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Applikationsbericht

Achieving Comprehensive Lipid Profiling with a CCS, Retention Time, and MS/MS Library

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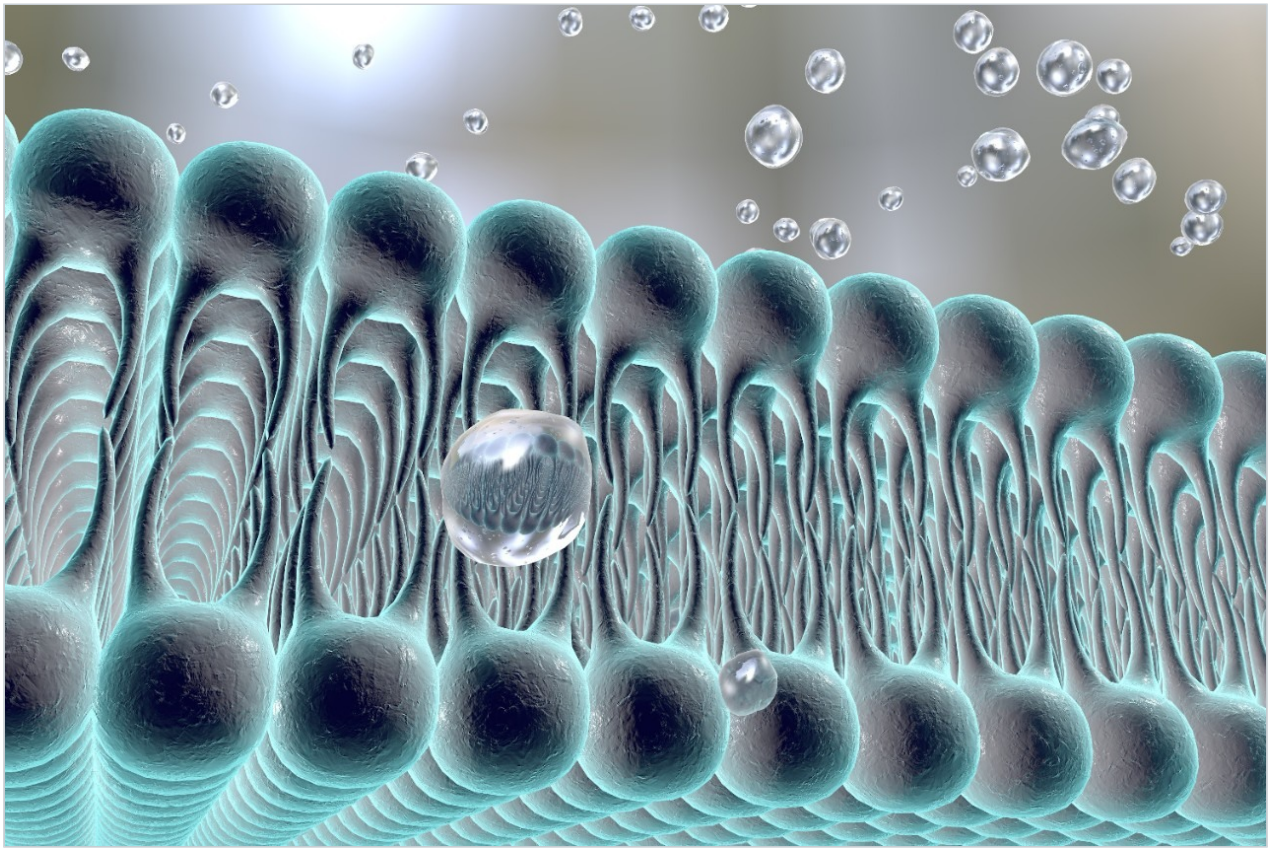

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Abstract

In this application note, a standardized protocol is used to measure RT and CCS information from a sample set consisting of 100 certified standards from multiple lipid classes to validate a predictive TWCCSN_2 Model by Broeckling *et al*.

Benefits

- An easy to deploy method and library, providing greater confidence for the identifications returned and enabling increased productivity
- A curated lipidomic database containing over 3200 lipid species which have been previously reported for human plasma and tissue samples
- Lipid standards allowed for the validation of a CCS predictive model, which allowed for the incorporation of *in-silico* values to increase coverage
- Assigned measured RTs to reduce false-positive identifications
- Comprehensive MS/MS information for multiple adducts built into the library for greater confidence
- Fast data processing and visualization using either UNIFI or Progenesis QI Informatics for maximum flexibility

Introduction

Lipids are well recognized as important biological molecules, playing a diverse role in various molecular functions, including signal transduction, membrane anchoring, as well as energy storage. To gain insight into the lipidome, analytical technologies such as liquid chromatography coupled with mass spectrometry (LC-MS) have become a routine means of analysis, providing comprehensive, high throughput, and robust solutions. Discovery-based workflows utilizing high resolution strategies, allow complex samples to be profiled, providing a large number of potential lipid identifications. A significant hurdle encountered for discovery lipidomics is the ability to identify lipids of interest accurately and confidently from database searches. Additional physiochemical properties such as retention time (RT) and collisional cross section (CCS), as well as diagnostic MS/MS information can be incorporated into the search space to increase confidence in the identifications returned.

CCS is an important physicochemical property of lipid species that is closely related to its chemical structure and three-dimensional conformation, yet is independent of the type or complexity of sample matrix.[1] Therefore, CCS

values can be used in addition to other molecular identifiers, such as RT and *m/z* as an orthogonal attribute to increase the specificity of lipid identifications.[1] Several *in-silico* and experimentally derived CCS databases have been published to-date as reported by Paglia *et al.*[1] but suffer a high false-positive rate and often identifications lack biological relevance. Ideally, experimental measured CCS values should be used to populate CCS databases, but due to a limited number of commercially available lipid standards, *in-silico* measurements are needed to enable adequate coverage.

Here we used a standardized protocol to measure RT and CCS information from a sample set consisting of 100 certified standards from multiple lipid classes to validate a predictive TWCCSN$_2$ Model by Broeckling *et al.*[2] The high correlation established between the experimentally measured and predicted values then allowed for the remainder of the library to be populated with *in-silico* CCS values. Fragment ion information was also appended to the library, accounting for multiple adduct types. Extracts resulting from human plasma and tissues (*i.e.* heart and liver) were used as additional test samples for identifying endogenous species.

Experimental

Sample Information

To assess the accuracy of the CCS prediction tool, a variety of lipid standards were acquired. These consisted of 69 individual lipids (Avanti Lipids, Alabama, USA) from across 14 classes, which were constituted into sample groups of no more than 7 lipids/mix with distinct reversed-phase (RP) chromatographic behaviors (*i.e.* to prevent co-elution), in IPA. Samples were injected at three different concentration levels in triplicate as a custom mix of standards. Additionally, Odd Chain LIPIDOMIX, Light Splash LIPIDOMIX, Differential Ion Mobility System Suitability LIPIDOMIX, Ceramide LIPIDOMIX, Splash LIPIDOMIX, and Deutrated Ceramide LIPIDOMIX were diluted 1:10 and 1:100 in IPA and injected as replicates (n=3).

Library Building

Additional class-based lipid extracts (PS, PE, PG, Cer and SM mixes) were prepared at 0.1 mg/mL and 0.01 mg/mL and acquired as technical triplicates. These were used to verify and add confidence in the biological relevance of the lipids selected. To evaluate the utility of the library, extracts resulting from human plasma, liver, and heart tissues were screened.

LC Conditions

LC system:	ACQUITY Premier UPLC
Column(s):	ACQUITY Premier CSH $C_{18,}$ 2.1 x 100 mm, 1.7 µm
Column temperature:	55 °C
Flow rate:	0.4 mL/min
Mobile phase:	600/390/10 (Acetonitrile/water/1 M Ammonium formate) in 0.1% formic acid (A) and 900/90/10 (IPA/Acetonitrile/1 M Ammonium formate) in 0.1% formic acid (B)
Run time:	12 minutes
Injection volume:	1–4 µL

Gradient

Time [min]	Flow [mL/min]	%A	%B	Curve
Initial	0.4	50	50	initial
0.5	0.4	47	53	6
4.0	0.4	45	55	6
7.0	0.4	35	65	6
7.5	0.4	20	80	1
10	0.4	1	99	6
11	0.4	1	99	1
12	0.4	50	50	1

MS Conditions

MS systems:	SYNAPT XS

Ionization mode:	ESI (+/-)
Capillary voltage:	3 kV (+); 2.5 kV (-)
Acquisition mode:	HDMS^{E}
Acquisition range:	50–1200 Da
Collision energy:	Linear ramp (transfer CE) 25–45 eV
Cone voltage:	30 V
Source temperature:	120 °C
Desolvation temperature:	500 °C
Cone gas flow:	150 L/hr
Desolvation flow:	1000 L/hr
Helium cell gas flow:	180 mL/min
IMS gas flow (nitrogen):	90 mL/min
IMS wave velocity:	900 m/s
IMS wave height:	40 V

Informatics

MassLynx, UNIFI, and Progenesis QI

Results and Discussion

Validating predicted CCS values

Over 100 individual lipids from custom mixes and commercially available sources were used to validate the *in-silico* CCS predictions.[2] Lipid standards were chosen to cover all the major lipid classes and to ensure a wide cross section of lipid structures were accounted for. The mean observed CCS values of measured lipid standards is compared to the predicted values by calculating the percentage difference:

$$\frac{(\text{Obs CCS- Pred CCS})}{\text{Obs CCS}} * 100$$

Comparing the measured CCS with *in-silico* predicted values, provided high correlation for all classes. Figure 1 represents three example lipid classes, highlighting excellent linearity with r^2 >0.96 being generated.

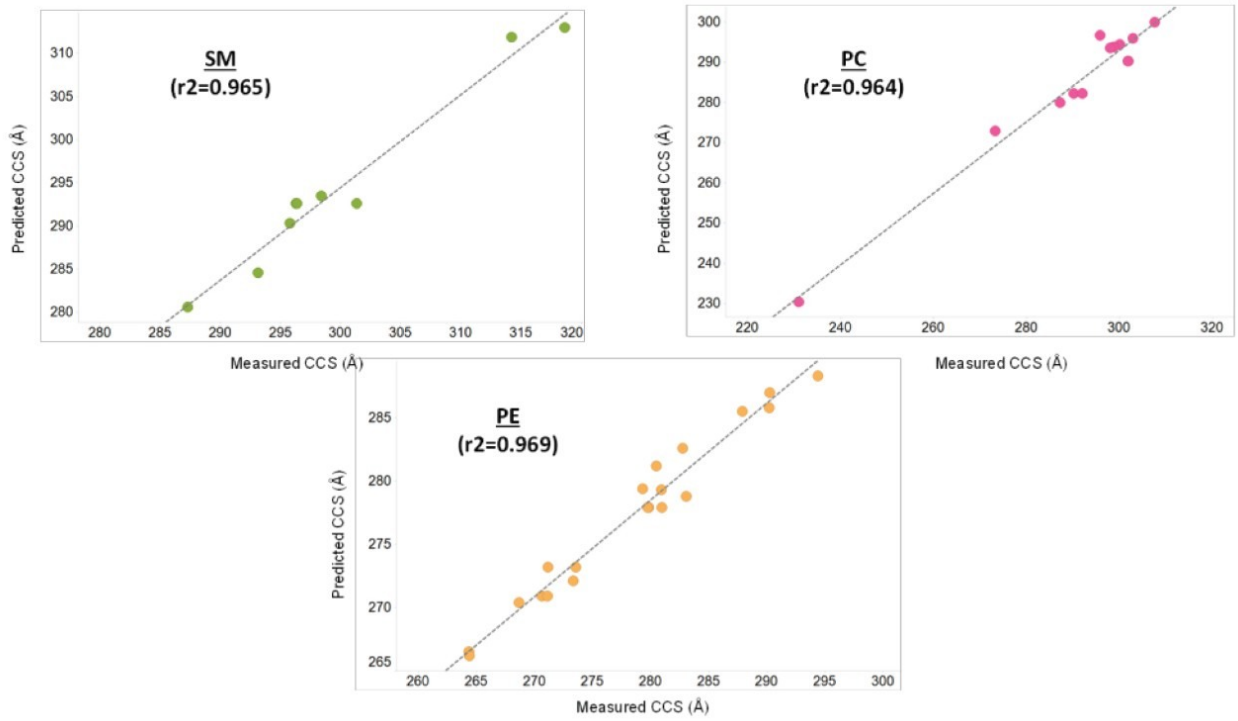


Figure 1. Assessment of predicted CCS values using measured CCS values of known lipid standards. Three example lipid classes are shown, representing sphingomyelin (SM), phosphocholine (PC), and phosphatidylethanolamine (PE). The data represents protonated adducts for both negative and positive ESI.

Peaks which were either saturated, had an error ≥5% between measured and predicted CCS values, or a retention time deviation ≥0.1 min were excluded. After correction for retention time, to reduce false-positive hits, 96.8% (positive ion) and 95.5% (negative ion) of the experimental data were found to be

within ±5% CCS tolerance when compared with their *in-silico* values, confirming the findings of previously reported data.[2]

A total of 32 and 16 lipid classes are represented with CCS values for positive and negative ion respectively. The CCS distribution ranges from 143–349 Å (positive ion) and 124–334 Å (negative ion). Exemplar CCS distributions for a subset of lipid classes corresponding to positive ion are provided in Figure 2.

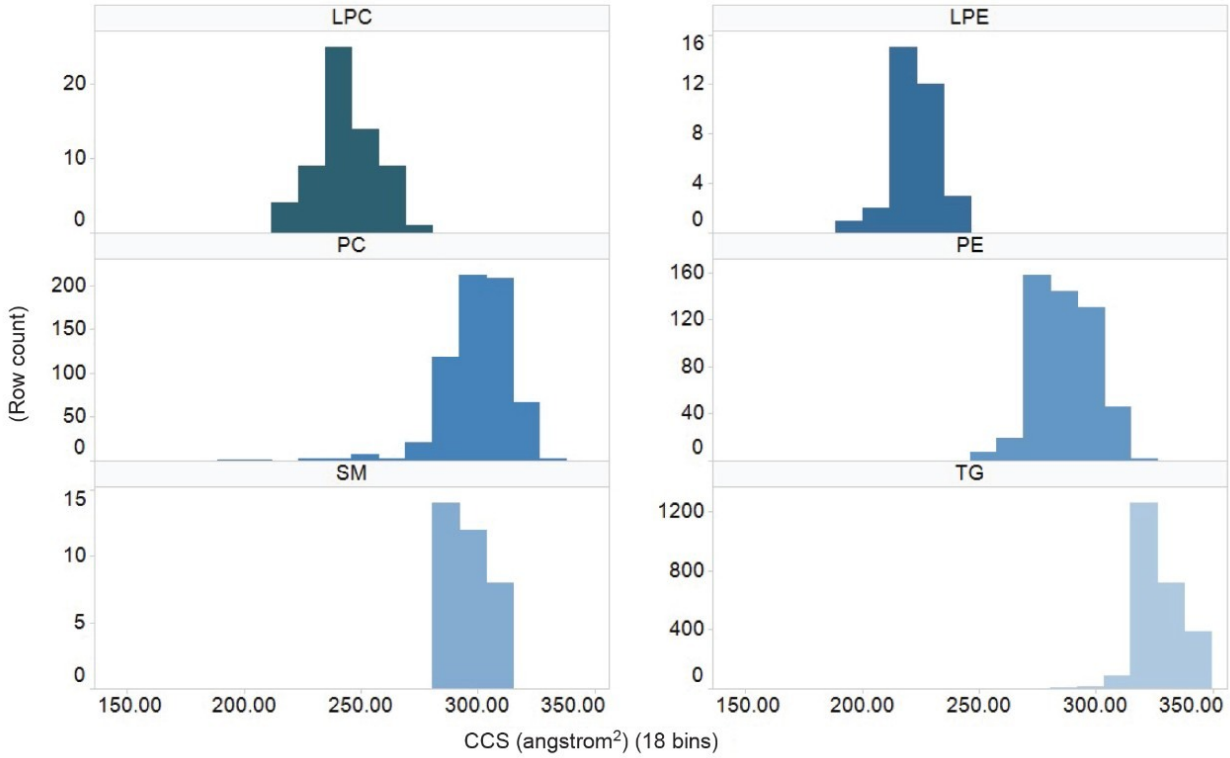


Figure 2. Example CCS distribution of six lipid classes (ESI+) from the library.

Database Overview

The availability of comprehensive, well-curated databases is important for ensuring compounds are accurately and confidently identified. Therefore, to address these needs, the comprehensive lipid database presented here, consists of 3200 lipid entries and include the most common adducts for both positive and negative ESI. Figure 3 shows the distribution of lipid entries between the ionization modes, whilst Figure 4 represents the adducts accounted for in both ionization modes. Of the 3200 lipids presented, 43% of the entries are represented by both ionization modes. This has the advantage of providing higher identification confidence. For example, when considering phospholipids, the ability to characterize both the head group and fatty acyl chain(s) is possible.

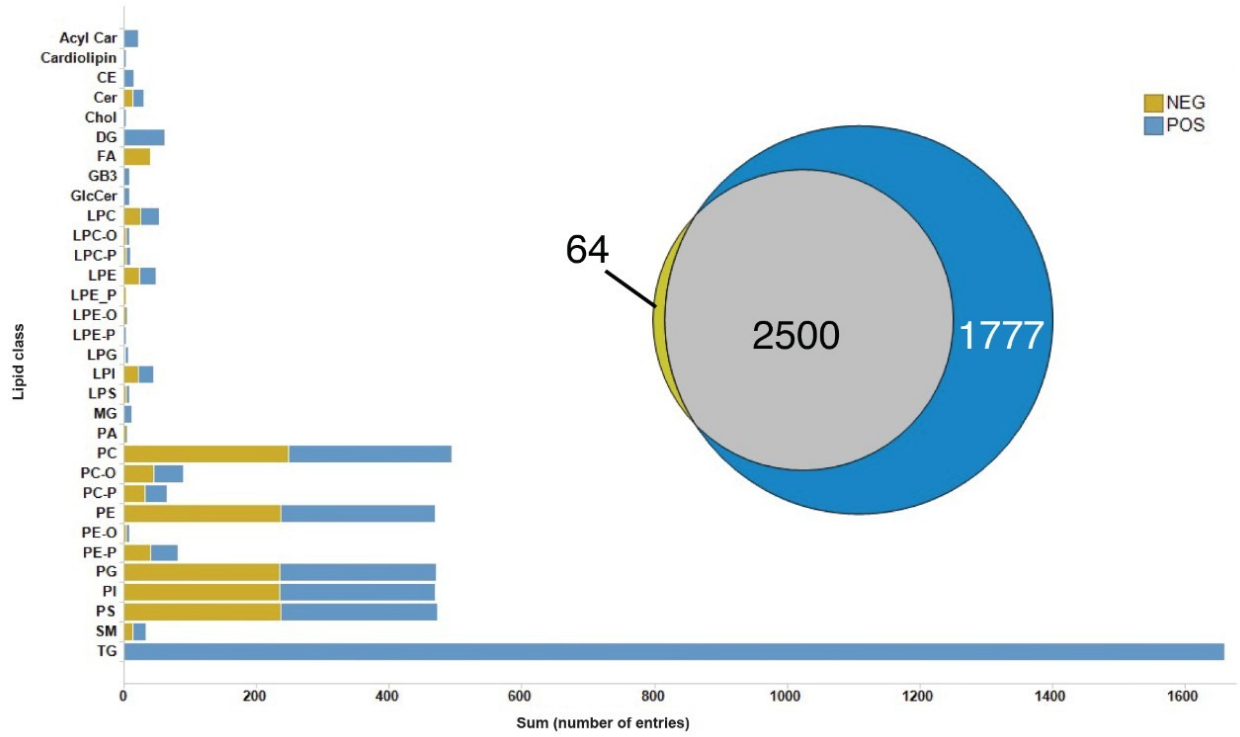


Figure 3. Lipid library composition for both positive (blue) and negative (yellow) ESI. A total of 3200 entries are represented with 43% common between both ionization modes.

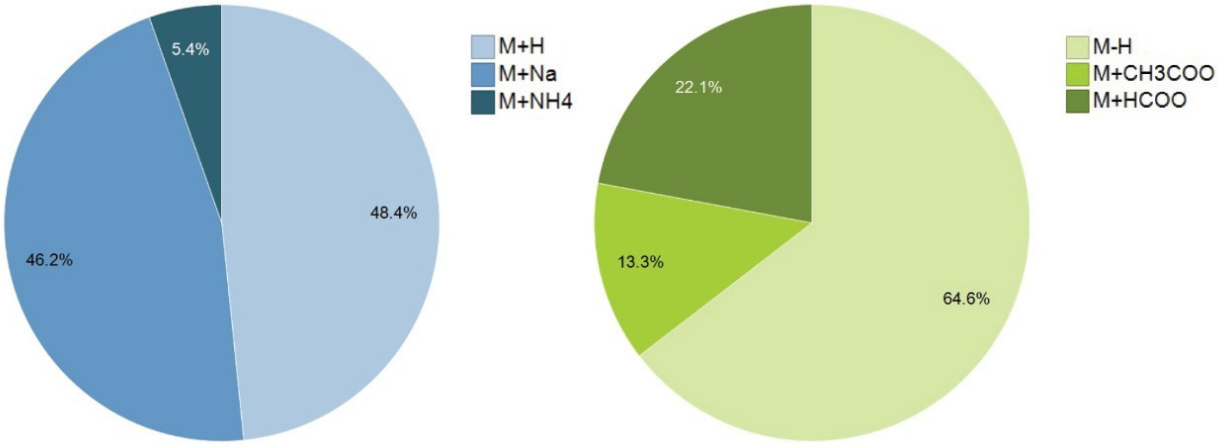


Figure 4. Distribution of adducts represented within the library for positive (blue) and negative (green) ionization modes.

The chromatographic method employed for generating the library consisted of a 12-minute cycle time, which has

previously been demonstrated as being specific, robust, and reproducible for large scale lipidomic studies.[3] Additionally, this methodology also utilized the benefits of the hybrid surface technology from the ACQUITY Premier System, whereby the sensitivity and recovery of phosphorylated and carboxylated lipid species are significantly improved (Application Note 720007092 <https://www.waters.com/nextgen/us/en/library/application-notes/2021/acquity-premier-lc-technology-significantly-improves-sensitivity,-peak-shape-and-recovery-for-phosphorylated-and-carboxylate-lipids.html>). Retention time values relating to the 100 lipid standards which were used for CCS validation, were also used for verification and appended to the database. Example chromatography for a plasma extract is provided in Figure 5, along with the RT distribution for the experimentally measured lipid standards.

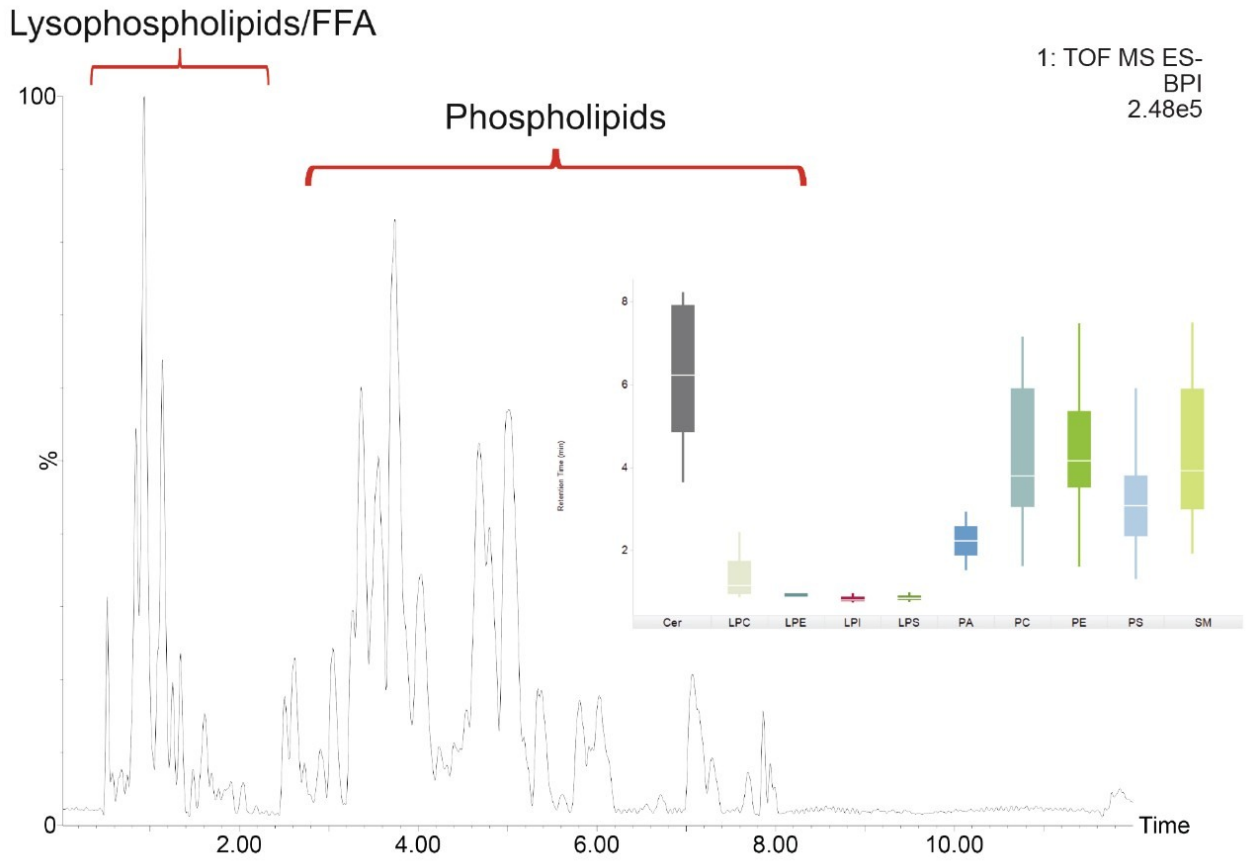
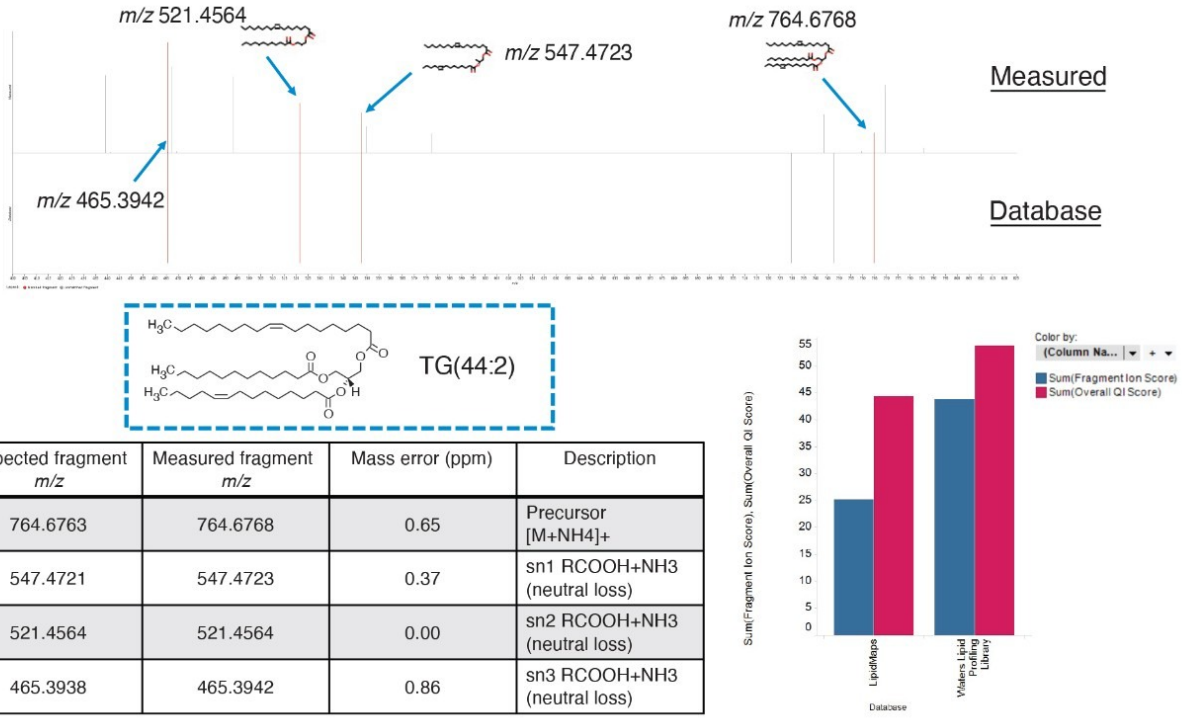


Figure 5. Chromatogram representing a plasma extract (ESI-) with the various lipid groups highlighted based on RT. Inset is the RT distribution gained for a number of the lipid standards measured.

Utility with Progenesis QI and UNIFI for Maximum Flexibility

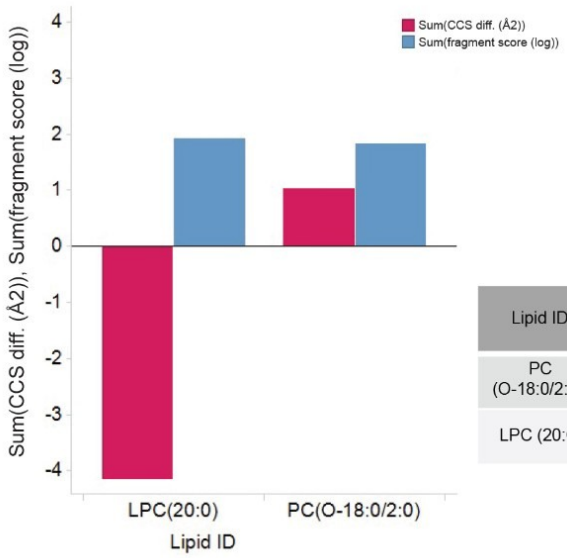
To illustrate the utility of the library, database searches were conducted for a variety of endogenous matrices. The library has been constructed to provide maximum flexibility, enabling searches to be performed using either

Progenesis QI or UNIFI Processing Software. Figure 6 shows an endogenous TG(44:2) identification resulting from a Progenesis QI Search. Comparing searches between the Waters Lipid Profiling Library and LipidMaps shows a 42% increase in fragment ion scores, whilst the overall identification score (inclusive of CCS and MS/MS) resulted in a 17% increase when using the Waters Lipid Library. The increases observed in identification score(s) is a result of the enhanced specificity provided with the inclusion of CCS and MS/MS information. Figure 7 highlights how implementing CCS can help to filter the number of identifications returned following a database search.



Expected fragment *m/z*	Measured fragment *m/z*	Mass error (ppm)	Description
764.6763	764.6768	0.65	Precursor [M+NH4]+
547.4721	547.4723	0.37	sn1 RCOOH+NH3 (neutral loss)
521.4564	521.4564	0.00	sn2 RCOOH+NH3 (neutral loss)
465.3938	465.3942	0.86	sn3 RCOOH+NH3 (neutral loss)

Figure 6. Example Progenesis QI Database Search of a human plasma extract. The NH4+ adducted TG(44:2) is presented, highlighting the increased scoring (fragment ion and overall scores) achieved when searching against the Waters Lipid Profiling Library. The mirror plot indicates matching fragment ions from the experimental data (measured) with those of the database.



Lipid ID	Adduct	CCS (Å²)	ΔCCS (Å²)	Overall score	Fragment score	Fragment score (log)	Mass error (ppm)
PC (O-18:0/2:0)	[M+H]	247.8	1.03	62.2	68.1	1.83	-0.57
LPC (20:0)	[M+H]	249.5	-4.14	59.2	83.4	1.92	-0.57

Figure 7. Filtering the number of potential identifications on the basis of CCS. The example provided, shows how implementing CCS as part of the database search can significantly help to highlight the most credible identifications.

Similarly, data corresponding to human plasma spiked with Odd Chain LIPIDOMIX was processed through UNIFI (Figure 8). The MS/MS relating to SM(d18:1/12:0) is shown for both positive and negative ion data with the library matched fragment ions clearly marked.

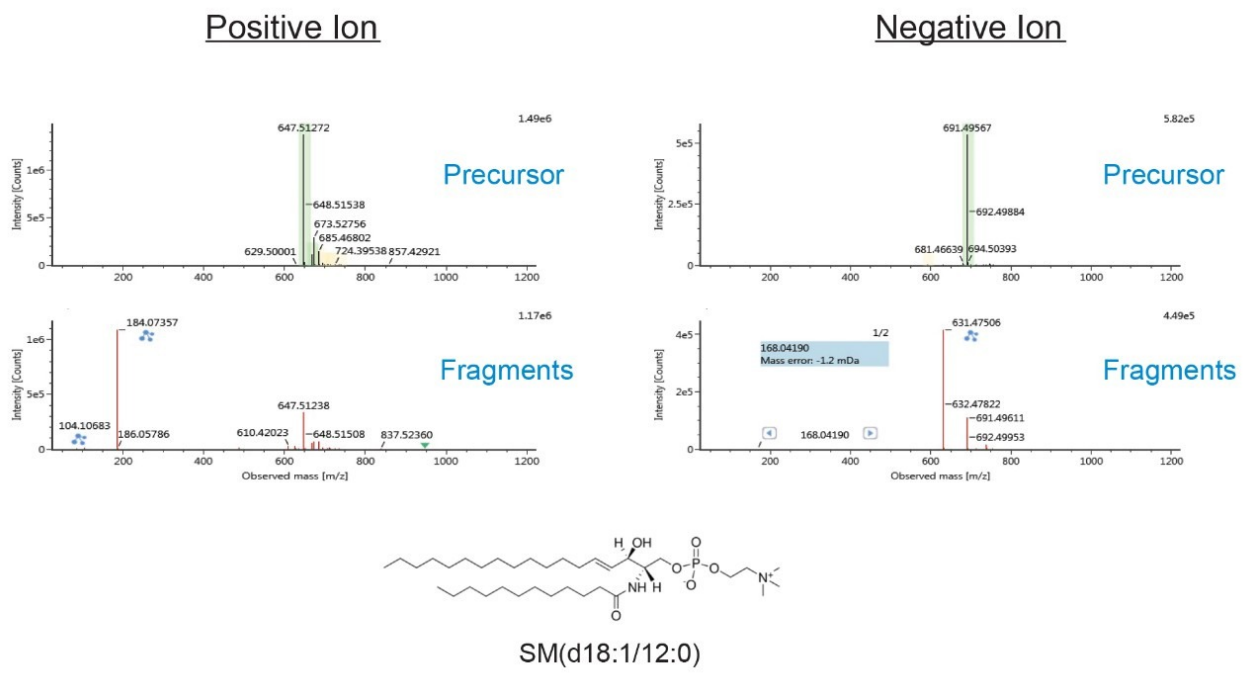


Figure 8. UNIFI processed data searched against the Waters Lipid Library. This example shows the positive and negative ion spectra, representing the precursor and fragment ions of SM(d18:1/12:0).

Conclusion

- A predictive algorithm which generates *in-silico* CCS values has been successfully validated using over 100 lipid standards across 14 lipid classes.
- Incorporation of CCS, MS/MS, and retention time information is shown to increase specificity, resulting in increased identification scores and thereby providing greater confidence.
- Extensive database curation based on biological relevance and measured values from extracts.
- The versatility of the library allows it to be used with UNIFI and/or Progenesis QI Informatics.

References

1. Paglia, G., Smith, A. J., Astarita, G. (2021) Ion Mobility Mass Spectrometry in the Omics Era : Challenges and

Opportunities for Metabolomics and Lipidomics. doi:10.1002/mas.21686.

2. Broeckling, C. D. *et al.* Application of Predicted Collisional Cross Section to Metabolome Databases to Probabilistically Describe the Current and Future Ion Mobility Mass Spectrometry. *J. Am. Soc. Mass Spectrom.* (2021) doi:10.1021/jasms.0c00375.

3. Giorgis Isaac, Nyasha Munjoma, Lee A. Gethings, Lauren Mullin, Robert S. Plumb. A Robust and Reproducible Reversed-Phase Lipid Profiling Method for Large Sample Sets, Waters Application Note, 720006959EN < https://www.waters.com/nextgen/us/en/library/application-notes/2020/a-robust-and-reproducible-reversed-phase-lipid-profiling-method-for-large-sample-sets.html> .

4. Giorgis Isaac, Robert S. Plumb. ACQUITY Premier LC Technology Significantly Improves Sensitivity, Peak Shape, and Recovery for Phosphorylated and Carboxylate Lipids, Waters Application Note, 720007092EN < https://www.waters.com/nextgen/us/en/library/application-notes/2021/acquity-premier-lc-technology-significantly-improves-sensitivity,-peak-shape-and-recovery-for-phosphorylated-and-carboxylate-lipids.html> .

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