BVB328 Practical Appendix 3 – Week 5

Calibration Curve and Analytical Figures of Merit in Skyline

Skyline is a Windows-based application developed in MacCoss Lab at the University of Washington. It is open source and freely available for academic and commercial use. It supports chromatography-based quantitation (both relative and absolute) from a range of data acquisition approaches (MRM, DDA, SWATH etc) and mass spectrometry platforms and works well with small molecule and proteomics workflows.

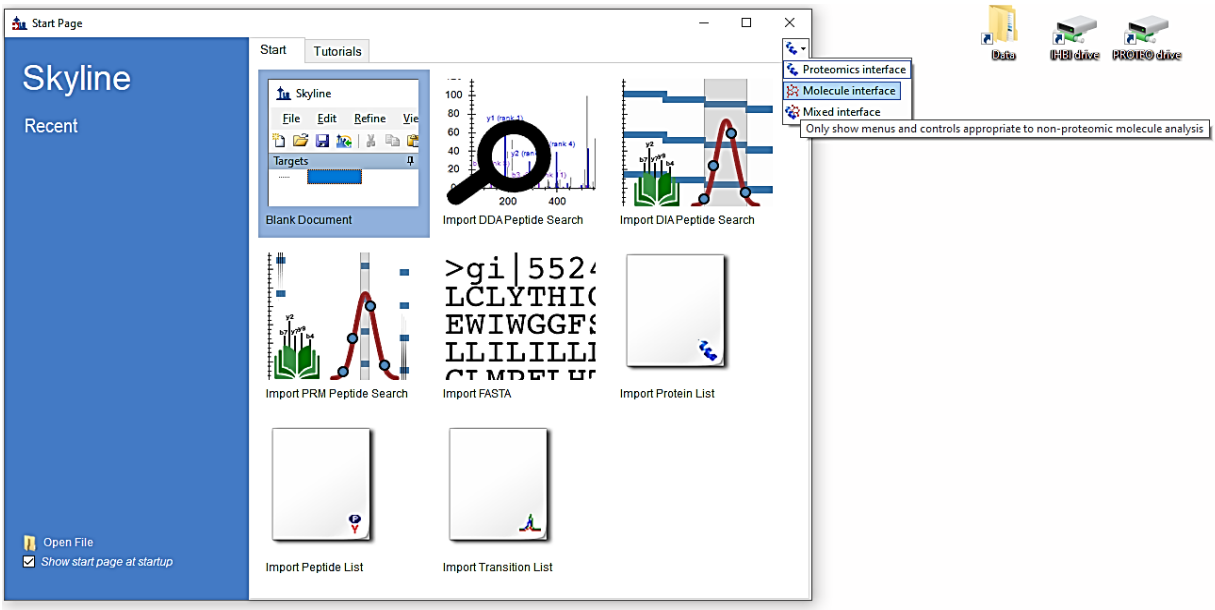
In this tutorial you will analyse quantitative mass spectrometry data collected on Shimadzu LC-MS 8050 instrument using MRM approach. Specifically, you will conduct absolute quantitation of D-asparagine using the standard curve method and normalisation to internal standard (L-valine-13C_5).

When it comes to regression analysis and determining figures of merit, Skyline offers several methods of fitting a line to calibration data including bilinear regression fit, which allows one to choose to calculate the limit of detection as the bilinear turning point. This method, introduced recently by MacCoss and co-workers (Galitzine *et al.;* 2018), was shown to provide more accurate estimate of limits of detection and more accurate line fit to experimental mass spectrometry data, that is often non-linear in the lower range, and we will apply it in this tutorial.

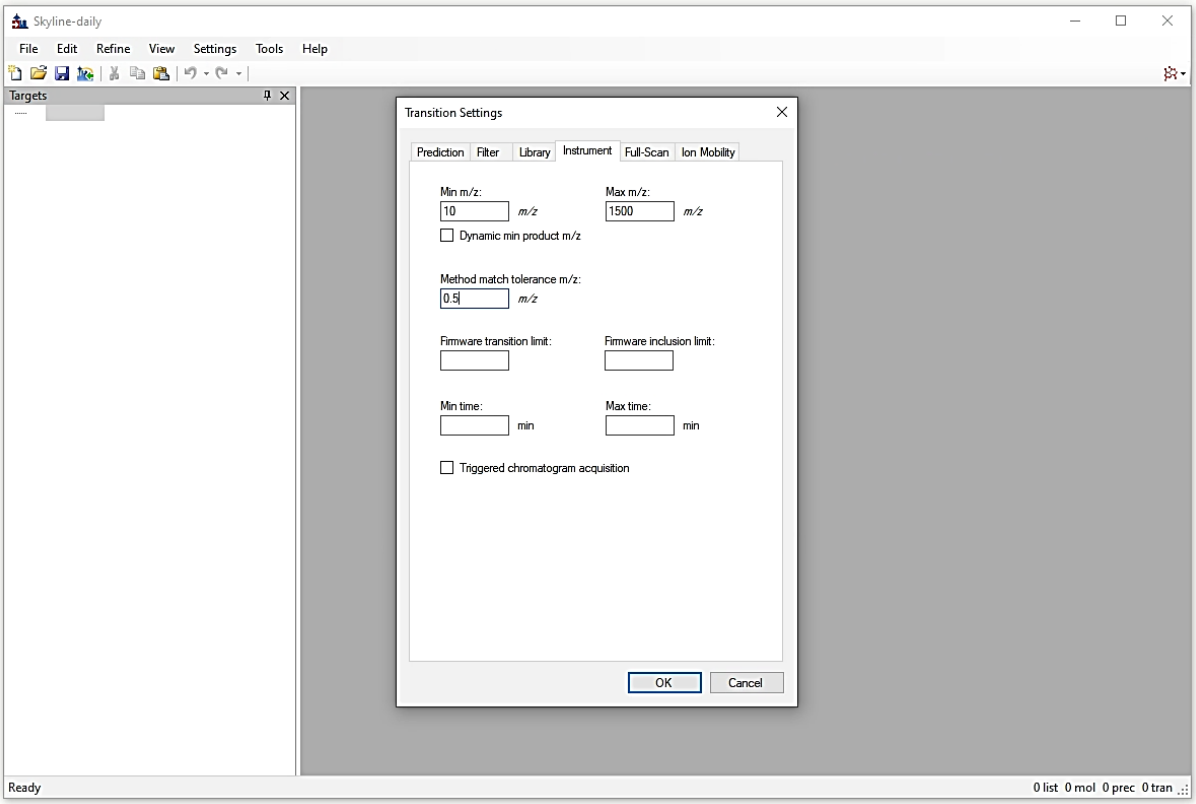
Galitzine C. *et al.,* (2018) Nonlinear Regression Improves Accuracy of Characterization of Multiplexed Mass Spectrometric Assays. *Mol Cell Proteomics*. 17(5):913-924. DOI: 10.1074/mcp.RA117.000322

Defining a list of *m/z* to be extracted

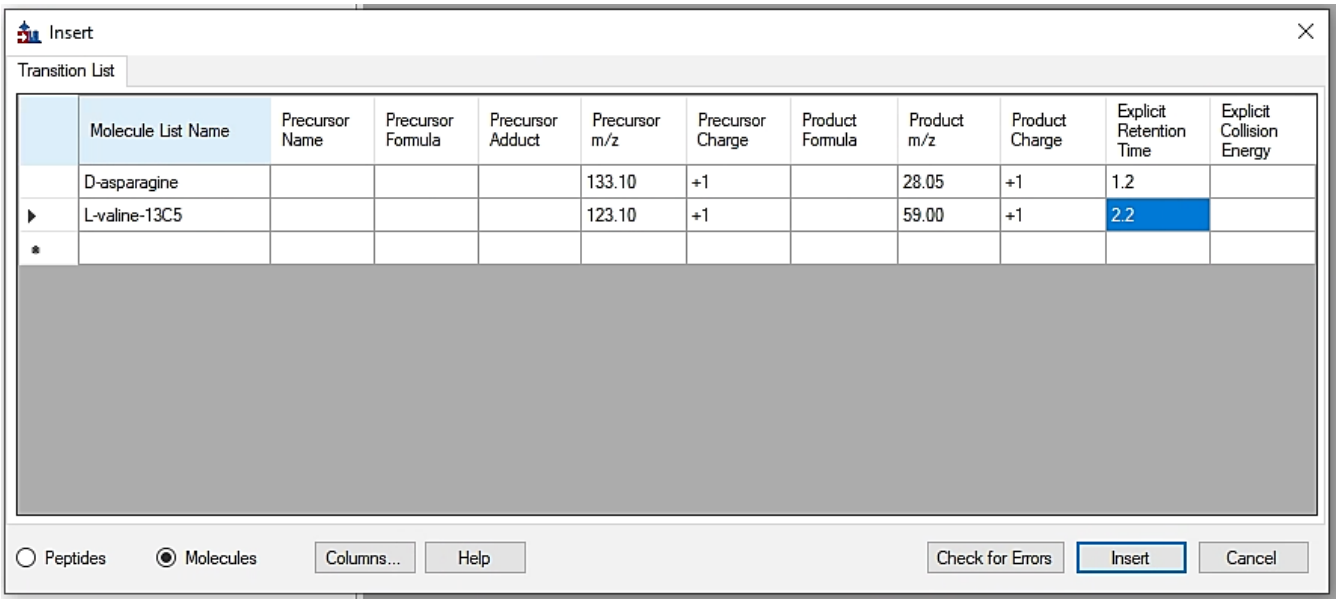
1. Open Skyline software
2. In Skyline's "Start Page" window, switch interface to "Molecule", and click on "Blank Document"



3. In "Settings" menu, select "Transition Settings", and there in "Instrument" tab, set **Min m/z** to "10" and **Method match tolerance m/z** to "0.5".

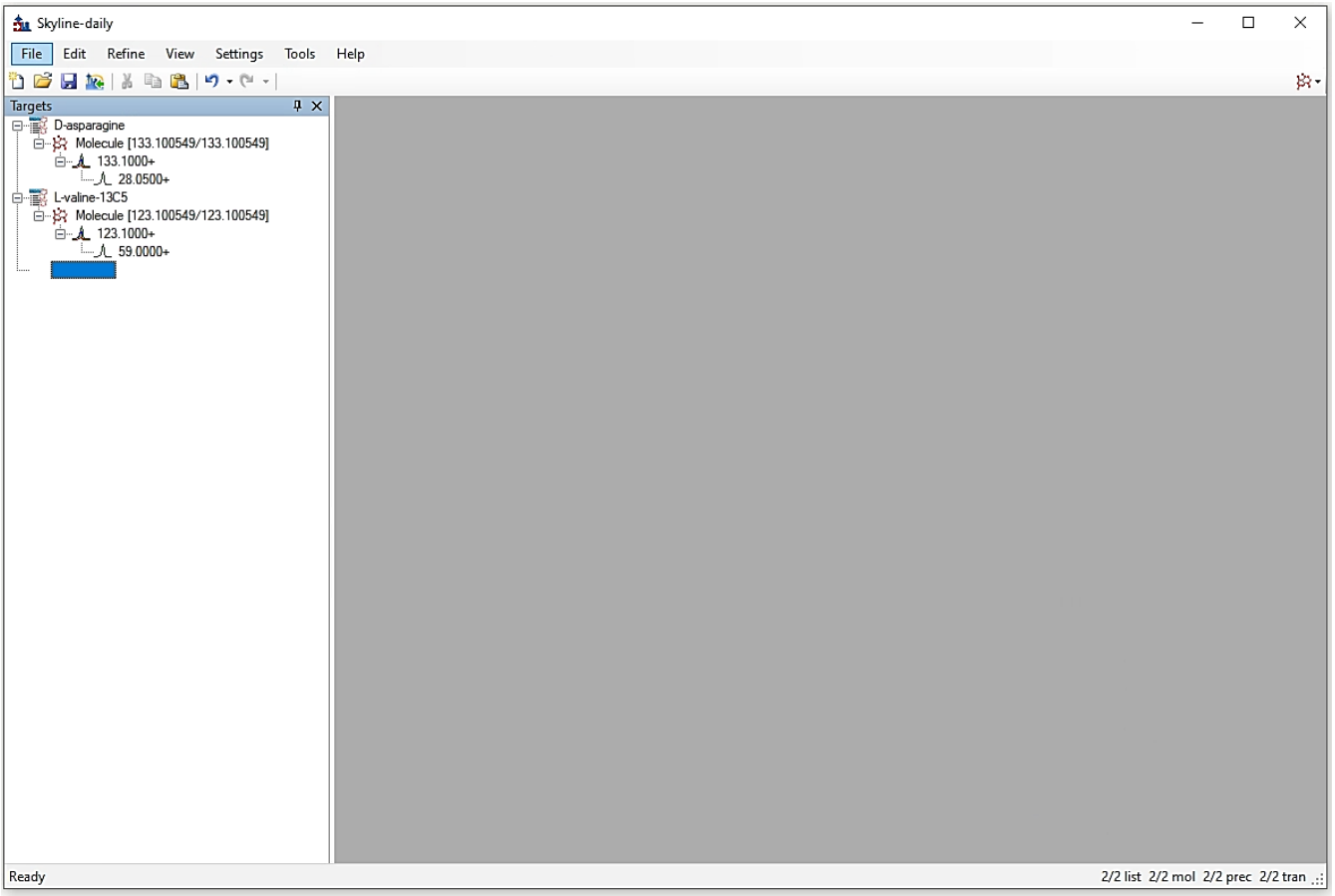


4. Click "OK" button.
5. In "Edit" menu, select "Insert", and then "Transition List", and update entries in "Insert" window as shown below:

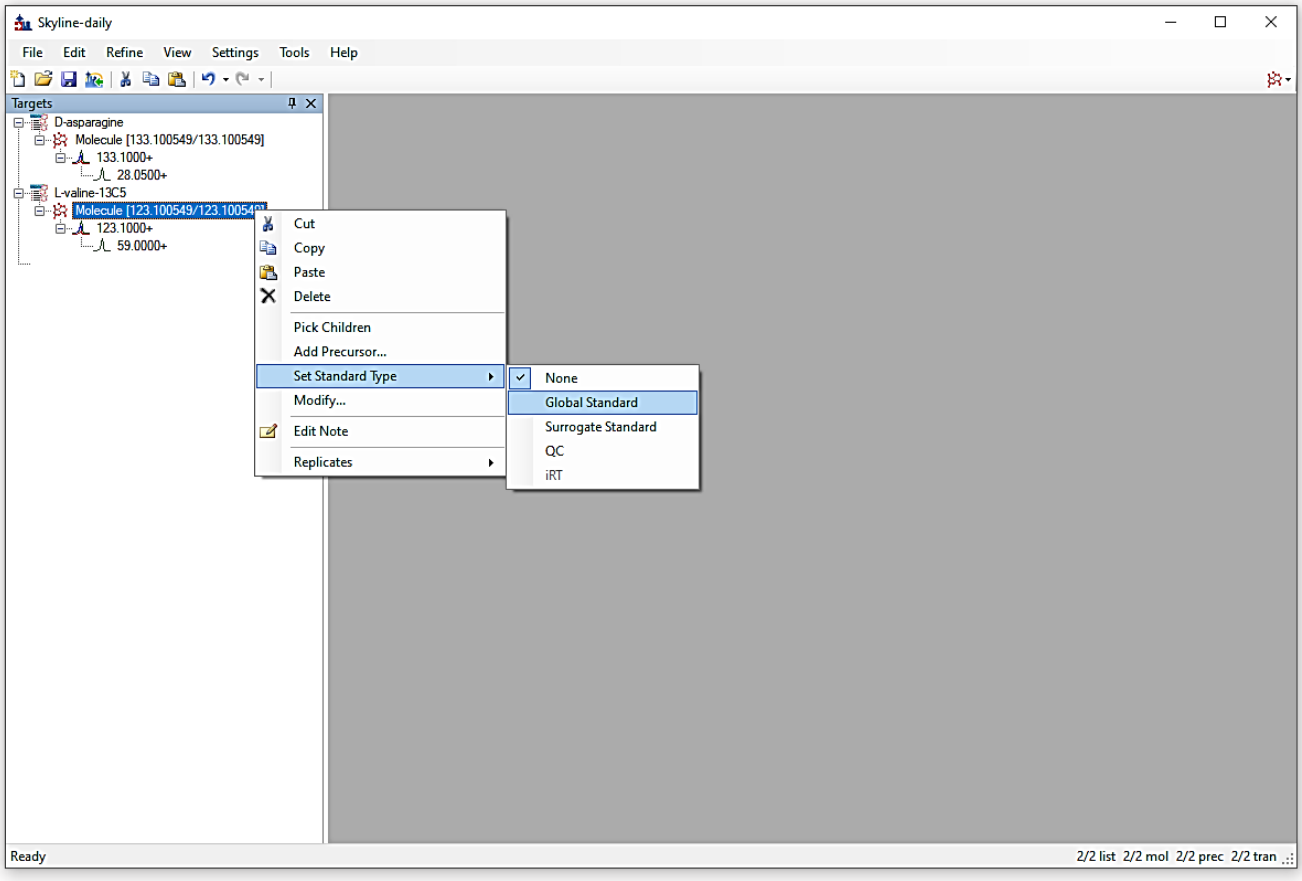


Note that if any required column is not displayed, you should click “Columns” button and adjust displayed columns accordingly.

6. Click “Insert” button.
7. In “Edit” menu, select “Expand All”, and then “Precursors”. You should now see Skyline window look like this:



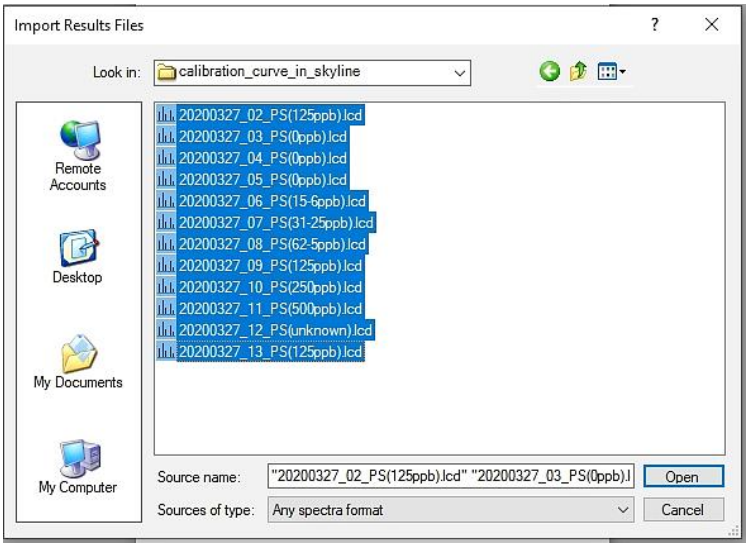
8. Right click on **Molecule [123.100549/123.100549]** under L-valine-13C5 and select “Set Standard Type”, and then “Global Standard”.



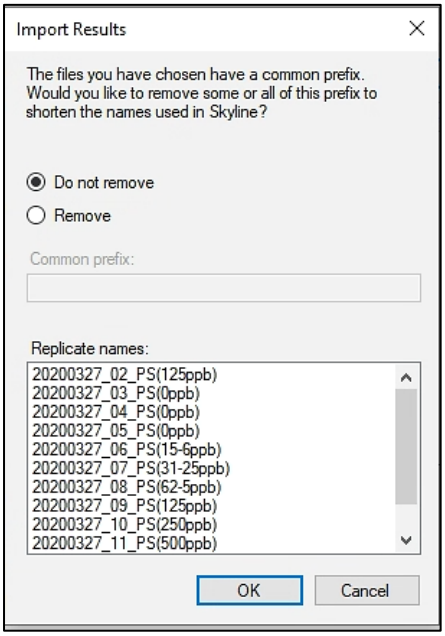
9. In “File” menu, select “Save As”, and save Skyline document with your name of choice i.e. Sample calibration curve. The document is now ready for importing data.

Importing and annotating data

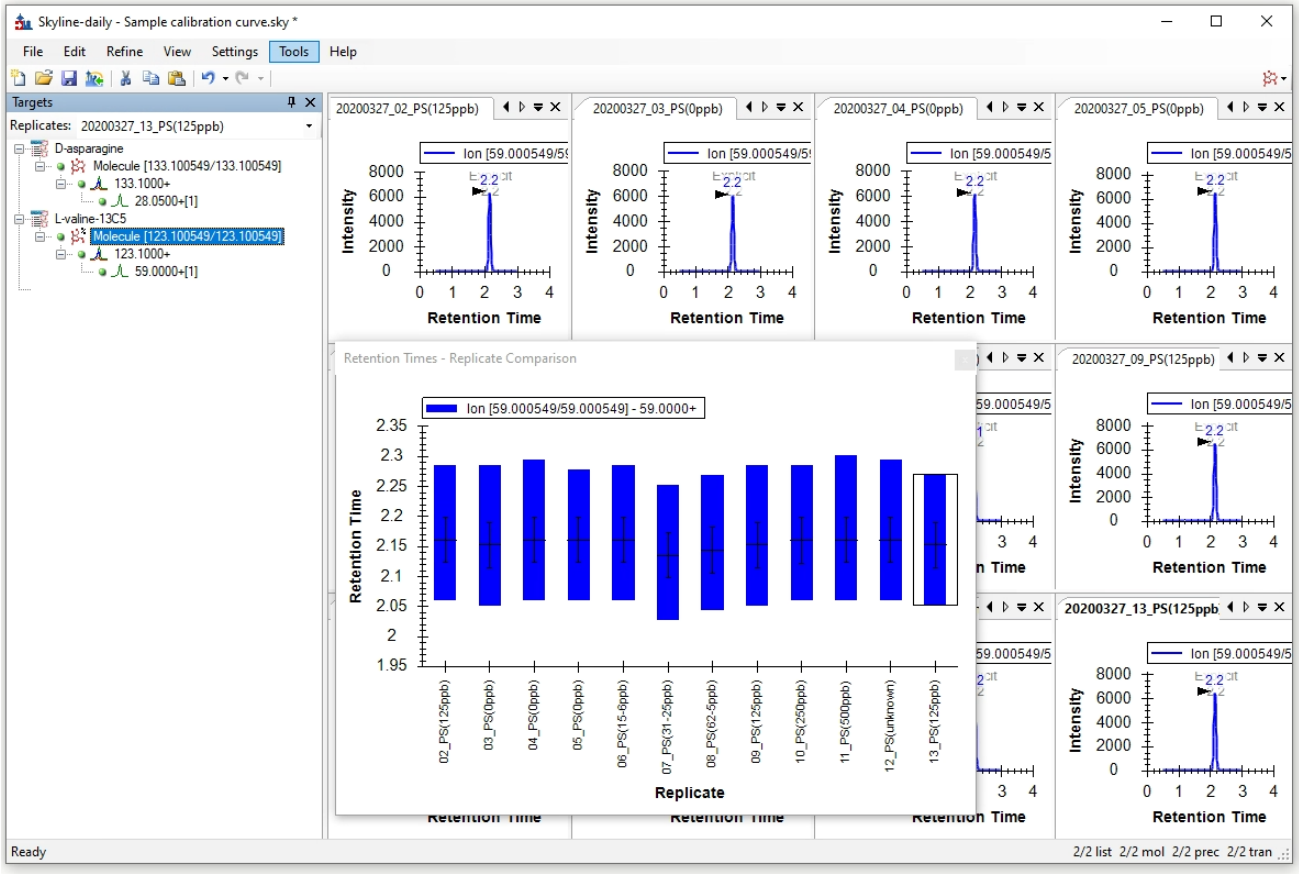
10. In “File” menu, select “Import”, and then “Results”, and click “OK” button.
11. Browse to the folder containing the downloaded sample data, and select all files in that folder, and click “Open” button.



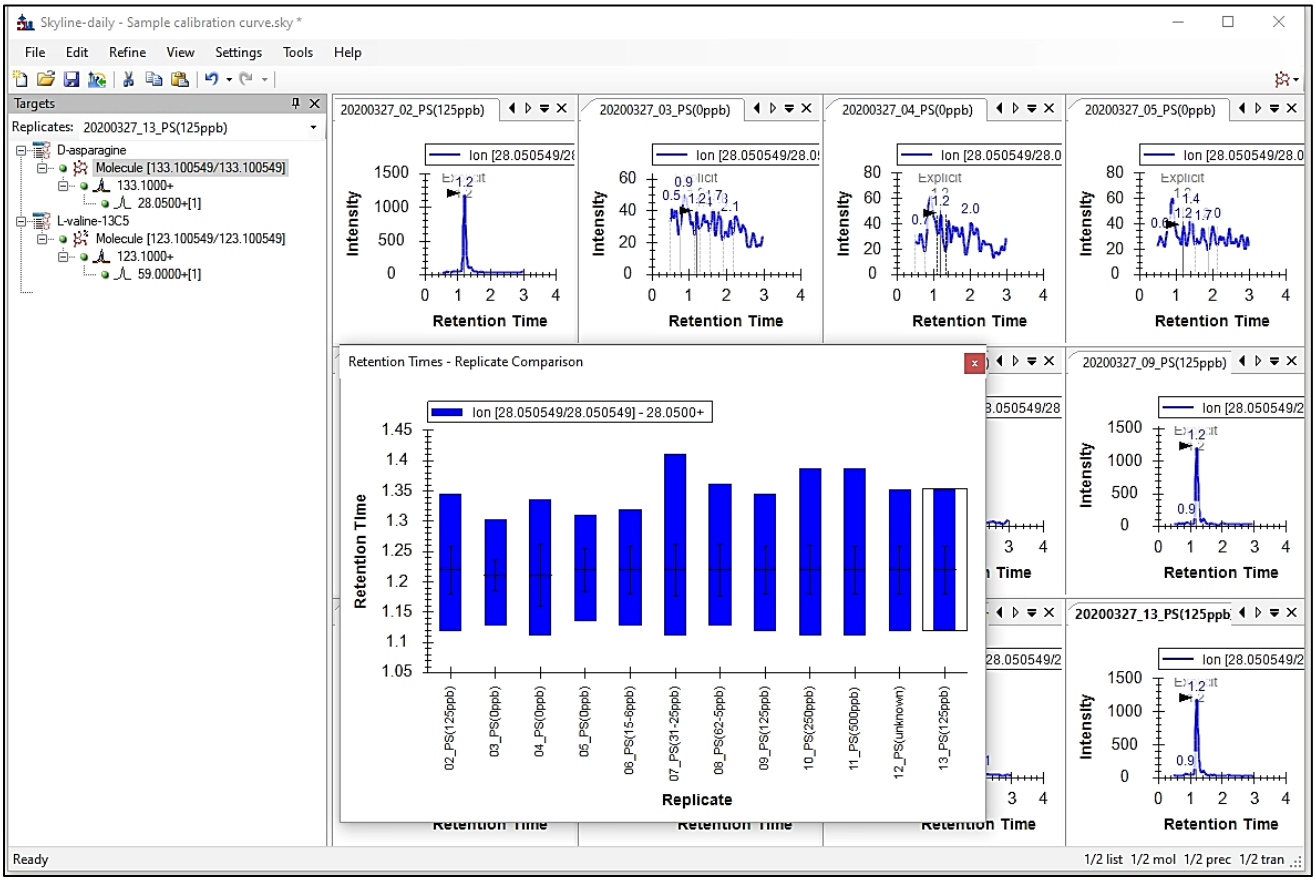
12. In “Import Results” window, select “Do not remove” option and click “OK”.



13. Wait until the status bar at the bottom of Skyline window states “Ready”.
14. In “View” menu, select “Arrange Graphs”, and then “Tiled”.
15. In “View” menu, select “Retention Time”, and then “Replicate Comparison”
16. Now if you click on **Molecule [123.100549/123.100549** under **L-valine-13C5**, you should see chromatographic data together with “Retention Time - Replicate Comparison” plot for this internal standard like this:



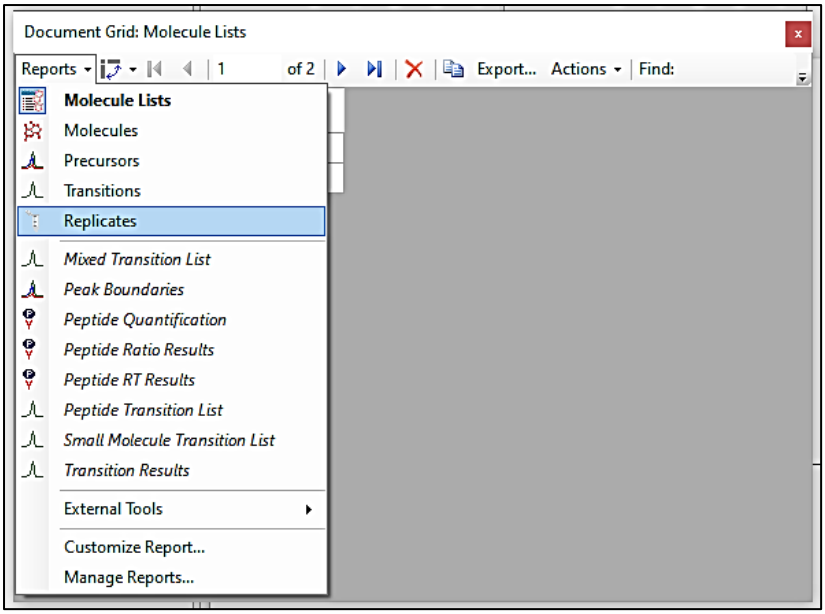
17. And if you click on **Molecule [133.100549/133.100549]** under **D-asparagine,** you should see chromatographic data together with “Retention Time - Replicate Comparison” plot for this target analyte like this:



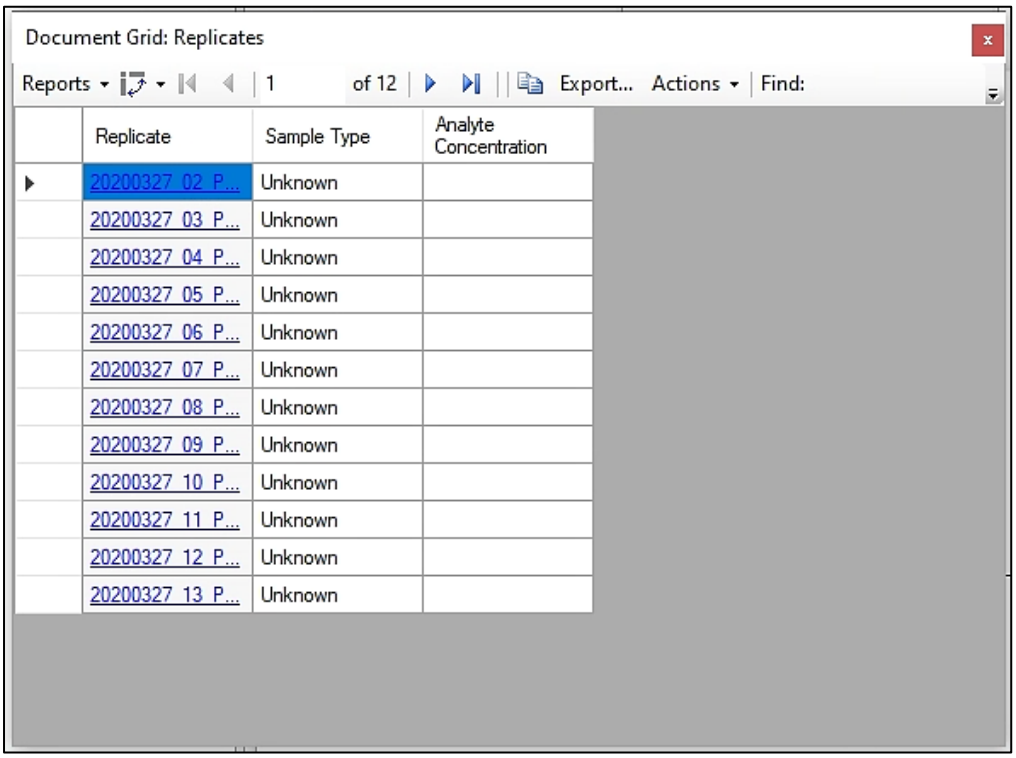
Note that the “Retention Time - Replicate Comparison” plots allow you to check if the same area of a chromatogram was integrated for the same analyte in all samples. If you notice any inconsistency, you should click on problematic sample and conduct manual adjustment of peak boundaries. Manual integration may be necessary in the case of non-gaussian peaks, or blank samples, or samples with very low signal, or samples in which a presence of interference signal prevents Skyline from picking the right peak.

Also note that Skyline interface is fully customizable. You can drag and drop windows in different location/arrangement.

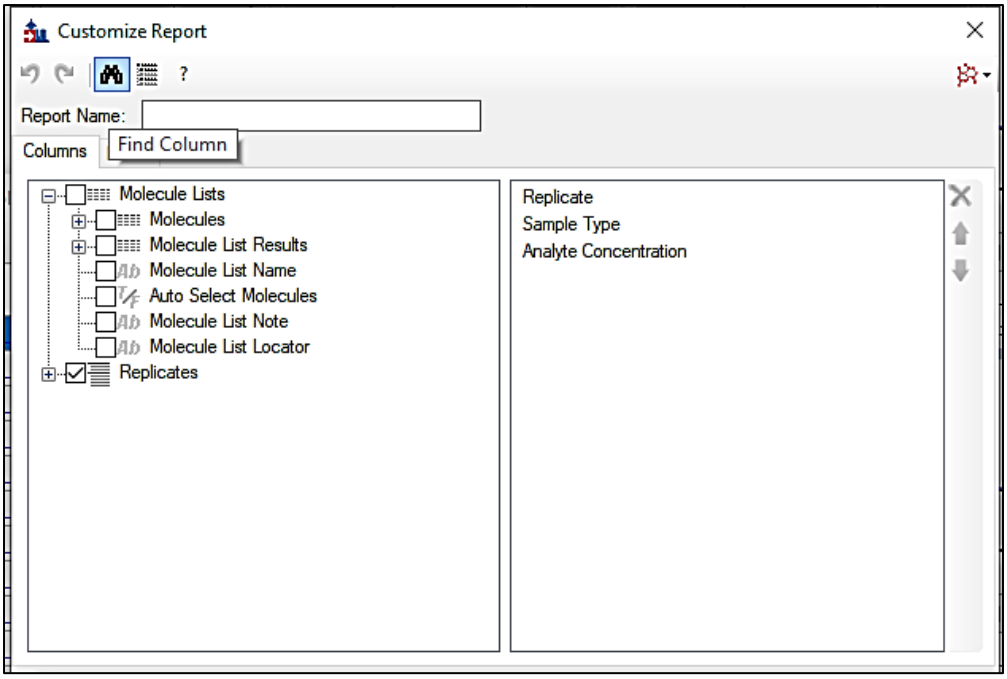
18. In “View” menu, select “Document Grid”.
19. In “Document Grid” window, select “Reports”, and then “Replicates”.



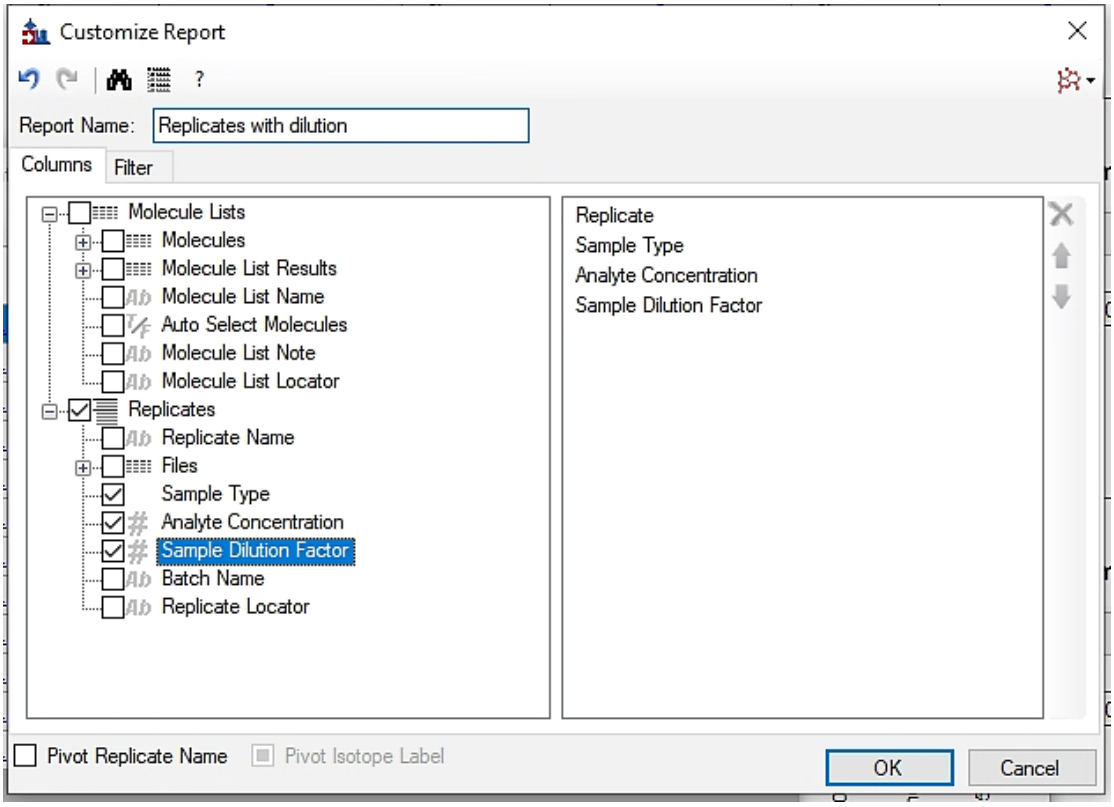
You should see “Document Grid” window look like below:



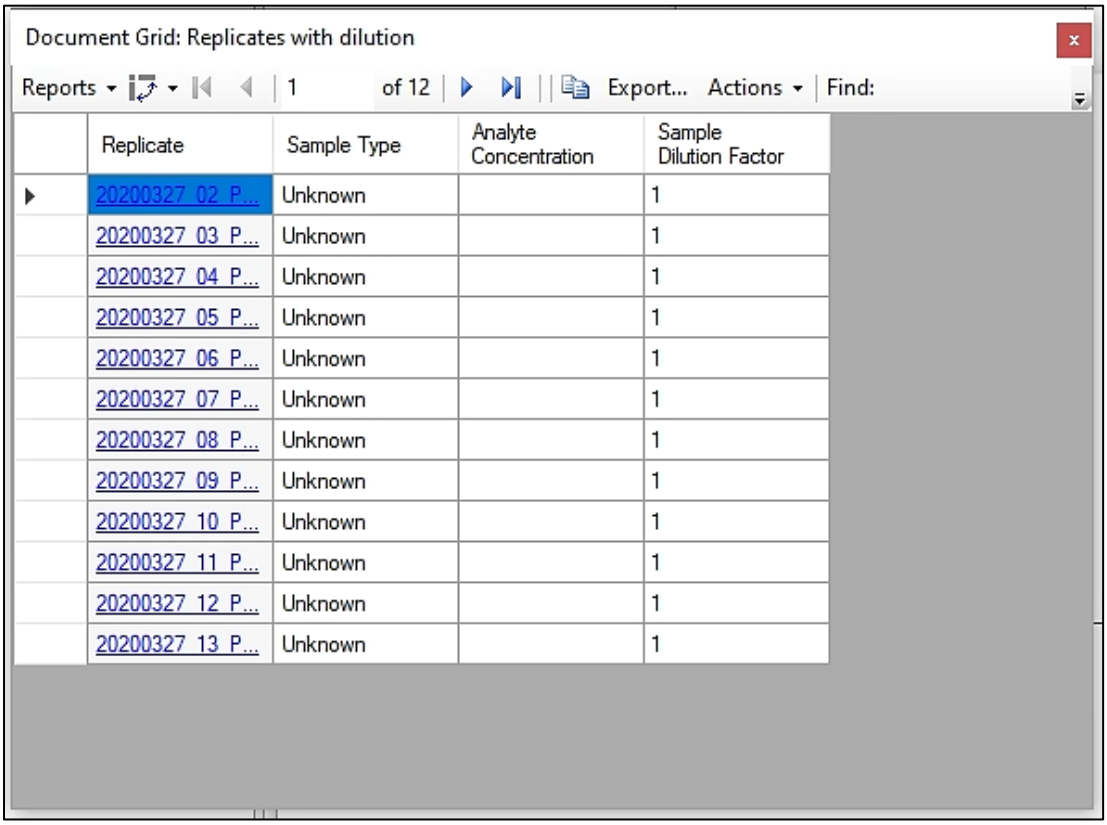
20. To display an additional column corresponding to dilution factor, in “Document Grid” window, select “Reports”, and then “Customize Report”.
21. In “Customize Report” window, click on **binoculars** icon.



22. In **Find what** field of “Find Column” window, type “dilution”, and then click “Find Next” button and then click “Close” button.
23. In **Report Name** field of “Customize Report” window, type “Replicates with dilution”, and then tick the checkbox next to the “Sample Dilution Factor”, and then click “OK” button.

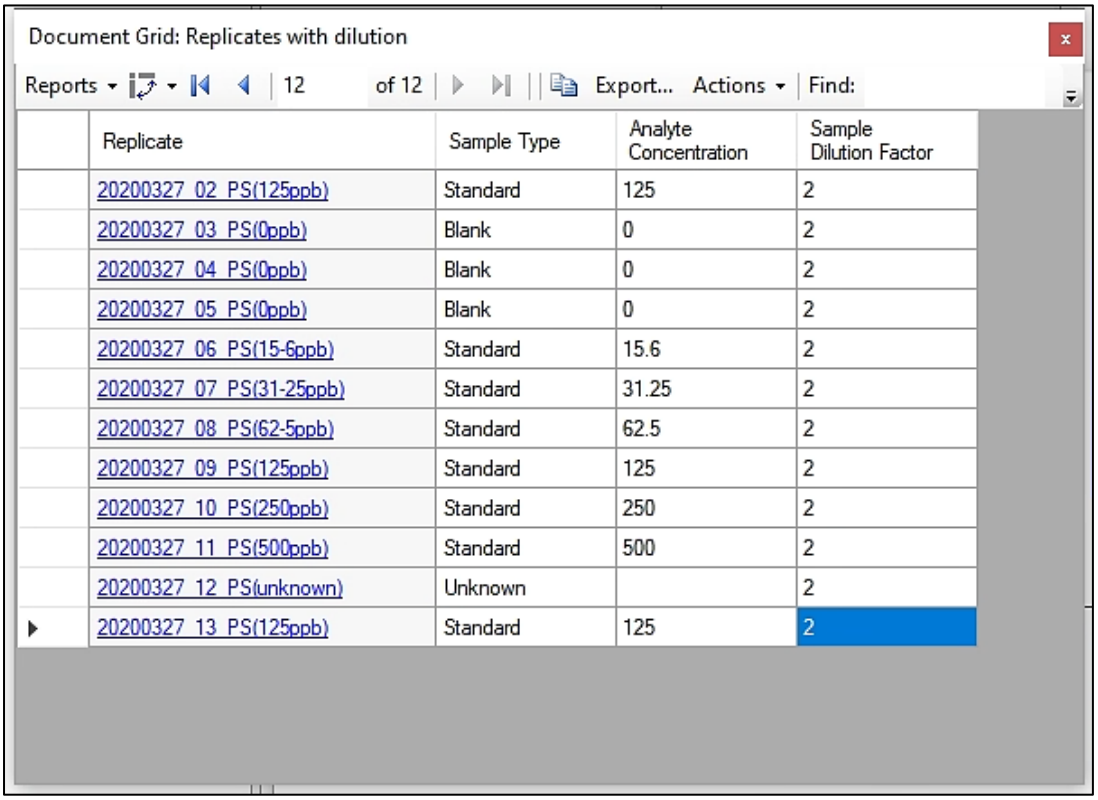


The “Document Grid” window should now look like below:



	Replicate	Sample Type	Analyte Concentration	Sample Dilution Factor
▶	20200327_02_P...	Unknown		1
	20200327_03_P...	Unknown		1
	20200327_04_P...	Unknown		1
	20200327_05_P...	Unknown		1
	20200327_06_P...	Unknown		1
	20200327_07_P...	Unknown		1
	20200327_08_P...	Unknown		1
	20200327_09_P...	Unknown		1
	20200327_10_P...	Unknown		1
	20200327_11_P...	Unknown		1
	20200327_12_P...	Unknown		1
	20200327_13_P...	Unknown		1

24. Now update the entries in “Document Grid” window as shown below:



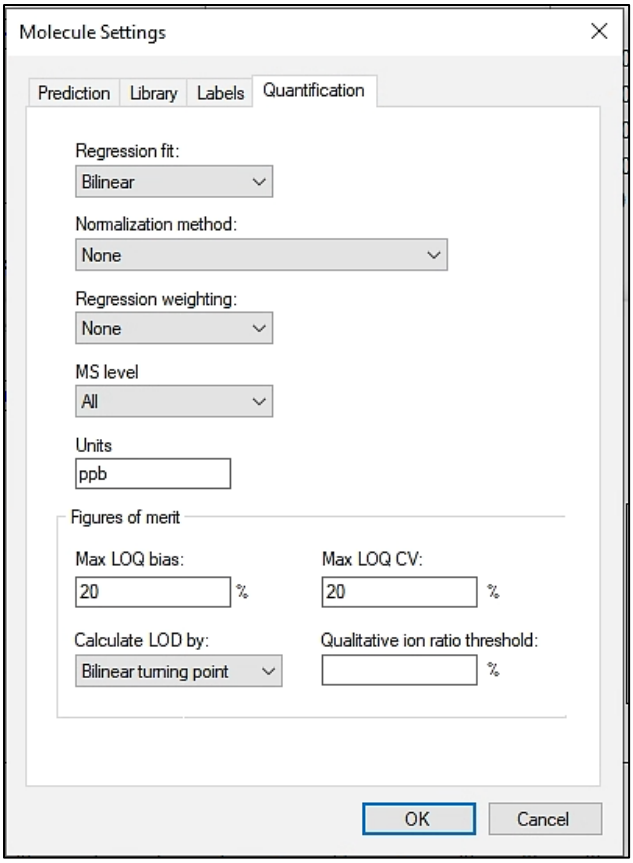
	Replicate	Sample Type	Analyte Concentration	Sample Dilution Factor
	20200327_02_PS(125ppb)	Standard	125	2
	20200327_03_PS(0ppb)	Blank	0	2
	20200327_04_PS(0ppb)	Blank	0	2
	20200327_05_PS(0ppb)	Blank	0	2
	20200327_06_PS(15-6ppb)	Standard	15.6	2
	20200327_07_PS(31-25ppb)	Standard	31.25	2
	20200327_08_PS(62-5ppb)	Standard	62.5	2
	20200327_09_PS(125ppb)	Standard	125	2
	20200327_10_PS(250ppb)	Standard	250	2
	20200327_11_PS(500ppb)	Standard	500	2
	20200327_12_PS(unknown)	Unknown		2
▶	20200327_13_PS(125ppb)	Standard	125	2

Note that dilution factor of ‘2’ was also applied to calibration curve samples and this is because they were diluted 2x when spiking in with internal standard.

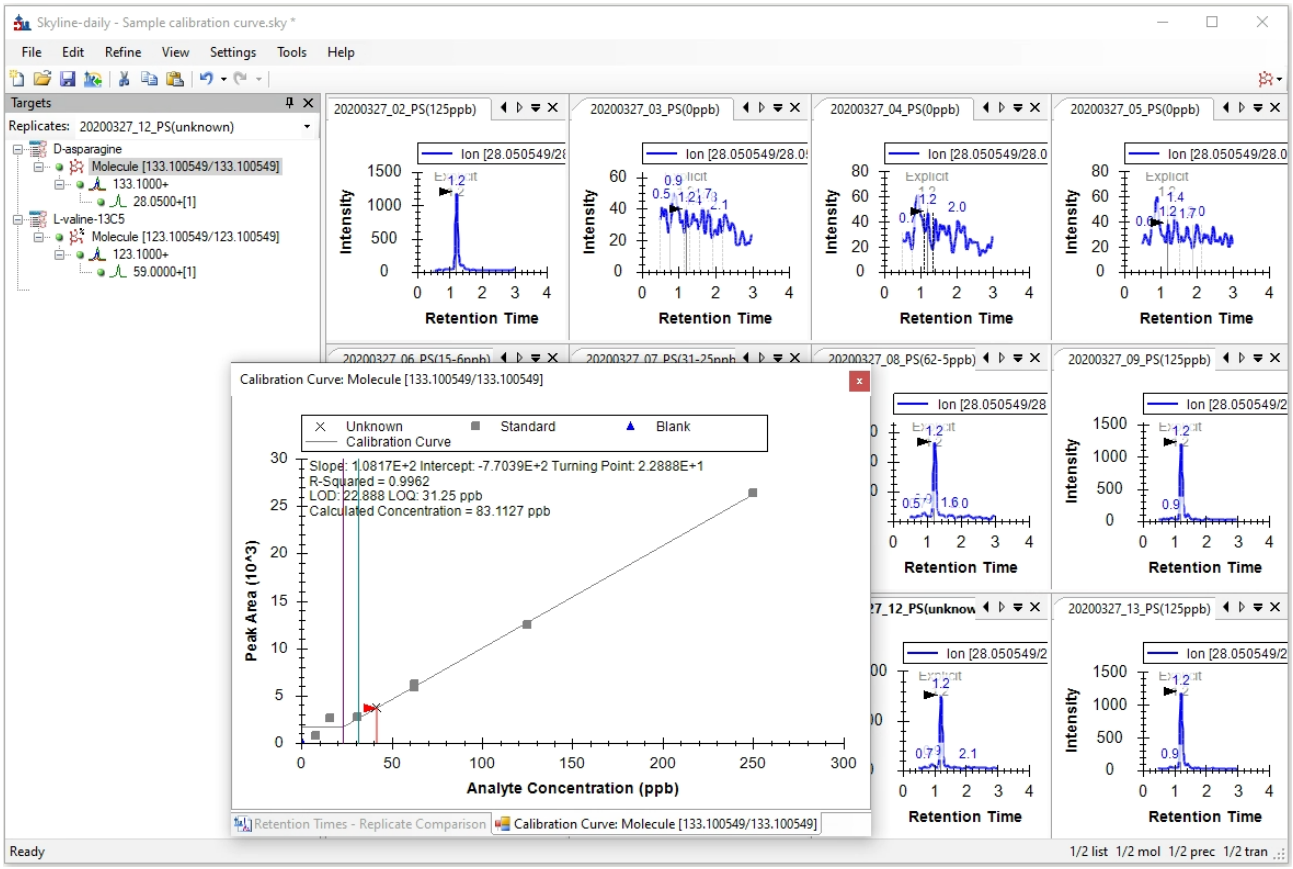
25. Click “X” button to close “Document Grid” window.
26. In “File” menu, select “Save”. The document is now ready for regression analysis.

Fitting line and determining concentration

27. In “Settings” menu, select “Molecule Settings”, and there in “Quantification” tab adjust the entries as shown below:



28. Click “OK” button.
29. In “View” menu, select “Calibration Curve”.
30. Now if you click on **Molecule [133.100549/133.100549]** under **D-asparagine,** you should see calibration curve plot for this analyte like this:



Note that you can adjust what is displayed on the plot by right clicking on it, and selecting “Show Legend”, “Show Selection”, and “Show Figures of Merit”. Also, in “Show Sample Type”, select “Unknown”, “Standard” and “Blank”.

31. To read concentration in test sample, simply click on “x” sample on calibration curve plot and read value from the plot.
32. To generate calibration curve with normalisation to internal standard, in “Settings” menu, select “Molecule Settings”, and there in “Quantification” tab select “Ratio to Global Standards” in **Normalisation method** field.

    Note that the type of regression fit, normalisation method and regression weighing will have an impact on figures of merit. You should test different combinations and choose the one that works best for your dataset (results in the most accurate predictions in the concentration range of interest).