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Next generation microsampling towards sustainable forensic analysis: Volumetric DBS for cocaine and metabolites

Sarah Palano [a,1], Dorota Turoňova [b,1], Michele Protti [a,1], Lenka Kujovská Krčmová [b,c], Roccaldo Sardella [d], Přemysl Mladěnka [e], Roberto Mandrioli [f], Stefano Girotti [a], Laura Mercolini [a,*]

[a] Research Group of Pharmaco-Toxicological Analysis (PTA Lab), Department of Pharmacy and Biotechnology (FaBiT), Alma Mater Studiorum - University of Bologna, Via Belmeloro 6, 40126 Bologna, Italy
[b] Department of Clinical Biochemistry and Diagnostics, University Hospital Hradec Králové, Sokolská 581, 50005 Hradec Králové, Czech Republic
[c] Department of Analytical Chemistry, Faculty of Pharmacy in Hradec Králové, Charles University, Akademika Heyrovského 1203, 50003 Hradec Králové, Czech Republic
[d] Department of Pharmaceutical Sciences, University of Perugia, Via Fabretti 48, 06123 Perugia, Italy
[e] Department of Pharmacology and Toxicology, Faculty of Pharmacy in Hradec Králové, Charles University, Akademika Heyrovského 1203, 50003 Hradec Králové, Czech Republic
[f] Department for Life Quality Studies, Alma Mater Studiorum - University of Bologna, Corso d'Augusto 237, 47921 Rimini, Italy

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ABSTRACT

This study introduces for the first time a reliable whole blood microsampling method for forensic analysis of cocaine and its metabolites using quantitative dried blood spot (qDBS) technology and UHPLC-MS analysis. This methodology offers accurate and less invasive sampling and aligns with the current trend towards sustainable and accessible analytical methods. Microsampling is subject-friendly, improves logistics, stability and efficiency, marking a shift towards modern forensic practices with wide-ranging application potential.

The qDBS-UHPLC-MS method underwent comprehensive validation, confirming its linearity, sensitivity, precision, extraction efficiency and stability. UHPLC-MS achieved effective chromatographic separation and suitable sensitivity, with detection limits between 1.0 and 2.5 ng/mL, and quantitation limits from 2.5 to 7.5 ng/mL. Analytes showed over 85.1 % extraction yield and less than 7.5 % relative standard deviation in precision. Stability tests indicated superior analyte preservation in qDBS at room temperature versus refrigerated plasma, while minimal matrix effect highlighted the sample clean-up efficiency. Application to real samples yielded consistent quali-quantitative results between qDBS and plasma samples, confirming method suitability for forensic cocaine bioanalysis, thus addressing critical needs in drug testing and pharmaco-toxicological analysis.

1. Introduction

Cocaine (methyl (1*R*,2*R*,3*S*,5*S*)-3-benzoyloxy-8-methyl-8-azabicyclo [3.2.1]octane-2-carboxylate, COC, Fig. 1a) is an alkaloid extracted from coca leaves (*Erythroxylon* or *Erythroxylum coca*) and typically isolated in hydrochloride form as a fine, crystalline white powder [1]. It is a potent CNS stimulant and one of the most widely abused natural substances, second only to *Cannabis* [2,3]. Its addictive effects arise from its psychoactive properties, increasing synaptic dopamine concentrations and causing intense pleasure [4]. Effects from a single dose appear quickly and last from 20 min to several hours, depending on dosage, purity and administration route [5], while chronic use leads to tolerance, craving and severe health risks such as strokes, heart attacks and respiratory issues [1]. COC is also listed in the World Anti-Doping Agency (WADA) Prohibited List under Section S6 (Stimulants) [6].

COC metabolism, mainly in the liver, is rapid with a mean half-life of 30 min to 1.5 h. Detection usually relies on its main inactive metabolites: benzoylecgonine (BEG, Fig. 1b) and ecgonine methyl ester (EME, Fig. 1c) [7,8,9,10,11]. Notably, cocaethylene (CET, Fig. 1d) forms by transesterification in the presence of ethanol, is more cardiotoxic than

* Corresponding author.
E-mail address: laura.mercolini@unibo.it (L. Mercolini).
[1] Sarah Palano, Dorota Turoňova and Michele Protti contributed equally to this work.

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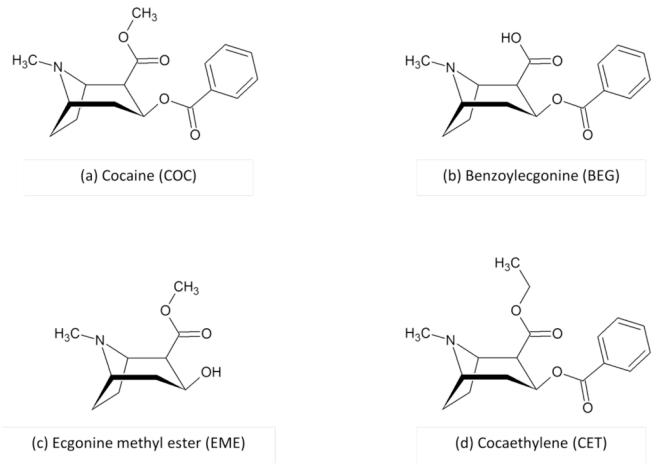

Fig. 1. Chemical structures of: (a) cocaine (COC), (b) benzoylecgonine (BEG), (c) ecgonine methylester (EME) and (d) cocaethylene (CET).

COC [12] and has been proposed as a marker of the concomitant consumption of COC and ethanol [13]. However, CET presence in illicit COC samples has challenged its reliability as a marker of co-consumption [14,15], yet its detection remains significant due to its toxicity [14].

The need for innovative detection methods in forensic, healthcare and workplace fields is driven by COC widespread abuse. Blood detection indicates recent exposure, vital for clinical toxicology and driving under the influence of alcohol or other drugs (DUI) investigations [8,16]. However, traditional blood collection for COC analysis requires specialised phlebotomy and proper handling due to COC instability [4,17,18]; an alternative relies on dried blood spot (DBS) sampling via finger pricking [4]. Microsampling technologies, like dried matrix spotting (DMS) and volumetric absorptive microsampling (VAMS), enable small volume collections (a few μL) suitable for scenarios where conventional methods are impractical, such as roadside testing and in-competition anti-doping testing, while enhancing analyte stability over time [19–21]. DBS samples can be collected easily without special equipment, apart from disposable needles and spotting cards [8]. One advanced DBS technology, Capitainer® qDBS, accurately samples 10 μL of blood from a fingertip drop [22] (Fig. 2a). This technology, defined as "volumetric" microsampling, ensures an accurate blood volume through an automatically filled capillary channel, regardless of the blood drop initial volume and density [23,24] (Fig. 2b). This technology minimizes sampling errors and provides real-time feedback for valid sample collection [25] (Fig. 2c) while, like other microsampling methods, being rapid, feasible, minimally invasive and reducing analysis times and costs. Dried blood microsamples simplify transport and storage, which can be safely performed at room temperature due to increased stability, thus minimising contamination, manipulation and biological risks [26–28]. Compared to classical DBS sampling, volumetric blood collection technologies are more accurate and less sensitive to haematocrit effects [29]. Microsampling not only enhances sample collection efficiency and practicality, but also aligns with green analytical chemistry (GAC) and in particular green sample preparation (GSP) principles. It reduces the environmental impact compared to traditional methods by minimising resource use, eliminating the need for refrigeration or freezing, and reducing biohazardous waste. The dried nature of microsamples halts enzymatic and chemical reactions, ensuring analyte stability and simplifying logistics.

Few methods exist for the analysis of COC and its metabolites in microsamples [30]; some of them use capillary blood by means of classical DBS or VAMS [4,3]. Recent investigations have employed calibrated capillaries [31] or three-dimensional embossed hydrophobic paper [32]; however, no studies have yet applied microfluidic volumetric technologies like qDBS for COC and metabolites analysis in whole blood. This innovative method aims at the analysis of COC, BEG, EME and CET in stable, haematocrit-independent microsamples.

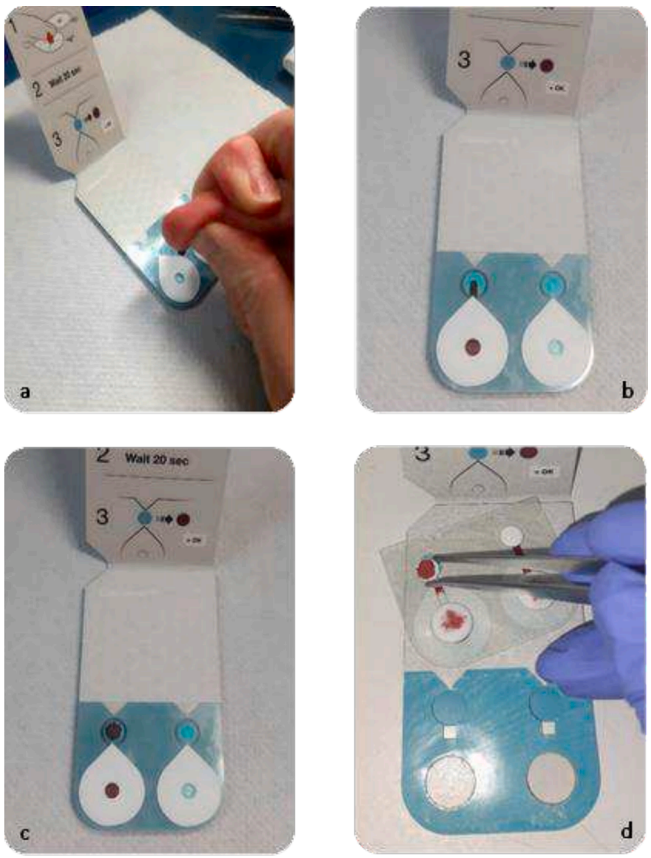

Fig. 2. Schematic representation of qDBS sampling process.

2. Materials and methods

2.1. Chemicals and standard solutions

Certified methanolic stock solutions (1 mg/mL) of COC, BEG, EME, CET, BEG-D3 (IS1), COC-D3 (IS2), were from Cerilliant (Saint Louis, MO, USA). Analytical grade acetonitrile (ACN), methanol (MeOH), formic acid (FA) were purchased from Merck (Milan, Italy). Ultrapure water (18.2 MΩ•cm) was obtained from a Milli-Q system from Merck Millipore (Darmstadt, Germany). Standard solutions were freshly prepared each day and stored in MS amber glass vials to protect them from light.

2.2. Instrumentation and conditions

The method employed a UHPLC system integrated with a refrigerated autosampler and coupled to single quadrupole mass spectrometry equipped with electrospray ionisation. Namely, the system was composed of a Vanquish UHPLC pump, a Vanquish autosampler, a Vanquish column compartment, and an ISQ EC single quadrupole mass spectrometer (all from Thermo Fisher Sci., Waltham, MA, USA), while data processing was achieved using Thermo Fisher Scientific Dionex Chromeleon 7.3 software. Chromatographic separation exploited an Agilent (Santa Clara, CA, USA) Zorbax SB-C18 column (50 × 2.1 mm ID, 3.5 μm) maintained at 25 °C and coupled with a Zorbax SB-C18 guard column. Mobile phase consisted of a mixture of 0.1 % aqueous FA and 0.1 % FA in ACN at a constant flow rate of 0.3 mL/min with an optimised gradient: 0.0–1.5 min, 3 % B; 1.5–2.5 min, 3–30 % B; 2.5–4.5 min, 30 % B; 4.5–5.0 min, 30–3 % B; 5.0–6.0 min, 3 % B. Optimised MS parameters

were: positive ion source (ESI+) voltage, 3.5 kV; vaporiser temperature, 172 °C; desolvation temperature, 300 °C; desolvation gas flow, 23.0 psig; auxiliary gas pressure 4.0 psig; sweep gas pressure, 0.5 psig. Chromatograms for each compound were extracted from their $[M + H]^+$ ions in single ion monitoring (SIM) mode (mass scan range 50–500): *m/z* 304.27 for COC, *m/z* 290.16 for BEG, *m/z* 200.18 for EME, *m/z* 318.24 for CET, *m/z* 293.18 for IS1, *m/z* 307.24 for IS2.

2.3. Sample collection and pretreatment

Capitainer qDBS devices (from Capitainer, Solna, Sweden) use a combination of paper, microfluidic polymer and a double-valve design to sample a fixed 10-μL volume from a drop of blood. The sampling procedure begins by placing a single blood drop obtained from a finger prick (multiple drops could cause volumetric inaccuracies) into contact with the device inlet slot (contact time 1 s) (Fig. 2a). In about 20 s, the blood flows into the dosing channel, reaching the outlet disc and changing its colour to red, thus confirming the correct sampling (Fig. 2b). The process takes place through capillary action and using dissolvable membranes as valves. When a sufficient volume of blood is put in contact with the inlet port of the device, the microchannel fills automatically through capillary action. The inlet dissolvable valve is then activated, emptying excess blood into a waste paper piece, so that the microchannel contains an accurately controlled volume of blood which will also activate the outlet dissolvable valve (Fig. 2c). The blood will then be absorbed onto the paper pad and allowed to dry to generate a volumetric DBS (qDBS) sample [30] (Fig. 2d).

After sampling as previously described and following a 60-min drying time, qDBS samples were taken from the device with the help of tweezers and fortified by adding a standard mixture containing ISs at the concentration of 50 ng/mL (and the analytes at known concentrations for method development and validation purposes). After 30 min, the dried sample was placed in 1 mL of a solution composed of MeOH/ACN (3:1) and extracted by ultrasound-assisted extraction (UAE) at 40 kHz for 5 min, then centrifuged. The supernatant was then dried, redissolved in mobile phase (initial composition), and injected into the UHPLC-MS system.

2.4. Method validation

The qDBS-UHPLC-MS method was validated following International Conference on Harmonization (ICH) [33] and Food and Drug Administration [34] guidelines. The tested parameters were linearity, including limit of detection (LOD) and limit of quantitation (LOQ), precision, extraction efficiency, stability, and matrix effect. Blank qDBS samples were fortified with standard mixtures, subjected to the pretreatment protocol and analysed. The assay was performed in triplicate for each concentration to evaluate linearity (by the least square method), its range and correlation coefficient (r^2). For sensitivity, LOD and LOQ values were defined as the concentrations generating peaks whose heights were 3 and 10 times the background noise, respectively.

Precision (as percent relative standard deviation, RSD%) was evaluated by intraday (n = 6 in the same day) and interday (n = 6 in different days) studies on blank samples fortified with three different analyte concentrations. Extraction efficiency was calculated at three different concentrations, by comparing fortified sample peak areas with those from standard mixtures.

Matrix effect was evaluated by means of post-extraction fortification of blank samples at three different concentrations, and peak areas were compared with those from standard mixtures.

The stability of analytes fortified into qDBS blank samples stored at RT was compared with that in fluid plasma at −20 °C. Blank qDBS and plasma samples were fortified and analysed at 1-week intervals for two months and the results were expressed as remaining analyte (%). For reference plasma analysis, extraction was performed using a solid phase extraction (SPE) protocol based on the use of C8 cartridges (50 mg/1 mL): activation, 2 × 1 mL of MeOH; conditioning, 2 × 1 mL of ultrapure water; loading, 400 μL plasma + 400 μL ultrapure water; washing, 2 × 1 mL of ultrapure water + 1 mL of a water/MeOH mixture (90:10, V/V); elution, 1 mL of MeOH. The extract was dried under vacuum, redissolved with 100 μL of a water/ACN (70:30) mixture containing 0.1 % FA and analysed.

3. Results and discussion

3.1. Initial chromatographic assays

The primary chromatographic variables, including sorbent material, FA concentration, solvent ratio gradient, and flow rate, were carefully examined and fine-tuned to ensure optimal analyte separation within reasonable run times and to maximize MS analyte ionisation efficiency. Specifically, the gradient percentage of solution B was varied from 3 to 50 % and the gradient times were changed from ± 30 to 60 s; the flow rate was tested between 0.1 and 0.4 mL/min. Ammonium formate and FA were tested as mobile phase additives in the 0.02–0.50 % range.

Under the optimised conditions detailed in the Experimental section, all analytes were successfully eluted and separated in under 6 min, yielding resolved and symmetrical peaks.

3.2. Evaluation of the sampled volume

Gravimetric tests were performed to define the accuracy of the volume of whole blood sampled by the Capitainer qDBS technology. qDBS devices (n = 6) were weighed, using a balance with a resolution of 0.01 mg, before and after normal microfluidic sampling by finger prick and the results were compared with the weight of the same matrix weighing 10 μL of whole blood sampled with a micropipette (n = 6). Collected qDBS volumes gave statistically indistinguishable values from pipetted ones (~10 μL), thus demonstrating the good sampling accuracy using the volumetric microfluidic device (10.1 ± 0.3 μL).

3.3. Volumetric DBS sampling and sample preparation procedure development

Preliminary drying time assays by gravimetric analysis (n = 3) were carried out by measuring every 10 min (25 °C, 55 % humidity) the changes in weight of the qDBS, on an analytical balance with a maximum capacity of 220 g and a precision of 0.1 mg, until at least three consecutive readings were constant. This showed that the time required to produce a constant weight, 10-μL qDBS is 60 min (Fig. 3a). Using the same procedure, the drying time of the standard analyte and IS mixture used to fortify either blank or real samples (5 μL) was tested; this fortified spot resulted to be completely dry in 30 min (Fig. 3b).

The analytes were extracted from qDBS samples by means of solvent treatment. Samples were pretreated evaluating different extraction conditions to optimise the protocol.

Different extraction solvents were tested, including mixtures composed of MeOH/ACN in different ratios (from 3:1 to 1:2, V/V) and ammonium acetate or FA in water and in water/MeOH, water/ACN and water/MeOH/ACN mixtures. Best results were obtained using exclusively organic solvent mixtures at high ratios of MeOH, i.e., with the 3:1, V/V MeOH/ACN mixture. Regarding the extraction procedure itself, two different workflows were tested: one based on UAE and one based on vortex-assisted extraction (VAE), applied for different times (1–10 min). As can be seen from Table 1, good extraction efficiency values were obtained using both UAE and VAE, but slightly better for the former, which was then selected, and 5 min were enough to achieve satisfactory extraction efficiency for all the analytes (>85.1 %) and ISs (>89.9 %).

The chromatogram of a blank qDBS sample, also fortified with the analytes and the ISs, after sample pretreatment and UHPLC-MS analysis, is shown in Fig. 4. As can be seen, both resolution, peak symmetry and retention times were deemed satisfying and the baseline signal was clean

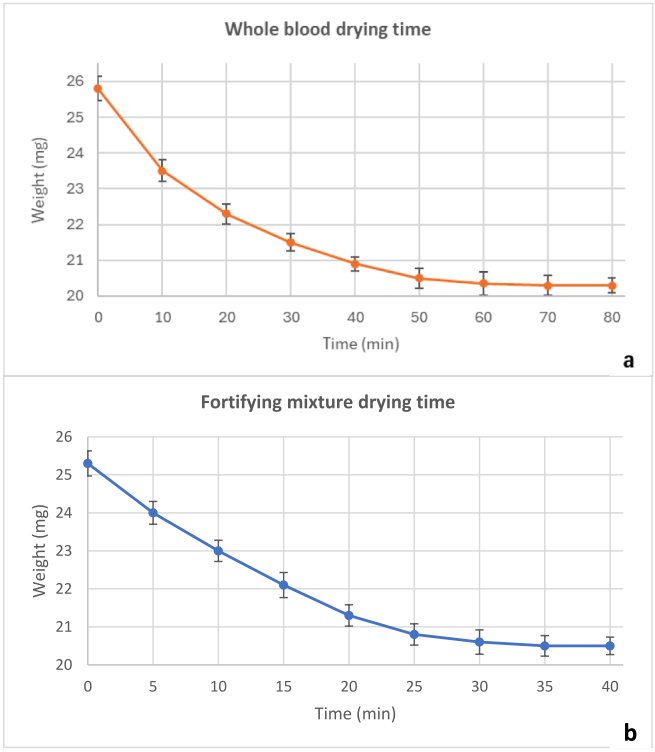

Fig. 3. Drying time assay results: (a) whole blood; (b) standard solution of the analytes and the ISs (5 μL) deposited on a qDBS.

Table 1
Comparison between UAE and VAE extraction procedures.

Compound	Extraction time (min)	Extraction efficiency (%)[a]	
		UAE	VAE
EME	1	81.6	64.9
	5	95.3	82.5
	10	95.9	85.7
BEG	1	82.5	61.2
	5	99.7	80.3
	10	99.1	83.6
COC	1	76.1	62.8
	5	89.7	85.7
	10	89.5	86.1
CET	1	70.7	48.6
	5	85.2	62.0
	10	84.9	64.2
IS1	1	85.9	71.4
	5	99.0	90.9
	10	99.2	90.0
IS2	1	81.9	73.8
	5	90.0	90.8
	10	89.9	90.1

[a] $n = 3$.

and stable.

3.4. Method validation

3.4.1. Linearity and sensitivity

To determine linearity, blank samples fortified with standard analyte mixtures at seven different concentrations for the analytes and at a constant 50 ng/mL concentration for the ISs were analysed. The calibration curves were plotted, and the respective linear correlation coefficients (r^2) calculated, which were always higher than 0.9987. The observed linearity ranges were 7.5–500 ng/mL for EME, 2.5–500 ng/mL for BEG, 5.0–500 ng/mL for COC, and 5.0–500 ng/mL for CET.

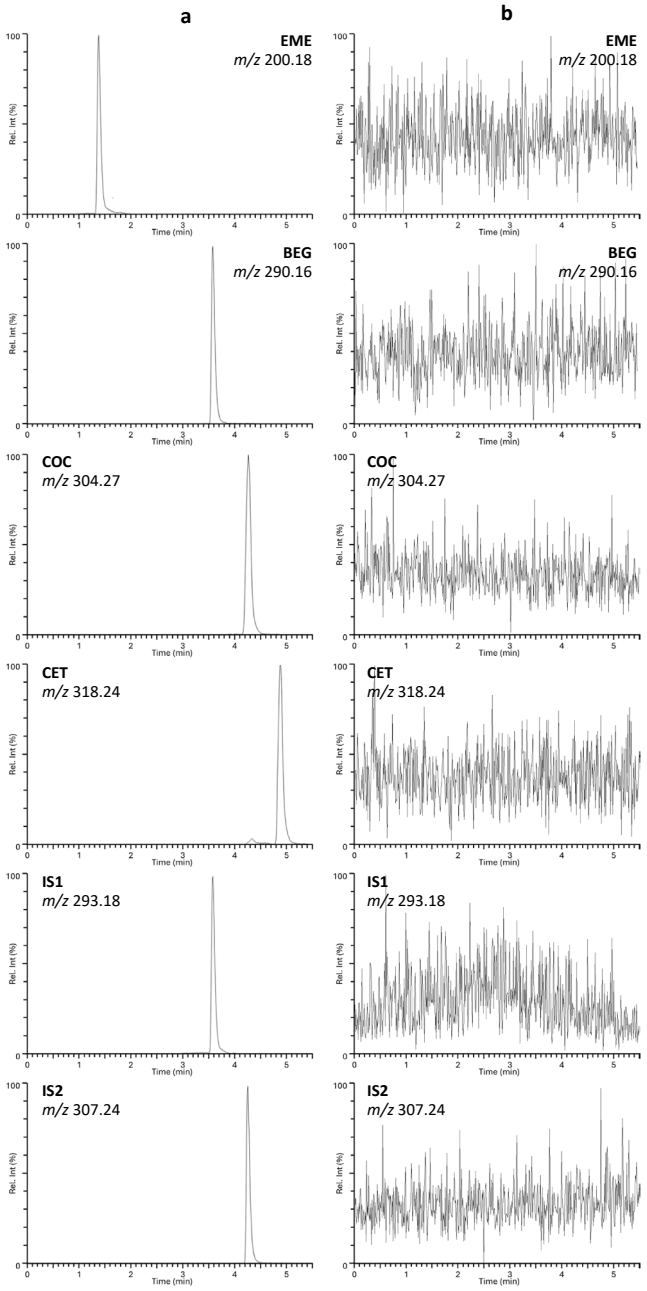

Fig. 4. UHPLC-MS chromatograms obtained from the analysis of a) blank qDBS fortified with the analytes at the concentration of 100 ng/mL and the ISs at 50 ng/mL each and b) blank qDBS sample.

The sensitivity of the method was evaluated by calculating the respective LOD and LOQ for each analyte; results ranged between 2.5 and 7.5 ng/mL in terms of LOQ and 1.0 and 2.5 in terms of LOD (see Table 2).

3.4.2. Extraction efficiency and precision

The results of extraction efficiency assays were good, in the 85.2–99.7 % range for all analytes at all concentration levels. Six replicates were analysed in the same day for intraday precision (repeatability) and on six different days for interday (intermediate) precision, expressed as RSD%. As can be seen, all results complied with the established acceptance criteria (RSD < 20 % for both intraday and interday precision). Complete results are shown in Table 2.

Table 2
Validation results on blank fortified samples.

Compound	Linearity range (ng/mL)	r^2	LOQ (ng/mL)	LOD (ng/mL)	Level (ng/mL)	Precision (RSD%)[a]		Extraction efficiency (%)[b]	Matrix effect (RE %)[b]	Stability at 60 days (%)[b]	
						Intraday	Interday			qDBS (RT)	Plasma (−20 °C)
EME	7.5–500	0.9991	7.5	2.5	7.5	7.1	7.4	86.4	14	84	75
					100	6.6	6.9	90.8	12	88	77
					500	5.6	5.6	91.6	15	88	76
BEG	2.5–500	0.9989	2.5	1.0	2.5	6.3	6.4	91.4	16	89	78
					100	5.8	6.0	99.7	14	90	80
					500	5.1	5.3	98.2	14	89	80
COC	5.0–500	0.9993	5.0	1.5	5.0	5.8	6.0	89.7	11	91	80
					100	5.4	5.4	92.7	9	91	83
					500	4.8	5.2	95.3	10	90	81
CET	5.0–500	0.9988	5.0	1.5	5.0	6.1	6.2	85.2	15	85	76
					100	5.5	5.5	88.6	12	86	78
					500	5.2	5.3	92.3	14	90	80
IS1	/	/	/	/	50	6.0	6.1	99.0	13	/	/
IS2	/	/	/	/	50	5.2	5.6	98.0	8	/	/

[a] n = 6.
[b] n = 3.

3.4.3. Stability

Dried microsamples are often stored at RT, since they usually maintain good stability, thanks to the lack of water in the blood spots, which prevents the progress of many chemical and enzymatic reactions and in general stabilises most analytes [35]. In particular, although COC stability in biological samples is usually low, recent studies have found that a drying step significantly increases it [31]. To test analyte stability, the analyte peaks obtained by processing freshly dried and analyte-fortified samples at three different concentrations, representing low, medium and high values within the respective linearity ranges were compared at 1-week intervals, for two months, to those of fluid plasma stored at −20 °C. As one can see in Table 3, analyte stability in qDBS samples at RT is better than their stability in plasma at lower, controlled temperatures; in any case, analyte recovery from qDBS in stability assays after two months was never lower than 84–91 % for the four analytes.

3.4.4. Matrix effect

Matrix effect was evaluated on blank anonymised leftover samples from healthy volunteers, fortifying the samples with the analytes and the ISs after extraction, analysing them and comparing the results with standard solutions at the same nominal concentration.

Matrix effect was always satisfactory, highlighting the effectiveness of the optimised sample clean-up: mean error was always in the 12–15 % range for EME, 14–16 % for BEG, 9–11 % for COC and 12–15 % for CET.

Table 3
Stability test results.

Time point (weeks)	Mean analyte recovery (%)	
	qDBS (RT)	Plasma (−20 °C)
0	100	100
1	98	96
2	97	93
3	95	88
4	93	86
5	92	83
6	90	81
7	89	79
8	88	78

3.5. Application to real samples

The established method was applied to leftover, anonymised whole blood samples from COC users, enabling a comparison of the results obtained from dried qDBS samples with those from classic blood plasma. For qDBS samples, ISs were added directly to the microsamples as previously described, then they were dried and subjected to sample treatment before UHPLC-MS analysis. Plasma samples were obtained from whole blood by centrifugation, fortified with the ISs and analysed after SPE sample treatment as described. Table 4 presents the comprehensive quantitative information acquired through the analysis of both qDBS and plasma samples from four subjects. Plasma concentration for each target analyte was converted to whole blood concentration using the respective tabulated whole blood/plasma concentration ratios which accounts for analyte dilution correlated to hematocrit, and for red blood cell/plasma partitioning. Constant hematocrit levels were employed, set at 38 % for females and 48 % for males, alongside consistent whole blood to plasma ratios for COC (0.8), BEG and EME (1.0) across all instances, in accordance with the literature [35,36].

Quali-quantitative results obtained from qDBS samples were always in good agreement with those found in fluid plasma. Therefore, the use of new microsamples generated via microfluidic technology coupled to UHPLC-MS analysis proved to be effective for the determination of COC and metabolites in dried samples, thus confirming the applicability of the method.

4. Conclusion

Our study introduces for the first time a promising approach to the forensic bioanalysis of cocaine and its metabolites by leveraging Capitainer qDBS technology that exploits a combination of paper, microfluidic polymer and a double-valve design to sample volumetrically accurate DBS samples from a drop of whole blood. The implementation of this innovative microsampling technology not only facilitates precise and reproducible sample collection but also aligns seamlessly with the principles of GSP. In this framework, qDBS microsampling inherently enables the collection of small volumes of samples, thus minimising the need for resource-intensive procedures and materials. Moreover, the

Table 4
Method application to real qDBS samples and comparison with plasma.

Subject	Concentration found ± SD (ng/mL)[a]							
	COC		BEG		EME		CET	
	qDBS	Plasma[b]	qDBS	Plasma[b]	qDBS	Plasma[b]	qDBS	Plasma[b]
1	235 ± 9	258 ± 11	324 ± 16	349 ± 18	116 ± 6	126 ± 8	21 ± 4	23 ± 5
2	308 ± 10	283 ± 12	298 ± 8	272 ± 21	141 ± 7	126 ± 9	n.d.[c]	n.d.[c]
3	131 ± 6	141 ± 7	286 ± 10	260 ± 12	98 ± 6	91 ± 6	n.d.[c]	n.d.[c]
4	77 ± 4	89 ± 5	182 ± 8	162 ± 10	41 ± 3	46 ± 4	47 ± 6	43 ± 7

[a] n = 3.
[b] After conversion using blood/plasma concentration ratios.
[c] < LOD.

dried nature of microsamples eliminates the necessity for refrigeration or freezing during transport and storage, further contributing to environmental friendliness, as analyte stability in qDBS samples stored at RT was significantly better than their stability in plasma at lower, energy-intensive controlled temperatures. The ease of use, minimal invasiveness, and reduced environmental impact position qDBS as a valuable tool in advancing both the science and sustainability of bioanalytical research with high applicability potential for forensic investigations, anti-doping and workplace testing. Further studies are warranted to expand the scope of applications and explore additional avenues for automation and self-sampling, anticipating continued advancements in the field of microsampling for comprehensive forensic analyses.

CRediT authorship contribution statement

Sarah Palano: Writing – original draft, Validation, Formal analysis, Conceptualization. **Dorota Turoňova:** Methodology, Formal analysis. **Michele Protti:** Writing – review & editing, Validation, Supervision, Investigation, Data curation, Conceptualization. **Lenka Kujovská Krčmová:** Supervision. **Roccaldo Sardella:** Data curation, Conceptualization. **Přemysl Mladěnka:** Funding acquisition. **Roberto Mandrioli:** Writing – review & editing, Validation, Methodology, Conceptualization. **Stefano Girotti:** Data curation, Conceptualization. **Laura Mercolini:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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