A Systematic Approach to Development of Analytical Scale and Microflow-based LC-MS Metabolomics Methods to Support Drug Discovery and Development

Sarah Geller[1], Harvey Lieberman[1], Alla Kloss[1], and Alexander R. Ivanov[2]

[1]Sanofi, Waltham, Massachusetts, USA; [2]Barnett Institute of Chemical and Biological Analysis, Department of Chemistry and Chemical Biology, Northeastern University, Boston, Massachusetts, USA

OVERVIEW

Purpose

- To develop and optimize ion-pair free global metabolomics LC-MS profiling methods
- To scale down optimized methods to microflow LC-MS separation for additional gains in sensitivity
- To test an alternative ESI interface for microspray in microflow LC-MS applications

Methods

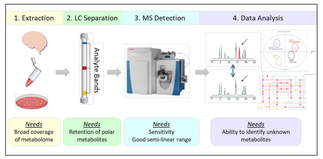
- Untargeted global profiling methods were carried out using LC separation coupled with high resolution accurate mass spectrometric detection (HRAM)

Results

- Analytical flow LC-MS methods were developed and optimized
  - Standard mixture of 62 metabolites was developed for evaluation and optimization of LC-MS based profiling methods
  - Eighteen LC columns from seven vendors were evaluated under acidic, neutral, and basic conditions
  - The best ion pair free methods were selected for global metabolomics profiling in a positive and negative ion modes
- These selected methods were successfully scaled down to a microflow-based LC separation
- A gain in sensitivity, semi-quantitative range, and sample size requirements were observed with the microflow based LC-MS method as compared to the analytical flow method

INTRODUCTION

Within drug discovery and development there is an increased reliance on metabolic biomarkers to monitor disease, establish phenotypic screens, understand mechanisms of action for active compounds, and monitor in vivo and clinical studies. Therefore, there is an increased demand for global metabolomics methods to provide coverage and sensitivity towards differences in metabolite expression and reproducibility. The requirements for a global LC-MS profiling method are more complex than those encountered during the development of a targeted LC-MS/MS based methodology. The method must cover a broad range of metabolites that are chemically diverse and present at varying concentrations within a sample. A profiling method must also separate and retain hydrophilic analytes while also providing good ionization of hydrophilic analytes. The method must also be sensitive and have a semi-quantitative range for up to tens of thousands of metabolites. Due to these demands, a systematic approach is necessary for the development and evaluation of LC-MS based metabolomics methods, using either conventional techniques or when establishing new methods that allow for additional gains in sensitivity and a reduction in sample size requirements, such as those seen with methods based on microseparations coupled to mass spectrometry.



METHODS

Instrumentation: An Acquity UPLC I-Class (Waters Corporation) was used for the analytical scale methods and an Acquity UPLC M-Class (Waters Corporation) was used for all microseparations. A Q Exactive™ MS (Thermo Fisher Scientific) was used for all experiments.

Processing: Data processing was performed using Compound Discoverer™ (Thermo Fisher Scientific) and GeneData Expressionist (GeneData) software packages.

Optimized LC-MS Methods: Samples were analyzed in both positive and negative modes with a m/z range 67-1000 and a resolution setting of 70,0000 at m/z 200

Selected Analytical and Microflow Methods:

Positive Ion Mode:

- Columns: Waters HSS SB C18, 2.1 x 100 mm and 0.3 x 100 mm, 1.7 µm
- Mobile Phase A: 0.1% FA in water
- Mobile Phase B: 0.1% FA in acetonitrile

Negative Ion Mode:

- Columns: Imtakt Scherzo SM-C18, 2.0 x 100 mm and 0.3 x 100 mm, 3 µm
- Mobile Phase A: 5 mM AmAc in water, pH 5.8
- Mobile Phase B: Acetonitrile

Gradient: Hold 0.1% B for 1 min; ramp to 99% B to 21 min; wash, re-equilibrate
Column Temp: 40 °C; Sample Temp: 10 °C

Ion-Pairing Method:

Positive Ion Mode:

- Column: Phenomenex Luna C18(2)-HST, 3.0 x 100 mm, 2.5 µm
- Mobile Phase A: 0.25% TFA, 0.15% HFBA in water
- Mobile Phase B: Acetonitrile

Negative Ion Mode:

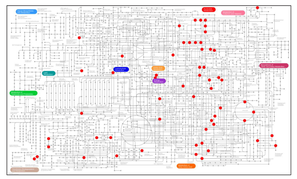
- Column: Waters Acquity UPLC BEH C18, 2.1 x 100 mm, 1.7 µm
- Mobile Phase A: 5 mM hexylamine, 5 mM AmAc in water, pH 9
- Mobile Phase B: Acetonitrile

Gradient: Hold 0.1% B for 0.75 min; ramp to 80% B to 8.0 min; ramp to 95% B to 9.0 min; wash, re-equilibrate
Column Temp: 30 °C: Sample Temp: 10 °C

RESULTS

A standard mixture was developed to use for the evaluation of all analytical and microscale methods and consisted of the following

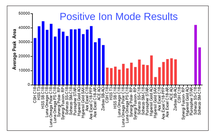
- 62 analytes representing different metabolite classes and pathways
  - Represented analyte classes include (but are not limited to) amino acids, carnitines, neurotransmitters, nucleotides, and steroids
- Positional isomers (leucine/isoleucine and 1-/3-methylhisitidine) to test for separation
- A broad LogP range (-6.69 – 6.09) to cover both hydrophilic and hydrophobic analytes

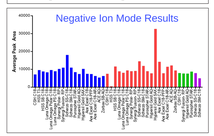


Analytes, highlighted with red circles, are visualized using iPath 3.0, a cellular pathway map of global metabolism.

Eighteen LC columns from seven vendors were evaluated under acidic, neutral, and basic conditions using the standard mixture

Mobile Phase A	Mobile Phase B
0.1% FA in H_2O, pH 2.8	0.1% FA in ACN
10 mM AmAc in H_2O, pH 5.8	ACN
10 mM AmF in H_2O, pH 9	ACN
0.1% FA in H_2O	30 mM AmF in 30:70 H_2O:ACN





A two step scoring system was developed to identify the column-mobile phase conditions that performed the best

- Points were assigned based on pre-determined criteria in two rounds
- The points were tallied at the end of each round for a total score

First round

- Isomer separation
- Coverage
- Retention factor
- Average peak area

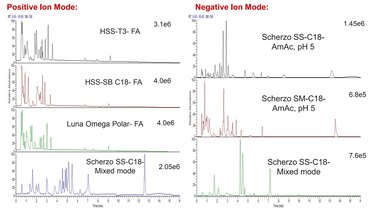
Second round

- Those with a score of 3 or above were evaluated for peak shape

Category	Description	Parameters	Possible Points
Isomer Separation	Ability to separate positional isomers	1-/3-Methylhistidine	1
		Leucine/Isoleucine	1
ID'ed	Coverage- The total number of analytes identified	> 51	2
		48 - 51	1
		30 - 47	0
		< 30	-1
Retention Factor	The percent of analytes with k' < 1.0	< 30	2
		30 - 39	1
		40 - 45	0
		> 45	-1
Avg Peak Area	Total Average Peak Area	Top 15% of all conditions tested	2
		Bottom 15% of all conditions tested	-1
Peak Shape	The percent of analytes with an undesired peak shape	< 50 %	1

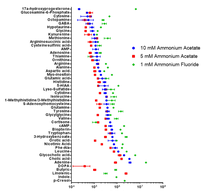
Conditions with a final score of 4 or above were chosen for further optimization studies

Extracted Ion Chromatograms of Chosen Conditions:



XIC of all 62 analytes in the standard mixture with 10 ppm mass accuracy
Standard concentration: 500 ng/mL
LC run time: 20 min
Injection volume: 10 µL
Flow rate: 0.5 mL/min

Selected column-mobile phase combinations underwent further optimization of column temperature, mobile phase composition, and mobile phase pH



Differences in analyte coverage and peak height are seen due to changing MPA composition in the negative ion mode

Analytes are shown in the order of increasing peak intensities with 10 mM Ammonium Acetate MPA

- Imtakt Scherzo SM-C18 optimization
  - Negative Ion Mode
  - Std Mix: 100 ng/mL
  - Column temp: 40 °C
  - MPB: ACN
  - Flow Rate: 0.4 mL/min
  - Run Time: 26 min

After evaluation of all conditions tested, the following were chosen for scale down to microflow LC separation

- Column temp: 40 °C
- LC gradient: Hold 0.1% B for 1 min; ramp to 99% B to 21 min; wash, re-equilbrate
- Microscale column dimensions: 0.3 x 100 mm
- Flow rates: 6-10 µL/min
- Columns and mobile phases:

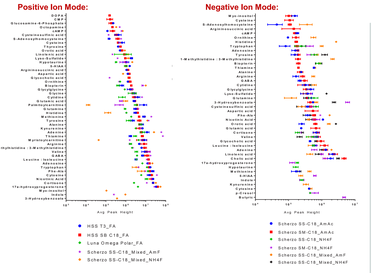
Positive Ion Mode:

Column	Mobile Phases A	Mobile Phases B
HSS T3	0.1 % Formic acid in H_2O	0.1% Formic acid in ACN
HSS SB C18	0.1 % Formic acid in H_2O	0.1% Formic acid in ACN
Luna Omega Polar C18	0.1 % Formic acid in H_2O	0.1% Formic acid in ACN
Scherzo SS-C18	0.1 % Formic acid in H_2O	20 mM Ammonium formate in 10:90 H_2O:ACN
	0.1 % Formic acid in H_2O	1 mM Ammonium fluoride in 10:90 H_2O:ACN

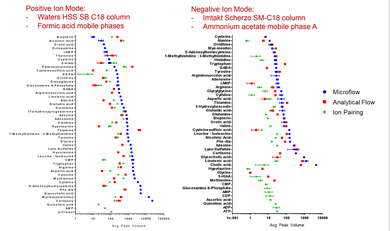
Negative Ion Mode:

Column	Mobile Phases A	Mobile Phases B
Scherzo SS-C18 and Scherzo SM-C18	10 mM Ammonium acetate in H_2O, pH 5.8	Acetonitrile
	1 mM Ammonium Fluoride in H_2O, pH 5.4	Acetonitrile
Scherzo SS-C18	0.1 % Formic acid in H_2O	20 mM Ammonium formate in 10:90 H_2O:ACN
	0.1 % Formic acid in H_2O	1 mM Ammonium fluoride in 10:90 H_2O:ACN

Results of Microflow LC Separation:



A positive and negative ion mode method was chosen and compared to the respective analytical flow method as well as an in house ion-pairing reagent containing method

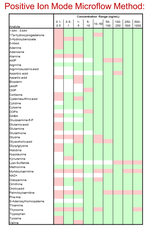


Standard Mixture concentration: 100 ng/mL Injection volume: 1 µL

Since the goal of global profiling studies is identification of metabolites with changes in expression associated with phenotypic differences, knowing the semi-quantitative capabilities of an LC-MS metabolomics method is necessary.

We are estimating these capabilities by examining those metabolites which exhibit a linear concentration dependence in peak height.

Positive Ion Mode Microflow Method:

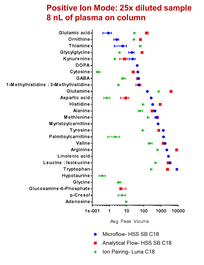


- In this study all optimized methods were evaluated using the 62 analyte standard mixture at ten concentration levels
- The linear response of each analyte was determined by comparing the theoretical ratio of the response at each neighboring standard concentration
- Those with exhibiting a linear response are highlighted in green while those with a non-linear response are highlighted in pink as shown to the left

	Semi-quantitative range (ng/mL)	
	Positive Ion Mode	Negative Ion Mode
Microflow	0.5-1000	5-1000
Analytical Flow	5-1000	5-1000
Ion Pairing	5-1000	50-1000

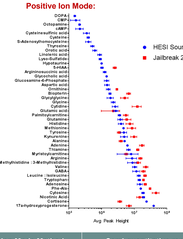
The performance of the optimized methods was also tested and compared to the performance observed in the presence of a complex biological matrix

- WT mouse plasma was analyzed at three dilution levels (25x, 50x, and 100x)
- The analyte coverage from the standard mixture was compared across all optimized methods
- The linear response of all clusters (m/z-RT pairs) was determined by comparing the theoretical ratio of the response for each neighboring dilution
- The percent of these clusters with a linear response, between the 50x and 25x diluted plasma samples, are reported

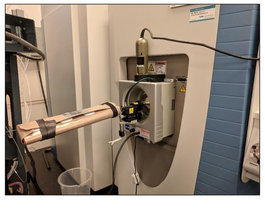


	% Clusters with a Linear Response	
	Positive Ion Mode	Negative Ion Mode
Microflow	44	32
Analytical Flow	35	35
Ion Pairing	34	35

The Phoenix S&T Jailbreak 2.0 source was evaluated as an alternative ESI interface for improvements in method sensitivity and linearity of response as compared to the standard HESI source with a low flow needle using the 62 analyte standard mixture



Negative Ion Mode Method	Semi-quantitative range
HESI Source	5-1000 ng/mL
Jailbreak Source	50-1000 ng/mL



CONCLUSIONS

- Ion-pair free global metabolomics profiling methods were developed and optimized that resulted in improvements in analyte coverage, sensitivity and expanded quantitation ranges especially significant in the negative ion mode.
- These methods were successfully scaled down from a 2.1 mm i.d column with a 0.5 mL/min flow rate to a microflow-based LC separation using a 0.3 mm i.d. column and 6-10 µL/min flow rates
- The microflow methods generally outperformed the analytical flow methods and improved the sensitivity 5-10 fold on average and also expanded the list of metabolites with a linear concentration dependence in the positive ion mode
- The increase in percentage of clusters with linear response observed in the plasma samples suggest that transfer of the profiling methods to a microflow based separation has the potential for reducing sample size requirements
- While the Phoenix S&T Jailbreak 2.0 source provided better sensitivity for most analytes in this study, the analyte coverage and semi-quantitative range that we have achieved with this source were significantly less than that seen with the conventional HESI source with a low flow needle
- The reasons for this discrepancy have not yet been established and further optimization may be required. This will be attempted in the future experiments.

FUNDING

- Sanofi
- NIH NIGMI 1R01GM120272 (ARI); NIH NCI R01CA218500 (ARI); NIH NIGMI 1R43GM117932-01 (ARI), and ASMS 2015 Research Award (ARI)