Skyline Small Molecule iRT Retention Time Normalization

Normalizing retention time variation can improve the accuracy and throughput of small molecule and peptide analysis. There is an existing iRT Retention Time Prediction tutorial for peptides however this tutorial will specifically address small molecule normalization and synchronized integration.

In this tutorial, you will learn how to synchronize peak integration, build an iRT calculator for a previously published CE-MS dataset and use the calculator to normalized retention time variation for analytes with substantially varying retention times. You will also see how the use of normalized retention times with synchronized peak integration can improve the throughput and accuracy of manual peak picking.

Study information

The Skyline document for this tutorial is a modified Skyline assay from Panorama Public (https://panoramaweb.org/tczcoD.url), a Capillary Electrophoresis - High Res Mass Spectrometry analysis of a series of dried blood spot, plasma, and brain samples from animals which had been dosed with stable-isotope labeled arginine[1]. Although migration time is a more appropriate term to use for CE-MS, here we will be using "retention time" instead. For the purposes of speed, we have reduced the number of files

Getting Started

To start this tutorial, download the following ZIP file:

https://skyline.ms/wiki/home/software/Skyline/events/2022-10%20Skyline%20Online/page.view?name=session2

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

C:\Users\cashwood\Documents

This will create a new folder:

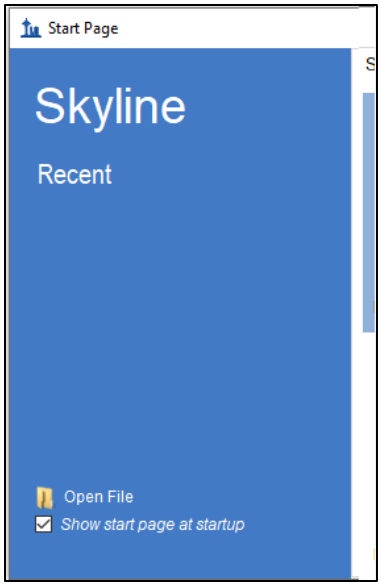
C:\Users\cashwood\Documents\SmallMoleculeiRT

It will contain all the files necessary for this tutorial.

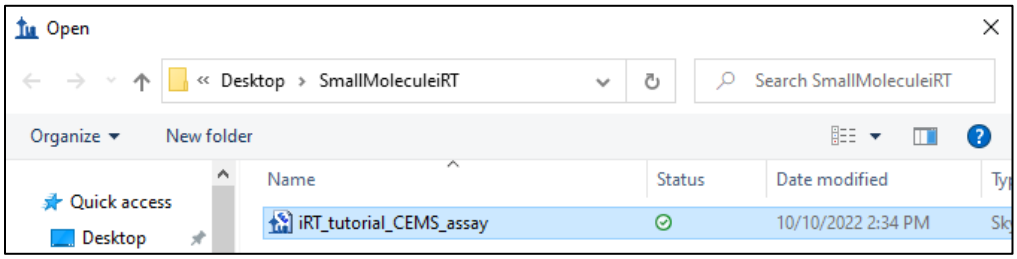
If you have been using Skyline prior to starting this tutorial, it is a good idea to revert Skyline to its default settings. To do so:

- Start Skyline.

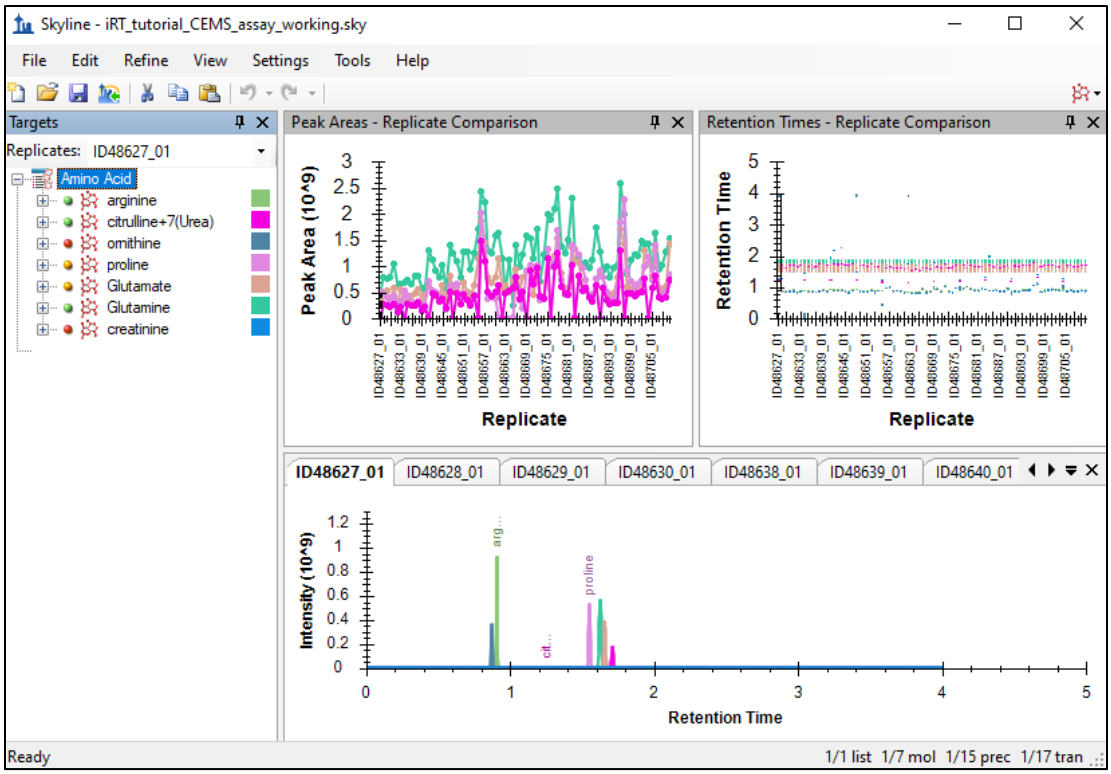
- On the **Start Page,** click **Open File** which looks like this:



- On the **Open** window, double-click the **iRT_tutorial_CEMS_assay** to open the Skyline assay**.**



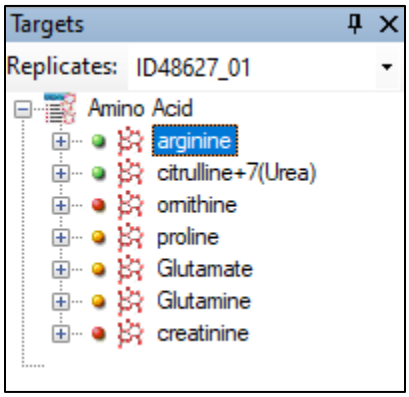
- Your Skyline window should now look like this:



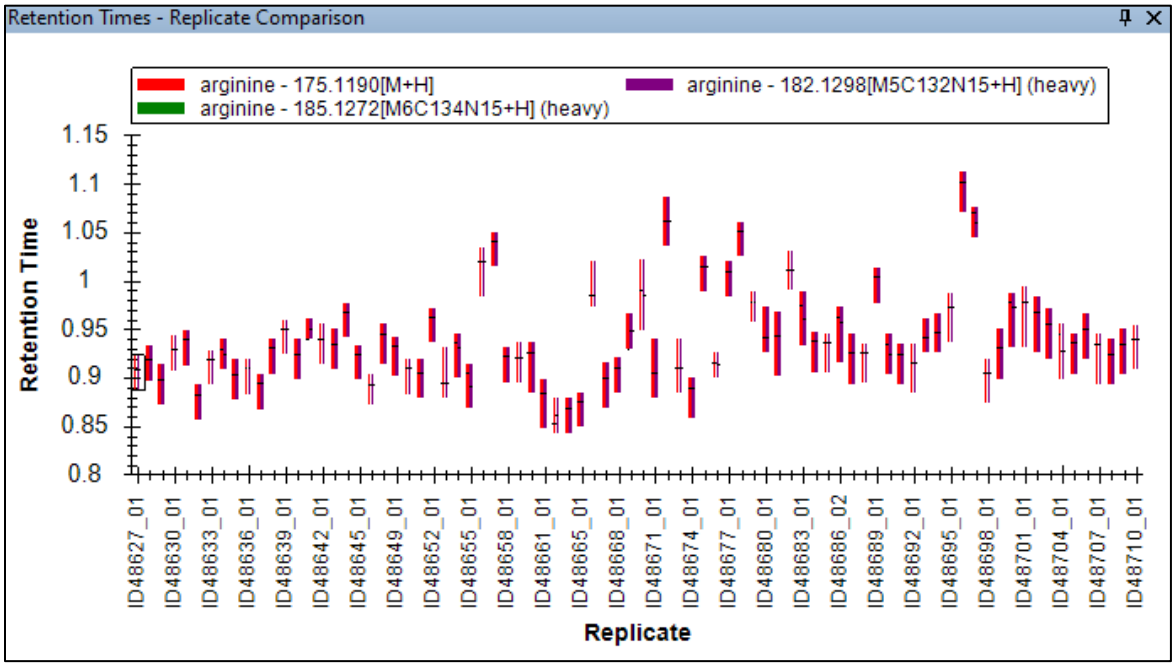
Choosing molecules to be classified as iRT standards

For this tutorial, our retention times will be normalized to five molecules whose peaks were picked well by Skyline's peak picking algorithm. Importantly, the correct peaks should be assigned for these five molecules so we will first evaluate the peak picking for iRT alignment. If the incorrect peaks are picked, the retention time calculator will not be able to be sufficiently calibrated.

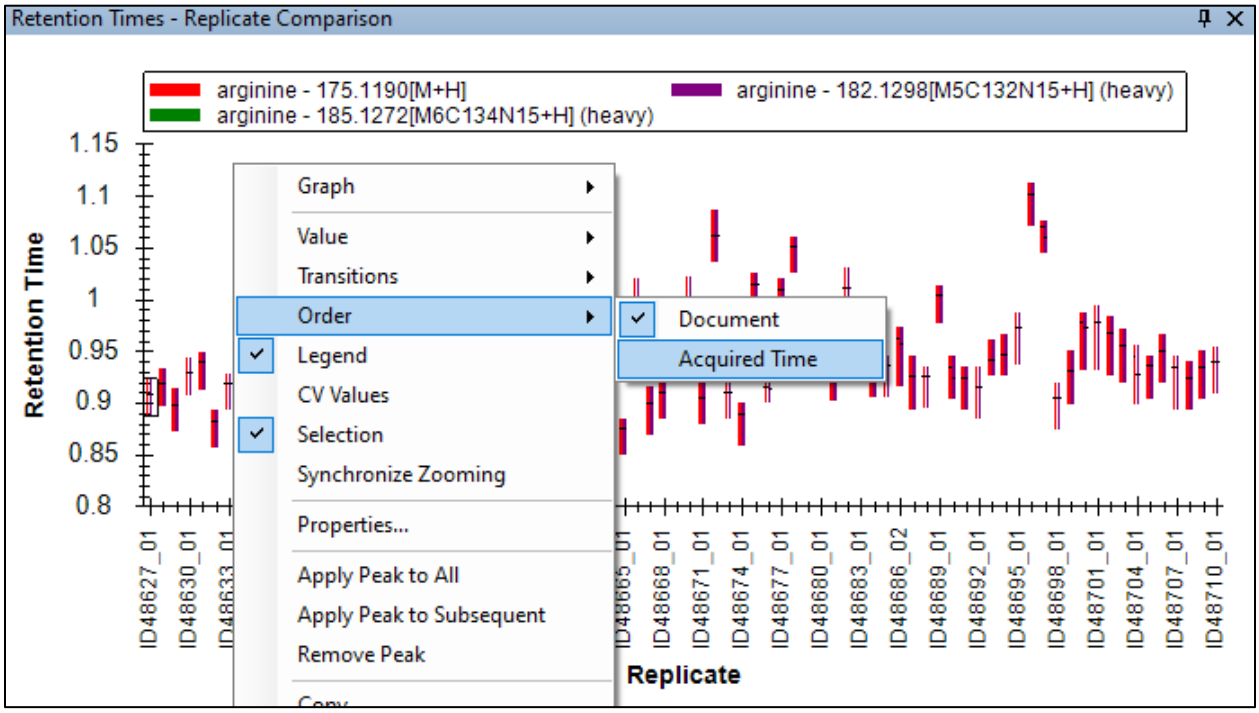
- First, select the target **arginine** in the target list



- As you can see in the **Retention Times – Replicate Comparison** window, there is a minor amount of retention time variation for arginine

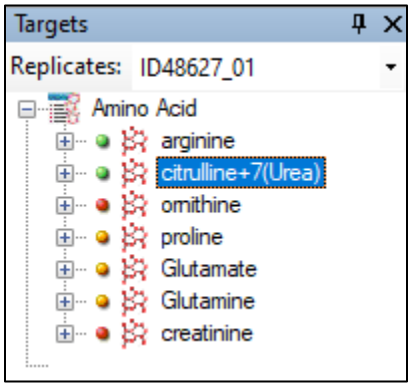


- To evaluate if the observed drift is related to acquisition time, right click in the **Retention Times – Replicate Comparison** window, hover over the **Order** option and select **Acquired Time**

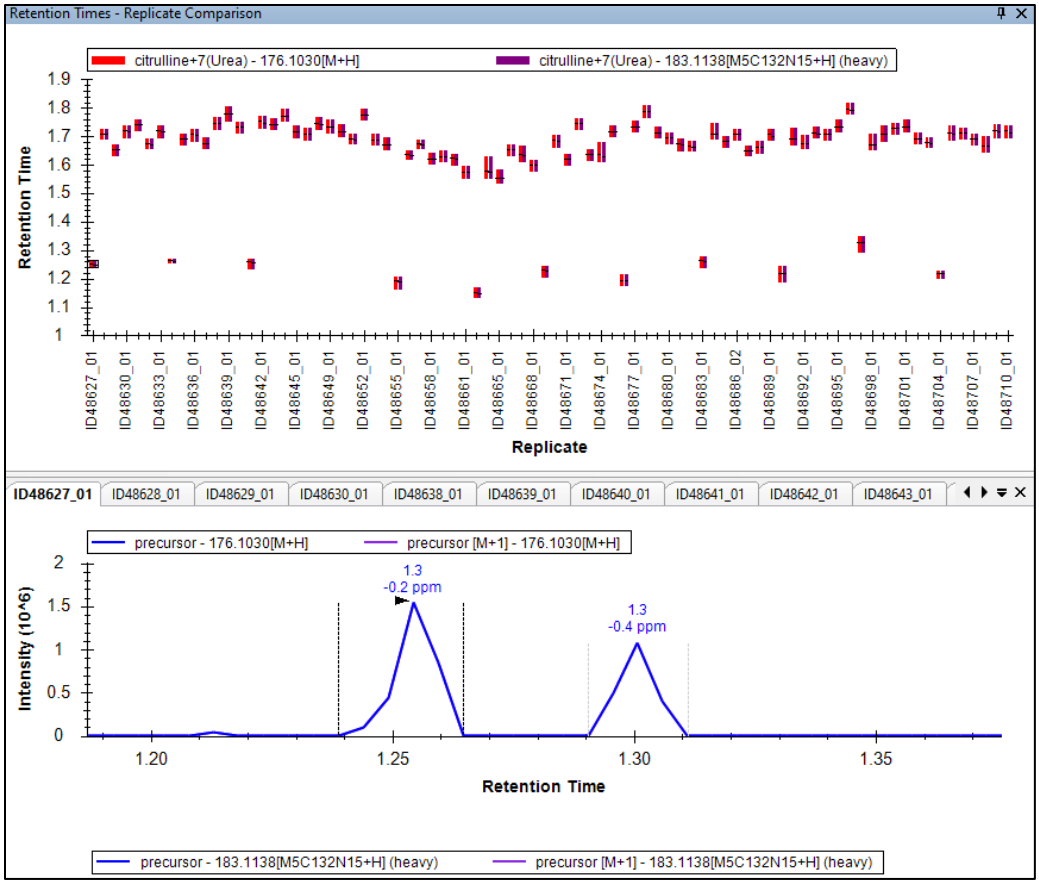


In this example, there is no change in the order of the replicates as the Document order reflects the time these data were acquired. This molecule is a good candidate for retention time normalization due to the low relative retention time variation.

- Select the target **citrulline+7(Urea)**



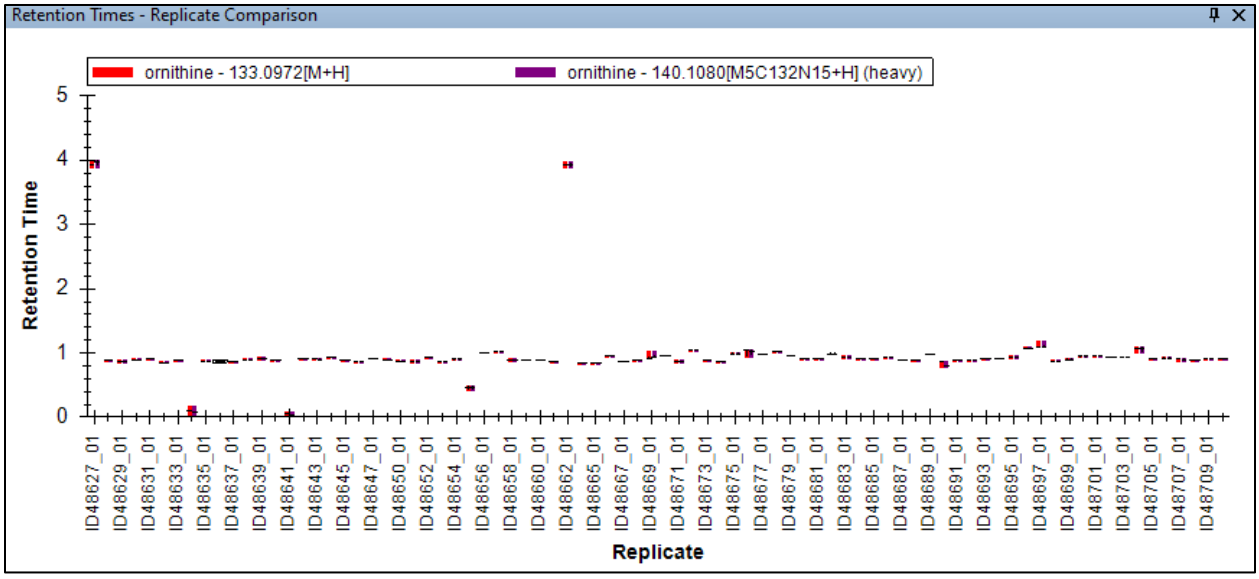
Here we can see that there is greater variation for this molecule, and multiple peaks are present in each chromatogram. This could be challenging to analyze without retention time normalization. For these reasons, we will not use this molecule as a standard for retention time normalization. It is important to note that the retention time standards do not need to be actual standards, endogenous analytes can also be used like in this tutorial.



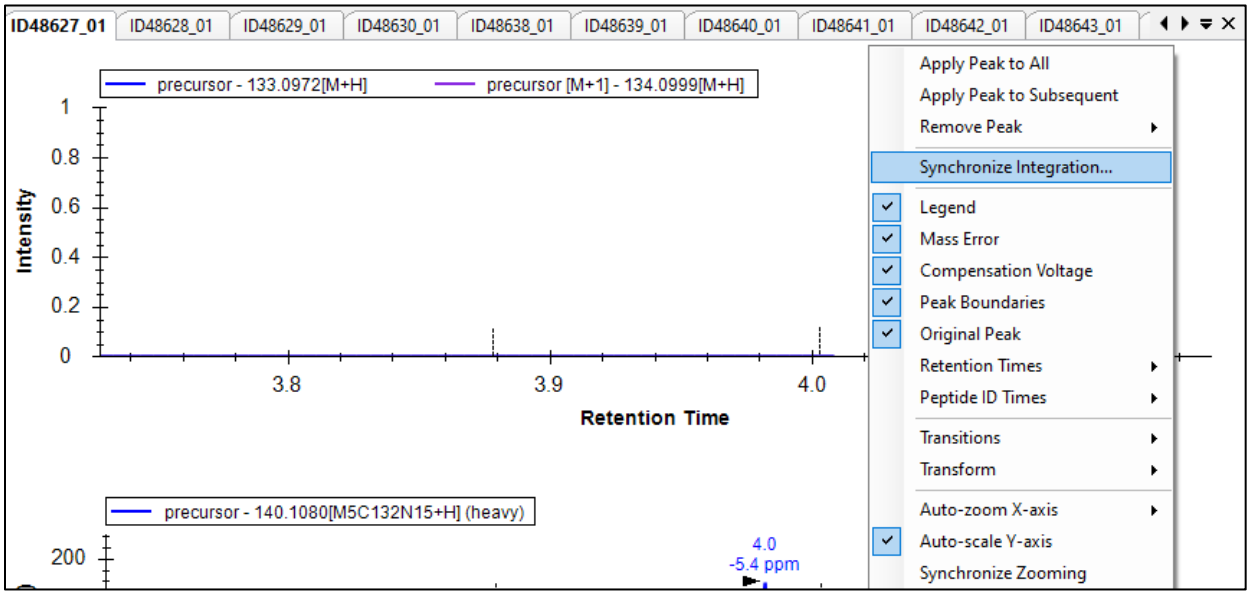
Synchronizing retention times for high throughput peak picking

This sample set contains 84 runs and while picking the peaks manually is feasible, we can integrate multiple replicates at once with the **Synchronize Integration** feature. Here, we will use synchronized integrations to define the integration bounds for Ornithine across all 84 replicates in a single click.

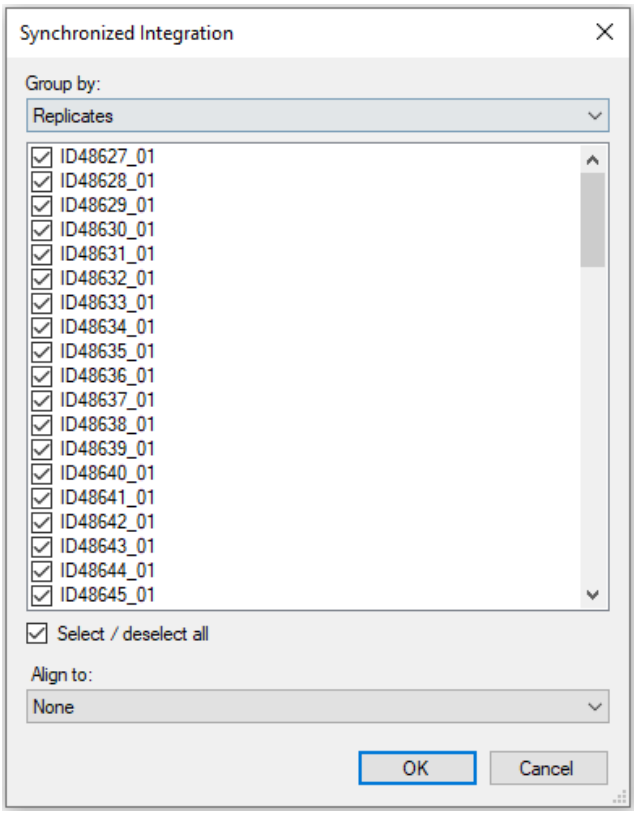
- Select the target **Ornithine** in the target list. Your **Retention times – Replicate Comparison** window should look like below with a few runs having peaks beyond the 0.8 – 1 minute window.



- Right click in the Chromatogram window and select **Synchronize Integration**

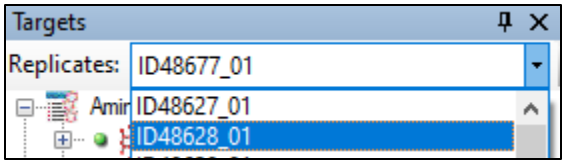


A window will appear to select replicates for synchronized integration. The replicates that are selected here will have their integration synchronized. For this example, we will be synchronizing the integration for all replicates.

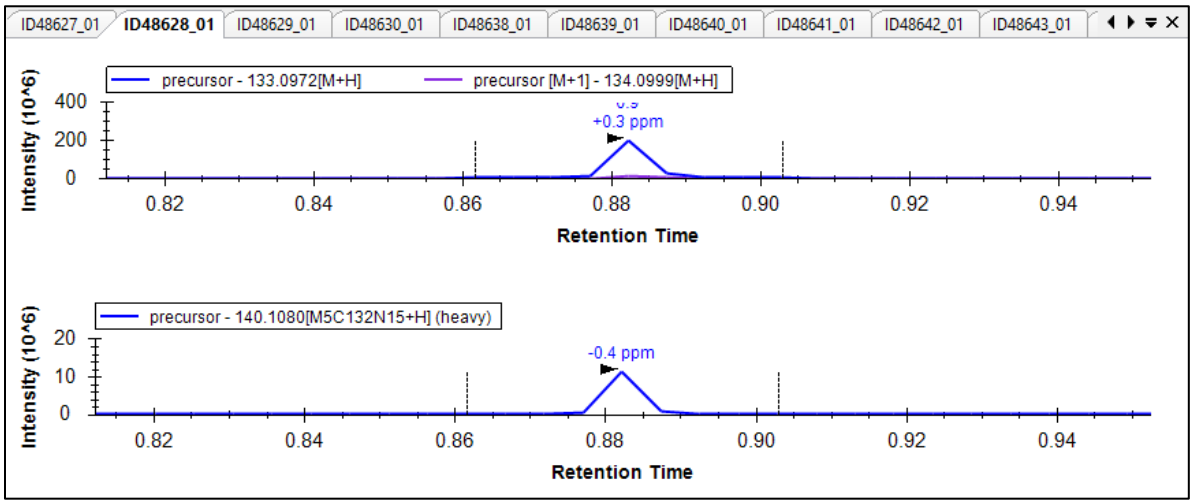


- Click **OK**

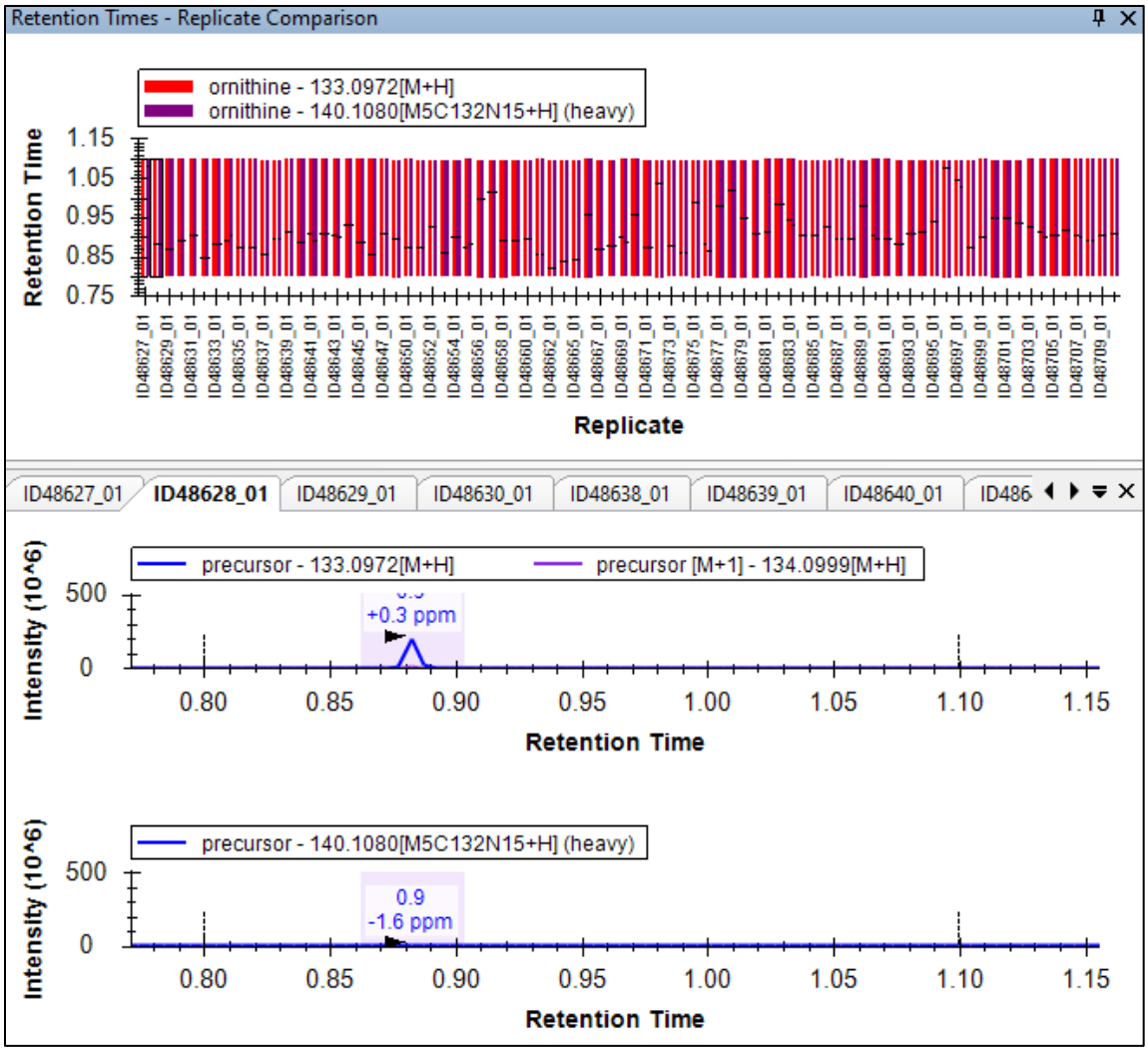
As our integrations are now synchronized for all replicates, we want to ensure that our start and end retention times cover all the peaks observed in the run. For this tutorial, we have determined the start retention time of 0.8 min and end retention time of 1.1 min to be suitable integration bounds for all replicates.



- Select replicate **ID48628_01** in the targets window. Your chromatogram view should look like below:



- Zoom out from the chromatogram until the retention times of 0.8 and 1.1 min are visible and use your cursor on the retention time axis to integrate the 0.8 to 1.1 min window. Your **Retention Times – Replicate Comparison** window and **Chromatogram** window should now look like this:

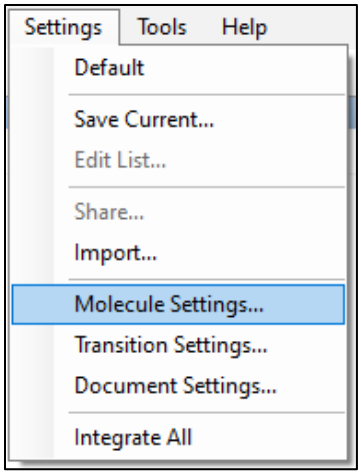


As you can see in the **Retention Times – Replicate Comparison** window, all of the replicates for **ornithine** now have the same integration bounds. There are no longer any outlier retention times so it is now suitable for use as an iRT standard.

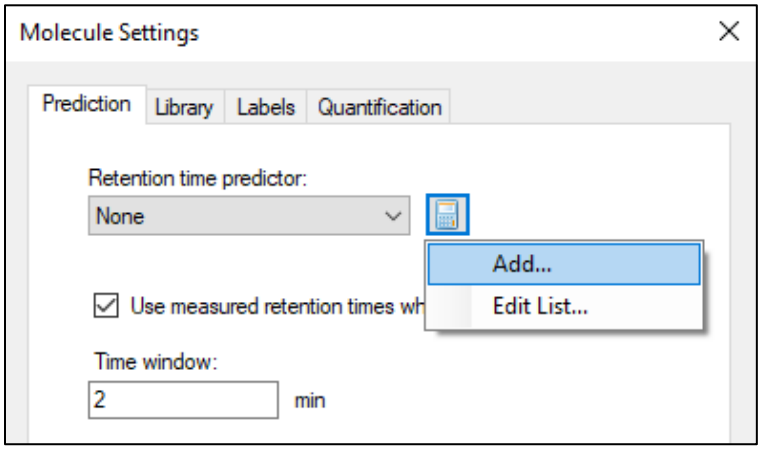
Building an iRT calculator

Now that we have a set of molecules that are suitable for retention time alignment, we can build the iRT calculator.

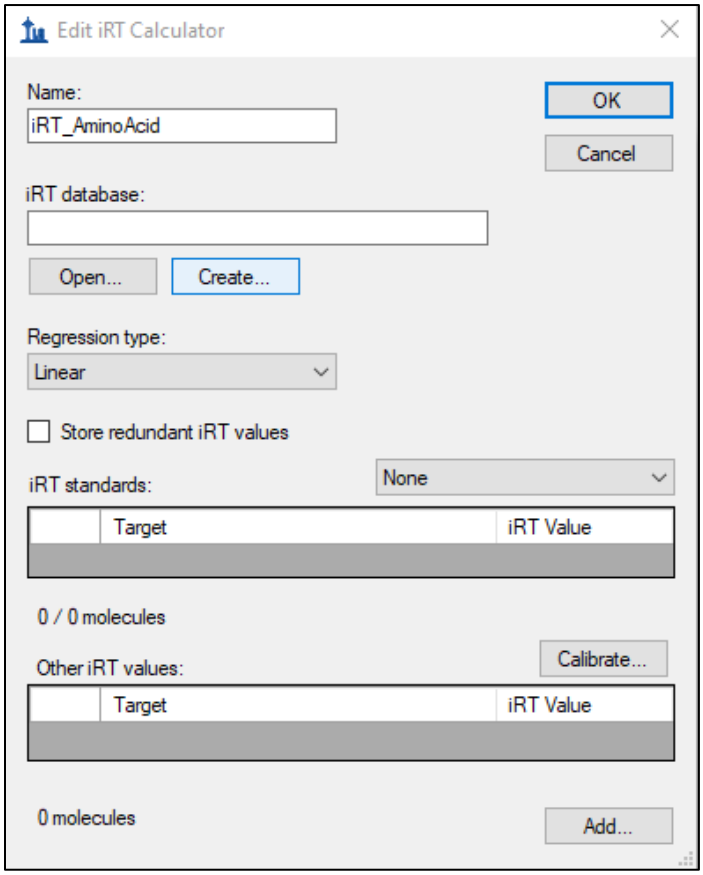
- Open the **Molecule Settings** and select the **Prediction** tab



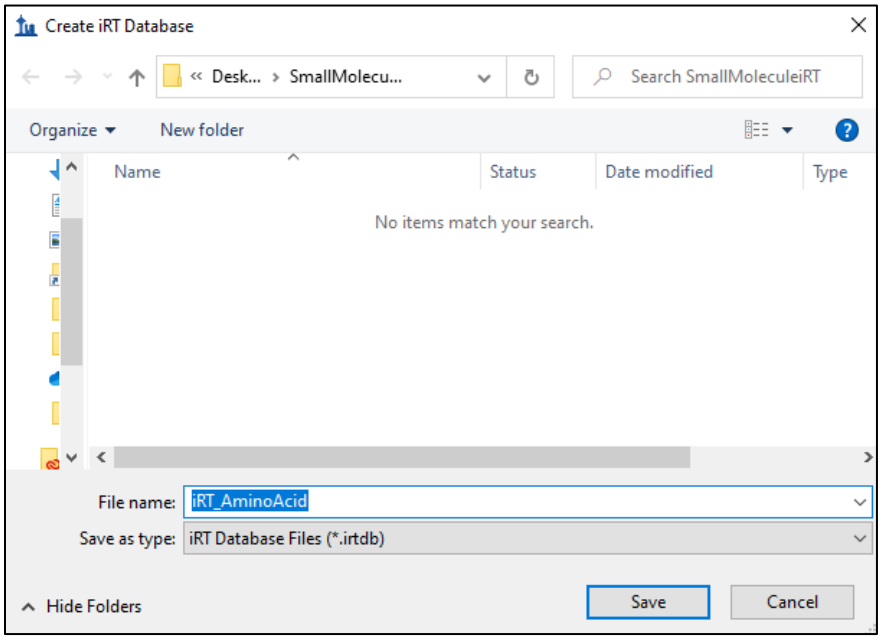
- On the **Prediction** tab, click the **calculator icon**, and click **Add.** This will open the **Edit iRT Calculator** form so we can start our retention time normalization process.



- Name our iRT calculator **iRT_AminoAcid** and then click the **Create** button underneath iRT database



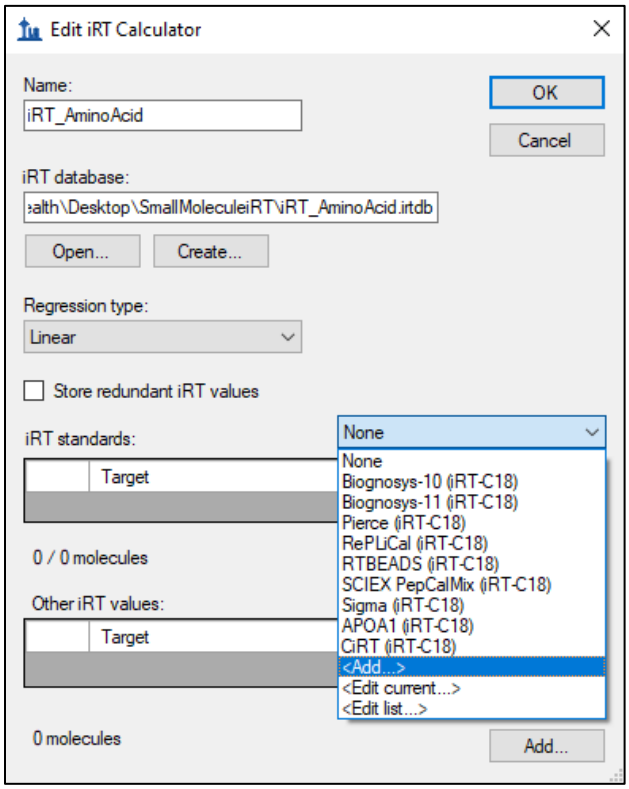
- Name our iRT database as **iRT_AminoAcid** and click **Save**. This will have the file type of .irtdb.



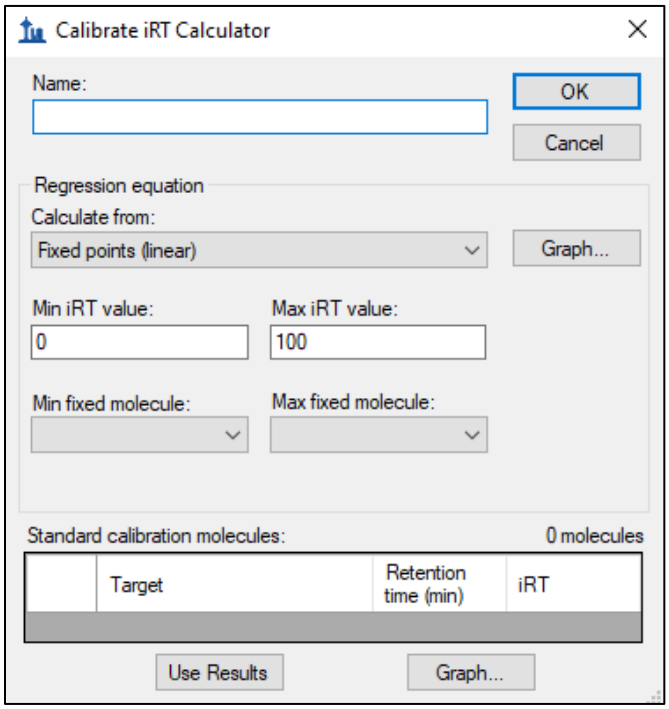
You will now be returned to the **Edit iRT Calculator** window.

Now that we have created our iRT Calculator, it is time to add our iRT standards

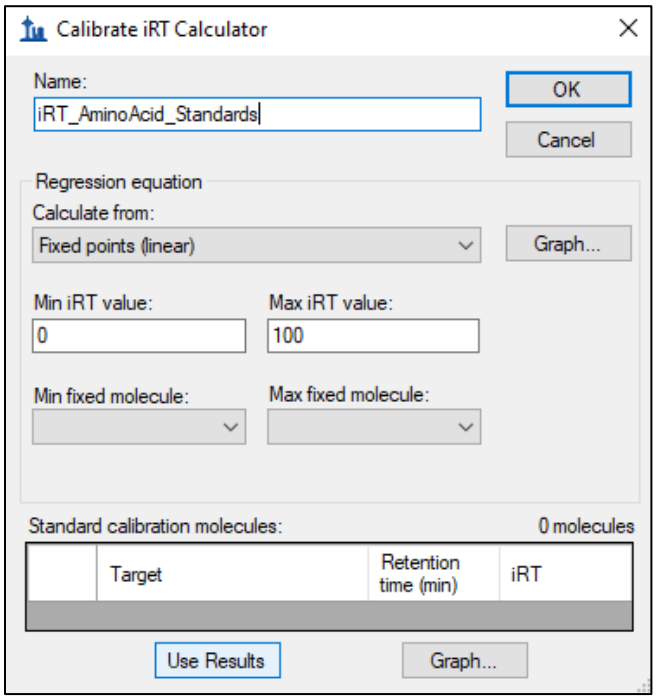
- Click on the dropdown box next to iRT standards and click on **Add**



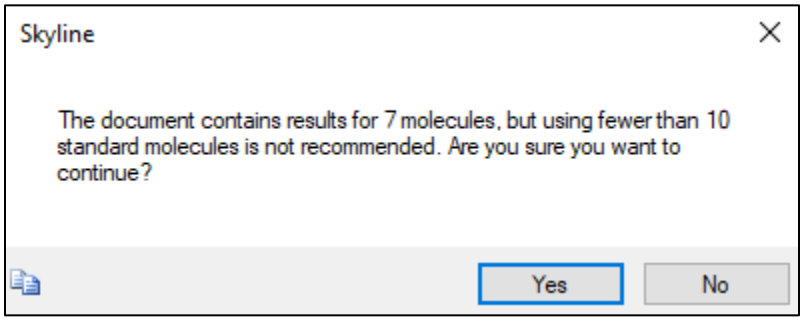
A new window will appear, **Calibrate iRT Calculator**, which will look like this:



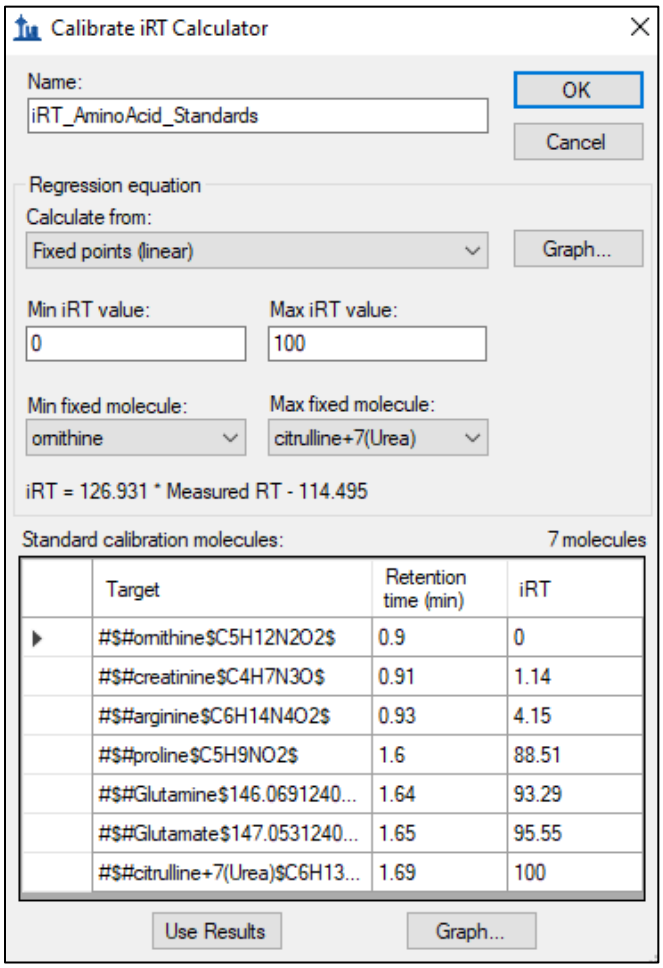
- Name our iRT Calculator **iRT_AminoAcid_Standards** and click on the **Use Results** button



- A warning will appear saying that using fewer than 10 standard molecules is not recommended. As we have limited analytes for this study, we will only be using five but 10 or more should be used if possible. Click **Yes.**

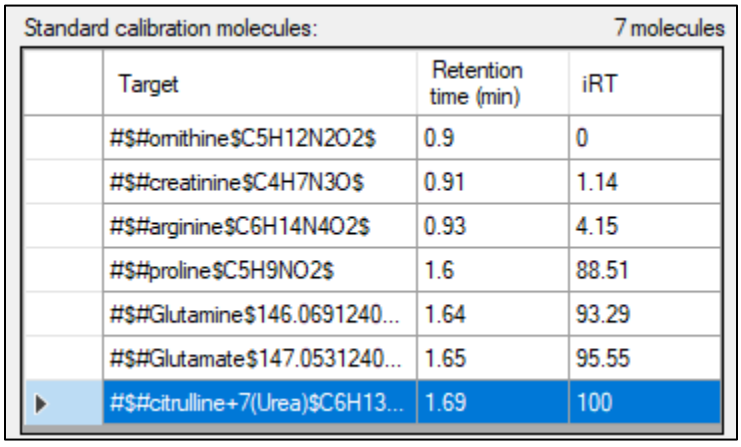


Your **Calibrate iRT Calculator** window should now be populated with every molecule measured in this dataset, similar to below:

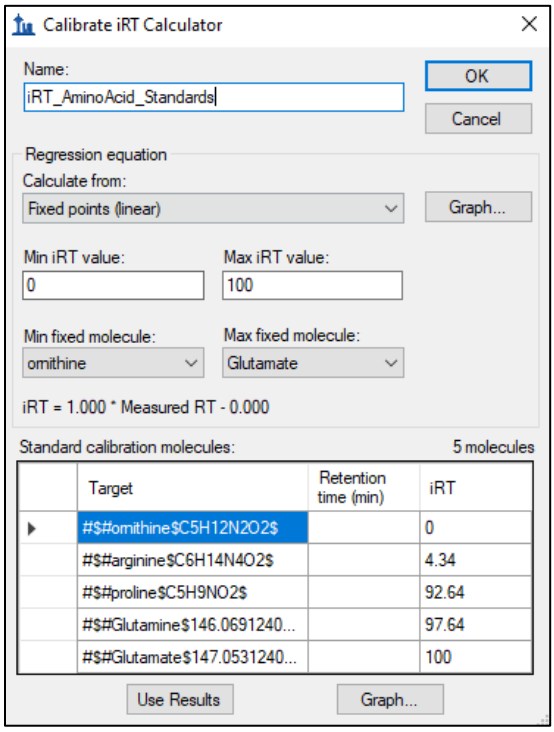


Earlier in this tutorial, we saw at least one molecule that had sizable variation and was therefore not suitable as a retention time standard. We will now remove those molecules before normalizing retention times.

- Click the tab next to the row containing “citrulline+7(Urea)” and remove the molecule from list by pressing Delete on your keyboard. Do the same for creatinine.

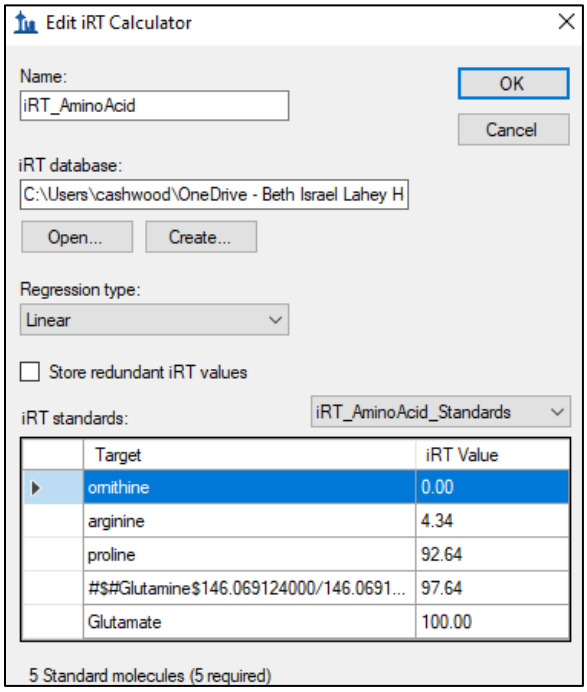


Your Standard Calibration molecules should now look like this:

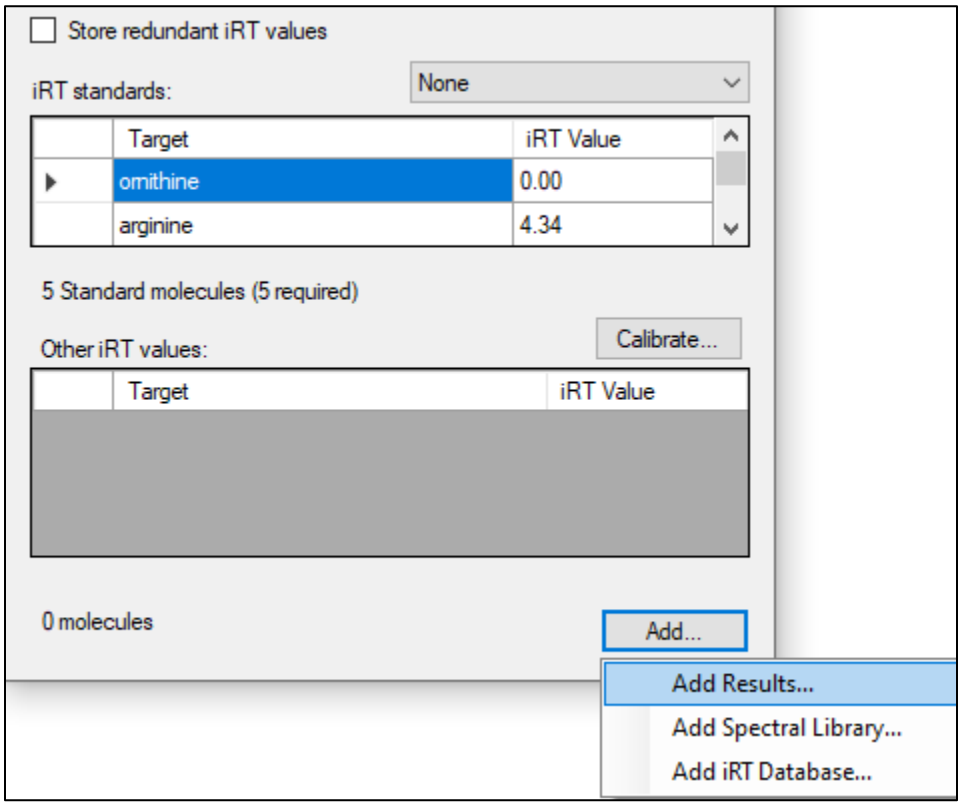


Ornithine is the earliest analyte to elute with a retention time of 0.9 min and glutamate is the last to elute with a retention time of 1.66 min. The iRT values assigned to each of these molecules are arbitrary values reflecting the retention times. The minimum and maximum iRT values can be changed depending on your preferences.

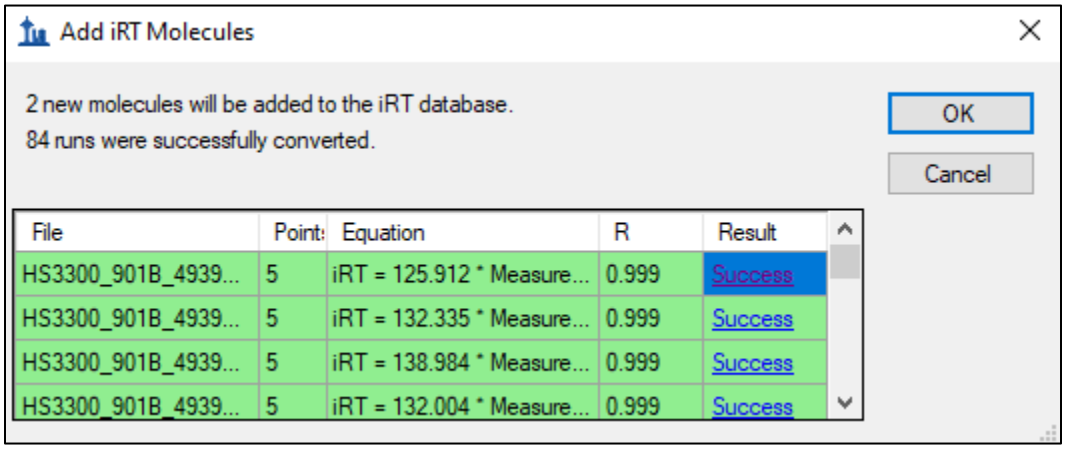
- Click **OK** on the **Calibrate iRT Calculator** window. You should be back to the **Edit iRT Calculator** window and it should look as follows. Note that 5 standard molecules is the absolute minimum for creating an iRT calculator.



- Verify iRT was successfully calibrated by clicking **Add** at the bottom of the window and click **Add Results**



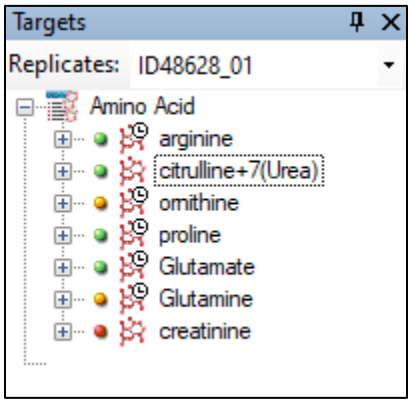
- A prompt will appear confirming that 84 runs were successfully converted. The blue hyperlink **Success** can be clicked to review the calibration for each raw file.



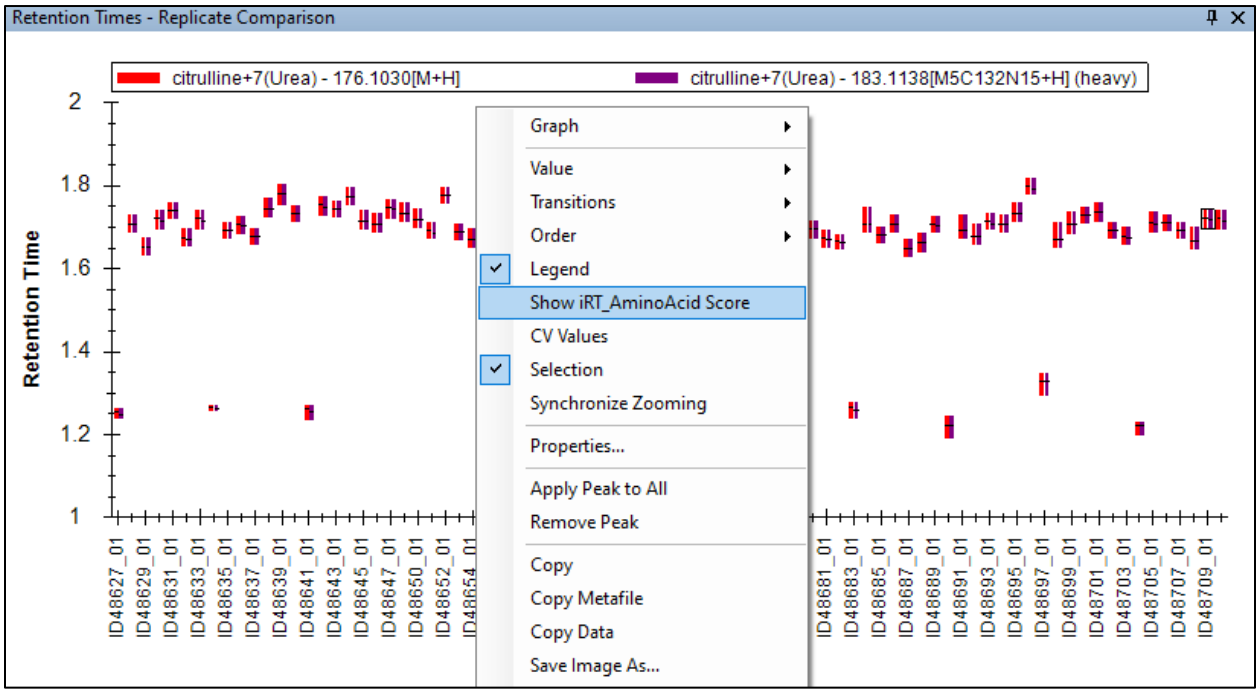
- As we do not want to add two molecules to the iRT database, click **Cancel**. Then, click **OK** on the **Edit iRT Calculator** window and click **Yes** for the prompt asking if you want to continue with only 5 standard molecules. Then click **OK** on the **Molecule Settings** window to be returned back to your Skyline document.

Using the iRT calculator

Your Target list should now have a time icon for the molecules selected as retention time standards, as shown below. We will now evaluate the effectiveness of the retention time normalization by our five retention time standard molecules.

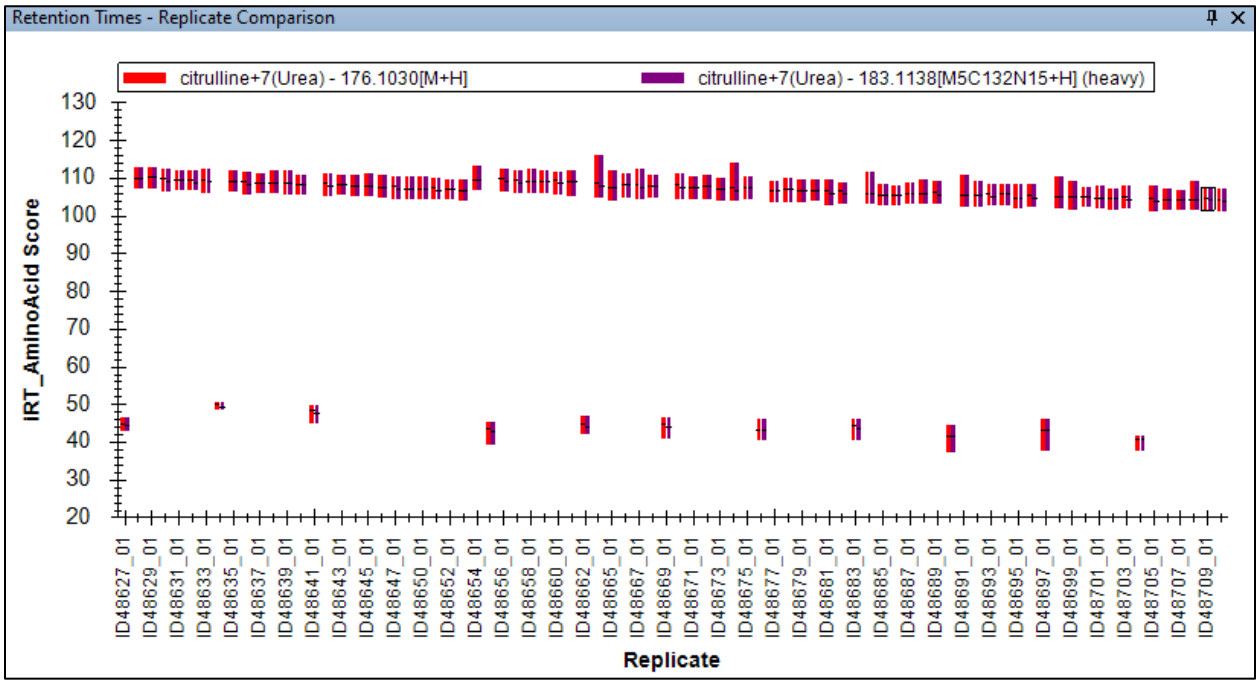


- Select **citrulline+7(Urea)** from the target list, you should see the following **Retention Times - Replicate Comparison** window. If you remember, this molecule was not included as a retention time standard because there was substantial retention time variation.
- Right click on the **Retention Times - Replicate Comparison** plot and a new option will appear in the drop down menu – **Show iRT_AminoAcid_Score**. Select it.

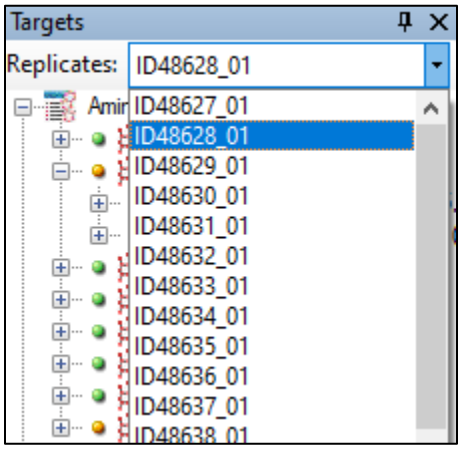


The iRT calculator you previously created has now been used to normalize the retention times into the **iRT_AminoAcid Score**. Ideally, this would reduce the retention time variation between replicates.

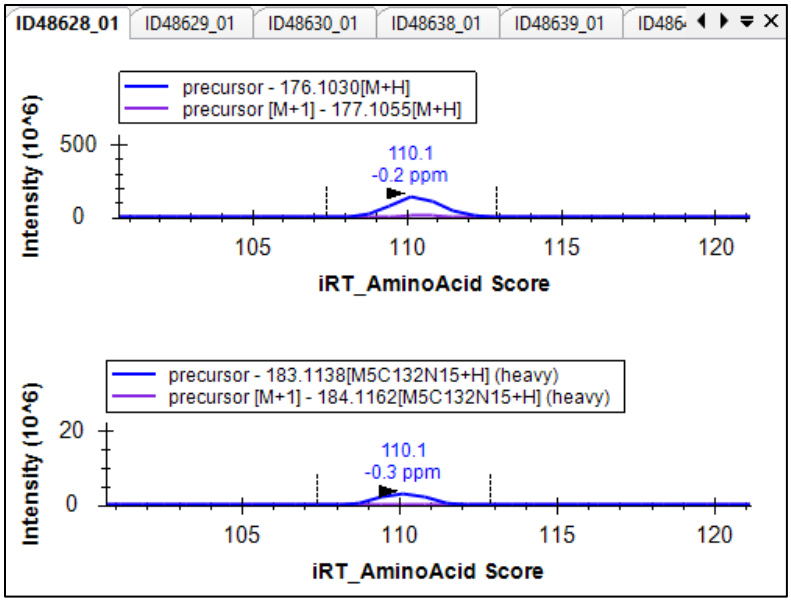
The **Retention Times – Replicate Comparison** plot should now look like below. We can see that the outlier peaks, likely incorrectly picked peaks, are clearly separated from the peaks subjected to the original retention time variation. This retention time variation has been normalized for a more consistent **iRT_AminoAcid Score**. We can now re-integrated these peaks with the previously used synchronized integration feature.



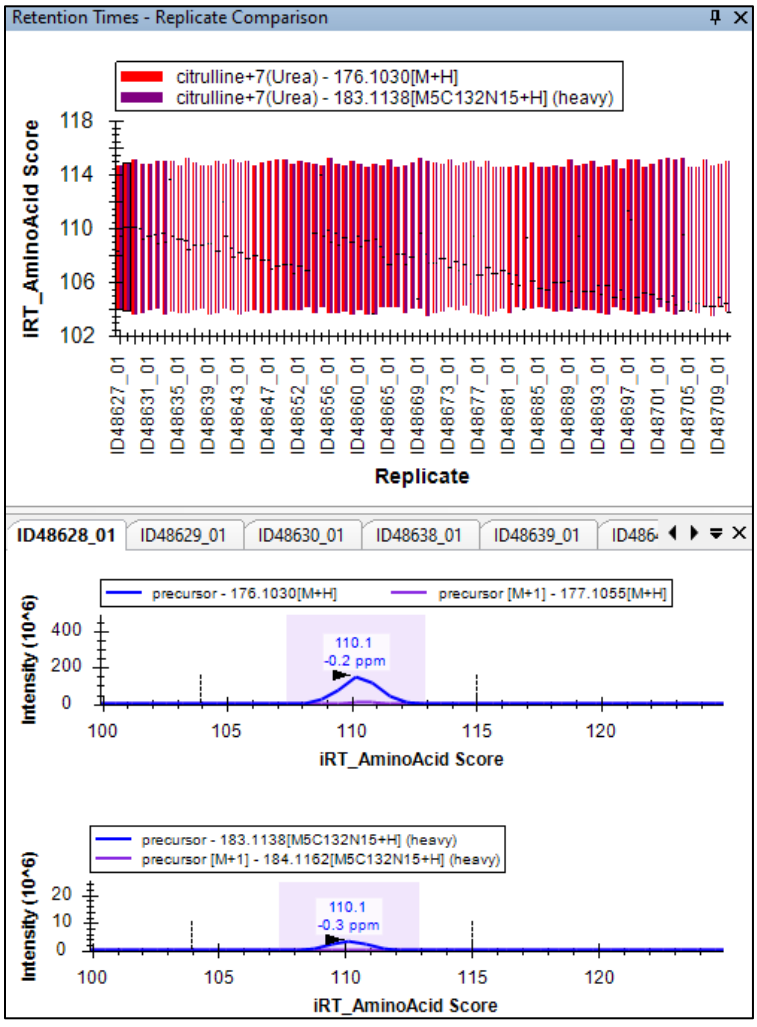
- Select replicate ID48628_01 in the **Targets** window.



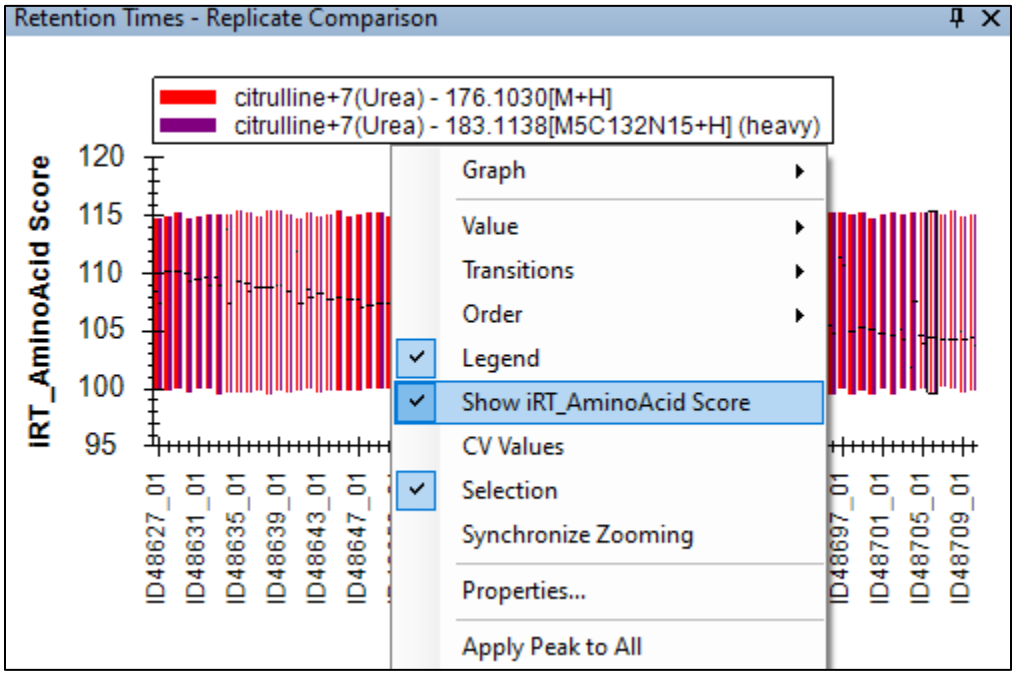
- Adjust your X-axis on the **Chromatogram** view to ensure the peak is observed at 110.1 **iRT_AminoAcid Score**. Note that the previous **Retention Time** label has been replaced by **iRT_AminoAcid Score** and it reflects the above **Retention Times – Replicate Comparison** window above.



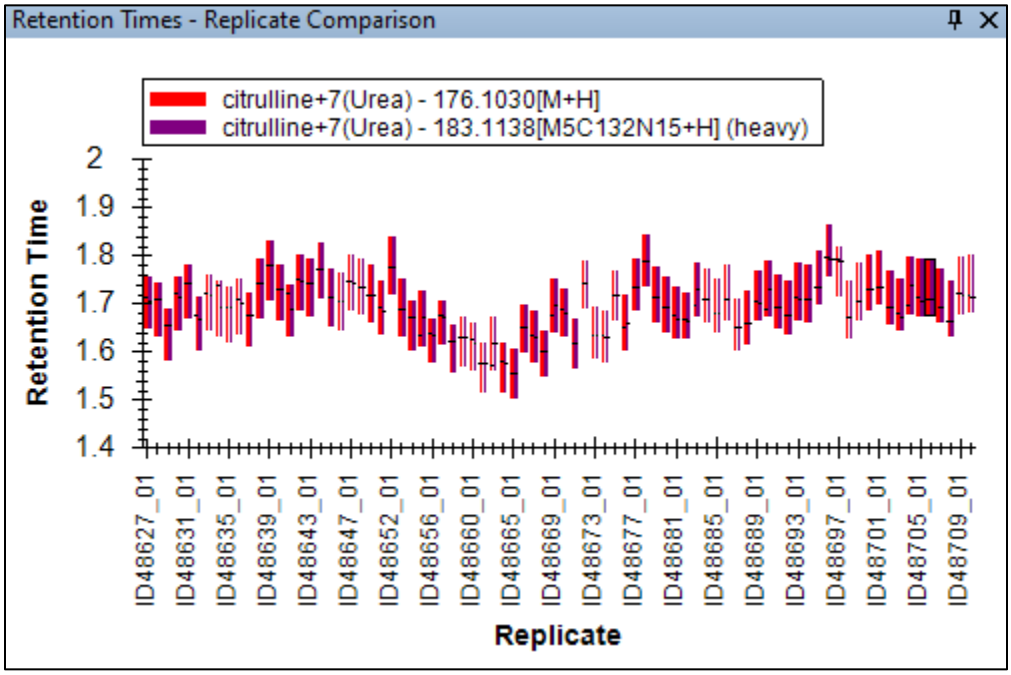
- Adjust the integration window to start at 100 to 115 **iRT_AminoAcid Score**. Your window should now look like below, with all replicates now having the same integration window.



- Evaluate the effectiveness of the retention time normalization by switching back to our retention time view. Right click on the **Retention Times – Replicate Comparison** plot and uncheck **Show iRT_AminoAcid Score**.

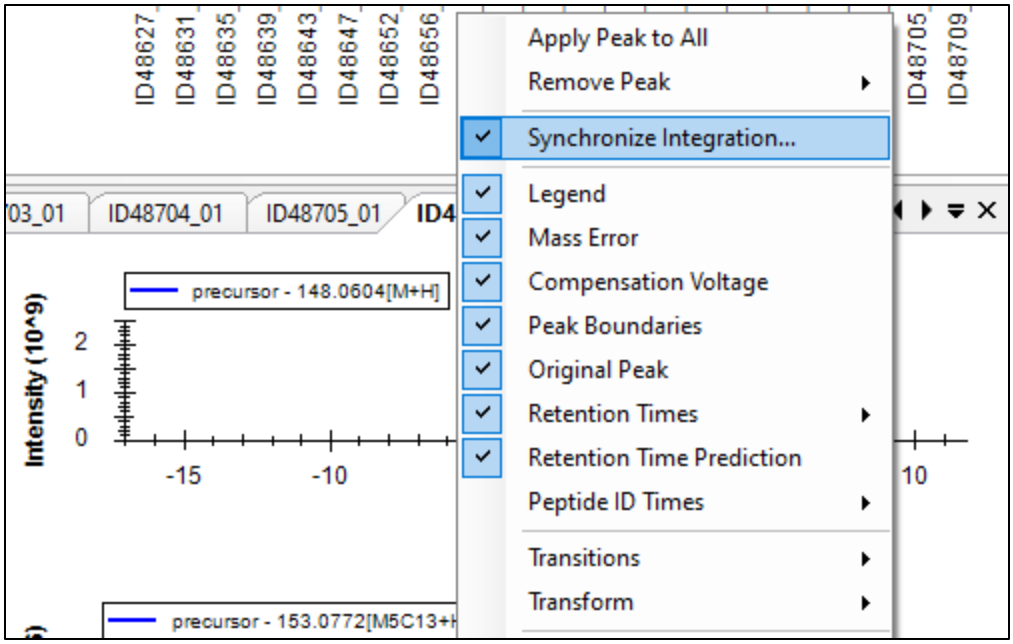


- The following plot should be updated as shown below:

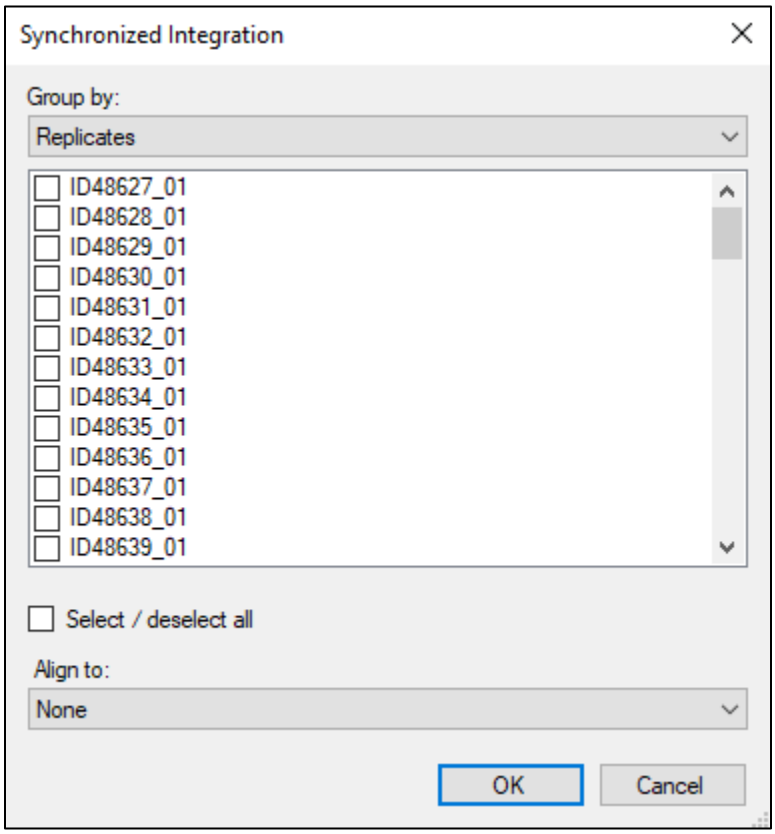


Here we can see that using iRT has allowed us to maintain tight integration windows while also ensuring complete peak integration across all replicates, something that would not have been possible with Synchronized Integration alone. The last step for this tutorial is to turn off the **Synchronized Integration** feature.

- Right click on the **Chromatogram** plot and select **Synchronize Integration**



- Uncheck the **Select / deselect all** checkbox and your window should change like below:



- Click **OK**. Your integrations are no longer synchronized.

Conclusion

In this tutorial, you have learned how to use the synchronize integration feature to integrate multiple replicates in a single click, build an iRT calculator using small molecules, and use iRT normalization with synchronized integration to ensure consistent and specific peak picking across a dataset with variable retention times. While we have successfully used the minimum number of molecules for this tutorial, we recommend using additional standard molecules for iRT if the retention time variation has not been sufficiently addressed in your own datasets.

Bibliography

1. Adams KJ, *et al*. Capillary Electrophoresis-High Resolution Mass Spectrometry for Measuring In Vivo Arginine Isotope Incorporation in Alzheimer's Disease Mouse Models. J Am Soc Mass Spectrom. 2021 Jun 2;32(6):1448-1458. doi: 10.1021/jasms.1c00055. Epub 2021 May 24. PMID: 34028275.