A Wellness Study to Investigate the Effects of Diet and Exercise on the Metabolome

Targeted Metabolomics using the M3 MicroLC and QTRAP® 6500+ System

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Identification of metabolites from urine and plasma is necessary for investigation of the affected metabolic pathways in dietary assessment studies[1], precision medicine and research on validating potential disease biomarkers[2] and large population-based research to evaluate the effects of nutritional, pharmaceutical, and environmental exposures[3]. Self-testing is on the rise as more people become interested in monitoring their health. This is evident from the rise of wearable technology and related applications (apps) to advance this process into more simplified and meaningful data to the everyday consumer.

Liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis has proven to be an essential tool for identification and quantitation of metabolites in complex sample matrices due to its inherent sensitivity gains[4,5]. Here dried blood spots samples (DBS) have been analyzed using a previously described sample extraction method[6] for a small wellness study using a previously described microflow targeted metabolomics method which monitors over 300 polar metabolites covering all major metabolic pathways[7].

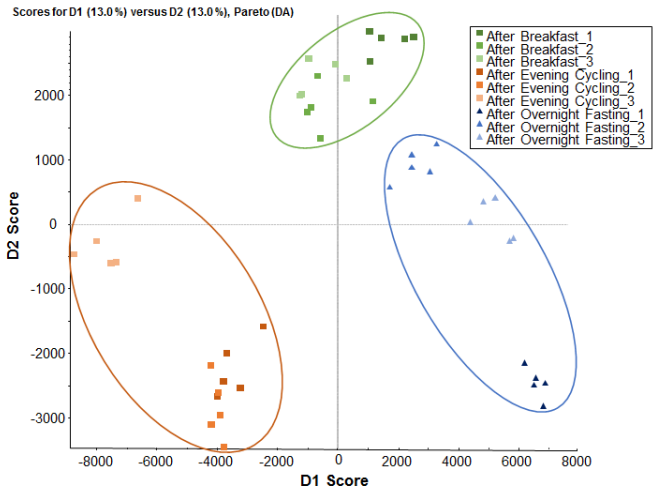

Figure 1: Principal Component Analysis (PCA-DA) of Targeted Metabolomics Results from a Wellness Study. Polar metabolites from DBS extracts from three different sample groups (after overnight fasting, after breakfast and after evening cycling) were analyzed. Three sample replicates per wellness group and the accompanying 5 LC-MS replicates cluster together. Each of the three different metabolic conditions studied are differentiated based on their polar metabolite profile.

This study design was chosen for its simplicity in highlighting the strength of metabolomics as a tool to differentiate three different metabolic states; namely at fasting, fed and active. By leveraging the improved sensitivity provided by microflow LC, the three different conditions monitored during this wellness study could be clearly differentiated from the metabolomic profiles. The method also demonstrates that sample provided in a droplet of blood on dried blood spot cards is sufficient for metabolomics analyses.

Key Feature of Microflow LC for Metabolomics

- This microflow LC method provides improved sensitivity with S/N improvement of up to 60X with to up to 50% higher coverage of the metabolome than traditional analytical approaches[7]
- This method is a single LC-MS/MS targeted method allowing detection of 312 polar metabolites across multiple biochemical pathways enabling classifications of these sample groups
- Microflow Luna-NH2 HILIC chromatography provides excellent chromatographic separation of polar, hydrophilic metabolites
- M3 MicroLC reduces solvent consumption and costs
- The sensitivity and speed of the QTRAP® 6500+ system with IonDrive™ Technology allows an efficient high throughput assay by using +/- polarity switching (5 msec) in a single sample injection

Methods

Sample Preparation: A 6-mm dry blood spot (DBS) disk collected on Whatman 903TM filter paper cards was used. DBS were collected for all three metabolic states in triplicate. Each DBS card was punched using a GE Healthcare Uni-Core punch with ID 6 mm (or 2X3 mm) ID. Each disk was transferred to 2 mL Eppendorf tubes. 150 µL of pre-cold extraction solvent (3:3:2) isopropanol/acetonitrile/water) was added to extract metabolites. 5 µL of internal standard solution (2 µg/mL heavy labeled leucine and alanine) was added. The samples were vortexed briefly; sonicated for 5 minutes, and allowed to stand at room temperature for 30 min. Samples were centrifuged at 14,000 RPM for five minutes. 100 µL of the supernatant were collected and dried using a TurboVap evaporator to a pellet using no heat with 10 psi N2 gas flow. The fresh pellet was dissolved in 100 µL of HILIC (hydrophilic interaction liquid chromatography) sample resuspension buffer, mixed well by vortexing and centrifuged at 14,000 RPM for 10 min. 90 µL of supernatant was transferred to deactivated QsertVials (Waters) for microflow targeted metabolomics analysis following our previously described method[7]. Injection volume was 5 µL with 5 replicate injections.

LC-MRM Analysis: Samples were analyzed using a M3 MicroLC system coupled with a QTRAP® 6500+ System. LC and MS conditions have been previously described here[7].

Data Processing: Data was analyzed using MarkerView™ Software.

Results

A total of 312 polar metabolites were monitored using this microflow LC-MS assay for this wellness study. Screening these polar metabolites across the three metabolic states (after overnight fasting, breakfast and cycling) identifies several metabolites that change during these conditions. The PCA of the three sample groups clearly shows that they are differentiated based on their metabolic profile (Figure 1). The five LC-MRM replicates of each sample cluster together well, highlighting the reproducibility of the targeted assay. The loadings plot highlights the metabolites responsible for the differentiation of these wellness groups (Figure 2).

Examples of metabolites which are altered during these different metabolic conditions are highlighted in Figure 3. Acetylcarnitine and choline are seen in the top left corner of the loadings plot and are observed to increase after exercise.

In contrast, on the bottom right corner of the loadings plot aspartic acid and betaine are observed, showing the opposite effect of decreasing after exercise (Figure 3, right).

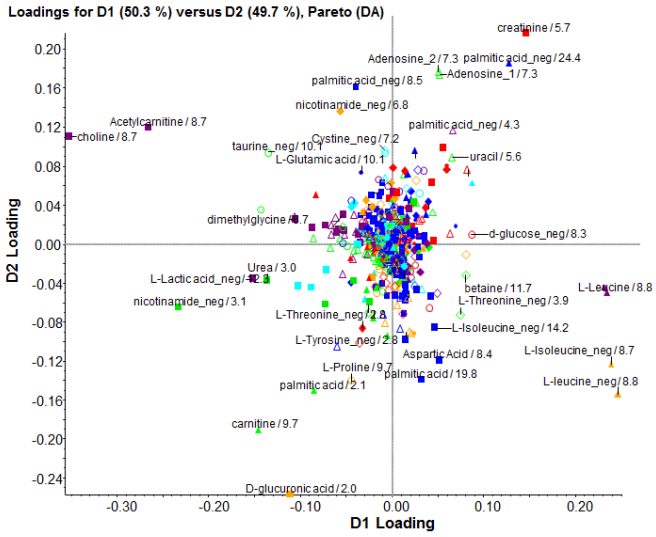

Figure 2: Metabolites Responsible for Differentiation between the Metabolic Conditions. The loadings plot highlights the metabolites responsible for the differentiation of the three different sample groups (after overnight fasting, after breakfast and after evening cycling).

It is known that during exercise protein degradation occurs, releasing amino acids into the body[8]. Of these, tyrosine is released from human skeletal muscle in the presence of insulin hence the spike in tyrosine concentrations in the samples after the cycling activity was completed (Figure 4). Three other metabolites were observed to show a similar pattern of change to tyrosine.

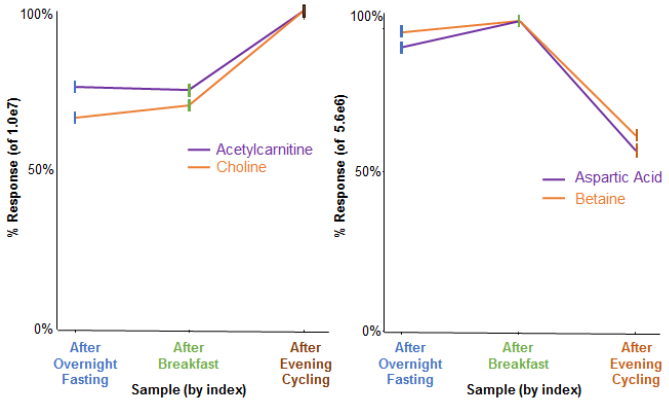

Figure 3. Profile Plots for Selected Metabolite Changes from MarkerView™ Software. These plots highlight the fluctuation of certain metabolites under the three different metabolic conditions. Increases were observed in acetylcarnitine and choline after the exercise activity while a decrease in aspartic acid and betaine was observed after exercise.

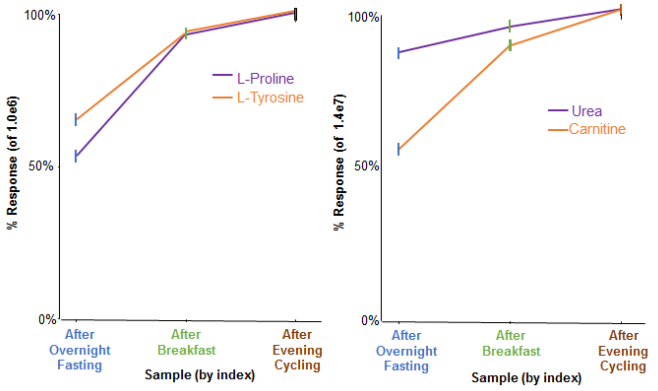

Figure 4. Increase in Plasma Levels of Amino Acids after Eating and Exercise. Two amino acids were also observed to have yet another different pattern, showing fairly consistent levels during daily activity but being reduced after overnight fasting. Urea and carnitine follow a similar pattern.

Conclusions

DBS is a minimally invasive way of collecting blood samples with minimal cost and training, and without the requirement for refrigeration or freezing during transport. Sample collection using dried blood spots is used for screening newborns for inborn errors of metabolism for many years now. Metabolite extraction procedure from DBS samples is easy and fast and does not require protein precipitation as compared to traditional blood sampling as proteins stay on these protein cards.

The microflow targeted metabolomics analysis from DBS samples is a rapid, sensitive, and accurate method for profiling polar metabolites. This workflow demonstrates the possibility of utilizing this targeted metabolomics method for the monitoring these targeted polar metabolites in other wellness-based studies.

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Document number: RUO-MKT-02-7164-A

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