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RESEARCH ARTICLE

Analysis of doping control samples using supercritical fluid chromatography-tandem mass spectrometry: Ready for routine use

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Supercritical fluid chromatography is proving to be a good separation and sample preparation tool for various analytical applications and, as such, has gained the attention of the anti-doping community. Here, the applicability of supercritical fluid chromatography hyphenated to tandem mass spectrometry for routine doping control analysis was tested. A multi-analyte method was developed to cover 197 drugs and metabolites that are prohibited in sport. More than 1000 samples were analyzed by applying a "dilute and inject" approach after hydrolysis of glucuronide metabolites. Additionally, a comparison with routinely used liquid chromatography-mass spectrometry was performed with 250 of the 1000 samples and a number of past positive anti-doping samples. It revealed some features where supercritical fluid chromatography-tandem mass spectrometry was found to be complementary or advantageous to liquid chromatography-mass spectrometry for anti-doping purposes, such as better retention of analytes that are poorly retained in reversed-phase liquid chromatography. Our results suggest that supercritical fluid chromatography-tandem mass spectrometry is sensitive (limit of detection <50% relevant minimum required performance level required by the World Anti-Doping Agency for anti-doping analysis), reproducible, robust, precise (analytes of interest area coefficient of variation <5%; retention time difference coefficient of variation <1%) and complementary to

Article Related Abbreviations: MeOH, methanol; MRM, multiple reaction monitoring; MRPL, minimum required performance levels; NH_4OAc, ammonium acetate; QC, quality control; t_R CV, coefficient of variation of retention time; THC-COOH, 11-nor-9-carboxy-Δ9-tetrahydrocannabinol; WADA, World Anti-Doping Agency.

existing techniques currently used for routine analysis in the World Anti-Doping Agency accredited laboratories.

KEYWORDS

bioanalysis, drugs, doping agents, method performance, reliability

1 | INTRODUCTION

Chromatography, both gas and liquid, coupled with MS are the two main anti-doping analytical techniques employed in World Anti-Doping Agency (WADA) accredited laboratories for testing for the presence of prohibited substances in biological matrices such as urine [1–4]. Compounds to be tested by these laboratories are reviewed annually by WADA and published in the "List of Prohibited Substances and Methods" [5]. The list contains over 200 named compounds, which are grouped into 10 different categories (S0–S9), according to their pharmacological actions. The analytical techniques used by the accredited laboratories must meet the detection and identification criteria outlined in various WADA Technical Documents such as TD2022MRPL ("Minimum Required Performance Levels and Applicable Minimum Reporting Levels for Non-Threshold Substances Analysed by Chromatographic - Mass Spectrometric Analytical Methods") and TD2021IDCR (Minimum Criteria for Chromatographic-Mass Spectrometric Confirmation of the Identity of Analytes for Doping Control Purposes) [6, 7].

Although SFC was invented in the 1960s, it was not until a few years ago that it gained more attention among analytical chemists [8] when advances in the mechanics of the instruments, particularly better control of the back pressure, made routine use more reliable. Initially, SFC resembled normal phase chromatography and employed silica as the stationary phase and only supercritical CO_2 as the mobile phase [9, 10]. Supercritical CO_2 is a non-polar solvent and is kept under pressure at or over its so-called critical point. Alcohols (methanol [MeOH], ethanol, or isopropanol) may be added to the supercritical CO_2 to modify its polarity which, together with the appropriate stationary phase, allows the separation of various types of an analyte. SFC can be coupled to different detectors such as UV-visible or flame ionization detectors, although, especially for bioanalytical applications, coupling to MS seems to be the most desirable choice due to its sensitivity, selectivity, mass accuracy, and the possibility to provide structural and quantitative analysis [11]. Successful SFC-MS hyphenation, i.e. with suitable interfacing, requires the maintenance of supercritical CO_2 compressibility, which is successfully achieved by the back-pressure regulator. The design of SFC-MS interfaces has been improved over the years and, with the advancement of column technology, namely the availability of sub-2 μm particle size ultra-high-performance columns, the SFC-MS technology proved to be a good tool for both separation and sample preparation [12–14]. There are examples of employing SFC-MS for the analysis of pesticides, plant materials, pharmaceuticals, or metabolomic studies [15–21].

Since 2013, SFC-MS has shown the potential for application in the anti-doping field. SFC is especially valued for enantioseparations [22]. With respect to doping control analysis, Parr et al. performed the enantiomeric separation of clenbuterol in urine to distinguish deliberate misuse from accidental consumption through ingestion of contaminated meat [23, 24]. Subsequently, SFC-MS has gained more attention among researchers in the anti-doping community. SFC-MS was proposed as a method for the screening of various classes of compound from the WADA Prohibited List. Some practical considerations were discussed as well as their advantages over more conventional LC and GC-based methods [25–28]. Applications of SFC-MS/MS for doping control analysis including method details are summarized in a recent review [29].

Here we describe the SFC-MS analysis of more than 1000 samples (anti-doping samples plus additional quality control [QC] samples and standard solutions) over an extended period of time (10 weeks) and discuss the performance with respect to the technique being "fit for purpose" for routine use by anti-doping laboratories particularly for the initial testing procedures but also for dealing with samples giving relatively poor retention in RP-LC. Furthermore, we provide the performance comparison of SFC-MS/MS with the widely applied LC-MS/MS-based approach.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

All analytes were purchased as reference materials from various suppliers or provided by other anti-doping laboratories. The internal standard d_3-ephedrine (as hydrochloride 1 mg/ml ampoule) was bought from Sigma-Aldrich (Gillingham, UK), d_3-salbutamol (as hydrochloride, 4-[2-(tert-butylamino)-1-[$^2H_1$]-

1-hydroxyethyl]-2-[$^2H_2$]-hydroxymethylphenol) and d_3-testosterone glucuronide (16,16,17-[$^2H_3$]-testosterone glucuronide) were purchased from the National Measurement Institute (Canberra, Australia), and mefruside was purchased from Bayer (Leverkusen, Germany). β-Glucuronidase K 12 from *Escherichia coli (E. coli)* and ammonium acetate (NH_4OAc) were obtained from Sigma-Aldrich (Gillingham, UK), ammonium formate and formic acid from VWR (Darmstadt, Germany). Carbon dioxide (CO_2, 4.8) was purchased from Air Liquide (Berlin, Germany). Solvents, additives, and modifiers were of LC-MS grade and purchased from VWR (Darmstadt, Germany). Fresh ultrapure water was obtained from a LaboStar 2-DI/UV system (SG Wasseraufbereitung und Regeneration GmbH, Barsbüttel, Germany) equipped with LC-Pak Polisher and a 0.22-μm membrane point-of-use cartridge (Millipak; Th Geyer, Berlin, Germany).

2.1.1 | Reference solutions

All internal standard compounds were dissolved in MeOH volumetrically making the final concentration of 10 μg/ml. Stock solutions of the reference substances of the analytes were prepared in MeOH at concentrations of 1 mg/ml and diluted with MeOH to yield working standard solutions.

2.1.2 | QC samples

QC 1 and QC 2 were prepared by spiking urine with compounds and concentrations as presented in Table 1. At the time of doing this research, WADA TD2019MRPL [30] was in force and served as a guide for QC preparation. There is currently no minimum required performance levels (MRPL) for steroid sulfates and the concentration was chosen considering instrument sensitivity. QCs as well as non-spiked blank urine samples were run with every batch.

2.1.3 | Urine samples

Aliquots of regular doping control urine samples (n = 1000) were utilized. Ethical approval for the use of these samples for this study was obtained from the King's College London Research Ethics Committee (LRS-17/18-7119). Only samples collected from athletes who had provided research consent on the doping control form, completed when the sample was collected, were used. Once the samples were collected from the athletes, they were analyzed in the Drug Control Centre for the presence of prohibited substances. The samples were stored frozen at -20°C for at least 3 months after the anti-doping analytical report had been issued and were then anonymized before use in this study. Additionally authenticated positive control urines (n = 26) and negative controls (n = 5) were provided by the Italian anti-doping laboratory in Rome. Out of these samples, 250 including the positive controls were also analyzed by LC-MS/MS, and results of SFC- and LC-MS/MS were compared.

2.2 | Sample preparation

The method used for sample preparation was adapted from Parr et al. [31]. In brief, aliquots of 200 μl of urine were spiked with the 10 μl of the internal standards solution, and 25 μl of *E. coli* β-glucuronidase in an Eppendorf tube. Hydrolysis was performed at 50°C for 1 h in an Eppendorf Thermomixer (Wesseling-Berzdorf, Germany) with gentle mixing. After cooling to ambient temperature tetrahydrofuran (765 μl) was added and the samples were centrifuged at 13.9 g force for 8 min using an Eppendorf MiniSpin centrifuge (Wesseling-Berzdorf). The supernatant was transferred into glass vials and stored in the autosampler or in the freezer (T = –18°C) until analysis. Out of this solution, 5 μl were injected into the SFC-MS/MS. For LC-MS/MS analysis the samples were diluted analogously, however using H_2O instead of tetrahydrofuran for dilution.

2.3 | Instrumentation

2.3.1 | SFC-tandem mass spectrometry

The analyses were performed on an Agilent 1260 Infinity II SFC system (Agilent Technologies GmbH, Waldbronn, Germany) coupled to an Agilent Ultivo triple quadrupole mass spectrometer in splitless mode through a specially designed SFC-MS interface. The interface design allows the user to change between split and splitless modes by switching the position of two capillaries. Here we use splitless mode where the effluent from the SFC is completely transferred into the MS. Data acquisition and evaluation were performed by Agilent MassHunter software package version B.10.00. Autosampler temperature was kept at 4°C throughout the procedure.

Chromatographic separation was accomplished using a Waters ACQUITY UPC^2 BEH 2-EP column (100 x 3.0 mm, 1.7 μm particle size, Waters GmbH, Eschborn, Germany) using supercritical CO_2 with a mixture of NH_4OAc (5 mM) in MeOH:H_2O (96.5:3.5, v:v) as a modifier (eluent B). The back pressure regulator for supercritical CO_2 was set to 150 bar and 60°C in order to minimize retention time fluctuations along the gradient as recommended by the manufacturer. A flow rate of 1.4 ml/min was used

TABLE 1 Quality control (QC) 1 and QC 2 composition

QC 1	Concentration (ng/ml)	QC 2	Concentration (ng/ml)
Amiloride	100	1,3-DMB	100
Amphetamine	100	2-Amino-6-methylheptane	100
p-Hydroxy amphetamine	100	Bendroflumethiazide*	100
Atenolol	100	Dexamethasone	30
Benzoylecgonine	100	Oxilofrine	100
Betamethasone	30	Probenecid	100
Buprenorphine	5	Salmeterol	20
Cathine	100	Tuaminoheptane	100
Codeine	50	Cortisol	30
Ephedrine	100	Prednisone	30
Etilefrine	100	20β-Hydroxy prednisone	20
Fenoterol	20	THC-COOH	150
Fentanyl	2	Norfenefrine	100
Formoterol	20	Octopamine	1000
FPCAM	30	Pseudoephedrine	100
Hydrochlorothiazide*	100	19-Norandrosterone sulfate	50
Ketoconazole	50	Androsterone sulfate	200
Methamphetamine	100	DHEA sulfate	200
Morphine	50	5α-DHT sulfate	50
Nikethamide	100	Ethyl sulfate	500
Oxymorphone	50	19-Noretiocholanolone sulfate	50
Prednisolone	30	Meldonium	200
Propranolol*	50	Octopamine sulfate	500
Ritalinic acid	100	Nandrolone sulfate	50
Salbutamol	500	Epitestosterone sulfate	50
Tramadol	50	Testosterone sulfate	50
Cortisone	30		
20β-Hydroxy prednisolone	30		

*Compound spiked at 50 % MRPL, all others at their MRPL (TD2019MRPL [30]) except for salbutamol which was spiked at 50 % threshold and THC-COOH at the threshold (WADA TD2015DL [31]). The threshold is the WADA maximum permissible level of the concentration, ratio, or score for a threshold substance in a sample [32].

Abbreviations: 1,3-DMB, 1,3-dimethylbutylamine; DHEA, dehydroepiandrosterone; DHT, 5α-dihydrotestosterone; FPCAM, fluticasone propionate-17-carboxylic acid; QC, quality control.

by applying segmented linear gradients with different slopes (starting at 2% B, 3.0 min 5% B, 8.0 min 20% B, 11.0 min 50% B, 12.0 min 60% B, and 13.5 min 62.5%B 2.5 min post-time), resulting in a total run time of 15 min. Chromatography was performed at 28°C. The focused, extended, extra-control, delay-volume-free injection was performed by injecting 5 µl of the sample.

The MS was operated in electrospray positive ionization mode at a capillary voltage of 4000 V and in negative mode at 3500 V. The nozzle voltage was set at 500 V in positive and 1000 V in negative mode. A drying gas flow of 5 L/min at 150°C, a sheath gas flow of 12 L/min at 375°C, and a nebulizer pressure of 30 psi were used.

Multiple reaction monitoring (MRM) conditions, including collision energy and retention time details, are available in the supplemental information (Table S1). All transitions were recorded in "unit" resolution which was 0.7 Da. The detection and identification criteria used were as outlined in the WADA Technical Document TD2015IDCR [32].

2.3.2 | LC-tandem mass spectrometry

The analyses were performed on an Agilent 1260 Infinity II SFC system (Agilent Technologies GmbH, Waldbronn, Germany) coupled to an Agilent Ultivo triple quadrupole mass spectrometer where the LC/SFC system was set up as a hybrid system allowing the switch between SFC and LC mode without reconnecting columns or capillaries. To utilize the hybrid mode an additional 1290 Infinity II quaternary pump and a switching valve were added.

The SFC autosampler can be switched from focused, extended, extra-control, delay-volume-free mode (SFC) to standard loop mode (LC) via the MassHunter control software including the necessary flushing procedures. Additional solvent channels enabled fully automatic switching between modes. The LC-MS/MS method was adapted from the initial testing procedure (screening method) currently used in the WADA-accredited laboratory in Rome [33] using an Agilent Eclipse plus C18 column (100 x 2.1 mm, 1.8 μm particle size) with a mobile phase of water containing 5 mM ammonium formate and 0.1% formic acid (eluent A) and ACN and formic acid (1000:1, v:v, eluent B) running segmented linear gradients with different slopes (starting at 2% B and after 0.5 min increasing to 35% B at 6 min, to 98% B at 9.5 min, then hold at 98% B for 2.4 min, returning to 2% B in 0.1 min, followed by 1.5 min re-equilibration at 2% B) at a flow rate of 0.45 ml/min and ambient temperature.

Mass Hunter software (version 10; Agilent Technologies Inc. Santa Clara, CA, USA) was used for data acquisition and (qualitative and semi-quantitative) data processing. MS optimization was undertaken using Mass Hunter Optimizer. Statistical analysis was performed using Microsoft Excel (Microsoft, Munich, Germany) and Minitab Statistical Software (version 19.2020.1, RRID:SCR_014483, Coventry, UK).

2.4 | Method validation

Method validation was performed with respect to the intended use of the method in anti-doping laboratories. For the vast majority of the prohibited compounds in sports, qualitative detection is sufficient. WADA-accredited laboratories are required to meet the limits of detection specified in a regularly updated MRPL technical document (the appropriate version in force was TD2019MRPL [30]). Thus, our method aimed at covering multiple analytes for qualitative detection, basically intended for screening and not for quantitation. All validation criteria were determined using spiked urine samples or reference substances in a neat solvent.

Selectivity was evaluated by analyzing ten different blank urine samples for interfering signals. In addition, apart from solvent blanks, additional urine blanks (containing only internal standards) and double urine blanks (with nothing added apart from β-glucuronidase) were injected to check for carryover after every 10 samples and before and after standard mixes (urines spiked with QC compounds).

Area precision (calculated as the coefficient of variation) and retention time stability (given as t_R CV) were determined from multiple injections of spiked urine samples ($n = 5$ per day) over three consecutive days.

Calibration was performed with a matrix-matched calibration series, using four different concentrations from 25% MRPL to 200% MRPL, in order to get an estimate of concentrations of the individual analytes.

Recovery was calculated by comparing spiked urine samples mimicking real urine samples ($n = 10$) with spiked reference samples for which water was used instead of urine ($n = 10$).

A calibration series with neat solvent and reference substances as well as a calibration series containing urinary matrix were measured. Matrix effect (ME%) calculations were carried out according to Matuszewski et al. [34] at each calibration level.

$$\text{ME\%} = \frac{\text{Peak area matrix matched calibration}}{\text{Peak area neat solvent calibration}} \times 100$$

Limits of detection were calculated from serial dilutions of the analytes in urine with S/N ratios of the quantifier and qualifier S/N > 3 and their ratio within the criteria required by WADA [32].

Carryover was evaluated by injection of a blank solvent directly after high calibration standards (500 and 1000 ng/ml).

2.5 | Peak symmetry

Based on the approaches used in the United States, European, and Japanese Pharmacopoeias, peak symmetry was calculated at 10% peak height by dividing the area of the integrated peak after the peak maximum and the peak area before the peak maximum.

3 | RESULTS AND DISCUSSION

3.1 | Method development

Method development followed the principles of analytical lifecycle management [35, 36]. Based on the earlier developed method for screening of polar analytes in doping control [31], the target analytes were extended to cover various classes of drugs. The total number of compounds included in the method was 197 (Table 2 and Table S1). Suitable chromatography is often a good means of avoiding matrix interference in the MS. Because of the better chromatography observed from aglycones, samples were deglucuronidated by enzymatic hydrolysis. The same method could then be used for SFC, LC, or GC, and the prepared aliquots were used for the SFC/LC comparison. Instead of using the optimization conditions reported in our previous paper [31], optimization was redone since a newer MS, an Ultivo MS, was used for this study and

TABLE 2 Compound classes and the number of analytes targeted per class

WADA Compound Class	Number of analytes
Anabolic steroids	24
β_2-Agonists	7
β_2-Blockers	23
Cannabimimetics	1
Diuretics	26
Glucocorticoids	17
Hypoxia-inducible factors	1
Internal standard	5
Modulators	17
Monitoring program	5
Narcotics	5
Opioids	6
Stimulants	60
Total	**197**

additional analytes were included. Detailed information about ion transitions and retention times are presented in Table S1 and representative chromatograms of urines spiked with QC compounds and blanks are presented in Figure S2A–M.

The updated analytical target profile was included to check the suitability of the method as a screening procedure (initial testing procedure) for doping control, meeting the required limits of detection at 50% MRPL for all target analytes. As glucuronide metabolites are known to be challenging in SFC analysis, enzymatic cleavage with β-glucuronidase was implemented and the aglycones were included in the SFC-MS procedure. Also investigated in the initial method development was the inclusion of NH_4OAc as a mobile phase additive and MeOH as a modifier, which demonstrated their suitability for the target analytes. The protonation state of several analytes included in the method is strongly influenced by pH. Ammonium acetate demonstrated its suitability to improve the chromatographic behavior of these analytes. However, West et al. [37] report that an apparent "pH"~5 is maintained in SFC and the addition of acids or bases does not change the acidity of the mobile phase significantly. It is hypothesized that the improved peak shape rather results from the masking of residual silanol groups present on the silica-based stationary phase, thus suppressing the interaction of basic analytes with these groups [38] or from the generation of ion pairs that are jointly chromatographed. A relatively large amount of modifier was used to wash off late eluting compounds from the chromatographic column. This was considered important as the sample preparation did not involve any sample cleanup being a simple dilute and inject approach.

Finally, the MS conditions needed adaptation to allow the inclusion of the new analytes and to deal with the large number of ion transitions that need to be covered. A dynamic MRM method was used to cover all relevant ion transitions (Table S1). This method provided sufficient data points for good peak integration of all substances with the chromatographic conditions employed. For most of the compounds, two transitions were found to be sufficient and support the calculation of the co-elution score as a criterion of quality of both chromatography and identification. For some 20 compounds, three transitions have been used to enable the user to choose between them in case of matrix influence. Those 20 compounds are mainly endogenous compounds or natural product compounds such as 11-nor-9-carboxy-Δ9-tetrahydrocannabinol (THC-COOH).

Although optimization was needed as compounds were added, the method proved to be very flexible and further compounds can be added as the need arises. Nevertheless, care needs to be taken not to upset the optimized chromatography. At the time of doing this research, WADA TD2015IDCR [32] was in force and our analytes of interest met the detection and identification criteria outlined in this document; the subsequent TD2021IDCR [7] does not make any significant difference to the criteria we have used.

3.2 | SFC-MS/MS method performance characterization

Method validation was performed with respect to the intended use of the method in anti-doping laboratories.

3.2.1 | Robustness

The method was found to be robust for the analysis of the different urine samples over the whole project period. As the critical factor for separation, the modifier composition was slightly altered: Robustness of the method with respect to the ionic strength of the modifier (NH_4OAc concentration = 5 ± 1 mM) and water content (3.5 ± 1%) was shown not to affect separation over this range.

3.2.2 | Selectivity

For the majority of target analytes such as morphine, codeine, propranolol, ethyl sulfate, meldonium, and testosterone sulfate no interfering peak was detected in blank urines (Figure S2A–F). However, a few compounds showed non-Gaussian chromatography and the presence of endogenous co-eluting components not removed by the

simple dilute and inject approach; examples of this are amiloride, fentanyl, and probenecid (Figure S2G–I). This endogenous co-elution is related to sample preparation, which was not optimized in this study. Whether this could be improved was not evaluated but is not considered to be a limitation of the SFC-MS technique. Other issues that were not clearly linked to endogenous co-elution were observed. Tramadol has a shoulder seen on the main peak (Figure S2J). The signal size for fluticasone propionate-17-carboxylic acid has a low abundance and could be missed even at the MRPL if variables in the processing method (relating to peak integration) are not carefully set (Figure S2K). There is hardly any difference between urine spiked with 20β-hydroxy prednisolone, 20β-hydroxy prednisone and blank (non-spiked) urine, most likely due to coeluting isomers (Figure S2L,M).

3.2.3 | Limits of detection

Limits of detection were calculated using serial dilutions of the matrix-matched standards, with a S/N of the qualifier and quantifier >3 and their ratios as required by WADA [7, 32]. Calculated limits of detection were all less than 50% of the relevant MRPLs [30] in anti-doping control (e.g., MRPL urinary concentrations, in general, are 20 ng/ml for β_2-sympathomimetics, 100 ng/ml for stimulants and β-blockers, and 50 ng/ml for narcotics).

3.2.4 | Calibration

Using matrix-matched calibrants, unweighted linear calibrations for all analytes were obtained from 25% to 200% of their respective MRPLs.

3.2.5 | Accuracy

The accuracy of the concentrations was found by comparison of spiked urine samples with neat standard calibration curves. The values for trueness ranged between 90 and 110% for most of the compounds. Although the method was not intended to be used for quantitation, it was found that an estimate of concentration was reasonably reliable.

3.2.6 | Precision

Method precision was tested in terms of area precision and retention time stability. The majority of compounds showed area CVs < 5% (CV_{max} = 10.5%) and retention time differences t_R CV was << 1% (CV_{max} = 2.1%), thus meeting the criteria set by WADA [32]. In contrast to earlier instrumentation, which was mainly used for enantioseparation [22, 39], the new SFC-MS/MS devices yield very constant retention times (t_R CVs far below 1% for the majority of compounds). A typical batch of 50 samples plus QCs and reference standard injections was run in 18 hours. No trend in area values was detected within or between sample batches. Thus, no significant sample instability was observed at least for one week while samples were stored in the autosampler (T = 4°C). It can be seen from Figure S1 that batches of larger sizes were sometimes used and the gap in time between batches varied; this was considered important to replicate real-life conditions experienced in the typical anti-doping laboratory.

3.2.7 | Recovery

Recovery of the analytes was 100% as no extraction was performed. The success of the cleavage of the glucuronides was monitored using the internal standard d_3-testosterone glucuronide. Hydrolysis was considered to have been effective if no d_3-testosterone glucuronide peak was detected in the corresponding acquisition window with S/N > 3:1.

3.2.8 | Matrix effects

No significant ion suppression or ion enhancement was observed in matrix-matched standards for any of the analytes.

3.2.9 | Carryover

No relevant signal was detected in blank samples after injection of the highest calibrants.

3.2.10 | Peak characteristics

Retention time stability

Figure 1 shows the excellent stability of the retention times of a range of the analytes investigated in 19 different batches of urine samples over the whole 10 week study period. Note that typically even the far outside retention time values for analytes such as tramadol, THC-COOH, probenecid, and oxymorphone lie within a retention time window of ±0.15 min. For example, THC-COOH as the poorest example has the majority of the data points within just 0.02 min with a few extreme values. These far-outside values would normally be reviewed (but were deliberately not excluded from this data set) to check the chromatog-

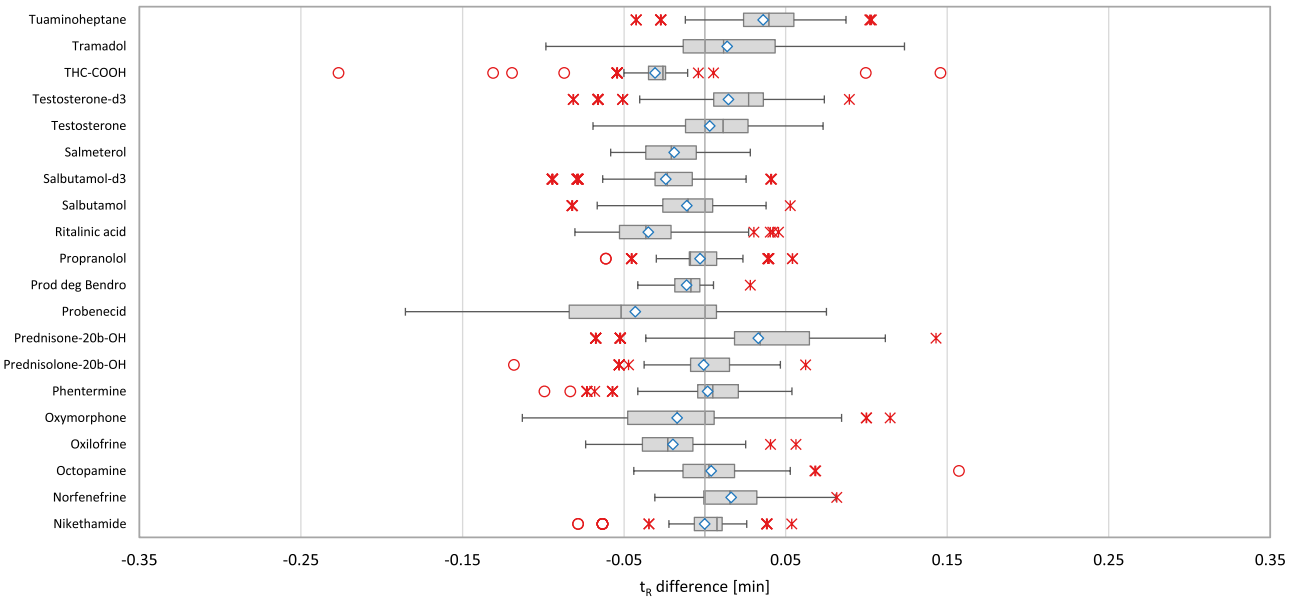

FIGURE 1 Retention time differences of selected compounds from reference values (obtained from standard solutions run at the start of the project) over some 19 different batches of urine samples run over 10 weeks. "Prod deg Bendro" refers to bendroflumethiazide degradation product, that is, 4-amino-6-(trifluoromethyl)-benzene-1,3-disulfonamide.

Key to symbols

The shaded rectangle shows the interquartile range between the first and third quartiles; within each rectangle, the vertical line is the median of the data and the diamond is the mean; whiskers represent the variability of the data outside of the interquartile range; stars represent outside values or outliers falling between 1.5 and 3.0 interquartiles outside of the inner quartiles; circles represent far outside values, which fall more than 3.0 interquartiles outside of the inner quartiles.

TABLE 3 Mean peak symmetry and measure of the variation of the internal standards over the whole study

Internal standard	Number of results	Mean peak symmetry	Peak symmetry %CV
d_3-ephedrine	1418	1.08	8.9
d_3-salbutamol	1425	1.21	6.9
mefruside	1411	1.01	9.3
d_3-testosterone	1422	1.17	7.1

raphy and integration of these analytes to confirm that the peak identification and integration are correct.

Peak symmetry

The software calculated peak start and end reliably, independent of noise and signal height. Peak symmetry data for internal standards for the whole study are presented in Table 3. Gaussian peak shapes for some compounds such as morphine, codeine, propranolol, ethyl sulfate, meldonium, and testosterone sulfate are presented in Figure S2A–F. Generally, the peak symmetry for all the standards was very good.

3.3 | Reporting

A data reduction system was employed to curate spurious signals from the final output. This was performed as follows: First, for each compound based on the data obtained from reference material, any integrated peak area which was less than 30% of the area obtained from the standard at 25% MRPL was rejected. Then the maximum tolerance windows for relative abundances published by WADA (TD2015IDCR [32]) were applied to the remaining peak areas. These rules specify a 10% absolute window for ions greater in intensity than 50%, a 20% relative window for ions of relative abundance of greater than 25% up to 50%, and an absolute window of 5% for ions of relative abundance between 1% and 25%. Then, a standard MassHunter script was used to remove integrated peak areas of target peaks where there was no qualifier of sufficient signal to be integrated. The output report is thereby reduced to show only the relevant peaks greatly facilitating data review and reducing the output (number of pages) by 75%. For example, with a negative sample, only the peaks for the internal standards will be displayed.

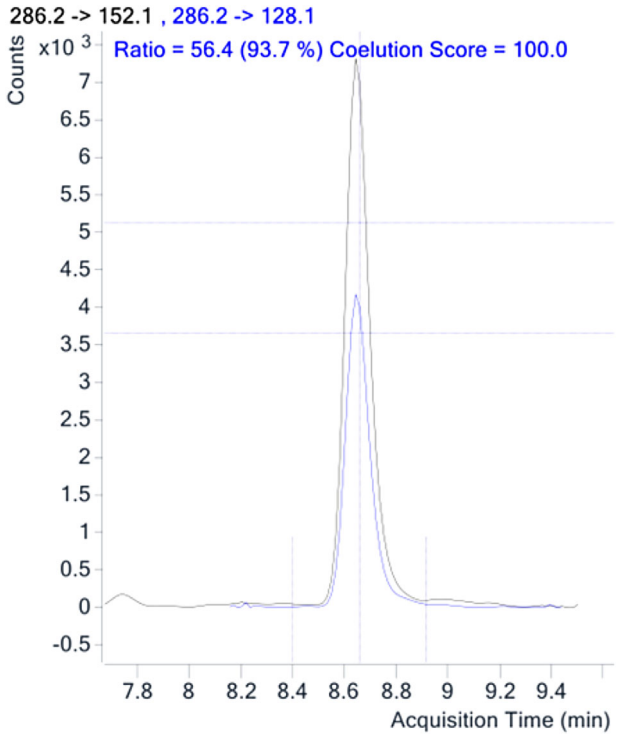

FIGURE 2 Representative chromatogram of morphine using SFC, showing the good peak shape and longer retention compared with that obtained by RP-LC. Vertical lines show the expected retention time with the appropriate permitted retention time limits. The horizontal lines indicate the limits according to the World Anti-Doping Agency (WADA) TD2015IDCR for the uncertainty bands of the qualifier ratios of the multiple reaction monitoring (MRM) chromatograms displayed. The co-elution score is also displayed above each compound window.

The displayed chromatogram of morphine (Figure 2) shows vertical lines with the expected retention time with the appropriate permitted retention time limits. The horizontal lines indicate the limits according to WADA TD2015IDCR for the uncertainty bands of the qualifier ratios of the MRM chromatograms displayed. The Agilent co-elution score [40] is also displayed above each compound window. This score gives an indication of peak purity and is based on time and MRM intensity (for morphine m/z 286 → 152 and m/z 286→ 128). The co-elution score should be greater than 90%. Two MRMs are used as a minimum and three for endogenous or natural compounds.

3.4 | Comparison of the SFC-MS/MS method with RP-HPLC-MS/MS

The comparison of the adapted SFC-MS/MS method with RP-based methods showed similar performance for most of the substances analyzed. Retention times and elution orders are different due to the orthogonality of SFC. Very polar compounds such as etilefrine, octopamine, morphine, meldonium, or ethyl sulfate show almost no retention under RP conditions, while our SFC-MS/MS method shows good retention for these compounds. This is of advantage, especially for direct injection methods with high matrix load as ion suppression is thereby drastically reduced. Retention times and elution orders using the SFC method are different from both reversed-phase and HILIC separations due to the orthogonality of the SFC technique. As displayed in Figure 3, the example of octopamine sulfate, the retention times show the large difference between SFC and LC methods further demonstrating the orthogonality. The retention time shifting may help to "compensate" for possible co-eluting components. Figure 4 shows the retention time relationship between SFC and LC for more than 190 analytes. Although statistically negatively correlated, there is sufficient orthogonality to provide valuable additional identification information by using both methods. Reversed-phase LC-MS/MS is routinely used for the quantitation of threshold substances in anti-doping laboratories. Some of our preliminary experiments to evaluate SFC-MS for quantitation of threshold substances (e.g., morphine, salbutamol, and ephedrine) confirm the potential applicability of SFC-MS for this purpose.

3.5 | Proof-of-concept

Analysis of 1,000 authentic doping control urines as well as a number of external quality assurance samples was performed as a proof of concept that the method described in this paper was fit for purpose and ready for use in routine anti-doping analysis.

Specifically, the performance of the system met all expectations showing very consistent performance in both retention time stability and chromatography over the duration of the whole experiment (Figure 1, Figure 2, and Figure S1 for the timing of the different batches). Of note, the system was shut down for one month and it was observed from the start-up of the system that an equilibration time of at least 12 hours was important whereafter very stable performance was resumed.

4 | CONCLUDING REMARKS

Prior work has documented [29] the suitability of SFC-MS/MS for the separation of polar and nonpolar analytes in the field of doping control and forensics. However, suitability in routine settings, i.e. analysis of large numbers of samples run over 10 weeks has rarely been demonstrated

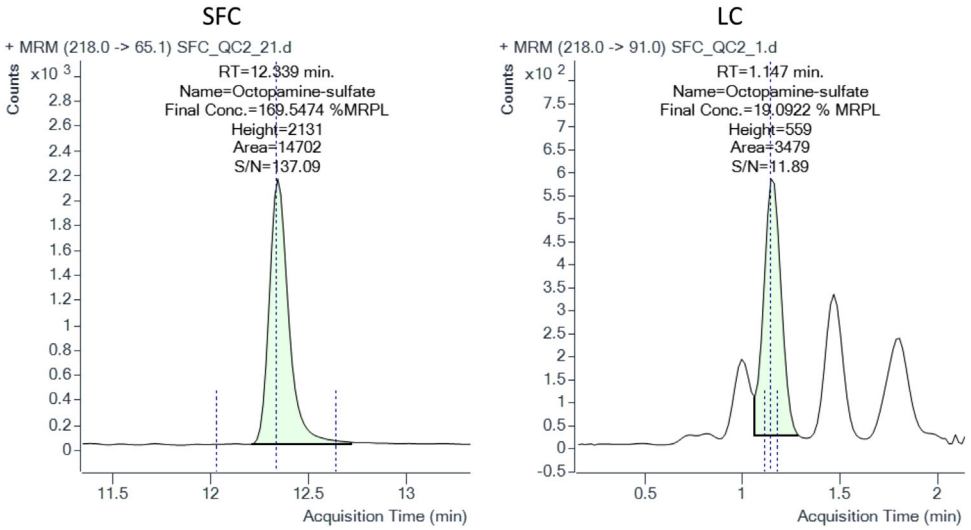

FIGURE 3 Chromatographic comparison of both SFC and LC for octopamine sulfate (matrix-matched quality control [QC] standard).

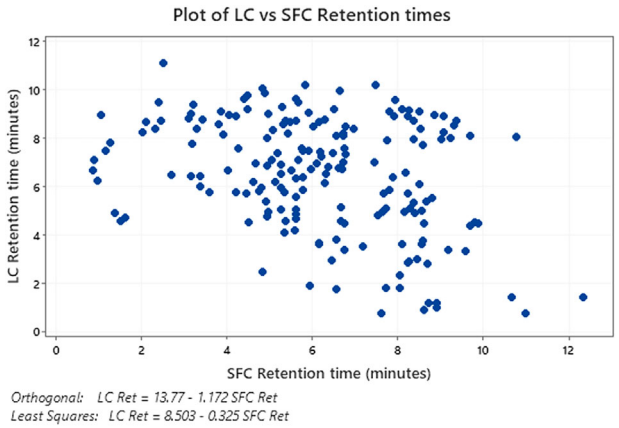

FIGURE 4 Plot of retention times of analytes using SFC and reversed-phase LC. Excellent orthogonality is demonstrated by orthogonal regression analysis, which shows a very limited negative correlation between the retention times of the two methods ($p >$ 0.05 for the first six minutes and $p = 0.047$ for the first seven minutes of the SFC analysis).

until now. For the purposes of our work, the SFC-MS acquisition method adapted from the literature [31] was validated and utilized for the analysis of more than 1000 samples including past-positive anti-doping samples. Samples were simply diluted and injected prior to analysis, a quick and cost-effective way of sample preparation. While doing this work, two WADA Technical Documents were effective TD2019MRPL and TD2015IDCR [30, 32]. By the time this research was completed, new corresponding Technical Documents became effective, TD2022MRPL and TD2021IDCR [6, 7]. The MRPL reduction was more significant, but in our view did not have an impact on our results and conclusions, since we tested linear calibration for all analytes over the range of 25%–200% of the 2019MRPL [30]. All validation criteria were satisfied, and the method proved to be robust for routine anti-doping analysis. SFC-MS proved to be complementary to the widely used LC-MS/MS approach. SFC-MS proved advantageous for the better retention of early eluting LC-MS/MS compounds (e.g., morphine). Potential limitations of the method relate mainly to the simple sample preparation and to the quantitative analysis of threshold substances. Sample preparation could be further improved and optimized to avoid endogenous co-elution. According to our preliminary experiments, the use of SFC-MS for the quantitation of threshold substances appears to be straightforward although still to be proven. The quantitative analysis of threshold substances by SFC-MS was not within the scope of this work, but with proper optimization, it should be possible.

It remains to be seen whether similar performance can be achieved from other instruments on the market. Our early work is promising and will be the subject of further communication.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data are availabe from the authors upon request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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