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RESEARCH ARTICLE

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Detection of doping substances in paired dried blood spots and urine samples collected during doping controls in Danish fitness centers

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Abstract

The use of dried blood spot (DBS) in anti-doping can be advantageous in terms of collection, transportation, and storage compared with the traditional anti-doping testing matrices urine and venous blood. There could, nonetheless, be disadvantages such as shorter detection windows for some substances compared with urine, but real-life comparison of the detectability of prohibited substances in DBS and urine is lacking. Herein, we present a liquid chromatography–high resolution mass spectrometry (LC-HRMS)-based screening method for simultaneous detection of 19 target analytes from the doping substance categories S1–S5 in a single spot. Ninety-eight urine and upper-arm DBS (Tasso-M20) sample pairs were collected from fitness centers customers notified for doping control by Anti Doping Denmark, and three sample pairs were collected from active steroid users undergoing clinical evaluation and treatment at a Danish hospital. The analytical findings were cross compared to evaluate the applicability of the developed DBS testing menu in terms of feasibility and analytical performance. To our knowledge, this is the first study to compare the detectability of prohibited substances in DBS and urine samples collected in a doping control setting. Twenty-seven of the urine samples and 23 DBS samples were positive, and we observed a very high concordance (95%) in the overall analytical results (i.e., positive or negative samples for both urine and DBS). Collectively, these results are very promising, and DBS seems suitable as a stand-alone matrix in doping control in fitness centers likely because of the high analyte concentration levels in these samples.

KEYWORDS
anabolic androgenic steroids, doping control analysis, dried blood spots (DBS), fitness centers, urine

1 | INTRODUCTION

From September 1, 2021, dried blood spots (DBS) were approved as a new sample matrix in doping control by the World Anti-Doping Agency (WADA).[1] The DBS method has shown advantages in collection, transportation, and storage compared with the traditional anti-doping testing matrices urine and venous blood.[2,3] In a recent study, we illustrated that DBS sampling from fingertip and upper arm was

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easy, quick, comparably painless, and preferred by both Doping Control Officers and athletes over conventional sample collection methods.[4] The average sample collection time was only 2 min. This is of great benefit compared to traditional urine collection, which can last for hours in cases where spontaneous urination is not possible.[4] Furthermore, various compounds have shown high analyte stability in DBS,[3,5–12] which suggests that DBS testing enables the collection of blood specimens for routine doping analysis without the need for temperature-controlled and time-sensitive shipment. Based on this, it appears that the implementation of DBS testing has the potential to improve the time- and cost-efficiency in anti-doping and make the doping control more athlete friendly, while concurrently enabling increased testing frequencies, for example, large-scale testing at training venues or at competitions, which could enhance both detection and deterrence.

Despite a lower sample volume, DBS testing also offers benefits from an analytical perspective, and the portfolio of promising DBS applications in anti-doping has grown to include representative target analytes from all classes of prohibited substances on the WADA Prohibited List.[13] Because the drug or active metabolites are transported in the bloodstream to target tissues and organs, DBS testing can add information on the presence of pharmacologically relevant blood concentrations.[14–16] This may be particularly relevant for substances prohibited by WADA in competition only, where, for example, simultaneously collected DBS samples can support in the results management process for adverse analytical findings derived from urine samples.[17] Additionally, DBS could be the preferred matrix for detection of substances that are not easily detectable in urine, such as parent compounds and drugs that may not be excreted in urine because of their size or chemical properties. In this regard, DBS testing has been shown to be applicable for detection of intact anabolic steroid esters,[6,18,19] which are rapidly hydrolyzed into free steroids in plasma.[20] This is of high relevance because anabolic agents are the doping substances most frequently detected in doping control samples from athletes, especially in power/strength sports.[21,22] The current approach in doping analysis to monitor and screen for doping with testosterone, its precursors, or metabolites is based on indirect detection through evaluation of urinary steroid profiles, and subsequent confirmation through a laborious and expensive gas chromatography-combustion-isotope-ratio mass spectrometry (IRMS) analysis.[23] The presence of anabolic esters in blood, like DBS, represents a more convenient method to confirm the exogenous origin of the steroids.[24]

Based on the above, DBS testing undoubtedly offers many possibilities as a complementary matrix in anti-doping. Nevertheless, there could be disadvantages such as shorter windows of detection for some substances because of the longer persistence of drug metabolites in urine compared to blood, as well as the limited DBS sample volume, which may influence the assay sensitivity and the number of analyses that can be performed on a sample. Hence, the pros and cons must be thoroughly considered when anti-doping authorities plan DBS testing as part of their testing programs. In the short term, one possibility is the implementation of a targeted multi-analyte screening assay for single-spot DBS analysis.[11,12,25] As an example, Thomas et al. proposed a method for the detection of 26 selected model compounds from different drug classes (anabolic agents [S1], beta-2 agonists [S3], hormone and metabolic modulators [S4], diuretics [S5], stimulants [S6], cannabinoids [S8], glucocorticoids [S9], and beta-blockers [P1]).[25] This study was an inspiration to the mixed menu of this study, and since then methods comprising a large number of analytes from different compound–classes have been published.[11,26] However, real-life comparison of the detectability of substances in DBS and urine is lacking.

In the beginning of the COVID-19 pandemic, normal testing of athletes was not possible. Looking for alternative approaches to continue testing, DBS appeared as a suitable matrix as devices facilitating self-testing and physical distancing became available. Therefore, Anti Doping Denmark (ADD), Anti-Doping Norway, and the Norwegian Doping Control Laboratory decided to develop a limited DBS screening menu for fast implementation in the anti-doping authorities' testing programs during this challenging period. A pilot program was initiated by ADD to evaluate the applicability of the developed method in terms of feasibility and analytical performance. In Denmark, a unique possibility exists for such an evaluation because ADD has the authority to carry out doping controls in numerous fitness centers located around the country that have cooperation agreements with ADD. For the fitness centers, the cooperation with ADD is on a voluntary basis and subject to a small annual fee. Nevertheless, when the contract between the fitness center and ADD has been signed, all members of the fitness center are subject to doping control (urine sample collection). This is written in the membership agreement between the fitness center and the member. Through ADD's national doping prevention efforts in these fitness centers, the fitness consultants conduct site visits where they maintain ongoing dialog with staff and members, and carry out doping controls (i.e., as a doping control officer) with urine collections when relevant to remove potentially unhealthy and inappropriate role models. Members who refuse to provide a sample or test positive are sanctioned with a 4-year ban from organized sport and a 2-year ban from all fitness centers who cooperate with ADD. In the present pilot program, fitness center customers notified for doping control were asked if they, in addition to the standard urine sample, would be willing to provide a DBS sample. The DBS findings were used for a cross-comparison with urine findings, providing information regarding the developed DBS testing menu's analytical performance and its applicability in fitness centers and potentially also in power/strength sports.

2 | MATERIALS AND METHODS

2.1 | Chemicals, reagents, and materials

Anastrozole was obtained from Astra Zeneca (Cambridge, England); andarine, metenolone, and ostarine were obtained from TRC (Toronto, Canada); boldenone, oxandrolone, stanozolol, and trenbolone were obtained from Steraloids (Newport, USA); clenbuterol and

clenbuterol-d_6 were obtained from RIVM (Bilthoven, Netherlands); clomifene, ethoxyzolamide, hydrochlorothiazide, nandrolone, tamoxifen, and terbutaline were obtained from Sigma (Schnelldorf, Germany); dehydrochloromethyltestosterone (DHCMT), metandienone, and testosterone-d_3 were obtained from NMI (Australia); furosemide was obtained from Apothekernes Laboratorium (Norway); ibutamoren was obtained from AusPep (Australia); and letrozole was obtained from Novartis (Basel, Switzerland).

DBS cards (FTA® DMPK-C) were obtained from Whatman™ GE Healthcare (Uppsala, Sweden), and Tasso-M20 OnDemand devices were obtained from Tasso Inc. (Seattle, USA). Tamper-evident urine sampling equipment for doping control were purchased from Berlinger Special AG (Ganterschwil, Switzerland) and Lockcon AG (Wil, Switzerland). Liquid chromatography–mass spectrometry (LC–MS) grade methanol (MeOH) and acetonitrile (ACN) were purchased from Sigma (Schnelldorf, Germany). 30K Amicon® Ultra centrifugal filters were obtained from Merck Millipore Ltd. (Cork, Ireland).

2.2 | Stock solutions

Standard stock solutions of the different analytes were prepared in MeOH and stored at −20°C. Working solutions were freshly prepared in whole blood by dilution of the stock solutions. A solution of 50 pg/mL testosterone-d_3, 50 pg/mL ethoxyzolamide, and 10 pg/mL clenbuterol-d_6 was used as internal standards (ISTDs).

2.3 | Preparation of DBS samples

DBS samples for method development and validation were prepared with venous whole blood collected from healthy volunteers into tubes containing ethylenediaminetetraacetic acid (EDTA). The whole blood samples were fortified with the analyte working solutions to different concentrations and rotated for 20 min before spotting 20 µL on the DBS sampling cards. The DBS samples dried for at least 2 h at room temperature before the cards were stored at −20°C until analysis.

2.4 | Sample preparation

For DBS analysis, the whole DBS spot was cut out from the Whatman DMPK-C cards, or one pebble (20 µL) of dried blood was removed from the Tasso-M20 sample pod and placed in a 2-mL Eppendorf tube. 1 mL extraction solvent consisting of the ISTDs in a mixture of ACN:MeOH (50:50 *v*/*v*) was added to each tube. The samples were incubated for 1 h at 40°C at 1400 rpm. The extract was then filtrated (2 × 500 µL) using Amicon 30 K centrifugal filters at 20°C at 10.000 × *g* for 15 min. The filtrate was evaporated to dryness under nitrogen gas at 40°C and redissolved in 50 µL 10% ACN in deionized water before analysis.

For urinary analysis, the samples were prepared by the in-house routine method for analyzing doping substances. Briefly, 2.5- or 5-mL (when SG < 1.010) urine was enzymatically hydrolyzed by β-glucuronidase and subsequently liquid extracted with tert-butyl methyl ether (TBME) at pH 9.5–9.8. The extract was divided in two aliquots, of which one was derivatized and analyzed by gas chromatography–with tandem mass spectrometry (GC–MS/MS), while the other aliquot was subjected to liquid chromatography–with tandem mass spectrometry (LC–MS/MS) analysis as described below.

2.5 | Urinary analysis

The GC–MS/MS screening of the urine samples was performed using the 7890A GC coupled to the 7000C mass detector (Agilent Technologies, Santa Clara, CA, USA). The separation was carried out on a BPX5 column (0.25 mm × 15 m, 0.25 µm) (SGE Analytical Science, Melbourne, Australia), with helium as carrier gas and a 20.5-min temperature gradient from 160 to 310°C. A volume of 0.5 µL of derivatized sample was injected using pulsed splitless mode at 280°C. The GC–MS/MS method was based on multiple reaction monitoring (MRM) using positive polarization.

Additionally, 10% of the TBME extract was analyzed by the 1290 series HPLC combined with the 6490-mass spectrometer (both Agilent Technologies, Santa Clara, CA, USA). After evaporating and redissolving in 125 µL 10% MeOH, 10 µL of the extract was loaded on the Zorbax SB-C18 (2.1 × 50 mm, 1.8 µm, Agilent Technologies, Santa Clara, CA, USA) column. Mobile phase A consisted of 10-mM ammonium formate and 0.1% formic acid in water, whereas mobile phase B consisted of pure ACN. A 15-min gradient was used to separate the compounds. The gradient started with 10% mobile phase B, raised to 35% in 5 min, then raised to 98% within 5 min before switching back to 10% mobile phase B. The LC–MS/MS method was based on MRM using positive and negative polarization.

The urine analysis of fitness samples included anabolic androgenic steroids, other anabolic agents, and limited hormone and metabolic modulators according to the agreed testing menu between Anti Doping Denmark and the Norwegian Doping Control Laboratory. In agreement with Anti Doping Denmark, confirmatory analysis was performed for one analytical finding per sample.

2.6 | DBS analysis by liquid chromatography–high resolution mass spectrometry (LC-HRMS)

The chromatographic separation and detection were carried out using a Dionex Ultimate 3000 chromatographic system connected to a Q Exactive mass spectrometer equipped with a heated electrospray ionization (HESI) ion source (Thermo Scientific, Bremen, Germany). Twenty microliters of each sample was injected onto the Zorbax SB-C18 analytical column (2.1 × 50 mm, 1.8 µm, Agilent) at 250 µL/min. The column compartment was set to 40°C. Mobile phase A consisted of 0.1% FA and mobile phase B consisted of ACN. The samples were analyzed using two different gradients and ionization modes: one for analytes in the positive mode (I) and one for analytes in the negative

TABLE 1 Selected substances in the DBS testing menu and main LC-HRMS related characteristics.

Analyte	Polarity	Retention time (min)	Precursor ion (*m/z*)	Collision energy	Product ions (*m/z*)
S1. Anabolic agents					
Andarine	−	7.1	440.1075	20	150.0540 261.0475
Boldenone	+	7.2	287.2006	35	121.0640 135.1157
Clenbuterol	+	4.3	277.0869	25	203.0120 259.0743
Dehydrochloromethyltestosterone (DHCMT)	+	9.3	335.1772	25	149.1310 317.1636
Metandienone	+	8.1	301.2162	25	149.1312 121.0639
Metenolone	+	9.0	303.2318	40	83.0490 187.1465
Nandrolone	+	7.2	275.2006	50	109.0642 145.0999
Ostarine	−	7.7	388.0915	15	118.0278 269.0527
Oxandrolone	+	7.2	307.2268	25	289.2135 271.2028
Stanozolol	+	8.3	329.2587	70	81.0448 107.0850
4β-hydroxy-stanozolol	+	6.2	345.2536	30	309.2292 327.2396
16β-hydroxy-stanozolol	+	6.1	345.2536	70	81.0446 107.0848
Trenbolone	+	6.8	271.1693	65	107.0486 133.0636
S2. Peptide hormones, growth factors, related substances, and mimetics					
Ibutamoren	+	6.1	529.2479	20	267.1155 235.1431
S3. Beta-2 agonists					
Terbutaline*	+	2.0	226.1438	35	152.0691 125.0586
S4. Hormone and metabolic modulators					
Anastrozole	+	6.0	294.1713	40	225.1364 198.1256
Clomifene	+	12.7	406.1932	30	100.1127 72.0816
Letrozole	−	6.5	284.0942	45	242.0689 127.0273
Tamoxifen*	+	13.5	372.2322	35	72.0811 129.0692
S5. Diuretics and masking agents					
Furosemide	−	6.1	329.0004	45	204.9811 77.9631
Hydrochlorothiazide	−	2.9	295.9572	45	204.9811 77.9630
Internal standards					
Clenbuterol-d_6	+	4.3	283.1246	25	204.0186 265.1122
Ethoxyzolamide	−	6.2	257.0060	25	178.0301 77.9631

(Continues)

TABLE 1 (Continued)

Analyte	Polarity	Retention time (min)	Precursor ion (*m/z*)	Collision energy	Product ions (*m/z*)
Testosterone-d_3	+	7.9	289.2162	40	97.0654 109.0652

Note: Please note that for urine, the screening menu allowed the detection of both parent compound and one or more metabolites, whereas the DBS screening menu included parent compounds only. DBS, dried blood spot; LC-HRMS, liquid chromatography–high resolution mass spectrometry; WADA, World Anti-Doping Agency.

*Additional substances that are not prohibited in Danish fitness centers but prohibited according to WADA's list of prohibited substances.

mode (II). Gradient I was held at 5% B for 1 min, then increased to 40% B in 4 min, to 45% B in 5 min, and to 55% B in 3 min, before 2 min at 98% B and then back to 5% B for 3 min to regenerate the column. Gradient II was held at 5% B for 1 min, then increased to 35% B in 5 min, to 98% B in 5 min and then held at 98% B for 1 min before switching back to 5% B for 3 min to regenerate the column. The total analysis time per run was 18 min with gradient I and 15 min with gradient II. The spray voltage was set to 3.5 kV. Nitrogen was used as collision gas and as source gas. The heated capillary was kept at 350°C. Parallel reaction monitoring (PRM) was performed in the mass spectrometer at a resolution of 17500 FWHM (full width at half maximum), monitoring the precursor ions in a 2 Da mass window. The diagnostic product ions and the collision energy used for each precursor ion are listed in Table 1. The instrument was calibrated according to the manufacture's specification enabling mass accuracies of better than 5 ppm. The Thermo Scientific TraceFinder software version 4.0 was used for both acquisition and data interpretation.

The concentrations were estimated using a single point calibrator (SPC) at the urine minimum required performance level (MRPL). The SPC samples were prepared by adding reference materials to fresh whole blood and applying 20 µL to each of the Tasso-M20 sampling pods. The SPC samples were left to dry in room temperature for at least 2 h and then stored at −20°C for up to 3 months.

2.7 | Results management

The term "Positive Finding," corresponding to an Adverse Analytical Finding (AAF) in doping control in sports, was used when evaluating the analytical results according to the regulations in the fitness program because the term "AAF" is not normally used within the fitness program. Further, the term "Positive Sample" was used for samples with at least one detected substance. When evaluating the analytical results according to the WADA regulations (the Prohibited List, the Technical Document on MRPLs [TD MRPL], and the TD IRMS) applicable for urine samples in elite sports, the term "AAF" was used for urine positive cases, whereas the term "Presumptive Adverse Analytical Finding" was used if the initial testing procedure showed the presence of a prohibited substance in the DBS sample because no confirmatory procedures were performed for DBS.

Boldenone has a minimum reporting level (MRL) in urine, and levels below 30 ng/mL must be forwarded to IRMS to establish whether the origin is exogeneous or endogenous. No equivalent level exists for boldenone in DBS. When a DBS boldenone finding in a fitness sample was accompanied by a boldenone in urine above 30 ng/mL, the DBS was considered a "Positive Sample." When a DBS boldenone finding in a fitness sample was accompanied by a urine where boldenone was either undetectable or below 30 ng/mL, the DBS was considered an "Atypical Sample."

For urine samples with multiple findings in the screening analysis, only one selected finding was confirmed (indicated by "c" in Table 3) and the remaining reported as screening findings, in agreement with Anti Doping Denmark. Samples in which no prohibited substances were detected were reported as "Negative."

2.8 | Method validation of DBS analysis

The method validation was conducted using DBS samples prepared with EDTA whole blood, fortified with the different analytes, and spotted on Whatman DMPK-C sampling cards. The following parameters were considered: selectivity, limit of detection (LOD), precision, robustness, recovery, matrix effects, and carryover. Because of limited availability of Tasso-M20 devices at the time of validation, a more restricted validation protocol with a limited number of replicates (four replicates on 1 day as compared to 2 days for DMPK-C) was used.

2.8.1 | Selectivity

The selectivity was investigated by the analysis of 10 different blank DBS samples obtained from healthy volunteers (non-fasting status, three females, seven males). For DMPK-C, the blank DBS samples were collected by finger-prick, whereas the blank Tasso-M20 samples were prepared with EDTA whole blood. The samples were analyzed to look for the presence of interfering signals at the respective retention times of the analytes.

2.8.2 | LOD

The technical document for DBS in force at the time of analysis (TD2021DBS) only covered non-threshold substances without MRLs.[1] According to the International Standard for Laboratories (ISL) and the TD MRPL, the estimated LOD for non-threshold substances in urine shall be ≤ 50% of the corresponding MRPL.[27] Relevant MRPLs for DBS

were not available at the time of method development and validation, and limited knowledge was available regarding analyte concentrations in DBS. Therefore, each analyte's LOD for DMPK-C was investigated using seven different concentration levels relative to the urine MRPL: 1 × MRPL, 0.5 × MRPL, 0.25 × MRPL, 0.125 × MRPL, 0.05 × MRPL, 0.025 × MRPL, and 0.01 × MRPL. For Tasso-M20, the LODs were estimated using six different concentration levels (1 × MRPL, 0.5 × MRPL, 0.25 × MRPL, 0.125 × MRPL, 0.05 × MRPL, and 0.025 × MRPL). Diuretics followed the same dilution protocol but starting at 0.1 × MRPL. The LOD was estimated using a detection response curve and defined as the 95% positive detection rate.

2.8.3 | Precision and robustness

Precision and robustness were investigated at four concentration levels from the urine MRPLs of the analytes down to 12.5% of the urine MRPLs (10 times lower for diuretics). Three replicates were analyzed on two consecutive days, by different engineers. The intra- and inter-day precisions were calculated as the peak area of the analytes relative to the peak area of the ISTDs, expressed as relative standard deviation in percent. For Tasso-M20, only intra-day precision was evaluated.

2.8.4 | Recovery

The recovery was investigated at four concentration levels from the urine MRPLs of the analytes down to 12.5% of the urine MRPLs (10 times lower for the diuretics). The peak area of the different analytes was compared to that of the extract from a negative DBS sample fortified with the analytes at the end of the sample preparation, that is, before evaporation (100% sample). Recovery from Tasso-M20 was investigated at urine MRPL.

2.8.5 | Matrix effects

The matrix effects were investigated at four concentration levels from the urine MRPLs of the analytes down to 12.5% of the urine MRPLs (10 times lower for the diuretics). The peak areas from negative DBS extracts fortified with the different analytes were compared to a pure standard solution of the analytes at the corresponding concentration levels. Matrix effects from Tasso-M20 were investigated at urine MRPL.

2.8.6 | Carryover

The carryover effect was evaluated by the analysis of a blank sample directly after the analysis of a high concentration sample (500% of the urine MRPLs, 10 times lower for the diuretics). The peak areas were compared.

2.9 | Applicability study with collection of real-life doping control samples

The applicability of the developed DBS testing menu for doping control analysis in fitness centers was evaluated by means of a pilot program as part of ADD's national doping prevention efforts in fitness centers that have cooperation agreements with ADD. The pilot program was conducted in the largest Danish fitness chains—SATS and Fitness World—from September 2020 to October 2022. Twelve of ADD's fitness consultants were trained in collecting upper-arm DBS samples using the Tasso-M20 device, and the fitness centers customers notified for doping control were asked if they would be willing to provide an upper-arm DBS sample (Tasso-M20) for research purposes in addition to the standard, mandatory urine sample. Additionally, active steroid users undergoing clinical evaluation and treatment at the medical Department of Endocrinology at Herlev–Gentofte University Hospital, Copenhagen, Denmark were asked if they would be willing to provide paired urine and upper-arm DBS samples (Tasso-M20) for the study. The study did not require an ethical approval from the local ethics committee, but all participants provided informed, written consent prior to the sample collections.

The doping control urine samples were collected according to the WADA's guidelines under witness of a fitness consultants from ADD, but a minimum urine volume of 65 mL per sample was allowed. Urine sample collection from patients was not witnessed. Parallel DBS samples (~80 µL per sample pod consisting of four pebbles) were collected by a fitness consultant using an automatic microneedle-based Tasso-M20 device, which was applied to the deltoid muscle of the participants. DBS sampling from the patients was performed by a medical doctor.

In total, 101 sample pairs were collected in the pilot program, of which 98 from males and three from females. Three of the sample pairs were collected from active steroid users undergoing clinical evaluation and treatment at a Danish hospital and 98 from fitness center customers. The samples were transported to the Norwegian Doping Control Laboratory in Oslo, Norway, which is accredited by WADA and Norwegian Accreditation (ISO/IEC 17025). The samples were analyzed as described earlier, and the analytical results of the paired DBS and urine samples were compared. For a fair comparison, only analytical findings included in the DBS testing menu were evaluated when comparing the urine and DBS analytical results.

3 | RESULTS AND DISCUSSION

3.1 | Analyte selection

A limited DBS screening menu consisting of several substance groups covering analytical findings in Danish fitness centers and routine analytical findings in the Norwegian Doping Control Laboratory in recent years was developed. The resulting LC-HRMS-based screening method covered the simultaneous detection of 19 selected prohibited substances from S1 to S5 (Table 1). In Danish fitness centers, the list of prohibited substances is connected to the Danish "Act Prohibiting Certain Doping Substances" (Act No. 232 of April 21, 1999 as

amended by Act No. 69 of February 4, 2004 and by Act No. 352 of May 6, 2009), and most of the previous urine findings indicated the administration of anabolic agents. Therefore, the DBS screening menu mainly consisted of anabolic agents (11) and hormone modulators (4) often used in combination with anabolic androgenic steroids (AAS). An additional anabolic agent, drostanolone, was initially part of the menu, but this analyte could not be satisfactorily validated with the chosen method and was excluded.

Because of limited knowledge regarding analyte concentrations and metabolite patterns in venous blood and DBS, the DBS method consisted of parent compounds only, with the exception of two stanozolol metabolites: 4β-hydroxy-stanozolol and 16β-hydroxy-stanozolol. Furthermore, the multi-analyte assay included three ISTDs: testosterone-d3, ethoxyzolamide, and clenbuterol-d_6.

3.2 | Method development

We aimed to develop a method that was applicable in routine doping control analysis in terms of both feasibility and analytical performance. This meant using more generic procedures, herein limiting the number of sample preparations steps, and using solvents that are better to work with/less hazardous, while still producing sufficient analytical sensitivity. Several elution conditions (50:50 MeOH:ACN, 50:50 TBME:MeOH, 100% TBME, and 50:50 acetone: MeOH) and redissolution solvents (5% and 10% MeOH; 5%, 10%, and 15% ACN) were investigated, with 50:50 MeOH:ACN and 10% ACN being the most promising options.

3.3 | DBS method validation

A screening method for a selection of analytes from DBS was optimized and validated (see Section 2.8). The validation was performed using the standard DMPK-C cards and the Tasso-M20 device. The main validation results from the highest concentration level are summarized in Tables 2a and 2b.

3.3.1 | Selectivity

Selectivity is the ability of the method to differentiate the analyte of interest from endogenous matrix components or from other substances present in the sample. The selectivity was acceptable for all included substances as 100% (10/10) of the blank DMPK-C and Tasso-M20 samples analyzed resulted in a negative screening result.

3.3.2 | LOD

The analytical sensitivity was determined by estimating LODs for the different analytes. The estimated LODs, based on a signal-to-noise

TABLE 2a Main DBS method validation results: DMPK-C.

Analyte	LOD (ng/mL)	Intra-day	Inter-day	Recovery	Matrix effect
Anastrozole	0.20	2%	8%	92 ± 7%	−11 ± 20%
Andarine	0.08	12%	28%	89 ± 18%	−19 ± 8%
Boldenone	0.15	5%	9%	80 ± 7%	−18 ± 15%
Clenbuterol	0.03	4%	7%	74 ± 8%	−17 ± 8%
Clomifene	0.85	2%	19%	44 ± 3%	88 ± 82%
DHCMT	1.17	10%	15%	80 ± 1%	23 ± 34%
Furosemide	0.20	9%	16%	65 ± 10%	−3 ± 22%
Hydrochlorothiazide	0.20	5%	13%	80 ± 11%	10 ± 8%
Ibutamoren	0.25	3%	4%	61 ± 3%	−8 ± 28%
Letrozole	0.20	10%	23%	86 ± 20%	−29 ± 11%
Metandienone	0.20	1%	5%	83 ± 9%	2 ± 25%
Metenolone	0.58	3%	9%	77 ± 8%	−12 ± 23%
Nandrolone	0.58	3%	10%	77 ± 9%	−21 ± 17%
Ostarine	0.23	10%	24%	77 ± 13%	−27 ± 7%
Oxandrolone	2.25	6%	17%	82 ± 15%	2 ± 18%
Stanozolol	0.23	3%	18%	64 ± 4%	−39 ± 1%
4β-hydroxy-stanozolol	0.23	1%	10%	73 ± 4%	−20 ± 12%
16β-hydroxy-stanozolol	0.42	2%	10%	66 ± 4%	−21 ± 12%
Tamoxifen	1.90	2%	12%	51 ± 3%	159 ± 116%
Terbutaline	0.75	1%	7%	82 ± 7%	−67 ± 12%
Trenbolone	0.58	2%	5%	70 ± 7%	−9 ± 24%

Note: DBS, dried blood spot; LOD, limit of detection.

TABLE 2b Main DBS method validation results: Tasso-M20.

Analyte	LOD (ng/mL)	Intra-day	Recovery	Matrix effect
Anastrozole	0.50*	3%	104 ± 23%	−23 ± 5%
Andarine	0.22	3%	90 ± 15%	−89 ± 3%
Boldenone	0.13*	2%	104 ± 23%	−21 ± 8%
Clenbuterol	0.03	2%	96 ± 18%	−30 ± 4%
Clomifene	0.50*	17%	88 ± 16%	NA
DHCMT	0.23	6%	108 ± 25%	−39 ± 11%
Furosemide	0.50*	5%	89 ± 13%	−36 ± 6%
Hydrochlorothiazide	0.50*	7%	95 ± 18%	−9 ± 10%
Ibutamoren	0.50	9%	37 ± 10%	65 ± 22%
Letrozole	0.50*	3%	96 ± 17%	−73 ± 3%
Metandienone	0.06	3%	102 ± 24%	−27 ± 6%
Metenolone	0.13*	4%	98 ± 16%	−13 ± 6%
Nandrolone	0.13*	3%	100 ± 17%	−12 ± 6%
Ostarine	0.42	4%	84 ± 28%	−85 ± 12%
Oxandrolone	0.54	5%	108 ± 24%	−21 ± 20%
Stanozolol	0.50*	2%	86 ± 8%	−63 ± 4%
4β-hydroxy-stanozolol	0.09	3%	93 ± 7%	−35 ± 5%
16β-hydroxy-stanozolol	0.10	3%	87 ± 5%	−39 ± 10%
Tamoxifen	0.50*	20%	99 ± 20%	NA
Terbutaline	0.93	6%	52 ± 6%	−20 ± 28%
Trenbolone	0.23	2%	88 ± 20%	−22 ± 8%

Note: DBS, dried blood spot.
*Indicated is the lowest concentration level tested. NA = not possible to get a reliable result because of high ion enhancement.

ratio of 3 and a 95% detection rate of the different analytes, ranged from 0.03 to 2.25 ng/mL for DMPK-C (Table 2a). The LODs for Tasso-M20 ranged from 0.03 to 0.54 ng/mL (Table 2b).

Because DBS testing has been included in the anti-doping testing program for a comparably short period, no MRPLs have yet been established for DBS. However, our obtained detection limits are within the recently proposed tentative laboratory performance levels, which were based on relevant therapeutic plasma concentration ranges, DBS detection limits, and administration results in the literature.[3] Thus, the obtained analytical sensitivity in 20 µL blood was regarded satisfactory for anti-doping purposes for both DMPK-C and Tasso-M20.

3.3.3 | Precision and robustness

Precision and robustness were investigated on four concentration levels; however, only the results from the highest concentration level are shown in Table 2a. The intra-day precision was high for most analytes in both matrices (range 1% to 17% RSD), as illustrated in Tables 2a and 2b. The inter-day precision (DMPK-C only) was slightly lower for several analytes (range: 4% to 28% RSD). A similar variation is also observed for the screening method from urine. Nonetheless, the precision was considered satisfactory given the qualitative analytical approach.

3.3.4 | Recovery

Different extraction solvents and solvent mixtures were tested, and the selected mixture of ACN:MeOH (50:50 *v*/*v*) yielded the best recoveries for both DMPK-C and Tasso-M20. The extraction recoveries of the different analytes are listed in Tables 2a and 2b. For the DMPK-C card (Table 2a), clomifene and tamoxifen had the lowest recoveries (44–51%), whereas all others had recoveries above 60%. For the Tasso-M20 device, ibutamoren and terbutaline had the lowest recoveries (37–52%), whereas the other analytes had recoveries above 80%. A similar variation in extraction recoveries is also observed for the screening method from urine. As a comparison, Thomas et al. extracted the DBS samples (DMPK-C, 20 µL per spot) twice using ultrasonication, first with a mixture of methanol and TBME and subsequently with acetone. Method validation yielded corresponding recoveries in the range 58–77%.[25] More recently, Chang et al. extracted the volumetric dried blood tips (Mitra device, Neoteryx, USA, 20 µL per tip) in water and TBME using ultrasound-assisted extraction followed by liquid–liquid extraction, which yielded recoveries between 43% and 74% for the anabolic steroids that are also included in our testing menu.[12] The Tasso-M20 device and the Mitra device are both volumetric microsampling devices constituted by porous polymer tips, and Mazzarino et al. obtained similar results for the two microsampling matrices despite differences in the polymer substrates.[11]

3.3.5 | Matrix effects

Varying ion suppression was observed for the analytes. For DMPK-C, terbutaline experienced the highest ion suppression (Table 2a), whereas for Tasso-M20, andarine and ostarine experienced the highest ion suppression (Table 2b). The highest ion enhancements were observed for tamoxifen and clomifene in DBS from both devices (Tables 2a and 2b).

3.3.6 | Carryover

No carryover effect was observed for any of the analytes in the subsequent blank sample after the analysis of a high concentration sample.

3.4 | Applicability study with real-life doping control samples

To our knowledge, this is the first study to compare the detectability of prohibited substances in DBS and urine samples collected in a doping control setting. The analytical results are presented in Figure 1 and Table 3.

3.4.1 | Comparison of urine and DBS analytical results

For a fair comparison, only analytes included in the DBS testing menu were evaluated when comparing the urine and DBS analytical results. However, as metabolites for most of the parent compounds included in the DBS menu are part of the urine method, findings for only metabolites in urine samples were also included. Twenty-seven (27) of the urine samples and 23 of the DBS samples were positive samples, and one DBS sample was atypical (Figure 1a,b).

We observed concordance between urine and DBS in 95% (96/101) of the overall analytical results (positive vs. negative samples; Figure 1c). Four of the urine samples with analytical findings (samples 24 to 27) returned negative results for the paired DBS samples (Figure 1c and Table 3). Further, when evaluating the individual analytical findings (analyte level) and not only the overall analytical results, nine urine samples (10 if including sample pair 30 with terbutaline) contained one or more prohibited substances that were not detected in the matched DBS samples. Nonetheless, the paired DBS samples still lead to positive samples as they contained detectable levels of other prohibited substances on the DBS menu (Table 3). If conducting doping controls with DBS only, these DBS samples would thus still have resulted in doping sanctions.

Noteworthy, the substances detected in the urine samples but not in the accompanying DBS were either parent compounds or metabolites of low concentrations, which could indicate that the sample collection took place during the tail end of the excretion curve or after administration of low doses: For example, four samples (samples 1, 7, 13, and 24) contained trenbolone metabolites (1.7–8.7 ng/mL), two samples (samples 6 and 27) contained DHCMT metabolites, three samples (samples 12, 20, and 26) contained metandienone metabolites (≤ 71 ng/mL), and one sample (sample 17) contained clomifene (0.4 ng/mL) (Table 3). As discussed in the Sections 3.4.2 and 3.4.3, this information is of relevance when anti-doping authorities implement DBS testing in their testing programs and evaluate to which extent doping controls can be conducted with DBS only. In forthcoming work, the collected DBS samples will be exploited to evaluate metabolite patterns for inclusion of relevant metabolites in the DBS testing menu. This could potentially prolong the detection windows in DBS for some substances, although the detectability will still depend on the doses administered and the time between sample collection and administration.

Furthermore, the inclusion of other relevant substances in the DBS testing menu will be explored. In Table 3, only analytical findings included in the DBS testing menu are presented. Noteworthy, four urine samples were positive based on GC/C/IRMS results or detection of methasterone and oxymetholone only (data not shown), which were not part of the DBS menu, whereas three of the urine samples (samples 24 to 26) contained a combination of substances that were

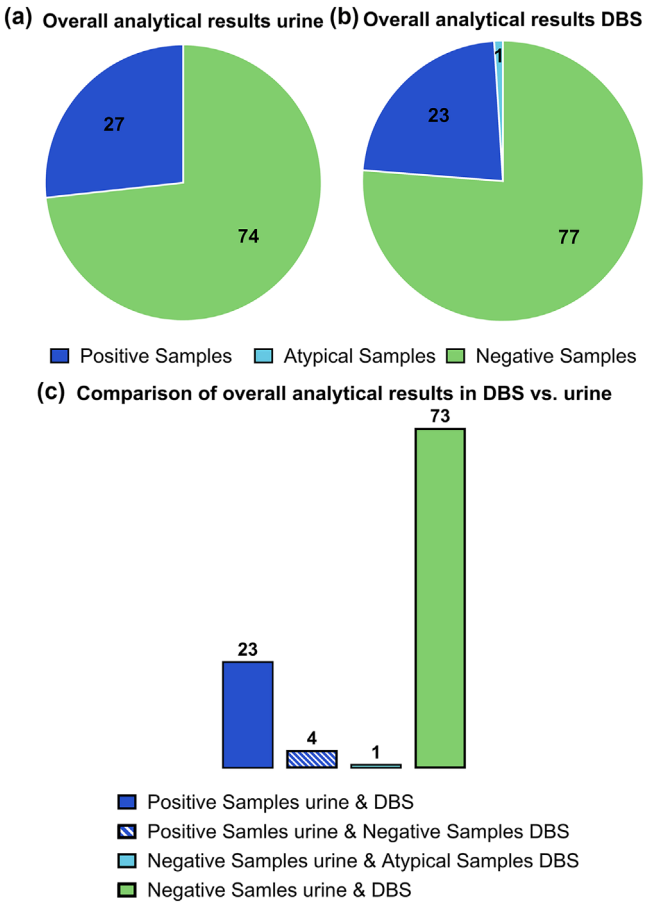

FIGURE 1 Summary of the overall analytical results in (a) urine and (b) DBS and comparison of the (c) overall analytical results. DBS, dried blood spot.

TABLE 3 Comparison of urine and DBS analytical results.

Sample pair	Findings urine	Target analytes in urine	Estimated conc. urine (ng/mL)*	Findings DBS	Estimated conc. DBS (ng/mL)*	Overall analytical result urine	Overall analytical result DBS
1	Boldenone (S1)	Boldenone	2163	Boldenone (S1)	15	Positive sample	Positive sample
	Boldenone metabolite (S1)	Boldenone M (5β-androst-1-en-17β-ol-3-one)	2414				
	Metandienone metabolites (S1)	5β-THMT	20	Metandienone (S1)	0.7		
		Epimetandienone	1				
		Epimetendiol	0.4				
		6β-hydroxy-metandienone	0.4				
		Metandienon M3 (17β-hydroxymethyl-17α-methyl-18-norandrosta-1,4,13-trien-3-one)	Pos				
	Nandrolone metabolites (S1)[C]	19-NA (19-norandrosterone)[C]	9709	Nandrolone (S1)	16		
		19-NE (19-noretiocholanolone)[C]	2937				
	Tamoxifen metabolites (S4)	Tamoxifen M (3-hydroxy-4-methoxytamoxifen)	4	Tamoxifen (S4)	12		
		Tamoxifen M2 (carboxy-tamoxifen)	Pos				
	Trenbolone metabolite (S1)	Epitrenbolone	8.7				
2	Clenbuterol (S1)[C]	Clenbuterol[C]	17	Clenbuterol (S1)	1.6	Positive sample	Positive sample
	Letrozole (S4)	Letrozole	51	Letrozole (S4)	41		
	Letrozole metabolite (S4)	Letrozole M (bis-4-cyanophenylmetanol)	140				
	Nandrolone metabolites (S1)	19-NA (19-norandrosterone)	17	Nandrolone (S1)	1.4		
		19-NE (19-noretiocholanolone)	3.5				
	Tamoxifen metabolites (S4)	Tamoxifen M (3-hydroxy-4-methoxytamoxifen)	14	Tamoxifen (S4)	144		
		Tamoxifen M2 (carboxy-tamoxifen)	Pos				
3	Ostarine (S1)	Ostarine	4.9	Ostarine (S1)	29	Positive sample	Positive sample
	Ostarine metabolites (S1)	Ostarine glucuronide	Pos				
		Hydroxyostarine glucuronide	Pos				
4	Boldenone (S1)	Boldenone	10			Positive sample	Positive sample
	Boldenone metabolite (S1)	Boldenone M (5β-androst-1-en-17β-ol-3-one)	54				
	Metandienone metabolites (S1)[C]	Epimetandienone[C]	1403	Metandienone (S1)	55		
		Epimetendiol[C]	435				
		5β-THMT[C]	27				

(Continues)

TABLE 3 (Continued)

Sample pair	Findings urine	Target analytes in urine	Estimated conc. urine (ng/mL)*	Findings DBS	Estimated conc. DBS (ng/mL)*	Overall analytical result urine	Overall analytical result DBS
		Metandienone M3 (17β-hydroxymethyl-17α-methyl-18-norandrosta-1,4,13-trien-3-one)	Pos				
	Stanozolol (S1)	Stanozolol	207	Stanozolol (S1)	7.7		
	Stanozolol metabolites (S1)	16β-hydroxy-stanozolol	259	4β-hydroxy-stanozolol (S1)	0.7		
		3′hydroxy-stanozolol	148				
		4β-hydroxy-stanozolol	38				
5	Nandrolone metabolites (S1)[c]	19-NA (19-norandrosterone)[c]	2391	Nandrolone (S1)	8.9	Positive sample	Positive sample
		19-NE (19-noretiocholanolone)[c]	216				
6	Boldenone (S1)	Boldenone	23			Positive sample	Positive sample
	Boldenone metabolite (S1)	Boldenone M (5β-androst-1-en-17β-ol-3-one)	29				
	DHCMT metabolites (S1)	DHCMT M3 (4-chloro-18-nor-17β-hydroxymethyl,17a-methyl-5b-androsta-1,13-dien-3a-ol)	Pos				
		DHCMT M4 (4-chloro-18-nor-17β-hydroxymethyl,17α-methyl-5α-androsta-13-en-3α-ol)	Pos				
	Nandrolone metabolites (S1)	19-NA (19-norandrosterone)	16				
		19-NE (19-noretiocholanolone)	6				
	Trenbolone metabolite (S1)	Epitrenbolone	1316	Trenbolone (S1)	23		
7	Clenbuterol (S1)	Clenbuterol	8.3	Clenbuterol (S1)	0.9	Positive sample	Positive sample
	Nandrolone metabolites (S1)	19-NA (19-norandrosterone)	13				
		19-NE (19-noretiocholanolone)	5.9				
	Trenbolone metabolite (S1)	Epitrenbolone	3.6				
8	Anastrozole (S4)[c]	Anastrozole[c]	6.4	Anastrozole (S4)	1.1	Positive sample	Positive sample
9	Boldenone (S1)	Boldenone	400	Boldenone (S1)	5.2	Positive sample	Positive sample
	Boldenone metabolite (S1)	Boldenone M (5β-androst-1-en-17β-ol-3-one)	156				
	Letrozole (S4)	Letrozole	1.6	Letrozole (S4)	0.1		
	Letrozole metabolite (S4)	Letrozole M (bis-4-cyanophenylmetanol)	8.6				
	Nandrolone metabolites (S1)[c]	19-NA (19-norandrosterone)[c]	3149	Nandrolone (S1)	14		

TABLE 3 (Continued)

Sample pair	Findings urine	Target analytes in urine	Estimated conc. urine (ng/mL)*	Findings DBS	Estimated conc. DBS (ng/mL)*	Overall analytical result urine	Overall analytical result DBS
		19-NE (19-noretiocholanolone)	2225				
	Trenbolone metabolite (S1)	Epitrenbolone	226	Trenbolone (S1)	1.6		
10	Metandienone metabolites (S1)[C]	Epimetandienone[C]	4884	Metandienone (S1)	52	Positive sample	Positive sample
		6β-hydroxy-metandienone[C]	3250				
		Epimetendiol[C]	901				
		5β-THMT[C]	358				
		Metandienone M3 (17β-hydroxymethyl-17α-methyl-18-norandrosta-1,4,13-trien-3-one)[C]	Pos				
	Trenbolone metabolite (S1)	Epitrenbolone	314	Trenbolone (S1)	2.4		
11	Boldenone (S1)[C]	Boldenone[C]	2739	Boldenone (S1)	13	Positive sample	Positive sample
	Boldenone metabolite (S1)	Boldenone M (5β-androst-1-en-17β-ol-3-one)[C]	1374				
	Tamoxifen metabolites (S4)	Tamoxifen M (3-hydroxy-4-methoxytamoxifen)	320	Tamoxifen (S4)	Pos		
		Tamoxifen M2 (carboxy-tamoxifen)	Pos				
12	Anastrozole (S4)	Anastrozole	94	Anastrozole (S4)	15	Positive sample	Positive sample
	Metandienone metabolite (S1)	Metandienone M3 (17β-hydroxymethyl-17α-methyl-18-norandrosta-1,4,13-trien-3-one)	Pos				
	Trenbolone (S1)[C]	Trenbolone[C]	302	Trenbolone (S1)	7.5		
	Trenbolone metabolite (S1)[C]	Epitrenbolone[C]	1014				
13	Stanozolol (S1)	Stanozolol	0.8			Positive sample	Positive sample
	Stanozolol metabolites (S1)	16β-hydroxy-stanozolol	5.3	16β-hydroxy-stanozolol (S1)	0.1		
		3′hydroxy-stanozolol	2.4	4β-hydroxy-stanozolol (S1)	0.2		
	Trenbolone (S1)[C]	Trenbolone[C]	0.1				
	Trenbolone metabolite (S1)[C]	Epitrenbolone[C]	1.8				

(Continues)

TABLE 3 (Continued)

Sample pair	Findings urine	Target analytes in urine	Estimated conc. urine (ng/mL)*	Findings DBS	Estimated conc. DBS (ng/mL)*	Overall analytical result urine	Overall analytical result DBS
14	Boldenone (S1)	Boldenone	15	Boldenone (S1)	0.1	Positive sample	Positive sample
	Boldenone metabolite (S1)	Boldenone M (5β-androst-1-en-17β-ol-3-one)	15				
	Nandrolone metabolites (S1)[C]	19-NA (19-norandrosterone)[C]	17897	Nandrolone (S1)	12		
		19-NE (19-noretiocholanolone)	6000				
	Trenbolone metabolites (S1)	Epitrenbolone	901	Trenbolone (S1)	6.2		
15	Clenbuterol (S1)	Clenbuterol	83	Clenbuterol (S1)	0.6	Positive sample	Positive sample
16	Boldenone (S1)[C]	Boldenone[C]	8196	Boldenone (S1)	30	Positive sample	Positive sample
	Boldenone metabolite (S1)[C]	Boldenone M (5β-androst-1-en-17β-ol-3-one)[C]	1685				
	Metandienone metabolites (S1)	5β-THMT	179	Metandienone (S1)	1.7		
		6β-hydroxy-metandienone	140				
		Epimetendiol	106				
		Epimetandienone	86				
		Metandienone M3 (17β-hydroxymethyl-17α-methyl-18-norandrosta-1,4,13-trien-3-one)	Pos				
	Stanozolol metabolites (S1)	16β-hydroxy-stanozolol	49	Stanozolol (S1)	3.6		
		3′-hydroxy-stanozolol	18				
	Trenbolone metabolite (S1)	Epitrenbolone	502	Trenbolone (S1)	9.6		
17	Boldenone (S1)[C]	Boldenone[C]	3970	Boldenone (S1)	19	Positive sample	Positive sample
	Boldenone metabolite (S1)[C]	Boldenone M (5β-androst-1-en-17β-ol-3-one)[C]	2231				
	Clomifene (S4)	Clomifene	0.4				
	Clomifene metabolites (S4)	M19 (4-hydroxy-clomifene)	Traces				
		M20 (3-hydroxy-4-methoxy-clomifene)	Pos				
18	Boldenone (S1)	Boldenone	3.8	Boldenone (S1)	0.3	Positive sample	Positive sample
	Boldenone metabolite (S1)	Boldenone M (5β-androst-1-en-17β-ol-3-one)	1.9				
	Nandrolone metabolites (S1)	19-NA (19-norandrosterone)	31000	Nandrolone (S1)	15		
		19-NE (19-noretiocholanolone)	11000				
19	Nandrolone metabolites (S1)	19-NA (19-norandrosterone)	122			Positive sample	Positive sample
		19-NE (19-noretiocholanolone)	60				

TABLE 3 (Continued)

Sample pair	Findings urine	Target analytes in urine	Estimated conc. urine (ng/mL)*	Findings DBS	Estimated conc. DBS (ng/mL)*	Overall analytical result urine	Overall analytical result DBS
	Trenbolone metabolite (S1)	Epitrenbolone	433	Trenbolone (S1)	2.1		
20	DHCMT (S1) [C]	DHCMT[C]	350	DHCMT (S1)	9.0	Positive sample	Positive sample
	DHCMT metabolites (S1)	DHCMT M (1 6β,17β-dihydroxy-4-chloro-17α-methylandrosta-1,4-dien-3-one)	Pos				
		DHCMT M2 (4-chloro-17a-methyl-5b-androstan-3a,16,17b-triol)	Pos				
		DHCMT M3 (4-chloro-18-nor-17β-hydroxymethyl,17a-methyl-5b-androsta-1,13-dien-3a-ol)	Pos				
		DHCMT M4 (4-chloro-18-nor-17β-hydroxymethyl,17α-methyl-5α-androsta-13-en-3α-ol)	Pos				
		5β-THMT	71				
	Metandienone metabolites (S1)	5β-THMT	71				
		Epimetandienone	2.1				
		Epimetendiol	1.9				
		Metandienone M3 (17β-hydroxymethyl-17α-methyl-18-norandrosta-1,4,13-trien-3-one)	Pos				
21	Stanozolol (S1)	Stanozolol	31	Stanozolol (S1)	16	Positive sample	Positive sample
	Stanozolol metabolites (S1)[C]	16β-hydroxy-stanozolol[C]	69				
		3′hydroxy-stanozolol	36				
		4β-hydroxy-stanozolol	15	4β-hydroxy-stanozolol	0.4		
22	Metenolone (S1)[C]	Metenolone	1015	Metenolone (S1)	13	Positive sample	Positive sample
	Metenolone metabolite (S1)[C]	Metenolone M (3α-hydroxy-1-methylene-5α-androstan-17-one)[C]	254				
23	Anastrozole (S4)	Anastrozole	97	Anastrozole (S4)	9.2	Positive sample	Positive sample
	Boldenone (S1)[C]	Boldenone[C]	15558	Boldenone (S1)	48		
	Boldenone metabolite (S1)[C]	Boldenone M (5β-androst-1-en-17β-ol-3-one)[C]	1267				
	Nandrolone metabolites (S1)	19-NA (19-norandrosterone)	4240	Nandrolone (S1)	5.7		
		19-NE (19-noretiocholanolone)	828				
	Trenbolone metabolite (S1)	Epitrenbolone	1600	Trenbolone (S1)	20		

(Continues)

TABLE 3 (Continued)

Sample pair	Findings urine	Target analytes in urine	Estimated conc. urine (ng/mL)*	Findings DBS	Estimated conc. DBS (ng/mL)*	Overall analytical result urine	Overall analytical result DBS
24	Boldenone (S1)	Boldenone	2.3			Positive sample	Negative sample
	Boldenone metabolite (S1)	Boldenone M (5β-androst-1-en-17β-ol-3-one)	2.1				
	Trenbolone metabolite (S1)	Epitrenbolone	1.7				
25	Nandrolone metabolites (S1)	19-NA (19-norandrosterone)	19			Positive sample	Negative sample
		19-NE (19-noretiocholanolone)	4				
	Tamoxifen metabolites (S4)	Tamoxifen M (3-hydroxy-4-methoxytamoxifen)	166				
		Tamoxifen M2 (carboxy-tamoxifen)	Pos				
26	Metandienone metabolite (S1)	Metandienone M3 (17β-hydroxymethyl-17α-methyl-18-norandrosta-1,4,13-trien-3-one)	Pos			Positive sample	Negative sample
27	DHCMT metabolites (S1)[C]	DHCMT M4 (4-chloro-18-nor-17β-hydroxymethyl,17α-methyl-5α-androsta-13-en-3α-ol)[C]	Pos			Positive sample	Negative sample
		DHCMT M2 (4-chloro-17a-methyl-5b-androstan-3a,16,17b-triol)	Pos				
28				Boldenone (S1)	0.6	Negative sample	Atypical sample[§]
29	Terbutaline (S3)	Terbutaline	34	Terbutaline (S3)	0.6	Negative sample[#]	Negative sample[#]
30	Terbutaline (S3)	Terbutaline	15			Negative sample[#]	Negative sample
31	Tamoxifen metabolite (S4)	Tamoxifen M (3-hydroxy-4-methoxytamoxifen)	1.3	Tamoxifen (S4)	0.5	Negative sample[#]	Negative sample[#]
		Tamoxifen M2 (carboxy-tamoxifen)	Pos				
	Terbutaline (S3)	Terbutaline	153	Terbutaline (S3)	3.2		
32	Tamoxifen metabolites (S4)[C]	Tamoxifen M (3-hydroxy-4-methoxytamoxifen)[C]	521	Tamoxifen (S4)	56	Negative sample[#]	Negative sample[#]
		Tamoxifen M2 (carboxy-tamoxifen)	Pos				

Note: C, confirmed finding, DBS, dried blood spot; DHCMT, dehydrochloromethyltestosterone, MRL, minimum reporting level; Pos, the analyte was detected but the concentration could not be calculated; TD IRMS, Technical document for isotope-ratio mass spectrometry; TD MRPL, Technical document on minimum required performance level; WADA, World Anti-Doping Agency.

*Please note that for many analytes, the findings in urine are based on metabolites and not the presence of parent compound, whereas for DBS all analytes are screened for parent compound only. The concentrations were estimated using a single point calibrator (SPC). The reported amounts in urine and DBS should be compared with caution because the urine concentrations are estimated based on a combination of glucuronides and free fraction, whereas the reported DBS concentrations are estimated based on free fraction only.

[§]Estimated amount in DBS is below the MRL for boldenone in urine.

[#]Finding that is not prohibited according to the Danish "Act Prohibiting Certain Doping Substances" in Danish fitness centers but would result in an AAF/Presumptive Adverse Analytical Finding according to WADA regulations (the Prohibited List, the TD MRPL and the TD IRMS) applicable for urine sample in elite sports.

not detected (i.e., metabolite of metandienone, metabolite of nandrolone and tamoxifen, and metabolite of trenbolone, respectively) and substances not analyzed (i.e., desoxymethyltestosterone, oxymetholone and methasterone, drostanolone, and drostanolone, respectively) in the DBS samples. In total, 18 urine samples contained drostanolone, which was part of the DBS testing menu during method development but excluded because of unsatisfactory validation results. Based on these observations, a future step would be to expand the DBS testing menu for fitness samples to include desoxymethyltestosterone, drostanolone, methasterone, and oxymetholone (all S1), as well as develop an extensive steroid ester menu. In this regard, Jing et al. recently proposed a fully automated DBS sample preparation and detection method for 13 anabolic steroid esters.[19] Further, because the development of the present multi-analyte screening method, considerable anti-doping research and development work into DBS testing and analyses have been carried out,[8,19,28–37] including the development of comprehensive multi-analyte screening methods covering all substance classes on the WADA Prohibited List.[11,26]

The technical document for DBS in force at the time of analysis (TD2021DBS) only covered non-threshold substances without MRLs.[1] Despite being subject to an MRL, boldenone, nandrolone, and clenbuterol were still included in the DBS menu as they are frequently used doping substances in fitness centers, and we wanted to compare the detectability of these in urine versus DBS. Interestingly, one of the DBS samples (sample 28) contained boldenone (0.6 ng/mL), whereas boldenone or metabolite were not detected in the paired urine sample (Table 3 and Figure 1c). As a future step, including boldenone esters in the steroid ester menu can help verify the origin (exogenous or endogenous) of boldenone in DBS.

3.4.2 | Applicability of the developed DBS testing menu in fitness centers

Based on our findings, DBS as matrix and the developed DBS testing menu seem to be highly applicable for doping controls with collection of only DBS in fitness centers, where the goal of doping controls is to remove potentially unhealthy and inappropriate role models, and thereby primarily active users of doping agents.

3.4.3 | Applicability of the developed DBS testing menu in elite sports

The results herein represent samples collected in fitness centers analyzed with a tailormade and limited DBS screening menu containing mainly anabolic agents. Therefore, caution should be taken when interpreting the results and extrapolating the applicability of the developed menu to elite sports. Here, lower doses are expected to be administered than among fitness center customers, and our results are, thus, likely not directly transferable. Further, in urine, we can detect a large number of metabolites, some of which have very long detection windows. This may not be the case in DBS, and we need more data regarding metabolite patterns in blood.

In total, we observed 69 analytical findings in urine and 50 analytical findings in DBS when interpreting the analytical results according to the WADA regulations in elite sports (Table 3). These results are very promising, but whether to conduct doping controls with collection of DBS only or in addition to urine and/or venous blood must likely be very carefully evaluated if extending the observed results to elite athletes in disciplines where the developed DBS testing menu may be relevant (e.g., power/strength sports). Here, the doses administered are expected to be lower than among fitness center customers, which reduces the analyte detectability. Nevertheless, DBS testing has the potential to improve the time and cost efficiency in anti-doping and thereby allows for higher frequency of testing and rapid mass testing/whole-team testing, which could increase detection and deterrence. Therefore, the pros and cons of using DBS as a stand-alone matrix must be thoroughly considered when anti-doping authorities plan efficient testing programs.

Noteworthy, urine samples collected in-competition do not necessarily reflect whether an athlete competed under the systemic influence of a prohibited substance. Urine is a liquid by-product of metabolism to which substances accumulate before excretion, while blood—and hence, DBS—on the contrary enables the determination of circulating concentrations at the time of competition. DBS is therefore well suited as an in-competition test for physiologically relevant levels of substances only prohibited in competition either alone or combined with urine to support anti-doping authorities in the results management and decision-making process.[17] Future studies should compare the analytical results of real, matched DBS and urine samples from athletes, and administration studies are necessary to establish limits for substances prohibited in competition only.

4 | CONCLUSION

Herein, we developed a feasible, sensitive, and robust method for simultaneous detection of 19 selected prohibited substances from S1 to S5 in DBS, and, to our knowledge, this is the first study to compare the detectability of these prohibited substances in paired DBS and urine samples collected in a doping control setting. We observed promising results, with a very good (95%) agreement between the overall analytical results (positive vs. negative samples) of the 101 matched urine and upper-arm DBS samples collected from fitness center customers and patients. The substances detected in the urine samples but not in the paired DBS samples—a total of 19 findings—were detected at low concentrations and/or by metabolites only in the urine samples. Thus, the developed DBS testing menu seems to have sufficient sensitivity to detect recent administrations/doping use, and thereby be applicable for detection of doping in fitness centers. As a next step for use in elite sports, metabolite patterns in DBS and the inclusion of metabolites and additional relevant analytes in the DBS testing menu should be explored, followed by comparison of

the analytical results of real sample pairs collected during doping controls in sports.

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CONFLICT OF INTEREST STATEMENT

Sara A. Solheim and Jakob Mørkeberg were employed by one of the funding organizations, Anti Doping Denmark, during the sample collection and the preparation of this manuscript.

DATA AVAILABILITY STATEMENT

The data are available on request because of privacy/ethical restrictions.

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