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Does volumetric absorptive microsampling eliminate the hematocrit bias for caffeine and paraxanthine in dried blood samples? A comparative study

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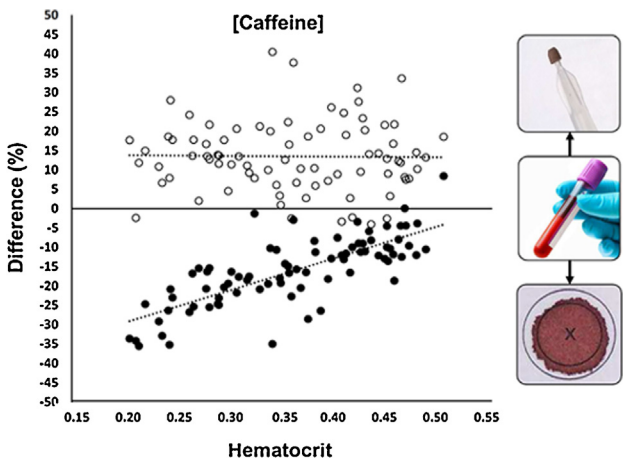
HIGHLIGHTS

- Evaluation of the potential of VAMS to overcome the hematocrit effect.
- Successful validation of LC-MS/MS method for caffeine–paraxanthine in VAMS samples.
- Comparative study between human VAMS, DBS and blood samples with varying hematocrit.
- VAMS results not affected by bias that changed over evaluated hematocrit range.
- For the same samples, VAMS concentrations slightly overestimated whole blood concentrations.

GRAPHICAL ABSTRACT



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ABSTRACT

Volumetric absorptive microsampling (VAMS) is a novel sampling technique that allows the straightforward collection of an accurate volume of blood (approximately 10 μL) from a drop or pool of blood by dipping an absorbent polymeric tip into it. The resulting blood microsample is dried and analyzed as a whole. The aim of this study was to evaluate the potential of VAMS to overcome the hematocrit bias, an important issue in the analysis of dried blood microsamples. An LC-MS/MS method for analysis of the model compounds caffeine and paraxanthine in VAMS samples was fully validated and fulfilled all pre-established criteria. In conjunction with previously validated procedures for dried blood spots (DBS) and blood, this allowed us to set up a meticulous comparative study in which both compounds were determined in over 80 corresponding VAMS, DBS and liquid whole blood samples. These originated from authentic human patient samples, covering a wide hematocrit range (0.21–0.50). By calculating the differences with reference whole blood concentrations, we found that analyte concentrations in VAMS samples were not affected by a bias that changed over the evaluated hematocrit range, in contrast to DBS results. However, VAMS concentrations tend to overestimate whole blood concentrations, as a consistent positive bias was observed. A different behavior of VAMS samples prepared from incurred and spiked blood, combined with a somewhat reduced recovery of caffeine and paraxanthine from VAMS tips at high hematocrit values, an effect that was not observed for DBS using a

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very similar extraction procedure, was found to be at the basis of the observed VAMS-whole blood deviations. Based on this study, being the first in which the validity and robustness of VAMS is evaluated by analyzing incurred human samples, it can be concluded that VAMS effectively assists in eliminating the effect of hematocrit.

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1. Introduction

Owing to distinct advantages over traditional liquid blood samples (whole blood, serum and plasma), dried blood spot (DBS) sampling has been increasingly applied over the last years in various bioanalytical areas including preclinical animal studies [1–3] and clinical trials [4] in drug development programs, therapeutic drug monitoring [5,6] and toxicology [7,8]. Implementing dried blood microsamples in an analytical workflow may simplify sample collection, transport, storage and processing. Furthermore, it enables collection of representative samples in the patient's home setting or in resource-limited areas. Nevertheless, several issues are still limiting the generalized use of DBS in routine bioanalysis, amongst which the influence of hematocrit (Hct) is one of the most addressed topics, along with the volume of blood spotted onto the filter paper cards and the spot homogeneity. As Hct is directly related with blood viscosity, a drop of blood with high Hct will spread less on filter paper, resulting in spots with a smaller diameter. Consequently, a fixed-sized sub-punch taken from a high Hct DBS will contain a higher amount of blood (and analyte) than a punch taken from a low Hct DBS, giving rise to a Hct-dependent assay bias [9–11]. Apart from the latter phenomenon, high Hct levels may negatively impact the recovery of an analyte from DBS, an effect that can be minimized by optimizing the extraction conditions [12,13].

Several strategies to cope with the Hct effect caused by differential spreading of blood on filter paper have been proposed in the past few years [10,14]. In general, for every sub-punch DBS method, the impact of Hct can be minimized by preparing the calibration line using blood with a Hct value close to the expected median Hct of the study population. Furthermore, by analyzing quality control (QC) samples prepared from blood with varying Hct values during method validation, a Hct interval can be established in which assay bias is within acceptable limits. To verify whether the Hct of a given DBS actually lies within this interval, potassium (K^+) concentrations can be measured in extracts of DBS as they allow to predict the Hct of the blood used to prepare the DBS [15]. These predicted Hct values, or the K^+ concentrations as such, can actually be used to correct for the Hct-induced bias [16,17]. An alternative approach is to use devices, consisting of a multilayered membrane filtration system, that generate dried plasma spots (DPS) upon application of blood [18,19]. Although also these systems hold promise to overcoming the Hct issue, their validity and practical applicability needs to be supported by more data.

Analyzing complete volumetrically applied DBS is perhaps the simplest way to avoid the Hct effect [20]. Here, the blood can be applied on pre-punched paper discs [12,21,22] or, alternatively, the entire DBS can be punched [23]. However, these approaches require the accurate and precise application of a fixed volume of blood onto the filter paper substrate using pipettes or micro-capillaries. Whereas this is a feasible strategy when sampling is to be carried out by trained personnel, it precludes, e.g., home-sampling by non-experienced individuals.

Recently, two interesting new approaches that allow to collect a fixed volume of blood from a non-volumetrically deposited sample have been developed. The first is a microfluidic device consisting of a foldable support system that holds a DBS card on one side and a microfluidic plate with sized capillaries on the other [24]. The second, termed volumetric absorptive microsampling (VAMS), allows straightforward collection of an accurate volume of blood without the need for specialized devices, such as pipettes or capillaries. The device consists of a plastic handler with attached to it an absorbent polymeric tip, which, when dipped into blood, wicks up an accurate volume of blood (~10 μL) [25]. By sampling blood with Hct values ranging from 0.20 to 0.70, it has been shown that the absorbed blood volume was independent of Hct [25]. In addition, analysis of VAMS samples with a similar Hct range (0.20–0.65) in six different laboratories revealed an acceptable variability in absorbed blood volume (8.7% CV) [26]. Very recently, VAMS was used in a toxicokinetic (TK) study in rats. Although controlling the blood flow rate from the tail vein appeared to be an issue, leading to overfilling of the VAMS tips, the authors concluded that TK parameters for paracetamol obtained by VAMS were comparable to those measured in conventional diluted whole blood microsamples [27]. Based on these studies, VAMS shows promise to overcome the Hct and volume bias in the analysis of dried blood microsamples, while maintaining the benefits of collecting dried samples. Furthermore, in contrast to sub-punch DBS methods, potential sample inhomogeneity is no longer an issue as the entire tip is extracted. However, up to now, only artificial human samples (obtained via spiking blood that was prepared to have a certain Hct) have been used to demonstrate the ability of VAMS to overcome the Hct effect [25–27]; the impact of Hct on the analysis of real life human samples remained to be established. Therefore, the aim of this study was to evaluate the potential of VAMS to eliminate the Hct effect by analyzing over 80 incurred human patient samples with a wide Hct range (0.21–0.50). This is the first in-human study in which analyte concentrations obtained by VAMS are compared to concentrations measured in corresponding whole blood and DBS samples. The analyzed samples originated from patients admitted to 18 different hospital departments, including critical units, such as surgery or burn wound center, and consultation departments of diverse disciplines, such as radiology, cardiology, nephrology or oncology. Therefore, in addition to deviating Hct values, it was expected that this study batch includes a wide variety of sample characteristics, representing a highly relevant study batch to evaluate the practical applicability and robustness of VAMS. Caffeine and paraxanthine, determined using a validated LC-MS/MS method, were selected as model compounds since we previously observed that DBS sub-punch concentrations of both compounds are subject to a Hct-induced bias [16,28].

2. Materials and methods

2.1. Chemicals and stock solutions

Caffeine, paraxanthine, their internal standards (IS) caffeine-$^{13}C_3$ and paraxanthine-$^{13}C_4$–$^{15}N_3$ and formic acid were purchased from Sigma–Aldrich (Diegem, Belgium). LC–MS grade methanol was obtained from Biosolve (Valkenswaard, The Netherlands). Ultrapure water was provided by a Synergy® Water Purification System (Merck Millipore, Overijse, Belgium). Stock solutions of caffeine and paraxanthine in water (1 mg mL^{-1}) and the IS in methanol (100 μg mL^{-1}) were prepared as described before [29].

2.2. Sample collection

Caffeine- and paraxanthine-free venous whole blood from a caffeine abstinent healthy volunteer was collected in ethylenediaminetetraacetic acid (EDTA) tubes (Venosafe® 9 mL VF-109SDK, Terumo, Leuven, Belgium) and used to prepare calibrators and QC samples. Patient whole blood samples ($n = 96$) with a wide Hct range (0.21–0.50) were obtained by taking aliquots (400 μL) from EDTA blood samples (Venosafe® 4 mL VF-054SDK, Terumo) admitted to the Laboratory of Clinical Biology of Ghent University Hospital for routine analysis. All samples were anonymized and, apart from Hct values and hospital departments, no clinical parameters or patient data were available. Hct was measured using a Sysmex XE-5000 hematology analyzer (Sysmex, Kobe, Japan). This procedure was approved by the Ethics Committee of Ghent University Hospital.

VAMS devices (brand name Mitra™) were purchased from Phenomenex (Utrecht, The Netherlands). VAMS samples were generated by dipping the upper part of the tip into a volume of whole blood contained in 2 mL tubes, thereby ensuring that the tips were not completely immersed into the blood to prevent overfilling. Upon turning completely red, the tips were held in place for an additional 2 s. Subsequently, the devices were positioned in a dedicated rack to prevent samples from touching each other while being air-dried for 2 h at room temperature. DBS samples were prepared by spotting 25 μL whole blood on WHA10334885 Whatman 903 filter paper (GE Healthcare, Dassel, Germany) using a calibrated pipette and dried under the same conditions as VAMS samples. Whole blood samples were obtained by transferring 50 μL blood to 2 mL tubes. Until analysis, dried samples were stored at room temperature in the presence of desiccant (two 5 g Minipax® absorbent packets, Sigma–Aldrich) in a closed plastic box or a zip-closure plastic bag for VAMS and DBS samples, respectively. Liquid blood samples were stored at −20 °C for 41 days.

2.3. Sample preparation and LC-MS/MS procedures

VAMS tips were separated from the handler and placed in 2 mL cups before 140 μL of a methanol/water (80/20, v/v) mixture, containing 0.01% formic acid and the isotopically labeled IS of caffeine and paraxanthine, was added. The tips were extracted for 10 min in a Thermomixer® comfort (Eppendorf, Hamburg, Germany) set at 1000 rpm and 22 °C. Following a centrifugation step at room temperature (10 min; 10,000 × g), 90 μL of the supernatant was isolated and mixed with 390 μL of water, containing 0.01% formic acid. The resulting mixture was transferred to an LC vial and 10 μL was injected onto the UPLC® column. DBS and whole blood samples were processed using previously developed and validated methods [29].

All samples were analyzed by LC-MS/MS using an Acquity UPLC® (Waters, Milford, MA, USA)—API 4000™ triple quadrupole mass spectrometer (AB SCIEX, Framingham, MA, USA) configuration. Chromatography and mass spectrometry parameters for DBS and whole blood analysis are described elsewhere [29]. Instrument settings for analysis of VAMS samples were identical as those for DBS.

2.4. Validation of the VAMS method

Validation of the VAMS method was based on European Medicines Agency (EMA) and U.S. Food and Drug Administration (FDA) guidelines for bioanalytical method validation [30,31] and included evaluation of selectivity, carry-over, lower limit of quantification (LLOQ), linearity, precision, accuracy, matrix effect, recovery and stability.

Selectivity was assessed by analyzing VAMS samples that were prepared using blank whole blood from six different healthy volunteers. Blank samples, along with zero (IS-spiked blank matrix) samples, were also analyzed at the start of every analytical run to verify whether the IS potentially contributed to the responses of the analytes as a result of impurity. All resulting chromatograms were inspected for interfering peaks, which were considered acceptable if responses were less than 20% of the LLOQ for the analytes and 5% for the IS [30]. Blank samples injected after the highest calibrators were used to determine carry-over, thereby applying identical criteria as for assessment of selectivity.

On four non-consecutive days, eight-point calibration lines were constructed using blank whole blood and analyzed to evaluate linearity. The concentrations of the calibrators were 0.050, 0.075, 0.25, 0.50, 1.0, 2.5, 5.0, 10.0 μg mL^{-1} for caffeine and 0.025, 0.050, 0.100, 0.25, 0.50, 1.0, 2.5, 5.0 μg mL^{-1} for paraxanthine. The LLOQ was the lowest calibrator and was defined as the lowest concentration of caffeine and paraxanthine which could be measured with %RSD and %bias below 20%. To evaluate whether the obtained calibration data were homoscedastic, an F-test was performed at the 99% confidence level and residual vs. nominal concentration plots were constructed [32]. Both unweighted and weighted linear regression were applied to find the best fitted-model. The following weighting factors were tested: $1/x$, $1/x^2$, $1/\sqrt{x}$, $1/y$, $1/y^2$ and $1/\sqrt{y}$. The obtained models were compared by calculating the sum% relative error (RE) and plotting %RE against nominal concentrations [32]. In order to accept the selected model, mean back-calculated concentrations of the calibrators should be within ±15% of the nominal value or within ±20% for the LLOQ [30].

QCs at four concentration levels were prepared and analyzed in duplicate on four non-consecutive days to determine precision and accuracy. The nominal concentrations were 0.05 and 0.025 (LLOQ), 0.12 and 0.06 (low), 4.0 and 2.0 (medium), 8.0 and 4.0 (high) μg mL^{-1} for caffeine and paraxanthine, respectively. Independent stock solutions were used for QCs and calibrators. For all spiked samples, the volume of non-matrix solvents never exceeded 5% of the total sample volume. A single factor ANOVA was used to calculate intra- and interbatch precision values (%RSD) [33]. Accuracy (%bias) was calculated by dividing the difference between the obtained concentration and the nominal value by the nominal value and multiplying it by 100. Precision and accuracy should be within ±15% (±20% for the LLOQ) [30].

Blank whole blood originating from six different sources with Hct values ranging from 0.40 to 0.46 (0.43 ± 0.02; mean ± SD) was used to determine matrix effect and recovery. Blood samples were spiked at low and high concentration level before (C) or after (B) extraction. Solutions of caffeine and paraxanthine in the starting eluent at the same concentrations (A) were prepared as well. To prepare solutions A and B, a nominal blood volume of 10 μL, absorbed by the VAMS tips when dipped into the blood samples, was taken into account. The ratios of peak areas of (B) to those of (A) were multiplied by 100 to obtain absolute matrix effect values, while absolute recovery values were calculated by multiplying the ratios of peak areas of (C) to those of (B) by 100. Relative matrix effect and recovery were obtained by the %RSD of absolute matrix effect and recovery values [34]. Relative matrix effect should not exceed 15% [30]. To evaluate the impact of Hct on recovery and matrix effect, blood samples with different Hct values (0.21, 0.42 and 0.62) were prepared starting from blood with Hct 0.48, as described before [15]. For all four Hct levels, recovery and matrix effect were determined in triplicate on low and high concentration level. Here, 10 μL of blank or spiked blood was spotted onto the upper surface of the VAMS tips using a calibrated pipette.

Stability of VAMS samples was evaluated by analyzing low and high QCs in triplicate at time point zero and after 4 days of storage

Table 1
Calibration and sensitivity data for the determination of caffeine and paraxanthine in VAMS samples using LC-MS/MS ($n = 4$).

	Weighting	Slope, mean ± SD [95% CI]	Intercept, mean ± SD [95% CI]	R^2	Standard deviation of residuals	Range ($\mu g\,mL^{-1}$)
Caffeine	$1/y^2$	2.021 ± 0.215 [1.833–2.209]	0.036 ± 0.010 [0.028–0.045]	0.996	0.250	0.050–10.0
Paraxanthine	$1/x^2$	4.456 ± 0.480 [4.035–4.877]	0.025 ± 0.014 [0.012–0.037]	0.995	0.284	0.025–5.0

Table 2
Intra- and inter-batch precision and accuracy ($n = 4 \times 2$) for QCs of caffeine and paraxanthine at four concentration levels in VAMS samples.

QC	Intrabatch precision (%RSD)		Interbatch precision (%RSD)		Accuracy (%bias)	
	Caffeine	Paraxanthine	Caffeine	Paraxanthine	Caffeine	Paraxanthine
LLOQ	8.08	6.65	14.31	13.36	8.96	1.77
Low	7.82	5.68	9.03	7.73	3.48	4.21
Medium	4.84	3.22	5.70	5.38	−0.64	1.07
High	3.12	4.30	5.42	5.85	1.63	2.17

at 60 °C and 82 days at room temperature. VAMS samples were placed in dedicated clamshells and stored in zip-closure plastic bags with dessicant until analysis. Processed sample stability was assessed by re-injecting extracts of low and high QCs ($n = 3$) after 4 days of storage in the autosampler (4 °C) and 30 days at −20 °C. All stability samples were analyzed against a freshly prepared calibration curve. The obtained mean concentrations at a given time point should be within ±15% of the concentration measured at time point zero and of the nominal concentration [30].

2.5. Comparative study involving VAMS, DBS and whole blood sampling

Caffeine and paraxanthine concentrations were determined in VAMS, DBS and whole blood samples originating from 96 hospital patient blood samples. All samples were analyzed against freshly prepared matrix-matched calibration curves, prepared from blank blood from a single donor with a Hct of 0.48. To examine the impact of Hct on VAMS and DBS results, differences (%) between VAMS or DBS concentrations and whole blood concentrations were plotted against Hct values. Percentage differences were calculated by dividing the difference between VAMS or DBS and whole blood concentrations by the whole blood concentrations and multiplying the result by 100. Using least squares regression analysis, linear regression lines were fitted to the resulting data. Slopes, intercepts and their 95% confidence intervals (CI) were calculated by the Analysis Toolpack of MS Excel® 2013 (Microsoft, Redmond, WA, USA). VAMS and whole blood concentrations were also compared by constructing Bland–Altman plots using Medcalc statistical software version 12.7.5 (Medcalc Software bvba, Ostend, Belgium).

Furthermore, caffeine and paraxanthine concentrations were determined in VAMS and whole blood samples ($n = 3$) originating from spiked and incurred blood. Therefore, blank EDTA blood from a healthy volunteer was spiked with caffeine and paraxanthine to obtain nominal concentrations of 1.0 and 0.8 $\mu g\,mL^{-1}$, respectively. These target concentrations were based on the results of a previously conducted CYP1A2 phenotyping study [28] and should approximate whole blood caffeine and paraxanthine concentrations 6 h after the intake of a 150 mg caffeine test dose. Hence, on the same day, a second blood sample was collected from the same volunteer 6 h after oral ingestion of a capsule containing 150 mg caffeine. Spiked and incurred samples were analyzed in the same analytical run. Calibration curves for VAMS and whole blood samples were prepared using a single pool of blank blood.

3. Results and discussion

3.1. Validation of the VAMS method

No unacceptable interfering peaks were observed in VAMS samples prepared from blank blood from 6 individual sources and the IS did not contribute to the responses of caffeine and paraxanthine. Furthermore, no carry-over was found in blank samples injected after the highest calibrators. Calibration data for caffeine and paraxanthine were found to be heteroscedastic. Weighted linear regression considerably improved %RE values compared to unweighted regression. The selected weighting factors for both compounds are shown in Table 1, together with calibration and sensitivity data. Using these regression parameters, mean back-calculated concentrations of the calibrators deviated less than 5 and 7% from the nominal concentrations for caffeine and paraxanthine, respectively. The chosen models fulfilled the acceptance criteria and the calibration lines were linear. Intra- and interbatch precision (%RSD) and accuracy (%bias) values for caffeine and paraxanthine, displayed in Table 2, were below 15% and, therefore, also met the acceptance criteria.

Table 3 shows matrix effect and recovery data for caffeine and paraxanthine, determined in VAMS samples prepared from

Table 3
Absolute and relative matrix effect and recovery data ($n = 6$) for caffeine and paraxanthine at two concentration levels in VAMS samples prepared using whole blood from six different individuals with Hct values ranging from 0.40 to 0.46 (0.43 ± 0.02; mean ± SD).

	Absolute matrix effect (mean ± SD, %)		Relative matrix effect (%RSD)		Absolute recovery (mean ± SD, %)		Relative recovery (%RSD)	
QC	Without IS	With IS	Without IS	With IS	Without IS	With IS	Without IS	With IS
Caffeine								
Low	101.71 ± 3.23	98.27 ± 3.87	3.17	3.94	99.10 ± 4.33	116.42 ± 6.10	4.37	5.24
High	102.50 ± 1.23	100.08 ± 1.18	1.20	1.18	94.04 ± 2.60	108.73 ± 2.77	2.77	2.55
Paraxanthine								
Low	99.79 ± 5.09	99.81 ± 5.68	5.10	5.69	90.16 ± 3.71	100.20 ± 2.66	4.11	2.66
High	101.09 ± 1.08	99.44 ± 1.71	1.07	1.72	87.74 ± 2.34	95.74 ± 2.01	2.66	2.10

Table 4
Absolute recovery and matrix effect data ($n = 3$) for caffeine and paraxanthine at two concentration levels in VAMS samples prepared using whole blood with varying Hct values.

	Hct	Caffeine		Paraxanthine	
		Low QC	High QC	Low QC	High QC
Absolute recovery (mean ± SD, %)	0.21	101.45 ± 2.26	101.79 ± 0.67	86.98 ± 1.52	87.14 ± 1.75
	0.42	101.30 ± 1.28	100.53 ± 2.67	84.70 ± 3.53	84.33 ± 1.38
	0.48	93.86 ± 0.89	91.08 ± 2.03	75.48 ± 0.60	75.93 ± 1.09
	0.62	92.01 ± 4.80	92.91 ± 4.74	73.51 ± 1.60	77.72 ± 5.69
Absolute matrix effect (mean ± SD, %)	0.21	104.29 ± 3.37	100.49 ± 1.54	101.46 ± 2.49	99.69 ± 2.01
	0.42	101.16 ± 0.53	99.52 ± 1.71	98.28 ± 3.28	99.48 ± 0.34
	0.48	97.46 ± 0.50	97.48 ± 0.99	99.03 ± 2.73	99.10 ± 1.31
	0.62	103.07 ± 2.09	98.36 ± 0.31	100.58 ± 4.26	99.48 ± 1.78

6 individual lots of blood with an average Hct of 0.43 ± 0.02. The absolute matrix effect for both compounds was close to 100%, with or without compensation by the IS. Relative matrix effect, being lower than 4 and 6% RSD for caffeine and paraxanthine, respectively, was well within the predefined acceptance limits (15% RSD). High recovery values (>87%) were obtained for both compounds. As recovery was somewhat lower for the IS than for the analytes, a slight overcompensation for caffeine was observed when the IS was taken into account. However, it should be noted that, in order to calculate the amount of analyte that was spiked after extraction, a nominal absorbed blood volume of 10 μL was assumed. According to the manufacturer, the average absorbed volume of the VAMS tips used in this study was actually 10.7 μL. Therefore, the reported recovery values in Table 3 represent a limited overestimation of the real recovery. Importantly, recovery for both compounds was reproducible (<6% RSD). As the blood samples used in the latter experiment had a narrow Hct range (0.40–0.46), the effect of Hct on recovery and matrix effect was further evaluated using blood samples with deviating Hct values (0.21–0.62). Absolute recovery and matrix effect values, determined in triplicate at low and high concentration levels, are presented in Table 4. For these data, compensation by the IS was already taken into account. While matrix effect was not affected by Hct recovery for both compounds was lower at higher Hct values (0.48 and 0.62). A similar trend was observed by Denniff et al., who found a reduced recovery for low paracetamol concentrations in VAMS samples, with recovery values ranging from 92.9 to 70.6% over a comparable Hct range (0.20–0.69) [27]. It seems that as Hct increases, the higher relative amount of erythrocytes trapped in the VAMS tips renders it more difficult for compounds to be desorbed from the tips. Since 10 μL of blood was directly spotted onto the VAMS tips in this experiment, it can be excluded that lower volumes of blood absorbed by the tips were at the basis of the reduced recovery at high Hct. Although beyond the scope of this study, this recovery issue can most probably be resolved by optimizing the extraction conditions, for example by using sonication [12] or elevated extraction temperatures [13], as demonstrated in DBS analysis. Here, we opted to apply a very similar protocol as previously developed and validated for the extraction of caffeine and paraxanthine from DBS and for which a suchlike influence of Hct on recovery was not observed [29]. Stability data for low and high QCs, analyzed in triplicate, are presented in Table 5 and demonstrate that caffeine and paraxanthine were stable in VAMS samples for at least 82 days when stored at room temperature and for at least 4 days when stored at 60 °C, the latter representing potential shipping conditions. Furthermore, processed samples were stable when stored for at least 4 days in the autosampler set at 4 °C and for at least 30 days at −20 °C. Stock solutions of both compounds and the IS were stable for at least 6 months at −20 °C [29].

Table 5
Stability data for caffeine and paraxanthine in VAMS samples at two concentration levels ($n = 3$). Data are presented as the percentage of the concentrations measured at time point zero.

QC	Room temperature 82 days (mean ± SD, %)		60 °C 4 days (mean ± SD, %)	
	Caffeine	Paraxanthine	Caffeine	Paraxanthine
Low	100.10 ± 11.24	97.44 ± 10.38	97.58 ± 8.64	90.94 ± 8.28
High	99.11 ± 3.50	99.41 ± 4.92	94.09 ± 7.08	95.50 ± 5.09

3.2. Comparative study involving VAMS, DBS and whole blood sampling

In 81 out of 96 corresponding VAMS, DBS and whole blood samples, caffeine concentrations above the LLOQ (0.050 μg mL^{-1}) could be measured, while paraxanthine concentrations above the LLOQ (0.025 μg mL^{-1}) were measured in 83 samples. As we relied on daily consumption of caffeine containing beverages or food to determine caffeine and paraxanthine concentrations in the collected samples, no data on caffeine dose or time since the last intake were available. The latter, combined with the availability of a single blood sample per subject, did not allow to calculate pharmacokinetic parameters. Hct values in the analyzed samples ranged from 0.21 to 0.50, with a median of 0.36. It should be noted that all samples were analyzed against calibration curves prepared using blood with a Hct of 0.48. We specifically opted to use a calibration curve generated from blood with a relatively high Hct to clearly visualize the Hct effect on DBS concentrations and create a worst case scenario, both for DBS and VAMS samples. This allows a challenging assessment of the Hct effect. The differences (%) between VAMS or DBS concentrations and whole blood concentrations, plotted against Hct values, are shown in Fig. 1 for caffeine (a) and paraxanthine (b). A similar Hct-induced bias was observed for DBS concentrations of both compounds, as regression lines fitted to the differences between DBS and whole blood concentrations had a slope of 83.67 (95% CI; [67.80–99.54]) and an intercept of −46.30 (95% CI; [−52.17 to −40.44]) for caffeine and a slope of 82.25 (95% CI; [67.61–96.90]) and an intercept of −52.16 (95% CI; [−57.57 to −46.75]) for paraxanthine. Measured DBS concentrations markedly decreased with decreasing Hct values. These results are in accordance with previous findings for both compounds [16]. With differences between DBS and whole blood concentrations ranging from −35.52 to 8.39% for caffeine and from −41.44 to 4.16% for paraxanthine over the evaluated Hct range, the assay bias was unacceptable at low Hct levels. However, as we previously demonstrated [16], DBS concentrations of both compounds can be corrected for the Hct bias in a convenient way by means of an algorithm based on K^+ concentrations measured in

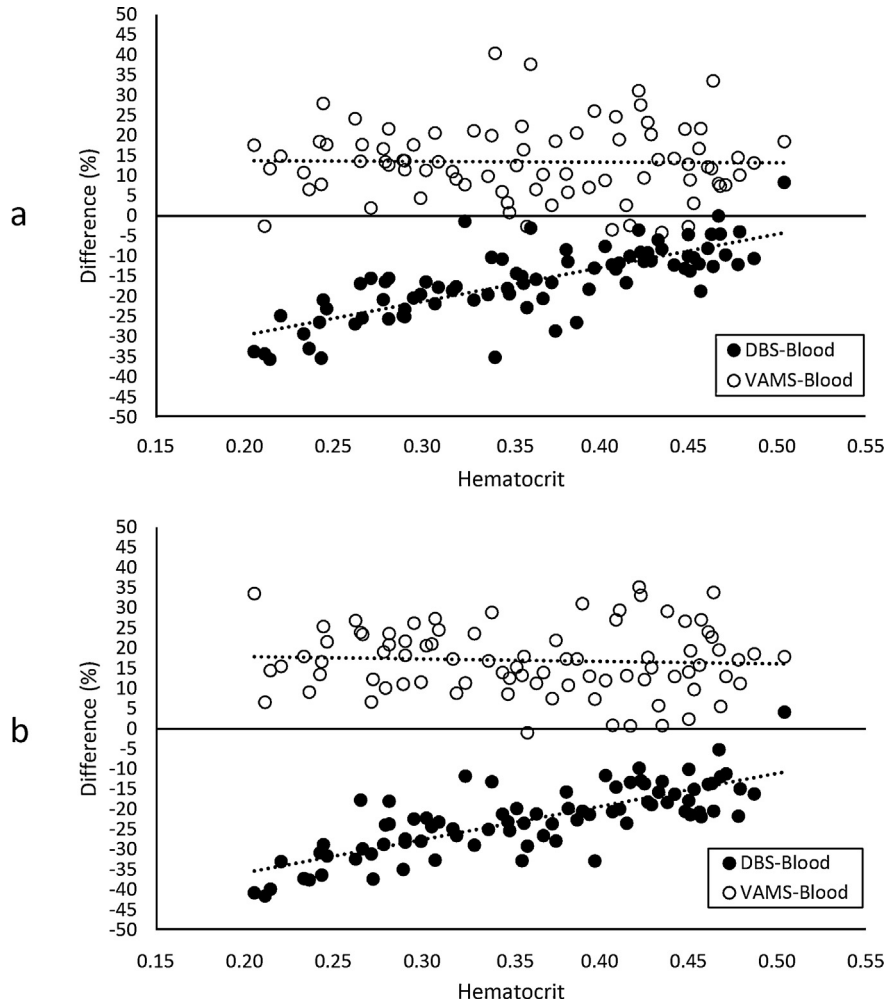

Fig. 1. Differences (%) between VAMS (white dots) or DBS (black dots) concentrations and whole blood concentrations plotted against hematocrit for (a) caffeine ($n = 81$) and (b) paraxanthine ($n = 83$). Broken lines represent linear regression lines.

DBS extracts. VAMS concentrations, on the other hand, were not affected by a suchlike Hct-effect. The regression lines fitted to the differences between VAMS and whole blood concentrations had a slope of −1.70 (95% CI; [−26.96–23.56]) and an intercept of 14.09 (95% CI; [4.76–23.43]) for caffeine and a slope of −5.98 (95% CI; [−28.42–16.47]) and an intercept of 19.13 (95% CI; [10.84–27.42]) for paraxanthine. As, for both compounds, the 95% CIs of the slopes included the 0 value, it can be concluded that the differences between VAMS and whole blood concentrations did not change in function of Hct (in the range between 0.21 and 0.50). These findings on real patient samples lend support to other studies using artificial samples, demonstrating that VAMS devices are able to collect an accurate volume of blood over a wide Hct range [25,26].

Remarkably, VAMS concentrations were subject to a consistent positive bias, irrespective of Hct. Bland–Altman plots for the differences between VAMS and whole blood concentrations (Fig. 2) revealed a mean positive difference of 12.3% (95% CI; [10.5–14.1%]) and 15.4% (95% CI; [13.9–16.9%]) for caffeine and paraxanthine, respectively. Thus, analyte concentrations determined in VAMS samples tend to overestimate whole blood concentrations, although deviations were relatively limited, taking into account that results from different matrices (i.e., dried VAMS tips vs. liquid whole blood), obtained by different extraction procedures and analyzed in separate analytical runs, were compared. For example, for 86 and 74% of the samples for caffeine and paraxanthine, respectively, differences between VAMS and whole blood concentrations were below 20%, thereby fulfilling the acceptance criteria of incurred sample reanalysis, intended for reanalysis of a single matrix [30]. Interestingly, a positive bias for VAMS results was also described by Denniff et al. [27]. In a paracetamol TK study in rats, mean C_{max} was up to 40% higher when measured in VAMS samples compared to diluted whole blood samples. The authors concluded that this bias was caused by a combination of overfilled VAMS tips, as a result of the rate of blood flow from the tail vein being higher than the rate at which tips could absorb the blood, and the fact that VAMS and whole blood samples were collected from different groups of animals. In another study, the authors found a significant positive bias for midazolam when VAMS tips were intentionally submerged into blood past the shoulder [25]. In our study, we paid special attention to avoid overfilling of the tips. All VAMS samples were prepared by dipping the tip into a small volume of blood (±400 μL) contained in 2 mL plastic tubes, enabling good visibility of the blood surface, by operators experienced in handling blood (micro) samples. Care was taken to prevent that VAMS tips were dipped too deeply into the blood samples.

The differences observed in our study may be attributed to a combination of factors. First, from an analytical point of view, the recovery of caffeine and paraxanthine from VAMS samples was reduced at higher Hct values, as discussed in Section 3.1. Data in Table 4 show that the recovery of both compounds was lower

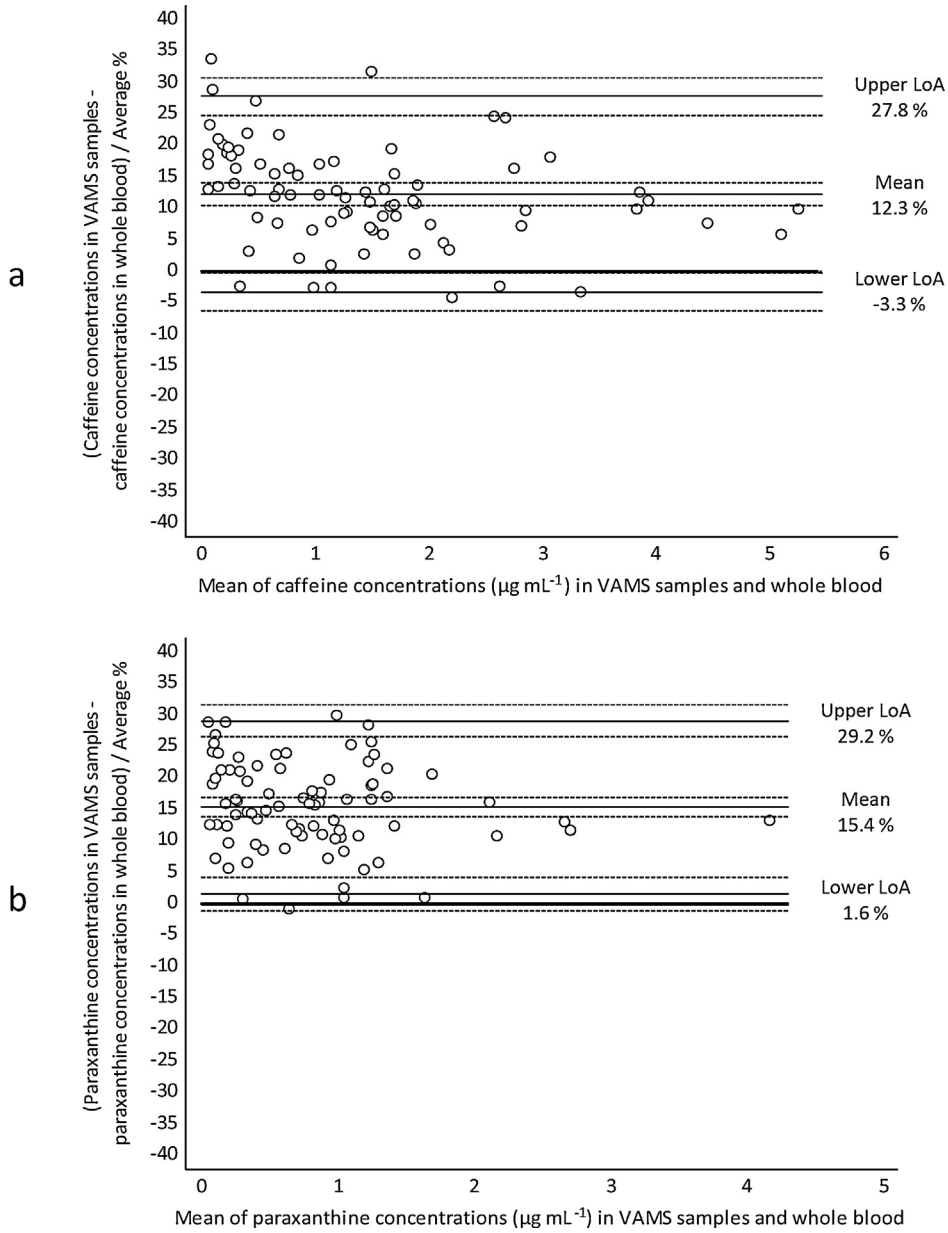

Fig. 2. Bland–Altman plots for the comparison between VAMS and whole blood concentrations for (a) caffeine ($n = 81$) and (b) paraxanthine ($n = 83$). Mean differences and limits of agreement (LoAs) are represented by full lines, 95% confidence limits by broken lines.

starting from Hct 0.48, with highly comparable values at Hct 0.48 and 0.62. The latter also applies for the recovery data at the lower evaluated Hct levels (0.21 and 0.42). Importantly, the patient samples in our study were analyzed against calibrators prepared from blood with Hct 0.48. However, all but two patients samples had Hct values below 0.48, with 0.21 being the lowest Hct. Consequently, analyte concentrations in patient VAMS samples will have been consistently overestimated, albeit to a limited extent, due to the difference in recovery between calibrators and study samples. Hence, although VAMS results were not subject to a "traditional Hct effect", with lower concentrations measured at low Hct levels and higher concentrations at high Hct values, as seen in DBS analysis, Hct did have an impact on the analysis of VAMS samples, by affecting the recovery. We consider it unlikely that other analytical factors contributed to the disparity between VAMS and whole blood concentrations. Hct had no effect on ion suppression or enhancement for VAMS samples. The different types of samples were analyzed against calibration curves prepared in the respective matrices. Accuracy values (%bias) for QCs of caffeine and paraxanthine in liquid whole blood and VAMS samples were within ±5% (±9% at LLOQ level). Furthermore, under the storage conditions used here, both compounds were stable in whole blood and VAMS samples for prolonged periods of time, exceeding the storage times the actual study samples were subject to. Apart from the evaluation of the stability of caffeine and paraxanthine in VAMS samples reported here, a whole blood stability experiment was previously performed and described elsewhere [29]. Caffeine and paraxanthine proved to be stable in whole blood for at least 172 days when stored at −20 °C. From a methodological point of view, several additional factors may have played a role. First of all, the observed mean differences between VAMS and whole blood concentrations (12.3 and 15.4% for caffeine and paraxanthine, respectively) are highly comparable to the mean differences previously found between plasma and whole blood concentrations (15.2 and 16.6 for caffeine and paraxanthine, respectively) [28]. This might lead one to hypothesize that VAMS

Table 6
Caffeine and paraxanthine concentrations in VAMS and whole blood samples ($n = 3$) originating from spiked and incurred blood. Blank EDTA blood from a healthy volunteer was spiked with caffeine and paraxanthine to obtain nominal concentrations of 1.0 and 0.8 μg mL^{-1}, respectively. Incurred blood was collected on the same day from the same volunteer 6 h after oral ingestion of a capsule containing 150 mg caffeine.

		VAMS (μg mL^{-1}) mean ± SD (%CV)	Whole blood (μg mL^{-1}) mean ± SD; (%CV)	Difference VAMS-whole blood (%)
Caffeine	Spiked samples	1.03 ± 0.01 [1.36]	1.01 ± 0.01 [1.31]	1.49
	Incurred samples	1.12 ± 0.01 [1.07]	1.04 ± 0.02 [1.74]	6.86*
Paraxanthine	Spiked samples	0.81 ± 0.01 [1.45]	0.81 ± 0.01 [0.62]	0.35
	Incurred samples	0.82 ± 0.02 [1.95]	0.77 ± 0.01 [0.88]	6.35*

* Significant difference ($p < 0.05$).

tips might preferentially absorb the plasma component of blood and that the resulting VAMS concentrations would reflect plasma rather than whole blood concentrations. However, by comparing the hemoglobin content of VAMS tips prepared using the standard dipping technique to that of tips to which 10 μL of blood was spotted, Denniff et al. demonstrated that there is no indication for such an effect [25]. Furthermore, the positive bias of VAMS vs. whole blood may result from the fact that measured whole blood concentrations underestimated the actual values, instead of VAMS concentrations overestimating whole blood concentrations. In this light, we verified whether whole blood samples were completely hemolyzed by the applied sample preparation procedure [29]. During validation of the whole blood method, QCs of caffeine and paraxanthine at four concentration levels, stored at −80 °C for 90 min and thawed at room temperature, were analyzed in duplicate on three different days along with freshly prepared QCs. As for dried VAMS samples, hemolysis was considered to be complete in frozen and thawed blood samples. The resulting caffeine and paraxanthine concentrations are summarized in Supplementary Table S1. No significant differences between caffeine and paraxanthine concentrations in fresh and hemolyzed blood were found; all differences were within ±3%. These results exclude that potential incomplete hemolysis of blood samples may have contributed to the observed deviations. Interestingly, we observed an apparently differential behavior of VAMS samples prepared from incurred vs. spiked blood. Table 6 shows caffeine and paraxanthine concentrations measured in VAMS and whole blood samples ($n = 3$) originating from either spiked blank blood or blood collected 6 h after the administration of a 150 mg caffeine dose. For both compounds, no significant differences were found between spiked whole blood and corresponding VAMS samples. Analyte concentrations in incurred blood, however, were higher when measured in VAMS compared to whole blood samples. Although differences were limited (6.86 and 6.35% for caffeine and paraxanthine, respectively), these were statistically significant, as determined by independent samples *t*-tests ($p = 0.005$ and 0.008 for caffeine and paraxanthine, respectively). Therefore, we concluded that the latter effect, for which the underlying reason is unknown, combined with the somewhat reduced recovery of caffeine and paraxanthine from VAMS samples at the Hct of the calibration line used for analysis of the study samples, was at the basis of the observed positive difference between VAMS and whole blood results.

4. Conclusion

VAMS, a novel sampling technique that allows to accurately collect a fixed volume of blood, represents a promising approach to overcome the effect of deviating Hct values in the analysis of dried blood microsamples. In this study, the potential of VAMS to effectively eliminate the Hct bias was evaluated by analyzing over 80 VAMS samples prepared from incurred whole blood collected from hospital patients displaying a wide Hct range (0.21–0.50). Therefore, an LC-MS/MS method for the determination of caffeine and paraxanthine in VAMS tips was fully validated. All evaluated parameters met the pre-established criteria. Analyte concentrations in VAMS samples were compared to corresponding DBS and whole blood concentrations. VAMS results were not affected by a bias that changed in function of Hct as differences between caffeine and paraxanthine concentrations in VAMS and whole blood samples did not vary over the evaluated Hct range, in contrast to the observed DBS-blood differences. However, on the other hand, it should be noted that measured VAMS concentrations consistently overestimated whole blood concentrations. It was found that this effect was caused by a combination of a different behavior of VAMS samples prepared from incurred or spiked blood and a reduced recovery of the analytes from VAMS tips at high Hct values. Interestingly, in a previous study, the latter effect was not observed for DBS, although very similar procedures were used to extract caffeine and paraxanthine from DBS and VAMS samples. On the basis of this study, it can be concluded that VAMS indeed allows to overcome the Hct bias for caffeine and paraxanthine, although care should be taken when protocols developed for DBS analysis are transferred to VAMS samples. As this study is the first in which the usefulness of VAMS is evaluated based on results obtained from real incurred human samples, our findings should be compared to those for other compounds with varying characteristics, determined using validated procedures, in order to further accept VAMS as a reliable sampling technique in bioanalysis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aca.2015.04.056.

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