Microflow Chromatography Provides Improved Sensitivity and Coverage of Polar Metabolites for Targeted Metabolomics

Improved Detection of Polar Metabolites in Complex Matrices Using M3 MicroLC and QTRAP® 6500+

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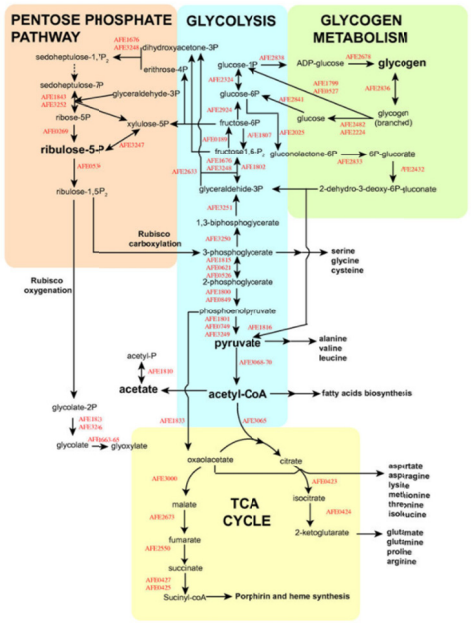
Metabolomics analysis allows discovery of novel therapeutics, screening for drug toxicity and efficacy, and monitoring diet and environmental exposure effects on health. Identifying metabolites from urine and plasma is essential for validating potential disease biomarkers for research and interrogating the 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 quantitation of metabolites in complex sample matrices due to its inherent sensitivity gains (1, 2). 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. Here, we describe a robust and sensitive workflow using a M3 MicroLC coupled to a QTRAP® 6500+ mass spectrometer for screening over 300 polar metabolites in biological samples. We have implemented a single HILIC microflow LC-MS/MS method for profiling over 300 polar metabolites using multiple reaction monitoring (MRM) with positive/negative polarity switching in a single injection workflow (3). While microflow has become increasingly popular for many applications (4), microflow for metabolomics has not been readily employed 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 5 µL onto the microLC column, while maintaining peak shape. We have identified 263 unique metabolites out of 312 targets (296 out of 363 Q1/Q3 transitions monitored) from plasma/urine and MDCK (Madin-Darby Canine Kidney) cell line extracts, covering all major metabolic pathways, with up to 60X improved sensitivity for some metabolites. The sample preparation takes ~2 hours with an additional 1 hour for sample run and data analysis.



Central Carbon Metabolism

Key Benefits of this Workflow

- 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
- Microflow Luna-NH2 HILIC chromatography provides excellent chromatographic separation of polar, hydrophilic metabolites
- A single LC-MS/MS targeted method allowing detection of 312 polar metabolites across multiple biochemical pathways
- M3 MicroLC reduces solvent consumption and costs
- 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

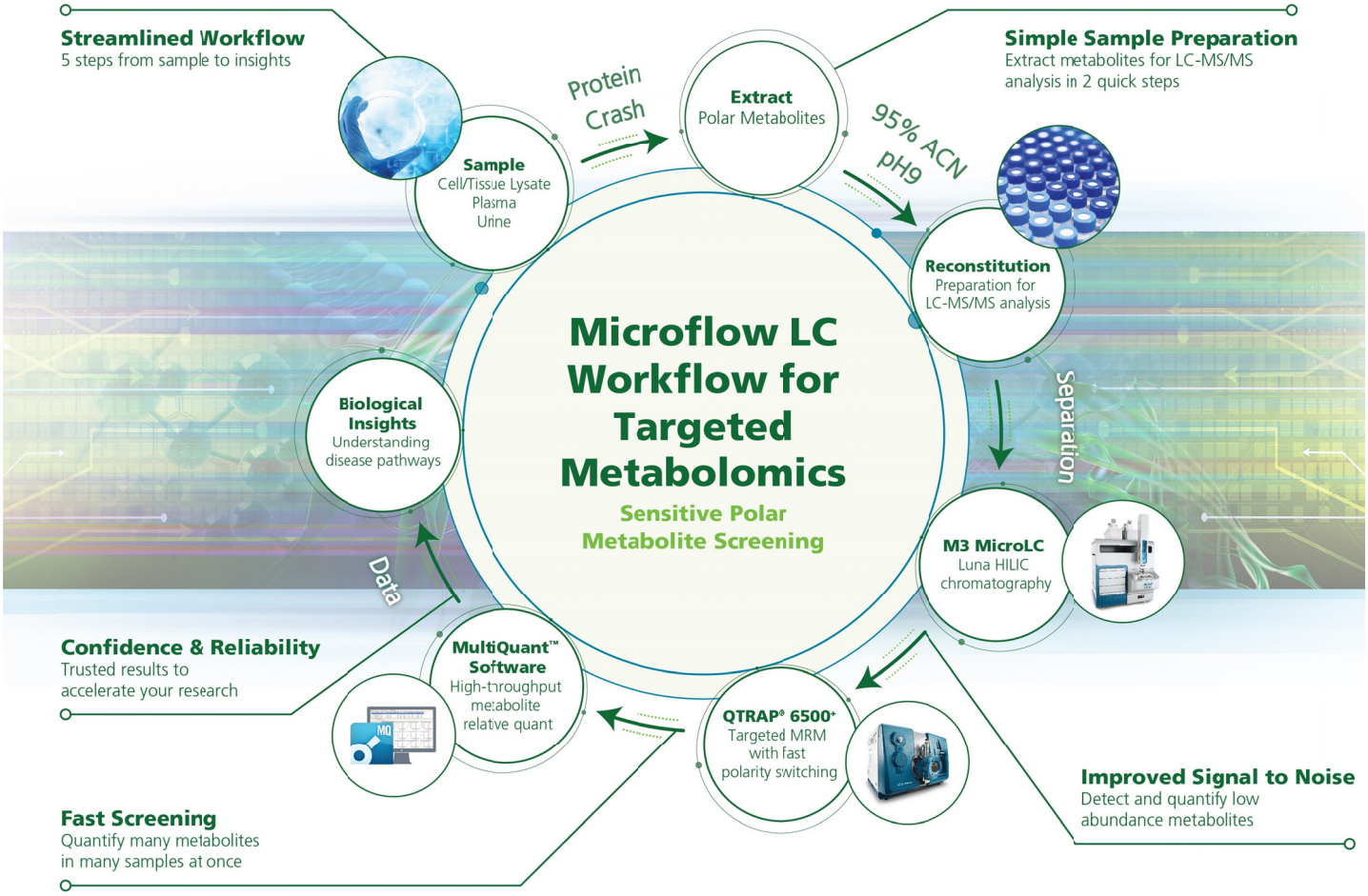


Figure 1. Microflow LC Workflow for Targeted Metabolomics

Experimental Design

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) 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 at 14,000 RPM for 10 min. An 800 µL aliquot of the supernatant which contained 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 N2 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 (Figure3).

Analytical Flow 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 NH2 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.

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. 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 (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 analytical and microflow (SCIEX) and 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 transitions) with 3 msec dwell time and 50 ms settling time for polarity switching to monitor these endogenous metabolites across different metabolic pathways.

Table1. HILIC 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

Table2. 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 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 and Discussion

The typical used (aqueous) sample solvent in analytical flow LC does not allow for injecting larger volumes of samples without sacrificing HILIC chromatographic resolution. Here, by simply reconstituting the sample in organic solvent (100% mobile phase B), we were able to inject up to 5 µL on to the microLC column, while maintaining 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 4).

For each detected metabolite in urine, the lowest observed S/N (calculated by MultiQuant), was plotted versus the number of replicates that the metabolite was detected in (Figure 2). 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. Based on our dataset metabolites with S/N ratio of ≥ 20 are detected metabolites with high confidence and require no need of manual validation or replicate analysis.

The microflow HILIC LC-MRM method improved the S/N ratio by up to 60X. 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).

Improved Sensitivity and Coverage

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 3), which resulted in detection of up to 50% more targeted polar metabolites with high confidence when compared to analytical flow LC-MRM method (Figure 6).

Table 3 (right). Increased Metabolite Identification by M3 MicroLC. 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.

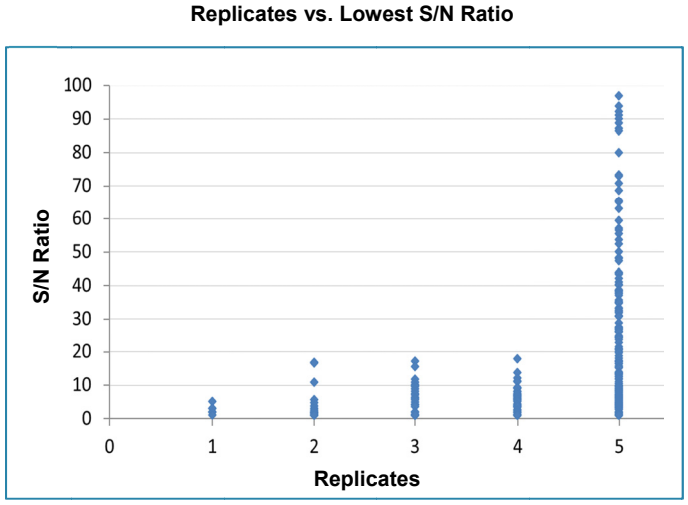


Figure 2. Minimum S/N Ratio Across Replicate Analysis.

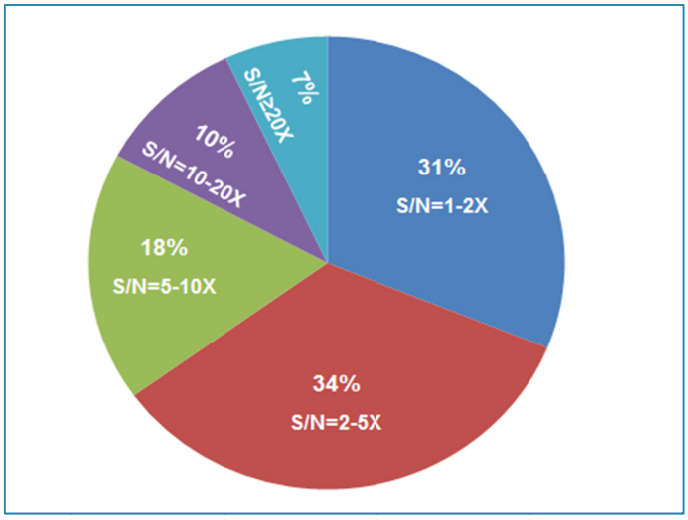


Figure 3. Microflow LC S/N Ratio Improvement. 35% of detected metabolites from urine showed S/N ratio improvement of 5-≥20 fold resulting in identification of these metabolites with higher confidence using microflow LC.

Identified Metabolites and (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%

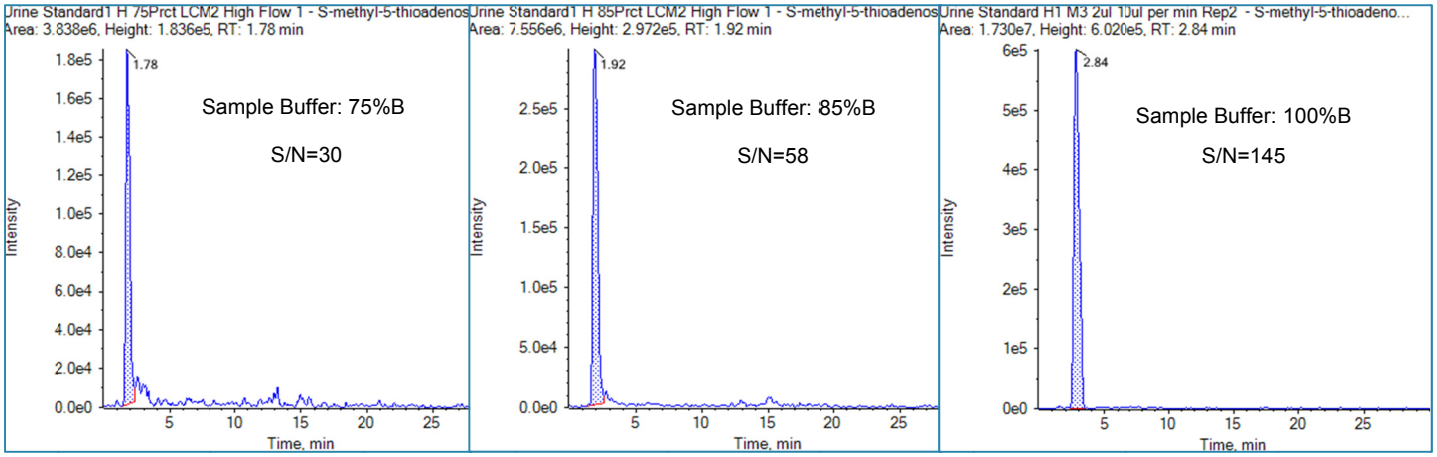


Figure 4. Polar Metabolite Solubility Optimization. S/N ratio improves with increasing organic content (100% B) for metabolite extract reconstitution, as compared to 75 and 85% Mobile Phase B, which demonstrate optimized solubility and HILIC affinity of polar metabolite in 100% B.

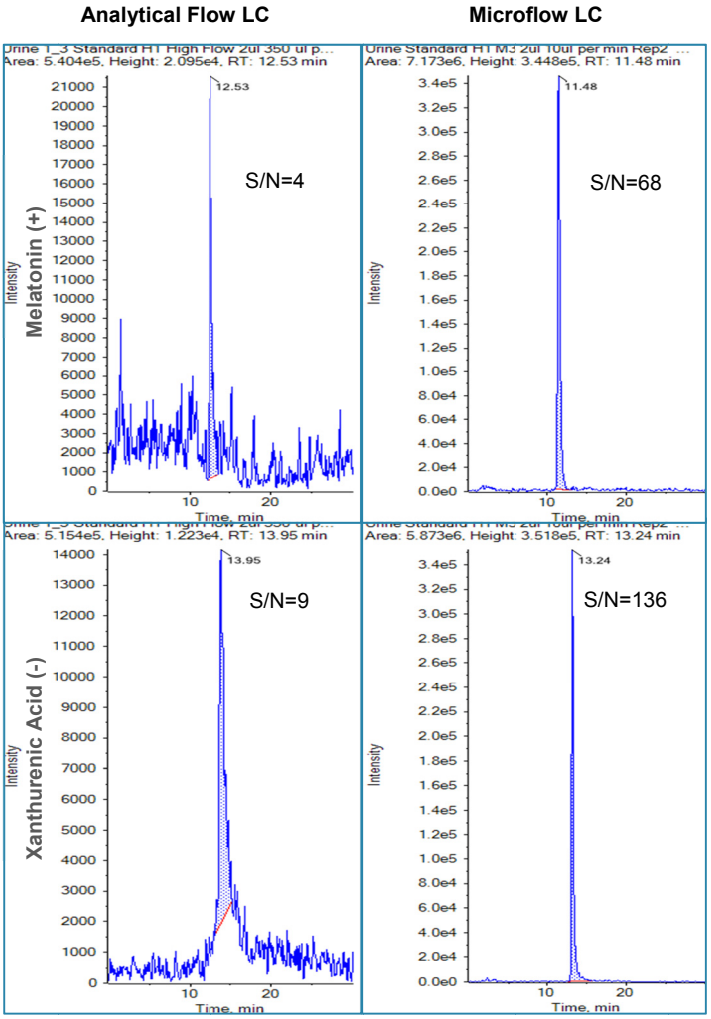


Figure 5. Microflow LC S/N Ratio Improvement. Integrated peak for Melatonin (+) and Xanthurenic Acid (-) in urine shows up to 17X improvement in S/N with microflow LC as compared to analytical flow LC.

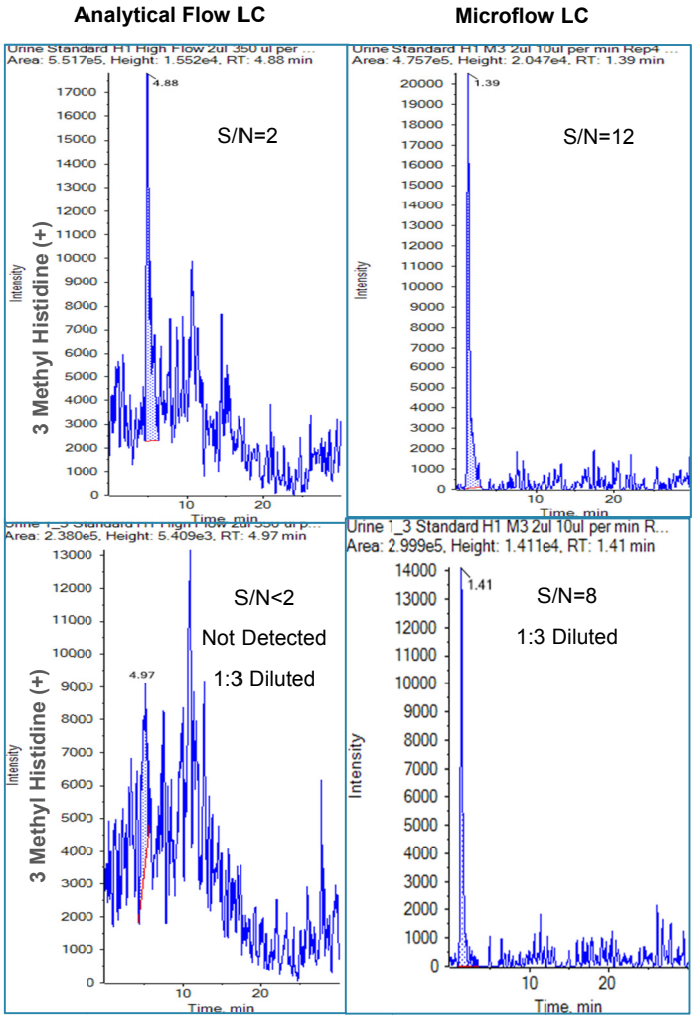


Figure 6. Microflow LC Improved Sensitivity. Peaks for 3-Methylhistidine (+) in urine extract and 1:3 diluted sample shows up to 8X improvement in S/N with microflow LC as compared to analytical flow LC.

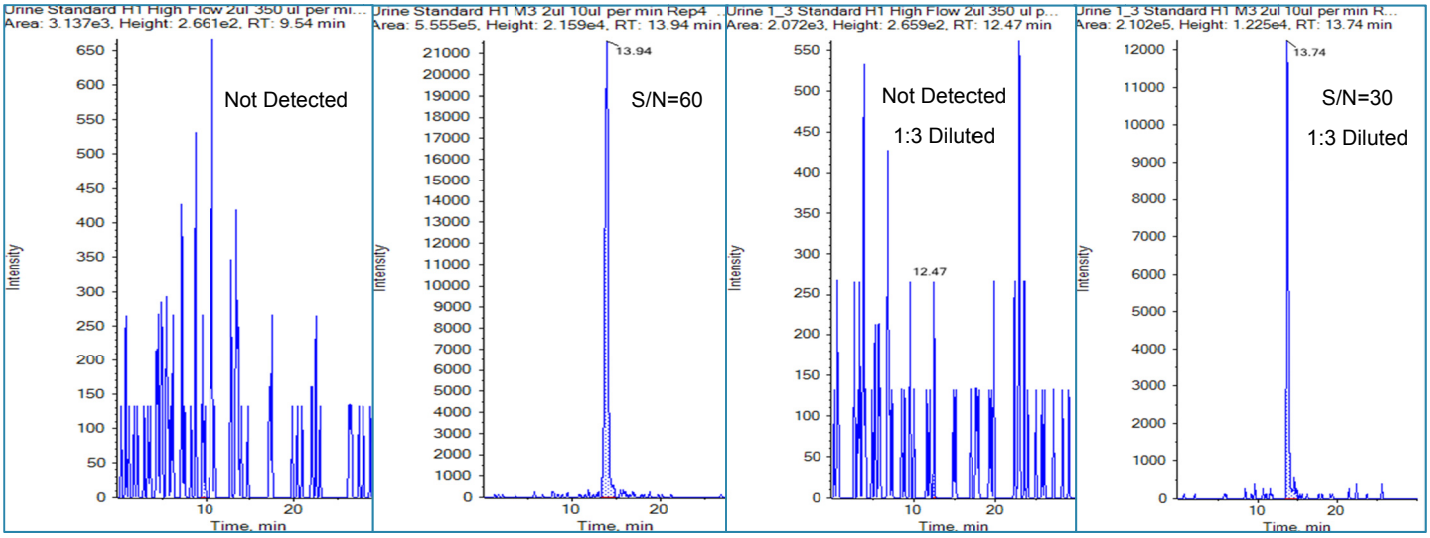


Figure 7. Microflow LC Method Sensitivity. The peak for udP (-) shows the improved sensitivity of microflow HILIC–MRM vs. the analytical flow LC-MRM. With microflow LC, udP (-) in both 1:3 diluted and undiluted sample is identified where it was not identified in data from analytical flow LC.

Figures 5-7 clearly show improved S/N ratio for metabolites (Melatonin (+), Xanthurenic Acid (-), 3-Methylhistidine (+) and udP (-) using the microflow LC-MRM vs. analytical LC-MRM.

The improved S/N ratio provided by microflow HILIC, LC-MRM method provides solid detection of 263 metabolites and up to 50% improvement in detection when compared to data obtained with the analytical flow HILIC, LC-MRM 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, using this 30 min microflow HILIC LC-MRM workflow we were able to detect 263 polar metabolites across urine, plasma and MDCK cells and improve throughput by up to 4X.

Metabolites not identified from our targeted list either had a minimum S/N of <10 in all five replicates or were low to be detected (MultiQuant output of N/A), which could be due to degradation, for example, -coA metabolite families which degrade quickly and are more difficult to detect than other metabolites.

	Identified Unique Metabolites (% of Targeted Metabolites)	
Samples	**Analytical Flow LC**	**Microflow LC**
Urine	*50%*	*70%*
Plasma	*56%*	*62%*
MDCK Cell Lines	*28%*	*42%*

Table 4. Improved Metabolite Coverage with M3 MicroLC. 312 unique metabolites in three types of samples were targeted in this method and percentage metabolite identification with minimum S/N of ≥10, shows improved coverage with microflow approach.

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.

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.

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Document number: RUO-MKT-02-6110-A

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