# A Wellness Study Using Microflow Targeted Metabolomics to Investigate the Effects of Diet and Exercise on the Metabolome

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## INTRODUCTION

Identification of metabolites from plasma and urine 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 and more people become interested in monitoring their health. This is evident from the rise of wearable technology and related applications (apps) available today 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].

Here dried blood spots samples (DBS) have been analyzed using a previously described sample extraction method[5] for a small wellness study using a previously described microflow targeted metabolomics workflow shown in Figure1 which monitors over 300 polar metabolites covering all major metabolic pathways[6]. 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.

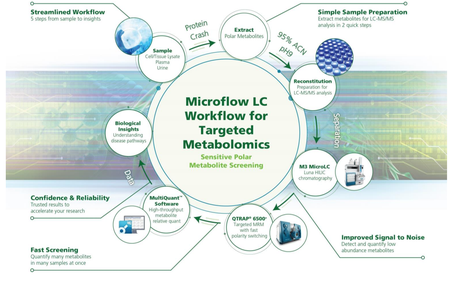


**Figure 1.** Microflow LC Workflow for Targeted Metabolomics.

## MATERIALS AND 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[6]. Injection volume was 5 µL with 5 replicate injections.

**Microflow Liquid Chromatography:** A SCIEX M3 MicroLC system, with an integrated autosampler, was used in direct injection mode, in combination with a source mounted column oven (SCIEX). A Luna 3 µm NH2 100 Å, 150 x 0.3 mm analytical column (Phenomenex) was used with a micro filter 1 µm SS (Upchurch Scientific) before the column to prolong column life time. The column temperature was set to 40° C. Injection volume was 5 µL, and the autosampler needle and valve wash consisted of 1 cycle using mobile phase A, followed by two cycles using mobile phase B.

Mobile phase A=95% water, 5% acetonitrile, 20mM ammonium acetate and 20mM ammonium hydroxide, pH=9, Mobile phase B = 95% acetonitrile and 5% mobile phase A and 20 mM ammonium hydroxide, were used for both analytical flow and microflow method. Flow rate of 10 µL/min was used. The gradient method used for both analytical flow and microflow method is listed in Table 1A.

**Table 1 A-B. LC-MS Method Parameters.** (Left) Table A shows the HILIC LC gradient and (Right) Table B lists the source and MS parameters used for Microflow Method .

**(A)**

| Time | %B |
|---|---|
| 0 | 100 |
| 2 | 100 |
| 5 | 85 |
| 15 | 30 |
| 18 | 2 |
| 20 | 2 |
| 22 | 100 |
| 30 | 100 |

**(B)**

| | Microflow LC | |
|---|---|---|
| MS Parameters | (+) | (-) |
| Electrode ID | 25 µm | 25 µm |
| Curtain Gas | 30 | 30 |
| Collision GAS | High | High |
| IonSpray Voltage | 5000 | -4500 |
| Temperature | 300 | 300 |
| Ion Source Gas 1 | 30 | 30 |
| Ion Source Gas 2 | 35 | 35 |
| DP | 93 | -93 |
| EP | 10 | -10 |
| CXP | 10 | -10 |

**Mass Spectrometry:** A SCIEX QTRAP® 6500+ system with IonDrive™ Turbo V source was used with a 25 µm ID electrode (SCIEX). MS source parameters were optimized including CE, EP, DP and CXP. The source, gas and MS parameters are listed in Table 1B. A total of 187 positive ion mode MRM's and 176 negative ion mode MRM's for a total 312 unique polar metabolites were combined into a single +/- switching experiment (363 total MRM's) with 3 msec dwell time and 50 ms settling time for polarity switching to monitor these endogenous metabolites across different metabolic pathways.

**Data Processing:** MultiQuant™ 3.0.2 Software (SCIEX) was used for data analysis with MQ4 peak integration algorithm, gaussian smooth width of 1.0 points, RT half window of 30 sec and min peak width of 8 points. Integrated peaks with minimum signal to S/N of 10 or more in all replicates were selected and manually validated. Five replicate LC-MS/MS injections were acquired for the analysis. MarkerView™ Software 1.3 was used for differential data analysis using Principal Component Analysis (PCA).

## RESULTS

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.

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).

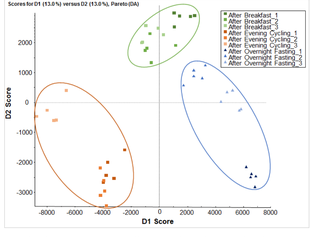


**Figure 1: Principal Component Analysis (PCA-DA) of Targeted Metabolomics Results from a Wellness Study.** Polar metabolites from DBS extracts from three different sample groups (after overnight fasting, after breakfast and after evening cycling) were analyzed. Three sample replicates per wellness group 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.

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).

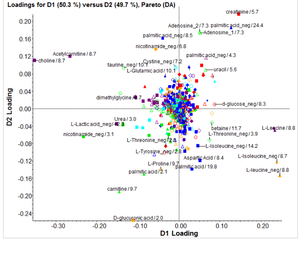


**Figure 2: Metabolites Responsible for Differentiation between the Metabolic Conditions.** The loadings plot highlights the metabolites responsible for the differentiation of the three different sample groups (after overnight fasting, after breakfast and after evening cycling).

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).

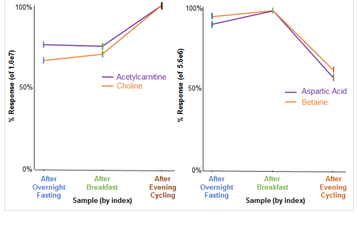


**Figure 3. Profile Plots for Selected Metabolite Changes from MarkerView™ Software.** These plots highlight the fluctuation of certain metabolites under the three different metabolic conditions. Increases were observed in acetylcarnitine and choline after the exercise activity while a decrease in aspartic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

It is known that during exercise protein degradation occurs, releasing amino acids into the body[7]. 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.

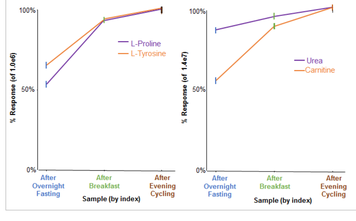


**Figure 4. Increase in Plasma Levels of Amino Acids after Eating and Exercise.** Two amino acids were also observed to have yet another different pattern, showing fairly consistent levels during daily activity but being reduced after overnight fasting. Urea and carnitine follow a similar pattern.

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.

## CONCLUSIONS

- 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
- 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[6]
- 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

## REFERENCES

1. O'Gorman A, Gibbons H, and Brennan L. Metabolomics in the identification of biomarkers of dietary intake. Comput Struct Biotechnol J. (2013) 4: e201301004.
2. Beger RD, et al. Metabolomics enables precision medicine: "A White Paper, Community Perspective". Metabolomics. (2016) 12(9), 149.
3. Su LJ, et al. The Use of Metabolomics in Population-Based Research. Adv Nutr. (2014) Nov 5(6): 785–788.
4. Ivanisevic J, et al. Toward 'Omic Scale Metabolite Profiling: A Dual Separation−Mass Spectrometry Approach for Coverage of Lipid and Central Carbon Metabolism. Anal Chem. (2013) Jul 16,85(14) 6876-84.
5. Drolet J et al. Integrated Metabolomics Assessment of Human Dried Blood Spots and Urine Strips. Metabolites. (2017) Jul 15;7(3).
6. Motamedchaboki K and Ubhi BK. Microflow Chromatography Provides Improved Sensitivity and Coverage of Polar Metabolites for Targeted Metabolomics. SCIEX Technical Note RUO-MKT-02-6110-A.
7. Poortmans JR, Carpentier A, Pereira-Lancha LO, Lancha, Jr A. Protein turnover, amino acid requirements and recommendations for athletes and active populations. (2012) Brazilain Journal of Medical and Biological Research. Sep; 45(10): 875–890.

## TRADEMARKS/LICENSING