



Chromatogram Analysis User's Manual

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Introduction

Byos hosts a number of workflows incorporating Chromatogram analysis that can be used 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 released 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, Byos provides efficient and comprehensive interactive workflows.

Chromatogram Analysis in Byos 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.

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

Windows Support

Byos Desktop is currently supported on Windows 10 and Windows 11. **Note that for Windows 10, you must have version 1809 or later.**

System Specifications

- **Recommended PC:**
 - Windows 10/11 64-bit
 - 32 GB RAM
 - 1TB disk space (Solid State SSD)
 - Recent version of Intel Core i7 or i9 / AMD Ryzen 7 or 9 (with AVX support)
 - Oracle JRE or OpenJDK
 - C++ compiler version 16 or higher
- **Recommend PC for *high performance computing* (e.g. 32+ cores)**
 - Windows Server 2022 or Windows 10/11

- 64 GB RAM
- 2 TB disk space (Solid State SSD)
- Xeon CPU(s) (at least 16 physical cores) (with AVX support)
- Oracle JRE or OpenJDK
- C++ compiler version 16 or higher

Note that standalone applications are no longer supported outside of Byos and that Chromatogram Analysis is performed using Byos workflows.

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

Workflows

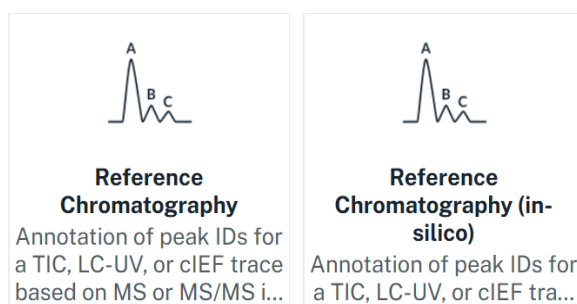


Figure 1: Peptide and Glycan Annotation (Reference) Workflow

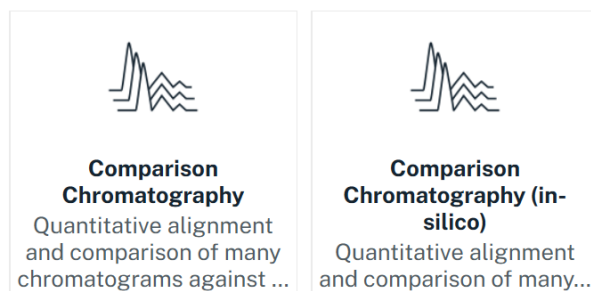


Figure 2: Peptide and Glycan Annotation (Comparison) Workflow

Tour of Chromatogram Analysis

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

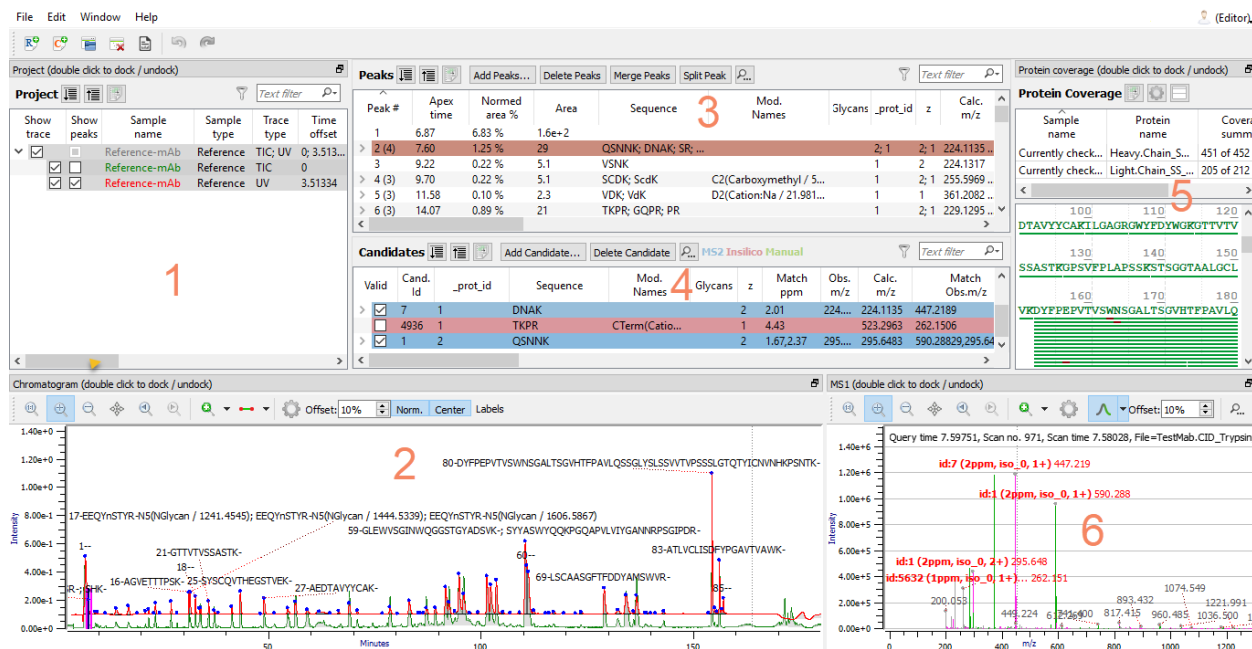


Figure 3: Six Chromatogram Analysis views: (1) Project table with input MS files, (2) Trace plot with selected peak, (3) Peaks table, (4) Candidates table (elution peaks), (5) Protein Coverage tables, and (6) MS1 spectrum (summed spectra).

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 trace plot and the trace peak lists displayed in Views 2 and 3.
2. The **Trace plot** 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 trace plot 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 trace plot. 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 trace peak selected in Views 2 and 3. By default The a single MS1 spectrum is shown, associated with the trace time designated by the black bar in the trace plot view. 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. When the **EnableMS1Sum = true** advanced command is included, the displayed MS1 will be the sum of all scans between the two pink bars that mark the selected trace peak boundaries.

Note that four of these views (Project, Trace plot, 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 Trace plot 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.

Project Creation

Chromatogram Analysis can be used to analyze a single sample or to compare several 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 the **Reference Chromatography** system workflow.

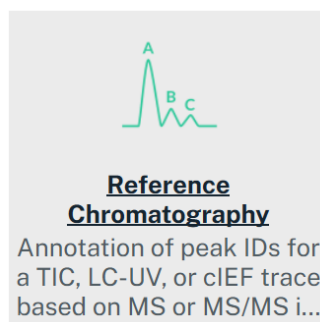



Figure 4: New Reference Project

Samples tab (Reference Chromatography)

The **Samples** 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 *.byrs1t 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**.

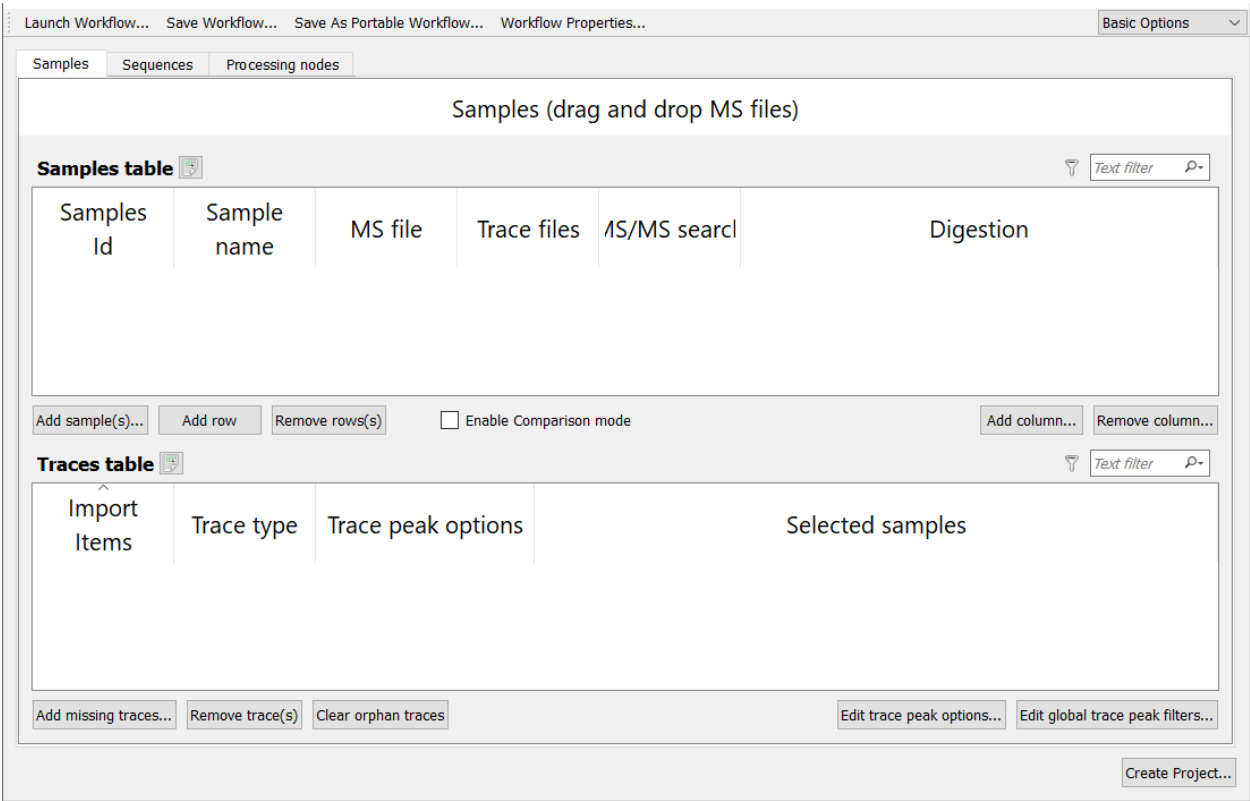



Figure 5: Adding samples and their traces in the Samples tab

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 the 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)**.

Adding 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:

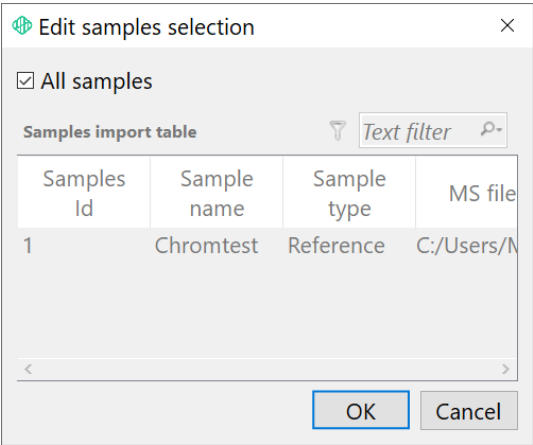



Figure 6: Edit samples selection dialog to associate traces with sample names

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:

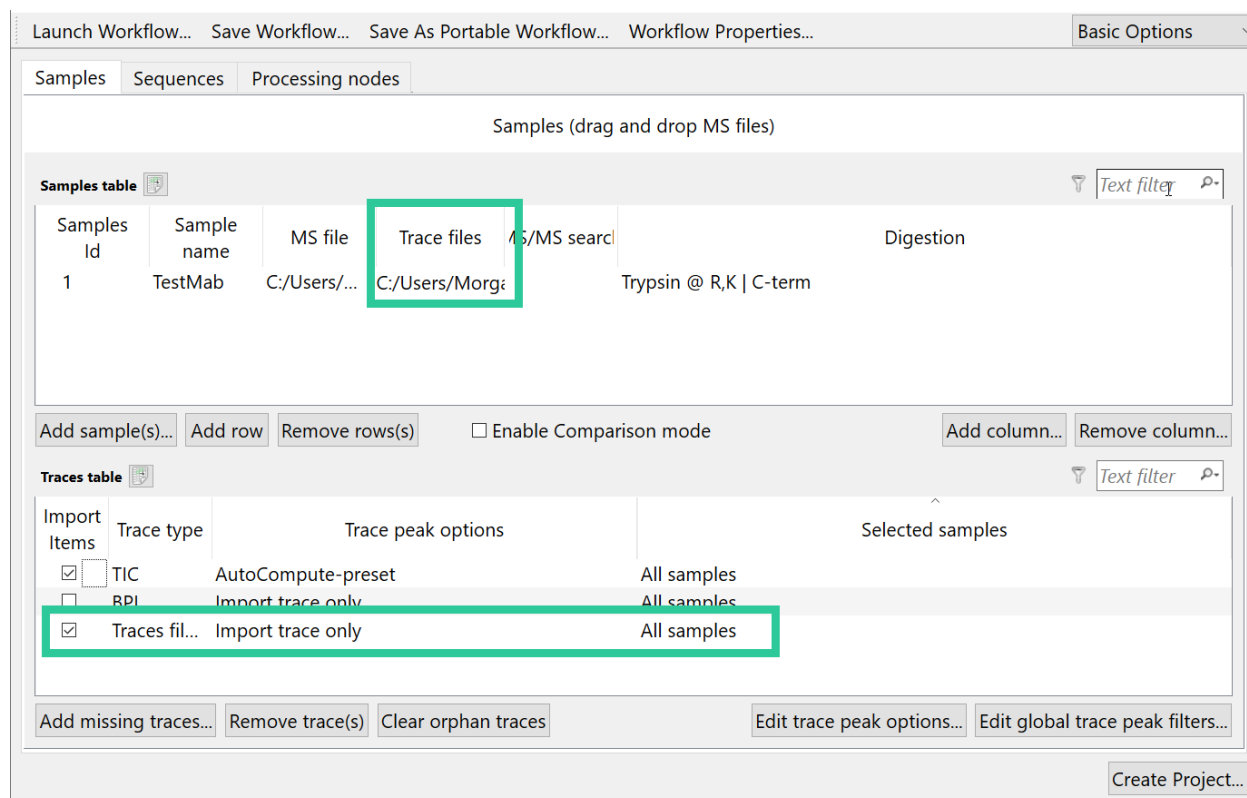



Figure 7: Traces imported from a *.csv file

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:

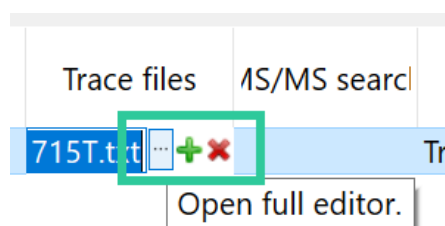


Figure 8: Adding multiple trace files to a sample

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

Byonic MS/MS search files in *.byrs1t 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:

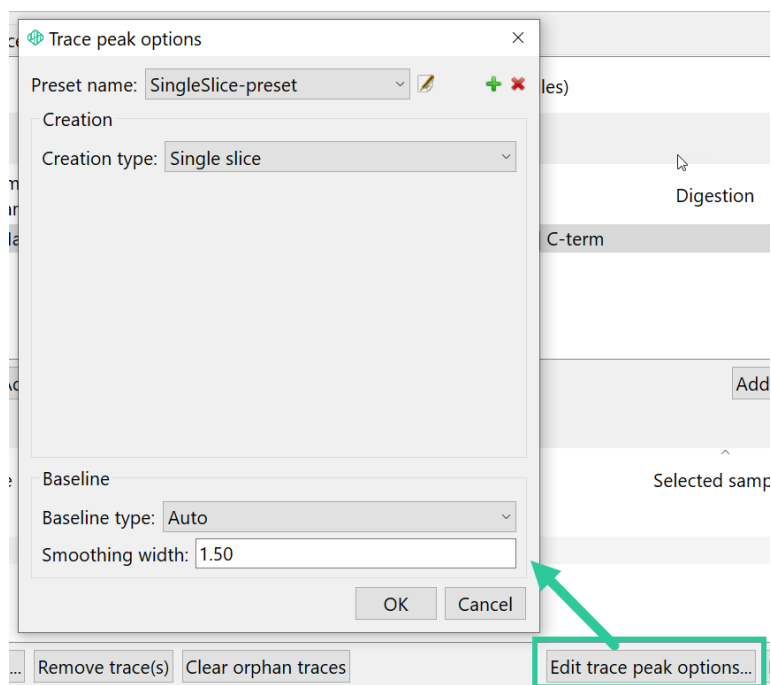


Figure 9: Trace peak options presets

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:

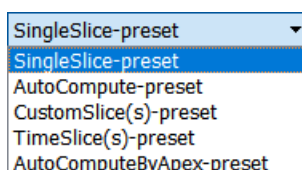



Figure 10: Default trace peak presets

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 **x** button to delete a selected preset.

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

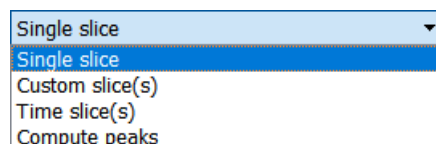


Figure 11: Trace peak Creation types

These Creation types use the following peak processing methods:

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

Creation type: Custom slice(s) ▼

Start time	End time
1.20	2.78
3.12	4.57

Add row Delete row

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:

Creation type: Time slice(s) ▼

First slice range: 0.00 5.00

Time step between slices: 2.50

Number of slices (optional):

Show example

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 trace.
- **Number of slices** sets the maximum number of defined slices. If blank, the last slice is determined by the end of the trace x-axis range.
- **Show Example** displays an example fixed width slice configuration with definitions and explanations:

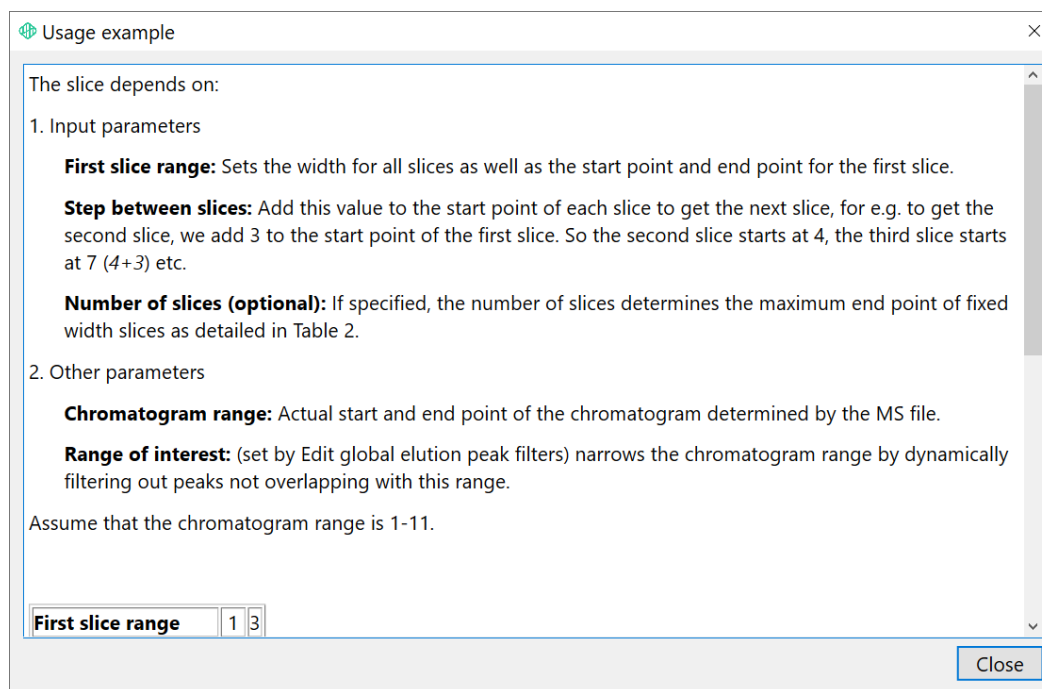


Figure 14: Fixed width slice Usage example

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

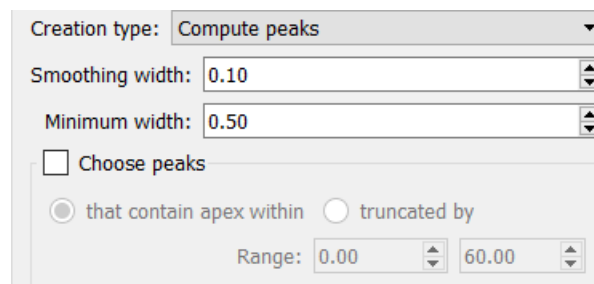


Figure 15: Trace peak Compute peaks option

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.

Global trace peak filters can also be applied. To set peak property filters for all the traces, click **Edit Global trace peak filters**:

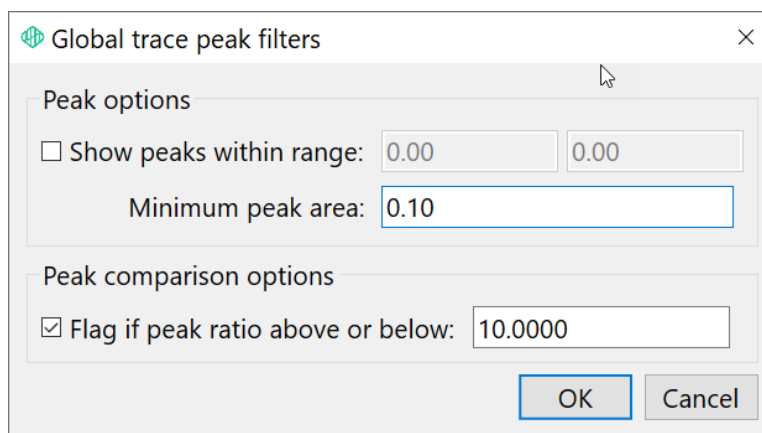


Figure 16: Global trace peak filters

Check **Show peaks within range** to manually set start and end points. **Minimum peak area** sets a Normed area % cutoff filter. Normed area % is calculated by dividing the peak area of an individual trace peak by the sum of all trace peak areas within the Global filter retention time range, set with **Show peaks within range**. In the example above, any trace peak with a **Normed area %** less than 0.1% is filtered out. Flag if peak ratio above or below: sets a peak ratio (relative to the reference sample) threshold flag and is only applicable for comparison mode projects.

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 Analysis and Chromatogram Analysis projects created in versions 3.8 and above will not correctly open in older versions of these applications.

Samples tab (Comparison Chromatography)

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

To create a comparison project in Byos, select the **Comparison Chromatography** system workflow. Project Creations for Comparison Chromatography workflows share the same general settings as the Reference Chromatography workflows. However, in the Samples tab of Comparison Chromatography workflows, **Enable Comparison mode** is checked on and an additional **Sample type** column exists in the Samples table. The **Sample Type** column allows the user to designate Samples as **Reference** or **NonReference** for Comparison Mode analysis.

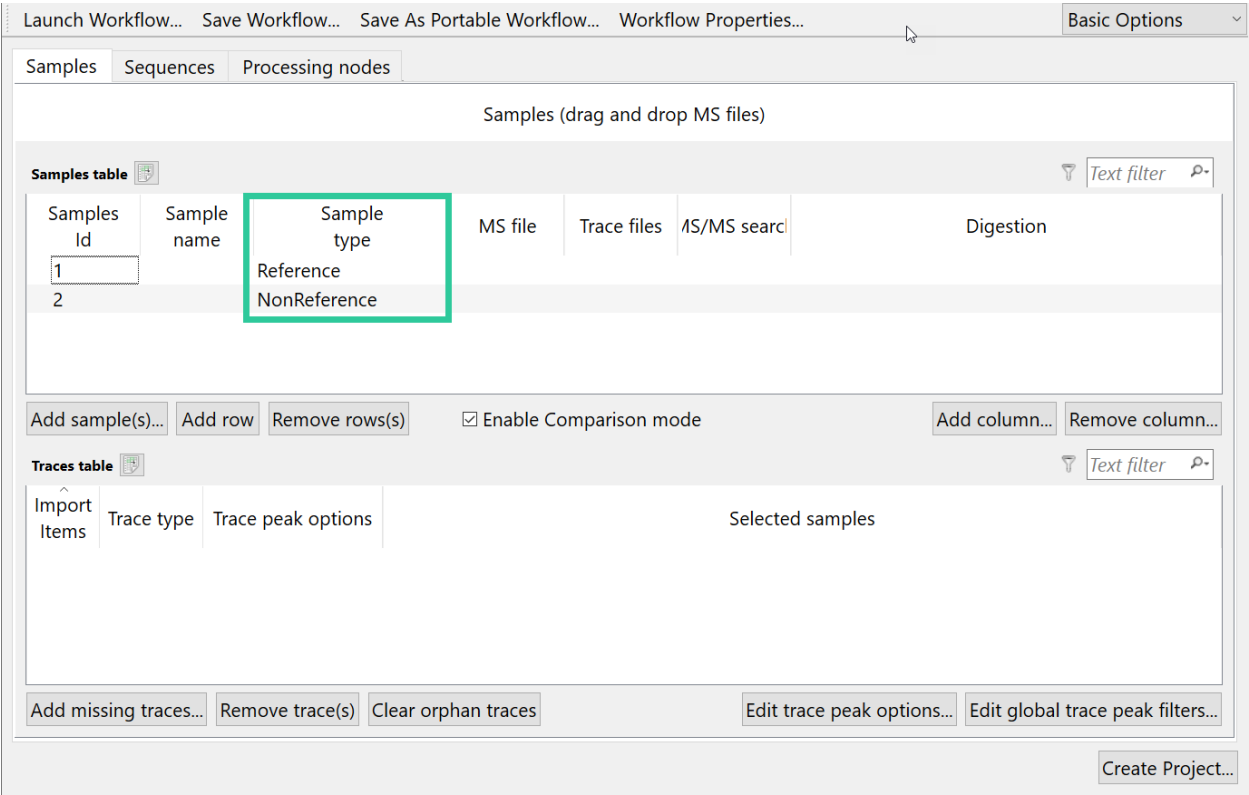


Figure 17: Sample Type column in Comparison Chromatography

The **Enable Comparison mode** option is checked by default for Comparison Chromatography workflows.

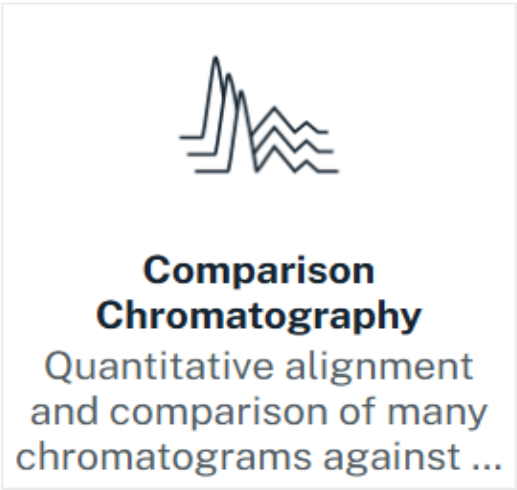


Figure 18: Comparison Chromatography workflow

Sequences tab

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

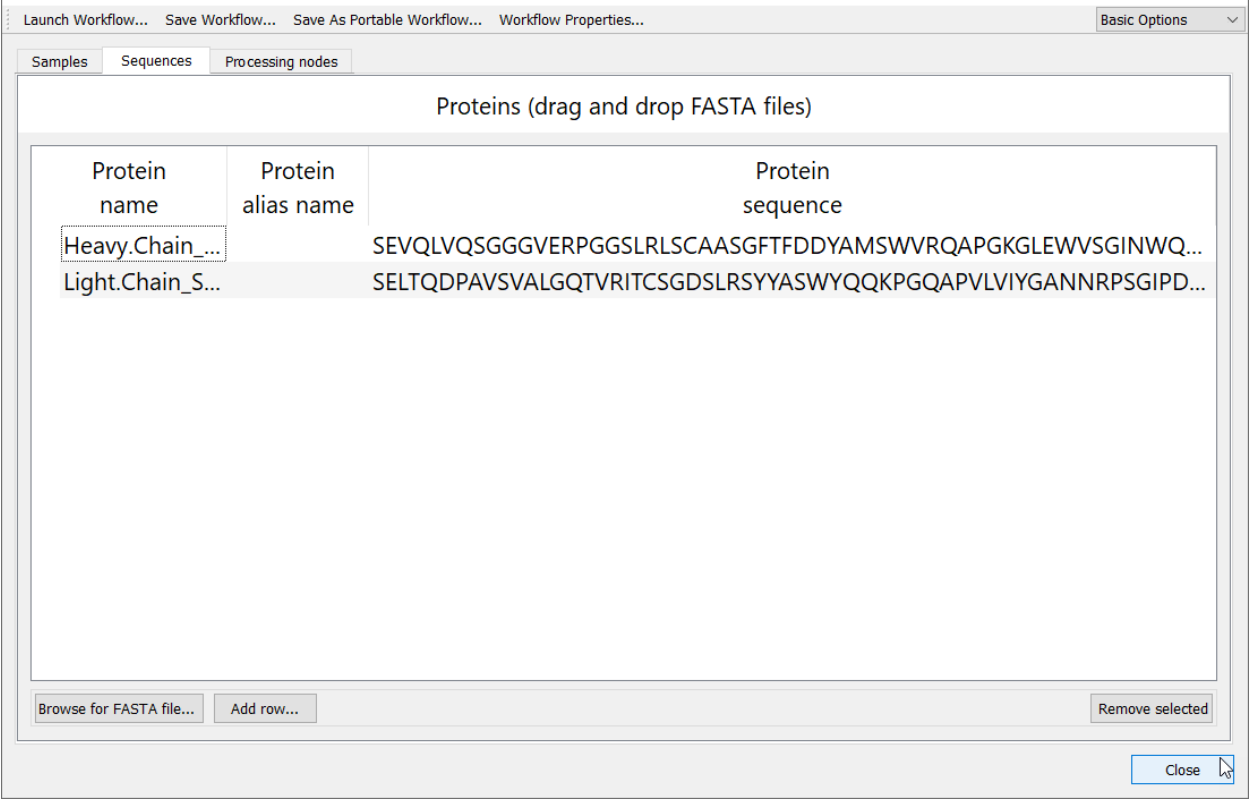


Figure 19: Sequences tab in Chromatography projects

Processing nodes

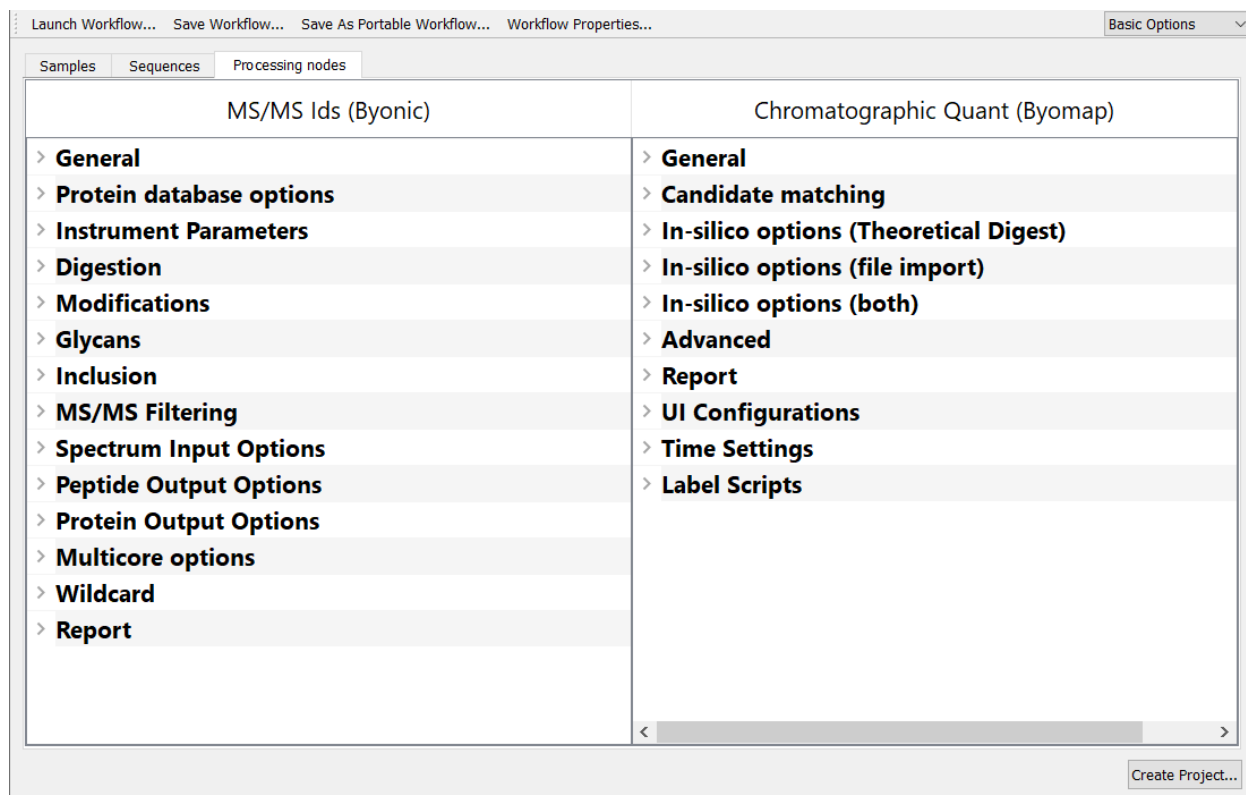


Figure 20: Processing nodes parameters – Reference Chromatography

MS/MS Ids

NOTE: These parameters are not part of the Reference Chromatography (in-silico) or Characterization Chromatography (in-silico) workflows

- **General**

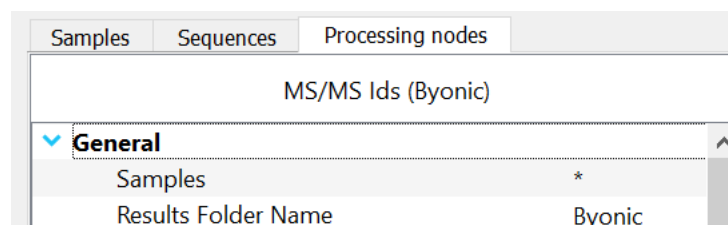


Figure 21: General

- **Samples** - The "*" will apply all parameters to all samples dragged and dropped into the Samples tab.
 - **Results Folder Name** - This is automatically set to "Byonic" and will be applied to the additional processing (as defined in the workflow).
- **Protein database options**

▼ Protein database options	
Add Common Contaminants	— No
Add Decoys	✓ Yes

Figure 22: Protein database options

The protein database options **Add Common Contaminants** (default setting = No) and **Add Decoys** (default setting = Yes) were added to the Byonic Processing node in v. 3.9. The protein database should contain both targets and decoys (recognized by protein names beginning >Reverse or >Decoy) for false discovery rate (FDR) estimation. Byonic will automatically add decoys if the **Add decoys** box is checked and contaminant proteins (e.g., trypsin, bovine serum albumin, and human keratins) if the **Add common contaminants** box is checked. The common.contaminants.fasta database is located here: C:\Program Files\ProteinMetrics\PMI-Suite\Tools\Byonic\data

- **Instrument Parameters**

▼ Instrument Parameters	
Precursor Mass Tolerance	6.00 ppm
Fragmentation Type	QTOF / HCD
Fragment Mass Tolerance 1	0.60 Da
Fragment Mass Tolerance 2	0.60 Da
Recalibration (from Preview)	None
Recalibration (lock mass)	None

Figure 23: Instrument Parameters. Optimize for the experiment completed.

In the figure above, the user set 6.0 ppm **Precursor Mass Tolerance**, 0.60 Da **Fragment Mass Tolerance**, and QTOF/HCD as the **Fragmentation Type**. Both Dalton and ppm mass tolerances for precursors and fragments are supported, along with several fragmentation types. The Dalton tolerance applies to measured mass for precursors but measured m/z for fragments. The way scoring is completed changes at fragment tolerances of 0.1 Da or 100 ppm or less: high-resolution MS/MS is assumed, meaning resolution sufficient to distinguish charge states of fragment ions. For this reason, fragment tolerances larger than 0.1 Da should be used with low-resolution (ion trap) MS/MS analysis.

Internal models for most fragmentation types are included – CID low energy (ion trap), QTOF / HCD (beam-type CID), and ETD / ECD (electron transfer and electron capture dissociation), as well as a number of combinations of types. These internal models determine which fragment peak types will be scored and annotated. For example, prominent c- and z-ions and small y-ions are expected for ETD. Prominent oxonium ions are expected from glycopeptides with QTOF / HCD fragmentation, but small or missing oxonium ions from CID low energy.

- **Precursor Mass Tolerance**

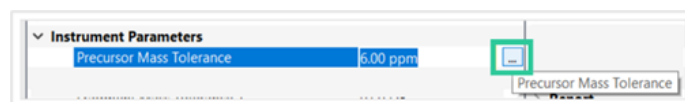


Figure 24: Precursor Mass Tolerance

The user can change this value by clicking within the text box and then clicking on the activated blue “...” square. The user can then modify the text value and mass accuracy, as required. This is shown in the figure below.

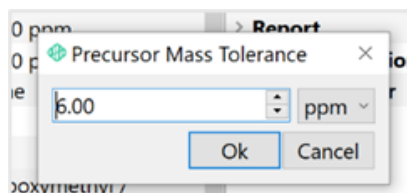


Figure 25: Modify the text value and mass accuracy

- **Fragmentation Type**

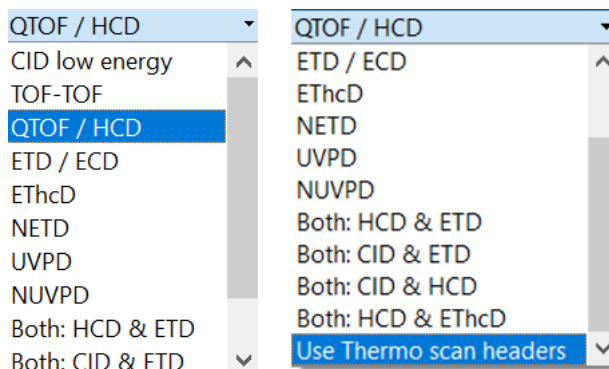


Figure 26: Fragmentation type.

The user can select from the available options using the drop-down menu. Additional options are visible using the scroll bar, including “Both:” for spectrum file(s) containing more than one fragmentation type.

- **Fragment Mass Tolerance 1** - The user can set the value used to acquire data in either ppm or Da.
- **Fragment Mass Tolerance 2** - If necessary, the user can set the value used to acquire data in either ppm or Da. This is only required for the “Both:” Fragmentation Types. No value will be applied if only a value for “Fragmentation Mass Tolerance 1” was entered.
- **Recalibration (lock mass)**

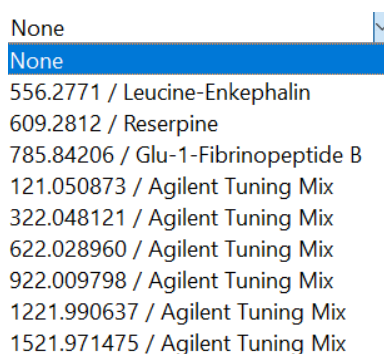


Figure 27: Lock mass calibration options

The user can select from the available options using the drop-down menu.

- **Digestion**

▼ Digestion	
Cleavage Site(s)	RK
Cleavage Side	C-terminal
Digestion Specificity	Fully specific (fastest)
Missed Cleavages	2

Figure 28: Digestion options

The Digestion settings allow the user to set the residues recognized by the digestion enzyme. In this example, the enzyme is trypsin, so the user entered RK for arginine and lysine for the Cleavage Site(s) and chose C-terminal for the Cleavage Side.

- **Cleavage Site(s)** - The user can change this value by entering text. If the user leaves the Cleavage Site(s) box empty, the only specific cleavage sites are protein termini.
- **Cleavage Side**

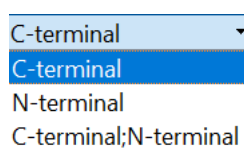


Figure 29: Cleavage Side options

The user can change this by selecting another option from a drop-down menu. Click on the current selection to activate the available options.

- **Digestion Specificity**

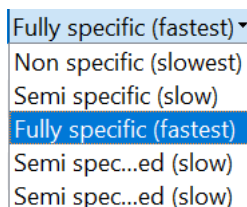


Figure 30: Digestion Specificity

Here the user chose “Fully specific (fastest)” search, meaning that both the N- and C-terminal cleavages must be C-terminal to R or K. Nonspecific cleavage at either or both endpoints is supported. A nonspecific search with RK in the Cleavage Site(s) box searches all peptides but favors tryptic peptides; the user must leave the Cleavage Site(s) box empty for a true no-enzyme search. Digestion Specificity can be changed by selecting another option from a drop-down menu. The user can click on the current selection to activate the drop-down menu to view the available options.

- **Missed Cleavages** - The user selected 2 Missed Cleavages, as shown in Figure 28. This limits the maximum number of internal Rs and Ks not followed by P to 2; leaving Missed Cleavages at its default value of -1, which means any number of internal Rs and Ks. Missed Cleavages can be changed by entering text.

- **Modifications**

▼ Modifications	
Modifications	Carboxymethyl / +58.005479 @ C fixed
Total Common Max	2
Total Rare Max	1

Figure 31: Modifications options

Like most proteomics search engines, two types of modifications are supported: 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.

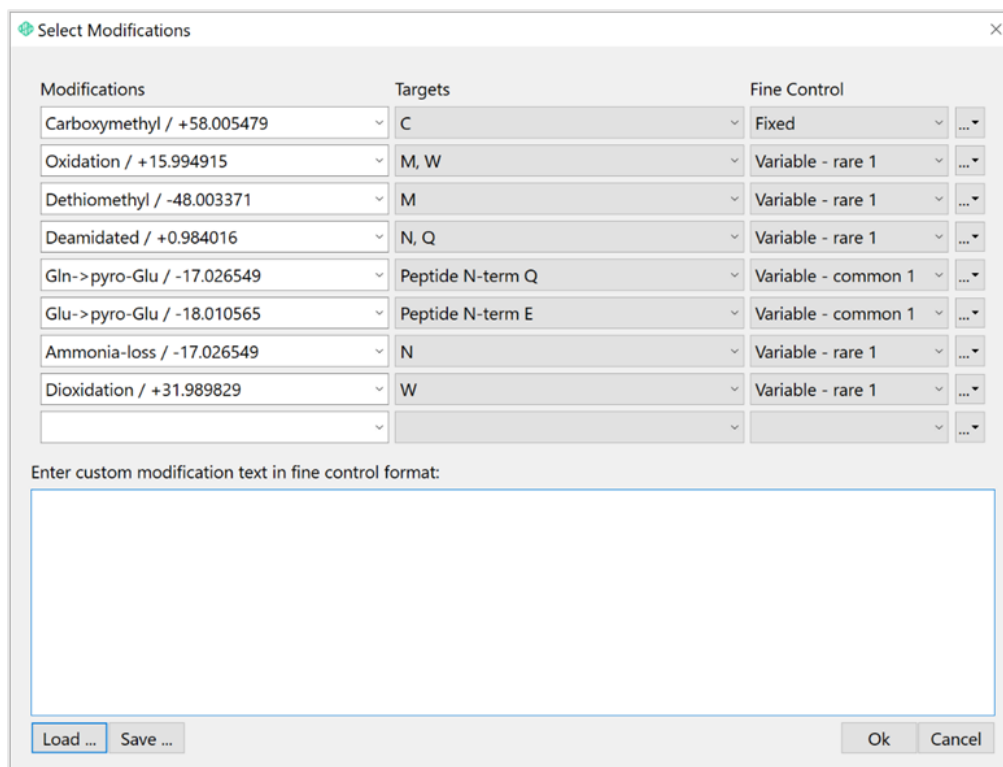
- **Modifications**



Figure 32: Click within the text box to activate the light blue “...” button

To view the list of modifications included with the default workflow, the user can click within the text box to activate the light blue “...” button. It will take a second or two for the window to open.

The user can then specify any number of modification rules via a pull-down menu containing all the modifications listed in www.unimod.org. For convenience, frequently used modifications are listed twice, at the top and again in the complete list. The three pull-down menus in each row select modification type, target residues, and fine control. There is a fourth pull-down, which lets the user delete, invert (as in (De)Carbamidomethyl), or add “attributes” to modifications. Attributes allow the user to define protein-specific modifications.



Select Modifications

Modifications	Targets	Fine Control
Carboxymethyl / +58.005479	C	Fixed
Oxidation / +15.994915	M, W	Variable - rare 1
Dethiomethyl / -48.003371	M	Variable - rare 1
Deamidated / +0.984016	N, Q	Variable - rare 1
Gln->pyro-Glu / -17.026549	Peptide N-term Q	Variable - common 1
Glu->pyro-Glu / -18.010565	Peptide N-term E	Variable - common 1
Ammonia-loss / -17.026549	N	Variable - rare 1
Dioxidation / +31.989829	W	Variable - rare 1

Enter custom modification text in fine control format:

Figure 33: Select Modifications window

- **Total Common Max and Total Rare Max** - A unique feature not found in other search engines is offered: the user designates each variable modification as either “common” or “rare”. Separate limits on the number of occurrences of each variable modification (rare/common) can be set. Please review the Knowledge Base article “Byonic Modification Fine Controls” for details.

Conceptually, there is one modification “slot” for each residue, along with slots for the peptide’s N- and C-termini. A variable modification such as +0.984016 @ N uses up the residue slot; a nonspecific terminal modification such as +57.021464 @ NTerm uses up the terminal slot; but residue-specific N-terminal modifications, such as -17.026549 @ NTerm Q, use up both the residue and the N-terminal slots.

The big open box is a space for the user to type in custom modifications not listed in Unimod. The manual fine control format has the form:

```
Modification_Name / Mass_Delta @ Targets | Fine_Control
```

Modification_Name / is optional. 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.

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

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

A limited number of nonstandard amino acid residues is supported by redefining one-letter amino acid abbreviations using fixed modifications. B, Z, U, O, J, and X are accepted in FASTA protein databases, with masses, respectively, of 114.042927 (same as N), 128.058578 (same as Q), 150.95363 (selenocysteine), 237.052645 (pyrrolysine), 100.0, and 110.05 (close to averagine). By placing, for example, a fixed modification of +13.04768 on J, the user can make J in a FASTA database have mass 113.04768, correct for hydroxyproline. However, amino acid sequence is used to predict peak intensity, so this fixed modification on J will not give the same scores as a +15.9949 variable modification on P.

For comprehensive sequence variant searches, or other searches with large numbers of modifications, it is more convenient to paste in a list of modifications in the custom modification box than to add all the modifications via the drop-down menus. Sequence variant lists are available from Protein Metrics by contacting support@proteinmetrics.com.

• Glycans

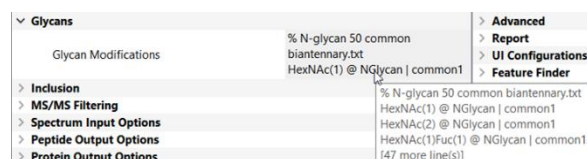


Figure 34: Glycans are loaded as a list of records

Three ways to define glycan modifications are offered: internal preset tables, external glycan databases, and user-defined glycans. Clicking on the activated light blue “...” button pops up a window labeled **Select Glycans** as shown below.

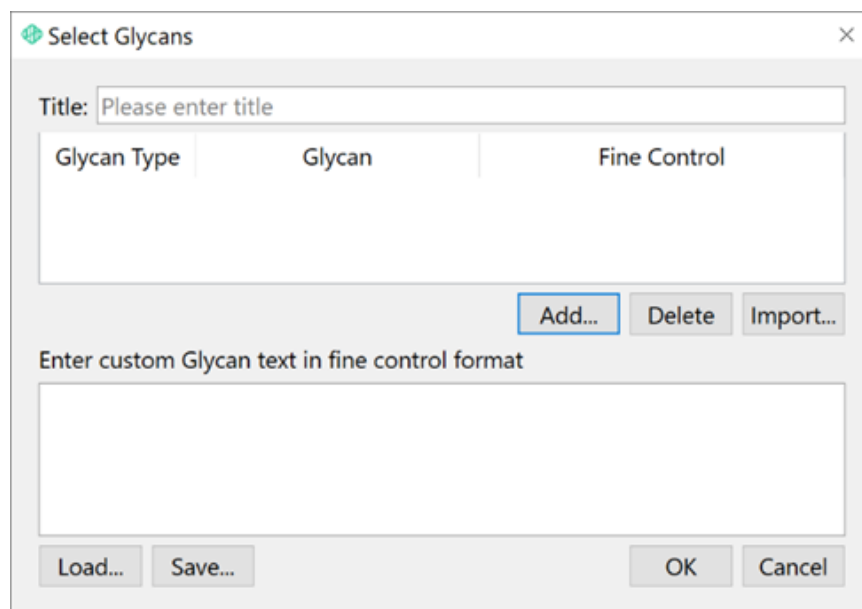


Figure 35: The Select Glycans window

Enter glycan database(s) allows the user to input a database of glycans. Clicking on the drop-down menu and selecting **Browse** takes the user to the C:\Program Files\ProteinMetrics\PMI-Suite\Base\data\GlycanDatabases folder where the user can select from the databases currently included by default with installation. These databases are editable and the user can also add customized databases to this folder to select instead. We continually add to this set of glycan databases based on customer feedback – please reach out to support@proteinmetrics.com to request additional content.

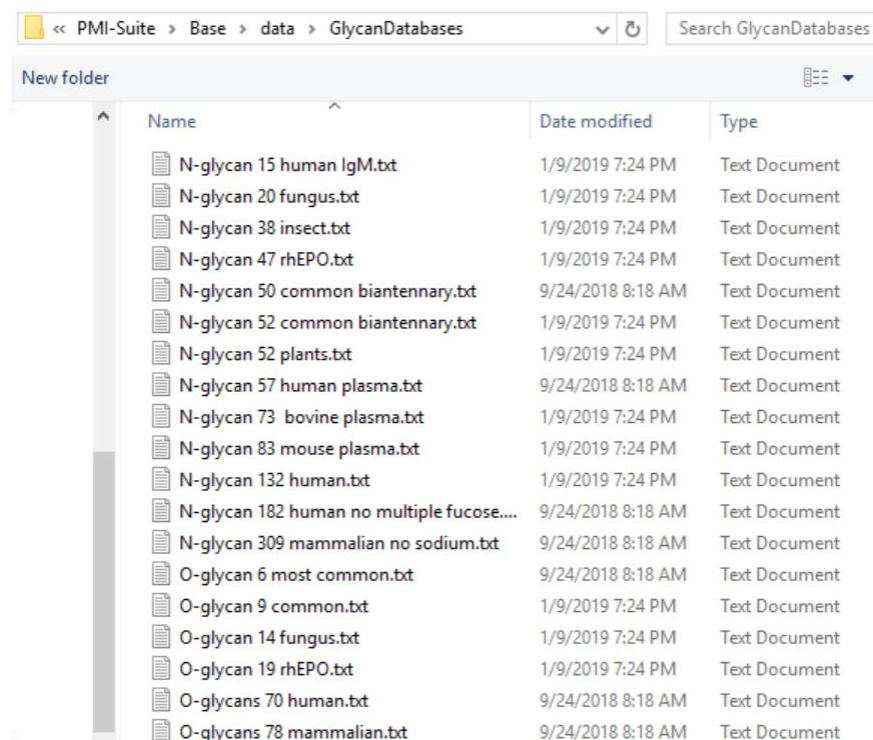


Figure 36: Glycan databases included with installation

The user can choose Glycan type (N- or O-linked), browse for a text file of glycan compositions, and then set the Fine Control (rare1, common2, etc.).

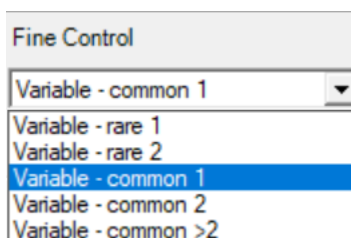


Figure 37: Modification Fine Control options

The text files include one glycan composition per line; for example, the following gives five of the most common human O-glycans. Spaces between monosaccharides are optional, and unused monosaccharides can be left out or included with zero (0) occurrences.

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

Enter specific glycan(s) is located in the middle part of Select Glycans and allows the user to input glycans one at a time by specifying monosaccharide compositions.

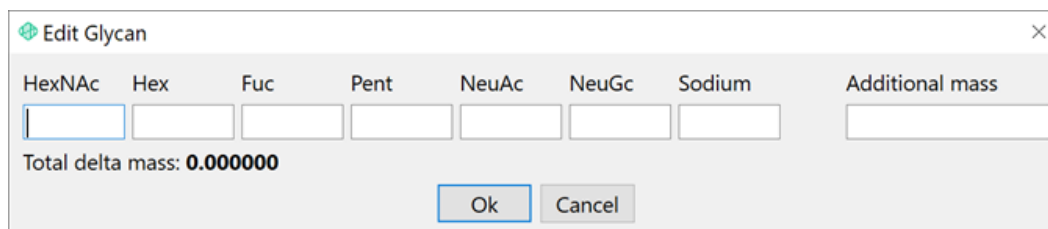


Figure 38: Enter specific glycans

Here the user chooses Glycan type (N- or O-linked) from the drop-down menu, builds a custom glycan by clicking on the white “...” box and entering values in the **Edit Glycan** window, and then sets the Fine Control (rare1, common2, etc.). Six monosaccharide residues are allowed: HexNAc, Hexose, Fucose, Pentose (common in plants), NeuAc, and NeuGc (common in non-humans). A box for Sodium is also included because this is a common adduct on sialic acids. Other glycan masses and modifications such as sulfation and acetylation can be defined with the Additional mass box; this mass is added to the mass of the monosaccharides. The total delta mass will automatically populate. The user selects OK to close the window and include the value.

In the third section, the user can add additional glycans using the same format as within the glycan database text files.

For some helpful examples and best practices for conducting N-linked and O-linked glycan searches, see our Application Notes at <https://www.proteinmetrics.com/resources/>.

- **Spectrum Input Options**

These parameters help Byos cope with imperfect inputs. For example, on many MS instruments, precursor ion charges are uncertain for some or all spectra.

Spectrum Input Options	
Apply Charges To	unassigned spectra
Charge States	
Precursor Isotope Off by X	No error check
Maximum Precursor Mass	12,000
Precursor and Charge Assignments	Compute from MS1
Maximum Number of Precursors per Scan	1
Smoothing Width (m/z)	0.01

Figure 39: Spectrum Input Options

- **Apply Charges To** - By default, the assigned charge will be used for all spectra with assigned charges and +1, +2, +3 will be used for all CID spectra and +2, +3, +4 for all ETD spectra without assigned charges. The Apply Charges To parameter allows the user to override this default setting by instead selecting unassigned spectra.
- **Charge States** - All comma-separated charges detailed to each spectrum will be applied (based on the values the user entered into the Charge States box).
- **Precursor Isotope Off By X**

Precursor Isotope Off by X	No error check
Maximum Precursor Mass	No error check
Precursor and Charge Assignments	Too high (narrow)
Maximum Number of Precursors per Scan	Too high (wide)
Smoothing Width (m/z)	Too high or low (narrow)
Smoothing Width (m/z)	Too high or low (wide)

Figure 40: Precursor Isotope Off By X

Similarly, on many instruments the nominal precursor mass may actually be the mass of a ^{13}C isotope peak rather than of the base (all ^{12}C monoisotopic) peak, so the true precursor mass will within 10 ppm of 2350.120 Da or within 10 ppm of 2351.123 Da. Precursor Isotope Off By X is a pulldown menu with several options.

No error check will use only the assigned precursor; **Too high (narrow)** will allow the assigned precursor to be up to 2 Da too high; **Too high (wide)** will allow the assigned precursor to be up to n Da too high for a precursor of mass at least 1000n Da; **Too high or low (narrow)** will allow the assigned precursor to be up to 2 Da too high or 2 Da too low; **Too high or low (wide)** will allow the assigned precursor to be up to n Da too high or 2 Da too low for a precursor of mass at least 1000n Da.

- **Maximum Precursor Mass** - The user can set the Maximum Precursor Mass to be considered.
- **Precursor and Charge Assignments**

Precursor and Charge Assignments	Compute from MS1
Maximum Number of Precursors per Scan	Originally assigned
Smoothing Width (m/z)	Compute from MS1

Figure 41: Precursor and Charge Assignments

The precursor and charge assignments can also be calculated directly from the MS1 data or use the originally assigned values. The user can set this using a drop-down menu.

- **Maximum Number of Precursors per Scan** - Multiple precursors per scan is also considered – it is recommended for the user to set this to 2 for complex samples and 5-10 if processing MSe or DIA data.

- **Smoothing Width** - The user can enter a sigma value in Thomsons for Gaussian smoothing and centroiding of Waters or Sciex data. Half-width at peak half maximum (~ 0.01 m/z) works well and is the default value already entered for the user.
- **Peptide Output Options**

▼ Peptide Output Options	
Automatic Score Cut	✓ Yes
Manual Score Cut	0
Show All N-glycopeptides	— No

Figure 42: Peptide Output Options

The Peptide Output Options parameters offer options for filtering the peptide-spectrum matches (PSMs) by score. By default, PSM filtering is deferred until after protein ranking, and then filters to control PSM FDR on the “true” proteins—those ranked above the top-ranking decoy protein. This method gains sensitivity while simultaneously reducing both protein and PSM FDRs. ([Two-dimensional target decoy strategy for shotgun proteomics, Journal of Proteome Research 10 \(12\), 5296-5301, 2011.](#))

- **Automatic Score Cut** - To filter PSMs before protein ranking, the user can click **Yes** to activate Automatic score cut and type in a minimum score. When Automatic Score cut is set to Yes, the Manual Score Cut value is ignored. Autoscore cut=yes uses multiple fields (MS/MS score, MS1 accuracy, and several others) to filter the list of PSMs shown.
- **Manual Score Cut** – If Automatic Score Cut is set to No, Manual Score Cut is used. Manual Score Cut filters strictly on MSMS score.
- **Show All N-Glycopeptides** - The user also has the option to Show All N-Glycopeptides. This will show N-Glycopeptide matches regardless of score or FDR. This is recommended for simple samples only. This can be especially useful for low energy CID data.

- **Protein Output Options**

▼ Protein Output Options	
Protein FDR	1% FDR (or 20 reverse count)
Create Focused Database	— No
Export mzIdentML	— No

Figure 43: Protein Output Options

- **Protein FDR**

1% FDR (or 20 reverse count) ▼
1% FDR (or 20 reverse count)
2% FDR (or 50 reverse count)
No cuts

Figure 44: Protein FDR options

This gives the user control of the protein list cut-off. By default, the protein list is cut at 1% protein FDR or 20 decoy proteins, whichever comes last, but the user can ask for 2% protein FDR or a completely unfiltered (but still ranked) protein list, or No Cuts.

- **Create a Focused Database** - If the user clicks this to select **Yes**, the software is directed to output a new FASTA file (labeled focused and appearing in the output objs directory) containing only the proteins found in the search, along with suitable decoys (>Reverse) for

unbiased FDR estimation. The focused database can then be used for subsequent wide searches, including more modifications and/or a wildcard. Of course, the user can also create focused databases outside of the software by editing existing FASTA files.

- **Export mzIdentML** - The user can also select to export this file as a mzIdentML file.

- **Multicore Options**

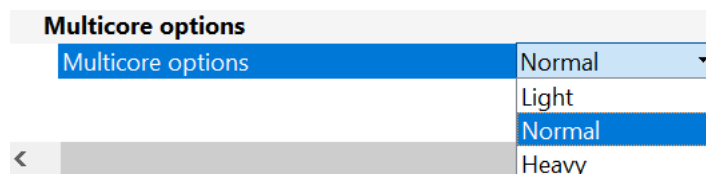


Figure 45: Multicore processing options

The user can control the number of computer cores of the CPU used through the drop-menu. The Light searches uses one core, Normal search uses all available cores minus two, and Heavy search uses all available cores.

- **Wildcard**

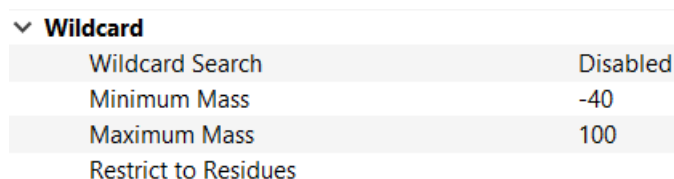


Figure 46: Wildcard parameters.

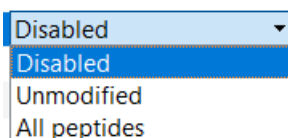


Figure 47: Wildcard Search drop-down options.

Wildcard lets the user turn on wildcard searches, set the range for the wildcard mass, and restrict the wildcard to certain residues if desired. The options Disabled, Unmodified, and All peptides are available through a drop-down menu.

The **Restrict to residues** box uses the common 20 single-letter residue abbreviations, and (lower case) n denotes peptide N-terminus and (lower case) c denotes peptide C-terminus (e.g. Kn searches lysine and N-terminus). Leaving the field blank searches all residues. A wildcard, even one with a mass range of only 50 or 60 Da, greatly increases the size of the search. It is best used with a focused database (see the Advanced tab section below) and used either alone or with only a few other modifications enabled. Most wildcard mass shifts will be recognizable by an expert; hence, a wildcard can be used to discover which known modifications should be enabled in a subsequent search. For more details about the wildcard search, see the application note “Byonic™: Wildcard Search™” at <https://www.proteinmetrics.com/resources/#application-notes>.

By specifying most modifications as rare, it is quite feasible to search for 10 – 20 modification types at once with Byonic. Even larger searches are possible with focused protein databases, for example with therapeutic proteins. Such a focused database easily allows efficient mutation searches with 200+ possible substitutions, or oxidative footprinting searches with 50+ types of oxidations. Glycans and wildcards can easily enlarge the search space by 2 to 3 orders of magnitude, so these options should be

used with care, and in conjunction with only the most common variable modifications (such as oxidized methionine or pyro-Glu N-terminus). **NOTE:** The single most important factor in search time is Total common max. Roughly speaking, the search time grows as C^T where C is the number of common modifications enabled and T is Total common max.

The **Appendix** of the Byonic Manual provides examples of frequently found modifications and appropriate syntax for including those modifications in a Byonic search.

Chromatographic Quant

NOTE: As mentioned in the above MS/MS Ions section, these parameters apply alone to the Reference Chromatography (in-silico) and Comparison Chromatography (in-silico) workflows.

- **General**

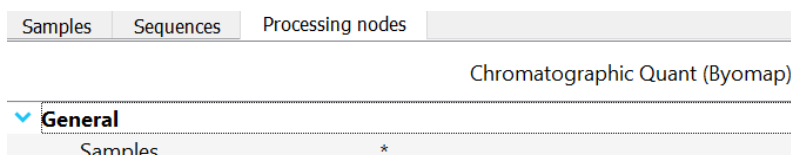


Figure 48: Samples to include in the project

- **Samples** - The "*" will apply all parameters to all samples dragged and dropped into the Samples tab.

- **Candidate Matching**

Candidate matching	
Mass assignments	Auto candidate assignment
Minimum charge	1.00
Maximum charge	5.00
Tolerance (ppm)	10.00
Minimum peak intensity percent	10.00
Minimum m/z	250.00

Figure 49: Candidate matching

These parameters allow the user to set mass assignment, charge range and other candidate matching filters before project creation.

- **In-silico options**

▼ In-silico options (Theoretical Digest)	
Enable in-silico digest	— No
Digestion	Trypsin @ R,K C-term
Missed Cleavages Max	0
Peptide Minimum Mass	300.00 da
Peptide Maximum Mass	9,000.00 da
Total Common Max	2
Total Rare Max	1
Glycans	
Modification options	
▼ In-silico options (CSV import)	
In-silico peptides CSV	
▼ In-silico options (both)	
Skip if in-silico peptide is duplicate of MS2	✓ Yes

Figure 50: In-silico options

Parameters are separated into three sections to clarify which pertain to a **Theoretical Digest**, **CSV import** and **both**.

- **Enable in-silico digest** - The user has the option to click **Yes** to enable this parameter to generate an in-silico list of peptides. The below additional parameters must then be set. NOTE: The option to add a list of in-silico peptides from a CSV file is offered through In-Silico Peptides CSV.
- **Digestion**

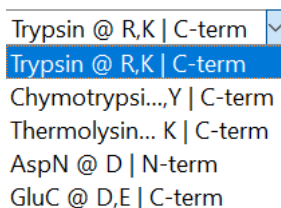


Figure 51: Digestion options

The user can view several options through the drop-down menu. These are detailed as one letter residue codes adjacent to cleavage points. The user may enter a customized digestion by typing using the following syntax: Name @ Amino acid letters | C-term or N-term.

- **Missed Cleavages Max**

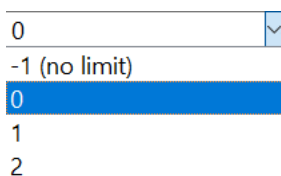
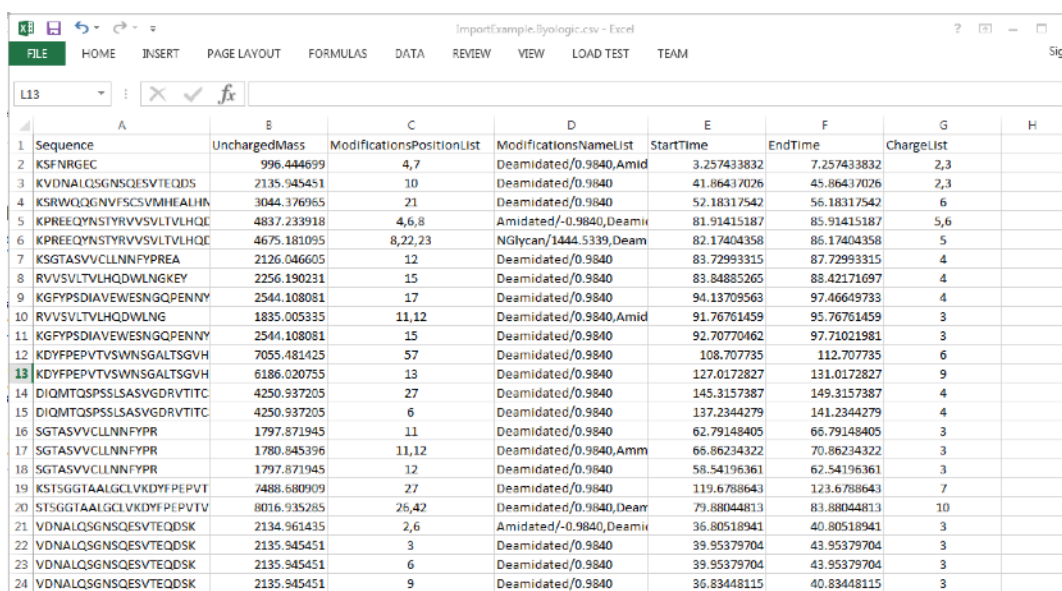


Figure 52: Missed Cleavages Max options

The user can set the maximum number of missed cleavages per peptide by selecting a value using the drop-down menu. A value of -1 allows any number of cleavages.

- **Peptide Minimum Mass** - The user can set the minimum value of the peptide mass range.
- **Peptide Maximum Mass** - The user can set the maximum value of the peptide mass range.

- **Total Common Max** - The user can set the maximum number of common modifications per peptide. The search size grows by an order of magnitude with each increase in Total Common Max.
- **Total Rare Max** - The user can set the maximum number of rare modifications per peptide. The search size grows by an order of magnitude with each increase in Total Rare Max.
- **Glycans** - The user can specify a glycan database or custom glycans to include in the in-silico generation. This is done the same as detailed in the MS/MS Ids section.
- **Modifications Options** - The user can specify modifications to include in the in-silico generation. This is done the same as detailed in the MS/MS Ids section.
- **In-Silico Peptides CSV** - This option allows the user to add a list of in-silico peptides from an imported CSV file, thus there is no need to run an MS2 search if there are known modifications with known masses and retention times. The format for the CSV is shown below.



	A	B	C	D	E	F	G	H
	Sequence	UnchargedMass	ModificationsPositionList	ModificationsNameList	StartTime	EndTime	ChargeList	
1	KSFNRGEC	996.444699	4,7	Deamidated/0.9840,Amid	3.257433832	7.257433832	2,3	
2	KVDNALQSGNSQESVTEQDS	2135.945451	10	Deamidated/0.9840	41.86437026	45.86437026	2,3	
3	KSRWQQGNVFCISVMHEALHN	3044.376965	21	Deamidated/0.9840	52.18317542	56.18317542	6	
4	KPREEQYNSTYRVVSVLTVHLQ	4837.233918	4,6,8	Amidated/-0.9840,Deamid	81.91415187	85.91415187	5,6	
5	KPREEQYNSTYRVVSVLTVHLQ	4675.181095	8,22,23	NGlycan/1444.5339,Deamid	82.17404358	86.17404358	5	
6	KSGTASVCLNNFYPREA	2126.046605	12	Deamidated/0.9840	83.72993315	87.72993315	4	
7	RVVSVLTVHLQDNLNGKEY	2256.190231	15	Deamidated/0.9840	83.84885265	88.42171697	4	
8	KGFYPSDIAVEWESNGQPENNY	2544.108081	17	Deamidated/0.9840	94.13709563	97.46649733	4	
9	RVVSVLTVHLQDNLNG	1835.005335	11,12	Deamidated/0.9840,Amid	91.76761459	95.76761459	3	
10	KGFYPSDIAVEWESNGQPENNY	2544.108081	15	Deamidated/0.9840	92.70770462	97.71021981	3	
11	KDYFPEPVTISWNSGALTSVGH	7055.481425	57	Deamidated/0.9840	108.707735	112.707735	6	
12	KDYFPEPVTISWNSGALTSVGH	6186.020755	13	Deamidated/0.9840	127.0172827	131.0172827	9	
13	DIQMTQSPSSLSASVGDRVTTC	4250.937205	27	Deamidated/0.9840	145.3157387	149.3157387	4	
14	DIQMTQSPSSLSASVGDRVTTC	4250.937205	6	Deamidated/0.9840	137.2344279	141.2344279	4	
15	SGTASVCLNNFYPR	1797.871945	11	Deamidated/0.9840	62.79148405	66.79148405	3	
16	SGTASVCLNNFYPR	1780.845396	11,12	Deamidated/0.9840,Amid	66.86234322	70.86234322	3	
17	SGTASVCLNNFYPR	1797.871945	12	Deamidated/0.9840	58.54196361	62.54196361	3	
18	KSTSGGTAALGCLVKDYFPEPVT	7488.680909	27	Deamidated/0.9840	119.6788643	123.6788643	7	
19	STSGGTAALGCLVKDYFPEPVT	8016.935285	26,42	Deamidated/0.9840,Deamid	79.88044813	83.88044813	10	
20	VDNALQSGNSQESVTEQDSK	2134.961435	2,6	Amidated/-0.9840,Deamid	36.80518941	40.80518941	3	
21	VDNALQSGNSQESVTEQDSK	2135.945451	3	Deamidated/0.9840	39.95379704	43.95379704	3	
22	VDNALQSGNSQESVTEQDSK	2135.945451	6	Deamidated/0.9840	39.95379704	43.95379704	3	
23	VDNALQSGNSQESVTEQDSK	2135.945451	9	Deamidated/0.9840	36.83448115	40.83448115	3	

Figure 53: Format for an In-Silico Peptides CSV

- **Skip if in-silico peptide is duplicate of MS2** - The user can click to select **Yes**. This will skip any generated in-silico peptide if one already exists from the MS2 data. It is recommended to make this selection when generating in-silico peptides.

Advanced

Advanced	
Enable Lock-Mass Calibration	No
Lock Mass (m/z)	1221.990637
Lock Mass tolerance (ppm)	100.00
Centroid Smoothing Width	0.02
Elution Prediction Score Min	300
Compute Fragment Coverage	Yes
Advanced configuration	

Figure 54: Advanced parameters

- **Enable Lock-Mass Calibration** - The user can click to select between **No** and **Yes**.

- **Lock Mass (m/z)**

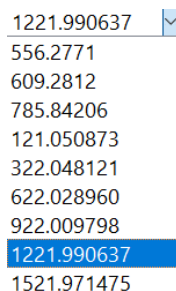


Figure 55: Lock mass m/z value options

The user can enter the calibrant m/z value. Several are also available using the drop-down menu or the user can type in a numerical value. If empty, no calibration will be applied.

- **Lock Mass tolerance (ppm)** - The user can enter the calibrant m/z mass tolerance value in ppm. The user can type in a numerical value or use the up and down arrow keys to increase or decrease the value. If empty, no calibration will be applied.
- **Centroid Smoothing Width** - The user can enter a sigma value in Thomsons for Gaussian smoothing and centroiding of Waters or Sciex data. Half-width at peak half maximum (~0.01 m/z) works well and is the default value already entered for the user.
- **Elution Prediction Score Min** - This filter will find wildtype peptides with scores above this value to adjust the elution time.
- **Compute Fragment Coverage** - The user can click between **Yes** and **No** to compute fragmentation coverage at project creation.
- **Advanced Configuration** - The user can enter text commands to complete advanced processing. These are detailed in the Release Notes included with each quarterly release. Please reach out to support@proteinmetrics.com for additional details.

- **Report**



Figure 56: Report Configuration Path

Each Byos default workflow includes a report template created by our Customer Success team that is optimized for the specific type of analysis. If the user prefers a customized report template, they can direct Byos to this file using the light blue “...” button. They will be prompted to select an .rptc file.

- **UI Configurations**

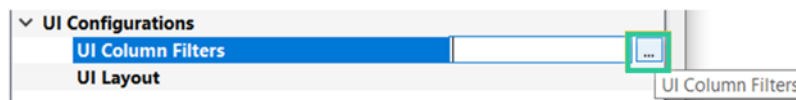


Figure 57: UI Configurations – UI Column Filters and UI Layout.

The user can import column filters as well as layout files. This is designed to standardize analyses across all users, labs, and sites. The user can direct Byos to the preferred file for each using the light blue “...” button. The user will be prompted to select a file, .cft (Filters) or .ini (Layout) file.

- **Time Settings**

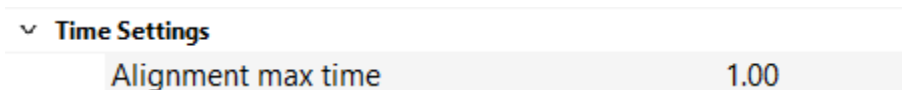


Figure 58: Time Settings

- **Alignment max time** sets the maximum alignment value between plots (for example, UV and TIC). This value will limit the allowed alignment time between the two signals.

The default values for both are set to 0.00, which means filtering will not be applied. The user can set them to apply the additional processing options. The default value for “Alignment max time” is set to 1.00.

- **Label Scripts**

This feature allows users to customize peak labels for Trace plot.

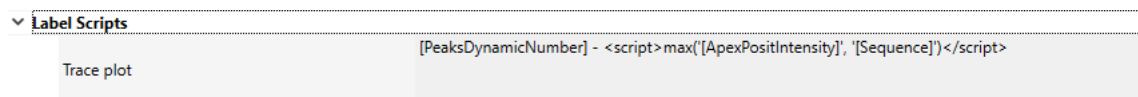


Figure 59: Label Scripts

Scripts related to trace plot are in C:\Program Files\ProteinMetrics\PMI-Suite\Base\labelscripts\traceplot folder. To load a script during project creation, select **Processing nodes**, expand **Label Scripts**, click on ... for Trace plot, then click **Load** to select a script, then click **OK** and **Create Project**. This will result in creation of the project with custom labels as specified in the script.

The user has an option to load scripts after project creation as well. To customize peak labels after project creation, select **Rendering options** icon, click **Edit Annotations**, then click **Load** to select a script, then click **Open** and click **OK** to display new custom labels.

- **Peak Construction options** (older versions of Byos)

The Peak Construction option parameters formerly found in Byos are now set directly in the Samples tab with the buttons **Edit trace peak options** and **Edit global trace peak filters**.

Main Menu Bar

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

File Menu

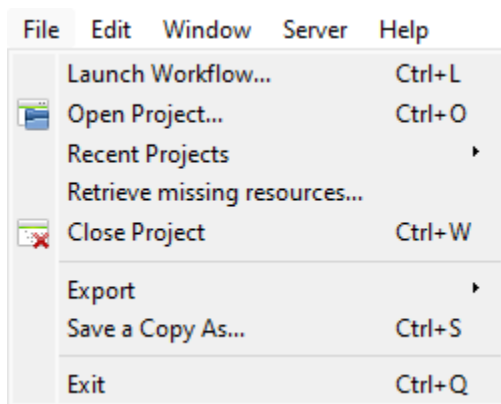


Figure 60: File menu

The **File** pull-down menus manage project files and export reports and data. The File menus include items 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 Byos. 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 **Open Project**, **Close Project**, and **Report**, respectively, are also available as icon buttons below the topmost menu bar.



Figure 61: Menus and toolbar buttons

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 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.

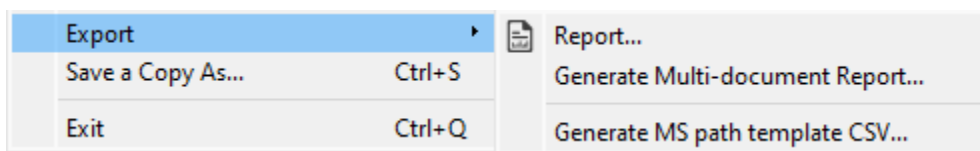


Figure 62: File > Export menu



Figure 63: 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.

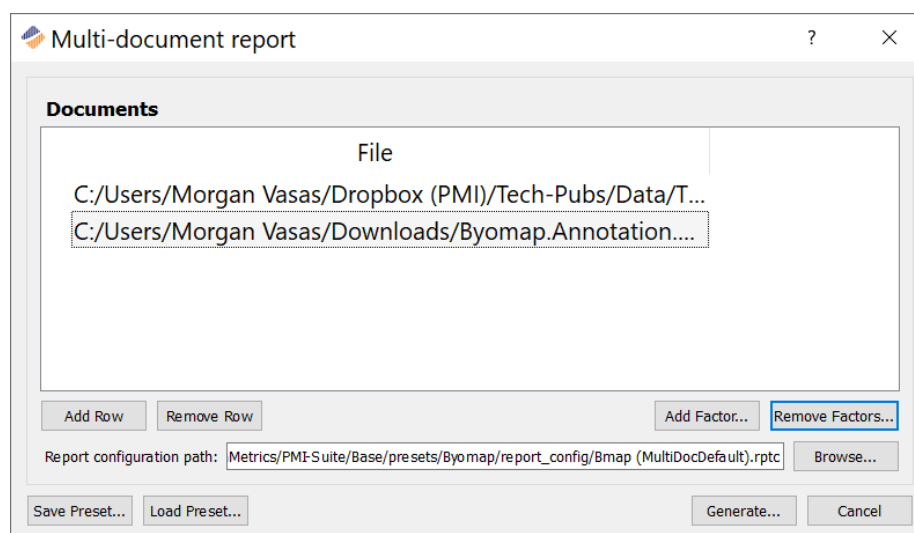


Figure 64: Multi-document report

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

Edit Menu

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

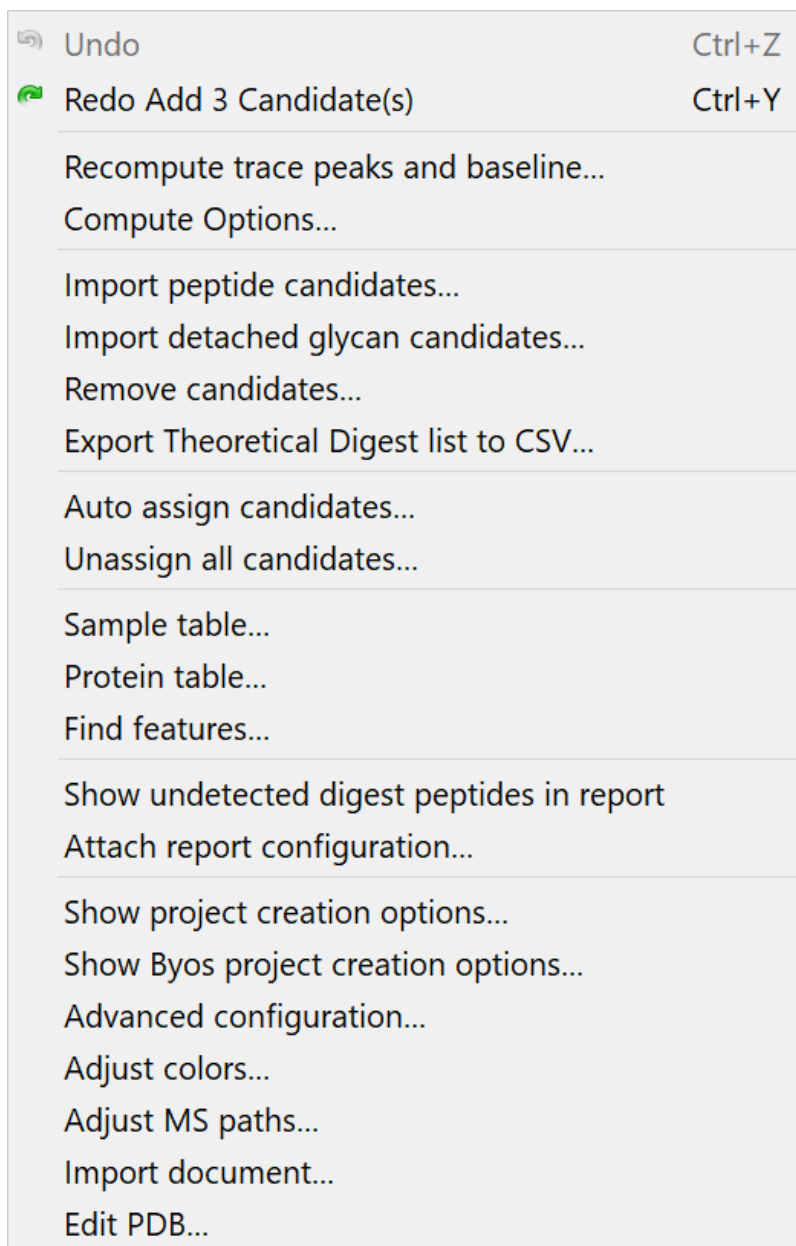


Figure 67: Edit menu

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

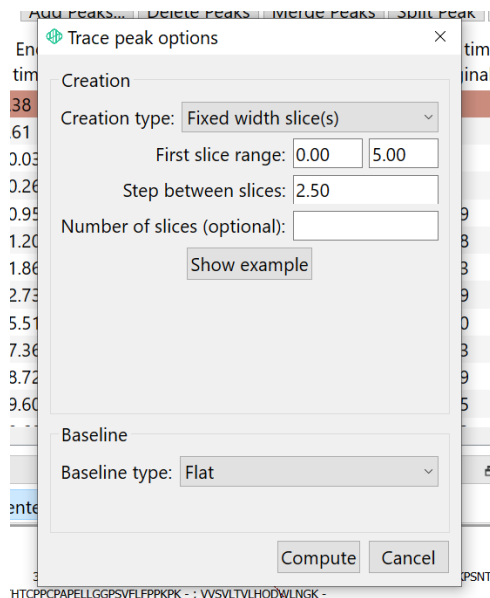


Figure 68: Trace peak options – post-processing

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 trace processing before and/or after project creation. For details, see the [Trace Peaks Options](#) section.

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

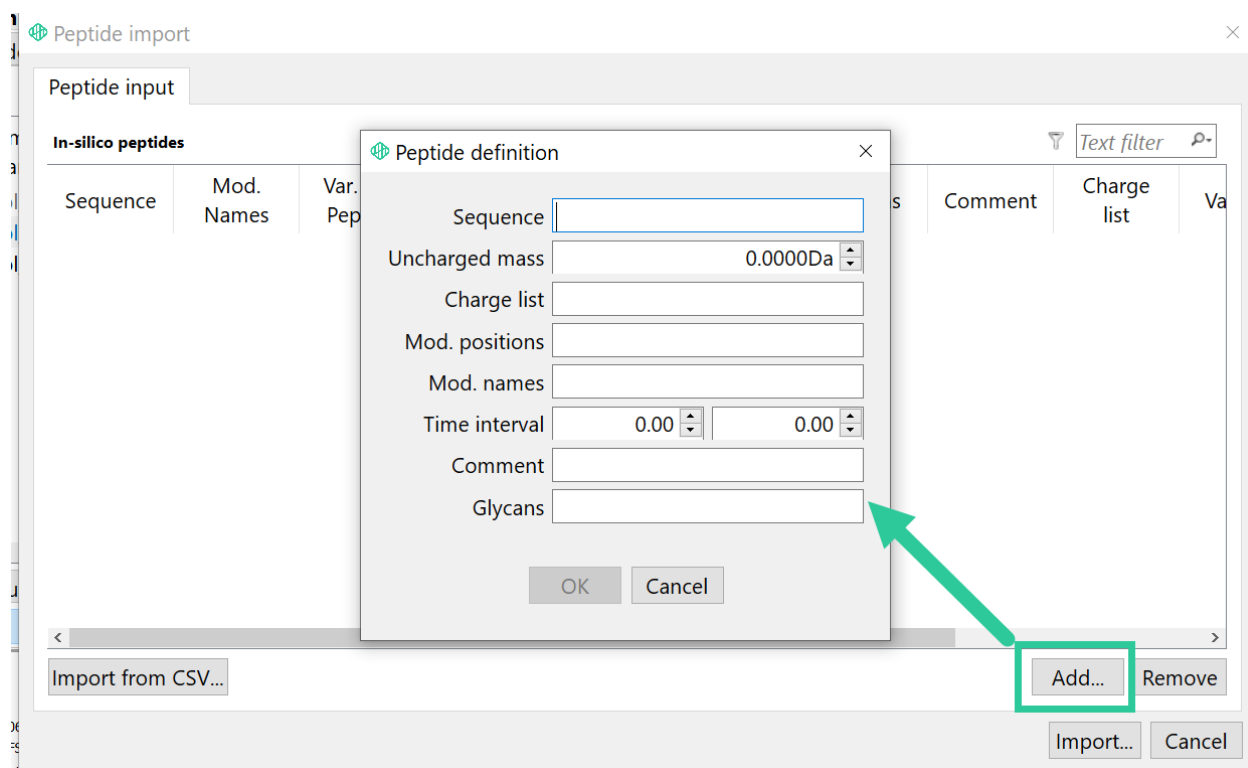


Figure 71: Peptide import and definition

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:

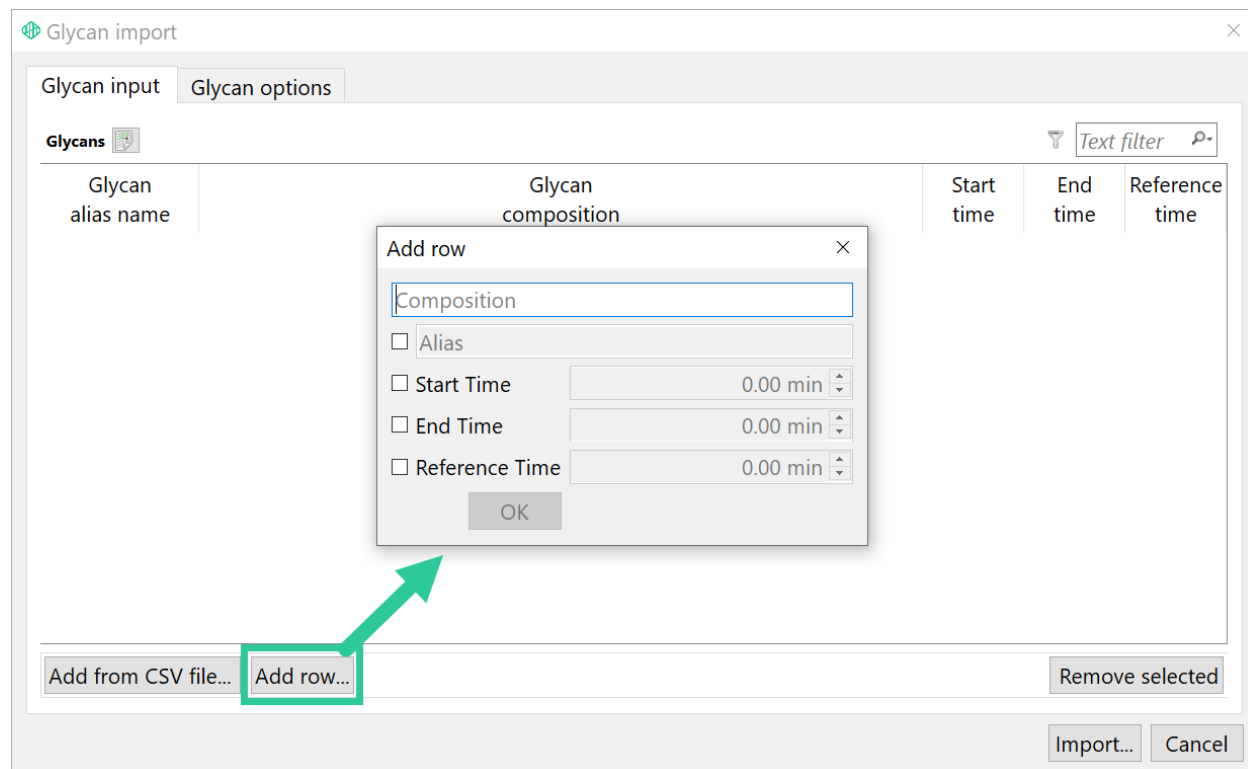


Figure 72: Glycan import – Glycan input

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 adducts for the glycans:

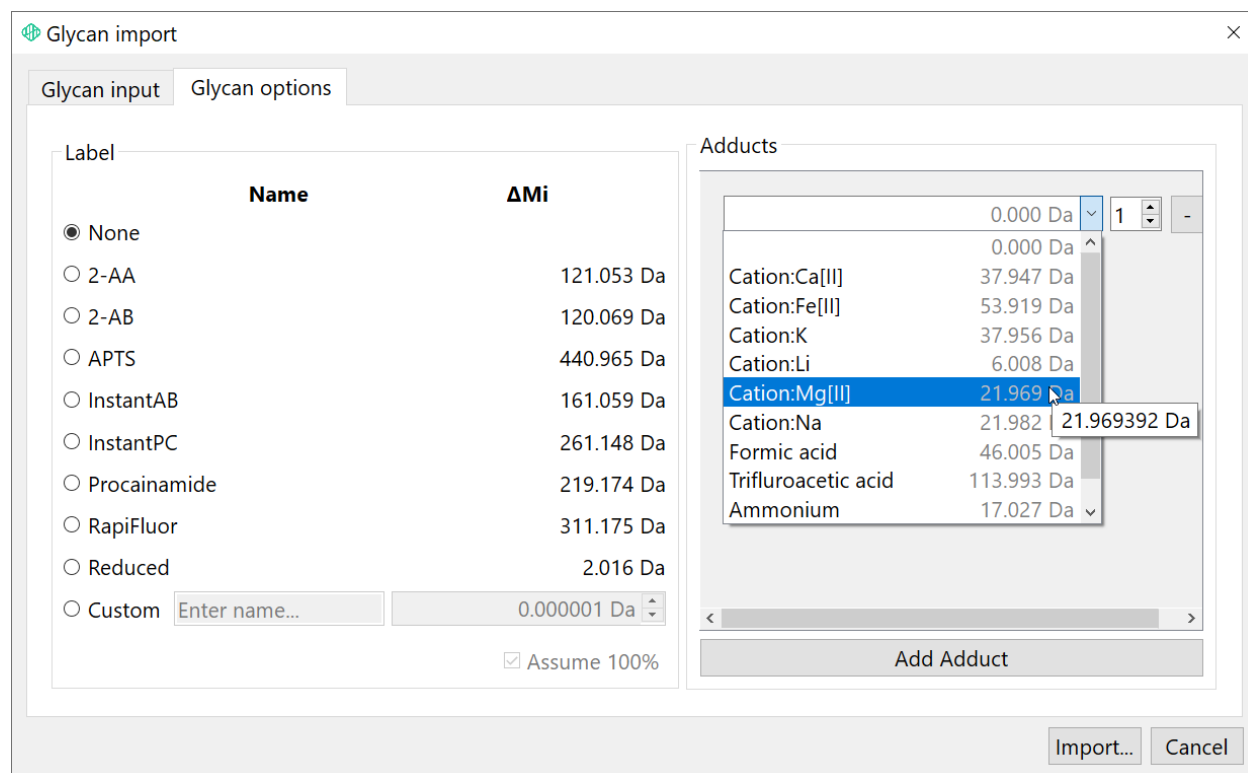


Figure 73: Glycan import - Glycan options

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 recommended to further 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.

Show undetected digest peptides in report allows either including or excluding the undetected peptides for reporting. Enabling this will result in a new label "ND" in the **Peak Comment** column in reporting tabs, denoting that the specific peptide is not detected in the analyses.

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 is obsolete and can be ignored.

Show Byos Project Creation options displays the Byos workflow Project Creation options used to generate the project. Users can edit the workflow settings, but these workflow edits are **not** applied to the current project. There is also no option to create projects from this view. Users can use this option to:

1. View the Project Creation options used to create the current project.
2. Click **Save workflow** to save the workflow settings used to create the project as a .wflw file.
3. Click **Save as Portable Workflow** to create a folder containing the .wflw and associated files (like .rptc).
4. Edit the Project Creation workflow settings and then click **Save workflow** to create a new workflow, using the current project creation settings as a starting point. The new workflow must be opened using **File>Launch Workflow** to create a new project.

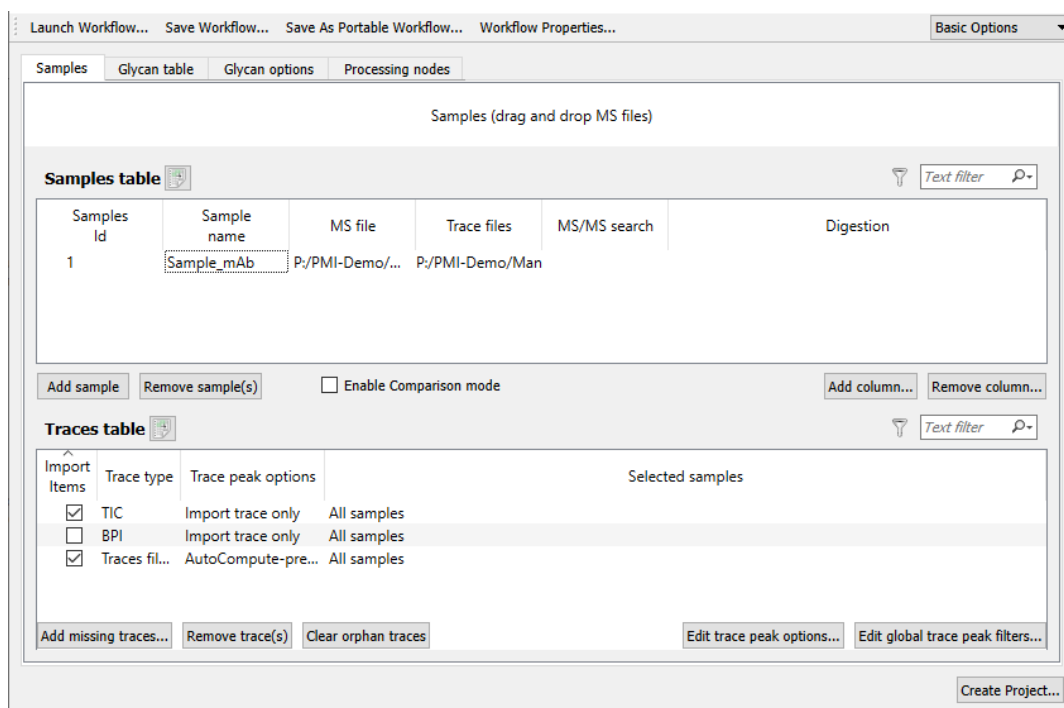


Figure 74: Show Byos project creation options

The **Advanced configuration** dialog is used to enter text commands, which are often new features still in beta testing. A list of available advanced commands can be found [here](#).

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

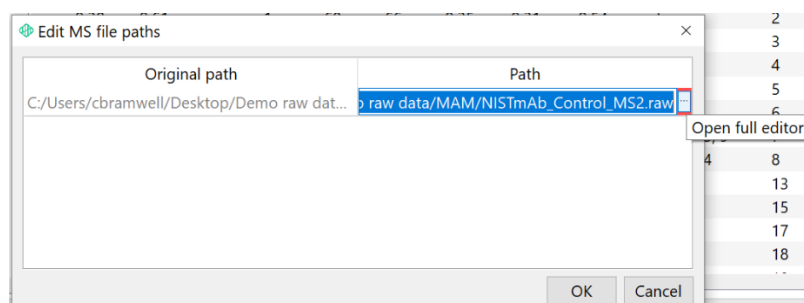


Figure 75: Edit MS file paths

Import document is used to import a Peptide Analysis (Byologic™) *.blgc project file.

Window Menu

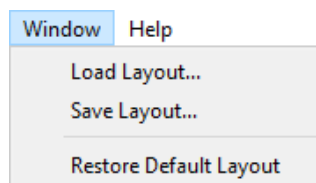




Figure 76: Window menu

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, Trace plot, 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.

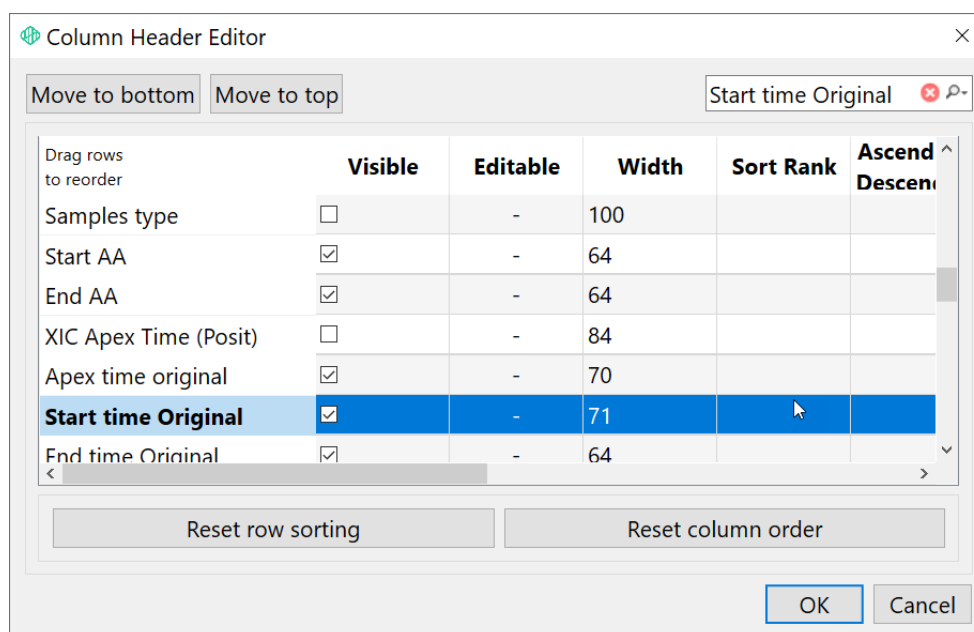


Figure 77: Column Header Editor

The **Search Box** can be used to search for specific values or variables, with options to filter for only whole values, with case-sensitivity, and to only search in vertical header columns.

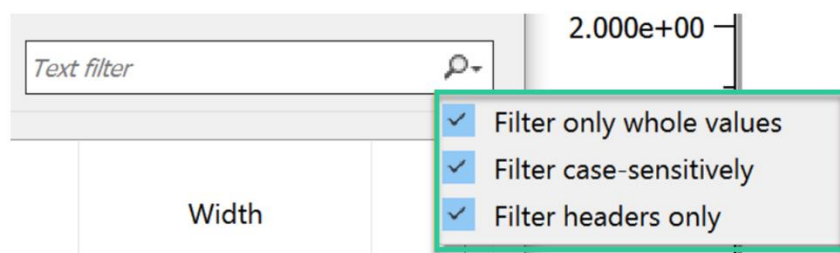


Figure 78: Search box filters

A selected row can be moved to the top or the bottom by clicking **Move to Bottom** or **Move to Top**; alternatively, the same actions can be performed by pressing Alt + t or Alt + b, respectively.

Help Menu

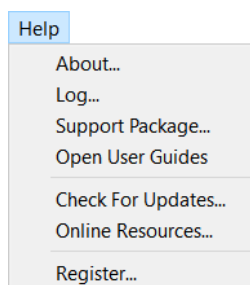






Figure 79: Help menu

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.)

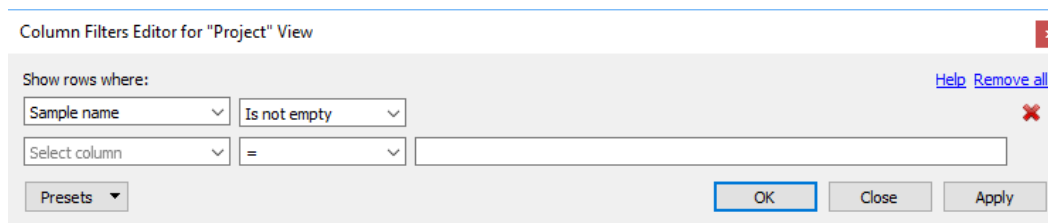





Figure 80: Column filters editor

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.

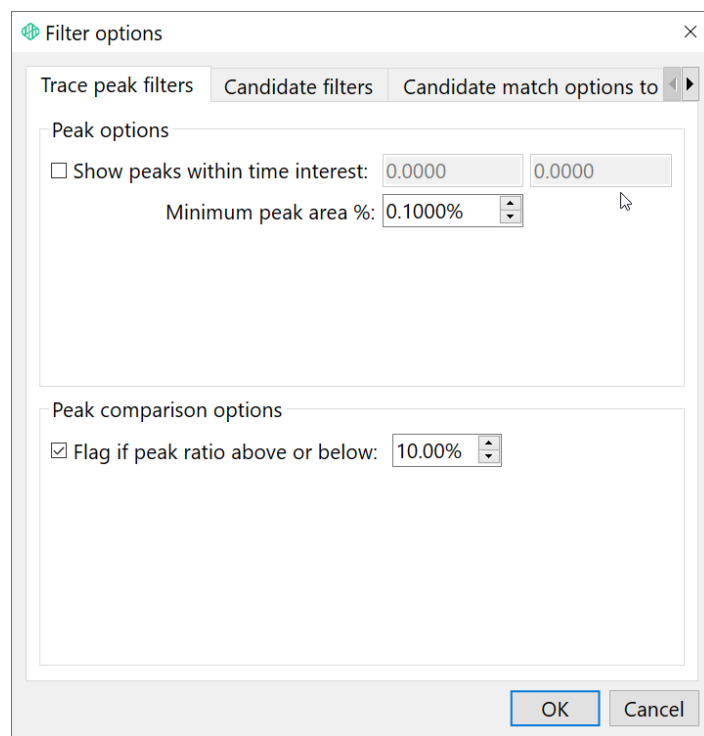
Filter Options

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:

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 trace. 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 Trace plot.



The dialog box is titled "Filter options" and has three tabs: "Trace peak filters", "Candidate filters", and "Candidate match options to". The "Trace peak filters" tab is selected. It contains two sections: "Peak options" and "Peak comparison options".

Peak options:

- ☐ Show peaks within time interest: 0.0000 to 0.0000
- Minimum peak area %: 0.1000%

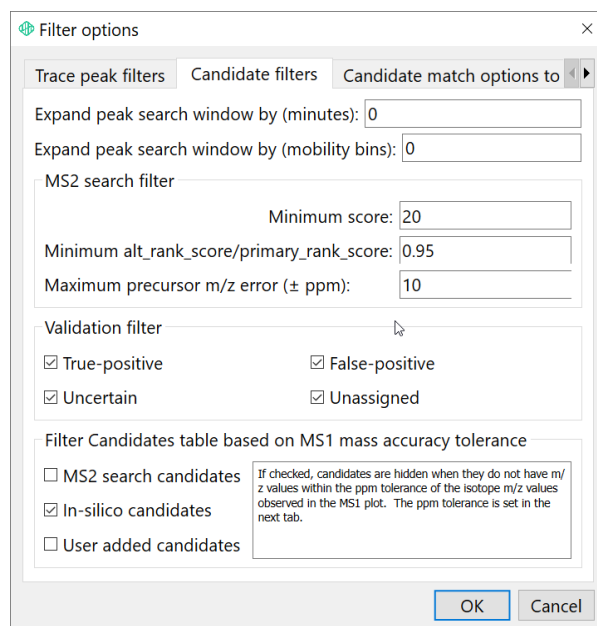
Peak comparison options:

- ☒ Flag if peak ratio above or below: 10.00%

At the bottom are "OK" and "Cancel" buttons.

Figure 81: Filter options – Trace peak filters tab

The **Candidate filters** 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.



The dialog box is titled "Filter options" and has three tabs: "Trace peak filters", "Candidate filters", and "Candidate match options to". The "Candidate filters" tab is selected. It contains several sections:

- Expand peak search window by (minutes):** 0
- Expand peak search window by (mobility bins):** 0
- MS2 search filter:**
 - Minimum score: 20
 - Minimum alt_rank_score/primary_rank_score: 0.95
 - Maximum precursor m/z error (\pm ppm): 10
- Validation filter:**
 - ☒ True-positive
 - ☒ False-positive
 - ☒ Uncertain
 - ☒ Unassigned
- Filter Candidates table based on MS1 mass accuracy tolerance:**
 - ☐ MS2 search candidates
 - ☒ In-silico candidates
 - ☐ User added candidates

A note box states: "If checked, candidates are hidden when they do not have m/z values within the ppm tolerance of the isotope m/z values observed in the MS1 plot. The ppm tolerance is set in the next tab."

At the bottom are "OK" and "Cancel" buttons.

Figure 82: Candidate filters tab

The **Candidate match options to MS1** 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.

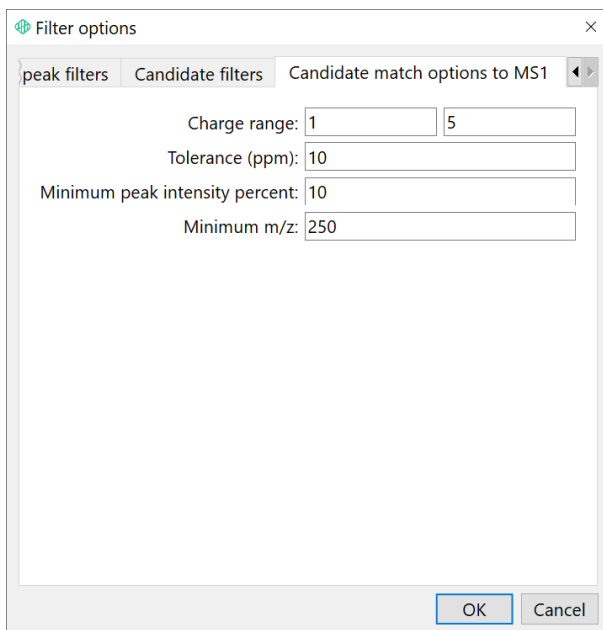
The image shows a 'Filter options' dialog box with three tabs: 'peak filters', 'Candidate filters', and 'Candidate match options to MS1'. The 'Candidate match options to MS1' tab is selected. It contains four input fields: 'Charge range' with values 1 and 5, 'Tolerance (ppm)' with value 10, 'Minimum peak intensity percent' with value 10, and 'Minimum m/z' with value 250. At the bottom right are 'OK' and 'Cancel' buttons.

Figure 83: Candidate match options to MS1 tab

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.)

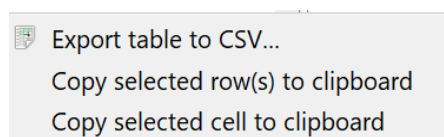


Figure 84: Table right-click menus

- **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.
- **Copy selected cell to clipboard** allows the user to copy the contents of the selected cell. It works just like CTRL+C.
- **Enable soft select (Project table only)** enables selection of rows in the Project table to display them individually in the Trace 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.

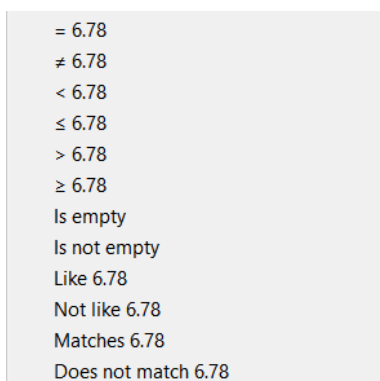
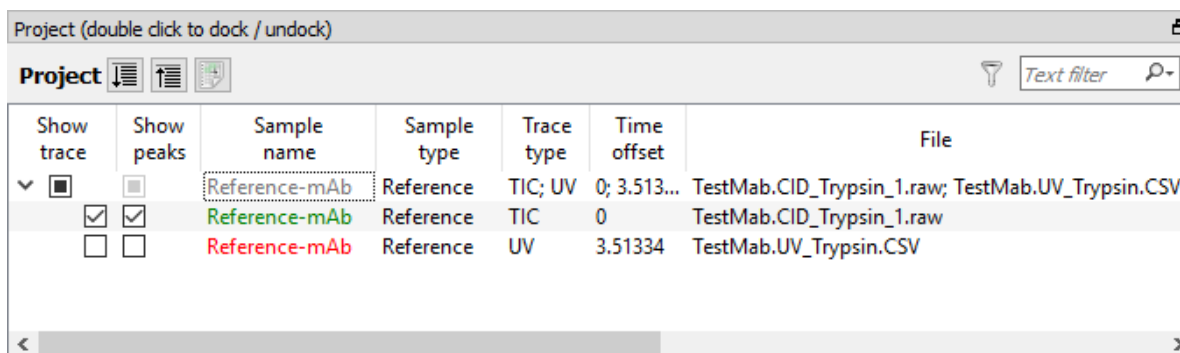


Figure 85: Right-click field searches

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:











Project (double click to dock / undock)						
Project   						
Show trace	Show peaks	Sample name	Sample type	Trace type	Time offset	File
<input checked="" type="checkbox"/>	<input type="checkbox"/>	Reference-mAb	Reference	TIC; UV	0; 3.513...	TestMab.CID_Trypsin_1.raw; TestMab.UV_Trypsin.CSV
<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	Reference-mAb	Reference	TIC	0	TestMab.CID_Trypsin_1.raw
<input type="checkbox"/>	<input type="checkbox"/>	Reference-mAb	Reference	UV	3.51334	TestMab.UV_Trypsin.CSV

Figure 86: Project table for reference project

To display one or all of the traces (channels) present, check the **Show trace** box at the left. Only one of these traces may have peak numbers for annotation (because different traces 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 spec channels.

Project (double click to dock / undock)

Project     Text filter 



Show trace	Show peaks	Sample name	Sample type	Trace type	Time offset	File
> 		Reference-mAb	Reference	TIC; UV	0; 3.513...	TestMab.CID_Trypsin_1.raw; TestMab.UV_Trypsin.C
<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	Sample A2.txt	Non-reference	UV	3.31333	Sample A2.txt
<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	Sample B1.CSV	Non-reference	UV	4.39333	Sample B1.CSV
<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	Sample B2.CSV	Non-reference	UV	4.2	Sample B2.CSV
<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	Sample C1.CSV	Non-reference	UV	2.82	Sample C1.CSV
<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	Sample C2.CSV	Non-reference	UV	2.92667	Sample C2.CSV

Figure 87: Project table for comparison project

Peaks Table and Menu

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

Peaks

Add Peaks...

Delete Peaks

Merge Peaks

Split Peak

Text filter

Peak #	Apex time	Normed area %	Area	Sequence	Mod. Names	Glycans	_prot_id	z	Calc. m/z	Obs. m/z	Obs.M
> 5 (6)	10.94 - 1...	0.05% - 0.13%	1.05e+0 - ...	VdK; VdK	D2(Cation:Na / 21.981...		1	1	0.0000 - 3...	0.0000 - 3...	0.0000 - ...
> 6 (6)	13.32 - 1...	0.85% - 0.92%	1.75e+1 - ...	TKPR; GQPR; PR		1	2; 1	0.0000 - 2...	0.0000 - 2...	0.0000 - ...	
> 7 (6)	16.04 - 1...	0.95% - 1.06%	2.08e+1 - ...	eYK; QAPGK; q...	E1(Glu->pyro-Glu / -1...	1	2; 1	0.0000 - 4...	0.0000 - 4...	0.0000 - ...	
> 8 (6)	18.48 - 1...	0.07% - 0.10%	1.50e+0 - ...	AKGQPR; cKVSNN	C1(Carboxymethyl / 5...	1	2; 1	0.0000 - 6...	0.0000 - 6...	0.0000 - ...	

Figure 88: 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.

The Peaks table also includes the following specialized right-click menu item:

- **Show protein summary** opens a table with protein data for the selected peaks.

Protein summary

Protein summary

text filter

Order	_prot_id	Protein name	Protein alias name	Sequence	Start AA	End AA	Var. Pos Protein
1	2	gij002 NIS...	HC	K.NQVVVK.V	78	83	

OK

Cancel

Figure 89: Protein summary

Candidates Table and Menu

The **Candidates** table contains all the imported candidates relevant to the selected trace peak. When records are selected, the corresponding peak labels in the MS1 plot are displayed in red:

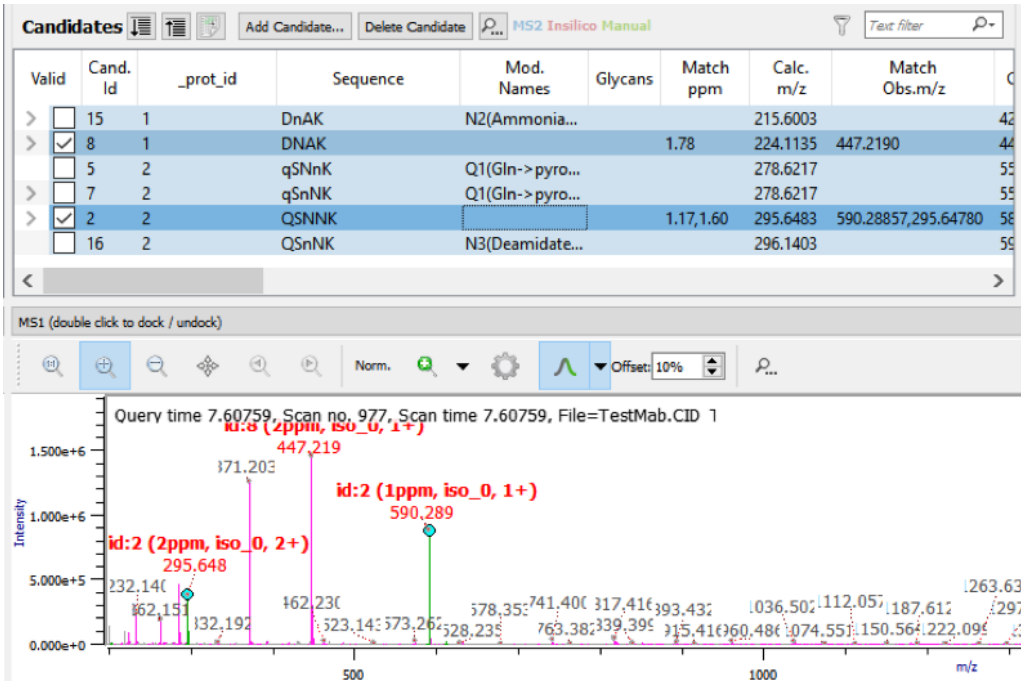
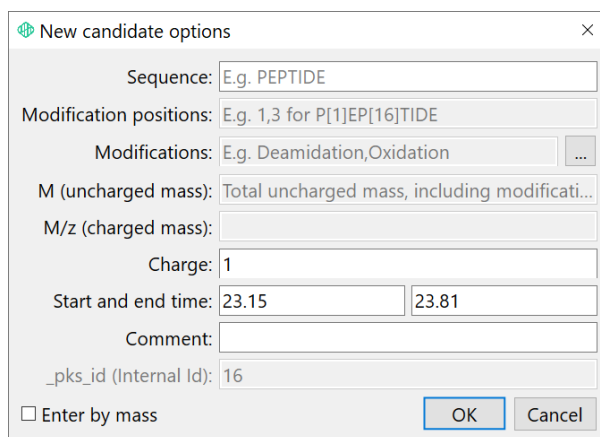


Figure 90: Candidates table matched with MS1 plot labels

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 *.byrsIt 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:



New candidate options

Sequence: E.g. PEPTIDE

Modification positions: E.g. 1,3 for P[1]EP[16]TIDE

Modifications: E.g. Deamidation,Oxidation

M (uncharged mass): Total uncharged mass, including modificati...

M/z (charged mass):

Charge: 1

Start and end time: 23.15 23.81

Comment:

_pks_id (Internal Id): 16

☐ Enter by mass

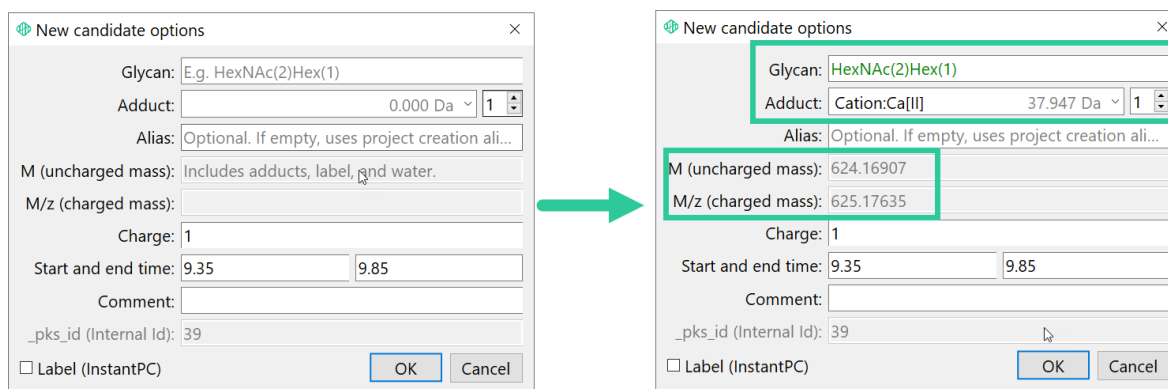
OK Cancel

Figure 91: New candidate options

Note that not all the fields require an entry.

For released glycans, Add Candidate allows for the manual import of a glycan sequence. After selecting a peak in the **Candidates** table, Clicking **Add Candidate** button will open a pop-up dialog to manually enter the glycans. **Glycan** field is mandatory to edit other fields within this **New Candidate options** window. Editing the **Glycan** and/or **Adduct** will update the **M (uncharged mass)** and **M/z (charged mass)** fields. Click **OK** to update the Candidates table with the new row.

Glycans column in the **Candidates** and **Peaks** tables will be updated with the new **Glycan** and **Adduct** information. **Sequence** column in the **Candidates** table will be updated with the information provided in the **Alias** field. If **Alias** field is empty, the **Sequence** column will be updated using the information provided in the Glycan table during the project creation. If there is no matching information in the Glycan table, Alias will simply be **Glycan::Adduct**, for example, HexNAc(2)Hex(1)::Ca[II].



New candidate options

Glycan: E.g. HexNAc(2)Hex(1)

Adduct: 0.000 Da 1

Alias: Optional. If empty, uses project creation ali...

M (uncharged mass): Includes adducts, label, and water.

M/z (charged mass):

Charge: 1

Start and end time: 9.35 9.85

Comment:

_pks_id (Internal Id): 39

☐ Label (InstantPC)

OK Cancel

→

New candidate options

Glycan: HexNAc(2)Hex(1)

Adduct: Cation:Ca[II] 37.947 Da 1

Alias: Optional. If empty, uses project creation ali...

M (uncharged mass): 624.16907

M/z (charged mass): 625.17635

Charge: 1

Start and end time: 9.35 9.85

Comment:

_pks_id (Internal Id): 39

☐ Label (InstantPC)

OK Cancel

Figure 92: Manual import of a glycan sequence

- **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 assigned, 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 unassign 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 colored lines below each sequence:

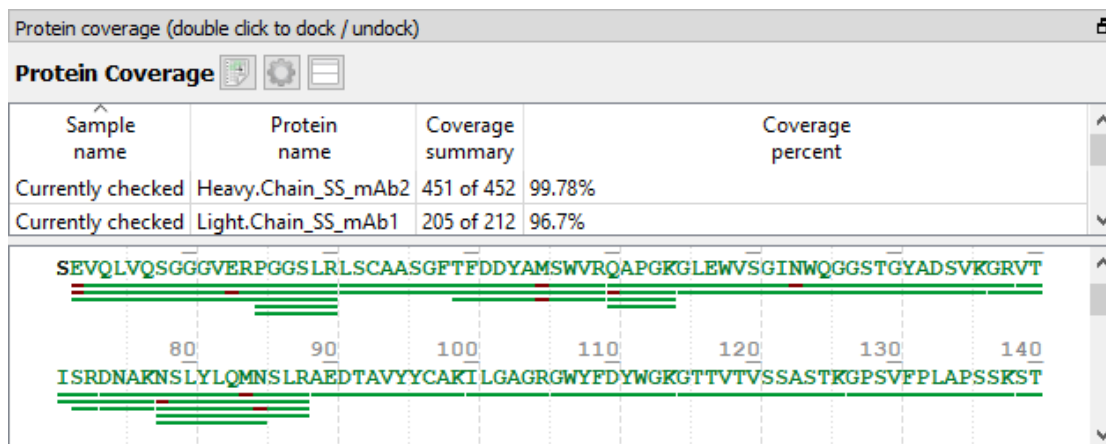




Figure 93: Protein Coverage table

If a highlighted trace peak contains that peptide sequence, it is highlighted in green. Modifications (such as oxidation or deamidation) to the peptide are highlighted in neon green. 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 trace 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:

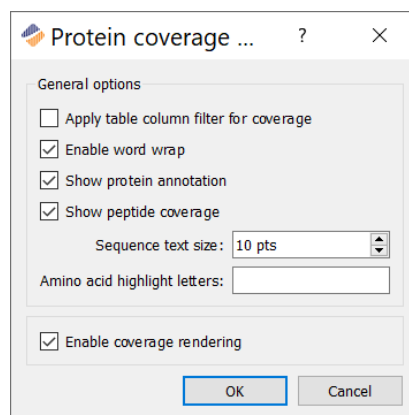


Figure 94: Protein coverage rendering

- 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:

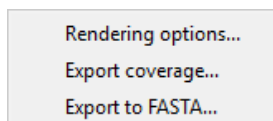










Figure 95: Protein Coverage right-click menu

- **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 Trace plot 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).

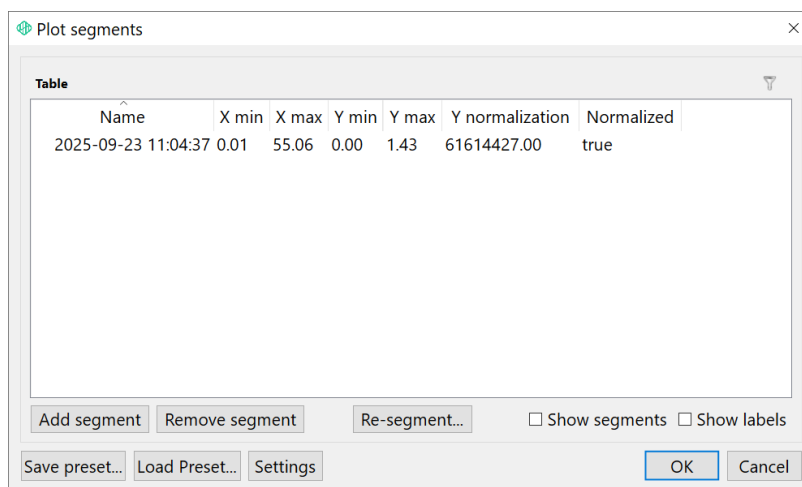



Figure 96: Plot segments

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 traces 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 trace 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.

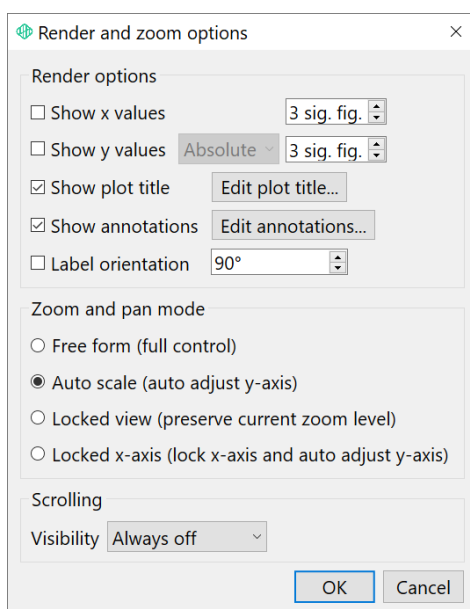


Figure 97: Render and zoom options

- **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 trace as part of the title for any of the plots.

- **Show annotations** (Trace plot 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** (Trace plot only) sets the angle for the label text, where 0° 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 Trace plot 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 Trace plot 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.

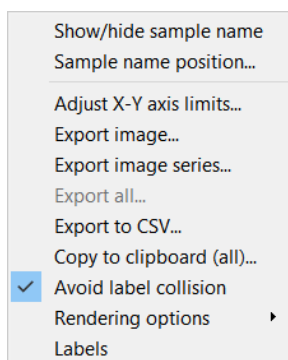




Figure 98: Plot right-click menu

- **Show/hide sample name** (Trace plot only) toggles the display of sample names beside their corresponding peaks.
- **Sample name position** (Trace 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 report or as a *.png, *.wmf or *.svg image 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.

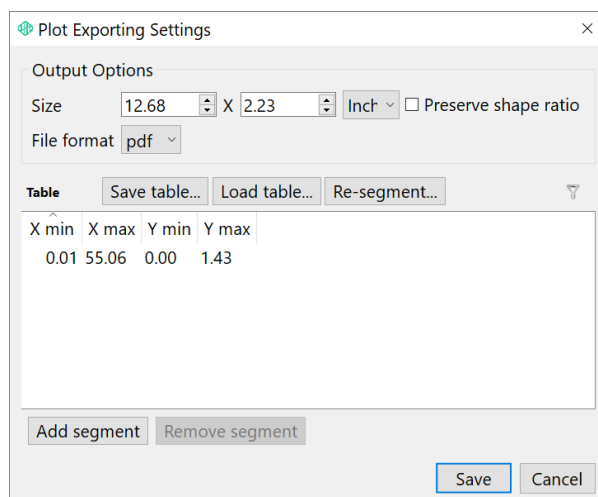


Figure 99: Plot Exporting Settings

Note: Image exports also support *.svg image formats. In the file name cell, change the selected extension to .svg and click **Save**.

- **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:

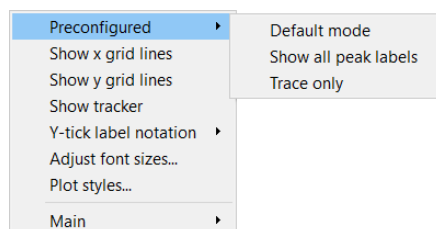


Figure 101: Rendering options sub-menu

- **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.
- **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.
- **Y-tick label notation** switches between display of **Absolute** y value, and **Percent** value as a function of the highest peak.
- **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.

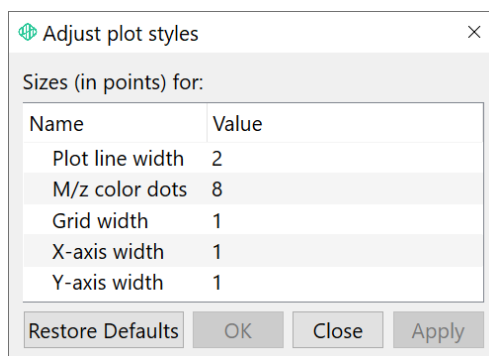


Figure 102: Adjust plot styles

- **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.

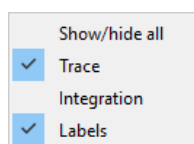


Figure 103: Plot name menu options

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

Trace Plot and Menus





The graphical trace of the chromatogram or electropherogram 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 trace 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 trace peak, first highlight the peak either by clicking on a row in the Peaks table or Alt-click the peak in the Trace 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 Trace 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 Trace when created

The Trace 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:

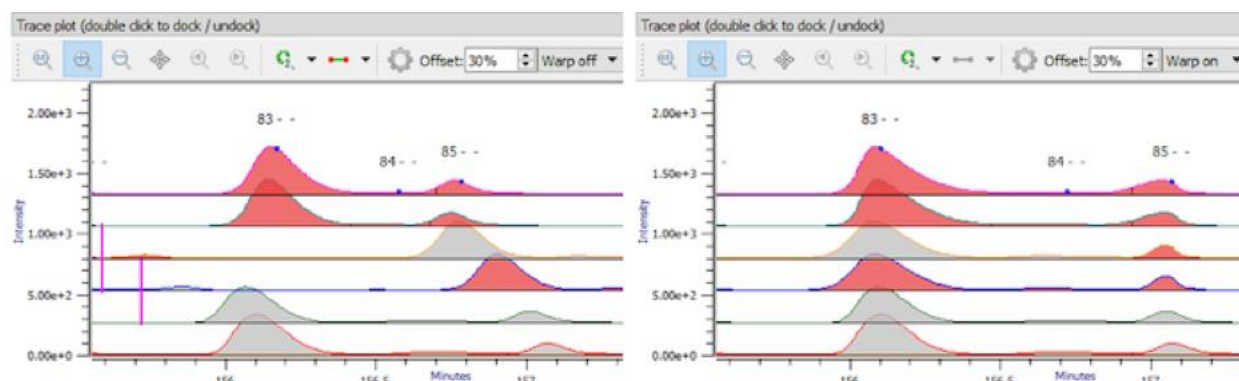


Figure 104: Comparing effects of Warp off and Warp on

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 traces 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 Trace plot view to lock together a particular region(s) that they know are the same molecule and thus constrain the nonlinear. The **Center** button pans the trace 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.
- The slider to the right of the Label button changes the allowable distance of labels from reference points. Moving the slider to the right will display more labels that are further apart. Dotted lines connect the extra

labels to their peaks. Moving the slider to the left will reduce the number and separation of the labels. For example, with slider to the left, there can be label collision:



Figure 105: Minimum label separation can hide or overlap labels

As the slider is moved to the right, more labels are shown, and they are spaced further apart:

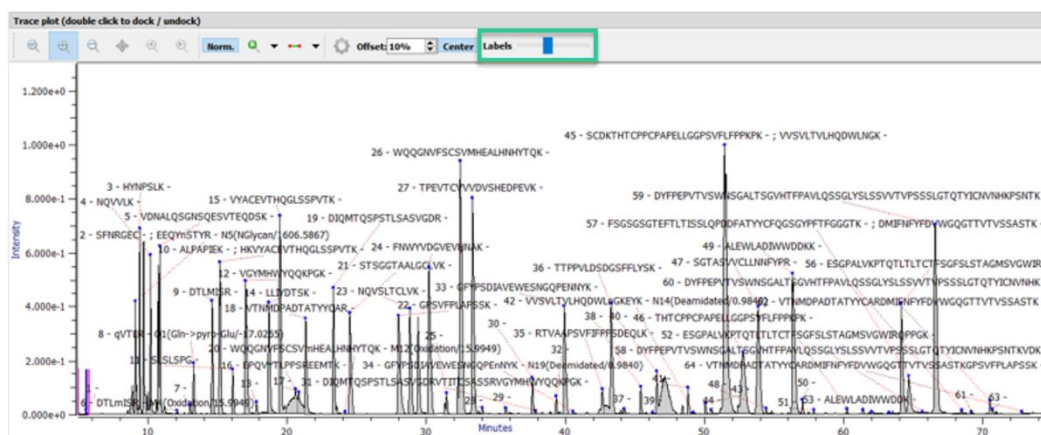


Figure 106: Increasing label separation shows more and clearer labels

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 Trace plot view.

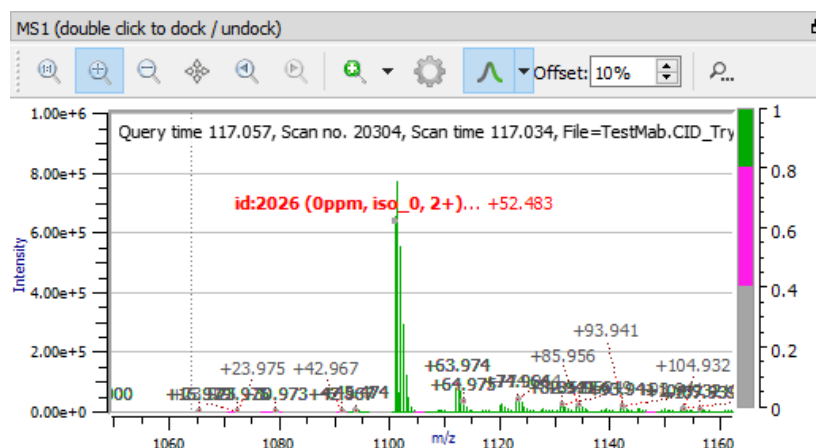




Figure 107: MS1 Plot

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 (1.0-0.8) are colored green. Less correlated peaks are colored magenta. Poorly correlated peaks (0.4-0.0) 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 Duplicates 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

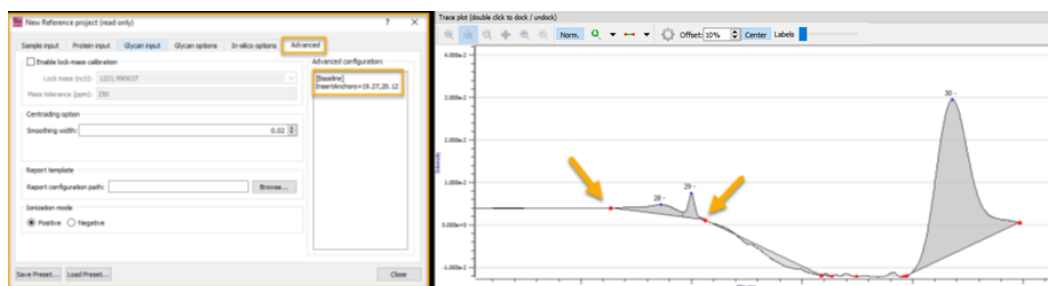


Figure 108: Insert baseline anchors

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 trace.

5. **EnableMS1Sum** performs MS1 scan summing with corresponding noise reduction. This makes it easier to find and assign low level glycans (and peptides) in noisy MS1 spectra:

[Byomap]

EnableMS1Sum = true

TimeCorrFilterValue = 0.1

With scan summing, the displayed MS1 will be the sum of all scans between the two pink bars that mark the peak boundaries. When disabled, the displayed MS1 scan will be a single scan taken at the point indicated by the blue bar (usually the peak apex).

TimeCorrFilterValue controls MS1 background noise reduction. The time correlations correspond to the color coding used in the MS1: well correlated peaks (coefficients > 0.8) are colored green, less well correlated peaks (coefficients between 0.4 and 0.8) are colored magenta and poorly correlated peaks (coefficients below 0.4) are

colored grey. The threshold setting removes any of these poorly correlated ions from the MS1 results that fall below that setting.

It is recommended to first try with `TimeCorrFilterValue = 0.1` to reduce noise levels without over filtering the data. Increasing this value to 0.4 removes most of the gray, poorly correlated peaks. Further increasing this value to 0.6, removes the pink non-correlating peaks. An example of a plot before and after scan summing noise reduction is shown below:

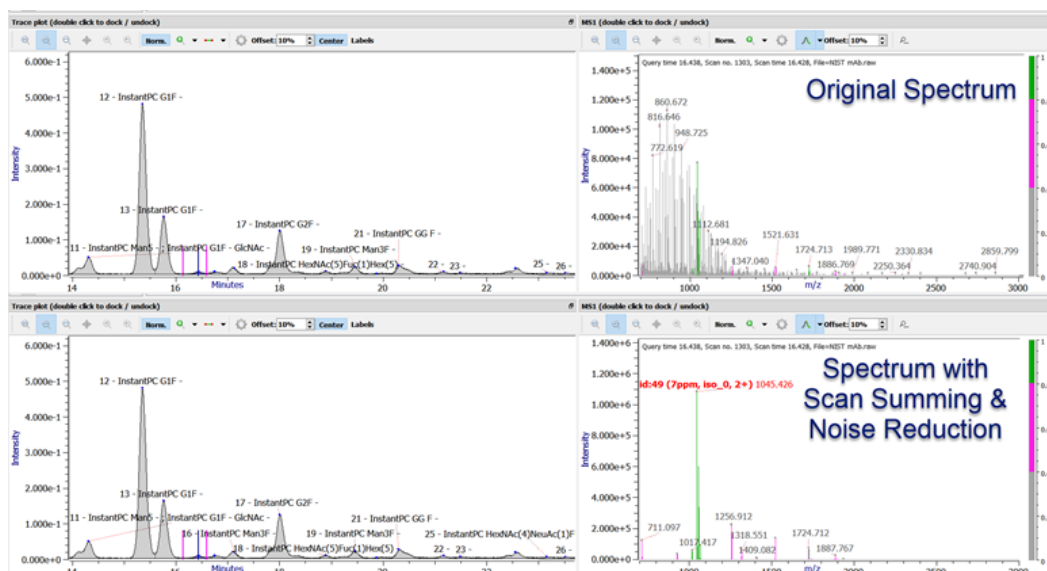


Figure 109: Scan summing for noise reduction

This advanced command also results in scan time range label for the MS1 plot title.

6. **AddDuplicates** control how homologous peptide sequences are annotated in the **Protein coverage** table. Two options allow Protein coverage to be digestion-aware:

```
[ProteinCoverage]
AddDuplicates = 1
```

The options for `AddDuplicates` are:

- 1 - duplication within the same protein
- 2 - duplication across all proteins
- 3 - duplication within the same protein applying protease specificity
- 4 - duplication across all proteins applying protease specificity

Note: if the advanced command is not used, the default is option 4. The protease is specified in the **Digestion** dropdown in the **In-silico** tab during project creation. If no protease is specified (i.e, **Digestion** is blank), the project will be created with `AddDuplicates = 2`.

7. **HideLabelsIfCollision** controls whether labels are hidden in the case of label collisions (the default setting is true):

```
[Plot]
HideLabelsIfCollision = true
```

8. **EnableGlycanCartoons** displays the peak labels as glycan cartoons. The size of the glycan cartoons can be controlled with **GlycanCartoonsSymbolSize** parameter. The default size is 4.

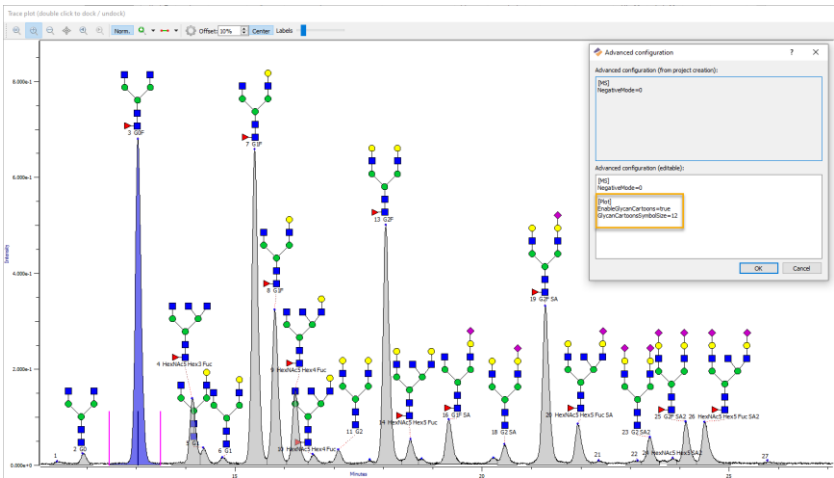


Figure 110: Glycan Cartoons

```
[Plot]
EnableGlycanCartoons=true
GlycanCartoonsSymbolSize=12
```

The above advanced commands will result in glycan cartoons with size set to 12.

The user has the option to choose from alternative representations of selected glycan structures by right clicking on a candidate that has a glycan composition, which will provide the option to **Show alternative glycan structures**.

Candidates							MS2 Insilico Manual	
Valid	Cand. Id	_prot_id	Sequence	Mod. Iame	Glycans	Me P		
<input checked="" type="checkbox"/>	26		~G0	HexNAc(4)Hex(3)		4.47		
<input type="checkbox"/>	38		~HexNAc(4)NeuAc(1)NeuGc(1)Fuc(1)Hex(5)	HexNAc(4)NeuAc(1)NeuGc(1)Fuc(1)Hex(5)		3.09		

Export table to CSV...

Copy selected row(s) to clipboard

Show alternative glycan structures...

Figure 111:Show alternative glycan structures

This will open a **Glycan Structure Selection** window from which the user can choose between a menu of structures that are maintained in a backend library and match the glycan composition. The example below shows that in addition to the G0 structure for HexNAc(4)Hex(3), there are two other bisecting GlcNAc options that the user can display.

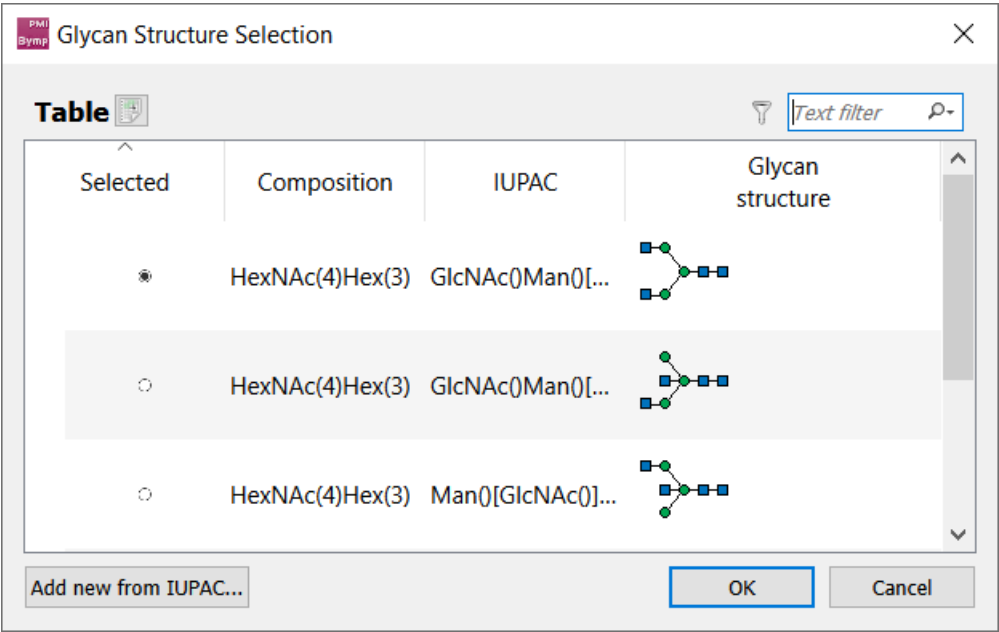


Figure 112:Glycan Structure Selection

If the user selects one of these alternatives and clicks 'OK', the structure shown in the chromatogram and reports will update to the user selection.

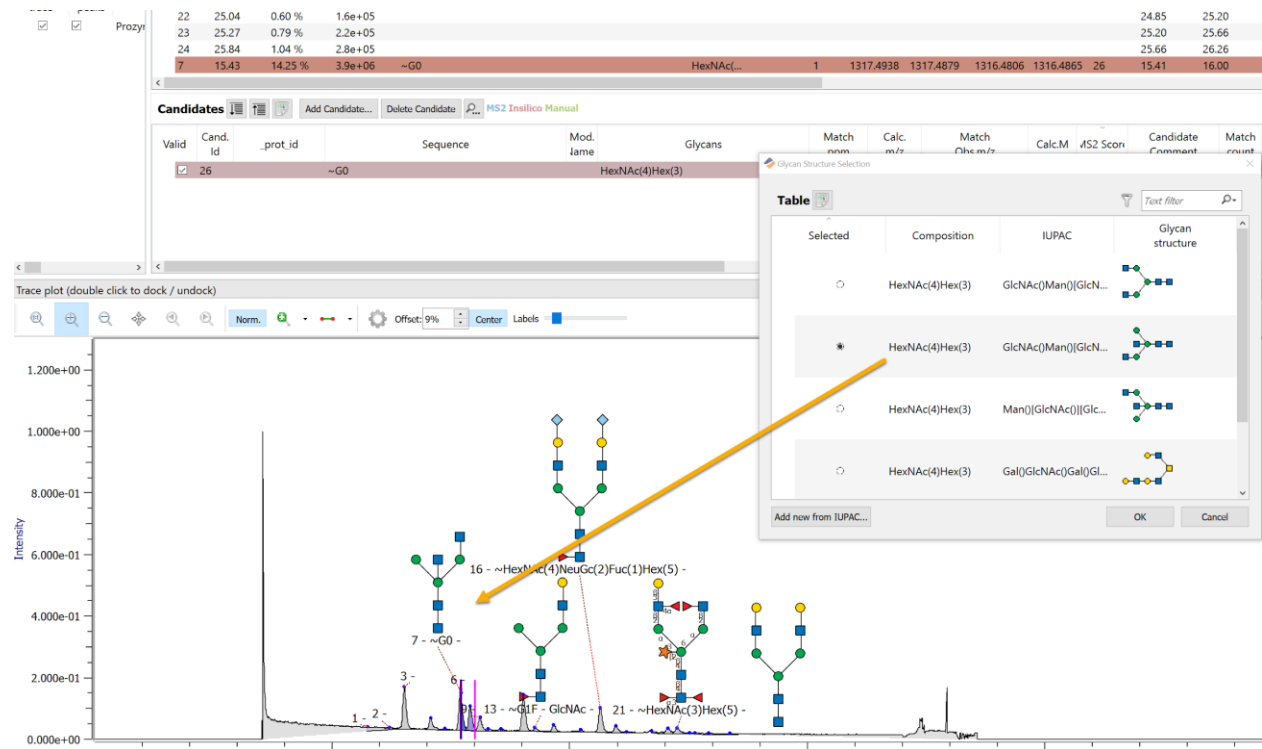


Figure 113: Updated glycan cartoon

Structures included in the table are maintained in a backend library, but a user can add additional structures by clicking **Add new from IUPAC**. This button will open a new window where the user can provide a text representation of the glycan structure in condensed IUPAC format.

Glycan Structure Selection

Selected	Composition	IUPAC	Glycan structure
<input checked="" type="radio"/>	HexNAc(4)Hex(3)	GlcNAc()Man()[...]	
<input type="radio"/>	HexNAc(4)Hex(3)	GlcNAc()Man()[...]	
<input type="radio"/>	HexNAc(4)Hex(3)	Man()[GlcNAc()...	

Add new from IUPAC...

Glycan Structure from IUPAC

Text filter

Draw

Add Cancel

Figure 114: Add new from IUPAC...

For example, if an O-linked type glycan is preferred, the user can input:

Glycan Structure from IUPAC

Gal()GlcNAc()Gal()GlcNAc()[Gal()GlcNAc()]GalNAc()

Draw

Add Cancel

Figure 115: O-linked type glycan input

Varying degrees of specificity are available within this feature. For example, if the user wishes to display complete specificity, they can annotate edges with anomeric and linkage information. Or, alternatively, if more less specificity is needed, brackets denoting ambiguous connectivity can be rendered.

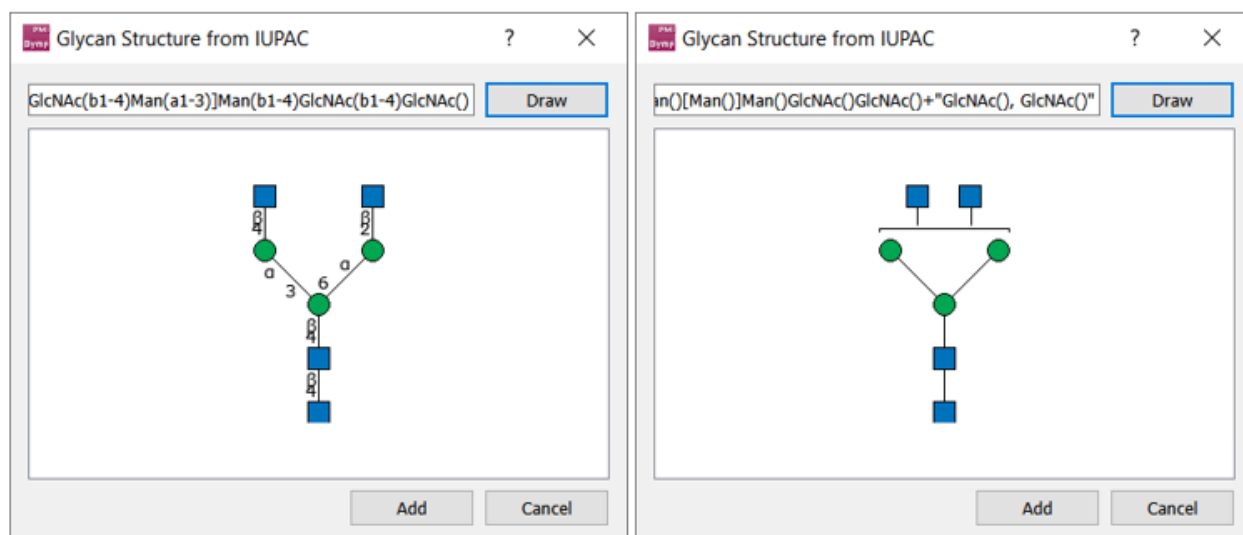
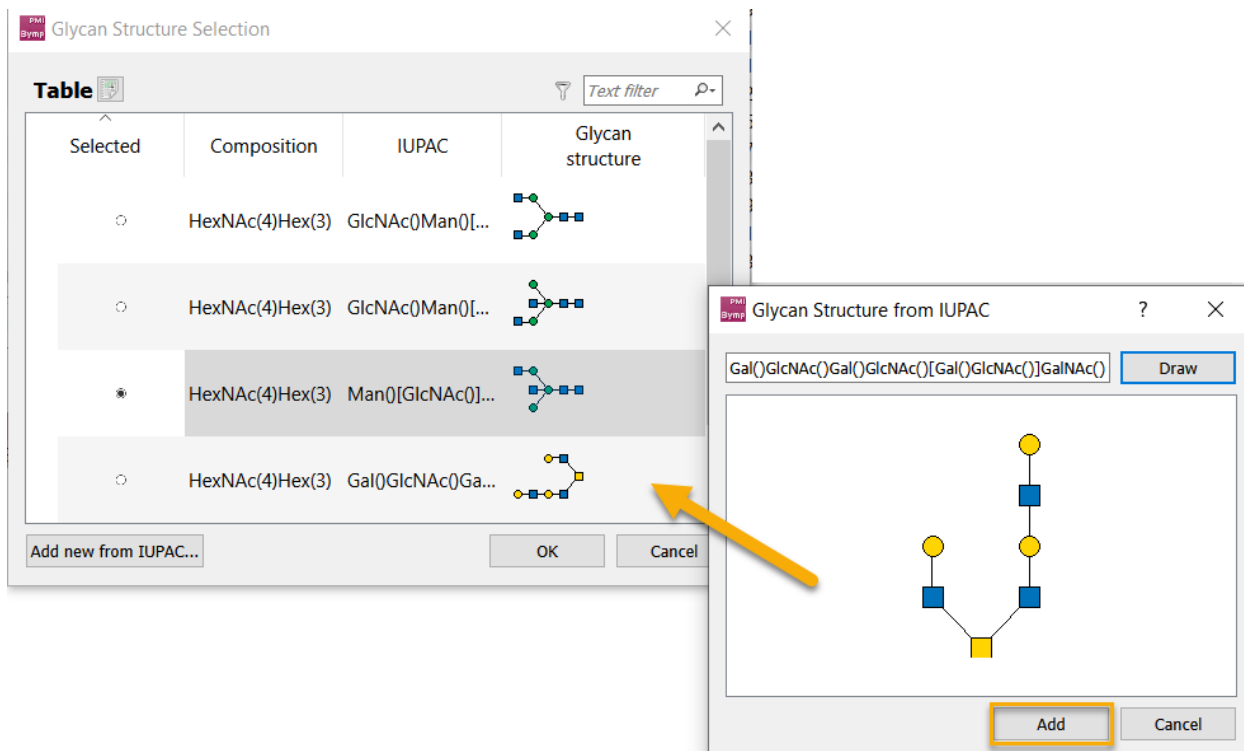


Figure 116: Additional structures

If the user has provided a proper IUPAC text that matches the corresponding composition, a structure preview will be displayed after clicking 'Draw'. Clicking 'Add' will add the new structure to the backend library and it will become available in the 'Glycan Structure Selection' menu.







Selected	Composition	IUPAC	Glycan structure
<input type="radio"/>	HexNAc(4)Hex(3)	GlcNAc()Man()[...]	
<input type="radio"/>	HexNAc(4)Hex(3)	GlcNAc()Man()[...]	
<input checked="" type="radio"/>	HexNAc(4)Hex(3)	Man()[GlcNAc()...]	
<input type="radio"/>	HexNAc(4)Hex(3)	Gal()GlcNAc()Ga...	

Figure 117: Adding IUPAC structure to library

If the user has provided an invalid IUPAC text or one that does not match the composition, a warning message will be displayed.

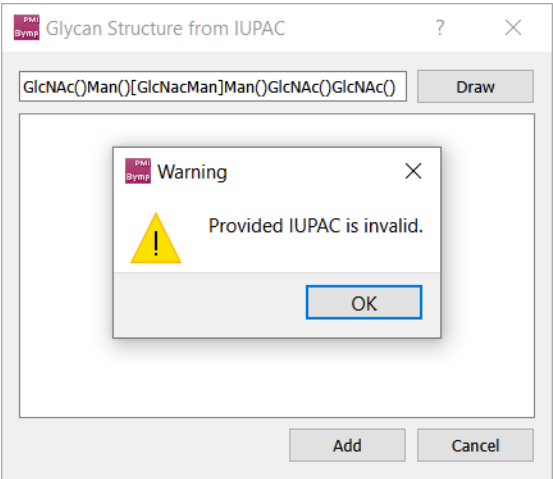


Figure 118: Invalid IUPAC

Online resources, such as [GlyConnect's Structure List](#), are available for the user to find condensed IUPAC text for various structures.

ID	IUPAC	Composition	Core	Glycan Type	GlyYouCan	UniCarb-DB	Cartoon	Biological associations
13	GlcNAc(b1-2)Man(a1-3)[Man(a1-6)]Man(b1-4)GlcNAc(b1-4)GlcNAc+-+HexNAc(b1-7)*	Hex3 HexNAc4	Complex	N-Linked	G66032DV			TAXONOMY (1) PROTEIN (1) SOURCE (0) STRUCTURE (1) COMPOSITION (1) DISEASE (0) REFERENCE (1) SITE (1) PEPTIDE (0)
256	GlcNAc(b1-2)Man(a1-6)[GlcNAc(b1-4)Man(a1-3)]Man(b1-4)GlcNAc(b1-4)GlcNAc	Hex3 HexNAc4	Complex	N-Linked	G42679AH			TAXONOMY (1) PROTEIN (1) SOURCE (1) STRUCTURE (1) COMPOSITION (1) DISEASE (1) REFERENCE (1) SITE (1) PEPTIDE (0)
647	GlcNAc(71-2)Man(a1-3)[GlcNAc(71-2)Man(a1-6)]Man(b1-4)GlcNAc(b1-4)GlcNAc	Hex3 HexNAc4	Complex	N-Linked	G90743AT			TAXONOMY (1) PROTEIN (1) SOURCE (1) STRUCTURE (1) COMPOSITION (1) DISEASE (0) REFERENCE (1) SITE (1) PEPTIDE (0)
670	GalNAc(71-7)GlcNAc(71-7)Man(a1-7)[Man(a1-7)]Man(b1-4)GlcNAc(b1-4)GlcNAc	Hex3 HexNAc4	Complex	N-Linked	G85085RX			TAXONOMY (1) PROTEIN (1) SOURCE (1) STRUCTURE (1) COMPOSITION (1) DISEASE (0) REFERENCE (1) SITE (1) PEPTIDE (0)
1744	GlcNAc(b1-2)[GlcNAc(b1-4)]Man(a1-3)[GlcNAc(b1-2)Man(a1-6)]Man(b1-4)GlcNAc	Hex3 HexNAc4	No-core	N-Linked	G14296HB			TAXONOMY (1) PROTEIN (0) SOURCE (6) STRUCTURE (1) COMPOSITION (1) DISEASE (1)

Figure 119: GlyConnect's Structure List site

However, if the user wishes to construct their own IUPAC text, the following instructions are available.

The condensed IUPAC format encodes the glycan's tree structure in a linear, text format, where the branching events are denoted by square brackets, []. The example below shows two GlcNAc()Man() branches are extended

off of the core Man()GlcNac()GlcNac(). This structure is represented by the IUPAC GlcNac()Man()[GlcNac()Man()]Man()GlcNac()GlcNac().

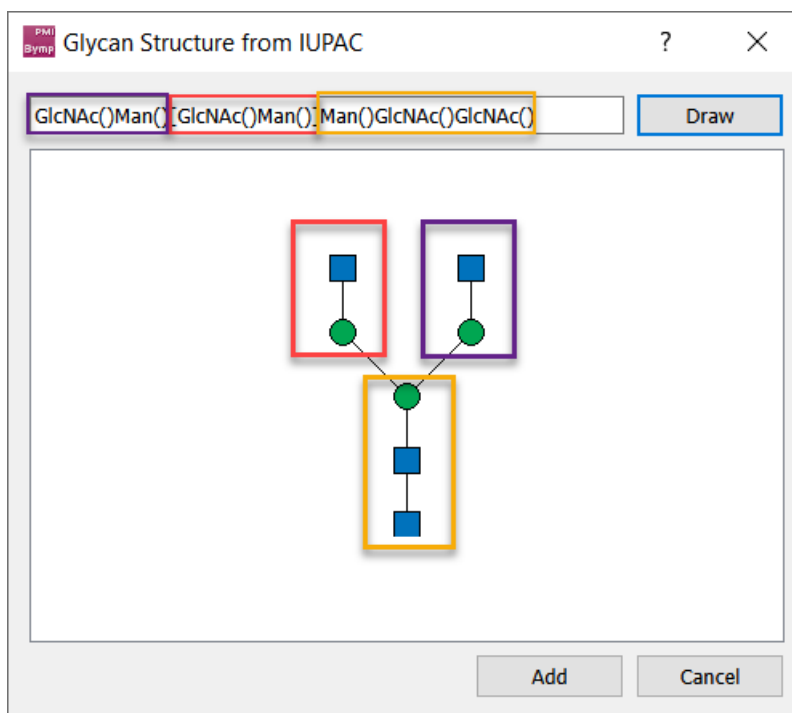


Figure 120: Preview for GlcNac()Man()[GlcNac()Man()]Man()GlcNac()GlcNac()

In the case of a bisecting GlcNAc structure, where three branches are extended off the core mannose, an additional set of brackets can be used:

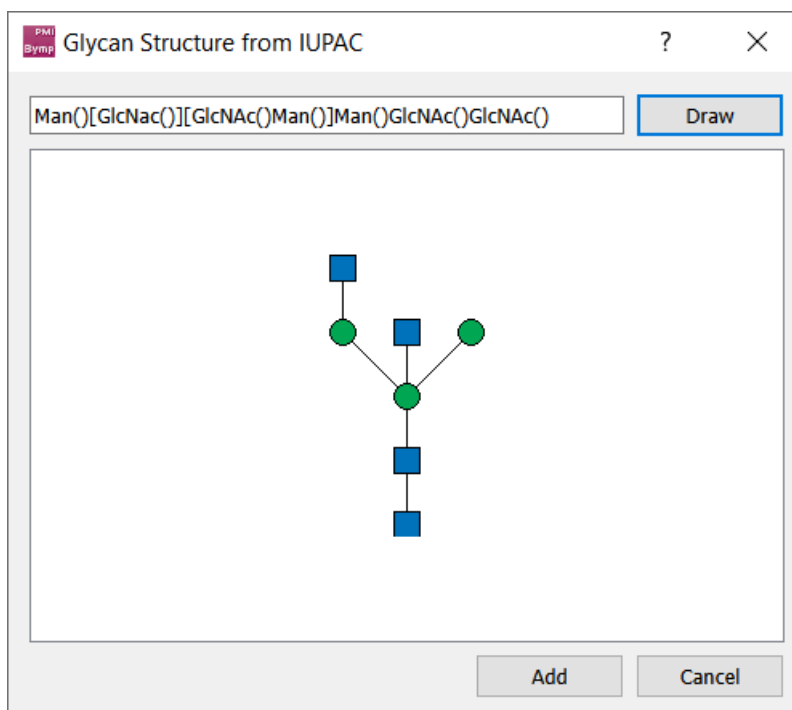


Figure 121: Bisecting GlcNAc structure

Anomeric and linkage annotation is provided in the parenthesis after each monomer in the format Xn-m, where X can be either 'a' (alpha) or 'b' (beta), n is always 1 and so is not rendered, and m is the carbon from which the monomer is extended.

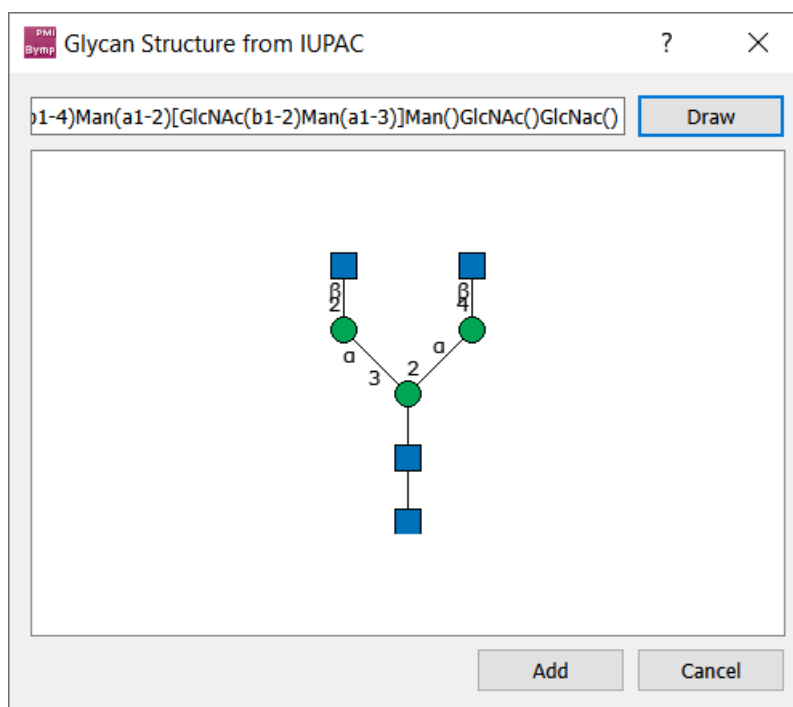


Figure 122: Annotation and linkage information included

For ambiguous connectivity displayed with brackets, the floating extensions are denoted in quotes after a '+' from the main tree structure (i.e., [coreStructure]+[bracketStructure1], [bracketStructure2], etc.)

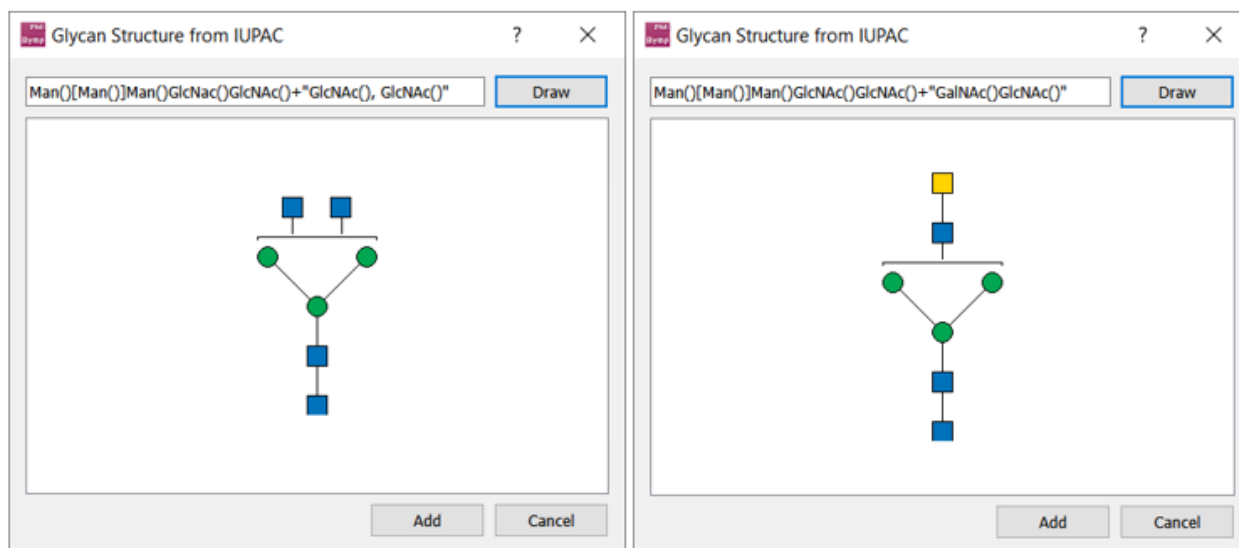


Figure 123: Additional types of connectivity

An option to include the intact cartoon within annotated glycan MS2 spectra is available as of Byos v5.6. It can be found under the same control as the peptide fragment map.



A list of supported monosaccharides that can be used in condensed IUPAC interpretation is shown below:

Name	Composition	IUPAC	Shape	Color
Allose	Hex	All()	Circle	Purple
Altrose	Hex	Alt()	Circle	Pink
Arabinose	Pent	Ara()	Star	Green
Deaminated neuraminic acid	Kdn	Kdn()	Diamond	Green
Deoxyaltrose	dHex	6dAlt()	Triangle	Pink
Deoxygulose	dHex	6dGul()	Triangle	Orange
Deoxytalose	dHex	6dTal()	Triangle	Light blue
Fucose	dHex/Fuc	Fuc()	Triangle	Red
Galactose	Hex	Gal()	Circle	Yellow
Generic deoxyhexose	dHex	dHex()	Triangle	Unfilled
Generic hexose	Hex	Hex()	Circle	Unfilled
Generic N-acetylhexosamine	HexNAc	HexNAc()	Square	Unfilled
Generic pentose	Pent	Pent()	Star	Unfilled
Generic sialic acid	NeuAc	Sia()	Diamond	Red
Glucose	Hex	Glc()	Circle	Blue
Gulose	Hex	Gul()	Circle	Orange
Idose	Hex	Ido()	Circle	Brown
Lyxose	Pent	Lyx()	Star	Yellow
Mannose	Hex	Man()	Circle	Green
N-acetylallosamine	HexNAc	AlINAc()	Square	Purple
N-acetylaltrosamine	HexNAc	AltNAc()	Square	Pink

N-acetylgalactosamine	HexNAc	GalNAc()	Square	Yellow
N-acetylglucosamine	HexNAc	GlcNAc()	Square	Blue
N-acetylgulosamine	HexNAc	GulNAc()	Square	Orange
N-acetylidosamine	HexNAc	IdoNAc()	Square	Brown
N-acetylmannosamine	HexNAc	ManNAc()	Square	Green
N-acetylneuraminic acid	NeuAc	NeuAc()	Diamond	Purple
N-acetylaltosamine	HexNAc	TalNAc()	Square	Light blue
N-glycolylneuraminic acid	NeuGc	NeuGc()	Diamond	Light blue
Neuraminic acid	Neu	Neu()	Diamond	Brown
Quinovose	dHex	Qui()	Triangle	Blue
Rhamnose	dHex	Rha()	Triangle	Green
Ribose	Pent	Rib()	Star	Pink
Talose	Hex	Tal()	Circle	Light blue
Xylose	Pent	Xyl()	Star	Orange

Table 1: Supported monosaccharides for condensed IUPAC

Note: This feature was designed for one glycan/composition/IUPAC per row, so it does not allow editing for multiply glycosylated peptides.

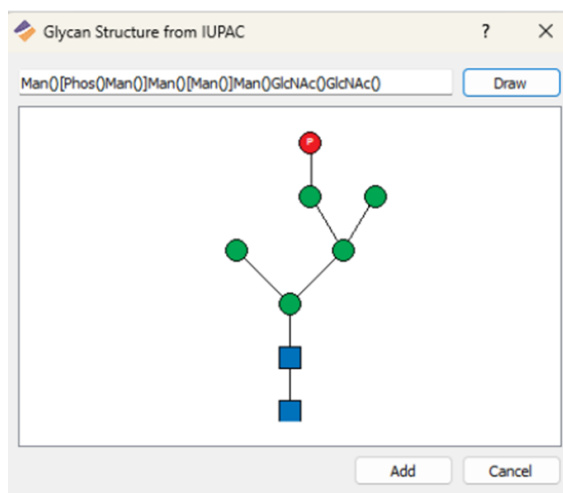
Glycan Name	Composition
AGly	Null
GlcNAc	HexNAc(1)
GlcNAc2	HexNAc(2)
GlcNAc F	HexNAc(1)Fuc(1)
GlcNAc2 F	HexNAc(2)Fuc(1)
Man1	HexNAc(2)Hex(1)
Man2	HexNAc(2)Hex(2)
Man2F	HexNAc(2)Hex(2)Fuc(1)
Man3	HexNAc(2)Hex(3)
Man3F	HexNAc(2)Hex(3)Fuc(1)
Man4	HexNAc(2)Hex(4)
Man5	HexNAc(2)Hex(5)
Man5F	HexNAc(2)Hex(5)Fuc(1)
Man6	HexNAc(2)Hex(6)
Man7	HexNAc(2)Hex(7)
Man8	HexNAc(2)Hex(8)
Man9	HexNAc(2)Hex(9)
Man3 +GlcNAc	HexNAc(3)Hex(3)
G0F - arm	HexNAc(3)Hex(2)Fuc(1)
G0 -GlcNAc	HexNAc(3)Hex(3)
G0 - arm	HexNAc(3)Hex(2)
G0F -GlcNAc	HexNAc(3)Hex(3)Fuc(1)
G1 -GlcNAc	HexNAc(3)Hex(4)
G1F -GlcNAc	HexNAc(3)Hex(4)Fuc(1)
G0	HexNAc(4)Hex(3)
G0F	HexNAc(4)Hex(3)Fuc(1)

G1	HexNAc(4)Hex(4)
G1F	HexNAc(4)Hex(4)Fuc(1)
G2	HexNAc(4)Hex(5)
G2F	HexNAc(4)Hex(5)Fuc(1)
G1F SA	HexNAc(4)Hex(4)Fuc(1)NeuAc(1)
G2 SA	HexNAc(4)Hex(5)NeuAc(1)
G2F SA	HexNAc(4)Hex(5)Fuc(1)NeuAc(1)
G2 SA2	HexNAc(4)Hex(5)NeuAc(2)
G2F SA2	HexNAc(4)Hex(5)Fuc(1)NeuAc(2)
G2 SA1 NA1	HexNAc(4)Hex(5)NeuAc(1)NeuGc(1)
G2F SA1 NA1	HexNAc(4)Hex(5)Fuc(1)NeuAc(1)NeuGc(1)
G2 NA	HexNAc(4)Hex(5)NeuGc(1)
G2 NA2	HexNAc(4)Hex(5)NeuGc(2)
G2F NA2	HexNAc(4)Hex(5)Fuc(1)NeuGc(2)
G2F NA	HexNAc(4)Hex(5)Fuc(1)NeuGc(1)

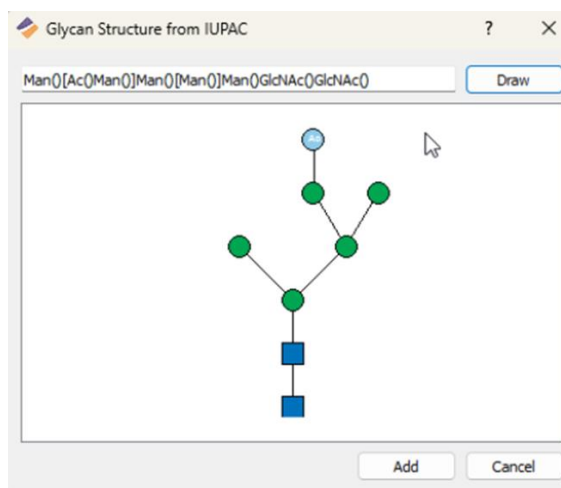
Table 2: List of Common Glycans and their Compositions

Also added in v5.6 is the ability to include relevant modifications into the glycan structure. These include phosphorylation, sulfation, acetylation, N-acetylation, and the additions of phosphocholine and pyruvate. They are denoted in IUPAC string as Phos(), Sulfo(), Ac(), NAc(), Pyr(), and PC() and represented by red, brown, blue (Ac and Nac), orange, and pink circles, respectively, with mod acronyms written in white lettering.

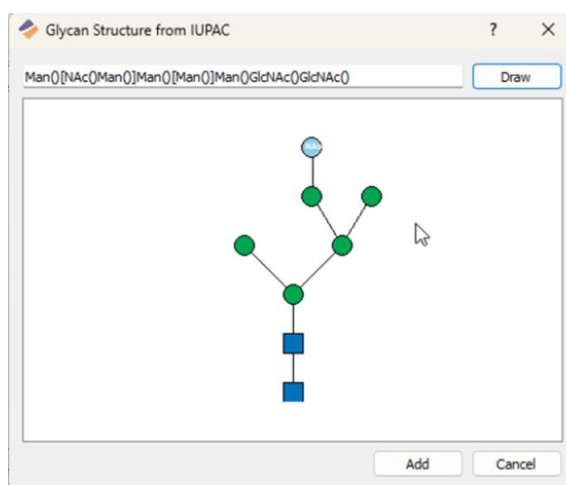
Phospho:



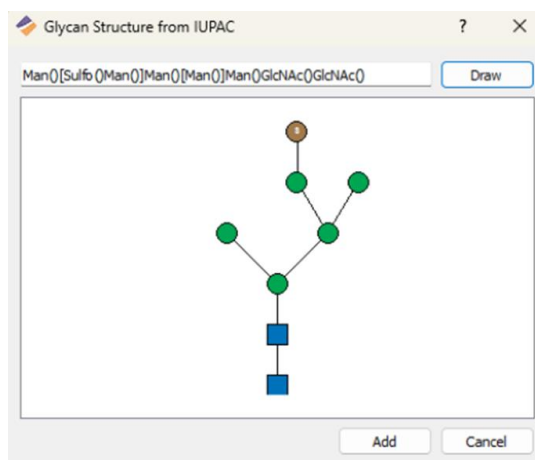
Acetylation:



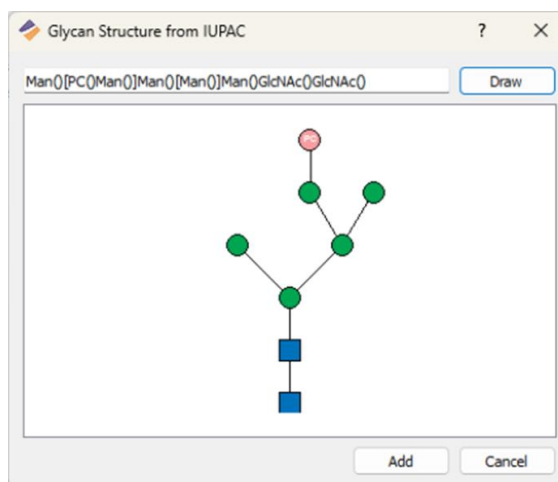
N-Acetylation:



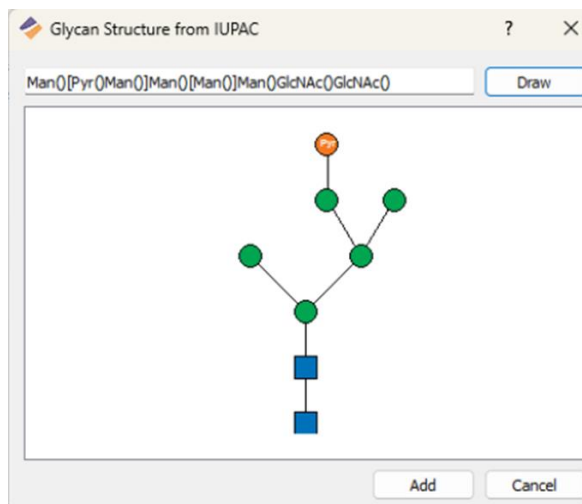
Sulfation:



Phosphocholine:



Pyruvate:



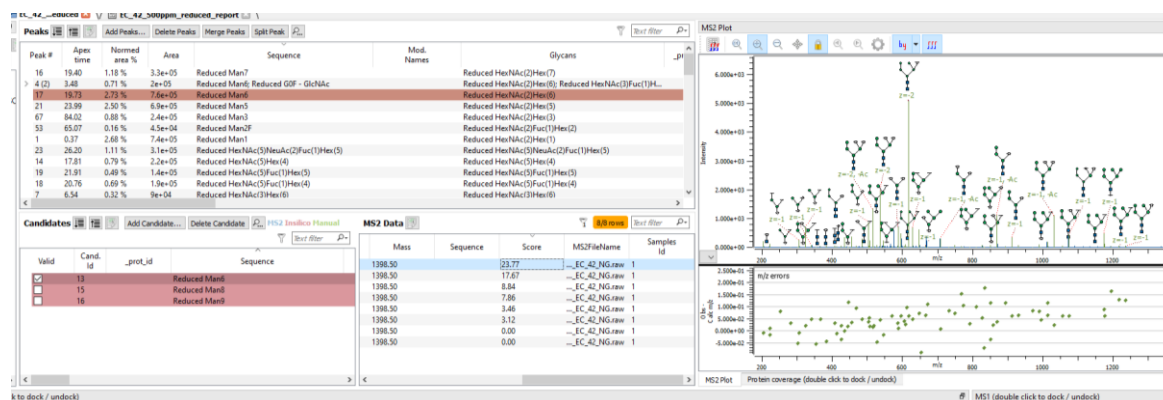
9. Released Glycan workflows currently match glycan compositions to elution peaks by accurate mass matching at the MS1 level. Tandem MS of released glycans can provide further confidence to peak annotations, as well as evidence of a glycan's higher order structure. We now provide the option to annotate and score tandem MS spectra to aid in their characterization. This functionality is enabled with the advanced commands listed below:

```
[Byomap]
EnableMS2=true
Ms2Tolerance=0.2 da

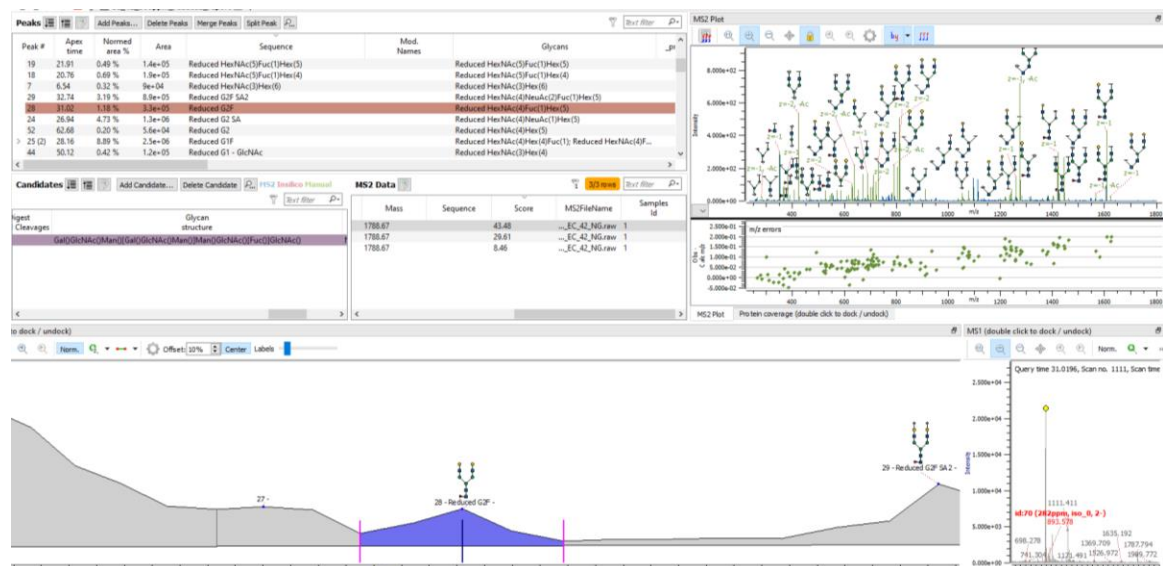
[Plot]
EnableGlycanCartoons=true
GlycanCartoonsSymbolSize=4
```

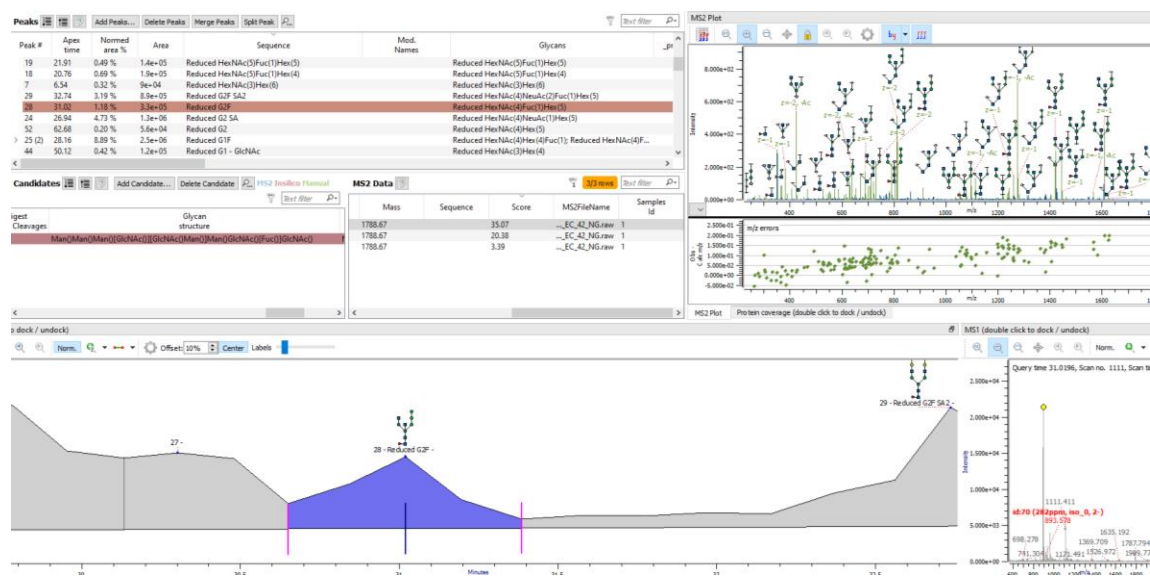
The value of the **Ms2Tolerance** should be in line with the mass accuracy of the mass analyzer used.

If the input data contains tandem MS spectra, the user will be provided a list of scans matching the candidate's intact mass within the time limits of the peak in the 'MS2 Data' table. Selection of a row in this table will then display the spectrum in the 'MS2 Plot' window, annotated with glycan fragments in SNFG format.



The B, C, Y, and Z-type candidate fragments ions originating from up to 3 cleavage events, as well as associated acetyl losses, are generated from the IUPAC glycan structure associated with candidate. This allows the user to view the differential coverage of the tandem MS with different glycan structures by choosing between different structure options. A probability score based on a cumulative distribution function is provided to help in the assignment. Below is an example where the MS spectra contains more evidence for G2F (score = 43.48) than a bisecting GlcNAc structure with the same composition (score = 35.07).





10. Charge and monoisotope checks have been added to glycan fragment annotation. The implementation requires profile scan data; therefore, if a user has centroid data, this check should be turned off with the following advanced command:

```
[MS2]
ChargeDetermination=false
```