Improved Sensitivity, Resolution and Speed for Lipid Profiling using Microflow Liquid Chromatography

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ABSTRACT

To improve the sensitivity, resolution and speed for lipid profiling and quantitation, a high flow LC with high resolution mass spectrometry method was converted to a microflow method where significant sensitivity improvement was observed with an average of 24-fold improvement for monitored internal standards with 1/10 of on column injectoin. Besides the sensitivity gain, better chromatographic resolution, 2-fold throughput improvement and less sample and solvent consumption were also observed.

INTRODUCTION

Lipids are essential for life, playing diverse and important roles in nutrition and health. Alterations in lipid metabolism are associated with various human diseases including obesity, heart disease, and diabetes mellitus[1].

During recent years, advances in liquid chromatography combined with mass spectrometry (LC/MS) technology has resulted in the introduction of useful tools and powerful workflows for the analysis of complex lipid mixtures in various biological samples[2,3].

The key challenges of lipid profiling in biological samples are specificity, sensitivity and speed of analysis. Specificity is critical for lipid profiling especially for individual lipids where isomeric and isobaric species are difficult to separate by chromatography or mass spectrometry alone. Good sensitivity and dynamic range is required for the detection of lower abundant lipids. Speed of analysis is also important in lipidomics; large studies often require a compromise between throughput and lipidome coverage.

Here, the utilization of microflow chromatography for lipid profiling was assessed and compared to the traditional high flow strategy. The sensitivity of detection of low abundant lipids was improved using the microLC 200 system. It was also found that smaller injection volumes of sample results in less frequent instrument cleaning and improved column lifetimes.

MATERIALS AND METHODS

Sample Preparation:
Cold methanol (225 µL) containing a mixture of odd chain and deuterated lipid internal was added to a 20 µL sample aliquot, which was placed into a 1.5 mL Eppendorf tube and vortexed for 10 seconds. Then, 750 µL of cold methyl tert-butyl ether (MTBE) containing CE(22:1) (internal standard) was added, followed by vortexing for 10 seconds and shaking for 6 minutes at 4 ºC. Phase separation was induced by adding 188 µL of MS-grade water. Upon vortexing for 20 seconds the sample was centrifuged at 14,000 rpm for 2 minutes. The upper organic phase was collected in two 300 µL aliquots and evaporated. Dried extracts were re-suspended by vortexing for 10 seconds using 65 µL of a mixture of methanol/toluene (9:1, v/v) containing an internal standard 12-[[(cyclohexylamino)carbonyl]amino]-dodecanoic acid (CUDA). Finally, the sample was centrifuged at 14,000 rpm for 2 minutes prior to LC/MS analysis.

HPLC Conditions:
The separation was performed on an Eksigent ekspert™ microLC 200 system equipped with a high microflow module (20–200 µL/min). Lipids were separated using an Acquity CSH C18 column (1 × 50 mm, 1.7 µm, 130 Å) maintained at 60 ºC, and with a flow rate of 100 µL/min. The injection was 1 µL loop in full loop mode. Mobile phases were: 60:40 acetonitrile:H_2O (A) and 90:10 isopropanol:acetonitrile (B) both with 10 mM ammonium formate and 0.1% formic acid.

MS/MS Conditions:
Lipid analysis was performed on the TripleTOF® 5600 System (AB SCIEX) equipped with the DuoSpray™ Ion Source plumbed with the 65 µm electrode for microflow. The source conditions were optimized for the microflow rates as follows: spray voltage (ISVF) of 5500 V, nebulizing gas (GS1) of 30, heating gas (GS2) of 70, curtain gas (CUR) of 30, and heater temperature (TEM) of 300 ºC.

A TOF survey scan experiment (80 ms) was performed acquiring from m/z 100–1600. Information dependent acquisition (IDA) was used for MS/MS analysis with the top 8 candidate ions collected per cycle. The MS/MS spectra was acquired from m/z 100-1600 at an accumulation time of 30 ms with a collision energy of 45 eV and a collision energy spread of +25 eV.

Automated calibration was performed using an external calibrant delivery system (CDS) which infuses calibration solution prior to sample introduction.

Data Processing:
Data was processed using PeakView® Software.

RESULTS

Method Development
The microflow LC method was built based on a well-established higher flow LC method using a 2.1 × 100 mm (1.7 µm, 130 Å) column at a flow rate of 600 µL/min, maintained at 65 ° C. The high flow separation was performed using an Acquity UPLC system (Waters). Method transfer was simple: the gradient program was proportionally reduced to half the run time with reduced linear mobile phase velocity to improve chromatographic performance and to maintain lower system pressure. The intention was to improve the sample throughput of the method, the chromatographic performance and at the same time to reduce operational costs such as solvent consumption and waste disposal.

The MS acquisition method consisted of both a TOF MS survey scan and information dependent scans with high resolution, accurate mass MS/MS spectra collected at fast cycle times, such that it was compatible with the micro LC speed and high resolution.

High Flow Method

Column:	CSH C18 2.1 × 100mm, 1.7µm
Flow Rate:	600 µL/min
Injection :	5 µL
Gradient:	

Time	%A	B%
0	85	15
2	70	30
2.5	52	48
11	18	82
11.5	1	99
12	1	99
12.1	85	15
15	85	15

Micro Flow Method

Column:	CSH C18 1.0 × 50mm, 1.7µm
Flow Rate:	100 µL/min
Injection :	1 µL* (5 µL sample overload 1uL loop)
Gradient:	

Time	%A	B%
0	85	15
1	70	30
1.3	52	48
5.5	18	82
5.8	1	99
6	1	99
6.1	85	15
7.5	85	15

Method Performance
With a microflow approach, the overall sensitivity for lipid species was significantly improved. Firstly, the extracted ion chromatograms (XIC) of all used internal standards (ISTD) from both regular flow and microflow methods were compared (data not shown here). The average intensity improvement was ~24X across the 13 lipid internal standards. The total chromatography run time was reduced from 15 minutes to 7.5 enabling higher sample throughput. Also worth noting is that the amount injected on column was reduced with the lower flow rate strategy where only 1/10 of the sample was loaded on-column compared with the regular flow method. Finally, at 100 µL/min, only 750 µL of solvent was used for the microflow method, as opposed to the 9000 µL of solvent consumed with high flow, a reduction in mobile phase consumption by nearly 91%.

To compare in detail these improvements, several internal standards were randomly selected based on their retention times and ion chromatograms were extracted (XICs) from TOF MS spectra (Figure 1). Overall the peak widths observed using the microflow LC method (0.6 to 1.62 seconds PWHM) was on average half of those observed with high flow method (1.56 to 3.96 seconds PWHM). The XIC peak intensity improvement observed for all 13 internal standards (22 different adducts) ranged from 2 to 117 fold.

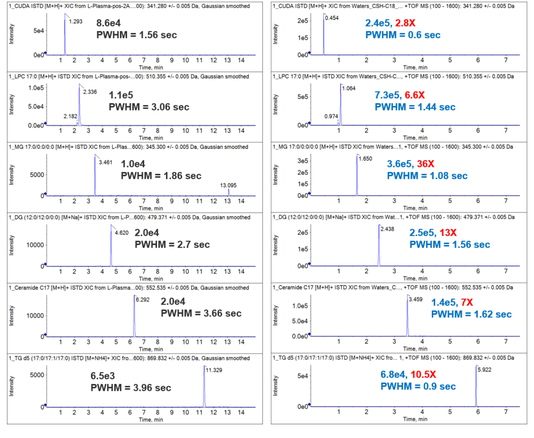

Figure 1. Comparison of Microflow and Regular Flow LC Performances.

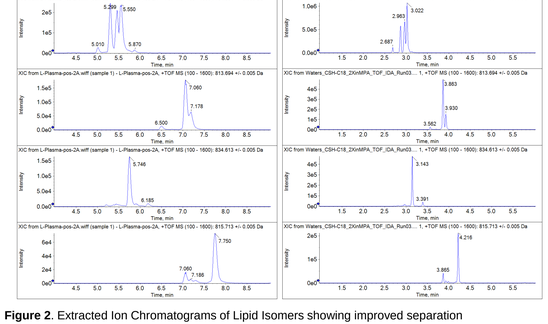

Figure 2. Extracted Ion Chromatograms of Lipid Isomers showing improved separation

Besides the significantly improved peak shape and sensitivity, the chromatographic resolution was also noticeably better with microflow, as seen in Figure 2. With improved separation and higher resolution this enabled more confidence in identification and better quantitation of the lipid species.
For different species/adducts identified for the same lipid analyte, different intensity improvements may be observed, and the extent of improvements usually followed the order: ammonium adduct > protonated molecule > sodium adduct.

Good reproducibility is another key requirement for lipidomics research, both for retention time and for peak intensity. Retention time can also be used for analyte identification confirmation when reproducibility is solid. Excellent reproducibility was observed with the microflow method for retention time, chromatographic resolution and MS signal intensity (Figure 3). An overlay of 5 repeated injections showed very good reproducibility. For the 22 internal standard adducts monitored, the %CV for retention time ranged from 0.01% to 0.18%, and the %CV for peak area ranges from 2.9% to 18%.

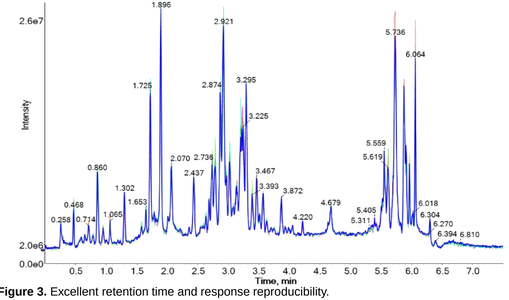

Figure 3. Excellent retention time and response reproducibility.

CONCLUSIONS

The work here describes the use of microflow LC with high resolution, accurate mass spectrometry for fast lipid profiling. Here, the Eksigent ekspert™ microLC 200 system coupled with the TripleTOF® 5600 system provided a powerful solution, with an average of 24X improved sensitivity with only 1/10 of the injected amount, improved chromatographic resolution for confidence in identity confirmation and 2 × improved throughput, as well as more than 90% savings on solvent consumption.

REFERENCES

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TRADEMARKS/LICENSING