A Novel Targeted Metabolic Profiling Workflow for Simultaneous Reverse Phase and Normal Phase LC-MS/MS Analysis on a M3 MicroLC System

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ABSTRACT

Recent advances in HPLC technology have inspired investigators to explore increasing throughput of data acquisition by multiplexing. This ultimately leads to faster processing times and overall simplification of the process, especially in the field of metabolomics, where often several different strategies need to be employed for discovery metabolomics.

Here we explore the concept of "multiplexing" a true simultaneous dual column strategy combining reverse phase with normal phase (NH2) chromatography. Unlike other methods recently reported[1], this is not a "trap and elude" approach, but rather a single injection, dual-column, HPLC method. The speed of the mass spectrometer also allows for several hundred MRM's to be monitored by positive/negative switching in one experiment, so potentially up to four separate analyses are merged into one.

INTRODUCTION

Many of the compounds of interest in the study of metabolism are extremely polar and therefore often unable to be analyzed through traditional coupling of reversed phase chromatography and mass spectrometry. The development of HILIC mode and similar amide (NH2) chromatography has allowed polar, hydrophilic compounds to be adequately chromatographed, although each sample now must be injected multiple times and the HPLC mobile phases, columns, and experiments must be reconfigured prior to analysis. Often a single sample set must be run in reverse phase, normal phase, MS+, and MS- modes in order to ensure detection of all potential metabolites. This requires two seperate LC methods, and two MS polarity methods, up to four analyses for the same sample. The scope of this project is to evaluate the feasibility of utilizing a dual gradient microflow LC system to perform simultaneous reversed phase and normal phase LC-MS/MS analysis improving the throughput of a metabolomics assays.

MATERIALS AND METHODS

Sample Preparation: Serum sample preparation method for a 10 ppm metabolite mix standard: Placed 200 µL of human serum (3X charcoal stripped) in a 1.5 ml micro-centrifuge tube, centrifuged at 14000 g for 10 min at 4 ºC. Transferred the supernatant (170 µL) to a new 1.5 mL micro-centrifuge tube. Added 4 times volume of methanol (cooled to -80 ºC) to the supernatant, making a final 80% (*Vol/Vol*) methanol solution. Gently mixed and incubated overnight at -80 ºC. Centrifuged at 14000g for 10 min (4 ºC). Transferred the supernatant (680 µL) to a new 1.5 mL micro-centrifuge tube. SpeedVac to a pellet using no heat (5.5 hr). Added 1500 µL (1475 µL of water + 75 µL of a 200 ug/mL stock) metabolites standard (80% Acetonitrile for normal phase) metabolites standard (H_2O for C18AQ) into the pellet and centrifuged at 14000g. Prepared the 2 ppm QC by taking 100 µL of the 10 ppm standard and adding 400 µL of water, spin/shake. Keep sample at -20 ºC for further usage.

HPLC Conditions:
Mobile Phase A = 99.9% water, 0.1 % formic acid, 5 mM NH_4OAc.
Mobile Phase B = 97.9% ACN, 2% water, 0.1 % formic acid, 5 mM NH_4OAc.
Reverse Phase column: SCIEX ChromXP C18-CL, 3µm, 120Å, 150 x 0.5 mm.
Normal Phase column: YMC Pack NH2 , 3µm, 120Å, 100 x 0.5 mm.
Flow Rate: 20 µL/min on each column, 40 µL/min total column eluent entering the MS.
Column Oven: 40ºC. Injection volume: 1 µL.
Figure 2 shows the dual gradient profiles and settings parameters used on the Micro M3 System.

MS/MS Conditions:
A SCIEX QTRAP® 6500+ system equipped with IonDrive™ source mass spectrometer coupled to a SCIEX M3 MicroLC system was used for this study. A 65 µm ID micro flow electrode was utilized to minimize extra column peak broadening. **Figure 1** shows how the M3 MicroLC system was configured for the multiplexing dual column method. 185 positive mode MRM's and 162 negative mode MRM's were combined into a single pos/neg switching experiment (347 total MRM's) to monitor a wide range of endogenous metabolites (a complete list of all metabolites monitored in this study is available upon request).

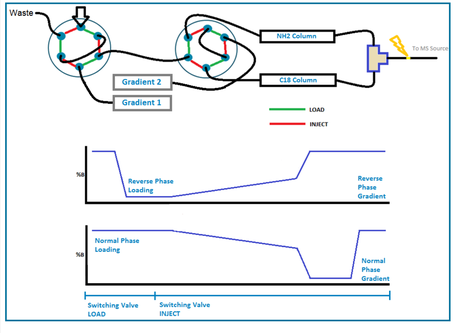

Figure 1 shows how the M3 MicroLC system was configured for the multiplexing dual column method.

Injection 1 (HILIC/NH2 Phase) Injection 2 (Reverse Phase)

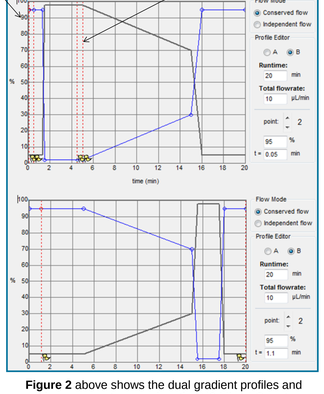

Figure 2 above shows the dual gradient profiles and settings parameters used on the M3 MicroLC system

The autosampler method was programmed to aspirate 6 µL from a single HPLC vial then dispense two staggered 3 µL volumes into a 1 µL loop for injection on each column. The first sample aliquot is dispensed into the loop and injected onto the normal phase column using gradient channel 1. Next, the switching valve turns directing the sample loop output towards the reverse phase column. The second sample aliquot is dispensed into the loop while gradient 1 begins conditioning the reverse phase column with mobile phase A. Gradient 2 is now running mobile phase B through the normal phase column to focus the previous sample injection. The second sample aliquot is injected onto the reverse phase column and both gradients begin simultaneous but opposite elution flow profiles. A timed wait was used to synchronize the sample placement with the injection and switching valve commands. The eluent from both columns is mixed in a ZDV tee prior to entering the mass spectrometer.

Figure 3 The plumbing set up on the M3 MicroLC system

Proposed Benefits

Improved Throughput:
a. Condense Normal and Reverse Phase methods into a single LC/MS analysis run.
b. Eliminate the need to re-configure and equilibrate HPLC column & mobile phase.
c. Extremely low system volume of the M3 MicroLC system reduces long analysis and column equilibration time.

Improved Sensitivity:
a. Performing microbore LC/MS on 0.5mm ID columns promotes higher ion sampling efficiencies and better MS response.
b. Combining the normal and reversed phase HPLC eluent before entering the ESI electrode ensures an ideal mixture of organic solvent and H_20 promoting a more stable and efficient electrospray ionization.

RESULTS

Figures 4,5, and 6 at left top, center, bottom respectively, show examples of the data we obtained using this novel method.

Figure 4 shows the TIC (top pane) of 8 replicate injections of the 2ppm standard. XIC's of Hippuric acid in negative and positive ion modes (center panes). L-Tryptophan in negative and positive ion modes are shown in the bottom panes.

Figure 5 shows the TIC (top pane) of 8 replicate injections of the 2ppm standard. XIC's of L-Leucine and L-Isoleucine in positive and negative modes. As one can see the HILIC column was able to resolve the two compounds, whereas the Reverse Phase C18 column did not resolve them (peak at ~6.0 min vs. the resolved NP (NH2) peaks at ~9.2 and ~9.5 min).

Figure 6 shows the TIC (top pane) of 8 replicate injections of the 2ppm standard. XIC of Tetradecanedioic acid is a good example of the extreme variance of RP vs. NP (NH2) column retention times.

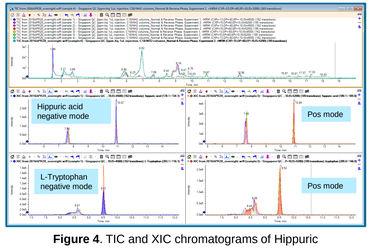

Figure 4. TIC and XIC chromatograms of Hippuric acid and L-Tryptophan.

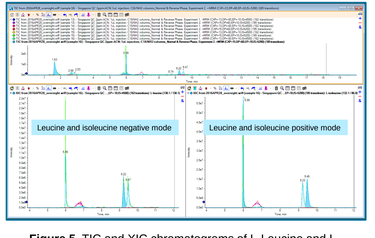

Figure 5. TIC and XIC chromatograms of L-Leucine and L-Isoleucine in positive and negative modes.

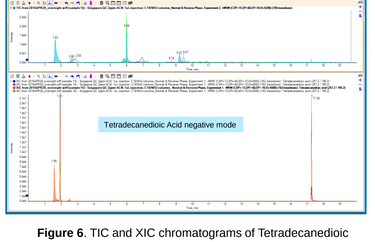

Figure 6. TIC and XIC chromatograms of Tetradecanedioic acid in negative mode.

NEGATIVE MODE transitions observed with significant S/N					
Q1	Q3	dwell (msec)	name	CE	RT
275	97	3	6-phospho-D-gluconate	-13	5.8
179.1	89.1	3	d-glucose	-12	5.84
289	97	3	D-sedoheptulose-1-7-phosphate	-27	5.7
339	97.004	3	fructose-1,6-bisphosphate	-30	5.79
259.01	241	3	glucose-1-phosphate	-16	11.26
178.05	134.06	3	hippuric acid	-25	10.87
186	142.03	3	Indoleacrylic acid	-20	9.51
191.02	117	3	isocitrate	-19	5.34
130.08	130.08	3	L-isoleucine	-10	9.34
130.081	130.081	3	L-leucine	-10	9.2
145.004	101	3	Phenylpropiolic acid	-18	2.11
257.17	195.17	3	Tetradecanedioic acid	-30	17.26
203.08	116.05	3	L-Tryptophan	-30	9.51
180.09	180.09	3	tyrosine	-10	8.96
167.001	124	3	Uric acid	-17	6.41

POSITIVE MODE transitions observed with significant S/N					
Q1	Q3	dwell (msec)	name	CE	RT
67	45	3	1,4-diaminobutane	21	10.02
104	86	3	3-amino isobutanoate	25	5.9
104.01	69	3	4-aminobutyrate	22	10.05
90.1	44.2	3	alanine	13	11.3
159	99	3	allaontoin	11	2.8
118.02	58	3	betaine	36	9.1
104	60	3	choline	21	5.85
114	44.2	3	creatinine	14	9.86
195.0499	195.0499	3	D-glucuronic acid	10	8.45
104.02	58	3	dimethylglycine	21	5.85
154	137	3	Dopamine	16	6.61
323	81	3	dTMP	19	5.76
104.1	86	3	gamma-Aminoisobutyrate	16	9.97
148.1	84.1	3	glutamate	17	5.97
258.1	104	3	Glycerophosphocholine	16	12.92
284.1	135	3	guanosine	20	17.84
180.065	105.03	3	hippuric acid	30	10.88
137	110	3	hypoxanthine	10	6.58
118	91	3	indole	26	9.52
269	137.01	3	inosione	14	7.47
104	58	3	L-alpha-Aminobutyrate	17	5.85
223	149	3	L-Cystathyonine	29	17.33
132.1	86	3	leucine	13	9.23
206	189	3	lipoamide	15	9.5
132.11	86.01	3	L-isoleucine	13	9.47
150.1	133	3	methionine	12	9.56
137.001	94	3	methylnicotinamide	20	6.58
123.1	80	3	nicotinamide	22	6.93
257.25	257.25	3	palmitic acid	10	9.32
130	84	3	Pipecolic acid	18	17.08
116.1	70.1	3	proline	13	10.13
90.04	44.1	3	sarcosine	20	11.29
106	60	3	serine	15	12.31
202.1	129.1	3	spermine	19	16.63
120	74	3	threonine	35	5.92
205	146	3	tryptophan	18	9.5
182.1	77	3	tyrosine	39	10.87
118.1	55.2	3	valine	13	6.01

Table I. Negative mode compounds with MRM transitions, dwell time, collision energies and retention times observed in the human serum samples spiked with 2 ppm standards (shown in green highlight).

Table II. Positive mode compounds with MRM transitions, dwell time, collision energies and retention times observed in the human serum samples spiked with 2 ppm standards (shown in green highlight).

CONCLUSIONS

Here we have demonstrated a true simultaneous dual-column LCMS method can provide data in both reverse phase and normal phase while monitoring several hundred endogenous compounds in both positive and negative ionization modes. Sensitivity and peak shapes are not compromised and the method increases the productivity by factors of 2X-4X over conventional methods requiring separate injections and batch acquisitions.

Proposed Future/Follow Up: This initial investigation showed us a few areas where we would like to improve upon this methodology. We would like to explore injecting less sample volume onto the system. The Micro M3 System combined with the QTRAP® 6500+ system has great sensitivity so lower sample volumes could increase the robustness and use less sample. Internal standards of metabolites with serial dilutions will give us the opportunity to explore statistical analyses as well as absolute quantitation, our next project evaluation.

REFERENCES

1. J.D. Netto, S. Wong, M. Ritchie, *High Resolution Separation of Phospholipids Using a Novel Orthogonal Two-Dimensional UPLC/Q-Tof MS System Configuration*, Waters Application Note, 2013 Waters Corporation. January 2013 720004546EN AG-PDF.

TRADEMARKS/LICENSING

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