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A solution to the remote sampling problem? Comparison of metabolite stability with different dried blood sampling methods

Time-dependent metabolite stability and general suitability of dried blood spots (DBS) and volumetric absorptive microsampling (VAMS®) using Mitra® tips for quantitative metabolomics

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1 Introduction

In recent years there has been a growing interest in home sampling for nutrient or fitness assessment. This approach to sample collection can be convenient and non-invasive for subjects, but how reliable are the results?

While home sampling can involve many different sample types such as urine, blood, saliva, breath, and feces, in this application note we focus on the coverage and stability of dried blood samples for different health applications. We compare our own results obtained with dried blood spots (DBS) and Mitra tips and review the literature to find out how other scientists approached this topic and see what differences may be expected for metabolites routinely measured in plasma. A summary of our findings is presented in Table 1.

Blood sampling at home

Blood sampling at home usually involves harvesting capillary blood via a finger prick or from the upper arm. There are commercial solutions that can stabilize the blood as dried whole blood, dried plasma, or liquid serum/plasma. This application note focuses on dried whole blood.

Since multipanel metabolomics equipment is not yet available at point-of-care (POC) facilities, stability of samples and analytes during sample transport is a major concern.

Transporting liquid plasma, serum, or whole blood for metabolomic analyses requires samples to be stabilized with a buffer (usually K3-EDTA, sodium citrate, or lithium heparin) and frozen with dry ice. These logistics are

Category	Plasma	DBS	Mitra tips
Metabolites quantitatively detected on collection day	508	433	473
Metabolites quantitatively detected after three days at room temperature	N/A	415	448
Of these, number of small molecules | lipids	N/A	69 | 346	84 | 364

Table 1: Comparison of metabolite detectability (at least in 50% of the samples) in ethylenediaminetetraacetic acid (EDTA) plasma, DBS, and dried blood Mitra tips under different conditions.

unrealistic for home sampling and would compromise the affordability of frequent sample collection and timely analysis. For these reasons, non-liquid blood sampling methods are very appealing for home sampling.

DBS sampling on filter paper has been used since the 1960s. Today, volumetric methods are also available, for example hemaPEN®, Capitainer®B, or HemaXis DB. Alternatives include volumetric absorptive microsampling (VAMS) devices, of which Mitra tips are the most tested and widespread, and blood collection in sorbent/stabilizing/separating material (Tasso®).

VAMS gained attention because it facilitates straightforward collection of a small, defined blood volume by untrained sample donors. Furthermore, studies have shown that blood VAMS sampling is usually not sensitive to hematocrit variability (Kesel et al. 2015; Mandrioli et al. 2020). Hematocrit-related issues, which particularly affect liquid chromatography and mass spectrometry (LC-MS) analysis, remain a major barrier to regulatory and general acceptance of classical DBS analysis in the bioanalytical and clinical field (Velghe et al. 2019).

Previous studies have tested the stability of metabolites in VAMS using an untargeted metabolomics approach, which lacked absolute quantification of metabolites and did not fully identify spectral features (Volani et al. 2017; Volani et al. 2023). This approach naturally leads to better numbers as degrading metabolites are not recognized as being partly degraded when they are still qualitatively measurable. Nevertheless, the authors provided a good foundation for our stability study by comparing capillary blood with serum and plasma, and a basis for our hypothesis that VAMS is a good tool to solve the home sampling problem. Additionally, different studies have assessed the stability of 26 metabolites in the kynurenine-tryptophan pathway (Protti et al. 2022), and of amino acids and other organic acids (Kok et al. 2019) in a very comprehensive way.

This application note builds on this initial work to assess the stability of 630 metabolites in VAMS and DBS over several days (three to seven days) at room temperature to find out if common logistics constraints for home sampling can be met, and therefore, if the sampling is compatible with the biocrates quantitative metabolomics workflow.

2 Methods

Whatman 903™ filter paper was used to generate the DBS samples. Mitra tips were obtained from Neoteryx. Initial tests were conducted with 10 µL Mitra tips and 20 µL Mitra tips. Since the larger variant did not result in a marked increase in the number of detected metabolites, it was decided to conduct the stability study with 10 µL Mitra tips, as a smaller sample volume is associated with less discomfort for the user.

Figure 1: Mitra tip and DBS blood sampling devices. ©MarekPhotoDesign.com | Adobe Stock | #626298463

Three healthy individuals were enrolled to create several samples of DBS and dried blood Mitra tips simultaneously. Each individual washed their hands with warm water for at least one minute to increase blood flow. The fingertips were dried, disinfected with an alcohol wipe and pricked with Unistick 3 Normal lancets. Each individual produced six DBS samples with three drops of blood per DBS, corresponding to 75-80 µL. Each individual also produced nine Mitra tip samples of 10 µL each.

DBS samples were dried for four hours at room temperature in the dark. Mitra tip samples were dried for two hours at room temperature in the dark. After the drying time, all samples were transferred to small plastic bags with silica gel to keep them dry. From each matrix, three samples from each individual were then frozen at -80°C right away or kept in the dark at room temperature for three days (DBS and Mitra tips) or five or seven days (Mitra tips only), before being transferred to -80°C until measurement.

The biocrates MxP® Quant 500 kit for absolute quantification of up to 630 predefined metabolites (small molecules and lipids) was used for sample measurement. For the DBS samples, 3 mm punches were generated from DBS cards and placed as samples in the wells of the 96-well plate directly. For measurement of Mitra tip samples, the blood-filled sponges at the tip were carefully stripped off directly into the wells of the 96-well plate. Sample measurement was conducted on an Agilent 1290 UHPLC – SCIEX QTRAP 5500 instrument. Measurement results were quantified with Sciex Analyst® and the biocrates MetIDQ software.

Detectability was assessed with a 50% threshold: a metabolite was rated detectable when present in at least 50% of the samples. Stability was assessed by comparing measured concentrations after a set number of days at room temperature with concentrations in samples frozen directly after the drying period. Stability was rated acceptable when the mean of the measured concentrations (mean of triplicate samples kept at room temperature for a fixed number of days) over all individuals was between 85% and 115% of the mean concentration of triplicates frozen right after the drying time. In addition, the coefficient of variance (CV) was calculated for the triplicates. The CV was rated acceptable when the mean CV over all individuals was according to the specifications of the kit validation for plasma samples. This indicated a CV below 15% for all 7-point calibrated metabolites, and below 30% for all 1-point calibrated metabolites (except for the metabolites rated "quantitative with restrictions", for which the acceptable mean CV was below 20%). An additional criterion was that the mean of the maximum CVs over all individuals had to be below 40%.

3 Results

A total of 273 metabolites met the acceptance criteria outlined above in DBS samples stored for three days at room temperature before being frozen at -80°C until measurement. This corresponds to 43% of the metabolite portfolio of the MxP® Quant 500 kit. With the matrix Mitra tip, 249 metabolites met the acceptance criteria, which corresponds to 40% of the metabolites assessed (Figure 2). Thus, classical DBS and dried blood Mitra tips enable quantification of a similar number of stable metabolites.

Of the 630 metabolites assessed in the MxP® Quant 500, 142 were detectable in less than 50% of the samples in plasma. Most of these had the same detectability in dried blood samples. Of the 381 metabolites not initially classified as stable and detectable in dried blood Mitra tip samples, 60 were found to be stable but detectable in less than 50% of the samples. This suggests that almost half of the targeted metabolites are stable in this matrix, albeit with variable detectability.

A total of 74 metabolites were so rarely detected that an assessment of stability was not possible. We could not identify stable but

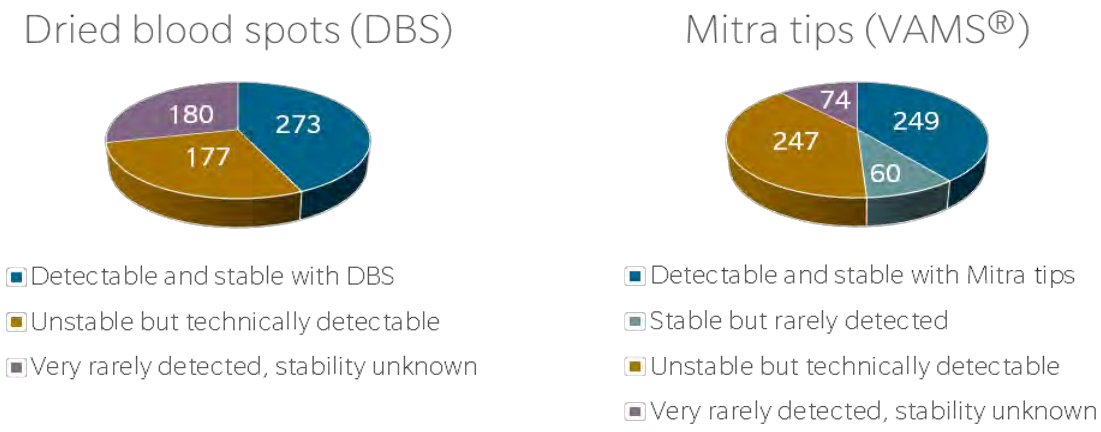

Figure 2: Comparison of detectable and stable metabolites in DBS and Mitra tips when stored for three days at room temperature

rarely detected metabolites using DBS because fewer experiments were conducted.

A total of 23 of the 630 metabolites were detected in less than 10% of all plasma samples in the healthy population and were never detected in any of the dried blood samples. Levels of these metabolites usually increase in certain disease conditions, which may increase detectability in dried blood samples. However, the stability of these and other rarely detectable metabolites remains unknown because it could not be assessed. The total number of rarely detected metabolites with unknown stability is much higher in DBS than in Mitra tips.

Table 2 provides a comparison of the metabolites that were detectable and stable at room temperature for three days in each matrix per class. The left half (including vitamins and cofactors) focuses on the small molecules, and the right half on the lipids. DBS appears to detect a slightly higher number of lipids than Mitra tips; 219 lipids are stable and detectable with DBS compared to 180 with Mitra tips. For the polar small molecules, Mitra tips enable accurate quantification of a larger number of metabolites than DBS (69 versus 52).

A transport time of three days until freezing may not be feasible in all cases. For that reason, we also tested stability and detectability in Mitra tips after five and seven days of storage at room temperature. Figure 3 shows the results. Without any storage time, 473 metabolites were detectable in dried blood Mitra tips. This is only slightly less than the number of metabolites detectable in EDTA plasma.

Table 2: Comparison of detectable and stable metabolites in DBS and Mitra tips summarized per metabolite class when stored for three days at room temperature.

	Alkaloids	Amine oxides	Amino acids	Amino acid-related	Bile Acids	Biogenic amines	Carbohydrates	Carboxylic acids	Cresols	Fatty acids	Hormones	Indoles derivatives	Nucleobase-related	Vitamins & cofactors	Acylcarnitines	Ceramides	Cholesterol esters	Diacylglycerols	Dihydroceramides	Glycerophospholipids	Glycosylceramides	Sphingolipids	Triacylglycerols
DBS	1	1	14	15	8	4	1	2	1	2	1	3	1	0	5	17	11	3	2	49	19	14	99
Mitra tips	1	0	17	17	12	7	0	3	1	4	2	3	1	1	18	24	4	4	5	35	28	14	48

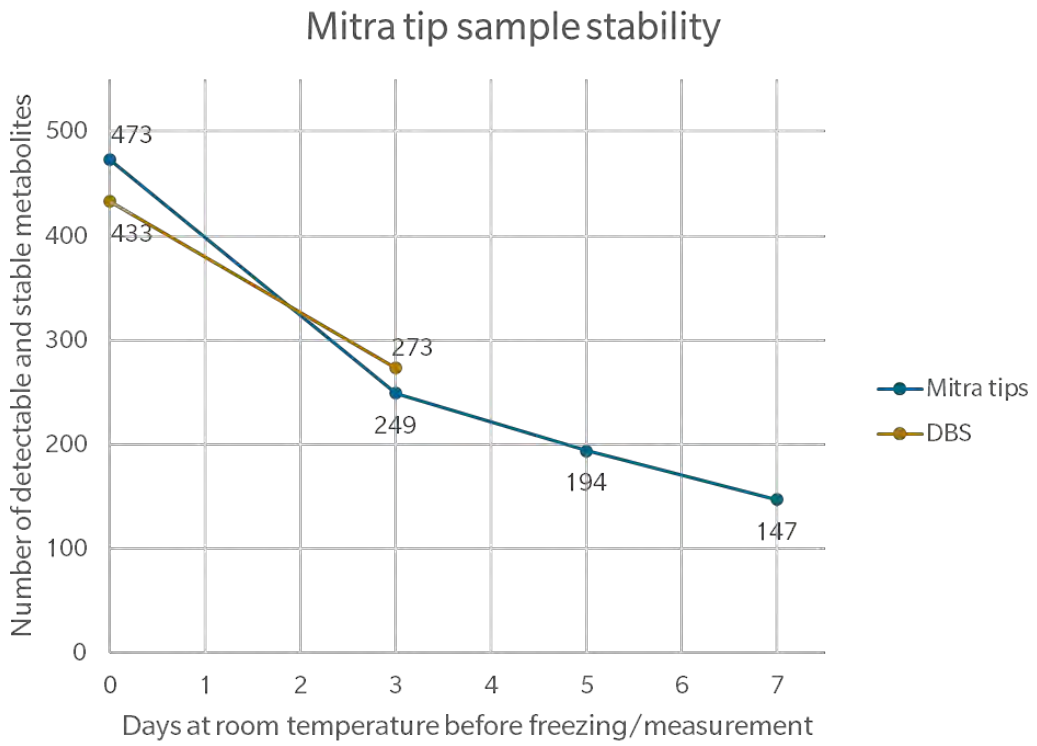

Figure 3: Number of detectable and stable metabolites in Mitra tips depending on storage time at room temperature.

After three days, a little more than 50% were still detectable and were present in the same concentration range as defined in the acceptance criteria. After five days, only 194 metabolites were still detectable and displayed stable concentrations, corresponding to 41% of the originally detected metabolites.

As expected, metabolites that are structurally sensitive to decay showed greater changes in concentration with increased storage time. Reduced stability was more common in lipids than in polar small molecules because lipids are prone to both dissociation and oxidation. Oxidation depends on the sample surface area, which is much smaller in DBS than in the porous, sponge-like Mitra tips. This explains why lipids were more stable when assessed with DBS. However, the larger surface area in Mitra tip samples accelerates drying compared to classical DBS, reducing the time for enzymes to metabolize sample molecules.

As an example, the amino acid arginine was readily detectable in dried blood Mitra tips but not in DBS. Red blood cells contain enzymes that quickly and efficiently metabolize free arginine to ornithine, but the Mitra tip samples dry fast without much hemolysis so that arginine concentrations remain stable. Table 3 compares the metabolites that were detectable and stable at room temperature for three to seven days compared to zero days in each matrix per class.

The stability of metabolites in DBS for different storage times was not tested. However, we know from previous experiments that the detectability of metabolites decreases in DBS in a similar way as in dried blood Mitra tips.

Table 3: Comparison of detectable and stable metabolites in dried blood Mitra tips summarized per metabolite class when stored for three or five days at room temperature (RT).

	Alkaloids	Amine oxides	Amino acids	Amino acid-related	Bile Acids	Biogenic amines	Carbohydrates	Carboxylic acids	Cresols	Fatty acids	Hormones	Indoles derivatives	Nucleobase-related	Vitamins & cofactors	Acylcarnitines	Ceramides	Cholesterol esters	Diacylglycerols	Dihydroceramides	Glycerophospholipids	Glycosylceramides	Sphingolipids	Triacylglycerols
Mitra tips, 0 days RT	1	1	20	23	12	7	1	6	1	7	3	3	2	1	19	26	16	10	5	87	34	14	174
Mitra tips, 3 days RT	1	0	17	17	12	7	0	3	1	4	2	3	1	1	18	24	4	4	5	35	28	14	48
Mitra tips, 5 days RT	1	0	17	16	12	6	0	3	1	1	2	3	1	1	14	24	3	3	3	26	27	13	17
Mitra tips, 7 days RT	1	0	10	11	12	3	0	2	1	1	1	2	1	1	14	23	2	3	3	13	24	13	6

4 Discussion

The MxP® Quant 500 kit can quantify up to 630 metabolites. About 500 metabolites are usually detectable in human plasma. It is generally accepted that plasma samples need to be frozen right after processing to maintain the metabolite concentrations found *in vivo*. Because many metabolites are more stable at room temperature in dried blood samples than in plasma, dried blood samples are a preferred alternative to plasma when there are no medical personnel to collect or process the sample, or when the sample cannot be frozen right away. Our research confirms this for a significant fraction of the metabolites assessed with the MxP® Quant 500 kit, depending on the storage time at room temperature. Assuming the dried blood sample can be transported to the laboratory within three days, where it can be frozen until measurement, 273 metabolites are detectable and stable in DBS. Mitra tips quantify slightly fewer metabolites but can detect more polar small molecules. This may make Mitra tips a better sampling choice for many applications, especially given the advantages of easier handling and smaller blood volume requirements. Once stored at -80°C, the metabolome of dried blood samples is stable for at least six months (Volani et al. 2017).

Unlike plasma or serum samples, the extraction method for dried blood samples has a marked effect on metabolite concentrations. As a result, comparing concentrations between laboratories can be problematic, which should not be overlooked. The most suitable extraction method will depend on the analytes targeted, so there is no generally accepted extraction method for dried blood samples. For example, an acetonitrile/water solution has been recommended for polar metabolome extraction (Volani et al. 2017), whereas ethanol- or isopropanol-containing extraction solutions are more suitable for lipid extraction. At biocrates, we use a methanol-based extraction solution for dried blood samples because it has proven to be suitable for both polar small molecules and lipids. Consideration should be given to the pH of the extraction solution, as alkaline conditions are more effective for extracting red blood cell metabolites (Volani et al. 2017), which may be undesired. Other parameters can also influence extraction efficiency: research has shown that sonication of samples during extraction is superior to vortexing or shaking for metabolite recovery and minimizes the impact of hematocrit differences (Mano et al. 2015).

VAMS using Mitra tips is a good choice for home sampling when conducting quantitative metabolomics to monitor potential health status indicators. For example, biogenic amines are used in health assessments because of their essential signaling roles as neurotransmitters or hormones, or as source molecules for the biosynthesis of such (Purves et al. 2001).

Of the nine biogenic amines, only four are stable and detectable in DBS, against seven

in Mitra tips. Researchers assessing liver health (Farooqui et al. 2022) and the interplay between gut health and the microbiome (Kumar et al. 2022) may be interested to note that of the 14 bile acids assessed with the MxP® Quant 500, only eight were detectable and stable in DBS, against 12 in Mitra tips. Mitra tips are also the better choice when acylcarnitines are of interest. While long-chain acylcarnitines have been linked to poor clinical status, many studies have observed that short-chain acylcarnitines are associated with positive effects in the treatment of various disorders, including kidney disease (Andrianova et al. 2020). Mitra tips enable quantification of 18 acylcarnitines, while only five are stable and detectable in DBS.

Generally, small molecules yield more information per metabolite about an individual's health status than lipids. This is because health issues tend to have the same effect on all lipids within an ontology class, and because more is known about the functional roles of metabolites for small molecules than for lipids. Therefore, the higher number of stable and detectable small molecules in Mitra tips versus DBS is More relevant to the interpretation of the overall panel than the higher number of detectable and stable lipids in DBS compared to Mitra tips. This makes dried blood Mitra tips the more informative matrix to assess and monitor the health status of an individual.

In contrast, DBS sampling is preferable for triacylglycerols, which may be used to assess overall blood lipid content as a marker for certain cardiovascular diseases (CVD) (Aberra et al. 2020). With more than 90 stable and detectable triglycerides, DBS clearly surpass Mitra tips in this regard. Additionally, blood levels of trimethylamine oxide (TMAO) are associated with CVD risk (Zheng und He 2022), and this amine oxide is stable in DBS but unstable in Mitra tips.

DBS sampling is also more suitable for monitoring blood sugar levels. Concentrations of hexose, a sum signal derived mainly from glucose, are stable in DBS for three days. The acceptance criteria were not met when using Mitra tips.

It must be acknowledged that some metabolites that are relevant for health monitoring, like the omega-3 and omega-6 fatty acids (DiNicolantonio und O'Keefe 2021), are not stable in either DBS or Mitra tips. Adding certain stabilizers to the sample matrix can solve this problem but may negatively affect other metabolites assessed. The issue is usually resolved by collecting a separate sample with a pretreated sampling device specifically for this analysis. For fatty acid analysis, silica gel-coated DBS with antioxidant and a chelating agent has been described as effective (Liu et al. 2014).

Our experiments show that pretreatment with an antioxidant stabilizes fatty acids in Mitra tips as well. However, polyunsaturated fatty acids degrade at similar rates, so the ratio of omega-3 to omega-6 fatty acids is stable in dried blood Mitra tip samples for up to five days at room temperature without addition of stabilizers even though the single metabolites are not.

5 Conclusions

As interest in remote sampling grows, dried blood sampling devices are gaining in popularity. The ability to derive meaningful insights from reliable results obtained from home and remote sampling aligns with "4P medicine," a concept that characterizes the future of medicine as predictive, personalized, preventive and participatory (Alonso et al. 2019).

With the MxP® Quant 500 kit, roughly 250 metabolites can be detected and quantified in dried blood samples kept for up to three days at room temperature. This is consistent with both classical DBS and the more recently established Mitra tips. From an analytical point of view, the markedly reduced hematocrit bias and the superior stability of many small molecules in dried

blood Mitra tips is a marked advantage for this matrix. For the user, the low sample volumes required and the high reliability of the measured concentrations add convenience in the sampling process and trust in the results.

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