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Evaluation of the Capitainer-B Microfluidic Device as a New Hematocrit-Independent Alternative for Dried Blood Spot Collection

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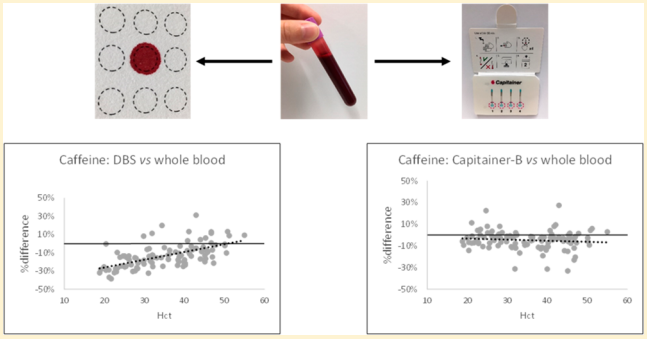
ABSTRACT: The hematocrit-bias still remains one of the most discussed issues when it comes to dried blood spot (DBS) analysis. Therefore, many attempts to cope with this issue have been made, among which the development of novel sampling tools such as the Capitainer-B (further referred to as MF (microfluidic)-DBS) devices. These are designed to allow a straightforward absorption of a fixed volume (13.5 μL) of blood by a preperforated paper disc, which can be analyzed afterward. The aim of this study was to evaluate the potential of these devices to nullify the hematocrit-based area bias and to investigate whether the amount of blood applied has an influence on the device performance. An LC-MS/MS method for the quantification of caffeine and paraxanthine in MF-DBS was fully validated, meeting all preset acceptance criteria. In a next step, using a set of 133 authentic, venous patient samples with a hematocrit range of 18.8−55.0, concentrations of both compounds in MF-DBS were compared to those in corresponding partial-punch pipetted DBS (PI-DBS) and liquid blood samples. When compared to blood as a reference, the concentrations obtained in MF-DBS were not affected by a bias in function of the evaluated hematocrit, in contrast to those obtained from partial-punch PI-DBS. Furthermore, analysis of samples resulting from spiking different volumes of whole blood at different hematocrit levels, revealed that the amount of blood applied at the device inlet has no influence on the performance of the devices. Therefore, it can be concluded from this study, being the first in which the impact of the hematocrit and the applied volume is evaluated by analyzing authentic, venous patient samples, that MF-DBS devices effectively assist in eliminating the hematocrit-based area bias, independently from the applied blood volume.



Lately, many efforts have been made in an attempt to overcome the well-discussed hematocrit (Hct)-issue coupled to classical dried blood spot (DBS) analysis. Theoretically, the overall Hct-based bias can be seen as a 3-fold problem, that is, a distinction can be made between a Hct-based area bias, a Hct-based recovery bias, and a Hct-based matrix bias.[1] The Hct-based recovery bias is resulting from the fact that the internal standard is typically only added during the extraction step for conventional DBS extraction, leading to the inability to correct for variations in recovery from the dried blood. Furthermore, a Hct-based matrix bias can be the result of the fact that a DBS sample with a different Hct can be considered as being a different matrix. Therefore, for the evaluation of recovery and matrix effects during method validation, the inclusion of blood samples with a broad Hct range is very valuable. The Hct-based area bias is best documented and is the consequence of the different spreading of whole blood—with a varying Hct—over classical DBS cards because of differences in the viscosity of the blood: blood with a higher Hct (e.g., 50%) will spread less compared to the same amount of blood with a lower Hct (e.g., 30%). This phenomenon gives rise to DBS with a substantially different area, which in turn leads to a difference in the amount of analyte sampled when using a fixed diameter subpunch. Logically, a whole spot analysis after volumetric application of a fixed amount of blood should nullify this area bias. However, volumetric application can only be achieved when using a dedicated device.

Volumetric application by using anticoagulant-coated micro-capillaries or calibrated pipettes will in clinical practice only be possible in situations where dedicated staff (e.g., an experienced nurse or trained laboratory personnel) is available (e.g., patients in a hospital setting, post-mortem sampling or preclinical studies).[2] When this volumetric application needs to be performed by nonexperienced people (e.g., patients at home), this nondirect application of a drop of blood constitutes a non-negligible disadvantage. Therefore, to render volumetric dried blood sampling at home possible, different strategies have been proposed to volumetrically generate the dried samples, without the necessity of using a calibrated pipet. The HemaPEN technology (Trajan Scientific and Medical, Australia), the volumetric absorptive microsampling (VAMS) technology (Neoteryx, USA), the volumetric absorptive paper disc (VAPD), mini-disc (VAPDmini), and the HemaXis DB

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device (DBS System SA, Gland, Switzerland) are examples of techniques proposed for a user-friendly generation of volumetric dried blood samples.[3−9] However, although direct application of a blood drop from a fingertip onto the above-mentioned devices may be relatively straightforward, proper instructions still need to be given to self-sampling patients in order to avoid incorrect sampling, leading to erroneous results. Here, we focus on the Capitainer-B (further referred to as MF (microfluidic)-DBS) device, recently designed by the KTH Royal Institute of Technology (KTH, Stockholm, Sweden) and commercialized by Capitainer AB (Stockholm, Sweden). The device is equipped with an inlet port to which a drop of blood (e.g., obtained via a fingerprick) is added, resulting in the filling of a capillary microchannel with a fixed volume of 13.5 μL. Upon completely filling this capillary channel, a thin film at the inlet dissolves, resulting in the absorption of the excessive amount of blood by a paper matrix, leading to the separation of the excess blood and the filled channel. Finally, upon dissolving of a thin film at the outlet, the capillary channel is emptied through capillary forces, resulting in the absorption of 13.5 μL of blood by a preperforated paper disc (Ahlstrom 222 filter paper).[10] Spooner et al. readily provided a proof of concept in which it was demonstrated that the devices are able to precisely dispense an average blood volume of 13.5 μL across an artificially generated broad Hct range (25−65%).[11] Furthermore, a good agreement was found when comparing the results of the MF-DBS devices with those of liquid whole blood, making use of the direct alcohol marker phosphatidylethanol 16:0/18:1.[12] On the basis of these proof of concepts, the MF-DBS devices show promise to overcome the Hct-based area bias, while maintaining the benefits coupled to classical DBS analysis. However, although for phosphatidylethanol 16:0/18:1 determinations, authentic, venous patient samples were used, no information was provided on the Hct levels of the used patient samples, meaning that no conclusion could be made on the ability of the MF-DBS devices to overcome the Hct bias. Therefore, the impact of the Hct on the analysis of authentic patient samples remained to be established. Furthermore, it remained to be investigated whether the amount of blood dispensed at the device inlet has an impact on the accuracy and precision. Therefore, the aim of this study was to evaluate the potential of MF-DBS devices to eliminate the Hct-based area bias by analyzing 133 left-over patient samples across a wide Hct range (18.8−55.0), with caffeine and paraxanthine as model compounds. To do so, concentrations measured in MF-DBS were compared to corresponding whole blood and partial-punch pipetted DBS (PI-DBS). Moreover, different volumes of blood (25, 30, 35, 40, and 50 μL) were applied, originating from patient samples with a very low or very high Hct to check whether the amount of blood added at the inlet port has an influence on the performance of the devices. Since the included samples originated from patients admitted to 17 hospital departments (including surgery, radiology, rheumatology, endocrinology, and nephrology, among others), the samples used in this study include a wide variety of characteristics, which is highly relevant to assess the device's practical relevance and robustness.

EXPERIMENTAL SECTION

Chemicals and Stock Solutions. Formic acid, caffeine, paraxanthine, and the internal standards (IS) caffeine-$^{13}C_3$ and paraxanthine-$^{13}C_4$-$^{15}N_3$ were obtained from Sigma-Aldrich (Diegem, Belgium). LC-MS grade methanol was purchased from Biosolve (Valkenswaard, The Netherlands). A Synergy Water Purification System (Merck Millipore, Overijse, Belgium) provided ultrapure water. For caffeine and paraxanthine, stock solutions of 1 mg/mL were prepared by dissolving 10 mg of the compound in 10 mL of water. For the IS, an appropriate dilution of a commercially available 1 mg/mL solution of caffeine-$^{13}C_3$ in methanol and a dilution of 2 mg of paraxanthine-$^{13}C_4$−$^{15}N_3$ in 20 mL of methanol provided a 100 μg/mL stock solution. Working solutions of the standards and the IS were prepared on the day of analysis by diluting the stored (−20 °C) stock solutions with water. Independently prepared stock solutions were used to prepare the calibrators and quality control samples (QCs) as described before.[13]

Sample Collection. Blank venous whole blood from a caffeine abstinent healthy, female volunteer was collected in EDTA tubes (BD Vacutainer with BD Hemogard closure 10 mL) and used for the preparation of calibrators and QC samples. Whole blood samples were generated by transferring 50 μL of blood into 2 mL Eppendorf tubes. MF-DBS devices were generously provided by Capitainer AB (Stockholm, Sweden). MF-DBS were generated by pipetting 35 μL of whole blood at the inlet port of the device using a calibrated pipet. After completing sampling, the devices were left to dry for approximately 3 h at room temperature prior to storage at ambient temperature in the presence of desiccant (two 5 g Minipaxabsorbent packets, Sigma-Aldrich) in zip-closure plastic bags until analysis. PI-DBS were prepared by pipetting 25 μL of whole blood onto Whatman 903 filter paper (GE Healthcare, Dassel, Germany) using a calibrated pipet. The drying conditions were the same as described above for the MF-DBS.

When necessary, a Sysmex XE-5000 hematology analyzer (Sysmex Corp., Kobe, Japan) was used to determine the (adapted) Hct.

Sample Preparation and UPLC-MS/MS Method. For the MF-DBS, sample preparation was performed by removing the preperforated paper discs using tweezers and transferring these into 2 mL Eppendorf tubes, before adding 225 μL of a methanol/water (80/20, v/v) mixture, containing 0.01% formic acid and both labeled IS, at 33 ng/mL and 16.5 ng/mL for caffeine-$^{13}C_3$ and paraxanthine-$^{13}C_4$-$^{15}N_3$, respectively. Subsequently, the samples were shaken for 10 min at 1000 rpm and 60 °C on a Thermoshaker TS-100C (BioSan, Riga, Latvia), followed by a centrifugation step at ambient temperature for 10 min at 10 000*g*. Before injection of 10 μL onto the UPLC column, 90 μL of the supernatant was diluted with 550 μL of water, containing 0.01% formic acid of which 250 μL was transferred to a vial with plastic insert. For quantification of caffeine and paraxanthine in PI-DBS (Whatman 903 filter paper) and whole blood samples, readily available fully validated methods were used, with a central 3 mm punch being used for PI-DBS.[13]

A Waters Acquity UPLC system (Waters, Milford, MA, USA) coupled to a SCIEX API 4000 mass spectrometer (SCIEX, Framingham, MA, USA) was used to analyze all samples. The LC-MS/MS system was controlled by SCIEX analyst 1.6.2 and by the Waters Acquity console software. Mobile phases A and B consisted of 0.01% formic acid in water and methanol, respectively. The same chromatography and mass spectrometry parameters as described elsewhere were used for the analysis of PI-DBS, whole blood, and MF-DBS.[13]

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Validation of the MF-DBS Method. Method validation was based on U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) guidelines for bioanalytical method validation[14,15] and included the evaluation of accuracy, precision, carry-over, selectivity, homoscedasticity, calibration model, stability, matrix effect, recovery, and Hct effect. At the start of each series, control blanks (i.e., MF-DBS prepared with blank blood and analyzed without IS in the extraction solvent) and zero samples (i.e., MF-DBS prepared with blank blood and extracted using the regular solvent) were analyzed.

Accuracy and precision were determined by analyzing QCs at four concentration levels (lower limit of quantification (LLOQ), low, mid, and high) in duplicate on three different days. The used concentration levels for caffeine and paraxanthine, respectively, were 0.05 and 0.025 μg/mL (LLOQ), 0.12 and 0.06 μg/mL (low), 4.0 and 2.0 μg/mL (mid), and 8.0 and 4.0 μg/mL (high). The within day and total assay precision (%relative standard deviation, %RSD) were determined by using a single factor analysis of variance (ANOVA), while the accuracy (%bias) was evaluated by dividing the difference between the obtained concentration and the nominal value by the nominal value, this multiplied by 100.[16,17] Both, the %bias and the %RSD should be within ±15%, except for the LLOQ (within ±20%).[15]

Carry-over was assessed by injecting 2 blank samples after the highest calibrators, on four different days (n = 8). Carry-over is considered acceptable when the obtained responses for the analytes were less than 20% of the LLOQ peak area and less than 5% for the IS.[15] For selectivity, identical acceptance criteria were applied. Selectivity was evaluated by analyzing blank MF-DBS, prepared with whole blood originating from 8 different volunteers.

Eight-point calibration lines were measured in duplicate on each of 4 days to evaluate homoscedasticity and the calibration model. The nominal concentrations of the calibrators were 0.050, 0.10, 0.25, 0.50, 1.0, 2.5, 5.0, and 10.0 μg/mL for caffeine and 0.025, 0.050, 0.10, 0.25, 0.50, 1.0, 2.5, and 5.0 μg/mL for paraxanthine. An F-test (α = 1%) at the lowest and highest calibrators was used to test the homoscedasticity. Furthermore, both unweighted and weighted ($1/x$, $1/x^2$, $1/\sqrt{x}$, $1/y$, $1/y^2$, and $1/\sqrt{y}$) linear and quadratic regression were applied to find the best fitting model. The sum% relative error (RE) and plotting of the %RE against the nominal concentrations was used to compare the obtained models. A back-calculation, in which the mean concentrations of the calibrators should be within ±15% of the nominal value or within ±20% for the LLOQ, was performed before accepting a selected model.[15]

Former experiments did not reveal any problems with the (processed sample) stability of caffeine and paraxanthine.[5,13] However, since the DBS paper included within the MF-DBS device is not completely identical to the paper used within the previously validated PI-DBS method, a limited stability study was carried out here. Stability was assessed by analyzing low and high QCs in triplicate after 4 days of storage at 60 °C and after 3 months of storage at −20 °C and at room temperature in zip-closure plastic bags containing two 5 g packages of desiccant. A freshly prepared eight-point calibration line was used at each day of analysis to calculate the concentration of the stored MF-DBS. To be acceptable, the mean concentration of the QCs at a particular time point should not deviate more than ±15% from the nominal value.[15]

Two concentration levels (low or high QC) together with the IS were spiked to a blank blood extract (originating from 7 different donors) (A) or to a neat MeOH/H_2O (80/20, v/v) + 0.01% formic acid solution (B) to investigate the matrix effects. Furthermore, the Hct range was broadened by adding or removing a specific amount of plasma from whole blood, this by centrifuging an aliquot of blood with a Hct of 38.7 in an Eppendorf 5804R centrifuge (Hamburg, Germany) for 5 min at 1000g. In this way, 10 different blank blood extracts could be obtained with a Hct ranging from 24.4 to 50.5. The IS-corrected matrix effect is calculated as the ratio of the peak areas of (A) to those of (B), multiplied by 100. Overall, the % RSD of this IS-corrected matrix effect should be less than 15%.[15]

The recovery was evaluated for low and high QCs (n = 6) at three different Hct levels (target values at 21.0, 42.0, and 62.0), prepared by adding or removing a certain amount of plasma from whole blood with a Hct of 40.3. Two sets of MF-DBS were compared: (C) MF-DBS obtained by pipetting 13.5 μL of spiked blood directly onto the preperforated paper discs and (D) MF-DBS generated by pipetting 13.5 μL of blank whole blood and to which the analytes were only spiked after the extraction. The average of the peak area of (C) divided by that of (D) multiplied by 100, revealed the absolute recovery values (%).

Where relevant, statistical analyses were performed using the Minitab software.

Application. To get a complete view on the performance of the MF-DBS devices, a comparative study between MF-DBS, partial-punch PI-DBS and whole blood samples was carried out. Caffeine and paraxanthine concentrations were determined in 133 hospital patient blood samples (collected in EDTA tubes). Approval for this study (the use of left-over venous blood samples from patients and control blank blood samples from volunteers) was provided by the Ethics Committee of Ghent University Hospital (EC2018/0519). Blank blood, from a single donor (Hct 46.2), was used to prepare matrix-matched calibration curves. The impact of the Hct on the MF-DBS and PI-DBS results was evaluated by plotting the percentage difference between MF-DBS or PI-DBS and whole blood in function of the different Hct levels. The differences between MF-DBS or PI-DBS and whole blood concentrations, divided by the whole blood concentrations, multiplied by 100, represented these percentage differences. Furthermore, Medcalc statistical software, version 14.12.0 (Medcalc Software bvba., Ostend, Belgium), was used to create Bland−Altman plots to compare MF-DBS and whole blood samples.

Additionally, to check whether the amount of blood added at the inlet port of the device has an influence on the measured caffeine and paraxanthine concentrations, different volumes (25, 30, 35, 40, and 50 μL) of blood, originating from patient samples with a very low or high Hct, were applied.

Furthermore, to better reflect realistic conditions, the use of capillary blood (collected via a fingerprick), obtained from 4 healthy volunteers, was tested. Here, a comparison was made between the caffeine and paraxanthine concentrations obtained via the use of MF-DBS devices with those in liquid capillary blood. A BD Microtainer contact activated safety lancet (BD, Franklin Lakes, USA) was used to perform the fingerprick. Approval for this study was provided by the Ethics Committee of Ghent University Hospital (EC2018/0519) and a written informed consent was obtained from each volunteer.

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Table 1. Within Day and Total Precision and Accuracy (n = 3 × 2) for QCs of Caffeine and Paraxanthine at Four Concentration Levels (LLOQ, Low, Mid, and High) in MF-DBS

	within day precision (%RSD) (n = 3 × 2)		total precision (%RSD) (n = 3 × 2)		accuracy (%bias) (n = 3 × 2)	
QC	caffeine	paraxanthine	caffeine	paraxanthine	caffeine	paraxanthine
LLOQ	6.40	9.42	11.88	9.42	−1.03	3.13
low	3.07	3.69	3.31	4.35	1.67	6.14
mid	3.35	7.48	3.35	7.80	2.92	1.92
high	7.39	5.16	8.84	5.37	4.54	5.92

Table 2. Stability Data for Caffeine and Paraxanthine in MF-DBS at Low and High QC (n = 3)[a]

	4 days at 60 °C (%difference) (n = 3)		3 months at −20 °C (%difference) (n = 3)		3 months at RT (%difference) (n = 3)	
QC	caffeine	paraxanthine	caffeine	paraxanthine	caffeine	paraxanthine
low	3.33	8.00	7.78	12.39	15.28	14.94
high	7.17	13.5	7.54	0.33	5.04	−0.25

[a]Data are presented as the percentage difference between the concentration measured at the specific time points and the nominal values.

RESULTS AND DISCUSSION

Method Validation. As can be concluded from Table 1, the overall %bias was below 6.15%, meeting the acceptance criterion for accuracy. Furthermore, with a within day and total assay precision (%RSD) below 12%, the acceptance criterion for precision was also met for both compounds.

No carry-over was found when injecting 2 blank samples after the highest calibrators. For selectivity, no unacceptable interferences were observed in blank MF-DBS, prepared with whole blood from 8 different volunteers (Hct range: 37.1−43.1).

Calibration data for both caffeine and paraxanthine were found to be heteroscedastic. For both compounds, weighted regression did improve the %RE, therefore weighted linear regression was selected, using a weighting factor $1/x^2$. When using these models, the mean back-calculated concentrations did not differ more than ±10%. Hence, a linear calibration model could be accepted for both compounds.

As can be concluded from Table 2, both caffeine and paraxanthine were stable for at least 4 days in MF-DBS when stored at 60 °C (representing an accelerated stability experiment) and for at least 3 months when stored at room temperature or −20 °C.

As displayed in Table 3, the values for the analyte matrix effect indicate a relevant (>15%) ionization enhancement for paraxanthine. However, since the IS-corrected matrix effect was within 98.9−107.1% for both compounds, it can be concluded that the IS compensates for the differences in ionization. Furthermore, the preset acceptance criterion (<15% %RSD) for the IS-corrected matrix effects was met.

To evaluate the recovery, low and high QCs (n = 6) were prepared in blank blood with three different Hct levels (21.5, 40.7, and 58.3), which was used to generate MF-DBS. High recovery values were obtained for both compounds, at 105.97 ± 3.48% for caffeine and 97.36 ± 9.06% for paraxanthine, these values corresponding to the averages calculated from all values obtained at all Hct levels and at both QC levels. Furthermore, when the 40.7 Hct level is normalized to 100%, all recoveries were within ±15% of this 40.7 Hct reference sample, demonstrating that the Hct has no statistical significant effect (p > 0.1, one-way ANOVA test) on the recovery (Figure 1). This confirms the Hct-independence of the MF-DBS devices, as also observed by Spooner et al., who evaluated the recovery of radiolabeled material.[11]

Application. The validated method was applied on 133 authentic, venous left-over patient samples with a Hct ranging

Table 3. Analyte Matrix Effect and IS-Corrected Matrix Effect for Caffeine and Paraxanthine

	caffeine		paraxanthine	
	low QC	high QC	low QC	high QC
	analyte matrix effect			
mean of 10 donors (%)	98.9	84.9	128.7	115.8
%RSD	4.83	2.27	5.48	2.69
	IS-corrected matrix effect			
mean of 10 donors (%)	107.1	104.6	105.8	98.9
%RSD	6.80	2.44	6.62	2.10

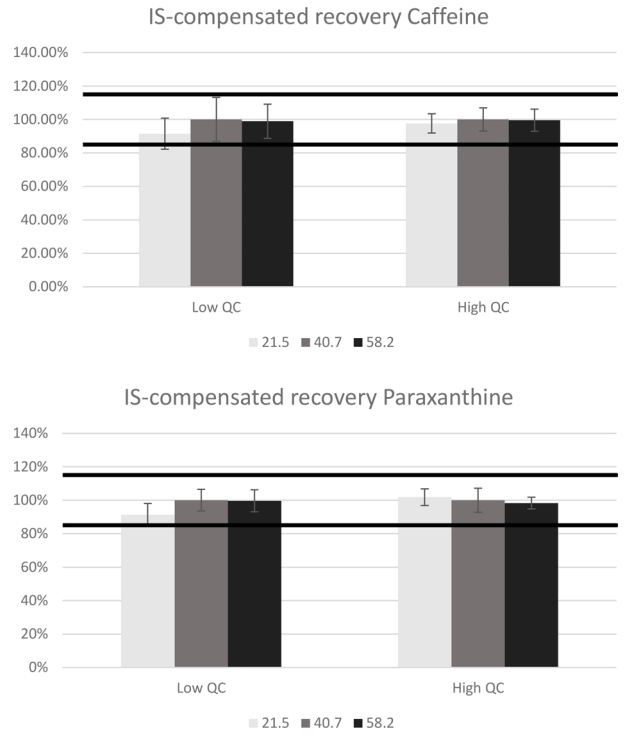

Figure 1. IS-compensated recovery (%) at low and high QC level (n = 6) for caffeine and paraxanthine measured in MF-DBS at 3 different Hct levels (21.5, 40.7, and 58.2), with the 40.7 Hct sample being normalized to 100%. The full lines indicate the ±15% deviation limits.

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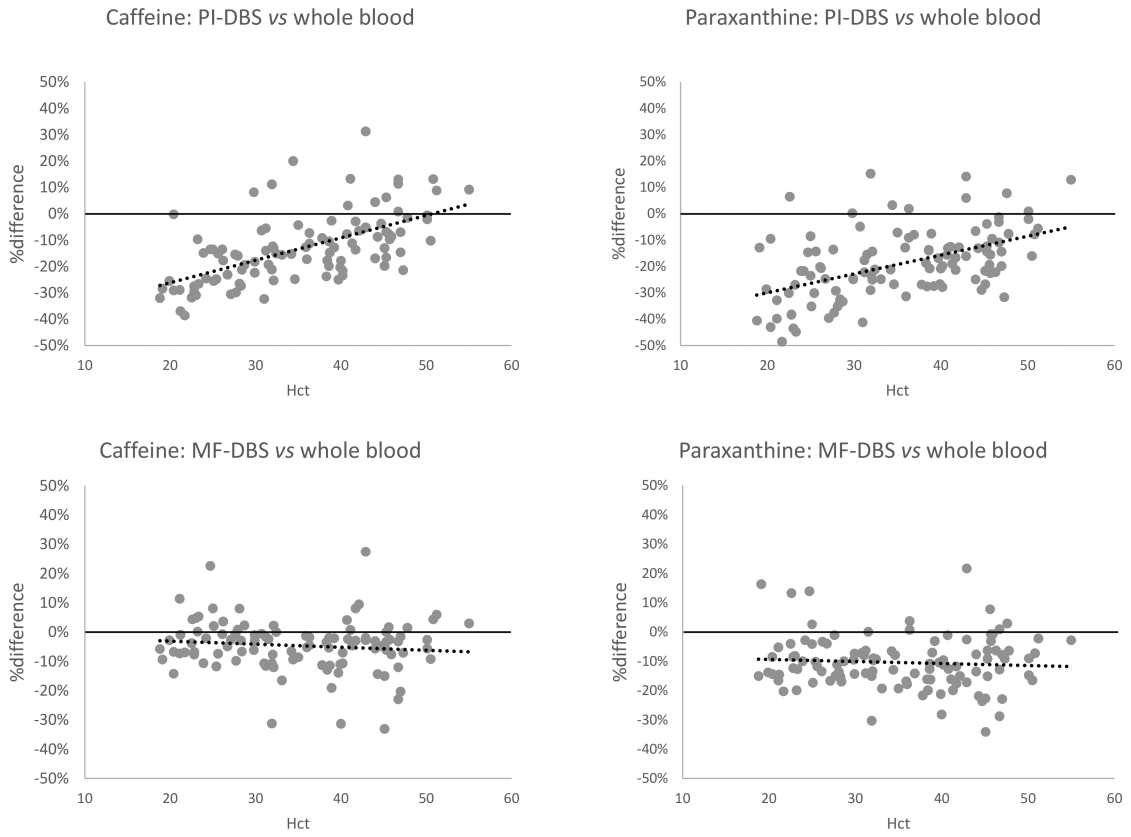

Figure 2. Percentage difference between MF-DBS or PI-DBS concentrations and whole blood concentrations, plotted against hematocrit for caffeine (n = 105) and paraxanthine (n = 110). Broken lines represent linear regression lines.

from 18.8 to 55.0, with 35.95 being the median. In 105 respectively 110 of the 133 patient samples (corresponding MF-DBS, PI-DBS and whole blood samples), caffeine and paraxanthine concentrations were above the respective LLOQs (0.05 and 0.025 μg/mL). All samples were analyzed against a calibration curve prepared using blood with a Hct of 46.2. This relatively high Hct was deliberately chosen, since in this way the Hct effect on PI-DBS concentrations can be clearly visualized. Furthermore, it enables to create a worst case scenario for MF-DBS and PI-DBS, allowing an exhaustive assessment of (a possible) Hct effect. Figure 2 depicts the percentage difference between MF-DBS or PI-DBS concentrations and whole blood concentrations in function of the Hct. Regression lines fitted to the differences between PI-DBS and whole blood concentrations had a slope of 0.851 (95% CI; [0.628−1.08]) and 0.716 (95% CI; [0.480−0.952]) and an intercept of −43.2 (95% CI; [−51.3 to −35.0]) and −44.3 (95% CI [−52.9 to −35.7]) for caffeine and paraxanthine, respectively, revealing for both compounds a similar Hct-induced bias, impacting PI-DBS concentrations. PI-DBS concentrations clearly decreased with a decreasing Hct, which is in line with previous findings for both compounds.[5,18] In contrast, the concentrations obtained from the MF-DBS were not affected by a suchlike Hct-effect (Figure 2, lower panels). The regression lines fitted to the differences between the MF-DBS and whole blood concentrations had a slope of −0.103 (95% CI; [−0.288 to 0.0819]) and −0.0707 (95% CI; [−0.256 to 0.114]) and an intercept of −1.07 (95% CI; [−7.77 to 5.63]) and −7.92 (95% CI; [−14.7 to 1.18]) for caffeine and paraxanthine, respectively. Incurred sample reanalysis resulted in regression lines with a slope of 0.00398 (95% CI; [−0.164 to 0.172]) and −0.00122 (95% CI; [−0.232 to 0.230]) and an intercept of −3.79 (95% CI; [−9.85 to 2.27]) and −2.59 (95% CI; [−11.0 to 5.81]) for caffeine and paraxanthine, respectively. Since the 95% CIs of the slopes for both compounds, for both analyses, included 0, it can be concluded that the differences between the MF-DBS and whole blood concentrations did not change in function of the Hct, this within the Hct range of 18.8 to 55.0. Therefore, the Hct-independence of the devices, as readily suggested by Spooner et al., who evaluated the recovery of radiolabeled material, is supported by these findings on authentic, venous patient samples.[11]

Bland−Altman plots for the differences between MF-DBS and whole blood concentrations (Figure 3) revealed a mean negative bias of −5.2% (95% CI; [−6.87 to −3.29%] and −11.5% (95% CI; [−13.23 to −9.39%]) for caffeine and paraxanthine, respectively. Incurred samples reanalysis revealed a mean negative bias of −4.0% (95% CI; [−5.47 to −2.42%] and −3.3% (95% CI; [−5.23 to −1.08%]) for caffeine and paraxanthine, respectively. From this it can be concluded that in our experiments the concentrations were slightly underestimated in MF-DBS versus whole blood. However, taking into account that results from different matrices (i.e., dried MF-DBS versus liquid whole blood), obtained by different extraction methods and analyzed in separate analytical runs on different days, were compared, it can be concluded that these deviations were limited. For example, 93.3% and 89.1% of the caffeine and paraxanthine samples, respectively, did not differ more than 20% from the whole blood concentrations. For the incurred sample reanalysis, this was the case for 97.1% and 93.6% of the caffeine and paraxanthine samples, respectively,

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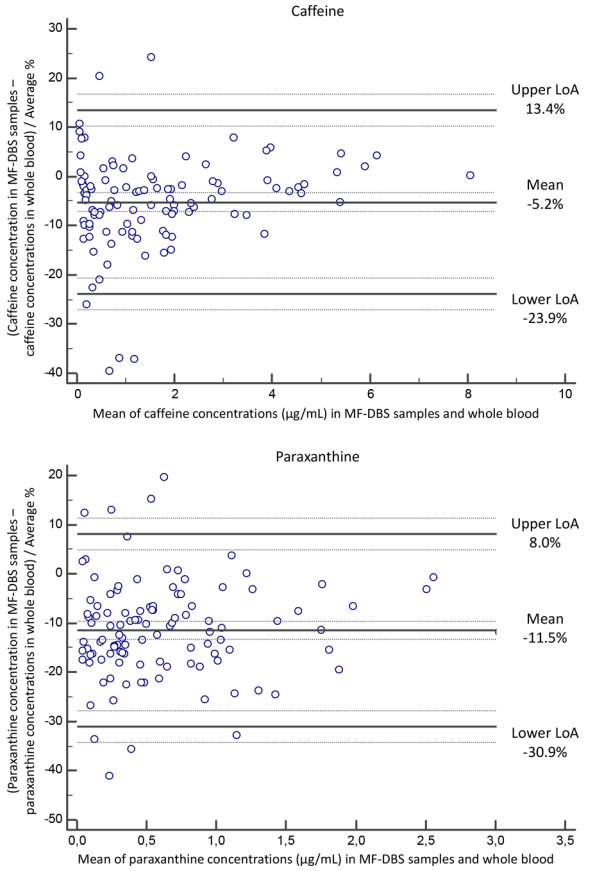

Figure 3. Bland–Altman plots for the comparison between whole blood and MF-DBS concentrations for caffeine (n = 105) and paraxanthine (n = 110). Mean differences and limits of agreement (LoAs) are represented by full lines, 95% confidence limits by broken lines.

meeting the acceptance criterion for incurred sample reanalysis.[15] Moreover, when comparing the incurred sample reanalysis to the original MF-DBS analysis, 97 out of the 105 caffeine samples, and 90 out of the 110 paraxanthine samples were within ±20% of the mean, meeting the acceptance criterion for incurred samples reanalysis.[14] Furthermore, the different types of samples were analyzed against calibration curves prepared in the respective matrices, with accuracy values (%bias) for QCs of caffeine and paraxanthine within ±6% and ±5% (±14% at LLOQ level) for MF-DBS and whole blood samples, respectively. The storage conditions used during the actual study (1 day at 4 °C for whole blood samples and 1 day at room temperature for MF-DBS) did definitely not exceed the storage conditions in which both analytes were stable in both whole blood (stable for at least 7 days at 4 °C) and MF-DBS devices (stable for at least 3 months when stored at room temperature).[13] Moreover, Hct had no effect on ion suppression or enhancement for MF-DBS. Therefore, we believe that a possible explanation for the positive bias observed in our study may be found in the fact that the patient samples were analyzed against a calibration curve prepared from blood with a Hct of 46.2, which was rather high, since only 15 out of the 133 included patient samples had a Hct value above 46.2. Although our recovery experiments had not revealed a Hct-dependent impact on recovery during method validation, this limited bias could still be induced by a small difference in recovery, taking into account that recovery experiments were performed using a small number (n = 6) of spiked samples, while here a number of 133 authentic, venous patient samples were evaluated. As noted earlier, here, we wished to "stress" the system, by using calibrators set up in a Hct at the higher end of the normal range, rather than using calibrators at about the median Hct of the anticipated range, as we recommend for dried blood analysis.[2,19]

In reality, samples will be collected by applying an undefined volume of blood, resulting from a finger prick, rather than by pipetting an amount of blood by the help of a calibrated pipet. We therefore evaluated whether the amount of blood added at the inlet port of the device influences the measured analyte concentrations. To this end, we applied different volumes (25, 30, 35, 40, and 50 µL) of blood, originating from authentic, venous patient samples with a Hct of 18.8, 22.8, 50.1, and 55. Results are depicted in Figure 4.

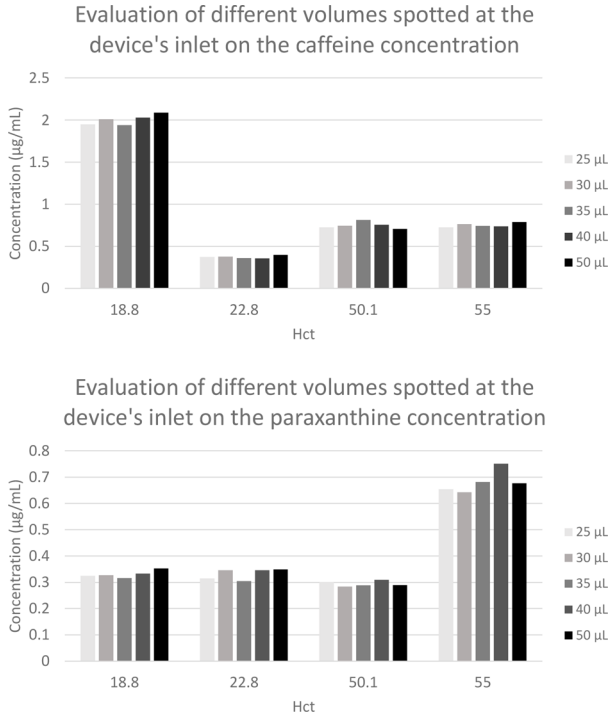

Figure 4. Evaluation of the impact of applying different volumes of blood, originating from patient samples with a Hct of 18.8, 22.8, 50.1 and 55, at the device's inlet.

When comparing the concentrations obtained by pipetting different volumes, a CV% lower than 6.20% is obtained for both compounds. Therefore, it can be concluded that the amount of blood applied at the inlet of the device does not have an impact on the caffeine and paraxanthine concentration measured, this independent from a patient's Hct. Lastly, to exclude that coagulation may pose a problem, a small preliminary study was set up in which the use of non-anticoagulated capillary blood (obtained via a fingerprick) was evaluated. Capillary blood, obtained from 4 healthy volunteers, was pipetted onto MF-DBS devices and as a reference measurement, liquid capillary blood obtained from the same fingerprick was analyzed. In all cases, the difference between MF-DBS and capillary liquid blood concentrations was below ±13.5% for both compounds, suggesting that also with nonanticoagulated blood the specifications are met. Further, large-scale, experiments in which the capillary blood is applied

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Anal. Chem. 2018, 90, 12893–12899

directly onto the devices are necessary to substantiate this conclusion.

CONCLUSION

MF-DBS devices, one of the recently proposed strategies to help overcoming the Hct-based area bias allow an accurate collection of a fixed volume (13.5 μL) of blood. In this study, the potential of the devices to effectively nullify the Hct-based area bias was evaluated by analyzing 133 samples, prepared from whole blood covering a wide Hct range (18.8−55.0) originating from hospital patients. To this end, an LC-MS/MS method for the quantification of caffeine and paraxanthine, making use of MF-DBS devices, was completely validated, including the evaluation of both bioanalytical and dried blood sample specific parameters, with all preset acceptance criteria being met. Analyte concentrations measured in MF-DBS samples were compared to those measured in corresponding partial-punch PI-DBS and liquid whole blood samples. This comparison revealed that there was no Hct-dependent impact on the concentrations measured in MF-DBS, in contrast to parial-punch PI-DBS concentrations. However, we did observe a limited mean negative bias for both analytes in MF-DBS, when compared to whole blood. The use of blank blood with a rather high Hct (46.2) for the preparation of the calibrators might explain this, although no impact of Hct on recovery was observed during method validation. Given the results obtained and pending confirmation by others, using the same or other analytes, we conclude on the cautious side, that also for the MF-DBS devices it may be advisable to set up calibration lines at the anticipated median of the population-to-be-investigated. Evaluation of a potential volume impact by analyzing caffeine and paraxanthine concentrations in MF-DBS, derived from patient samples with a very low (18.8 and 20.8) or a very high (50.1 and 55) Hct, demonstrated that the amount of blood added at the inlet of the devices has no influence on the performance of the device, independently from the blood's Hct. Moreover, a preliminary study using capillary blood of 4 healthy volunteers indicated that the use of nonanticoagulated blood did not cause a problem.

On the basis of these findings, obtained by applying fully validated methods on authentic, venous patient samples, we conclude that the MF-DBS devices effectively assist in nullifying the Hct-based area basis for caffeine and paraxanthine and that the volume added at the inlet of the device does not have an impact on the measured analyte concentrations. It is important that these findings, which seem promising, should be corroborated by others, using other compounds with different features. In addition, further evaluation of the device by using capillary microsamples, applied directly from a fingertip, is essential for evaluating its robustness in real practice. The latter is important to accept MF-DBS devices as a reliable alternative for whole blood analysis in existing and emerging applications.

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Notes

The authors declare no competing financial interest.

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