

# Skyline High Resolution Metabolomics

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The Skyline Targeted Mass Spectrometry Environment provides informative visual displays of the raw mass spectrometer data you import into your Skyline documents. Originally developed for proteomics use, Skyline has been extended to work with generalized molecules. This tutorial explores using Skyline for targeted quantification of small molecules using high resolution mass spectra.

In this tutorial, you will learn about Quantification of Polyunsaturated Fatty Acids in Plasma using NIST SRM-1950 as a single-point external calibrant, used along with stable-isotope labeled internal standards.

You will explore:

- Use of a molecular formula and adduct to assign a precursor accurate mass.
- Analysis of high resolution accurate mass precursor quantification data in Skyline.
- Quantification using single-point external calibration with SIL internal standards and surrogate standards.
- Use of a Concentration Multiplier to adjust the calibration range for each analyte.

Skyline aims to provide a vendor-neutral platform for targeted quantitative mass spectrometry research. It can import raw data from the instrument vendors Agilent, SCIEX, Bruker, Shimadzu, Thermo-Scientific, and Waters. The ability to import data across various instrument platforms facilitates cross-instrument comparisons and multi-site studies. This remains equally true in using it to target small molecules, as it has been for years in the field of proteomics.

If you have not already looked at the “[Skyline Small Molecule Targets](#)” tutorial you should do so now, in order to pick up a few basics about how Skyline works with small molecule descriptions including chemical formulas and adducts.

## Getting Started

To start this tutorial, download the following ZIP file:

<https://skyline.ms/tutorials/HiResMetabolomics.zip>

Extract the files in it to a folder on your computer, like:

C:\Users\bspratt\Documents

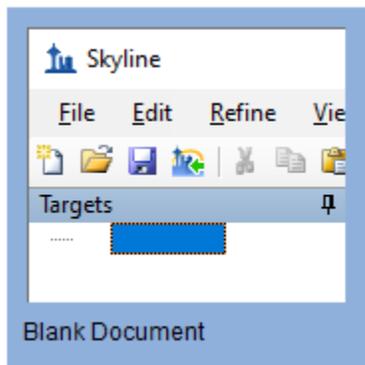
This will create a new folder:

C:\Users\bspratt\Documents\HiResMetabolomics

It will contain all the files necessary for this tutorial.

If you have been using Skyline prior to starting this tutorial, it is a good idea to revert Skyline to its default settings. To do so:

- Start Skyline.
- On the **Start Page**, click **Blank Document** which looks like this:

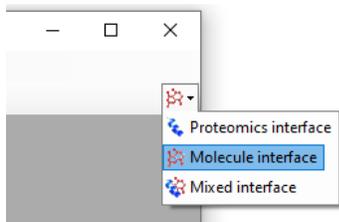


- On the **Settings** menu, click **Default**.
- Click **No** on the form asking if you want to save the current settings.

The document settings in this instance of Skyline have now been reset to the default.

Since this tutorial covers a small molecule topic, you can choose the molecule interface by doing the following:

- Click the user interface control in the upper right-hand corner of the Skyline window, and click **Molecule interface** which looks like this:



Skyline is operating in molecule mode which is displayed by the molecule icon  in the upper right-hand corner of the Skyline window. Its original proteomics menus and controls are now hidden, allowing you to focus on small molecule analysis.

## Importing a Molecule Transition List into a Skyline Document

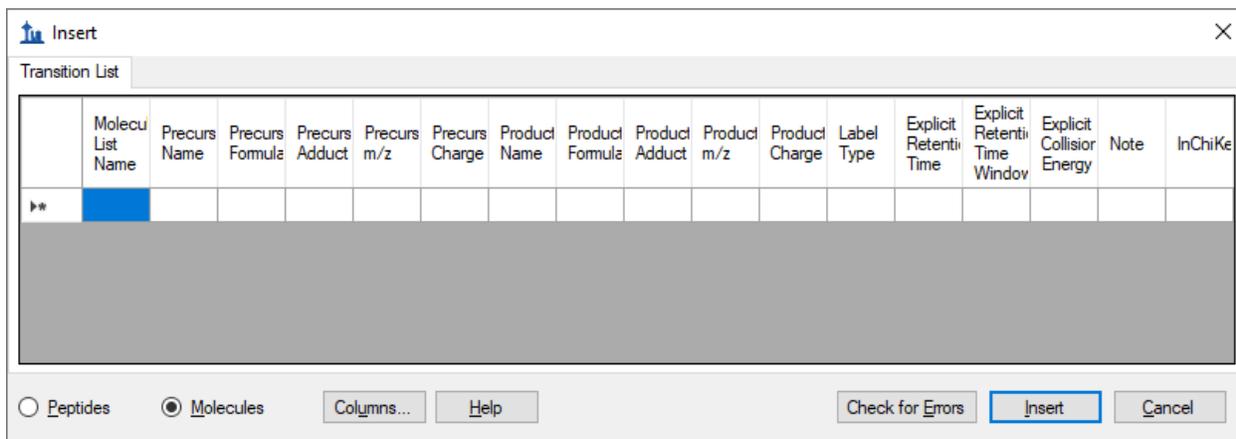
To add a set of target transitions for the small molecules in this experiment, do the following:

- If you are an Excel user, open the provided PUFA\_TransitionList.xlsx file. Otherwise, you can open the CSV version PUFA\_TransitionList.csv in any text editor.

Review the column headers in the first row of this file, and then switch back to Skyline and do the following:

- On the Skyline **Edit** menu, choose **Insert** and click **Transition List**.

Skyline will show the **Insert** form, looking something like this (you may have a different column selection and order from previous uses of Skyline):

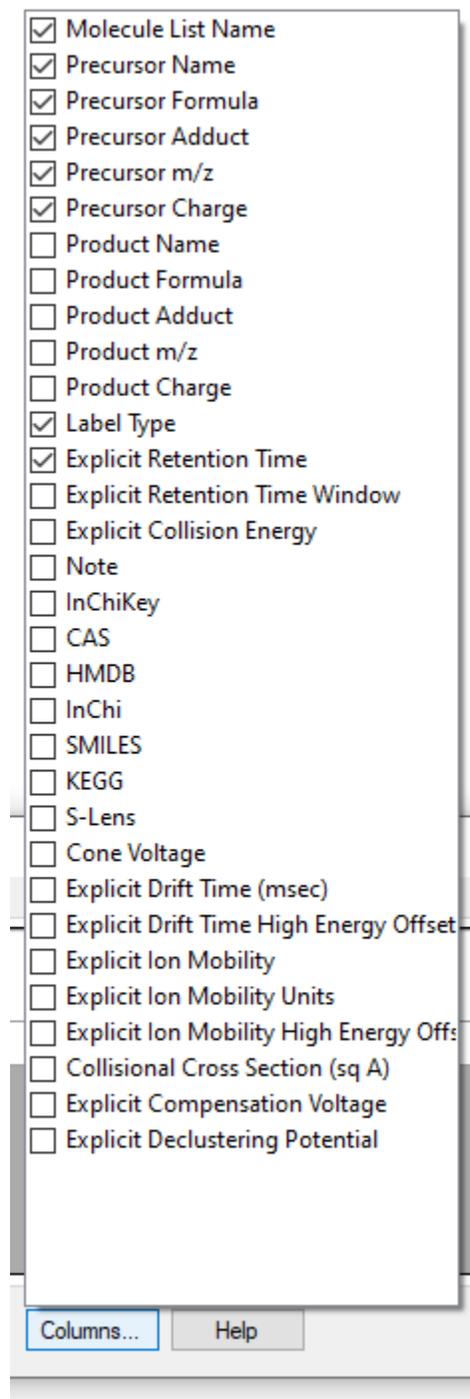


The screenshot shows the 'Insert' dialog box in Skyline. The title bar reads 'Insert' with a close button. Below the title bar is a tab labeled 'Transition List'. The main area contains a table with the following headers: Molecule List Name, Precurs Name, Precurs Formula, Precurs Adduct, Precurs m/z, Precurs Charge, Product Name, Product Formula, Product Adduct, Product m/z, Product Charge, Label Type, Explicit Retention Time, Explicit Retention Time Window, Explicit Collision Energy, Note, and InChIKey. The first row of the table is highlighted in blue. Below the table, there are radio buttons for 'Peptides' and 'Molecules' (which is selected), a 'Columns...' button, and a 'Help' button. At the bottom right, there are buttons for 'Check for Errors', 'Insert', and 'Cancel'.

You can see that there are some extra column headers in the **Insert** form, and the column order is not the same in the form as in the spreadsheet. Both issues are easy to correct:

- Click the **Columns** button.
- Uncheck the columns that do not appear in the spreadsheet.

This should result in a column picking menu like the one shown below:



A screenshot of a column picking menu. The menu is a vertical list of items, each with a checkbox. The items are: Molecule List Name (checked), Precursor Name (checked), Precursor Formula (checked), Precursor Adduct (checked), Precursor m/z (checked), Precursor Charge (checked), Product Name (unchecked), Product Formula (unchecked), Product Adduct (unchecked), Product m/z (unchecked), Product Charge (unchecked), Label Type (checked), Explicit Retention Time (checked), Explicit Retention Time Window (unchecked), Explicit Collision Energy (unchecked), Note (unchecked), InChiKey (unchecked), CAS (unchecked), HMDB (unchecked), InChi (unchecked), SMILES (unchecked), KEGG (unchecked), S-Lens (unchecked), Cone Voltage (unchecked), Explicit Drift Time (msec) (unchecked), Explicit Drift Time High Energy Offset (unchecked), Explicit Ion Mobility (unchecked), Explicit Ion Mobility Units (unchecked), Explicit Ion Mobility High Energy Offset (unchecked), Collisional Cross Section (sq A) (unchecked), Explicit Compensation Voltage (unchecked), and Explicit Declustering Potential (unchecked). At the bottom of the menu are two buttons: 'Columns...' and 'Help'.

Next do the following to reorder the columns in the **Insert** form:

- Click and drag each column header you want to move to the order matching the spreadsheet.

Once you have selected and arranged your columns, the **Insert** form should look like this:

The 'Insert' dialog box shows a 'Transition List' table with the following columns: Molecule List Name, Precursor Name, Precursor Formula, Precursor Adduct, Label Type, Precursor m/z, Precursor Charge, and Explicit Retention Time. The first cell of the first row is highlighted in blue. Below the table, the 'Molecules' radio button is selected, and the 'Columns...' button is highlighted. Other buttons include 'Peptides', 'Help', 'Check for Errors', 'Insert', and 'Cancel'.

To add the transitions specified in the spreadsheet, do the following:

- Select the contents of the spreadsheet, excluding the first row containing the headers.
- Click the **Copy** button on the toolbar (or press Ctrl-C on your keyboard).
- Switch back to Skyline.
- With the first cell in the form highlighted blue, press Ctrl-V on your keyboard to paste.
- Click the **Check for Errors** button.

Note: If you accidentally copied the header row or got the column order wrong, then you will see an error at this point.

There is an intentional error in the transition list: charge is given as 1, but the adduct is [M-H] resulting in the error message shown below:

The 'Insert transition list' dialog box displays an error message: "Adduct [M-H] charge -1 does not agree with declared charge 1". Below the error message, the 'Transition List' table contains the following data:

	Molecule List Name	Precursor Name	Precursor Formula	Precursor Adduct	Label Type	Precursor m/z	Precursor Charge	Explicit Retention Time
▶	Fatty Acid	FA 18:2 omega-...	C18H32O2	[M-H]			1	1.3
	Fatty Acid	FA 18:2 omega-...	C18H28H'4O2	[M-H]			1	1.3
	Fatty Acid	FA 18:3 omega-...	C18H30O2	[M-H]			1	1.1
	Fatty Acid	FA 20:4 omega-...	C20H32O2	[M-H]			1	1.2
	Fatty Acid	FA 20:4 omega-...	C20H24H'8O2	[M-H]			1	1.2
	Fatty Acid	FA 22:6 omega-...	C22H32O2	[M-H]			1	1.1
	Fatty Acid	FA 22:6 omega-...	C22H27H'5O2	[M-H]			1	1.1
*								

Below the table, the 'Molecules' radio button is selected, and the 'Columns...' button is highlighted. Other buttons include 'Peptides', 'Help', 'Check for Errors', 'Insert', and 'Cancel'.

- Change the charge value to -1 in each row.
- Click the **Check for Errors** button again.

The **Insert** form should look like this:

Insert transition list

No errors

Transition List

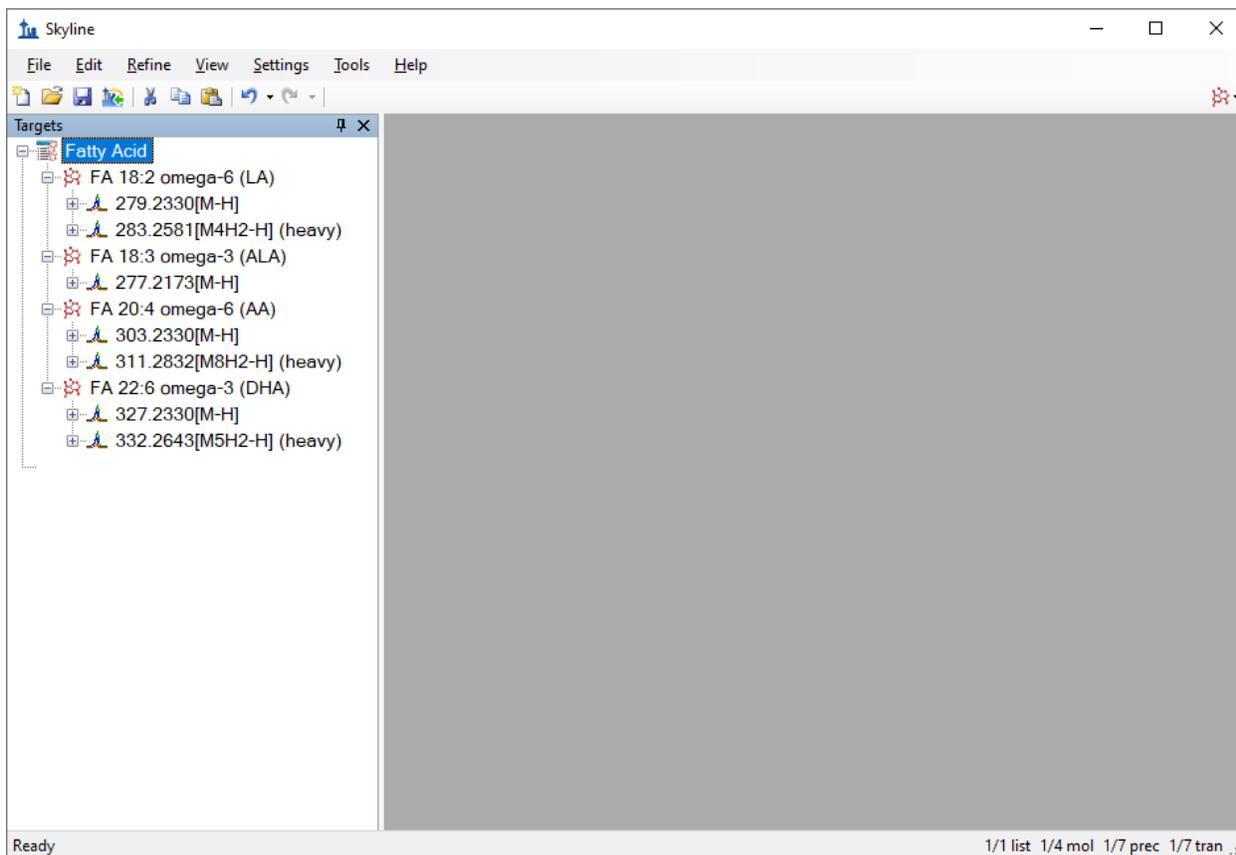
	Molecule List Name	Precursor Name	Precursor Formula	Precursor Adduct	Label Type	Precursor m/z	Precursor Charge	Explicit Retention Time
▶	Fatty Acid	FA 18:2 omega-...	C18H32O2	[M-H]		279.23295438	-1	1.3
	Fatty Acid	FA 18:2 omega-...	C18H28H'4O2	[M-H]	heavy	283.258061356	-1	1.3
	Fatty Acid	FA 18:3 omega-...	C18H30O2	[M-H]		277.21730431	-1	1.1
	Fatty Acid	FA 20:4 omega-...	C20H32O2	[M-H]		303.23295438	-1	1.2
	Fatty Acid	FA 20:4 omega-...	C20H24H'8O2	[M-H]	heavy	311.283168332	-1	1.2
	Fatty Acid	FA 22:6 omega-...	C22H32O2	[M-H]		327.23295438	-1	1.1
	Fatty Acid	FA 22:6 omega-...	C22H27H'5O2	[M-H]	heavy	332.2643381	-1	1.1
*								

Peptides   
 Molecules   
   
   
   
   

Notice that Skyline has automatically filled in the **Label Type** column, having determined that the first two entries are a heavy/light labeled pair based on having the same name and formulas that differ only in isotopic labeling (four of the hydrogens are replaced by Deuterium in the second formula).

- Click on the **Insert** button.
- On the Skyline **Edit** menu, choose **Expand All** and click **Molecules**.

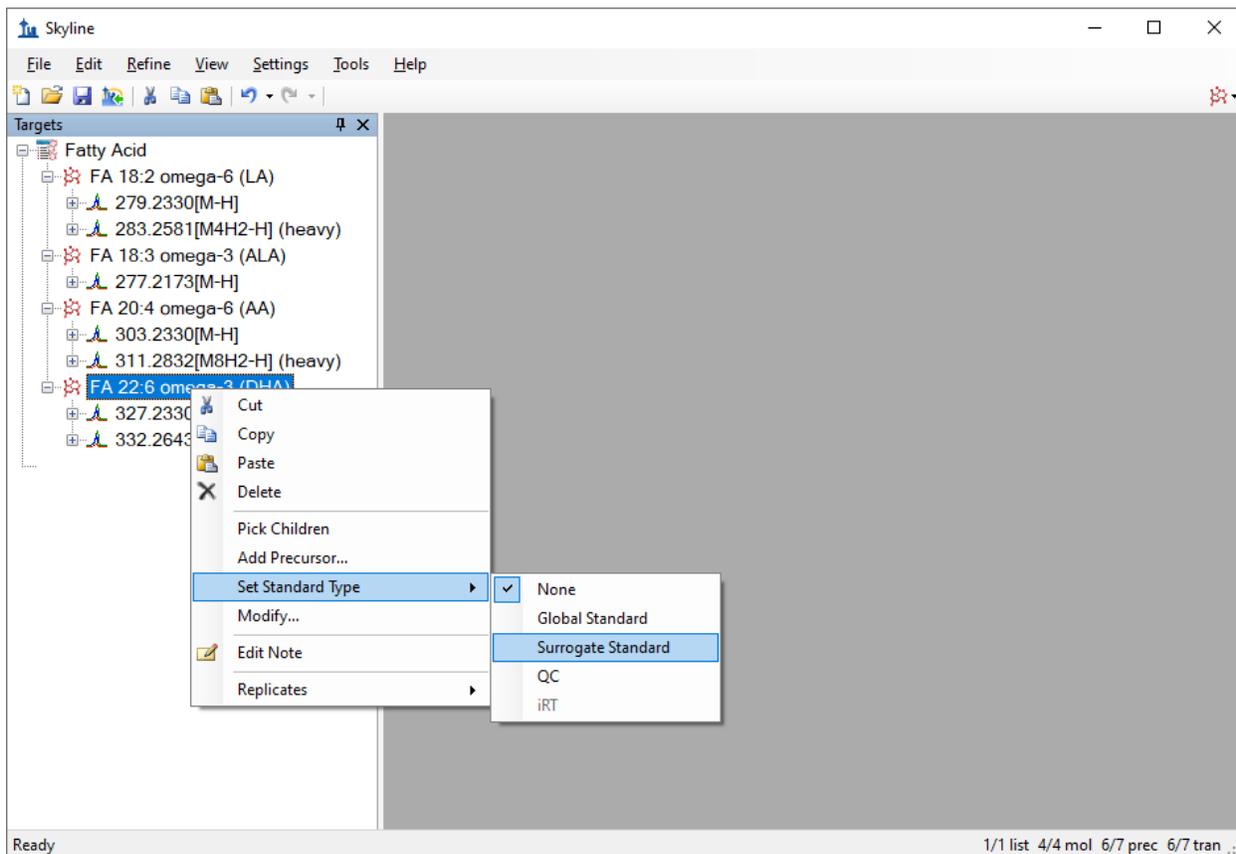
Your Skyline window should now look like this, displaying a tree of polyunsaturated fatty acids, along with their stable-isotope internal standards (as applicable):



Note that for the molecules with a heavy labeled precursor, the label is expressed as part of the adduct description for the heavy variant. The “[M4H2-H]” adduct tells you that four hydrogen atoms in the molecule are replaced by deuterium or H2 (“M4H2”) and that it is ionized by a proton loss (“-H”).

You will notice that ALA (alpha-linoleic acid) does not have a heavy-labeled precursor. Instead, it will be assigned a different stable-isotope labeled molecule as its surrogate standard. In this case d5-DHA will be used because it is the closest in retention time. To prepare for this association, do the following:

- Right-click the “DHA” target, choose **Set Standard Type**, and click **Surrogate Standard**.



## Transition Settings

Next, you will review the transition settings before importing the experimental mass spectrometer results. To do this, perform the following steps:

- On the **Settings** menu, click **Transition Settings**.
- Click the **Filter** tab.
- In the **Ion types** field, enter “f, p”.

The **Transitions Settings** form should look like this:

The screenshot shows the "Transition Settings" dialog box with the "Filter" tab selected. The "Molecules" section contains three input fields: "Precursor adducts" with "[M+H]", "Fragment adducts" with "[M+]", and "Ion types" with "f,p". Below this is a "Precursor m/z exclusion window" field with "m/z" to its right. At the bottom of the "Molecules" section is a checked checkbox labeled "Auto-select all matching transitions". The dialog has "OK" and "Cancel" buttons at the bottom right.

NOTE: The "f,p" setting in **Ion Types** on the **Filter** tab means you are interested in both fragment and precursor ion transitions. This Skyline document contains only precursor transitions, but the "f" is harmless.

- Click to the **Full-Scan** tab in the **Transitions Settings** form.
- Set the **Isotope peaks included** field to "Count".
- Set the **Precursor mass analyzer** field to "Orbitrap".
- In the **Peaks** field, enter "2".
- Set the **Resolving power** field to "70,000", At "200" m/z.
- In the section **Retention time filtering** section, choose **Include all matching scans**.

The **Transitions Settings** form should look like this:

The image shows a software dialog box titled "Transition Settings" with a close button (X) in the top right corner. The dialog has four tabs: "Prediction", "Filter", "Library", and "Instrument", with "Full-Scan" currently selected. The "MS1 filtering" section contains: "Isotope peaks included:" set to "Count", "Precursor mass analyzer:" set to "Orbitrap", "Peaks:" set to "2", "Resolving power:" set to "70000", "At:" set to "200 m/z", and "Isotope labeling enrichment:" set to "Default". The "MS/MS filtering" section contains: "Acquisition method:" set to "None", "Product mass analyzer:" (empty), "Isolation scheme:" (empty), and "Resolution:" (empty) m/z. Below this is a checkbox for "Use high-selectivity extraction" which is unchecked. The "Retention time filtering" section has three radio button options: "Use only scans within 5 minutes of MS/MS IDs", "Use only scans within 5 minutes of predicted RT", and "Include all matching scans" (which is selected). At the bottom are "OK" and "Cancel" buttons.

Now the experimental mass spectrometer results can be imported.

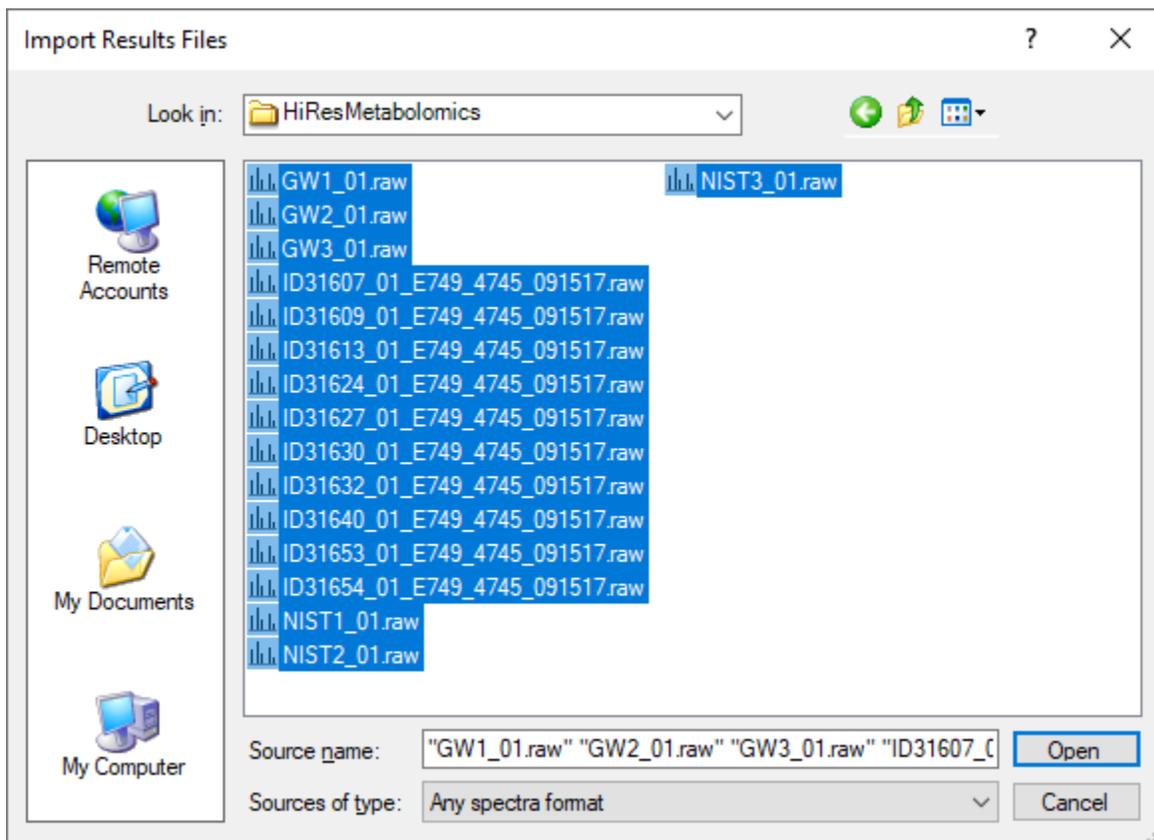
## Importing Mass Spectrometer Runs

Perform the following steps:

- On the **File** menu, click **Save**. (Ctrl-S)
- Save this document as "SM\_HiRes\_v1.sky" in the tutorial folder you created.
- On the **File** menu, choose **Import** and click **Results**.
- In the **Import Results** form, choose **Import single-injection replicates in files**.

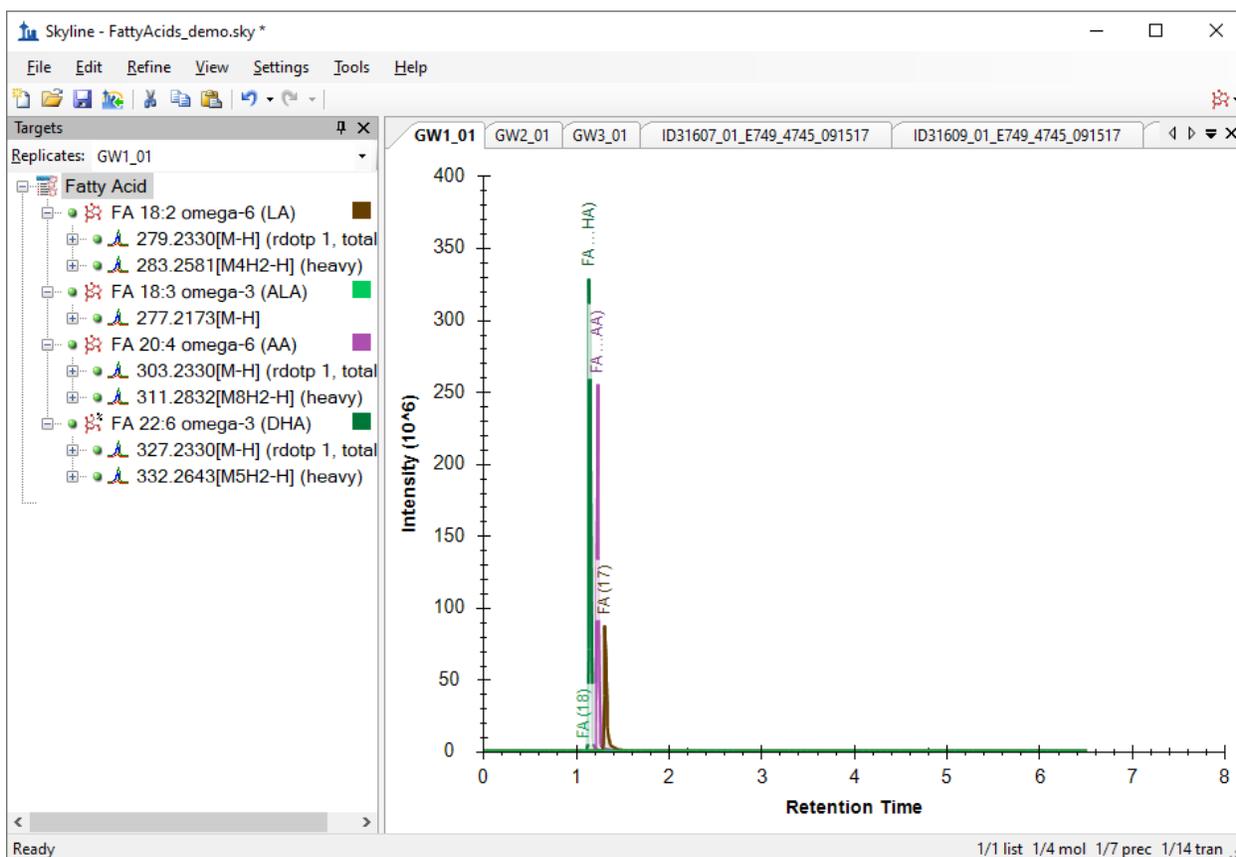
- In the **Files to import simultaneously** dropdown list at the bottom of the form, click **Many** which will provide the best import performance.
- Click the **OK** button.
- Select all 16 raw data folders in the tutorial folder by clicking the first listed and then holding down the Shift key and clicking the last.

The **Import Results Files** form should look like this:



- Click the **Open** button.

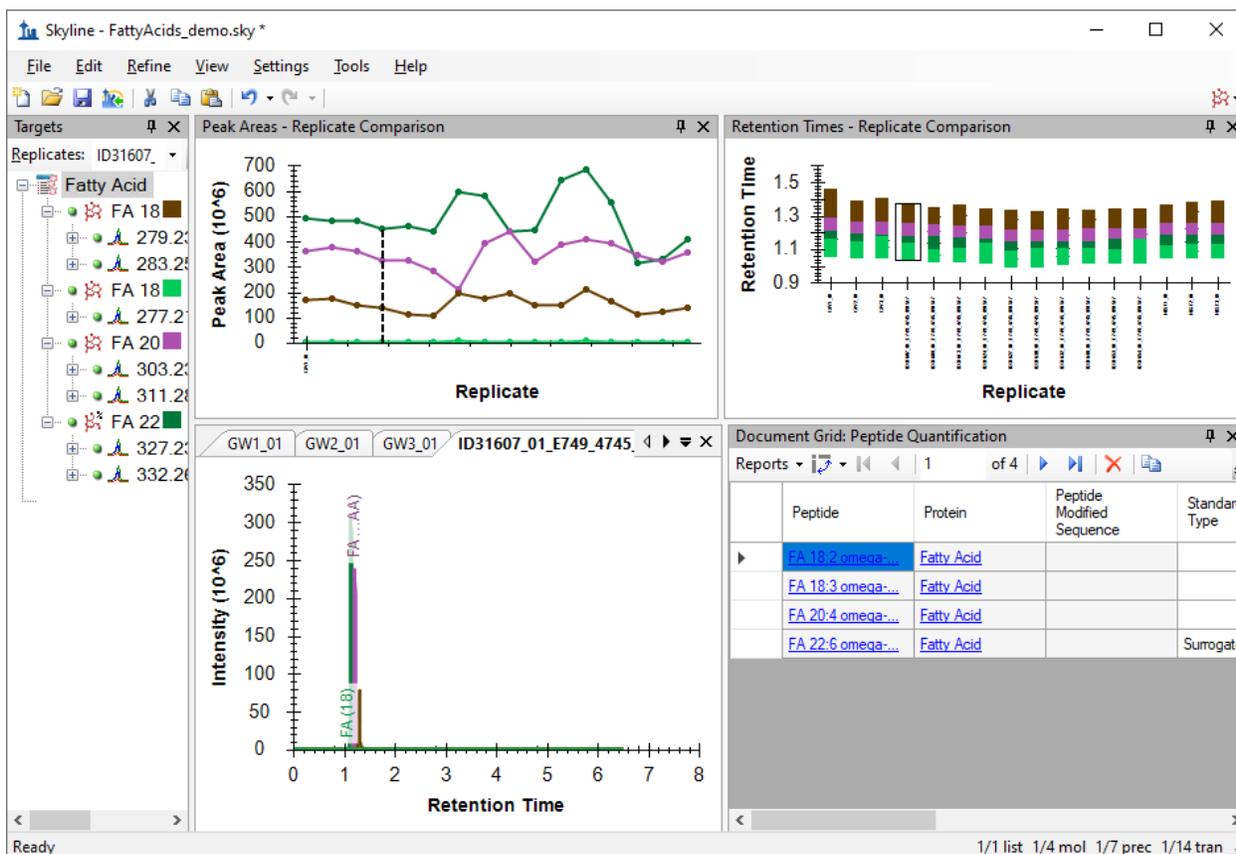
The files should import within 30 seconds or so, leaving your Skyline window looking like this:



To take advantage of the Skyline summary graphs for viewing individual targets, do the following:

- On the **View** menu, choose **Peak Areas** and click **Replicate Comparison**.
- On the **View** menu, choose **Retention Times** and click **Replicate Comparison**.
- Click and drag these views to dock them above the chromatogram graphs.
- On the **View** menu, choose **Document Grid**.
- In the **Document Grid** view, click the **Reports** menu, and then click **Peptide Quantification**.
- Click and drag the **Document Grid** view and dock it next to the chromatogram graphs.

The Skyline window should now look like this:



## Checking Peak Integration

Looking at the **Retention Times – Replicate Comparison** window, you can see by the lack of outliers that Skyline did not have any problems with peak integration.

## Preparing for ‘Single Point Quantification’

Next, follow these steps to prepare Skyline to produce calibrated quantitative values for the targeted molecules based on an external single-point calibration run:

- On the **Settings** Menu, click **Molecule Settings**.
- Click the **Quantification** tab.
- In the **Regression fit** field, select “Linear though zero”.
- In the **Normalization method**, select “Ratio to Heavy”.
- Leave the **Regression weighting** field set to “None”.
- Leave the **MS level** field set to “All”.
- In the **Units** field enter “uM”.

The **Molecule Settings** form should look like this:

The screenshot shows a dialog box titled "Molecule Settings" with a close button (X) in the top right corner. The dialog has four tabs: "Prediction", "Library", "Labels", and "Quantification". The "Quantification" tab is selected and highlighted with a dashed border. Inside the dialog, there are several settings:

- Regression fit:** A dropdown menu set to "Linear through zero".
- Normalization method:** A dropdown menu set to "Ratio to Heavy".
- Regression weighting:** A dropdown menu set to "None".
- MS level:** A dropdown menu set to "All".
- Units:** A text input field containing "uM".
- Figures of merit:** A sub-dialog containing:
  - Max LOQ bias:** An empty text input field followed by a percent sign (%).
  - Max LOQ CV:** An empty text input field followed by a percent sign (%).
  - Calculate LOD by:** A dropdown menu set to "None".

At the bottom of the dialog, there are two buttons: "OK" and "Cancel". The "OK" button is highlighted with a blue border.

- Click the **OK** button.

Return to the **Document Grid** view and do the following:

- Click the **Reports** menu, and then click **Replicates**.

To establish the role of each of the samples (replicates) in the study, as a standard, an unknown, or a quality control run, edit the Sample Type and Analyte Concentration cells for each row as necessary so the grid looks like this:

Replicate	Sample Type	Analyte Concentration
<a href="#">GW1_01</a>	Quality Control	
<a href="#">GW2_01</a>	Quality Control	
<a href="#">GW3_01</a>	Quality Control	
<a href="#">ID31607_01 E7...</a>	Unknown	
<a href="#">ID31609_01 E7...</a>	Unknown	
<a href="#">ID31613_01 E7...</a>	Unknown	
<a href="#">ID31624_01 E7...</a>	Unknown	
<a href="#">ID31627_01 E7...</a>	Unknown	
<a href="#">ID31630_01 E7...</a>	Unknown	
<a href="#">ID31632_01 E7...</a>	Unknown	
<a href="#">ID31640_01 E7...</a>	Unknown	
<a href="#">ID31653_01 E7...</a>	Unknown	
<a href="#">ID31654_01 E7...</a>	Unknown	
<a href="#">NIST1_01</a>	Standard	1
<a href="#">NIST2_01</a>	Standard	1
<a href="#">NIST3_01</a>	Standard	1

In this situation, the concentration given for the NIST sample (NIST-SRM-1950) is entered as “1”, since all analytes have a different concentration. Think of this as establishing your standard as “One NIST Unit”. Therefore, in the next step, the concentration multipliers will each be given as the actual concentration of the analyte in the NIST SRM-1950 in order to adjust the target concentration of those analytes to their respective reference values.

- In the **Document Grid**, click on the **Reports** menu and then click **Peptide Quantification**.
- Set the Concentration Multiplier and Normalization Method cells for each target as shown below.

Document Grid: Peptide Quantification

1 of 4

Export... Actions Find: A

Peptide	Protein	Peptide Modified Sequence	Standard Type	Internal Standard Concentration	Concentration Multiplier	Normalization Method	Calibration Curve
FA 18:2 omega-6	Fatty Acid				2838		<a href="#">Slope: 7.5236E-5</a>
FA 18:3 omega-3	Fatty Acid				54	Ratio to surrogat...	<a href="#">Slope: 6.0312E-5</a>
FA 20:4 omega-6	Fatty Acid				984		<a href="#">Slope: 1.1368E-4</a>
FA 22:6 omega-3	Fatty Acid		Surrogate Stand...		118		<a href="#">Slope: 3.9464E-4</a>

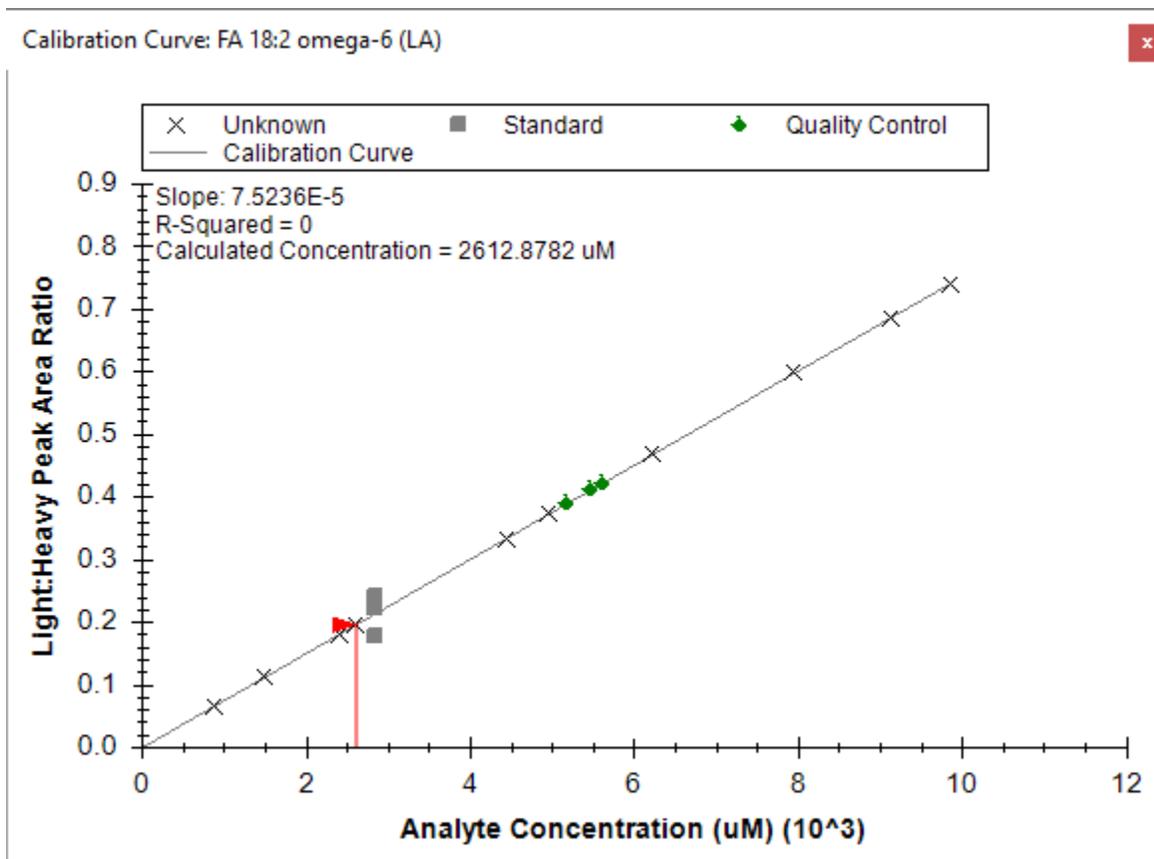
The default normalization method is “Ratio to Heavy” when using stable-isotope internal standards, but you need to set the “FA 18:3” analyte to use the previously-defined surrogate standard as the denominator in the analyte to standard ratio.

## Inspecting the Calibration Curves

Each entry in the Calibration Curve column is a clickable link that shows and activates the **Calibration Curve** view for the molecule in that row.

- Click on the calibration curve link for “FA 18:2”

The **Calibration Curve** view will appear looking like this:



Note that the calibration curve by default has an intercept of 0, as defined by the method of quantification (**Settings** menu, **Molecule Settings, Quantification** tab). Using a single concentration in the external calibration runs defines a “single point” at that concentration and the average Light:Heavy ratio of the measured runs. This point and the zero intercept, thus, define the linear equation used to calibrate all other runs. The **Calibration Curve** graph shows the Unknown and Quality Control points plotted on that line, because you did not enter known concentrations for them. In some experiments, quality control runs are used where the concentrations are known, but in this case the quality control runs are technical replicates of a sample pooled from the unknown samples.

The **Document Grid** or **Report Grid** can be used to export quantitative data (in micromolar) for each sample analyzed.

## Conclusion

In this tutorial, you have learned how to create a Skyline document that targets small molecules specified as precursor ion chemical formulas and adducts. You imported a multi-replicate data set collected on a Thermo Q Exactive Orbitrap mass spectrometer for a set of plasma samples, and saw how many existing Skyline features created initially for targeted proteomics use can now be applied to small molecule data. You applied external single-point calibration to derive micromolar values for each analyte in each run where the concentration was unknown.