Improved dried blood spot-based metabolomics using a novel microsampling device

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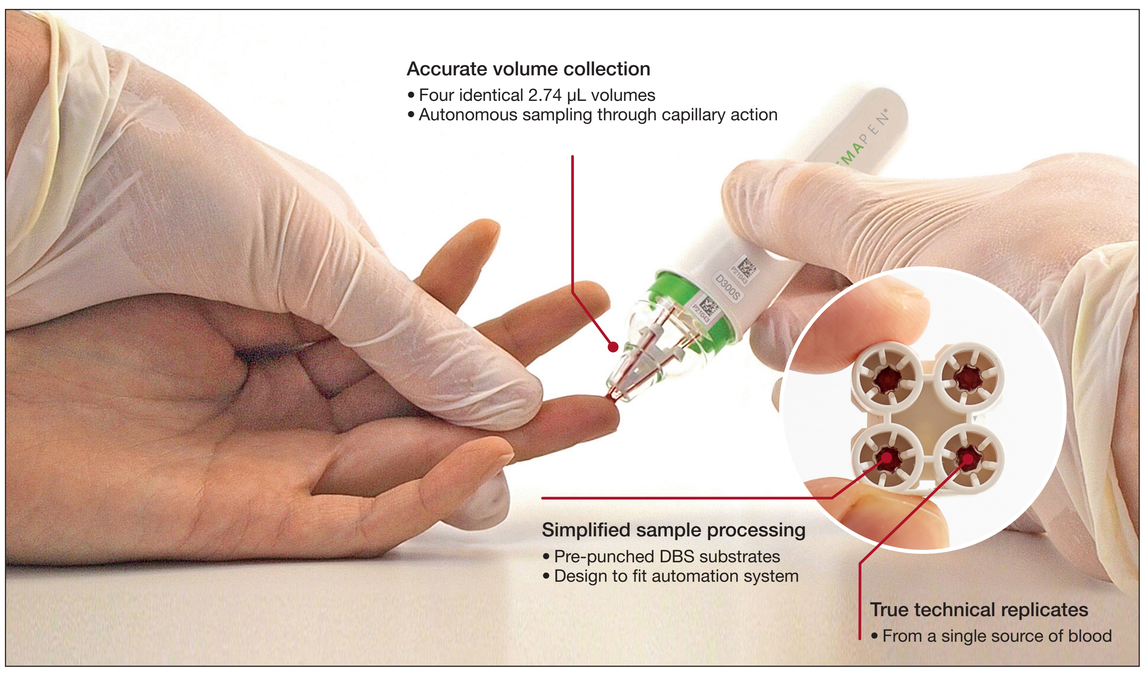
Overview

Dried blood spots (DBS) have been used for screening inborn errors of metabolism in millions of newborns since the 1960s. There is an increasing interest in the use of DBS for a wider range of applications that exploit the benefits of DBS over traditional whole blood, plasma and serum samples such as remote sampling and independence over cold chain logistics. Herein we present three studies associated with a novel blood microsampling device – the hemaPEN® - using a DBS workflow:

- **Study 1 –** Targeted metabolomics analysis of 2.74 µL blood biological and technical replicates.
- **Study 2 –** Stability of metabolites under different storage conditions for DBS replicates.
- **Study 3 –** Fatty acid methyl ester (FAME) stability with and without BHT stored under different conditions, using both Guthrie and DBS from the microsampling device.

The device collects an accurate blood volume controlled using precision bore glass capillaries of a pre-determined volume. It provides 4 x 2.74 µL replicates from the same blood source, which are then transferred to a DBS substrate all encapsulated in a controlled desiccated environment to maintain sample integrity. Hence, the utility of the device is ideal for longitudinal (time-course) metabolomic profiling.



The hemaPEN blood collection and storage device shown here collecting a capillary blood sample:
- The device does not contain a lancet and can be used with any blood source.
- DBS cartridge pre-loaded with paper or polymer substrates.
- Embedded desiccant for rapid drying (90 min) – stable for 7 days at 95% humidity and 35°C.

Methods

Study 1 – Targeted metabolite analysis of 2.74uL biological and technical DBS replicates.

Four separate hemaPEN were used to collect DBS samples from one healthy donor (including 4 x 2.74 µL technical replicates) by fingerstick capillary blood onto pre-punched grade Whatman 903 paper – labelled HP1, HP2, HP3 and HP4. Samples were allowed to dry at room temperature for four hours, and stored in a -80° C freezer until analysis. Sample extraction consisted of a thawing step at room temperature for 30 mins, before 100 µL of chilled methanol (containing internal standards – 13C-valine and 13C-sorbitol) was added to extract polar metabolites in 1.5 mL Eppendorf tubes. Samples were then vortexed for 30 secs, thermomixed at 4°C for 10 mins and centrifuged at 15,000 rpm and 4°C for 5 mins.

Supernatants were collected into an insert and placed in another 1.5 mL Eppendorf tube for drying in a vacuum concentrator at room temperature for about two hours. Once dried, the insert was then transferred to 2 mL GC vials and online derivatized using 20 µL methoxyamine hydrochloride and 20 µL BSTFA + 1% TMCS. A 1 µL split injection was made into a Shimadzu GCMS-TQ8050 (QQQ) utilizing the Shimadzu Smart Metabolite Database (containing 475 MRM transitions), using a 30 m x 0.25 mm x 1 µm 5% phenyl polysilphenylenesiloxane column and providing a total analysis time of 37 mins. Data was processed and exported into Metaboanalyst (www.metaboanalyst.ca) as a matrix using Shimadzu's Insight software (log transformed; median normalized).

Study 2 – Stability of metabolites under different storage conditions for DBS replicates.

Dried blood spot samples (4 x 2.74 µL technical replicates) were collected from one healthy donor by fingerstick capillary blood and allowed to dry at room temperature for four hours and stored under four different conditions: i) -20°C, ii) 4 °C, iii) room temperature, iv) room temperature with prior addition of butylated hydroxytoluene (BHT) to the DBS before blood collection. Four technical replicates were utilized for each storage condition, apart from the BHT group which utilized three technical replicates. Samples were thawed at room temperature for 30 mins, before 100 µL of chilled methanol (containing internal standards – 13C-valine and 13C-sorbitol) was added to extract polar metabolites in 1.5 mL Eppendorf tubes. Samples were then vortexed for 30 secs, thermomixed at 4°C for 10 mins and finally centrifuged at 15,000 rpm and 4°C for 5 mins. Supernatants were collected into an insert and placed in another 1.5 mL Eppendorf tube for drying in a vacuum concentrator at room temperature for about two hours. Once dried, the insert was then transferred to 2 mL GC vials and online derivatized using 20 µL methoxyamine hydrochloride and 20 µL BSTFA + 1% TMCS. A 1 µL split injection was made into a Shimadzu GCMS-TQ8050 (QQQ).

Study 3 – Fatty acid methyl ester (FAME) stability with/without BHT, different storage conditions using hemaPEN DBS.

The hemaPEN device, with and without the addition of antioxidant BHT on the pre-punched DBS substrates, was used to collect fingerstick capillary blood from one healthy donor and allowed to dry at room temperature for four hours. hemaPEN were stored under three different storage conditions:
i) room temperature, ii) 4°C, iii) -20°C over 4 weeks. After storage of each condition, DBS samples were added to 100 µL of 1% (v/v) H_2SO_4 in anhydrous methanol. Transmethylation reaction was performed at 70°C for 3 h. FAMEs were extracted into 100 µL of n-heptane and analysed by GC-MS/MS (Thermo 1310 – TSQ 8000 Evo). DBS samples (3.5 mm sub-punch) from Guthrie card (time 0) was also transmethylated as described above and used as the baseline for all DBS workflows.

Results

Study 1

Overall, 203 metabolites were detected from one 3.5 mm spot consisting of 2.74 µL of dried blood from the hemaPEN device. No significantly different (P-value < 0.05, fold change > 2) metabolites were found between the biological replicates HP1, HP2, HP3 and HP4 – nor between the four technical replicates generated from each hemaPEN sample (i.e. R1, R2, R3 and R4). This demonstrates the ability of the device to accurately collect reproducible dried blood spots – and is shown by the overlapping groups in the PCA (Figure 1) and dendrogram (Figure 2).

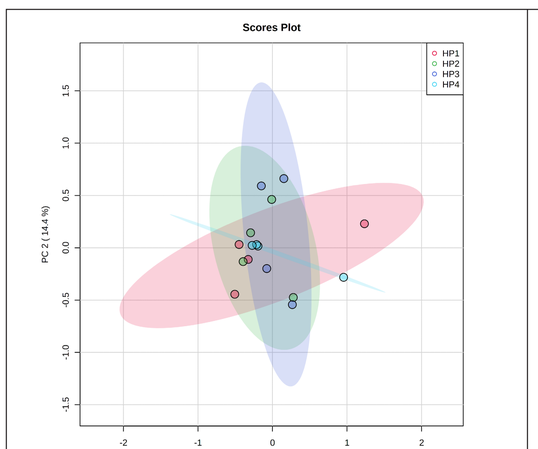


Figure 1. PCA of overlapping hemaPEN groups.

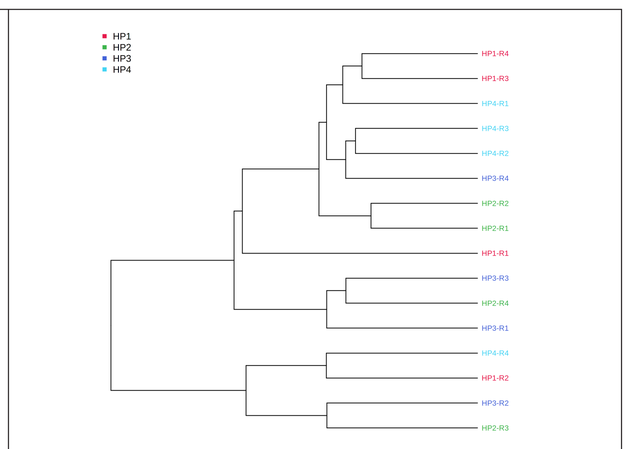


Figure 2. Dendrogram of hemaPEN grouping.

The biochemical pathway enrichment below (Figure 3) describes the top 50 pathways in which the 203 metabolites are found, as well as their coverage in KEGG pathways (Figure 4) (red spot = detected metabolite).

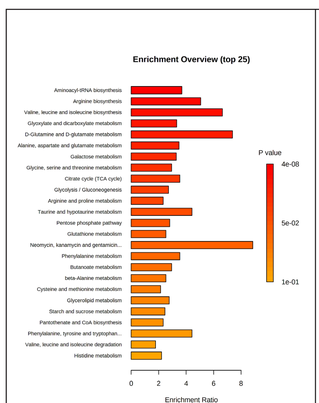


Figure 3. Top 50 enrichment pathways.

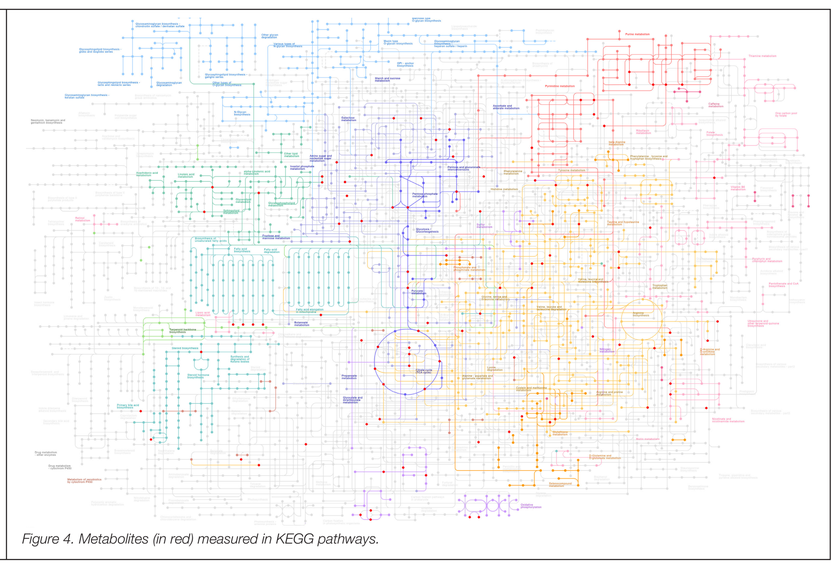


Figure 4. Metabolites (in red) measured in KEGG pathways.

Study 2

The results obtained indicated that most polar metabolites were preserved in fridge/freezer temperatures - apart for fatty acids which were preserved better at room temperatures (these results correlate well with results in Study 3). Unexpectedly, an increase in the recovery of amino acids at room temperature was also observed – this may be due to proteolysis occurring from protease action. Future experiments will focus on preventing proteolysis by quenching metabolism at the sampling step by the addition of methanol/acetone.

Overall, 56 out of 186 metabolites detected were significantly affected (p-value < 0.05, fold change > 2) by the different storage conditions i.e. freezer temperature ('-20°C' group), fridge temperature ('2_8°C' group), room temperature ('RT' group) and room temperature and BHT ('BHT' group). The heatmap below best demonstrates the preservation of most metabolites in cold storage – except for unexpected changes observed in the amino acids (Figure 5); most likely due to proteolysis.

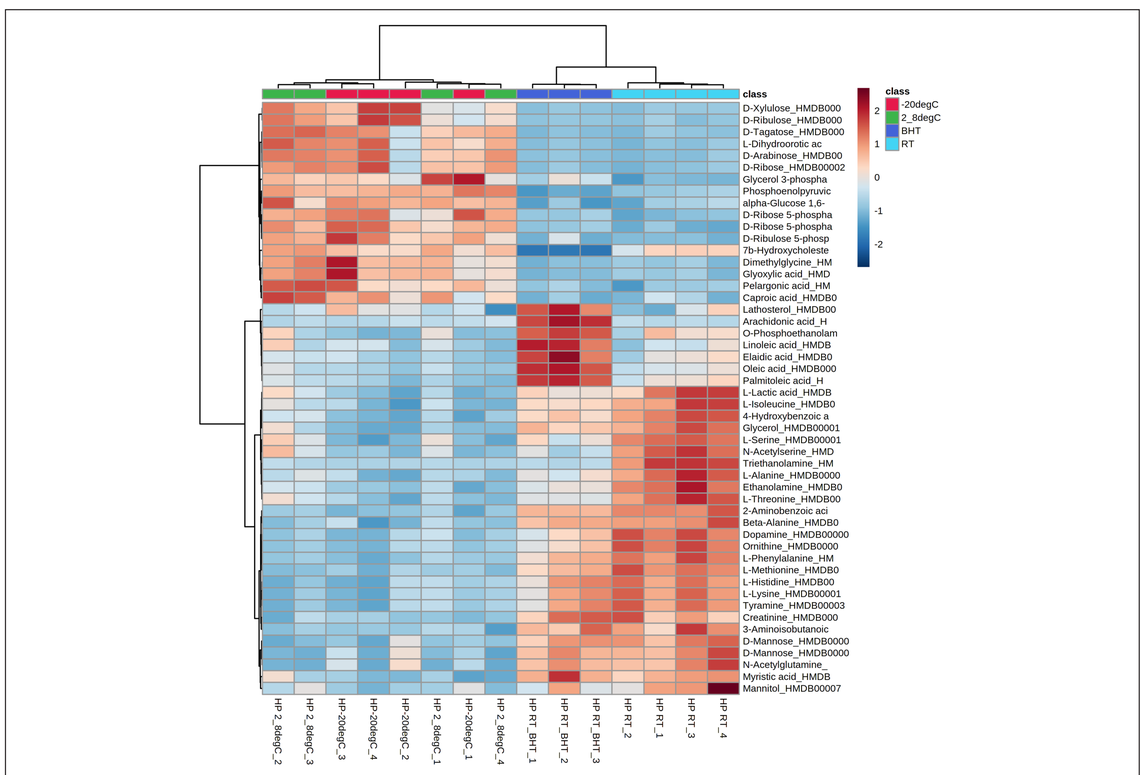


Figure 5. Heatmap of significantly altered metabolites according to storage condition

Study 3

Results of study 3 indicate that BHT can provide substantial protection against fatty acids degradation in DBS samples stored under different conditions. Without BHT on the DBS substrates, estimated omega-3 index percentages decreased by 33% after 1 week, 44% after 2 weeks and 53% after 4 weeks stored in room temperature. The AA:EPA, omega-6 : omega-3 and estimated omega-3 index for DBS samples coated with BHT in hemaPEN are within 20% variation after stored in R.T., 4°C and -20°C for four weeks.

Figure 6. Relative percentage (% of total fatty acids) of different fatty acid groups in BHT coated and uncoated hemaPEN during storage over 4 weeks.
**Asterisk indicates %Total Fatty Acid or Index vary more than 20% compared to baseline (day 0).*

Conclusion

DBS microsampling is well suited to metabolomics studies and here we have demonstrated the advantages of a novel collection device, the hemaPEN, in controlling the quality of the sample collected. Despite the well recognized advantages of DBS sampling we have demonstrated that depending on the target metabolite cohort the sample storage and pre-treatment must be taken into account to minimize artefacts such as proteolysis and oxidation of sensitive analytes, highlighting the importance of storage studies.

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