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RESEARCH ARTICLE

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# Optimizing detection of erythropoietin receptor agonists from dried blood spots for anti-doping application

Carmel E. Heiland[1,2] | Magnus Ericsson[1,3] | Anton Pohanka[1,2] | Lena Ekström[1,2] | Alexandre Marchand[3]

[1]Department of Laboratory Medicine, Karolinska Institute, Stockholm, Sweden

[2]Clinical Pharmacology, Karolinska University Laboratory, Karolinska University Hospital, Stockholm, Sweden

[3]Laboratoire AntiDopage Français (LADF), University of Paris-Saclay, Châtenay-Malabry, France

**Correspondence**
Carmel E. Heiland, Department of Laboratory Medicine, Karolinska Institute, Stockholm, Sweden.
Email: carmel.heiland@ki.se

**Funding information**
Karolinska University Hospital Clinical Pharmacology department

## Abstract

The World Anti-Doping Agency (WADA) has recently implemented dried blood spots (DBSs) as a matrix for doping control. However, specifications regarding the analysis of the class of prohibited substances called erythropoietin (EPO) receptor agonists (ERAs) from DBSs are not yet described. The aim of this study was to find optimal conditions (sample volume and storage) to sensitively detect endogenous erythropoietin (hEPO) and prohibited ERAs from DBSs and compare detection limits to WADA-stipulated minimum required performance levels (MRPLs) for ERAs in serum/plasma samples. Venous whole blood was spotted onto Whatman 903 DBS cards with primarily 60 μl of blood, but various volumes from 20 to75 μl were tested. All samples were immunopurified with MAIIA EPO Purification Gel kit (EPGK) and analysed with sodium *N*-lauroylsarcosinate polyacrylamide gel electrophoresis (SAR-PAGE) and Western blot. Sixty-microliter DBSs allowed the detection of the four main ERAs (BRP, NESP, CERA and EPO-Fc) at concentrations close to WADA's MRPLs described for 500 μl of serum/plasma. Different storage temperatures, from −20°C to 37°C, were evaluated and did not affect ERA detection. A comparison of the detection of endogenous EPO from the different anti-doping matrices (urine, serum and DBSs produced from upper arm capillary blood) from five participants for 6 weeks was performed. Endogenous EPO extracted from DBSs showed intra-individual variations in male and female subjects, but less than in urine. Doping controls would benefit from the stability of ERAs on DBSs: It can be a complementary matrix for ERA analysis, particularly in the absence of EPO signals in urine.

**KEYWORDS**
DBS, doping control, EPO, SAR-PAGE, volumetric blood sampling device

## 1 | INTRODUCTION

Erythropoietin (EPO) is the main circulating hormone regulating red blood cell count by promoting differentiation and maturation of erythroid cells into reticulocytes in the bone marrow in response to hypoxic conditions.[1–4] Therapeutic drugs based on endogenous EPO (hEPO) called erythropoietin receptor agonists (ERAs) were introduced to the drug market in the 1980s[5] and are still used nowadays to treat various anaemic conditions (e.g., chronic kidney disease, cancer-associated anaemia and HIV-associated anaemia) due to their

ability to bind to the EPO receptor and activate erythropoiesis.[3] First-generation ERAs (known as recombinant EPOs [rEPOs], e.g., epoetin alpha and epoetin beta) were produced after cloning the human EPO gene in mammalian cells.[3,5–7] New generations of ERAs with additional modifications followed: first by increasing the number of glycosylations (darbepoetin α, NESP),[8] adding pegylated moieties (e.g., methoxypolyethylene glycol epoetin beta, CERA),[6] or creating EPO fusion proteins (e.g., EPO-Fc).[9] These new generations of EPO-modified molecules have longer half-lives and require less frequent administration for treatment. Concomitant to the appearance of rEPO drugs, the sports scene saw the improvement of athletes' endurance and an increase in the abuse of these drugs in the 90s–2000s, before the implementation of a detection method in the anti-doping laboratories. Although prohibited by the World Anti-Doping Agency (WADA),[10] rEPOs and the following generations of ERAs are still used by some athletes and need constant surveillance.

Although the staple doping control matrices are urine and serum, dried blood spots (DBSs) from capillary blood, which were introduced for phenylketonuria (PKU) detection in new-borns in the 1960s,[11] have been proposed as a complementary matrix useful for anti-doping. The limited volume collected on DBSs can be sufficient to detect substances of interest because of a stabilization of substances after the drying process and because of the increase in sensitivity of the detection techniques in recent years.[12–18] The benefits also include simplified collection of blood and the possibility to increase the frequency of collection, simplicity of transport and storage, and supplying an additional information source for doping testing.[19] Since the analysis of PKU in the 1960s, DBS collection has evolved from blood drops on filter papers, like Whatman® and FTA® cards that are still commonly used for therapeutic drug monitoring,[20,21] to complex collection devices, including volumetric devices using paper (e.g., Capitainer and Hemaxis), polymers (e.g., Mitra® or volumetric absorptive microsampling [VAMS] from Neoteryx, Tasso M-20 from Tasso Inc.), or blood reservoirs (e.g., TAP from SeventhSense Biosystems), that collect a specific volume and reduce haematocrit effects that can arise from using the common filter paper technique. These often collect 10–20 µl of blood, which may be sufficient for detecting many analytes,[14,17,18,22–26] but it may not suffice for ERAs, which, like hEPO, circulate at relatively low concentrations in blood,[2] thus requiring larger sample volumes (usually 10- to 15-ml urine and 0.5-ml serum) for sensitive detection. Also, with the spread of micro-doses among athletes, it is an additional analytical challenge to identify micro-doses of ERAs.[27–30] The development of semi-automated upper arm DBS collection devices (e.g., TAP: 100-µl blood and Tasso-M20: four spots of 20-µl blood) and some VAMS (e.g., Mitra: 20- to 30-µl blood) offers a potential solution to increase the dried blood volume collected for analysis. These devices have been tested as sample collection methods and have been well-received among athletes and doping control officers.[31]

Despite DBSs considered as unfeasible for ERA detection in 2016,[32] recent studies successfully used this matrix for ERA detection: a validated method for hEPO, rEPO, NESP and CERA detection from 25-µl modelled blood DBSs using polyacrylamide gel electrophoresis (PAGE) was described in 2018[15] and a method to successfully detect CERA from patients by using 20-µl modelled blood DBSs and a commercial ELISA kit in 2021.[33] However, in these studies, therapeutic doses of ERAs were investigated, a far cry from the minimum required performance levels (MRPLs) that need to be reached in blood for the four main generations of ERAs recently published by WADA.[34]

The aim of this study was to find optimal conditions for sensitive and specific ERA detection from DBSs and to evaluate if WADA's MRPLs could be reached using a single DBS for the four main ERAs (BRP, NESP, CERA and EPO-Fc). Various conditions were tested, including different DBS collection supports (Whatman, Tasso), different blood sample volumes (20–180 µl), detection limit evaluations for the four ERAs, and ERA stability for up to 6 days. We also compared endogenous EPO distribution in three matrices (DBSs, serum, and urine) from in vivo samples to demonstrate that the method is fit for purpose.

## 2 | MATERIALS AND METHODS

### 2.1 | Chemicals

Reference standards used were as follows: methoxypolyethylen-glycol-epoetin beta, MIRCERA (CERA; Roche, Basel Switzerland), darbepoetin alfa, Aranesp (Novel erythropoietin stimulating protein, NESP; Amgen; CA, USA), human EPO-alpha Fc (EPO-Fc; ProSpec; Rehovot, Israel), and biological reference protein (BRP—a reference substance for rEPOs; European Directorate for the Quality of Medicines and HealthCare, Strasbourg, France). Methanol was from J.T. Baker (NJ, USA). Bromophenol blue and Immobilon® ECL Ultra Western HRP Substrate were from Merck Millipore Ltd (MA, USA). Sodium *N*-lauroylsarcosinate (SAR), sodium dodecyl sulphate (SDS), 3-(*N*-morpholino)-propane sulfonic acid (MOPS), ethylenediaminetetraacetic acid (EDTA), glycine, glycerol, tris (hydroxymethyl)aminomethane and DL-dithiothreitol (DTT) were from Sigma-Aldrich (MO, USA). NuPAGE antioxidant and Gibco phosphate-buffered saline (PBS) tablets were purchased from Thermo Fisher Scientific (MA, USA). Powdered milk was purchased from Carl Roth (Karlsruhe, Germany). Extra thick blot paper was from Bio-Rad (CA, USA). Human EPO biotinylated antibody monoclonal mouse IgG2A clone #AE7A5 was from R&D Systems (MN, USA), and Streptavidin-POD conjugate was from Roche (Basel, Switzerland).

### 2.2 | Experimental set-up

#### 2.2.1 | In vitro samples

A venous whole blood pool (BD Vacutainer K2E EDTA tubes, Becton, Dickson and company, NJ, USA) from three anonymous volunteers was used for all in vitro DBS experiments described here. The DBS volume of venous blood pipetted on Whatman 903 cards

(GE Healthcare, IL, USA) was 60 μl, unless otherwise stated, and DBSs were air-dried for approximately 2 h before storage at room temperature (RT) in plastic bags with desiccant (unless otherwise stated). A urine pool of six anonymous volunteers was used for stability testing.

#### 2.2.2 | Sample volume

To compare different blood sample volumes for EPO detection capabilities, one to three DBSs were added to the MAIIA column and immunopurified together (one spot = 60 μl, two spots = 120 μl, and three spots = 180 μl; $n = 3$ for each volume). A second approach was taken by testing extraction from a single spot loaded with different non-spiked blood volumes (20, 25, 40, 50, 60 and 75 μl; $n = 3$ for each volume) or spiked blood volumes (25, 50 and 75 μl; $n = 3$ for each volume). Blood samples were spiked with the four ERAs: BRP 125 pg/ml (about 15 IU/L), NESP 25 pg/ml, CERA 1000 pg/ml and EPO-Fc 250 pg/ml.

#### 2.2.3 | Detection limit

To determine the lowest detectable concentration of the four ERAs in a 60-μl DBS, whole blood was spiked and diluted with blank blood in a twofold series. The concentrations analysed were 500, 250, 125 and 62.5 pg/ml whole blood for CERA ($n = 3$, analysed on three occasions); 248, 124, 62 and 31 pg/ml for EPO-Fc ($n = 3$, analysed on three occasions) and BRP (equivalent to about 30–4 IU/L, $n = 4$, analysed on two occasions); and 50.4, 25.2, 12.6 and 6.3 pg/ml for NESP ($n = 3$, analysed on three occasions).

#### 2.2.4 | Stability

To test the stability of EPO, blank and spiked whole blood (at concentrations known to be easily detectable: BRP 336 pg/ml [~40 IU/L], NESP 40 pg/ml, CERA 100 pg/ml, EPO-Fc 100 pg/ml) were pipetted onto Whatman 903 filter paper and stored at −20°C, 4°C, RT, and 37°C for approximately 40 h and for 6 days ($n = 5$ per temperature). A blank urine pool was stored in glass bottles at the same conditions as DBSs. Urine samples were filtered through HPF Millex PVDF 0.45-μm filters (Merck Millipore Ltd, MA, USA) into the immunopurification columns. To statistically assess the stability of the ERAs in DBSs over the storage period, CERA, EPO-Fc and NESP signal intensities were normalized to the BRP/hEPO signal intensity in each gel lane because data were spread over four membranes.

### 2.3 | In vivo samples

Urine, DBSs from capillary blood (using Tasso-M20 devices), and serum samples (collected in BD vacutainer SST, Becton, Dickson and company, NJ, USA) from five healthy participants (three males, two females; ages 26–35) were collected to evaluate EPO immunodetection from DBSs compared with other well-established matrices. All participants gave their written informed consent, and the collection was approved by the Swedish Ethical Review Authority (Ethical permit Dnr 2020-04258). Samples were collected over a 6-week period: Serum was collected on Weeks 1, 4 and 7 and centrifuged at 18,615*g* for 15 min; urine and capillary blood were collected once a week in the morning.

### 2.4 | EPO extraction and immunopurification

The EPO Purification Gel Kit (EPGK) for Blood (MAIIA Diagnostics, Uppsala, Sweden) was used for extraction and immunopurification as per the manufacturer's instructions, with minor modifications. Briefly, one whole DBS, cut from the Whatman 903 card with scissors (or three 20-μl Tasso DBSs), and 5 ml of sample buffer were added to the column with a funnel attached and incubated for 90 min end-over-end at RT. The flow through was filtered through the column, the column was washed, and hEPO and the ERAs were eluted with 200 μl of elution buffer (centrifugation at 500*g* for 1 min). The eluate was then concentrated to about 15 μl using an Amicon 30-kDa molecular weight cut-off (MWCO) filter (Merck Millipore Ltd, MA, USA; 14,000*g* for 30 min). Immunopurified samples were stored at −20°C until analysis.

This same method was used for 500 μl of serum samples from the in vivo study with the difference that the 35-μl eluate was transferred to a MWCO filter and centrifuged at 10,000*g* for 10 min, giving a final volume of about 25 μl. Immunopurified samples were stored at −20°C until analysis.

For urine samples, the EPGK for Urine (MAIIA Diagnostics, Uppsala, Sweden) was used. In short, sample buffer was mixed into 10 ml of sample and incubated for 10 min. Samples were incubated for 90 min end-over-end at RT. The column was washed, and hEPO and ERAs were eluted with 50 μl of elution buffer (centrifugation at 500*g* for 1 min). Immunopurified samples were stored at −20°C until analysis.

### 2.5 | SAR-PAGE and Western blotting

The same procedure was followed as previously described,[35] with minor differences. Briefly, all 15 μl of the concentrated DBS eluate (10 μl of serum and urine eluates), together with sample loading buffer, were loaded onto the NuPAGE 10% Bis-Tris polyacrylamide gel (Thermo Fisher Scientific, MA, USA). ERA proteins were separated for about 90 min at 140 V using SAR running buffer. ERAs were transferred to an Immobilon-P PVDF membrane (Merck Millipore Ltd, MA, USA) using a semi-dry transfer system (Cytiva Amersham TE77 PWR blotter, MA, USA), then the membrane was incubated in reducing conditions for 45 min. After rinsing the membrane in PBS, it was blocked in 5% milk and incubated overnight at 4°C in biotinylated anti-EPO

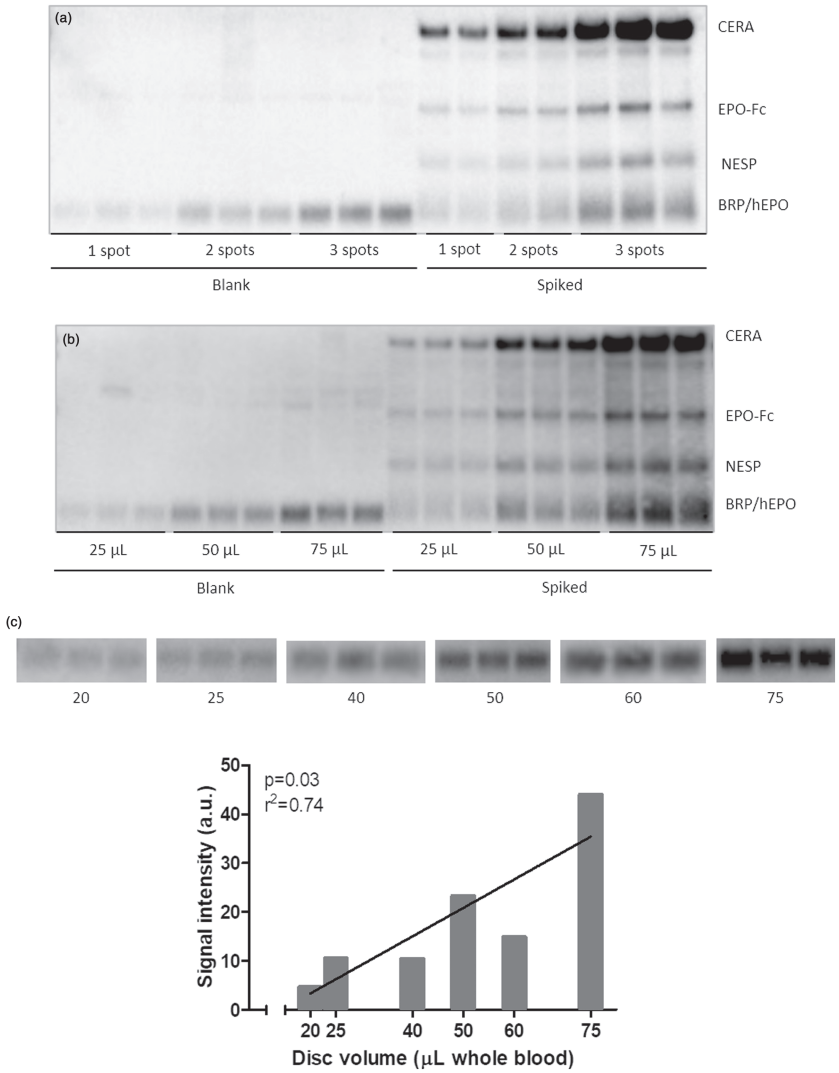


**FIGURE 1** Test of various sample volumes for detecting endogenous erythropoietin (hEPO) and erythropoietin receptor agonists (ERAs). Whole blood, blank or spiked with 125 pg biological reference protein (BRP) (~15 IU/L), 25 pg NESP, 1000 pg CERA, and 250 pg EPO-Fc per ml of blood, was pipetted onto Whatman dried blood spot (DBS) cards (60 µl/spot or 20, 25, 40, 50 or 75 µl/spot). Samples were analysed with sodium *N*-lauroylsarcosinate polyacrylamide gel electrophoresis (SAR-PAGE) and immunodetection. (a) hEPO and ERA signals obtained when increasing initial sample volume by the number of DBSs immunopurified simultaneously (one, two or three spots with 60 µl of blood). (b) hEPO and ERA signals obtained when increasing sample volume applied to a spot (25, 50 or 75 µl of blood). (c) Endogenous EPO signals obtained from 20 to 75 µl of whole blood on DBSs (triplicates per sample volume) at exposure time 60 s and graphic representation of the medians of signal intensities and linear regression calculations

antibody (0.5 µg/ml 1% milk), moved to RT to be washed in PBS, then incubated 1 h at RT in streptavidin-POD conjugate, and washed again. After enhanced chemiluminescence (ECL) substrate addition, images of the membrane were taken with a CCD camera (Bio-Rad, CA, USA), and results were analysed with Image Lab 6.0.1 (Bio-Rad, CA, USA) and GASepo 2.1 (Austrian Research Centers GmbH, Seibersdorf, Austria).

## 2.6 | Statistical analysis

To test the effects of different initial sample volumes on DBSs, Mann Whitney U tests and linear regression calculations were performed. To assess the stability of the ERAs, Mann Whitney U tests were used to determine significance of the signal intensities between the time points. *p* values were two-tailed, and significance was based on a *p* value ≤ 0.05. All statistical analyses were performed on the signal intensities of the EPO bands in GraphPad Prism 5.

# 3 | RESULTS

## 3.1 | Blood sample volume impact on ERA detection

To determine how the initial blood sample volume affects the ability to detect ERAs, experiments were done first by evaluating ERA detection in one to three spots of 60-µl, immunopurified to a single final eluate, then in one single DBS spotted with various blood volumes (Figure 1). ERA and hEPO signal intensities increased similarly with the number of simultaneously immunopurified spots (Figure 1a). A clear hEPO signal and all four ERAs were readily detected with a single spot of 60 µl. Single DBSs of 20–75 µl of blood were then evaluated, and overall, there was a positive association ($r^2 = 0.74$, $p = 0.03$) found between the signal intensity and the blood volume (Figure 1b,c). However, there were no statistically significant differences between the volumes when compared individually. hEPO and the ERAs could be detected in all conditions even with the lowest

sample volume tested (20 µl for hEPO, 25 µl for ERA-spiked samples) albeit faintly. Some non-specific bands (NSBs) were detected above EPO-Fc but did not interfere with ERA identification. A single DBS and a volume of 60 µl appeared to be a good compromise as a reasonably low volume for obtaining a good signal intensity and sensitivity for all ERAs, so this volume was used for the subsequent tests.

### 3.2 | Detection limits

The lowest concentration of ERAs that could be detected from one 60-µl spot was determined (Figure 2). CERA was detected down to 62.5 pg/ml in dried whole blood, EPO-Fc to 62 pg/ml, BRP to 31 pg/ml (3.7 IU/L), and NESP to 12.6 pg/ml. These concentrations are close to WADA's MRPLs for ERAs in serum/plasma (CERA: 25 pg/ml, EPO-Fc: 25 pg/ml, BRP: 10 IU/L, NESP: 10 pg/ml).[34] See Figure S1 for representative gel images of the detection limit tests.

### 3.3 | The stability of EPO in DBSs and urine

Spiked DBSs were stored at −20°C, 4°C, RT, and 37°C for about 40 h and for 6 days to investigate the stability of ERAs under these conditions (Figure 3). BRP/hEPO showed a significant difference between the two time points at RT ($p = 0.02$). After normalizing the CERA, EPO-Fc and NESP data, significant differences between 40 h and 6 days were found for EPO-Fc at −20°C and 4°C ($p = 0.01$). Results differed slightly when not normalized (Figure S2). An increasing trend of the signal intensities was observed when extracted after 6 days of storage compared with the ones obtained after storage for 40 h at −20°C, 4°C, or RT, but not at 37°C. A similar analysis was performed on urine samples (Figure 4a). When urine samples were stored for 6 days, the hEPO signal intensities decreased as the temperature increased, down to no signal at 37°C (a significant difference from the other temperatures; Figure 4c). Endogenous EPO signals from DBSs remained stable over the four temperatures (Figure 4b,c).

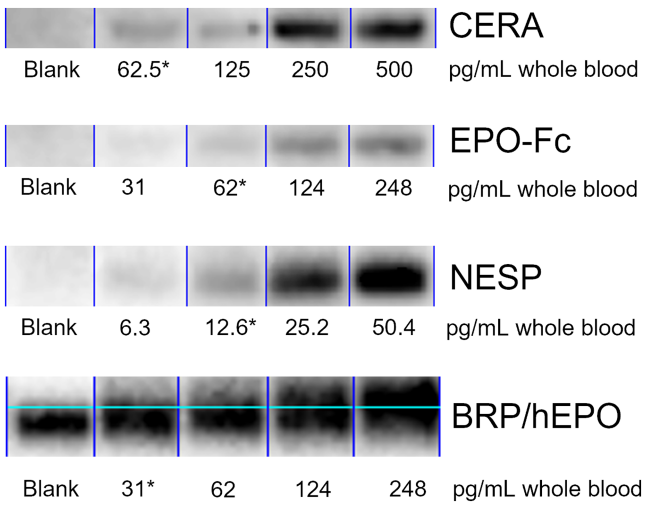


**FIGURE 2** Detection limits evaluated for CERA, EPO-Fc, NESP, and biological reference protein (BRP)/endogenous EPO (hEPO) from one 60-µl dried blood spot (DBS). DBSs were spotted with blank blood or blood spiked with the four erythropoietin receptor agonists (ERAs) at decreasing concentrations (as indicated in the figure; two-fold serial dilution performed with blank blood), and ERA analysis was performed in triplicate on two to three occasions. Samples were analysed with sodium *N*-lauroylsarcosinate polyacrylamide gel electrophoresis (SAR-PAGE) and immunodetection. The figure presents a representative image of each signal obtained with contrast adjustment performed with GASepo software. Asterisks indicate concentrations considered to be the detection limit [Colour figure can be viewed at wileyonlinelibrary.com]

### 3.4 | In vivo samples

Urine, serum, and capillary blood samples (Tasso device automatically producing four 20-µl blood spots) were repeatedly collected from five volunteers over a 6-week period to evaluate detection and intra-individual variations of endogenous EPO. Endogenous EPO was immunopurified and analysed from 500-µl serum, 10-ml urine, and three Tasso pebbles of 20 µl extracted together to start from 60 µl of dried blood (Figure 5a). Despite lower signal intensities in DBSs than for serum or urine, endogenous EPO was detected in all samples, indicating that the Tasso devices, like Whatman DBSs, were well-suited for ERA analysis. However, Tasso pebbles still had traces of blood after the extraction procedure, potentially indicating an incomplete desorption from the spot. Intra-individual variation of EPO signals was more pronounced in urine and capillary blood samples compared with serum over the collection period, but there were only three time points evaluated for serum per participant (Figure 5b). Correcting for specific gravity did not significantly change the variation in urine signal intensity (data not shown). Urinary specific gravity for the five participants over the collection period is shown in Table S1.

## 4 | DISCUSSION

This project aimed to better evaluate DBSs as a matrix to perform ERA detection and the conditions to obtain a sensitive detection of hEPO and ERAs.

One limitation often cited for DBSs compared with serum is the low volume of blood that can be analysed and the need for an efficient desorption step for the analyte of interest. In this work, the preparative steps were performed with the MAIIA EPGK kit for blood, which could be used for one or several DBSs on either paper or polymer material, while the subsequent steps of the ERA analysis were performed similarly to those for urine and serum analysis. With these analytical conditions, as low as 20 µl of dried blood was sufficient to detect hEPO, but 60 µl appeared appropriate to have better identification capabilities for ERAs at relatively low levels. Indeed, with 60 µl, detection limits are about 0.5–2.5 times greater than the MRPLs established by WADA for 500 µl of serum/plasma, which is satisfactory considering the small volume of blood analysed. Based on the

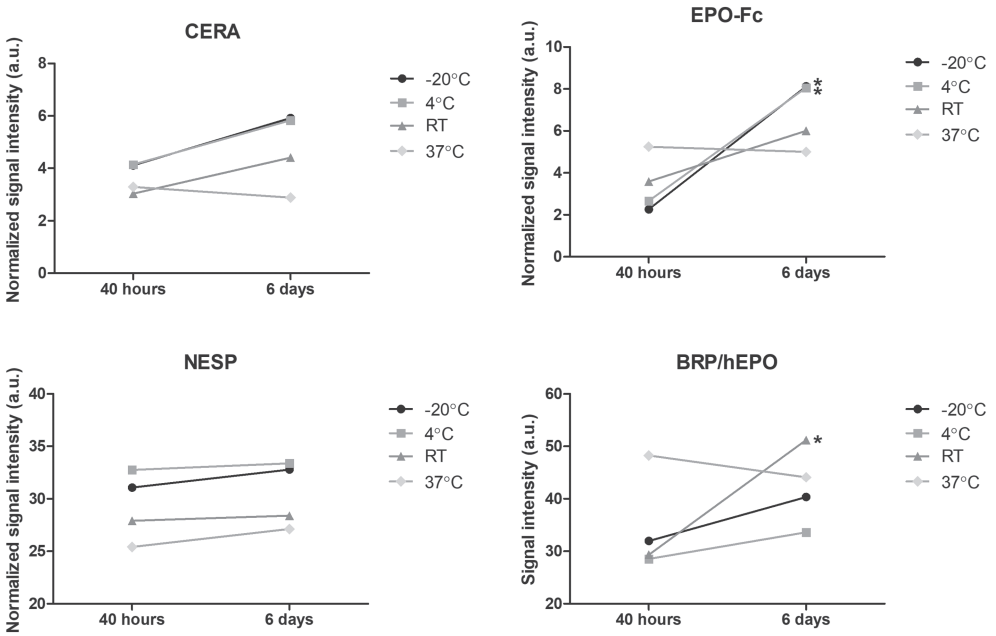


**FIGURE 3** Normalized stability of erythropoietin receptor agonists (ERAs) on dried blood spots (DBSs) stored at −20°C, 4°C, room temperature (RT), and 37°C between 40 h and 6 days. Samples were analysed with sodium *N*-lauroylsarcosinate polyacrylamide gel electrophoresis (SAR-PAGE) and immunodetection. Signal intensities for CERA, EPO-Fc, and NESP are normalized using biological reference protein (BRP)/endogenous EPO (hEPO) in each gel lane ($n = 5$). BRP/hEPO raw data are presented. ERAs spiked in DBSs (BRP 336 pg [~40 IU/L], NESP 40 pg, CERA 100 pg, EPO-Fc 100 pg/ml blood) were extracted, ERA detection was performed, and the signal intensities of the bands corresponding to each ERA were measured. Significant differences between 40 h and 6 days of storage are indicated with an asterisk ($p \leq 0.05$)

results obtained here, MRPLs for 60-µl DBS samples could be proposed: 150 pg/ml CERA and EPO-Fc (about six times higher than the serum MRPLs), 30 pg/ml NESP (about three times higher than the serum MRPL), and 10 IU/L rEPO (the same as the serum MRPL). This 60-µl sample volume is easily acquired, as multiple 20-µl spots can be collected in one session either using automated DBS collection of four 20-µl spots from the upper arm (Tasso device) or using other volumetric devices. Even a pipette can be used to load 60 µl on a paper DBS support after a finger prick or from another source of capillary blood (e.g., the 100-µl upper arm TAP device). Like for any doping analysis, there will be the need for enough matrix collected during the control to perform the initial screening and confirmation analyses and an additional sample to be stored in case of counter-analysis. Therefore, multiple collections during each doping control session will be required to get three separate samples with 60 µl of dried blood. A possibility to simplify collection would be to reduce the analytical sample volume to two 20-µl DBSs, allowing one Tasso device for ERA screening and confirmation procedures and a second Tasso device stored sealed for counter-analyses. However, the reduction in sample volume would reduce the sensitivity, and further testing is required to evaluate the detection limits in that case. DBSs may not be the preferred matrix for ERA analysis compared with urine and serum. However, even with a lower sensitivity compared with urine, ERAs will be stabilized and have reduced risk of degradation, which can occur in urine samples, leading to undetectable EPO signals. Compared with serum, the simplicity of DBS collection could allow increased frequency of doping control testing, especially considering that blood samples make up less than 20% of all collected samples.[36] Therefore, ERA analysis from DBSs could improve the chances of sample collection occurring during the detection window of doped athletes.

Stability experiments were performed to evaluate the possibility to send the samples to an anti-doping laboratory at RT and the best way to store them before initial screening. The results presented here confirm the good stability of hEPO and the ERAs for a week, independent of storage temperature, as suggested in a previous study with Whatman cards that demonstrated the stability of EPO for 30 days at 4°C and RT, and for about 3 months in samples with therapeutic doses of rEPO and NESP.[15] Therefore, DBS storage recommendations published by WADA (long-term at 4°C or colder and short term at RT or colder)[37] are well-adapted to ERA analysis for DBSs.

On the other hand, EPO signals in urine decreased with increasing temperature, a result to be considered because urine is currently the simplest sample to non-invasively collect from an athlete[32] and represents more than 80% of all collected doping control samples,[36] and it is not uncommon for urine samples to be transported at RT, occasionally over several days. This can promote bacterial and protease activity in the sample and ultimately result in inconclusive results because of degradation of ERAs, detection of NSBs, and/or absence of signal.[38,39] In our experiments, only 10 µl of the 50-µl final urine eluate was loaded and analysed (to avoid too many saturated signals for samples with high EPO concentrations), although the complete 15-µl eluate from the DBSs was loaded because of the low EPO content in such a small blood volume. A similar loading of the whole urine eluate after further concentration was not tested, but it may have allowed

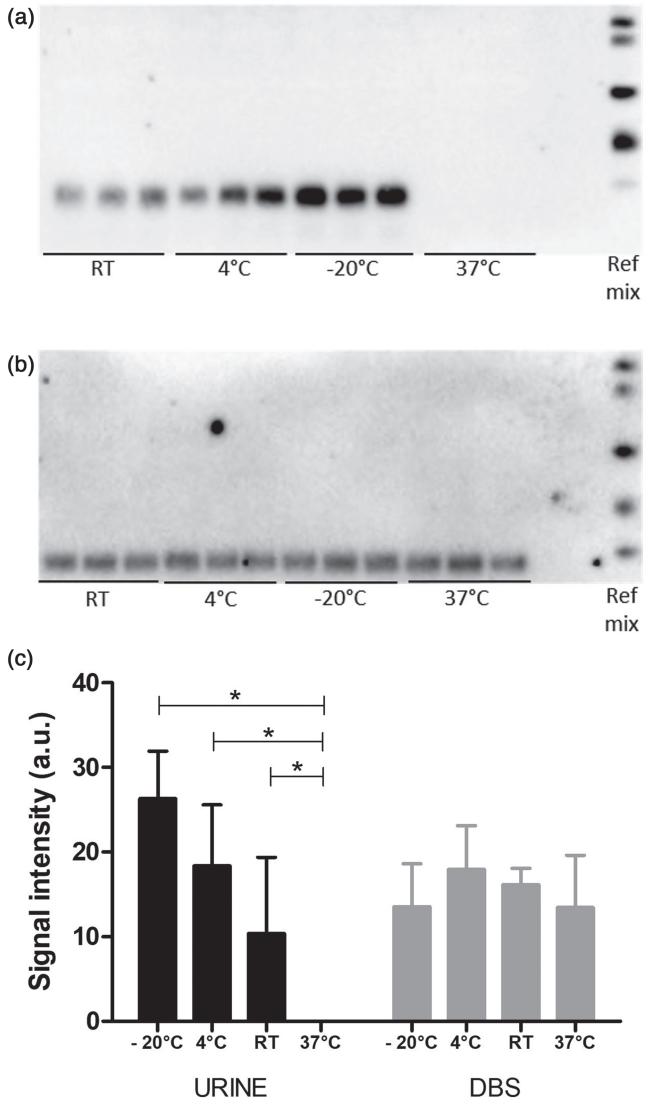


**FIGURE 4** Endogenous erythropoietin (hEPO) stability signal intensities from urine and dried blood spot (DBS) samples after 6 days stored at −20°C, 4°C, room temperature (RT), and 37°C. EPO was immunopurified from 10-ml urine and 60-µl DBS and analysed with sodium *N*-lauroylsarcosinate polyacrylamide gel electrophoresis (SAR-PAGE) and immunodetection. Gel images of urine (a) and DBSs (b) at the four temperature conditions ($n = 3$ per temperature). (c) hEPO signal intensities within each matrix at the four temperature conditions ($n = 5$ per temperature and matrix, median values are shown). Asterisks signify a significant difference within the urine matrix between temperatures

detection of EPO in the stability experiment at 37°C. However, EPO is clearly less stable in urine after a time in hot temperatures. DBSs from capillary blood would be an efficient complementary sample in cases of degradation issues with urinary EPO. If no EPO signal is detected in urine, then DBSs collected at the same time can be analysed for ERAs and provide conclusive results if an athlete is doped or not. It would also be a source of blood when there is a suspicious rEPO urine sample and the potential expression of the *EPO* c.577del variant must be checked[34] and, if necessary, as a source of DNA if further DNA sequencing is required.

Even though ERA detection from DBSs is not dependent on the drying time,[15] it would be important to standardize the drying conditions (e.g., packing with desiccant) by the doping control officers and storage conditions at the anti-doping laboratories with precautions to avoid direct light and humidity that could cause pre-analytical enzymatic degradation of EPO. One surprising observation in our experiments was the increased signal intensities observed for DBSs extracted after 6 days compared with 40 h. We did not find any clear explanation for this because all the analytical steps were the same, but small differences during sample preparation and variability due to the low DBS volumes might have caused slightly better desorption of ERAs from the DBSs.

Endogenous EPO was always detectable after extraction from DBSs in our experiments; however, NSBs were sometimes observed in the samples, which are also occasionally detected in serum/plasma samples because of the many proteins in blood. This should be avoided especially in case of a confirmation analysis. Although biotinylated AE7A5 antibody (used here) decreases artefacts in EPO blood analysis,[40,41] a double blotting method to reduce NSBs caused by a secondary probing antibody[42] or alternative extraction and/or immunopurification methods (e.g., ELISA or magnetic beads) should be tested to see if they would be appropriate for a confirmation procedure. Although DBSs on a filter paper support were well-extracted with the MAIIA EPGK, optimization in processing and detection are still possible, particularly for the Tasso-M20 pebbles, because some variations in the DBS intra-individual signal intensities may be from unevenness in blood desorption from the polymer, indicating that the 90-min rotation during extraction/immunopurification is not efficient enough to extract all the EPO from the polymer. Possible ways to improve extraction and sensitivity are prolonging the rotation time, or by sonication[33] or rinsing of the polymer with buffer before incubation with the sepharose gel beads.

When continuing to look at the intra-individual variations in EPO in vivo over a 6-week period, there was more variation with DBSs than with serum, possibly due to the lower EPO quantity extracted and analysed from 60 µl of whole blood compared to 500 µl of serum. In an administration study, therapeutic rEPO doses were more sensitively detected in DBSs from capillary blood than in venous blood.[15] Therefore, an evaluation of the sensitivity is necessary for any support authorized by WADA.

In this study, five of 37 Tasso-M20 devices (13.5%) failed to collect sufficient blood volumes for EPO analysis (either no or partial collection), which was quite a high failure rate, possibly due to insufficient pressure on the button that pushed the needle on the upper arm. In a study comparing upper arm and finger prick capillary blood collection, 2% of the Tasso-M20 devices failed.[31] Defective sampling devices could mean that re-sampling may be required during a doping control for sufficient sample volumes to be collected. However, the few publications with Tasso devices report positive feedback from study participants,[31,43,44] although in one study, some participants expressed hesitation in pressing the button to eject the needle

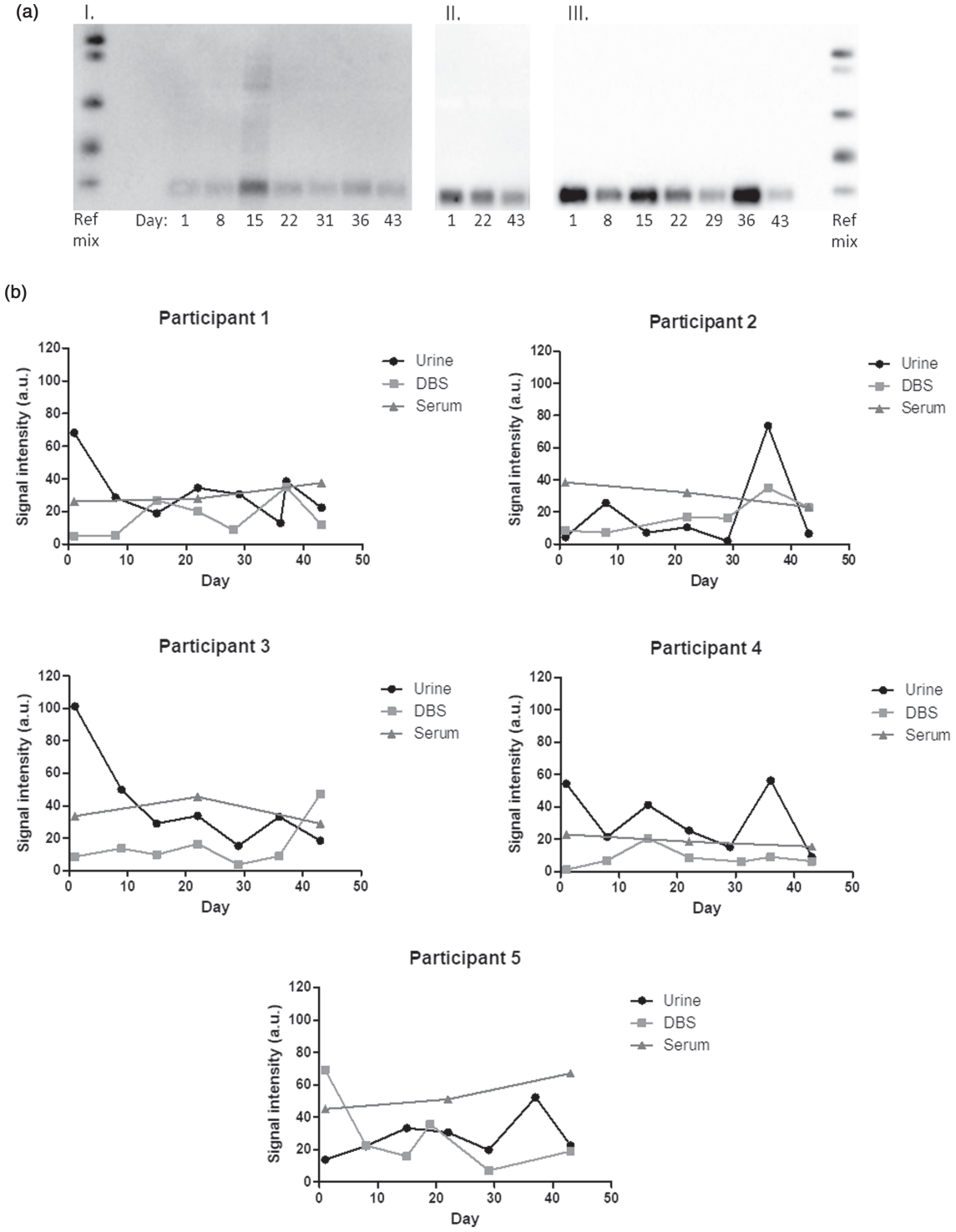


**FIGURE 5** Intra-individual variations of endogenous erythropoietin (hEPO) signals analysed from urine, serum, and dried blood spots (DBSs). Samples were analysed with sodium *N*-lauroylsarcosinate polyacrylamide gel electrophoresis (SAR-PAGE) and immunodetection. (a) Images of EPO signals obtained after analysing DBSs (I), serum (II), and urine (III) from Participant 4 over 6 weeks. (b) Graphs presenting the evolution of hEPO signal intensities from urine, DBSs, and serum samples for the five participants over 6 weeks. Urine and DBSs were collected seven times and serum three times during the collection period

for the painless sampling to begin,[44] but this could be performed by the doping control officer. If capillary blood collection by other methods is authorized by WADA, depending on the athlete's wishes, finger prick samples are still an efficient way to get blood that can be deposited onto filter paper or directly collected with VAMS or quantitative DBS devices (Capitainer, Hemaxis). The volumetric/quantitative

devices are a good way to standardize the volumes collected and remove haematocrit bias caused by uneven blood distribution on filter paper.[45] Experiments with CERA and VAMS have shown promising results, with DBSs being of particular interest for the detection of this ERA which is not easily filtered in urine.[33] Haematocrit bias does not affect ERA detection,[15] particularly when the entire spot is analysed, but collecting sufficient and precise volumes is important for detection.

## 5 | CONCLUSIONS

Sensitive detection of endogenous EPO and ERAs close to WADA's MRPLs in 60 µl of whole blood, reasonable storage conditions, and EPO detection from volumetric capillary blood devices demonstrate the possibility of DBSs as an analytical matrix for EPO analysis. This makes DBSs a valuable complementary matrix to urine for doping control.

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### DATA AVAILABILITY STATEMENT

Data available on request due to privacy/ethical restrictions.

### ORCID

*Carmel E. Heiland* https://orcid.org/0000-0001-8367-7364
*Lena Ekström* https://orcid.org/0000-0003-1053-2345
*Alexandre Marchand* https://orcid.org/0000-0002-2670-6939

### REFERENCES

1. Jelkmann W. Erythropoietin: structure, control of production, and function. *Physiol Rev*. 1992;72(2):449-489. doi:10.1152/physrev.1992.72.2.449.
2. Jelkmann W. Molecular biology of erythropoietin. *Intern Med*. 2004;43(8):649-659. doi:10.2169/internalmedicine.43.649.
3. Ng T, Marx G, Littlewood T, et al. Recombinant erythropoietin in clinical practice. *Postgrad Med J*. 2003;79(933):367-376. doi:10.1136/pmj.79.933.367.
4. Inoue N, Takeuchi M, Ohashi H, Suzuki T. The production of recombinant human erythropoietin. *Biotechnol Annu Rev*. 1995;1:297-313. doi:10.1016/S1387-2656(08)70055-3.
5. Kalantar-Zadeh K. History of erythropoiesis-stimulating agents, the development of biosimilars, and the future of anemia treatment in nephrology. *Am J Nephrol*. 2017;45(3):235-247. doi:10.1159/000455387.
6. Jelkmann W. Erythropoiesis stimulating agents and techniques: a challenge for doping analysts. *Curr Med Chem*. 2009;16(10):1236-1247. doi:10.2174/092986709787846668.
7. Drüeke TB, Massy ZA. Erythropoiesis-Stimulating Agents and Mortality. *J Am Soc Nephrol*. 2019;30(6):907-908. doi:10.1681/ASN.2019030266.
8. Egrie JC, Browne JK. Development and characterization of novel erythropoiesis stimulating protein (NESP). *Nephrol Dial Transplant* 2001;16 Suppl 3:3–13. doi:10.1093/ndt/16.suppl_3.3
9. Reichel C, Thevis M. Detection of EPO-Fc fusion protein in human blood: screening and confirmation protocols for sports drug testing. *Drug Test Anal*. 2012;4(11):818-829. doi:10.1002/dta.1381.
10. WADA. World anti-doping code. Prohibited list 2022. https://www.wada-ama.org/en
11. Guthrie R, Susi A. A simple phenylalanine method for detecting phenylketonuria in large populations of newborn infants. *Pediatrics*. 1963;32(3):338-343. doi:10.1542/peds.32.3.338.
12. Cox HD, Rampton J, Eichner D. Quantification of insulin-like growth factor-1 in dried blood spots for detection of growth hormone abuse in sport. *Anal Bioanal Chem*. 2013;405(6):1949-1958. doi:10.1007/s00216-012-6626-y.
13. Kojima A, Nishitani Y, Sato M, Kageyama S, Dohi M, Okano M. Comparison of urine analysis and dried blood spot analysis for the detection of ephedrine and methylephedrine in doping control. *Drug Test Anal*. 2016;8(2):189-198. doi:10.1002/dta.1803.
14. Moller I, Thomas A, Geyer H, Möller I, Schänzer W, Thevis M. Development and validation of a mass spectrometric detection method of peginesatide in dried blood spots for sports drug testing. *Anal Bioanal Chem*. 2012;403(9):2715-2724. doi:10.1007/s00216-012-6043-2.
15. Reverter-Branchat G, Ventura R, Ezzel Din M, Mateus J, Pedro C, Segura J. Detection of erythropoiesis-stimulating agents in a single dried blood spot. *Drug Test Anal*. 2018;10(10):1496-1507. doi:10.1002/dta.2418.
16. Solheim SA, Jessen S, Morkeberg J, et al. Single-dose administration of clenbuterol is detectable in dried blood spots. *Drug Test Anal*. 2020;12(9):1366-1372. doi:10.1002/dta.2872.
17. Thomas A, Geyer H, Schanzer W, et al. Sensitive determination of prohibited drugs in dried blood spots (DBS) for doping controls by means of a benchtop quadrupole/Orbitrap mass spectrometer. *Anal Bioanal Chem*. 2012;403(5):1279-1289. doi:10.1007/s00216-011-5655-2.
18. Lange T, Walpurgis K, Thomas A, Geyer H, Thevis M. Development of two complementary LC-HRMS methods for analyzing sotatercept in dried blood spots for doping controls. *Bioanalysis*. 2019;11(10):923-940. doi:10.4155/bio-2018-0313.
19. Cox HD. Dried Blood Spots May Improve Detection of Blood Doping. *Clin Chem*. 2019;65(12):1481-1483. doi:10.1373/clinchem.2019.311902.
20. Linder C, Wide K, Walander M, Beck O, Gustafsson LL, Pohanka A. Comparison between dried blood spot and plasma sampling for therapeutic drug monitoring of antiepileptic drugs in children with epilepsy: A step towards home sampling. *Clin Biochem*. 2017;50(7-8):418-424. doi:10.1016/j.clinbiochem.2016.12.008
21. Milosheska D, Grabnar I, Vovk T. Dried blood spots for monitoring and individualization of antiepileptic drug treatment. *Eur J Pharm Sci*. 2015;75:25-39. doi:10.1016/j.ejps.2015.04.008.
22. Lange T, Thomas A, Walpurgis K, Thevis M. Fully automated dried blood spot sample preparation enables the detection of lower molecular mass peptide and non-peptide doping agents by means of LC-HRMS. *Anal Bioanal Chem*. 2020;412(15):3765-3777. doi:10.1007/s00216-020-02634-4.
23. Peng SH, Segura J, Farre M, Farré Ḿ, de la Torre X. Oral testosterone administration detected by testosterone glucuronidation measured in blood spots dried on filter paper. *Clin Chem*. 2000;46(4):515-522. doi:10.1093/clinchem/46.4.515.
24. Thomas A, Geyer H, Guddat S, Schänzer W, Thevis M. Dried blood spots (DBS) for doping control analysis. *Drug Test Anal*. 2011;3(11–12):806-813. doi:10.1002/dta.342.
25. Tretzel L, Thomas A, Geyer H, et al. Use of dried blood spots in doping control analysis of anabolic steroid esters. *J Pharm Biomed Anal*. 2014;96:21-30. doi:10.1016/j.jpba.2014.03.013.
26. Cox HD, Eichner D. Mass spectrometry method to measure membrane proteins in dried blood spots for the detection of blood doping

practices in sport. *Anal Chem*. 2017;89(18):10029-10036. doi:10.1021/acs.analchem.7b02492.
27. Marchand A, Buisson C, Martin L, Martin JA, Molina A, Ressiot D. Report on an anti-doping operation in Guadeloupe: High number of positive cases and inferences about doping habits. *Drug Test Anal*. 2017;9(11–12):1753-1761. doi:10.1002/dta.2185.
28. Dehnes Y, Shalina A, Myrvold L. Detection of recombinant EPO in blood and urine samples with EPO WGA MAIIA, IEF and SAR-PAGE after microdose injections. *Drug Test Anal*. 2013;5(11–12):861-869. doi:10.1002/dta.1579.
29. Martin L, Ashenden M, Bejder J, et al. New insights for identification of doping with recombinant human erythropoietin micro-doses after high hydration. *Drug Test Anal*. 2016;8(11–12):1119-1130. doi:10.1002/dta.2004.
30. Mørkeberg J, Sharpe K, Karstoft K, Ashenden MJ. Detection of micro-doses of rhEPO with the MAIIA test. *Scand J Med Sci Sports*. 2014;24(4):634-641. doi:10.1111/sms.12049.
31. Solheim SA, Ringsted TK, Nordsborg NB, Dehnes Y, Levernæs MCS, Mørkeberg J. No pain, just gain: painless, easy, and fast dried blood spot collection from fingertip and upper arm in doping control. *Drug Test Anal*. 2021;13(10):1783-1790. doi:10.1002/dta.3135.
32. Thevis M, Geyer H, Tretzel L, Schänzer W. Sports drug testing using complementary matrices: Advantages and limitations. *J Pharm Biomed Anal*. 2016;130:220-230. doi:10.1016/j.jpba.2016.03.055.
33. Rocca A, Martin L, Kuuranne T, Ericsson M, Marchand A, Leuenberger N. A fast screening method for the detection of CERA in dried blood spots. *Drug Test Anal*. 2021;1-6. doi:10.1002/dta.3142
34. WADA. Harmonization of analysis and reporting of erythropoietin (EPO) and other EPO-receptor agonists (ERAs) by polyacrylamide gel electrophoretic (PAGE) analytical methods. TD2022EPO. https://www.wada-ama.org/en
35. Heiland CE, Masquelier M, Bhuiyan H, Ericsson M. A simple method to immunopurify erthyropoiesis stimulating agents from urine, aiming to optimize erythropoietin screening by SAR-PAGE. *Drug Test Anal*. 2019;11(11–12):1666-1674. doi:10.1002/dta.2730.
36. WADA. 2020 Anti-doping Testing Figures. https://www.wada-ama.org/en
37. WADA. Dried blood spots (DBS) for doping control. TD2021DBS. https://www.wada-ama.org/en
38. Lasne F, Martin L, Crepin N, de Ceaurriz J. Detection of isoelectric profiles of erythropoietin in urine: differentiation of natural and administered recombinant hormones. *Anal Biochem*. 2002;311(2):119-126. doi:10.1016/S0003-2697(02)00407-4.
39. Thevis M, Maurer J, Kohler M, Geyer H, Schänzer W. Proteases in doping control analysis. *Int J Sports Med*. 2007;28(7):545-549. doi:10.1055/s-2007-965159.
40. Martin L, Martin JA, Audran M, et al. An optimized SDS-PAGE protocol with a new blotting system for the initial testing procedure of ESAs in doping control. *Drug Test Anal*. 2022;14(1):181-187.
41. Reichel C, Erceg D, Lorenc B, et al. Data from a microdosed recombinant human erythropoietin administration study applying the new biotinylated clone AE7A5 antibody and a further optimized sarcosyl polyacrylamide gel electrophoresis protocol. *Drug Testing and Anal*. 2021;1-10.
42. Lasne F. Double-blotting: a solution to the problem of non-specific binding of secondary antibodies in immunoblotting procedures. *J Immunol Methods*. 2001;253(1–2):125-131. doi:10.1016/S0022-1759(01)00355-6.
43. Roadcap B, Hussain A, Dreyer D, et al. Clinical application of volumetric absorptive microsampling to the gefapixant development program. *Bioanalysis*. 2020;12(13):893-904. doi:10.4155/bio-2020-0074.
44. Williams KJ, Lutman J, McCaughey C, Fischer SK. Assessment of low volume sampling technologies: utility in nonclinical and clinical studies. *Bioanalysis*. 2021;13(9):679-691. doi:10.4155/bio-2021-0027.
45. Capiau S, Veenhof H, Koster RA, et al. Official international association for therapeutic drug monitoring and clinical toxicology guideline: development and validation of dried blood spot-based methods for therapeutic drug monitoring. *Ther Drug Monit*. 2019;41(4):409-430. doi:10.1097/FTD.0000000000000643.

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