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Leveraging Multidimensional Separations to Enhance Traditional LC-MS Lipidomics Workflows

Sarah M. Stow[1], Mark Sartain[1], Aivett Bilbao[2], Bryson C. Gibbons[2], Juli Salcedo[1], Xiangdong Li[1], Adithya Murali[1], Jeremy Koelmel[3], Robin H.J Kemperman[3], John C. Fjeldsted[1]

[1]Agilent Technologies, Santa Clara, CA

[2]Pacific Northwest Nation Laboratory, Richland, WA

[3]University of Florida, Gainesville, FL

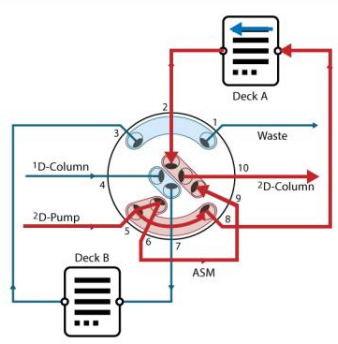
Introduction

Lipidomics Workflow

Lipidomics workflows that utilize MS strategies are challenging due to the high occurrence of lipid isomers, resulting in overlapping lipid ions for a single *m/z* value. LC separations prior to MS measurements help reduce sample complexity, but additional analytical techniques are needed to further elucidate the structural diversity that is present in lipid samples. In this study, ion mobility (IM) separations are evaluated in support of MS-based lipidomic workflows. All Ions IM-MS fragmentation that aligns fragment ions with their precursor using the drift dimension facilitates lipid identifications for data-independent acquisition. 2D-LC which combines different chromatographic separations is utilized to reduce sample complexity prior to analysis with All Ions IM-MS fragmentation.

Two Dimensional Liquid Chromatography

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). A diagram of the operating principle for the 2D-LC valving is shown for the sample loops and active solvent modulation.



Ion Mobility Mass Spectrometry

The Agilent 6560 Ion Mobility Q-TOF LC/MS system 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 aligns fragment ions with their precursors according to drift time.

Experimental

LC-IM-MS Experiments

LC methods were adapted from previously described methods for HILIC[1] and reverse phase[2], but briefly an RX-SIL HILIC column (3.0 x 100 mm, 1.8 micron, 0.36 mL/min flow rate) and a Agilent Poroshell 120 EC-C18 column (3.0 x 100 mm, 2.7 µm, 0.6 mL/min flow rate) were used for LC experiments. Ion mobility experiments were performed with the single field approach described previously[3]. Lipid standards were purchased from Avanti Polar Lipids (Alabaster, AL) and the NIST SRM 1950 human plasma from Millipore Sigma (St. Louis, MO).

HILIC

Min	Sol A: ACN (0.1% FA)	Sol B: ACN:MeOH:H_2O (50:20:30 v/v) (20 mM NH_4HCO_2)
0.0	70%	30%
2.0	40%	60%
4.0	30%	70%
5.0	0%	100%
8.0	0%	100%
9.0	70%	30%
12.0	70%	30%

Reversed Phase

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)
0.0	30%	70%
1.0	30%	70%
3.5	14%	86%
10.0	14%	86%
11.0	0%	100%
17.0	0%	100%
17.1	30%	70%
19.0	30%	70%

All Ions IM-MS Experiments

All Ions IM-MS experiments were performed on the Agilent 6560 IM-MS. A ramped collision energy was used as shown in the table to the right. Figure 1 displays the concept of aligning fragment ions with their precursor.

Drift Time (ms)	Collision Energy
0	10
20	15
30	20
40	25
50	35
59	50

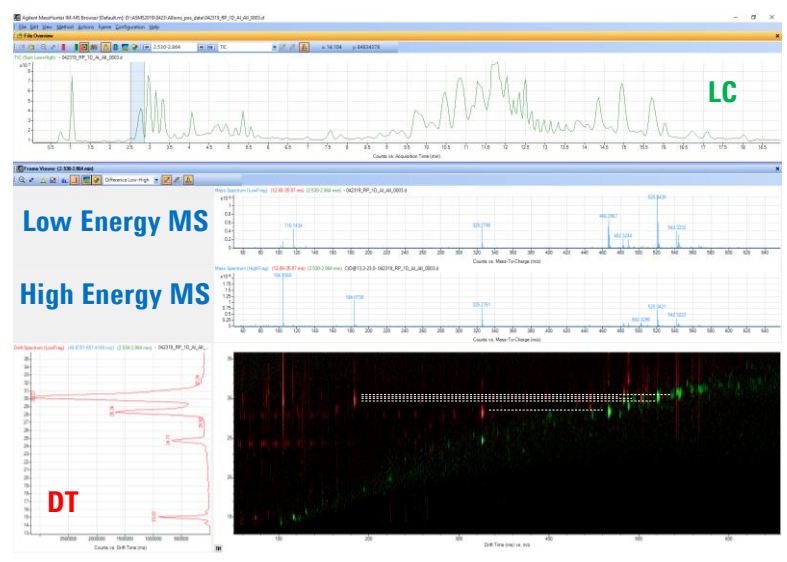


Figure 1. Fragment ions (red) aligned with precursor ions (green) from an All Ions IM-MS experiment

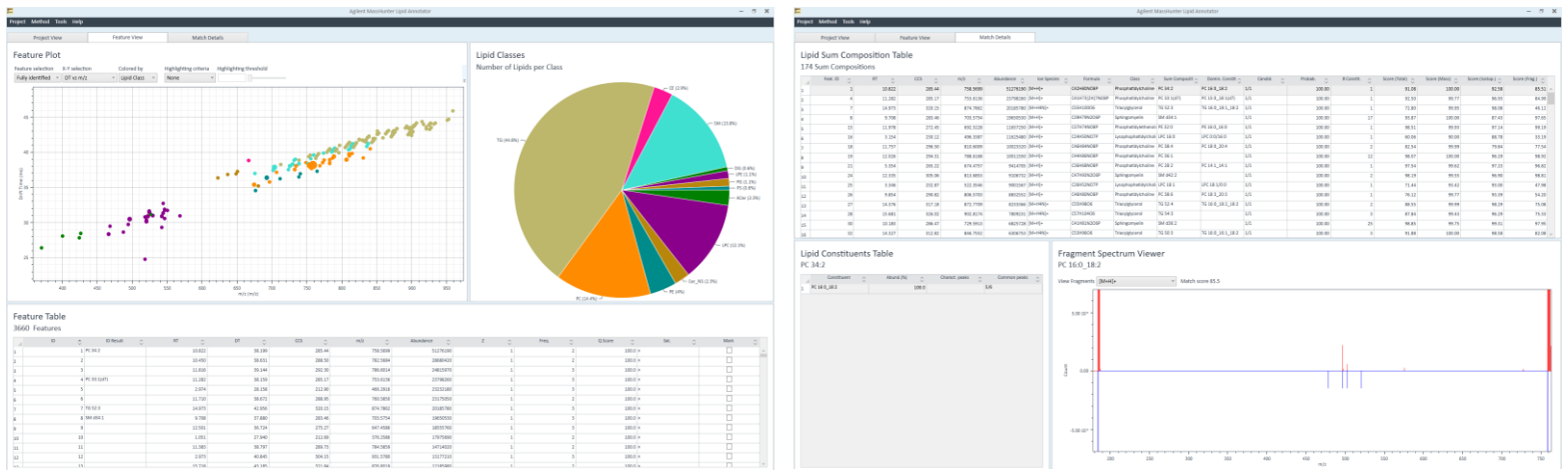


Figure 2. Feature View and Match Details View Results from Lipid Annotator for All Ions IM-MS Data. Feature View provides a high level preview of the lipid classes present in the sample in a pie chart and a feature plot which can show *m/z* vs. RT or *m/z* vs. DT (shown here). The Match Details View has a table that gives information about the lipid annotation including scoring details. There is a mirror plot that the user can inspect to build confidence in the annotations.

Lipid Annotator & All Ions IM-MS

Lipid Annotator supports All Ions IM-MS data where fragment ions are drift aligned with their precursor ion. The mass, isotope pattern, and MS/MS spectral agreement are all used to make confident lipid annotations.

Accurate Mass, RT, CCS, and MS/MS Spectra Database

Annotated lipids are then exported into a PCDL that contains accurate mass, retention time, collision cross section, and MS/MS spectra. This database can be used in an untargeted workflow with Agilent MassHunter Mass Profiler and ID Browser or the *m/z*, RT, and CCS can be used to created a list for a targeted extraction with Skyline[4]. The generated PCDL supports manual editing.

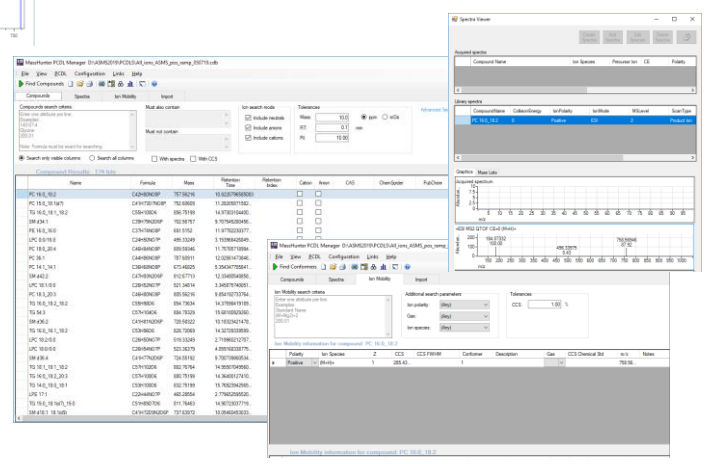


Figure 3. PCDL Export from Lipid Annotator with Accurate Mass, Retention Time, Collision Cross Section, and MS/MS Spectra

Aligning MS1 Features with Lipid Annotations

MS1 data is preferred for profiling data analysis investigations as it provides better peak shapes since time is not spent on the MS/MS level analysis. For IM data, there are two workflows to align the MS1 data with the annotation results from Lipid Annotator. Mass Profiler and ID Browser provide an untargeted approach to the alignment while Skyline performs targeted extraction on the MS1 data. Skyline provides data analysis capabilities, but both workflows can result in either a CEF or CSV format for lipid specific data analysis capabilities in Mass Profiler Professional.

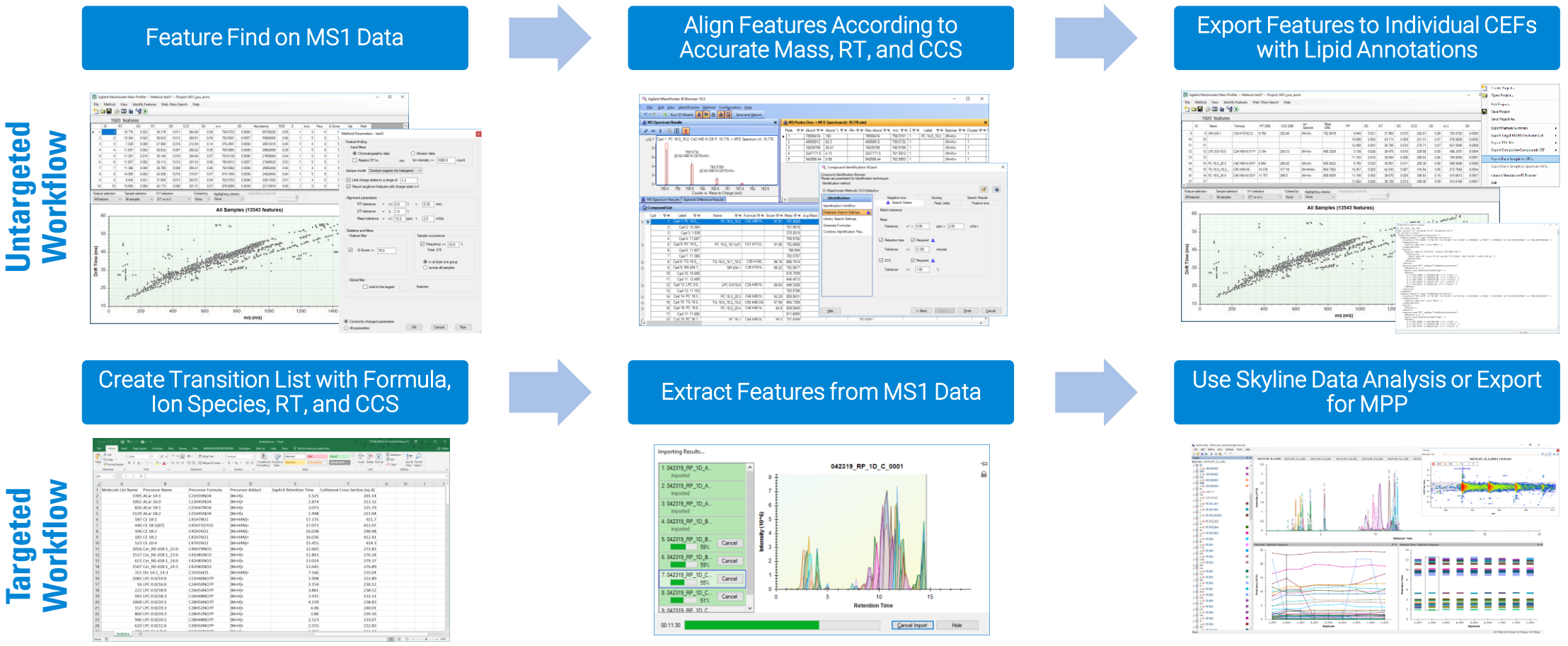
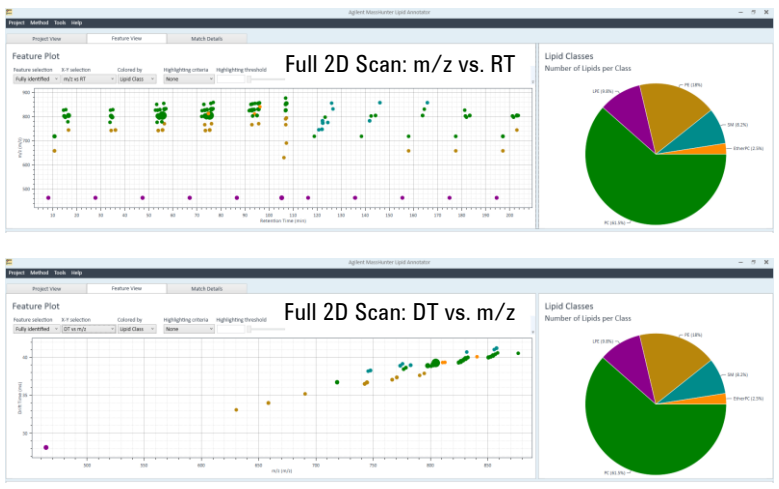


Figure 4. Workflow diagrams for aligning MS1 features with lipid annotations from Lipid Annotator. In both workflows accurate mass, retention time and collision cross section values are all used either as a targeted extraction with Skyline or as an untargeted annotation with Mass Profiler and ID Browser.

Results and Discussion

Benefits of 2D-LC for Lipid Workflows

2D-LC workflows are classified as either heart cutting or comprehensive. In a heart cutting experiment specified sections of the first dimension are cut and sent to the second dimension. Comprehensive workflows send the entire first dimension to the second to create a two dimensional depiction of the data. Traditionally the comprehensive approach uses fast value switching and thus very short 2D runs. By using a hybrid high resolution approach, the second dimension can be longer as performed for this lipid analysis. Current research efforts are investigating how to better visualize the multidimensional data which allow for the RT from both LC dimensions to be utilized. Additionally, the benefit of performing 2D-LC prior to All Ions IM-MS experiments was evaluated in terms of annotation number.



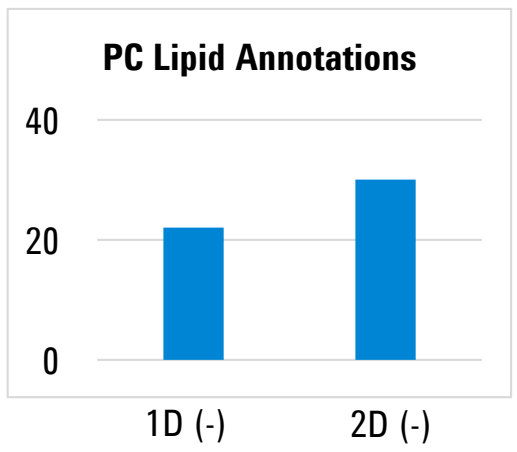


Figure 5. The *m/z* vs. RT (each of the 11 clusters representing a RP run) and DT vs. *m/z* options allow the user to visualize 2D-LC IM-MS data in 3D while still benefiting from increased separations from 2D-LC. The bar chart shows an increase in negative mode for PC annotations.

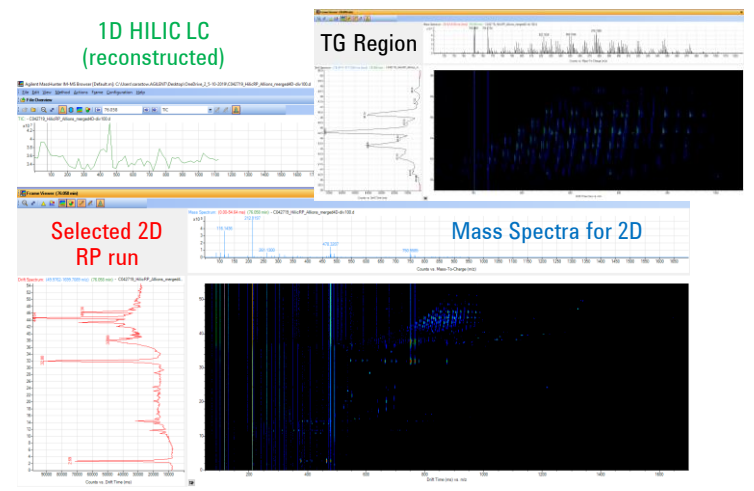


Figure 6. Repurposing the heat map feature in IM Browser for 2D-LC data allows the 2D data to be analyzed while referencing the 1D elution time. Here a complex region of TGs is highlighted.

Further Data Analysis with Mass Profiler Professional

Mass Profiler Professional provides a set of lipid specific data analysis options. These include internal standards normalization, lipid heat maps both across classes and within a class, a Kendrick mass defect plot, and a scatter plot colored by lipid class. For unknown lipids (grey in the plot below) using a combination of Kendrick mass defect, CCS, and RT provide confidence in potential new annotations. The lipid heat maps show how spiked in standards in sample groups A (PC, PG, PS), B (PE), and C (LPE) can be traced. The specific lipid standards can also be highlighted on the heat maps within each lipid class as shown here for PC lipids.

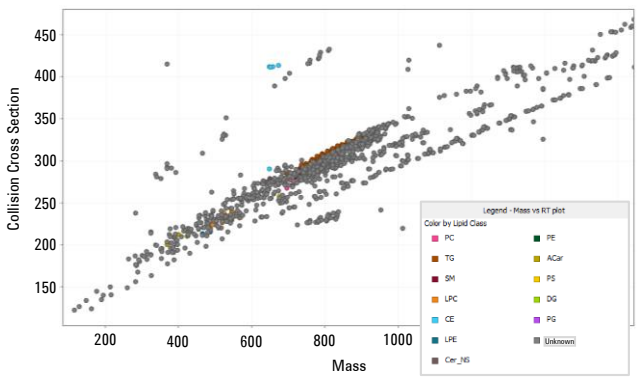
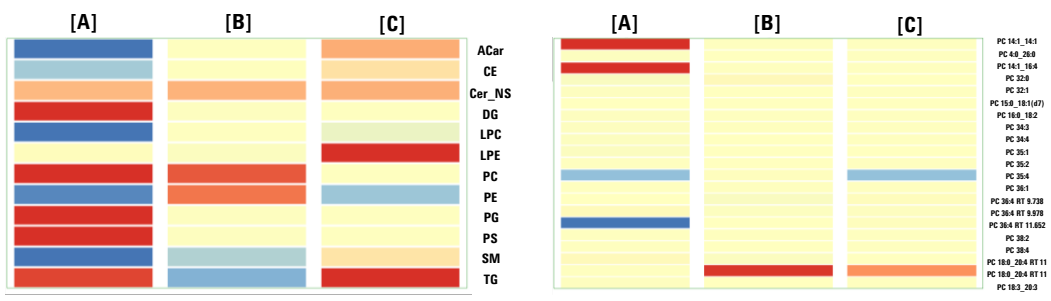


Figure 7. The colored by lipid class scatter plot for mass vs. CCS and the lipid heat maps across classes as well as within the PC lipid class are highlighted here.



Conclusions

- Lipid annotations based on All Ions IM-MS experiments can be aligned with MS1 data for profiling using either Mass Profiler and ID Browser or Skyline
- Performing 2D-LC prior to All Ions IM-MS can improve the number of lipid annotations by reducing the complexity introduced to the MS at a given time
- Future research efforts will evaluate the precursor-fragment alignment algorithm for All Ions IM-MS and optimize 2D-LC methods for optimal lipid separations

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.

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