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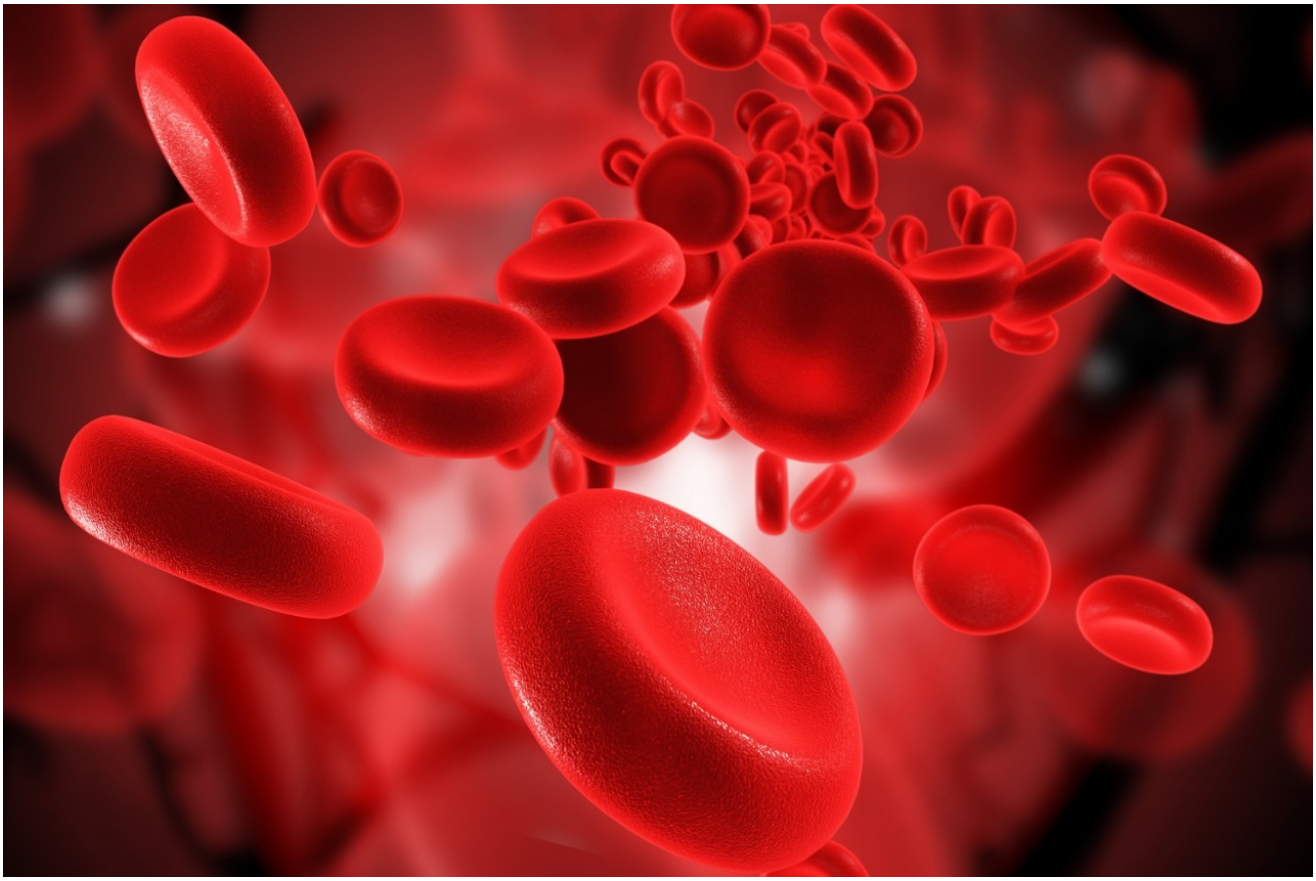
應用手冊

High Resolution Separation of Phospholipids Using a Novel Orthogonal Two-Dimensional UPLC-QTof MS System Configuration

Jeremy Dietrich Netto, Stephen Wong, Mark Ritchie

Waters Corporation



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Abstract

In this application note, we present a novel configuration of the Waters ACQUITY UPLC System with 2D Technology. This minimally modified system is able to utilize the advantages of both HILIC and reversed phase methods in tandem to provide a truly orthogonal and high resolution separation of amphiphilic lipids. We demonstrate the application of 2D UPLC-MS for the analysis of lipids in human plasma as this biofluid is a highly complex matrix with large lipid diversity covering many orders of concentration, thus presenting an analytical challenge when analyzed using traditional single dimensional liquid chromatography.

Benefits

- Improved peak capacity by using multiple columns for better characterization of complex mixtures such as lipids
- Low abundance components are chromatographically separated from high abundance components allowing high confidence in identification and accurate quantification due to the removal of isotopic interferences
- Ability to use combinations of different chemistries, such as IEX and SEC, for truly orthogonal 2D LC separations

Introduction

Lipids play many important roles in maintaining homeostasis of living organisms. These include energy storage, maintaining structural integrity of cell membranes, and acting as signaling molecules. Understanding these lipids may provide insights into mechanisms of disease, including the identification of biomarkers and potential drug targets. Lipids can be either hydrophobic or amphiphilic in nature, with phospholipids being the latter comprised of a hydrophilic phosphate head group and a lipophilic diglyceride tail.

The chemical behavior of amphiphilic lipids has led to the adoption of three main techniques for analysis by liquid chromatography including reversed phase, normal phase, and HILIC separation sciences. While reversed phase chromatography separates these lipids based on their lipophilicity (alkyl chain length and/or degree of saturation), it does not show class distinction especially between classes such as the phosphotidylcholines and sphingomyelins. Normal phase and HILIC chromatography, on the other hand, provide a separation based on the lipid's head group polarity but provides little separation within the given class.

In this application note, we present a novel configuration of the Waters ACQUITY UPLC System with 2D Technology. This minimally modified system is able to utilize the advantages of both HILIC and reversed phase methods in tandem to provide a truly orthogonal and high resolution separation of amphiphilic lipids. We demonstrate the application of 2D UPLC-MS for the analysis of lipids in human plasma as this biofluid is a highly complex matrix with large lipid diversity covering many orders of concentration, thus presenting an analytical challenge when analyzed using traditional single dimensional liquid chromatography.

Experimental

UPLC conditions

System:	ACQUITY UPLC with 2D Technology
First dimension column:	ACQUITY BEH HILIC 2.1 x 100 mm, 1.7 µm
Second dimension column:	ACQUITY CSH C_{18} 2.1 x 100 mm, 1.7 µm
Trap column:	ACQUITY UPLC BEH C_8 VanGuard 130Å 2.1 x 5 mm, 1.7 µm

Alpha pump

Mobile phase A:	95% ACN 5% H_2O 10 mM NH_4Ac (pH 5.0)
Mobile phase B:	50% ACN 50% H_2O 10 mM NH_4Ac (pH 5.0)
UPLC flow rate:	0.5 mL/min

Gradient:

Time(min)	%A	%B
Initial	100	0
10.0	80	20
10.1	20	80
13.0	20	80
13.1	100	0
16.0	100	0

Beta pump

Mobile phase A:	40% ACN 60% H_2O 10 mM NH_4Ac (pH 5.0)
Mobile phase B:	10% ACN 90% IPA 10 mM NH_4Ac (pH 5.0)
UPLC flow rate:	0.5 mL/min

Gradient:

Time(min)	%A	%B
Initial	100	0
Fraction Elute (FE)*	100	0
FE + 0.10	60	40
FE + 20.0	0	100

Time(min)	%A	%B
FE + 23.0	0	100
FE + 23.1	100	0
FE + 25	100	0

**Fraction Elute (FE) time will vary according to the RT of the lipid class of interest.*

Column Manager (CM-A)

First dimension column temp.:	30 °C
Second dimension column temp.:	65 °C

Valve Events Table:

Time (min)	Left valve	Right valve
0	Position 2	Position 2
Fraction Trap	Position 1	Position 1
Fraction Elute (FE)	Position 2	Position 2
FE + 25	Position 1	Position 1

MS conditions

Mass spectrometer:	Waters Xevo G2 QTof

Acquisition mode:	ESI +ve / -ve, MS^E
Capillary voltage:	2.0 kV (+ve) / 1.00 kV (-ve)
Sampling cone:	35.0 V
Extraction cone:	4.0 kV
MS collision energy:	4.0 V
MSE energy ramp:	20 to 45 V
Source temp.:	120 °C
Desolvation temp.:	500 °C
Desolvation gas flow:	1000 L/h
Cone gas flow:	10 L/h
Acquisition range:	*m/z* 50 to 1200
Lock mass (LeuEnk):	+ve, *m/z* 556.2771 and 278.1141 -ve, *m/z* 554.2615 and 236.1035

Sample description

Lipids were extracted from human plasma using the Waters Ostro sample preparation method.[1]

2D UPLC flow diagram

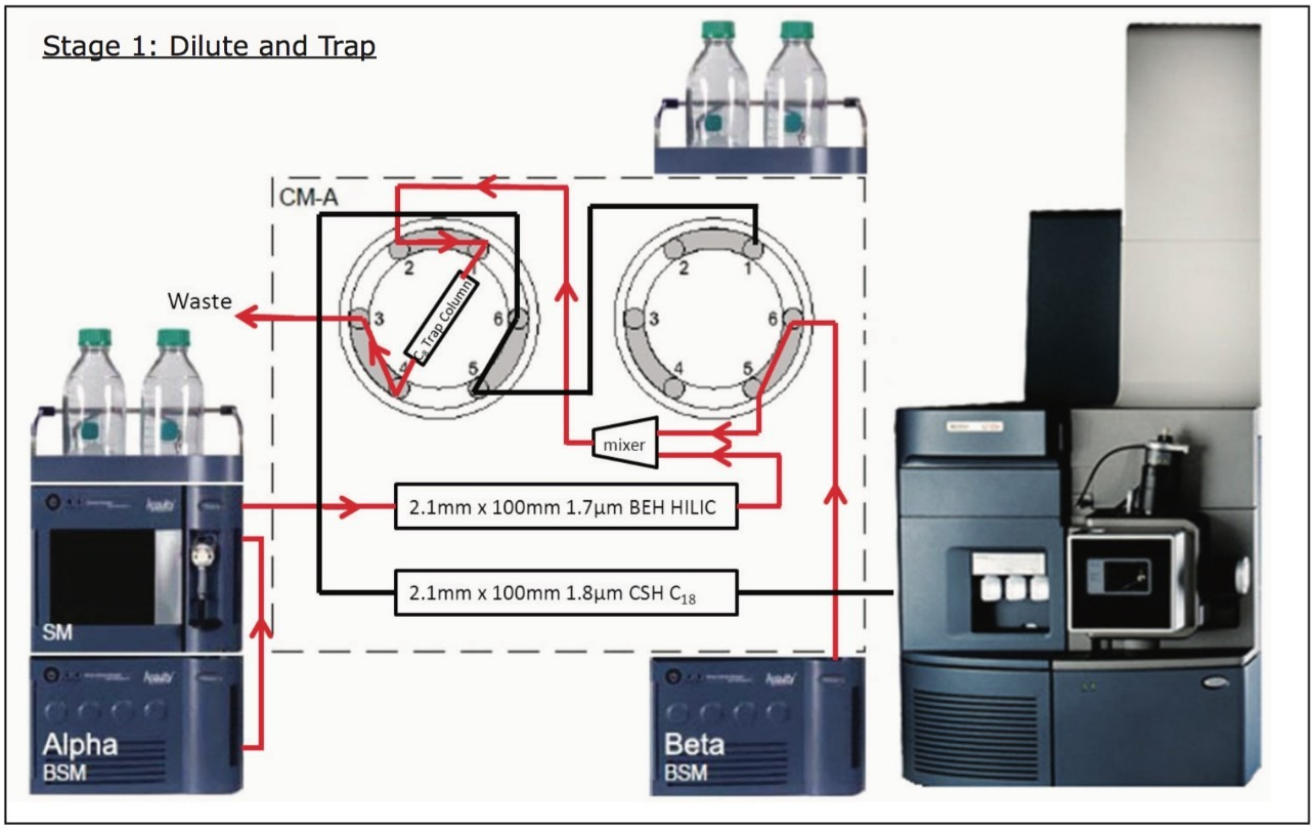

Figure 1. Flow diagram of the 2D UPLC-MS system showing stage 1 where the left and right valves are both in position 1, and trapping analytes eluting off the HILIC column is performed.

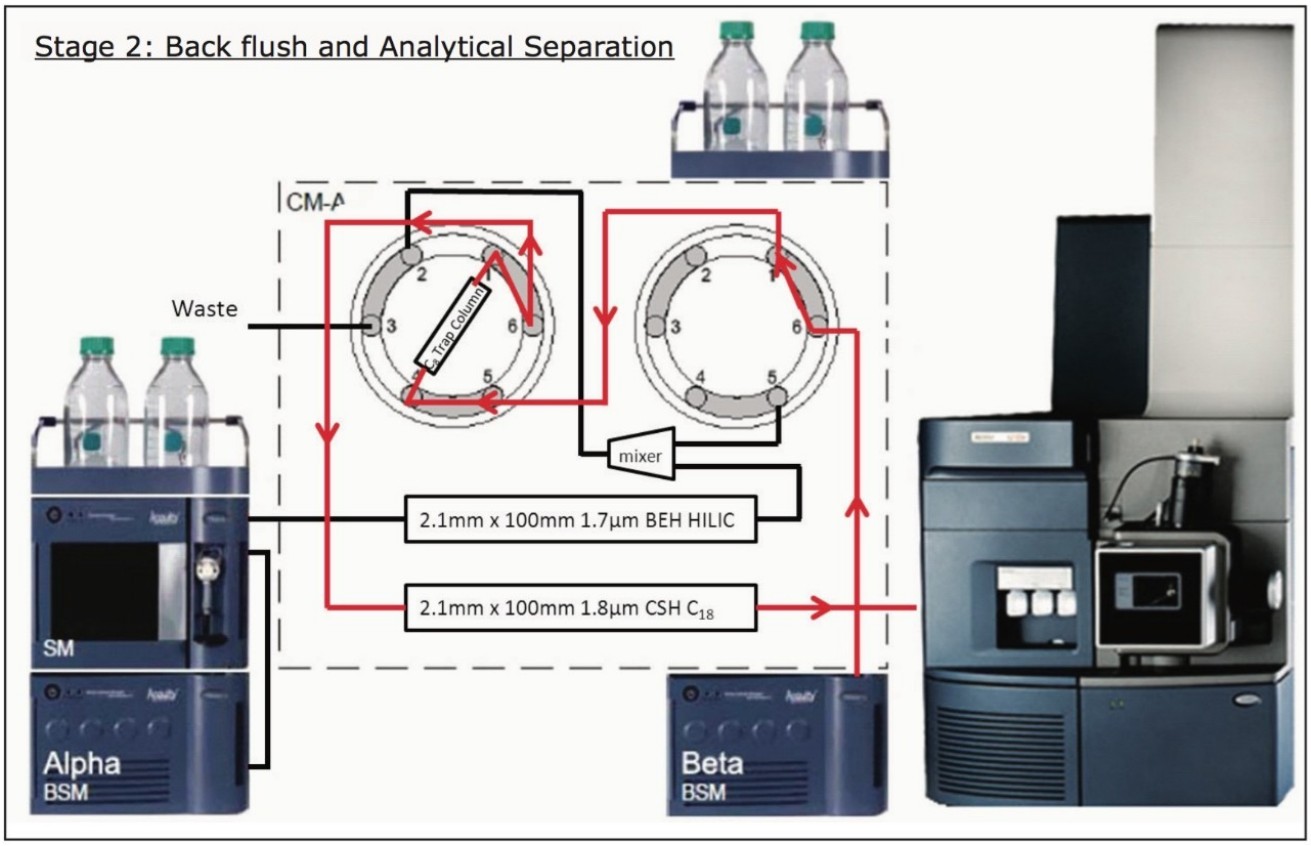

Figure 2. Flow diagram of the 2D UPLC-MS system showing stage 2 where the left and right valves are both in position 2 when the trapped analytes are back-flushed off the trapping column, and sent to the C_{18} column for reversed phase separation.

Results and Discussion

Comparison of separation between 1D and 2D UPLC-MS

By combining the orthogonality of the HILIC and reversed phase separations into a single tandem 2D UPLC-MS method, we overcame the challenges of inter-class co-elution posed by reversed phase liquid chromatography, as shown in Figure 3, between the PC and SM classes. The coelution of these two classes are particularly problematic, as they have only a single dalton difference between them, and co elution makes identification and accurate quantification difficult due to isotopic interferences.

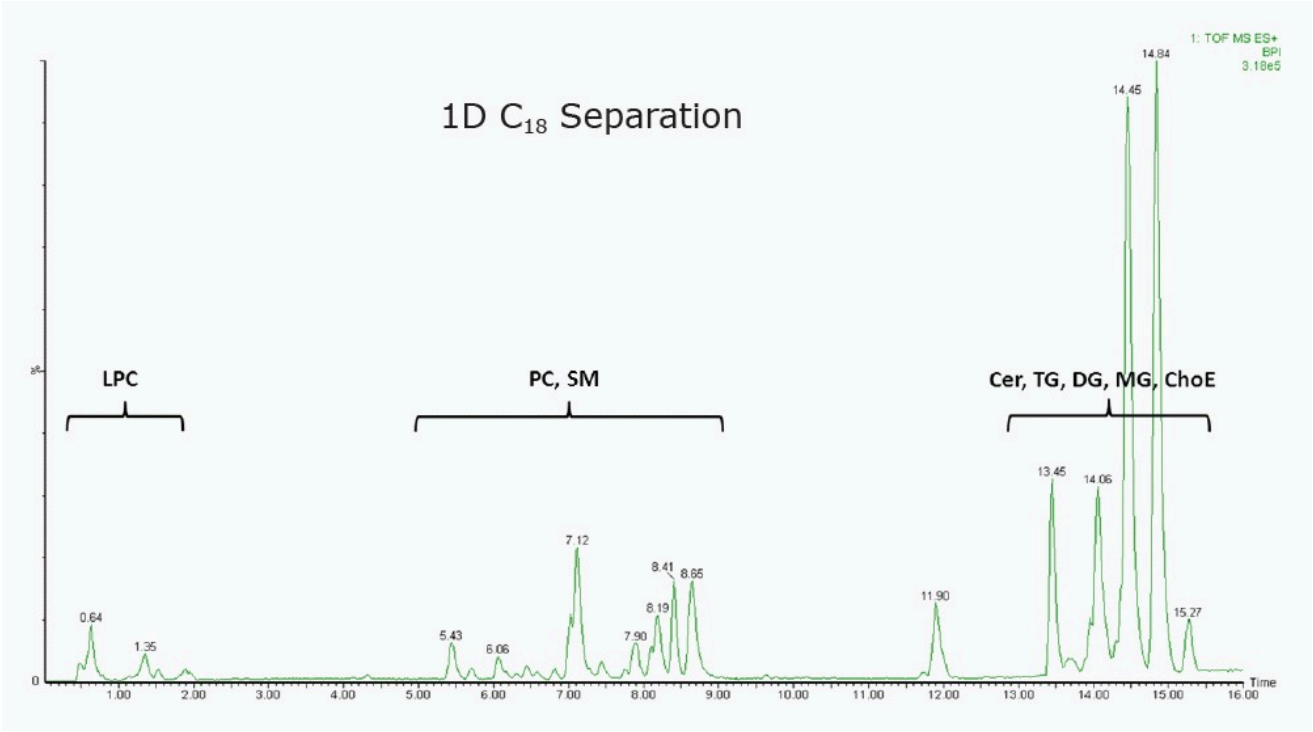

Figure 3. Chromatogram showing single-dimensional reversed phase separation of plasma lipids. SMs co-elute with PCs making identification and quantification difficult due to 1Da difference between species of the two classes.

In the HILIC method, inter-class separation is eliminated, as shown in Figure 4, with the PCs and SMs well separated. However, there is now intra-class co-elution which affects the peak capacity and, hence, the sensitivity of the method. In the 2D UPLC-MS method there was improved resolution of the individual lipids of each class using the PC and SM classes as examples, as shown in Figure 5.

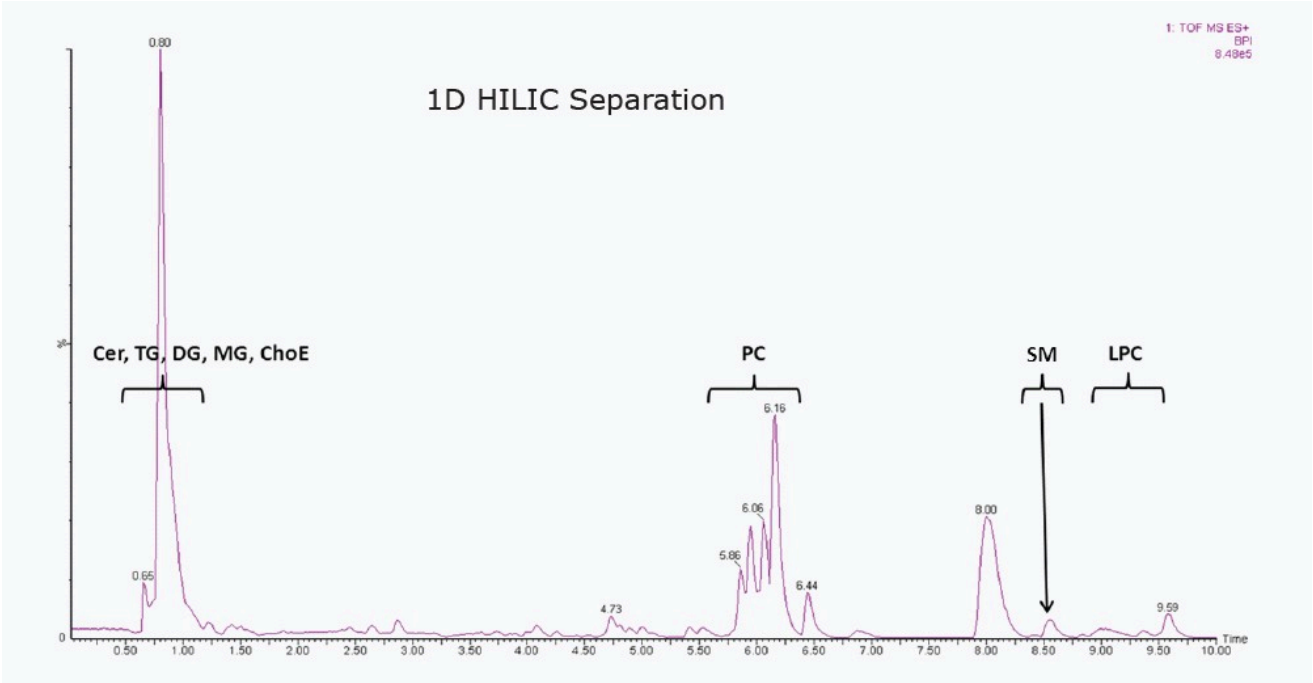

Figure 4. Chromatogram showing single-dimensional HILIC separation of plasma lipids. SMs are separated from PCs but individual species within each class co-elute, leading to poor resolution and dynamic range.

The increased peak capacity from the use of combined columns improved both the resolution, shown in Figure 5, as well as the dynamic range of the individual lipids within each class. Using the PC class as an example, the SimLipid (Premier Biosoft) lipid identification software was able to detect and identify 37% more PCs than the HILIC method, and 40% more PCs than the reversed phase C_{18} method. Generally, the ion intensities for the 2D UPLC-MS method were higher than the HILIC or C_{18} methods for the same samples run, which could be attributed to the removal of isotopic interferences.

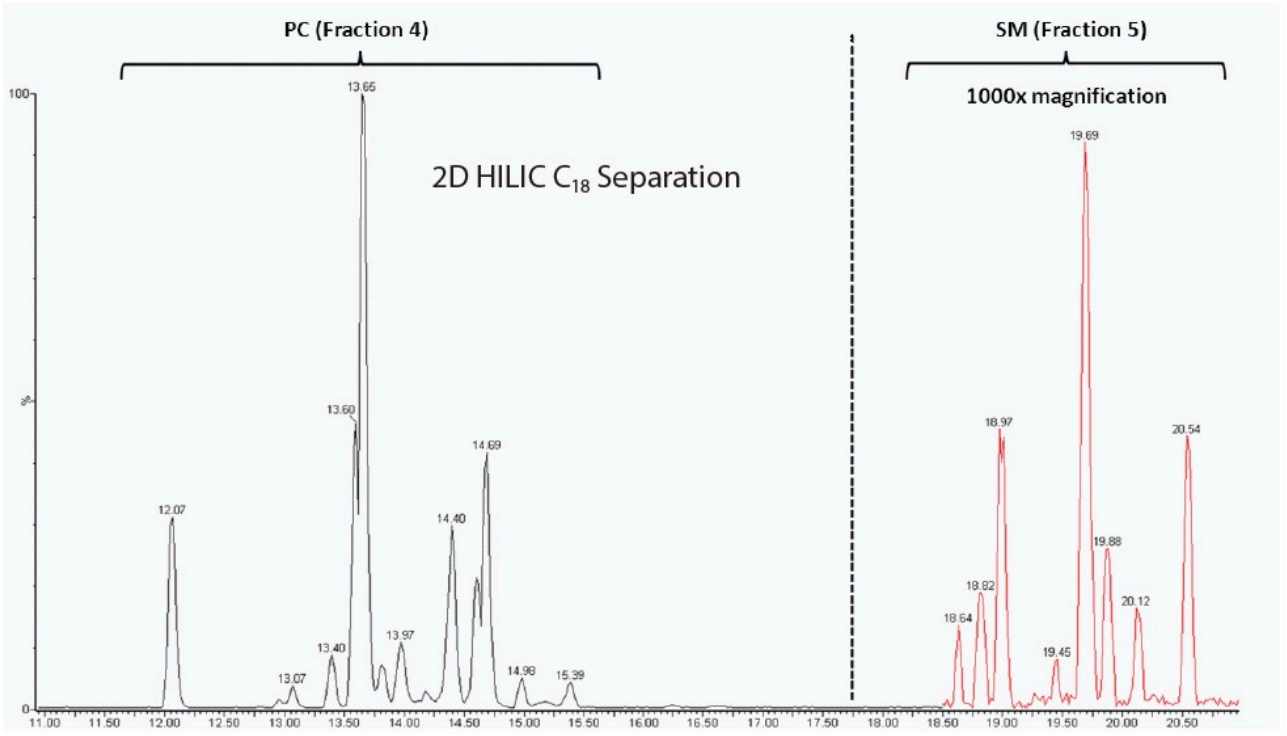

Figure 5. Overlay of fractions from two-dimensional separation of plasma lipids. SMs are now class separated from the PCs by HILIC, and the individual lipids within each class are separated further by reversed phase.

Predictable separation of the 2D UPLC-MS method

Since the second dimension of separation is reversed phase, the lipids were able to be further separated chromatographically, according to their hydrophobicity (alkyl chain length and degree of saturation), as shown in Figure 6. This was more advantageous than the HILIC method; whereby, residual isotopic interferences due to the co-elution could affect the confidence level for positive identification and quantification of these lipids.

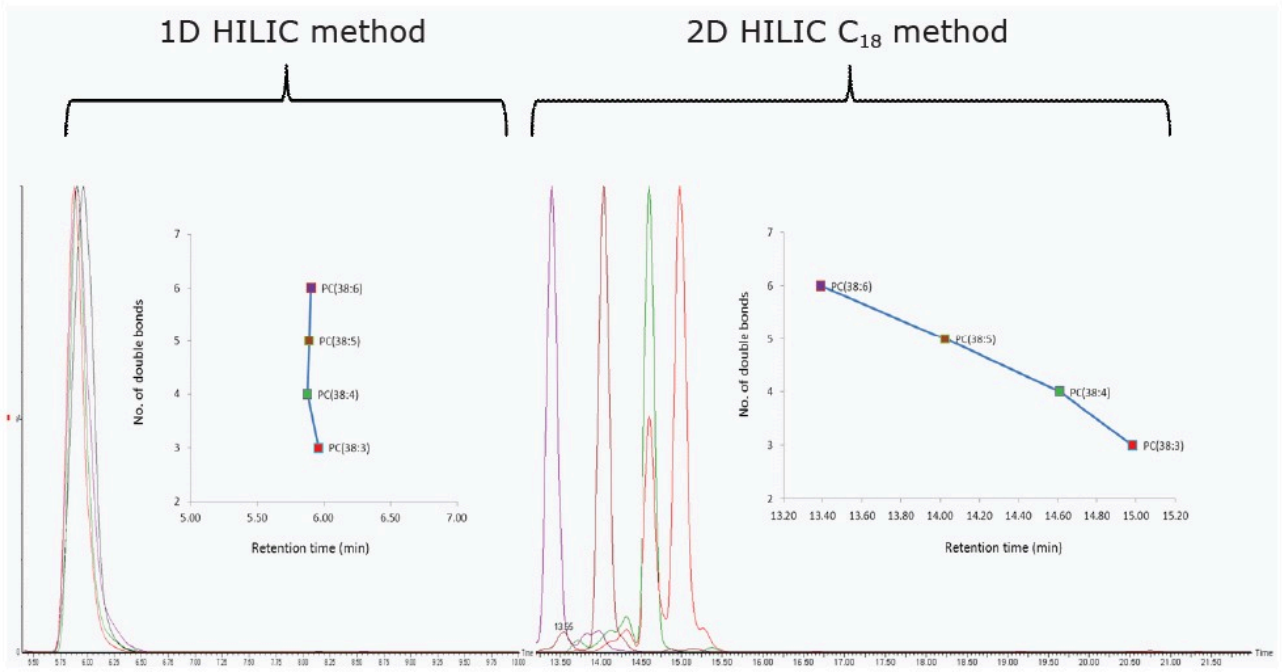

Figure 6. Comparison between 1D HILIC and 2D HILIC C_{18} of the degree of PC alkyl chain saturation (number of double bonds) against retention time.

Improved specificity of the 2D UPLC-MS method

"Heart cutting" a pure fraction of each lipid class using the HILIC column as the first dimension, followed by further separation using reversed phase in the second dimension eliminates errors in identification due to isotopic interferences from co-eluting peaks. This is especially true for the PC and SM classes which differ by 1Da, as shown in Figures 7 and 8.

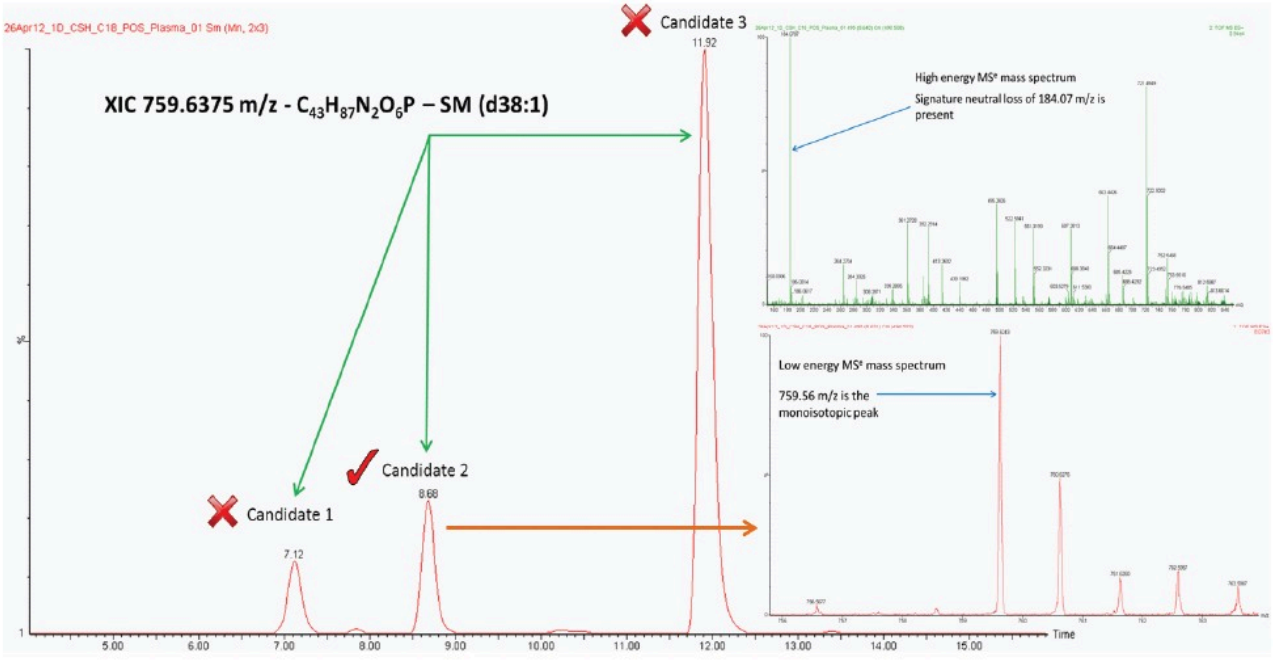

Figure 7. XIC of 759.6375 m/z from the 1D C_{18} TIC reveals three potential candidates for an SM. Further analysis of both the MS and MS^E data reveals candidate two to be the correct one.

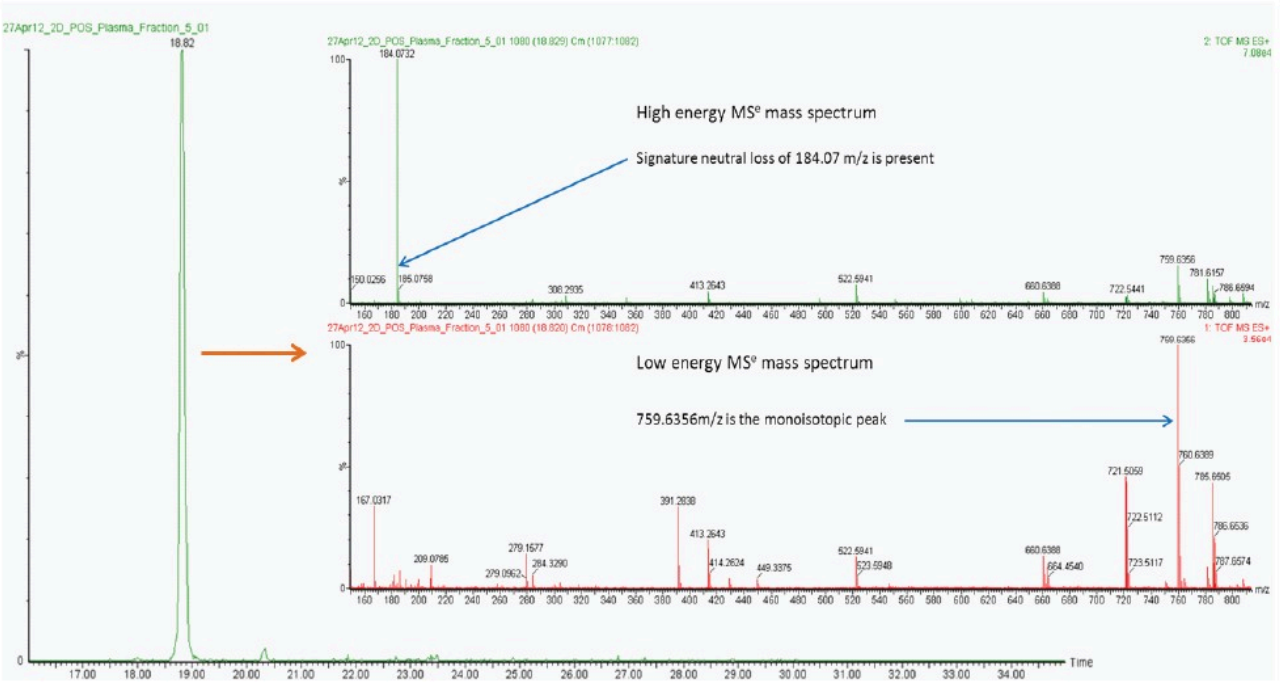

Figure 8. XIC of 759.6375 m/z from the 2D HILIC C_{18} TIC reveals only one potential candidate for the SM. Further analysis of both the MS and MS/MS data confirms this candidate is the correct one.

Conclusion

The limitations posed by traditional single-dimensional separations of HILIC or reversed phase were overcome by the introduction of this novel 2D UPLC-MS configured system which leverages the advantages of both types of methods. This resulted in improved chromatographic resolution, peak capacity, and specificity. In addition, the system is completely automated and UPLC technology provides the capability of high throughput, high resolution analyses compared to traditional HPLCs.

In this application note, we show the orthogonality of this system by pairing the HILIC and C_{18} chemistries; however, the system can also be easily adapted for other two-dimensional applications such as IEX-RP and SEC-RP. The 2D UPLC-MS system configuration described here uses commercially available components with little modification needed and can easily be switched to other UPLC with 2D technology modes, such as parallel column regeneration or conventional single-dimensional separation.

References

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