SHORT COMMUNICATION

Fatty Acids

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Estimation of the Volume of Blood in a Small Disc Punched From a Dried Blood Spot Card

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A specialized dried blood spot (DBS) collection system (PUFAcoat™) in combination with liquid chromatography-tandem mass spectrometry (LC-MS/MS) has enabled the measurement of numerous analytes in minimal volumes of blood. The current study aimed to determine the volume of blood in 3 and 6 mm discs obtained from our DBS system. The volume of blood in 3 and 6 mm discs obtained from DBS cards is estimated using four different methods: (i) gravimetric analysis; (ii) LC-MS/MS; (iii) a hemoglobin colorimetric assay; and (iv) GC. Differences in the estimated volume are compared between methods, and variations in estimated blood volume within and between individuals are determined. The average volume of blood in a DBS disc is calculated to be 1.6 ± 0.4 μL and 8.7 ± 1.9 μL for 3 and 6 mm discs, respectively. This estimate is similar between direct and indirect analytical methods and between DBS samples with different starting volumes independent of the method, but there is considerable variation in the volume of blood in comparably-sized DBS discs from different individuals. Current methods enable the estimation of the blood volume in a small disc obtained from a DBS but a method that can both accurately measure volume and store blood on a DBS is required.

***Practical Applications*: This study demonstrates that it is possible to evaluate the volume of blood contained in a small disc punched from a dried blood spot (DBS) card using a range of different methods, but there is considerable variation in the estimate of volume in samples collected from different individuals. These variations could potentially result in overestimation or underestimation of endogenous levels of various metabolites if presuming a fixed volume, which may be clinically significant. Although there are several ways to correct for blood volume contained on a DBS, the practicality and the universality of these methods are questionable. Ideally, the development of a tool to better determine the volume of blood and/or to precisely spot a volume of blood is required to ensure accuracy when expressing the results of DBS analyses per unit of blood.**

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1. Introduction

The dried blood spot (DBS) in combination with a range of analytical platforms has been widely used as a screening tool for clinical conditions, most notably screening for congenital metabolic diseases in newborn infants.[1,2] The DBS system involves spotting a few drops of blood onto a filter paper, which is air dried and transported to a laboratory for extraction and analysis. Typically only a small punch (3 or 6 mm disc) is taken from the DBS for the extraction of analytes.[3,4] Multiple analytes can be measured from a single disc of DBS simultaneously, mainly because of the utilization of sensitive instruments such as the mass spectrometer.[5]

There are many advantages of using the DBS system over using fresh blood or plasma samples, particularly in a clinical setting. These include the minimal volume of blood required (30–100 μL), ability to collect samples from a finger or heel prick, thus eliminating the need for venepuncture, ease of sample transport, and storage and stability of the compounds for months or years on the DBS once blood is dried.[5,6] However, there are limitations associated with the DBS system, with one of the most significant being the uncertainty regarding the volume of blood contained in a 3 or a 6 mm disc punched from the DBS, caused in part by the uneven coverage of blood on the card. A number of previous studies have reported that variations in blood hematocrit have a particularly significant effect on the spread of blood on the standard Whatman 903 paper, and ultimately on the volume of blood estimated to be in a given punch size.[7–9] Whereas a number of methods have been developed in an attempt to correct for these factors, including post-column infusion of an internal standard and on-card hematocrit analysis,[10–12] these approaches add further complexity to the procedure. In addition, the practicality, universality, and clinical validity of these methods for volume correction have been questioned.[13]

Our laboratory has recently adopted the use of a modified DBS system called PUFAcoat™ paper for routine fatty acid analyses.

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The PUFAcoat™ system, which utilizes a special silica-gel coated filter paper impregnated with an anti-oxidant and a chelating agent, is superior for stabilizing polyunsaturated fatty acids during storage at room temperature and was lower in contaminants compared to several other DBS systems.[14,15] However, it is unknown whether the volume of blood in a small disc obtained from the PUFAcoat™ paper would be the same as that taken from conventional 903 paper because PUFAcoat™ paper is not as absorbent as the standard 903 paper. It was also unclear that whether the volume of blood in punches collected from the same DBS is consistent.

With the increasing uses of DBS systems generally and the PUFAcoat™ paper specifically,[16] the volume of blood in a punched DBS disc needed to be evaluated in order to accurately quantify the levels of analytes in blood from different individuals. The objective of the current study was to determine the volume of blood in a small disc (3 or 6 mm) taken from a DBS collected onto a PUFAcoat™ paper using several different estimation methods, and to assess the variation in volume both within and between individuals.

2. Materials and Methods

2.1. Standards and Reagents

LC-MS/MS grade methanol was purchased from Merck (Darmstadt, Germany). Deuterated internal standard, d_5-docosahexaenoic acid (d_5-DHA) (≥99% purity), was purchased from Cayman Chemical Company (Michigan, USA). Tritridecanoin ($C_{13:0}$) and triheptadecanoin ($C_{17:0}$) fatty acid standards were purchased from Nu-Check Prep Inc. (Elysian, USA). A.C.S grade sulfuric acid was obtained from Sigma–Aldrich (Castle Hill, NSW, Australia). Hemoglobin colorimetric assay kits were purchased from Cayman Chemical Company (Michigan, USA). AR grade heptane was acquired from Rowe Scientific (South Australia, Australia).

2.2. Subjects and Sampling

Ethical approval for the collection of blood for this study was obtained from The University of Adelaide Human Research Ethics Committee (H–2016–088). Thirty healthy individuals (25 females and 5 males) aged between 22 and 70 years agreed to participate in the study. All participants provided written informed consent prior to blood collection, and venous blood from each participant was collected through the antecubital vein into dipotassium EDTA vacutainers (Becton, Dickinson and Company, South Australia, Australia). Blood was immediately spotted onto PUFAcoat™ paper (Xerion Limited, Victoria, Australia) and standard Whatman 903 paper (Sigma–Aldrich, NSW, Australia) and papers were air–dried at room temperature for 3 h in the dark. After drying, the paper was placed inside an aluminum zip lock bag (VWR International, QLD, Australia) containing silica gel desiccant and stored at −80 °C until use.

2.3. Methods for Volume Estimation

2.3.1. Gravimetric Analysis of DBS Discs

Separate PUFAcoat™ DBS cards, each containing 30 μL of blood from one of six different individuals, were weighed separately using a microbalance ($d = 0.01$ mg). In addition, three separate 3 mm DBS discs punched from the same 30 μL DBS were weighed individually. The volume of blood in each 3 mm disc was calculated for each of the DBS prepared from different individuals using the following equation:

$$\text{Volume of blood } (\mu\text{L}) = \text{Volume of blood spotted on DBS } (\mu\text{L}) \times \frac{\text{Weight of a 3 mm disc (mg)}}{\text{Weight of the DBS (mg)}}$$

The above experiment was repeated for DBS samples on standard 903 paper collected from the same six individuals.

2.3.2. LC-MS/MS Measurement

To establish a calibration curve, 18 separate 3 mm discs were first punched from blank PUFAcoat™ paper. Increasing volumes of blood (0, 0.5, 1, 1.5, 2, and 3 μL) from the same blood sample were then aliquoted onto the separate pre-punched 3 mm discs (three discs for each volume) and the discs were placed separately into a 96-well plate for analysis. A known volume (30 μL) of blood from three different individuals was also spotted onto three separate PUFAcoat™ cards. Five separate 3 mm DBS discs were punched from each of these three DBS cards and placed into a 96-well plate. Lipids were extracted from the DBS discs according to a previously described method,[17] using 80% aqueous methanol spiked with internal standard (0.01 ng μL^{-1} d_5-DHA). The plate was shaken for 30 min on a plate shaker and the supernatant transferred into wells of a fresh plate.[17] Endogenous concentrations of the long chain polyunsaturated fatty acid (LCPUFA), docosahexanoic acid (DHA), in each of the 3 mm DBS disc extracts were determined using LC-MS/MS. Briefly, analyses were conducted with an Agilent 1290 Infinity LC system (Agilent Technologies) equipped with a binary pump and thermostated autosampler held at 4 °C, connected to a 5500 triple quadrupole mass spectrometer (AB Sciex), using electrospray ionization in negative mode. The mass spectrometer conditions were as described in detail previously.[17]

A calibration curve was generated for DHA by plotting the concentration of the fatty acid measured on the pre-punched 3 mm discs (y-axis) against the volume of blood originally added to the disc (x-axis). The equation of the line of best fit was calculated and used (in combination with the measurements obtained for the fatty acid concentration) to determine the volume of blood in each 3 mm disc punched from the DBS cards.

2.3.3. Hemoglobin Colorimetric Assay

New calibration curves of hemoglobin were set up using 18 separate 3 mm discs with increasing volumes of blood for each individual. In addition, 20, 30, and 40 μL of blood from the

30 different individuals were spotted onto blank PUFAcoat™ cards along with a set consisting of 20 µL spotted twice (40* µL), and cards were dried for 3 h in the dark. On the following day, three separate 3 mm discs were punched from each of these DBS cards and placed into separate wells in a 96-well plate and a hemoglobin colorimetric assay was performed according to the manufacturer's instructions (Cayman Chemical, Michigan, USA), with slight modifications. Briefly, hemoglobin sample buffer (20 µL) was added to each well followed by hemoglobin detector (180 µL). The plate was then covered with aluminum foil and incubated at room temperature for 15 min, after which 150 µL of supernatant was transferred to a new 96-well plate. Absorbance was measured at 490 nm using a spectrophotometer (BioTek, Winooski, USA). The absorbance measured from the calibration curve was used to calculate the volume of blood in the 3 mm DBS discs.

2.3.4. GC Measurement

For estimating volume by GC with flame ionization detection (FID), exogenous tritridecanoin ($C_{13:0}$) fatty acid standard was spiked into separate ≈400 µL samples of whole blood from three different individuals. The samples were incubated at room temperature for 10 min, to allow the mixing of the spiked standard with blood, and 20, 30, and 40 µL of each spiked blood sample was spotted in triplicate onto PUFAcoat™ paper. One 6 mm disc was punched from each DBS and placed into a 5 mL glass vial. The remainder of each DBS (i.e., any remaining blood that was not included in the 6 mm disc, see **Figure 1**) was carefully cut out and placed into a separate 5 mL glass vial. Methylation solution (2 mL) containing 1% sulfuric acid in methanol, and triheptadecanoin ($C_{17:0}$) as an internal standard, was then mixed with the DBS sample in each glass vial, and samples were incubated at 70 °C for 3 h. The resultant fatty acid methyl esters (FAME) were extracted with heptane and quantified using a 6890 gas chromatograph (Hewlett-Packard, CA, USA) equipped with a BPX70 capillary column (30 m × 0.32 mm, film thickness 0.25 µm; SGE Analytical Science, VIC, Australia), and FID as described in detail previously.[17]

The ratios of spiked tritridecanoin ($C_{13:0}$) to internal standard triheptadecanoin ($C_{17:0}$) were calculated for each sample. The ratio values obtained for each 6 mm disc were added to the ratio values obtained for each corresponding DBS remainder which enabled the calculation of the total ratio values for the full DBS (see Figure 1). The volume of blood in a 6 mm DBS disc was then calculated according to the following equation:

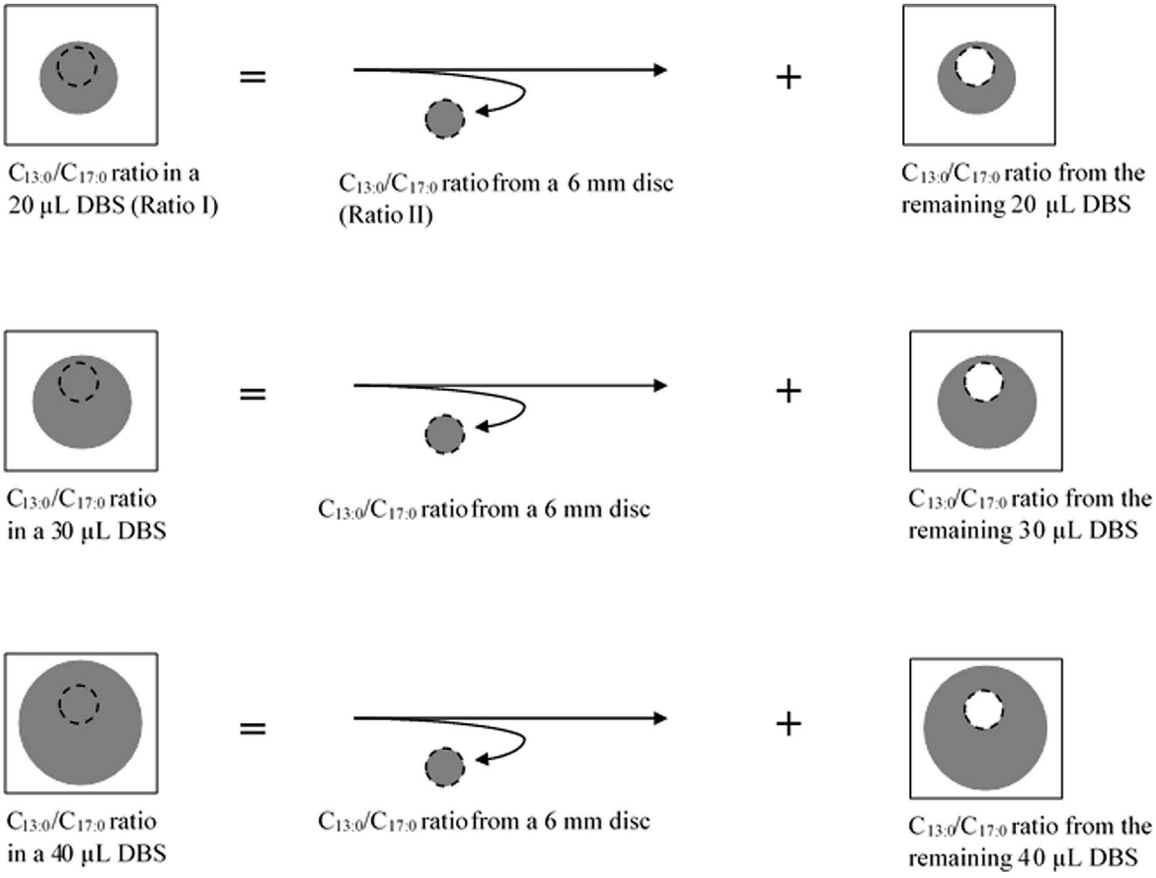

Figure 1. Illustration of the protocol used with GC-FID to assess the volume of blood in a 6 mm disc obtained from DBS containing 20, 30, and 40 µL of blood. The remainders of the DBS were also analyzed separately. The ratio in a whole DBS was equal to the sum of ratios obtained from a 6 mm disc and the corresponding remainder for a given initial spot size.

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$$\text{Volume of blood in a 6 mm disc (μL)} = \text{Volume of blood in DBS (μL)} \times \frac{\text{Ratio II}}{\text{Ratio I}}$$

Ratio I = ratio of $C_{13:0}/C_{17:0}$ obtained from the full DBS by adding the ratio values obtained from 6 mm disc and remaining DBS
Ratio II = ratio of $C_{13:0}/C_{17:0}$ in the 6 mm disc

2.4. Statistical Analysis

All data are reported as the mean ± SD unless otherwise indicated. All descriptive statistical analyses and linear regression of calibration curves were performed using Microsoft Excel 2013. One-way ANOVA was performed using GraphPad prism to compare different volume analysis methods and various volumes spotted on the PUFAcoat™ paper within each method. In addition, variations in blood volume between triplicate discs from the same DBS sample in an individual were determined using one-way ANOVA.

3. Results and Discussion

DBS sampling technology is a cost-effective, convenient sampling approach and used widely as an efficient screening tool. It has been also used for many years in the diagnosis of adrenoleukodystrophy (genetic disorder characterized by the loss of myelin) by measuring very long chain saturated fatty acids as biomarkers of this disease.[18,19] However, one of the limitations of DBS systems has been the variability in the estimation of the blood volume in discs obtained from a DBS.

In the current study, the volume of blood in 3 and 6 mm discs obtained from PUFAcoat™ paper was evaluated using gravimetric analysis, LC-MS/MS measurement, hemoglobin colorimetric assay, and GC. The results obtained for the volume of blood attributed to a 3 mm disc did not differ significantly between methods (**Table 1**, $p = 0.83$): discs obtained from a 30 μL DBS were estimated to contain 1.8 ± 0.3 μL using the gravimetric method for six individuals' blood and 1.4 ± 0.3 μL using LC-MS/MS with DHA calibration curves for three individuals' blood; discs obtained from samples from 20, 30, 40, and 40* μL DBS were estimated to contain 1.5 ± 0.5, 1.6 ± 0.6, 1.4 ± 0.3, and 1.6 ± 0.5 μL, respectively, with an average of 1.5 ± 0.5 μL, using the hemoglobin colorimetric assay for DBS samples from 30 individuals. Additionally, there were no differences in the volumes estimated using the hemoglobin method ($p = 0.40$) for 3 mm discs from PUFAcoat™ DBS containing different volumes of blood, suggesting that the amount of dried blood in a 3 mm punch is not impacted significantly by differences in the total volume of blood initially spotted onto the paper. This has particular relevance to the clinical setting, since the volume of blood that can be collected by a finger or heel prick from different patients may vary considerably, depending on their age and medical condition.[20]

For the GC determinations of the volume of blood, a 6 mm disc was used because detection of FAME with FID did not allow for an accurate measurement of fatty acid content from a 3 mm DBS disc. Using this approach, in combination with indirect measurement of blood volume via quantification of an exogenous fatty acid standard, indicated that a 6 mm disc from a DBS contained an average of 8.7 ± 1.9 μL of blood. As with the 3 mm DBS punches assessed by weight (gravimetric), the calibration curve (LC-MS/MS) or hemoglobin assay, the volume of dried blood in a 6 mm disc estimated using GC did not vary significantly ($p = 0.17$, **Table 2**) according to the total volume of blood in the DBS: 8.2 ± 1.5 μL, 7.6 ± 1.1 μL, and 10 ± 2.0 μL for 6 mm discs obtained from 20, 30, and 40 μL DBS, respectively. Although there appeared to be variation, the blood volume estimates between different individuals were not significantly different ($p = 0.19$, Table 2), although this may be due to the small sample size for this experiment ($n = 3$).

Other studies have reported that blood volume in a small disc is significantly influenced by the hematocrit of that blood sample.[8,9,21] As a result, we adjusted for each individuals' hematocrit by using individual standard curves for each participant in the hemoglobin colorimetric assay. Despite this, however, there was still considerable inter-individual variation in the estimated volume of blood in 3 mm DBS punches from 30 different individuals using this assay (CV ≈ 32%) (**Figure 2**). This could be due to a number of factors including differences in the spread of blood onto the paper and uneven coverage of the DBS.[22] Our findings are in line with previous studies using other DBS systems that have also reported variations in

Table 1. Estimation of volume of blood (μL) determined for 3 mm discs obtained from DBS and assessed by three different methods using PUFAcoat™.

Method	Subjects (*n*)	Volume of blood spotted	Estimated volume of blood in 3 mm discs μL[a]
Gravimetric	6	30	1.8 ± 0.3
Calibration curve (LC-MS/MS)	3	30	1.4 ± 0.3
Hemoglobin colorimetric assay	30	20	1.5 ± 0.5
		30	1.6 ± 0.6
		40	1.4 ± 0.3
		40[b]	1.6 ± 0.5

[a]Calculated from three replicates per individual; [b]Produced from two applications of 20 μL of blood on the same card.

Table 2. Volume of blood determined using GC-FID for 6 mm discs obtained from DBS from three individuals.

	Calculated volume of blood in a 6 mm disc (μL)[a]			
Volume of blood spotted on PUFAcoat™ paper (μL)	Individual 1	Individual 2	Individual 3	Average
20	8.9 ± 1.9	7.0 ± 1.4	8.6 ± 0.6	8.2 ± 1.5
30	7.3 ± 0.6	6.9 ± 0.4	9.4 ± 1.1	7.6 ± 1.1
40	11.2 ± 2.2	8.2 ± 0.8	11.1 ± 1.2	10 ± 2.0
Average	8.9 ± 2.3	7.5 ± 1.0	10 ± 1.5	8.7 ± 1.9

[a]Calculated from three replicates per individual.

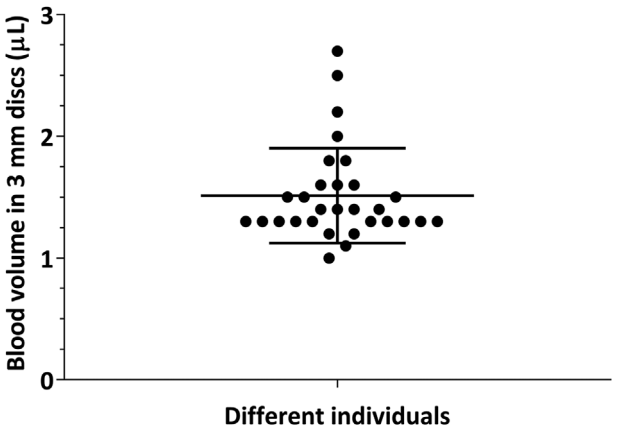

Figure 2. Illustration of the distribution in the blood volume observed between different individual's blood samples when evaluated using the hemoglobin assay of a 3 mm disc. Results expressed as mean ± SD, $n = 30$.

estimated blood volumes in DBS discs according to DBS size and punch location.[8,11,23]

The estimated volume of blood in a 3 mm disc obtained from a conventional newborn screening blood collection paper (903 paper) in a previous study was ≈3 µL,[5] which is notably higher than the estimates calculated for the 903 paper from the gravimetric analysis in the current study (2.2 ± 0.1 µL). This may be due to several factors. First, there may be variations in the type of 903 paper (i.e., thickness) used in different laboratories which will affect the absorbance of blood on the paper, therefore affecting the blood volume. Secondly, we used a 30 µL DBS for the gravimetric analysis but spotting different volumes may have an impact on the volume of blood in a 3 mm disc.

Comparing the different DBS papers, the estimated volume of blood in a 3 mm disc punched from the PUFAcoat™ paper was lower than that for 903 paper as reported previously[5] and calculated from the gravimetric analysis in the current study. PUFAcoat™ paper differs from standard 903 paper by being thinner,[14] and by using a silica gel coated paper that is much less porous and is expected to have a lower rate of absorption for a given area of paper. These differences are expected to affect the spread of blood, leading to a lower volume of blood in the same area of PUFAcoat™ paper compared to conventional filter papers. The slower absorbance of blood by the silica-gel paper in the PUFAcoat™ card also has the potential to result in differences in the volume of blood in 3 mm discs punched from different parts of the same DBS sample, however there was little variation in the blood volume estimated in triplicate 3 mm DBS punches from the same DBS sample in the current study (CV <9%). Our use of strategies to minimize the differences between the range of DBS assessed, such as avoiding punching discs from the middle of the DBS where blood may be more concentrated, have likely prevented greater variation.

Variations in the blood volume of a small DBS disc may result in underestimation or overestimation of the fatty acid concentrations in blood. For instance, a 25% difference in blood volume in a small disc can result in ≈22% difference in free DHA concentrations in blood. Data should therefore be interpreted with caution when expressed as absolute concentrations of fatty acids per unit volume of blood. Nonetheless, a previous study showed relatively strong positive correlations ($r = 0.7$–0.8) between polyunsaturated fatty acid concentrations in samples extracted from a 6 mm DBS and whole blood using an identical method.[24] This may indicate that the DBS methods can be used for comparison of lipid profiles between groups, but further work is required to verify this.

The results of the current study are in line with previous reports of conventional DBS systems in suggesting that it is difficult to avoid inter-individual variation in the volume of blood contained in a DBS disc. As a result, several methods have been developed to account for such DBS volume variations.[10–12,21] One such method is an on-card approach to assess the hematocrit of DBS in order to correct for sample volume, which has previously been shown to improve the precision and the accuracy of the results.[10] However, this method only accounts for the variations in volume because of hematocrit but volume is reportedly impacted more by certain physical characteristics such as the DBS diameter and shape, which has led to the suggestion that DBS samples outside a given range should be excluded from these analyses.[11]

Despite attempts to correct for variations in the blood volume of individual DBS discs, the intra-individual variability in this measure remains an ongoing issue that needs to be overcome to ensure accuracy of results expressed per unit volume of blood. One potential approach to limit the impact of variations in volume would be to use the whole DBS sample based on a known volume of blood spotted on the filter paper. However, this is not practical to determine the volume in a clinical setting and leaves no DBS available for other analyses. A similar, but more attractive option, would be to use a device that can ensure a known and consistent volume of blood is used to generate multiple blood spots on the filter paper, and to use entire spots for analysis. Alternatively, an analytical approach which measures blood volume in a 3 mm punch prior to determination of analyte concentrations could also be applied. In combination with the current DBS technology, such approaches will ensure that DBS samples provide a robust collection system for accurate and sensitive screening.

4. Conclusions

In this study, we have determined the volume of blood in a small disc (3 and 6 mm) obtained from a DBS collected onto our PUFAcoat™ system using both endogenous and exogenous reference compounds. While there was consistency in the estimate of the average blood volume between methods and within individuals, there was considerable variation in the estimated volume between individuals. These results are in line with a similar study undertaken using conventional 903 paper DBS systems, and suggest that there is a need for new devices that enable a consistent and known volume of blood to be collected for spotting onto DBS paper.

Abbreviations

DBS, dried blood spot; DHA, docosahexaenoic acid; FAME, fatty acid methyl esters; FID, flame ionization detector; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LCPUFA, long chain polyunsaturated fatty acids.

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Conflict of Interest

RAG serves on the advisory board for the Fonterra group. All associated honoraria are paid directly to his host organization, and used to support travel and professional development for students and early career researchers. RAG and LG are the inventors of PUFAcoat system, the patent for which is owned by the University of Adelaide. The license for PUFAcoat has been granted to Xerion Ltd Melbourne. BSM serves on the advisory board for the Nestle Nutrition Institute and has given talks for Danone Nutricia and Aspen Nutrition. All associated honoraria are paid directly to her host organization and used to support travel and professional development for students and early career researchers.

Keywords

PUFAcoat paper, volume estimation, dried blood spot, hematocrit, comparison of methods

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