration in the Acquity UPC^2, only a small part of the flow is entering the MS, while a significant part is

Table 3
Results of the QC samples for IRMS, concentrations determined by SFC-MS/MS and the $\delta^{13}C$ values (vs VPDB) after SFC purification in comparison with the expected $\delta^{13}C$ values after HPLC purification. (ND = not detected in GC/C/IRMS).

	PQC			NQC		
	[ng/mL]	$\delta^{13}C$ (SFC)	$\delta^{13}C$ (HPLC)	[ng/mL]	$\delta^{13}C$ (SFC)	$\delta^{13}C$ (HPLC)
T	541	-30.0	-30.5	37	-25.0	-24.3
E	52	ND	-29.7	21	ND	-25.6
A	3988	-28.4	-28.4	2129	-24.5	-24.0
Etio	3070	-30.0	-28.6	1116	-26.0	-24.8
5αAdiol	67	-29.2	-29.3	62	-25.5	-25.1
5βAdiol	288	-28.4	-28.8	73	-25.4	-25.0
PD	93	-23.1	-23.6	248	-23.9	-24.0
16EN	328	-23.2	-23.6	749	-23.4	-23.8
11-O-Etio	90	-23.5	-24.4	122	-23.8	-24.6
DHEA	12	ND	-23.1	25	ND	-24.5

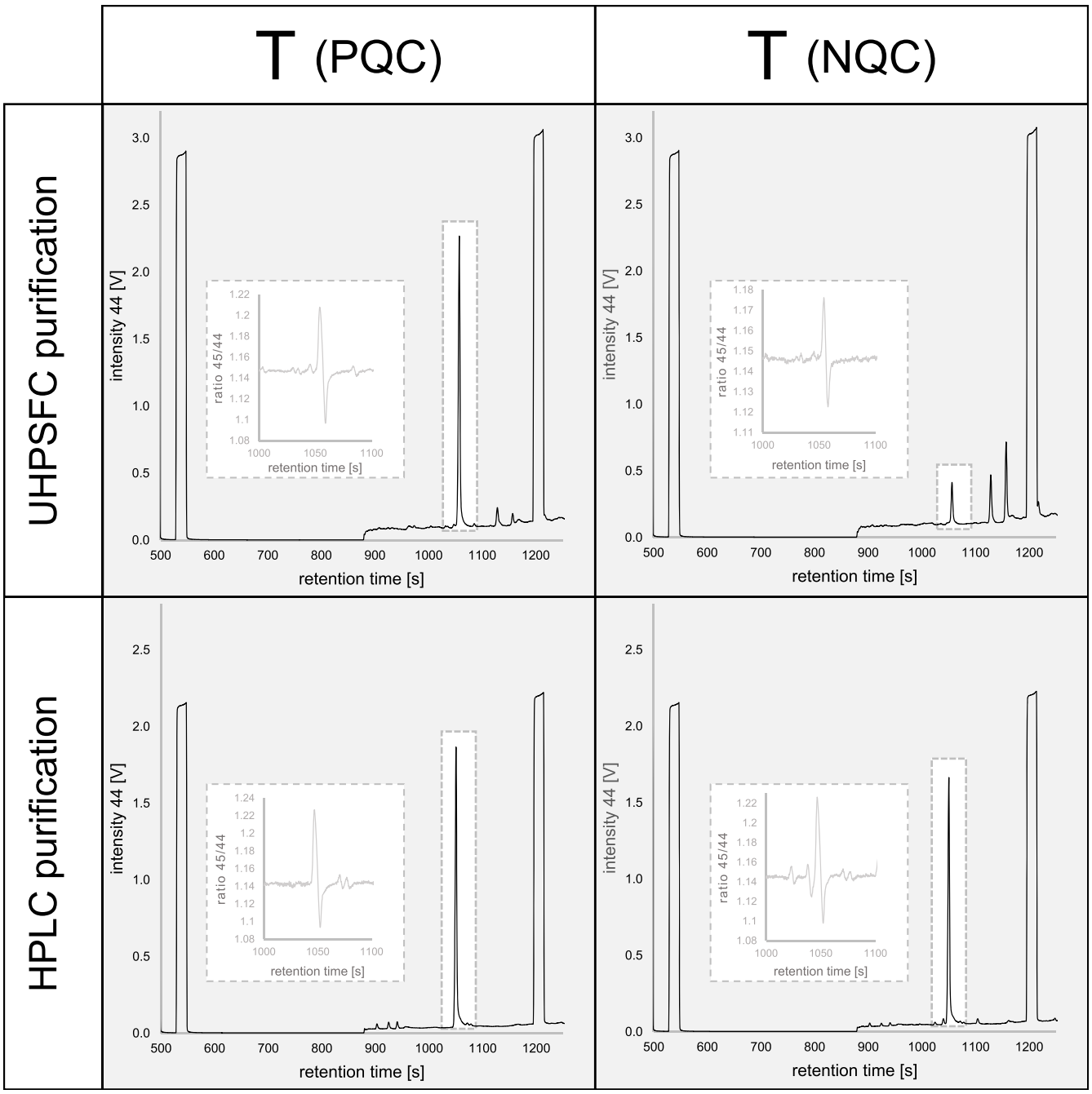

Fig. 5. GC/C/IRMS chromatograms of T after single UHPSFC purification compared with double HPLC purification using PQC and NQC as samples. The peak of interest is highlighted with a dashed border and the corresponding ratio 45/44 is shown to demonstrate the peak purity.

diverted through the BPR capillary to the waste [28]. This setup would therefore allow to collect purified fractions while performing MS analysis at the same time. However, GC/C/IRMS notoriously lacks sensitivity, therefore higher sample volumes are needed to achieve acceptable signals. For this reason, the previously described extraction in 96-well-plates could not be performed and an established sample preparation protocol for IRMS analysis was adapted and used for this preliminary study dealing with fraction collection [31]. While the acetylation was necessary for sensitive MS quantification, it would not have been needed for semi-preparative UHPSFC. However, the separation was developed for acetylated steroids and the acetylation is beneficial for the subsequent GC/C/IRMS analysis regarding their chromatographic properties.

Experimentally, it was necessary to inject the maximal possible amount of sample to increase the sensitivity. To prevent excessive peak broadening due to the higher injection volumes, the reconstitution solvent was changed to cyclohexane. Since no sensitive MS quantification was required and as ammonium formate was not soluble in cyclohexane the modifier composition was also changed to pure MeOH to prevent precipitations of ammonium formate. To prevent sample loss due to rapid evaporation at the end of the BPR capillary and increase the solubility of the analytes, the make-up flow was increased to 0.4 mL/min. For this proof-of-concept study, the fractions were collected manually and the delay between the MS detector and the exit of the BPR-capillary was determined by collecting fractions with fixed time intervals and reanalysing their contents afterwards. With the estimated delay, entire

peaks with a margin before and afterwards could be collected, to prevent isotopic fractionation from partial peak collection [46].

Results showed that, despite the use of the longer column (250 mm instead of 100 mm), it remained impractical to sufficiently separate all substances to collect individual substance fractions. The isomers 5αAdiol and 5βAdiol had to be collected together and to ensure the proper collection of PD, the collection time overlapped with the subsequent fraction. Therefore, Etio was split in two fractions, and its δ^{13}C-value did not match with the expected value. The CIRs of E and DHEA could not be determined as their concentrations were too low, as the final sample extract was only partially injected on the semi-preparative UHPSFC, whereas its entire volume was injected on the semi-preparative HPLC [31].

Despite these limitations, the δ^{13}C values of the most sensitive markers of T misuse (T, 5αAdiol and 5βAdiol) as well as three ERCs (PD, 16EN and 11-O-Etio) could be determined as shown in Table 3. Differences between the expected δ^{13}C values and the obtained δ^{13}C values after SFC purification below 1 ‰ were seen as satisfying results. The peaks that were used to calculate the δ^{13}C-values were identified as the corresponding substances *via* their EI-MS spectra (data not shown). Furthermore, these peaks had no significant interferences, indicated by a clean sigmoidal swing of the 45/44 ratio, as demonstrated for T in Fig. 5. The GC/C/IRMS chromatograms of the other substances were compiled in supplementary material S3. Since routine doping controls mainly relies on the sensitive markers and PD as primary ERC, and omits the analysis of the other fractions, this proposed UHPSFC purification method could be fit for purpose. However, GC/C/IRMS after UHPSFC fraction collection was less sensitive with the herein described set-up in comparison to the regular semi-preparative HPLC purification and would not fulfil the required sensitivity criteria from WADA [3]. Therefore, the method should be further optimised with special attention to the injection and fraction collection, before proposing it for implementation into routine analyses of doping control samples.

4. Conclusion

To the best of our knowledge, we developed the first UHPSFC-MS/MS method capable of urinary steroid profiling for the ABP. Furthermore, the sample preparation was modernised to facilitate high-throughput analyses. Quantification of urinary steroids was performed by 1/x weighted quadratic regression over the concentration range that is expected for athletes. The method proved to be sensitive and accurate, as the prevailing criteria for LOQs and measurement uncertainties were met. Comparison of results from real urine samples analysed by the herein presented UHPSFC-MS/MS method and the gold standard GC–MS demonstrated comparability of the results obtained by either of the two methods. Considering these results, we would propose to apply UHPSFC-MS/MS for anti-doping analyses in general and evaluate further its capabilities for quantitative analysis. In the future, after more tests have been conducted, this technique could be used as an alternative initial testing procedure for the determination of the urinary steroid profile for the ABP. While the steroid concentrations were up to 20 % overestimated by UHPSFC-MS/MS the steroid ratios showed differences below 10 % in comparison to the values obtained by GC–MS. Since the ABP is considering the steroid ratios for the evaluation of steroid profiles, and especially the most sensitive markers T/E and 5αAdiol/E, the sensitivity of the ABP would be maintained.

Furthermore, the possibility of using UHPSFC for sample purification was explored. With a simple manual fraction collection set-up, we could demonstrate that semi-preparative UHPSFC could be used as a fast alternative to semi-preparative HPLC. No isotopic fractionation was observed on correctly collected fractions, and the purity of the fractions was satisfactory, therefore UHPSFC could be used to purify urine samples prior to GC/C/IRMS analysis. However, to maximise sensitivity and robustness, the injection parameters should be further optimised, and we would recommend the use of an automated fraction collector.

CRediT authorship contribution statement

Tobias Langer: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Raul Nicoli:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Formal analysis, Conceptualization. **Davy Guillarme:** Writing – review & editing, Supervision. **Carine Schweizer-Grundisch:** Investigation. **Serge Rudaz:** Writing – review & editing. **Silke Grabherr:** Writing – review & editing, Resources. **Tiia Kuuranne:** Writing – review & editing, Resources. **Alessandro Musenga:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2024.465224.

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