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Informatics Solutions for Improved Identifications in 2D-LC/MS and IM-MS Lipidomics Workflows

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Introduction

Lipidomics Workflows

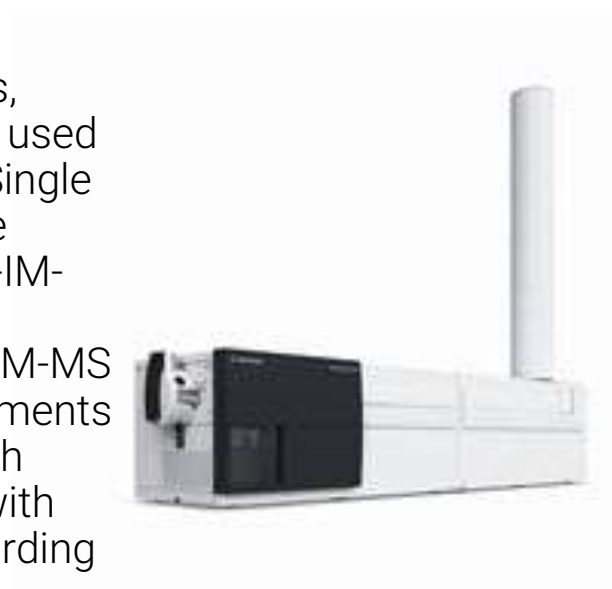
Confident identifications are a challenge in the field of MS based lipidomics due to high occurrence of lipid isomers and isobars. Chromatographic separations prior to MS analysis can remove some signal overlap by separating lipid classes according to head group as well as separating lipids based on fatty acid saturation. Combining these two separations with a 2D-LC platform removes ambiguity for many ion signals. Performing data-independent MS/MS experiments after ion mobility (IM) separations allows fragment ions to be linked back to their precursor ion in the drift time dimension. These technologies offer key advances in confidently identifying more lipid species in omics workflows. In this study we apply a novel twist on the contemporary online high resolution 2D-LC experiment as well as evaluate the ability to make confident identifications with the All Ions IM-MS workflow with both 1D and 2D LC separations.

Two Dimensional Liquid Chromatography

1D and 2D LC experiments were performed on a commercial LC (1290 Series), Agilent Technologies, Santa Clara, CA. Multiple heart cutting and high resolution 2D LC experiments were performed with orthogonal LC methods (HILIC and Reverse Phase). The instrument was equipped with the 2 pos/4 port duo 2x6 loops.

Ion mobility mass spectrometry

The 6560 IM-Q-TOF (Agilent Technologies, Santa Clara, CA) was used for IM experiments. Single field CCS values were calculated for the LC-IM-MS experiments. Additionally, All Ions IM-MS fragmentation experiments were performed which align fragment ions with their precursors according to drift time.



Experimental

LC Experiments

LC methods were adapted from a previously described method[1], but briefly, all first dimensional experiments were performed with an RX-Sil HILIC column (3.0 x 100 mm, 1.8 micron, 360 µL/min flow rate). Second dimension experiments were performed with a Zorbax SB-C18 column (2.1 x 100 mm, 1.8 micron, 0.2 mL/min flow rate). Ion mobility experiments were performed with the single field approach described previously[2]. Lipid standards were purchased from Avanti Polar Lipids (Alabaster, AL) and were run as both a mixture of standards as well as spiked into a bovine heart extract (Avanti).

1D-LC

Min	Sol A: ACN (0.1% FA)	Sol B: ACN:MeOH:H_2O (50:20:30 v/v) (20 mM NH_4HCO_2)
0	70%	30%
2	40%	60%
4	30%	70%
5	0%	100%
8	0%	100%
9	70%	30%

2D-LC

Min	Sol A: MeOH:H_2O (10:90 v/v) (0.1% FA & 20 mM NH_4HCO_2)	Sol B: ACN:MeOH:IPA (20:30:50 v/v) (0.1% FA & 20 mM NH_4HCO_2)
0	60%	40%
2	40%	60%
4	20%	80%
5	15%	85%
6	10%	90%
7	5%	95%
8	0%	100%
12	0%	100%
13	60%	40%

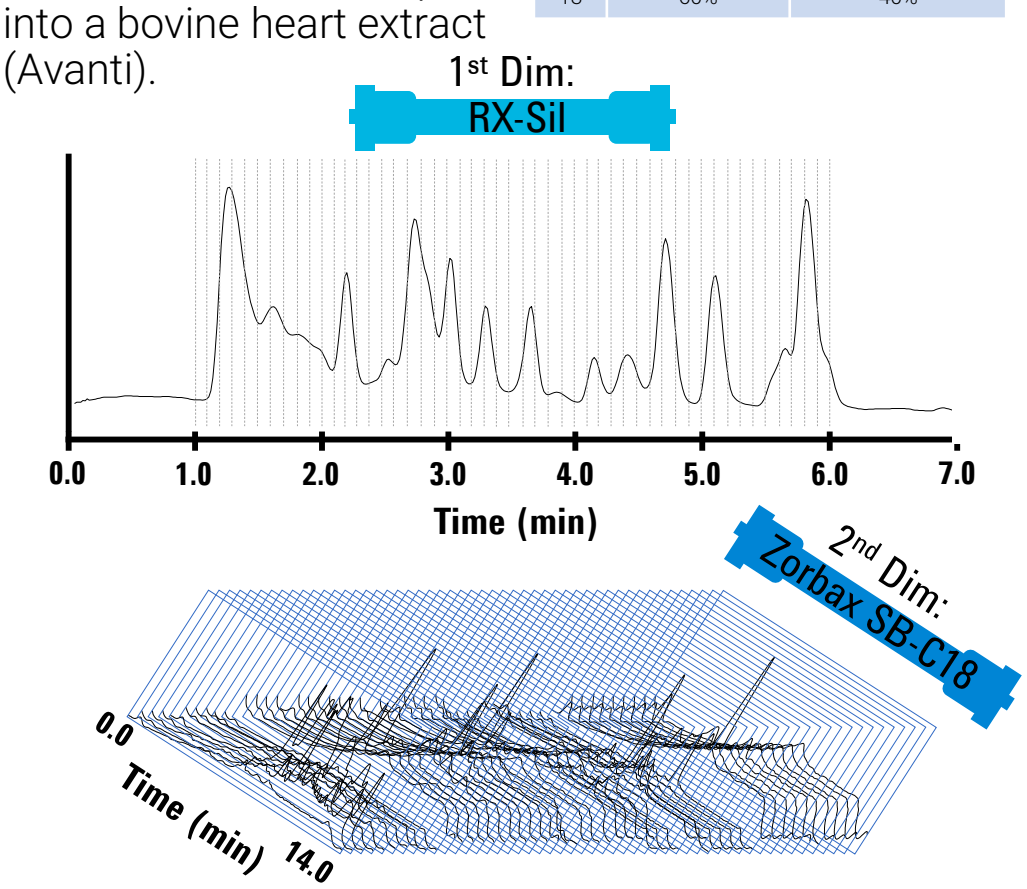


Figure 1. Due to poor absorbance of the lipid species with the UV detector, total ion chromatograms from MS were used to guide multiple heart cutting 2D-LC experiments. High resolution experiments with 10 - 6 second cuts were performed across 5 different 1-minute regions of the first dimension chromatogram. This resulted in 50 second dimension chromatograms each with a 14 minute gradient which is effectively a comprehensive experiment, but with longer 2D runs than with the typical comprehensive workflow.

1D LC-MS

Results for the lipid standards with the first LC separation with the RX-Sil HILIC column. Lipids generally elute according to class. Overlap is observed for lipids in the same class especially for Ceramides where 6 co-elute at the same retention time.

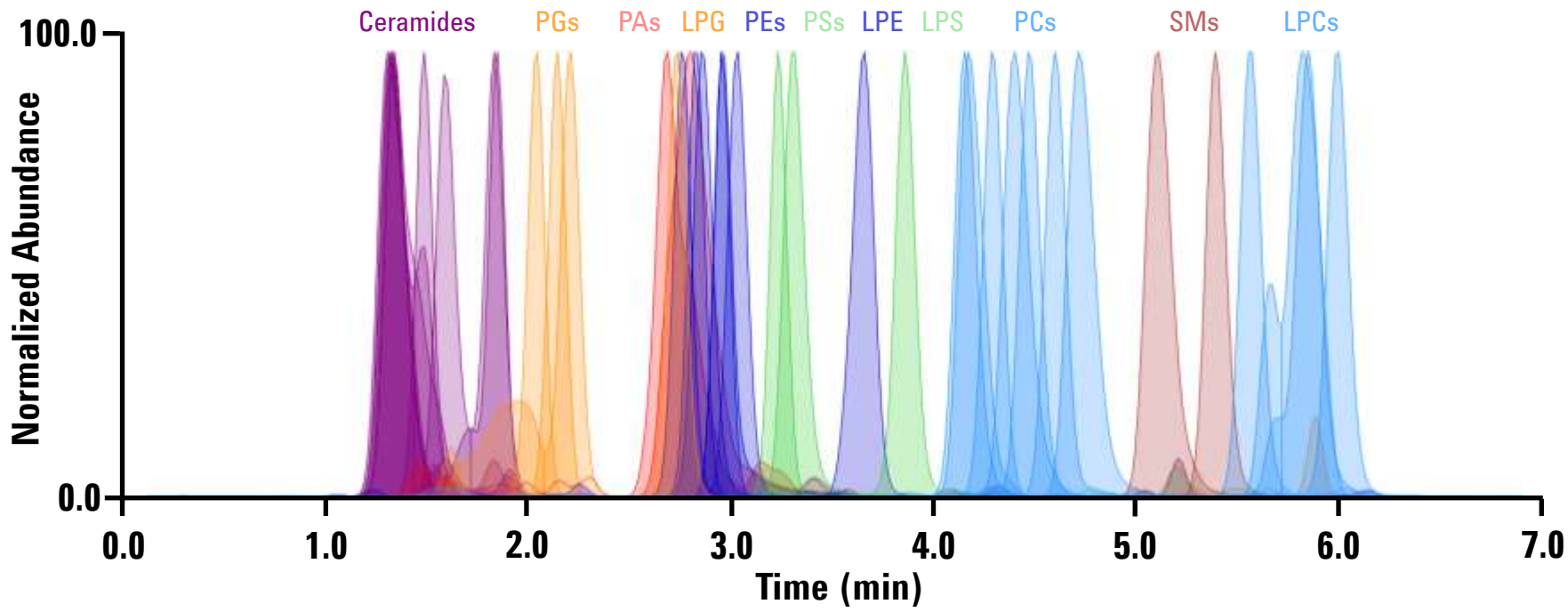


Figure 2. 1D Extracted chromatograms of lipid standards

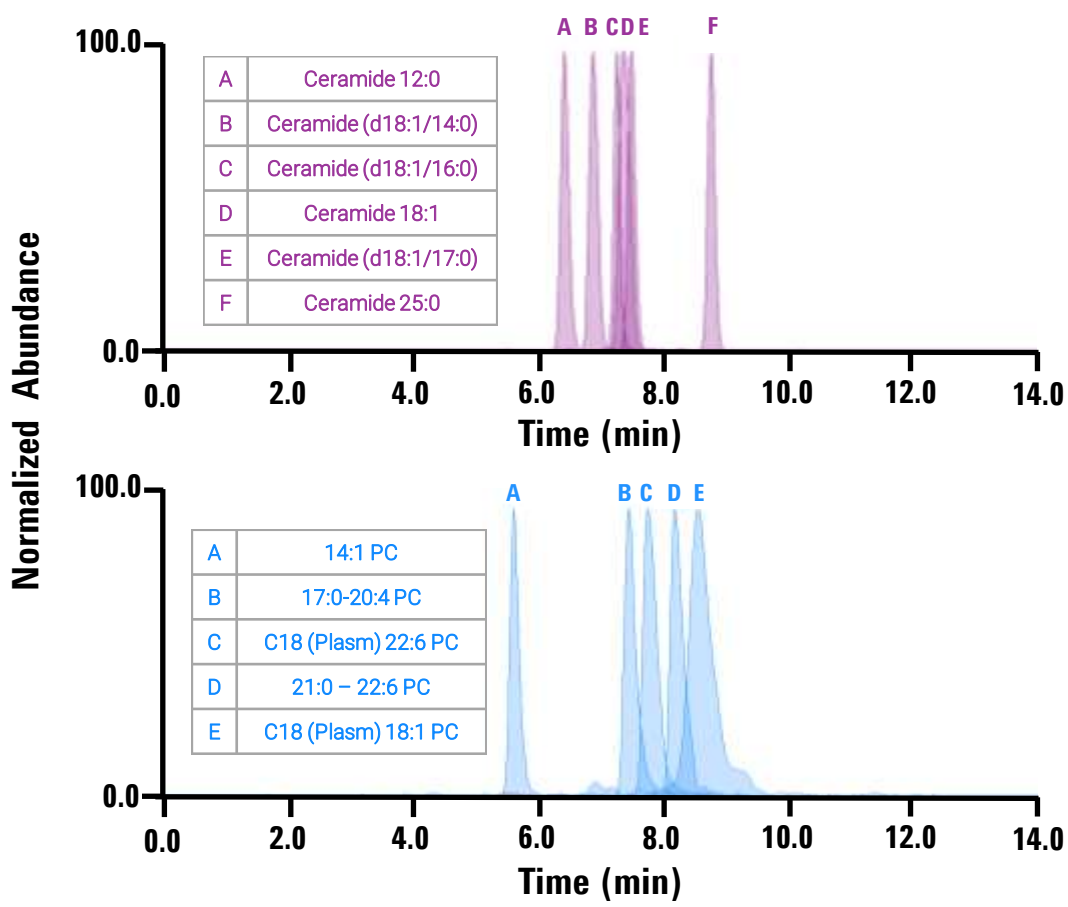
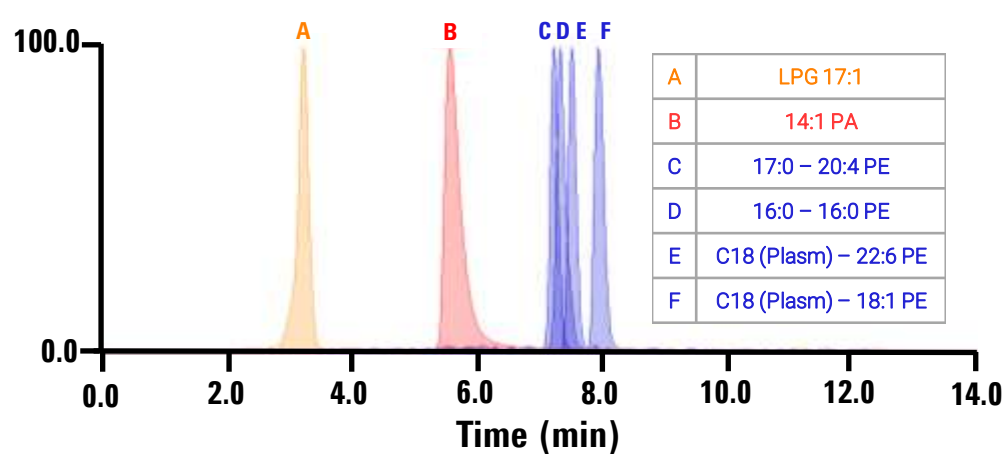


Figure 3. 2D Extracted chromatograms for selected lipids



2D LC - MS

Several lipid standards from the same class which co-eluted in the first dimension were further separated with Zorbax SB-C18 column in the second dimension which separated according to lipid tail length and degree of saturation. Second dimension separations are shown for Ceramides and PCs as well as a high density region including a LPG, a PA, and PEs.

IM-MS

Ion mobility was also evaluated for lipid separations. Plotting m/z vs. CCS creates a conformational space for the lipid standards. Lipids generally group according to class. Ceramides occupy a larger conformational space than the lyso lipids with similar *m/z*. The conformational space for the other lipids follows the trend of PC > PE > PG > PS which mimics their head groups in size. Additionally we see lipids with longer tails having larger CCS values. Degree of saturation also plays a role in the trends observed.

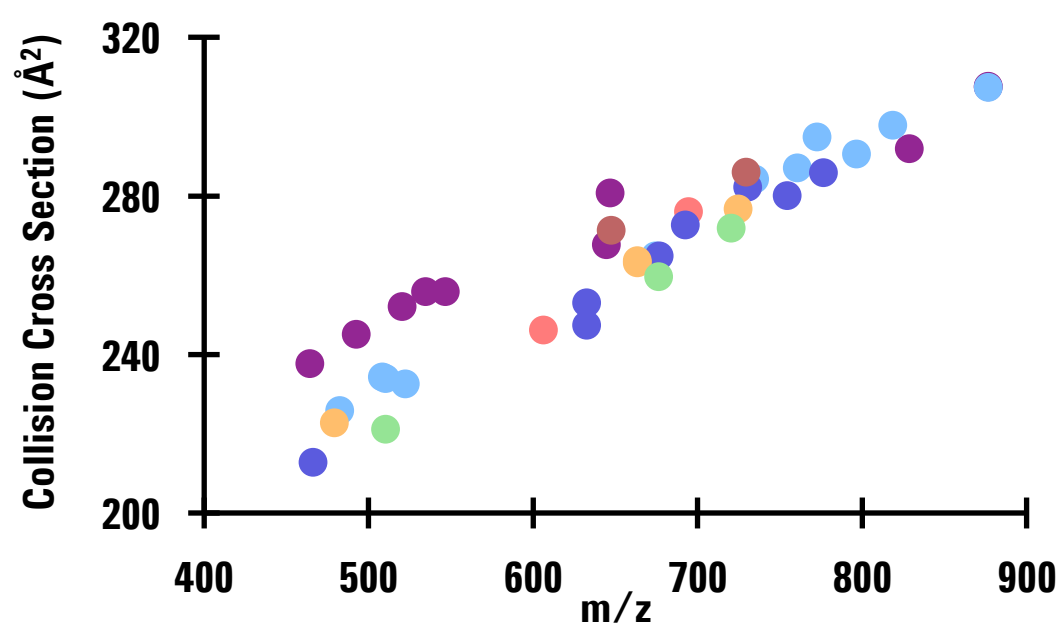
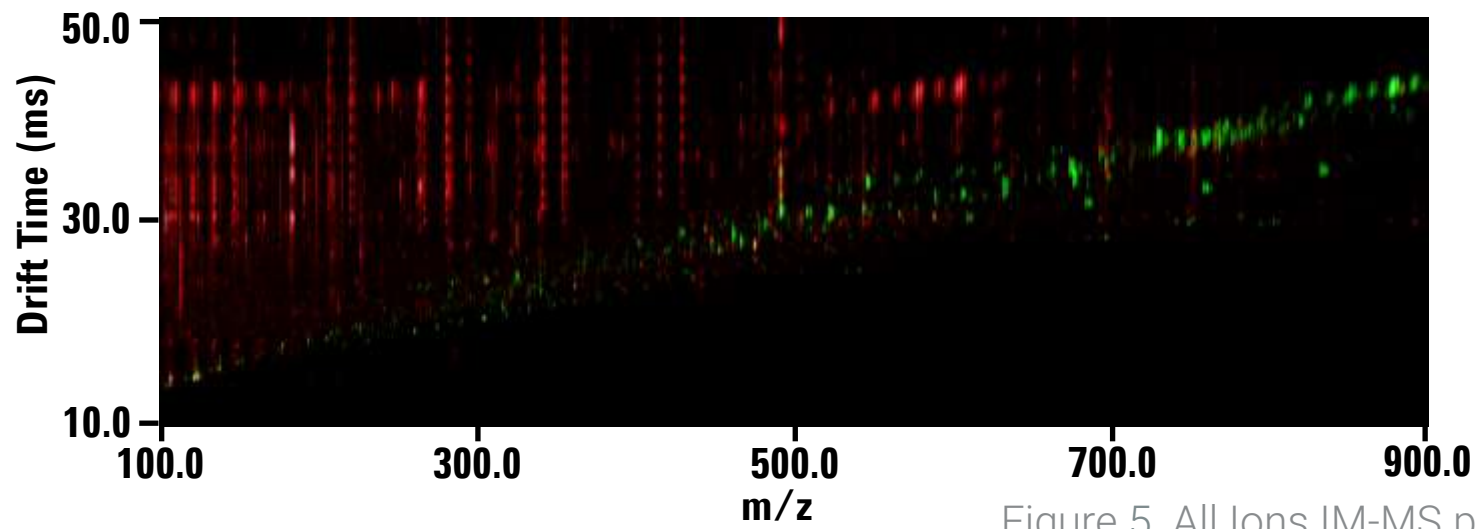


Figure 4. Conformational space plot of the lipid standards.

Results and Discussion



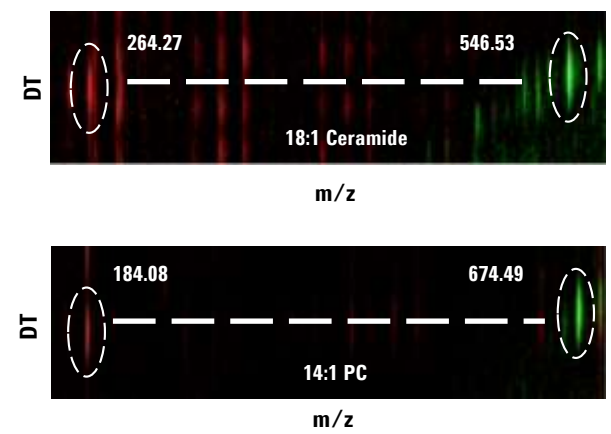


Figure 5. All Ions IM-MS plot with overlaid High CE data (Red) and Low CE Data (Green). 18:1 Ceramide and 14:1 PC are shown as examples with their signature fragments of 264 for Ceramides and 184 for PCs.

Identifications with All Ions IM-MS Fragmentation

The lipid standards analyzed in this study were spiked into a bovine heart extract to evaluate the ability of the all ions workflow to make identifications in a complex sample matrix. Both 1D and 2D chromatogram separations were performed prior to the All Ions IM-MS experiment and the results are shown to the left. Database matching (DB) based on accurate mass (15 ppm) and CCS (<1%) were evaluated as well as MS/MS spectral library matching (Lib.). A PCDL with experimental MS/MS spectra and calibrated single field CCS values was used for the identification workflow. Over 90% of lipid standards were identified by at least DB or spectral match in either positive or negative mode for both 1D and 2D LC. Unidentified species are most likely due to low signal intensity. More Ceramides (6 vs. 3) were identified with both DB and Lib. match in both positive and negative mode following 2D-LC separations as shown by the larger purple region in the pie chart for 2D-LC. One additional PC and PE were also identified following 2D-LC separations.

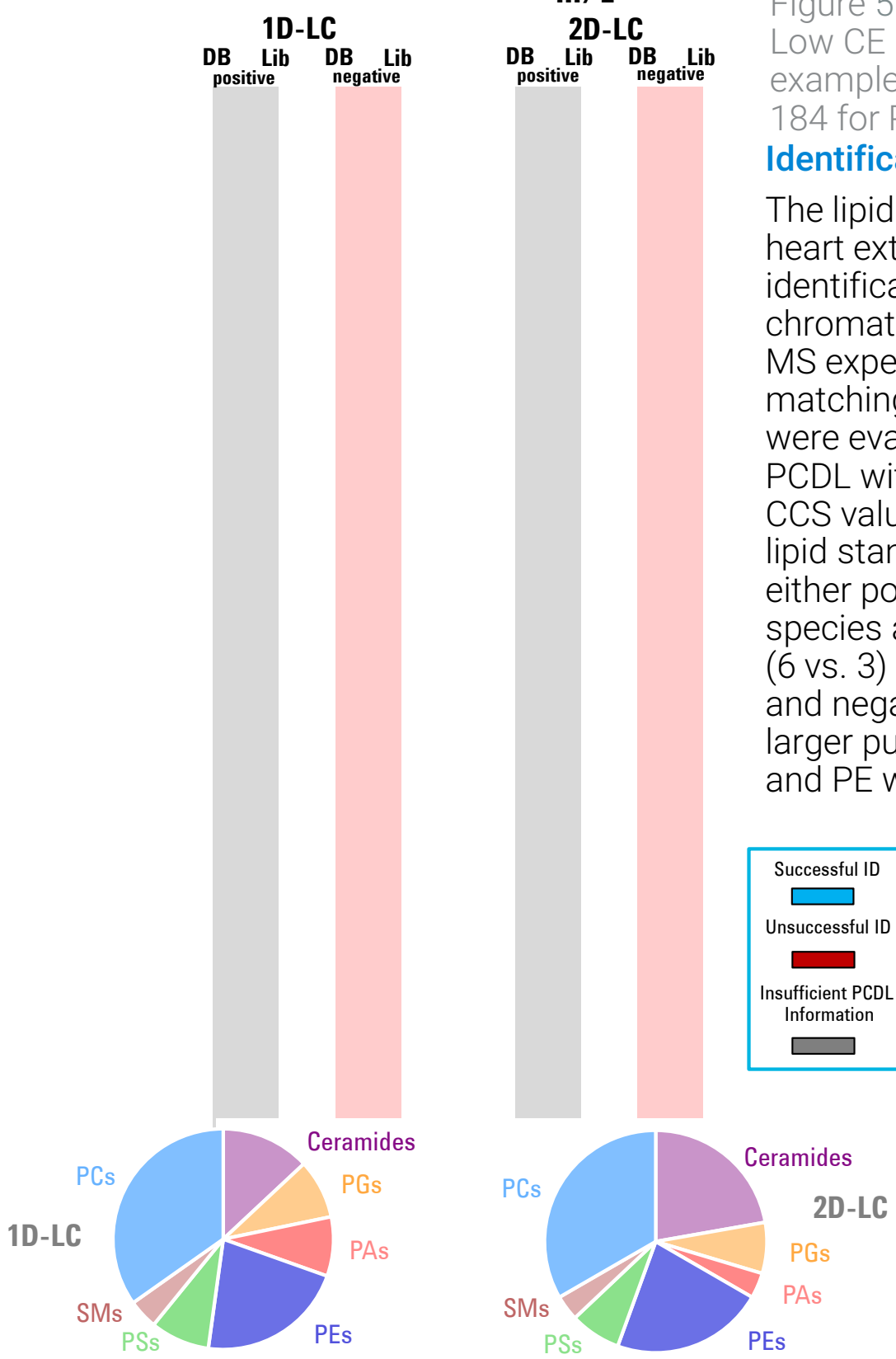


Figure 6. Identification results for internal standards spiked in the bovine heart extract are shown in the bar chart. Identifications were based on a database search (DB) and library MS/MS spectral match (Lib) for both positive and negative mode. The pie charts summarize lipid class results for those lipids identified with both DB and Lib. match in both positive and negative mode.

Conclusions

- 2D-LC allows lipid separations based both on head group and fatty acyl tail length and degree of saturation
- Performing high resolution 2D-LC separations in a comprehensive fashion provides a promising technique for complex samples
- All Ions IM-MS Fragmentation successfully identifies lipids in a complex matrix
- Combining 2D-LC and All-Ions IM-MS Fragmentation provides confident identifications for lipids

References

[1] *Unraveling the Complexity of Lipidomes by Multiple Heart Cutting Q-TOF LC-MS with the Agilent 1290 Infinity 2D-LC Solution,* G. Vanhoenacker, R. t'Kindt, F. David, P. Sandra, and K. Sandra, Application Note: Biotherapeutics and Biosimilars, 2015.

[2] *An Interlaboratory Evaluation of Drift Tube Ion Mobility Collision Cross Section Measurements*, S. M. Stow, T. J. Causon, X. Zheng, R. T. Kurulugama, T. Mairinger, J. C. May, E. E. Rennie, E. Baker, R. Smith, J. A. McLean, S. Hann and J. C. Fjeldsted, Anal. Chem. 2017, 89(17), 9048-9055.

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