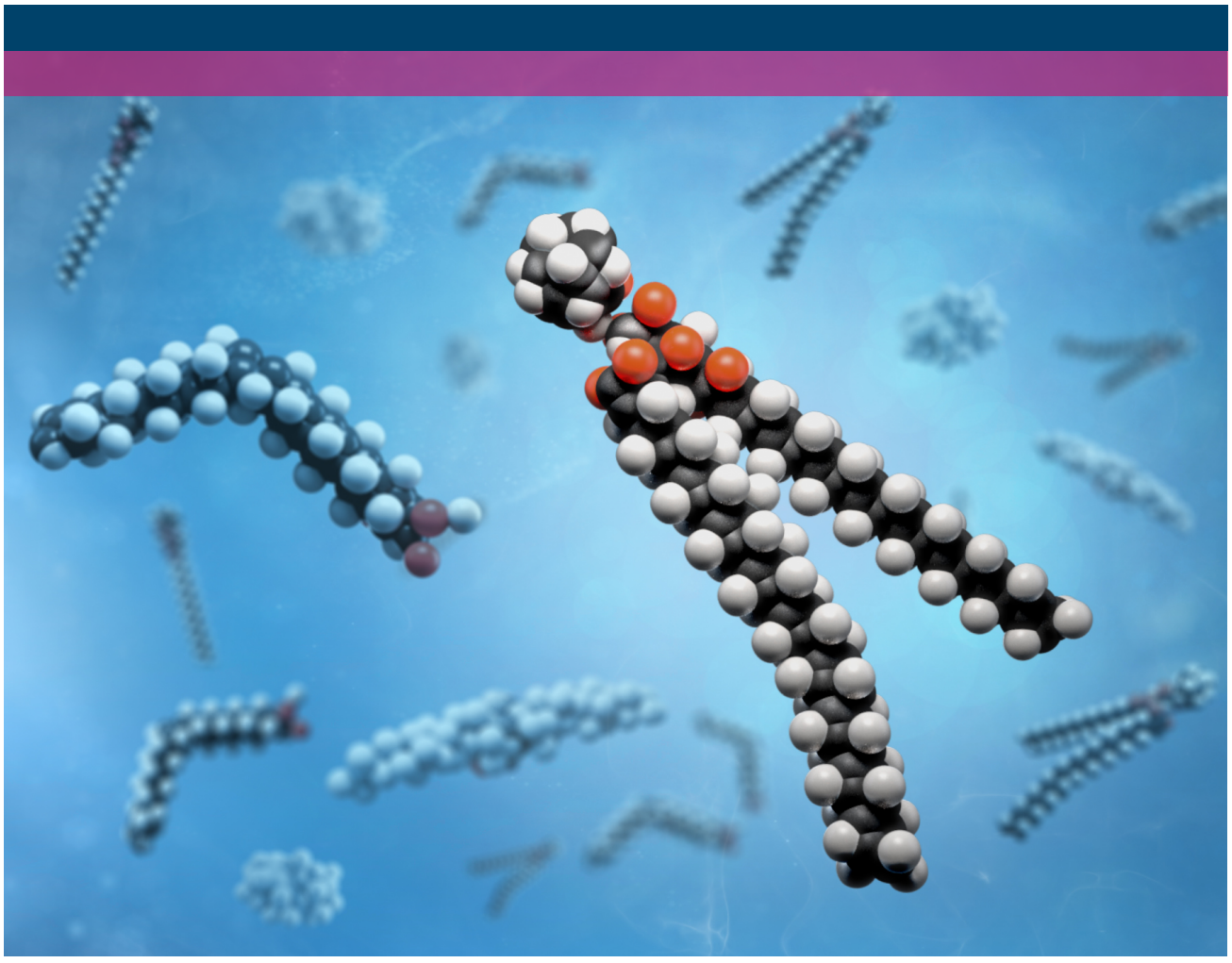
Lipidomics Workflow Guide

Agilent 6560 Ion Mobility LC/Q-TOF



Notices

Manual Part Number

D0003136 Revision A.00
July 2021

Copyright

Agilent Technologies, Inc.
5301 Stevens Creek Blvd.
Santa Clara, CA 95051

Warranty

The material contained in this document is provided "as is," and is subject to being changed, without notice, in future editions. Further, to the maximum extent permitted by applicable law, Agilent disclaims all warranties, either express or implied, with regard to this manual and any information contained herein, including but not limited to the implied warranties of merchantability and fitness for a particular purpose. Agilent shall not be liable for errors or for incidental or consequential damages in connection with the furnishing, use, or performance of this document or of any information contained herein. Should Agilent and the user have a separate written agreement with warranty terms covering the material in this document that conflict with these terms, the warranty terms in the separate agreement shall control.

Technology Licenses

The hardware and/or software described in this document are furnished under a license and may be used or copied only in accordance with the terms of such license.

Restricted Rights Legend

U.S. Government Restricted Rights. Software and technical data rights granted to the federal government include only those rights customarily provided to end user customers. Agilent provides this customary commercial license in Software and technical data pursuant to FAR 12.211 (Technical Data) and 12.212 (Computer Software) and, for the Department of Defense, DFARS 252.227-7015 (Technical Data - Commercial Items) and DFARS 227.7202-3 (Rights in Commercial Computer Software or Computer Software Documentation).

Safety Notices

CAUTION

A **CAUTION** notice denotes a hazard. It calls attention to an operating procedure, practice, or the like that, if not correctly performed or adhered to, could result in damage to the product or loss of important data. Do not proceed beyond a **CAUTION** notice until the indicated conditions are fully understood and met.

WARNING

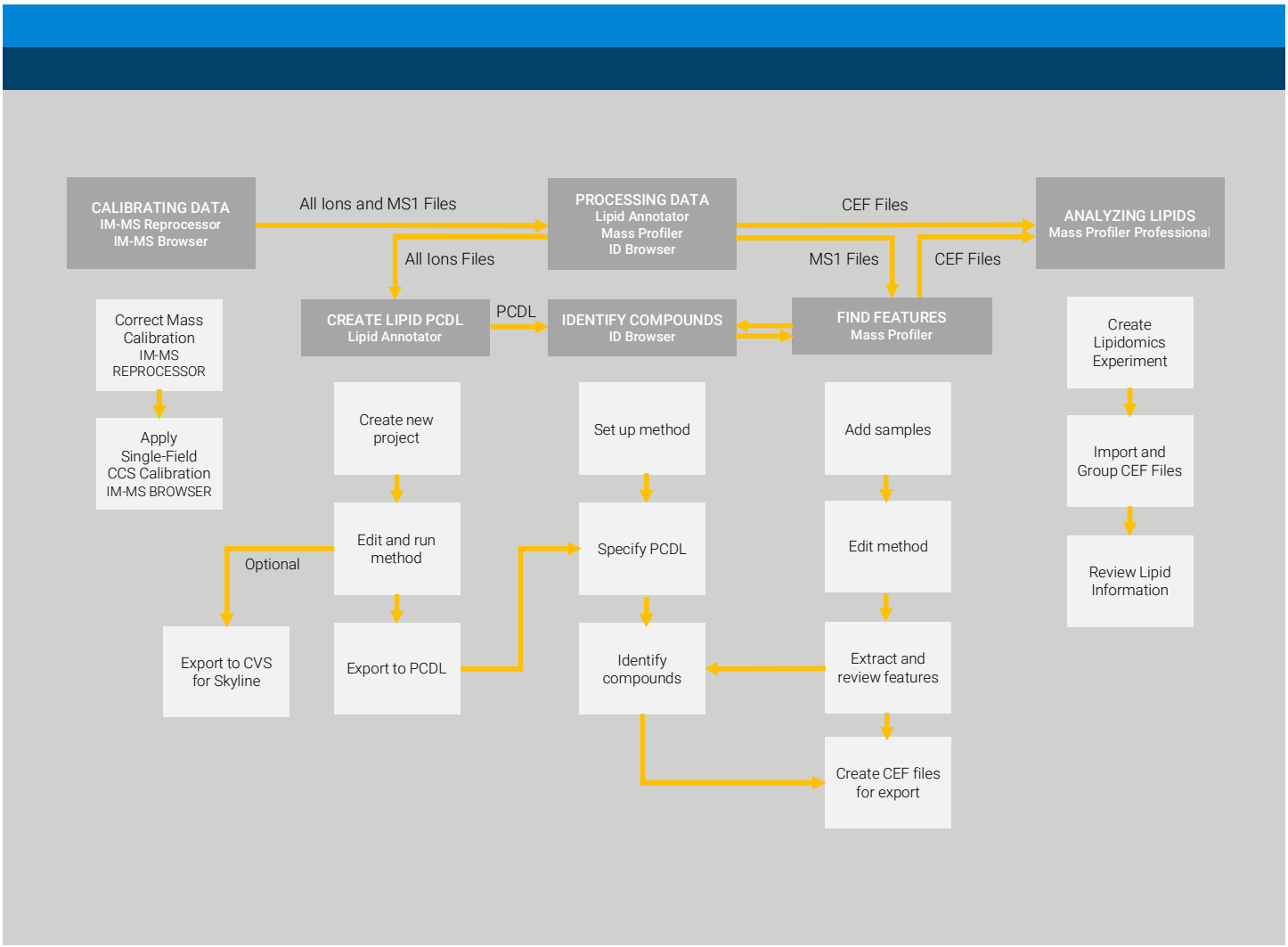
A WARNING notice denotes a hazard. It calls attention to an operating procedure, practice, or the like that, if not correctly performed or adhered to, could result in personal injury or death. Do not proceed beyond a WARNING notice until the indicated conditions are fully understood and met.

Content

1 Before You Begin

Make sure you read and understand the information in this chapter and have the necessary computer equipment, software and example data files before you start your analysis.

Introduction



To understand the specifics of the Primary Lipidomics Workflow, first familiarize yourself with lipids, lipidomics and the advantage of ion mobility mass spectrometry for lipidomics studies.

What are lipids?

Lipids are a very broad, diversified group of hydrophobic or amphipathic (both hydrophobic and hydrophilic) small molecules including fats, oils, hormones and certain components of membranes grouped together because they do not interact appreciably with water. One type of lipid, the triglycerides, is sequestered as fat in adipose cells, which serve as the energy-storage depot for organisms and also provide thermal insulation. Some lipids such as steroid hormones serve as chemical messengers between cells, tissues and organs. Others communicate signals between biochemical systems within a single cell.

The amphipathic nature of some lipids lets them form structures such as vesicles, micelles, liposomes, or membranes in an aqueous environment. Triglycerides, phospholipids and sphingolipids are amphipathic and consist of a polar “head” and a non-polar acyl, or fatty acid, tail or tails attached via an ester or amide bond to the head group. See an example below:

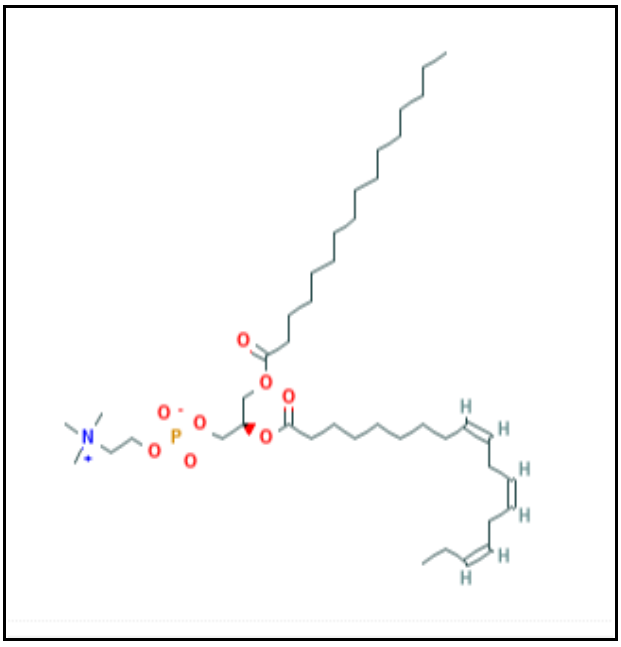


Figure 1. Example of an amphipathic lipid - phosphatidylcholine 34:3

https://pubchem.ncbi.nlm.nih.gov/compound/24778702#section=2D-Structure

Sterol lipids and prenol lipids, on the other hand, are hydrophobic and are derived from isoprene units. Cholesterol is an example of a sterol lipid.

See **"Lipids"** on page 89 to understand how to name the lipids with fatty acid chains.

See also **www.lipidmaps.org**.

What is lipidomics?

Lipidomics is one of the four components that make up the field of "metabolomics" research. Metabolites are the end products of all biological/cellular processes, and "metabolome" refers to the collective set of all metabolites generated in a biological system (cell, tissue, organ or organism). Metabolomics is the scientific study to characterize and identify the metabolome. The metabolome is comprised of four major classes of biological molecules: sugars, amino acids, nucleotides, and lipids.

The systematic study of the entire lipid profile of a cell/tissue/organ/organism is referred to as "lipidomics". Mass spectrometry is one of the most widely used technologies in lipidomics research for the identification as well as quantitation of thousands of lipid molecules that constitute the cellular "lipidome", the full lipid complement of a biological system.

Why ion mobility mass spectrometry for lipids?

Resolving isomeric species in lipidomics workflows is a huge challenge. For example, the possible permutations of fatty acid tail length and double bond locations that could describe a TG 49:1 sum composition are quite large, and several isomers could be present within a sample. These permutations have the same mass and potentially the same RT, making it difficult to differentiate them with traditional LC-MS methods. Performing LC-MS with ion mobility provides an additional dimension of separation to these experiments.

Following LC separation and before MS detection, ions are separated based on their size and shape during an ion mobility experiment, resulting in different drift times. These drift times are used to calculate a Collision Cross Section (CCS) value or a rotationally averaged surface area for the ion. Adding CCS values to accurate mass and RT values, which are both available from traditional LC-MS experiments, provides better specification and more confidence when identifying lipids.

Lipidomics workflows also require MS/MS fragmentation to annotate lipids confidently. Detecting specific fragment ions can tell you that 15:0, 16:1, and 18:0 are the three fatty acid tails for the TG 49:1 lipid. All Ions fragmentation is a data-independent acquisition mode where all precursor ions are fragmented. When All Ions fragmentation is used without ion mobility separation, the resulting data can be hard to interpret because of the difficulty of linking precursor and fragment ions. When the technique is used with ion mobility, the fragments from the All Ions experiment are drift-aligned with their precursor ion, which allows for easier data interpretation.

See **"Lipid sum composition"** on page 89.

What will you learn from this guide?

Agilent has several software packages commonly used for compound discovery and identification. The newly developed Lipid Annotator generates a lipid database specific to your data set, which you can use in these existing software packages.

Additional tools have been added to Mass Profiler Professional (MPP) that allow you to evaluate the distribution of lipid classes, types, and abundances in different samples. See **Chapter 4**, "Analyzing Lipids".

Using a sequence of these software tools, you can execute the Primary Lipidomics Workflow, described on the next pages. Agilent calls this the primary workflow because, although other lipidomics workflows do exist, their instructions do not appear in this guide.

Primary Lipidomics Workflow

This guide takes you through the Primary Lipidomics Workflow, which uses both All Ions and MS1 data files. All Ions data is collected for a pooled QC of all your samples, and this data file is used to create a custom lipid database with Lipid Annotator. Mass Profiler finds features in each individual MS1 sample data file. These features are then identified in ID Browser using the lipids database created with Lipid Annotator based on the pooled QC sample. Or you can export the lipid database to a CSV file for import into Skyline for a targeted data analysis workflow.

Workflow Steps

After acquiring the data, follow these Major Steps to eventually assess the lipid composition and abundance differences between sample sets. Each chapter gives you additional instructions to complete a primary task or tasks in the workflow.

Step 1. Calibrate Data (Chapter 2)

Step 1. Calibrate data

First, correct the mass calibration with IM-MS Reprocessor. Next, apply a single-field CCS calibration in IM-MS Browser.

Steps 2-4. Process Data (Chapter 3)

Step 2. Create lipid PCDL

Use Lipid Annotator to annotate lipids in the All Ions data files when fragment ions match the theoretical library, generating a database that contains accurate mass, retention time and CCS values. You can either export the database to a PCDL file for use with ID Browser or to a CSV file for import into Skyline.

Step 3. Find features

Using Mass Profiler on MS1 data files, perform feature finding, which results in a feature list that includes a mass, retention time and CCS value for each feature.

Step 4. Identify compounds

Using the PCDL from Lipid Annotator, identify the MS1 features as lipids with ID Browser based on accurate mass, RT and CCS values. Then return the feature list to Mass Profiler, where you create CEF files for export.

Step 5. Analyze Lipids (Chapter 4)

Step 5. Analyze lipids

With Mass Profiler Professional (MPP), lipid assessment tools are provided to compare the compound and abundance distribution between samples (exported CEF files imported to MPP).

You can also use MPP to filter out features, leaving only the most relevant features. Normalizing the data in MPP corrects data for changes in RT or response so a single feature common to several samples is not treated. MPP also provides you with statistical data analysis tools for discovering significant differences between sample sets, although instructions are not provided in this guide. See **"Resource Apps"** on page 94.

References

Chapter 5 gives you distinctions for the important terms in chapters 1-4 and a listing of guides, interactive tutorials and video tutorials to supplement your knowledge.

Uses of the lipidomics workflow

The lipidomics workflow may be used to do the following analyses:

- Compare two or more biological groups
- Find and identify potential biomarkers
- Look for biomarkers of toxicology
- Integrate the lipidomics data with other multi-omics data sets to better understand the systems biology
- Discover new lipids
- Develop data mining and data processing procedures that produce characteristic markers for a set of samples
- Construct statistical models for sample classification

Agilent enables lipidomics research for a variety of applications:

- Basic research - Identify and validate lipid biomarkers that correlate with disease states, as well as provide fundamental insights into biology
- Pharmaceuticals - Identify lipids and markers of toxicity for drug discovery and development
- Agriculture - Identify and understand metabolic pathways to optimize crop development, improve yields, and avoid pesticide/herbicide resistance
- Environmental studies - Identify lipids that relate to the effects of chemicals and other stressors in the environment on a biological system
- Biofuels - Identify lipid profiles to optimize fermentation processes and biofuel production
- Food/Nutrition - Identify the presence or absence of lipids that correlate with major food traits, such as quality, authenticity, taste, and nutritional value, and aid in the development of nutraceuticals

Required Items

You need this computer equipment, software and the specified data files to implement the Primary Lipidomics Workflow:

Computer Equipment

4 Physical Cores, 32-64 GB memory and 3.5 GHz processor speed

Software

Use these software programs and utilities for each Major Step of the Primary Lipidomics Workflow.

Step 1. Calibrate data IM-MS Reprocessor 10.0 and IM-MS Browser 10.0

Step 2. Create lipid PCDL Lipid Annotator 1.0 and PCDL Manager 8.0

Step 3. Find features Mass Profiler 10.0

Step 4. Identify compounds ID Browser 10.0

Step 5. Analyze lipids Mass Profiler Professional 15.1 (MPP)

Example Data Files

The data sets that you can use to work through the instructions in this guide are positive ion mobility LC/Q-TOF lipid data (both MS1 and All Ions data). You can find the files at **Agilent SubscribeNet** in the IM Lipidomics Data folder. See the part numbers and file names/descriptions in **Table 1**.

Table 1. IM Lipidomics Data Files on Agilent SubscribeNet

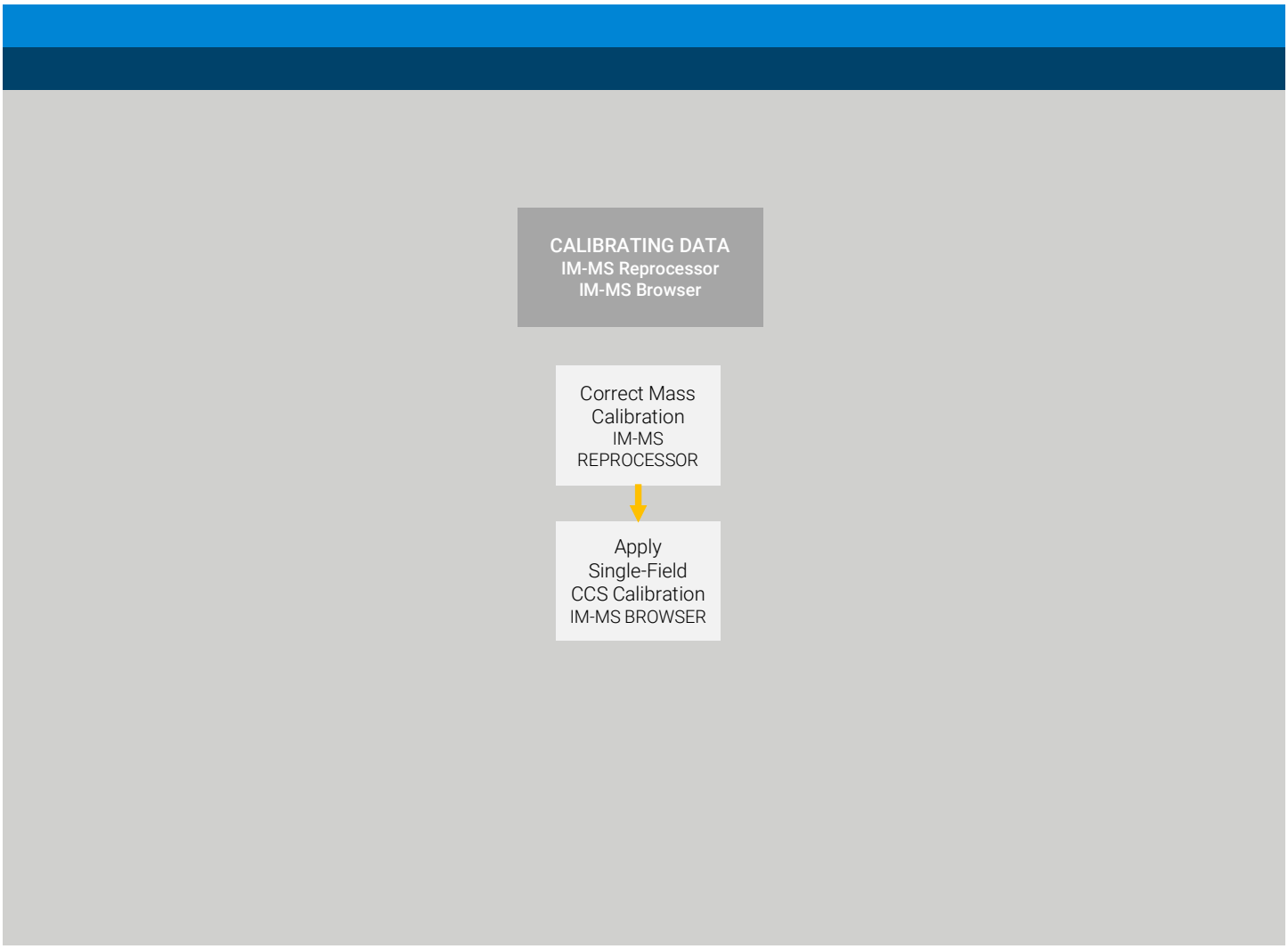
Agilent Part Number	File Name in SubscribeNet	Description	Data File Folder Name
G3335-10004	All Ions.zip	Fragmentation Sample Data	All Ions
G3335-10005	Stnd0_1017.zip	MS1 Sample Data with no standards	Nist0_1017
G3335-10006	Stnd2_1017.zip	MS1 Sample Data with standards	Nist2_1017
G3335-10007	Stnd4_1017.zip	MS1 Sample Data with twice the amount of standards as in previous folder	Nist4_1017
G3335-10008	Tune Mix.zip	One All Ions file with tuning data One MS1 file with tuning data	Tune Mix

This page intentionally left blank.

2 Calibrating Data

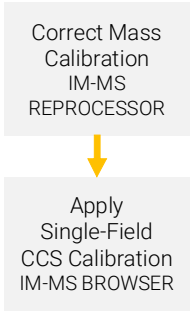
Calibrating data is the first Major Step in the Ion Mobility Primary Lipidomics Workflow. This chapter takes you through the steps to calibrate the raw data for creating a PCDL and finding features. This chapter focuses on correcting mass calibration and applying a single-field CCS calibration to the corrected data.

Before you analytically process the data -- create a custom PCDL, extract features and identify compounds -- you must first calibrate the raw data. To do this, you correct mass calibration and then apply single-field CCS calibration.



For standard-mode data (All Ions and MS1 non-multiplexed data) you can use the PNNL PreProcessor (**https://omics.pnl.gov/software/pnnl-preprocessor**) to smooth and repair saturated data, resulting in improved performance of the data analysis carried out in this guide. For simplicity, this guide processes the raw data files with mass and CCS calibration and does no preprocessing with the PNNL Preprocessor.

Additionally, if you have acquired multiplexed data, the lipid workflow in this guide cannot be completed until you use the PNNL Preprocessor for demultiplexing the data before CCS calibration.



Calibrating standard-mode data involves two steps:

- Step 1. Correct mass calibration using IM-MS Reprocessor

  The Reference Mass Calibration cannot be applied to IM-MS data during data acquisition as it can be for regular QTOF experiments. To recalibrate IM-MS data you use the IM-MS Reprocessor. Reference mass ions must be present and elute throughout the entire data file for this utility to apply a mass recalibration.

- Step 2. Apply single-field CCS calibration

  After loading a tune-mix data file into the IM-MS Browser, you determine the Beta and TFix values and save them to the tune-mix file and to the sample data files acquired under the same settings as the tune-mix data file. During custom PCDL creation in Lipid Annotator and feature finding in Mass Profiler the CCS values are calculated using these Beta and TFix values.

Correct Mass Calibration

Correcting mass calibration involves five major steps and an optional one:

- Step 1. Start IM-MS Reprocessor
- Step 2. Load selected file
- Step 3. mSet up recalibration
- Step 4. Run recalibration
- Step 5. Recalibrate remaining files
- Optional Step. Restore original file

NOTE

You may not need to correct mass calibration if your data file already recalibrated with good results (fewer than half the scans are missing). To find out, do **step 4** page 21. To learn how to correct mass calibration using these instructions, assume you need to recalibrate.

Step 1. Start IM-MS Reprocessor

IM-MS Reprocessor is installed during the installation of IM-MS Browser. To access the reprocessor for the first time, follow these instructions:

1 In the Windows Search field, type `reprocess`.

2 Right-click the IM-MS Reprocessor “Best Match” app.

3 Select **Open**.

OR

1 In the folder **\Program Files\Agilent\MassHunter\Workstation\IMS,** open the **Bin** folder.

2 Right-click the **imsreprocess** application and select **Create shortcut**.

3 Make sure you place the shortcut icon on the Desktop.

4 Double-click the **IM-MS Reprocessor** icon,  .

You can now double-click the IM-MS Reprocessor icon whenever you want to open the Reprocessor.

The IM-MS Data File Reprocessing Utility main window appears with the Operations tab open.

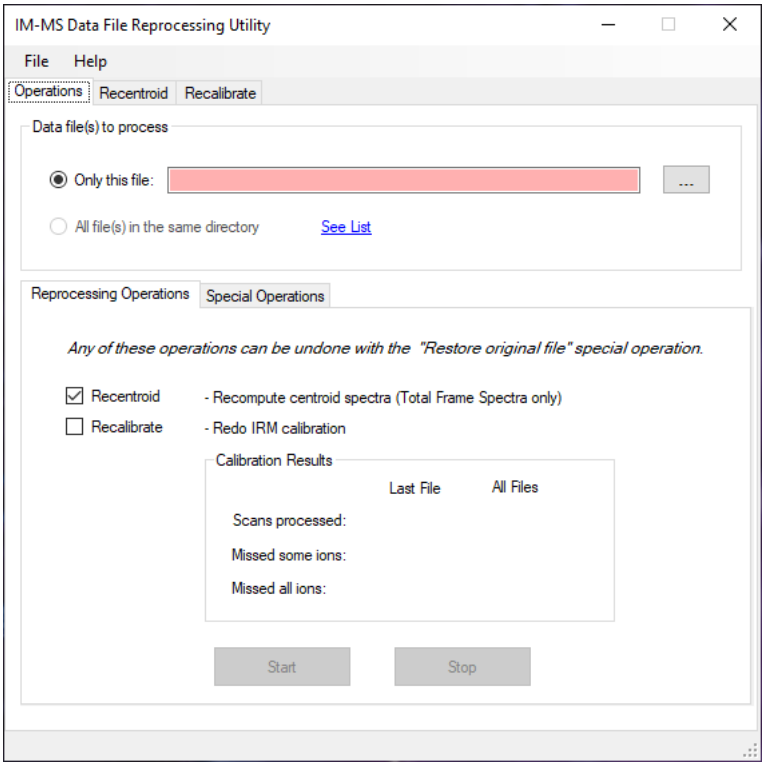


Figure 2. Operations tab for the Reprocessing Utility

Step 2. Load selected file

You can recalibrate the file sets in a major folder in any order -- tune mix, All Ions, MS1. Follow the instructions below to load the files from the data folder.

1 Click the **...** button (**Browse** button) next to the **Only this file** text field**.**

2 From your file folder, select any file in the folder you intend to reprocess.

This guide uses the All Ions tune-mix file, but you can select any file in any folder. You now see the file name as the Data file(s) to process, and you see another selection appear, letting you reprocess all the files in the folder.

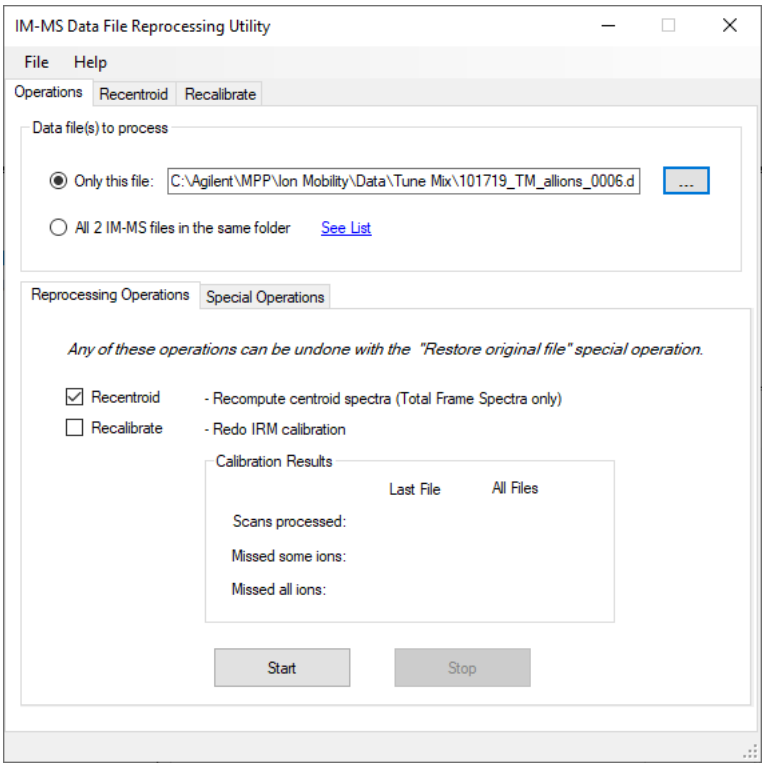


Figure 3. Operations tab with only one file in the folder loaded

Step 3. Set up recalibration

In setting up the Recalibrate operation, make sure you have selected the correct masses for the tune mix or the samples.

Set up the operation

1. To recalibrate more than one file at a time, select **All 2 IM-MS files in the same folder**, or the selection that specifies how many files are in the folder.
2. Mark the **Recalibrate** check box.
3. Clear the **Recentroid** check box.

Select the recalibration values

1 Click the **Recalibrate** tab.

The Recalibrate dialog box appears. See **Figure 4**.

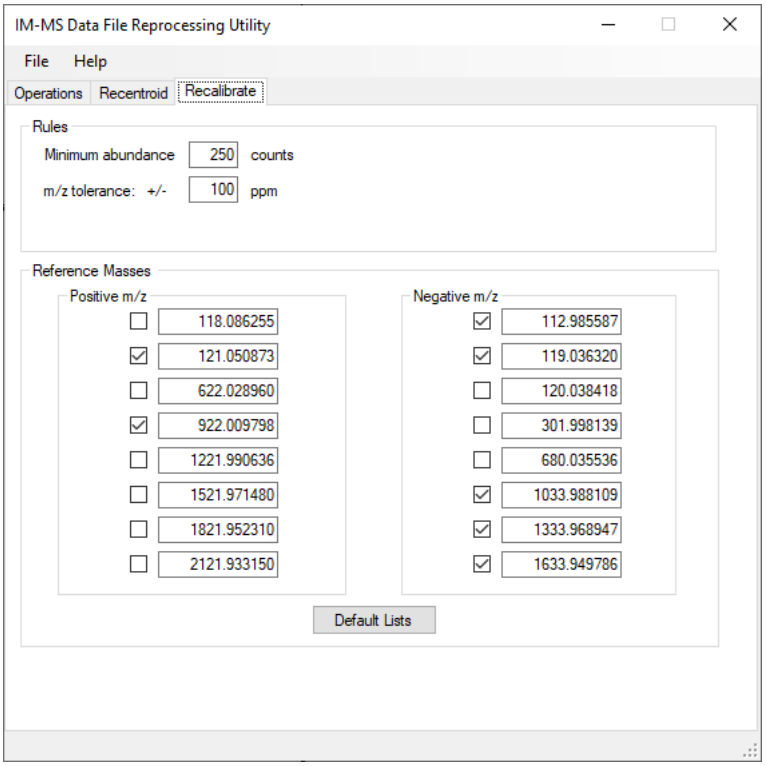


Figure 4. Recalibrate tab with positive ions selected for the sample files (default)

2 Keep the **Minimum abundance** and **m/z tolerance** values the same.

3 Mark the **Positive m/z** check boxes for the values you want as Reference Masses for either the tune-mix files or the sample files. (See Note on **page 15**.)

NOTE

Make sure to edit the positive mass values when switching between a tune-mix file (118, 322, 622, 922,1221and 1521) and a sample data file, which likely has only 121 and 922 present. Replace the 121 value with 322.04873 for the tune-mix files. Don't worry about marking the Negative m/z check boxes at this time. Use **Figure 4** on page 18 for the sample files and **Figure 5** for the tune-mix files.

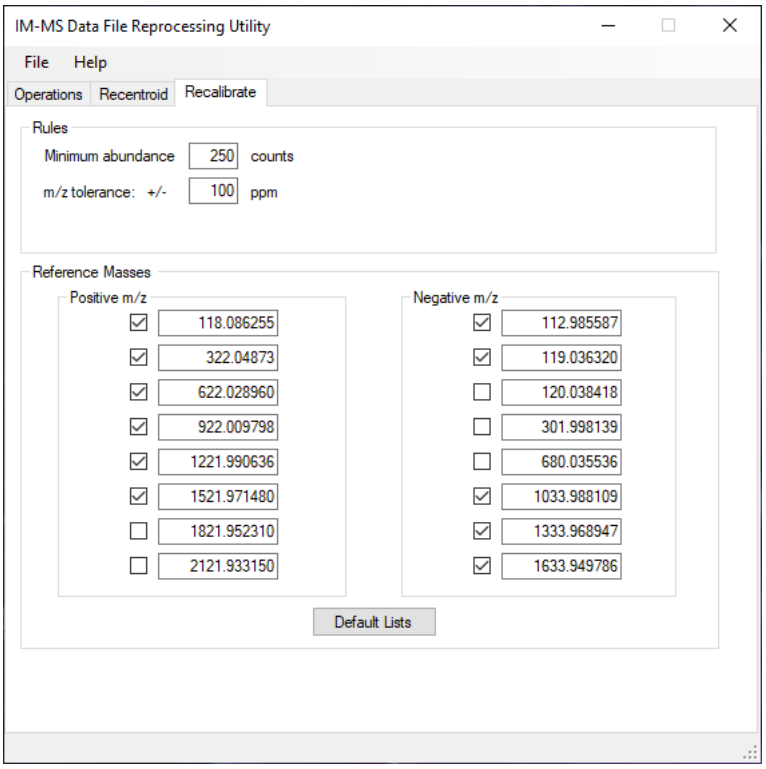


Figure 5. Recalibrate tab with positive ions selected for the tune-mix files

Step 4. Run recalibration

Your new recalibration results will overwrite the previous ones.

Start the recalibration

1 Select the **Operations** tab.
2 Click **Start**.

When you see the Done message at the bottom of the screen, the masses in the data files have been recalibrated.

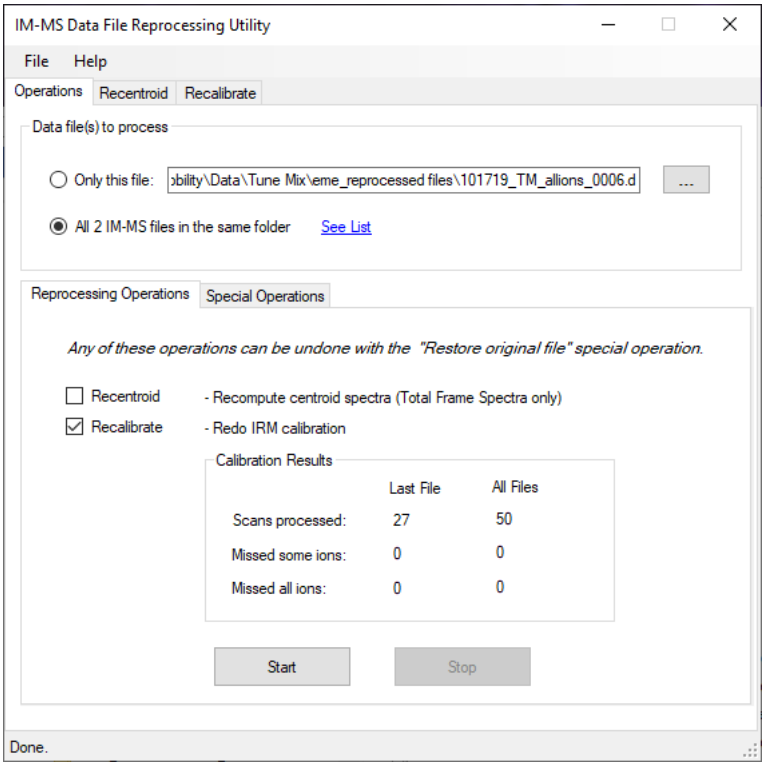


Figure 6. Completed recalibration for both tune-mix files

3 If you applied the mass calibration to sample files, check them for missed ions.

For both tune-mix files and sample files, the zeros indicate all the marked ions are present in all the scans. Missing ions are not likely to appear for tune-mix files. For a sample file, if more than half the scans are missing ions, you likely have a bad recalibration.

4 (optional) For a bad recalibration, do these steps:

a Restore the original data file, which you can do with only one data file at a time, and then recalibrate. See **"To restore original file"** on page 21.

b If more than half the scans are still missing ions, check the data file in IM-MS Browser to verify that the reference mass ions meet the Rules values specified on the Recalibrate tab. See **Figure 4** on page 18.

Step 5. Recalibrate remaining files

- Repeat Steps 2-4 for the files in other folders.

  Remember that for sample files you mark the positive ions in **Figure 4** on page 18 and for tune-mix files you mark those in **Figure 5** on page 19.

To restore original file

Follow these steps if you think you need to revert back to the original data file after a bad mass recalibration.

1 Click the **Special Operations** tab.

Figure 7 appears.

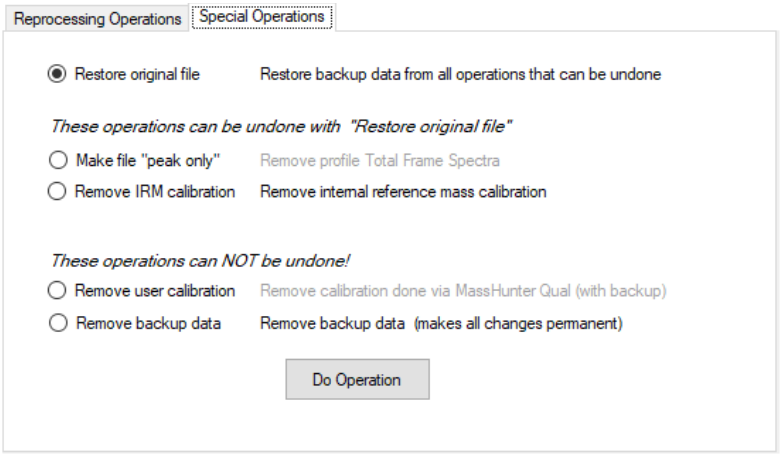


Figure 7. Special Operations Tab

2 Make sure **Restore original file** is marked, or mark any of the other operations you want to do.

3 Click **Do Operation**.

NOTE

These special operations will work only on the data file loaded into the Only this file field. If you mark the description for all the files in the folder, the operations will not be performed on the other files.

4 (optional) To see all the reprocessing and restoration operations that have been applied to the data file, open the ReprocessLog.txt file in the AcqData folder under the .d folder. See **Figure 8**.

```
7/19/2020 9:42:45 AM    Reprocessing started.
7/19/2020 9:42:45 AM            Recalibration requested
7/19/2020 9:42:45 AM                    Positive ion reference m/z values = 118.086255, 322.048730, 622.028960, 922.009798, 1221.990636, 15
7/19/2020 9:42:45 AM                    m/z tolerance (ppm) = 100
7/19/2020 9:42:45 AM                    Minimum abundance = 250
7/19/2020 9:42:45 AM                    Peak min valley = 0.70
7/19/2020 9:42:45 AM                    IRM statistics: 23 scans        0 missing some ions     0 missing all ions
7/19/2020 9:42:45 AM    Reprocessing finished.
12/20/2020 9:16:32 AM   Reprocessing started.
12/20/2020 9:16:32 AM           Recalibration requested
12/20/2020 9:16:32 AM                   Positive ion reference m/z values = 118.086255, 322.048730, 622.028960, 922.009798, 1221.990636, 15
12/20/2020 9:16:32 AM                   m/z tolerance (ppm) = 100
12/20/2020 9:16:32 AM                   Minimum abundance = 250
12/20/2020 9:16:32 AM                   Peak min valley = 0.70
12/20/2020 9:16:33 AM                   IRM statistics: 23 scans        0 missing some ions     0 missing all ions
12/20/2020 9:16:33 AM   Reprocessing finished.
12/20/2020 9:31:21 AM   Restore original data - begin
12/20/2020 9:31:21 AM           Operation succeeded
12/20/2020 9:31:21 AM   Restore original data - end
```

Figure 8. ReprocessLog.txt file in Notepad

Apply Single-Field CCS Calibration

In this step you take the mass-calibrated tune-mix data into IM-MS Browser to determine the single-field collision cross section (CCS) constants, Beta and TFix, which the Lipid Annotator and Mass Profiler programs use to calculate CCS values. You apply the constants to the tune-mix files and the sample files corresponding to each tune-mix file. Follow these instructions for the All Ions and MS1 tune-mix and sample files.

Step 1. Load the All Ions tune-mix file

Step 2. Sum the signals

Step 3. Calculate the Beta and TFix values

Step 4. Save the values to the tune-mix and sample files

Step 5. Repeat steps for MS1 tune-mix and sample files

NOTE

If you have multiplexed data you must demultiplex it before doing the single-field calibration. You can demultiplex it in the PNNL Preprocessor.

Step 1. Load the All Ions tune-mix file

Be sure you have downloaded Agilent IM-MS Browser 10.0.

Start IM-MS Browser

- Click the IM-MS Browser icon  .

The Open Data File dialog box opens on top of the IM-MS Browser main screen.

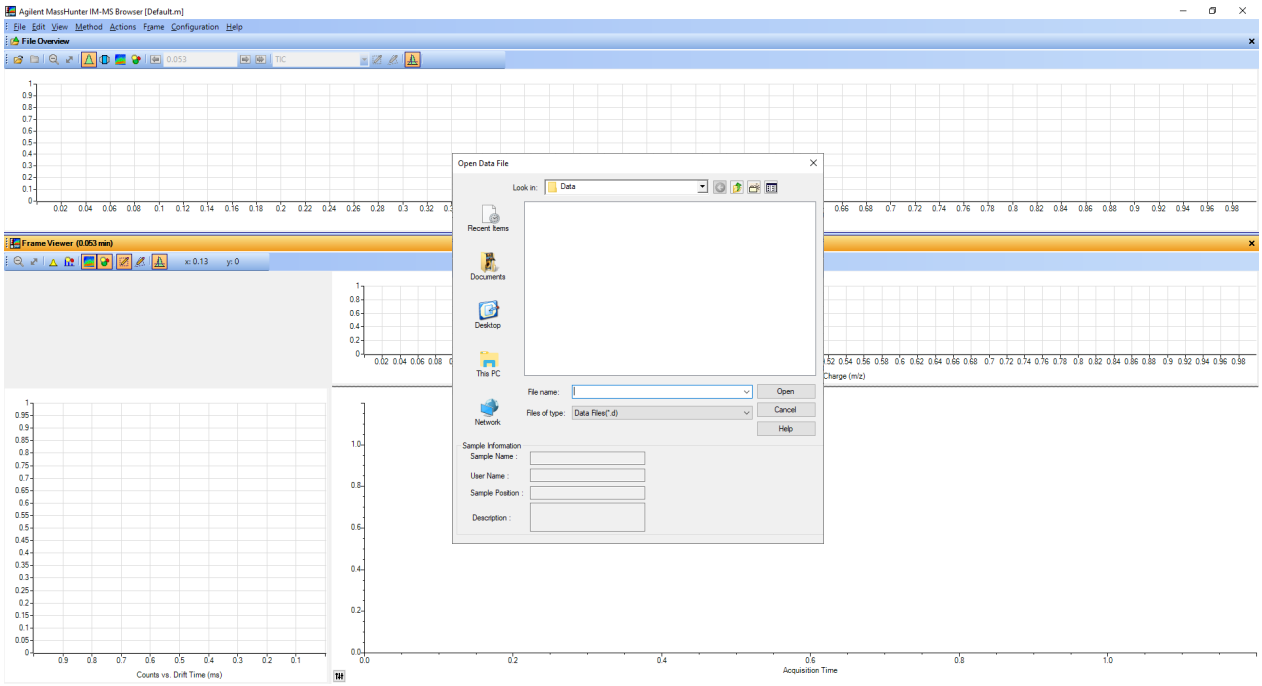


Figure 9. Open Data File dialog box on top of IM-MS Browser main window

Open a tune-mix file

In the tune-mix files folder, open the All Ions mass-calibrated tune-mix file.

1 Select **101719_TM_allions_0006.d**.

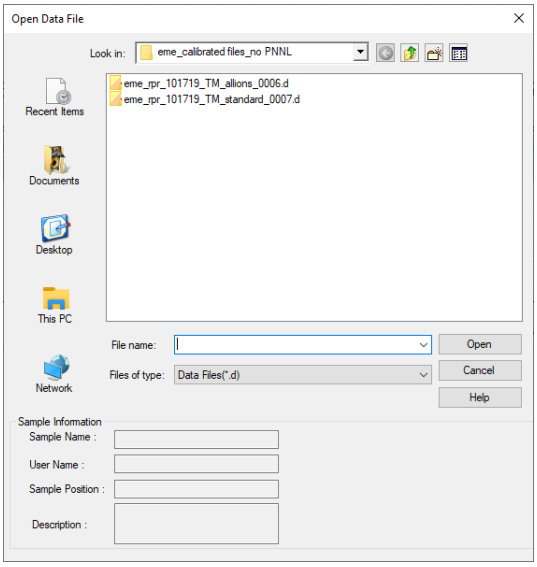


Figure 10. Open Data File dialog box with All Ions mass-calibrated tune mix selected

2 Click **Open** to add the file to IM-MS Browser.

IM-MS Browser now contains the All Ions tune-mix data file that has been mass-calibrated.

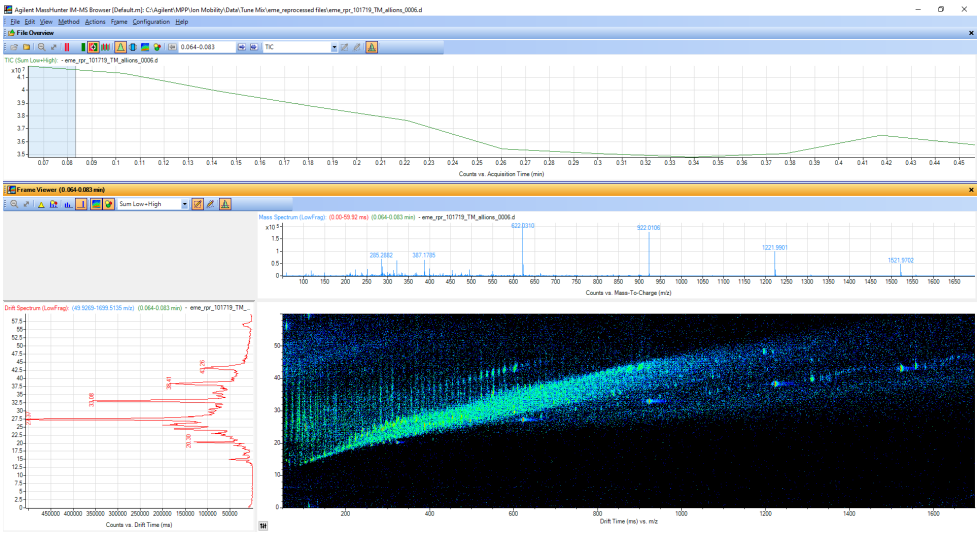


Figure 11. IM-MS Browser with All Ions mass-calibrated tune-mix data file

Step 2. Sum the signals

To sum the signals you must first select the entire TIC, then extract the frame.

1 In the **File Overview** pane, select the entire TIC.

2 To sum all the signals together, right-click the selected TIC.

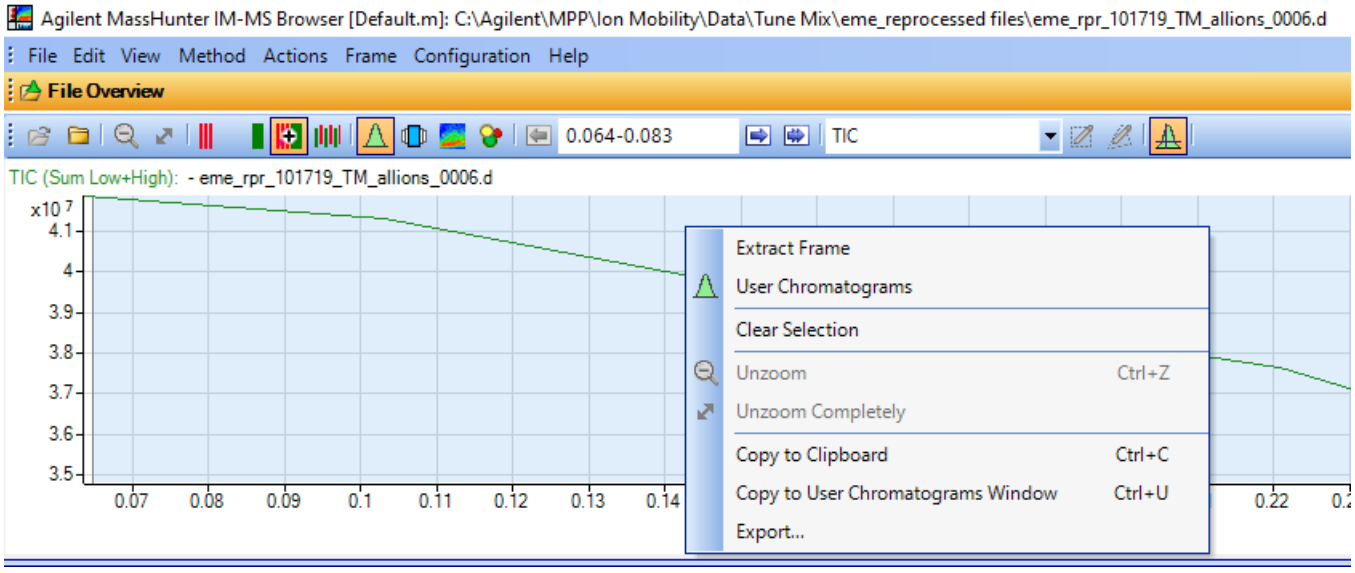


Figure 12. Selected All Ions TIC with short-cut menu

3 Select **Extract Frame**.

You can now see the summed signal in the Drift Time vs m/z pane.

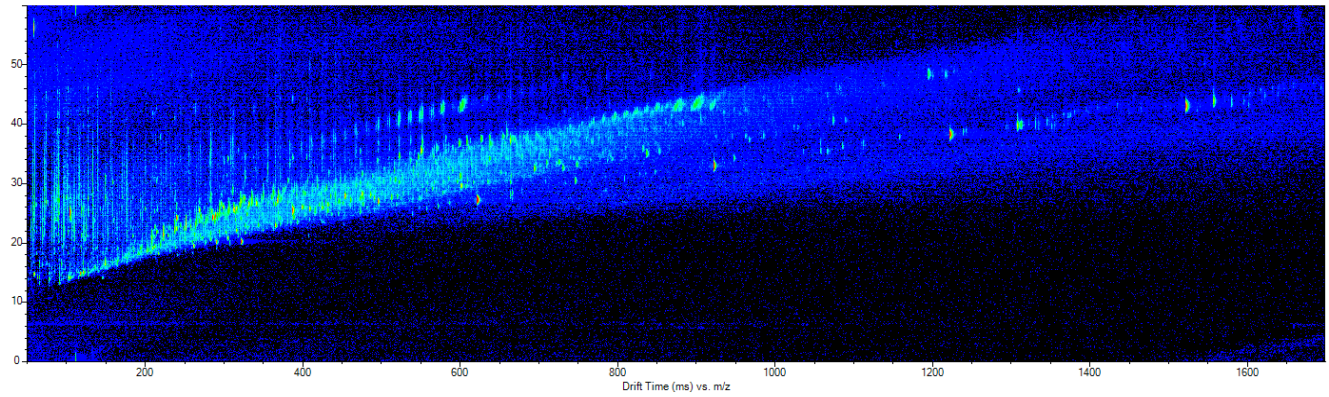


Figure 13. Summed signal after frame extraction

See *Agilent 6560 Ion Mobility LC-QTOF Fundamentals Guide* to learn about "frames".

Step 3. Calculate the Beta and TFix values

You calculate these values through the CCS Calibration (Single-Field) pane.

Display the CCS Calibration (Single-Field) pane

1 If the pane is visible when you first open IM-MS Browser, hide it and then follow the next instructions.

2 Click the **View** menu.

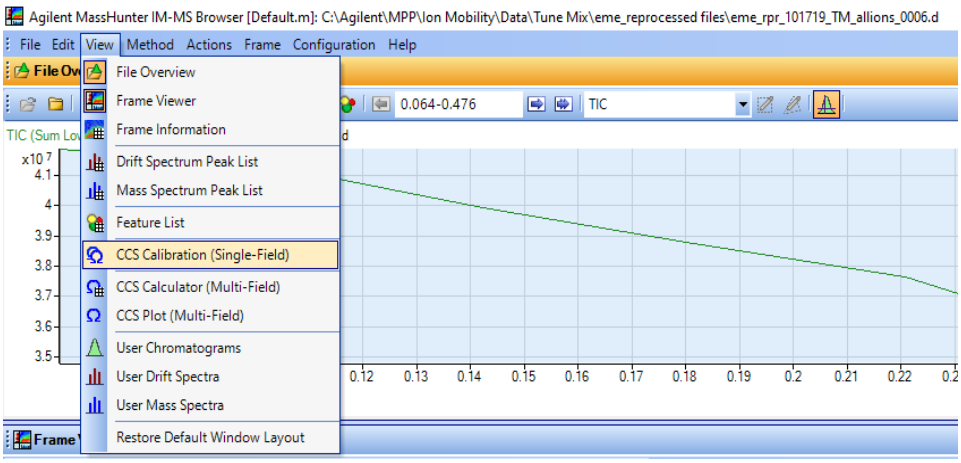


Figure 14. View menu

3 Select **CCS Calibration (Single-Field)**.

The CCS Calibration (Single-Field) pane appears. Note the bottom part of the pane.

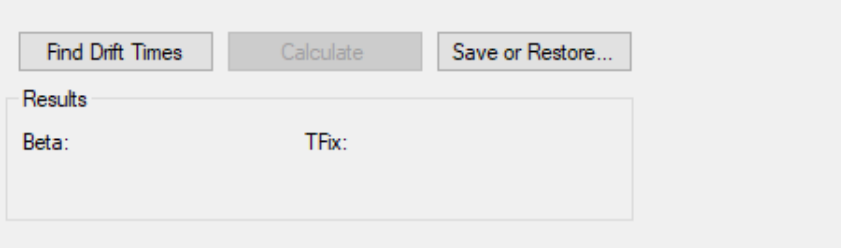


Figure 15. Location for calculating Beta and TFix values for the CCS Calibration

Find the drift times

- Click **Find Drift Times.** See the results below.

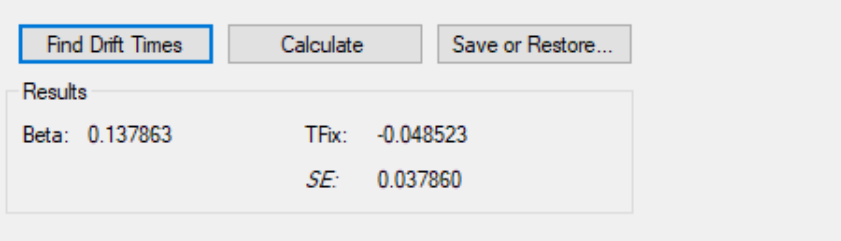


Figure 16. Calculated Beta and TFix values

Step 4. Save the values to the tune-mix and sample files

Now you save these values to the current tune-mix file and its corresponding sample files, in this case, the All Ions sample files.

1 Click **Save or Restore**.

The Save or Restore dialog box appears.

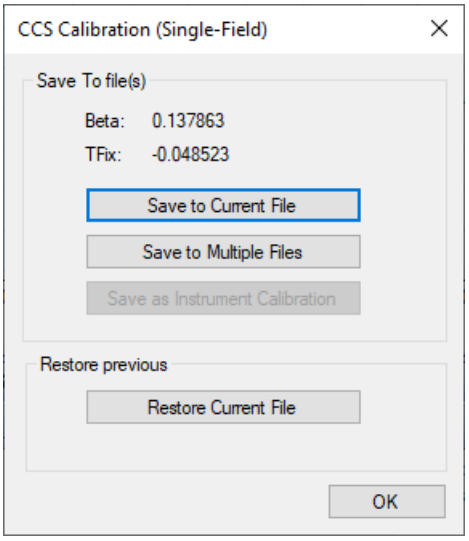


Figure 17. Save or Restore dialog box - Save to Current File highlighted

2 Click **Save to Current File**.

The Beta and TFix values are saved to the tune-mix file, in this case the All Ions tune-mix file.

3 Click **Save to Multiple Files**.

The Open Data Files dialog box appears.

a Open the folder holding sample files corresponding to the tune-mix file used for calculating the constants, in this first instance, the All Ions sample folder.

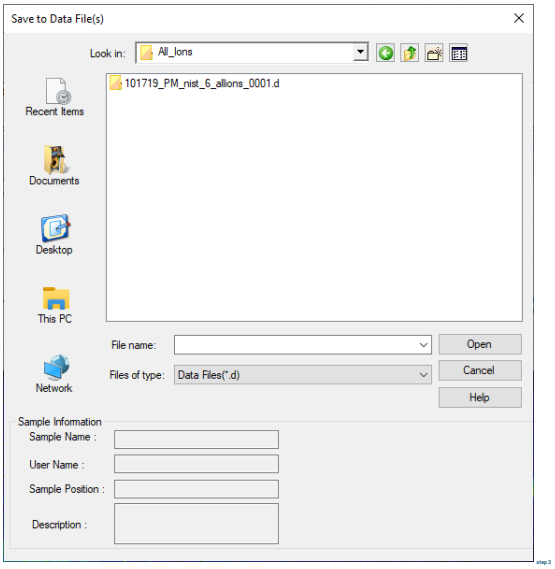


Figure 18. Save to Data File(s) dialog box for saving Beta and TFix values to sample files

b Select all the sample files that were acquired under the exact same experimental conditions as the tune-mix data file you used to calculate the drift times and constants. (If you used the All Ions tune mix, then select the All Ions sample files. Only one file is present in this example All Ions sample folder.)

c Click **Open**.

4 If more than one folder contains sample files you intend to save with the tune-mix file, repeat **step 3** for each folder.

5 To close the **Save or Restore** dialog box, click **OK**.

Step 5. Repeat steps for MS1 tune-mix and sample files

- If you began these instructions with the All Ions tune-mix and sample files, repeat Steps 1-4 outlined on the previous pages with the MS1 files.

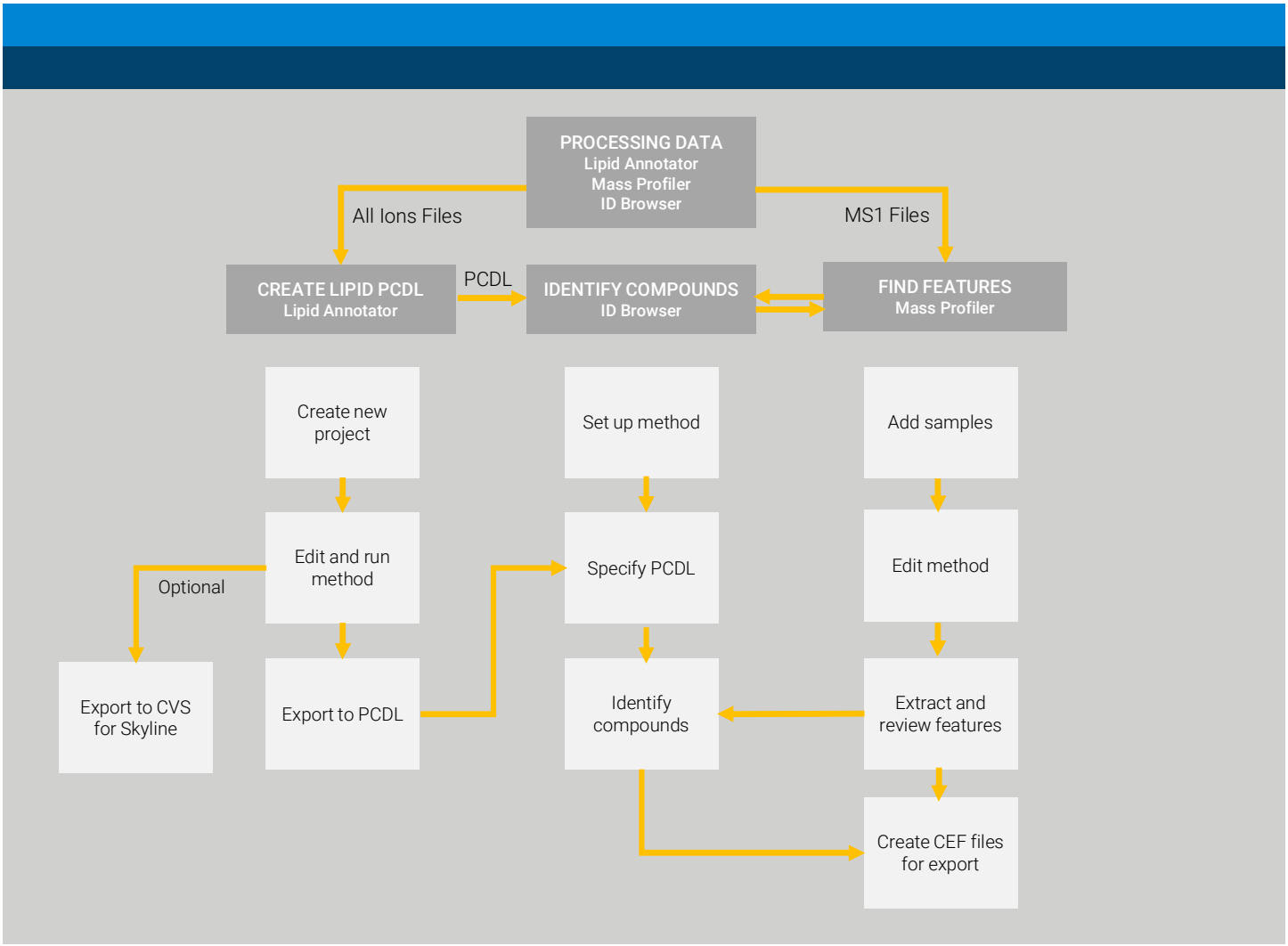
  The MS1 tune-mix file is the remaining file in the Tune Mix folder. The MS1 sample files reside in the three folders with the NIST prefix.

You are now ready to use the calibrated All Ions sample file for creating a custom lipid database and the calibrated MS1 sample files for finding features and identifying compounds.

3 Processing Data

This chapter takes you through the steps for processing already calibrated data. This chapter focuses on using All Ions data and Lipid Annotator to create a custom lipids PCDL (Personal Compound Database and Library), and using MS1 data and Mass Profiler to extract features, which are identified with the custom lipids database and ID Browser.

This chapter presents the Major Steps and sub-steps in the processing section of the lipidomics workflow. It assumes you have already calibrated the raw data for Step 1, following the instructions in **Chapter 2**, "Calibrating Data".



After acquiring and calibrating the data, you follow these 3 Major Steps of the Primary Lipidomics Workflow to identify the lipids, which data you then pass on to MPP to assess the lipid composition differences between sample sets.

Each section gives you additional instructions to complete the primary tasks in the processing part of the workflow.

Step 2. Create lipid PCDL

Use Lipid Annotator to annotate lipids in the All Ions data files when fragment ions match the theoretical library, generating a PCDL that contains accurate mass, retention time and CCS values. The CCS values are calculated from the Beta and TFix values determined in **Chapter 2**. You can also generate a CSV file to use in Skyline for targeted data analysis.

Step 3. Find features

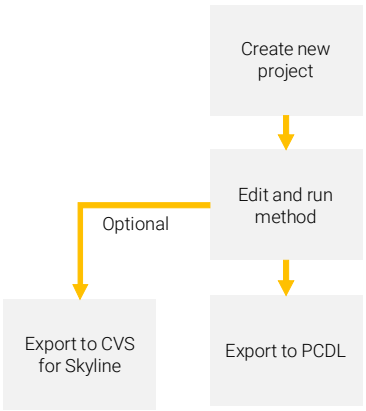
Find features according to mass, retention time and CCS values using Mass Profiler on MS1 data files.

Step 4. Identify compounds

Use the PCDL from Lipid Annotator with ID Browser to identify the MS1 features as lipids and return the compound information to Mass Profiler, where you create CEF files for export.

In **Chapter 4** you learn how to analyze the lipid data (**Step 5**) with Mass Profiler Professional (MPP), which provides lipid assessment tools with which to compare the compound distribution between samples (CEF files from **Step 4** imported to MPP).

Create Lipid PCDL



The second Major Step in the Primary Lipidomics Workflow, Create Lipid PCDL (Personal Compound Database and Library), includes three sub-steps:

- Step 1. Create new project
- Step 2. Edit and run method
- Step 3. Export to PCDL
- Optional Step. Export to CVS for Skyline

With these steps you create a custom lipid database used to identify the compounds in the feature extraction list produced in Mass Profiler (Export to PCDL) or used to further analyze ion mobility data in Skyline (Export to CSV for Skyline).

Step 1. Create new project

You must use calibrated All Ions data to create your custom lipid database.

Name the new project

1 Open Lipid Annotator.

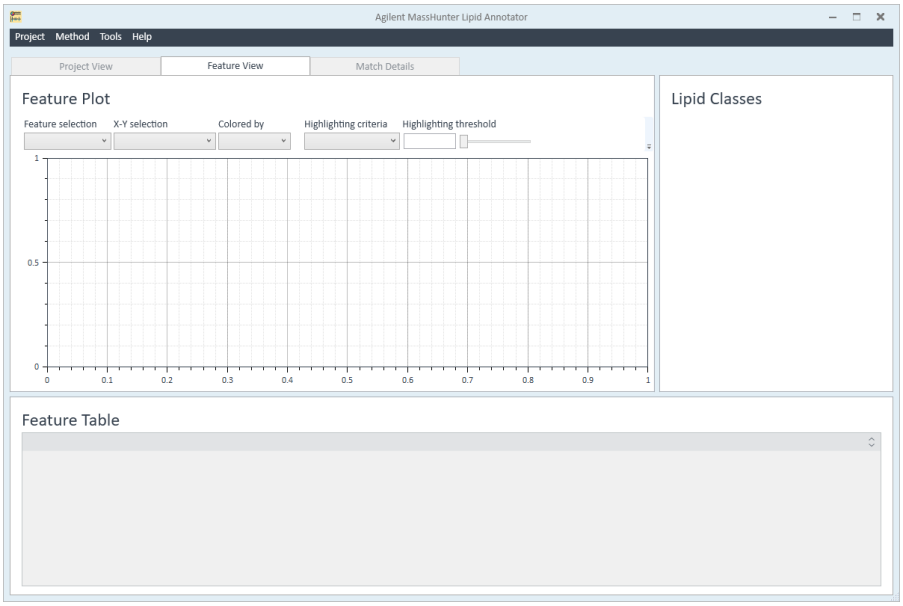


Figure 19. Lipid Annotator main window

2 Select **Project > Create** to bring up the Create a Project dialog box.

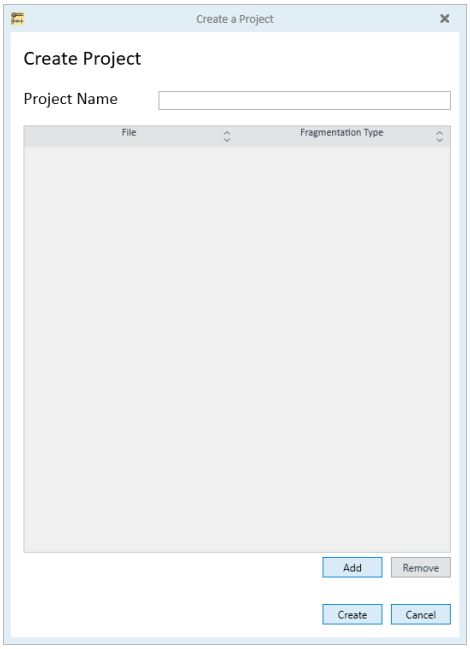


Figure 20. Create a Project dialog box

3 Type any name you want.

Add data files and create the project

1 Click **Add** and open the All Ions calibrated data file.

If you have multiple All Ions data files that contain different lipids you will need to process these separately in Lipid Annotator and then manually merge the PCDLs together in PCDL Manager.

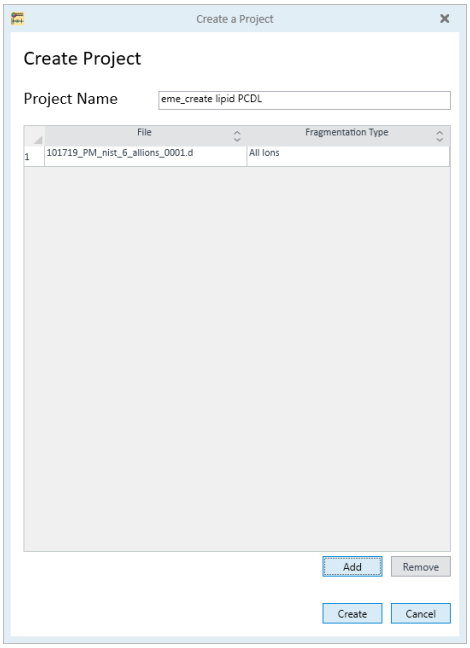


Figure 21. File added to custom lipid database project

2 Click **Create**.

The Project View - TIC Chromatogram Viewer appears with the data files listed.

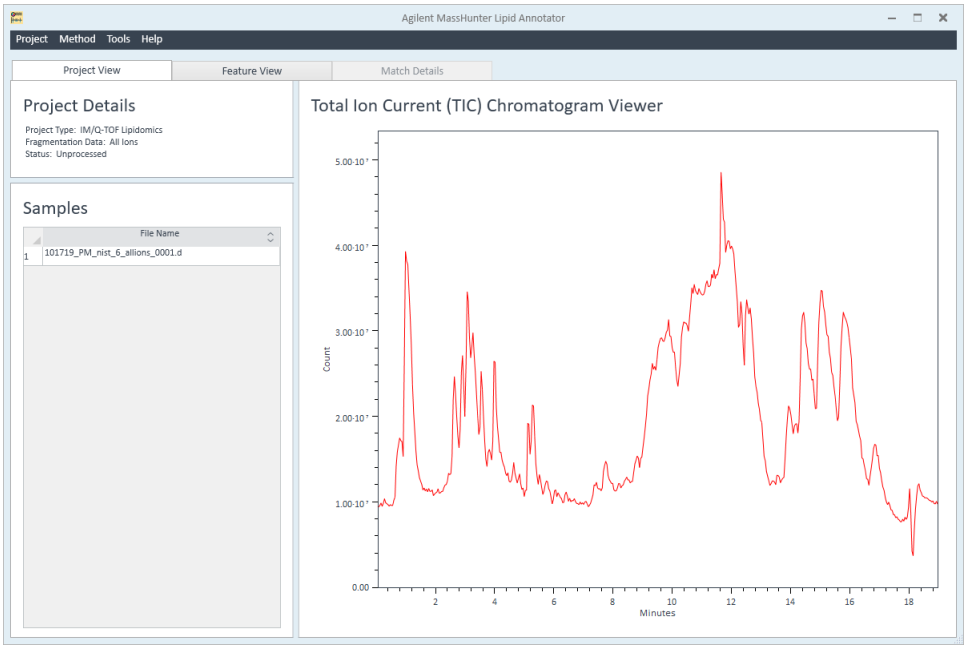


Figure 22. Project View - TIC Chromatogram Viewer

You are now ready to edit and run the method.

Step 2.Edit and run method

This exercise shows you how to edit the method should you choose to change parameter values, but you will keep the default values for this exercise. If possible, use the Default Method that comes with the program because these parameters were selected after analyzing over 60 lipid standards and achieved good results.

Edit the method

1. To make sure the default method is loaded, select **Method > Reset Method to Default**.
2. Select **Method > Edit Method** from the top menu.

The Method Parameters tabs appear with the default parameter values. The first tab contains values for filtering features.

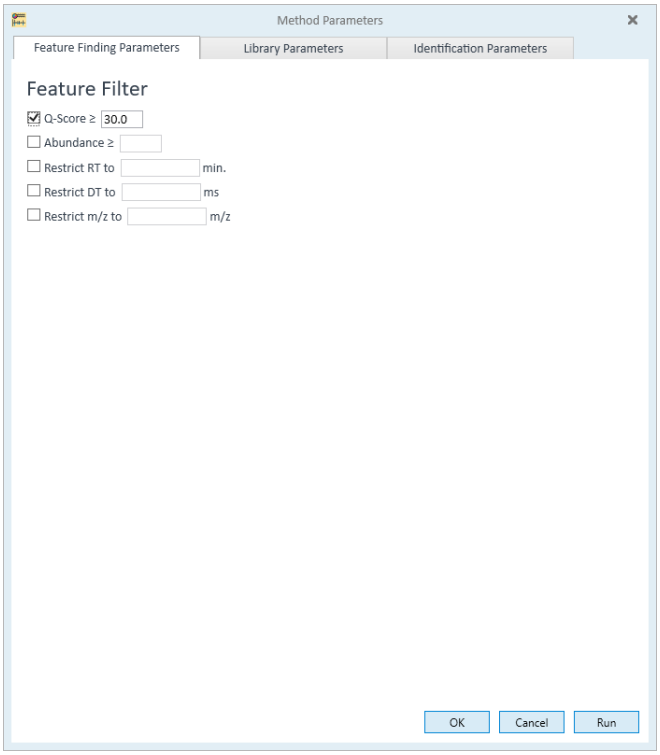


Figure 23. Feature Finding Parameters (default settings)

3 For this exercise keep the default values for Feature Finding Parameters.

4 Click **Library Parameters** and view the parameter values.

This tab lets you set the positive/negative ion filters and the lipid class filters.

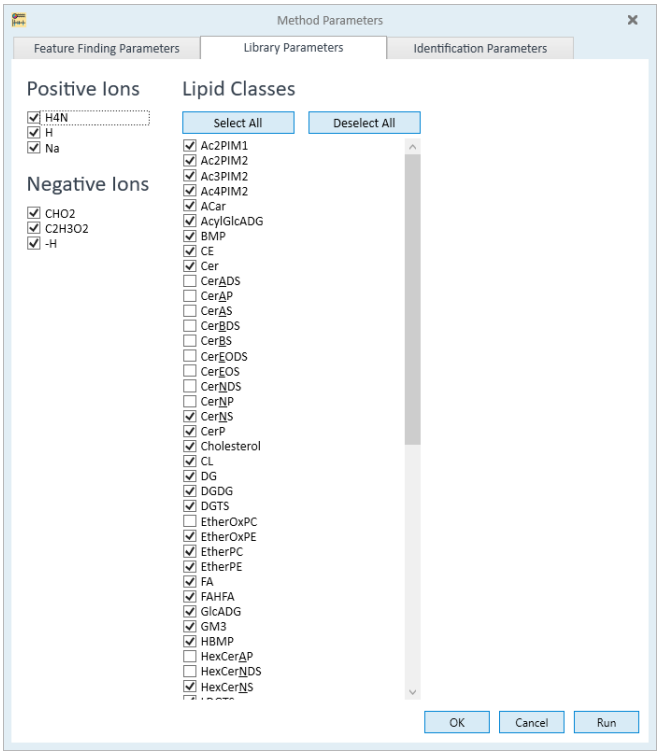


Figure 24. Library Parameters (default settings)

5 For this exercise keep the default values for the Library Parameters.

6 Click **Identification Parameters** and view the parameter values.

This tab lets you choose which lipids to report.

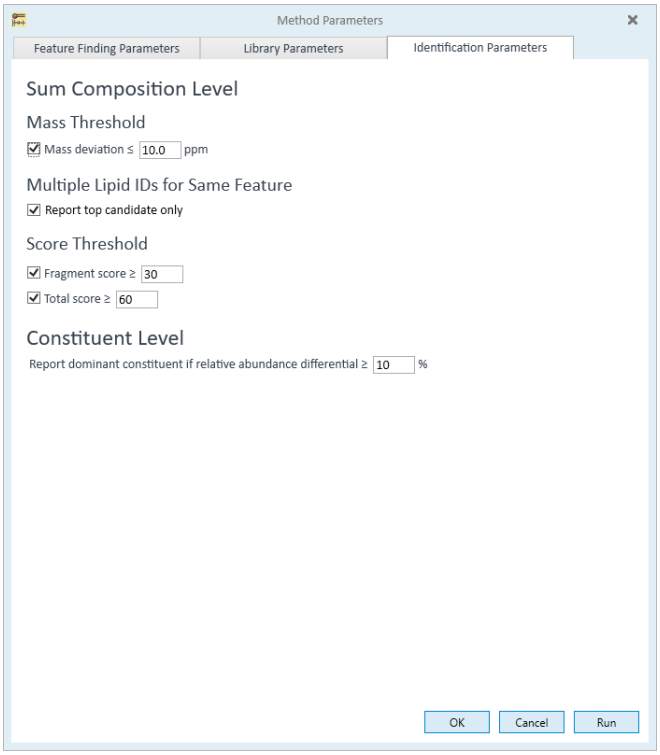


Figure 25. Identification Parameters tab (default settings)

NOTE

Amphipathic lipids are identified by lipid class, sum composition and lipid constituents, if fragmentation information confirms specific fatty acid tail fragments. See **"Lipids"** on page 89 for the definitions of these terms and for lipid naming protocols.

7 For this exercise keep the default values for the Identification Parameters tab.

If you intend to save the values you change, then save the method. If you intend to bring up the new method when you open a project, then save the project to a new name before you exit Lipid Annotator.

Run the method

Notice every tab has a Run button.

- Click **Run** after you complete editing the method.

  Processing time should be a minute or so with the example file. With your own All Ions file, the time will be longer because your file contains no results folder from the previous user's run.

The Processing Data message appears.

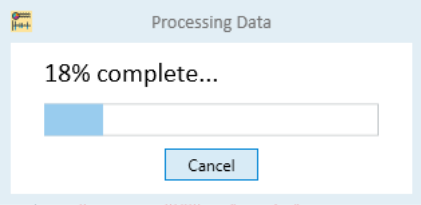


Figure 26. Processing Data message

When processing completes, the results appear in the Feature View.

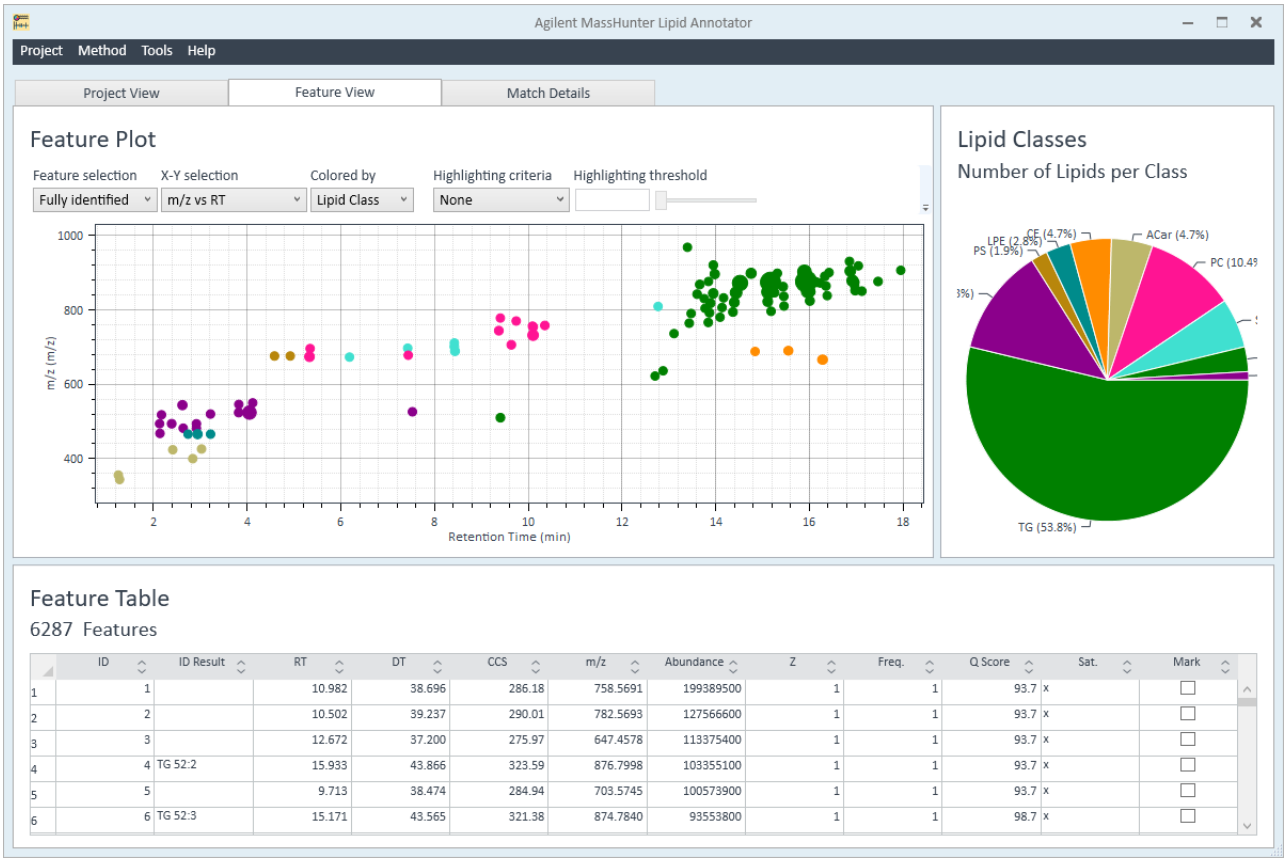


Figure 27. Feature View after run completion

To learn how to change the display and use the Lipid Annotator Tools, see **"Resource Apps"** on page 94 and find the tutorial for Lipid Annotator.

Review CCS values

Notice the calculated CCS values in the Feature Table CCS column. The program calculated the values based on the Beta and TFix constants determined in **"Apply Single-Field CCS Calibration"** on page 22 of **Chapter 2**.

Step 3.Export to PCDL

At this point you export the data as a custom lipid PCDL file.

Export to PCDL

1 Select **Project** from the top menu.

Figure 28. Project menu

2 Select **Export Match Result to PCDL...**

The dialog box of the same name appears.

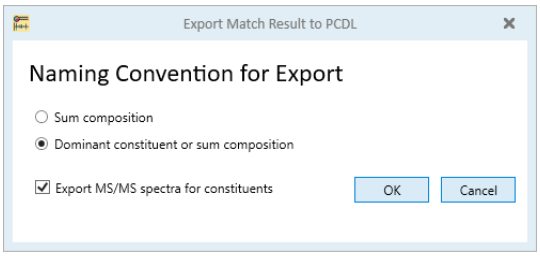


Figure 29. Export Match Result to PCDL dialog box

3 Click **OK**.

The Save As window appears. You now have the ability to name and save the database file.

4 Type *iii*_custom lipid PCDL, where *iii* refers to your initials.

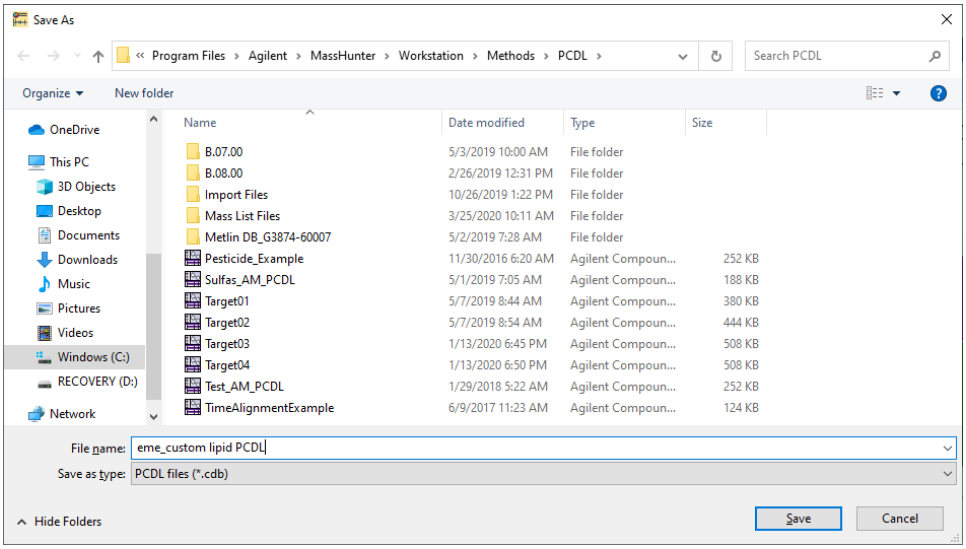


Figure 30. Save As window

5 Click **Save.**

The Export Successful message appears.

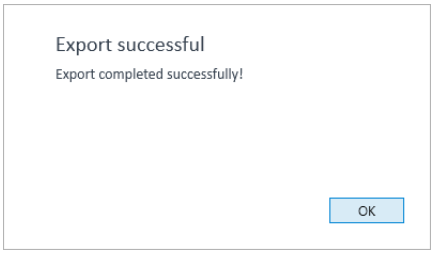


Figure 31. Export successful message

6 Click **OK**.

NOTE

If you want to add or delete compounds from the database you just created, load the file into PCDL Manager, which is included in your MassHunter software kit.

You are now ready to use this new custom lipid PCDL as a means to identify the features extracted with Mass Profiler in the next section.

To export to CSV for Skyline

If you want to use Skyline for targeted feature extraction instead of Mass Profiler for untargeted feature extraction, export the file to CSV.

Export to CSV

1 Select **Project** from the top menu.

See **Figure 28** on page 39.

2 Select **Export Features to CSV...**

The dialog box of the same name appears.

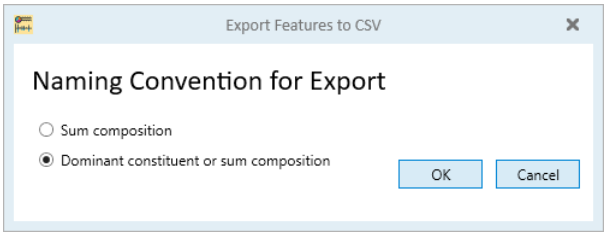


Figure 32. Export Features to CSV dialog box

3 Click **OK**.

The Save As window appears. You now have the ability to name and save the database file.

4 Type *iii*`_custom lipid CSV`, where *iii* refers to your initials.

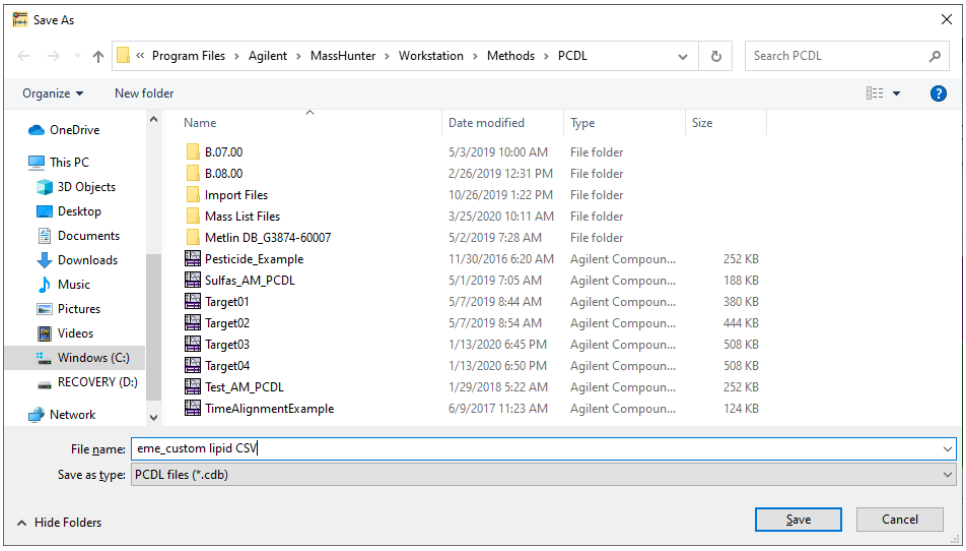


Figure 33. Save As window

5 Click **Save.**

The Export Successful message appears.

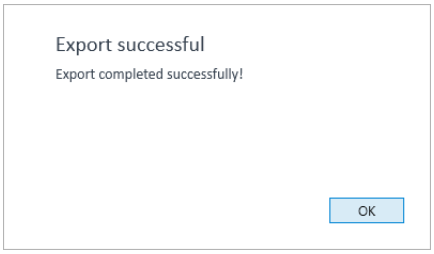


Figure 34. Export successful message

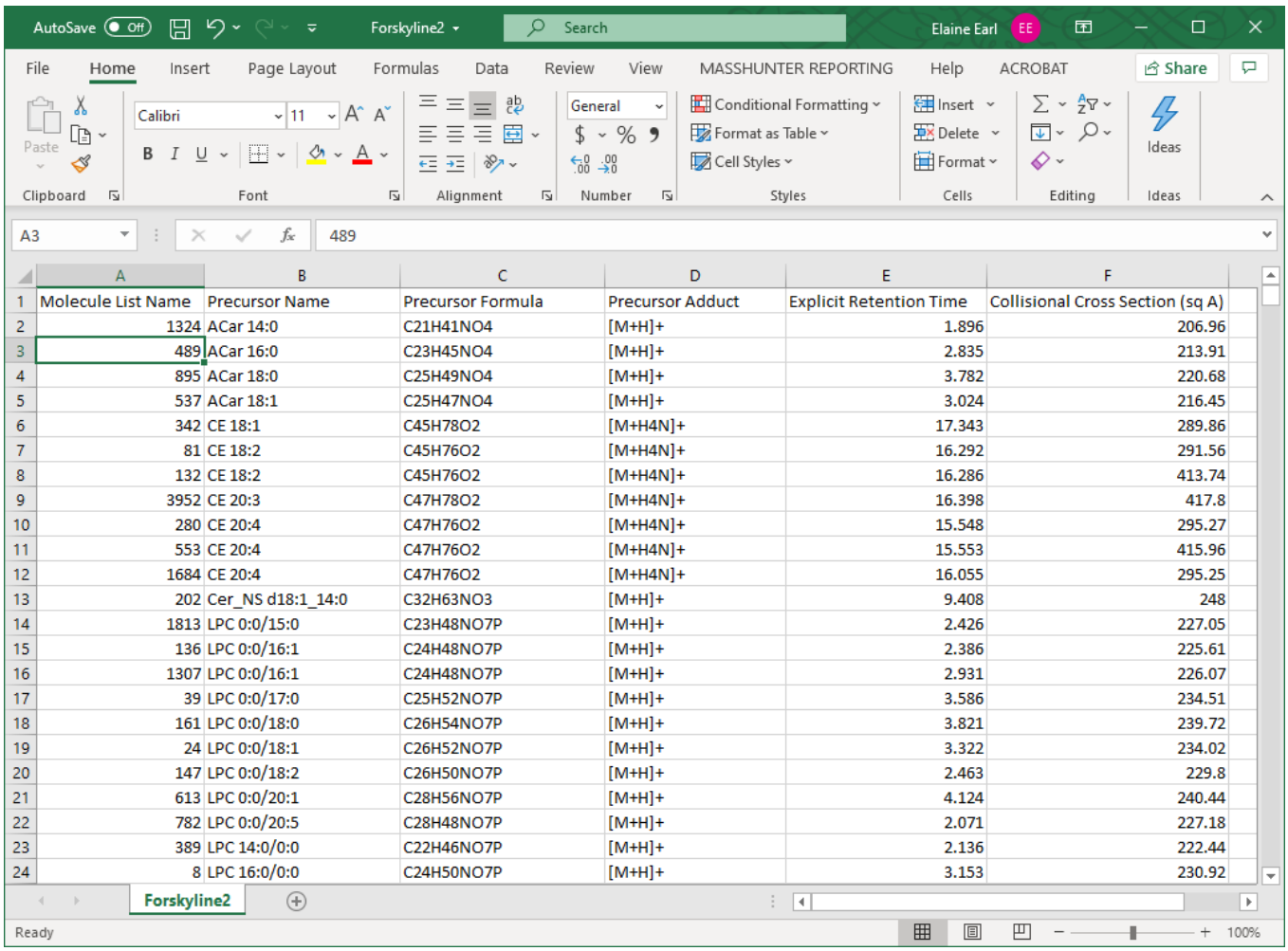
6 Click **OK**.

Prepare CSV file for Skyline import

You cannot import the CSV file into Skyline unless you do the following steps.

1 Copy the CSV file as another name, say, forskyline.csv, and open it in Excel.

You must do the steps below to have the spreadsheet look like **Figure 35**.



	A	B	C	D	E	F
1	Molecule List Name	Precursor Name	Precursor Formula	Precursor Adduct	Explicit Retention Time	Collisional Cross Section (sq A)
2	1324	ACar 14:0	C21H41NO4	[M+H]+	1.896	206.96
3	489	ACar 16:0	C23H45NO4	[M+H]+	2.835	213.91
4	895	ACar 18:0	C25H49NO4	[M+H]+	3.782	220.68
5	537	ACar 18:1	C25H47NO4	[M+H]+	3.024	216.45
6	342	CE 18:1	C45H78O2	[M+H4N]+	17.343	289.86
7	81	CE 18:2	C45H76O2	[M+H4N]+	16.292	291.56
8	132	CE 18:2	C45H76O2	[M+H4N]+	16.286	413.74
9	3952	CE 20:3	C47H78O2	[M+H4N]+	16.398	417.8
10	280	CE 20:4	C47H76O2	[M+H4N]+	15.548	295.27
11	553	CE 20:4	C47H76O2	[M+H4N]+	15.553	415.96
12	1684	CE 20:4	C47H76O2	[M+H4N]+	16.055	295.25
13	202	Cer_NS d18:1_14:0	C32H63NO3	[M+H]+	9.408	248
14	1813	LPC 0:0/15:0	C23H48NO7P	[M+H]+	2.426	227.05
15	136	LPC 0:0/16:1	C24H48NO7P	[M+H]+	2.386	225.61
16	1307	LPC 0:0/16:1	C24H48NO7P	[M+H]+	2.931	226.07
17	39	LPC 0:0/17:0	C25H52NO7P	[M+H]+	3.586	234.51
18	161	LPC 0:0/18:0	C26H54NO7P	[M+H]+	3.821	239.72
19	24	LPC 0:0/18:1	C26H52NO7P	[M+H]+	3.322	234.02
20	147	LPC 0:0/18:2	C26H50NO7P	[M+H]+	2.463	229.8
21	613	LPC 0:0/20:1	C28H56NO7P	[M+H]+	4.124	240.44
22	782	LPC 0:0/20:5	C28H48NO7P	[M+H]+	2.071	227.18
23	389	LPC 14:0/0:0	C22H46NO7P	[M+H]+	2.136	222.44
24	8	LPC 16:0/0:0	C24H50NO7P	[M+H]+	3.153	230.92

Figure 35. Excel .csv file with the correct entries.

2 Remove all the columns that are not included **Figure 35** on page 42.
3 Remove all rows that have no Molecule List Name and Precursor Name.
4 Rename columns to the names above.
5 Change “(“ to “[“ and “)” to “]”.
6 If Deuterium is present in the sample, change the “[2H]” in the Precursor Formula to “D”.
7 Import the file into Skyline and refer to its user guides to perform the tasks you want to do.

Find Features

This is the third Major Step in the Primary Lipidomics Workflow. It uses Mass Profiler and comprises three sub-steps.

- Step 1. Add samples

  After you launch Mass Profiler, you add the calibrated MS1 samples.
- Step 2. Edit method

  This step gives you instructions for editing the method in Mass Profiler to extract features based on retention time, mass and CCS value.
- Step 3. Extract and review features

  Ion mobility features are unidentified compounds represented by retention time, mass and CCS value. In this step you run the method you created in Step 2 to extract these features from the data. In the next section you identify the compounds with ID Browser.

Step 1. Add samples

You must first create a project and add samples before you edit a default method for the feature extraction.

Create new project

1 Launch **Mass Profiler**.

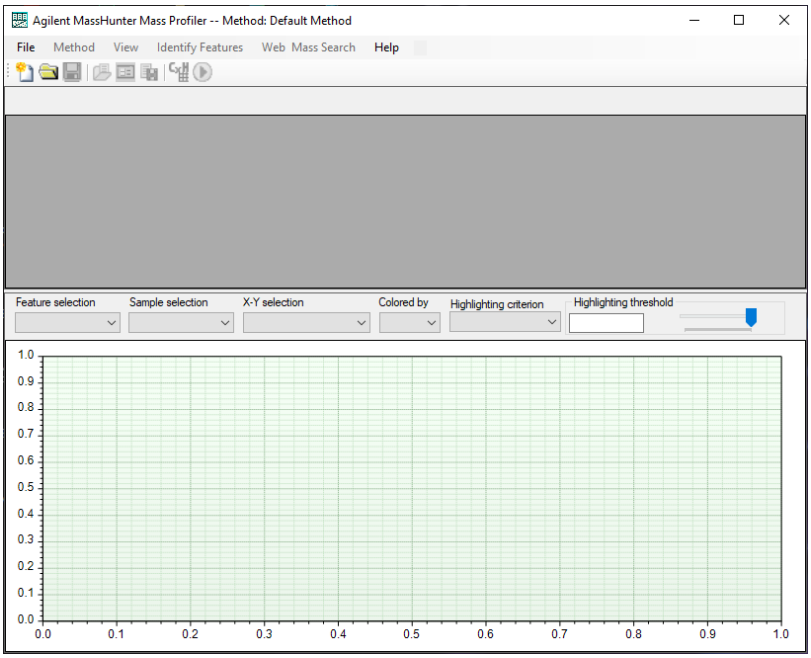


Figure 36. Agilent Mass Profiler main window

2 Note the method whose name is on the Title Bar.

3 Select **File > Create Project**.

4 For Number of groups, select **1** (**Figure 37**).

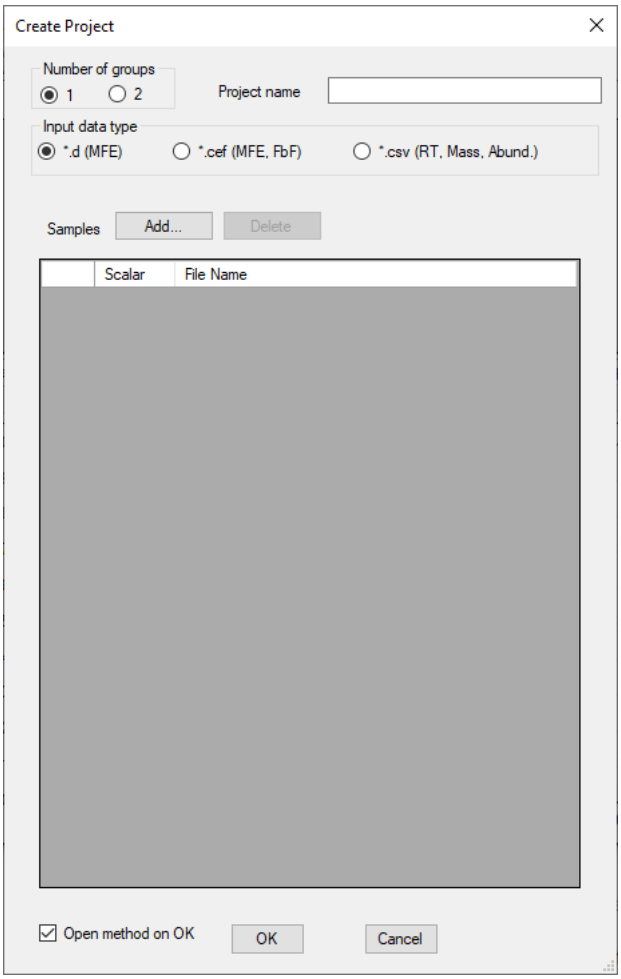


Figure 37. Create Project dialog box

Add calibrated MS1 samples

Because there are no control samples in the MS1 files, use the following instructions:

1 Type the project name, *iii*`_find lipids`, where *iii* refers to your initials.

2 Click **Add**.

3 Select the calibrated files in the Nist0_1017 folder, and click **Open**.

4 Repeat **step 2** and **step 3** for the calibrated files in the other two Nist folders.

The Create Project dialog box now contains the sample files.

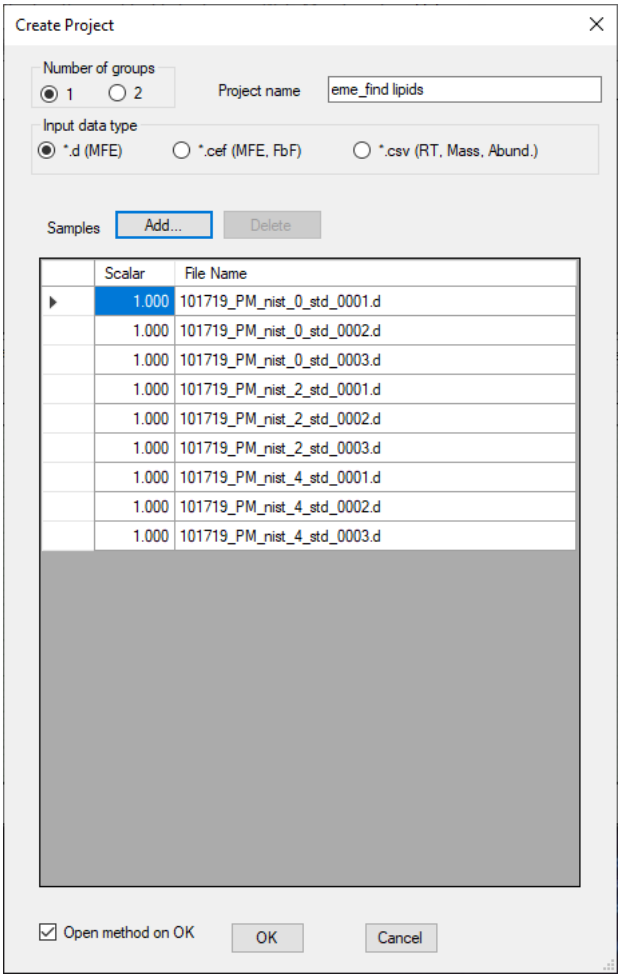


Figure 38. Mass Profiler Create Project dialog box with added files

5 If the default method's name is in the Title Bar of the main window:
   a Make sure the **Open method on OK** check box is **marked**.
   b Click **OK**.

At first, you see the dialog box with only commonly changed parameters (**Figure 39**).

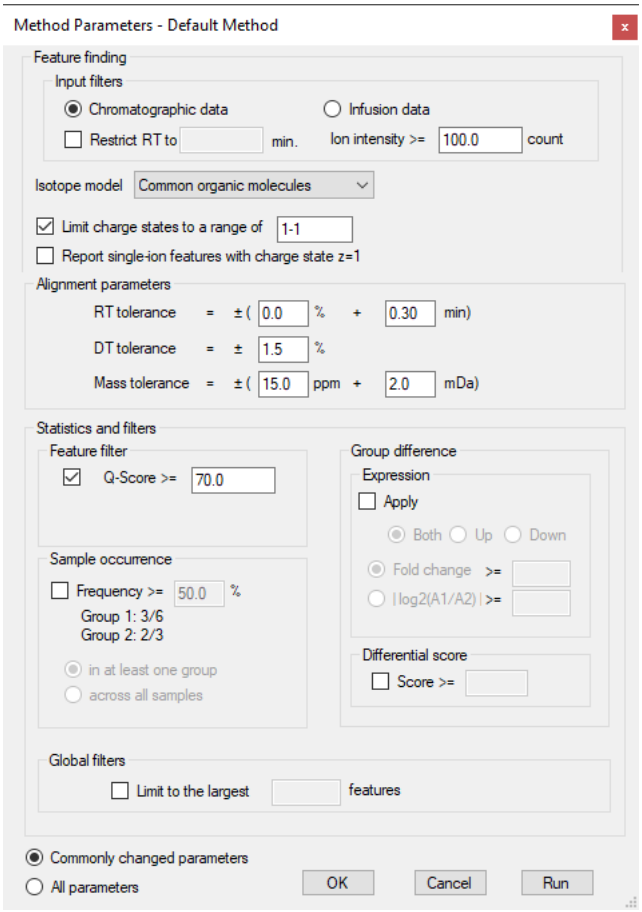


Figure 39. Default method with commonly changed parameters

c Go to **"Step 2. Edit method"** on page 48.

If a different method has been loaded:

a Make sure the **Open method on OK** check box is **clear**.

b Click **OK**.

c Select **Method > Reset Method to Default**.

d Select **Method > Edit Method**.

Figure 39 appears.

e Go to **"Step 2. Edit method"** on page 48.

Step 2. Edit method

One of the parameters you are going to edit is not on **Figure 39** on page 47.

Edit parameters

1 Select **All parameters**.

You now see the default method dialog box with three tabs.

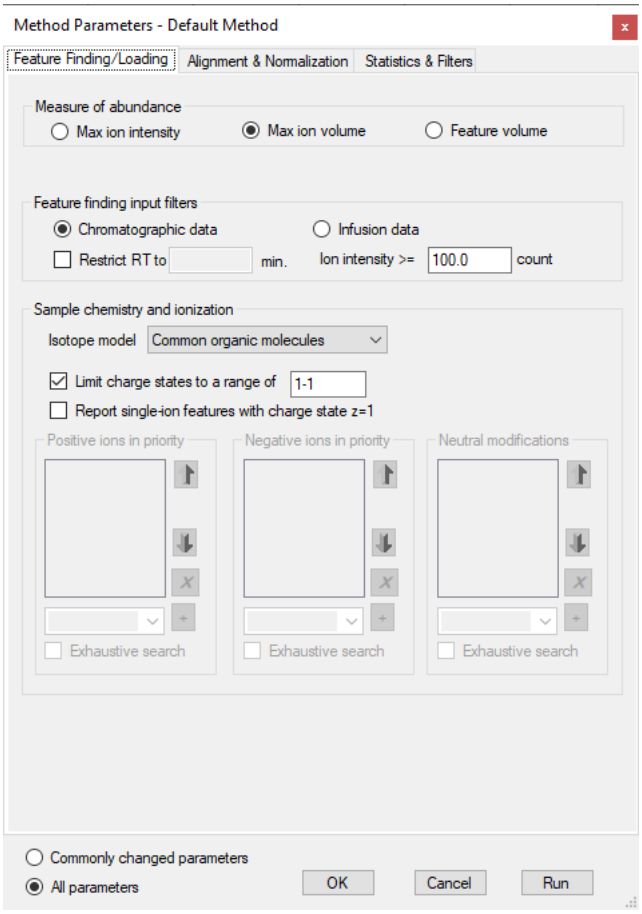


Figure 40. Default method with all parameters

2 Click a tab, and for each tab and parameter take the action described in **Table 2**.

Table 2 Parameters

For This Tab and Parameter:	Take This Action:
Feature Finding/Loading Tab:	
Isotope Model	Select Common organic (no halogens).
Limit charge states to a range of	Type `1-3`
Report single ion features with charge state z=1	Check box marked automatically when above Isotope Model selected
Alignment & Normalization Tab:	No edits
Statistics and Filters Tab:	
Abundance >	`5000`

The Feature Finding/Loading Tab now looks like this.

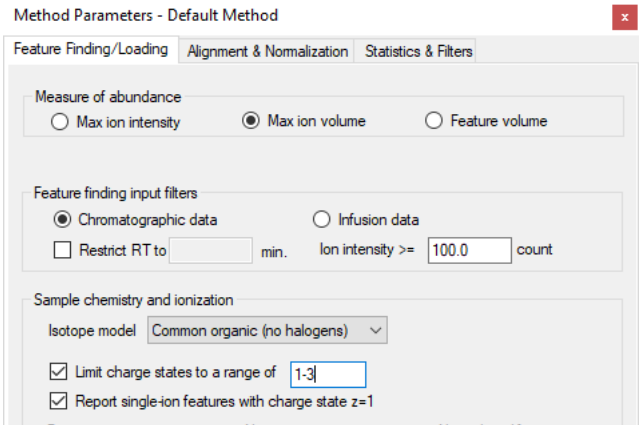


Figure 41. Edited Feature Finding/Loading Tab

The Statistics and Filters Tab now looks like this.

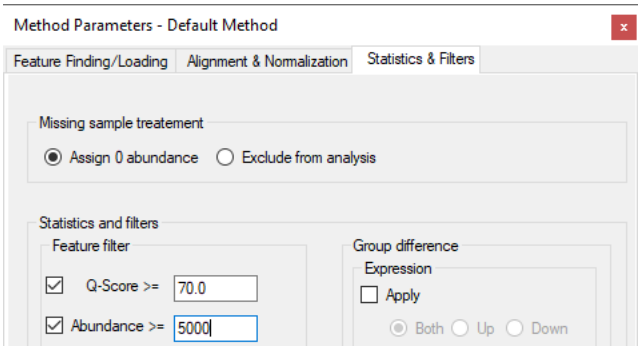


Figure 42. Edited Statistics and Filters Tab

3 Click **OK**.

NOTE

Changing a parameter value that is not commonly changed does not automatically place the parameter on the Commonly changed parameters dialog box. The Commonly changed parameters (not values) are always the same.

Save the method and project

You can start a run from any tab of the method dialog box, but it's wise to save both the method and project at this point.

1 Select **Method > Save Method As**.
2 Type the new method, *iii*`_find lipids.mpm`., and click **Save**.
3 Select **File > Save Project**.

Step 3. Extract and review features

Now you can run the method to extract features. Do not worry if the number of features varies a bit from run to run. The lipid matrix results in MPP should be the same as shown in the next chapter.

Run method

- Select **Method > Run Current Method**, or

  Open the method and click **Run**.

NOTE

For these example files, the run will take around a minute or so because Results folders already exist (from previous user's run).

Review features

When the run is complete, the Mass Profiler main window looks like this:

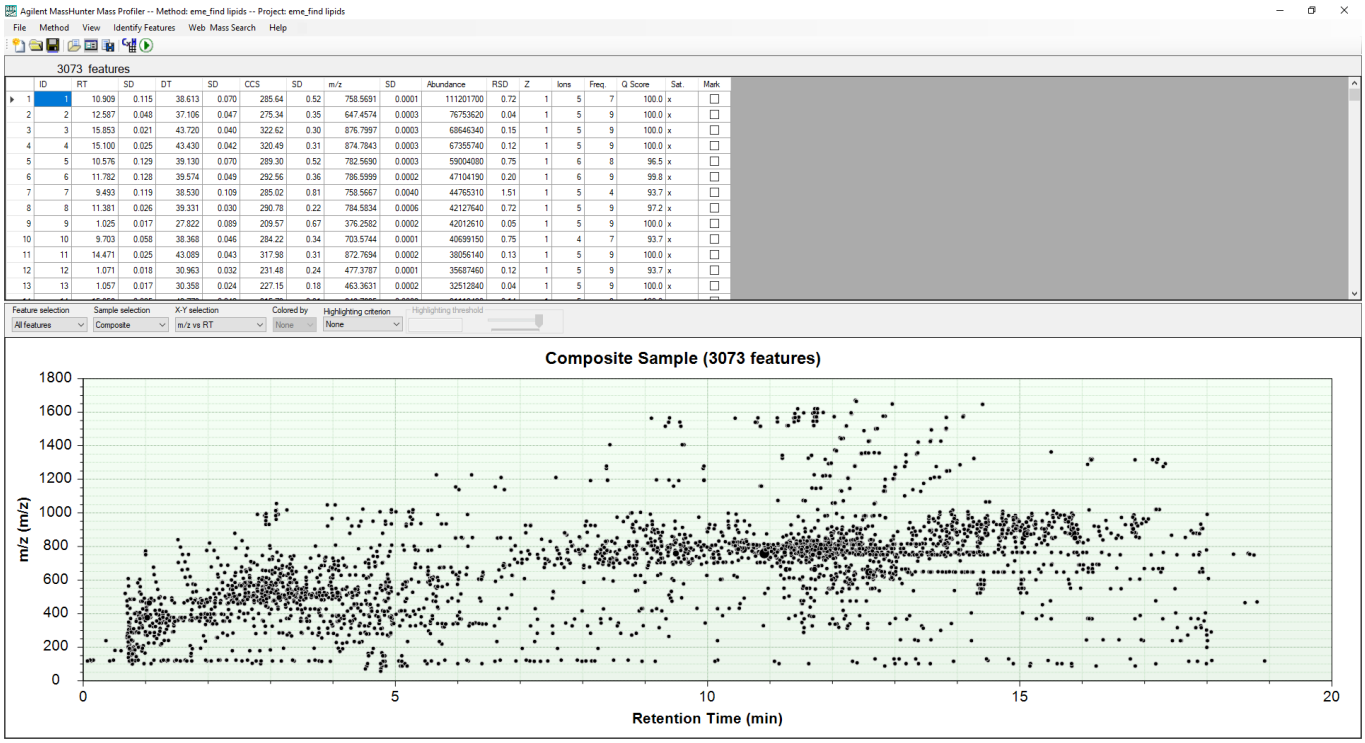
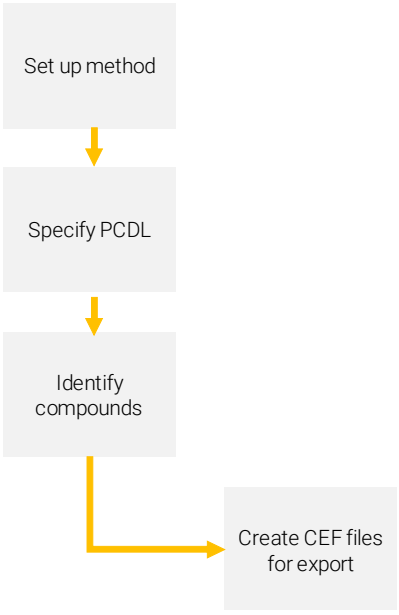


Figure 43. Mass Profiler main window with feature extraction results

Identify Compounds



In this fourth Major Step you identify features using the ID Browser within Mass Profiler and the custom lipid PCDL you created with Lipid Annotator. This Major Step comprises four sub-steps.

- Step 1. Set up method

  After you launch ID Browser from Mass Profiler containing the extracted MS1 features, you edit the parameters in the ID Browser method.
- Step 2. Specify PCDL

  This is where you enter the custom lipid PCDL file you created.
- Step 3. Identify compounds

  You then run the method to match the MS1 features with the compounds in the PCDL and return the data to Mass Profiler, where the CEF files are created.
- Step 4. Create CEF files for export

  In Mass Profiler you create the CEF files for export and then import them to Mass Profiler Professional (MPP) in the fifth Major Step, Analyze Lipids.

Step 1. Set up method

After launching ID Browser from Mass Profiler, you set up the method to identify the lipid compounds in the features you extracted.

Enter database search criteria

1 Launch ID Browser from Mass Profiler.

a Click the **Identify Features** menu.

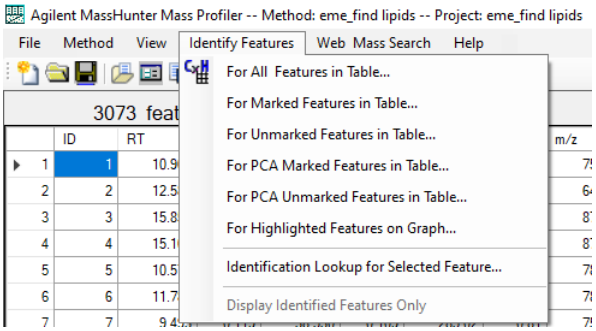


Figure 44. Identify Features menu for Mass Profiler

b Select **For All Features in Table...**

The ID Browser Wizard appears on top of the main window.

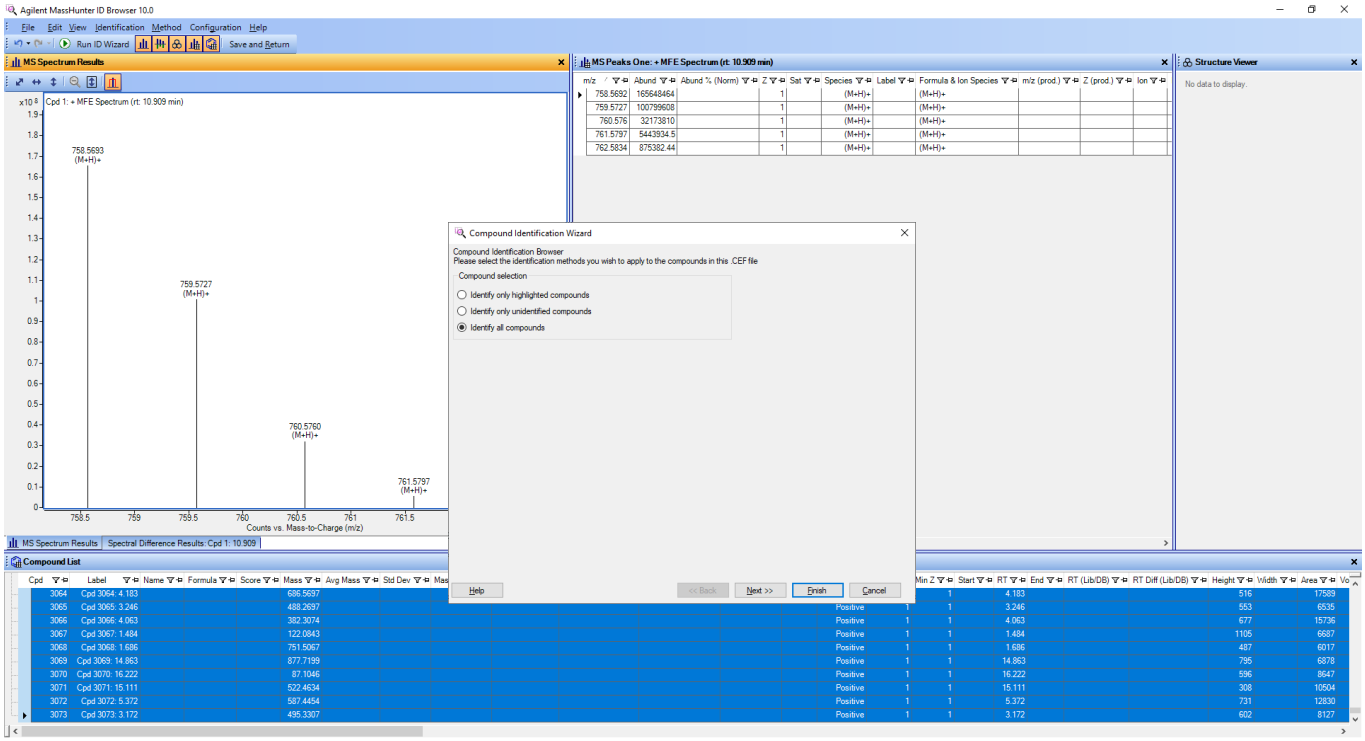


Figure 45. ID Browser Wizard on top of default identification results

2 In the Compound Identification Wizard, click **Next**.

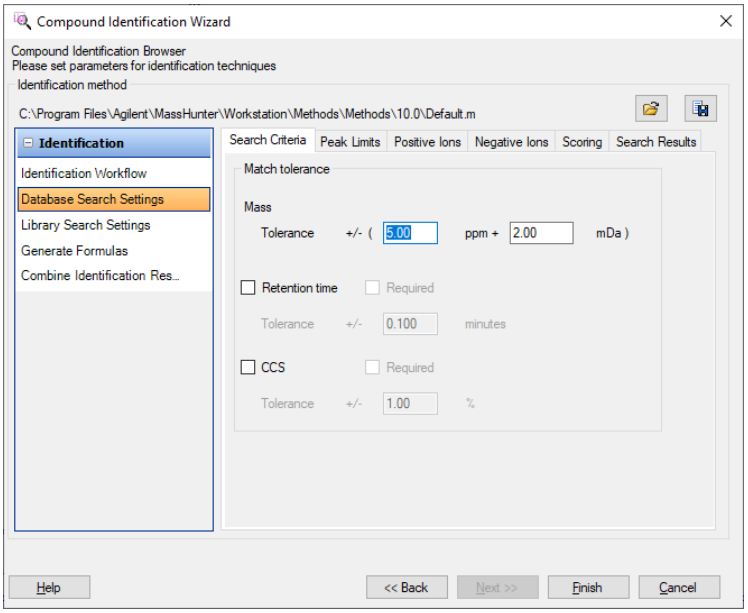


Figure 46. Database Search Settings - Search Criteria Tab

3 If the default method is listed as the Identification method, go to **step 4**.

If the default method is *not* listed as the Identification method:

a Click the **Open** icon.

b In the ...\Workstation\Methods\Methods\10.0 folder, select **Default.m**.

c Click **Open**.

4 Select **Database Search Settings** and click the **Search Criteria** tab.

5 Mark the **Retention Time** check box and the **Required** check box next to it.

6 Mark the **CCS** check box and the **Required** check box next to it.

7 Retain default values for the other parameters.

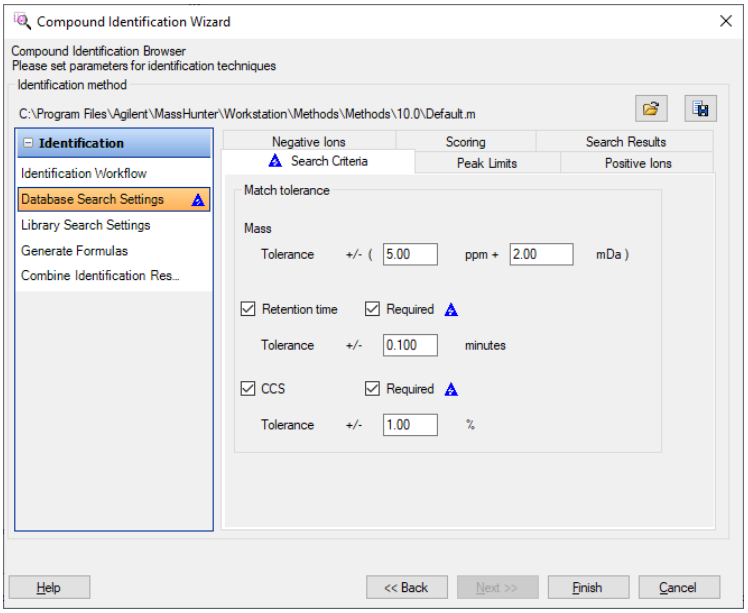


Figure 47. Search Criteria selections made

Enter additional positive ion

1 In Database Search Settings, click the **Positive Ions** tab.

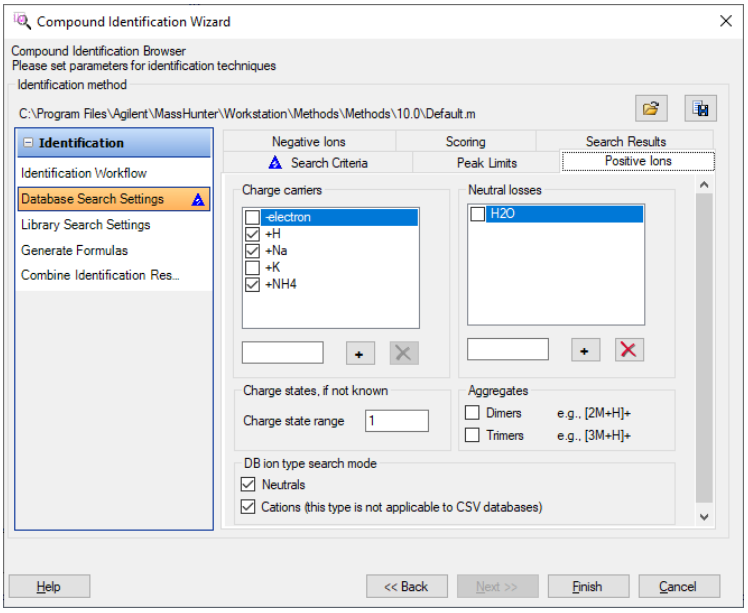


Figure 48. Positive Ions tab

2 Type `H4N` and click **+** to add it to the list of **Charge carriers**.

Because the PCDL from Lipid Annotator writes H4N, you need to add that to ID Browser, which is looking only for NH4.

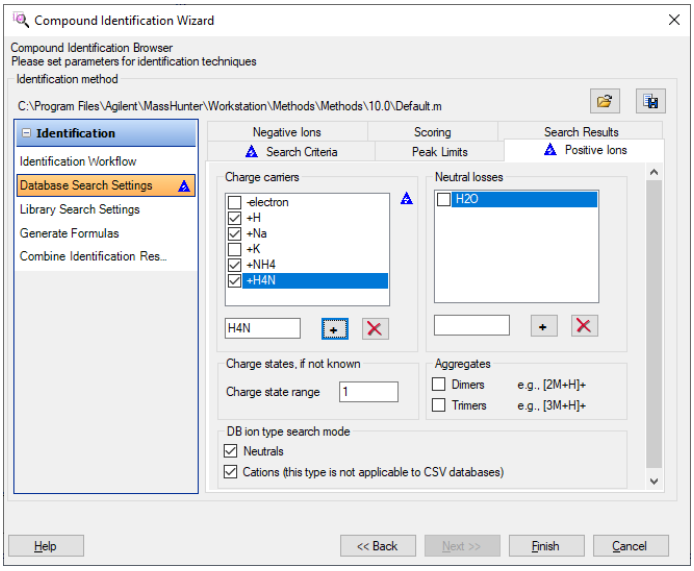


Figure 49. Positive Ions tab modified

Step 2. Specify PCDL

Before saving the method, as the last set-up step you link to the custom lipid PCDL you created previously.

Set up identification workflow

1 Select **Identification Workflow**.

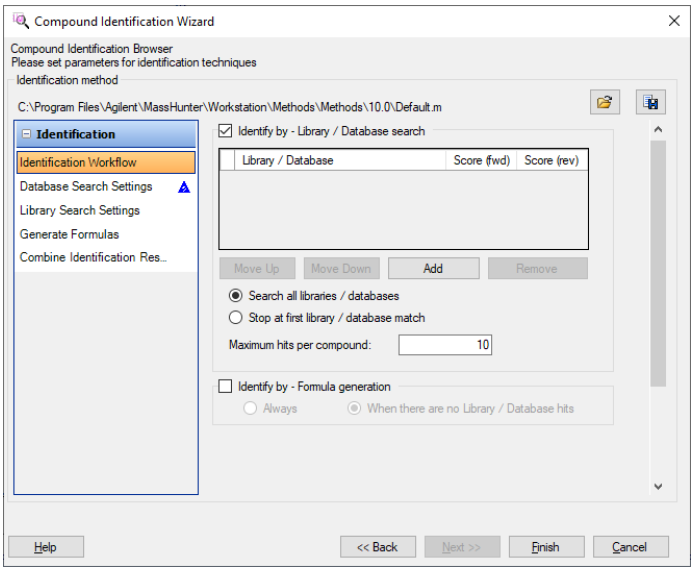


Figure 50. Identification Workflow without specified PCDL

2 To add the lipid database you created earlier in this chapter, click **Add**.

3 Select ***iii*_custom lipid PCDL**, and click **Open**.

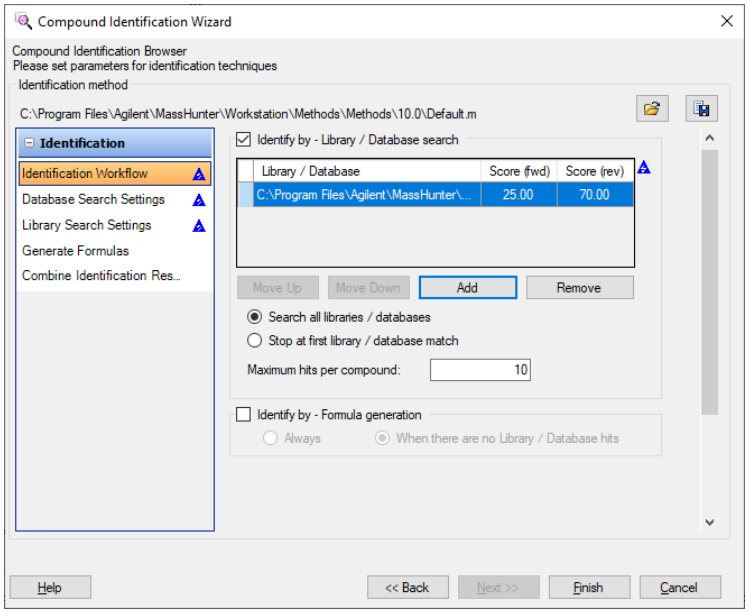


Figure 51. Identification Workflow with custom lipid PCDL added

Save the method

1 Click the **Save** icon.

2 Type `iii_ID Method - Lipids`, and click **Save**.

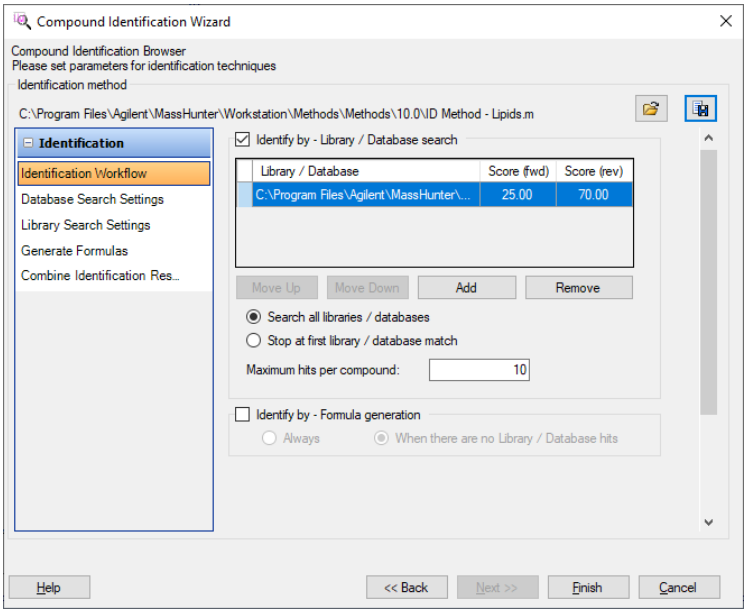


Figure 52. Identification Workflow with new saved method.

Step 3. Identify compounds

Now you are ready to run the method to identify compounds.

Run the method

1 To run the ID method, click **Finish**.

After ID Browser completes the identification, you see the same main screen as in **Figure 45** on page 53 but with a few identified compounds in the table.

2 To view identified and unidentified features in Mass Profiler, click **Save and Return**.

You now see the main Mass Profiler window showing the list of identified and unidentified features.

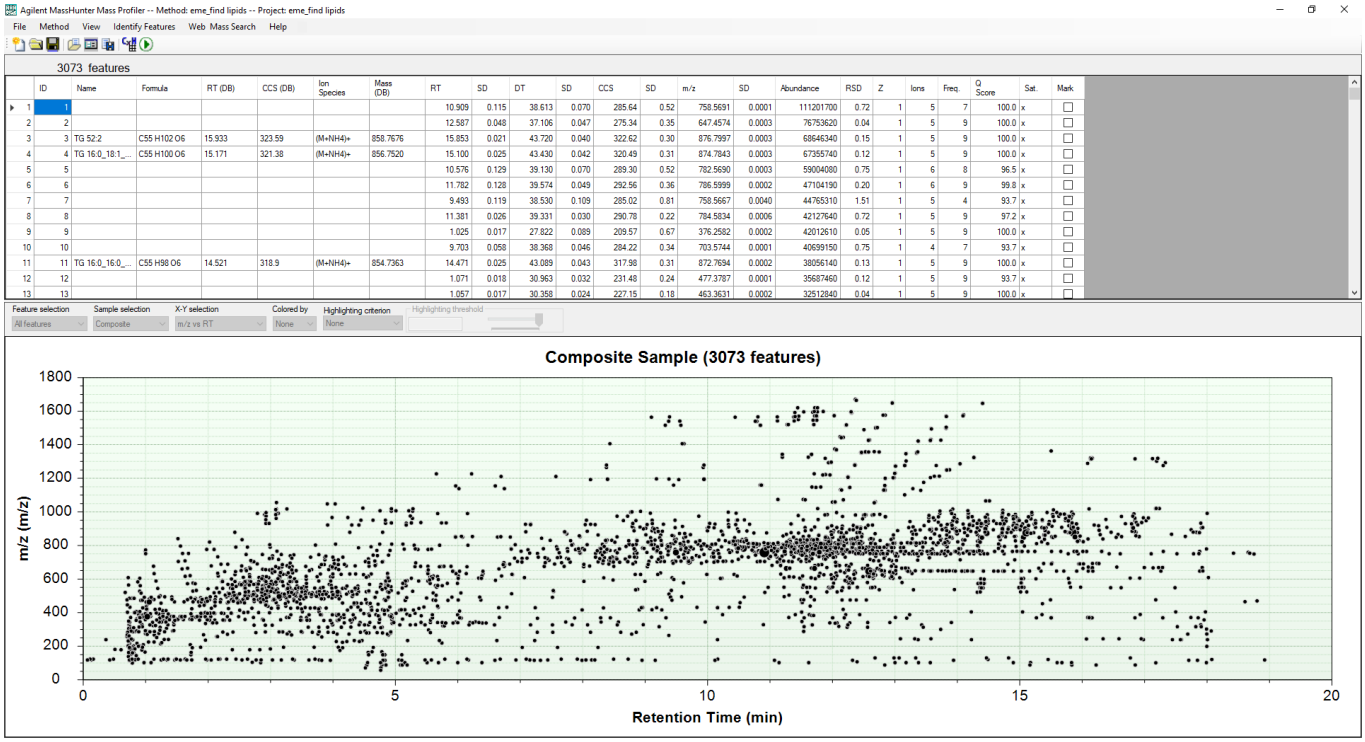


Figure 53. Mass Profiler main window with table of identified and unidentified features

Display identified compounds only

1 Click **Identify Features**.

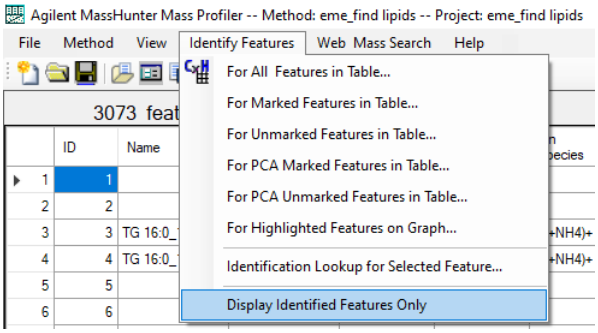


Figure 54. Identify Features menu

2 Select **Display Identified Features Only.**

The identified compounds appear in the table and graphic (**Figure 55**).

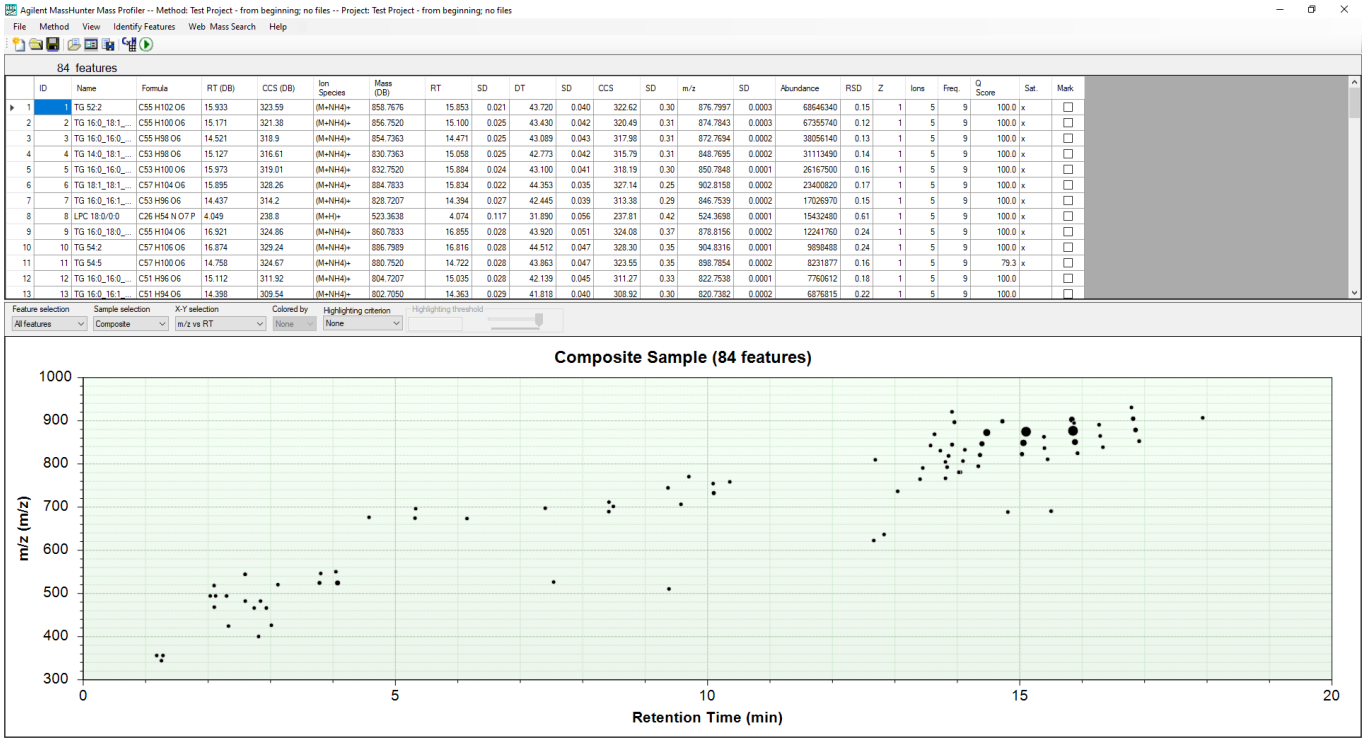


Figure 55. Identified features only in three sample sets

At this point you may want to save the project with the feature finding and identification results.

Step 4. Create CEF files for export

If you intend to analyze the sample files with the 84 identified lipid compounds, you must convert the files to CEF files and import them into MPP. See **Chapter 4**.

1 To see the menu item for creating CEF files for export, click **File**.

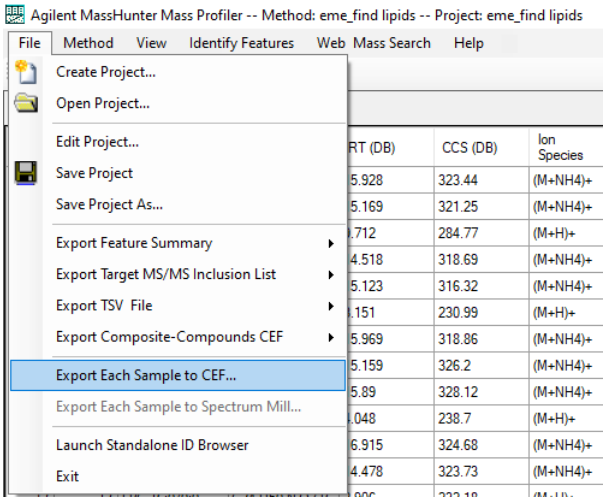


Figure 56. File menu

2 Select **Export Each Sample to CEF**.

A dialog box appears asking if you want to average sample values.

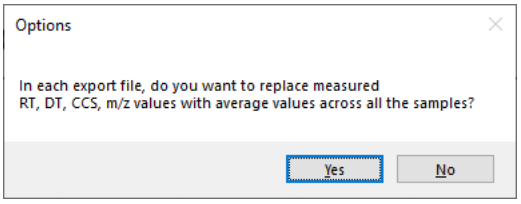


Figure 57. Dialog box for averaging sample values

3 Click **No**.

A dialog box appears locating the CEF files.

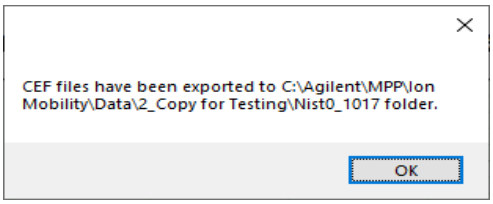


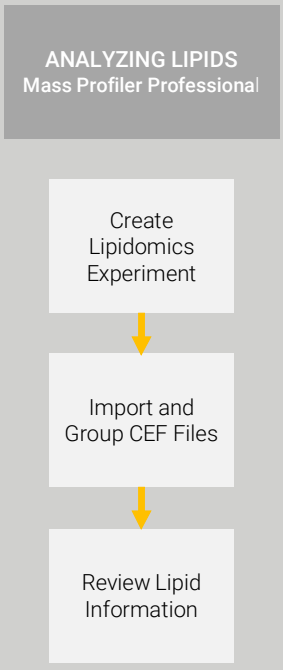
Figure 58. Dialog box for locating the CEF files

4 Click **OK**.

You can now import and analyze the files in MPP, as described in **Chapter 4**.

4 Analyzing Lipids

This chapter takes you through the tools you need to compare lipid classes in different sample sets using Agilent Mass Profiler Professional.



In the final Major Step within the Primary Lipidomics Workflow you work with MPP lipid tools to compare lipid compositions between sample sets. This workflow comprises three sub-steps.

- Step 1. Create lipidomics experiment

  After you launch MPP, you create a lipidomics experiment into which you load the CEF files.

- Step 2. Import and group CEF files

  In this step you load and group the CEF files you created in Mass Profiler, filter the data and produce an abundance vs sample plot.

- Step 3. Review lipid information

  You can review the lipids spreadsheet to take into account the ion mobility data. You can also distinguish among your sample sets by looking at the compound and abundance distribution of the lipid standards in the interpretation groups you've set up, which contain either averaged or non-averaged sample data. And you can generate a CCS vs Mass plot to see the lipid class differences for ion mobility data.

This fifth Major Step, Analyzing Lipids, completes the Primary Lipidomics Workflow.

Create Lipidomics Experiment

To create a lipidomics experiment you first create a new project and then a new experiment.

Step 1. Create new project

After launching Mass Profiler Professional (MPP), you set up a project.

1 Launch MPP.

The MPP main window appears with the Startup dialog box.

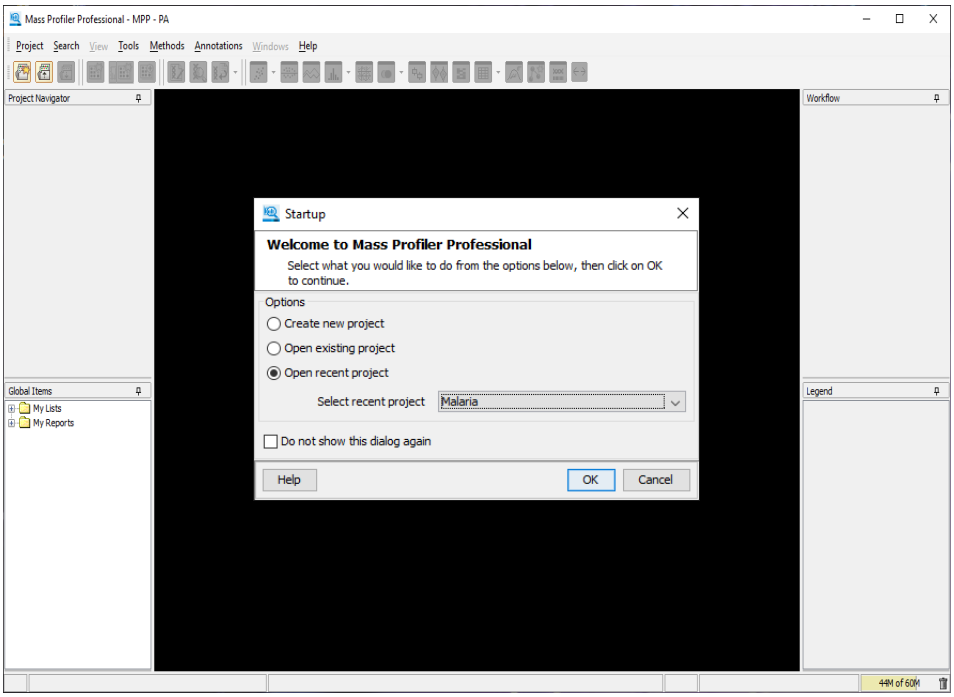


Figure 59. Startup dialog box in MPP main window

2 Select **Create new project** and click **OK**.

The Create New Project dialog box appears.

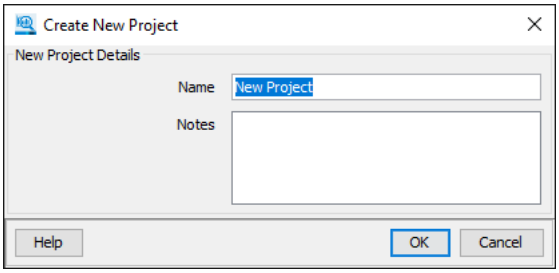


Figure 60. Create New Project dialog box

3 Type the new project name, *iii*_IM Lipid Project, where *iii* represents your initials.

4 Click **OK**.

Step 2. Create new experiment

The Experiment Selection Dialog appears.

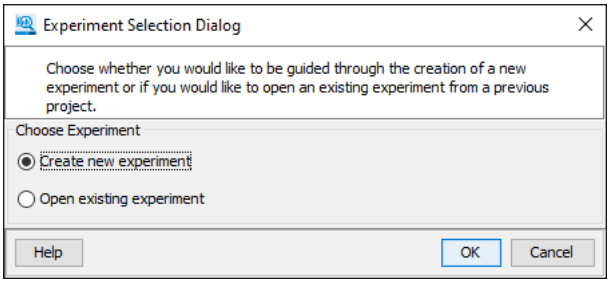


Figure 61. Experiment Selection Dialog box

1 Click **OK** to create a new experiment.

The Experiment description page of the New Experiment dialog box appears with no entries.

2 Type the **Experiment name** as *iii*_IM Lipid Expt.

3 For the **Experiment type**, select **Lipidomics** from the list.

4 For the **Workflow type**, select **Data Import Wizard** from the list.

You now see the Experiment description page with entries from **step 2** to **step 4** (**Figure 62** on page 65).

If you intend to do any statistical analyses on the data, you can select Analysis: Significance Testing and Fold Change from the Workflow type list now, or wait to select it from the Workflow menu when the main window appears after completing the Data Import Wizard.

The instructions that follow assume you have selected Data Import Wizard as the Workflow type.

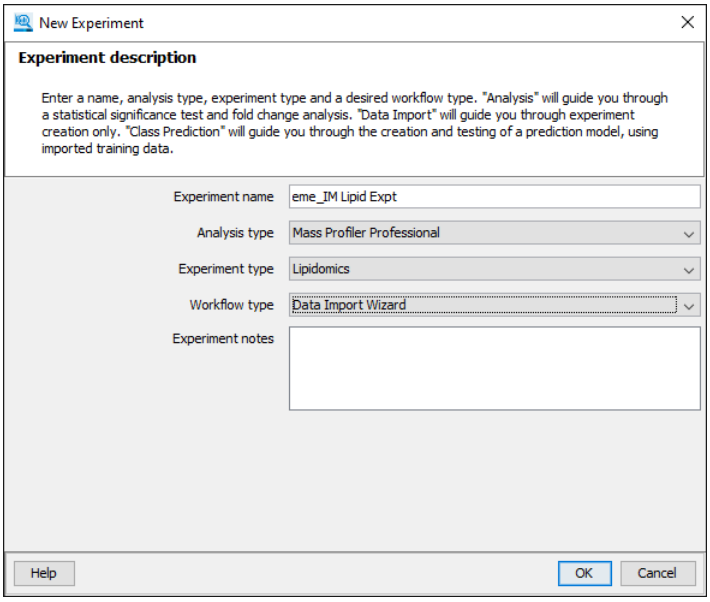


Figure 62. Experiment description with entries

5 Click **OK**.

The first step of the Experiment Creation Wizard appears.

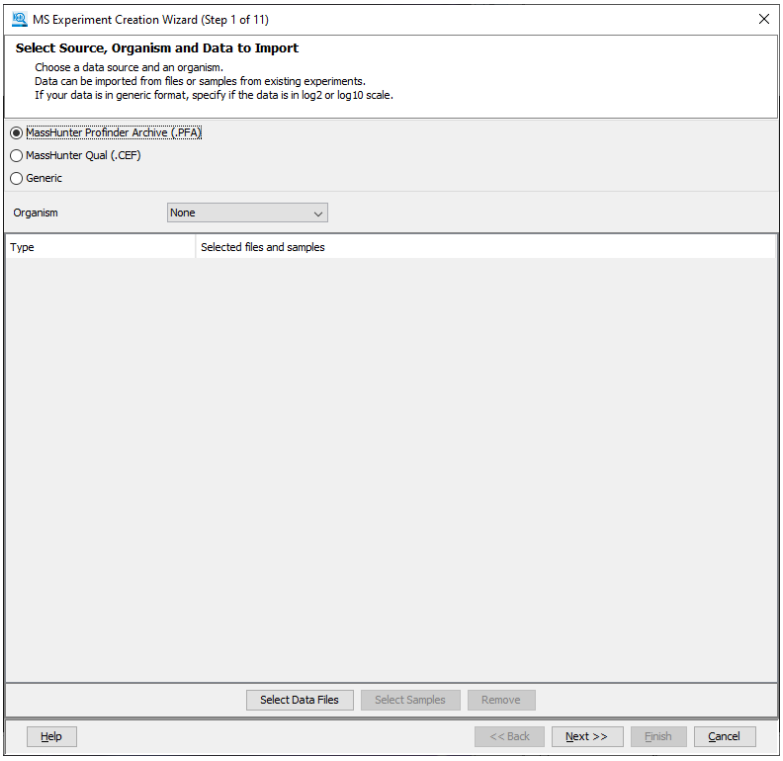


Figure 63. Step 1 of MS Experiment Creation Wizard

Import and Group CEF Files

After naming and describing the new project and experiment, you go through the Experiment Creation Wizard (Data Import Wizard) to import and group the CEF files you created in Mass Profiler. You then filter the data.

Step 1. Select data to import

You now work with the Experiment Creation Wizard that appeared after you clicked OK in New Experiment dialog box. You first select the data source, the organism and the data to import, as shown below.

1 Select **Masshunter Qual (.CEF)** as the **Data Source**.

2 For the **Organism**, select **Homo sapiens**.

3 Click **Select Data Files**, select the MS1 files in the **Nist0_1017** folder and click **Open**.

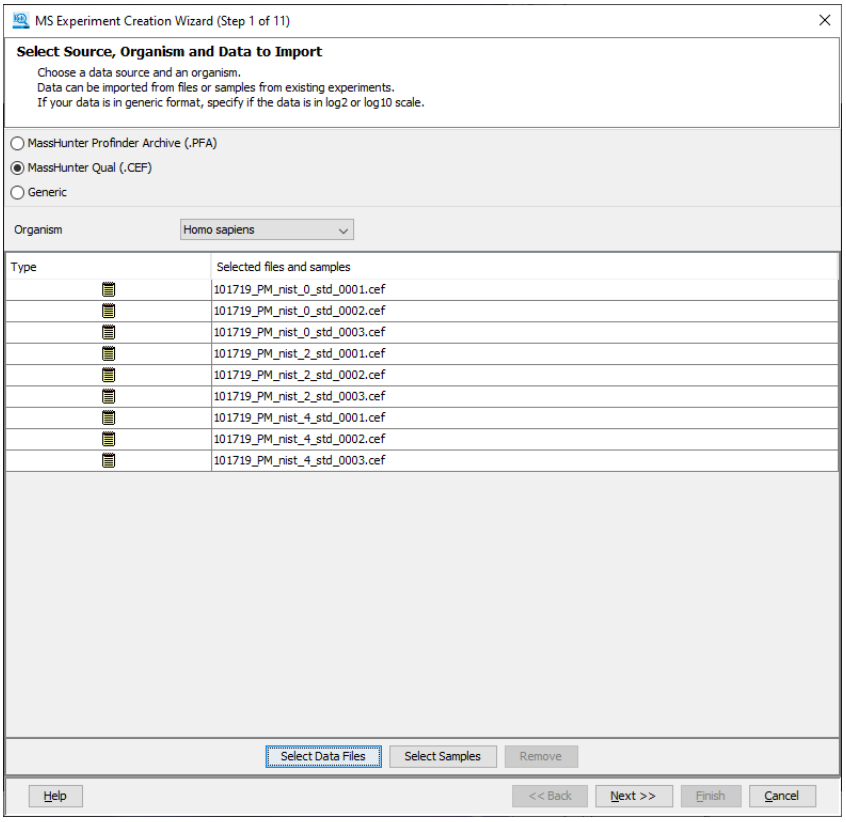


Figure 64. Step 1 of MS Experiment Creation Wizard with data files

4 Click **Next** twice.

The Experiment Grouping dialog box appears.

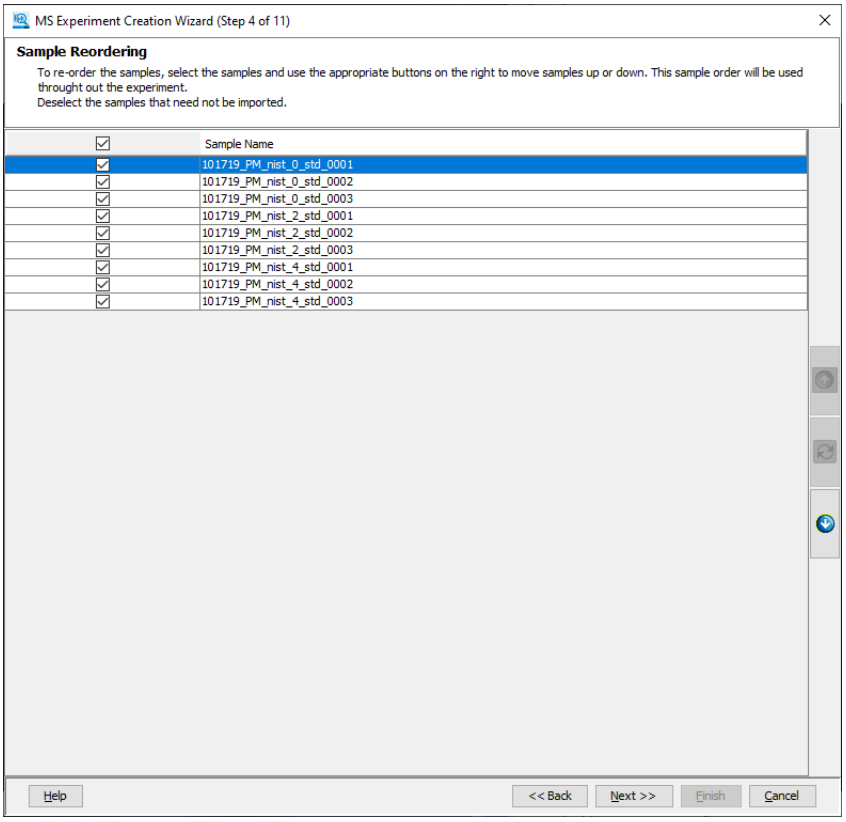


Figure 65. Experiment Grouping (Step 5)

Step 2. Group samples

1 Click **Add Parameter**.

The Edit Parameter Values dialog box appears.

2 For the **Parameter name**, type `Standard Concentration`.

You now group the nine samples into sets, each with a different standard. See **Figure 66** on page 69 for the entries given below.

3 Select the first three samples with no standards, and click **Assign Values**.

4 Type `No standard` and click **OK**.

5 Select the second set of samples, and click **Assign Values**.

6 Type `Standard 2` and click **OK**.

7 Select the third set of samples, and click **Assign Values**.

8 Type `Standard 4` and click **OK**.

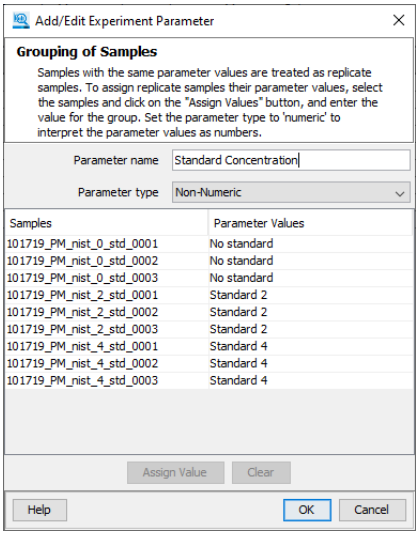


Figure 66. Add/Edit Experiment Parameter dialog box

9 Click **OK** to see a listing of the groups.

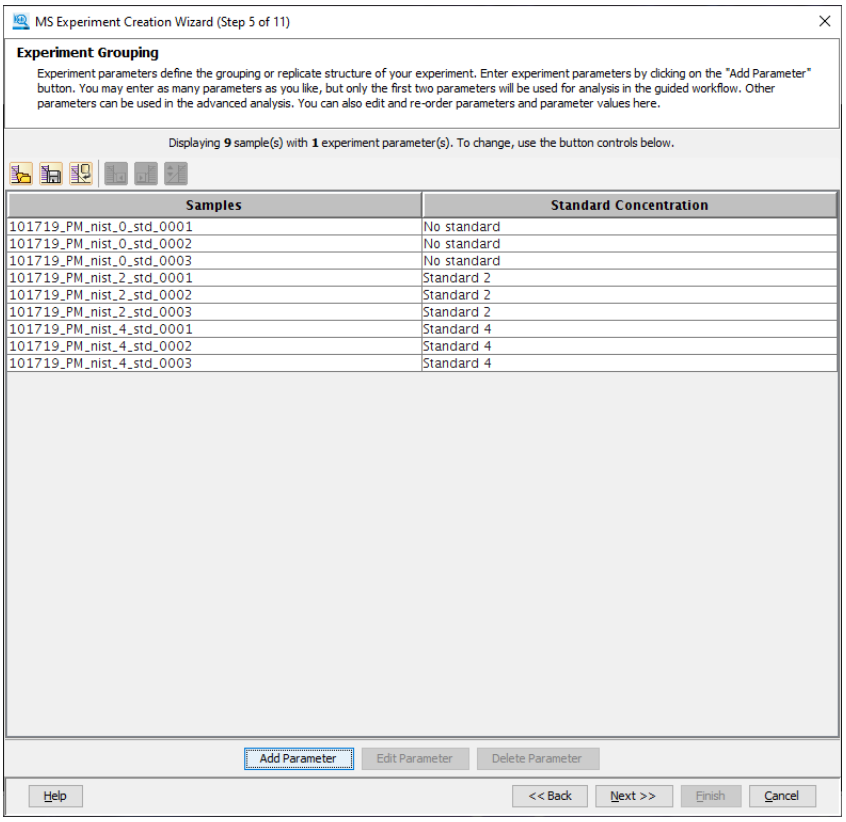


Figure 67. Experiment Grouping after adding groups

Step 3. Filter data

1 Click **Next**.

The Filtering step appears. Make the changes below. See **Figure 68**.

2 Clear the **Minimum absolute abundance** check box.

3 Change the **Minimum number of ions** to 1.

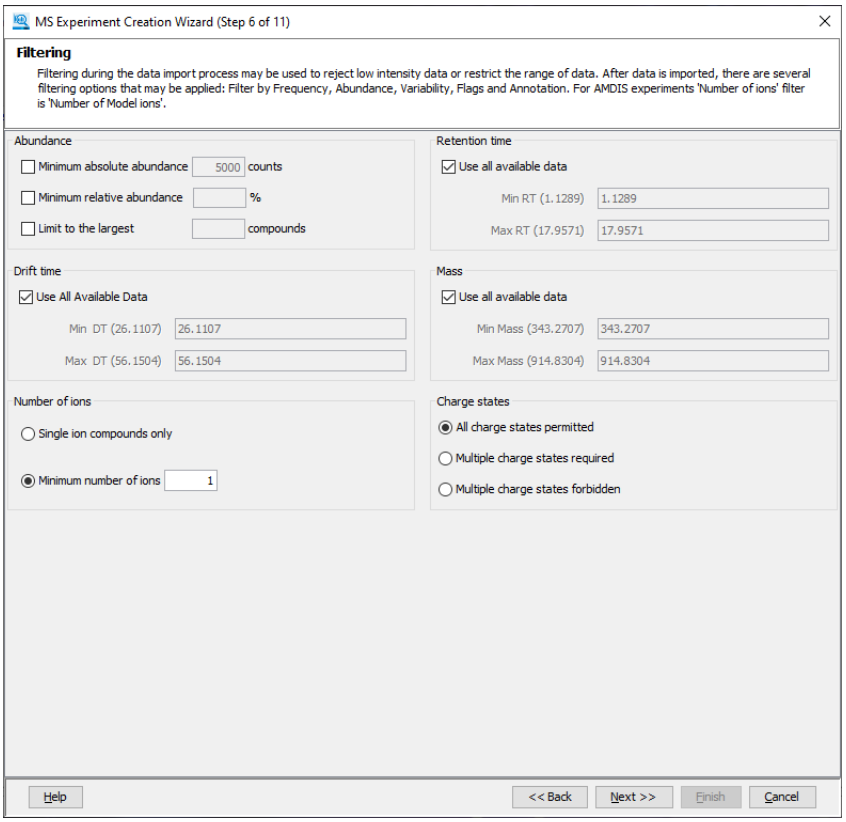


Figure 68. Filtering Step after changes

4 Click **Next** four times, then click **Finish**.

The MPP main window appears with an abundance vs sample profile on the display.

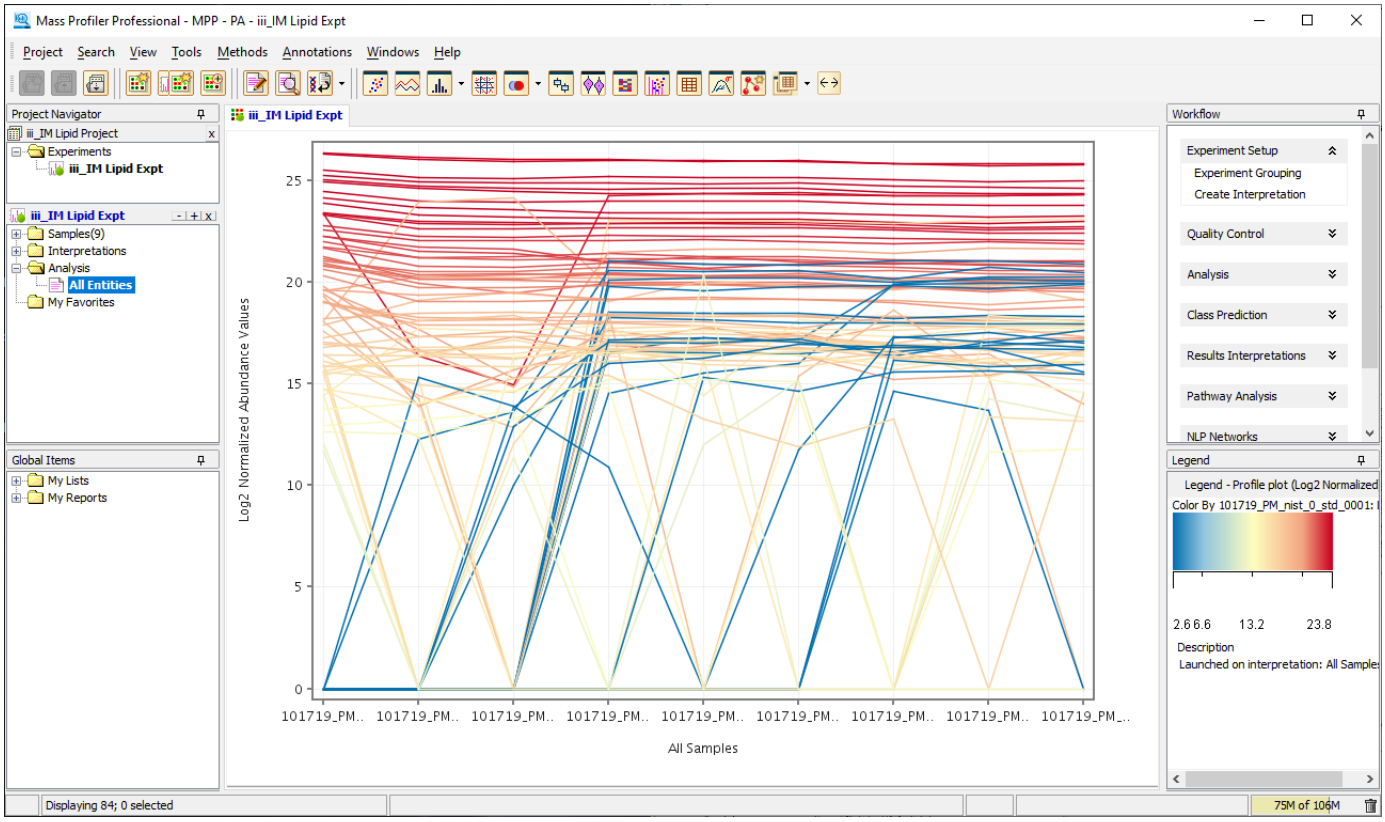


Figure 69. MPP main window with log normalized abundance vs samples

For these samples, normalization cannot be set up because internal standards were not included in the study.

You are now ready to move on to the next section, **"Review Lipid Information"** on page 72.

Review Lipid Information

This section shows you how to review the Drift Time (DT), the CCS value and the ion species for each lipid. Once you create interpretations with your experiment groupings, you also learn to use the lipid matrix tool to view the relative abundance of the standards and other lipids in the samples.

Step 1. View lipid ion mobility data

1 In the top icon toolbar, click the **Spreadsheet** icon, 

The spreadsheet with all the sample data appears.

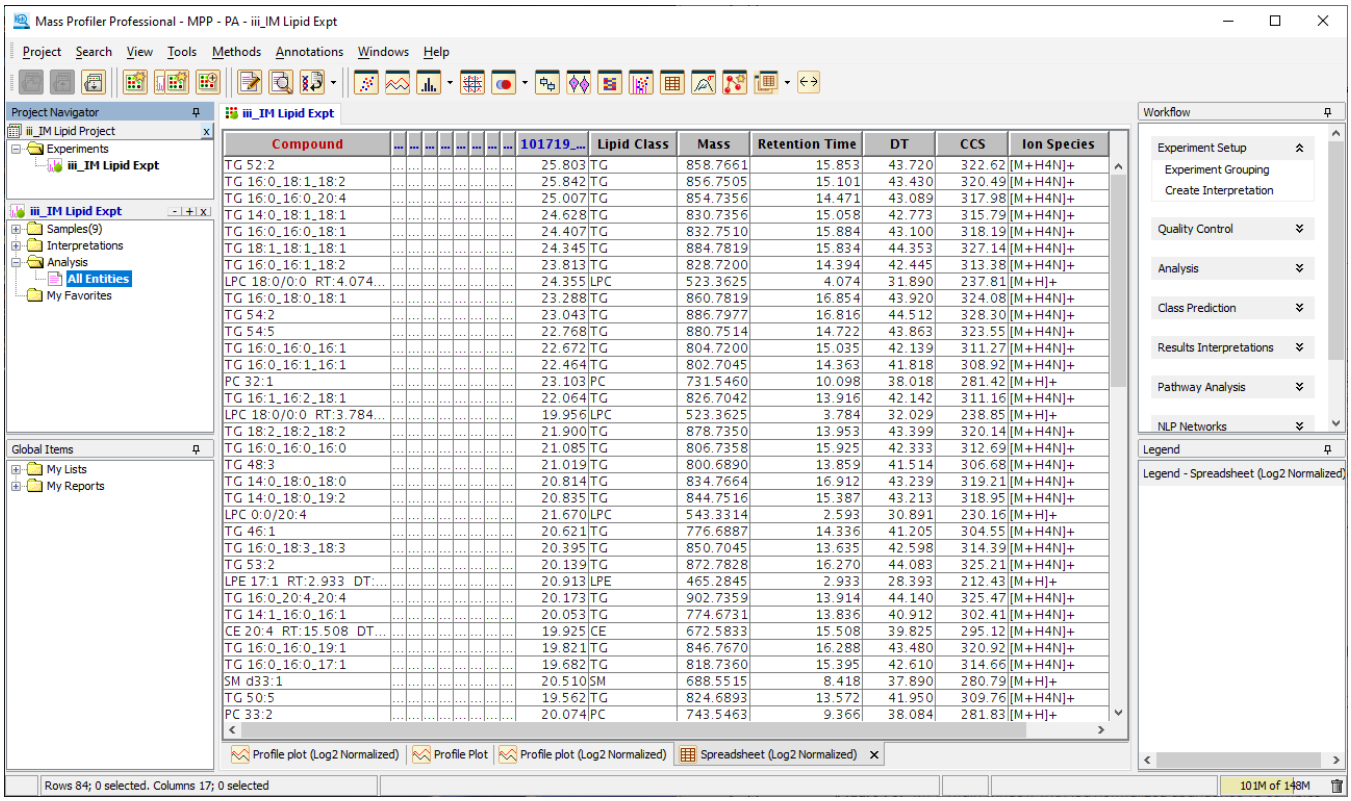


Figure 70. Spreadsheet View of the main MPP window

2 Note the Drift Time (DT), CCS values and ion species for each lipid.

Each ion species listed is the one whose CCS has been calculated for the corresponding lipid.

To add columns to the Spreadsheet View, right-click the **All Entities** item in **Project Navigator** on the left and then select **Inspect List**.

Step 2. Create interpretations

An interpretation is a grouping of experiment conditions for display and analysis. In this step you create two interpretations -- averaged and non-averaged.

1 In the **Workflow** menu under **Experiment Setup**, click **Create Interpretation**.

Step 1 of the Create Interpretation wizard appears.

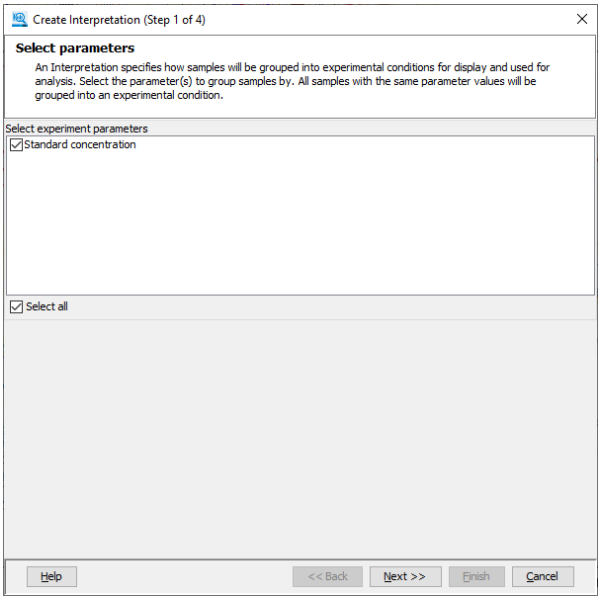


Figure 71. Step 1 of the Create Interpretation wizard - Select parameters

2 Click **Next** until you reach the next to last page (Step 3).

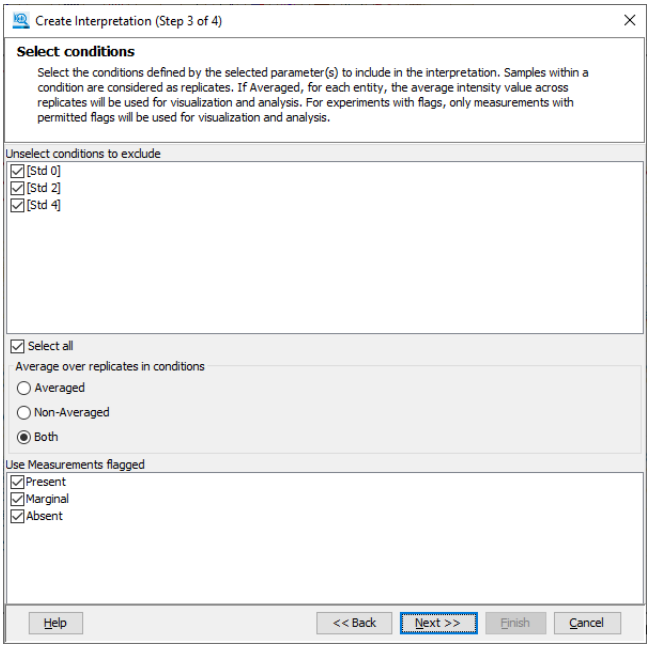


Figure 72. Step 3 of the Create Interpretation wizard - Select conditions

Notice that both Averaged and Non-Averaged options have been selected. This selection of default settings creates two separate interpretations.

3 Click **Next** to reach the last page (Step 4).

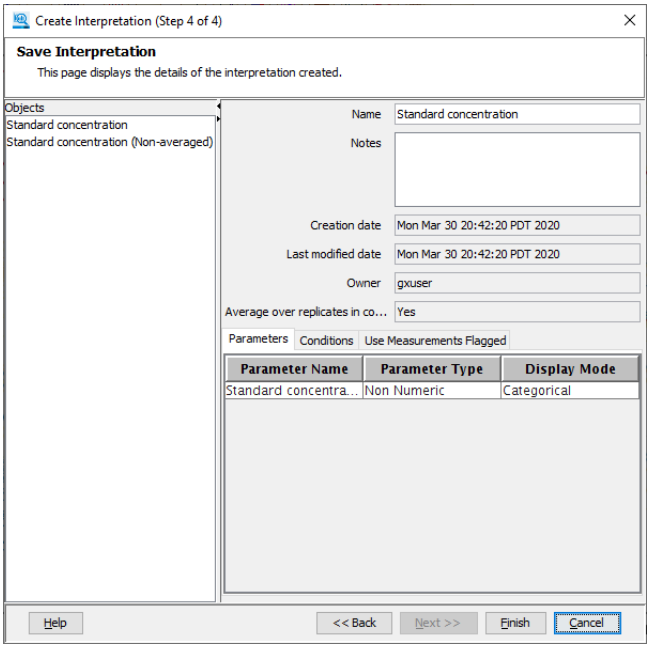


Figure 73. Last Step of Create Interpretation wizard - Save Interpretation

4 Click **Finish**.

Two interpretations are created, non-averaged and averaged.

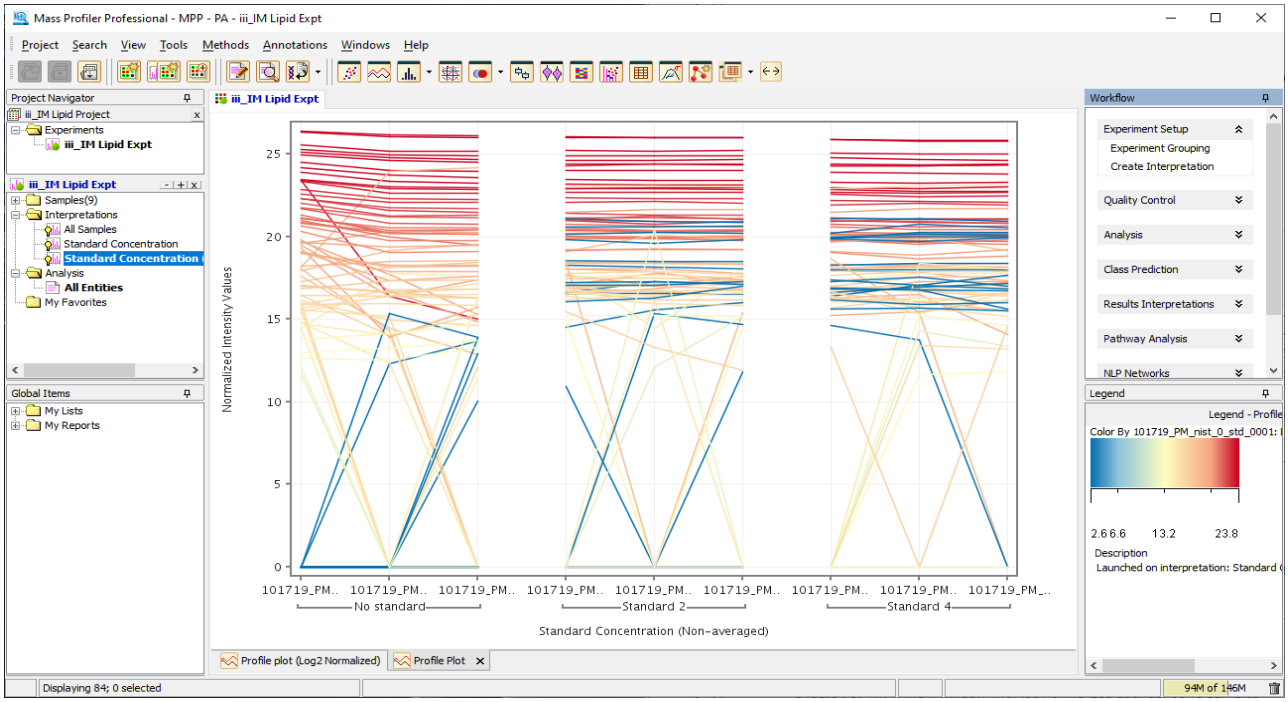


Figure 74. MPP main window - non-averaged interpretation, Standard concentration (Non-averaged)

Step 3. Create a lipid matrix

To view the abundance of different lipids, you use the lipid matrix tool.

1 In the Interpretation list, double-click the averaged interpretation, **Standard concentration**.

Figure 75. MPP main window - averaged interpretation, Standard concentration

2 Under **Results Interpretations** in the Workflow menu, select **Create Lipid Matrix**.

Step 1 of the Create Lipid Matrix wizard appears.

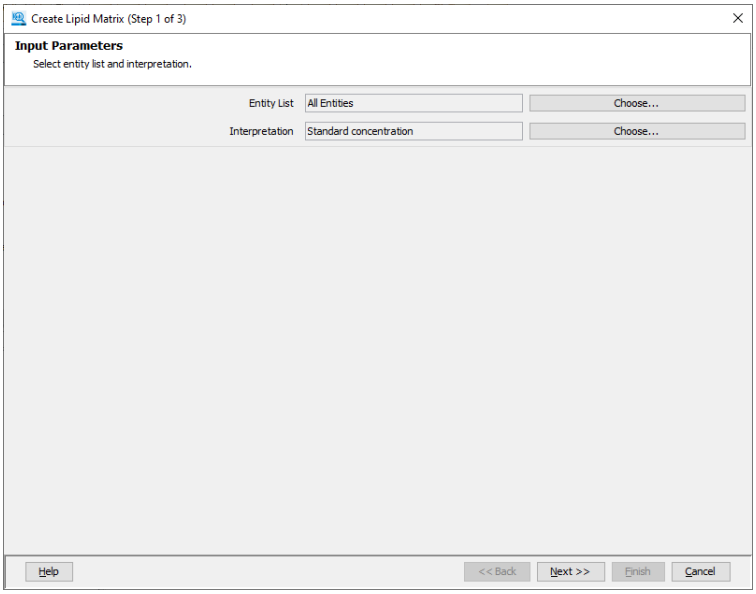


Figure 76. Create Lipid Matrix wizard - Input Parameters

The interpretation you see is the interpretation highlighted in the Project Navigator, which in this case is the averaged Standard concentration.

3 Click **Next**.

Step 2 of the wizard appears, which displays 3 graphics showing relative abundance in the sample sets. The first that appears is the Sum Composition.

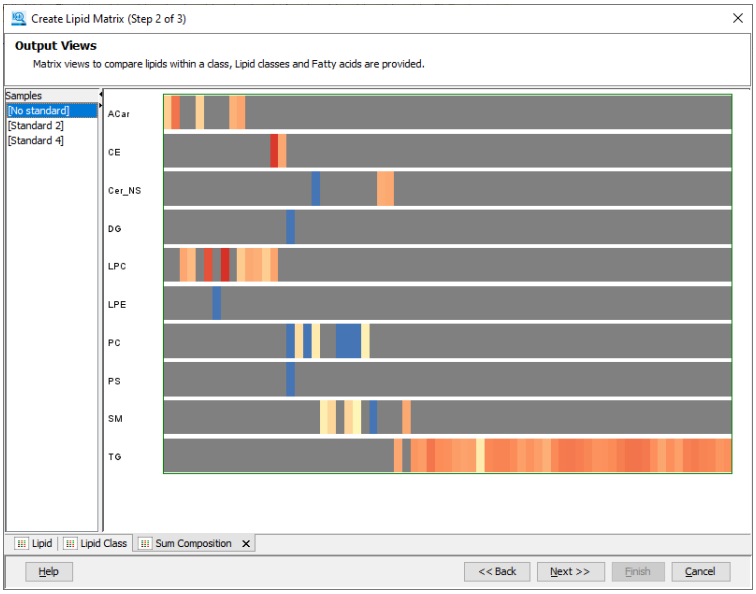


Figure 77. Create Lipid Matrix wizard - Output Views - Sum Composition

To see these graphics more clearly, you will view them in the main window.

4 Click **Next**.

The Save Lipid Matrix dialog box appears.

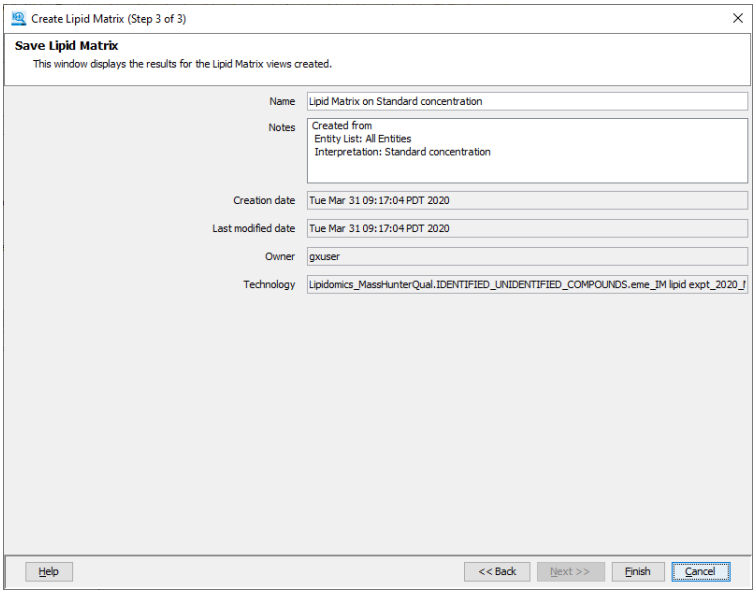


Figure 78. Save Lipid Matrix dialog box

5 Click **Finish**.

The main window appears with the Lipid display.

6 From the **Lipid Classes** list, select **PC**.

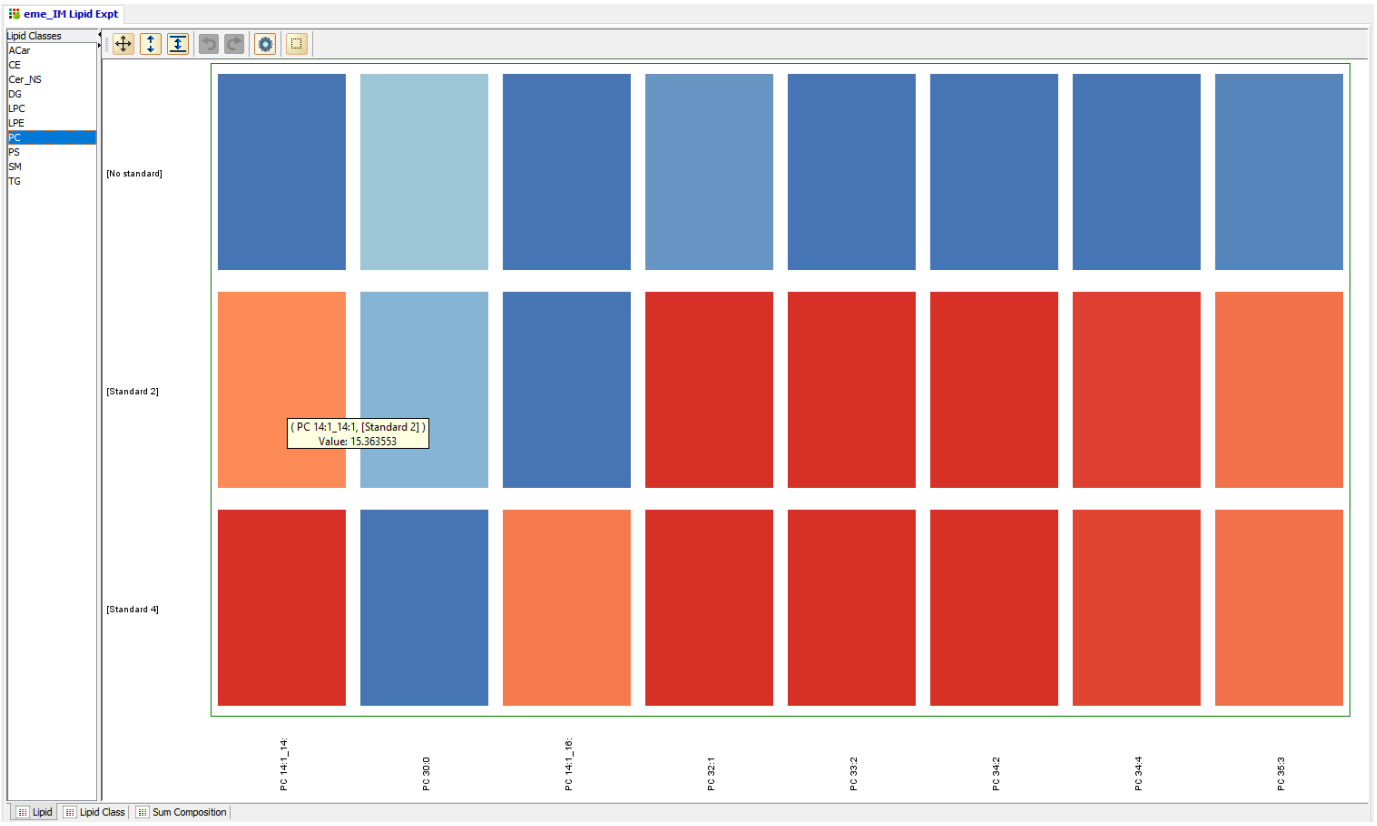


Figure 79. MPP main window - Lipid display for averaged concentration interpretation of the PC lipid class

This graphic display shows the different levels of abundance in the standard-spiked samples for each of the lipids in the PC class. Blue means less abundance. Red means strong abundance, and the colors between blue and red mean increasing degrees of abundance, from yellow to orange to red. See the Legend to the right of the matrix.

7 To see the full name of a lipid in the matrix and its abundance, pass the mouse over the rectangle of interest.

Notice that for the standard lipid PC 14:1_14:1, the abundance increases from no standard > standard 2 > standard 4.

8 Click the **Lipid Class** tab.

A different graphical display appears.

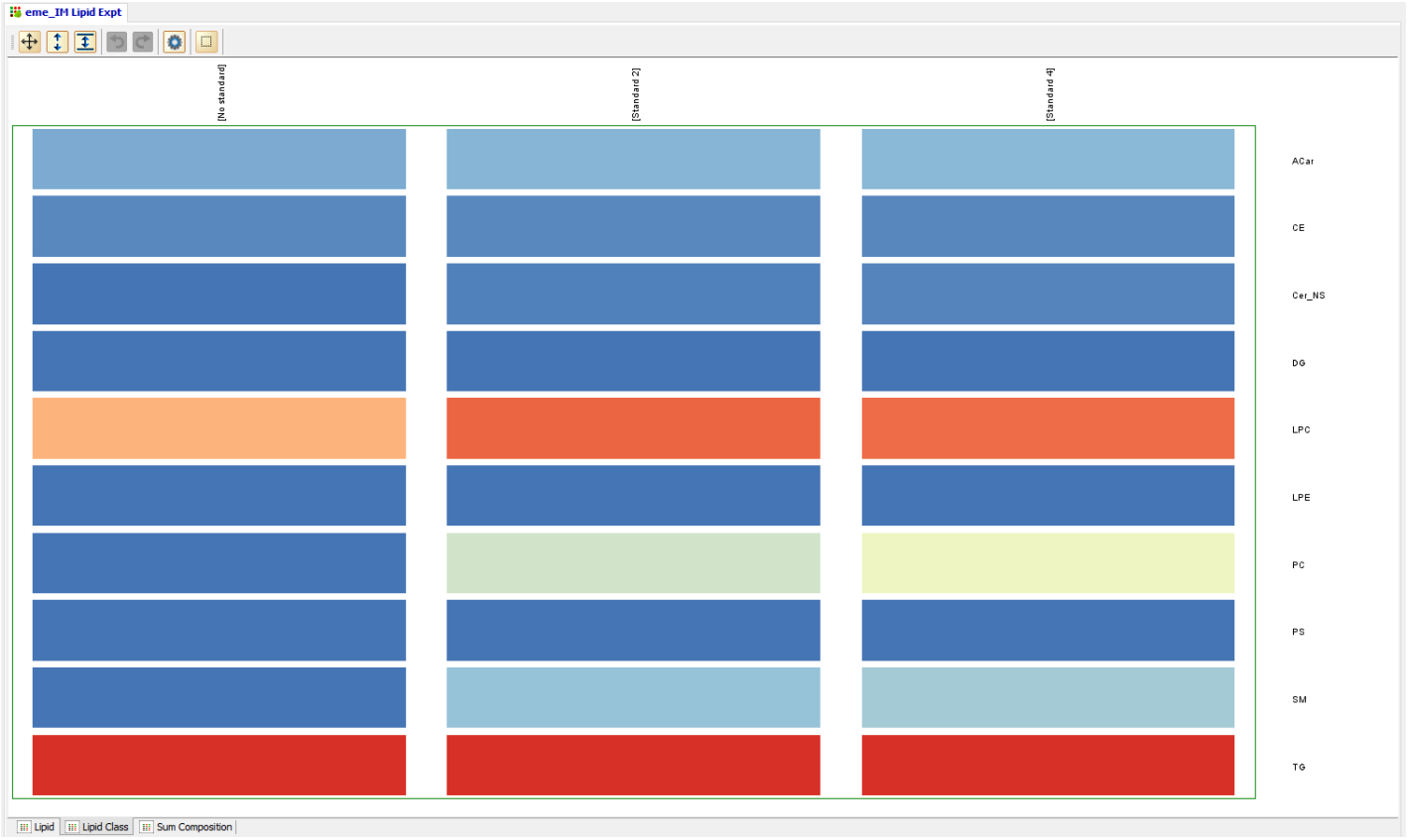


Figure 80. MPP main window - Lipid class display for averaged concentration interpretation

Even though there is one lipid standard spiked and found for the PC and SM classes, the results are muted because in this graphic you are viewing the results for all the lipids together in the class. Blue means less abundance, yellow or orange means some abundance, and red means strong abundance. See the Legend to the right of the matrix.

9 Click the **Sum Composition** tab.

Yet another graphical display appears.

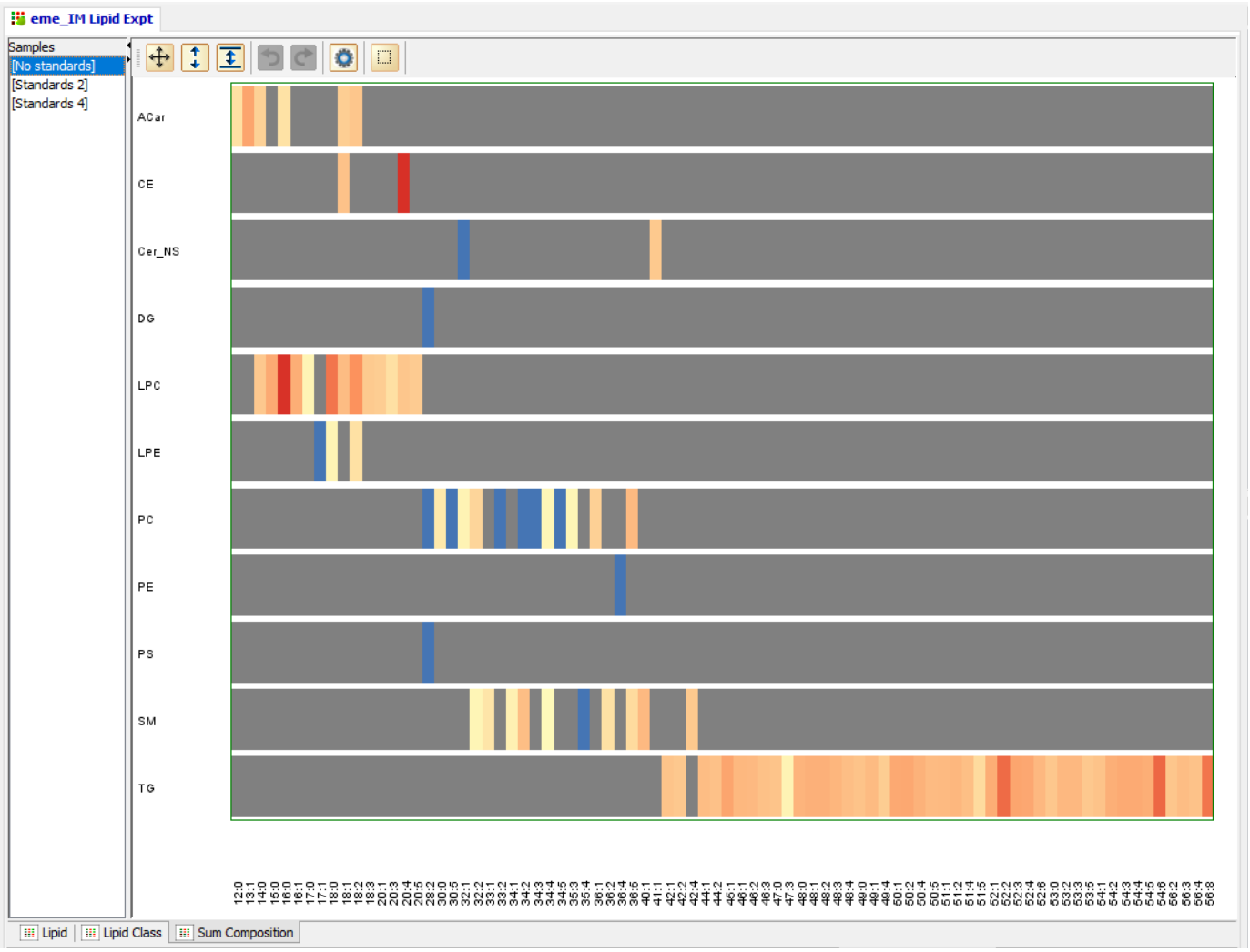


Figure 81. MPP main window - Sum Composition display for an averaged concentration interpretation

The sum composition shows the abundance (log of normalized abundance) of each lipid in a class for each sample set. Again, see the Legend to the right of the matrix for the color meanings.

The x-axis shows the number of carbons in the acyl tail groups for each lipid in a class. For example, the lipids in the ACar class have fewer carbons in their tails than the lipids in the other classes, and the lipids in the TG class have more carbons than the others, primarily because they have 3 tails and the ACar lipids have only one.

Also notice that PC 28:2 increases its abundance from No standard > Standard 2 > Standard 4.

Step 4. Plot CCS vs Mass

In addition to viewing lipid and lipid class relationships in MPP, you can generate plots with ion mobility data.

In this step you learn how to generate a CCS vs Mass plot.

1 In the MPP main window, click the **Mass vs RT Plot** icon, 

The plot display appears in the MPP window.

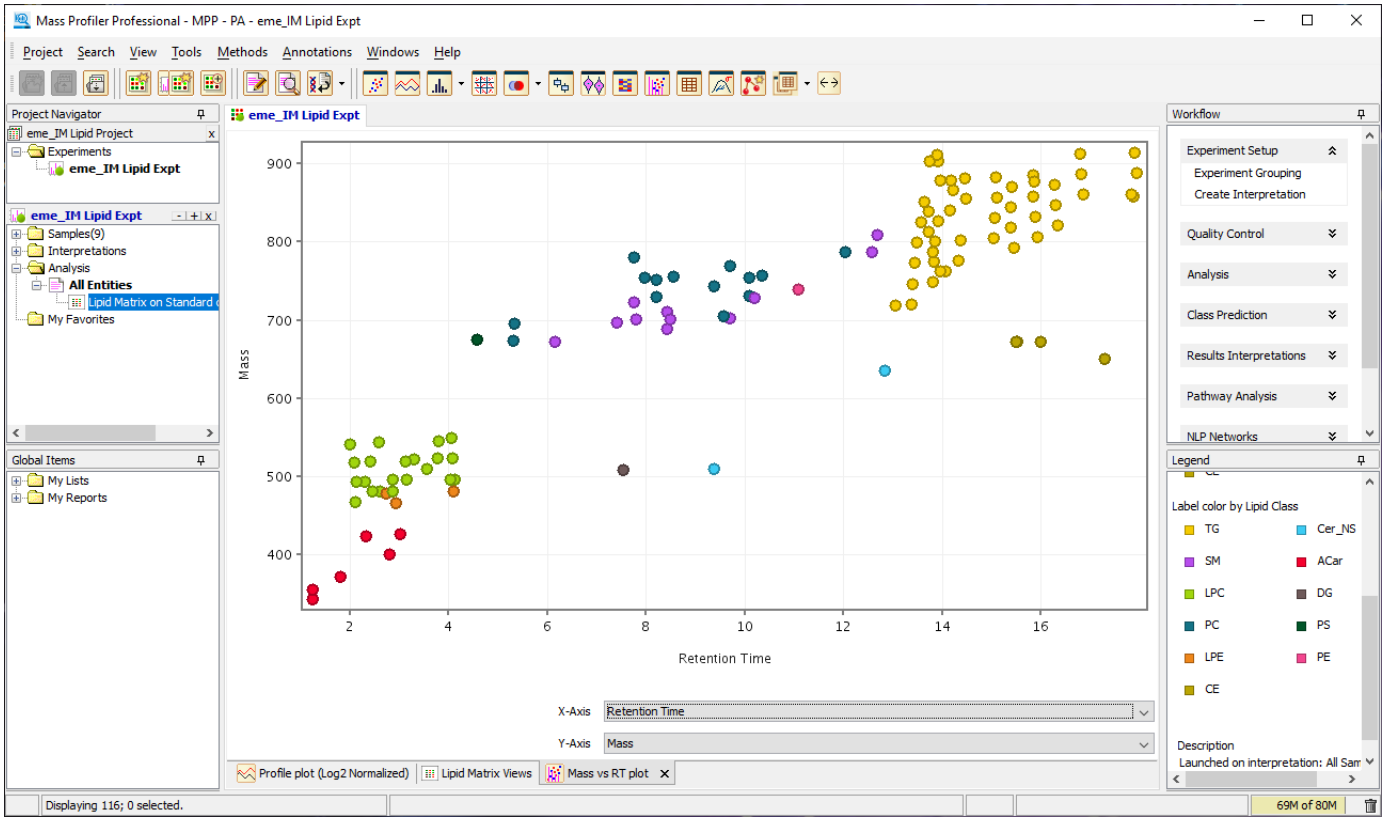


Figure 82. Mass vs RT plot in MPP window

2 From the X-Axis list, select **Mass**.

3 From the Y-Axis list, select **CCS**.

You now see the CCS vs Mass plot.

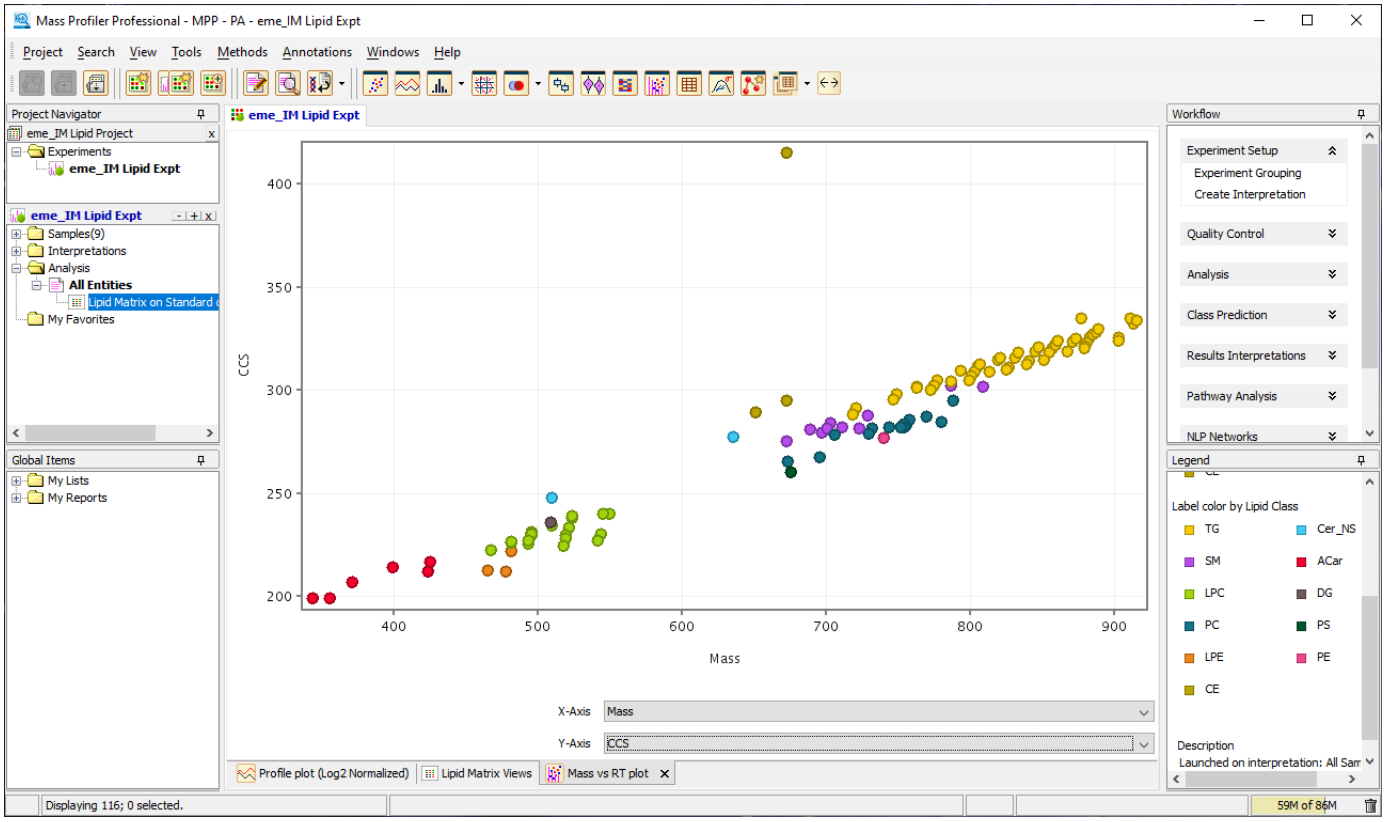


Figure 83. CCS vs Mass plot in MPP main window

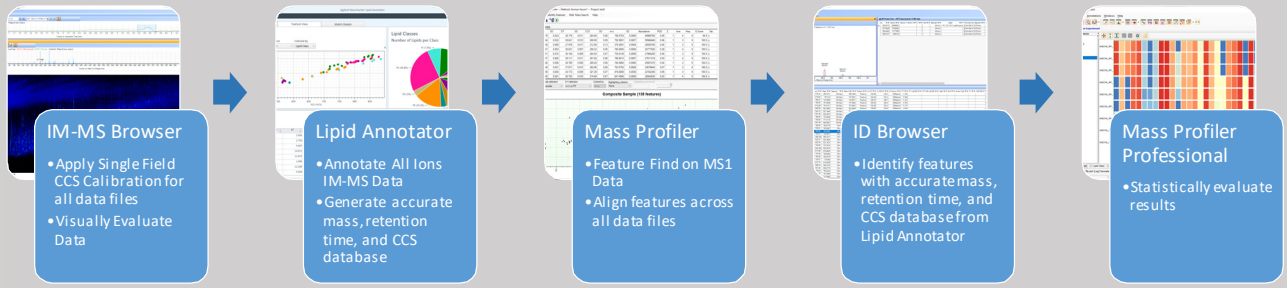
NOTE

Each lipid class is color-coordinated according to the legend on the right. Note how the lipids are grouped together according to class and structure (CCS values).

This page intentionally left blank.

5 Reference Information

This chapter consists of definitions of ion mobility, lipidomics and software terms, as well as references to Agilent publications that can help you use Agilent products to perform lipidomics analyses.



Definitions

Review of the terms and definitions presented in this section helps you understand the Agilent software wizards and the lipidomics workflow. Because this guide does not show you MPP statistical tools, refer to the *Agilent Metabolomics Workflow Guides* mentioned in **"Resource Apps"** on page 94 for these definitions.

Abundance

When mentioned in this guide, it usually refers to the log normalized abundance, or concentration, of the identified lipids in the sample set.

Alignment

Adjustment of the chromatographic retention time of eluting components to improve the data correlation among data sets, based on the elution of specific component(s) that are (1) naturally present in each sample or (2) deliberately added to the sample through spiking the sample with a known compound or set of compounds that do not interfere with the sample.

All Ions data

Ion mobility data files containing both MS1 and fragmentation data, an option specified for acquisition. All Ions data is used in Lipid Annotator because the fragmentation data is necessary for lipid annotation. See **"MS1 data"** on page 91.

Beta and TFix values

Constants similar to the slope and intercept of a straight line, determined with tune-mix data and used to determine CCS values for either All Ions data or MS1 data.

Biomarker

An organic compound that is an indication of a biological state and which by analytical measurement of its presence and concentration in a biological sample indicates a normal or altered function of higher level biological activity.

Calibration

Using a standard with known accuracy to determine the values of other compounds present in the sample.

CCS

Collision cross section (CCS) is the rotationally averaged surface area of the ion. The measured drift time of an ion from an ion mobility experiment is used along with other experimental parameters (temperature, pressure, drift tube length and voltage), information about the ion (mass and charge) and buffer gas (mass and number density) to calculate the CCS value. CCS values serve as additional descriptors in analytical workflows, adding not only more specificity, but also unique structural information about the ion that is not reflected in retention time or mass from traditional LC-MS experiments.

CEF file

A binary file format called a Compound Exchange Format (CEF) that is used to exchange data between Agilent software programs and between Agilent programs and third-party programs. In the lipidomics workflow, CEF files are used to share molecular features between Mass Profiler and Mass Profiler Professional.

Charge carriers

Specific ions, either positive or negative, such as $+NH_4$ or -Cl.

Charge state

Actual charge, either +N or -N, where N is the number of the charge.

Composite sample

In Mass Profiler, features from all the samples plotted together on the X-Y plot (i.e., m/z vs RT) are called the "composite sample".

Compound A lipid that may be individually referred to as a compound, feature, element, or entity during the various steps of the lipidomics data analysis.

Condition Another term for one of several values within a parameter for which exist correlating samples. Condition may also be referred to as a parameter value during the various steps of the lipidomics data analysis.

Database A saved set of information that includes all of the important qualities of a feature used to identify a feature as a particular compound. One of the lipidomics workflow steps is to create a custom lipid database for identifying unknown lipids.

Demultiplexed data As you set up to acquire ion mobility data, you can select either standard IM mode or multiplexed IM mode. To take multiplexed data through the lipidomics workflow you must first demultiplex the data. This process combines the signals from the multiple ion pulses, which increases the sensitivity of the measurement. Also see **"Multiplexed data"** on page 91 and **"Standard-mode data"** on page 93.

Dependent variable An element in a data set that can only be observed as a result of the influence from the variation of an independent variable. For example, in a typical discovery experiment the lipid profiles of a set of human serum samples make up the dependent variables of a study whose independent variable is the disease state.

Element A lipid that may be individually referred to as a compound, feature, element, or entity during the various steps of the lipidomics data analysis.

Entity A lipid that may be individually referred to as a compound, feature, element, or entity during the various steps of the lipidomics data analysis.

Experiment Data acquired in an attempt to understand causality, where tests or analyses are defined and performed on an organism to discover something that is not yet known, to demonstrate proof of something that is known, or to find out whether something is effective.

Experiment grouping Grouping samples into sets with the same qualities, conditions or treatments.

Externality A quality, attribute, or state that originates and/or is established independently from the specimen under evaluation.

Extraction The process of retrieving a deliberate subset of data from a larger data set whereby the subset of the data preserves the meaningful information as opposed to the redundant and less meaningful information. Also known as data extraction.

Feature Independent, distinct characteristics of phenomena and data under observation. Features are an important part of the identification of patterns within the data, whether processed by human intelligence, or artificial intelligence programs such as Agilent MassHunter Mass Profiler and Agilent Mass Profiler Professional. In lipidomics analysis a feature is a lipid and may be individually organized (extracted) by mass, abundance and CCS values before it is identified.

Feature extraction

The reduction of data size and complexity through the removal of redundant and non-specific data by using the important variables (features) associated with the data. Careful feature extraction yields a smaller data set that is more easily processed without any compromise in the information quality. This is part of the filtering and statistical analysis processes employed by Agilent Mass Profiler for the lipidomics workflow.

Frame

In ion mobility mass spectrometry, data acquisition is the ultra-high-speed signal digitization in the detector, which acquires arrival-time spectra, called transients, at a rate of one per 120 usec. To capture the speed of peaks being separated by the ion mobility drift tube, it is necessary to record each transient individually. A full drift spectrum of 60 msec is the result of 500 TOF transients, often referred to as the Frame transient. The acquisition system then sums Frame transients until the period specified by the Frame data rate for saving the file is reached. The mass and drift time data within this period is known as the Frame. See the *Agilent 6560 Ion Mobility LC/QTOF Fundamentals Guide* for a visual explanation of a Frame.

Frame extraction

The process of summing the signals within a selected region of the Frame as implemented in the IM-MS Browser.

Filtering

The process of establishing criteria by which entities are removed (filtered) from further analysis during the lipidomics workflow.

ID Browser

Agilent software that annotates the feature lists by assigning compound names based on database searching and spectral matching. It can be launched from either Mass Profiler or Mass Profiler Professional to assign compound names to features.

Identified compound

Chromatographic components that have an assigned identity, such as compound name and molecular formula, based on prior assessment or comparison with a database. See also **"Unidentified compound"** on page 93.

IM-MS Browser

Agilent software that displays and lets you analyze ion mobility data.

IM-MS Reprocessor

Agilent software designed to help you correct mass calibration in ion mobility data.

Independent variable

An essential element, constituent, attribute, or quality in a data set that is deliberately controlled in an experiment. For example, in a typical discovery experiment the lipid profiles of a set of human serum samples make up the dependent variables of a study whose independent variable is the disease state. An independent variable may be referred to as a parameter and is assigned a parameter value during the various steps of the lipidomics data analysis.

Interpretation

Groupings of samples based on the experimental parameters. By default in MPP, when you open an experiment, the "All Samples" interpretation is active. You can click another interpretation to activate it. To learn how to create an interpretation, see **"Step 2. Create interpretations"** on page 73.

Ion mobility

An analytical technique that separates ions based on their mobility through a drift gas. For drift tube IM, the velocity of the ion is the result of counteracting forces. The driving force is an electric field established along the length of the drift tube. The decelerating force arises from collisions with the drift gas within the tube, affected by the ion's shape and size, as well as by the number density of gas molecules in the drift tube. Ions that manifest a higher velocity have higher ion mobility.

Isotope model

The kind of isotopic envelope pattern a given data set may contain. The isotope model controls how the assignment algorithm uses peak abundances in addition to peak spacing when it tries to group peaks into isotope clusters (from which it can then determine the charge state). For example, an isotope model could be "Common organic compounds" or "Common organic (No Halogens)". See **"Method"** on page 91.

Lipid Annotator

Agilent software designed to annotate lipids based on matching fragmentation data with theoretical fragmentation spectra. Lipid Annotator provides a pie chart summary of lipid classes present in the sample and creates a custom lipid database, or PCDL.

Lipids

Lipids are a very broad, diversified group of hydrophobic or amphipathic (both hydrophobic and hydrophilic) small molecules that are critically involved in maintaining structural integrity of cellular membranes, serve as cellular energy stores and are also key intermediates of several signal transduction pathways. The amphipathic nature of some lipids lets them form structures such as vesicles, liposomes, or membranes in an aqueous environment. These lipids are **named** according to their "head group" component and their acyl tails.

Lipid class Categories of lipids organized by their component compounds. Hydrophobic lipids, such as Cholesterol, belong in their own class. Amphipathic lipids contain the same backbone or "head group" component, such as Carnitine, Glycerol or Sphingosine, and 1-3 long-chain acyl groups from fatty acids attached to an alcohol group or an amine on the "head group". Some examples of lipid classes for these three "head groups" are Acylcarnitine (ACar), Triacylglycerol (TG) Ceramide Non-hydroxy Fatty Acid Sphingosine (CerNS).

Lipid constituent A lipid constituent of a lipid class is a lipid whose name includes its class abbreviation and the individual number of acyl chains, carbons and double bonds. For example, the name PC 16:0_18:2, indicates this constituent is a phosphatidylcholine with two attached acyl tails, one of 16 carbons and no double bonds and one of 18 carbons and 2 double bonds. TG 16:0_16:1_18:1 is a triacyl glycerol with 3 acyl tails, one with 16 carbons and no double bonds, one with 16 carbons and one double bond and one with 18 carbons and one double bond.

Lipid sum composition A lipid or set of lipids can also be identified by their class and the total number of carbons and double bonds in the acyl chain or chains -- their sum composition. For example, PC 34:2 is a sum composition of phosphatidyl-choline with a total of 34 carbons and two double bonds in the attached acyl groups. Another example of a sum composition is TG 50:2, which has a total of 50 carbons and 2 double bonds in the attached acyl groups.

For a given sum composition one or multiple lipid constituents are possible based on evidence in the fragmentation spectra. These are listed in the bottom table on the Match Details view of Lipid Annotator. If only one lipid constituent is listed, evidence for only those specific fragment ions was found in the fragment spectra (e.g., for PC 34:2 if only PC 16:0_18:2 is listed, then only fragments for the acyl groups 16:0 and 18:2 were found). If multiple constituents are listed, then multiple fragment ions were detected in the fragment spectra for all the acyl groups (e.g., for TG 50:2 if TG 16:0_16:1_18:1, TG 15:0_17:1_18:1 and TG 16:0_16:0_18:2 are listed, then fragments were detected for the 15:0, 16:0, 16:1, 17:1, 18:1, and 18:2 acyl groups).

The percentages in the table represent the comparative abundance of each of the unique fragment ions, and the dominant constituent is the one present in the greatest abundance.

Lipidome The complete set of lipids found within a biological sample.

Lipidomics Identification and quantification of cellular lipids from an organism in a specified biological situation. The study of lipids is a subset of metabolomics.

Mass Profiler

Agilent software for extracting features from data files. This software serves as the batch processing software tool for ion mobility data. Once extracted, the features can be identified using the embedded ID Browser software tool. Mass Profiler also supports differential analysis for two sample groups and exports CEF files for more statistical analysis in MPP.

Mass Profiler Professional (MPP)

Agilent software with many statistical and display tools for analyzing identified and unidentified compounds. Its lipid matrix tool is especially useful for comparing abundances of compounds within and between lipid sample sets of differing qualities, conditions or treatments.

Mass calibration

In mass spectrometry, tuning compounds are used to adjust the mass scale and relative intensities of the mass spectral peaks.

Mass variation

The mass to charge (m/z) resolution of the mass spectral data enables the identification of compounds with nearly identical, or identical, chromatographic behavior by adjusting the m/z range for extracting ion chromatograms.

Mean

The numerical result of dividing the sum of the data values by the number of individual data observations.

Metabolism

The chemical reactions and physical processes whereby living organisms convert input compounds into living compounds, structures, energy and waste.

Metabolites

Small organic molecules that are intermediate compounds and products produced as part of metabolism. Metabolites are important modulators, substrates, by-products and building blocks of many different biological processes. They are typically in the range of 50 to 600 Da.

Metabolome The complete set of small-molecule metabolites that may be found within a biological sample.

Metabolomics The process of identification and quantification of all metabolites of an organism in a specified biological situation. The study of the metabolites of an organism presents a chemical "fingerprint" of the organism under the specific situation. See **"Metabonomics"** for the study of the change in the metabolites in response to externalities.

Metabonomics The metabolic response to externalities such as drugs, environmental factors, and disease. The study of metabonomics by the medical community may lead to more efficient drug discovery and to individualized patient treatment. Meaningful information learned from the metabolite response can be used for clinical diagnostics or for understanding the onset and progression of human diseases. See **"Metabolomics"** for the identification and quantitation of metabolites.

Method A procedure using specified, but modifiable, values of known parameters to process data.

MS1 data Data file containing only MS level data (i.e., no fragmentation data).

Multiplexed data IM data can be acquired in multiplexed mode, which is specified during data acquisition. In multiplexed mode multiple packets of ions are sent into the drift tube for each frame vs. a single packet during standard mode. Both 3-bit and 4-bit multiplexing are available options that result in either 4 or 8 packets of ions per frame. (See **"Frame"** on page 88.) Trap fill time must be reduced to 3-4 ms to fit the multiple packets within a typical maximum drift time frame of 60 ms. Also see **"Demultiplexed data"** on page 87 and **"Standard-mode data"** on page 93.

Multi-omics Biological analysis approach in which the data sets are multiple "omes", such as the genome, proteome, metabolome or lipidome. In other words, it is the use of multiple "omics" technologies to study life in a concerted way. By combining these "omes", researchers analyze complex biological big data to find novel associations between biological entities, pinpoint relevant biomarkers and build elaborate markers of disease and physiology. Ion mobility is an excellent tool for multi-omics studies because it provides an additional dimension of separation for these complex samples. Additionally, each "omics" class of compounds contains distinct structural characteristics that manifest in class separation on the plot of CCS values vs. mass.

Parameter Another term for an independent variable. Referred to as a parameter or parameter name and is assigned a parameter value during the various steps of the lipidomics data analysis.

Parameter value Another term for one of several values within a parameter for which exist correlating samples. Parameter value may also be referred to as a condition during the various steps of the lipidomics data analysis. See also **"Condition"** on page 87.

PCDL

PCDL stands for Personal Compound Database and Library. Agilent has designed a number of programs for creating and managing PCDL's, including Pathways to PCDL, PCDL Manager and Lipid Annotator. PCDL's are then used for database searching and spectral library matching with the Agilent ID Browser software.

PNNL Preprocessor

A software program designed by the Pacific Northwest National Laboratory (PNNL) in conjunction with Agilent for the purposes of preprocessing IM data prior to data analysis. Smoothing, saturation repair, and demultiplexing are a few of the techniques available in the PNNL Preprocessor.

Polarity

Positive or negative mode used for mass spectral data acquisition.

Quality

A feature, attribute, and/or characteristic element of a sample. The quality's analytical result shows the sample is representative of the larger specimen to a high degree of certainty.

Recalibration

If an application requires the best possible mass accuracy, a recalibration may need to be applied to the data file. To perform a recalibration, reference mass ions must be present throughout the entire analytical run. An IRM (internal reference mass) recalibration can then be carried out with the IM-MS Reprocessor utility, which stores the new mass calibration coefficients in the data file for use in other software applications.

Reduction

The process whereby the number of variables in a data set is decreased to improve computation time and information quality, e.g., an extracted ion chromatogram obtained from GC/MS and LC/MS data files. Reduction provides smaller, viewable and interpretable data sets by employing feature selection and feature extraction. Also known as dimension reduction and data reduction. This is part of the filtering and statistical analysis processes employed by Agilent Mass Profiler and by Agilent Mass Profiler Professional.

Replicate

Collecting multiple identical samples from a population to obtain a measured value that more closely approximates the true value.

Sample

A part, piece, or item that is taken from a specimen and understood as being representative of the larger specimen (e.g., blood sample, cell culture, body fluid, aliquot) or population. An analysis may be derived from samples taken at a particular geographical location, taken at a specific period of time during an experiment, and taken before or after a specific treatment. A small number of specimens used to represent a whole class or group.

Single-field CCS calibration

A method for calculating reliable CCS values. A data file is acquired for tune-mix ions that utilizes the exact same IM-MS settings used for the analytical run. This tune-mix data file is processed in IM-MS Browser to determine the Beta and Tfix single-field CCS calibration coefficients. These values are then applied to the analytical run so each feature's drift time, mass, and charge can be easily converted into a CCS value during feature finding.

Specimen An individual organism, e.g., a person, animal, plant, or other organism, of a class or group that is used as a representative of a whole class or group.

Spike The specific and quantitative addition of one or more standard compounds to a sample.

Standard A chemical or mixture of chemicals selected as a basis of comparing the quality of analytical results or of measuring and compensating for the precise offset or drift incurred over a set of analyses.

Standard deviation A measure of variability among a set of data that is equal to the square root of the arithmetic average of the squares of the deviations from the mean. A low standard deviation value indicates that the individual data tend to be very close to the mean, whereas a high standard deviation indicates that the data is spread out over a larger range of values from the mean.

Standard-mode data This term refers to IM data that is acquired without multiplexing. See **"Multiplexed data"** on page 91.

State A set of circumstances or attributes characterizing a biological organism at a given time. A few sample attributes may include temperature, time, pH, nutrition, geography, stress, disease, and controlled exposure.

TIC Total Ion Current

Tune mix A solution made up of compounds of known masses; used to optimize conditions for producing the best resolution and highest signal in a mass spectrometer. Used with IM-MS Reprocessor to correct the mass calibration of raw IM data. Used for single-field CCS calibration to calculate the Beta and TFix constants.

Unidentified compound Chromatographic components that are only uniquely denoted by their mass, abundance and retention times, or in the case of ion mobility data, their CCS values, and that have not been assigned an identity, such as compound name and molecular formula. Unidentified compounds are typically produced by feature finding and deconvolution algorithms. See also **"ID Browser"** on page 88.

Variable An element in a data set that assumes changing values, i.e., values that are not constant over the entire data set. The two types of variables are independent and dependent.

Wizard A sequence of dialog boxes presented by Agilent software programs that guide you through well-defined steps to enter information, organize data, and perform analyses.

Resource Apps

MassHunter software includes a Resource App from where you can access relevant resource material. Some of these guides can also be found on **www.agilent.com**.

These guides, tutorials and videos help you use Agilent products for lipidomics analyses. For citations to Agilent application notes, presentations, product brochures, technical overviews, and software written especially for metabolomics analyses but also useful for lipidomics analyses, see the *Agilent Metabolomics Discovery Workflow Guide*.

MassHunter Mass Profiler Professional Resources

- Agilent G3835AA MassHunter Mass Profiler Professional Software Quick Start Guide
- Agilent G3835AA MassHunter Mass Profiler Professional Software Familiarization Guide
- Agilent G3835AA MassHunter Mass Profiler Professional Software Application Guide
- Agilent Mass Profiler Professional - User Manual
- Agilent Getting Started with MPP
- Agilent MPP Statistics and Machine Learning
- Agilent MPP Training Videos
- Agilent Metabolomics Discovery Workflow Overview
- Agilent Metabolomics Discovery Workflow Guide

TOF and Q-TOF LC/MS Resource App

- Agilent Lipid Annotator eFam

Agilent Web Site

This guide is available from **www.agilent.com.**

- Metabolomics: Approaches Using Mass Spectrometry (Agilent publication 5990-4314EN, October 27, 2009)

Agilent Community

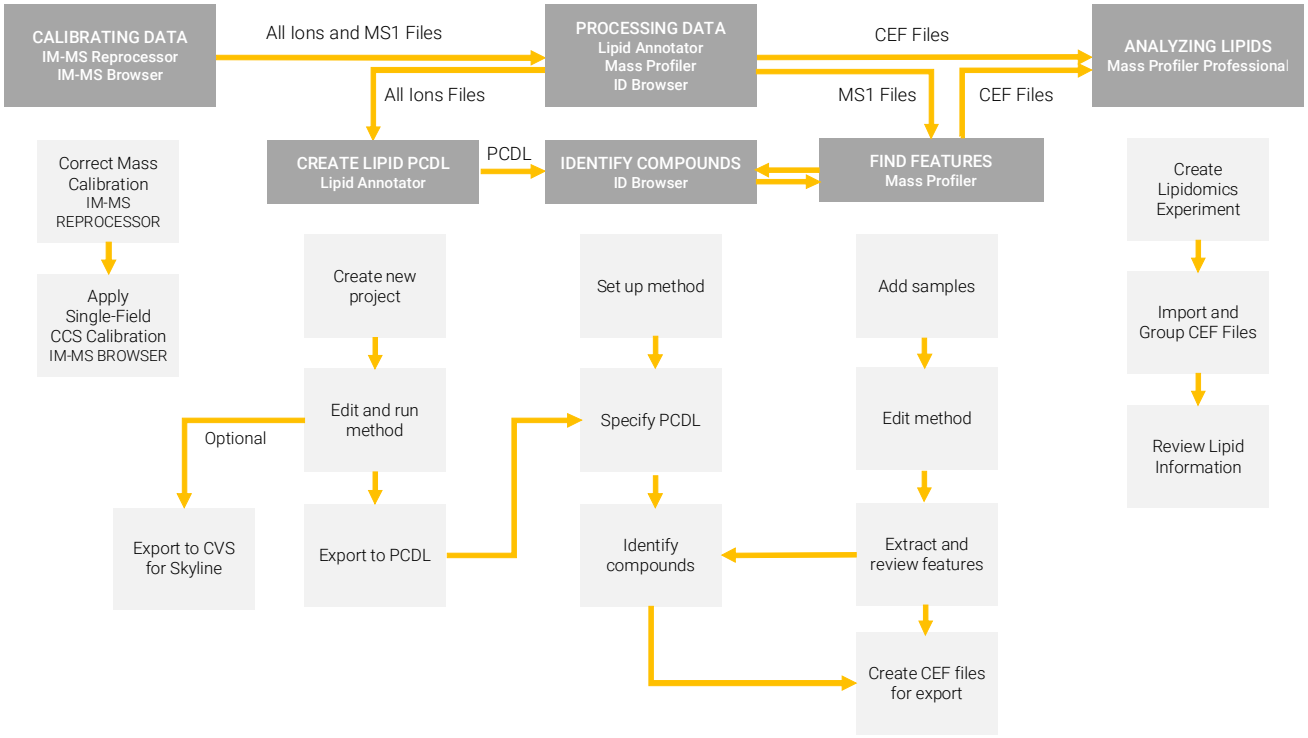
To get answers to your questions, join over 10,000 users in the Agilent Community. Review curated support materials organized by platform technology. Ask questions to industry colleagues and collaborators. Get notifications on new videos, documents, tools, and webinars relevant to your work.

https://community.agilent.com

This page intentionally left blank.

In This Guide

This guide describes the Lipidomics workflow using the Agilent 6560 Ion Mobility Mass Spectrometer and MassHunter software.



Learn more:
www.agilent.com

Buy online:
www.agilent.com/chem/store

Get answers to your technical questions and access resources in the Agilent Community: **community.agilent.com**

U.S. and Canada
1-800-227-9770
agilent_inquiries@agilent.com

Europe
info_agilent@agilent.com

Asia Pacific
inquiry_lsca@agilent.com

July 2021, Revision A.00
D0003136

Agilent