

## LC-MS/MS measurement of endogenous steroid hormones and phase II metabolites in blood volumetric absorptive microsampling (VAMS) for doping control purposes

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### ABSTRACT

**Background:** Volumetric Absorptive Microsampling (VAMS) is emerging as a valuable technique in the collection of dried biological specimens, offering a potential alternative to traditional sampling methods. The objective of this study was to assess the suitability of 30  $\mu$ L VAMS for the measurement of endogenous steroid hormones.

**Methods:** A novel LC-MS/MS method was developed for the quantification of 18 analytes in VAMS samples, including main endogenous free steroids and phase II metabolites of androgens. The method underwent validation in accordance with ISO/IEC 17025:2017 and World Anti-Doping Agency (WADA) requirements. Subsequently, it was applied to authentic VAMS samples obtained from 20 healthy volunteers to assess the stability of target analytes under varying storage conditions.

**Results:** The validation protocol assessed method's selectivity, matrix effect, extraction recovery, quantitative performance, carry-over and robustness. The analysis of authentic samples demonstrated the satisfactory stability of monitored steroids in VAMS stored at room temperature, 4 °C, -20 °C and -80 °C for up to 100 days and subjected to up to 3 freezing-thawing cycles.

**Conclusions:** The validated LC-MS/MS method demonstrated its suitability for the measurement of steroids in dried blood VAMS. The observed stability of steroidal compounds suggests promising prospects for future applications of VAMS, both in anti-doping contexts and clinical research.

### 1. Introduction

Volumetric Absorptive Microsampling (VAMS) is an innovative miniaturized sampling technology that has rapidly become an

established tool for the collection of dried biological fluids samples, such as urine, blood and saliva. It is now considered a viable alternative to non-volumetric blood microsampling, overcoming the hematocrit effect (volume percentage of blood cells in whole blood) and homogeneity

**Abbreviations:** 11-DF, 11-deoxycortisol; 17-OHP, 17-hydroxyprogesterone; A4, Androstenedione; ABP, Athlete Biological Passport; A-G, Androsterone glucuronide; A-S, Androsterone sulphate; BSP, Blood Steroid Profile; CORT, Corticosterone; DBS, Dried Blood Spots; DHEA-S, Dehydroepiandrosterone sulphate; EAAS, Endogenous Anabolic Androgenic Steroids; EpiA-S, Epiandrosterone sulphate; EpiT-S, Epitestosterone sulphate; ESI, Electrospray ionization; Etio-G, Etiocholanolone glucuronide; Etio-S, Etiocholanolone sulphate; F, Cortisol; IS, Internal standard; ISL, International Standard for Laboratories; LLOQ, Lower limit of quantification; LOD, Limit of detection; MeOH, Methanol; NaCl, Sodium chloride; NH<sub>4</sub>F, Ammonium fluoride; PD, Percentage difference; RCV, Reference Change Value; RT, Room temperature; T, Testosterone; TDM, Therapeutic drug monitoring; T-G, Testosterone glucuronide; T-S, Testosterone sulphate; VAMS, Volumetric Absorptive Microsampling; WADA, World Anti-Doping Agency.

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issues associated with dried blood spots (DBS) [1]. The collection devices consist of a porous, absorbent tip, made of a hydrophilic polymer, attached to a plastic handle that allows hematocrit-independent collection of capillary blood by accurately capturing a volume (currently available in 10, 20 and 30  $\mu\text{L}$  formats) from a single blood drop through capillary action. Following the drying process and adequate storage, the VAMS device tip is suitable for analytical purposes [2] and analytes of interest can be extracted with different solvents or solvent mixtures using various methods [3–5]. Giving its resilience to blood density and viscosity variations and user-friendly nature, VAMS has found success in various clinical applications, including pharmacokinetic studies [6,7], therapeutic drug monitoring (TDM) [8], drug abuse monitoring [9], routine clinical biochemistry analysis [10], biomarker discovery [11] and metabolomic studies [12]. In the domain of anti-doping research, VAMS has proven effective in the detection of small molecules [13,14], such as steroids in general and endogenous anabolic androgenic steroids (EAAS) using GC–MS/MS [15]. Building on the promising results obtained in detecting EAAS in dried blood matrices, the anti-doping scientific community is actively exploring innovative markers and analytical approaches for EAAS abuse detection, which is most often performed by GC/MS-based methods in urine and more recently also by LC/MS methods in serum [16]. The measurement of endogenous steroids levels has emerged as a crucial component in the field of steroid profiling research. Recent studies have shown that longitudinal monitoring of serum concentrations of testosterone (T) and androstenedione (A4), via the T/A4 ratio, along with dihydrotestosterone (DHT), could provide complementary information to the currently used urinary steroidal module of the Athlete Biological Passport (ABP) [16]. This is pivotal in circumventing the impact of potential confounding factors associated with the UGT2B17 polymorphism, while simultaneously enhancing sensitivity to detect doping with transdermal T in both male and female population [17,18]. Furthermore, an untargeted steroidomic approach has revealed more sensitive markers among phase II androgen metabolites (glucuro- and sulpho-conjugates), widening the detection window of doping with oral T [19]. The potential of DBS has been explored for the measurement of circulating levels of endogenous steroid hormones: while proving useful, it acknowledges subtle concentration differences between DBS and serum samples, which should be taken into account when devising strategies for longitudinal monitoring [20]. In this context, the use of dried blood samples as a sample matrix has been approved by World Anti-Doping Agency (WADA) in 2021 for doping control analysis. A dedicated Technical Document (TD2023DBS) is now available and provides specific requirements and procedures for the analytical testing and storage of DBS samples, aiming at the standardization DBS testing [21].

Despite the good results obtained with the use of DBS, VAMS procedures have attracted increasing interest in recent years and are recognized as an innovative and superior sampling method for quantitative purposes [2]. This method is currently becoming an established tool for obtaining dried samples of biological fluids and, at a research level, is demonstrating its validity as an alternative to urine, whole blood, and serum sampling in anti-doping analysis [17,22,23]. Although similar to DBS, VAMS offers several noteworthy advantages. It has the potential to enhance quantitative performance through accurate sample volume, ensuring extraction yield and reproducibility unaffected by hematocrit (HCT). Ideally, this leads to an improved analytical performance and a stronger correlation with plasma values. Moreover, VAMS can also simplify collection procedures by delivering homogeneous samples with a reduced reject rate, which is essential in scenarios with limited sample material availability. Additionally, the use of 96-well formats enhances accessibility to automation.

The aim of this work is to evaluate the suitability of VAMS, in a 30  $\mu\text{L}$  format, as an innovative matrix for the measurement of novel blood markers to unveil potential EAAS doping. The experimental work has been divided into two sub-projects. The first step involves the development and validation of a novel LC-MS/MS method. This method,

which is a first example of extended steroid profiling in whole blood VAMS microsamples, aims to quantify a comprehensive panel of circulating steroid hormones and phase II glucuro- and sulpho-conjugated metabolites of androgens. Mass spectrometric parameters and transitions were fine-tuned to achieve maximum sensitivity for the detection and quantification of the 18 target analytes in the final method. Additionally, the chromatographic separation was optimized to differentiate between various isomers, balancing resolution and overall analysis time and a comprehensive validation of the quantitative method was performed in compliance with ISO/IEC 17025:2017 and WADA requirements.

The second part of the study involves for the first time to date a stability assessment of steroid hormones and metabolites in dried whole blood VAMS microsamples, with the aim of exploring the potential advantages of VAMS in terms of sample transport and storage. Whole blood samples were collected from 20 healthy volunteers and a total of 17 VAMS aliquots were prepared for each individual. These aliquots were stored under four distinct conditions (room temperature, 4  $^{\circ}\text{C}$ ,  $-20^{\circ}\text{C}$ ,  $-80^{\circ}\text{C}$ ), and the effect of up to three freezing-thawing cycles was also evaluated.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Certified reference materials and labelled internal standards (IS) for all target steroidal compounds were obtained from three different providers. 11-deoxycortisol (11-DF),  $17\alpha$ -hydroxyprogesterone (17-OHP), A4, androsterone glucuronide (A-G), androsterone sulphate (A-S), corticosterone (CORT), cortisol (F), cortisone, dehydroepiandrosterone sulphate (DHEA-S), DHT, epiandrosterone sulphate (EpiA-S), epitestosterone sulphate (EpiT-S), etiocholanolone glucuronide (Etio-G), etiocholanolone sulphate (Etio-S), Progesterone, T, testosterone glucuronide (T-G), testosterone sulphate (T-S) as well as  $17\alpha$ -hydroxyprogesterone d8, A4 d7, CORT d8, DHT d3, progesterone d9, testosterone d3 were supplied by Steraloids (Newport, RI, USA); A-S d4, cortisol d4, cortisone d8, EpiA-S d3, Etio-G d5, Etio-S d5, T-G d3, T-S d3 were provided by LGC Standards (Teddington, United Kingdom); 11-DF d5 and A-G d4 were obtained from Merck KGaA (Darmstadt, Germany). UHPLC/MS grade methanol (MeOH) was purchased from Carlo Erba Reagents (Cornaredo, Italy), ultra-pure water (18.2 M $\Omega$  x cm) was obtained with the Smart2pure<sup>®</sup> system (Thermo Scientific, Waltham, MA, USA), ammonium fluoride (NH<sub>4</sub>F) for MS was supplied by Merck KGaA (Darmstadt, Germany), while saline solution (NaCl 0.9 %) was acquired from B. Braun Melsungen AG (Melsungen, Germany). Mitra<sup>®</sup> VAMS dried blood microsampling kits in the 30  $\mu\text{L}$  format were obtained from Neoteryx (Torrance, CA, USA) and Charcoal Stripped Human Serum was purchased from DivBioScience (Ulvenhout, The Netherlands).

Stock solutions of all analytes and IS were prepared at a concentration of 1 mg/mL (2 mg/mL for A-S, DHEA-S, EpiA-S and Etio-S) in MeOH and stored in 1.5 mL amber glass vials at  $-80^{\circ}\text{C}$ . For each compound, intermediate solutions at appropriate concentrations (1 mg/mL, 100  $\mu\text{g}/\text{mL}$ , 10  $\mu\text{g}/\text{mL}$ , 1  $\mu\text{g}/\text{mL}$ , 100 ng/mL, 10 ng/mL) were prepared by means of consecutive dilutions of stock solutions and stored in 10 mL glass tubes at  $-20^{\circ}\text{C}$ . A mixture containing all IS (IS mix) was prepared spiking different volumes of each IS intermediate solution at appropriate concentration for each IS (details in [Supplementary Material Table S1](#)) in a MeOH/H<sub>2</sub>O solution (90/10, v/v), which was stored in 10 mL glass tubes at  $-20^{\circ}\text{C}$  and used for the extraction procedure.

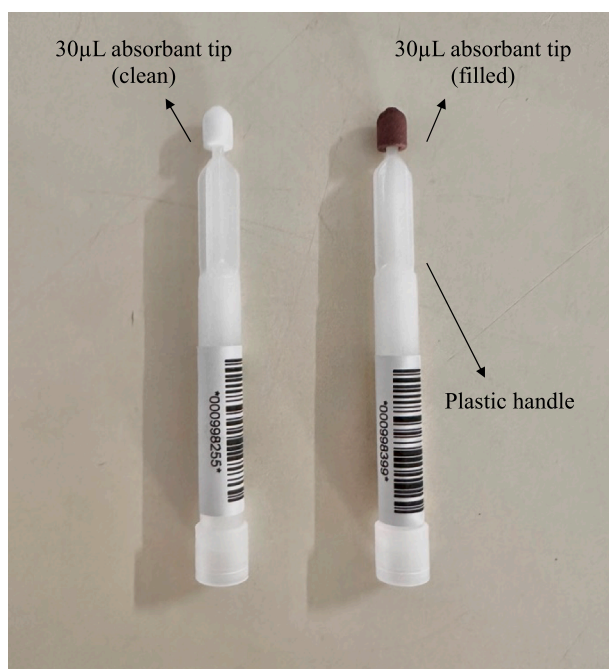
### 2.2. Calibration and validation samples

For the preparation of different calibration and validation samples, working solutions containing all target analytes were prepared in MeOH. Steroid-free artificial whole blood was prepared using a pool of whole blood samples collected from healthy female volunteers and using

the procedure reported by Higashi et al. [24] with slight modifications. In brief, pooled whole blood was firstly centrifuged at 1500g for 10 min and the resulting separated plasma was discarded. The erythrocyte fraction was then washed with sodium chloride (NaCl) 0.9 % solution, centrifuged at 1500g for 10 min, and the supernatant was discarded. Such procedure was repeated three times and the “washed” erythrocyte fraction was finally combined with charcoal stripped serum to obtain a steroid-free whole blood with a hematocrit of 50 %. Calibration and validation samples were then prepared by spiking the artificial blood with working solutions containing all target analytes to obtain desired final concentrations (details presented in [Supplementary Material Table S2](#)) and 30  $\mu$ L of spiked blood were pipetted on VAMS absorbent tips. The prepared VAMS calibration and validation samples were then dried at room temperature for a minimum of 30 min and stored in plastic bags with desiccator at 4 °C until their analysis.

### 2.3. VAMS samples extraction

Healthy individual as well as calibration and validation VAMS samples were collected spiking 30  $\mu$ L of collected whole blood on the VAMS support. An example of clean VAMS tip and after blood absorption is presented in [Fig. 1](#). The polymer tip was then placed in 1.5 mL polypropylene Eppendorf tubes and sample preparation procedure was carried out by following five consecutive steps: 1) 700  $\mu$ L of the MeOH/H<sub>2</sub>O solution (90/10, v/v) containing IS mix was added into each tube; the latter were then placed in an ultrasonic bath under a hood for 30 min at room temperature. 2) VAMS polymer tips were removed from the tubes and the extracts (700  $\mu$ L) were transferred to a 1 mL 96-well collection plate by manual pipetting. 3) Extracts were subjected to complete evaporation under a stream of nitrogen for approximately 30 min at 50 °C. 4) Extracted samples were reconstituted adding 100  $\mu$ L of a MeOH/H<sub>2</sub>O solution (50/50, v/v) to each well of the collection plate, which was shaken for 30 min at 600 rpm. 5) Reconstituted extracts were transferred into 1.5 mL Eppendorf tubes and centrifuged for 10 min at 13000 rpm and finally 80  $\mu$ L of supernatant were transferred into glass vials with conical inserts.



**Fig. 1.** Volumetric absorbent microsamplers in 30  $\mu$ L format before and after filling with blood.

### 2.4. LC-MS/MS analysis

Quantitative analyses were performed employing a Nexera X2 UHPLC system (Shimadzu, Tokyo, Japan) coupled to a Citrine Triple Quad MS/MS system (AB Sciex, Ontario, Canada). System was controlled with Analyst software, while data analysis (peak integration and quantification) was performed by using MultiQuant software (AB Sciex, Ontario, Canada). Luna® Omega C18 analytical column (100  $\times$  2.1 mm, 1.6  $\mu$ m; Phenomenex, Torrance, USA) set at 55 °C was employed to achieve a satisfactory chromatographic separation of target analytes. Mobile phase A was 2 mM NH<sub>4</sub>F in H<sub>2</sub>O and mobile phase B was MeOH. The chromatographic gradient started with a 1 min increase from 10 % to 55 % B, followed by a 2.5 min isocratic step at 55 % B, a further increase to 65 % B in 5 min and a second 2.5 min isocratic step at 65 % B; the gradient continued with a last increase from 65 % to 98 % B in 3 min followed by a washing step at 98 % B for 3 min; the column was finally re-equilibrated at initial conditions for 2 min. The injected volume was 20  $\mu$ L and the flow rate was set at 300  $\mu$ L/min, while the total run time was 19 min.

MS/MS analysis was performed in polarity switching mode using an electrospray ionization (ESI) source with the following parameters: source temperature was set at 550 °C while Ion spray voltage was set at 5500 in positive ionization mode and at -4500 V in negative mode; curtain gas, nebulizer gas and heater gas pressures were set at 35, 45 and 60 psi, respectively. One quantifier and one qualifier transition were selected for each target analyte, except for the sulphated steroids negatively ionized for which only one fragment (96.0 *m/z*) was monitored, while one MS/MS transition was selected for each IS. Transition-specific MS parameters, such as declustering potential, entrance potential, collision energy and cell exit potential were finely optimized for each monitored compound and IS by infusing standard solutions at 100 ng/mL in reconstitution solvent. A summary of the optimized MS/MS parameters for all target analytes is presented in [Supplementary Material Table S3](#).

### 2.5. Method validation procedure

The LC-MS/MS method was validated in accordance with ISO/IEC 17025:2017 [25] requirements and the WADA International Standard for Laboratories (ISL v10.0) [26], following the technical documents regarding the decision limits for the quantification of threshold substances in urine samples (TD2022DL) and the minimum criteria for compounds identification in chromatography (TD2023IDCR) [27,28]. WADA requirements were also taken into consideration for planning the experiments and for setting targets and acceptance criteria, as summarized in [Supplementary Material Table S4](#). The employed validation protocol included the assessment of selectivity, matrix effect, extraction recovery, quantitative performance (trueness, repeatability, intermediate precision, combined uncertainty, linearity range, limit of detection (LOD), lower limit of quantification (LLOQ)), carry-over and robustness.

#### 2.5.1. Selectivity

Selectivity was assessed extracting and analyzing five Level 4 calibration samples, five real female whole blood samples and five real male whole blood samples. Fragments ions' ratios measured in calibration samples were compared with the ones observed in real blood samples. Furthermore, five VAMS samples were created by spiking artificial steroid-free whole blood with a methanolic solution containing 60 exogenous steroids at concentration between 2 and 5 ng/mL. These samples were subjected to sample preparation procedure and analyzed to investigate the potential presence of chromatographic interferences occurring in the selected MS/MS transitions of all target analytes.

#### 2.5.2. Extraction recovery and matrix effect

Extraction recoveries and matrix effects were measured for all target analytes using the approach of Matuszewski et al. [29]. In detail,

extraction recoveries were calculated as the ratio between peak areas of steroid-free whole blood VAMS samples spiked before the sample preparation procedure and during the reconstitution step with a solution containing all monitored steroids. The assessment of matrix effects was performed by comparing the peak area of steroid-free whole blood VAMS samples spiked during the reconstitution step with that of the corresponding methanolic standard solution containing all the target analytes. These experiments were carried out in quadruplicate using the solution used for creating Level 4 calibration samples. More in detail, for VAMS samples in which Level 4 calibration solution was spiked before extraction the procedure described in Section 2.2 was carried out: Level 4 calibration solution was spiked in steroid-free whole blood and then 30  $\mu\text{L}$  of the prepared samples were pipetted on VAMS absorbent tips and then dried at room temperature for a minimum of 30 min before extraction.

### 2.5.3. Quantitative performance

Quantitative performances of the method (trueness, repeatability, intermediate precision, combined uncertainty and linearity range) were assessed on three different analytical series. For each validation series, five calibration and five validation samples were prepared and analyzed in duplicate and quadruplicate, respectively. Calibration and validation samples were prepared as described in Section 2.2 and five-point linear calibration curves were calculated using a  $1/x^2$  weighted regression using the peak area ratio of the quantifier transition to that of the corresponding IS. To be accepted, calibration curves should have a determination coefficient ( $R^2$ ) > 0.98, and all calibrator levels should result in a  $\pm 15\%$  maximum deviation from nominal concentration except for the less concentrated one, for which the maximum accepted deviation was set to  $\pm 20\%$ . In accordance with WADA Technical Document TD2022DL, combined measurement uncertainty (uc) was also assessed by quadratic combinations of the intermediate precision and the root mean square of the bias estimates and an acceptance criterion for this parameter was set at 20%, considering the mean value obtained at each concentration level of calibration/validation samples. Bias was determined as the mean absolute percentage difference between the concentrations measured in validation samples (analyzed in quadruplicate in each of the three validation days) and their nominal concentration taken as the reference.

### 2.5.4. Limit of detection (LOD) and lower limit of quantification (LLOQ)

The LOD was estimated as the lowest concentration at which each monitored steroid could be detected with a signal to-noise ratio greater than 3, while LLOQ was determined as the lowest concentration at which calculated combined uncertainty was below 20%.

### 2.5.5. Carry-over

Investigations on carry-over effect were performed by analyzing three extracted artificial steroid-free whole blood VAMS samples immediately after the most concentrated calibration sample (Level 5). Carry-over was evaluated as negligible when the mean target analytes' peak area in steroid-free samples was lower than 1% of the one measured in the Level 5 calibration sample.

### 2.5.6. Robustness

The robustness of the method was assessed over the three-day quantitative validation protocol, evaluating the influence of minor changes. These changes included variations in the operator performing sample extraction, mobile phase preparation, analytical column lot, and the effects of multiple instrumental maintenance procedures. The objective was to appraise the method's resilience to these minor variations and ensure its robust performance under different operational conditions.

## 2.6. Stability study

### 2.6.1. Samples collection and storage

To assess the stability of endogenous hormones in dried blood microsamples collected with VAMS technology, real whole blood samples were collected from twenty healthy volunteers, 10 males (mean age 58.5 years) and 10 females (mean age 48.4 years), who were recruited at the Blood Bank of the City of Health and Science University Hospital of Turin. The study protocol was approved by the local ethics committee (Protocol n. 488789) and the volunteers who were enrolled in the study met five inclusion criteria: age between 18 and 65 years; body weight higher than 50 kg; blood pressure below 180/100 mmHg; hemoglobin values higher than 12.5 and 13.5 g/dL in females and in males, respectively; anamnesis free from diagnosis of diabetes, cardiovascular diseases, epilepsy, neoplasms and/or autoimmune diseases.

Each participant provided a single whole blood sample on the same day, collected between 7:30 and 9:00 a.m. in a fasting state using K2-EDTA BD Vacutainer tubes (Becton, Dickinson & Company, Franklin Lakes, USA) in the 4 mL format. Immediately after sample collection, 17 VAMS aliquots were created for each participant by spiking 30  $\mu\text{L}$  of whole blood on different VAMS tips, which were left to air dry for 30 min at room temperature. One aliquot per individual was then analyzed on the same day of sample collection (baseline sample), while the other 16 aliquots were stored at four different conditions (room temperature (RT), 4  $^{\circ}\text{C}$ , -20  $^{\circ}\text{C}$  and -80  $^{\circ}\text{C}$ ) and analyzed according to the schedule outlined in Table 1.

### 2.6.2. Stability assessments

The evaluation of steroid hormones' stability in VAMS samples was carried out combining two different strategies and investigating all monitored analytes separately. The normal distribution of steroids' concentration values obtained in each day of analysis (20 samples per analyte) was checked by Shapiro-Wilk normality test. Depending on the test results, either a paired samples *t*-test or Wilcoxon signed-ranked test were employed to assess if significant differences existed between concentration values measured in samples stored in different conditions. In addition to this approach, Reference Change Value (RCV) was employed to assess whether observed differences in steroids' concentration between different aliquots could be attributed to the analytical variability of the method [30]. For this purpose, the mean percentage difference (PD) between the concentration values measured in each storage condition and the ones measured in baseline aliquots were calculated and were then compared to the RCV value obtained for each target analyte applying the following equation:

$$RCV = \sqrt{2} * Z * \sqrt{CV_a^2}$$

where  $Z = 1.9$  and it is referred to the 95% confidence interval and  $CV_a$  is the coefficient of analytical variability that was estimated for each analyte during quantitative validation protocol. If the mean PD between concentrations measured in baseline aliquots and aliquots stored in different storage conditions falls below the RCV, it could be assumed that such difference does not exceed the analytical variability of the

**Table 1**  
Storage conditions and analysis schedule of VAMS aliquots.

Storage conditions	Days									
	0	1	2	3	5	9	15	30	60	100
Baseline (BL)	X									
Room temperature (RT)		X			X		X		X	X
4 $^{\circ}\text{C}$					X	X	X			
-20 $^{\circ}\text{C}$							X	X	X	
-80 $^{\circ}\text{C}$									X	X
Freeze and thaw (FT)		X	X	X						



method and therefore the analyte could be considered stable in the investigated conditions.

### 3. Results & discussion

#### 3.1. Method validation

The method underwent validation in accordance with ISO/IEC and WADA regulations. Prior to conducting validation experiments, preliminary assessments were performed on a limited number of real serum samples to determine the presence or absence of all 18 steroidal compounds initially included in the developed method, using a previously validated method already in use in the laboratory [31]. Such preliminary tests (data not shown) highlighted the presence of selected target analytes in all analyzed samples at sufficiently high concentrations in larger blood volumes (200  $\mu$ L) compared to the volumes of whole blood sampled by VAMS devices (30  $\mu$ L). Therefore, all 18 steroidal compounds were maintained in the final quantitative method, and their analytical performance was thoroughly tested during the validation process.

##### 3.1.1. Selectivity

Regarding the selectivity, extraction process together with the optimized chromatographic separation and the selection of two MRM transitions for each compound established the initial level of selectivity of the method. The chromatographic separation, shown in Fig. 2, shows that all analytes are well separated. Moreover, the optimization allowed to effectively discriminate the isobaric isomers among the analytes of interest: CORT/11-DF, T-S/DHEA-S/EpiT-S, EpiA-S/A-S/Etio-S, Etio-G/A-G). Details on selected MRM transitions and retention times can be found in Supplementary Material Table S3 and Table 2, respectively.

The absence of chromatographic interferences in the selected MRM transitions of all target analytes was verified by analyzing five negative control VAMS samples and five negative VAMS samples spiked with a solution containing approximately 60 exogenous steroids at concentration between 2 and 5 ng/mL. The observation of MRM chromatograms of negative VAMS sample in the elution region of each steroid did not show any notable interferences (<20 % LLOQ). Furthermore, ten real VAMS samples (5 females and 5 males) were also extracted and analyzed in "Product Ion Scan" mode with a fixed collision energy, comparing the obtained MS/MS spectra with a Level 4 calibration sample. Real VAMS

samples as well as calibration VAMS samples were also analyzed with the developed method comparing the measured fragment ion ratio, calculated by dividing the area of the peak obtained in the two MRM transitions, and for all target steroids the results obtained in both female and male samples were within acceptable criteria (data presented in Supplementary Material Table S4).

##### 3.1.2. Extraction recovery and matrix effect

Extraction recoveries and matrix effects were measured for all target analytes using the approach of Matuszewski. From Table 2, where obtained results are presented, it is possible to observe satisfactory extraction recoveries for all investigated compounds ranging from 68.8 % (A-G) to 94.1 % (T) and achieved thanks to the applied sample preparation procedure. Concerning matrix effects, the steroid extraction procedure resulted in ion suppression of 20–30 % for all monitored analytes, highlighting a drawback of VAMS extraction if compared to more complex purifying techniques, such as SPE, LLE, and SLE. These findings, resulted from different batches of analysis performed employing VAMS from different production lots, suggest that the observed low ion suppression effect could be related to the nature of polymeric tip. Indeed, Salamin et al. did not report for a similar panel of steroidal compounds did not show a comparable effect when extracted from DBS samples, with matrix effects ranging from 90 % to 105 % [32]. Furthermore, in a recent work on the quantification of 25-hydroxyvitamin D2 and D3 in dried blood VAMS, a similar ion suppression effect of around 20 % was observed for the investigated steroidal analytes [33]. The outcomes of our study therefore support the use of high-end LC-MS instruments as well as the inclusion in the method of isotopically labelled IS for the analysis of dried blood micro-samples treated with organic solvents using a simple sonication step. To verify if the used IS mix could correct the observed ion suppression, experiment for the assessment of extraction recoveries and matrix effects of the employed isotopically labelled IS were performed and the obtained results, showing the similarity of recovery and matrix effect between target analytes and relative IS, are presented in Supplementary Material Table S5.

##### 3.1.3. Quantitative performance

Quantitative performances of the method were assessed on three analytical series by two different operators, testing duplicate samples at each of the five calibration curve levels, as well as quadruplicate samples

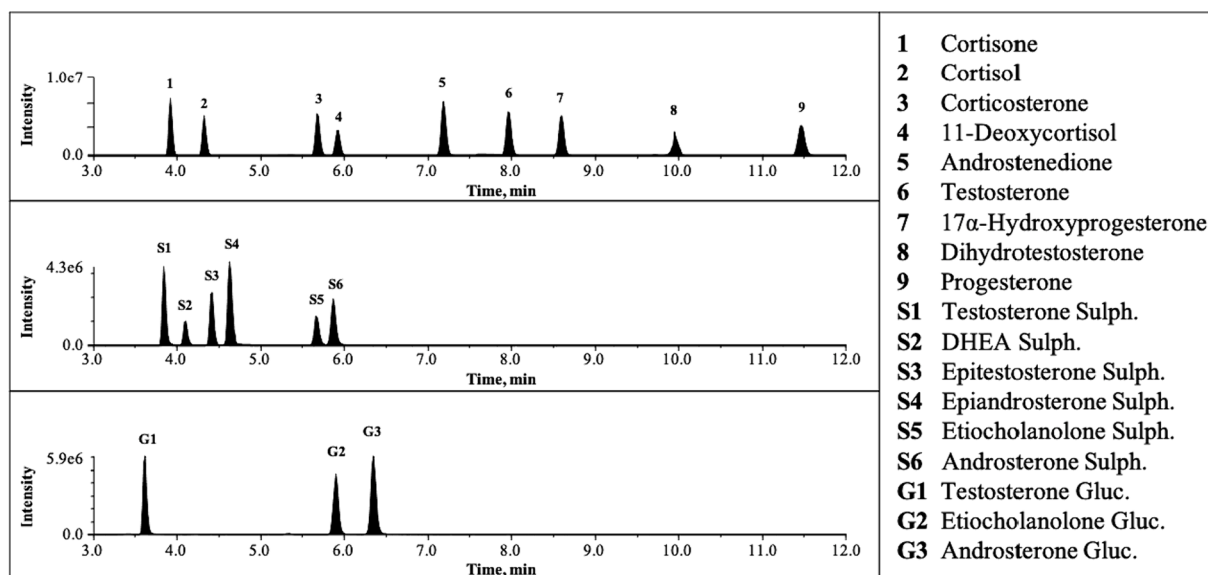


Fig. 2. Chromatograms obtained with the optimized chromatographic gradient for a methanolic solution containing all target analytes at a concentration of 10 ng/mL.

**Table 2**  
Summary of quantitative validation results.

Compound	rT [min]	Bias [%]	Repeatability [%]	Intermediate precision [%]	Combined uncertainty [%]	Linearity range [ng/mL]	LLOQ [pg/mL]	LOD [pg/mL]	Extraction recovery (CV) [%]	Matrix effect (CV) [%]
11-Deoxycortisol	5,92	5,6-6,4	4,1-7,6	4,0-7,0	7,6-10,2	0,1-5	100	50	87,4 (8,2)	68,2 (1,4)
17 $\alpha$ -Hydroxyprogesterone	8,59	4,9-8,1	5,0-8,7	5,9-8,2	9,5-11,6	0,15-7,5	150	75	88,3 (4,9)	74,8 (2,5)
Androstenedione	7,19	5,6-7,0	4,6-8,4	5,4-8,1	8,8-11,2	0,1-6	100	50	92,5 (5,8)	77,7 (3,1)
Corticosterone	5,68	6,4-7,9	5,9-8,3	6,7-8,9	10,3-12,3	0,15-10	150	50	92,3 (7,3)	74,6 (2,6)
Cortisol	4,32	6,2-8,9	3,1-7,2	3,4-8,8	7,5-12,2	5-300	5000	500	91,9 (11,8)	70,8 (2,4)
Cortisone	3,92	5,9-8,1	5,2-9,6	5,6-9,2	9,8-13,0	0,5-25	500	50	81,1 (1,7)	76,2 (1,3)
DHT	9,95	4,8-8,6	5,0-7,0	5,6-8,3	8,0-11,9	0,1-2	100	50	78,1 (11,7)	74,4 (3,3)
Progesterone	11,46	4,0-8,4	4,1-8,6	3,9-8,3	6,6-12,0	0,05-12,5	50	20	90,4 (5,3)	76,1 (1,4)
Testosterone	7,96	5,9-8,9	5,5-10,4	5,8-9,9	8,8-13,9	0,05-10	50	20	94,1 (5,4)	70,3 (1,2)
Androsterone Glucuronide	6,35	5,4-8,1	2,8-9,9	2,7-9,4	6,6-13,0	1-50	1000	250	68,8 (9,5)	76,0 (2,6)
Etiocholanolone Glucuronide	5,90	4,8-6,3	3,7-7,0	5,3-7,3	7,7-10,1	1-50	1000	200	70,6 (8,7)	83,9 (2,7)
Testosterone Glucuronide	3,58	5,6-7,8	6,0-9,0	6,4-8,4	9,2-11,7	0,05-4	50	25	73,5 (11,8)	75,3 (2,0)
Androsterone Sulphate	5,88	6,3-7,6	4,6-7,9	4,9-7,5	9,3-11,1	5-500	5000	1500	91,2 (10,3)	85,9 (2,6)
DHEA Sulphate	4,18	5,3-6,4	3,5-7,2	4,6-7,4	7,5-10,4	20-4000	20,000	7500	88,2 (8,4)	80,2 (3,1)
Epiandrosterone Sulphate	4,65	5,9-9,0	4,6-8,4	6,1-8,5	9,2-13,0	5-300	5000	2000	77,9 (12,3)	77,9 (2,6)
Epitestosterone Sulphate	4,41	5,4-7,6	4,7-9,3	5,5-8,9	9,4-12,4	0,1-5	100	50	78,3 (7,9)	76,6 (2,2)
Etiocholanolone Sulphate	5,68	5,0-7,4	3,6-8,2	3,3-8,6	6,6-12,0	2,5-250	2500	1250	91,2 (10,3)	85,9 (2,6)
Testosterone Sulphate	3,84	4,7-7,7	3,9-8,4	4,8-7,9	8,0-11,6	0,1-5	100	50	74,5 (8,7)	82,8 (2,1)

for each of the five validation levels.

Table 2 provides a comprehensive summary of the quantitative validation outcomes, including retention times of target analytes, extraction recoveries and matrix effects. Precision values were obtained for intermediate precision and repeatability, spanning between 2.8 % and 10.4 % and from 2.7 % to 9.9 %, respectively. Notably, an inverse relationship was found between analyte concentration and precision. In accordance with WADA Technical Document TD2022DL and Laboratory Guidelines for the quantification of endogenous steroids in blood for the ABP [34], combined uncertainty at the five concentration levels was also calculated, accounting for both random (precision) and systematic (accuracy) error by combining the two. Obtained results show values ranging from 6.6 to 13.9 %, which are all below the predefined threshold acceptance value of 15 % for all analytes at all concentration levels. The lower limit of quantitation (LLOQ) for each analyte was established as the lowest concentration at which a combined uncertainty of less than 20 % was observed, consistent with the lowest concentration found in the calibration/validation samples for all targeted steroid compounds. This resulted in satisfactory trueness and precision values for all targeted analytes. The chromatograms of all analytes at the LLOQ value are provided in [Supplementary Material Figure S1](#).

Upon further examination of the LLOQ values acquired for each targeted steroid, it is evident that the validated method displayed satisfactory levels of sensitivity for the majority of monitored analytes. However, an increased sensitivity would be required to efficiently measure DHT, T-G, T-S and EpiT-S in the majority of the female population, as well as to achieve P quantitation in male population. With this objective in mind, it may be envisaged to develop specific ultra-sensitive assays.

### 3.1.4. Carry-over

Negligible carry-over effects were observed for all target analytes by analyzing three extracted artificial steroid whole blood VAMS samples immediately after the most concentrated calibration sample (Level 5), with values below 0.1 % except for most concentrated analytes DHEA-S (0.6 %) and A-S (0.6 %) for which the most concentrated calibration sample was at 4 and 0.5 ug/mL, respectively.

### 3.1.5. Robustness

The robustness of the method was evaluated over the three days of quantitative validation protocol. Two different operators analyzed distinct batches each day, employing varied mobile phases and procedures for sample preparation solutions. Two distinct lots of analytical LC columns were employed and instrument maintenance (ESI source cleaning) was performed before each analytical batch. Despite these minor variations, the calibration lines (displayed in [Supplementary Material Table S6](#)) were deemed satisfactory with an  $R^2$  above 0.99. Finally, the analysis of uncertainty yielded values below 20 % for all target analyte compounds. Consequently, the developed method was judged to be robust within the linearity range for each compound.

### 3.2. Stability study

A stability study was carried out to explore the potential advantages that VAMS could offer in terms of sample transportation and storage. For this purpose, whole blood samples were collected from 20 healthy volunteers and VAMS aliquots were prepared by directly pipetting 30  $\mu$ L of whole blood on a single VAMS tip. A total of 17 VAMS aliquots were prepared for each individual, stored at different conditions for a predetermined number of days as described in [section 2.6.1](#). and analyzed according to the predetermined schedule in [Table 1](#). Once the entire schedule of analysis was completed, the stability of steroid hormones in VAMS samples was evaluated using two different strategies, examining each monitored analyte separately.

Steroids' concentration measured in baseline samples of the 20 recruited individuals are reported in [Table 3](#). It is worth noting that the

**Table 3**  
Summary of steroid concentrations measured in baseline aliquots from male and female investigated populations.

Compound	VAMS concentrations [ng/mL]	
	Mean (SD)/Median (IQR, Q1-Q3)	
	Males (n = 10)	Females (n = 10)
11-Deoxycortisol	0,24 (0,09)	0,27 (0,11)
17 $\alpha$ -Hydroxyprogesterone	0,35 (0,25–0,80)	0,32 (0,26–0,49)
Androstenedione	0,40 (0,27–0,63)	0,30 (0,26–0,35)
Corticosterone	1,76 (1,01)	0,79 (0,52)
Cortisol	83,03 (56,50)	39,59 (30,90)
Cortisone	8,58 (5,76)	4,91 (2,96)
DHT	0,23 (0,09)	not detectable
Progesterone	0,23 (0,18–0,31)*	1,47 (0,97–5,53)*
Testosterone	1,97 (1,387)	0,14 (0,09)*
Androsterone Glucuronide	6,85 (4,03–12,00)	4,21 (2,17–5,53)
Etiocolanolone Glucuronide	13,99 (11,72–15,83)	3,49 (2,84–6,40)
Testosterone Glucuronide	0,22 (0,15)	not detectable
Androsterone Sulphate	193,64 (85,86–220,79)	32,89 (19,63–59,16)
DHEA Sulphate	193,87 (124,41–397,52)	73,31 (65,60–125,49)
Epiandrosterone Sulphate	29,34 (15,11–49,31)	12,43 (10,90–16,03)
Epitestosterone Sulphate	0,17 (0,14–0,27)	not detectable
Etiocolanolone Sulphate	16,67 (8,74–21,69)	9,17 (6,82–14,74)
Testosterone Sulphate	0,44 (037–0,45)	not detectable

\* = n < 10.

developed and validated method was capable of measuring the majority of target analytes in all analyzed samples, with the exceptions of DHT, T-G, EpiT-S and T-S that were not detectable in female samples due to their low circulating levels. These results highlight the suitability of the presented method for steroid measurement also in clinical context, indeed it could be easily employed for the diagnosis of Congenital Adrenal Hyperplasia in which elevated levels of 17-OHP, A4 and T are observed [35], as well as for the monitoring of dexamethasone suppression test by measuring F circulating levels the morning after drug administration [36]. These are just a few examples of possible clinical application of steroid measurement by means of VAMS technology, however it is worth considering that a direct correlation of the obtained results with serum or plasma concentrations of target analytes reported in literature is not

**Table 4**  
Results of stability study obtained for Testosterone.

Storage condition	Day	Concentration [ng/mL] [mean (SD)]	p-value	PD [%]	Threshold [%]
RT	0	1,55 (1,44)	–	–	–
	1	1,56 (1,41)	0,840	1,11 %	19,40 %
	5	1,76 (1,61)	0,004*	5,85 %	19,40 %
	15	1,76 (1,62)	0,003*	10,31 %	19,40 %
	61	1,72 (1,58)	0,024*	5,14 %	19,40 %
4 °C	100	1,72 (1,52)	0,043*	9,71 %	19,40 %
	0	1,55 (1,44)	–	–	–
	5	1,65 (1,50)	0,032*	1,81 %	19,40 %
–20 °C	9	1,69 (1,54)	0,012*	5,36 %	19,40 %
	15	1,68 (1,57)	0,052	1,62 %	19,40 %
	0	1,55 (1,44)	–	–	–
–80 °C	15	1,76 (1,62)	0,005*	6,50 %	19,40 %
	30	1,76 (1,63)	0,016*	5,33 %	19,40 %
	60	1,72 (1,65)	0,042*	1,79 %	19,40 %
FT	0	1,55 (1,44)	–	–	–
	60	1,71 (1,52)	0,065	5,20 %	19,40 %
	100	1,61 (1,50)	0,414	–3,20 %	19,40 %
FT	0	1,55 (1,44)	–	–	–
	1	1,79 (1,65)	0,005*	7,84 %	19,40 %
	2	1,72 (1,56)	0,027*	2,23 %	19,40 %
	3	1,62 (1,48)	0,180	–7,88 %	19,40 %

\* Significant difference ( $\alpha = 0,05$ ).

**Table 5**  
Results of stability study obtained for Etiocolanolone Glucuronide.

Storage condition	Day	Concentration [ng/mL] [median (IQR)]	p-value	PD [%]	Threshold [%]
RT	0	9,45 (4,40–15,01)	–	–	–
	1	8,62 (4,40–14,83)	0,012*	–2,17 %	17,46 %
	5	9,13 (4,28–14,86)	0,099	–1,33 %	17,46 %
	15	8,70 (4,32–14,63)	0,181	–2,15 %	17,46 %
	61	8,99 (4,20–14,14)	0,043*	–1,22 %	17,46 %
	100	8,80 (4,09–14,49)	0,001*	–4,00 %	17,46 %
4 °C	0	9,45 (4,40–15,01)	–	–	–
	5	8,98 (4,26–14,58)	0,248	–1,56 %	17,46 %
	9	8,98 (4,22–14,93)	0,708	–0,98 %	17,46 %
–20 °C	15	9,16 (4,15–15,15)	1,000	–2,04 %	17,46 %
	0	9,45 (4,40–15,01)	–	–	–
	15	9,00 (3,93–15,09)	0,005*	–5,38 %	17,46 %
–80 °C	30	8,87 (4,32–14,71)	0,196	–5,56 %	17,46 %
	60	8,43 (4,31–15,00)	0,048*	–3,47 %	17,46 %
	0	9,45 (4,40–15,01)	–	–	–
FT	60	8,60 (4,31–14,57)	0,181	–3,95 %	17,46 %
	100	8,82 (4,22–14,28)	0,021*	–3,95 %	17,46 %
	0	9,45 (4,40–15,01)	–	–	–
FT	1	8,95 (3,92–15,13)	0,067	–2,20 %	17,46 %
	2	9,15 (3,86–14,80)	0,003*	–4,41 %	17,46 %
	3	9,08 (4,06–14,36)	0,002*	–3,93 %	17,46 %

\* Significant difference ( $\alpha = 0,05$ ).

**Table 6**  
Results of stability study obtained for Androsterone Sulphate.

Storage condition	Day	Concentration [ng/mL] [median (IQR)]	p-value	PD [%]	Threshold [%]
RT	0	70,53 (28,77–197,48)	–	–	–
	1	71,67 (30,28–201,68)	0,495	2–23 %	18,59 %
	5	70,26 (29,68–197,72)	0,865	0,22 %	18,59 %
	15	71,77 (29,75–195,95)	0,799	0,80 %	18,59 %
	61	72,88 (30,12–181,22)	0,734	–0,29 %	18,59 %
	100	73,67 (26,90–202,41)	0,246	–0,12 %	18,59 %
4 °C	0	70,53 (28,77–197,48)	–	–	–
	5	67,32 (26,50–180,13)	0,099	–3,74 %	18,59 %
	9	70,28 (25,68–196,30)	0,325	–3,45 %	18,59 %
	15	72,22 (28,59–196,71)	0,932	–0,71 %	18,59 %
–20 °C	0	70,53 (28,77–197,48)	–	–	–
	15	69,85 (27,38–203,57)	0,734	–0,22 %	18,59 %
	30	70,23 (28,06–196,39)	0,899	–0,22 %	18,59 %
	60	75,27 (27,22–187,01)	0,899	–1,45 %	18,59 %
–80 °C	0	70,53 (28,77–197,48)	–	–	–
	60	70,51 (26,94–199,35)	0,034*	–3,32 %	18,59 %
	100	73,55 (26,65–181,60)	0,246	–2,15 %	18,59 %
FT	0	70,53 (28,77–197,48)	–	–	–
	1	69,15 (28,87–204,87)	0,702	–1,22 %	18,59 %
	2	70,50 (27,92–199,59)	0,734	–0,55 %	18,59 %
	3	71,64 (28,62–200,32)	0,442	1,18 %	18,59 %

\* Significant difference ( $\alpha = 0,05$ ).

possible at the moment. In order to perform this task, since the majority of steroid hormones are bound to plasmatic protein, concentrations values measured in dried whole blood microsamples should be corrected for the hematocrit. Nevertheless, the great advantage of VAMS related to the ease of sample collection could be used to overcome such drawback by obtaining whole blood reference values of clinically relevant steroid hormones and metabolites, hence considering also variability due to hematocrit and being specific for capillary blood.

Based on the outcomes of normality tests, the distributions of measured concentrations of each analyte in a specific storage condition were described by mean and standard deviation or with median and interquartile ranges in case of normal or non-normal distributions, respectively. Comparison of these distributions, observed for each storage condition, with those obtained from the analysis of baseline samples was then performed to assess significant differences using paired sample *t*-test or Wilcoxon signed-ranked. The results obtained for T, Etio-G and A-S are presented in Tables 4,5 and 6, respectively, while the data collected for all other target analytes are shown in Supplementary Material Tables S7 to S21.

The data reported in the abovementioned tables reveal statistically significant differences ( $p$ -value < 0.05) when comparing T concentration distributions among the various aliquots for almost all storage conditions. Conversely, the opposite situation was observed for A-S,

with only the samples analyzed after 60 days storage at  $-80$  °C showing significant deviation from values measured in baseline samples. A third scenario was finally obtained for Etio-G, for which significant differences were mainly observed at the final time points of each storage condition, suggesting a possible slight degradation of the analyte collected in dried blood microsamples.

The stability of target analytes in stored VAMS samples was then further examined through a second approach. The latter was based on the calculation of the PD between steroids' concentration measured in baseline aliquots and in those stored under the various investigated conditions. In addition, for each steroid hormone the RCV was calculated using the equation described in Section 2.6.2 with the aim of determining a threshold that could reliably estimate the maximum PD that may be attributed to analytical variability rather than degradation. For each analyte and individual, PD obtained from concentration values of baseline samples and the ones obtained by analyzing all stored samples were calculated. Then, the mean PD calculated for each condition was plotted in vertical bar diagrams, highlighting also the calculated RCV as positive and negative stability threshold. The graphics obtained for T, Etio-G and A-S are presented in Fig. 3, while the outcomes obtained for all other monitored steroid hormones can be found in Supplementary Material Figures S2 to S16. Combining the data presented in Tables 4–6 and in Fig. 3, it is worth noting that the obtained PD between VAMS samples stored in different conditions and baseline samples never exceeded the analyte-specific RCV limit approximating the maximum acceptable analytical variability. Such outcome was not only observed for the three analytes discussed herein, but also for all other analytes included in the developed analytical method and it revealed that, although it was possible to detect statistically significant differences in concentration levels measured in baseline and VAMS samples stored at room temperature, 4 °C,  $-20$  °C and  $-80$  °C, such differences (when expressed as PD) always fall within the analytical variability range of the validated LC-MS/MS method.

To further corroborate the outcomes achieved by monitoring mean PD, individual graphs, representing measured steroid concentrations against days of storage, were generated for each volunteer enrolled in the stability study. In Fig. 4, the longitudinal graphics of T, Etio-G and A-S concentrations obtained for one individual enrolled in the study are presented. In the graphs, each storage condition is represented by a different series and it is possible to notice for all the three analytes that the concentrations measured in VAMS samples analyzed and stored at room temperature, 4 °C,  $-20$  °C, and  $-80$  °C for up to 100 days as well as aliquots subjected to up to three freeze and thaw cycles never exceeded the individual thresholds calculated by applying the RCV percentage threshold to the concentration measured in the baseline aliquot. This additional data treatment demonstrates that the results obtained from distribution-based calculations represent a bias-free approximation of degradation processes occurring in each volunteer's stored aliquots. Furthermore, such longitudinal monitoring highlighted once again the satisfactory stability of endogenous steroid hormones as well as androgen phase II metabolites in VAMS dried blood microsamples stored for up to 100 days.

#### 4. Conclusions

VAMS emerges as a cutting-edge technology for the collection of dried samples from various biological fluids, such as urine, blood and saliva. In particular, this innovative technology allows the collection of a fixed small blood volume (10,20 or 30  $\mu$ L) from a single blood drop, ensuring minimal invasiveness. Subsequent extraction and analysis of biomolecules of interest are then performed using diverse analytical platforms.

In this study, a novel LC-MS/MS method was developed and validated to measure a wide panel of 18 steroidal compounds, including main endogenous steroid hormones as well as most concentrated androgen phase II metabolites. The validation was conducted in



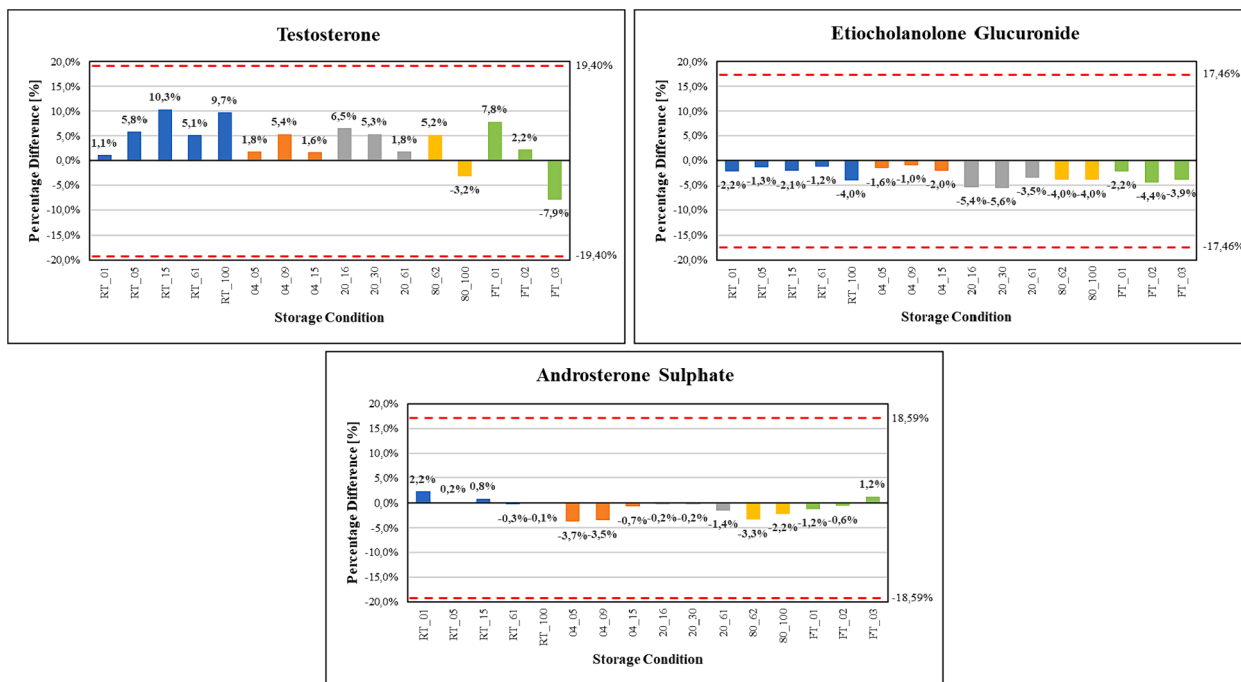


Fig. 3. Graphics of mean percentage difference in function of investigated storage conditions for Testosterone, Etiocholanolone Glucuronide and Androsterone Sulphate.

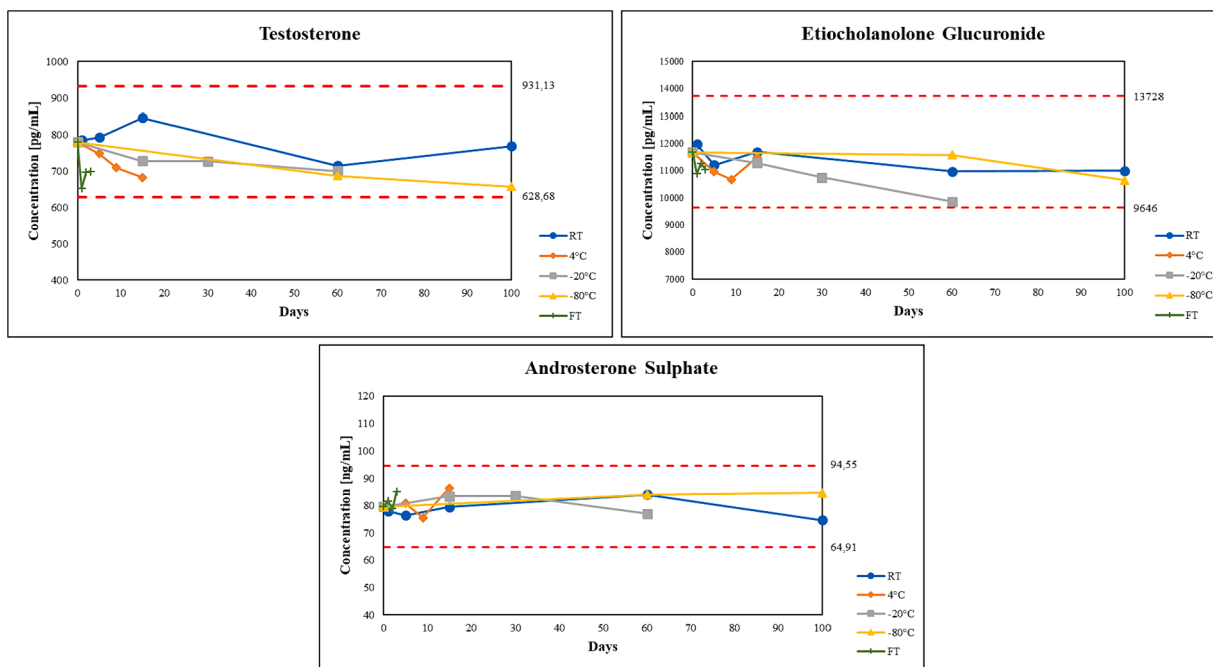


Fig. 4. Graphics of measured concentrations of Testosterone, Etiocholanolone Glucuronide and Androsterone Sulphate for a male individual in function of storage conditions.

accordance with ISO/IEC 17025:2017 standards and WADA requirements for quantitative analytical methods. This newly validated method represents a first example of an extended steroid profile measured in dried blood microsamples and collected with VAMS devices. While initially envisioned for the anti-doping field, the method's satisfactory quantitative performance during the three-day validation protocol, suggests potential applications in clinical research in the near future. In the context of doping control analysis, the validated method offers a valuable analytical platform for measuring all blood markers of

EAAS doping reported to date, including T and A4, currently included in the blood steroid profile (BSP), with satisfactory precision and accuracy. With the final aim of testing the suitability of VAMS for steroid hormones doping control analyses, the developed method was used to assess the stability of selected analytes in whole blood VAMS microsamples. Such aspect, never investigated in literature to date, it is of crucial importance because the long-term stability of steroids in different storage conditions, could represent a decisive advantage of VAMS sampling compared to serum/plasma classical sampling. It is

worth noting that in the stability study conducted on real samples in this research, whole blood was spiked on VAMS support by manually pipetting previously collected venous blood, rather than allowing capillary blood to absorb on the polymer tip. Therefore, before recommending the implementation of the developed analytical solution into the routine of WADA-accredited laboratories and ABP protocols, further studies are essential and should be performed focusing on two different aspects. Firstly, the correlation between the circulating levels of endogenous steroid hormones and their relative metabolites in venous and capillary blood should be assessed. Secondly, efforts focused on sample preparation would be a valuable asset in minimizing intra- and inter-laboratory variability, a crucial point for the potential future introduction of VAMS analysis for ABP purposes.

Nevertheless, the promising outcomes from the conducted stability study suggest that VAMS sampling and analysis could offer a substantial advantage in terms of sample collection and transportation. In fact, all monitored steroids did not show variations of measured blood concentrations accounting for more than analytical variability assessed during validation protocol. It is worth noting that steroid measurements remained unaffected by four different storage conditions (RT, 4 °C, -20 °C and -80 °C) over a period of up to 100 days, and the impact of three consecutive freeze-thaw cycles was found to be negligible. These reported findings provide valuable support for the advancement of BSP that was recently introduced by WADA, potentially facilitating the collection of a greater number of blood samples with a particular focus on research studies aiming to enhance understanding of possible confounding factors influencing circulating levels not only of T and A4, but also of all other blood markers indicative of EAAS doping.

Undoubtedly the minimized invasiveness of VAMS technology, along with the improved simplicity of sample collection and transportation due to the demonstrated stability of steroidal compounds in this study, makes this methodology fit not only for doping control purposes but also for clinical practice. Indeed, most of target analytes included in the validated LC-MS/MS method are currently monitored as diagnostic markers of an elevated number of endocrinological and metabolic pathologies [37–39]. The reliability of the presented method and the stability exhibited by steroid hormones collected on VAMS support could therefore serve as an important starting point for future clinical research endeavors. The latter would in particular benefit from the possibility of increasing the number of samples collected, supporting the recruitment of larger study cohorts (essential for investigating pathophysiological conditions of chronic non-communicable diseases which have a large epidemiological impact) as well as the addition of several collection time-points in studies aiming to monitor circadian variations of both novel and established biomarkers.

#### CRediT authorship contribution statement

**Federico Ponzetto:** Validation, Methodology, Funding acquisition, Conceptualization. **Mirko Parasiliti-Caprino:** Writing – review & editing, Resources. **Laura Leoni:** Writing – original draft, Validation. **Lorenzo Marinelli:** Writing – review & editing, Data curation. **Antonello Nonnato:** Writing – review & editing, Resources, Methodology. **Raul Nicoli:** Writing – review & editing, Methodology. **Tiia Kuuranne:** Writing – review & editing, Supervision. **Ezio Ghigo:** Writing – review & editing, Supervision, Project administration. **Giulio Mengozzi:** Writing – review & editing, Supervision, Resources. **Fabio Settanni:** Writing – review & editing, Supervision, 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

No data was used for the research described in the article.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cca.2024.117890>.

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