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Enhanced urinary stability of peptide hormones and growth factors by dried urine microsampling

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ABSTRACT

Volumetric absorptive microsampling (VAMS) and dried urine spot (DUS) strategies were applied for the collection of dried microsamples for anti-doping testing of low-stability peptide hormones and growth factors prohibited by the World Anti-Doping Agency (WADA). Drying, storage and transport conditions, as well as pretreatment steps, were optimised before liquid chromatography – tandem mass spectrometry (LC–MS/MS) analysis. The analytical method has been fully validated in terms of sensitivity (limits of quantitation 0.3–10 ng/mL), precision (RSD% < 6.6%) and extraction yields (78–91%). Dried microsample stability studies (90 days) have been performed and compared to fluid urine stability. Significantly higher losses have been observed in fluid urine stored at –20 °C (up to 55%) and –80 °C (up to 29%) than in dried urine microsamples stored at room temperature (< 19%). The final microsampling and analysis protocols allow the collection of urine microvolumes, unlikely to be tampered, stably storable and shippable with no particular precautions for possible anti-doping testing of prohibited peptides and hormones.

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1. Introduction

Doping in sports currently represents a tangible, protean problem, which can put athletes' health at serious risk, threatening their moral integrity and discrediting sports reputation in general. The constant update and the introduction of new regulations in doping testing activities, as well as the evolution of analytical techniques, have succeeded only in part in discouraging the use of prohibited substances and methods. Some challenges are still open within sampling and analysis procedures, including the inherent limitations of the elective biological fluid in doping control, i.e., urine. For example, analysis replication, transportation procedures at controlled temperature, data reliability are all critical points; in particular, the need to store relatively high volumes of biological fluid, at controlled and certified temperatures, is probably one of the most expensive and problematic aspects of the chain of cus-

tody. This is particularly significant in relation to collected sample stability during transportation and mid- and long-term storage of "B samples" for re-analysis after an adverse analytical finding (AAF) [1].

Doping control athlete urine samples should be shipped refrigerated to World Anti-Doping Agency (WADA) accredited laboratories, but this is not always the case. Commensal urethral microbiota, urinary pathogens and environmental bacterial species may contaminate urine samples, as collection procedures are not carried out under sterile conditions. Therefore, ongoing enzymatic activity by microbial contamination or other sources can cause modifications to the excreted compounds, particularly in the case of doping-relevant peptides such as hormones and growth factors.

For example, the fast nicking of human chorionic gonadotropin (hCG) hormone and its subsequent dissociation into free subunits have been extensively studied and demonstrated in urine samples, even when stored at controlled temperature [2]; enzymatic activity in urine can cause changes in the analytical behaviour of erythropoietin (EPO) [3]; as for luteinizing hormone (LH) analysis, time and storage conditions can rapidly cause urinary LH degra-

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dation [4]; moreover, the synthetic human adrenocorticotrophic hormone (ACTH) analogue tetracosactide was found to be unstable in solution when stored at room temperature or refrigerated [5]; also regarding human growth hormone (GH), whose urinary detection poses a challenge for analytical scientists, lack of stability was demonstrated both for GH itself [6], and for GH-responsive biomarkers (e.g. IGF-1) [7].

These data are extremely important for sample collection, transport and preparation procedures. Degradation during the sample preparation timeframe and preservation of the prepared solutions could represent critical sources of error and potentially result in a high occurrence of false negative or irreproducible results.

The availability of increasingly advanced instrumentations and the progressive automation of laboratory processes [8] are not sufficient to ensure reliable analytical results, as they need to be supported by specific, suitable pre-analytical phases, in order to avoid undermining result validity. Sample collection, transfer and storage are indeed crucial steps for the success of anti-doping controls.

This study involved two sampling techniques featuring miniaturisation and matrix drying, namely dried urine spot (DUS) sampling and volumetric absorptive microsampling (VAMS) to assess the stability in these micromatrices of peptide hormones included in the WADA Prohibited List [9]. The collection and processing of dried matrix spots (DMS), such as DUS, are promising tools for significantly improving current doping control strategies [10,11], since this technique addresses issues related to:

- compounds instability in aqueous samples that could lead to false negative results;
- limited detectability time frame that could lead to delayed re-analysis (B sample) issues;
- timely detection of pharmacologically relevant concentrations of substances that are not always prohibited;
- the need for rapid, reliable, easily automatable and high-throughput protocols.

On the other hand, VAMS approach is based on the absorption of a liquid sample onto a porous polymeric tip connected to a handler, by wicking; the volume of sample absorbed is accurately calibrated and related to the properties and size of the tip substrate itself. VAMS offers a simpler collection workflow than other currently available approaches, since the device can be directly applied to the sample surface and does not require the collected material to be transferred into containers or further processed [12]. Moreover, microsample inhomogeneity is kept to a minimum or uninfluential, differently from what happens for DUS. VAMS devices are designed to be similar in shape and dimension to pipette tips, allowing easy integration into automated sample processing procedures in the analytical laboratory.

For both DUS and VAMS, absorption on the card or device becomes an integral part of sample pretreatment, since many interfering matrix components are strongly retained by the absorbent, generally avoiding the need for other forms of purification after extraction [13]. Moreover, sample replicates can be easily collected at the same time and stored for re-analysis, with minimal space requirements [14].

For this project, DUS and VAMS techniques have been implemented for the collection of microvolumes of urine, in order to:

- enhance the stability of labile compounds;
- simplify sample collection, without the need for highly specialised personnel and dedicated facilities;
- allow logistics savings due to the small volume to be transported (e.g., by air shipments and through customs);

- enable the simplification and automation of all pre-analytical steps;
- minimise sample contamination and tampering risks, as well as operator biohazards, thanks to minimal sample handling;
- decrease overall analysis costs due to the possibility to store and transport samples without the need for special precautions (e.g., freezing and dry ice).

Of course, even if all of these goals are met, several steps are still missing before microsampling, and DUS and VAMS in particular, can be used for actual anti-doping workflows. The main drawback is probably related to sensitivity, which is generally lower for microsamples due to the impossibility of concentrating the analytes during sample preparation. Related to this is the need to use multiple microsamples for a complete analysis of all multiple classes of prohibited substances, where a single macroscopic urine sample would be sufficient.

The thorough development of the sampling and sample preparation procedures has been followed by their implementation into reliable workflows for high-throughput LC-MS/MS analysis.

The above described original microsampling strategies have been studied for the proof-of-concept stability assessment of several doping-relevant peptide hormones in dried urine microsamples stored under different conditions. The peptide compounds studied were: GHRP-1, GHRP-2, GHRP-6, hexarelin, alexamorelin, triptorelin, AOD9604, CJC-1293, desmopressin, TB-500, hCG and ACTH (tetracosactide) (Table 1). All of them are included in Section “S2. Peptide hormones, growth factors, related substances, and mimetics” of WADA's Prohibited List aside from desmopressin, included in Section “S5. Diuretics and masking agents” [9]. Stability has been investigated also regarding classic fluid urine samples stored under temperature-controlled conditions in order to carry out thorough comparisons with dried microsamples. Regarding stability studies of small peptides in dried microsamples, a fully automated DBS sample preparation as a multi-analyte initial testing approach for 46 lower molecular mass peptide and non-peptide compounds was developed as proof-of-concept demonstrating good stability in blood microsamples [15].

Different approaches than microsampling have of course been devised and successfully applied in the recent past for peptide analysis and for the discrimination of endogenous presence from exogenous administration. For example, for erythropoietins, sarcosyl-polyacrylamide gel electrophoresis (SAR-PAGE) followed by western blotting to differentiate erythropoietins based on their molecular weights is currently considered best practice [16]. As a consequence, erythropoietins were not included in the analyte panel for this study: recent advances seem to have largely solved most analytical problems related to their testing.

Moreover, recent studies have suggested that single-peptide detection of, e.g., hCG is not sufficient for the reliable monitoring of exogenous hCG administration, and that intact hCG, nicked hCG, free β -subunit and β -subunit core fragment should all be determined, and their ratios studied [17,18]. However, since the present study is a proof-of-concept regarding peptide stability, only some representative peptides were considered, to verify the possible stability increase obtained by dried microsampling using DUS and VAMS. So, inclusion of all fragments for all possible analytes was not deemed necessary and is left to future development and application studies. Moreover, a 2021 technical document from WADA [19] specifies that “A non-confirmed [hCG] PAAF [Presumptive Adverse Analytical Finding] may also be associated with doping but results from intact hCG degradation during sample storage”. This highlights that urine sample storage is still a critical issue, recent advances notwithstanding, and that stability enhancement studies can bring significant benefits to anti-doping testing.

Table 1
Peptide compounds considered in the study.

Compound	Peptide sequence ^a	MW (g/mol)	Category	WADA list
GHRP-1	H-Ala-His-(D-βNal)-Ala-Trp-(D-Phe)-Lys-NH ₂	955.13	GHRP	S2 - 2.3
GHRP-2	H-(D-Ala)-(D-βNal)-Ala-Trp-(D-Phe)-Lys-NH ₂	817.97	GHRP	S2 - 2.3
GHRP-6	H-His-(D-Trp)-Ala-Trp-(D-Phe)-Lys-NH ₂	873.03	GHRP	S2 - 2.3
Hexarelin	H-His-Mrp-Ala-Trp-(D-Phe)-Lys-NH ₂	887.04	GHRP	S2 - 2.3
Alexamorelin	H-Ala-His-Mrp-Ala-Trp-(D-Phe)-Lys-NH ₂	958.14	GHRP	S2 - 2.3
Triptorelin	Pyr-His-Trp-Ser-Tyr-(D-Trp)-Leu-Arg-Pro-Gly-NH ₂	1311.10	GnRH analogue	S2 - 2.1
AOD9604	H-Tyr-Leu-Arg-Ile-Val-Gln-Cys-Arg-Ser-Val-Glu-Gly-Ser-Cys-Gly-Phe-OH	1815.10	Growth hormone fragment	S2 - 2.3
CJC-1293	H-Tyr-(D-Ala)-Asp-Ala-Ile-Phe-Thr-Gln-Ser-Tyr-Arg-Lys-Val-Leu-Ala-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Leu-Ser-Arg-(MPA-Lys)-NH ₂	3355.8	GnRH analogue	S2 - 2.3
Desmopressin	Mpr-Tyr-Phe-Gln-Asn-Cys-Pro-(D-Arg)-Gly-NH ₂	1069.22	Vasopressin analogue	S5
TB-500	Ac-Leu-Lys-Lys-Thr-Glu-Thr-Gln-OH	890.00	Thymosin β4 fragment	S2 - 2.3
hCG	Val-Leu-Gln-Gly-Val-Leu-Pro-Ala-Leu-Pro-Gln-Val-Val-Cys-Asn-Tyr-Arg	1869.92	Human chorionic gonadotrophin	S2 - 2.1
ACTH (tetracosactide)	H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-OH	2933.57	Corticotropin	S2 - 2.2

^a Mrp: 2-methyl-D-Trp; Pyr: Pyroglutamyl; Mpr: 3-Mercaptopropionyl; MPA: Maleimidopropionyl.

The study proposed herein is the first one taking into consideration the urinary matrix (as matrix of choice in anti-doping practices) in the form of dried spots for a stability study of doping-relevant peptides and the first study ever considering the stability of this type of peptides in urine sampled using VAMS technology.

2. Materials and methods

2.1. Chemicals and standard solutions

MS-grade solvents, reagents and Whatman 903 protein saver cards were purchased from Sigma-Aldrich (St. Louis, USA). A Whatman (Maidstone, MA, USA) Harris Uni-Core Punch, 7 mm was used for punching the dried urine spot discs out of the spotting cards. Mitra® VAMS™ microsamplers (10 µL) were purchased from Neoteryx (Torrance, CA, USA). Ultrapure water was obtained by means of a MilliQ apparatus by Millipore (Milford, MA, USA). GHRP-1, GHRP-2, GHRP-6, hexarelin, alexamorelin, triptorelin, AOD9604, CJC-1293, desmopressin, TB-500, hCG and ACTH (tetracosactide) in powder form (> 95 % purity) were custom-synthesised by D.B.A. Italia S.r.l. (Milano, Italy). hCG, ACTH (tetracosactide), hexarelin and desmopressin pure powders were also purchased from Sigma-Aldrich. All custom-synthesised peptides were subjected to identity and purity confirmation carried out by high-resolution mass spectrometry (HRMS) analysis.

2.2. Instrumental platform

LC-MS/MS analysis was performed on a Waters Alliance e2695 chromatographic system with autosampler coupled to a Waters Micromass Quattro Micro triple-quadrupole mass spectrometer equipped with an electrospray ion source (ESI). Data processing was performed using Waters MassLynx 4.1 software.

The chromatographic separation was carried out on a Waters X-Select Peptide C18 (2.1 mm × 150 mm, 2.5 µm) reversed-phase column, maintained at RT and equipped with an X-Select Peptide C18 guard column. The mobile phase was a mixture of 0.1 % formic acid (FA) in acetonitrile (solvent A) and 0.1 % FA in water (solvent B) flowing at a 0.1 mL/min rate in gradient mode. The gradient was programmed as follows:

- 0.0–1.0 min, isocratic elution at 10 % of solvent A;
- 1.0–5.0 min, linear gradient from 10 % to 75 % of solvent A;
- 5.0–10.0 min, isocratic elution at 75 % of solvent A;
- 10.0–14.0 min, linear gradient from 75 % to 10 % of solvent A;

- 14.0–15.0 min, column equilibration with isocratic elution at 10 % of solvent A.

Tandem mass spectrometry acquisition was carried out in multiple reaction monitoring (MRM) mode, using an electrospray ionisation source operating in positive mode (ESI+). The optimised parameters were as follows: ion source voltage 3.60 kV, ion source temperature 125 °C, desolvation gas temperature 150 °C; desolvation gas flow rate 350 L/h. Nitrogen was used as the desolvation gas and was generated from pressurised air by an N2 LC-MS (Claind, Lenno, Italy) nitrogen generator; collision gas was 99.995 % argon (SIAD, Bergamo, Italy). The precursor ion and the product ions, with cone voltage and collision energy, were optimised by analyte methanolic solution infusion at 10 µL/min and are displayed in Table 2, together with the optimised quantitative and qualitative *m/z* transitions. The dwell times per channel were set at 300 ms for each analyte. Subsets of samples throughout the whole study were analysed for confirmation by means of a HRMS platform exploiting an Orbitrap mass spectrometer (ThermoFisher, San Jose, USA) operated in positive ion mode, also coupled to liquid chromatography. Full-scan spectra were acquired in the range of 40–3500 *m/z* for non-targeted peptide analysis to detect all analyte *m/z* signals. The used ion source parameters used were as follows: ESI capillary voltage 1500 V, cone voltage 22 V, drying gas: 2 L/min, nebulizer gas: 80 psi and temperature: 40 °C. A Thermo Fisher Ultimate 3000 UHPLC apparatus was employed before mass spectrometric analysis. A chromatographic PFP column (50 × 2.1 mm, 1.9 µm) was used for separation. The mobile phase components were: 0.1 % FA in acetonitrile (solvent A) and 0.1 % FA in water (solvent B) flowing at 0.1 mL/min rate. A binary gradient was used: 10 % of A was maintained for 0.6 min, in 3 min A% was raised to 75 and kept for 3 min; 10 % of A was reached in 2 min and the column was re-equilibrated at the starting conditions for 2 min. The chromatographic flow rate was 0.1 mL/min. The injection volume was 10 µL.

2.3. Samples

Urine samples, used as blank matrices, were represented by surrogate urine (Synthetic Urine e.k., Eberdingen-Nußdorf, Germany) and freely accessible anonymised pooled urine from non-traceable donors. Urine aliquots of 1 mL (for fluid urine pretreatment) and 200 µL (for microsample collection) were spiked with 10 µL of analyte standard mixtures at known concentrations and subjected to different pretreatment procedures according to the desired matrix: fluid urine, DUS, VAMS.

Table 2
Mass spectrometry parameters.

Compound	MW	MS/MS			HRMS			
		Parent ion	Main charge state	Daughter ions ^a	Cone Voltage (V)	Collision Energy (eV)	Main charge state	Exact mass
GHRP-1	955.13	478.57	[M+2 H] ²⁺	110.7 129.7	21.00 21.00	47.00 21.00	[M+2 H] ²⁺	478.5660
GHRP-2	817.97	818.94	[M+H] ⁺	170.6 269.5	43.00 43.00	71.00 43.00	[M+H] ⁺	818.9794
GHRP-6	873.03	874.09	[M+H] ⁺	324.5 856.1	47.00 47.00	45.00 29.00	[M+2 H] ²⁺	437.5155
Hexarelin	887.04	888.01	[M+H] ⁺	248.5 110.7	49.00 49.00	45.00 75.00	[M+2 H] ²⁺	444.2565
Alexamorelin	958.14	959.19	[M+H] ⁺	209.6 110.7	53.00 53.00	55.00 75.00	[M+2 H] ²⁺	479.7546
Triptorelin	1311.10	1312.15	[M+H] ⁺	159.7 249.6	93.00 93.00	75.00 75.00	[M+2 H] ²⁺	656.5541
AOD9604	1817.12	908.58	[M+2 H] ²⁺	136.7 854.3	55.00 55.00	51.00 21.00	[M+H] ⁺	1817.1204
CJC-1293	3355.80	672.18	[M+5 H] ⁵⁺	84.8 136.6	33.00 33.00	69.00 39.00	[M+5 H] ⁵⁺	672.1675
Desmopressin	1069.22	1070.26	[M+H] ⁺	70.6 120.7	73.00 73.00	75.00 71.00	[M+2 H] ²⁺	535.2210
TB-500	888.49	889.56	[M+H] ⁺	84.6 129.6	45.00 45.00	73.00 47.00	[M+2 H] ²⁺	445.4748
hCG	1869.92	963.84	[M+2 H] ²⁺	610.5 518.3	33.0 33.0	13.00 11.00	[M+2 H] ²⁺	963.8465
ACTH (tetracosactide)	2933.57	587.52	[M+5 H] ⁵⁺	671.5 223.0	33.00 33.00	21.00 21.00	[M+5 H] ⁵⁺	587.5244

^a In italics, confirmatory product ions.

2.3.1. Fluid urine

For fluid urine pretreatment, the procedure was based on SPE and adapted from a previously published paper [20]. Briefly, a polymeric reversed-phase, weak ion exchange mixed-mode SPE cartridge (Waters Oasis WCX30 mg, 1 mL) was conditioned with 1 mL of methanol and 1 mL of ultrapure water before loading 1-mL urine aliquot sample. The cartridge was then washed with 1 mL of ultrapure water and 1 mL of a 5 % methanol solution in ultrapure water. Analytes were then eluted with 1 mL of methanol containing 5 % FA. The eluate was evaporated under a gentle nitrogen stream, the residue was re-dissolved in 50 µL of a 50:50 (V/V) mixture of acetonitrile and water containing 0.1 % FA, and a 10 µL aliquot was injected into the analytical system. For hCG analysis, the dried extract was reconstituted in 50 µL of 50 mM NH₄HCO₃ solution and trypsin was added to reach a final concentration of 50 µg/mL. The mixture was then incubated at room temperature for 18 h before injection in the analytical system.

2.3.2. Dried urine spots (DUS)

Aliquots of 10 µL of matrix-analyte solution mixture were transferred onto a Whatman 903 protein saver card by means of micropipetting, the obtained spots were dried for 1 h at room temperature and protected from direct light sources, then stored at room temperature (RT) in the dark with suitable desiccant until analysis. To extract the analytes, a whole DUS was punched out from the card, extracted with 250 µL of pure methanol by combination of vortex-assisted extraction (VAE) and ultrasound-assisted extraction (UAE): 10 min UAE + 30 s VAE +10 min UAE. The solution was brought to dryness under a gentle nitrogen stream, re-dissolved with 50 µL of a 50:50 (V/V) mixture of acetonitrile and water containing 0.1 % FA and a 10 µL aliquot was injected into the analytical system. For hCG analysis, the dried extract was treated as reported in Section 2.3.1. before injection in the analytical system.

2.3.3. Volumetric absorptive microsampling (VAMS)

10 µL VAMS microsampler was put in contact with the surface of the sample mixture for 5 s, dried at room temperature (RT) for 1 h and protected from direct light sources, then stored at RT in the

dark with suitable desiccant until analysis. To extract the analytes, the micro-sampler tip was detached from the handle and subjected to 10 min UAE + 30 s VAE +10 min UAE in 100 µL of MeOH. The resulting solution was brought to dryness under a nitrogen stream, re-dissolved with 25 µL of a 50:50 (V/V) mixture of acetonitrile and water containing 0.1 % FA and a 10-µL aliquot was analysed.

2.4. Method validation

According to current official guidelines [21,22] the following validation parameters were tested: linearity, absolute recovery, precision, matrix effect and stability.

2.4.1. Linearity range, LOQ and LOD

Calibration samples were prepared by adding different known concentrations of the analytes to blank matrices. The resulting samples were subjected to sample preparation and analysis carried out in triplicate for each concentration. The obtained analyte peak areas were plotted against the corresponding nominal concentrations and the calibration curves set up by the least-squares method, using a $1/x^2$ weighing factor. Verification of the quality of fit to the calibration curves was evaluated by comparing back-calculated concentrations to the nominal ones. The limit of quantitation (LOQ) is defined as the lowest concentration of analyte in a sample which can be reliably quantified with an acceptable accuracy and precision, while the limit of detection (LOD) is the lowest concentration that can be detected. LOQ and LOD values for each analyte were determined by analysing 7 samples at different concentrations and assessed as the concentrations which gave rise to peaks whose heights were 10 and 3 times the baseline noise, respectively. In addition, for each analyte the Limit of Identification (LOI) was determined, defined by WADA as the lowest concentration assessed at no more than 5 % false negative rate.

2.4.2. Absolute recovery, precision and accuracy

Absolute recovery, precision and accuracy were evaluated by adding known amounts of the analytes (at four different concentrations, corresponding to the LOQ, the LOQ × 3, a middle point and a

high value of each calibration curve) to blank samples, then subjecting them to sample pretreatment and analysis. The analyte absolute peak areas were compared to those of injected standard solutions at the same nominal concentrations and the absolute recovery was calculated. The assays described above were repeated six times within the same day to obtain intraday precision and six times over six different days to evaluate interday precision, in both cases expressed as percentage relative standard deviation (RSD%). Accuracy was defined as the deviation from the nominal value expressed in percentage.

2.4.3. Matrix effect

Matrix effect of dried microsamples was evaluated on blank sample extracts, fortified post-extraction by adding known amounts of the analytes. The mean analyte peak area of extracts was then compared with the peak area from analyte solutions prepared in mobile phase at the same theoretical concentrations and the resulting percentage ratios were calculated.

2.4.4. Stability

The stability of the analytes in the different matrices was assessed as follows. For fluid urine, blank samples were spiked at a 1 µg/mL level, divided into separate aliquots, then stored in amber glass vials at −20 °C and −80 °C for 20 days. At regular intervals, a different sample vial was thawed, subjected to pretreatment and analysed by LC–MS/MS. Subsets of time point samples were analysed for confirmation by means of a HRMS platform exploiting an LTQ XL Orbitrap mass analyser. For dried matrices (DUS and VAMS), blank samples were spiked at a 1 µg/mL level, then stored in air-tight bags containing desiccant, protected from direct light sources at RT for 20 days. At regular intervals, a different sample was subjected to pretreatment and analysed. The measured analyte concentrations were compared to those of the same samples extracted and analysed immediately after preparation. Sub-optimal, short-term stability assessments were also carried out. Fluid urine, DUS and VAMS were sampled (or frozen in the case of fluid urine) after leaving the spiked fluid urine sample at RT for 3 h. Then, the samples were stored under optimal conditions (low humidity, protected from light and heat) and stability was assessed at regular intervals up to 25 h.

2.4.5. Additional validation parameters

Selectivity was evaluated using blank urine obtained from six healthy volunteers in order to demonstrate that no significant interference was observed at the retention times of the analytes object of this study in blank urine microsamples. Dilution integrity was evaluated in five replicates by pretreating and analysing VAMS and DUS samples spiked with analyte concentrations above the upper limit of quantification of each calibration curves. The obtained extracts were subjected to 10-fold dilution with the extract obtained from blank samples and precision and accuracy were evaluated.

Carryover was assessed in five replicates by injecting the extract obtained from the pretreatment of blank samples immediately after the analysis of VAMS and DUS samples fortified with analyte concentration corresponding to the upper limit of each calibration curve. Chromatograms were then checked for possible interfering signals.

3. Results and discussion

3.1. Optimisation of LC–MS/MS conditions

HRMS preliminarily confirmed the identity of all custom-synthesised peptides. Optimised mass spectrometry conditions

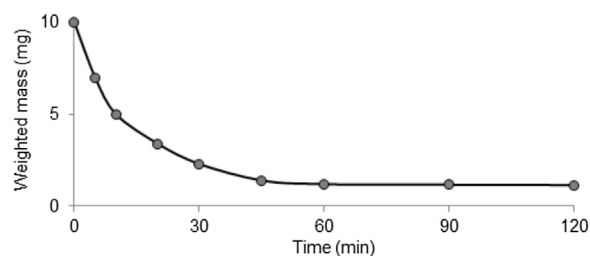


Fig. 1. DUS sample drying time assay results (n = 6).

were obtained by syringe infusion of analyte solutions directly into the ESI source.

A suitable chromatographic setup was investigated through the one-variable-at-a-time (OVAT) approach, to obtain good resolution and short retention times (while avoiding co-elution with residual matrix components); the main specific optimisation steps are specified in the Supplementary Materials (Table S1). At the end, good chromatographic separation was obtained by using a reverse-phase (RP) column specifically designed for intact mass peptide analysis (X-Select Peptide C18). An optimised composition gradient of MS-grade water and acetonitrile containing FA for optimal compound ionisation via electrospray ionisation (ESI) was applied, starting at 10 % organic modifier and going up to 75 % over 4 min and flowing at a constant rate of 0.1 mL/min that led to good peak shape and resolution within a chromatographic run of 15 min, including column re-equilibration. The MRM LC–MS/MS chromatogram obtained from the analysis of a blank VAMS sample spiked with the selected peptides is reported in Fig. S1.

3.2. Development of sample pretreatment

Different procedures were tested in order to compare the performance and suitability of the three urine-based matrices (fluid urine, DUS and VAMS). For fluid urine, liquid-liquid extraction (LLE) and solid phase extraction (SPE) were tested, using different solvents (for LLE) and different kinds of cartridges and procedures (for SPE). The best results, in terms of speed-cost-purification balance were obtained using SPE. A polymeric mixed-mode RP and weak ion exchange SPE cartridge (Waters Oasis WCX) provided satisfactory purification and extraction yield performances.

As regards DUS, drying time was the first tested parameter: at RT, complete drying (i.e., constant weight) was achieved in under 1 h (Fig. 1).

For a satisfactory analyte extraction from DUS, solvent type and volume were tested, and the following results were obtained: acetonitrile, acidified methanol and acetonitrile, and organic solvent/water mixtures were unsuitable, since acetonitrile provided low extraction yields, while acidified solvents and mixtures caused a relatively high matrix effect. Pure methanol, on the other hand, proved to be a very efficient extractor, and also provided satisfactory purification. Different extraction volumes in the 50–350 µL range were tested and extraction yields did not increase when volumes exceeded 250 µL, thus this volume was used for all subsequent assays. As regards the extraction mode, ultrasound-assisted extraction (UAE), vortex-assisted extraction (VAE) and a sequential combination of both modes were tested: while UAE gave better results than VAE, the combination of UAE and VAE improved extraction performances when compared to UAE alone (Fig. 2).

Regarding VAMS, the first optimisation step was the evaluation of the sampling volume, its variability and its overall suitability for urine sampling, since initially VAMS was developed specifically for blood sampling. Gravimetric experiments had already been carried out to establish the volume absorbed after 10-µL VAMS tip exposure to biological matrices other than blood, showing how the

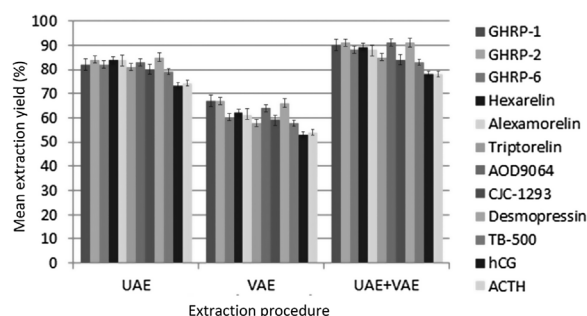


Fig. 2. DUS extraction procedure assay results (n = 6).

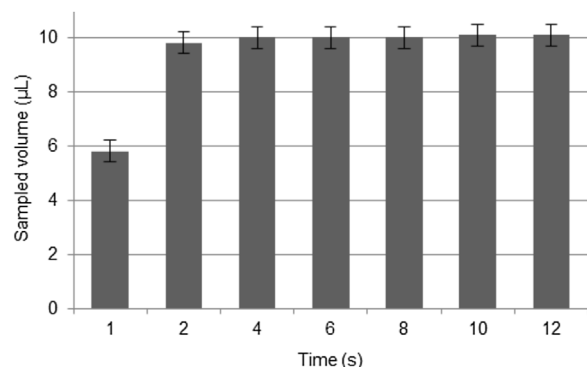


Fig. 3. VAMS sampling time assay results (n = 6).

collected volumes ($9.68 \pm 0.84 \mu\text{L}$) were statistically indistinguishable from those pipetted ($10.12 \pm 0.26 \mu\text{L}$), thus demonstrating good VAMS volume accuracy also for urine [13]. Sampling time was tested in the 1–12 s range. It was verified that no significant differences exist within this time range, suggesting the VAMS tip reaches saturation within a very short time, without further changes in the next few seconds. Thus, a 5 s sampling time was set for VAMS (Fig. 3).

Table 3
Linearity parameters.

Compound	Linearity range (ng/mL) ^a	r^2	LOD (ng/mL)	LOQ (ng/mL)
GHRP-1	1–5000	0.9990 ^b 0.9992 ^c	0.3	1
GHRP-2	1–5000	0.9992 ^b 0.9996 ^c	0.3	1
GHRP-6	1–5000	0.9989 ^b 0.9994 ^c	0.3	1
Hexarelin	1–5000	0.9990 ^b 0.9994 ^c	0.3	1
Alexamorelin	1–5000	0.9991 ^b 0.9992 ^c	0.3	1
Triptorelin	1–5000	0.9989 ^b 0.9992 ^c	0.3	1
AOD9604	1–5000	0.9985 ^b 0.9990 ^c	0.3	1
CJC-1293	0.5–5000	0.9990 ^b 0.9990 ^c	0.1	0.3
Desmopressin	1–5000	0.9993 ^b 0.9997 ^c	0.3	1
TB-500	1–5000	0.9989 ^b 0.9995 ^c	0.3	1
hCG	10–5000	0.9982 ^b 0.9985 ^c	3	10
ACTH	10–5000	0.9982 ^b 0.9983 ^c	3	10

^a n = 6.

^b DUS.

^c VAMS.

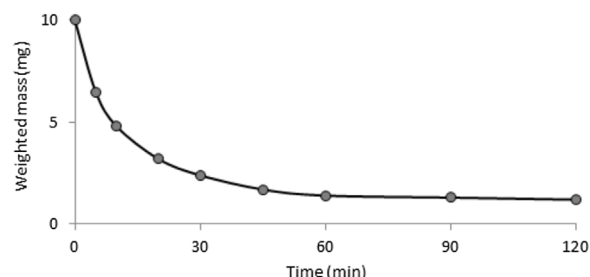


Fig. 4. VAMS sample drying time assay results (n = 6).

VAMS drying time can also be a significant source of time consumption; tests verified that constant weight (i.e., complete water evaporation) is reached in less than 1 h; the latter was chosen as the standard drying time (Fig. 4).

Then, extraction solvent and volume were also optimised; 100 μL of methanol gave the best results, although pure acetonitrile produced almost identical extraction yields (but a slightly higher matrix effect), while acidified solvents and water/organic solvent mixtures provided lower extraction yields and/or unacceptable matrix effect. As regards extraction means, similarly to DUS optimisation, a combination of UAE and VAE led to the best extraction performances.

3.3. Method validation

3.3.1. Linearity on spiked matrices

Standard solutions of the analytes at different concentrations were added to each blank matrix and the mixtures were subjected to the respective pretreatment procedure. Good linearity ($r^2 \geq 0.9981$) was obtained for all analytes over wide concentration ranges, as reported in Table 3. In addition to LOD and LOQ values reported in Table 3, for each analyte LOI value was determined, defined by WADA as the lowest concentration assessed at no more than 5 % false negative rate. The assessed LOI values were 0.5 ng/mL for GHRP-1, GHRP-2, GHRP-6, hexarelin, alexamorelin, triptorelin, AOD9604, desmopressin and TB-500; 0.2

Table 4

Precision, extraction yield and matrix effect.

Compound	Concentration Level	Interday precision (RSD%) ^a		Intraday precision (RSD%) ^a		Extraction yield (%) ^b		Matrix effect (%) ^b	
		DUS	VAMS	DUS	VAMS	DUS	VAMS	DUS	VAMS
GHRP-1	LOQ	4.9	4.7	4.5	4.4	83	84	5.9	5.7
	LOQ × 3	4.9	4.7	4.4	4.4	83	85	5.5	4.8
	ULOQ/2	4.7	4.6	4.2	4.2	85	85	4.9	4.6
	ULOQ	4.5	4.3	4.1	4.0	89	88	3.2	3.1
GHRP-2	LOQ	4.8	4.8	4.5	4.5	86	85	5.8	5.6
	LOQ × 3	4.5	4.5	4.4	4.5	86	90	5.0	3.3
	ULOQ/2	4.4	4.3	4.2	4.0	87	88	5.2	5.2
	ULOQ	4.0	4.0	3.7	3.7	89	90	4.7	4.8
GHRP-6	LOQ	5.0	4.8	4.7	4.5	84	86	6.5	6.3
	LOQ × 3	4.8	4.7	4.7	4.3	84	86	6.3	6.1
	ULOQ/2	4.7	4.5	4.3	4.2	88	88	6.0	6.1
	ULOQ	4.6	4.2	4.3	4.1	90	90	4.9	4.8
Hexarelin	LOQ	4.6	4.5	4.2	4.2	87	86	6.0	5.7
	LOQ × 3	4.4	4.3	4.0	4.0	89	89	5.5	5.2
	ULOQ/2	4.3	4.2	3.9	4.0	88	87	5.1	5.0
	ULOQ	4.0	4.0	3.8	3.6	91	89	4.2	4.0
Alexamorelin	LOQ	5.8	5.5	5.3	5.1	83	85	7.0	6.7
	LOQ × 3	5.6	5.3	5.1	5.1	81	88	6.7	6.6
	ULOQ/2	5.3	5.2	4.9	4.9	85	86	6.7	6.5
	ULOQ	5.0	4.8	4.7	4.6	88	87	5.9	5.8
Triptorelin	LOQ	4.8	4.6	4.3	4.4	85	87	6.2	6.2
	LOQ × 3	4.6	4.5	4.2	4.0	89	90	5.7	5.0
	ULOQ/2	4.3	4.2	4.0	3.9	89	90	4.9	4.7
	ULOQ	4.0	4.0	3.7	3.7	90	90	4.7	4.6
AOD9604	LOQ	6.2	6.0	6.1	5.9	78	79	7.4	7.3
	LOQ × 3	5.9	5.7	5.5	5.3	80	84	6.9	6.7
	ULOQ/2	5.6	5.2	5.2	5.1	82	82	6.6	6.5
	ULOQ	5.0	4.7	4.8	4.6	84	85	6.3	6.2
CJC-1293	LOQ	6.0	5.7	5.7	5.5	80	80	7.1	7.0
	LOQ × 3	5.6	5.2	5.6	5.2	80	81	7.0	6.8
	ULOQ/2	5.5	5.2	5.2	5.2	81	82	6.8	6.7
	ULOQ	5.0	5.0	4.9	4.8	81	84	6.3	6.2
Desmopressin	LOQ	4.8	4.6	4.4	4.3	87	88	6.9	6.8
	LOQ × 3	4.7	4.4	4.4	3.9	89	90	6.5	6.4
	ULOQ/2	4.5	4.3	4.0	3.9	91	91	6.0	6.0
	ULOQ	4.4	4.2	3.9	3.8	91	92	5.5	5.4
TB-500	LOQ	5.8	5.6	5.5	5.4	83	85	6.8	6.6
	LOQ × 3	5.7	5.6	5.5	5.4	84	88	6.6	6.2
	ULOQ/2	5.5	5.5	5.0	5.0	85	86	6.4	6.1
	ULOQ	5.1	5.0	5.0	4.7	88	88	6.1	6.2
hCG	LOQ	6.0	5.9	5.8	5.5	79	80	7.4	7.2
	LOQ × 3	5.9	5.7	5.7	5.3	82	84	7.2	7.2
	ULOQ/2	5.7	5.5	5.5	5.2	79	80	7.0	7.0
	ULOQ	5.2	5.2	5.0	4.9	83	82	6.1	6.5
ACTH	LOQ	6.5	6.3	6.2	6.0	78	79	8.0	7.6
	LOQ × 3	6.0	5.8	6.0	5.7	81	84	7.7	7.4
	ULOQ/2	5.8	5.5	5.7	5.6	80	82	7.5	7.3
	ULOQ	5.3	5.0	5.1	4.9	82	83	7.2	7.0

^a n = 6.

ng/mL for CJC-1293; 5 ng/mL for hCG and ACTH (tetracosactide). Commonly, hCG is assayed by immunological methods and its concentration expressed as IU/volume. For consistency, hCG concentrations were expressed in this work as mass/volume by taking into account the generally accepted conversion factor of 9.3 IU/μg hCG [23], although different conversion factors are reported by different sources. Based on this, the concentration range of hCG was 0.093–46.5 IU/mL, with a LOD value of 0.028 IU/mL and a LOQ value of 0.093 IU/mL.

3.3.2. Extraction yield, precision, accuracy and matrix effect

Extraction yield and precision were tested on blank matrices spiked with four different analyte concentrations; results are reported in Table 4. The resulting data were satisfactory, being always higher than 77 % for all analytes. The methodology granted also good precision for all matrices, with RSD values always lower than 6.6 %. Possible ion suppression or matrix effects were investigated at three concentration levels by comparing mean analyte peak area of each extract fortified post-extraction with analyte peak

area from standard solutions. The matrix effect results obtained were good, being always in the 3.1–8.0 % range (Table 4). Intra-day accuracy ranged from 4.2–8.9 %, whereas inter-day accuracy ranged from 4.0–8.7 % (Table S2).

3.3.3. Additional validation parameters

No interference was observed in the retention regions of the target analytes by observing the chromatograms of both blank VAMS and DUS microsamples obtained from six different sources, thus indicating high method selectivity.

Carryover assays, carried out by analysing the extract obtained from blank microsampling immediately after the highest calibration standards did not show any interfering signals influencing quantification. Dilution integrity was also determined, and it was found that a 10-fold dilution of samples spiked with concentrations higher than the upper calibration limits did not affect accuracy and precision (always within ±10 % for all the analytes)

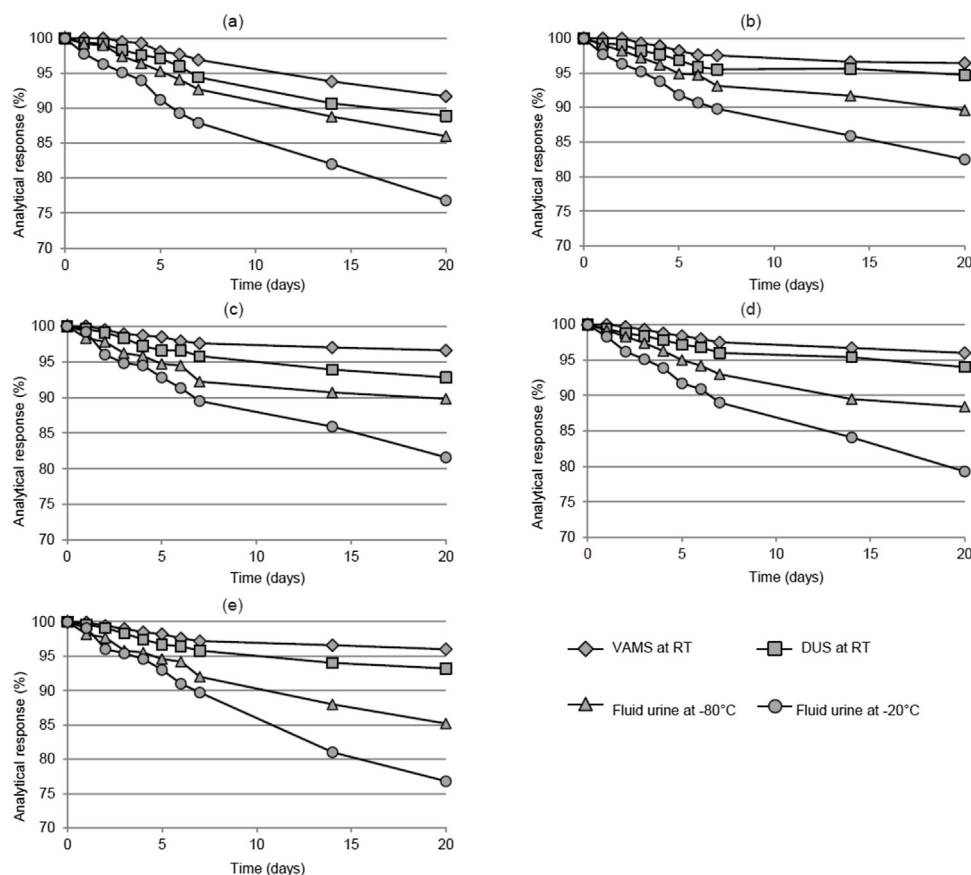


Fig. 5. Comparative mid-term stability assay results for (a) GHRP-1; (b) AOD9604; (c) CJC-1293; (d) alexamorelin; (e) ACTH.

3.3.4. Analyte stability and application to real samples

The stability of the analytes in the different matrices was assessed in parallel on urine, DUS and VAMS blank samples spiked at known concentrations.

The most significant results obtained from the comparative stability study by LC-MS/MS analysis and confirmed by HRMS analysis are shown in Fig. 5a-e for five representative peptides. Results for all peptides at relevant time points, together with the time (days) required to reach 80 % of the original concentration are shown in Table 5.

As can be seen, dried matrices always provided the highest stability, with results higher than 88.8 % after 20 days, even though they were stored at room temperature. Stability differences between DUS and VAMS were small and not always statistically significant. However, mean stability was higher for VAMS than for DUS at every time point. Fluid urine, on the contrary, provided significantly lower stability than dried matrices at all time points from 14 days on, although obviously samples stored at -80°C fared better than those stored at -20°C . The difference in mean recovery between dried and fluid matrices reached almost 20 % for GHRP-1. The data related to the other peptides object of this study did not significantly differ from those reported in the graphs.

It should be noted that this is the first report of a study regarding peptide stability in any dried urine microsamples.

The results obtained on fluid urine samples closely approximate those already available in scientific papers for similar storage [20,24,25], and pre-analytical conditions [26]. Desmopressin has been found to be “stable for more than 10 days at 4°C and more than 3 months at -20°C ” [27], and for GHRP and metabolites “at RT, 4°C and -20°C there was no degradation up to 1 day, 2 weeks and 2 months, respectively” [28]. No report is currently available

in the literature on the stability of CJC-1293. Moreover, additional stability assays were performed by using selected analytes stored for additional short-term (25 h) stability assessments, simulating sub-optimal operating conditions.

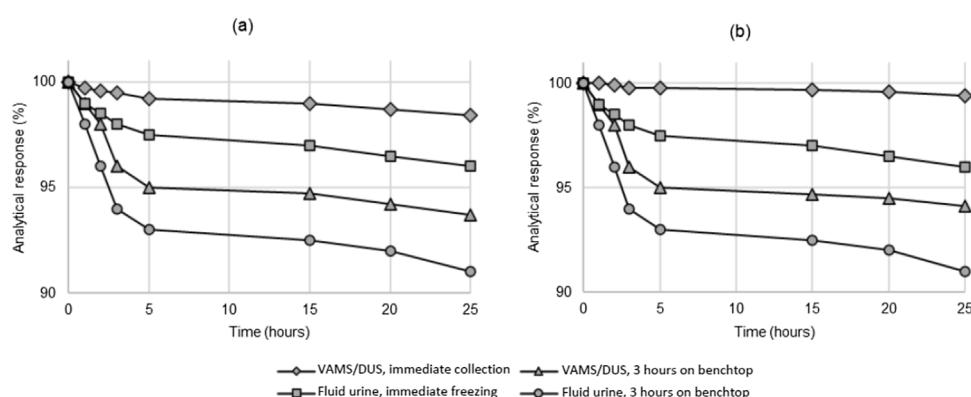
First, fluid urine, DUS and VAMS were sampled (or frozen in the case of fluid urine) after leaving the spiked fluid urine sample at RT for 3 h. These assays were aimed at simulating possible delays prior to microsampling/storage. An example of the results obtained from these assays on GHRP-1 used as test compound is shown in Fig. 6a-b (for DUS and VAMS, respectively). Similar trends were observed for the other target compounds (data not shown).

As could be expected, this sub-optimal scenario had a significant negative impact on analyte stability. However, after the urine sample was left at RT for 3 h, the subsequent data points of the short-term stability assay replicated the same trend as the optimal condition assays. This result highlighted that it is necessary to microsample and dry the matrix as early as possible, once the biological fluid has been collected. Dried matrix stability is confirmed to be higher than that of fluid urine, even in sub-optimal conditions.

As further evidence of the reliability of the proposed methodology, the developed urine microsampling and analysis approach was applied to a small set of real urine samples with expected high hCG concentrations from 3 volunteers. Both DUS and VAMS sample replicates were collected and dried as described in Sections 2.3.2. and 2.3.3., respectively. Samples were then subjected to pre-treatment and LC-MS/MS analysis immediately after sampling and drying and analyses were carried out in triplicate. The analysis of these urine microsamples from pregnant women allowed to identify and quantify the analyte in all of them, in the 0.43–2.99 $\mu\text{g/mL}$ (4.1–27.8 IU/mL) range and with a standard deviation always lower than 12 ng/mL (0.11 IU/mL) ($n = 3$). To assess and verify the stability

Table 5
Comparative stability.

Analyte	Intact analyte (%) ^a at the specified time point (days)										Time required to reach 80 % of the original concentration (days)					
	5					14						20				
	Fluid urine DUS (RT) (–20 °C)		VAMS (RT) Fluid urine DUS (RT) (–20 °C)		VAMS (RT) Fluid urine DUS (RT) (–20 °C)		VAMS (RT) Fluid urine DUS (RT) (–20 °C)		VAMS (RT) Fluid urine DUS (RT) (–20 °C)			VAMS (RT)				
GHRP-1	91.2	97.1	98.1	82.0	90.7	93.8	76.6	88.9	91.7	17	34	46				
GHRP-2	93.5	97.8	95.4	89.6	92.0	94.2	81.8	90.7	93.3	22	40	56				
GHRP-6	94.9	99.5	99.2	89.1	93.3	95.6	83.8	91.5	93.6	24	44	57				
Hexarelin	92.9	98.6	99.0	82.6	94.2	96.9	78.4	92.3	94.2	18	50	58				
Alexamorelin	91.7	97.2	98.4	84.1	95.4	96.7	79.3	94.0	95.0	19	55	60				
Triptorelin	90.8	97.6	98.5	82.4	92.5	94.2	77.0	89.2	93.0	17	37	51				
AOD9604	91.8	96.8	98.2	85.9	95.6	96.6	82.5	94.7	96.0	23	58	63				
CJC-1293	92.8	96.6	97.9	85.9	93.9	97.0	81.6	92.8	96.2	22	52	64				
Desmopressin	91.3	99.0	99.2	86.3	92.4	93.0	78.0	90.2	90.9	18	37	45				
TB-500	90.9	99.3	99.5	83.1	91.6	95.0	77.5	90.2	92.5	18	38	50				
hCG	89.6	96.4	97.1	81.8	90.8	92.6	76.7	89.1	90.5	17	36	41				
ACTH	93.0	96.7	98.2	81.0	94.1	96.6	76.8	93.2	96.0	17	52	63				

^a n = 6.**Fig. 6.** Results of delayed-storage short-term stability assays on GHRP-1 for (a) DUS and (b) VAMS.

of hCG in dried microsamples, an additional set of replicates was stored at RT in the dark with suitable desiccant and analysed after 20 days from sample collection. The analyses were carried out in triplicate. Sample stability, expressed as % of intact remaining compound, was always higher than 90.7 % for VAMS and higher than 89.5 % for DUS. These results from real samples showed high overlap with those obtained from spiked ones and represent a promising proof-of-concept regarding the application potential of the proposed microsampling strategies in terms of enhanced stability. One should note assay sensitivity is still far from the required levels regarding DUS and VAMS application to anti-doping testing of hCG. Aim of the present study was in fact the evaluation of possible doping-relevant peptide stability enhancement in dried microsamples, and further assays and refinements are still needed to actually apply dried microsampling to hCG anti-doping testing.

4. Conclusion

Innovative microsampling and pretreatment procedures based on dried matrices (DUS and urine VAMS) were optimised for the application to urine specimens for anti-doping purposes. Relevant experimental parameters were studied and specifically optimised.

An original LC–MS/MS method was developed for the simultaneous analysis of the peptides of interest in dried urine microsamples. After suitable study of the experimental conditions, the final methods provided good, solid performance within relatively short run times (15 min).

The LC–MS/MS method was fully validated according to current guidelines, with good results for all assays and all analytes.

The microsampling, pretreatment and analysis workflow was successfully applied to the study of peptide stability in dried matrices.

Short-term stability assays were carried out, both on dried and fluid urine-based matrices, with interesting results. In fact, analytes spiked in DUS and urine VAMS were remarkably stable, even though they were kept at RT, for the full duration of the stability study (20 days). The stability of the chosen peptides, which are known to be quite prone to degradation under common storage conditions, was greatly enhanced in comparison to fluid urine stored at freezing (–20 °C) and ultra-freezing (–80 °C) temperatures. The stability study revealed that the two dried matrices, DUS and VAMS, always provided very similar results, with all studied peptides recovered in the 89–98 % range at the end of the time period. This confirms that matrix drying effectively stops most forms of peptide degradation, and that these collection means could be suitable for anti-doping analysis purposes. What is more important, dried matrix stability performance regularly outclassed the corresponding results of fluid urine, be it stored at –20 °C or at –80 °C. Analyte loss in fluid urine at –80 °C was regularly 5–10 % larger at study end than the loss in dried matrices, with widely worse losses (up to 20 % more) for fluid urine kept at –20 °C.

Thus, dried urine micromatrices are very attractive and advantageous alternatives to fluid urine for the sampling, storage and testing of low-stability peptides during in- and out-of-competition controls. They are a mature platform, that is almost ready for routine application by selected laboratories, granting tangible advantages over traditional fluid sampling: enhanced stability for degradation-prone peptides; reduced storage requirements and

expenses; easier and cheaper transportation and shipping; equally reliable analytical performances; similar sensitivity and reproducibility.

It must be acknowledged that the dried microsampling approach has its drawbacks, first of all the decrease in sensitivity when compared to macroscopic sampling. However, if this is at least partially compensated by an increase in analyte stability, some steps are being made toward the technique maturity for anti-doping applications. It should also be noted that multiple (ostensibly tens of) DUS, or VAMS tips, would be needed to carry out complete initial and confirmatory anti-doping testing. This can be less than practical from a sample handling point of view. More work is still needed in this respect, for example testing the possibility of direct MSⁿ analysis of the solid dried matrices, or some other kind of non-destructive analysis that can be repeated multiple times on the same spot.

In order to advance studies on dried micromatrices for anti-doping peptide testing, several new activities are planned, including extended stability studies for periods up to 10 months, as well as more extensive stability studies under non-optimal conditions (high humidity, light exposure, high temperatures, and so on). Moreover, a natural expansion and continuation of this promising microsampling-based study will take into consideration all relevant metabolites identified to date for the selected compounds. This will allow to further expand the doping-relevant peptide detection window and to identify any differences in the stability profiles of the metabolites when compared to the respective parent compounds. Application to other biomatrices and other problematic peptides and proof-of-concept application to simulated anti-doping workflows are also in the planning phase. As a final consideration, WADA provides through its Technical Documents precise and sometimes rather complex analysis protocols in order to differentiate doping from natural occurrences (for example for hCG and hCG fragments). Considering that the objective of this specific work was centred around the concept of analyte stability in dried microsamples, the developed and validated instrumental analytical strategy envisaged straightforward protocols in order to carry out an in-depth, yet feasible stability study. In fact, the continuation of this research involves the inclusion of diagnostic peptide metabolites (e.g. D-Ala-D-(β-naphthyl)-Ala-Ala-OH, the main GHRP-2 metabolite in urine) and the integration of specific advanced instrumental protocols defined by WADA in order to obtain a miniaturised bioanalytical platform applicable as a whole to anti-doping activities.

Author statement

Michele Protti: Formal analysis, Validation, Writing of the Original Draft, Review & Editing, Funding acquisition. **Paolo M. Sberna:** Formal analysis, Validation, Writing of the Original Draft, Review & Editing. **Angelo E. Sberna:** Conceptualization, Data Curation, Review & Editing. **Renzo Ferrante:** Conceptualization, Data Curation, Review & Editing. **Roberto Mandrioli:** Resources, Supervision, Review & Editing, Funding acquisition. **Laura Mercolini:** Conceptualization, Resources, Supervision, Review & Editing, Funding acquisition, Project administration.

Declaration of Competing Interest

All authors declare they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi: <https://doi.org/10.1016/j.jpba.2021.114234>.

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