

PMI Chromatogram Analysis (Byomap™) User's Manual

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Contents

Introduction ..... **Error! Bookmark not defined.**
Workflows 1
Tour of Chromatogram Analysis 4
Creating a Reference Project 5
  Sample input Tab 5
    Trace Peaks Options 8
  Protein input tab 11
  Glycan input tab 14
  Glycan options tab 14
  In-silico options tab 15
    Modifications 16
    Glycans 18
    Use table 21
  Candidate matching tab 23
  Advanced and Log tabs 23
Creating a Comparison Project 24
Main Menu Bar 26
  File Menu 26
  File > Export Menu 27
  Edit Menu 29
  Window Menu 35
  Help Menu 36
Table Menu Bars 36
  Filter Options 37
  Table Right-Click Menus 39
  Project Table and Menu 39
  Peaks Table and Menu 40
  Candidates Table and Menu 41
  Protein Coverage Table Display and Menus 42
Plot Menu Bars 43
  Plot Right-Click Menus 46
  Chromatogram Plot and Menus 48
  MS1 Plot and Menus 50
Appendix 50
  Chromatogram Analysis: Advanced Commands 50

Introduction

Chromatogram Analysis (Byomap™) is unique interactive software developed in partnership with biopharmaceutical scientists for detailed, chromatogram-centric analysis of peptide and glycan samples. It is used from late discovery through clinical development to define design space and product profile and it supports Multi-Attribute Method (MAM) reporting. A map serves as a signature of a digested purified protein or detached glycans or any other separable chemical mixture.

Chromatogram Analysis enables researchers to quantitatively characterize their product down to low concentration components and reduces analysis time while increasing confidence. Flexible summary tables can quickly generate reports for non-mass spec stakeholders for internal review, or for application filings. For peptide or glycan mapping needs, Chromatogram Analysis provides an efficient and comprehensive interactive workflow.

Chromatogram Analysis has two modes of operation: annotation and comparison. **Annotation** (reference projects) assigns or "maps" identifications to the trace peaks in a liquid chromatogram (LC) or capillary electropherogram (CE). The reference (peptide or glycan) map comes from an annotation project. **Comparison** (comparison projects) aligns and quantitatively matches related chromatograms or electropherograms relative to a reference map.

Chromatogram Analysis can also be deployed in a fully automated workflow to allow for large numbers of samples to be analyzed and reported on in a consistent fashion.

Chromatogram Analysis features include:

- Support for multiple optical data channels such as UV absorption or fluorescence and TIC (total ion chromatogram) for LC or CE
- Automatic baseline correction and trace peak area integration
- Peptide and glycan identifications are made automatically by accurate mass (MS1) matching
- Identifications are made from candidates imported from MS/MS identifications, in-silico *.csv format table, in-silico protein digestion during project creation, and/or manual entry
- Annotations may be inspected in detail and evaluated by the analyst
- Simultaneous comparison of many LCs or CEs with optional trace time alignment by dynamic time warping
- Raw mass spec data are accepted from these vendors: Thermo Fisher, Waters, Sciex, Bruker, or Agilent. Optical data may be embedded in these files or imported via a *.csv file of time-intensity data pairs

Minimum System Requirements for Chromatogram Analysis:

- 2Ghz processor or faster
- 16 GB RAM or more
- Windows 7 (32 or 64-bit)
- One large monitor, or dual monitors

Workflows

Peptide and Glycan Mapping: Annotation Workflow

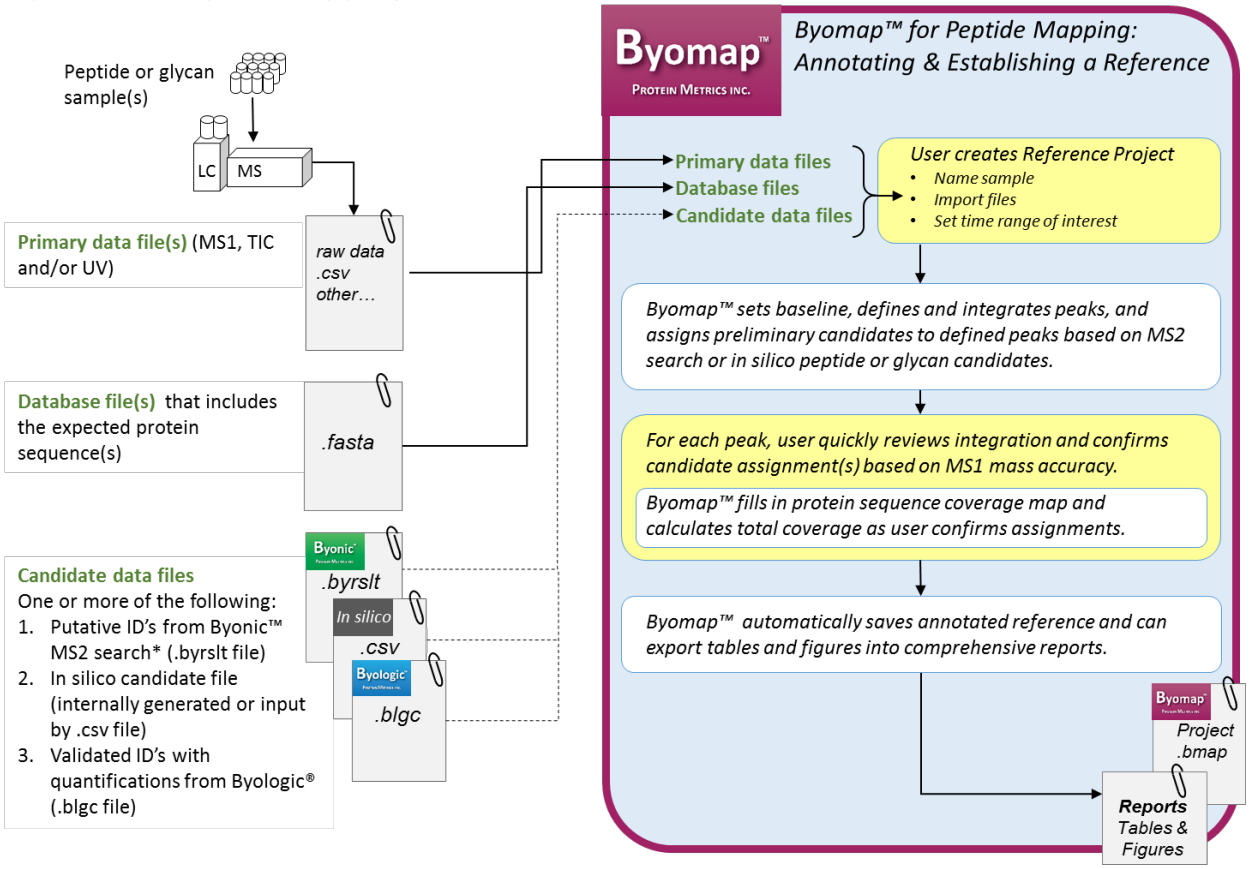


Figure 1: Peptide and Glycan Annotation (Reference) Workflow

Peptide and Glycan Mapping: Comparison Workflow

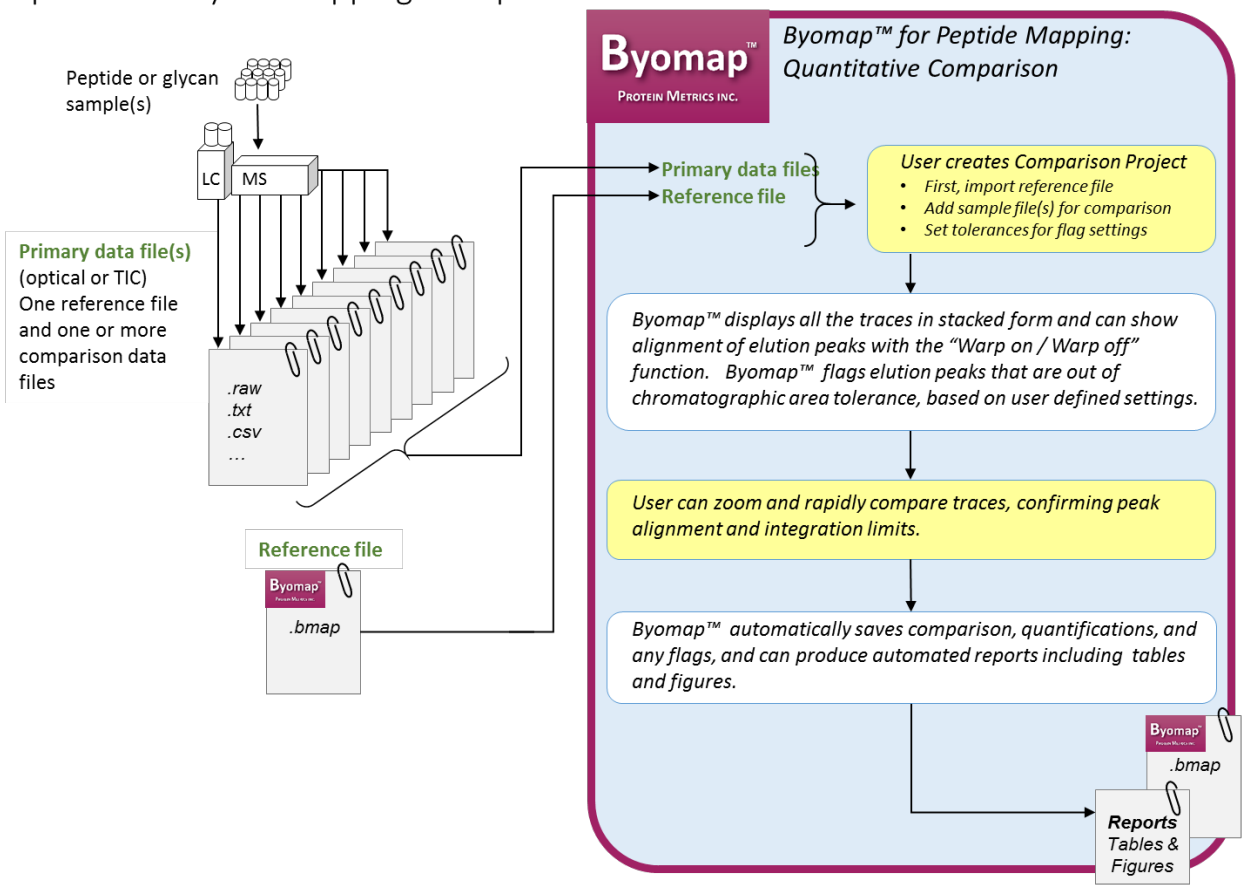


Figure 2: Peptide and Glycan Annotation (Comparison) Workflow

Tour of Chromatogram Analysis

The Chromatogram Analysis user interface (UI), or dashboard, shown in Figure 3 has six plot and table views:

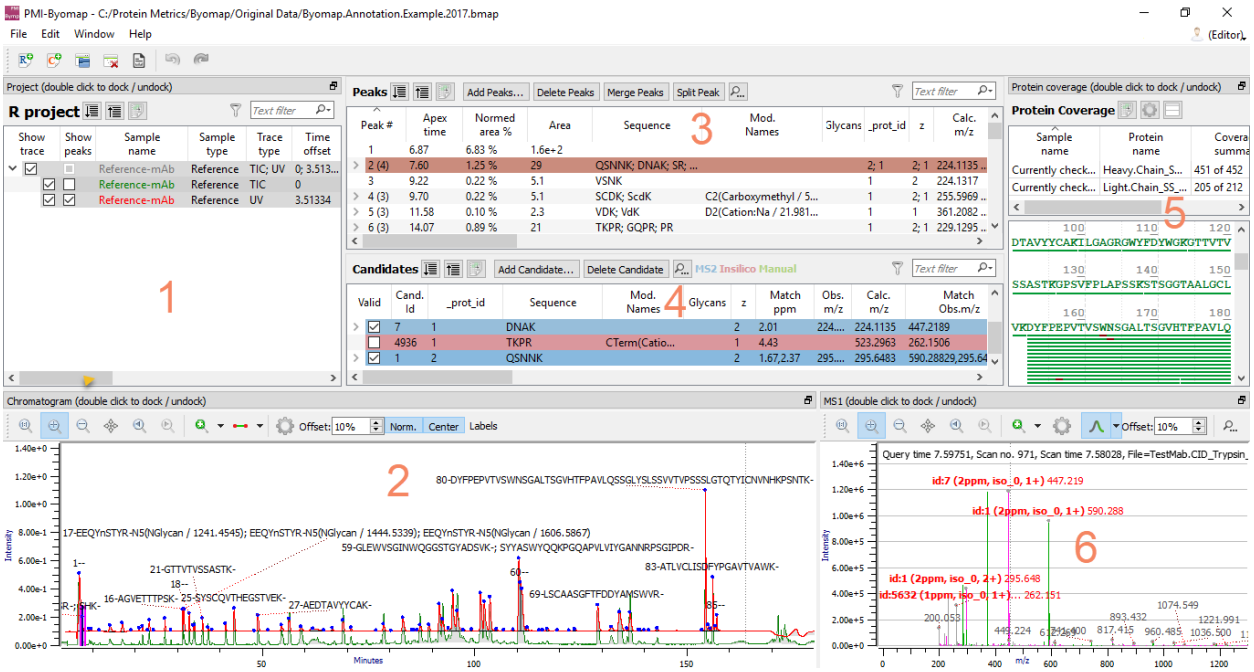


Figure 3: **Six Chromatogram Analysis views**: (1) Project table with input MS files, (2) Chromatogram with selected trace peak, (3) Peaks table, (4) Candidates table (elution peaks), (5) Protein Coverage tables, and (6) MS1 spectrum (summed spectra).

1. The **Project** view lists the data files in use. **R project** is a reference project, **M project** is a multi-sample project and **C project** is a comparison project. The **Show trace** and **Show peaks** check boxes can be used to turn on or off the chromatograms and the trace peak lists displayed in Views 2 and 3.
2. The **Chromatogram** shows the total ion chromatogram (TIC), base peak intensity (BPI), and/or UV traces for the peptide or glycan. In many ways, this is the heart of the interactive software. The adjustable magenta-colored integration limits in the chromatogram view highlight the trace peaks under study. The mass spectrum (MS1) displayed in View 6 is determined by the trace time designated by a black bar on this plot. This bar initially is set by Chromatogram Analysis to be at the peak apex.
3. The **Peaks** table summarizes the peptide or glycan peak information from the chromatogram. Selecting a row in the table selects a peak in View 2. Trace peaks can be merged, split, added, or deleted using the buttons at the top. Time limits can be adjusted by editing text in View 3 or dragging the magenta bars shown in View 2.
4. The **Candidates** table is where trace peak candidates are identified and assigned.
5. The **Protein Coverage** tables are where peptide sequences are identified and assigned to a peptide map peak. An assigned sequence appears in the Protein Coverage map. The sequence under current investigation is highlighted by the green bar becoming orange. The user can also click on any other green bar to highlight that sequence and change the other panes accordingly. A table, which may be hidden, reports the fraction of the protein(s) sequence covered by identification.

6. **MS1** shows the summed *m/z* plot for the chromatogram peak selected in Views 2 and 3. The spectrum is associated with the trace time designated by the black bar in the Chromatogram pane. The trace time of the MS1 spectra can be changed by moving the vertical black bar to a different time. Doing so can help increase understanding of the elution of peptides within the peptide peak.

Note that four of these views (Project, Chromatogram, Protein coverage, and MS1) may be detached by a double-click on the top of the view. The views can also be resized and rearranged. For example, the MS1 or Chromatogram view can be detached and moved to a second large monitor for easier viewing. These panes may be reinserted, in any available position to rearrange the layout. Layouts may be saved for later use with the **Window > Save Layout** menu.

Creating a Reference Project

Chromatogram Analysis can be used to analyze a single sample or to compare a number of related samples. Comparison of related samples starts with the analysis of a sample chosen to be the reference sample. To create a reference project, select **File** > **New Reference Project**. Alternatively, click the icon below the File menu. Chromatogram Analysis will open the **Sample input** tab for the new reference project:

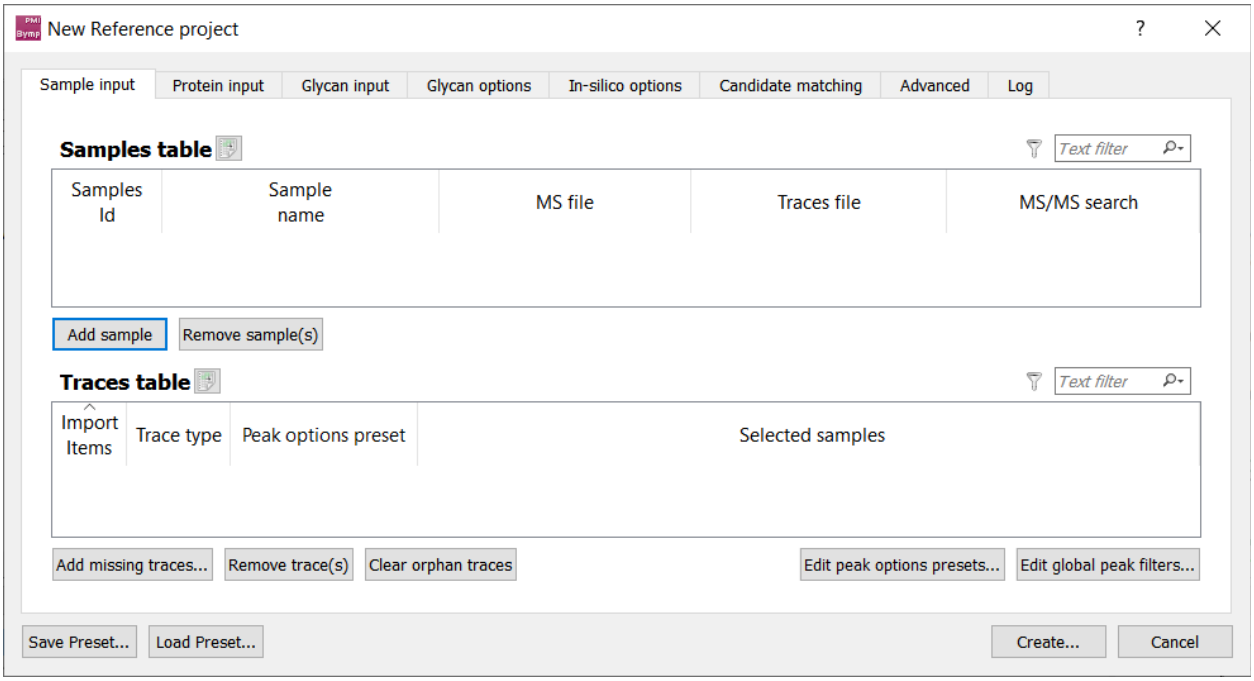


Figure 4: New Reference Project

Sample input Tab

The **Sample input** tab allows the user to load reference sample files into the project. Chromatogram Analysis accepts a variety of sample file types (Bruker: .d, Thermo: .raw, Waters: .raw, Sciex: .wiff, Agilent: .d). Alternatively, a project can be opened from a *.byrslt file generated from a previous Byonic™ search, or a Peptide Analysis (Byologic™) *.blgc project file. To load a file, drag and drop an MS file into the project window. Alternatively, double-click on **Enter filename**, click ... and browse to the file. Click **Open**.

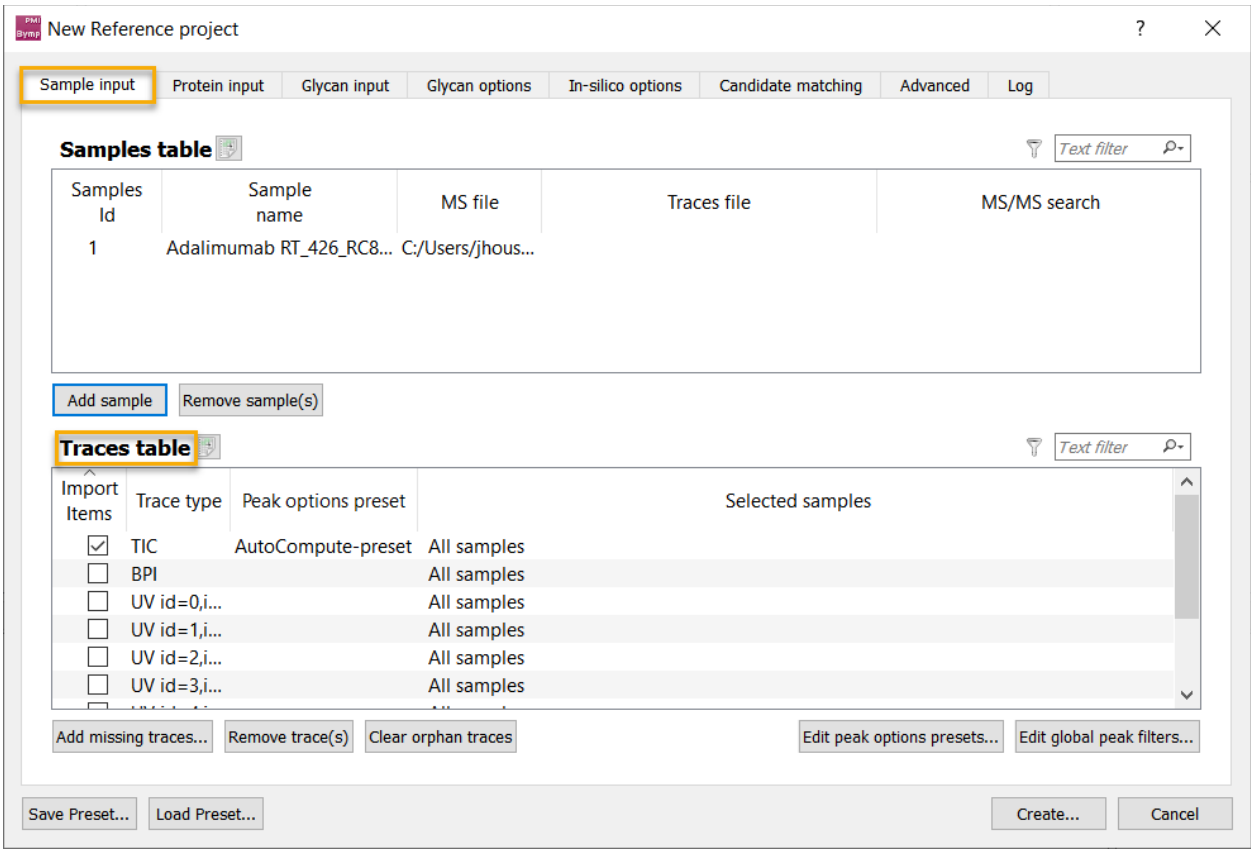


Figure 5: Adding samples and their traces in the Sample input tab

The sample is named for the file name; to change the sample name, click the default name twice and enter a new sample name. Click **Add sample** at left to add a new row to the **Samples table**. Then add the desired files as above. To remove an unwanted sample file row, select the row and click **Remove sample(s)**.

Starting with Chromatogram Analysis v. 3.8, the MS file populates both the **Samples table** and the new **Traces table** at the bottom. Selected (not checked) traces can be removed by clicking **Remove trace(s)**. Removed traces can be restored by clicking **Add missing traces**, check the trace to restore and click **OK**. To associate specific traces to the samples, double-click the text in the Selected samples column, then click the ··· button to open the Edit samples selection dialog:

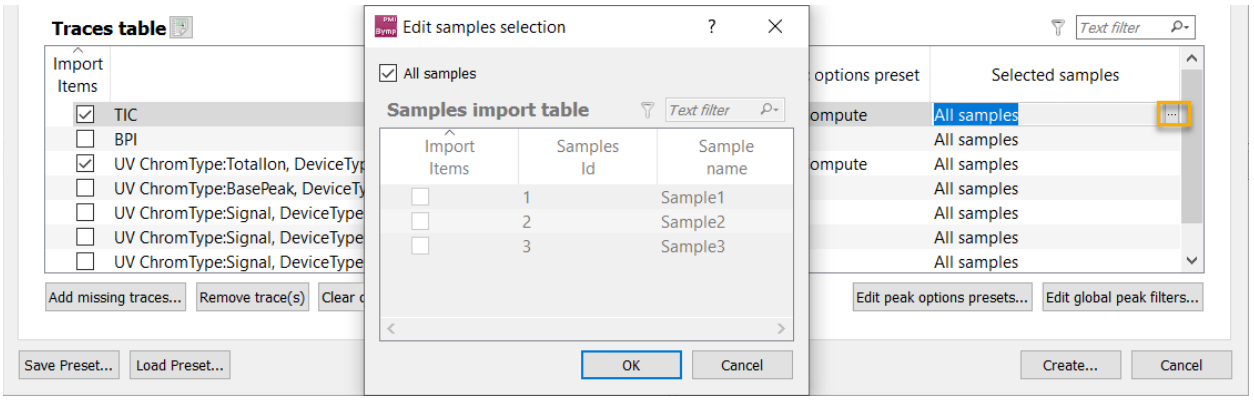


Figure 6: Edit samples selection dialog to associate traces with sample names

To associate the selected trace with an individual sample, uncheck **All samples**, check a single sample, and click **OK**. Note that multiple checked samples are not supported for traces.

To import a trace from a *.csv file, drag the file into the **Traces files** column in the Samples table. Alternatively, double-click in the Samples table row under the Traces file column, click ⋯ and browse to the sample file. Click **Open**. The trace file is added to the sample name and the trace is added to the Traces table:

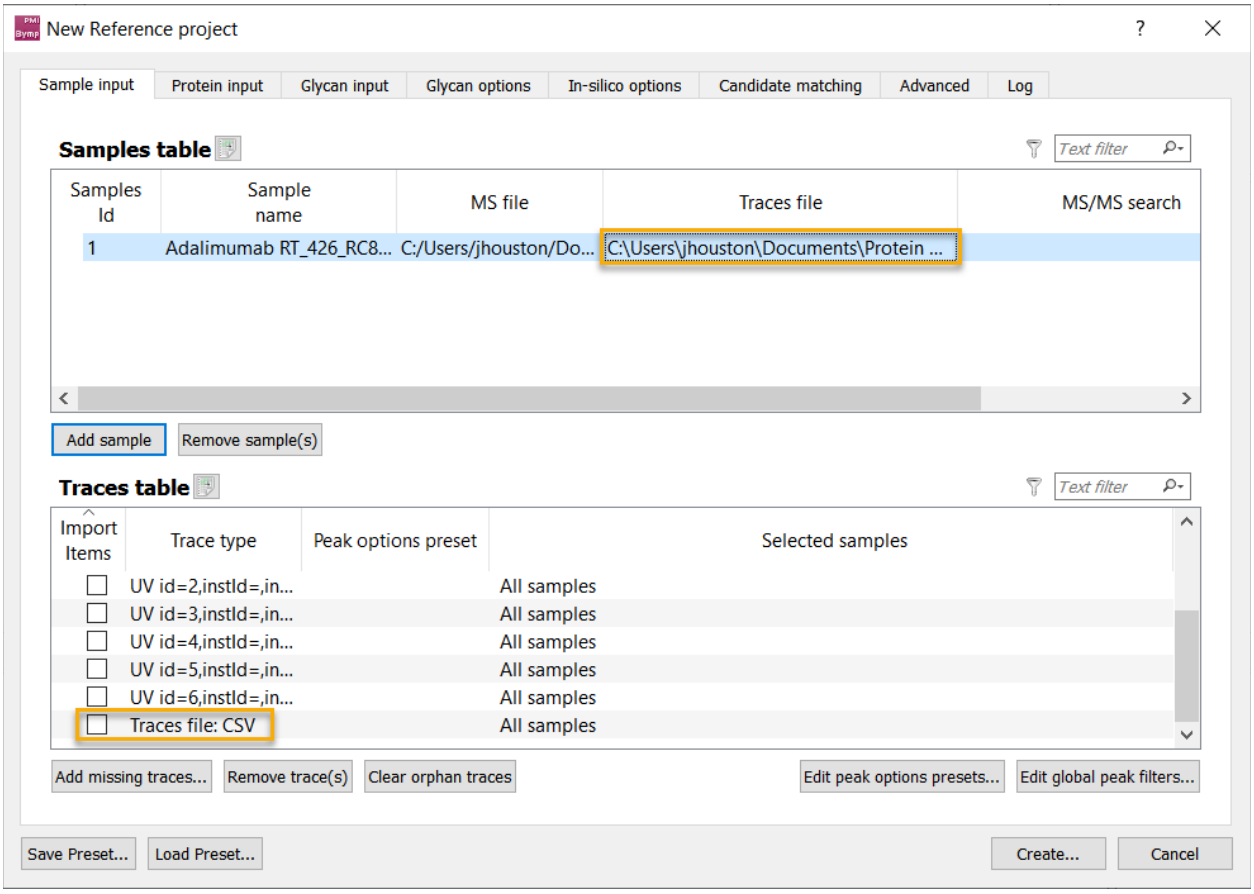


Figure 7: Traces imported from a *.csv file

Multiple traces can be added to a sample. To add additional trace files, click in the **Traces file** cell, click the ✚ icon and navigate to the new trace file, as before:

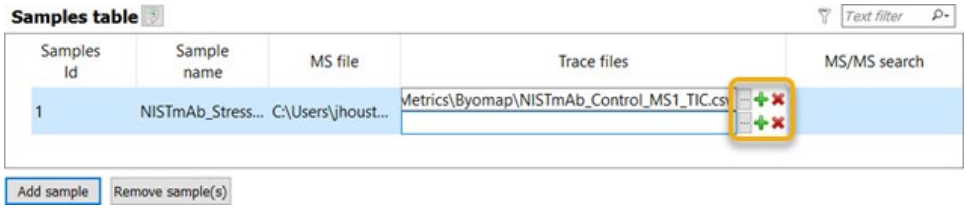


Figure 8: Adding multiple trace files to a sample

To remove a trace file, click in the **Traces file** cell to be removed and click the ✖ icon.

Byonic MS/MS search files in *.byrslt format can be dragged or navigated into the **MS/MS search** column.

Trace Peaks Options

To control how the trace peaks are processed, click **Edit peak options presets** at the bottom:

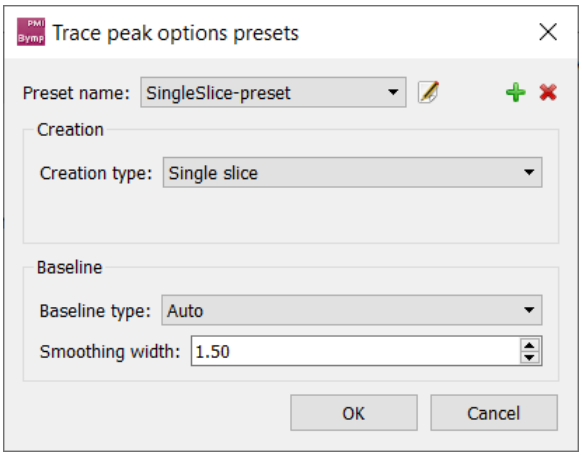


Figure 9: Trace peak options presets

At the bottom of the Trace peak options presets dialog are the Baseline parameters. The baseline type can be set to **Auto** or **Flat**. Baseline smoothing width is also set here.

The Trace peak options presets dialog contains five default peak processing presets:

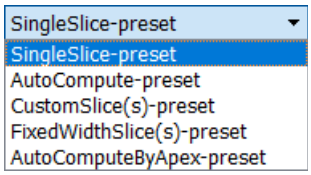


Figure 10: Default trace peak presets

Select a processing preset and edit the peak parameters. To create a custom preset, click the ✚ button, add a preset name, and click **OK**. Next, select a creation type and edit those peak parameters. The new preset is added to the list. Click the ✎ button to edit a selected preset name. Click the ✖ button to delete a selected preset.

The default and custom peak processing presets can use one of four **Creation types**:

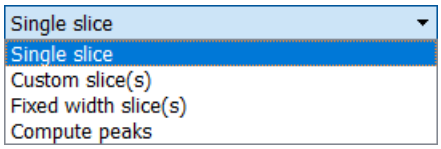


Figure 11: Trace peak Creation types

These Creation types use the following peak processing methods:

- **Single Slice** processes the entire chromatogram as single trace peak.
- **Custom slice(s)** divides trace peaks by user defined start and end points:

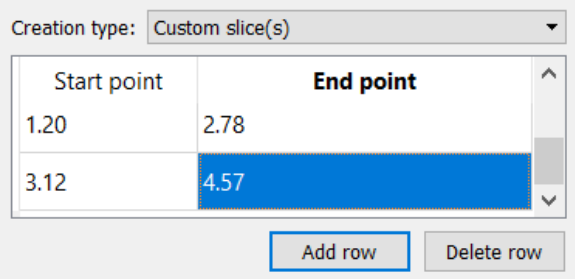


Figure 12: Trace peak Custom slice option

Click **Add row** and enter start and end points for each slice. To remove a slice, select it and click **Delete row**.

- **Fixed width slice(s)** divides trace peaks by regular intervals defined by the user:

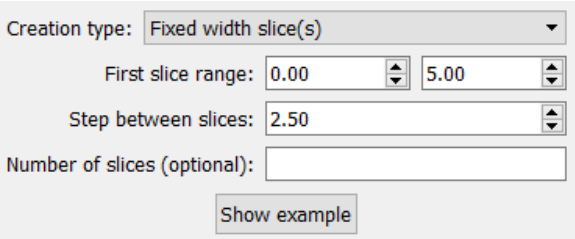
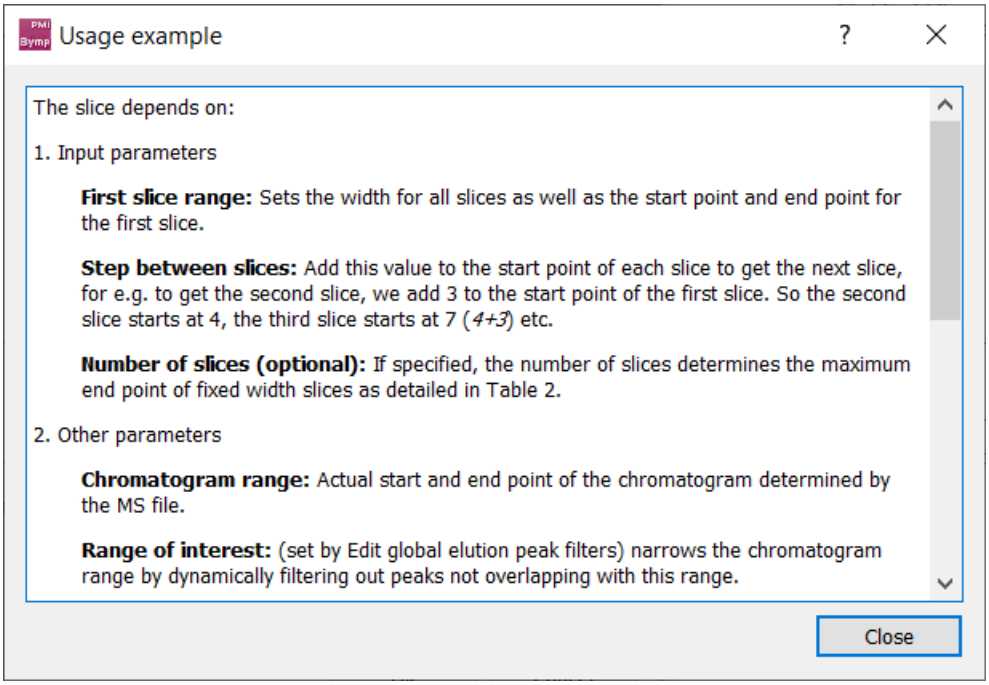


Figure 13: Trace peak Fixed width slice(s) option

- **First slice range** sets the start and end points for the first slice. This also sets the width for all slices.
- **Step** is the delta that sets the start of subsequent fixed width slices by adding the step value to the start of the preceding slices. For example, the values above define the first slice as 0 - 5, the second slice as 2.5 – 5, etc., through the end of the chromatogram.
- **Number of slices** sets the maximum number of defined slices. If blank, the last slice is determined by the end of the chromatogram x-axis range.
- **Show Example** displays an example fixed width slice configuration with definitions and explanations:



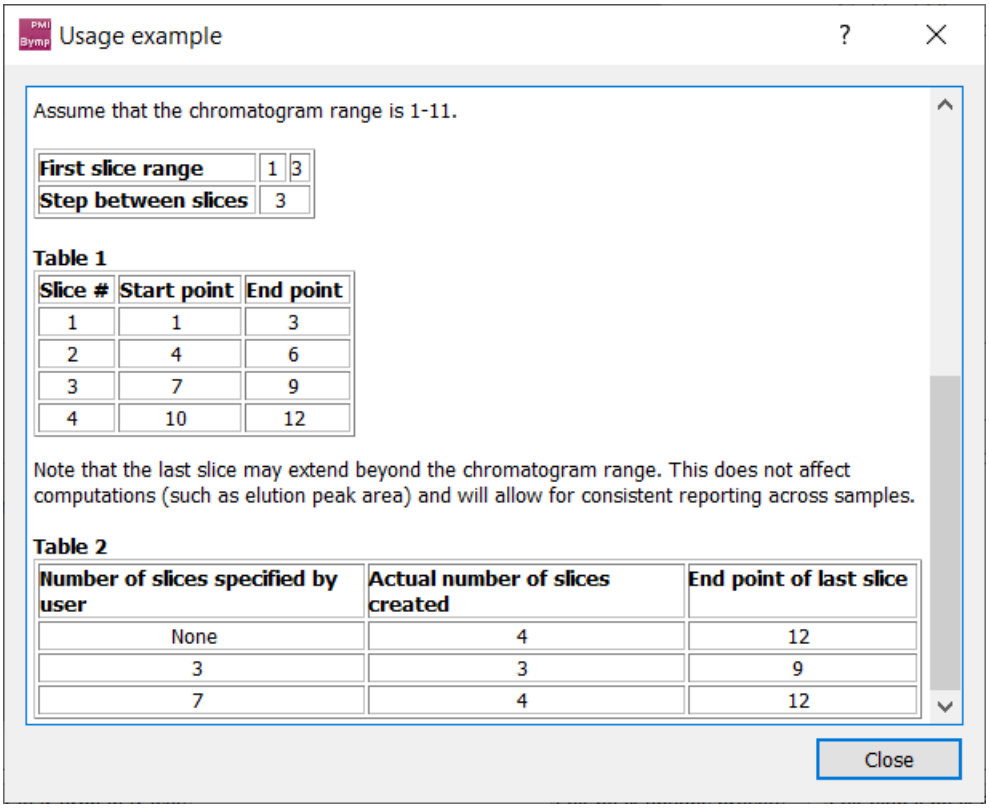


Figure 14: Fixed width slice Usage example

- **Compute peaks** automatically determines the trace peaks based on their minimum width, after smoothing:

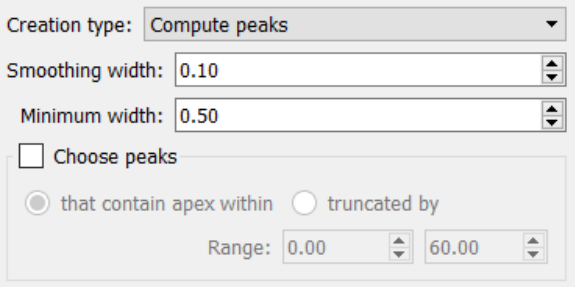


Figure 15: Trace peak Compute peaks option

Choose peaks, when checked, limits the detected peaks to the specified x-axis range. The option **that contain apex within** selects the whole peak of each apex within the specified range. The option **truncated by** trims the first and last peak to fit within the specified range.

Starting with Chromatogram Analysis v. 3.8, individual trace filters are replaced with a single peak filter across all traces. To set peak property filters for all the traces, click **Edit Global peak filters**:

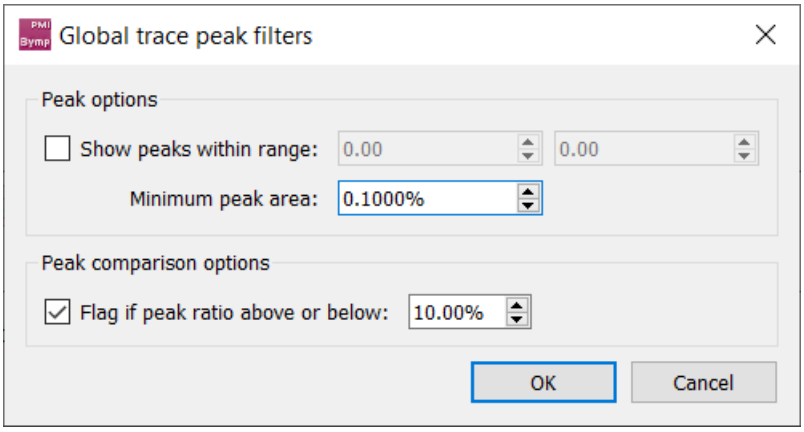


Figure 16: Global trace peak filters

Check **Show peaks within range** to manually set start and end points. **Minimum peak area** and a peak ratio threshold flag can be set in this dialog.

The Traces table and trace peak settings are backwards-compatible. Traces associated with samples in projects from software versions before 3.8 will correctly populate the Traces table in the current version. Trace peak settings and presets from versions before 3.8 will correctly migrate into the current version. However, Intact and Chromatogram Analysis projects created in versions 3.8 and above will not correctly open in older versions of those applications.

Protein input tab

The **Protein input** tab allows the user to enter a protein sequence either by adding a FASTA file or by typing it in directly.

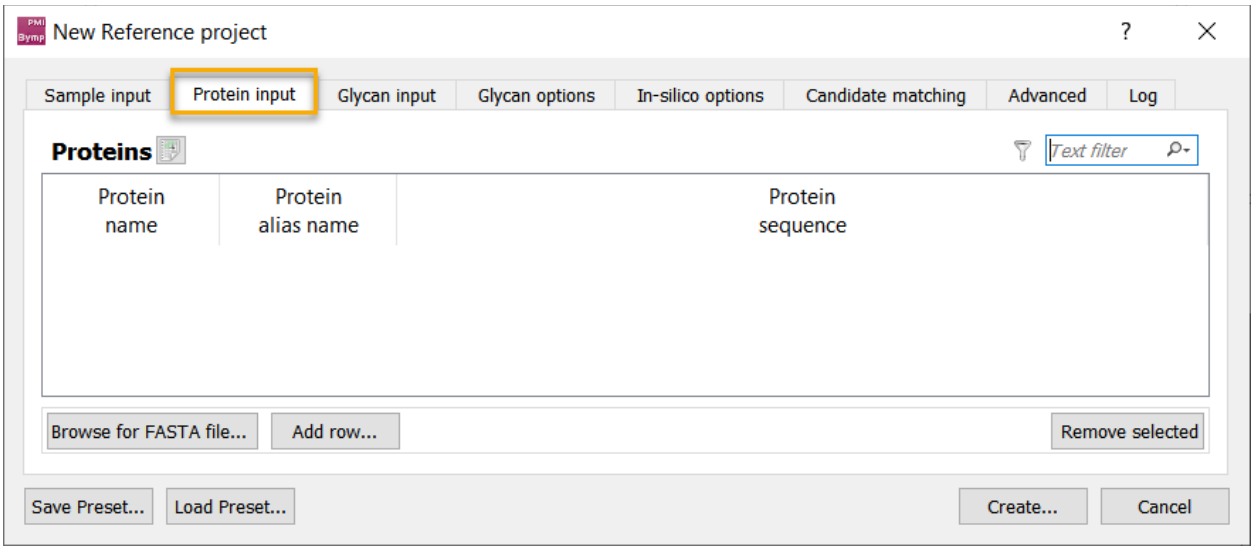


Figure 17: Protein input tab

To add a .fasta or .fa file, click **Browse for FASTA file**, locate the file to be added and click **Open**. FASTA files also can be dragged into this dialog directly.

The addition of a FASTA file opens the **Protein candidates** dialog:

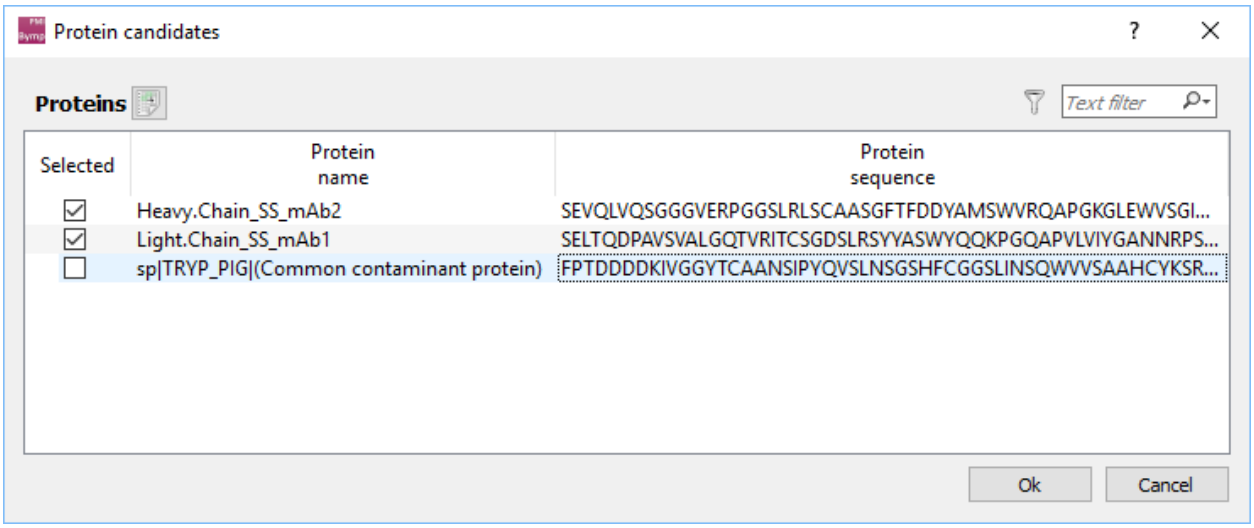


Figure 18: Protein candidates dialog

Check the box for each protein to include in the project. To edit Protein name, click on the name and enter a new one. When finished, click **Ok**. The Protein input tab is updated with the proteins and sequences for the project. To add a Protein alias name, click that field, and enter the desired value.

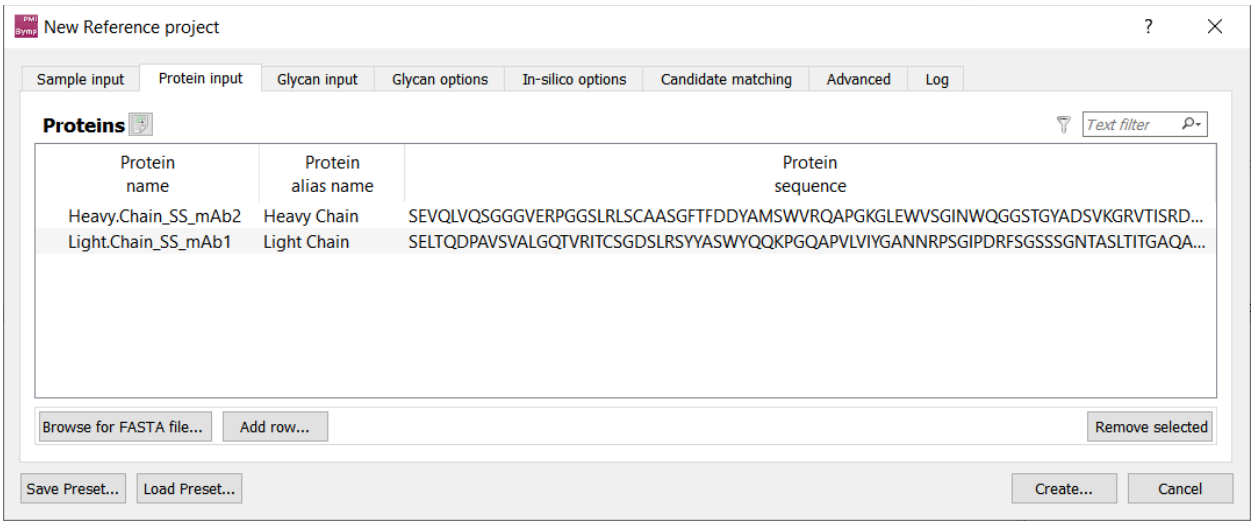


Figure 19: Protein input tab with proteins from a FASTA file

To add protein data from a URL or to enter it manually, click **Add row**. This opens the **Add Protein** dialog.

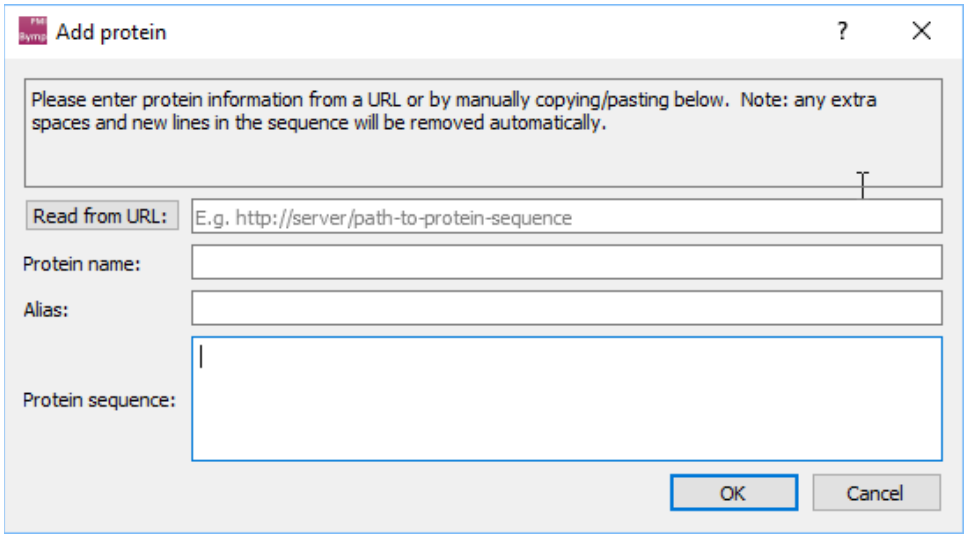


Figure 20: Adding a protein manually or from a URL

To add a protein from a URL address, enter the address and click **Read from URL**. To enter the protein data manually, enter or paste in the Protein name, Alias and Protein sequence. Click **OK** to add the protein as a row in the Protein input tab. To remove an added protein from the Protein input table, select that protein and click **Remove selected**.

The software automatically computes the average-isotope mass for each selected FASTA entry, and these masses are then available for automatic peak assignment. (The default computation assumes that Cys residues are disulfide bonded, but this default can be changed by the user.) Protein sequences can be arbitrary if the user inputs the average masses, overriding the computed masses. Average-isotope masses give the software reference masses so that it can assign mass peaks automatically based on mass deltas.

Glycan input tab

The **Glycan input** tab allows entry of glycan data. **Add from CSV file** imports glycans from a *.csv file. The file contains the glycan alias name, the glycan composition and optionally, the start and end times for the trace. An example of the *.csv file format is:

```
GlycanAliasName,GlycanComposition,StartTime,EndTime
Common N-linked core,HexNAc(2)Hex(3),2.34,8.76
Deoxyhexose,Deoxyhexose(1)HexNAc(2)Hex(3),2.34,8.76
```

Add row opens a dialog to manually enter glycans:

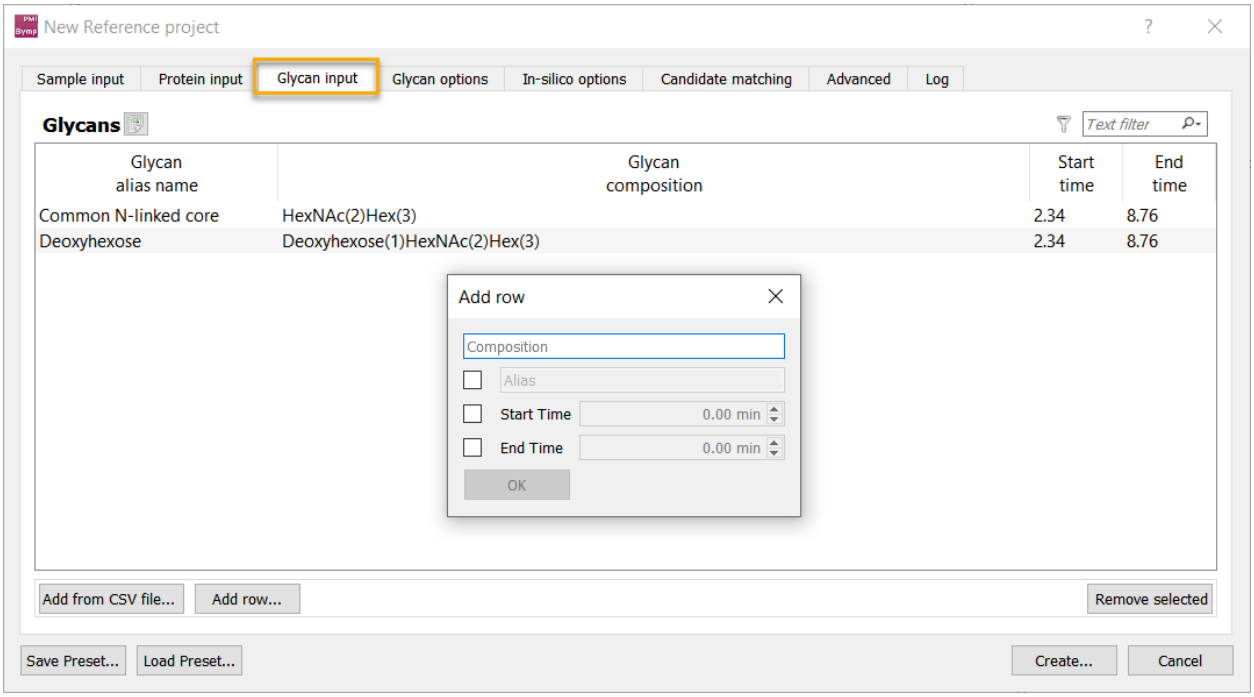


Figure 21: Glycan input tab to import or manually enter glycans

To delete glycan records, select the glycans and click **Remove selected**.

Glycan options tab

The Glycan options tab allows the user to identify the label used during the experiment. The delta mass of each tag is displayed and defined. The user can also use the checkbox to define 100% labeling.

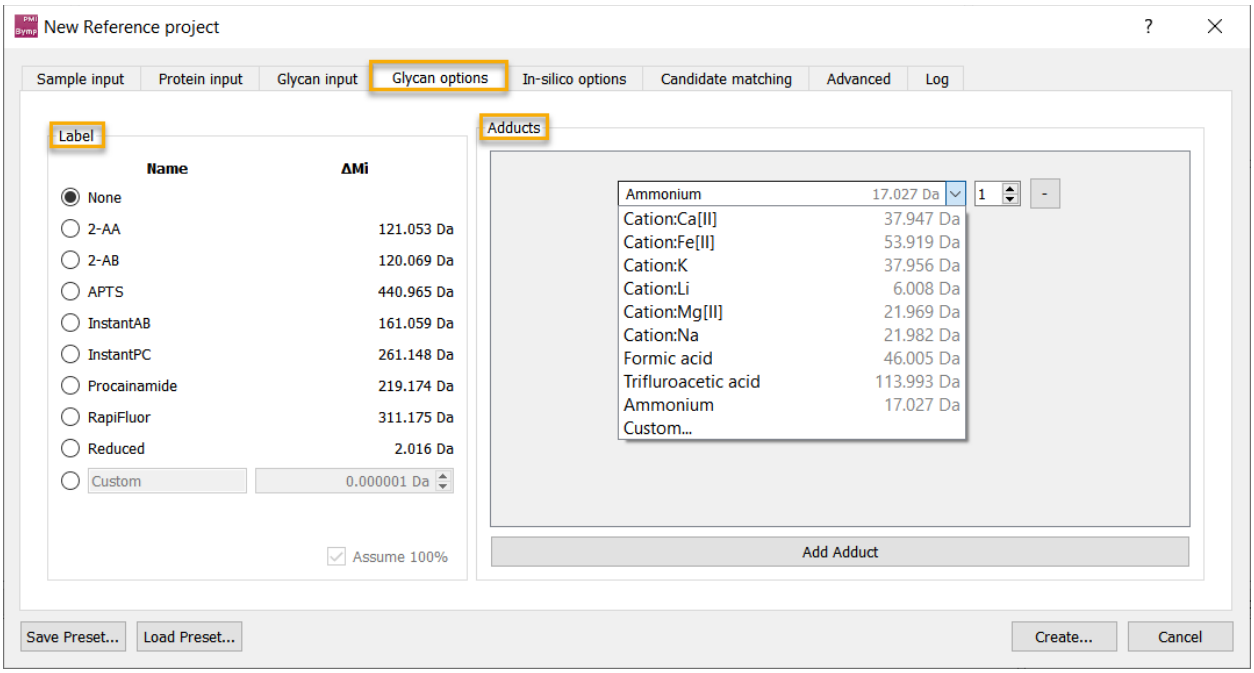


Figure 22: Glycan options tab – identify the label used and set Adducts

The user can include adducts to consider in the mass calculation in this same tab. Click **Add Adduct** to expose the drop-down menu in Figure 21. Click the button again to add more adducts.

In-silico options tab

The **in-silico options** tab allows the user to include in-silico information for a digested protein or glycan mixture in the analysis. Check the **In-silico** checkbox to enable this feature. There are two in-silico options: **Digest proteins** and **Use table**. The **Digest proteins** dialog box is similar to the Byonic™ user interface for those familiar with that software. As the name indicates, it is used to perform an in-silico digestion of the input proteins using specified digestion parameters in the Digestion options section.

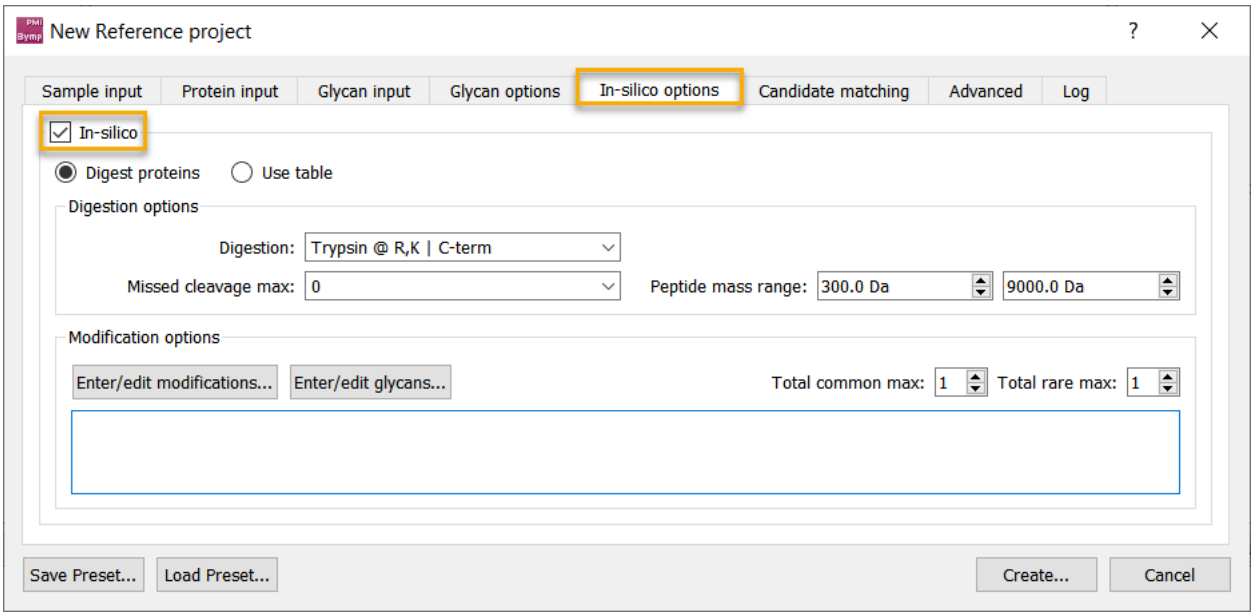


Figure 23: In-silico options tab in Digest proteins mode

Modifications

To enter modifications, click **Enter/edit modifications**:

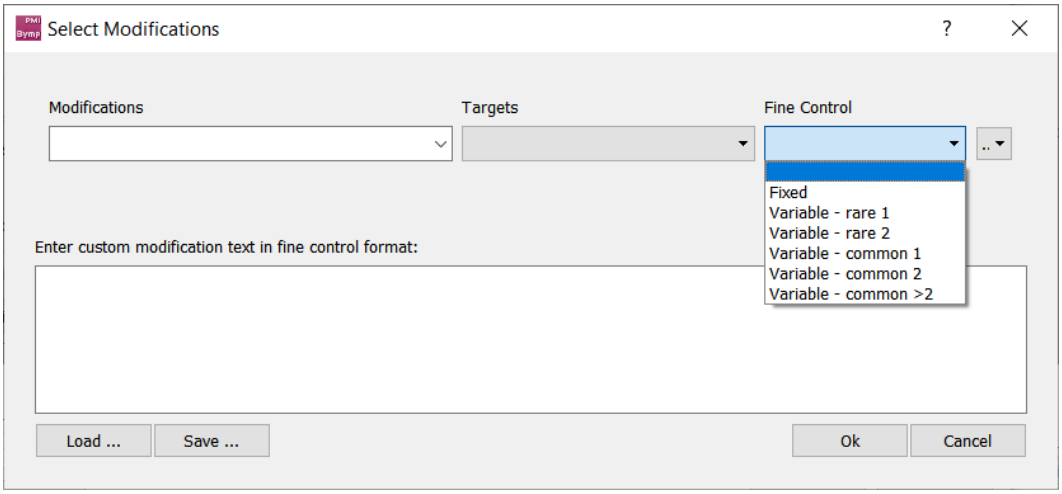


Figure 24: Select Modifications dialog

Chromatogram Analysis supports two types of modifications: fixed and variable. A **fixed** modification is assumed to occur on all the residues of that type, but a **variable** modification is optional, so that each site for a variable modification is considered with and without the modification.

The user can also designate each variable modification as either **"common"** or **"rare"**, with the names suggesting how they are used. The number of occurrences is defined for each variable modification, so that "common2" means at most two occurrences per peptide.

To set a modification, choose from the **Modifications** drop-down. Modifications include name and delta mass pairings, modification targets and fine control settings that set modifications as fixed or variable, as well as rare or common. Additional actions can also be performed on the modifications.

- **Modifications** displays a list of common modifications and their corresponding delta masses. The list includes all candidates found in www.unimod.org.

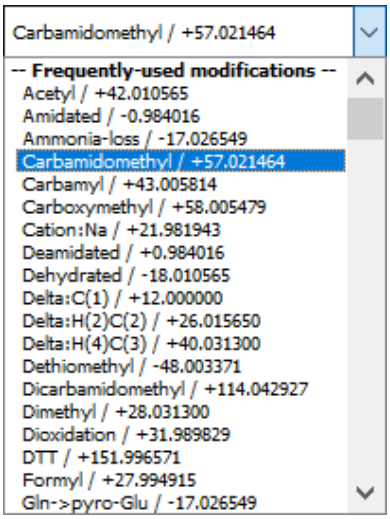


Figure 25: Modification Name / Mass Delta

For convenience, frequently used modifications are listed twice, once at the top and again in the complete list.

- **Targets** displays a list of possible target locations associated with the selected modification name. The Targets field allows the 20 one-letter amino acid abbreviations, as well as four special locations: NTerm, CTerm, Protein NTerm, and Protein CTerm. NTerm, CTerm, Protein NTerm, and Protein CTerm can also be used as modifiers of amino acid residues. Targets form a comma-separated list.

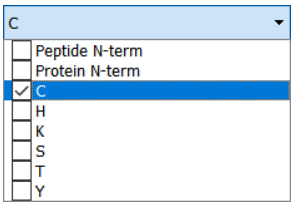


Figure 26: Modification Targets

- **Fine control** marks the modification as “Fixed” or “Variable”, whether the variable modification is considered “Rare” or “Common”, and the maximum count of that modification in the peptide.

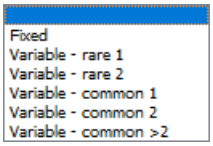


Figure 27: Fine Control

- The ... button in the last column exposes additional actions that can be performed on modifications:

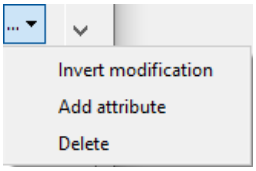


Figure 28: Additional Modification actions

  - Invert modification defines a variable modification that is the removal of a fixed modification (for example, under-alkylation. The modification is labeled with the prefix “(De)”, as shown in Figure 28:



Figure 29: Invert modification

  - **Add attribute** allow the user to define protein-specific modifications. For example, adding the attribute “ProteinLabel{collagen}” to Oxidation on P allows hydroxyproline only on proteins with “collagen” in their protein names:



Figure 30: Add modification attribute

- **Delete** removes that modification record.

Custom modifications can be manually entered in the box labeled **Enter custom modification text in fine control format**. The format for custom modifications is as follows:

Modification_Name / Mass_Delta @ Targets | Fine_Control

The following is an example of a real modification not (yet) in Unimod:

DehydroFormyl / +9.98435 @ NTerm S, NTerm T | rare1

Click **Save** to export the selected and custom modifications as a text file. This modifications text file can then be loaded into another Chromatogram Analysis project by clicking **Load**. Click **Ok** to add the defined and custom modifications to the **Modification options** section of the **In-silico options** tab:

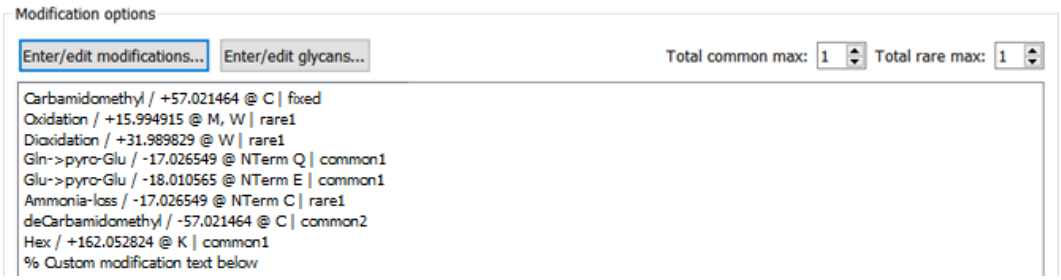


Figure 31: Example of loaded selected and custom modifications

To set separate limits on the **total number** of common and rare modifications per peptide, set **Total common max** and **Total rare max**. For example, to set a maximum of three phosphoserines per peptide, the user can set Total common modification max to 3 or split phosphorylated serine between two rules: common2 and rare1. For more information about modifications, see the **PMI Byonic Manual**.

Glycans

To enter glycans, click **Enter/edit glycans** in the **In-silico** tab (see Figure 22). The **Select Glycans** dialog opens:

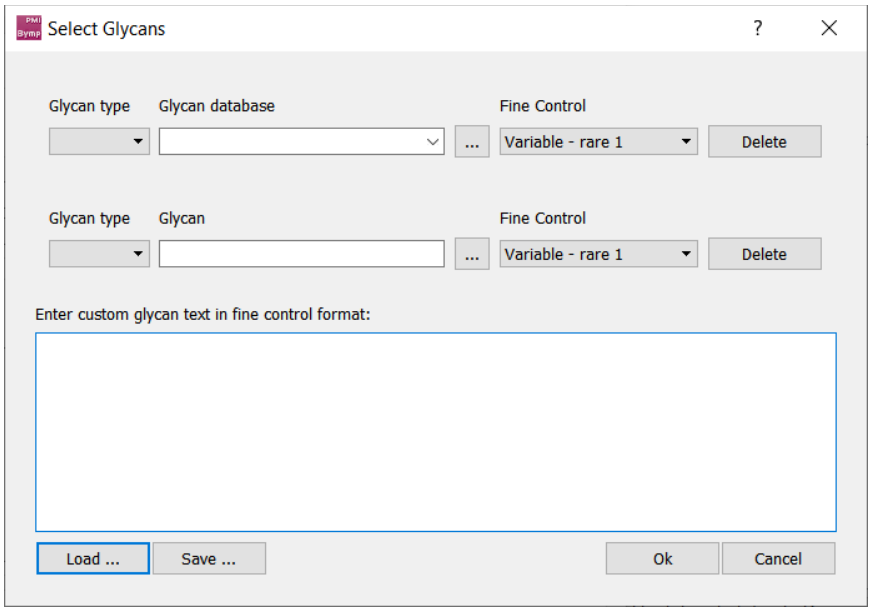


Figure 32: Select Glycans dialog

Select Glycans allows the user to set glycan types, choose a glycan database, and define variables as rare or common. The dialog also allows the free entry of glycans in the proper format.

- **Glycan type** sets it as either an N-glycan or an O-glycan. N-glycans are allowed on Nx{S/T} motif where x is anything except P. O-glycans are allowed on any S/T:

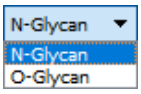


Figure 33: Glycan Type

- **Glycan Database** allows the user to input a database of glycans:

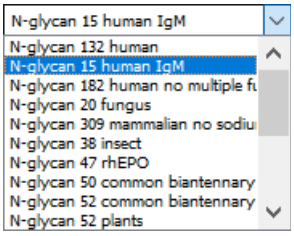


Figure 34: Glycan database options

The database dropdown references a list of glycan database text files found in **C:\Program Files\ProteinMetrics\PMI-Suite\Base\data\GlycanDatabases**. These text files can be edited, and new glycan database text files can be added to the directory, where they become available in the dropdown (after closing and reopening the application). This set of glycan databases is continually updated based on customer feedback. Please reach out to support@proteinmetrics.com to request additional content.

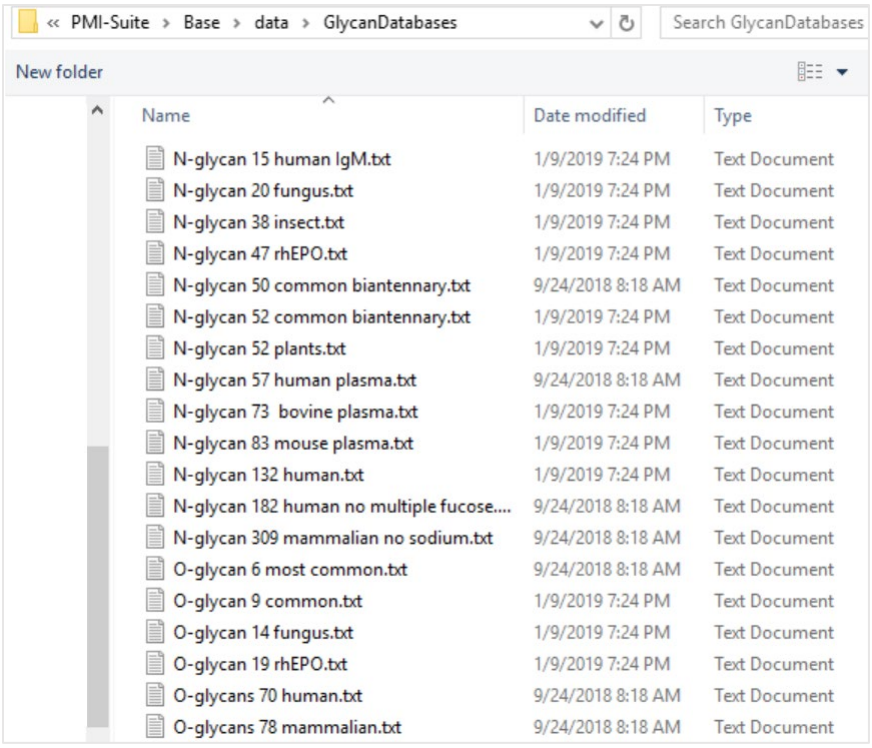


Figure 35: Glycan databases included with installation

Alternatively, a glycan database can be selected from another directory. To open a new glycan database, click the ... button, navigate to the desired directory, select database text file, and click **Open**.

- **Fine Control** designates the glycan as common or rare, and sets a limit on the count of that glycan per peptide:

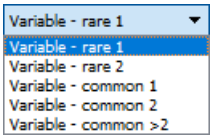


Figure 36: Glycan Fine Control options

- **Delete** removes the glycan record.

In addition to glycan databases, users can construct a glycan from monosaccharide units in the second section. To assemble a glycan, first select the Glycan type (N-Glycan and O-Glycan) from the dropdown. Next, click the ... button to the right of the Glycan box. This opens the **Edit Glycan** dialog:

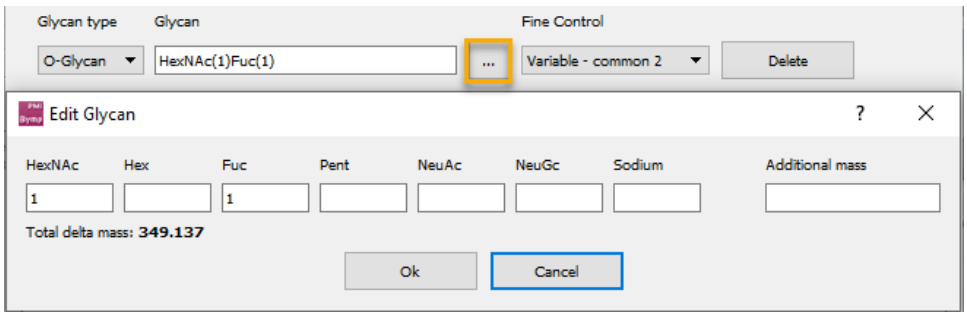


Figure 37: Enter a specific glycan

Enter the count of each used monosaccharide. Six monosaccharide residues are allowed: HexNAc, Hexose, Fucose, Pentose (common in plants), NeuAc, and NeuGc (common in non-humans). There is also a box for Sodium because this is a common adduct on sialic acids. Unused monosaccharides can be left blank or included with zero (0) occurrences. In the resulting text, spaces between monosaccharides units are optional. For example, five common human O-glycans are represented as:

HexNAc(1)
HexNAc(1)Hex(1)
HexNAc(1)Hex(1)NeuAc(1)
HexNAc(1)Hex(1)NeuAc(2)
HexNAc(1)Hex(1)Fuc(1)

Total delta mass displays below for the added monosaccharides. Glycan modifications such as acetylation, phosphorylation, or sulfation or less common monosaccharides such as deaminated neuraminic acid can be added by typing in the correct keywords, “Acetyl”, “Phospho”, “Sulfo”, and “Kdn” respectively. Click **Ok** to create the glycan:



Figure 38: Glycan assembled from monosaccharides

Assign a **Fine Control** value as with the glycan databases.

A third way to enter glycans is to enter or paste glycan text in the **Enter custom glycan text in fine control format** box at the bottom. These glycans are entered using the same format as for individually added glycans: Monosaccharide(count) @ 0 or NGlycan | fine control option

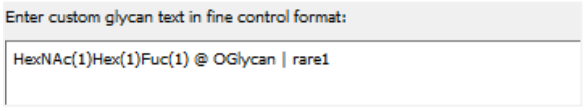


Figure 39: Manually entered or pasted glycan

As with modifications, glycans can be saved and reloaded in other Chromatogram Analysis projects. Click **Ok** to add the various glycans to the **Modification options** section of the **In-silico** tab:

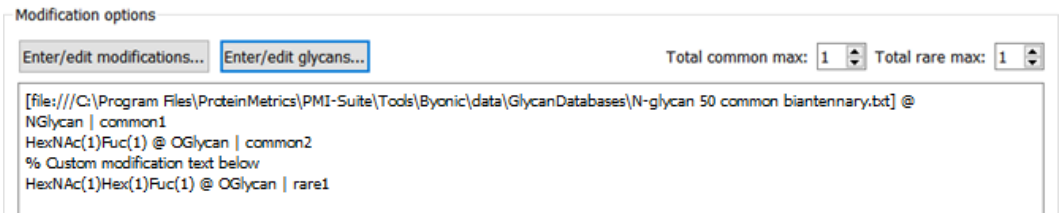


Figure 40: Example of loaded glycan database and individual glycans

Use table

The **Use table** dialog box allows the user to include in the analysis a table of any relevant molecular forms of peptides or glycans.

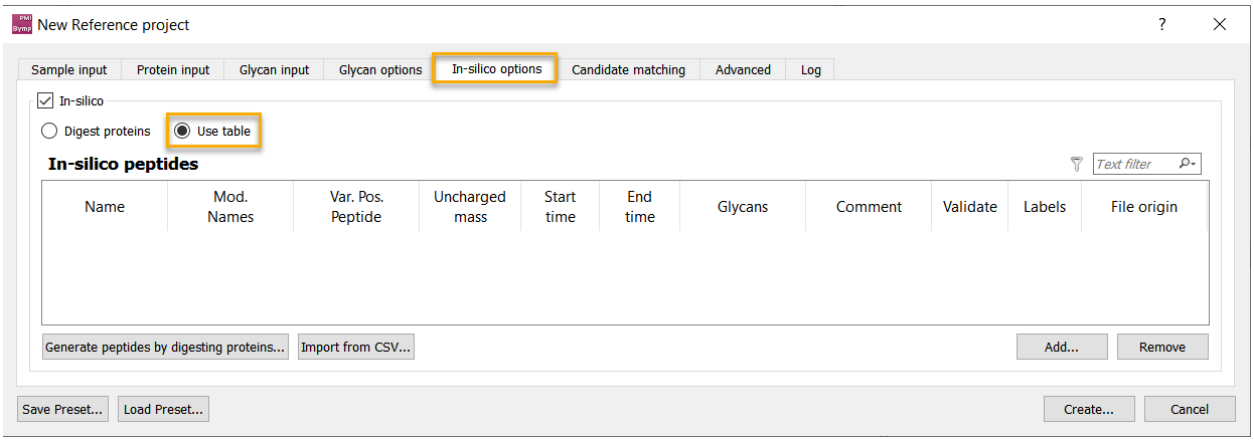


Figure 41: In-silico options tab User table mode

The **Generate peptides by digesting proteins** button at the lower left will bring up essentially the same dialog as for the **Digest proteins** mode, but the results of the digestion will be displayed in this table.

Click **Import from CSV** at the lower left and browse to an in-silico protein input file. Upon loading the file, the table is populated with the peptides and glycans. The format required for data entry can be seen in the file **ImportExample.Byomap.csv** located in **Program Files\ProteinMetrics\PMI-Suite\ExampleData**. This example is for a protein digest but the same format applies to a glycan library, where the glycan name is placed in the first column, currently named **Sequence**. Certain special characters, such as parentheses, are currently not allowed for glycan names. Only **Sequence**, **Uncharged mass**, **Start times**, and **End times** are required fields. The Start and End times sometimes define narrow time windows, and sometimes define large time windows when the detailed elution is not known.

To enter in-silico peptides manually, click **Add** at the lower right to open the **Peptide definition** dialog:

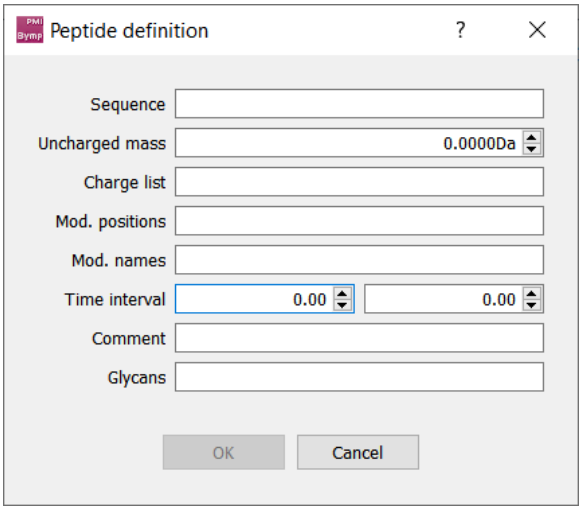


Figure 42: Peptide definition

Click in the fields in the table and enter or paste in the required information. Click **OK** to load the peptide. To delete in-silico peptide rows, select them, and click **Remove**.

Candidate matching tab

The Candidate **matching** tab allows the user to set mass assignment, charge range and other candidate matching filters before project creation:

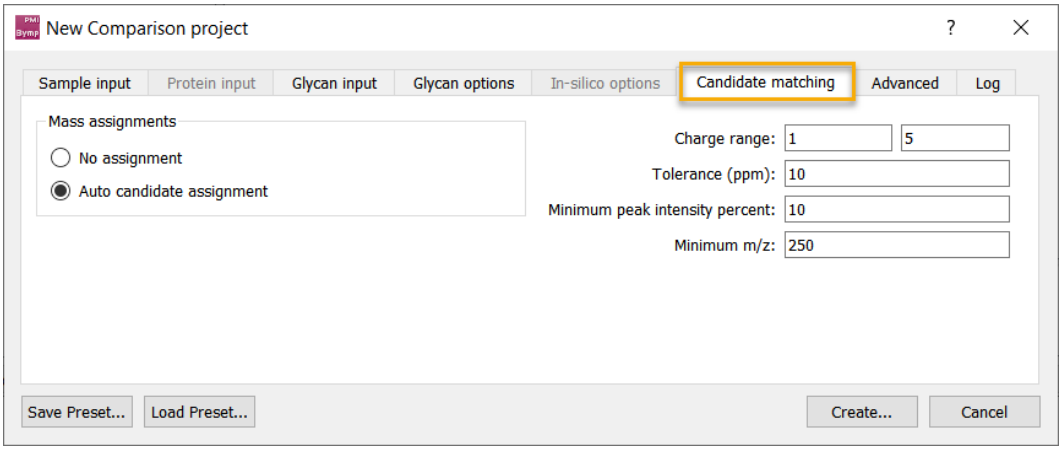


Figure 43: Candidate matching tab

Advanced and Log tabs

The **Advanced** tab contains less-commonly used features.

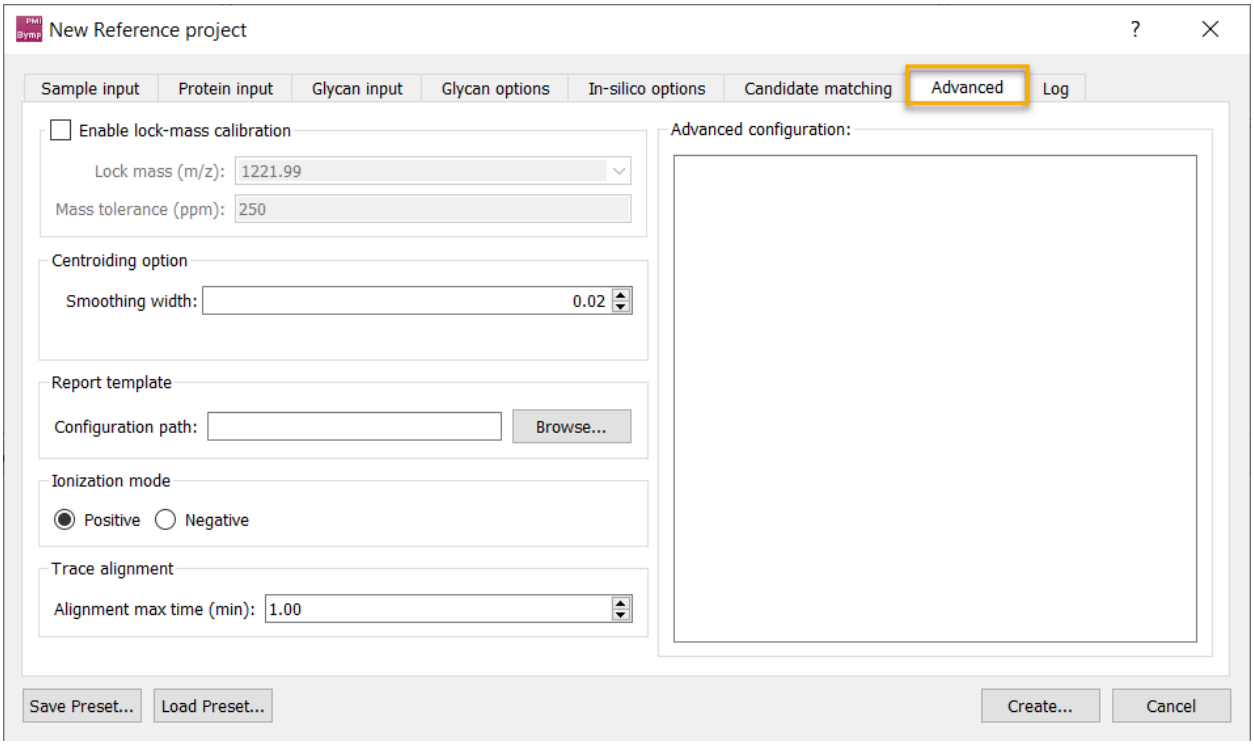


Figure 44: Advanced tab

The tab includes a checkbox labeled **Enable lock-mass calibration**. This option shifts *m/z* measurements based on an internal calibrant. Click the Enable lock-mass calibration dropdown to show a list of common lock masses; however, the user may type in any m/z value.

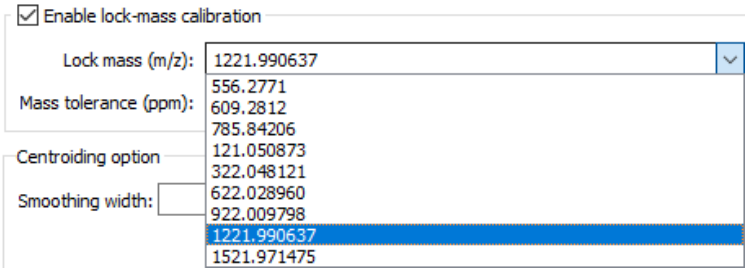


Figure 45: Lock mass options

Currently a single lock mass is used in recalibration. An m/z tolerance for locating the uncalibrated lock-mass m/z peak is required. For example, if the tallest peak in an *m/z* spectrum is at 1221.9771, shifting the *m/z* measurements so that this peak aligns to the correct value of 1221.9906 reduces the measurement error of the Adalimumab light chain monoisotopic mass from about 10 ppm to 1 ppm.

The Advanced tab includes the **Report template** section to preload a non-default **report** configuration to the project. A Report configuration also may be added after project creation. The report configuration function is covered in the section **File > Export Menu**. **Ionization Mode** has been moved from the advanced command area to this tab. The user can select between Positive and Negative values. **Trace alignment** sets the maximum time alignment in minutes for the traces.

The **Advanced configuration** box is used to enter text commands for less frequently used or newly developed options: monoisotopic mass, removing charge-one peaks, time-sliced trace peaks, use of isotope spacing to deduce charge, and many more advanced features.

The **Log** tab reports recent activity and is useful for troubleshooting problems. There is also a project creation progress bar on this tab

New reference project inputs can be saved to a file with extension *.bmapnpo using the **Save Preset** button at lower left. A saved file can then be reloaded with the **Load Preset** button. This is useful when creating multiple annotation reference projects with similar or the same data. Protein Metrics Batcher software can be used to run several data files with the same *.bmapnpo parameters; this is useful for automated workflows.

After all values have been entered, click the **Create** button to create the project. The **Save File** dialog will open to enter a folder location and file name for saving the project. Project creation takes less than a minute for a typical spectrum file of size 10 – 100 MB. The **Cancel** button will close the window without creating a project.

Creating a Comparison Project

Chromatogram Analysis supports comparison of multiple chromatograms on the same protein or detached glycan molecules for lot-to-lot comparability, biosimilarity, and degradation studies. Chromatogram Analysis also supports multi-sample projects for high-throughput analysis of many targets.

To create a comparison project, select **File > New Comparison Project**. Alternatively, click the icon below the Edit menu. In the **New Comparison project** dialog, drag in a previously saved reference project *.bmap file to the **MS file** column. Alternatively, double-click in the **MS file** first column cell, click the button and navigate to the reference file. Next, drag in or navigate to the files to be compared to the reference.

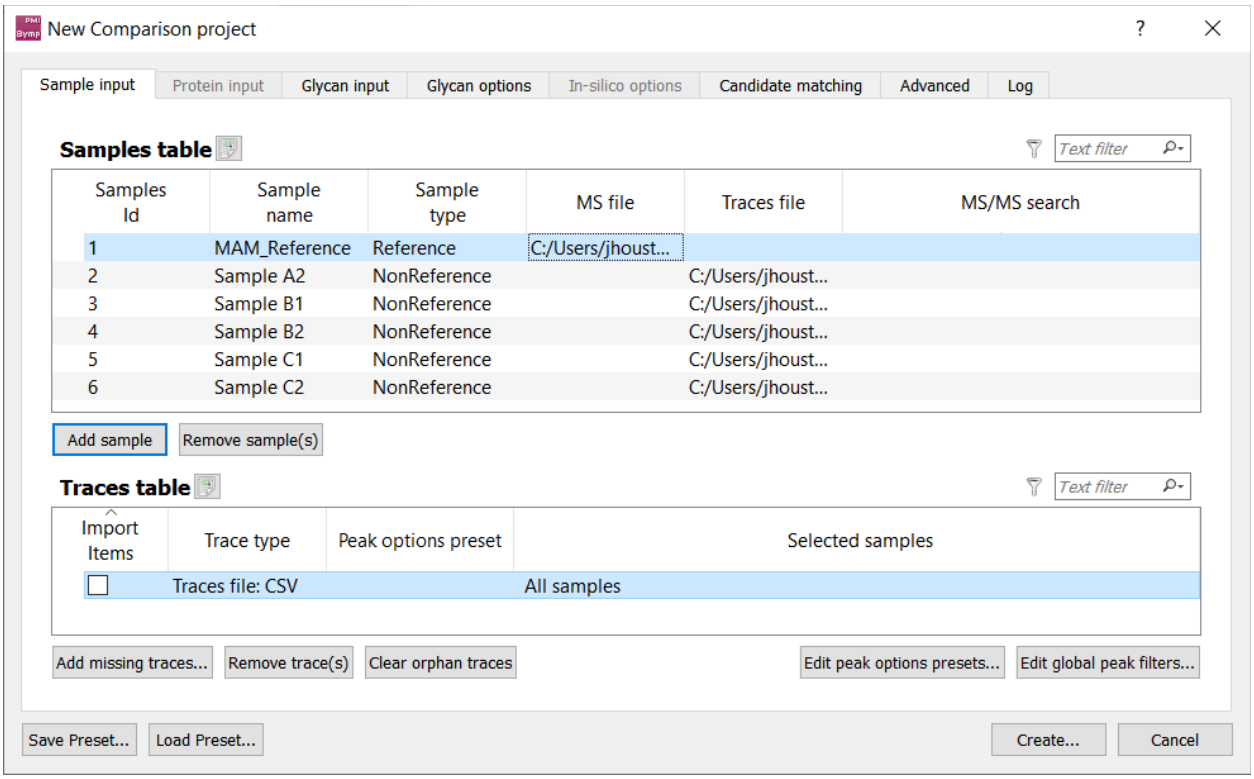


Figure 46: New Comparison project

In the above example, a reference project *.bmap file was added along with five additional chromatograms from imported UV traces in *.txt or *.csv file format. The user may also import raw data files from various instrument manufacturers.

As with reference projects, traces are displayed in the Traces table. Byonic MS/MS search files in *.byrslt format can be dragged or navigated into the **MS/MS search** column.

The **Glycan input**, **Glycan options**, **Candidate matching**, **Advanced** and **Log** tabs function the same way as when creating a reference project. Note that the Protein input and In-silico options tabs are greyed out because that information has already been entered and saved with the reference project *.bmap file.

Enter the initial settings as with a new reference project. The use of a saved preset also saves time when creating comparison projects. When ready, click **Create**. Enter a comparison project file name and location. Click **Create** to generate the new project.

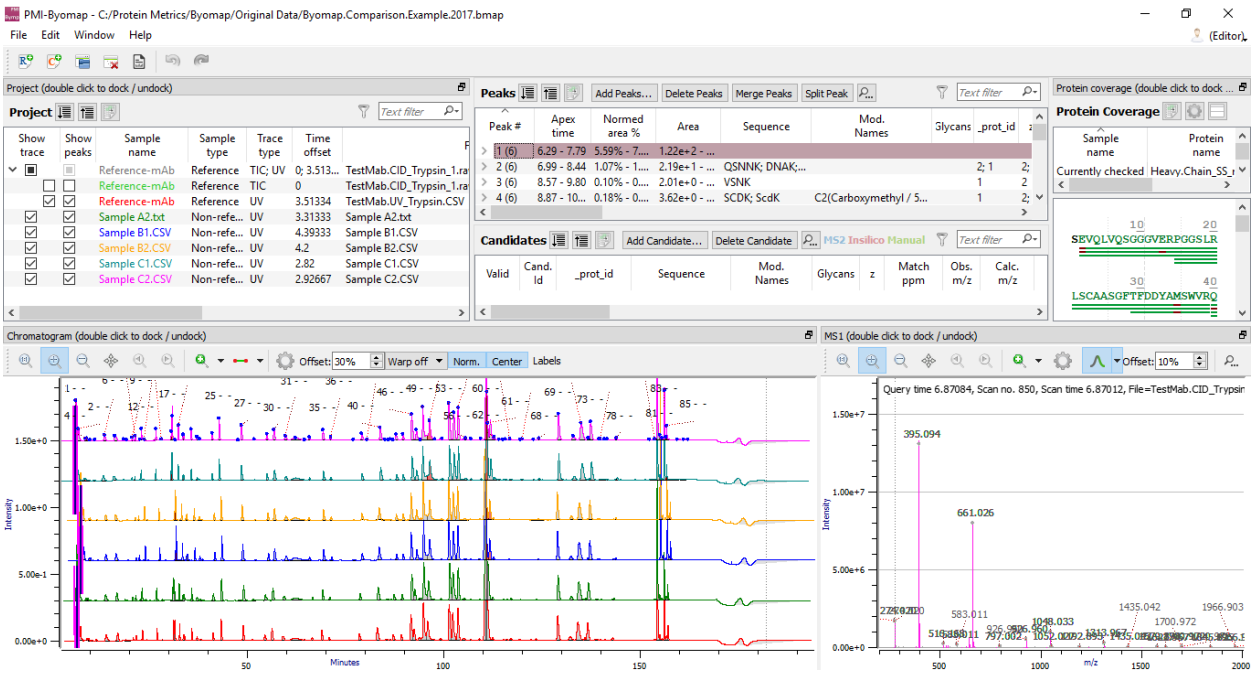


Figure 47: Comparison project

In the new comparison project, the **Project** table lists the raw and source files. The linear time offsets line up the TIC chromatograms with the optical channels for each sample. This happens automatically but the user can edit these values. The **Show trace** and **Show peaks** check boxes display or hide the files and peak subsets. The color coding used in the **Sample name** field corresponds to the colors of the traces in the **Chromatogram** plot.

The **Chromatogram** plot shows an overlay or stacked display of all TIC traces. The **Offset** percent controls the vertical spacing of the traces. For each peak identified in the reference project, the program attempts to identify the equivalent peak in each comparison file. Mismatched peaks, which should be rare, can be corrected manually.

Main Menu Bar

The topmost menu bar includes four items: **File, Edit, Window,** and **Help**.

File Menu

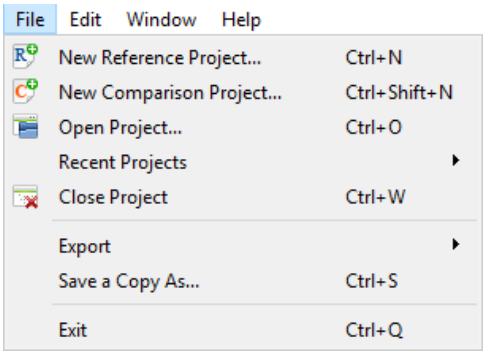


Figure 48: File menu

The **File** pull-down menus manage project files and export reports and data. The File menus includes items to create new reference or comparison projects, to open previously created projects from saved files, to save a copy of an open project, to close a project (yet leave the application open), and to exit Chromatogram Analysis. Mouse over the **Recent Projects** menu to open a side window with a list of recently visited projects that can be reopened.

The File menu functions **New Reference Project**, **New Comparison Project**, **Open Project**, **Close Project**, and **Export > Report**, respectively, are also available as icon buttons below the topmost menu bar.

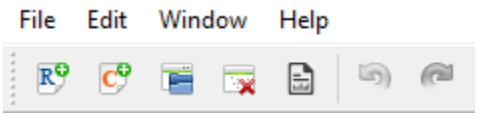


Figure 49: Menus and toolbar buttons

File > Export Menu

There are also five File sub-menu items to export reports and data.

File > Export > Report generates a report that includes a summary of the project files, one or more pivot tables (that can be visualized as bar charts, etc.), along with plot images. There are other valuable tools for making reports and regulatory filing documents. The general report is first opened in Chromatogram Analysis.

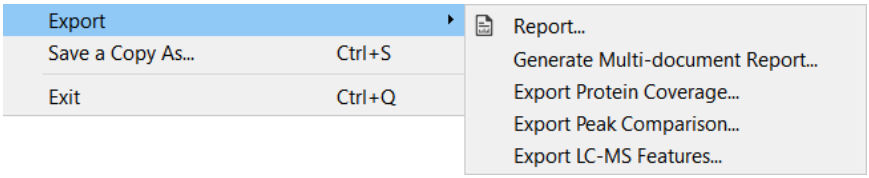


Figure 50: File > Export menu

File > Export > Report generates a report that includes a summary of the project files, one or more pivot tables (that can be visualized as heatmaps, bar charts, etc.), along with plot images.

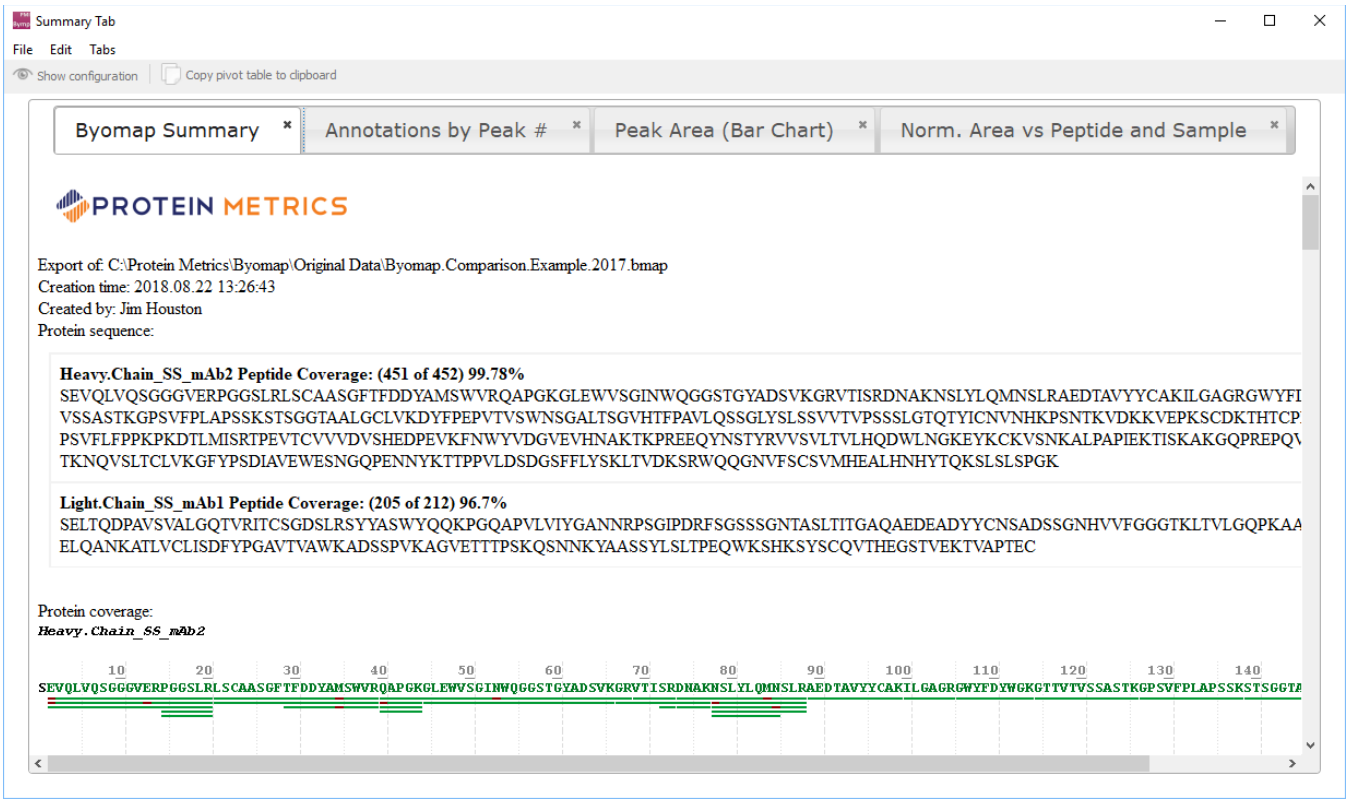


Figure 51: Default Chromatogram Analysis report

The **File > Export** sub-menu includes the item Generate Multi-document Report. The user can drag and drop in Chromatogram Analysis project *.bmap files to create a multi-document report displaying the table and graphical data from a series of reports.

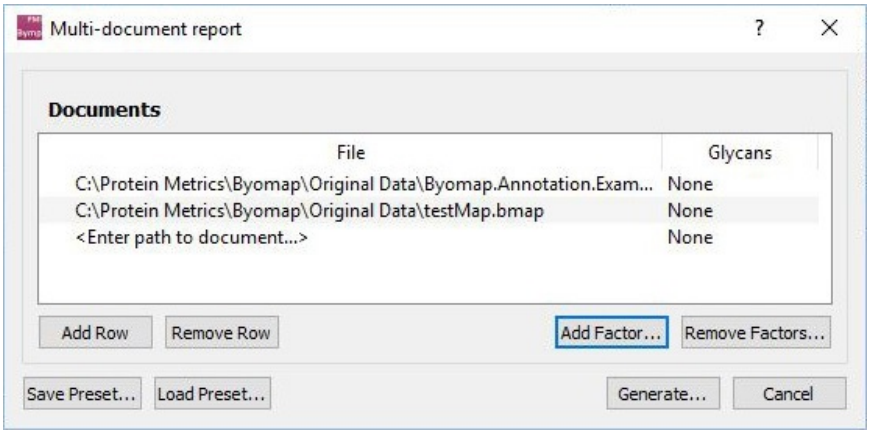


Figure 52: Multi-document report

To learn about reports in detail see the document **PMI Reporting Manual.pdf**.

File > Export > Export Protein Coverage opens a dialog identifying the sequenced proteins in reference projects. Additional proteins can be added from .fasta files or they can be added manually. The report can be customized for **Fixed modification**, **Digestion** or **Missed cleavage maximum** values.

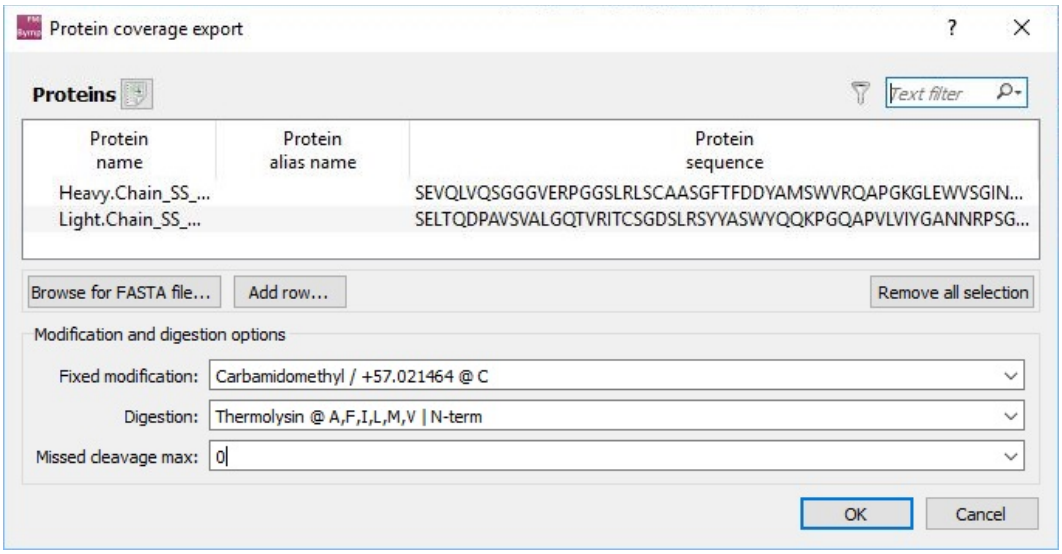


Figure 53: Protein coverage export

This dialog box is very similar to the in-silico generator box. It generates a .csv file with important overall project information followed by a table organized by the sequence of the protein.

File > Export > Export Peak Comparison exports a *.csv file containing a report of Peak table data from a comparison project

File > Export > Export LC-MS Features exports a *.csv file containing information about all observed molecular ions above a given intensity threshold, whether identified or not. This is useful to target unidentified molecular ions for further laboratory investigation by providing an inclusion list of unidentified ions for m/z and retention time (RT).

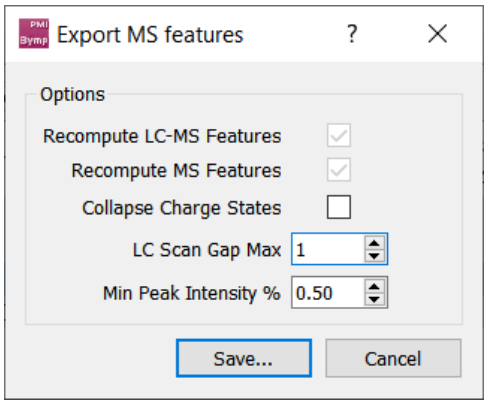


Figure 54: Export MS Features

Edit Menu

The **Edit** pull-down menu manages program settings.

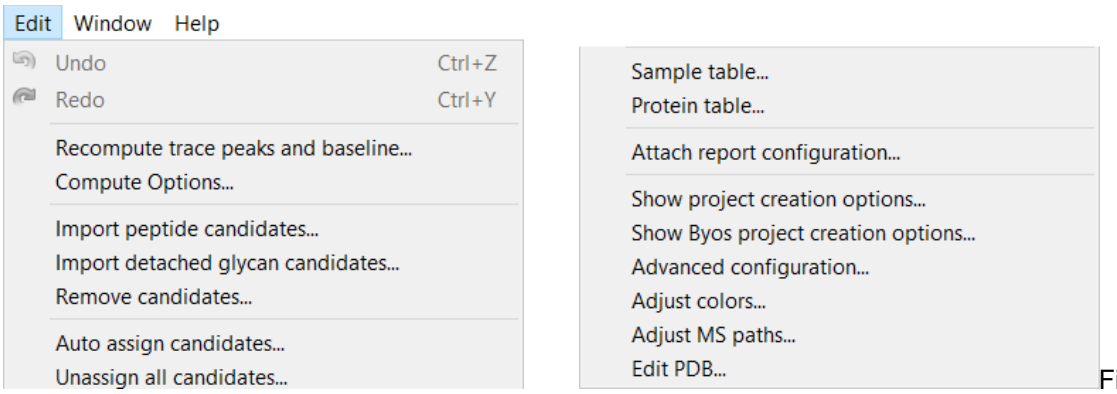


Figure 55: Edit menu

Undo and **Redo** allow the user to undo or redo changes. These Edit menu functions are also available as the arrow toolbar buttons bellow the menu bar to the right of the **Help** menu.

Recompute trace peaks and baseline opens the **Trace peak options** dialog that includes settings to modify and recompute the trace peaks.

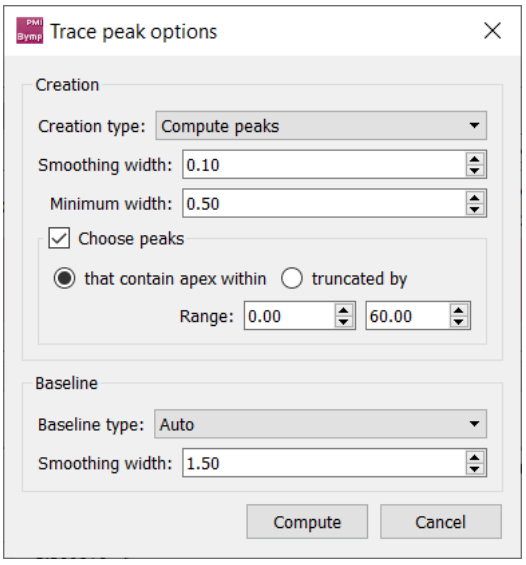


Figure 56: Trace peak options – post-processing

This is equivalent to the dialog opened by the **Edit trace peak options** button in the **Sample Input** tab when creating a new project. Thus, the user has the option to alter the chromatogram processing before and/or after project creation. For details, see the **Trace Peaks Options** section.

Compute Options opens the **Trace peaks compute options** dialog, which includes a list of trace peaks, the samples that contain these peaks, and the peak start and end times. To change the deconvolution parameters (*m/z* range, m range, etc.) for a specific trace peak, click **Edit** for that peak. This opens the **Intact options** dialog:

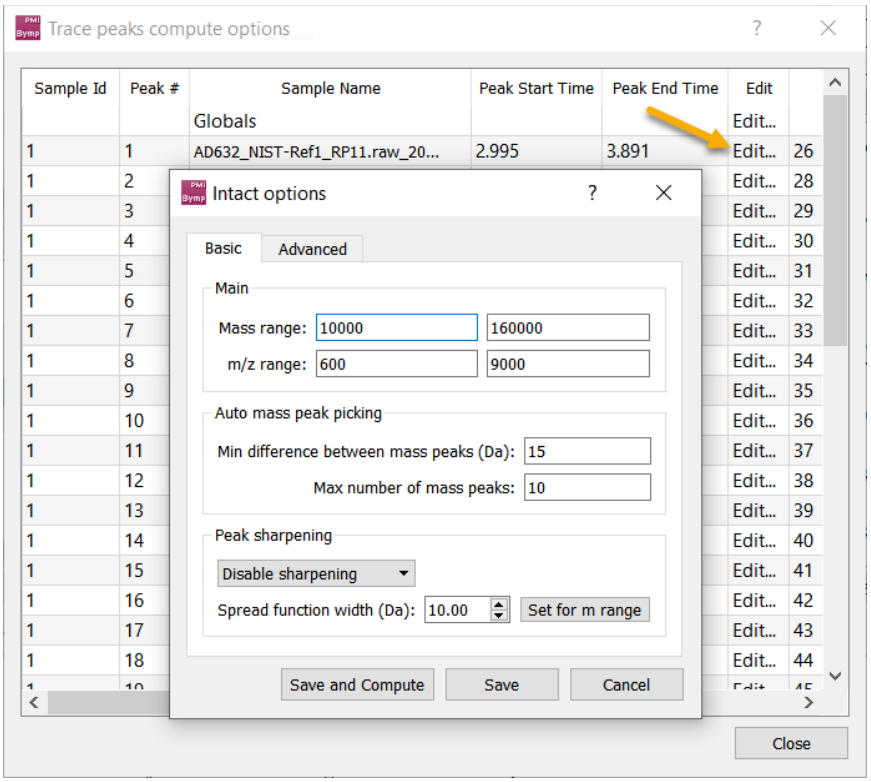


Figure 57: Intact options opened from the Trace peaks compute options dialog

The **Basic** tab contains the primary settings used in computing deconvolved masses. **Mass range** defines the range of neutral masses displayed in the neutral mass spectrum. ***m/z* range** defines the segment of the MS1 spectrum used to compute neutral masses. **Min difference between mass peaks** prevents the peak picker from picking multiple points on top of a ragged or isotope-resolved mass peak. **Max number of mass peaks** sets a limit on the number of picked peaks. The **Peak sharpening** dropdown controls an optional “super-resolution step” that sharpens peaks beyond what is seen in the data. **Spread function width** sets the expected width of a peak when peak sharpening is enabled. A reasonable width is the square root of the peak mass in kilodaltons, for example, 12 for a spectrum from 140,000 to 150,000 Da. A too-narrow width will not sharpen much, and a too-wide width might split peaks. Peak sharpening on mass spectra that span a range of more than about 20,000 Da is not recommended, because no single width will be optimal for the entire range. Peak sharpening should also be avoided on isotope-resolved mass spectra.

The **Advanced** tab of the **Intact options** dialog includes deconvolution and sharpening settings that are less commonly modified.

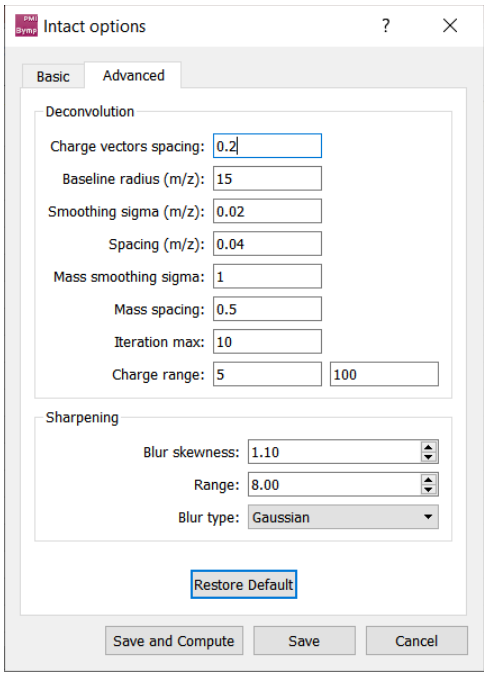


Figure 58: Intact options Advanced tab

After the settings are modified, click **Save and Compute**. The program computes an MS1 summed m/z spectrum and peak properties for each trace peak in the Peaks table.

Import peptide candidates opens a dialog to import or manually enter in-silico peptides:

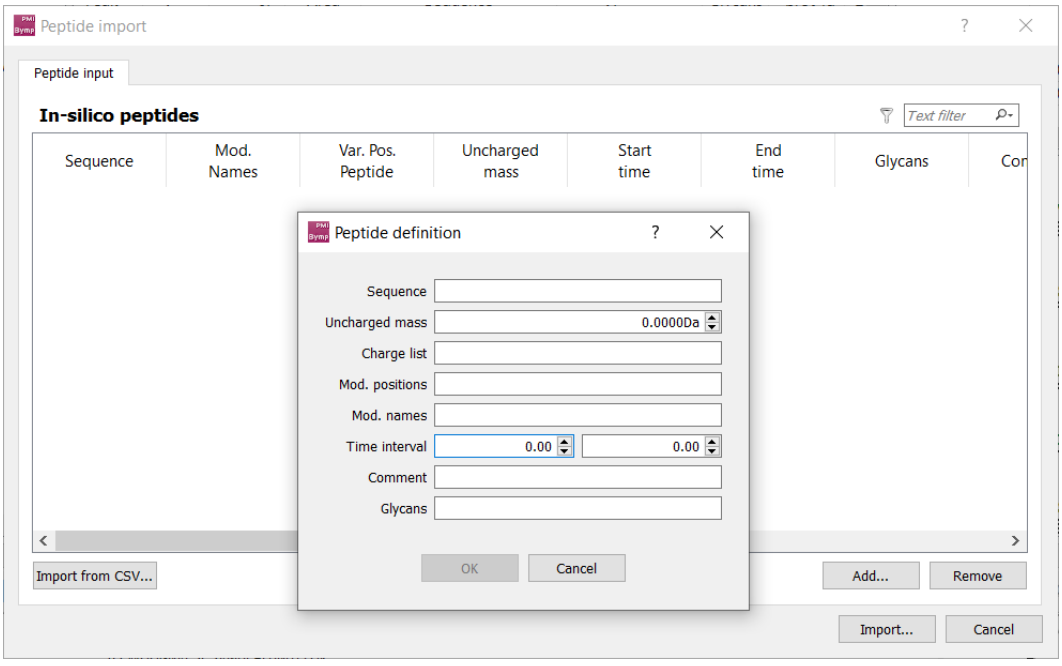


Figure 59: Peptide import and definition

Add from CSV file imports glycans from a *.csv file. **Add** opens the sub-dialog shown above, where the peptide sequence, mass and other properties can be added manually.

Import detached glycan candidates opens a dialog to import or manually enter glycans, similar to the **Glycan input** and **Glycan options** tabs during project creation:

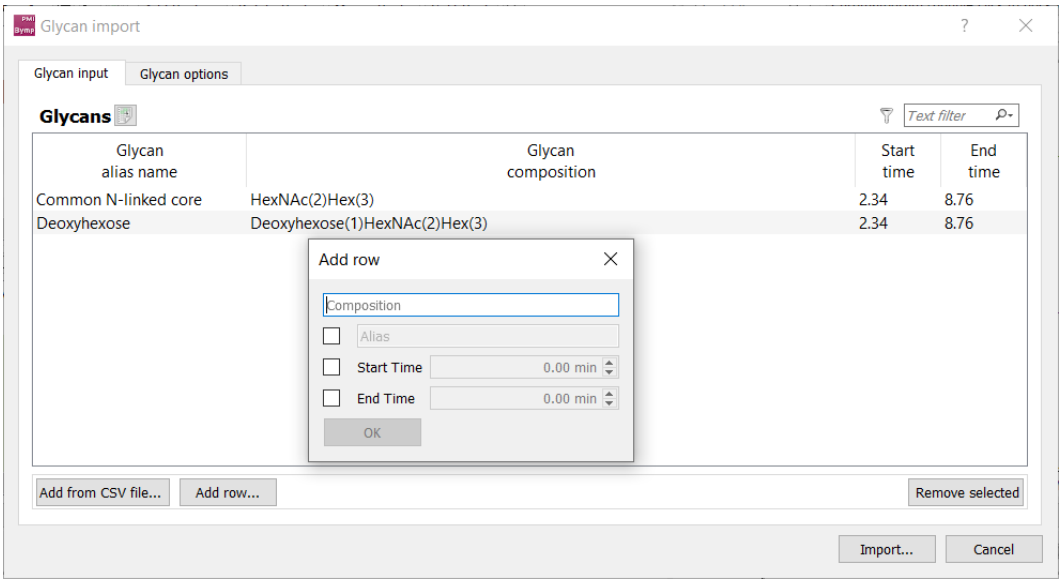


Figure 60: Glycan import – Glycan input

Add from CSV file imports glycans from a *.csv file. **Add row** opens the sub-dialog shown above, where a glycan composition can be added manually, along with optional entries for Alias, Start Time and End Time. The **Glycan options** tab defines labels and addicts for the glycans:

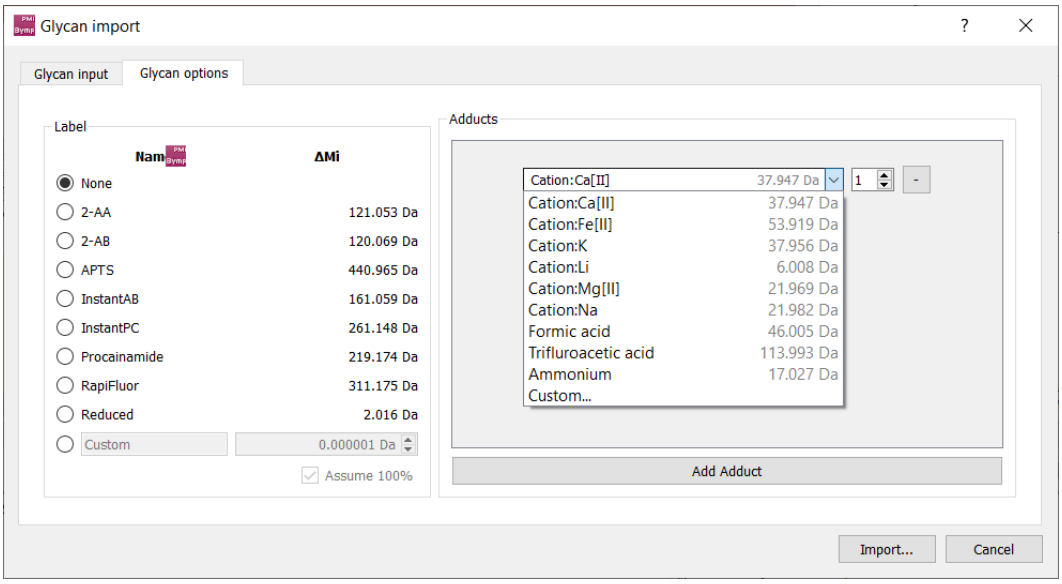


Figure 61: Glycan import - Glycan options

Remove candidates removes either unassigned or all in-silico candidates. This can be useful in cases where a wrong or incomplete candidate list has been imported. The user may then import a new set of candidates.

Auto assign candidates will match candidates with observed molecular ions within the m/z and RT tolerances set. These tolerances may be changed from their default values as shown in the **Advanced and**

Log tabs section. It is possible to manually assign candidates, but this tool is a reliable time saver and it is recommended. It is further recommended to review auto assigned candidates and edit them as needed.

Unassign all candidate removes and uncheck all candidate assignments. This function is useful when a newer set of candidates needs to be imported.

Sample table is used to customize the Project view. To add a comment for a sample, click in the **Comment** cell and enter text. To add a new column, click **Add custom column**, enter the column name and an optional default value and click **OK** twice. The column will appear after Comment in the Project View. To remove a custom column, click **Remove custom column**, select the column, and click **OK**.

Protein table is used to add proteins associated with a sample. Proteins are described by name (Accession name), sequence, and average mass. They are assigned to samples by the **Sample ID**. A protein defined in this table will automatically be labeled as **Reference** during automated mass assignments. As above, custom columns can be added and removed.

Attach report configuration attaches a report configuration file to the project, to be used instead of the default report configuration. Report configuration files (graphical layouts) are generated from **File > Export > Report** and have the extensions *.rptc or *.lvcfg. This menu can also be used to remove an attached report configuration file.

Show project creation options displays in a single window the values and inputs used to create the current project. This data is read-only in this window. To edit specific functions and values, use the corresponding Edit menu items described in this section. This feature allows the user to save the current settings used in the project with the **Save preset** button or load new ones with the **Load preset** button.

Show Byos project creation options displays the Byos workflow project creation options used to generate the project. This feature is only available for projects created through Byos. A read-only dialog similar to the **Byos Project Creation** dialog opens:

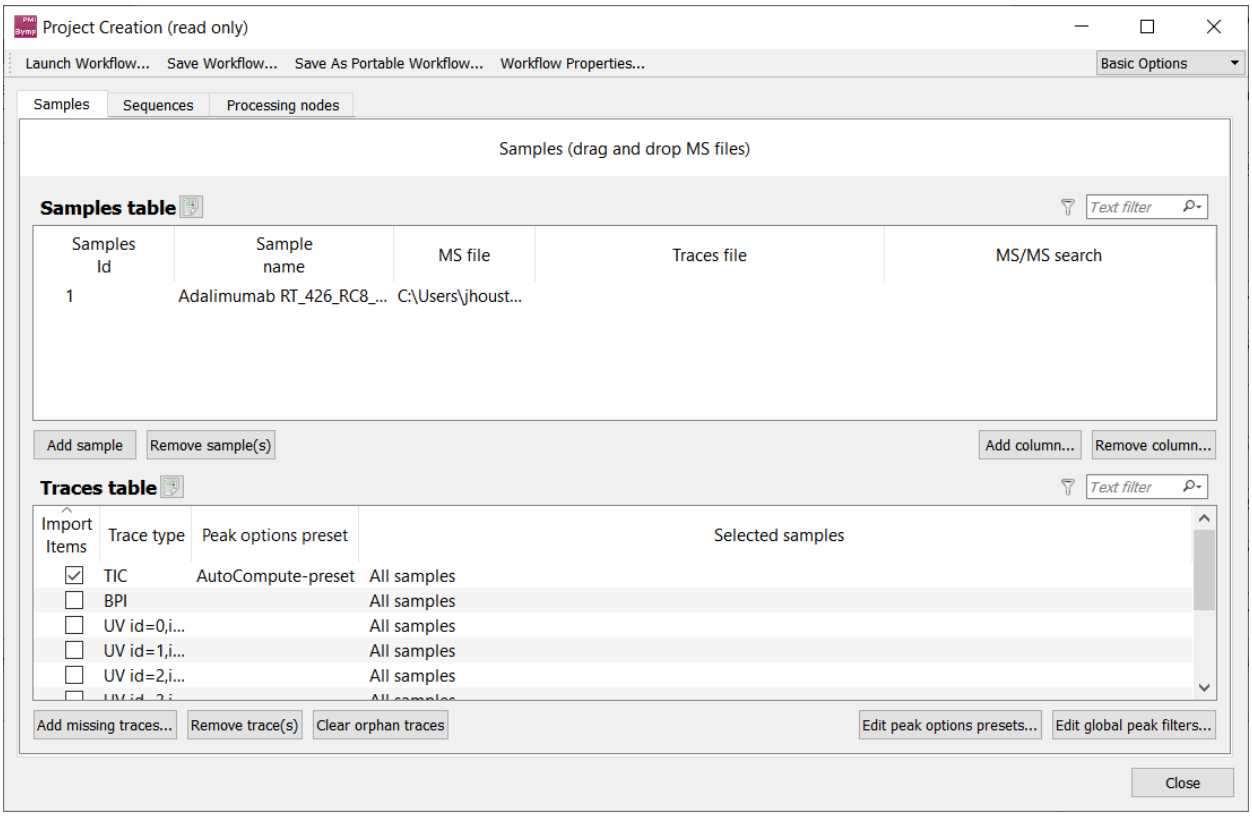


Figure 62: Show Byos project creation options

Advanced configuration is used to enter text commands, which are often new features still in beta testing. Text commands include controls for: use of isotope spacing to infer charge, removal of charge-one *m/z* peaks, expected mass differences between molecules (for example, 162 Da for hexose), and negative mode (for example, deprotonated proteins and DNA).

Adjust colors is used to modify the default colors associated with samples and chromatogram traces.

Adjust MS paths is used to modify the path to the source MS file, should the file be moved. Enter or navigate to the new directory containing the MS file.

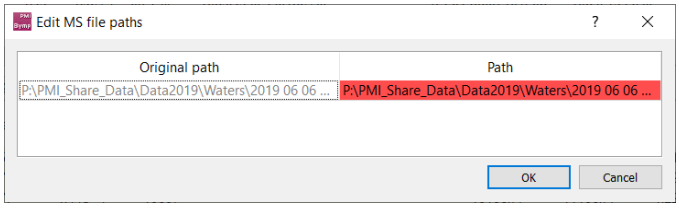


Figure 63: Edit MS file paths

Edit PDB loads protein database files for use by Protein 3D reports.

Window Menu

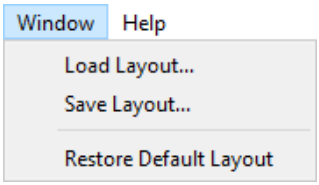


Figure 64: Window menu

The **Window** pull-down menu manages the visual layout, and the arrangement and sizes of the various table and plot views. **Load Layout** opens a saved layout stored to files with extension .ini. **Save Layout** saves the current layout to an .ini file. **Restore Default Layout** applies the layout used when the application is first opened.

The current layout can be customized by moving, resizing or editing the view panes. Many of the views (Project, Chromatogram, Protein coverage, and MS1) can be undocked by double-clicks on the tops of that view to create standalone windows that can be moved to another monitor. These views can be click-dragged to new locations in the layout. The view can also be resized by hovering over the vertical or horizontal edges between table and plot views so that the cursor changes to arrows:  or . Left click and drag the edges up or down or right or left to change the sizes of the views. Columns in the table views can be made larger or smaller in same manner. To reorder columns in a table, left click the name of the column, and then drag it to the new location. To do a simple sort, left click the header once to sort ascending and twice to sort descending. Mouse over a column header to see a description of that field.

Column headers can be modified through the **Column Header Editor** dialog, opened by a right-click anywhere on a table column header. Columns can be rearranged by dragging the header names up or down. To hide a column, uncheck the **Visible** box for that column. This dialog can also be used to specify number of significant figures and ranked sorting. The **Window > Save Layout** menu preserves these edits. The **Reset row sorting** and **Reset column order** buttons restores the default row and column properties.

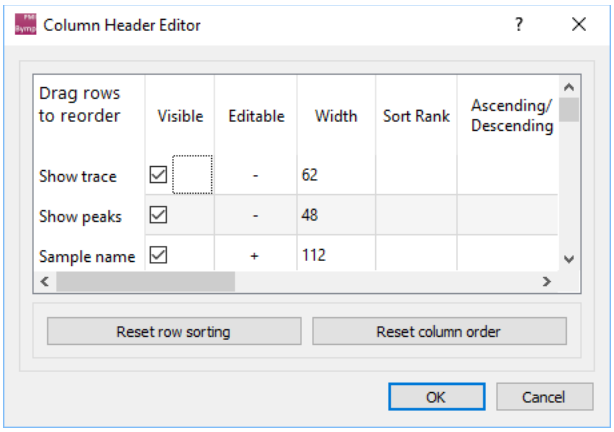


Figure 65: Column Header Editor

Columns can be rearranged by dragging the header names up or down. To hide a column, uncheck the Visible box for that column. This dialog can also be used to specify number of significant figures and ranked sorting. The Window > Save Layout menu preserves these edits for future use and sharing. The Reset row sorting and Reset column order buttons restores the default row and column properties.

Help Menu

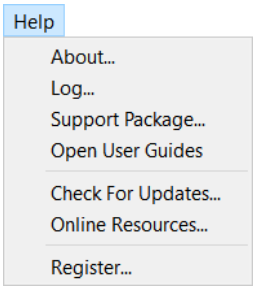


Figure 66: Help menu

The **Help** pull-down menu provides information about the software. The **About** menu shows the software version number, which is needed when reporting issues. The **Log** menu opens a log containing recent activity; this is helpful for troubleshooting problems together with Protein Metrics staff via support@proteinmetrics.com. **Support Package** collects all relevant information helpful for troubleshooting problems together with Protein Metrics staff via support@proteinmetrics.com. **Open User Guides** opens the installed folder with the manuals and quick start guides for Protein Metrics software, including PMI Chromatogram Analysis Manual. **Online Resources** opens the www.proteinmetrics.com/secure-resources webpage containing a variety of technical pages and tutorials related to PMI software. **Register** is used to activate the software upon first use.

Table Menu Bars

The menu bars at the top of the three table views, Project, Peaks, and Candidates, share icons that manage hierarchical lists and filter on data.

- The  icon expands rows to show “sub-rows”.
- The  icon collapses rows to hide sub-rows. The use of sub-row depends on the table. For example, in the Peaks table, a row is a trace peak and a sub-row is a mass peak within the spectrum.

- The  icon exports the table to a .csv file for opening with Excel.
- The  icon opens a dialog to create custom filters for the data table. (A single column filter can also be set by right-clicking on an entry in the column.)

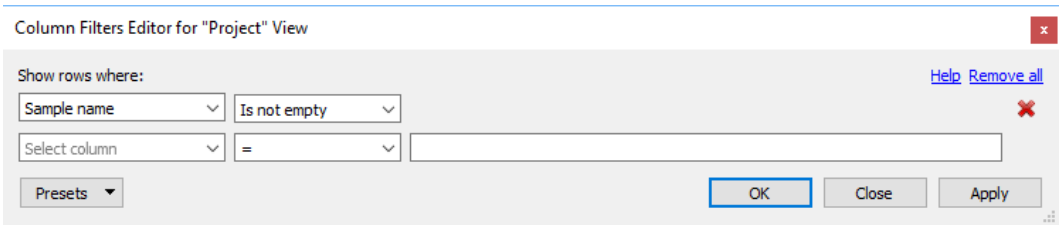


Figure 67: Column filters editor

Select a column in the first cell, select an operator in the second cell, and enter text in the third cell. A second filter row becomes available for further entry. Click the red X after a filter row to delete it. Custom filters allow masses to be filtered by mass range, annotation, intensity, and so forth. Custom filters can be stored with the project document or exported and imported using the **Presets** drop-down.

- The 

cell filters the entered text across the content of all columns in the protein and peptide tables. The records are filtered automatically as text is entered. Click the icon to search the string as a whole word or as case sensitive.
- To sort the contents of a column by ascending value, click the column header. Click the column header again to sort by descending value.

Filter Options

The  icon located on the Peaks, Candidates tables and MS1 plot opens a dialog that allows the user to filter peak and candidate data. The three views launch the same filter dialog, opening to the tab applicable to that view.

From the **Peaks** table, the magnifying glass icon opens the **Filter options** dialog:

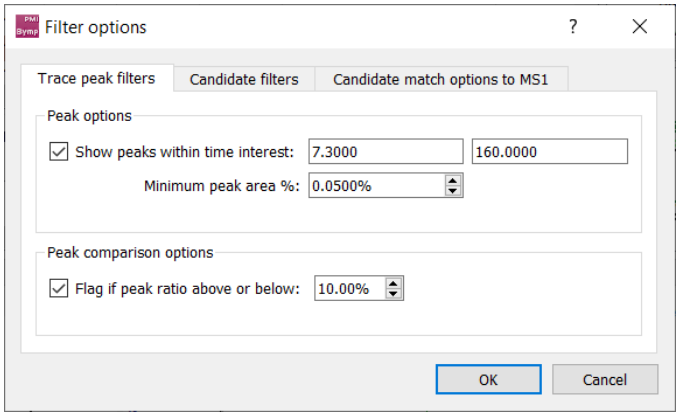


Figure 68: Filter options – Trace peak filters tab

Trace peak areas generally constitute a critical quality attribute (CQA) for product development and manufacture. One of the features of Chromatogram Analysis is the ability to automatically detect significant concentration changes between samples in a comparison project for each trace peak. The **Trace peak filters** tab sets the trace range and minimum peak area (percent of TIC, +/-0.10% by default) needed to

determine which trace peaks will be processed and assigned IDs. It is sometimes desirable to exclude ill-behaving areas at the beginning and end of a chromatogram. The **Peak comparison options** checkbox at the bottom flags comparison peaks that are above or below the reference peak by the percentage specified. Flagged comparison peaks are colored red in the Chromatogram plot.

From the **Candidates table**, the magnifying glass icon opens the **Candidate filters** tab.

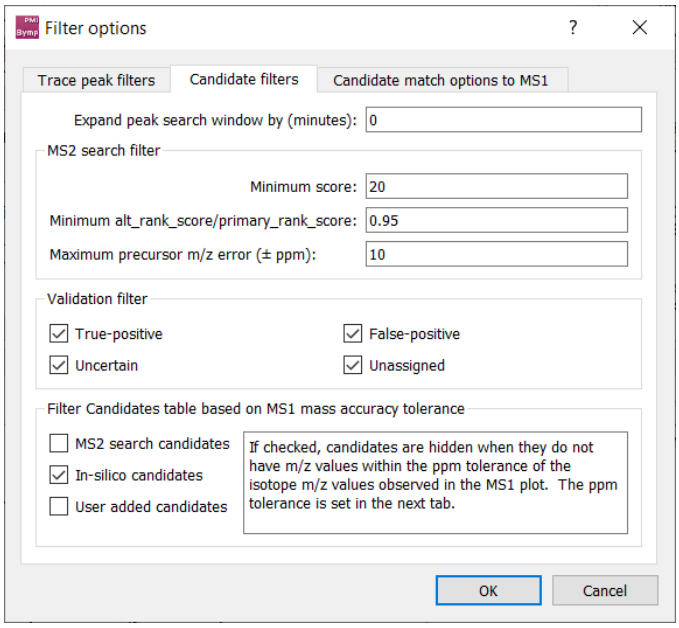


Figure 69: Candidate filters tab

This tab sets filters for candidates from the MS2 search results via the Peptide Analysis software or Byonic results. When filtering the MS2 results, there is the time uncertainty setting **Expand peak search window**, minimum score, and precursor m/z error setting and validation filters associated with the Peptide Analysis or Byonic software. For filtering on MS1 mass accuracy, the MS2 search identifications are already constrained by mass accuracy.

From the **Peaks** table, the magnifying glass icon opens the **Candidate match options to MS1** tab.

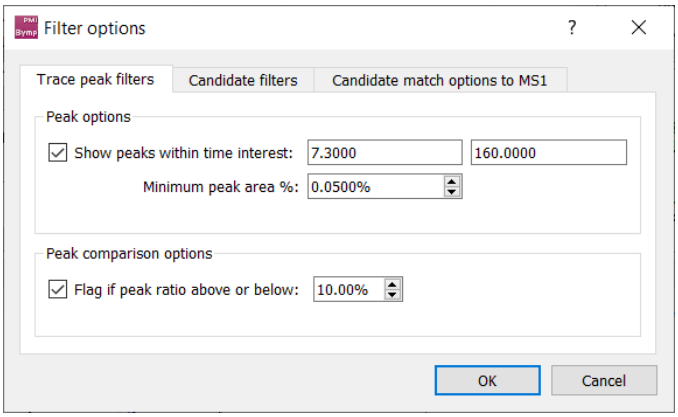


Figure 70: Candidate match options to MS1 tab

This tab controls the parameters associated with the molecular assignment of mass spectral peaks to a trace peak, and consequently the selection of candidates displayed in the Candidates table and MS1 plot.

There are ranges for charge states allowed for viewing (default is z =1 to 5), ppm tolerance (default 10 ppm), and minimum peak intensity percent in the MS1 view (default 10%). This last parameter applies to which molecular ions in the MS1 plot are sufficiently intense to be matched to a candidate. In this case, an isotope peak is eligible for matching if it is 10% or greater of the intensity of the most intense isotope in the MS1 plot, evaluated at the trace time apex of the peptide map peak. Mass spectral peaks below the Minimum m/z are ignored.

Table Right-Click Menus

The Project, Peaks and Candidates tables have context menu items revealed by a right-click on the rows of the tables. (Recall that a right-click on the header of these tables opens the Column Header Editor dialog.)

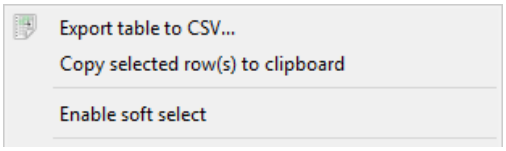


Figure 71: Table right-click menus

- **Export table to CSV** saves the table content and headers to a .csv file using parameters chosen in the **Export data** dialog. The user is also given the option to open the saved .csv file in Microsoft Excel.
- **Copy selected row(s) to clipboard** copies selected table rows, with their headers to be available for pasting into another application.
- **Enable soft select** (**Project** table only) enables selection of rows in the Project table to display them individually in the Chromatogram plot and other views, without the need to uncheck the **Show** checkboxes for the other rows.
- A set of query options for searching on the clicked text value using any of the available search operators. This is an easy way to filter a table based on a specific field value.

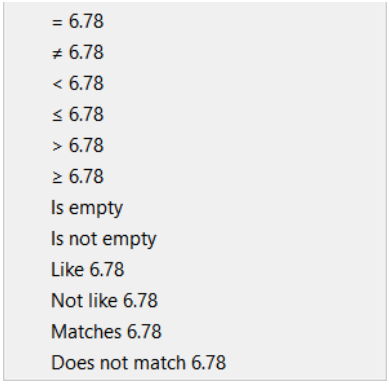


Figure 72:Right-click field searches

A table view's menus may also include options specialized for that table.

Project Table and Menu

The **Project** table contains information about the sample and data (trace) type. For a reference project, there is only one mass spec channel but there can be multiple optical channels:

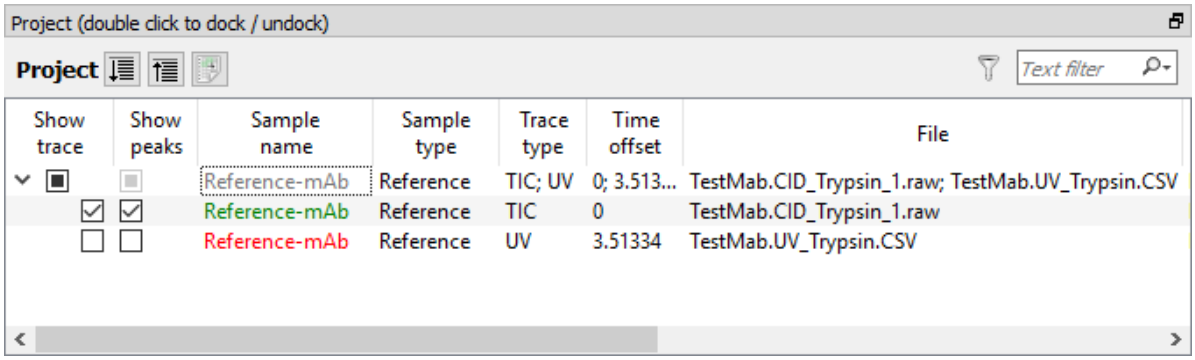


Figure 73: Project table for reference project

To display one or all of the chromatograms (channels) present, check the **Show trace** box at left. Only one of these traces may have peak numbers for annotation (because different chromatograms often have different numbers of peaks above the threshold). To choose the trace to annotate, check the **Show peaks** box. The Trace type shows the data type, and the Time offset displays the time difference between the XIC and optical trace times, through a linear time shift often needed for flow splitting. The Time offset can be edited by a click on the value, but this is seldom needed.

Comparison projects display multiple mass sec channels.

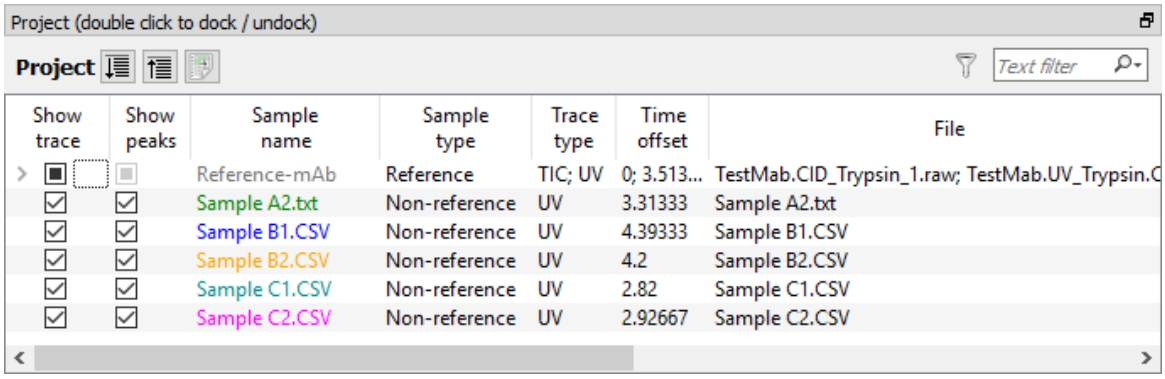


Figure 74: Project table for comparison project

Peaks Table and Menu

The **Peaks** table is where all important information is displayed in tabular form about the trace peaks and their molecular constituents, including analytical details:

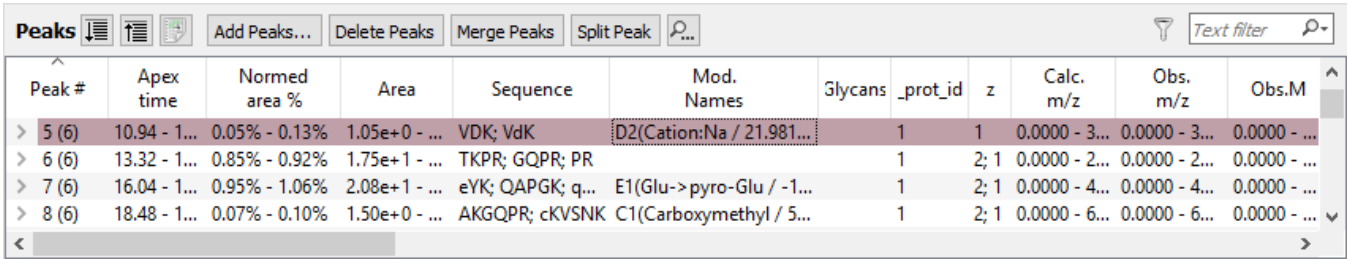


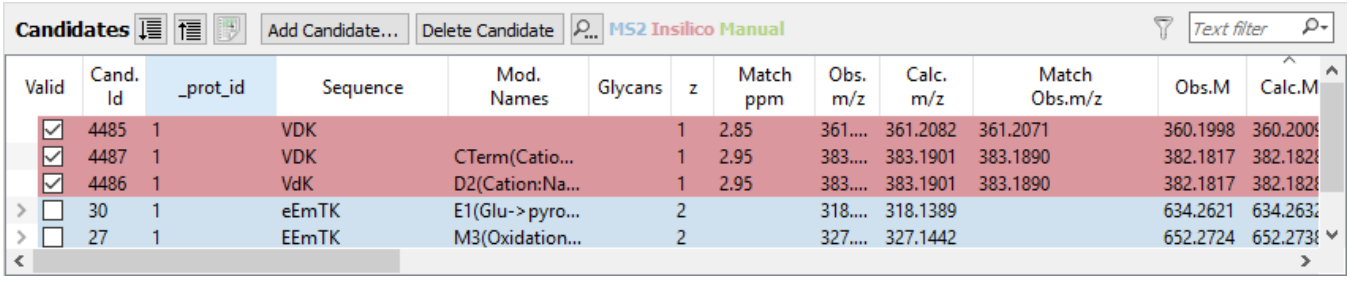
Figure 75: Peaks table

The Peaks table also includes the following specialized buttons:

- **Add Peaks** opens a dialog for the input of start and end integration times for new peaks to be added to the table. The user can choose between TIC or UV traces if both are active. This function is useful if a peak is slightly below the threshold for auto-assignment but the user still wishes to include it.
- **Delete Peaks** deletes the selected peak from the table.
- **Merge Peaks** merges a contiguous set of selected peaks into a single peak. Use Ctrl-click or Shift-click to select a set of peak rows.
- **Split Peak** splits a highlighted peak that has a local minimum into two peaks. The time values for the two peaks can then be adjusted separately.

Candidates Table and Menu

The **Candidates** table contains all the imported candidates relevant to the selected trace peak.



Valid	Cand. Id	_prot_id	Sequence	Mod. Names	Glycans	z	Match ppm	Obs. m/z	Calc. m/z	Match Obs.m/z	Obs.M	Calc.M
☑	4485	1	VDK			1	2.85	361....	361.2082	361.2071	360.1998	360.200
☑	4487	1	VDK	CTerm(Catio...		1	2.95	383....	383.1901	383.1890	382.1817	382.182
☑	4486	1	VdK	D2(Cation:Na...		1	2.95	383....	383.1901	383.1890	382.1817	382.182
☐	30	1	eEmTK	E1(Glu->pyro...		2		318....	318.1389		634.2621	634.263
☐	27	1	EEmTK	M3(Oxidation...		2		327....	327.1442		652.2724	652.273

Figure 76: Candidates table

As described earlier, candidates can be imported during project creation, and afterwards by using the **Edit > Import candidates** menu. The three sources for imported candidates are Peptide Analysis *.blgc files, Byonic .*.byrslt files, and in-silico *.csv files.

The Candidates table also includes the following specialized buttons:

- **Add Candidate** allows for the manual import of a candidate. It opens the **New candidate options** dialog:

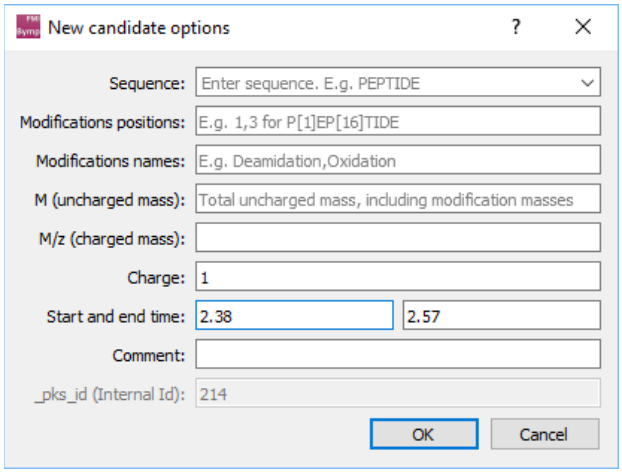


Figure 77: New candidate options

  Note that not all of the fields require an entry.

- **Delete Candidate** removes the selected candidate rows.

Note the color coding of the rows in the Candidates table as shown in the header: MS2 Insilico Manual. Blue rows represent MS2 search results, pink rows represent in-silico candidates, and green rows represent manual entry. When a candidate is assignment, the color takes on a darker shade and a check box appears in the **Valid** column.

As described in **Edit Menu**, there is an auto-assign function for candidates in Chromatogram Analysis. The user can manually assign or un-assign any candidate by checking/unchecking the **Valid** box. The criteria for assignment (matching) are found in the **Filter options** dialog box on the **Candidate filters** and **Candidate match options to MS1** tabs, as described in **Filter Options**. For example, for imported MS2 identifications, entering a time in minutes for the **Expand peak search window by** field can be useful.

Protein Coverage Table Display and Menus

The **Protein coverage** table includes protein and protein coverage data. The accompanying display maps the protein sequences of detected peptides to green lines below each sequence:

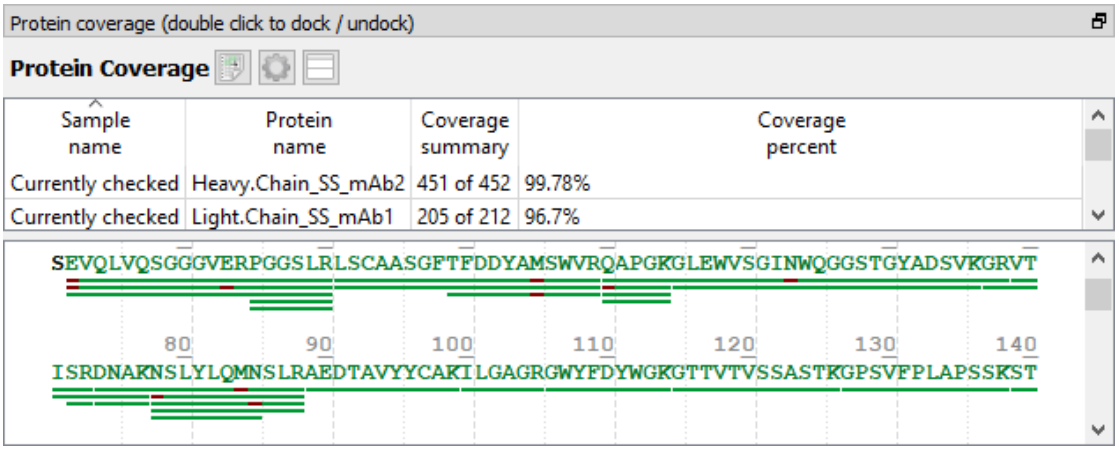


Figure 78: Protein Coverage table

If a highlighted trace peak contains that peptide sequence, it is highlighted in orange. Modifications (such as oxidation or deamidation) to the peptide are highlighted in red. The coverage map is interactive with other views. The user may select a row in the protein coverage table to highlight that peptide in the display. The user may highlight a trace peak in the Peaks table or Chromatogram plot and the corresponding peptide will become highlighted in the Protein coverage display.

The **Protein coverage** table includes the following specialized menu buttons:

- The  icon exports the protein coverage data in .csv format.
- The  icon opens the **Protein coverage rendering options** dialog:

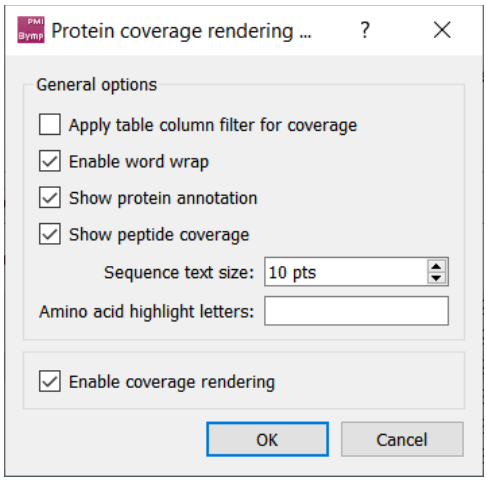


Figure 79: Protein coverage rendering

- The  icon turns off and on the Protein coverage table as a split screen with the sequence display.

Right click **Export table to CSV** in the Protein coverage table to save the table content and headers to a .csv file. The user is also given the option to open the.csv file in Microsoft Excel.

Right click in the Protein Coverage sequence display to enable a menu of three specialized options:

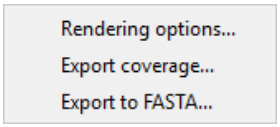


Figure 80: Protein Coverage right-click menu

- **Rendering options** opens the Protein coverage rendering options dialog shown directly above.
- **Export coverage** saves the protein sequence graphic as a *.png file. The user is also given the option to open it in the default graphic viewer.
- **Export to FASTA** saves the protein sequence graphic as a *.fasta file. The FASTA file is then available for import into new projects. The user is also given the option to open it in the default FASTA reader.

Plot Menu Bars

The menu bars at the top of the two plot views Chromatogram and MS1 spectrum share icons that support generic operations on plots. These icons appear in almost all of Protein Metrics's software products.

- The  icon resets the plot to default zoom level.
- The  icon enables zooming in. The cursor changes to this icon. Drag across the range of the plot to display to zoom to that x-range. By default, the plot's y-range scales according to the maximum y-value within the x-range, but the software also supports freeform zooming as described under the  icon.
- The  icon enables zooming out. Click anywhere in the plot to zoom out.

- The  icon enables moving (panning) across the plot. The cursor changes to this icon. Click the plot and drag up or down, right or left to view a part of the plot that is off-screen.
- The  icon performs an undo of the last zoom step.
- The  icon performs a redo of the last zoom step.
- The  icon manages plot segments (zoom states).

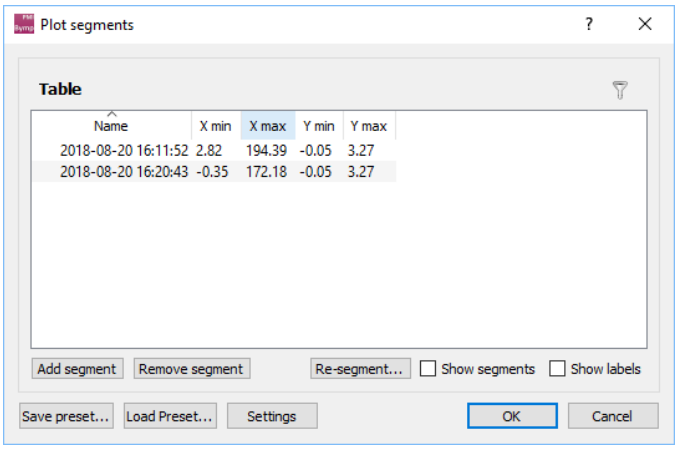


Figure 81: Plot segments

The number displayed in the icon shows how many plot segments are stored (named by the date and time of the save). To save the current zoom state as a plot segment, simply click the  icon. To switch to a previously stored plot segment, click the drop-down arrow and select the segment name to add, remove, rename or edit plot segments, click the drop-down arrow and then click **Edit**. The zoom state names, and x and y minima and maxima may be edited. To display all the plot segments at once, check **Show segments**. Once established, plot segment definitions can be saved and reloaded with the **Save preset** and **Load preset** buttons.

The **Plot Segment** dialog is useful for comparing two or more chromatograms that have large differences in appearances (e.g. a very heavily stressed sample versus a reference), sufficiently large that the time warping function may yield the wrong results. This tool allows the user to break up the chromatogram into segments, manually connecting one or more regions to ensure that the correct areas are aligned by the time warping function. For more detail about time warping see the **Chromatogram Plot and Menus** section.

- The  icon manages how plots are displayed (render options), as well as zoom modes.

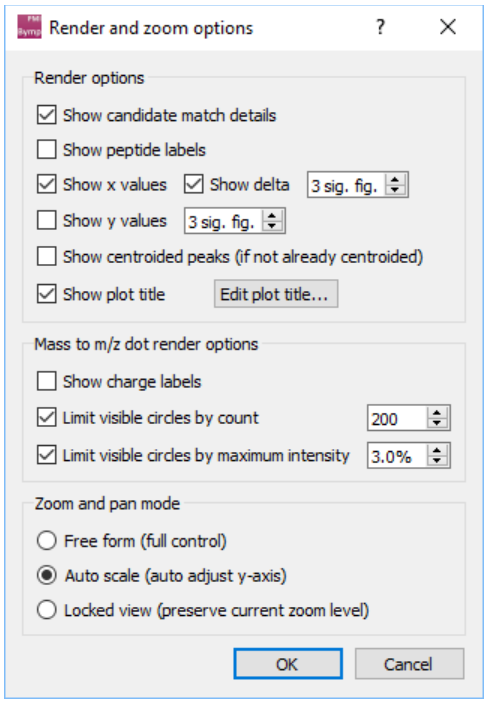


Figure 82: Render and zoom options

- **Show candidate match details** (MS1 only) displays the settings entered in the **Candidate match options** tab of the **Filter options** dialog (see the **Filter Options** section).
- **Show peptide labels** (MS1 only) displays peptide labels in red below the identifying labels for the corresponding peaks.
- **Show x values** and **Show y values** display the x- or y-coordinates beside plot peaks to the chosen number of significant figures. **Show delta** displays the x or y differences from a reference peak. To display the deltas, hover the cursor over the reference peak (which displays as 0). The other peaks show the positive or negative differences for that axis.
- **Show centroid peaks** (MS1 only) darkens the peaks that are determined to be centroid.
- **Show plot title** displays the title of the plot, as generated from field values. The information shown in the plot title can be customized by clicking the **Edit plot title** button. This opens the **Template Editor** dialog. The editor accepts free text or programmatic "short codes" that insert data values into the title. For example, "Time range [[StartTime] - [EndTime]]" displays the starting and ending times derived from the chromatogram as part of the title for any of the plots.
- **Show annotations** (Chromatogram only) displays text values beside peaks, which can be customized with the **Edit annotations** button. This opens the Template Editor dialog. The editor accepts free text or programmatic "short code" that use data values in the peak annotations. For example, [PeaksDynamicNumber]" displays the ID number for individual peaks in the Peaks table.
- **Label orientation** (Chromatogram only) sets the angle for the label text, where 0º is vertical.

- **Mass to *m/z* dot render options** (MS1 only). The three checkboxes, **Show charge labels**, **Limit visible circles by count**, and **Limit visible circles by maximum intensity** are intended for the Intact Analysis software. They are not active for Chromatogram Analysis.
- **Zoom and pan mode** options include **Free form** mode to select the desired y range as well as x range, **Auto scale** mode to select only the x range (the y range is then determined by the value of the highest peak), and **Locked view** mode to keep the current x range (for either *m/z* and/or m) when moving between trace peaks.

- The **Offset** entry on the Chromatogram and MS1 plot menu bars controls the vertical spacing for stacked plots. Offset of 30% means that plots overlap by 70%. Offset of 100% means that plots stack top-to-bottom without any overlap. An offset greater than 100% leaves white space between the stacked plots.

Plot Right-Click Menus

The Chromatogram and MS1 plots have a variety of context menus for plot styling and exporting revealed by a right-click on the plots. Figures exported can be useful for reports, publications, regulatory filings or internal communications. There are many options for rendering and exporting so that the user has much freedom to prepare a plot or figure style as needed.

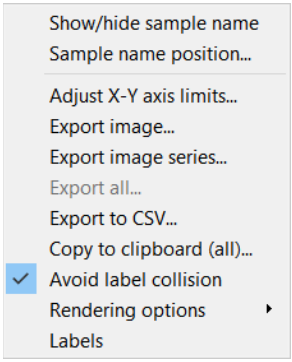


Figure 83: Plot right-click menu

- **Show/hide sample name** (Chromatogram plot only) toggles the display of sample names beside their corresponding peaks.
- **Sample name position** (Chromatogram plot only) toggles the display of sample names beside their corresponding peaks.
- **Adjust X-Y axis limits** opens a dialog for the user to manually edit the plots x and y maxima and minima. This is a less convenient but more precise alternative to the and icons.
- **Export image** allows the user to save the plot as a *.pdf or *.png file. It opens the **Plot Exporting Settings** dialog, which controls image size, file name and folder, and x and y minima and maxima. Sometimes a user wishes to display a wide range across the x-axis and yet retain significant detail. This can be done by breaking up the plot into a series of panels. The **Add segment** button creates segments with user-defined x and y values. A series of these segments that eliminates unneeded portions of the plot increases the effective detail in the image. This is also a useful function for automated reporting.

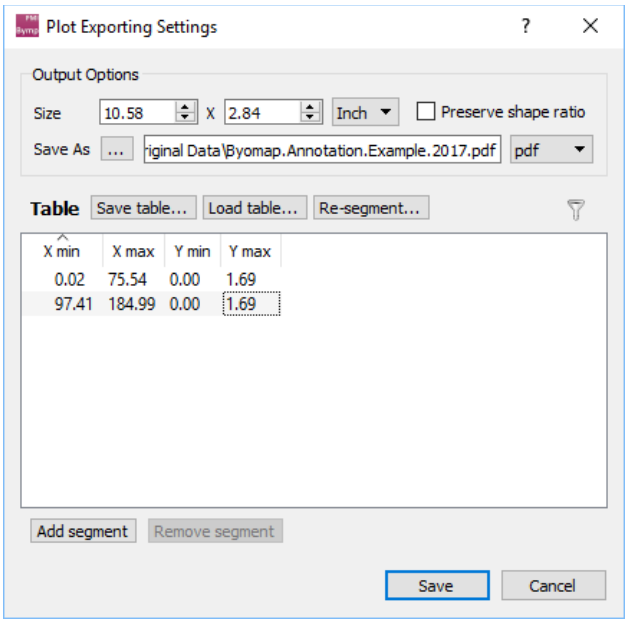


Figure 84: Plot Exporting Settings

- **Export image series** opens the Plot Export Settings dialog, except that this time it is prepopulated with six equal sized segments. This simplifies the edits of the segments.
- **Export all** also opens the **Plot Exporting Settings** dialog, except that the wildtype peptide plots are exported along with the peptide plots. Segment editing functions are disabled for this option.
- **Export to CSV** exports the plot trace x,y points to a .csv file.
- **Copy to clipboard (all)** enables pasting the plot image into another application.
- **Avoid label collision** staggers the label text to reduce overlap.
- **Rendering options** controls how a plot is displayed in the application. It includes several sub-menus:

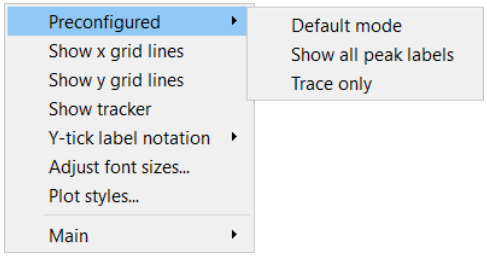


Figure 85: Rendering options sub-menu

  - **Preconfigured** sub-menus control what is displayed in the plots. **Default mode** displays the trace, peak labels and plot title. **Show all peak labels** does exactly that. **Trace only** turns off the peak labels and plot title.
  - **Trace only** turns off the peak labels and plot title.
  - **Show x grid lines** turns on and off the x grid lines.
  - **Show y grid lines** turns on and off the y grid lines

- **Show tracker** displays a vertical dotted line that follows the cursor when moved in the plot. This allows a more precise determination of the location of the cursor.
- **Adjust font sizes** controls the font size for each type of text on the plot.
- **Plot styles** allows changes to the graphic styling of the plot, including trace width, axis width and total m/z dot colors to be used.

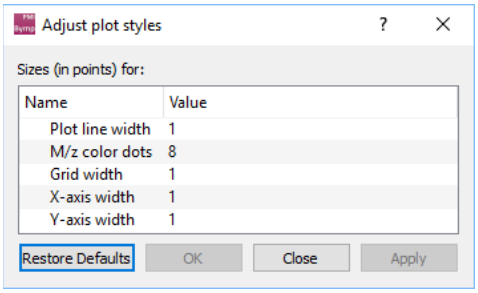


Figure 86: Adjust plot styles

- Plot name: The last of the **Rendering options** sub-menus displays the sample names of each displayed trace. Hover over the sample name to see a deeper sub-menu of plot choices. The user can turn on and off the trace, graphical integrations and peak labels for that particular sample. The **Show/hide all** option turns all of these options on or off.

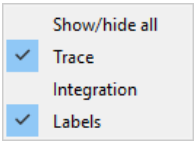


Figure 87: Plot name menu options

- **Label** (Chromatogram plot only) opens the Template Editor. The editor accepts free text or programmatic “short codes” that insert field data values into the labels.

Chromatogram Plot and Menus

The graphical trace of the chromatogram is a key part of the interactive software. It is recommended to begin the workflow with a close inspection of the chromatogram, especially the baseline and peak integration limits (elution peak definition). If necessary, re-compute using the menu **Edit > Recompute trace peaks and baseline**. After the chromatogram is computed, it is common to make some small manual adjustments to the peak integration limits and the baseline.

To edit the integration time limits for a trace peak, first highlight the peak either by clicking on a row in the Peaks table or Alt-click the peak in the Chromatogram plot. The integration limits will appear as two magenta vertical lines. Click one line at a time and drag it to a different trace time; the Peaks table will automatically update. Alternatively, the user may edit the **Start time** and **End time** fields in the Peaks table directly.

The chromatogram baseline is created with an algorithm that places anchor points on the baseline. These anchor points are editable. To manually adjust the baseline, zoom in on the peak of interest and use the baseline anchor options described below.

If the user turns on the “Snap to Plot” down-arrow button before inserting a new anchor point, then the newly created anchor point will snap to the chromatogram when created

The Chromatogram plot menu bar includes the following specialized controls:

- The icon shows the options for managing baseline anchors. Click this icon to expose three baseline anchor options:
    - The  icon moves the ends of a baseline anchor. Move the cursor to either end of the baseline to see the anchor displayed in green and the cursor changed to an arrow. Click, and drag the end of the baseline to the new location.
    - The  icon inserts a new baseline anchor (polygon vertex). Move the cursor to the baseline to see the anchor displayed in green, and the cursor changed to an arrow. Click anywhere within the baseline anchor to divide it into two parts at that point. The Move baseline anchor mode can be used to move the endpoints of the baseline anchors.
    - The  icon deletes a baseline anchor. Move the cursor to the end of the unwanted baseline to see the anchor displayed in green and the cursor changed to an arrow. Click to delete that baseline anchor.
- The **Warp off/Warp on** button (for comparison projects only). Warp on applies a dynamic nonlinear time warping to bring the trace time profile of each comparison sample into alignment with the reference file (bottom trace in the pane). The following two images show comparison traces with Warp off and then Warp on:

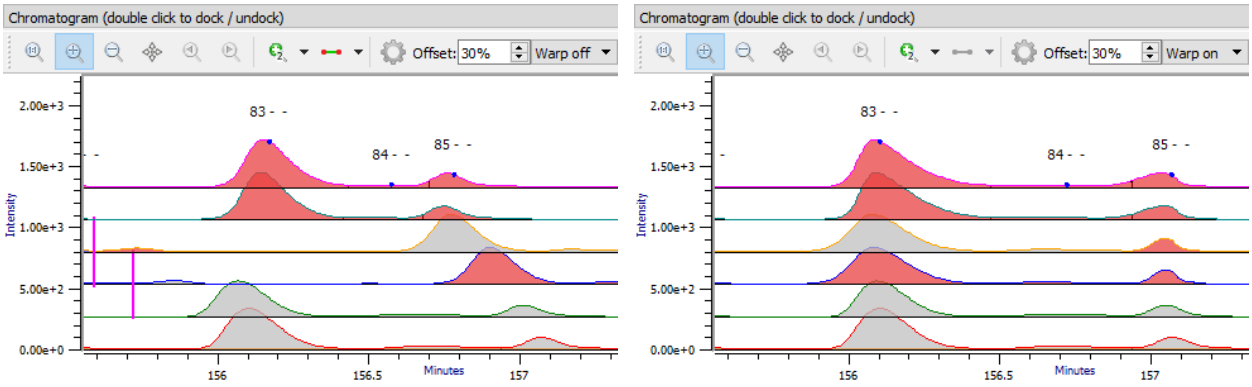


Figure 88: Comparing effects of Warp off and Warp on

The warping tool is essentially an inspection tool, to help the analyst judge if all the right peaks are being compared to the each other. The actual quantification for all peak areas is calculated in unwarped (original) time. The time warp algorithm is robust in terms of recognizing which trace peaks in different samples correspond to each other, but if the comparison involves chromatograms that begin to dramatically differ in appearance, as can be the case for example with highly stressed samples, then the time warp algorithm may sometimes make mistaken connections between the trace peaks in some regions. In such cases, the user may use the segment tool in the Chromatogram pane to lock together a particular region(s) that they know are the same molecule and in so doing constrain the nonlinear dynamic warping accordingly, allowing successful warping throughout the chromatogram.

- The **Norm** button scales the y-coordinates so that the tallest peak has intensity 1.
- The **Center** button pans the chromatogram to put a selected peak in the center.
- The **Label** button opens the Template Editor for peak labels. This functions the same way as the **Annotations** function in the **Rendering and zoom options** dialog. The editor accepts free text or programmatic “short codes” that can use field data as peak labels.

MS1 Plot and Menus

For the graphical display of MS1 data, the toolbar at the top has much in common with that described earlier for the Chromatogram view.

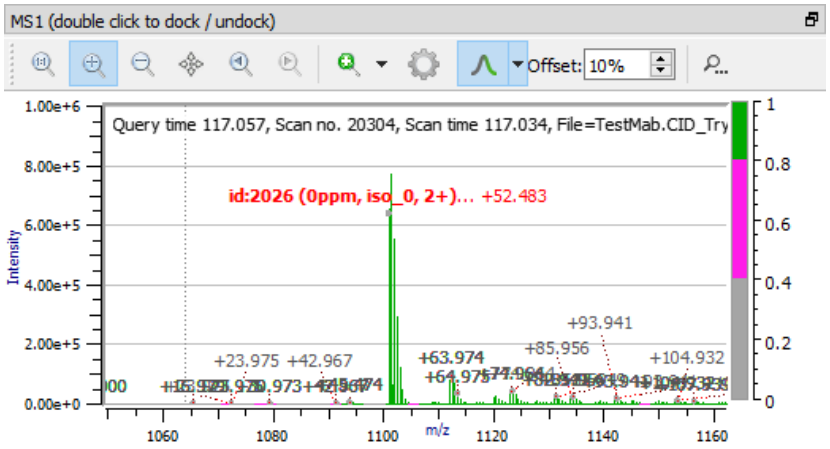


Figure 89: MS1 Plot

The MS1 plot menu bar includes the following specialized controls:

- The icon turns on and off the time-correlation color function. This function calculates the mathematical correlation coefficient of the MS1 intensity over the time-intensity profile of the trace peak as a function of m/z. Well correlated peaks are color green. Less correlated peaks are colored magenta. Poorly correlated peaks are colored grey. Click the icon to turn the correlation colors on or off. The dropdown arrow includes **Time profile similarity options** (which generally needs no adjustment and **Show/hide color map**. Click the latter to turn on and off the color bar scale on the right-hand side of the MS1 view.

  In the above figure, assigned candidates are shown in a bold font. The ID number displayed in red refers to the candidate shown in the Candidates table. Hover the mouse over a mass peak to display the mass differences of the other peaks relative to the inspected peak. This is useful to quickly check charge states or check masses associated with modifications like oxidation or sodiation.

- The icon shows the same dialog with the same options described in the **Filter Options** section. In the case of the MS1 plot, the filter options apply to displayed peaks, as well as to the rows of the Peaks and Candidates tables.

Appendix

Chromatogram Analysis: Advanced Commands

Chromatogram Analysis includes many ways to customize the functionality to fit specific needs. Protein Metrics uses Advanced Commands to test new ideas, beta-test new features, and enable specialized options, without adding complexity to the graphical user interface. This section describes several text-format Advanced Commands that will enable finer control over processing. Advanced commands may be entered during project creation in the **Advanced configuration** panel of the **Advanced** tab or after project creation by choosing **Edit -> Advanced configuration**.

1. **Assume Proline Cuts** allows the counting of cleavages to include cuts at proline.

```
[Byomap]
AssumeProlineCuts=1
```

2. **Remove Duplicates** ensures that candidates will only be assigned to the most abundant trace peak during auto-assignment. This is enabled (set to 1) by default. If disabled (set to 0), candidates may be assigned to more than one trace peak.

```
[AutoAssign]
RemoveDuplicates=0
```

3. **Remove Dupicates ISD** If enabled (set to 1), excludes in-source dissociation candidates from auto-assignment. A glycan is treated as a subcomposition of another candidate glycan for the same peak. This is disabled (set to 0) by default. If disabled (set to 0), in-source dissociation candidates will be treated no differently from other candidates during auto-assignment. A glycan is either the same composition or a supercomposition of the other candidate glycans for the same peak..If set to <blank>, the candidate does not represent a glycan.

```
[AutoAssign]
RemoveISD=1
```

Please note:

- Candidates with RemoveISD enabled (set to 1) will not be auto-assigned by **Edit > Auto assign candidates**
- Auto-assignment will only refrain from assigning ISD candidates; it will not unassign candidates that are already assigned. If auto-assignment has already been run for the project in a build without this feature, the user should first choose **Edit > Remove all candidate assignments** to unassign possible ISD candidates.

4. **Insert Anchors** injects manual baseline points when the baseline smoothing parameter alone is inadequate. This will help to keep big dips out of the calculated area if points just outside the time range are included, as depicted below:

```
[Baseline]
InsertAnchors=19.27,20.12
```

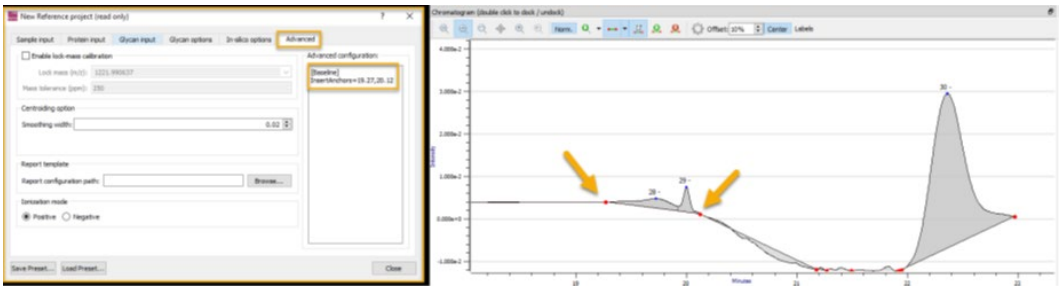


Figure 90: Insert baseline anchors

Note that this function requires the user to have knowledge of where to place the baseline points. The values 19.27 and 20.12 were observed from the chromatogram.