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Application Note

A Multidimensional Lipidomics Method: HILIC Coupled with Ion Mobility Enabled Time-of-Flight Mass Spectrometry

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Abstract

A HILIC-UPLC separation with ion mobility-Tof MS (SYNAPT G2-S HDMS) enables a multi-dimensional separation of complex biological mixtures, enhancing the information obtained from profiling lipids. HDMS Compare Software and TransOmics Informatics facilitate the comparison of the biological samples.

The combination of liquid chromatography, ion mobility, and oa-Tof mass spectrometry is a multi-dimensional separation strategy capable of analyzing complex biological mixtures to a depth not previously possible, enhancing the detail obtained from lipidomic profiling.

Benefits

Combining HILIC-UPLC liquid phase separation with gas phase ion mobility mass spectrometry to achieve a multi-dimensional characterization of lipids in complex mixtures enhances profiling of lipids in biological samples.

Introduction

One of the main challenges for a global lipid analysis (lipidomics) is the separation of the wide array of lipid species present in biological samples (Figure 1). Such a separation is not achievable using a single chromatographic dimension such as reversed- or normal-phase separation methods.[1-5] Normal-phase UPLC separates lipid classes based on their polar head group, whereas reversed-phase separates lipids according to their acyl chain length and number of double bonds.[1-6] Hydrophilic interaction chromatography (HILIC) separation has been proposed as an alternative to normal-phase separation, offering better MS compatibility and using less toxic solvents.[4-6] Recently, a two-dimensional separation using HILIC and reversed-phase has been proposed to maximize the separation of the lipidome before MS detection.[5,6]

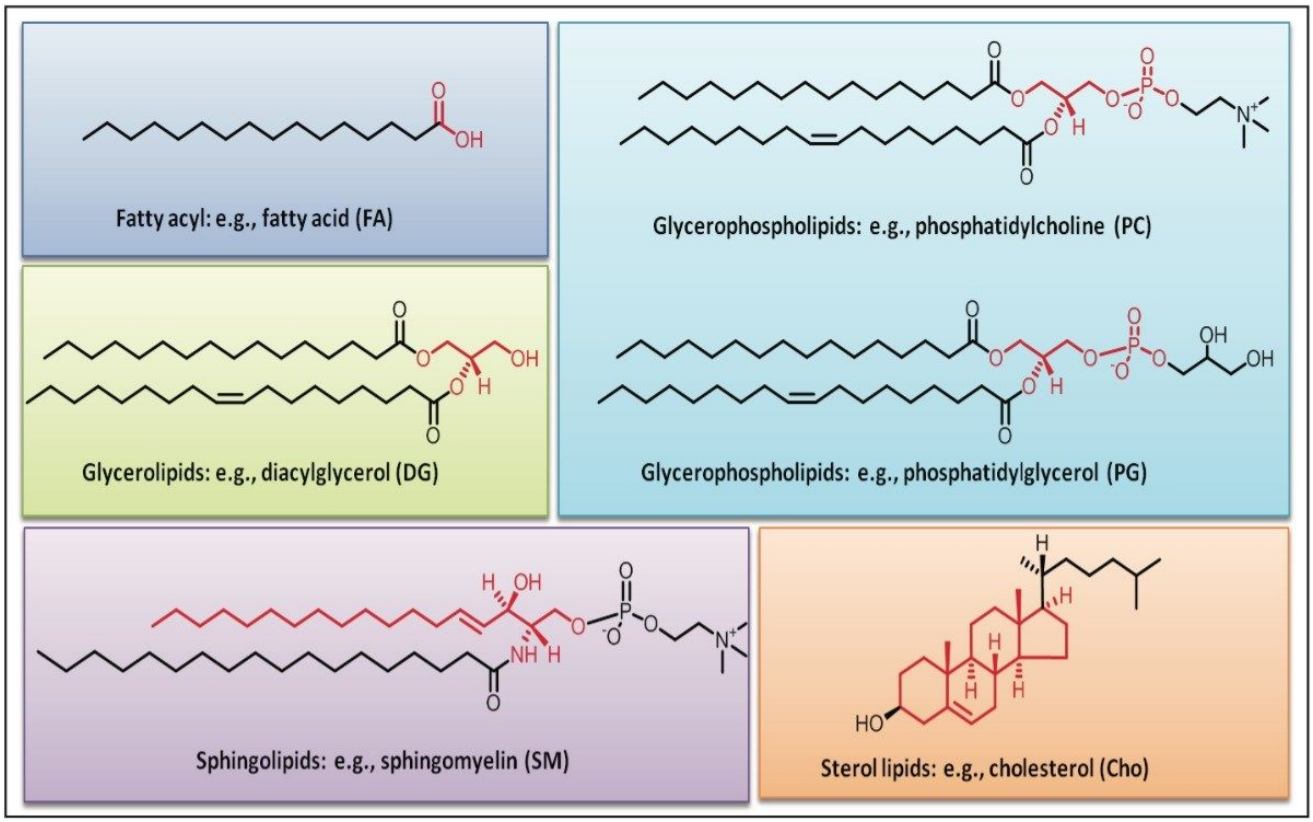

Figure 1. Lipid diversity. Lipids are divided into classes according to common structural moieties (in red), which may give rise to different chromatographic behaviors during HILIC.

In addition to chromatography, ion mobility can be used to separate lipid ions in the gas phase according to their size and molecular shape.[7,8] In this study, we apply the Waters Omics Research Platform with TransOmics Informatics. A HILIC-UPLC separation with ion mobility-Tof MS (SYNAPT G2-S HDMS) enables a multi-dimensional separation of complex biological mixtures, enhancing the information obtained from profiling lipids. HDMS Compare Software and TransOmics Informatics facilitate the comparison of the biological samples.

Experimental

Sample description

Lipid standards and total lipid extracts from bovine brain, heart, and liver were purchased from Avanti Polar Lipids. Non-natural lipids were spiked in the biological extracts and used as internal standards (Table 2).

UPLC conditions

System:	ACQUITY UPLC
Column:	ACQUITY UPLC BEH HILIC 2.1 x 100 mm
Column temp.:	30 °C
Mobile phase A:	10 mM ammonium acetate (pH 8.0) in 95% ACN
Mobile phase B:	10 mM ammonium acetate (pH 8.0) in 50% ACN

Gradient:

Time/min	%A	%B
0.00	99.9	0.1

Time/min	%A	%B
10.00	80.0	20.0
13.00	20.0	80.0
13.01	99.9	0.1
16.00	99.9	0.1
Flow rate:	0.5 mL/min	
Injection volume:	5 µL	

MS conditions

MS analyses were performed on a SYNAPT G2-S HDMS (Figure 2) with a conventional ESI source in LC-HDMS^E mode. Capillary voltages were optimized separately for positive (2.8 kV) and negative (1.9 kV) ion modes. Data were collected in two channels all of the time; low collision energy (6.0 V) for the molecular ions and high collision energy (20 to 35 V) for product ions. IMS gas: nitrogen; IMS T-Wave velocity: 900 m/s; IMS T-Wave height: 40 V.

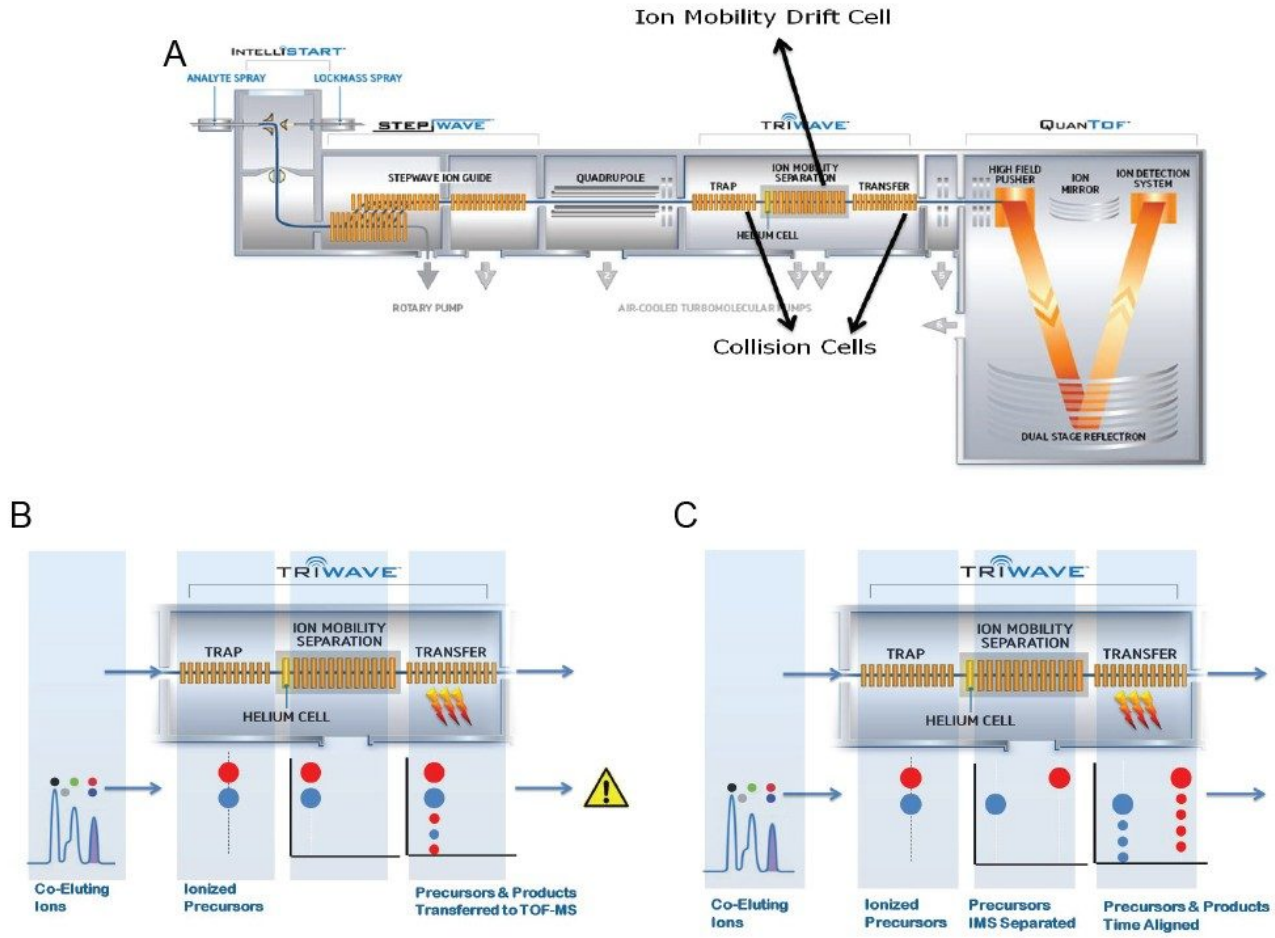

Figure 2. Ion mobility separation and fragmentation. A) Schematic of the SYNAPT G2-S HDMS System. B) MS^E can be extremely useful by itself, however when we consider a complex mixture of metabolites present in biological samples, they often co-elute. By fragmenting them, we only obtain a mixture of fragments which derive from various co-eluting precursors in this example. C) Ions can be separated in the ion mobility cell and subsequently fragmented in the transfer collision cell. The product ions generated in the collision cell have the same mobility drift time as their parent ions. Using this acquisition condition namely high-definition MS^E (HDMS^E), product ions can be aligned with their parent ions on the basis of mobility drift time as well as chromatographic retention time using the Waters proprietary Apex4D algorithm.

Data acquisition and processing

TransOmics Informatics and HDMS Compare Software

Results and Discussion

To separate lipids, we used hydrophilic interaction chromatography (HILIC) with an ACQUITY UPLC BEH HILIC 2.1 x 100 mm, 1.7 µm Column, and a reversed-phase solvent system (organic/aqueous) characterized by high organic mobile phase (>80% acetonitrile). This UPLC method was highly compatible with ESI, and separated lipids by classes, according to their polar properties (Figure 3 and Table 1).

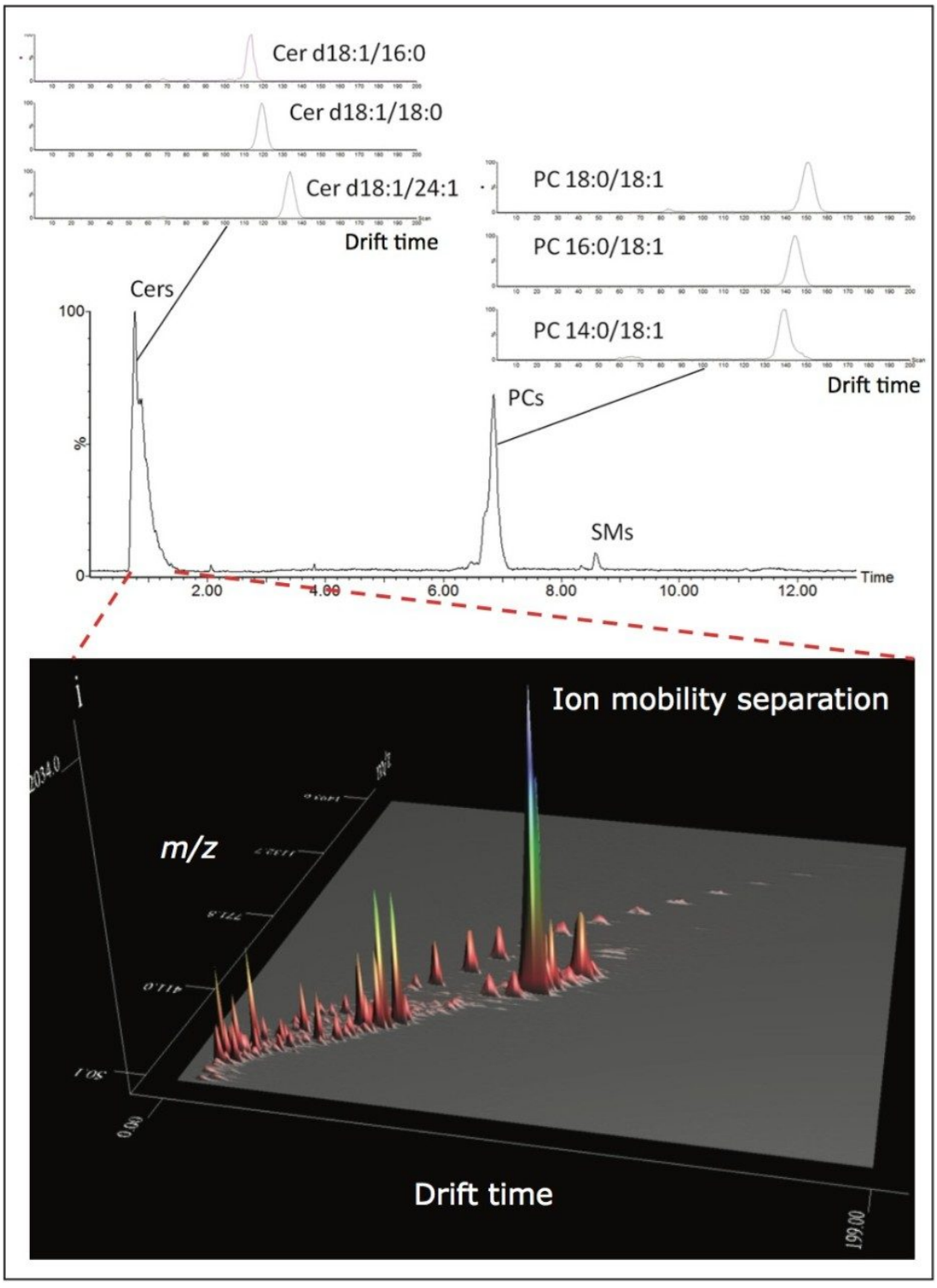

Figure 3. 2D separation by HILIC-ion mobility. Representative analysis of lipid standards using a combination of

HILIC separation and ion mobility (IM) separation (inserts) in positive ion mode. HILIC-IM analysis provides an additional degree of separation beyond chromatography, which is ideal for the analysis of complex lipid mixtures extracted from biological samples. After HILIC separation, ion mobility further separates ceramide species according to their molecular shapes. The molecular landscape visualized using an unbiased 3D representation (drift time, m/z, intensity) of a selected interval of retention time (0.5 to 1.5 minutes) allows the detection of many isobaric ceramide species. Such an approach highlights the power of ion mobility for the discovery of many low abundance molecular species that could otherwise be undetected.

Lipid class	ES polarity	RT window
FA	Neg	0-1
Cer	Pos	0-2
HexCer	Neg	0-2
ST	Neg	0-2
DiHexCer	Neg	2-4
PG	Neg	1-3
PE	Neg/Pos	5-7
PI	Neg	3-5
PS	Neg	4-6
PC	Pos	5-7
LPE	Neg/Pos	6-8
SM	Pos	7-9
LPC	Pos	8-10

Table 1. Lipid classes are separated by retention time (RT) windows in HILIC conditions.

Abbreviations: FA, fatty acids; Cer, ceramides;

HeXCer, HexosylCeramides; ST, sulfatides;

DiHexCer, DihexosylCeramides;

PG, phosphatidylglycerols;

PE, phosphatidylethanolamines;

PI, phosphatidylinositols;

PS, phosphatidylserines; PC, phosphatidylcholines;

LPE, lysophosphatidylethanolamines;

SM, sphingomyelins; LPC, lysophosphatidylcholines.

In addition to HILIC chromatography, the ion mobility capability of the SYNAPT G2-S HDMS Mass Spectrometer (Figures 2A-C and 3) was used to further discriminate lipid classes into their constituent components, based upon the different size and shape, that is, the ions collision cross section (Ω).[7,8] Lipid ions with different degrees of unsaturation and acyl length migrate with characteristic mobility times, due to their unique shape in the gas phase as they migrate through the ion mobility cell, which is filled with nitrogen gas at relatively high pressure (Figures 3 and 5). Ion mobility separations occur in the millisecond timeframe, making it ideal for situating between LC and MS, where LC separations upstream typically work in the second timeframe and Tof MS

downstream works in the nanosecond timeframe (Figures 2A and 3). The addition of ion mobility to the LC-MS provides enhanced peak capacity and improved signal-to-noise ratio (Figure 3).

To gain more structural information, we analyzed lipids employing LC-MS^E, which uses an alternating low and elevated collision energy in separate scans to acquire both precursor and product ion information in a single analytical run (Figures 2B and 4A). Ion mobility separation coupled with LC-MS^E (HDMS^E) improves the specificity for coeluting lipids by fragmenting ions after IMS separation (Figures 2C and 4B). Due to the complexity of the lipidome, the addition of ion mobility drift time as an orthogonal measurement to retention times provides complementary information regarding the lipid species, adding further specificity to lipid identification and data interpretation (Figure 4B).

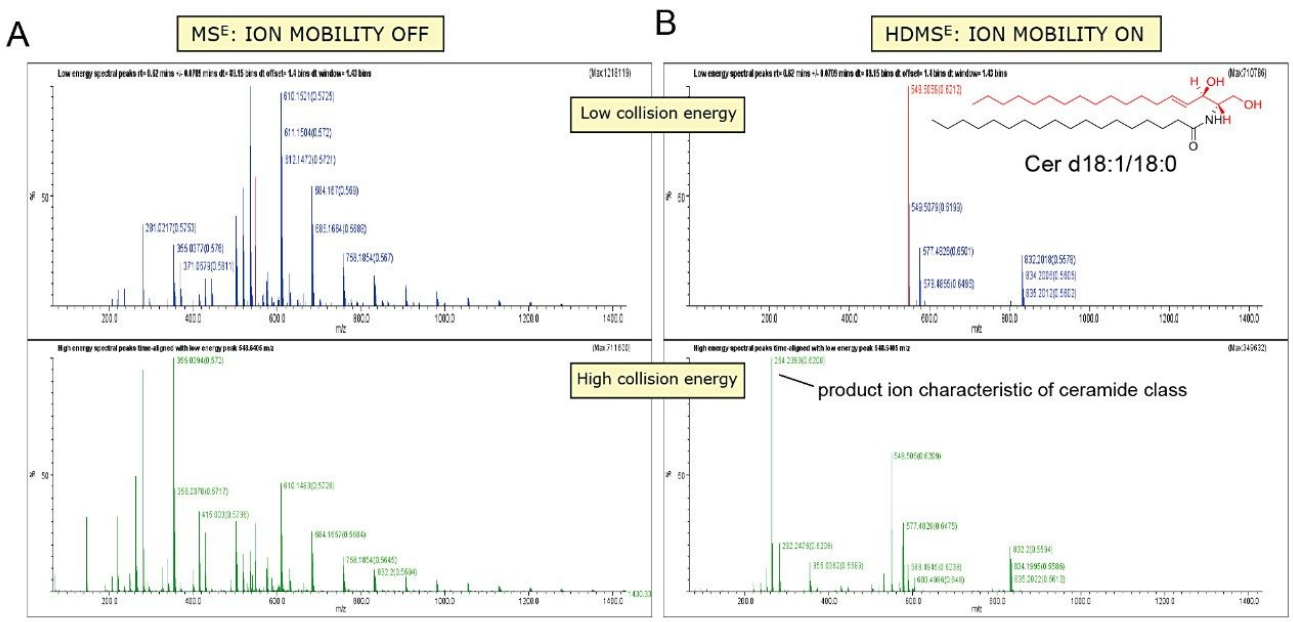

Figure 4. Structural characterization of lipid classes. Lipid classes generate characteristic fragments (product ions) upon collision-induced dissociation (CID). In positive ionization mode, ceramides are usually detected as dehydrated molecular ions using low collision energy; however, high collision energy generates all ceramide species with a common characteristic product ion. A) Waters instruments enable alternating low and high collision energy (MS^E), allowing to acquire precursor and product ion information in a single chromatographic run. The presence of co-eluting lipids, however, makes the interpretation of the high collision energy spectra difficult. B) By applying ion mobility separation, co-eluting lipids are separated based on their molecular size and shape before fragmentation in the transfer cell. This mode of acquisition ($HDMS^E$) results in cleaner fragmentation spectra and a more confident identification of lipid classes.

Using this novel technological approach, multidimensional molecular maps of lipids present in various animal tissues were generated. In these maps, each lipid is characterized by a combination of molecular coordinates including retention time, drift time, exact mass, fragment ions, and intensity (Figure 5). Such features highlighted the capacity of ion mobility to separate isobaric lipid species (i.e., species with the same mass). The molecular landscape visualized using multidimensional molecular maps also allows the detection of lipid species that could otherwise go unnoticed (Figures 3 and 5).

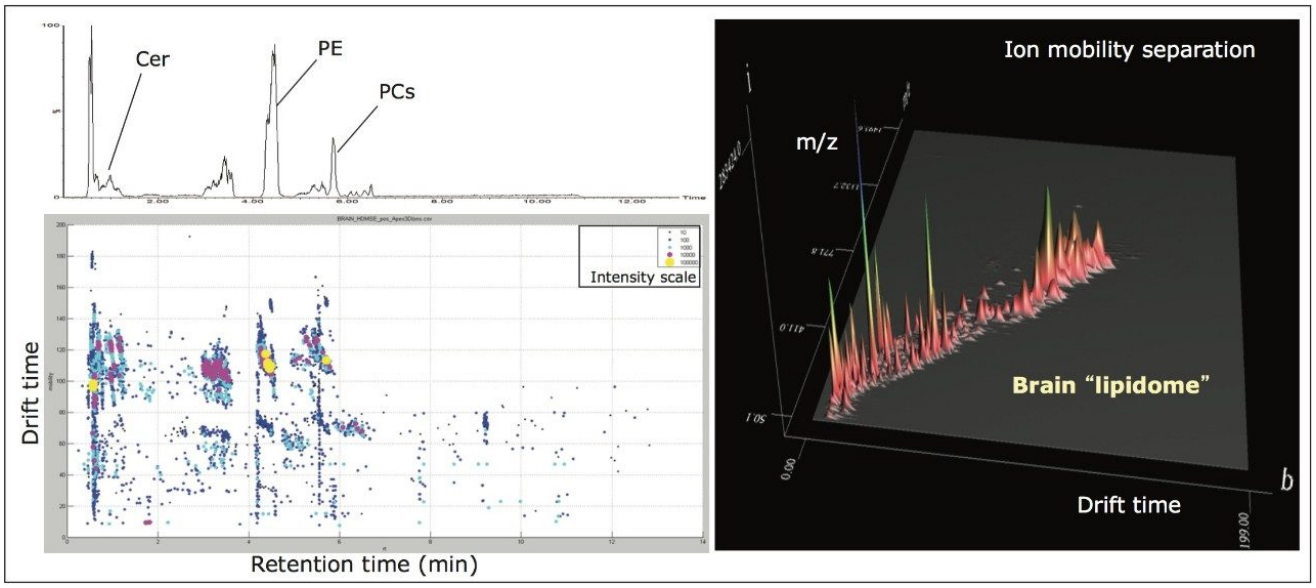

Figure 5. Mapping the brain lipidome using HILIC-ion mobility. Representative HILIC-ion mobility Tof analysis of total lipid extract from bovine brain. Lipids are separated by both retention time and mobility time (drift time). A multi-dimensional molecular map could be generated using unique coordinates such as retention times, mobility times, accurate masses, and intensities.

The comparison of molecular maps is facilitated by the use of HDMS Compare and TransOmics (Figures 6 and 7). HDMS Compare Software was used for a rapid comparison of different drift versus *m/z* plots at selected windows of retention times (Table 1). The drift time and spectral information associated with the components responsible for the differentiation can be extracted from the dataset and further analyzed (Figure 6). The use of TransOmics Informatics allows feature detection, alignment, and comparions across multiple samples using multivariate statistical approaches (PCA, dendrogram analysis) and database searching of discriminating features for the identification of the lipids alternating between samples. TransOmics uses ion mobility information to separate co-eluting isobaric lipids in the drift time dimension, increasing the specificity of identification and quantification (Figure 7). Lipid quantification was performed using appropriate internal standards for each lipid class (Table 2).

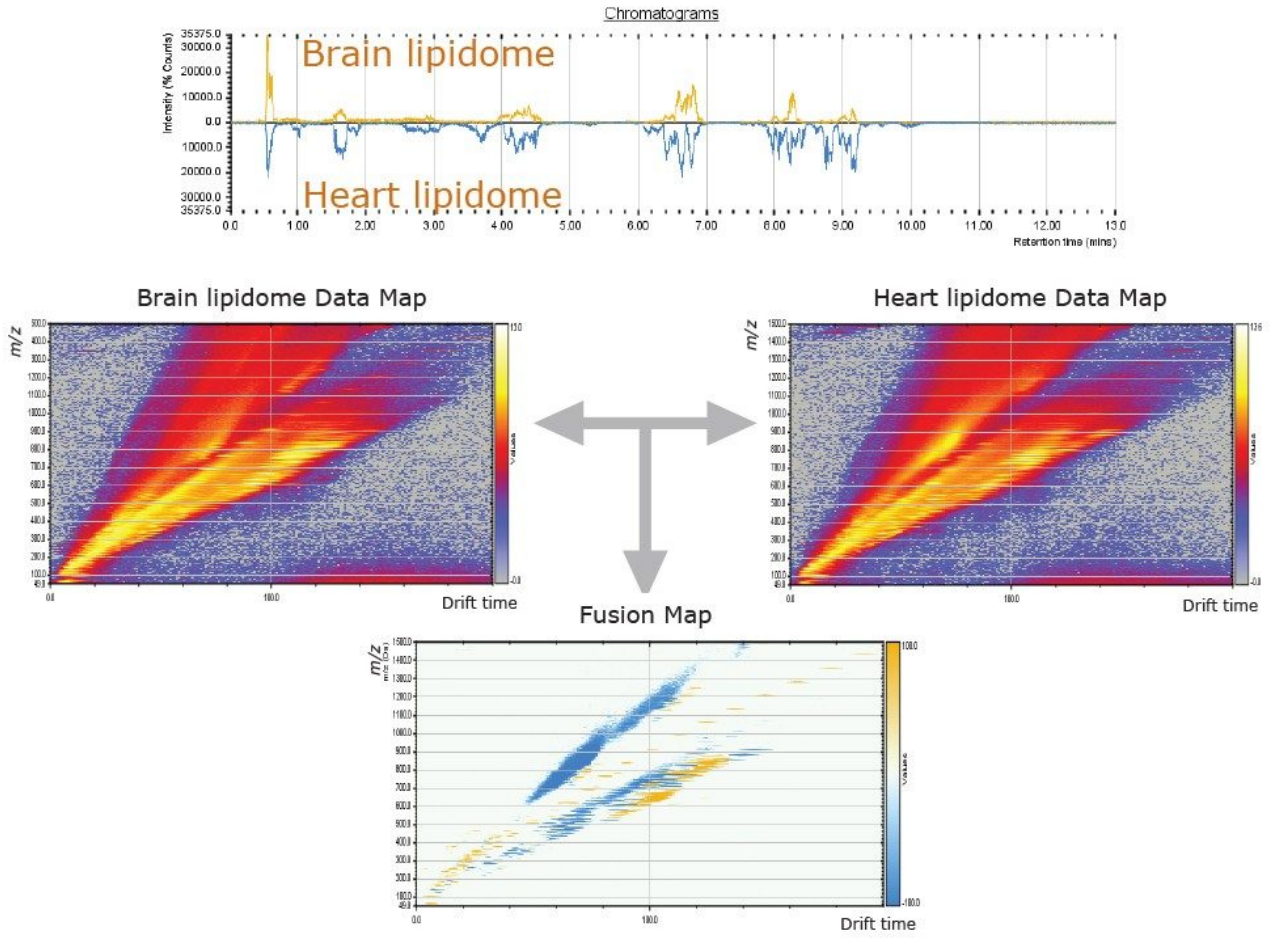

Figure 6. HDMS Compare Software for the comparison of lipid extracted from brain and heart tissues. HDMS Compare Software was used to overlay tissue-specific molecular maps. Key areas of significant differences between two samples were clearly visualized and identified with two different colors. Retention times, drift time, and mass information can be used for database searches and further identification of such molecular differences.

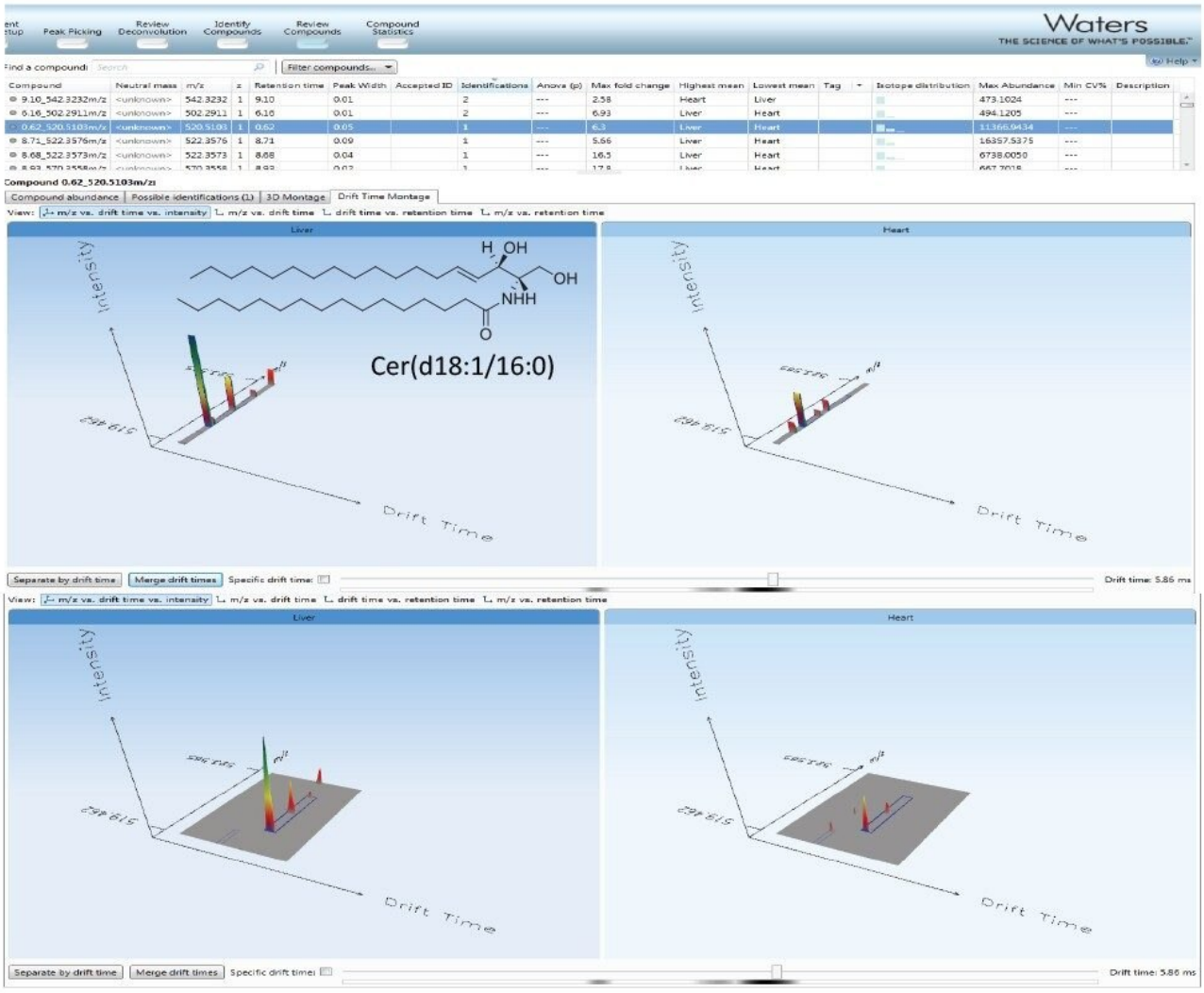

Figure 7. TransOmics for the comparison of lipid extracted from heart and liver tissues. TransOmics uses ion mobility information to separate co-eluting isobaric lipids in the drift time dimension, increasing the specificity of identification and quantification.

Lipid class	Internal standard	Abbreviation	Vendor	Catalog #
Sphigolipids				
Ceramides	N-Lauroyl-D-erythro-sphingosine	Cer(d18:1/12:0)	Avanti Polar Lipids	860512P
Sphingomyelin	N-(dodecanoyl)-sphing-4-enine-1-phosphocholine	SM (d18:1/12:0)	Avanti Polar Lipids	860583P
Glycerophospholipids				
Phosphatidylethanolamines	1,2- Dimyristoyl-sn-glycero-3-phosphoethanolamine	PE (14:0-14:0)	Avanti Polar Lipids	850745
	Avanti Polar Lipids			
Phosphatidylcholines	1,2- Dimyristoyl-sn-glycero-3-phosphocholine	PC (14:0/14:0)	Avanti Polar Lipids	850345
	Avanti Polar Lipids			
Phosphatidylserine	1,2- Dimyristoyl-sn-glycero-3-phosphoserine	PS (14:0/14:0)	Avanti Polar Lipids	840033
	Avanti Polar Lipids			
Phosphatidylglycerol	1,2- Dimyristoyl-sn-glycero-3-phosphoglycerol	PG (14:0/14:0)	Avanti Polar Lipids	840445
	Avanti Polar Lipids			
Phosphatidylinositol	1,2-DiHexanoyl-sn-glycero-3-phospho-(1'-myo-inositol)	16:0 PI	Avanti Polar Lipids	850141
	Avanti Polar Lipids			
Lysophosphatidylcholine	1-Heptadecenoyl-2-hydroxy-sn-glycero-3-phosphocholine	LPC (17:1)	Avanti Polar Lipids	LM-1601
Fatty acyl				
Fatty acids	10-heptadecenoic acid	FA (17:1)	Nu-Chek Prep	U-42-A

Table 2. List of lipids used as internal standards for selected lipid classes.

Conclusion

The combination of liquid chromatography, ion mobility, and oa-Tof mass spectrometry is a multidimensional separation strategy capable of analyzing complex biological mixtures to a depth not previously possible, enhancing the detail obtained from lipidomic profiling.

- HILIC separates lipid classes according to their polarity, providing stable retention time coordinates.
- Ion mobility separates lipids according to their difference in size and molecular shapes, providing Ω values

(drift time coordinates).

- LC-MS^E coupled with ion mobility separation (HDMS^E) allows the simultaneous collection of exact mass precursor and fragment ion information, providing structural information and improving the experimental specificity.
- HILIC coupled with LC-HDMS^E generates molecular maps with unique coordinates, including retention times, drift times, accurate precursor and fragment ion masses, as well as intensities.
- HDMS Compare and TransOmics provide informatics solutions to compare large numbers of molecular maps in a scalable fashion using multi-variate statistical approaches, adding further specificity and confidence to lipid identification and biological interpretation.

References

1. Rainville PD, Stumpf CL, Shockcor JP, Plumb RS, Nicholson JK. Novel Application of Reversed-Phase UPLC-oaTOF-MS for Lipid Analysis in Complex Biological Mixtures: A New Tool for Lipidomics. *J Proteome Res*. 2007 Feb; 6(2):552-8.
2. Lísa M, Cífková E, Holčapek M. Lipidomic profiling of biological tissues using off-line two-dimensional high-performance liquid chromatography-mass spectrometry. *J Chromatogr A*. 2011 Aug 5; 1218(31):5146-56. Epub 2011 May 30.
3. Isaac G, McDonald S, Astarita G. Lipid Separation: UPLC System for the Separation of Complex Biological Total Lipid Extracts. Waters Application Note 720004107en. 2011 Sept.
4. Netto J, Wong S, Ritchie M, Torta F, Narayanaswamy P. A Definitive Lipidomics Workflow for Human Plasma Utilizing Off-line Enrichment and Class Specific Separation of Phospholipids. Waters Application Note 720004521en. 2012 Dec.
5. Nie H, Liu R, Yang Y, Bai Y, Guan Y, Qian D, Wang T, Liu H. Lipid profiling of rat peritoneal surface layers by online normal- and reversed-phase 2D LC QToF-MS. *J Lipid Res.* 2010 Sep; 51(9):2833-44.
6. Netto JD, Wong S, Ritchie M. High Resolution Separation of Phospholipids Using a Novel Orthogonal Two-Dimensional UPLC/QTof MS System Configuration. Waters Application Note 720004546en. 2013 Jan.

7. Shvartsburg AA, Isaac G, Leveque N, Smith RD, Metz TO. Separation and classification of lipids using differential ion mobility spectrometry. *J Am Soc Mass Spectrom*. 2011 Jul;22(7):1146-55. Epub 2011 Apr 12. Erratum in: *J Am Soc Mass Spectrom*. 2011 Jul; 22(7):1156.

8. Kliman M, May JC, McLean JA. Lipid analysis and lipidomics by structurally selective ion mobility-mass spectrometry. *Biochim Biophys Acta*. 2011 Nov; 1811(11):935-45.

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