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Microflow LC-MS/MS Analysis Improves Sensitivity for Targeted Metabolomics

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Abstract

Metabolomics allows discovery of novel therapeutics, screening drug toxicity and efficacy, and monitoring diet and environmental exposure effects on health. Identifying metabolites from urine and plasma is essential for validating potential disease biomarkers and interrogating their affected metabolic pathways to further understand their biological implications. Liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis has become an essential tool for identification and quantification of metabolites in complex sample matrices due to its inherent sensitivity gains. Here, we describe a robust and sensitive workflow using a M3 MicroLC coupled to a QTRAP® 6500+ mass spectrometer for qualitative and quantitative analysis of polar metabolites (Figure 1). We have implemented a single HILIC microflow LC-MS/MS method for profiling polar metabolites using multiple reaction monitoring (MRM) with positive/negative polarity switching in a single injection workflow. While microflow has become increasingly popular for many applications, microflow for metabolomics has not been popular because the typically used (aqueous) sample solvent does not allow for injecting larger volumes of samples without sacrificing chromatographic resolution. However, by simply reconstituting the sample in an organic solvent (95% Acetonitrile, pH 9), we were able to inject up to 2 µL on to the microLC column, while maintaining peak shape, and have identified 263 unique metabolites out of 312 targets (296 out of 363 Q1/Q3 transitions monitored) from plasma/urine and MDCK cell line metabolite extracts, covering all major metabolic pathways, with up to 60X improved sensitivity for some metabolites. The sample preparation takes ~2 hrs with an additional 1 hour for sample run & data analysis.

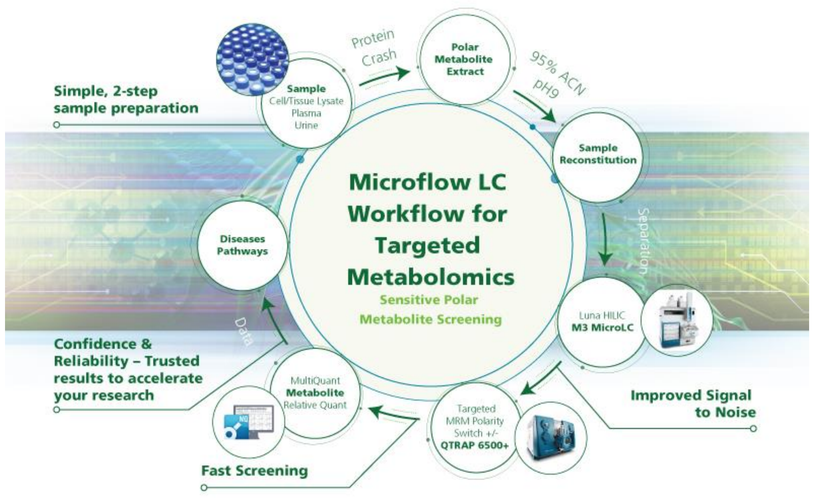


Figure 1. Microflow LC Workflow for Targeted Metabolomics.

Materials and Methods

Sample Preparation: Human urine was diluted in water (1:4) and 100 µL of diluted urine or 100 µL of control plasma for amino acid analysis (SCIEX) were transferred for the analysis 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 at 14,000 RPM for 10 min. An 800 µL aliquot of the supernatant which contains the extracted metabolites was transferred to a new 2 mL microtube. The sample was dried using a TurboVap evaporator to a pellet using no heat starting with 5 psi N_2 gas flow for 30 min and an extra 30 min at 10 psi pressure (~1hr). The fresh pellet was dissolved in 100 µL of HILIC sample resuspension buffer, mixed well by vortexing and centrifuged at 14,000 RPM for 10 min. Ninety microliters of supernatant was transferred to deactivated QsertVials (Waters) for LC-MS/MS analysis. Injection volume was 2 µl with 5 replicate injections. Samples were also diluted further (1:3) to check the method sensitivity. The HILIC sample resuspension buffer contained 95% acetonitrile and 5 % mobile phase A. 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. Madin-Darby Canine Kidney Epithelial Cells (MDCK Line) were extracted in 70% methanol and dried using a TurboVap evaporator 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 Liquid 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 NH_2 100 Å, 150 x 4.6 mm column (Phenomenex). 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, 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 method used is listed in Table 1.

Table 1. HILIC LC Gradient used for Analytical and Microflow Method.

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

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 NH_2 100 Å, 150 x 0.3 mm analytical column (Phenomenex) was used with a micro filter 1µm SS (Upchurch Scientific) before the column to prolong column life time. Mobile phases and LC gradient were identical to the analytical flow LC mobile phases (Table 1). 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.

Mass Spectrometry: A SCIEX QTRAP® 6500+ 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 microflow and analytical flow (SCIEX), CE, EP, DP and CXP values were kept constant for both analytical flow and microflow LC experiments.

The source, gas and MS parameters are listed in 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 +/- 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.

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
Collision GAS	High	High	High	High
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
DP	93	-93	93	-93
EP	10	-10	10	-10
CXP	10	-10	10	-10

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 ratio 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.

Results

For each detected metabolite in urine, the lowest observed S/N (calculated by MultiQuant™), was plotted versus the number of replicates that metabolite was detected in (Figure 2). 197 of metabolites detected with a 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.

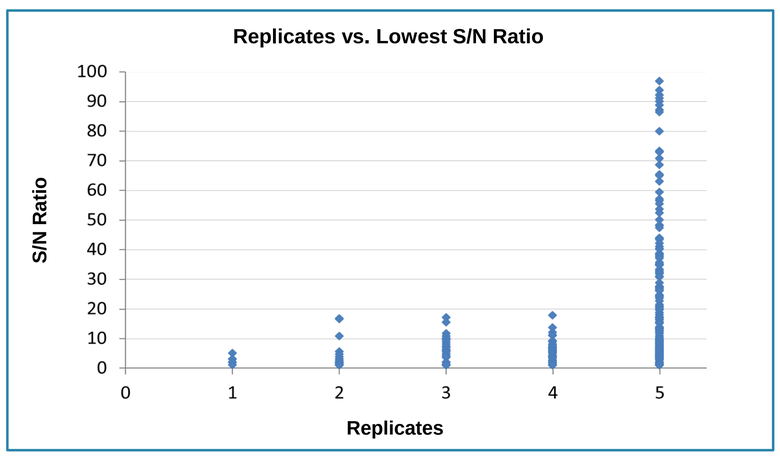


Figure 2. Lowest observed S/N Ratio Across Replicate Analysis. Metabolite with minimum S/N ratio of 10-20 10 were manually validated and listed in table. Based on our dataset metabolites with S/N ≥20 are detected metabolites with high confidence without requiring further manual validation.

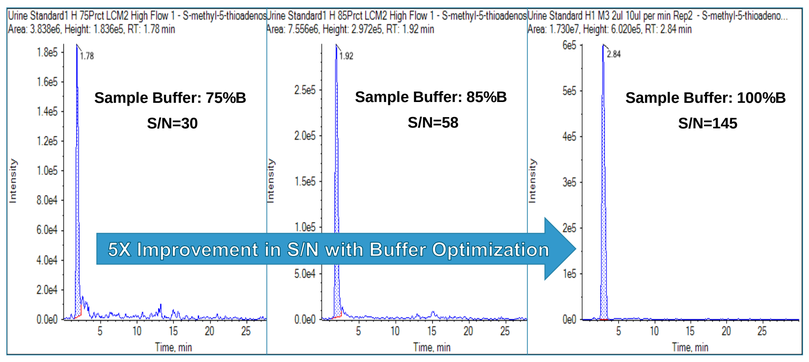


Figure 3. Metabolite Solubility Optimization. The MultiQuant™ integrated peak for urine S-methyl-5-thioadenosine shows improved S/N ratio when 100% B and 5% A was used for metabolite extract reconstitution when compared to 75 and 85% Mobile Phase B, which demonstrate optimized solubility and HILIC affinity of polar metabolite in 100% B.

Improved Signal to Noise Ratio (S/N)

The S/N ratio for all detected metabolites using this MRM method with both analytical flow LC and microflow LC were compared. S/N ratio was improved up to 60X with an average improvement of 10X (Figure 4), which resulted in detection of 50% more targeted polar metabolites with high confidence when compared to analytical flow LC-MRM method (Figure 5,6).

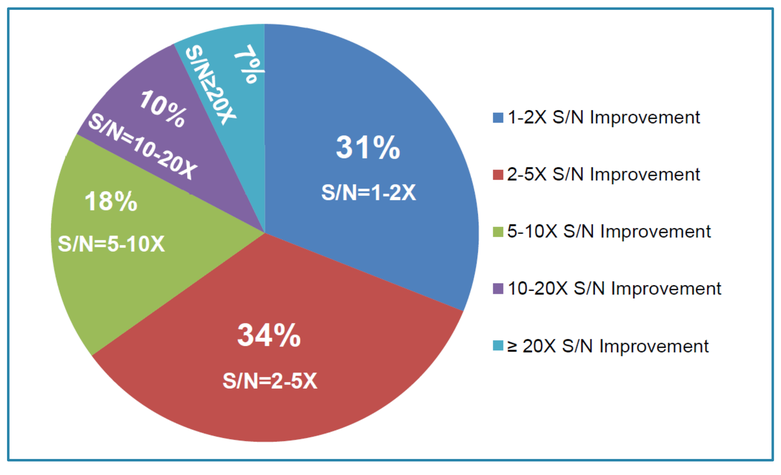


Figure 4. Microflow LC S/N Ratio Improvement. S/N ratio was improved using Microflow LC vs. Analytical flow LC. 35% of detected metabolites in both samples showed S/N ratio improvement of 5-≥20 fold resulting in identification of these metabolites with higher confidence using microflow LC.

Figure 5. Microflow LC S/N Improvement. The MultiQuant™ integrated peak for Melatonin (+) and Xanthurenic Acid (-) in urine shows improved S/N ratio with microflow LC as compared to integrated peaks in analytical flow LC.

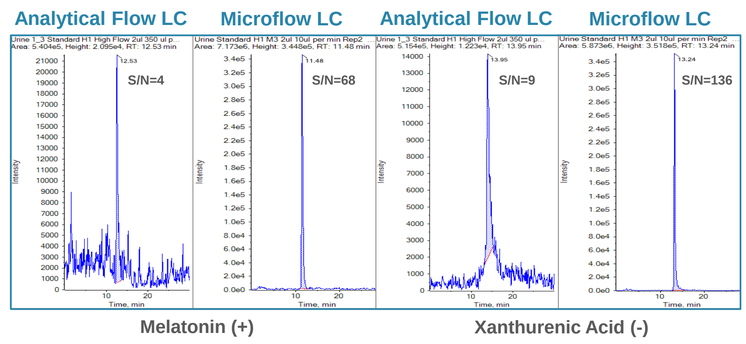
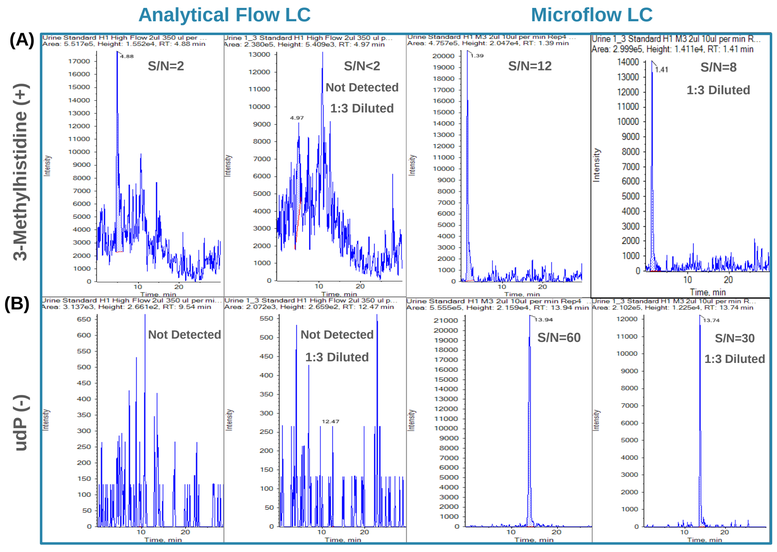


Figure 6. Microflow LC Improved Sensitivity. The MultiQuant™ integrated peak for 3-Methylhistidine (+) in urine extract and 1:3 diluted sample shows improved S/N ratio with microflow LC as compared to integrated peaks in analytical flow LC (Figure 6A). Figure 6B shows the improved sensitivity of microflow LC HILIC–MRM vs. the analytical flow LC-MRM for udP(-), the microflow LC-MRM identifies the udP (-) in both undiluted and 1:3 diluted sample but not the analytical flow LC-MRM.



Improved Metabolite Coverage

Often metabolite extracts must be run on reversed phase and normal phase, in negative and positive ion modes for detection of large number of polar metabolites whereas using this 30 min microflow HILIC LC-MRM workflow we were able to detect over 263 polar metabolites across urine, plasma and MDCK cells. Microflow HILIC LC-MRM method improved the S/N ratio by an average of 10X which resulted in higher number of metabolite detection with the larger improvement in urine by 35%, plasma by 11% and MDCK by 50% .

Table 3. Improved Metabolite Coverage by M3 MicroLC. Total number of unique metabolites and transitions identified with minimum S/N of ≥10 in three types of samples tested, showing improved metabolite coverage using the microflow LC method.

	Identified Unique Metabolites & (Transitions)		
Samples	**Analytical Flow LC**	**Microflow LC**	
MDCK Cell Lines	*87 (97)*	*131 (141)*	50%
Urine	*162 (182)*	*219 (242)*	35%
Plasma	*174 (196)*	*194 (218)*	11%

Conclusions

- 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 Luna-NH2 HILIC chromatography provides excellent chromatographic separation of polar, hydrophilic metabolites.
- 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.
- The sensitivity and speed of the QTRAP® 6500+ with IonDrive Technology allows an efficient high throughput assay by using +/- polarity switching (5 msec) in a single sample injection.
- M3 MicroLC reduces solvent consumption and costs.

References

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