Microflow chromatography provides improved sensitivity and coverage of polar metabolites for targeted metabolomics

Using M3 MicroLC System with the QTRAP® 6500+ System

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Metabolomics analysis in urine or plasma allows discovery of novel therapeutics, screening for drug toxicity and efficacy, and monitoring diet and environmental exposure effects on health. LC-MS/MS analysis has become an essential tool for identification and quantitation of metabolites in complex sample matrices due to its inherent sensitivity gains.[1,2,3] Many of the metabolites of interest in the study of metabolomics are extremely polar and therefore often unable to be analyzed through traditional coupling of reversed phase (RP) chromatography and mass spectrometry. Also to detect and quantify key metabolites from pathways of biochemical importance samples must be run on both reversed phase and normal phase, in negative and positive ion modes requiring a total of four injections.

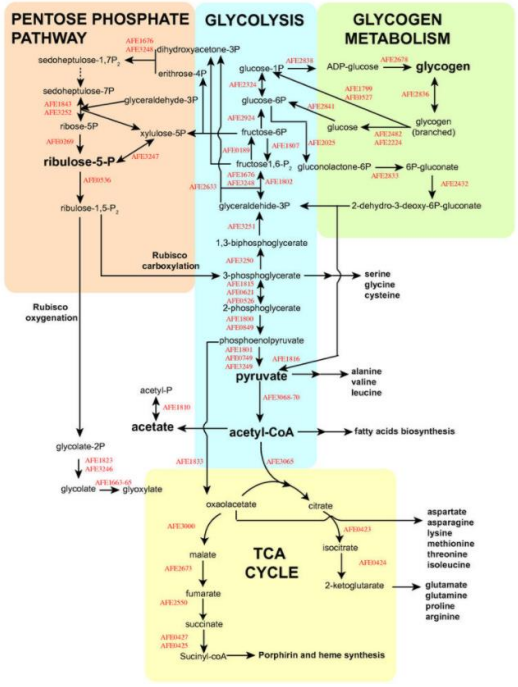

Figure 1. Central carbon metabolism. Targeted LC-MRM assay was developed to cover 312 metabolites across these key metabolic pathways.

Here, a robust and sensitive workflow using microflow LC coupled to a QTRAP 6500+ System for screening of over 300 polar metabolites (Figure 1) in biological samples is described. Using HILIC chromatography and polarity switching, a single injection workflow was developed and both analytical and microflow regimes were compared to investigate the impact of flow rate on metabolite detection.

Key features of microflow LC for targeted metabolomics

- A single LC-MS/MS targeted method allowing detection of 312 polar metabolites across multiple biochemical pathways
- 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
- M3 MicroLC system enables robust microflow chromatography, with reduced solvent consumption
  - Microflow Luna-NH2 HILIC chromatography provides excellent chromatographic separation of polar, hydrophilic metabolites.
- Improved sensitivity with S/N improvement of up to 60X with microflow LC
- Up to 50% higher coverage of the metabolome than traditional analytical approaches

Methods

Sample preparation: Urine was diluted in water (1:4) and 100 µL of diluted urine or 100 µL of control plasma for amino acid analysis (SCIEX) was transferred to a clear maximum recovery 1.5 mL microtube (Axygen). 20 µL of internal standards were spiked in the tube and 800 µL of cooled acetonitrile:acetone:methanol (8:1:1) was added to extract metabolites and precipitate the proteins. Samples were vortexed and incubated at 4 ºC for 30 min then centrifuged for 10 min. An 800 µL aliquot of the supernatant was transferred to a new 2 mL microtube then dried to a pellet.

The fresh pellet was dissolved in 100 µL of HILIC sample resuspension buffer (95% acetonitrile and 5 % mobile phase A), mixed well by vortexing and centrifuged for 10 min. Injection volume was 2 µL with 5 replicate injections. Samples were also diluted further (1:3) to check the method sensitivity.

Madin-Darby Canine Kidney Epithelial Cells (MDCK Line) were extracted in 70% methanol and dried to a pellet, dissolved in HILIC sample resuspension buffer. The solubility of the polar metabolites was evaluated in the HILIC sample resuspension buffer containing 100%, 85% and 75% mobile phase B (Figure 3).

Analytical flow chromatography: A SCIEX ExionLC™ AD HPLC system was used for the analytical flow part of the analysis. The columns used were a Luna 3 µm NH2 100 Å, 150 x 4.6 mm column (Phenomenex). Flow rate of 350 µL/min, wash solvent for the autosampler was 20/20/60 methanol/acetonitrile/isopropanol. Injection volume was 2 µL, and the column was kept at 40°C. The gradient used is described in Table 1.

Microflow flow chromatography: A SCIEX M3 MicroLC system was used in direct injection mode. 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. Flow rate of 10 µL/min was used. The column temperature was set to 40°C. Injection volume was 2 µL, and the autosampler needle and valve wash consisted of 1 cycle using mobile phase A, followed by two cycles using mobile phase B. The gradient used is described in Table 1.

Mass spectrometry: A SCIEX QTRAP 6500+ System with IonDrive Turbo V Source was used. For the microflow LC experiments, the standard 100 µm electrode was replaced with a 25 µm ID electrode (SCIEX). MS source parameters were optimized for analytical and microflow (Table 2). 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

Table 1. HILIC gradient used for analytical and microflow method.

Time (min)	%A	%B
0	*0*	*100*
2	*0*	*100*
5	*15*	*85*
15	*70*	*30*
18	*98*	*2*
20	*98*	*2*
22	*0*	*100*
30	*0*	*100*

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

+/- switching experiment (363 total MRM transitions) with 3 msec dwell time and 50 msec settling time for polarity switching to monitor these endogenous metabolites across different metabolic pathways.

Data processing: MultiQuant™ Software 3.0.2 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. Samples for both microflow and traditional flow LC-MS/MS analysis were prepared on the same day to exclude variations in response due to sample preparation. Five replicate LC-MS/MS injections were acquired for both analytical flow and microflow LC analysis.

Table 2. Source and MS parameters.

	Analytical flow LC		Microflow LC	
MS parameters	**(+)**	**(-)**	**(+)**	**(-)**
Electrode ID	100 µm	100 µm	25 µm	25 µm
Curtain Gas	30	30	30	30
IonSpray Voltage	5500	-4500	5000	-4500
Temperature	500	500	300	300
Ion Source Gas 1	35	35	30	30
Ion Source Gas 2	45	45	35	35

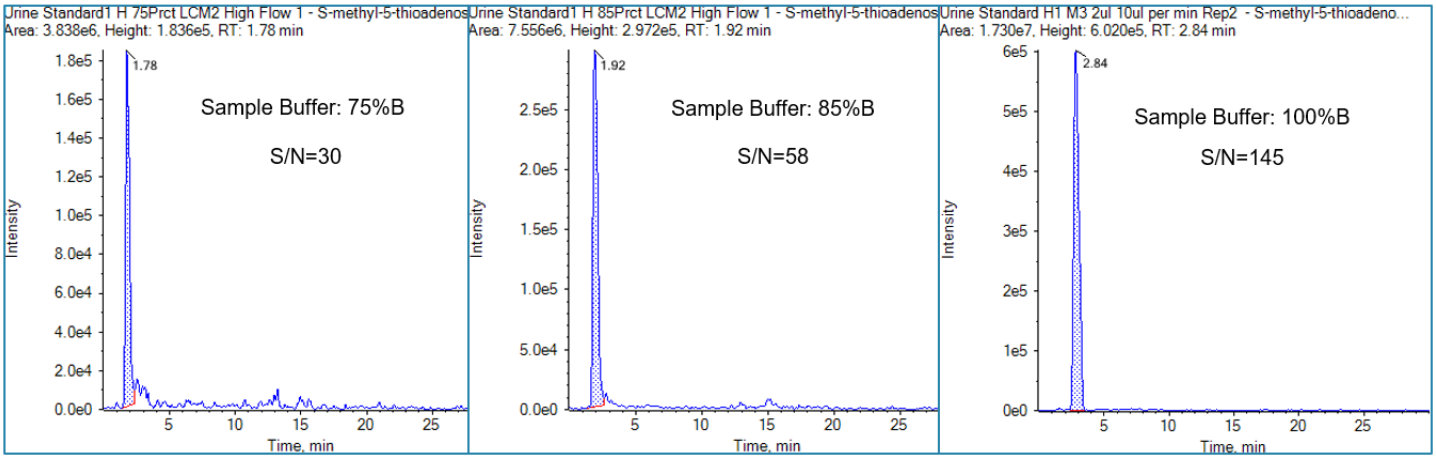

Figure 2. Polar metabolite solubility optimization. S/N ratio improves with increasing organic content (100% B) for metabolite extract reconstitution, as compared to 75 and 85% Mobile Phase B, which demonstrate optimized solubility and HILIC affinity of polar metabolite in 100% B.

Sample reconstitution

When using the typical aqueous sample solvent for sample reconstitution, it is difficult to inject larger volumes of samples without sacrificing HILIC chromatographic resolution. Here, by simply reconstituting the sample in organic solvent (100% mobile phase B), injection volumes up to 5 µL were possible on the microflow LC column, while maintaining good peak shape.

MultiQuant Software integrated peaks for the urine metabolites were compared where the metabolite pellet was reconstituted either in 75, 85 or 100% mobile phase B. The metabolite pellet dissolved in 100% B (the LC method starting buffer) shows improved sensitivity and S/N ratio for targeted polar metabolites (Figure 2).

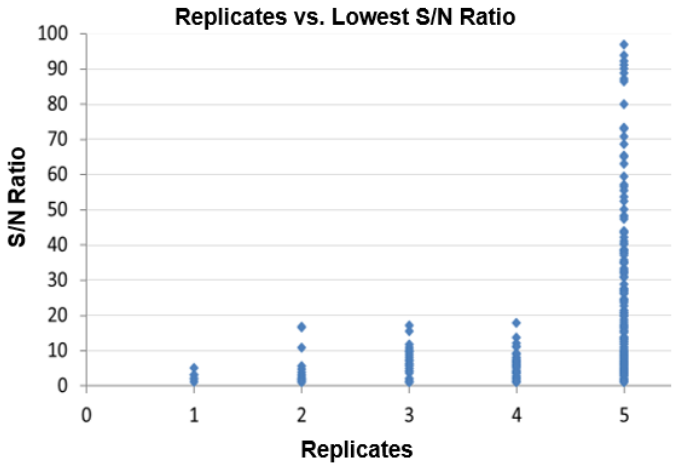

Figure 3. Minimum S/N ratio across replicate analysis.

Improved signal / noise

The S/N ratio for all detected metabolites using this MRM method with both analytical flow LC and microflow LC were compared. For each detected metabolite in urine, the lowest observed S/N (calculated by MultiQuant Software) was plotted versus the number of replicates that the metabolite was detected in (Figure 3). 197 metabolites that were detected with S/N ≥20 are seen in all 5 replicates, and therefore considered to be detectable with high confidence without requiring further manual validation. 22 of the metabolites detected with a lowest S/N of 10-20, were manually validated. All of these were detected in all 5 replicates with a manually determined S/N of at least 5.

The microflow HILIC LC-MRM method improved the S/N ratio by up to 60X with an average improvement of 10X (Figure 4). This improved sensitivity resulted in higher number of metabolite detection with a 50% improvement in MDCK cell line, 35% in urine and 11% in plasma when compared to analytical HILIC LC-MRM (Table 3).

Table 3. Increased metabolite identification by microflow LC. Total number of unique metabolites and transitions identified with minimum S/N of ≥10 in three types of samples were compared here, showing up to 50% more metabolite identification with microflow than analytical flow approach.

Samples	Analytical flow LC	Microflow LC	% gain in detected metabolites with microflow
MDCK Cell Lines	*87 (97)*	*131 (141)*	*50*
Urine	*162 (182)*	*219 (242)*	*35*
Plasma	*174 (196)*	*194 (218)*	*11*

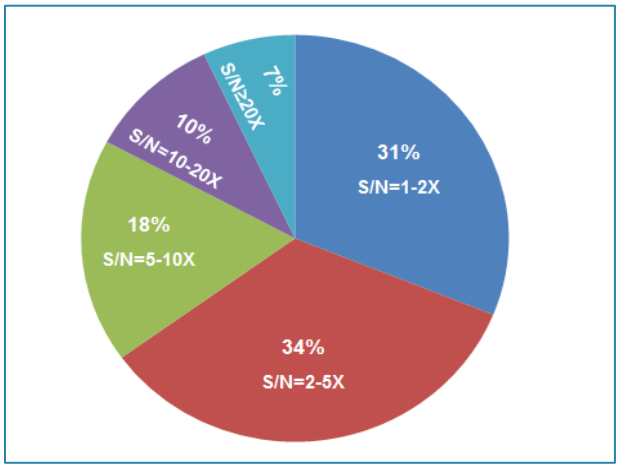

Figure 4. Microflow LC S/N ratio improvement. 35% of detected metabolites from urine showed S/N ratio improvement of 5-≥20 fold resulting in identification of these metabolites with higher confidence using microflow LC.

Improved detection of metabolites

Using this 30 min microflow HILIC LC-MRM workflow, a large # of metabolites were detected at a S/N ratio ≥10 in the 3 samples tested (Table 4). Figures 5 and 6 clearly show improved S/N ratio for select metabolites when using the microflow LC-MRM vs. analytical LC-MRM. The improved S/N ratio provided by microflow HILIC method provides solid detection of large numbers of metabolites and up to 50% improvement in detection when compared to data obtained with the analytical flow HILIC method.

Often metabolite extracts must be run on reversed phase and normal phase and both in negative and positive ion modes for detection of such large number of polar metabolites. Here, a single injection workflow with polarity switching detects high numbers of metabolites in biological samples.

Table 4. % of targeted metabolites with S/N ≥ 10. 312 unique metabolites in three types of samples were targeted in this method and percentage metabolites detected with minimum S/N of ≥10, shows improved coverage with microflow approach.

Samples	Analytical flow LC	Microflow LC
MDCK Cell Lines	*28%*	*42%*
Urine	*50%*	*70%*
Plasma	*56%*	*62%*

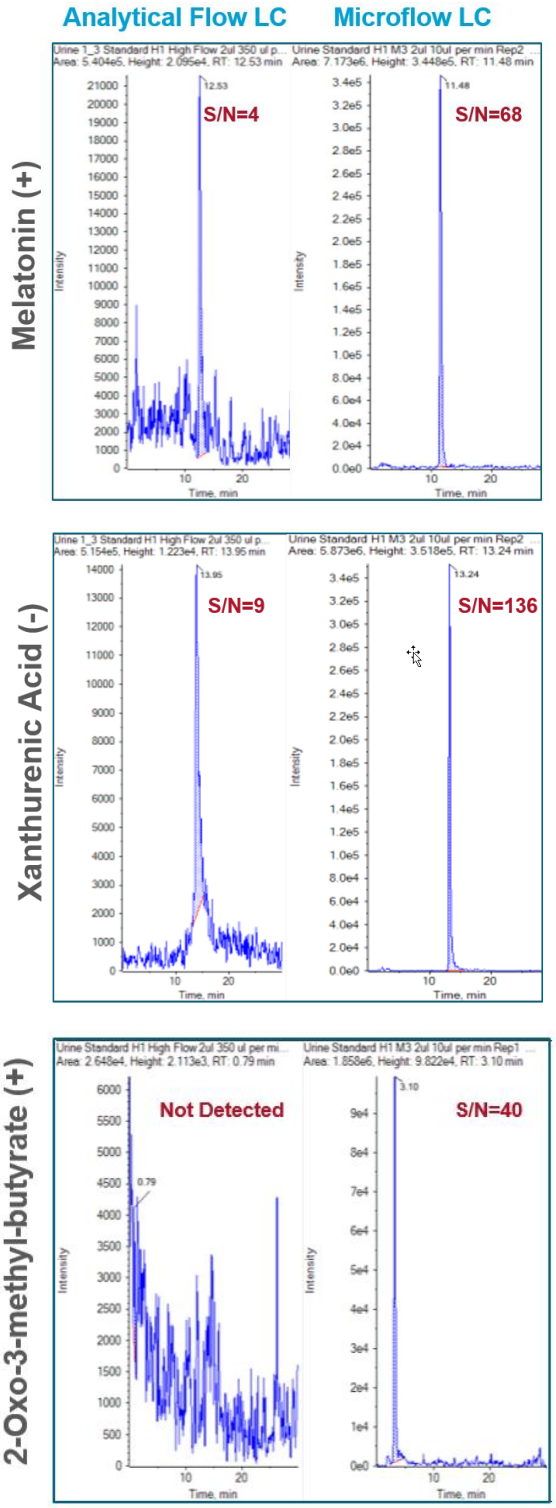

Figure 5. Improved S/N ratio improvement with microflow LC. Integrated peak for Melatonin (+), Xanthurenic Acid (-) and 2-oxo-3-methyl-butyrate (+) in urine shows up to 17X improvement in S/N with microflow LC as compared to analytical flow LC.

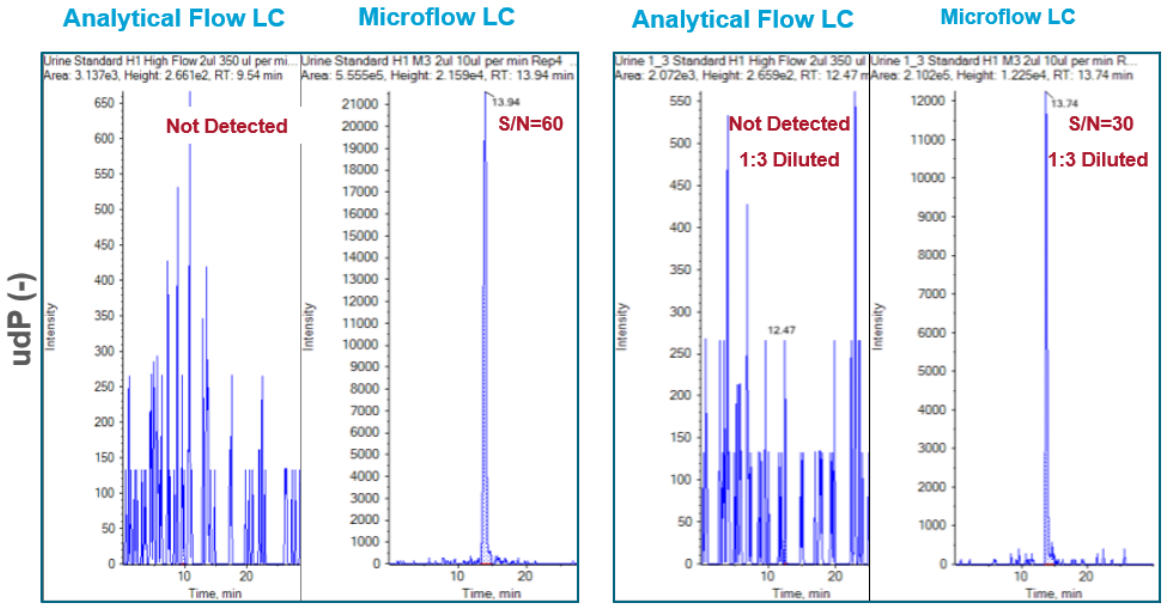

Figure 6. Microflow LC method sensitivity. The peak for udP (-) shows the improved sensitivity of microflow HILIC–MRM vs. the analytical flow LC-MRM. With microflow LC, udP (-) in both 1:3 diluted and undiluted sample is identified where it was not identified in data from analytical flow LC.

Conclusions

This 30 min method is a single LC-MRM targeted screening method allowing detection of over 300 polar metabolites across multiple biochemical pathways involved in cancer, cardiovascular, neurodegenerative, diabetes and obesity.

- This microflow LC-MRM workflow provides improved sensitivity and S/N ratio of up to 60X with an average improvement of 10X which offers a solution for detection of metabolites where metabolites need to be identified in low concentrations and/or when sample volumes are limited.
- This microflow method provides up to 50% increase in detection of polar metabolites.
- Microflow HILIC chromatography provides excellent chromatographic separation of polar, hydrophilic metabolites, with reduced solvent consumption and costs.

References

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2. Ivanisevic J, *et al.* (2013) Toward 'Omic Scale Metabolite Profiling: A Dual Separation−Mass Spectrometry Approach for Coverage of Lipid and Central Carbon Metabolism. *Anal Chem.* **85(14)**, 6876-84.
3. Yuan M, Breitkopf SB, Yang X, Asara JM. (2012) A positive/negative ion–switching, targeted mass spectrometry–based metabolomics platform for bodily fluids, cells, and fresh and fixed tissue. *Nat Protocols.* **7(5)**, 872-81.

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