Stability of metabolite profiles using dried blood spot cards

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Introduction

The use of dried blood spot (DBS) cards as a blood sampling technique is well suited for situations where the ultra-cold conditions (*i.e.* dry ice or refrigeration) necessary for plasma or serum are unachievable. They are also a viable alternative to in-clinic visits as they can be reliably prepared in an at-home, patient-driven collection process. Additionally, DBS cards are well suited for dealing with limited sample volumes and are a less invasive method, making longitudinal and pharmacokinetic studies requiring frequent collections more tenable. DBS collection typically consists of the deposition of a small volume of capillary blood onto dedicated paper cards. DBS cards have been successfully deployed in newborn screening (NBS) programs as a part of multiple public health policies (*i.e.* phenylketonuria, sickle cell disease, hypothyroidism, and HIV infection), viral infection monitoring, methylomics research, and drug monitoring (1-3). Given the high sensitivity, mass spectroscopy is well suited for DBS card analysis (4-6), and Metabolon has validated the use of Whatman 903 cards as a viable matrix for global untargeted metabolomics using our Precision Metabolomics™ platform. One of the main driving factors of using DBS cards, as noted above, is that immediate cold storage is not absolutely required. Here, we present our internal research on the stability of metabolite profiles based on various storage conditions and times, which drive our guidelines and recommendations for obtaining the best data from DBS cards.

Study Design

The short-term stability (STS) of metabolites in dried blood spots was assessed using a set of DBS cards prepared in bulk from a single pool of whole blood (WB). Cards were dried for ~3.5 hours at room temperature (RT), then were placed in gas-impermeable zip-top bags with desiccant packs and stored at various temperatures. The conditions tested were -80°C, -20°C, 4°C and RT, for either 1, 12, 21, or 28 days. Additionally, the same lot of WB was stored in single-use aliquots at 4°C (in liquid form) and analyzed alongside the DBS samples at each of the four timepoints. Five (5) replicates of DBS cards and 4 replicates of WB were analyzed at each time and temperature combination. *It is noted that we have found minimal differences between DBS cards prepared via pipetting venous whole blood compared to those prepared via finger-stick blood drop collection.*

Long-term stability (LTS) was assessed in 17 lots of DBS cards (25 µL spots of venous disodium EDTA blood) that were stored for up to 7 months at RT and -20°C, and analyzed after the initial preparation (T0), at 3 months, and at 7 months. The initial analysis (T0) was performed with six replicates per lot, while the latter timepoints were analyzed in singlet at each storage temperature. Additionally, a 'shipping' experiment was conducted to assess the impact that extreme heat and humidity exposure may have on DBS samples when shipped at ambient temperature (intentionally or unintentionally) during hot summer months after being stored at -80°C for ~28 days. One to four days prior to analysis (24-27 days at -80°C), DBS cards were removed from cold storage and then placed in a heated environment that was estimated

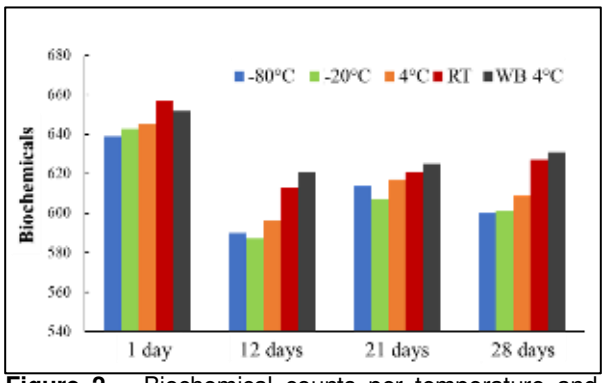

Figure 2 – Biochemical counts per temperature and timepoint. Each set of bars represents the number of biochemicals detected in at least 75% of samples at each temperature for DBS cards (color bars) and WB stored at 4°C (bar bars). *RT, room temperature; WB, liquid whole blood.*

to mimic possible (non-ideal) shipping conditions: approximately 35°C for between 1-4 days prior to analysis (but still in sealed bags with desiccant packs).

All STS, LTS, and Shipping study samples were processed using the standard extraction protocol developed by Metabolon for DBS cards (7). Briefly, 2 x 6 mm punches per card were soaked in water, followed by the addition of extraction solution and 4 minutes of shaking. After removal of the extraction solution to a fresh well, all proceeding steps followed Metabolon's normal workflow for sample analysis (UPLC-MS/MS, automated peak identification, data curation, statistics). We assigned a Stability Outcome to each biochemical by comparing adjacent timepoints to each other, looking at individual time-course plots, calculating the %CV across timepoints for biochemicals with >75% fill at all four timepoints, and imposing a fold-change requirement of ≤25% with significance of $p<0.05$). The following stability categories are used in analyses:

Stable – No significant changes occur between 1-28 days of storage.

Stabilize – Significant intensity changes at early times but none at later timepoints.

Limited stability - No significant changes at early timepoints but they are observed at later time points

Unstable – Significant changes throughout all time points (no signs of stabilization).

Inconclusive – Insufficient data. More timepoints necessary to establish a stability outcome.

Results

Short-term stability

First looking at the comparison of metabolite profile stability over time at a given temperature, we calculated the total number of biochemicals present in at least 3 of the 4 timepoints independently at each temperature. Between 587 and 657 biochemicals passed this criterion (**Figure 1**; *note scale of X-axis*). Using a more conservative level of 100% detection across timepoints, we calculated between 518 and 543 biochemicals with the same overall trends and proportions. At all temperatures, the lowest numbers were detected at the 12-day timepoint, and at all timepoints higher number of biochemicals were detected at higher storage temperatures. This is most likely due to degradation of labile biochemicals into one or more breakdown products. Regardless, in both cases the variation was relatively minor, with coefficients of variation (CV) between 1.1-2.5% across temperatures at each timepoint and 2.2-3.9% across timepoints per temperature.

The results (**Figure 2**) indicate that within the first 12 days of storage a "stabilization effect" occurred

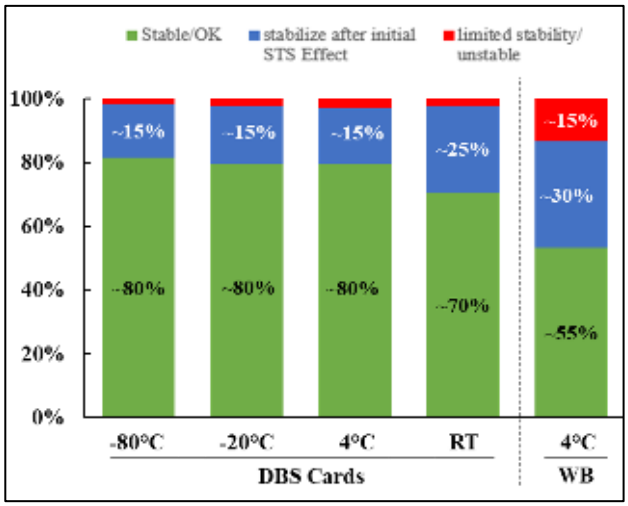

Figure 1 - Proportion of biochemicals at each temperature assigned to each Stability category (n~500 for being present in >75% of samples at all time points). Green indicates stability across days 1-28, blue indicates stability after 12-21 days of storage, and red indicates limited or no stability over the 28-day storage period. *RT, room temperature; WB, liquid whole blood. STS, short term stability effect.*

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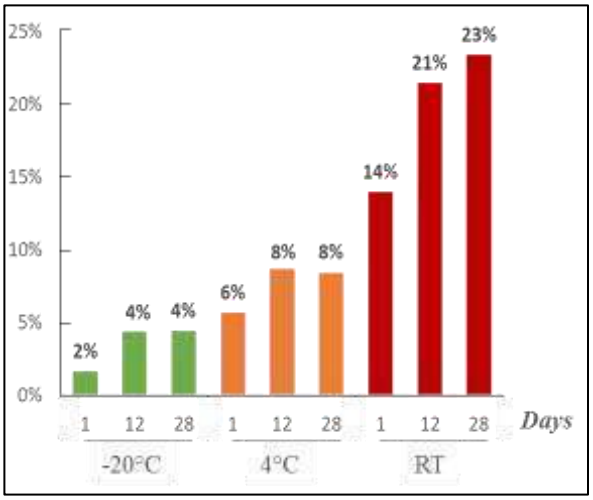

Figure 3 - Percentage of biochemicals with response differences >25% when compared to -80°C storage. There were approximately 575 biochemicals detected in at least 75% of samples at each temperature. *RT, room temperature.*

for approximately one-half to two-thirds of affected biochemicals at <4°C or RT, respectively, while in liquid form only one-third of affected biochemicals showed stability after the first 12 days. Combined with stable biochemicals, ~90% of the 500 biochemicals were stable or stabilized after the first 12 days when stored as DBS, regardless of storage temperature. The blue bars in Figure 2 include all biochemicals that demonstrated a stabilization effect within the first 2-3 weeks of storage, while the red bars illustrate the improved stability achieved when in dried form, as liquid WB contained ~10% more biochemicals with instability throughout the first 28 days (and showed no signs of stabilization). DBS stored cold or frozen resulted in the best stability, with ~10% more stable biochemicals than when stored at RT (80% vs 70%, respectively). We did note that RT storage affected more Lipids and Xenobiotics relative to other Super Pathways. However, most of the affected Lipids were among those that stabilized within the first 2–3 weeks.

We also considered the stability of DBS cards in comparison to the 'gold standard' condition of plasma: immediate storage (after drying) at -80°C (**Figure 3**). RT storage resulted in 14% of biochemicals showing significant differences overnight (Day 1, RT vs -80°C), while another 7% of biochemicals changed by Day 12. Less than 10% of biochemicals showed significant differences when stored at 4°C or -20°C, compared to -80°C for the same periods of time.

Long-term stability

Long-term storage stability was assessed by analyzing DBS cards stored for 3 months and 7 months. At -20°C, approximately 1.5-fold fewer biochemicals were affected within the seven-month test period compared to RT storage (27% vs 43% of 486 biochemicals analyzed; **Figure 4**). Around 83% and 64% of biochemicals were stable for at least three months (solid and hatched green bars) at -20°C and RT, respectively (<25% change, $p<0.05$). Another ~10–20% (blue bars) exhibited an initial stability effect after collection (at least within the first three months, but likely sooner as demonstrated in the STS evaluation), but then stabilized and remained stable up to seven months. Importantly, within most Super Pathways, greater than 50% of all biochemicals (with many over 70%) were stable or stabilized at 7 months of storage, even at RT. Only Cofactors/Vitamins (RT) and Peptides (-20°C) did not achieve this level of stability. While not formally analyzed, it is expected that the results for storage at 4°C would be intermediate to RT and -20°C, while storage at -80°C would be on par or likely better than

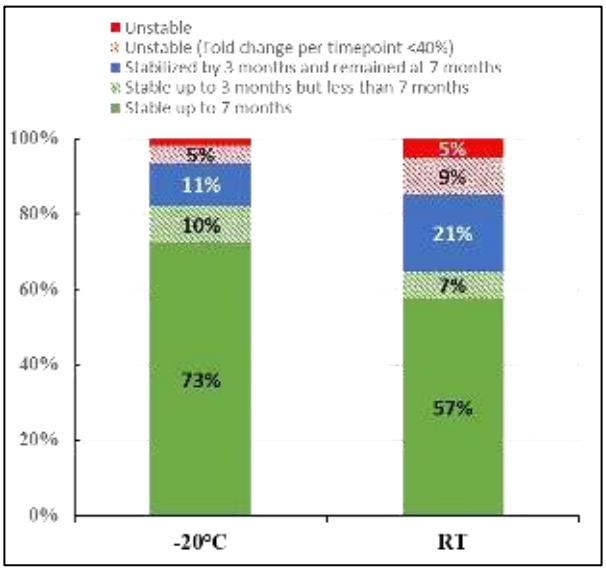

Figure 4 - Proportion of biochemicals (n=486) assigned to each long-term stability category at -20°C and RT (*room temperature*). Solid green indicates stability up to 7 months, hatched green indicates stability for at least 3 months but less than 7 months, blue indicates stabilization occurred between 0 and 3 months and remained stable through 7 months, and red indicates limited or no stability over the 7-month time course.

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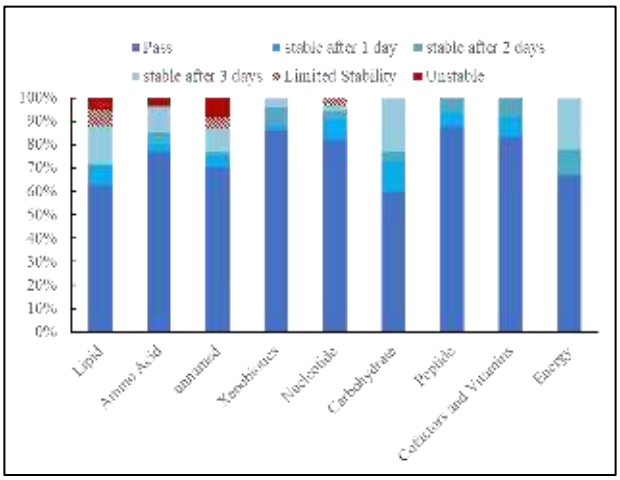

Figure 5 - Relative frequency of stability classes per Super pathway assessing non-ideal shipping conditions (high heat) compared to storage at -80°C. Samples were removed from -80°C and stored for up to 4 days at approximately 35°C to mimic unfavorable shipping conditions (*i.e.*, loss of dry ice during summer months).

storage at -20°C. Additionally, we have noted from internal usage that the stability of DBS cards stored at -80°C can extend up to several years.

Shipping Stability

An experiment was conducted to assess the impact of extreme heat exposure (*i.e.* shipped under non-ideal conditions): DBS cards were stored at approximately 35°C for 8-10 hours per day for 1-4 days prior to analysis. Remarkably, compared to continued storage at -80°C (and presumably shipping on dry ice), 93% (554/599) of biochemicals were either not affected (71%) or showed an initial change in intensity that stopped changing by the fourth day (22%). There were four Super Pathways that showed some increased instability due to these storage conditions: Lipids, amino acids, Unnamed, and Nucleotides. Of the only 7% of all biochemicals that had limited or no signs of stability, they constituted between 3–12% of these four Super Pathways. In other words, ≥88% of biochemicals in each Super Pathways were stable or stabilized within 4 days, as illustrated in **Figure 5**. Of the 175 total biochemicals that were altered with time, the majority exhibited an increase in signal intensity over time, a trend that was consistent across all Super Pathways.

Conclusions

The results presented herein from the STS, LTS, and Shipping experiments provide several guidelines regarding our recommended storage conditions for DBS cards. First, they indicate that after appropriate drying of the blood sample on the card (generally at least 3-4 hours at RT), the colder the storage temperature (down to -80°C), the better the data quality. *It is storage temperature rather than duration that has the largest influence on the frequency and magnitude of stability effects, and on the specific classes of biochemicals affected.* Free fatty acids, phospholipids, and biochemicals that are more prone to oxidation reactions are differentially altered at very low (-80°C) or more elevated temperatures (RT). We expect at least 3 months of stability at -20°C (94% of biochemicals) and RT (85% of biochemicals) when analyzed after the 3-week stabilization period. At colder temperatures, storage up to 7 months can also be a viable option (and may extend beyond this

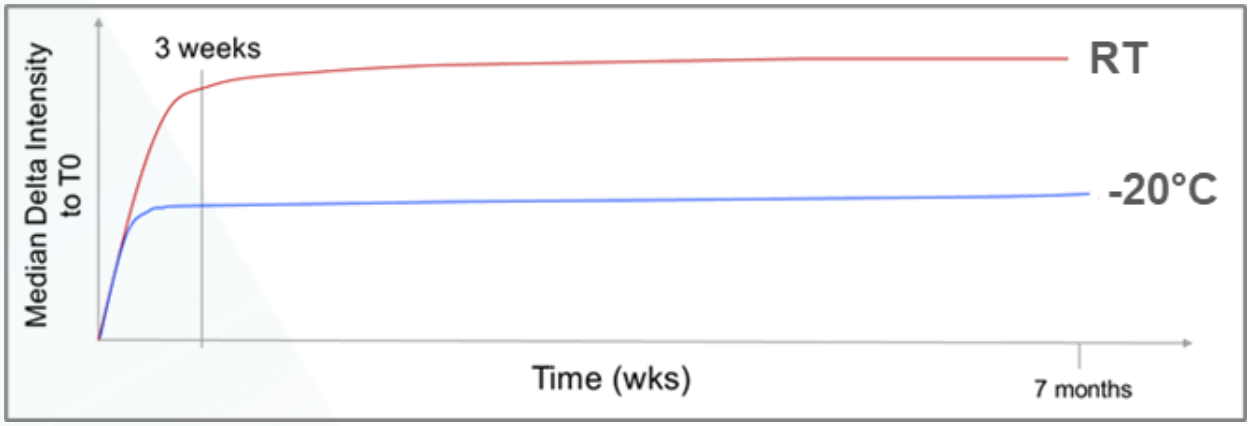

Figure 6 - Graph representing the effect of storage temperature on the magnitude of change and the stabilization effect by Week 3 that occurs for stability-sensitive biochemicals. *This image is a representation of the findings described in the document but is not a direct plotting of the specific data presented.*

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timeframe). Regardless of temperature, storage stability effects occur rapidly after collection and then plateau by week 3–4, resulting in >95% of biochemicals exhibiting stability after this initial "stabilization period". Thus, for prospective studies *it is recommended that samples be analyzed no earlier than this time interval after collection to mitigate potential changes that may occur as a result of differences in storage*. For previously collected samples, the storage time should not present issues. **Figure 6** shows a representation of the combined short term and long term stability studies data (although is not a direct plotting of the data itself) which indicates that while few biochemicals continue to change after 3-4 weeks at any given temperature, *samples stored under different conditions are not recommended to be compared to each other as the magnitude of early changes varies as a function of the storage temperature*. This is consistent with Metabolon's general sample preparation guidelines for all matrices, which emphasize sample collection consistency. If similar storage conditions cannot be guaranteed, increased group sizes would be recommended to help account for any increased sample variability. Regardless of the storage time and temperature, *shipping is recommended to be on dry-ice with desiccant packs*, if possible. The shipping experiment does provide evidence that if these conditions cannot be met, the effects of several days under non-deal conditions (heat and humidity) are unlikely to completely invalidate data from the samples as long as all samples experienced the same conditions. Combined, the results of these experiments indicate that DBS cards are a robust sample type for metabolomics analysis, with a range of storage conditions that allow for detection of a large number of metabolites. *Importantly, the variables tested (time, temperature) indicate that the biochemicals that do show instability are spread across all Super Pathways.* So long as all samples are collected and stored consistently across groups, then storage stability will have minimal impact on the analysis and interpretation of metabolomics data from DBS cards. As with any other matrix, if particular areas of metabolic focus are of interest or critical need, reaching out to Metabolon is advisable to ensure your expectations can be met adequately.

Ultimately the stability profile of DBS cards, as demonstrated in this validation, show that DBS cards are a viable option for patient sampling when either a cold storage pipeline is not feasible, or where the need for blood collection, in the absence of a medical professional, is desired or required. These may include at-home blood collections, remote location or vulnerable population research. *While cold storage conditions (-80°C) are optimal, they are not required for successful metabolomics analysis*.

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