Hands-on targeted lipidomics data:

Creation of a PRM transition list using LipidCreator and subsequent data analysis in Skyline

This short tutorial uses the PRM data acquired for **Cholesteryl esters (CE)** from a recently published study[1]. The authors used a complex mass spectrometry (MS)-based strategy combining untargeted and targeted workflows to generate a reference lipidome for human white adipose tissue (WAT). The sample set included subcutaneous (SAT) and visceral (VAT) WAT of lean and obese human. The mass spectrometry data were uploaded to MassIVE (MSV000086729) and are freely accessible for download https://massive.ucsd.edu/ProteoSAFe/dataset_files.jsp?task=96087cf567c44906ae4cd83e41 106d98#%7B%22table_sort_history%22%3A%22main.collection_asc%22%7D). In this tutorial, the provided PRM data was acquired on a Q Exactive Plus Hybrid Quadrupole Orbitrap mass spectrometer[1]. For further details the reader is referred to the publication the data origins from.

While acquisition methods such as single or multiple reaction monitoring (SRM/MRM) will record defined transitions (precursor to product ions), in PRM the pre-defined precursor ion is fragmented and the total MS^2 spectrum recorded. For all these methods, it is necessary to provide a list of precursors or transitions for which data should be acquired to the instrument. For PRM, such list usually contains the precursor (formula or m/z) and corresponding retention times (if performed in a scheduled manner).

In this tutorial, reported CE identifications from AdipoAtlas[1] are used to illustrate a targeted workflow for PRM data to perform a relative quantification of CEs aiming to answer the question how CE-abundances change between lean and obese patients. Therefore, this tutorial will start with the generation of a transition list with the help of **LipidCreator**[2] (Version 1.2.1 within **Skyline**). Afterwards, the available PRM files will be used to relatively quantify CEs using Skyline.

Within this tutorial you will explore:

- LipidCreator as a tool to rapidly generate PRM transition lists including isotopically labeled standards.
- Analysis of lipidomics PRM data in Skyline.
- Relative quantification by using a global standard.

[1] Lange, M. *et al.* AdipoAtlas: A reference lipidome for human white adipose tissue. *Cell Reports Medicine* **2**, 100407 (2021).
[2] Peng, B., Kopczynski, D., Pratt, B.S. et al. LipidCreator workbench to probe the lipidomic landscape. Nat Commun 11, 2057 (2020).

Experimental Design
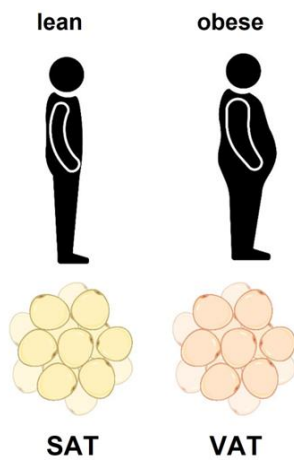
Lipids were extracted from approximately 50 mg of SAT and VAT WAT of lean and obese humans[1]. Organic phases were collected and dried under vacuum. Lipid extracts, rich in Triglycerides (TGs), were reconstituted in isopropanol and injected onto a reversed phase column (C_{30}) and detected on a Q Exactive Plus mass spectrometer. Prior to extraction, all samples were spiked with **SPLASH® LIPIDOMIX® Mass Spec Standard** (Avanti PolarLipids), containing deuterated lipid species. Of interest for this tutorial is the deuterated cholesteryl ester **CE 18:2 (D7)**, that will be used as global standard to relatively quantify endogenous CE levels.
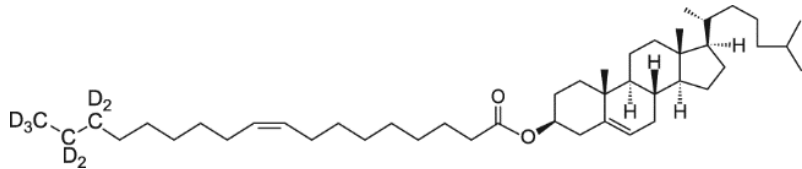
Identification of lipid species was achieved by a combination of software-assisted annotations together with manual curation of acquired MS^2 spectra from DDA runs of pooled samples (Top 10). As in WAT, TGs were the dominating neutral lipid class, for the accurate quantification of CEs, a PRM method was set-up, allowing a more sensitive detection of this group of lipids. The tutorial starts after that manual identification of CEs within the lipid extracts was successfully finished with several CEs identified as their ammoniated adducts ($[M+NH_4]^+$). Next, these CEs will be quantified by PRM. Therefore, in a first instance, a transition list for the targeted analysis will be created, and afterwards acquired PRMs will be analyzed using Skyline. Here, for the creation of the transition list, LipidCreator[2] will be used within Skyline, however, transition lists can also be generated manually or with other software tools.

[1] Lange, M. *et al.* AdipoAtlas: A reference lipidome for human white adipose tissue. *Cell Reports Medicine* **2**, 100407 (2021).
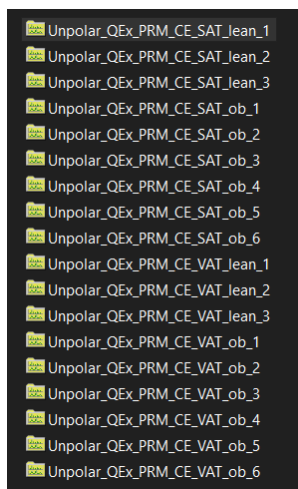[2] Peng, B., Kopczynski, D., Pratt, B.S. et al. LipidCreator workbench to probe the lipidomic landscape. Nat Commun 11, 2057 (2020).

Getting Started

(1) Install LipidCreator from the Skyline ToolStore
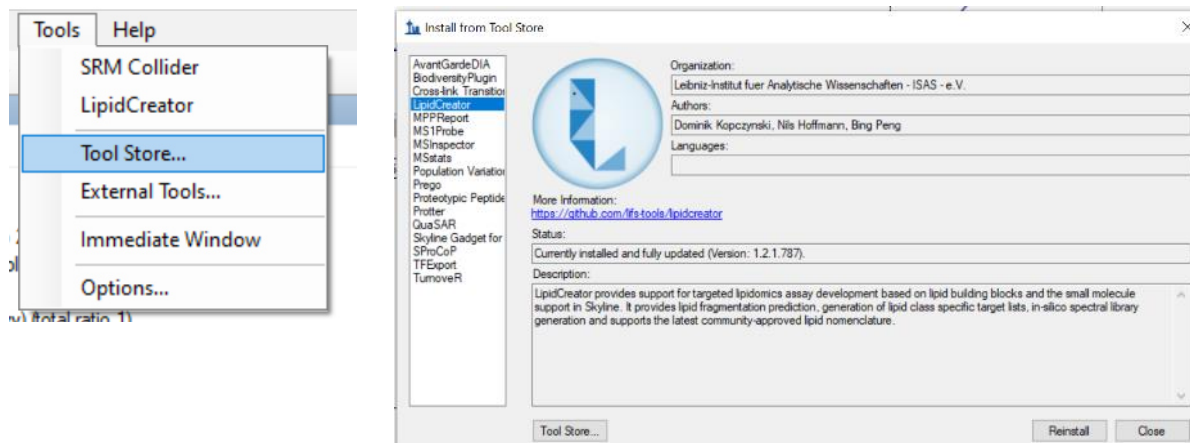
(2) Un- ZIP the folder that contains the RAW files and save these 18 files in a folder of your choice or download the corresponding RAW files from MassIVE.
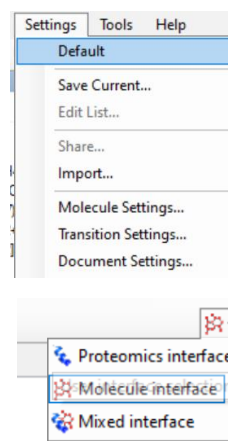

(3) If you have been using Skyline prior to starting this tutorial, you may want to reset Skyline to its default settings. To do so:

  • On the Settings menu, click Default.

  • You have the option to save your current settings here.

For the tutorial, Skyline was set to "**Default**".

(4) Select the small molecule interface.

Setting up Skyline

(1) Molecule settings

To change molecule settings, go to **Settings** and chose **Molecule settings** and under "**Labels**" include heavy.
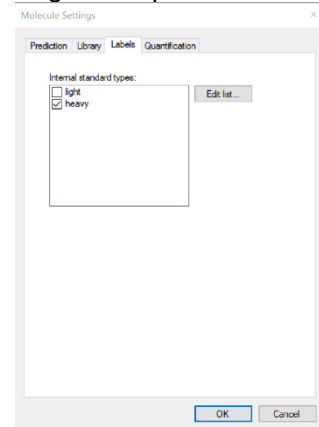
(2) Transition settings

To change transition settings, go to **Settings -> Transition settings** and change to the settings displayed in the screen shots:

In the *Filter tab* select as the Precursor adducts **[M+NH4]⁺**, as CEs are usually detected as this type of adduct. As fragment adducts please include **[M+]** and **[M+H].** Ion types only include fragments so can be set to "**f**".

On the *instruments tab*, please change the values for min m/z (200) and max m/z (1500) as well as the method match tolerance (0.005).

Under the *Full Scan* tab, select for **MS1 filtering** *Isotope peaks included* "**none**" and for **MS/MS filtering** set *acquisition method* to "**PRM**" with *Product mass analyzer* being "**Orbitrap**". As *Resolving power* select "**17,500 @ m/z 200**".

Optional: "Use high-selectivity extraction" can be used to extract a single-resolution width around targeted m/z, lowering the interference in complex sample matrices.
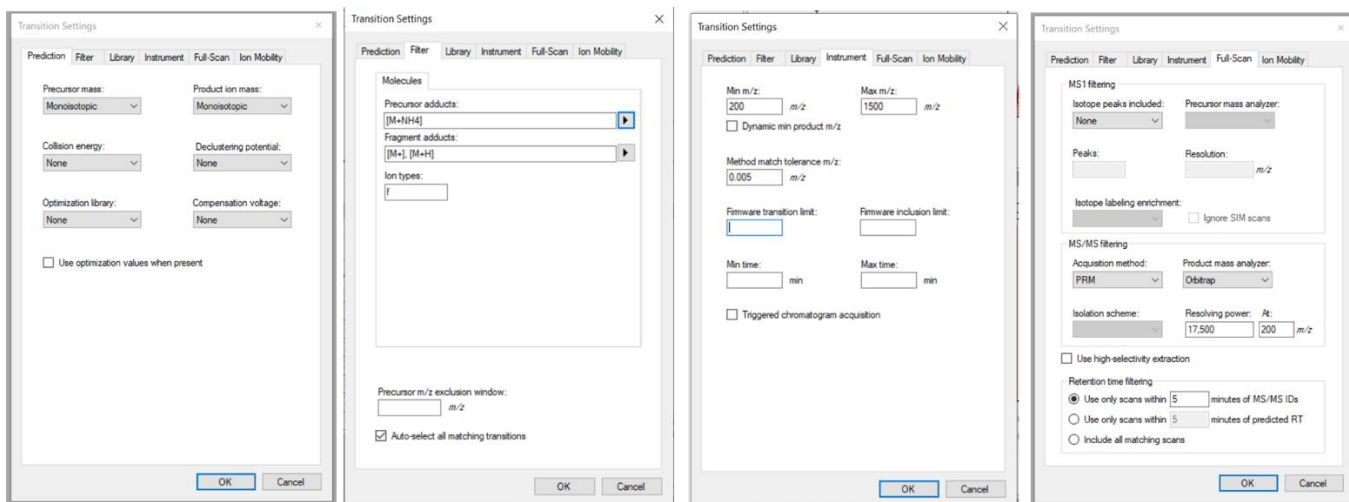
Transition settings used within this Tutorial. Values may change when data from a different instrument is used!

<u>Create a transition list with LipidCreator</u>

The following CEs should be included in the transition list:

CE	Retention time (min)
CE 18:1 (d7)	24.99
CE 18:1	24.99
CE 18:2	23.00
CE 20:3	23.29
CE 20:4	21.68
CE 22:4	23.71
CE 22:5	22.04
CE 22:6	20.96

(1) Transition for deuterated internal standard

- Open Lipid Creator (Tools → LipidCreator)
- You can select different Lipid classes from the tabs. Please select "**Sterol Lipids**" as the lipid class of interest. To generate Cholesteryl esters, select the *Type* "**ester**" and as *molecule* "**SE 27:1**" (Cholesterol will be annotated in Lipid creator as SE 27:1).

   *Note: Cholesteryl esters created with LipidCreator will be named "SE 27:1/FA X:Y". In shorthand notation CEs are annotated as **CE FA X:Y** and thus CEs will be referred to this nomenclature within the tutorial text.*

Sterol Lipids tab within LipidCreator.

- On the *Filters* tab change the settings in the following way : *Precursor filters to* "**Compute no precursor transitions**" and *heavy labeled isotope filters* to "**Compute with heavy labeled isotopes**". Note: *For CEs we are only interested in the cholestene fragment ion resulting from the loss of the fatty acid and this will be our only transition of interest.*

- Next, we have to define our isotopes via *Manage heavy isotopes.* As lipid class select again "**SE 27:1**". As *isotope suffix* add "**d7**", and as the *building block*, so the location of deuterium atoms, select "**fatty acyl**". In the *Elements table* add *Count (Isotopic)* "**7**" for Hydrogen. And confirm by "**Add Isotope**".

- In a next step, the deuterated CE 18:1 will be generated. Therefore, select for *Fatty acyl chain* "**18**" and *No DB* "**1**" and "**add sterol lipid**". Now your lipid list contains your internal standard.

Sterol Lipids tab within LipidCreator with deuterated CE added to the Lipid List.

(2) **Add unlabeled CEs to the transition list**

- Change *Filters* for *heavy labeled isotope filter* to "**Compute no heavy labeled isotopes**" and add the remaining CEs from the identification list. You can add them one by one, or for instance for all CEs with 18 Carbons you can compute all at the same time by setting *No DB* "**1-2**". Once done with this step, you will have a Lipid list that contains all CEs of interest.

- Now you can *Review* Lipids. Under options select **"Display precursors on species level (only containing class specific fragment)"**. This will be sufficient for CEs as their MS2 only contains the cholestene ion at m/z 369.35. Click continue.

Review Lipid list and select which information should be generated for the molecules on your list. Lipid transitions review shows all transitions that were created based on selected parameters.

(3) Modify Transition list

- You will now have a Transition list for CEs of interest. You can directly send the transition list to skyline (*Note: the list does not contain retention times yet*) or you can **store the transition** list as a **xlsx-file**. Please **store the transition list**.


Screenshot from the transition list opend in Excel. Retention time for each lipid were added manually. The labeled internal standard was marked as "ISTD").

- Open your transition list in Excel (or any other suitable program of your choice) and modify it in the following way (highlighted in red): For the internal standard please add "(ISTD)" to the precursor name and to all CEs add provided retention times.

- *Note: Alternatively, you can add retention times directly in Skyline. Therefore, send the transition list to skyline, right click on the molecule → Modify and add the retention time. For a small number of molecules this works fine, however, when you have a data set with hundreds of lipids, adding in a separate document will be less time-consuming.*
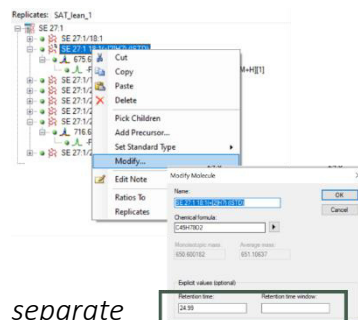
(4) Import Transition list and RAW data to Skyline

- For the import of your transition list go to *Edit* than *insert* and "transition list". Here you can simply copy and paste your transition list from Excel to Skyline. Check that all columns are assigned correctly and change if necessary.
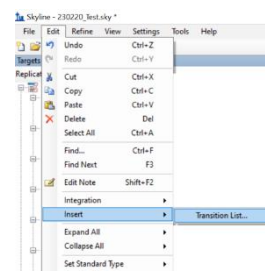
MoleculeGroup	PrecursorName	PrecursorFormula	PrecursorAdduct	PrecursorMz	PrecursorCharge	ProductName	ProductFormula	ProductAdduct	ProductMz	ProductCharge	retention time
Molecule Li...	Molecule Name	Molecul...	Precursor Adduct	Precursor m/z	Precursor Charge	Product Name	Product Formula	Product Adduct	Product m/z	Product Charge	Explicit Retenti...
SE 27:1	SE 27:1/18:1	C45H78O2	[M+NH4]1+	668.6340	+1	-FA 18:1(+HO)-C...	C27H44	[M+H]1+	369.3516	+1	24.99
SE 27:1	SE 27:1 18:1(+[2]...	C45H78O2	[M7H2+NH4]1+	675.6779	+1	-FA 18:1(+[2]H7)(...	C27H44	[M+H]1+	369.3516	+1	24.99
SE 27:1	SE 27:1/18:2	C45H76O2	[M+NH4]1+	666.6184	+1	-FA 18:2(+HO)-C...	C27H44	[M+H]1+	369.3516	+1	23
SE 27:1	SE 27:1/20:3	C47H78O2	[M+NH4]1+	692.6340	+1	-FA 20:3(+HO)-C...	C27H44	[M+H]1+	369.3516	+1	23.29
SE 27:1	SE 27:1/20:4	C47H76O2	[M+NH4]1+	690.6184	+1	-FA 20:4(+HO)-C...	C27H44	[M+H]1+	369.3516	+1	21.68
SE 27:1	SE 27:1/22:4	C49H80O2	[M+NH4]1+	718.6497	+1	-FA 22:4(+HO)-C...	C27H44	[M+H]1+	369.3516	+1	23.71
SE 27:1	SE 27:1/22:5	C49H78O2	[M+NH4]1+	716.6340	+1	-FA 22:5(+HO)-C...	C27H44	[M+H]1+	369.3516	+1	22.04
SE 27:1	SE 27:1/22:6	C49H76O2	[M+NH4]1+	714.6184	+1	-FA 22:6(+HO)-C...	C27H44	[M+H]1+	369.3516	+1	20.96

Transition list view within Skyline. List is added by copy and paste from the excel table.

- Your transition list will now be displayed in the *Target* view within Skyline.

  Note: If the import of your transition list worked, you can close LipidCreator. If you have issues with csv/excel files, send the transition list from LipidCreator directly to Skyline and add retention times manually as described under the Note in (3).

- Next, import PRM RAW files provided with this tutorial. Go to *File*, *Import* and select "**Results**". Here select "**Add single-injection replicates in files**" and *files to import simultaneously* "**Many**". Click OK. Select all files provided with this tutorial. You can remove the common prefix "**Unpolar_QEx_PRM_CE**" of the files by selecting "**Remove**" as file names are relatively long and important information will still be kept. Click OK and the data import will start.
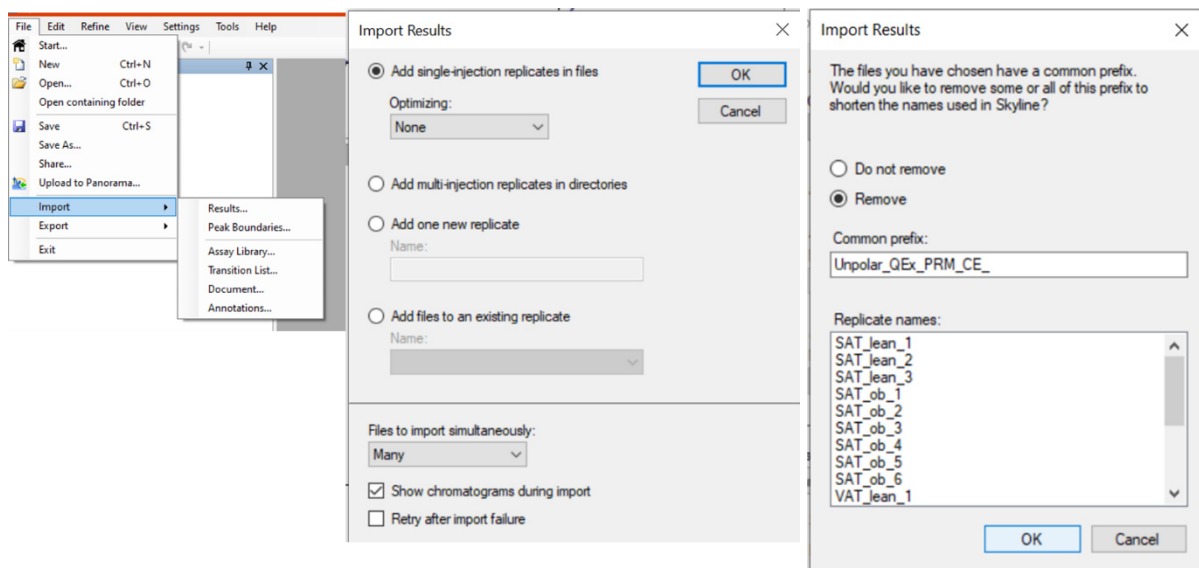

- Data import may take a few minutes and your Skyline window can look like this: *Note: You can transform/smooth data for better visualization of peaks. Therefore, right click on the extracted ion chromatogram and select transform. Here Savatzky-Golay Smoothing was used.*
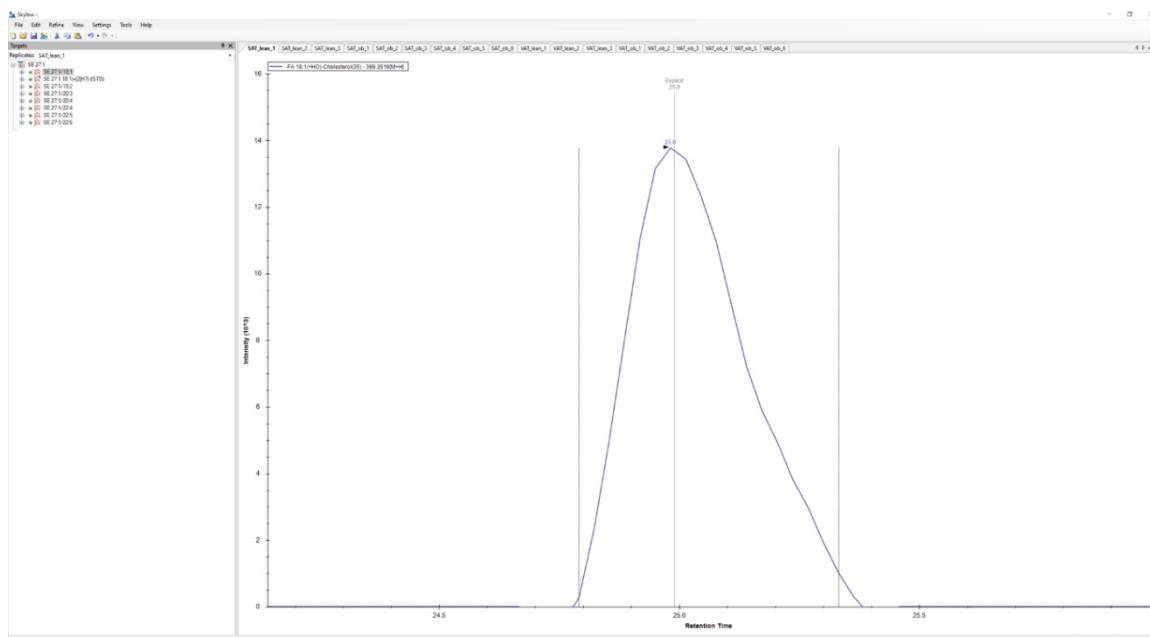
Data Validation

- On the *View* menu, choose *Peak Areas* and click "**Replicate Comparison**" and "**Molecule Comparison**".

- On the *View* menu, choose *Retention Times* and click "**Replicate Comparison**" and "**Molecule Comparison**".

- Click and drag these views to dock them on your screen below the extracted ion chromatogram.

- In the *Targets* view select the first Molecule "**SE27:1/18:1**"
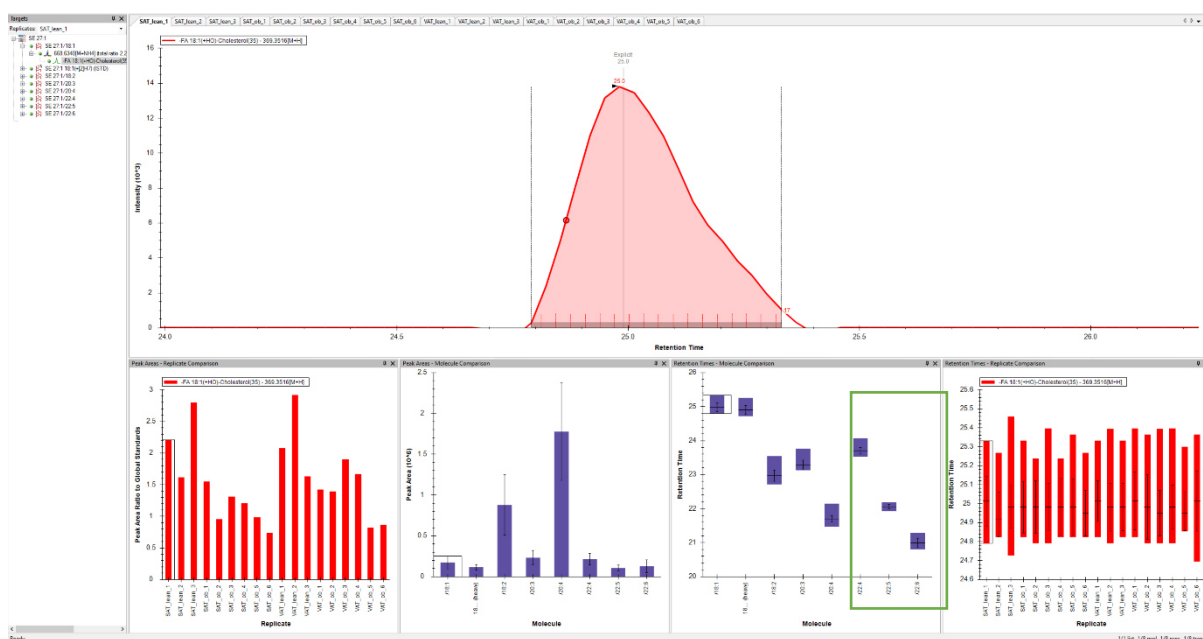
(1) Retention behavior of lipids

In the *Retention Times* "**Molecule Comparison**" window you can judge retention behavior of lipids. Here, lipids were analyzed on a reversed phase column. The labeled CE 18:1 (d7) and its endogenous non-labelled CE 18:1 should elute at very close retention times. Moreover, for lipid species of the same lipid class having the same number of carbons, here e.g., CE 22:4, CE 22:5, and CE 22:6 (green frame), the species with the highest number of double bonds (CE 22:6) will elute first and the species with least double bond last (CE 22:4). In the *Retention Times* "**Replicate Comparison**" you can check that retention time is similar within all replicates. It will directly show you obvious outliers if present.

*Note: If you select in the targets list "**SE 27:1**" you can also inspect the extracted ion chromatograms and "**retention time- replicate comparison**" for all the molecules contained on you transition list. This will directly show you, if your endogenous CE 18:1 and its deuterated standard elute at the same region of the chromatogram. For data sets with many lipids you will also be able to identify lipids with potential isotopic overlap.*
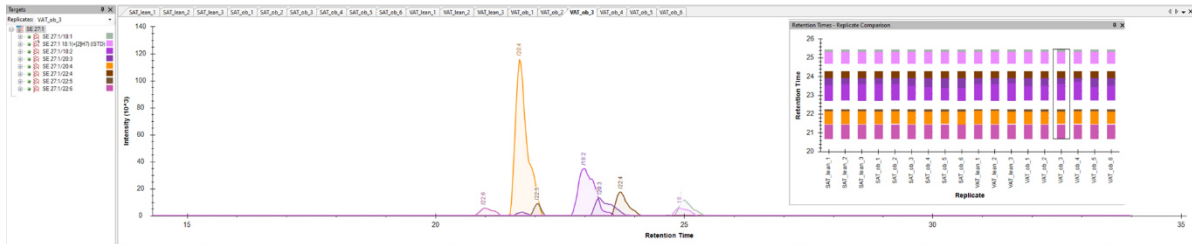
(2) Check and adjust Peak integration

As you can see for the first Molecule SE27:1/18:1 the peak integration is not optimal (left). Therefore, adjust Integrals to cover the whole peak (right). Check and adjust integration of each Molecule in each file.
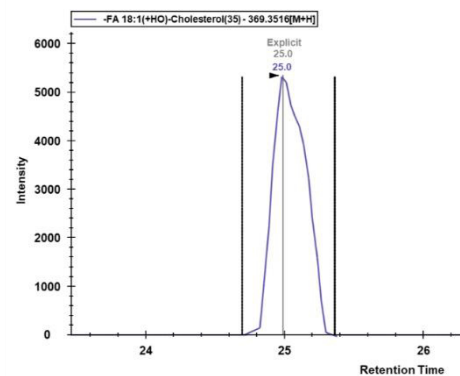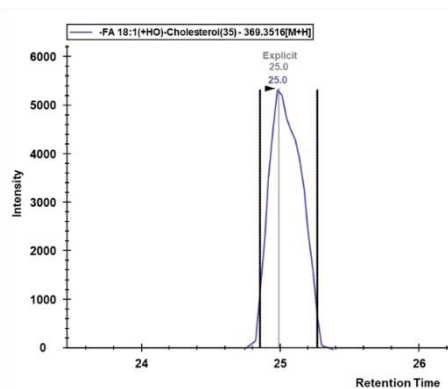
(3) Normalization to Internal standard

In Lipidomics data sets, usually one to two internal standards are spiked per lipid class, thus we can use the spiked deuterated CE standard to build a ratio of endogenous lipid to the internal standard. Skyline will calculate this ratio for you by applying the following steps:

- Define the deuterated CE as a "global standard". Therefore, right click on the lipid in the *target view* and select *Set Standard Type* "**Global Standard**".

- To let Skyline calculate the ratio for you, next you do right click again on the lipid in the *target view* and select *Ratio to* "**Global Standard**".

- In the *target view* you will now see the **ratios** Skyline calculated for each lipid.

- In the "**Peak Area-Replicate Comparison**" window you will now see the Peak area ratio to the global standard. This already gives you a first impression on your quantitative data.
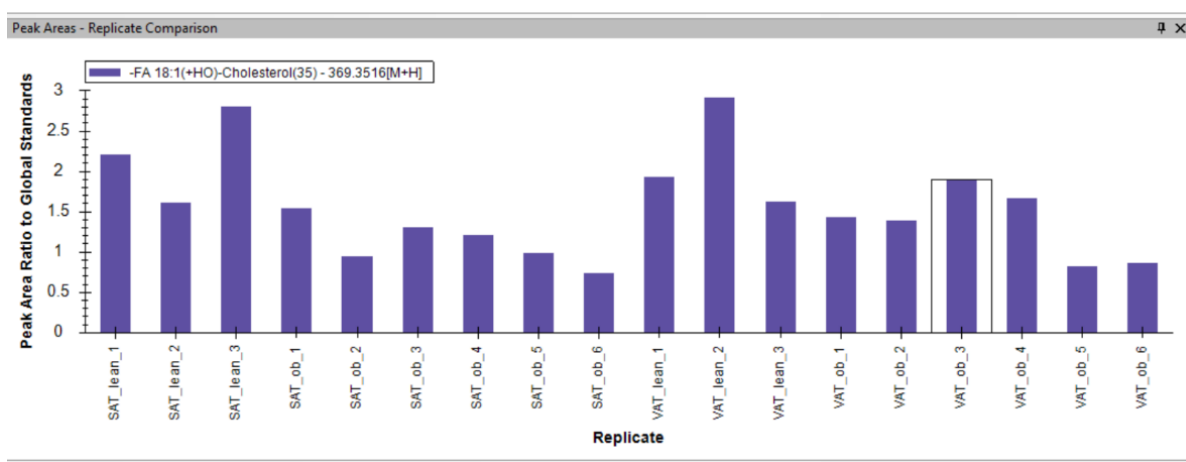
(4) Define groups for group comparison

As the experimental data was acquired from 4 different groups (VAT and SAT of lean and obese), you can add these conditions into your document to compare the peak area ratio of the four different groups. Therefore, go to *Settings* and select *Document Settings.* In the Annotations window select "**Add**". Define the annotation as "**CE group**" and as *type* select "**Value list**". As *values* please provide: **VAT_lean, VAT_ob, SAT_lean, SAT_ob**. These lists apply to "**Replicates**". Click ok and select the newly created

annotation. Next, go to *View* and select *document grid* and chose from the drop-down menu of *Reports* "Replicates". Here you now need to add the above defined groups to each replicate.

Addition of the groups now allows you to go the "Peak Areas- Replicate Comparison" view and change it to the groups by right click *group by* "CE groups". Skyline will now show you peak area group averages normalized to your internal standard. This representation indicates higher CE in the lean groups. Is this true for all CEs within this data set? *Note: Please keep in mind that this is only a relative quantification. And the data is not normalized to the amount of internal standard and tissue weight or protein amount. Therefore, conclusions must be drawn with caution!*
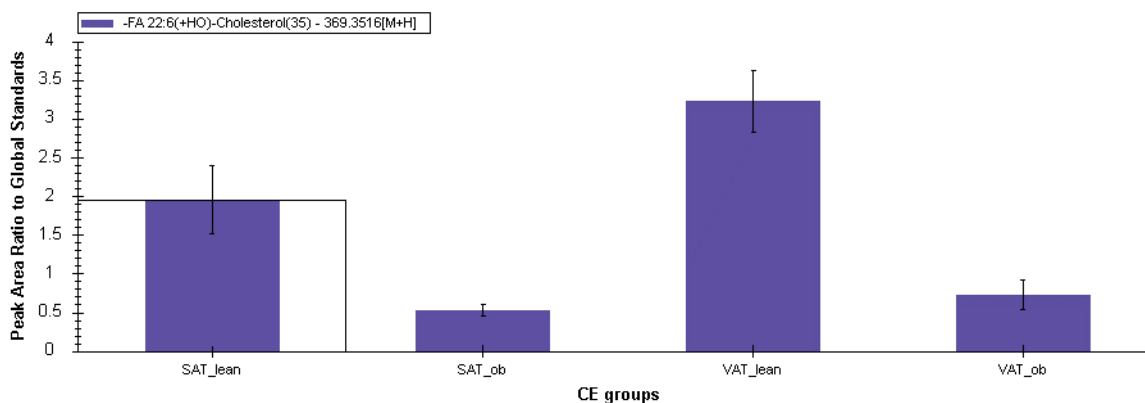
Data Export

The data is now ready to be exported. In order to export your data you first have to create a *document grid* that contains all information you want to be exported. Go to *View* and select *document grid*. Click on *Reports* and select the "Transitions" template. Next, click again on Reports and "Customize Report". We will modify this template in a way to contain all information we need. Fist Change the name of the Report to "Transitions_CE". Form the screenshot you can see which values we want to export. You can find these from the list by using the binoculars icon and type in the name.
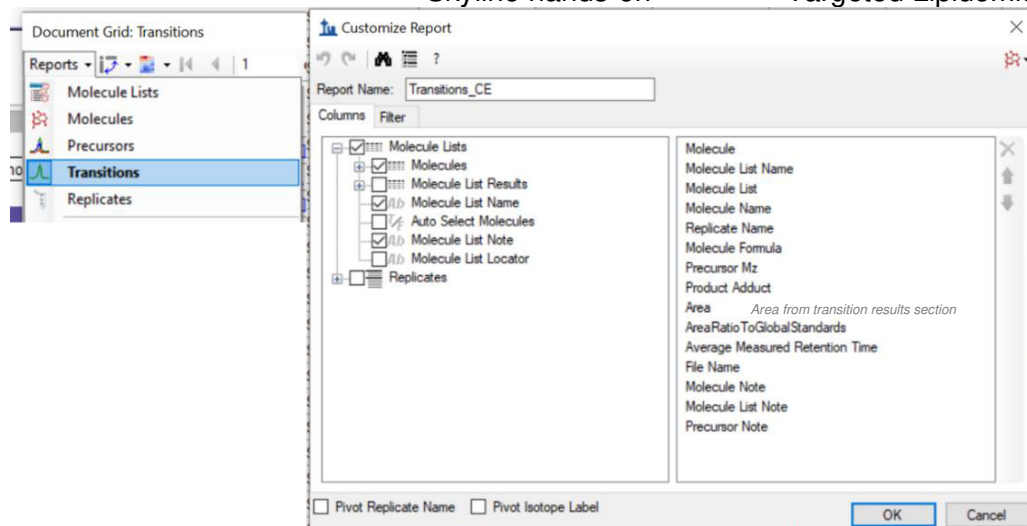
To export your final data matrix select "**Export**" in the *document grid* window. You are done with the Skyline Lipidomics PRM tutorial.

Note: You can process your data now further and for instance multiply with the concentration of the internal standard to obtain quantities and normalize these values to tissue weight or protein content. This, however, is beyond the scope of this tutorial that aimed to teach you how to generate transition list and analyze PRM data for lipids within skyline.