Potassium-based algorithm allows correction for the hematocrit bias in quantitative analysis of caffeine and its major metabolite in dried blood spots

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Published in:

Analytical and Bioanalytical Chemistry

406 (26): 6749-55 (2014)

doi: 10.1007/s00216-014-8114-z

Abstract

Although dried blood spot (DBS) sampling is increasingly receiving interest as a potential alternative to traditional blood sampling, the impact of hematocrit (Hct) on DBS results is limiting its final breakthrough in routine bioanalysis. To predict the Hct of a given DBS, potassium (K^+) proved to be a reliable marker. The aim of this study was to evaluate whether application of an algorithm, based upon predicted Hct or K^+ concentrations as such, allowed to correct for the Hct bias. Using validated LC-MS/MS methods, caffeine, chosen as a model compound, was determined in whole blood and corresponding DBS samples with a broad Hct range (0.18 – 0.47). A reference subset (n = 50) was used to generate an algorithm based on K^+ concentrations in DBS. Application of the developed algorithm on an independent test set (n = 50) alleviated the assay bias, especially at lower Hct values. Before correction, differences between DBS and whole blood concentrations ranged from -29.1 to 21.1 %. The mean difference, as obtained by Bland-Altman comparison, was -6.6 % (95 % CI; [-9.7 – -3.4 %]). After application of the algorithm, differences between corrected and whole blood concentrations lay between -19.9 and 13.9 % with a mean difference of -2.1 % (95 % CI; [-4.5 – 0.3 %]). The same algorithm was applied to a separate compound, paraxanthine, which was determined in 103 samples (Hct range 0.17 – 0.47), yielding similar results. In conclusion, a K^+-based algorithm allows to correct for the Hct bias in the quantitative analysis of caffeine and its metabolite paraxanthine.

Keywords: dried blood spots, hematocrit effect, bioanalytical methods, biological samples, clinical/biomedical analysis

1 Introduction

In recent years, dried blood spot (DBS) sampling has increasingly received interest as an alternative sampling strategy. However, despite the numerous DBS-based methods that have been developed and the many advantages associated with this sampling technique [1-2], its implementation in routine quantitative bioanalysis has to a great extent been limited by the hematocrit (Hct) issue [3]. This Hct issue is a problem that is both analytical and physiological in nature. The analytical facet comprises the impact of Hct on blood viscosity and, hence, the spreading of the blood on filter paper. Consequently, fixed size punches, taken from DBS with varying Hct values, will contain different amounts of blood. This facet also encompasses the influence of Hct on sample homogeneity, extraction efficiency and matrix effect [4]. The physiological facet of the Hct issue, on the other hand, relates to the influence of the Hct on the blood-to-plasma concentration ratio of an analyte. The latter is of key importance when DBS results are to be compared with established plasma- or serum-based reference intervals or therapeutic ranges [5].

As strongly deviating Hct values may have a significant impact on method accuracy and precision, as well as on the interpretation of DBS results, multiple strategies have been suggested to overcome this obstacle. These include analysis of whole, volumetrically applied DBS, the use of dried plasma spots instead of DBS or spotting on special filter substrates that are less prone to differential spreading of blood [3,6]. On the other hand, when DBS are prepared in a non-volumetric way, followed by analysis of partial punches, it is vital to define a Hct interval for every analyte in which method accuracy and precision are still adequate. Additionally, to minimize the Hct impact, calibration curves should be centered around a Hct value close to the median of the population of interest. However, even when such a Hct interval has been established, one still needs to confirm whether or not the Hct of a given DBS actually lies within this interval. To this end, a reliable marker is required that allows to predict the Hct of the blood used to prepare a given DBS. We previously demonstrated that potassium (K^+), an endogenous compound that correlates with the amount of red blood cells, allows to predict the Hct over a wide range with acceptable accuracy and precision [7]. This method was thoroughly validated and application on real patient samples showed a good correlation between the estimated Hct (based on the K^+ content) and the real Hct. However, hitherto, it has not been demonstrated if –and how– this Hct prediction may effectively assist in overcoming the Hct problem for a given DBS-based analytical method in real practice. Therefore, the aim of this study was to evaluate whether application of an algorithm, based

upon the predicted Hct or the K^+ concentrations as such, could adjust for the Hct-induced bias in the quantitative analysis of caffeine. Caffeine was selected as a model drug, since we previously observed a Hct effect on the accuracy of measured caffeine concentrations in DBS by analyzing quality control samples prepared from spiked blood with a wide Hct range (0.20 – 0.60) [8]. In this context, we also found that strongly deviating Hct values had no impact on matrix effect and recovery, using a similar Hct range (0.19 – 0.63) [9]. Furthermore, given the widespread consumption of caffeine-containing food products, a sufficient number of positive samples could be obtained without the need to set up a clinical study involving actual administration of a probe drug.

2 Materials and methods

2.1 Chemicals

Caffeine, caffeine-$^{13}C_3$, paraxanthine, paraxanthine-$^{13}C_4$-$^{15}N_3$ and formic acid were purchased from Sigma-Aldrich (Sigma-Aldrich, Diegem, Belgium), while KCl was obtained from UCB (UCB, Leuven, Belgium). LC-MS grade methanol was purchased from Biosolve (Biosolve, Valkenswaard, The Netherlands) and a Synergy® Water Purification System (Merck Millipore, Overijse, Belgium) provided ultrapure water.

2.2 Sample collection

Venous whole blood samples were obtained from both healthy volunteers (n = 61) and hospital patients (n = 117) and collected in Venosafe® 4 mL VF-054SHL or 9 mL VF-109SHL Li-heparin tubes (Terumo, Leuven, Belgium). Prior to blood collection, all healthy volunteers (age 24 – 48 years, 44 women and 17 men) were informed and signed informed consent. This study was approved by the Ethics Committee of Ghent University Hospital (Belgian registration number B670201111655 and B670201214201), which also approved the use of aliquots of routinely collected Li-heparin patient blood samples, admitted to the Laboratory of Clinical Biology of Ghent University Hospital (B670201319311). Because of an opting-out procedure, foreseeing the possibility of comparative analysis on left-over samples, no individualized informed consent was needed for the hospital patients. The samples were included in this study to obtain blood samples with deviating Hct values. As these were anonymized left-over samples, no additional data concerning age or sex of the donors could be traced back. On the day of venous blood sampling, corresponding DBS were prepared by spotting 25 µL whole blood on WHA10334885 Whatman 903 filter paper (GE

Healthcare, Dassel, Germany). The resulting DBS were air-dried for 2 hours and stored at ambient temperature in zip-closure plastic bags, containing two 5-g Minipax® absorbent packets as desiccant (Sigma-Aldrich, Diegem, Belgium). Liquid blood samples were stored at -20 °C until analysis.

2.3 Analyses

Concentrations of caffeine and its major metabolite paraxanthine were determined in whole blood and DBS using previously developed and validated methods on a Waters Acquity UPLC® system (Waters, Milford, MA, USA) coupled to an AB SCIEX API 4000™ triple quadrupole mass spectrometer (AB SCIEX, Framingham, MA, USA) [9]. For whole blood samples, a protein precipitation was performed by adding 100 µL of methanol, containing 0.01 % formic acid, to 50 µL of blood after the addition of 10 µL of an internal standard (IS) solution. DBS punches (3 mm) were extracted in 70 µL of a methanol/water (80/20, v/v) mixture, containing 0.01 % formic acid and the IS. After gentle shaking for 10 minutes, whole blood and DBS samples were centrifuged and the resulting supernatants were diluted with water, containing 0.01 % formic acid. Further details of the analytical methods are described elsewhere [9].

K^+ concentrations in DBS extracts were measured by indirect potentiometry, using the ion-selective electrode of a Roche Cobas 8000 routine chemistry analyzer (Roche Diagnostics, Mannheim, Germany), as previously described [7]. K^+ was extracted from 3-mm DBS punches by two-fold addition of 50 µL of a 2.5 mM KCl solution in ultrapure water and shaking for 5 minutes. Actual DBS K^+ concentrations were obtained by subtracting the value in the extraction solution from the measured value. These K^+ concentrations allowed to predict the approximate Hct of the blood used to prepare the DBS. Direct Hct measurement in whole blood was performed on a Sysmex XE-5000 hematology analyzer (Sysmex, Kobe, Japan).

2.4 Data analysis

Samples in which caffeine was measured were randomly assigned to a reference and test set of samples, thereby ensuring that Hct values were similarly distributed in both groups. For the reference set, the whole blood/DBS caffeine concentration ratios were plotted against K^+-based calculated Hct. A linear regression line was fitted to these data by applying the least squares method. The slope and intercept, together with their 95 % confidence intervals (CIs), were obtained using the Analysis Toolpack of MS Excel® 2013 (Microsoft, Redmond, WA,

USA). From the resulting linear model, a Hct-based correction algorithm was derived by a simple transformation. As the calculated Hct used to generate the algorithm was based on K^+ concentrations measured in DBS, a similar algorithm could be generated using the K^+ concentrations as such. The resulting algorithm was applied to the independent test set. To further evaluate the usefulness of this approach, the same algorithm that was constructed for caffeine was applied to another compound, paraxanthine, which was also determined in samples from healthy volunteers and hospital patients.

To assess the extent of the Hct effect before and after implementation of a correction algorithm, the differences (%) between DBS concentrations (uncorrected or corrected) and whole blood concentrations were plotted against measured and calculated Hct values for the caffeine reference set and against K^+ concentrations for the caffeine and paraxanthine test sets. Percentage differences were calculated by dividing the difference between DBS and whole blood concentrations by the whole blood concentration and multiplying the result by 100. To the data of the test sets, linear regression lines were fitted by applying the least squares method. Slope, intercept and corresponding 95 % CIs were obtained as described above. Additionally, uncorrected and corrected concentrations were compared with corresponding whole blood concentrations using Bland-Altman plots, which were generated with Medcalc version 12.7.5 (Medcalc Software bvba, Ostend, Belgium).

3 Results and discussion

Caffeine concentrations above the LLOQ (50 ng/mL) were measured in whole blood and DBS samples from 100 subjects, both healthy volunteers and hospital patients, displaying a wide Hct range (0.18 – 0.47). Figure 1a shows the Hct distribution in the entire sample set. The differences (%) between uncorrected caffeine concentrations in DBS and whole blood, plotted against Hct values measured in whole blood for the reference set (n = 50), are shown in Figure 2a. Lower DBS concentrations were found at low Hct levels, while higher concentrations were measured at high Hct levels, with differences ranging from -29.3 to 25.4 %. A similar pattern of decreasing uncorrected DBS concentrations with decreasing Hct was observed when differences (%) were plotted against calculated Hct values (Figure 2b), illustrating the validity of K^+-based Hct prediction [7].

The linear regression line fitted to the whole blood/DBS concentration ratios versus the calculated Hct for the reference set had a slope of -1.33 (95 % CI; [-1.74 – -0.93]) and an

intercept of 1.51 (95 % CI; [1.37 – 1.65]). From this linear relationship, an algorithm to correct the caffeine DBS concentrations, taking into account the calculated Hct of the DBS, was derived: *corrected DBS concentration = uncorrected DBS concentration * ((-1.33 * calculated Hct) + 1.51).* The impact of applying the developed algorithm can be deducted from Figure 2c, showing the differences between corrected DBS concentrations and whole blood concentrations plotted against the calculated Hct.

When whole blood/DBS concentration ratios were directly plotted against K^+ concentrations, as the latter were used to calculate the Hct, the resulting linear regression line had a slope of -0.41 (95 % CI; [-0.54 – -0.29]) and an intercept of 1.51 (95 % CI; [1.37 – 1.64]). From these data, the following algorithm was derived: *corrected concentration = uncorrected DBS concentration * ((-0.41 * K^+ concentration) + 1.51).* However, as this algorithm was based on the reference sample set, it is likely to work for this given set of samples. Therefore, the algorithm needed to be applied to the remaining samples (n = 50), representing an independent test set. The differences (%) between uncorrected DBS caffeine concentrations and whole blood caffeine concentrations versus measured K^+ concentrations in this test set are shown in Figure 3a. Before application of the algorithm, a Hct effect similar to that in the reference set was observed, as the regression line of the plotted variables, with K^+ concentrations representing a measure of Hct, had a slope of 32.03 (95 % CI; [21.38 – 42.68]) and an intercept of -41.05 (95 % CI; [-53.00 – -29.10]). Differences between uncorrected caffeine concentrations in DBS and whole blood ranged from -29.1 to 21.1 %. For 14 samples (i.e. 28 % of the data set), the difference was beyond ± 15 % limits. After application of the developed algorithm (Figure 3b), the regression line had a slope of -3.81 and an intercept of 2.44. The 95 % CIs of slope and intercept, respectively [-14.59 – 6.97] and [-9.65 – 14.53], included the zero-value. The differences between corrected and whole blood concentrations ranged from -19.9 to 13.9 %. Now, for only 4 samples (i.e. 8 % of the data set) the differences exceeded ± 15 % limits and none of the calculated concentrations deviated more than 20 % from the measured whole blood concentration. These findings were supported by the results of Bland-Altman comparisons, which clearly demonstrate the effect of the correction algorithm. The mean difference between uncorrected DBS caffeine concentrations and whole blood caffeine concentrations was -6.6 % (95 % CI; [-9.7 – -3.4 %]) (Figure 3c), while the mean difference between corrected concentrations and whole blood concentrations was -2.1 % (95 % CI; [-4.5 – 0.3 %]) (Figure 3d). As the latter 95 % CI contained 0, a consistent negative bias was no longer observed after correction.

Application of the same algorithm to paraxanthine, caffeine's major metabolite, further demonstrated the usefulness of the presented approach. Paraxanthine concentrations above the LLOQ could be measured in samples from 103 participants (Hct range 0.17 – 0.47). The Hct distribution in this sample set is shown in Figure 1b. The differences (%) between uncorrected DBS paraxanthine and whole blood paraxanthine concentrations versus measured K^+ concentrations are shown in Figure 4a. Also for this compound, uncorrected DBS concentrations were considerably affected by Hct, as the slope of the regression line of the plotted variables was 34.83 (95 % CI; [28.33 – 41.33]) and the intercept -45.12 (95 % CI; [-52.32 – -37.92]). The differences between uncorrected DBS and whole blood paraxanthine concentrations ranged from -35.2 to 15.8 %. Application of the algorithm yielded similar results as obtained for caffeine (Figure 4b), with the slope of the regression line being 1.58 and the intercept -4.56. Also here, the 95 % CIs of slope and intercept, respectively [-5.60 – 8.76] and [-12.51 – 3.39], included the zero-value. Differences between corrected and whole blood paraxanthine concentrations lay between -21.3 and 18.8 %. Before correction, the difference was beyond the ± 15 % limits for 22 samples (i.e. 21 % of the data set), while this was the case for only 8 samples (i.e. 8 % of the data set) after correction. With a single exception (-21.3 %), all corrected paraxanthine concentrations were within ± 20 % of the concentrations measured in whole blood. Also here, Bland-Altman comparisons supported the obtained results. Before correction, the mean difference was -8.2 % (95 % CI; [-10.6 – -5.9 %]) (Figure 4c). Again, the observed negative bias decreased considerably after correction, as the mean difference between corrected and whole blood paraxanthine concentrations was -3.2 % (95 % CI; [-4.8 – -1.6 %]) (Figure 4d).

4 Conclusion

Based on the findings described above, we conclude that we are able to correct for the Hct bias in the quantitative analysis of caffeine in DBS, using a K^+-based algorithm. The same algorithm, initially designed for caffeine, was applied to a separate compound, paraxanthine, yielding similar results. Caffeine and paraxanthine are weakly basic compounds that show low binding to plasma or red blood cell proteins [5]. To further support the broader applicability of the presented approach, its usefulness should be evaluated for more analytes, displaying varying physicochemical properties and binding characteristics, The latter should include, amongst others, neutral, acidic and basic compounds, hydrophilic and hydrophobic

substances and analytes with different plasma protein binding and blood cell association profiles.

Acknowledgements

The authors wish to acknowledge all volunteers who participated in the study and the skillful staff of the 24 h lab from Ghent University Hospital. S. Capiau wishes to acknowledge the FWO Research Foundation – Flanders for granting her a PhD Fellowship.

Conflicts of interest

The authors have no conflict of interest to declare. This study was financed by the Laboratory of Toxicology, Ghent University, Belgium. The authors received no additional funding directly related to the content of this study.

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Figures

Fig. 1 Hematocrit (Hct) distribution of blood samples collected from healthy volunteers and hospital patients in which caffeine (a) and paraxanthine (b) concentrations were measured *Calculated Hct: Hct of the blood used to prepare the DBS, as calculated using the K+ concentration measured in the DBS [7]

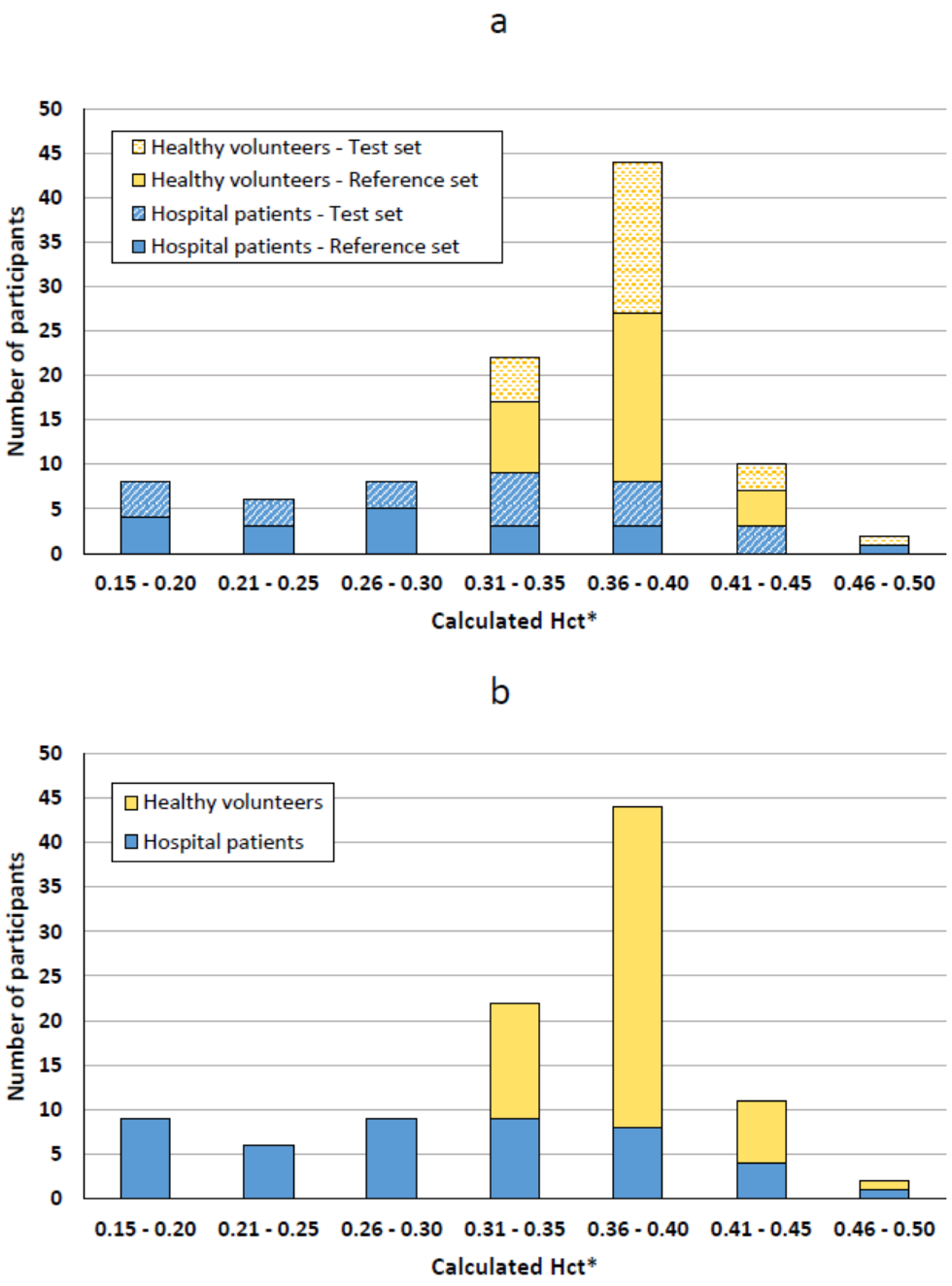

Fig. 2 Percentage differences (%) between uncorrected caffeine concentrations in dried blood spots (DBS) and whole blood plotted against hematocrit (Hct) measured in whole blood (a), against calculated Hct (b) and percentage differences (%) between corrected and whole blood caffeine concentrations plotted against calculated Hct (c). Dashed lines indicate ± 15 % difference limits, the full line indicates 0 % difference. All data were obtained from a reference set of whole blood and DBS samples (n = 50)

*Calculated Hct: Hct of the blood used to prepare the DBS, as calculated using the K+ concentration measured in the DBS [7]; [1]Measured Hct: Hct directly measured in whole blood using a Sysmex XE-5000 hematology analyzer

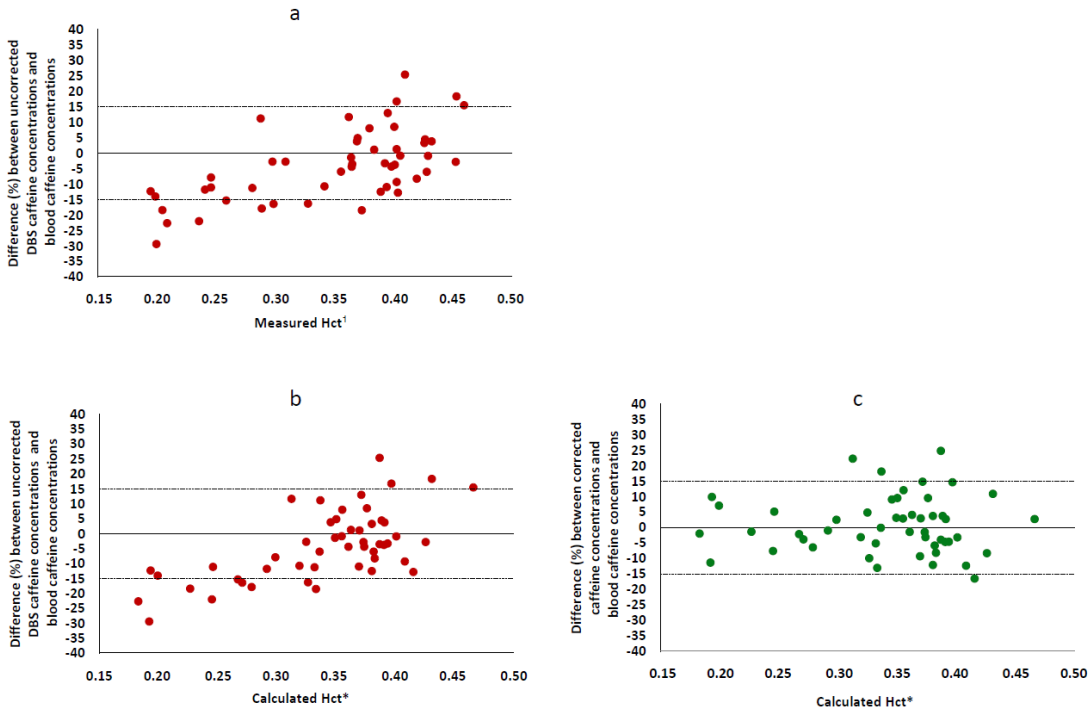

Fig. 3 Percentage differences (%) between uncorrected caffeine concentrations in dried blood spots (DBS) and whole blood (a) and between corrected and whole blood caffeine concentrations (b). Differences are plotted against potassium concentrations in DBS, dashed lines indicate ± 15 % difference limits and the full line indicates 0 % difference. Bland-Altman plot for the comparison between uncorrected DBS caffeine concentrations and whole blood caffeine concentrations (c) and between corrected and whole blood caffeine concentrations (d). The mean differences and the limits of agreement (LoAs) are displayed (full lines), together with their 95 % confidence limits (dashed lines). All data were obtained from a test set of whole blood and DBS samples (n = 50)

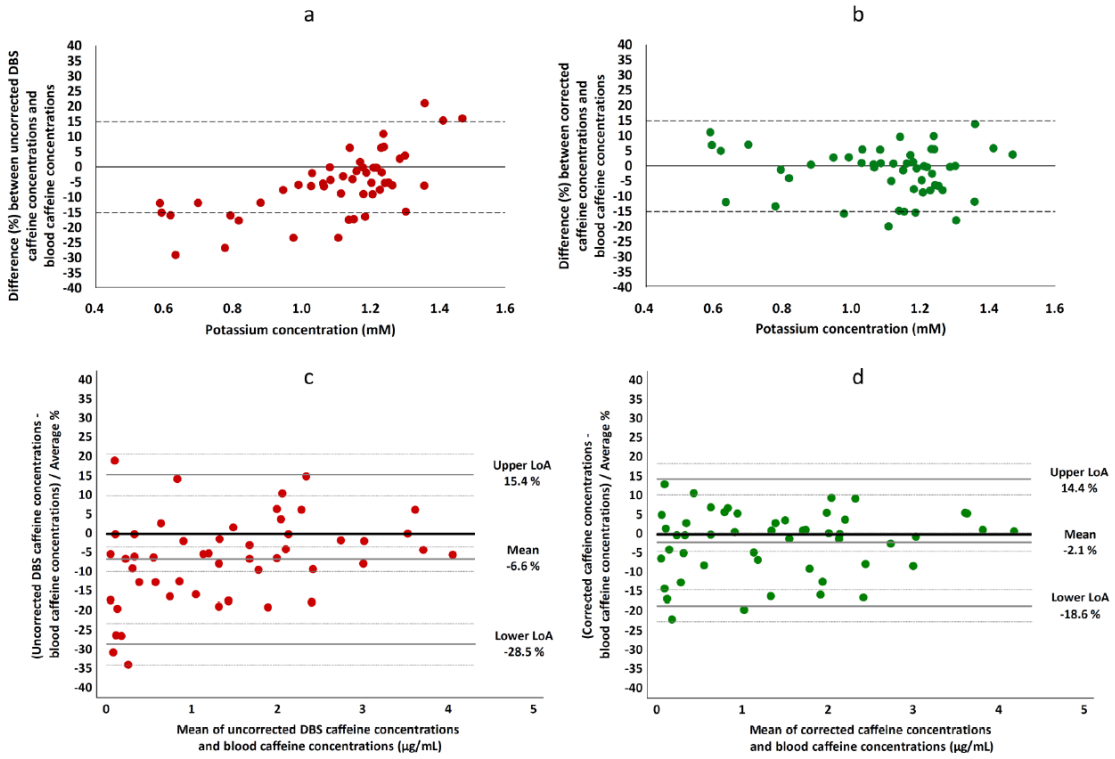

Fig. 4 Percentage differences (%) between uncorrected paraxanthine concentrations in dried blood spots (DBS) and whole blood (a) and between corrected and whole blood paraxanthine concentrations (b). Differences are plotted against potassium concentrations in DBS, dashed lines indicate ± 15 % difference limits and the full line indicates 0 % difference. Bland-Altman plot for the comparison between uncorrected DBS paraxanthine concentrations and whole blood paraxanthine concentrations (c) and between corrected and whole blood paraxanthine concentrations (d). The mean differences and the limits of agreement (LoAs) are displayed (full lines), together with their 95 % confidence limits (dashed lines). All data were obtained from a test set of whole blood and DBS samples (n = 103)