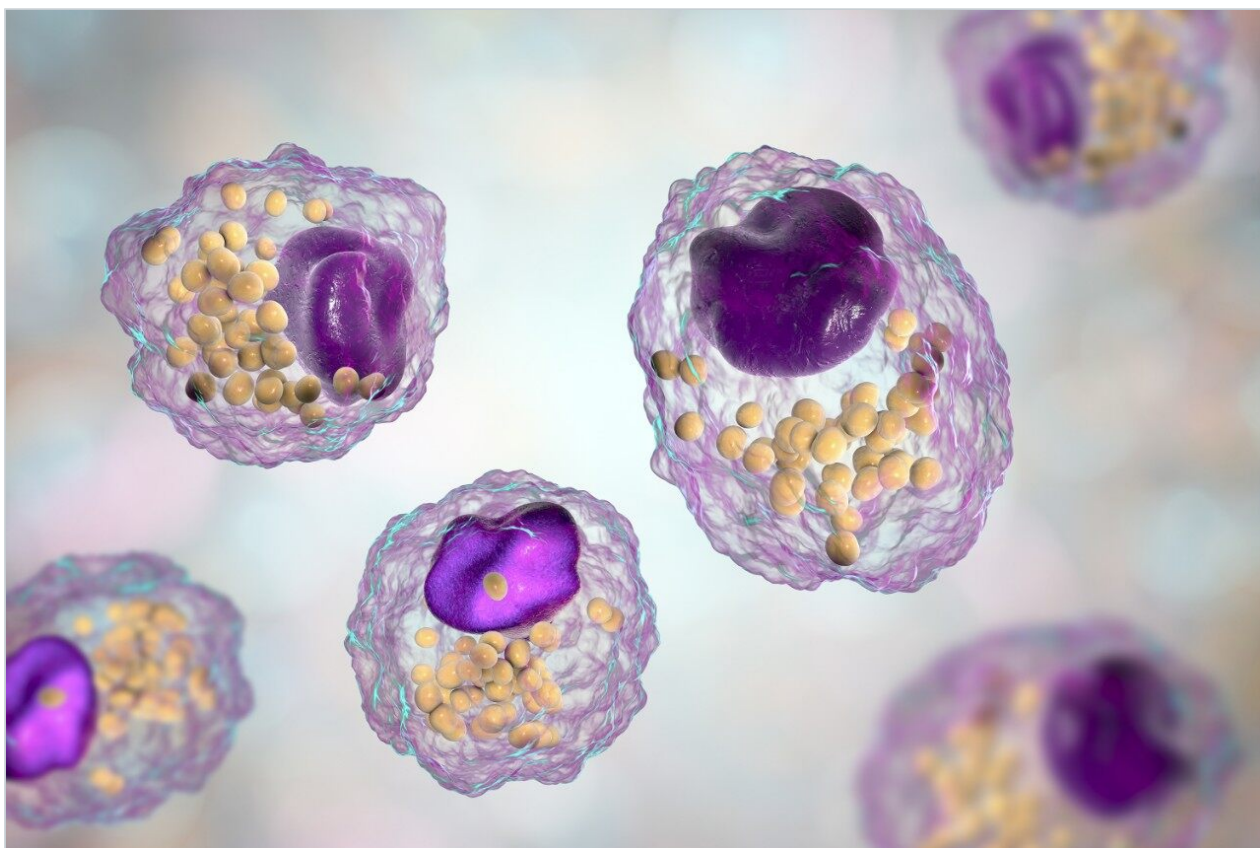


Lipid Class Separation Using UPC²/MS

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Abstract

A flexible universal method was developed for the analysis of inter-class separations of neutral and amphipathetic lipids. Since the goal of the experiment is to achieve flexible parameters providing the separation of lipids by class, the core methodology using the ACQUITY UPC² BEH column, methanol/acetonitrile modifier with ammonium formate as an additive provided the best results.

Benefits

- Provides ACQUITY UPC² methodology conditions for comprehensive lipid intra- and inter-class analysis
- Highlights the method parameters that aid as method development tools for comprehensive lipid profiling
- Provides a single separation technique and a single instrumentation approach for rapid lipids analyses

Introduction

The analysis of complex lipids has historically been a challenging task and may require a variety of analytical techniques. Lipids are generally recognized as hydrophobic compounds, but the properties of complex lipids containing phosphorus, sulfur, sugar, and nitrogen have a wide polarity range. Developing separation-based methodology utilizing a single technique is recognized as a common challenge for scientists researching lipidomic and metabolomic applications.

Recent advances in technology have revived the exploration of supercritical fluid chromatography (SFC) as a viable analytical technique. Research and development focused on improvements of SFC instrumentation has provided a holistically designed chromatographic system that utilizes liquid CO₂ as a mobile phase to leverage the chromatographic principles and selectivity of normal phase chromatography while providing the ease-of-use of reversed phase LC (RPLC). This new separation technique is referred to as UltraPerformance Convergence Chromatography (UPC²).

In this application note, UPC² technology is implemented for the analysis of lipid class separation. Method variables influencing the peak integrity and chromatographic separation for a mixture of lipids with different degrees of polarity are explored. The experiments were designed to understand the chromatographic behavior of lipids in a controlled setting using a variety of lipid extracts. Acyl chain length and a number of double bond influences were investigated using single moiety standards. The methodology parameters were

tested using lipid extracts composed of intra-class components. The method conditions are applied to biological lipid extracts, whereas method adjustments are investigated to manipulate the chromatography based on the goal of the analyst. Insights from these method variable manipulations help to scope the development of targeted lipid profiling and screening protocols.

Experimental

Lipid abbreviations

CER	Ceramides
SM	Sphingomyelin
PG	Phosphatidylglycerol
PE	Phosphatidylethanolamine
PC	Phosphatidylcholine
LPC	Lyso-Phosphatidylcholine
LPE	Lyso- Phosphatidylethanolamine

Sample description

Samples and standards were purchased from Avanti Polar Lipids. Mix 1 and Mix 2 were brain (porcine) extracts except LPC and PG which were egg (chicken) extracts. Stocks were prepared in 50:50 chloroform/methanol. Working lipid mixtures were prepared to the specified concentration.

Mix 1:	Ceramide, SM, (0.05 mg/mL) PG, PE, PC, (0.1 mg/mL)
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Mix 2: LPC, LPE, (0.05 mg/mL)

Mix 3: 1:1 of [mix 1] and [mix 2]

UPC² conditions

System: ACQUITY UPC²

Columns: ACQUITY UPC² BEH and HSS C₁₈ SB

Column temp.: 60 °C

Sample temp.: 10 °C

Injection volume: 1 µL

Flow rate: 1.85 mL/min

Back pressure: 1500 psi

Mobile phase A: CO₂

Mobile phase B: 50:50 methanol/acetonitrile with 1 g/L ammonium formate

Gradient: Refer to figures for detailed information

MS conditions

Mass spectrometers: ACQUITY SQD and SYNAPT G2 MS

Ionization mode: ESI positive

Acquisition range :	100 to 1500 Da
Capillary voltage :	3.5 kV
Cone voltage :	30 V
Informatics:	Empower 3 and MassLynx Software

Results and Discussion

MS optimization

The ACQUITY UPC² System was configured with an ACQUITY single quadrupole detector (SQD) for this preliminary investigation of chromatographic parameters. Injections of [Mix 3] were screened with different cone and capillary voltages to determine the best operating conditions. Based on the cone voltage screening results, 30 V was chosen for controlling overall in-source fragmentation of the optimal signal for the spectra of L- α -phosphatidylglycerol (egg PG) to provide precursor ion and valuable fragmentation information, as shown in Figure 1A. The optimal capillary voltage observed for all the peaks in the [Mix 3] was 3.5 to 4.0 kV, as shown in Figure 1B. The final MS conditions used 3.5 kV capillary voltage and 30 V cone voltage.

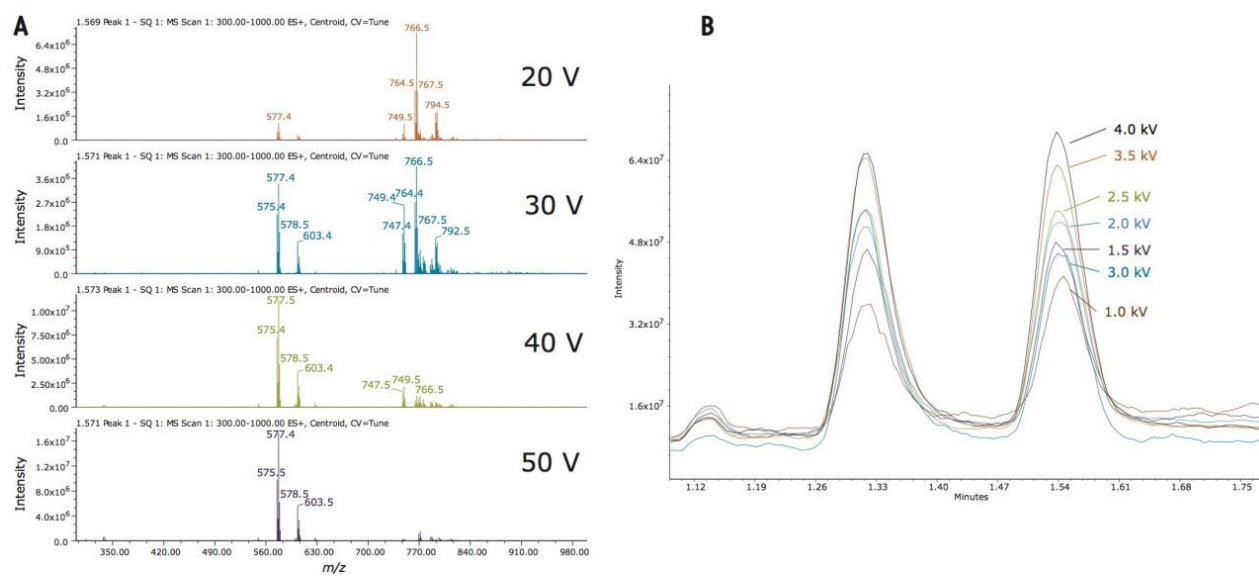


Figure 1. (A) Cone voltage screening results for L- α -phosphatidylglycerol; and (B) capillary voltage screening results for L- α -phosphatidylglycerol.

Chromatographic method development

The sample preparation workflow was very convenient for use with the ACQUITY UPC² System. The chloroform/methanol diluent provided good solubility without any noticeable adverse effects on peak shape. In a typical RPLC lipid analysis, the organic extract containing the lipids would have to be evaporated and re-constituted in a more compatible solvent. When using UPC², however, the organic extract containing the lipids can be directly injected onto the system, thereby saving time and costs when analyzing hundreds of biological samples. Screening different column stationary phases typically changes selectivity during method development. Therefore, our initial approach was to screen three stationary phases including UPC² CSH Fluoro-Phenyl, UPC² BEH 2-EP, and UPC² BEH. Optimal peak shape and selectivity was achieved on the UPC² BEH stationary phase for the inter-class separation of the lipid mixture, as shown in Figure 2.

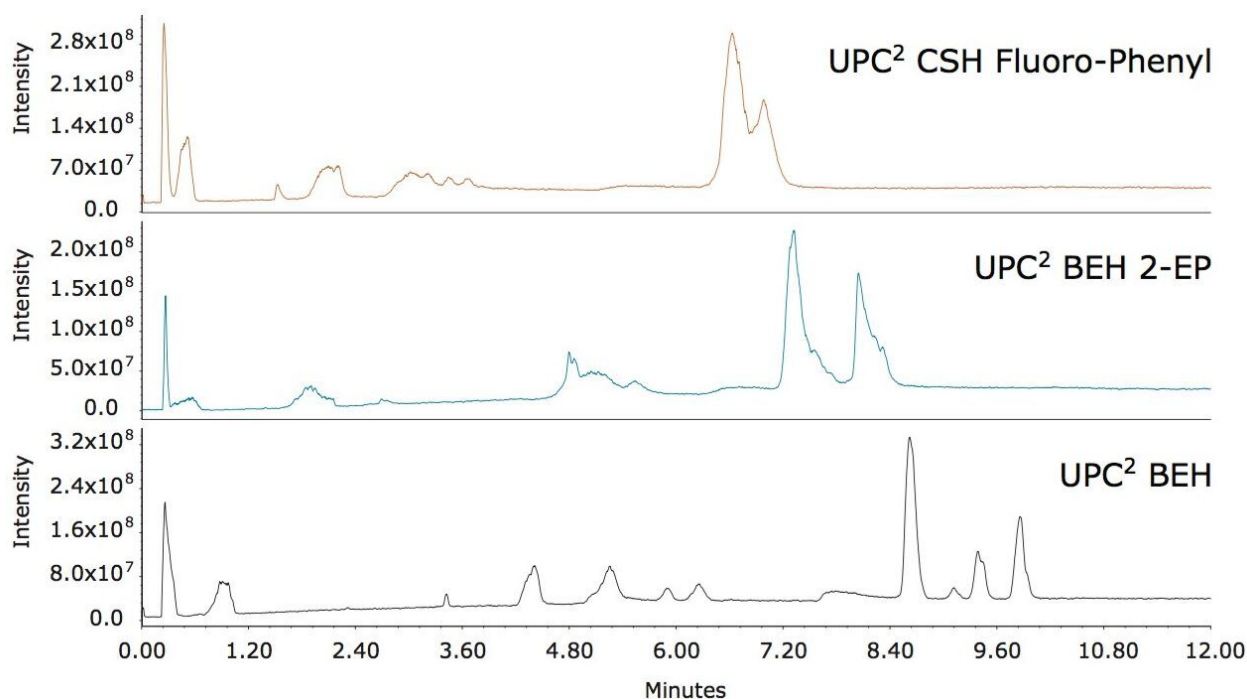


Figure 2. Column screening of the [metdev] mixture of neutral and polar lipids. A 12-minute 10% to 50% B screening method was used. Due to less retentivity of the BEH 2-EP and CSH Fluoro-Phenyl columns, the gradients were modified (10% to 30% B) to adjust for comparative use of the separation space. The mobile phase co-solvent was 1 g/L ammonium formate in MeOH derived from previously published reports.¹

Method development screening columns and modifier indicated the bridged ethylene hybrid (BEH) silica columns provided the best selectivity and peak shape. The lipid classes were identified by MS ESI+ utilizing the parameters determined by the MS optimization experiments. Injections of the individual lipid mixtures verified current peak assignments. The method used for screening the columns was optimized to focus on the inter-class separation of analytes in [Mix 3]. The previous 12-minute method was reduced and completed in 5 minutes, as shown in Figure 3.

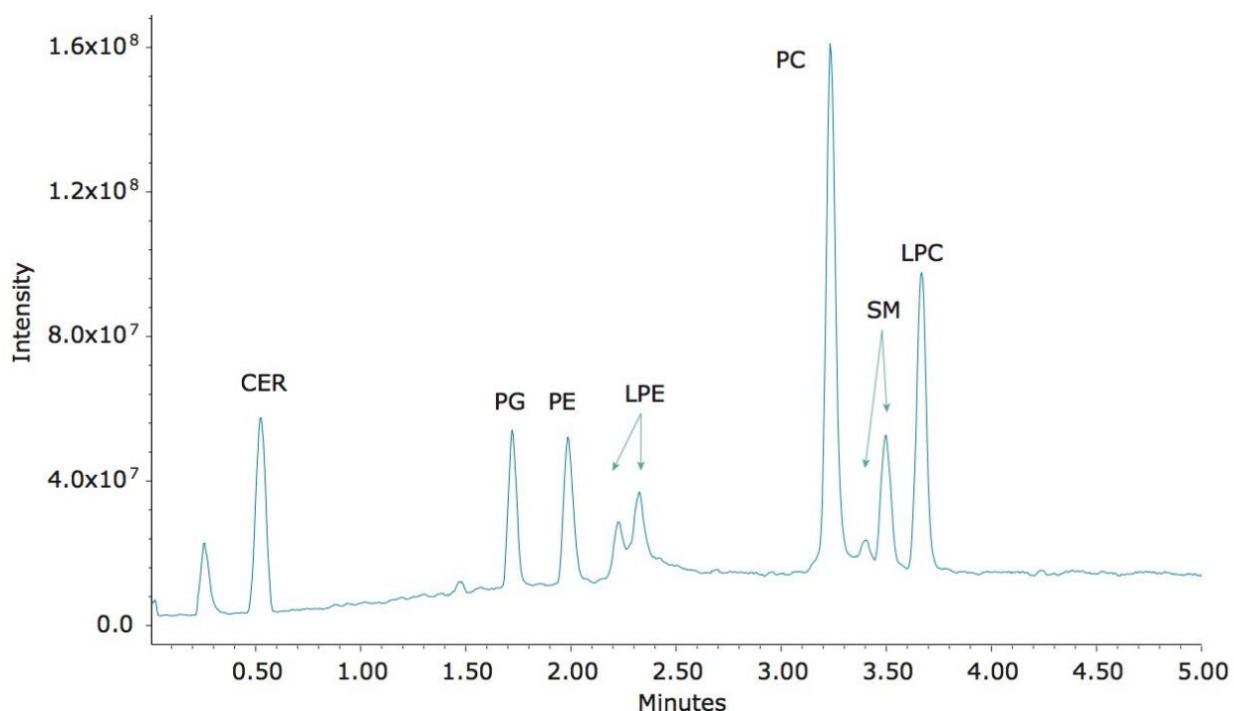


Figure 3. Injection of [Mix 3] mixture using the ACQUITY UPC² BEH column. The original gradient was 5% to 50% at 1.85 mL/min over 10 min with a 2 min hold at 50% B and 1-min re-equilibration at initial conditions before the next injection. The gradient was modified to 15% to 50% B at 1.85 mL/min over 3 min, held at 50% B for 2 min. The column was re-equilibrated at initial conditions before the next injection.

The lipid extracts purchased from Avanti provide documentation indicating various ratios of lipid intra-class constituents present within a respective standard extract. This was further confirmed when evaluating the mass spectral data for the two LPE peaks observed in chromatographic trace, as shown in Figure 4. It was found that the elution order of the molecular species within each lipid class depended on the number of double bonds on the acyl chain. Thus, the more saturated the acyl chain, the shorter the retention time. The acyl chain length has no effect on the elution order with each lipid class. Interestingly, the intra-class separation of LPE and SM standard extracts is observed when using the UPC² BEH stationary phase. The other stationary phases that provided broader peaks may offer a better intra-class separation of the Avanti extract standards. This hypothesis will be explored in future studies.

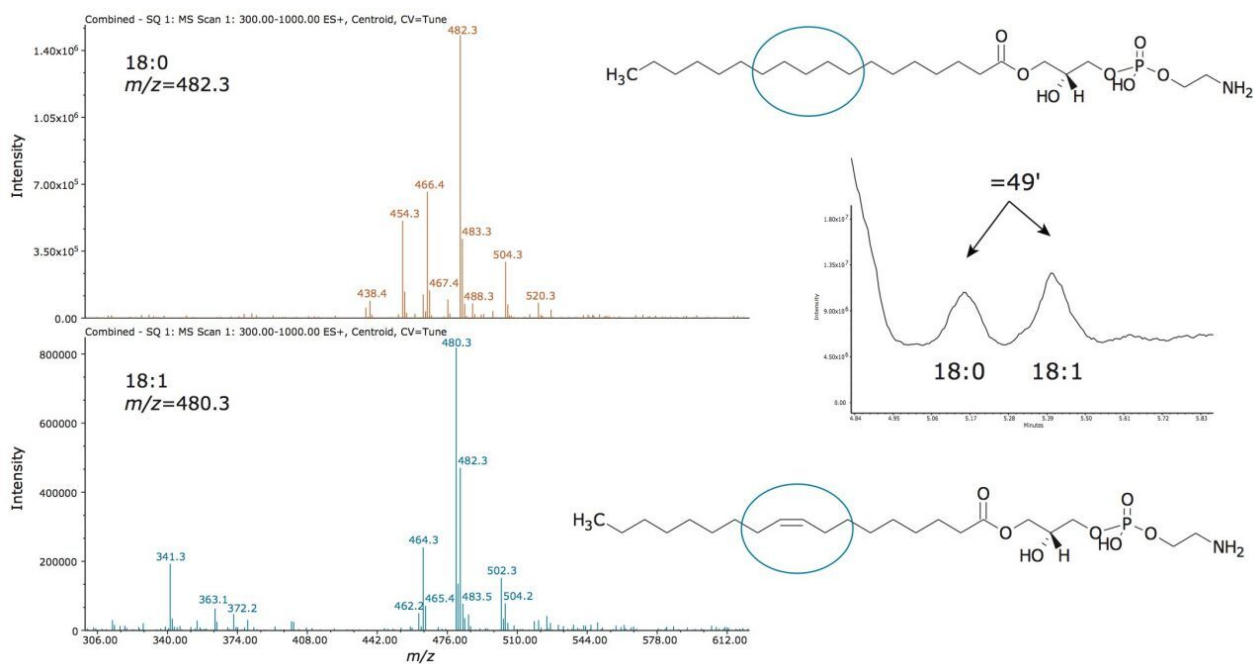


Figure 4. Interrogation of MS spectra for the two LPE peaks.

Manipulating retentivity

The method development process explored changes in gradient slope; however, changes in resolution were not significant. Experiments to manipulate retentivity were investigated. The variable which resulted in the greatest retentivity changes included the addition of a less polar solvent, such as acetonitrile, to the modifier. Two experiments were performed. The first was conducted with the original modifier composition of 100% methanol. The second experiment was conducted with modifier composition of 80% methanol and 20% acetonitrile. Both modifiers were doped with 1 g/L ammonium formate. In general, using acetonitrile in the modifier improved resolution, as shown in Table 1.

Peak ID	100% MeOH (RS)	80:20 MeOH:ACN (RS)	% Difference
PG	—	—	—
PE	2.69	3.54	+31.6
LPE 1	1.96	2.74	+39.8
LPE 2	1.67	2.17	+29.9
PC	10.81	8.56	-20.8
SM (1)	2.24	2.07	-7.6
SM (2)	1.10	1.06	+45.5
LPC	1.33	1.05	+12.8

Table 1. Impact of modifier composition on lipid resolution. Ceramide was excluded due to the large amount of resolution from the other peaks of interest.

Analysis of biological samples

The methodology was used to investigate two biological samples, each for distinctly different purposes. The first example examines a mouse heart extract using the six-minute method for the rapid inter-class targeted screening of lipids with different polarity. The objective was to determine if the methodology could detect the presence of specific lipids within the hundreds of samples collected from the diabetic treatment study. This proof of concept approach will be used for further studies utilizing MS/MS quantification assessing increases of phospholipids and sphingolipids during treatment. As seen in Figure 5, many of the phospholipids and sphingolipids were easily identified in the mouse heart extract by using this targeted UPC²-MS method.

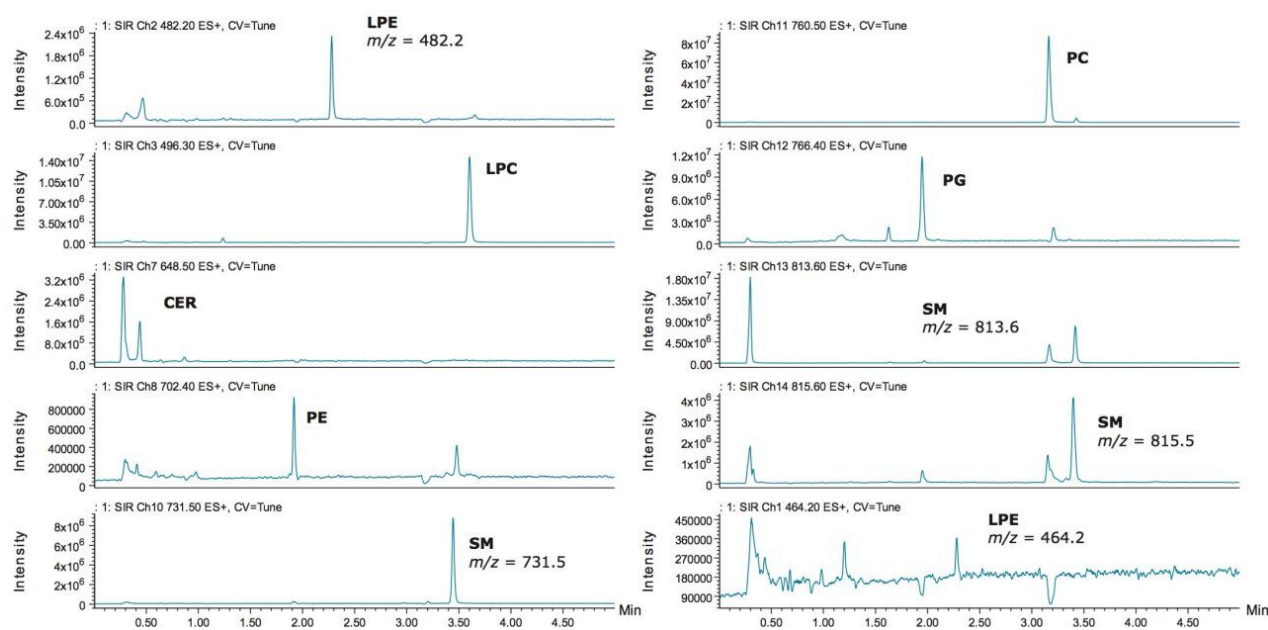


Figure 5. Targeted SIR analysis of a mouse heart extract. The gradient was 15% to 50% B at 1.85 mL/min over 3 min, held at 50% B for 2 min. The column was re-equilibrated at initial conditions for 1 min before the next injection.

Neutral lipids, such as acylglyceride and cholesterol esters, are characteristically non-polar. Typically, plant metabolomic profiling distinguishes heterogeneous distribution of neutral lipids, such as triacylglycerols and diacylglycerols² at different conditions. The example in Figure 6 investigates lipids present in a cotton seed oil extract. The data was collected using MS^E on a SYNAPT G2 Mass Spectrometer allowing for the characterization of the neutral lipids by precursor and product ion alignment. During the method development process, many neutral lipids eluted near the chromatographic void when starting the compositional gradient above 5% modifier using methanol (or methanol/acetonitrile). In this example, the UPC²-MS method was modified to retain the neutral lipids by reducing the starting percentage of modifier. The modifier was ramped to elute the polar lipids, known to be present as membrane lipid classes in cotton embryos.² By using this approach, the chromatography can be altered to provide greater retention, and often greater specificity for the neutral lipids. From an analytical technique perspective, the elution mechanisms are conceptually similar to performing a mobile phase gradient elution profile by liquid chromatography.

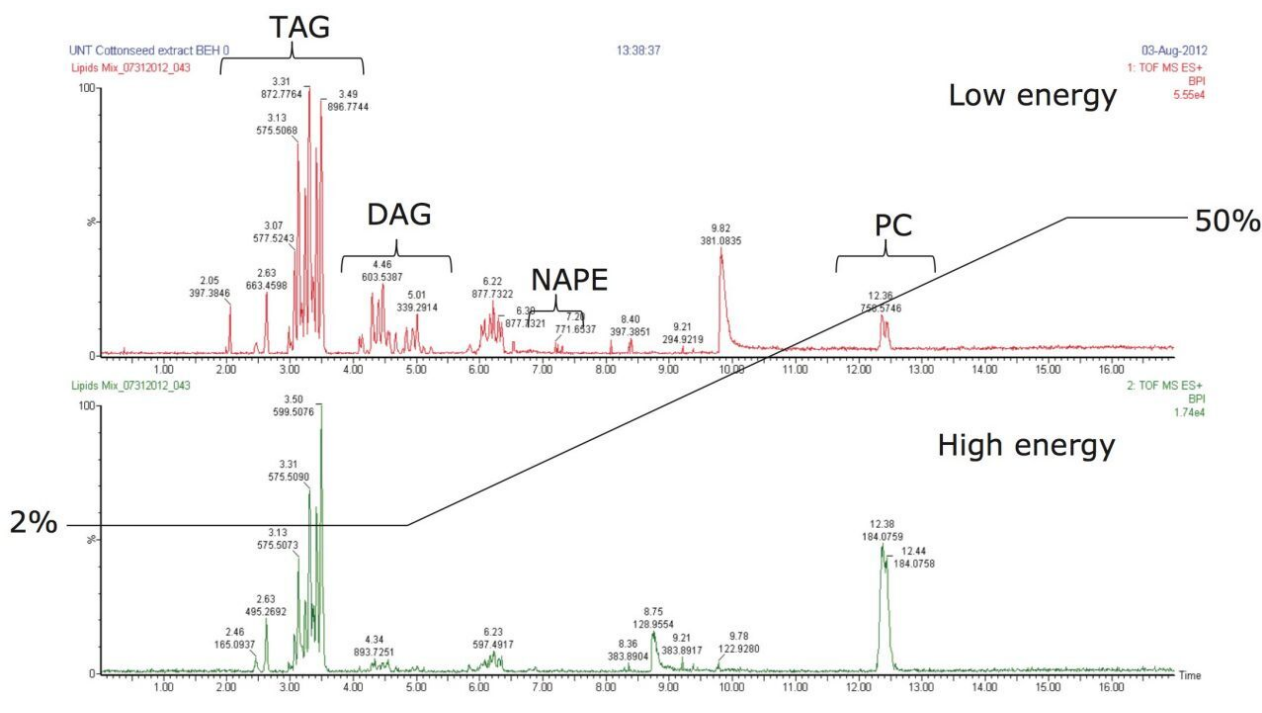


Figure 6. Comprehensive profiling of a cotton seed oil extract. The gradient was 2% to 5% B over 5 min, increased to 50% B over 10 min, and held at 50% B for 2 min using a flow rate of 1.85 mL/min. The column was re-equilibrated at initial conditions for 1 min before the next injection.

Conclusion

A flexible universal method was developed for the analysis of inter-class separations of neutral and amphipathetic lipids. Since the goal of the experiment was to achieve flexible parameters providing the separation of lipids by class, the core methodology using the ACQUITY UPC² BEH column, methanol/acetonitrile modifier with ammonium formate as an additive provided the best results. The gradient methodology and run time was adjustable to focus on rapid screening or comprehensive profiling of lipids in biological samples. The lipid sample preparation workflow is suitable for UPC². The organic phase of the biological lipid extract can be directly injected onto the ACQUITY UPC² System with MS detection, thereby saving time and operating costs. The rapid inter-class screening provided an analysis within seven minutes including re-equilibration. The MS spectral information confirmed instances of intra-class separation by distinguishing between the degrees of saturation, as demonstrated for the Lyso- Phosphatidylethanolamine separation on the ACQUITY UPC² BEH column. Resolution can be increased within the chromatographic

space by the addition of acetonitrile. For this methodology, an increase of 30% to 40% resolution can be observed between lipid classes for the majority of the analytes. The method development knowledge gained from these experiments build a foundation for the applicability of lipid analysis by convergence chromatography.

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 2. Horn PJ, Korte AR, Neogi PB, et al. Spatial Mapping of Lipids at Cellular Resolution in Embryos of Cotton. *Plant Cell.* 2012 Feb; 24(2): 622-636.
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