Impact of Chromatography on Lipid Profiling of Liver Tissue Extracts

Application Note

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Introduction

Lipidomics is a growing research area with a keen focus on characterizing and quantifying the diverse array of lipids within biological systems. The ability of mass spectrometry to successfully detect and identify the potentially thousands of lipid species within a given sample will directly hinge on the quality of lipid LC separation strategies.

Liver, brain, and cardiac tissue extracts are common biological samples used in lipidomics research. To demonstrate the impact of chromatography on lipidomics profiling, this Application Note summarizes the advantages of using reverse phase (RP) and normal phase (NP) chromatography for separating different classes of lipids using liver extract as an example.

This work was performed on an Agilent UHPLC 1290 system consisting of a binary pump, well plate autosampler, and thermostatted column compartment. The LC was coupled to an Agilent 6540 Q-TOF with an Agilent Jet Stream interface. The tissue extracts were separated by both RP and NP chromatography.

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Methods

Chromatography

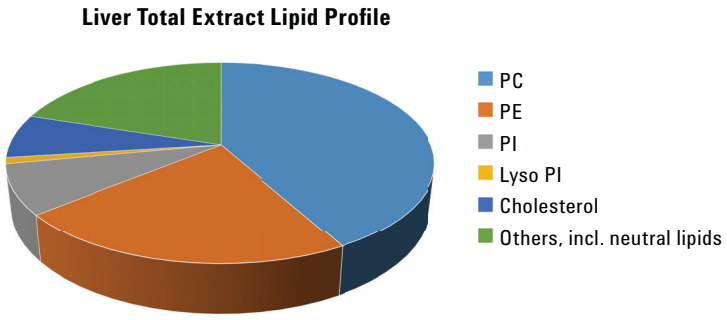
Reverse phase LC conditions	
Column	Agilent ZORBAX Eclipse Plus RRHD C18, 2.1 × 100 mm, 1.8 µm (p/n 959758-902)
Column temperature	50 °C
Injection volume	5.00 µL
Autosampler temperature	4 °C
Needle wash	15 seconds in wash port (50:50 methanol/isopropanol)
Mobile phase	A) 5:1:4 IPA/MeOH/H_2O with 5 mM NH_4OAc and 0.1 % CH_3COOH B) 99:1 IPA/H_2O with 5 mM NH_4OAc and 0.1 % CH_3COOH
Flow rate	0.350 mL/min
Gradient program	Time B (%) 0.00 0 3.00 0 5.00 20 25.00 30 35.00 95 36.00 95 38.00 0
Stop time	38 minutes
Post time	3 minutes

Normal phase LC conditions	
Column	Agilent ZORBAX Rx-Sil, 2.1 × 100 mm, 1.8 µm (p/n 828700-901)
Column temperature	25 °C
Injection volume	5.00 µL
Autosampler temperature	4 °C
Needle wash	15 seconds in wash port (50:50 methanol/isopropanol)
Mobile phase	A) 58:40:2 IPA/hexane/H_2O with 5 mM NH_4OAc and 0.1 % CH_3COOH B) 50:40:10 IPA/hexane/H_2O with 5 mM NH_4OAc and 0.1 % CH_3COOH
Flow rate	0.300 mL/min
Gradient program	Time B (%) 0.00 0 5.00 0 34.00 100 36.00 100 38.00 0
Stop time	38 minutes
Post time	5 minutes

Mass spectrometry

Agilent 6540 Q-TOF with Dual Agilent Jet Stream Source	
Instrument mode	2 GHz, extended dynamic range, *m/z* 1,700
Polarity	Positive or negative
Gas temperature	300 °C
Drying gas (nitrogen)	11 L/min
Nebulizer gas	35 psi
Sheath gas temperature	300 °C
Sheath gas flow	12 L/min
Capillary voltage	3,500 V (+), 3,000 V (–)
Nozzle voltage	0 V
Fragmentor	150 V
Oct 1 Rf Vpp	750 V
Acquisition speed	MS-only: 1 spectrum/second (MS) Auto MS/MS: 3 spectra/second (MS), 3 spectra/second (MS/MS)
Auto MS/MS parameters	Isolation width: Narrow (~1.3 amu) Collision energy: 20 eV
Reference correction	2 points at *m/z* 121.050873 (+), *m/z* 922.009798 (+) 2 points at *m/z* 112.985587 (-), *m/z* 980.016375 (-)

Distribution of lipid classes extracted from liver tissue



- Total lipid liver extracts were purchased from Avanti Polar Lipids (Alabaster, AL) with the lipid profile shown above
- Dried lipid extracts were reconstituted in 2:1 chloroform/methanol and further diluted in mobile phase A to a concentration of 200 ng/µL
- Injections of 5 µL (1 µg extract) were analyzed by reverse or normal phase LC/MS (see Methods section)

The first step in data analysis for profiling workflows is to extract molecular features from the results where features are defined by retention time and mass. A feature condenses the abundances from all the specified adducts and isotopes of a compound into a single compound. Molecular features were extracted with Agilent MassHunter Qualitative Analysis (Version B.07.00) using the Agilent Molecular Feature Extractor (MFE) algorithm, and lipids were annotated using the Agilent METLIN Lipids PCDL (B.07) as well as SimLipid software (Version 4.20, PREMIER Biosoft, Palo Alto, CA). The METLIN Lipids PCDL contains 640 MS/MS spectra acquired from chemical standards. SimLipid supports MS/MS theoretical matching of lipid spectra and contains 36,224 lipid entries.

Results and Discussion

A liver lipid extract separated with RP chromatography was analyzed with the MFE algorithm, resulting in 4,108 extracted compounds (features). The compounds were searched with the METLIN database, resulting in more than 1,000 compounds with lipid annotations from a single 38-minute LC/MS analytical run (Figure 1).

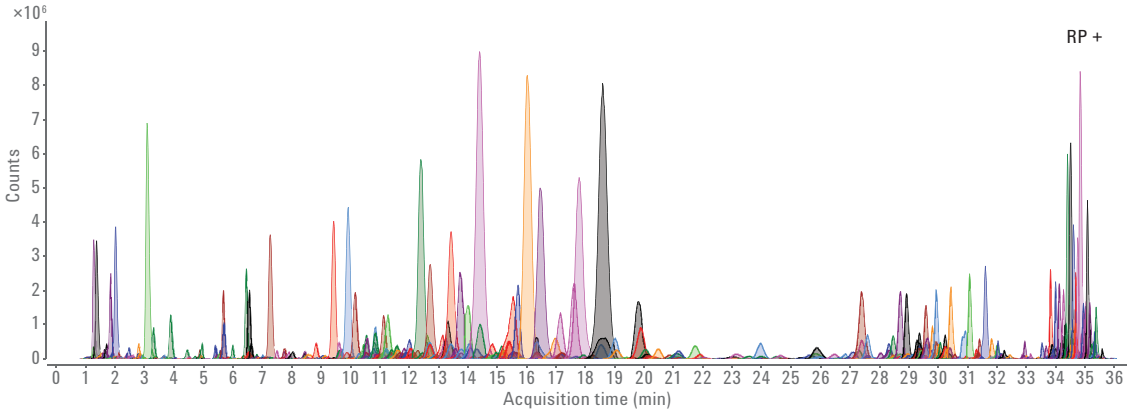


Figure 1. Overlaid extracted compound chromatograms (ECCs) corresponding to 1,061 annotated compounds with lipid annotations from a single LC/MS run.

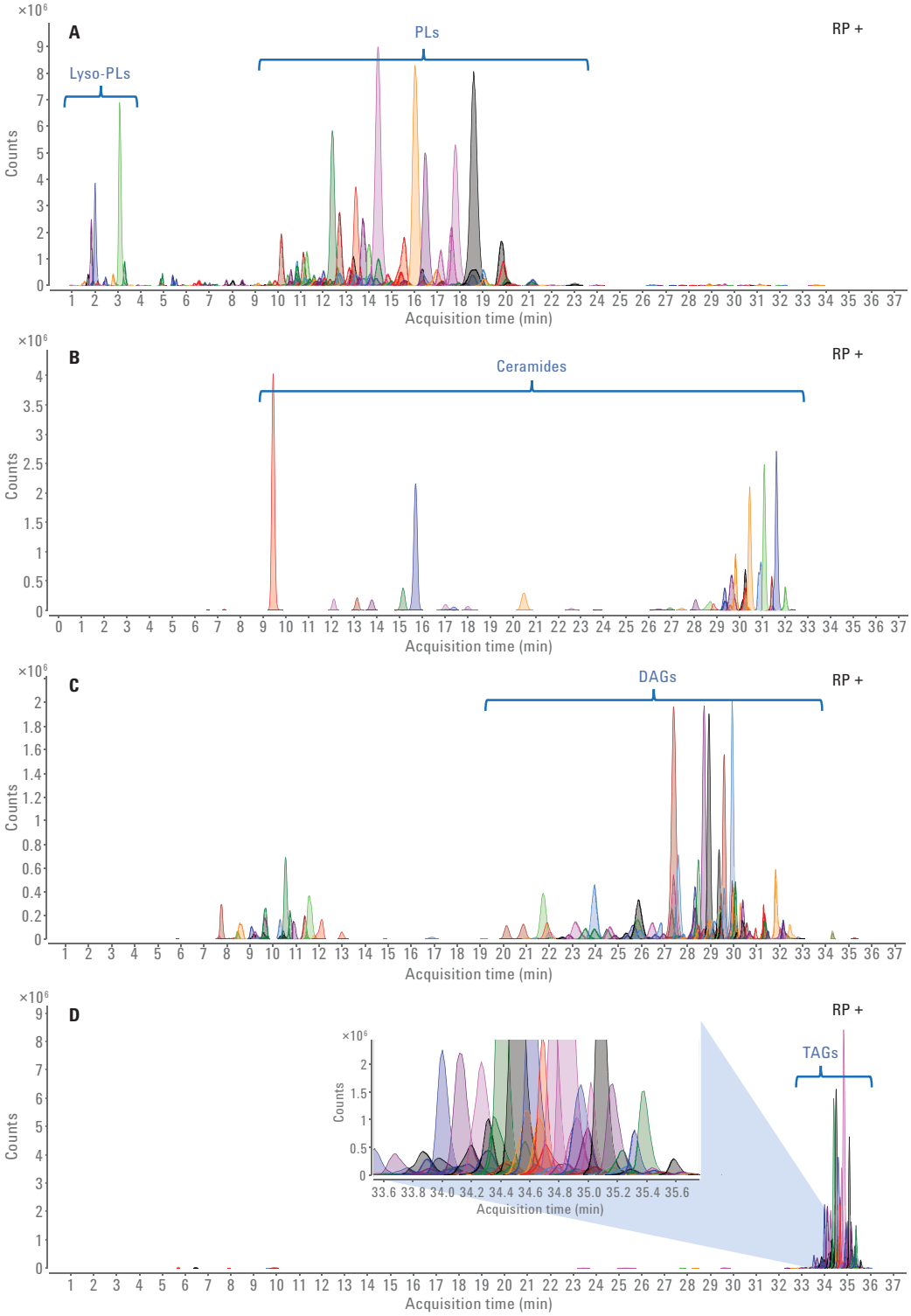


Figure 2. Examples of elution profiles of major annotated lipid classes from a single liver extract data file (RP, positive mode). A) Overlaid ECCs of 431 annotated phospholipids (PLs) and lyso-PLs; B) Overlaid ECCs of 48 annotated ceramides; C) Overlaid ECCs of 172 annotated diacylglycerols (DAGs); D) Overlaid ECCs of 123 annotated triacylglycerols (TAGs). A zoomed view of the 34–36 minute window demonstrates the advantage of using UHPLC to separate and annotate coeluting TAGs, as well as the dynamic range of detection of these coeluting TAGs.

In contrast to RP chromatography, where separation of lipids is based on lipophilic character primarily dictated by carbon chain length and the number of double bonds, NP chromatography primarily separates based on the polar nature of the lipid headgroup. Figure 3 demonstrates that NP chromatography was very effective in resolving relatively polar and charged lipids. However, neutral lipids such as sterols, DAGs, and TAGs were missing as they could not be chromatographically retained with these mobile phases. For the compounds that could be detected, a targeted database search based on user knowledge of lipid class behaviors is possible, and can lead to increased confidence in lipid annotation. For example, we could constrain the search to only PE lipids by querying compounds eluting within a confined retention time window and searching only PE entries with either a PE subset of the METLIN Lipid PCDL or a tailored SimLipid search.

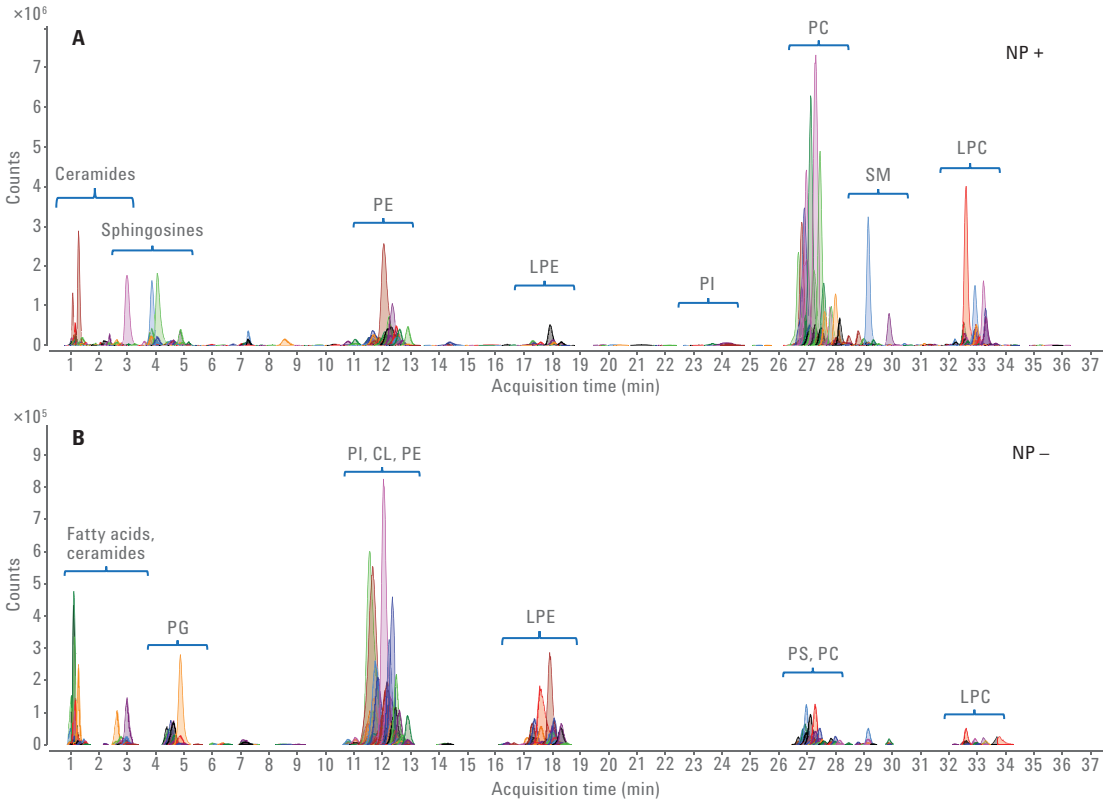


Figure 3. Elution profiles of lipid classes from a liver lipid extract resolved with NP chromatography and analyzed in positive and negative ion modes. CL: cardiolipin; LPC: lysophosphatidylcholine; LPE: lysophosphatidylethanolamine; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PG: phosphatidylglycerol; PI: phosphatidylinositol; PS: phosphatidylserine; SM: sphingomyelin.

Table 1 provides database search results comparing data files from the same liver extract acquired with differing chromatographies and ionization polarities. Data files acquired with MS1-only level data were processed with the MFE algorithm. The resulting compounds were annotated with the METLIN Lipids PCDL, or alternatively annotated with SimLipid software. For high-confidence lipid annotation, auto-MS/MS data files were also processed with the MFE algorithm, but only compounds that contained MS/MS data were used for database querying. Compounds were annotated either with library matching using METLIN Lipids PCDL or annotated with SimLipid based on MS/MS-only level data. It is important to note that in contrast to the METLIN PCDL format, which contains high-quality curated library spectra acquired from authentic chemical standards, SimLipid annotation is based on MS/MS pattern matching against theoretical *in-silico* generated MS/MS fragmentation patterns.

The examples above illustrate that chromatographic methods can be optimized to target different classes of lipids, and that RP and NP LC/MS each provide selective advantages in lipidomics analysis. RP LC/MS is more comprehensive, resolving many different lipid classes and resulting in a larger number of detected and annotated features. In addition, retention times are very reproducible, facilitating chromatographic alignment and differential profiling workflows. NP LC/MS also offers advantages, enabling more confident lipid annotations from the characteristic retention behavior of polar lipid classes. In addition, users can quickly assess gross lipid class differences between samples from chromatograms.

Data file type	MFE results no. of features	METLIN Lipids PCDL no. of annotated features	SimLipid 4.20 no. of annotated features
Reverse phase (+) MS only	4,108	1,061	1,172
Reverse phase (+) Auto-MS/MS	972 (612)	5	226
Reverse phase (–) MS only	1,399	586	430
Reverse phase (–) Auto-MS/MS	278 (247)	0	65
Normal phase (+) MS only	2,281	568	644
Normal phase (+) Auto-MS/MS	774 (308)	6	141
Normal phase (–) MS only	561	309	250
Normal phase (–) Auto-MS/MS	195 (126)	1	72

( ) Parentheses denote only compounds with MS/MS spectra and are used for querying.

Table 1. Summary of lipid annotation results from single liver lipid extract LC/MS data files comparing chromatographic methods (RP/NP), ionization modes (+/–), and MS data types (MS only/Auto-MS/MS). A mass match tolerance of 5 ppm was used for database searching. Note that for each annotated compound, there was often more than one possible matching lipid annotation.

Conclusions

The ability to comprehensively detect and profile a wide diversity of lipid classes from a complex biological sample was demonstrated. We have shown that the combination of lipid-specific UHPLC methods and an accurate-mass Q-TOF possessing a wide dynamic detection range, with advanced feature-finding software that enabled and automated lipid database searching, enabled the detection and annotation of over 1,000 lipids in a single LC/MS run. Chromatographic method development can be optimized to target different classes of lipids, as illustrated by liver extracts resolved by two alternative LC separation methods: reversed-phase and normal phase. Each provides selective advantages in terms of lipidomics analysis, resulting in deeper, more comprehensive lipid detection coverage.

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