MsMetrix

MsXelerator Tutorial:

Reactive Metabolite Detection using High Resolution Isotope Pattern Filtering (IPF II) and High Resolution Post Processing

1. **Detection of Reactive Metabolites of labeled GSH adducts using High Resolution Isotope Pattern Filtering (IPF II)**
2. **Removal of False Positives by applying High Resolution Post-Processing Filters**
3. **Metabolite ID and Detection of Cl containing compounds**
4. **Confirmation of IPF II results by Neutral Loss checking (IPF III)**

Introduction:

This document describes the Isotope Pattern Filtering II algorithm (IPF II) for detection of reactive metabolites trapped by **unlabeled/labeled** GSH, KCN or MOA. IPF II is a newly developed algorithm that operates directly on high resolution mass spectrometry data (Full Scans). Although the algorithms can be run at any mass accuracy, best results are obtained on high resolution instruments like the Orbitrap or Q-TOF instruments.

The main difference between our previously implemented algorithm IPF-I and IPF-II, is that the new algorithm directly uses the raw high resolution mass data (MS Mode), while IPF-I started by extraction of nominal ion currents (EIC Mode). Both algorithms can be used in a wide application range, which means that **any isotope pattern pair** can be defined and searched for. IPF-II currently includes a number of predefined searches:

Unlabeled/Labeled GSH,	mass difference 3.00374, ratio 1:1
Unlabeled/Labeled KCN,	mass difference 2.0040, ratio 1:1
Unlabeled/Labeled MOA,	mass difference 3.00190, ratio 1:1 (Methoxyamine, H_2N-O-CH_3 / H_2N-O-CD_3)
Detection of charge 2+ ions,	mass difference 0.5070, ratio flexible
Detection of charge 3+ ions,	mass difference 0.3345, ratio flexible
Detection of Chlorine,	mass difference 1.9970, ratio 3:1 (single) or 9:6 (double chlorine)

IPF I and II are very flexible; other isotope patterns can be created by setting the correct mass difference and ratios. IPF-II is a fairly simple but highly sensitive algorithm. It is guaranteed that I will not miss any hit when sensitive settings are used. However, the disadvantage (compared to IPF-I) is that it will also find a large number of false positives. These false positives will have to be removed by applying dedicated high resolution Post-Processing Filters. The Post-Processing algorithm is newly developed and contains about 12 powerful filters that can be run individually or automatically in series. For any of the available filters, the user has full control and can select to remove or mark the outcome of any filter. Filters are easily set and run from the Post-Processing GUI or directly from the IPeaks menu. Selection from the menu gives you a choice of two additional filters (m/z purity check and a Mass Defect Filter check).

The post-processing filters can be used on results from IPF-I, IPF-II or IPF III. In the case of IPF-I and IPF III you will probably end up with no that many false positives. This is caused by the fact that the engine of the IPF-I already uses one of the more powerful filters during the analysis (isotopes should co-elute or have a similar elution shape).

The IPeaks module of MsXelerator also contains a third algorithm to detect reactive metabolites. This algorithm is called IPF III and runs on MS^2 data. It can be used to directly search the MS^2 data (triggered by specified mass tag) on specific neutral losses (NL). Although the IPF III algorithm can be used as an individual algorithm, we use it in this tutorial to check if the most important hits from IPF II have indeed a neutral loss of 129 (loss of gamma glutamate from GSH) and to see if additional unique metabolites can be detected.

Figure 1 shows the overall workflow in reactive metabolite profiling studies using LC/MSMS (courtesy: Zhu and Ruan, ASMS 2008). An overview of IPF-I, IPF II and IPF-III is shown in Figure 2 and some of the Post Processing Filters are displayed in Figure 3. The workflows are shortly explained.

GHS Workflow:

Typically, 1:1 ratios of labeled/non-labeled GSH are used in rat liver microsomal incubation studies. Full scan data are acquired on a high resolution instrument. A data dependent MS^2 method is generally used (mass tag 3) to acquire MS^2 spectra for detection of possible hits in combination with Neutral Loss MS^3 scanning. On the Orbitrap one may setup MS^2 acquisition for both the light and heavy isotope (**paired MS^2 scans**) or use the light isotope only. The method can optionally be extended to acquire MS^3 spectra if specified neutral losses are detected (e.g. loss of gamma glutamate; m/z 129). IPF I and IPF II operate on full scan MS data (EIC mode or MS mode). They both use criteria based on the accurate mass difference between the light and heavy isotope. Furthermore, the ratio of mixing should be reflected in the MS data scans.

The IPF III algorithm uses MS^2 data. **Precursor ions having specified neutral losses (mass tag already known to be 3)** can be checked on the same criteria (delta m/z and ratio). Also shown in figure 1 is the use of the Post Processing Filters to be run at the end of each IPF analysis.

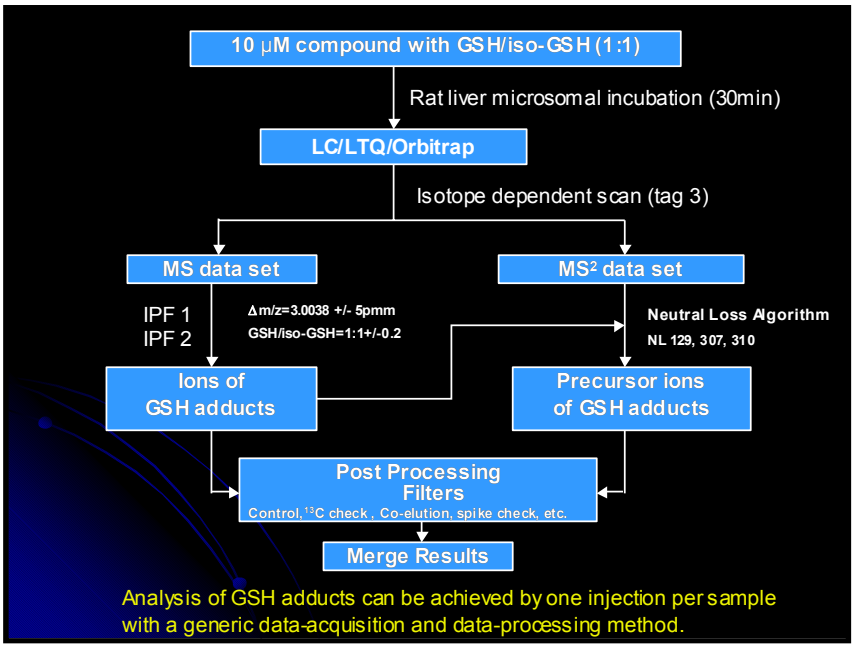


Figure 1: Flowchart of Analysis of GSH Adducts

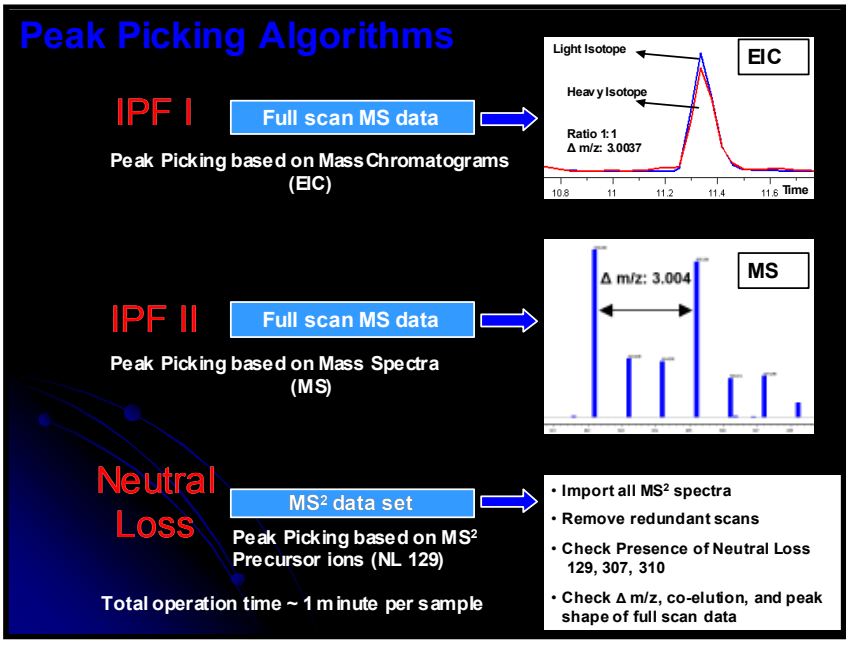


Figure 2: Overview of IPF I, IPF II and IPF III

IPF I is graphically shown in the top of Figure 2. Plotted are two Extracted Ion Currents (EIC). The accurate mass difference between the light and heavy isotope should be 3.0037, their ratio should be 1:1 +/- 0.3 and both EICs should co-elute! Normally, positive hits from IPF I should consist of a number of scans to define a chromatographic peak of a certain width. Single scan hits are not considered being real peaks.

IPF II is graphically shown in the middle of Figure 2. All ions in all full scan raw MS data are searched for a mass difference of 3.0037 and a ratio of 1:1 +/- 0.3 (both parameters and the margin on ratio can be specified). Hits from neighboring scans are compiled into one peak. The ratio can be based on the EIC areas or calculated in the top of the peak (based on a single scan).
In the example in Figure 2, IPF II will find the hit marked with the arrow, but it will also detect the ^{13}C isotopes as being a positive hit. In the case of a chlorine containing adduct, there will even be one more hit related to both ^{37}Cl isotopes. Other false positives often observed are: noisy co-eluting spikes that by chance have the proper mass difference, non co-eluting peaks, peaks also present in the control sample, charge 2+ ions and non-monoisotopic peaks. The Post-Processing Filter algorithm will take care of all these false positives.

IPF III is shown at the bottom of figure 2. As the MS^2 acquisition was triggered using a mass tag of 3, we already know that the correct nominal mass difference is present. IPF III reads all MS^2 spectra and precursor ions. Next it is checked that the precursor ions have the correct accurate mass difference. Also, all redundant MS^2 scans relating to the same peak are removed. Finally it is checked that the MS^2 spectrum contains the correct specified Neutral Loss. A small table of results will be presented. The Post Processing Filters can be used to remove false hits, similar to IPF II.

False Positives:
Figure 3 shows three examples of frequently observed false positives. These are all handled by the Post Processing Filter algorithm. The top shows the overlay of two Extracted Ion Currents (correct m/z difference and ratio) at a resolution of +/- 0.01 Da. It is a very small signal and only consists of one single scan. As we expect real peaks to be more than one scan wide, we will have to remove this hit. At very low detection levels, often many spikes will be found. The Post Processing Filter routine offers the possibility to check on spikes of 1 and 2 scans wide.
Attention: if your chromatography or data acquisition settings result in very narrow chromatographic peaks (2-3 scans wide), you should not use the spike detection filter. Please be aware that discriminating very small narrow peaks from noise is not easy. It is advised to set data acquisition parameters to give peaks being 5 scans wide or more (measured near the baseline). The second example of a false positive hit relates to a peak that is also present in the control sample. The example shows two EICs of a light and heavy isotope pair together with the light isotope of the control sample, plotted in mirror mode. The false positive should be removed from the result table.
The third example from Figure 3 shows that ^{13}C isotopes also will end up in the hit list. These should be removed because we are only interested in mono-isotopic hits to be kept in the result table.

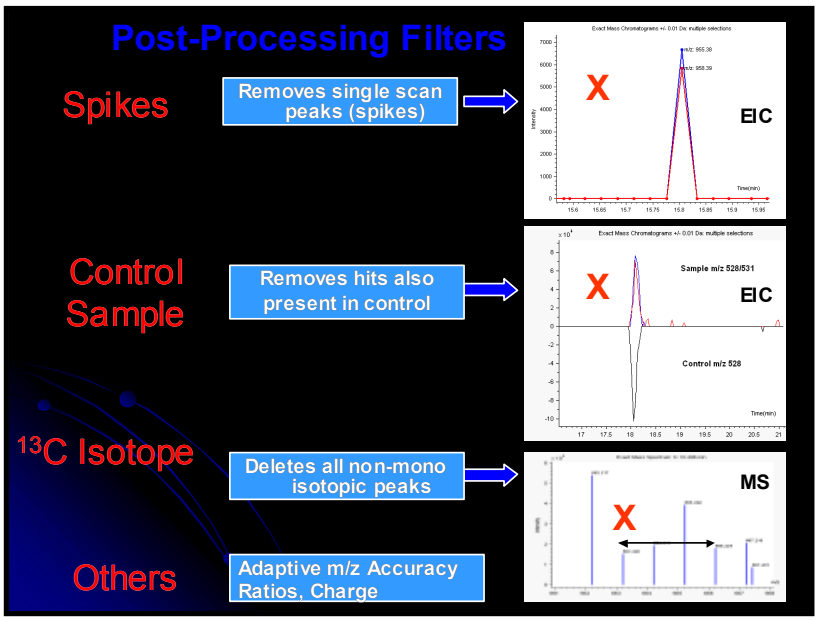


Figure 3: Examples of False Positive Hits: Spikes, peaks present in control sample and ^{13}C Isotopes

All algorithms typically run in less than 1 minute and are of course also capable to detect other Isotopic Patterns (Cl, Br etc.).

Tutorial example using Clozapine:

The example files used in this tutorial can be obtained from MsMetrix on request. The samples are measured on a Thermo Finnigan LTQ Orbitrap. Data acquisition settings were used as described in Figure 1 and shown below. The files were kindly provided by Dr. Mingshe Zhu, Bristol-Meyers Squibb, Princeton, New Jersey, USA.

Sample File description:

Name:	Clozapine-21.raw
Size:	12 Mb
Retention time range:	0.0 – 26 minutes
Mass Range Full Scan:	200 – 1000 Da
Mass Type:	Centroided
Scan events (3):	(1) ESI Full Scan, res=30.000, mass range 200-1000, (2): dep. MS^2 most intense ion from (1), (3): Dep. Neutral Loss from (2), NL in top 3. Isotopic data dependence enabled; mass tag =3.0038 +/- 0.3, expected ratio 0.85. Neutral Loss mass list 129.04
Number of Full Scans:	1191
Number of MS^2 Scans:	584
Number of MS^3 Scans:	104

Control Sample File description:

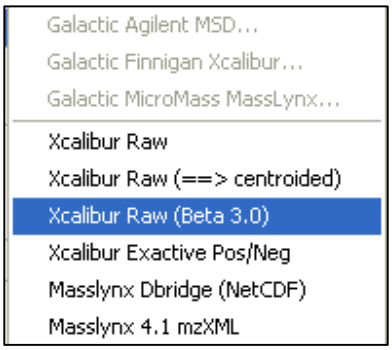
Name:	Control-21.raw

General Information:

MH^+ of Clozapine (calculated.):	327.1371
MH^+ of Clozapine GSH adduct (calculated):	632.2053
Mass difference between labeled and non-labeled GSH:	3.00374
Ratio of mixing (non-labeled : labeled)	1.0 : 0.85 (Ratio = 1.17)

Importing Sample and Control - Exploring the Sample in the Browser:

Start MsXelerator. The raw files can be imported simultaneously if they are located in the same folder. To import both files, select from the Menu: File > Import > Xcalibur (beta 3.0). The beta 3.0 convertor can also read the MS^2 scans needed in cases that IPF III will be used.



Important: the IPF II algorithm is based on centroided mass spectra. In case you have profile data, you should use the Xcalibur Raw (==> centroided) convertor.

When selecting multiple samples it is convenient to click the sample first, then press Ctrl and select the control sample. In this way the last selected file will be loaded into the Browser. Conversion will take about 30 seconds. Importing and conversion only has to be done once. The next time you can open the file using *Open* or select its name from the file history list.

The Browser can be used to explore a single sample and consists of a "summary window "in which the TIC or the BPC is displayed (Figure 4, bottom). In the top window the mass spectrum for the selected retention time is plotted. In the middle window the extracted EIC (Mass Chromatogram) will be plotted. The resolution at which the EIC is extracted can be set automatically by the user, the default value is 0.035. If Exact EIC plotting is not selected, nominal EICs will be plotted. Clicking on any position in the TIC or EIC window will extract the mass spectrum at this retention time. Automatically, the

EIC with the largest intensity from the mass spectrum will be extracted and plotted to the middle window. The Browser contains many keyboard shortcut keys to make zooming/scaling and other operations easy. **Press the *"h"* button to display the active keys.** For a general overview of the Browser, see the MsXelerator manual.

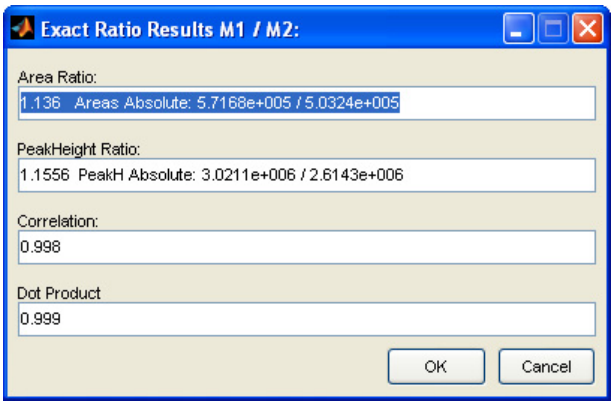
Some suggestions:

A good way to start is to enter the expected m/z value of the Clozapine GSH adduct in the *Select m/z Box*. In this case enter the value 632.205 (327.14+305.06) in the *Select m/z Box* on the right (marked by the circle in Figure 4). This action will extract and plot the accurate EIC 632.205 +/- 0.035 to the middle window.

Next, you can make the scans visible. Zoom in on the EIC and click the ***mark scans*** *icon*, found on the Icon Toolbar. As can be seen, this peak has a width near the baseline of about 4-5 scans. Remember this width. Click on the EIC to extract the MS spectrum at this position to the top window. Also be sure to turn on *AutoZoom MS* functionality, this will automatically zoom in on the region of interest. The number of labels and zoom range can all be set from the Options Menu. Also be sure that *exact MS* plotting is active. See icon on the right.

You can use the *Scan Slider*, located at the top right area of the MS spectrum to plot mass spectra at any position. This is nice if you want to explore MS spectra at other positions near the top of the peak. You can of course also click on any position in the EIC to extract the MS spectrum at the selected retention time.

From the MS spectrum displayed in the top window the characteristic labeling pattern of labeled and unlabeled GSH is recognized. The second isotope peak at m/z 635.2094 is lower in intensity compared to the light isotope peak. You can overlay the second accurate EIC by holding down the *CtrL key* followed by clicking the mouse near the second m/z peak in the top window.



To determine the ratio between both EICs select: *Menu > Task > Determine Ratio from Plot*. A crosshair cursor will be shown. Click to the left of the peak in the EIC window. Next, click on the right of the peak. You will be asked if ratios should be calculated as M2/M1 or M1/M2. M1 will be related to the first plotted EIC. Select M1/M2. The integration overview will be displayed as shown on the right. The calculated ratio based on area is 1.136 which is close to the theoretical expected value of 1.17 (1/0.85).
The integration results also show how well both EICs co-elute. This is determined by the Correlation Coefficient (CC) calculated between both EICs. A perfectly co-eluting pair, having identical peak shapes, has a CC value of 1.0. The CC is not intensity dependant. CC values less than 0.7 are suspicious or relate to noisy EICs.

It is advised to write down the absolute peak height of the first isotope, in this case 3.0211e+006. The intensity can be an important filter to remove very small hits. For instance, one can restrict the search to hits 1% in size compared to parent's adduct. One may also set an intensity restriction on the peak height of the parent Clozapine instead of Clozapine+GSH. The peak height intensity of Clozapine (327.1371) was found to be 2.44e+007, which means that the adduct peak is about 12% compared to the parent.

To plot the EICs at other resolutions, enter a different value in the *Res. Edit field*. The Browser uses a default value of 0.035 Da. The IPF II algorithm will use a default mass accuracy of 0.005. The Browser can be used to get a feeling about the accuracy that can be used. A too low value results in chromatographic peaks missing parts of the peak. The plot is immediately updated after selecting a different resolution value. These features are also available in IPeaks and MPeaks.

Basically, this is the most important information to be extracted from the Browser at this point (estimate of scan width, the peak area or peak height estimate, the ratio between light and heavy isotope and the accuracy to be used for extraction of accurate EICs). Of course you can explore other peaks in the Browser as well.

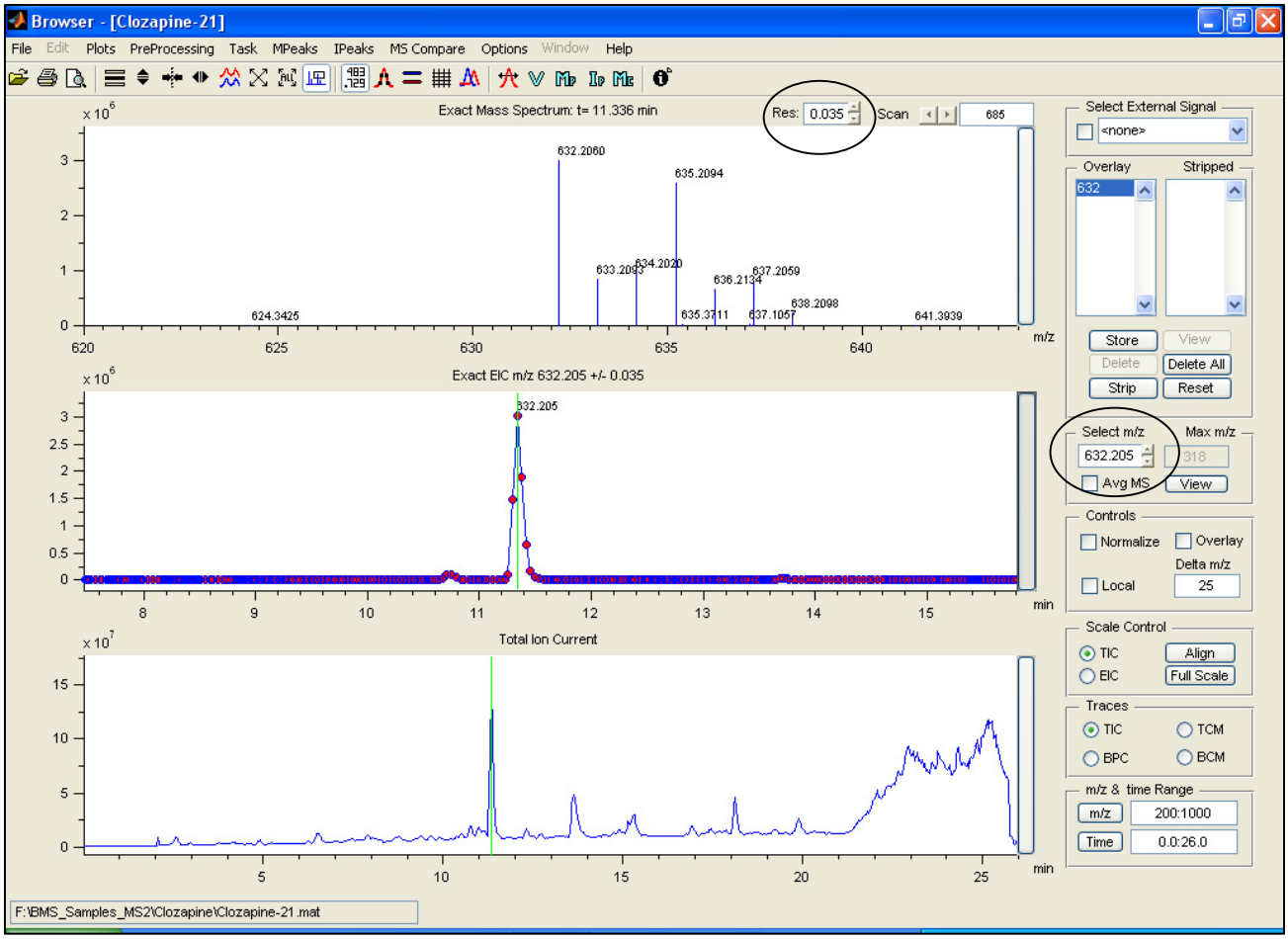


Figure 4: Browser Overview. Mass Spectra are plotted in exact mode, with auto zooming activated. The EIC plot was marked to make all scans visible. The m/z value of the Parent+GSH (m/z 632.205) was directly entered in the Select m/z Edit Box.

Running the IPF II algorithm:

To run IPF II, start IPeaks from the Browser by clicking on the ***IP icon*** or select: *Menu > IPeaks > Start IPeaks*. IPeaks will open and start with a window displaying the Total Ion Current.

Select from the menu: ***Isotopes > High Res. Isotope Pattern Filtering IPF2 (MS).*** You will get a warning that this application applies to Centroided Data only. Click Continue to proceed. The IPF II GUI will be displayed, as shown in Figure 5. The application will remember settings from the previous run. If you want to start from the beginning, press ***Load Defaults***. This will load default GSH settings using the full time and mass ranges.

The following options/parameters should be entered:

1. **Algorithm:** please select the first algorithm. The second algorithm was developed for very clean data. The Full Search algorithm searches every mass spectrum scan for ions having the correct mass difference and ratio.
2. **Mass Range:** you can select a limited mass range when needed. Often many false positives are found in the lower mass range; 200-300 Da. If you want to speed up the analysis and decrease the number of false positives you may want to restrict the search to a selected region. E.g., if the expected adduct of the parent has an m/z value of 632, you could use the range 332 – 932 Da (632 +/- 300 Da).
3. **Time Range:** specifies the time range to be used. The algorithm will start by using the TIC time limits displayed in IPeaks. To use the full range, press *Full*. To use the limits from the previous run, press *Prev*.

4. **Isotope Settings:** specify the delta m/z value to be used and the accuracy (margin). For Orbitrap data specify an accuracy of 0.001 - 0.005 Da. It is proposed to use an accuracy of 0.05 Da for Q-TOF data.
5. **Ratio Low – Ratio High:** enter the low and high ratio limits based on the mixing ratio used. The ratio is always calculated as Intensity Light / Intensity Heavy. Careful, ratios can deviate substantially for very low intensity ions. You might want to consider starting with wider limits (e.g. 1 +/- 0.4 => 0.6 and 1.4).
6. **Delta m/z List:** select a pre-defined labeling method from the list. After selecting a method, the proper settings will be used for the parameters: Delta m/z, ratio low and ratio high. Currently the following pre-defined choices are available:
   0.33 (3+), search for ions having charge 3+, Δ m/z: 0.33450, ratio 1, ratio high 10
   0.50 (2+), search for ions having charge 2+, Δ m/z: 0.50170, ratio 1, ratio high 10
   1.00 (1+), search for ions having charge 1+, Δ m/z: 1.00340, ratio low 1, ratio high 10
   1.99, search for peaks containing a single chlorine (Δ m/z: 1.997), ratio low 1.5, ratio high 6 (theoretically 3)
   2.00, KCN labeling (Δ m/z: 2.0040, ^{13}C ^{15}N), ratio low 0.70, ratio high 1.30
   3.00, GSH labeling (Δ m/z: 3.0037, $^{13}C_2$ ^{15}N) , ratio low 0.70, ratio high 1.30
   3.00, MOA labeling (Δ m/z: 3.019, D_3) , ratio low 0.70, ratio high 1.30
7. **Check and filter on ratios:** when checked, ratios will be applied during the search. For most applications this will be needed. If no ratios can be defined, you can uncheck this option. At the end, area ratios will be reported. You might see area ratios outside the specified limits. In this case the algorithm has detected that area ratio is outside limits, but peak height ratios still fall inside the specified limits.
8. **Add Second Isotope to result table:** when checked, both isotopes will be listed in the result table. This will allow easy overlay plotting of both EICs from the final result table (after post-processing).
9. **Thresholds: window width on peak maximum detection:** this value is related to the peak width in scans. For every hit it will be checked if hits before and after the current scan have larger intensities. The parameter is used to detect a local peak maximum. The width used is typically 5 scans. The value will also determine if closely eluting peaks can be discriminated from each other. As a start, use the value for the peak width estimate as determined in the Browser.
10. **Thresholds – Intensity:** sets the lower absolute intensity threshold to be used. Ions having intensities smaller than the specified value will be deleted from the analysis. Please set a lower threshold based on what is relevant to the analysis. If you don't use a threshold, the IPF II algorithm could take a long time to complete.
11. **Set to 0.1%:** this button will set the intensity threshold to 0.1% compared to the largest ion in the currently used mass and time range. Careful, the 0.1% might be related to some large background ion. It will be much better if you can input intensity thresholds based on either a known adduct or the parent ion.
    The default value (Orbitrap data) will be 10,000.
12. **Load Defaults:** pressing the load default button will set default values for a GSH search. The following parameters are set: Algorithm - Full search, delta m/z 3.00374, ratio limits 0.7 – 1.3, accuracy 0.005, filter on ratios - Yes, window width 5 scans, both isotopes will be listed in the output and an intensity threshold of 10,000 will be used.

Press *the Run button*, and the calculations will start. During the analysis you will see a number of processing bars in which the progress of analysis is reported.

The first message reports how many full scans are processed. The ions having the correct mass difference and ratio are determined. This step should be fast.
The second message displays how many redundant ions have to be checked. This step might take some time, especially if broad peaks are present. If the number of redundant ions exceeds a value of 50.000, you should consider using different parameters. If the correct intensity threshold was used, try to set a higher accuracy of 0.005. If this does not solve the problem, try to skip the low m/z region, or skip part of the chromatogram. Often in the start of the chromatogram and at the end, many ions are present (background). Alternatively, you can use a restricted m/z range, e.g. use the range 400 to 800 Da, if the adduct of the parent is expected to be in the middle of this range (m/z 632, in this example).
After this step the algorithm should be fast again. The third step performs peak integration and determines peak characteristics. In the final step the TIC is reconstructed based on the hits that are found. At the end of the run, you will be asked to enter an estimate of the peak width in minutes. This value is used to calculate some local noise characteristics and the correlation coefficient between both EICs in nominal mode. These peak characteristics are for information purposes only. Enter the estimated peak width in minutes.

The IPF II run, using the settings from Figure 4, results in at total of 406 hits. Most of these are false positives and we will need the Post-Processing Filters to remove many of them. You can have a look at the results by plotting the EICs from the IPeaks result table. First, sort on peak height. As you can see, many of the hits relate to spikes, ^{13}C isotopes and non co-eluting hits. However, when looking at the mass spectra we can indeed see that the criteria from the search comply (correct mass difference and ratio). In the first step the algorithm does not check for peaks that should co-elute. That's why area ratios often are correct, but peak height ratios are not. It is evident that the two criteria alone (delta m/z and ratio) do not define clear chromatographic peaks.
The run using settings from Figure 5 results in 406 hits. If we would use the full mass range a total of 983 hits would be found and if we had run the algorithm at a less high accuracy, e.g. 0.01 instead of 0.005, a total of about 2000 hits would be the result. Handling such amount of false positives must be done using a fast algorithm and cannot be done manually.

Please save the raw result table to disk when asked or you can do it manually: *Menu > File > Save Table* or press the *save icon* on the Icon toolbar. You will be asked to enter a comment which will be saved together with the results to recognize this run afterwards. Saving the raw results is necessary in case you want to experiment with different Post-Processing filters. You will be able to quickly try different settings by restoring the original result table.

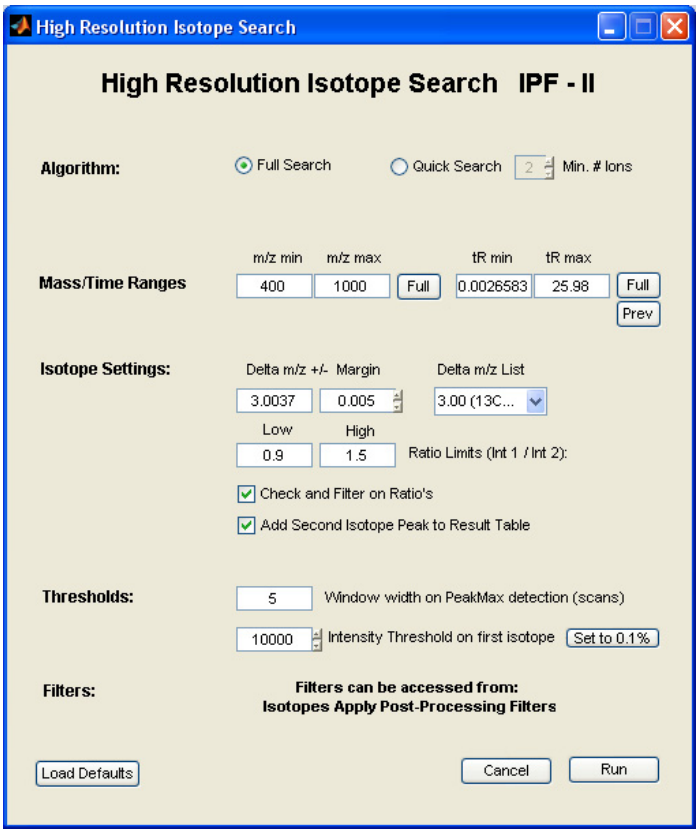


Figure 5: IPF II Interface, showing typical settings for GSH IPF Analysis

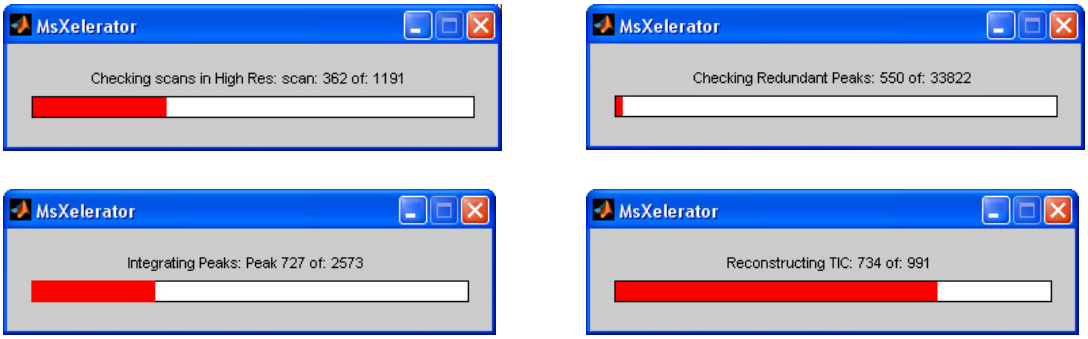


Figure 6: Different processing steps shown during the IPF II analysis. Carefully observe the second message. If the number of redundant ions to be checked is very large, e.g. > 50.000, consider using different parameters.

Removing False Positives by applying High Resolution Post-Processing Filters:

Additional filters are necessary to remove false positives from the initial hit list. Currently, the following checks can be applied:

- Peaks should be absent in one or more of the control samples
- Spikes (1 or 2 scan wide peaks) should be removed
- Only the monoisotopic peaks should appear in the list
- Only charge 1+ peaks are of interest
- Light and heavy EIC must co-elute (correlate)

Attention: High Resolution Post Processing can be applied on any result table from IPF I, IPF II or IPF III

To start the High Resolution Post-Processing Filter algorithm, select from the IPeaks Menu > *Isotopes* > *Apply Post Processing Filters.* Sometimes you will get a warning that you will have to switch to Mass Chromatogram (EIC) plotting mode. This is due to the fact that the GUI will open and as a default will use the displayed time limits as one of the filters. The information is extracted from the time range before starting the GUI. It is probably best to start the GUI with full scale EICs plotted, unless you already have a clear idea on the time ranges to use.

The Post Processing-GUI will display as shown in Figure 6. There are currently 12 filters available. You can apply all, or make a selection of filters. As a default, the filters will remove the false positives. However, by selecting *Label* instead of *Delete* you have full control regarding the output. If you are not sure about a specific filter, use *Label*. You can then afterwards see the actual results of each filter. All choices and parameters will be explained below:

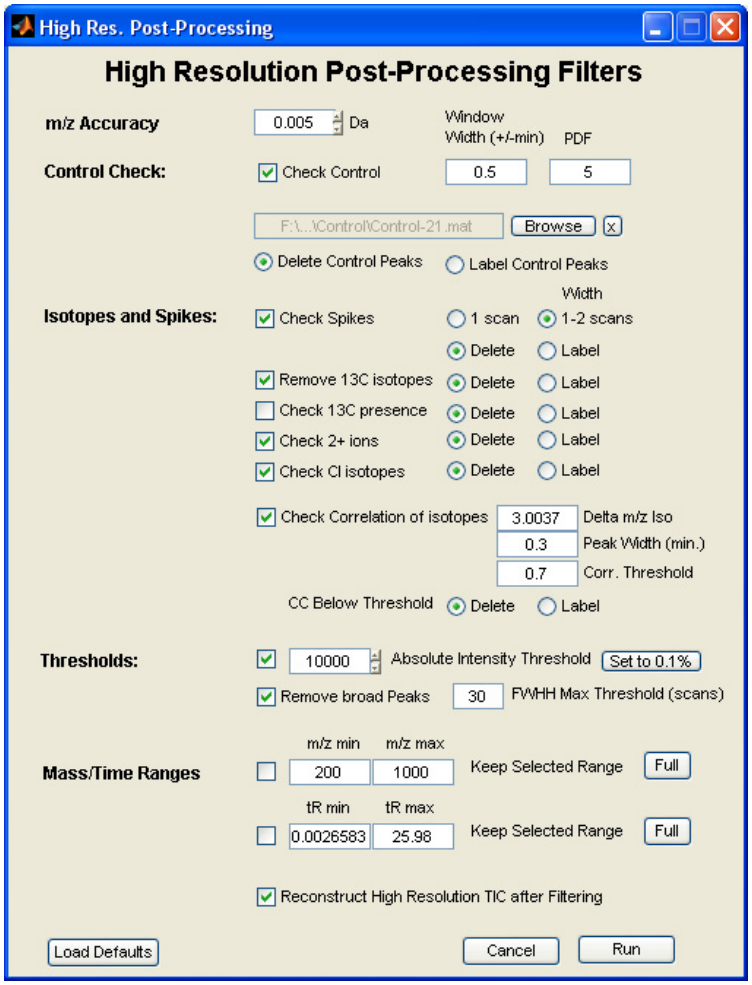


Figure 6: High Resolution Post-Processing GUI

Post-Processing Parameters:

1. **m/z Accuracy**: in principle you should use the same accuracy as was used during IPF II. The default value will be 0.005 Da. Most of the filters will apply to accurate EICs. The m/z accuracy determines EIC extraction width to be used, e.g., 632.15 +/- 0.005 Da.

2. **Control Check:** probably a large number hits will also be present in the control sample. For the IPF II application it is highly recommended to have (a) good control sample(s). Check or un-check the use of a control sample. You can select the control sample by using the *Browse button*. These settings will be remembered the next time you use the post-processing routine. The use of **multiple control samples** will be demonstrated at the end of this chapter.

   For every m/z value in the table the accurate EIC of the control sample will be extracted and compared to the sample peak. The comparison is based on a retention time window around the peak in the sample. As a default a value of 0.5 minutes will be used. Take care that the control sample has proper chromatographic alignment compared to the sample. The retention times of identical peaks should be within this window of 0.5 minutes. The algorithm will determine the ratio between the sample and control peak in the specified window. If the ratio is smaller than the Peak Difference Factor (PDF) the peak will be regarded as being present in both sample and control. The PDF factor is 1 for peaks equal in size, and infinite for peaks absent in the control sample. The default value for the PDF factor is 5. Peaks having ratios larger than 5 are regarded as unique peaks, not present in the control sample. If Delete was active, all peaks found in the control sample will be deleted. If Label was active the result table will show the outcome of each peak comparison (Control Peak or not a Control Peak). Also the ratio between sample and control will be listed in the result table.

   Figure 7 displays part of the result table if Control Labeling was selected. At the end of the table, for each m/z value, the result of the comparison is listed. Peaks absent in the control will have a very high ratio. If completely absent in the control sample, a infinite ratio would be the result. In this case a cutoff value of 999.00 will be used. Peaks in the table having ratios between 0 and 5 will be marked as “Control Peak”.

   Important: when the delete option was active only hits for which both the light and heavy isotope are present in the control sample will be deleted. If only one of them is found in the control, the peak is kept in the list. The user has to decide manually what to do. All filter operations can also be applied after Post Processing to recheck the table. E.g. first you will probably like to remove all false positives; next you might want to see which peaks are still present (light or heavy) in the control sample. The filter operations from the menu are available from *Menu > Isotopes*. More on this approach later.

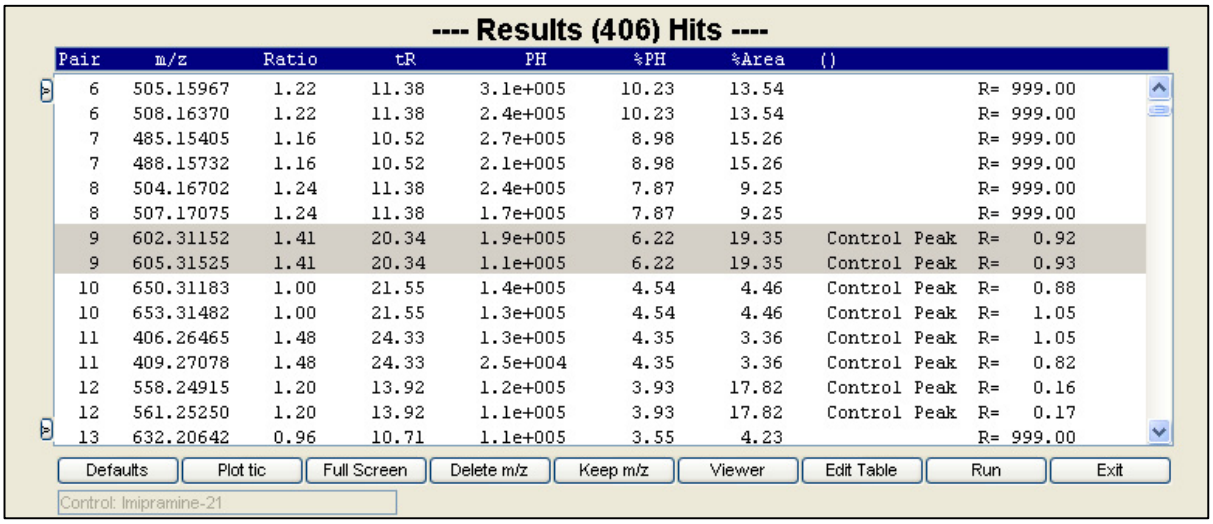


Figure 7: Part of the result table if post processing was run using the option: Label Control Peaks.

In IPeaks you can overlay the EICs of the sample with the EIC of a control. From the menu select: *Options > Overlay Reference/Control*. Browse to the control sample. The selected control file will be displayed in the *Control File name edit box* at the bottom of the IPeaks Screen as shown in Figure 7.
The first EIC in the result table will be plotted in blue, the heavy isotope in red and the control/reference EIC of the light isotope in black. You can optionally plot the control in mirror mode. This will make it easier to see differences. Also apply *Auto zooming* for easy viewing. Figure 8 shows the overlay plot of one of the hits (m/z 602.31/605.31) together with the light EIC (m/z 602.31) of the control sample. It is obvious that this peak is also present in the control sample and it should be deleted from the result table. The calculated ratio between sample and control is 0.92 for m/z 602.31 and 0.93 for m/z 605.31. At the end, it is advised to check all hits graphically and to check if alignment was as expected.

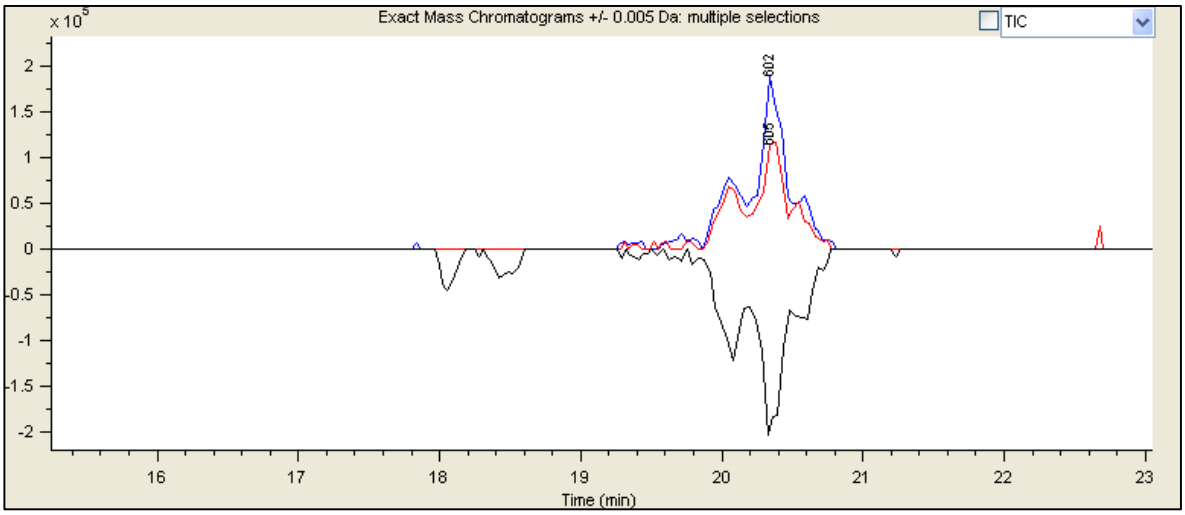


Figure 8: overlay of false positive hit 632.31 /635.31 (blue/red) with control sample m/z 632.31, plotted in black and mirror mode.

The resolution for plotting the EICs can be set or changed using the *Res. field* on the top left of the EIC window. The default value is taken from the resolution used in IPF II.

3. **Spike Detection:** at very low levels the chance of finding peaks that by chance have the correct mass difference and ratio substantially increases. It is often seen that these low level hits near the detection limit are actually spikes. The EICs are only 1-2 scans wide. Use spike detection to eliminate the very narrow hits.
   Important: if your chromatography results in very narrow peaks for real components, do not use spike elimination, as you might delete real peaks. It will then be very difficult to discriminate between real peaks and spikes. Besides the other filters, the intensity will be very important. It is advised to use chromatography that results in peaks having at least 4-5 scans across a real peak. As an example of a real spike see Figure 3.
   The spike algorithm gives you the choice of deleting or labeling spikes being 1 or 2 scans wide. The default value will be to check two scan wide spikes. Spikes will always have a high correlation for the co-elution check, so this parameter is not very useful in these cases.

4. **Remove ^{13}C Isotopes:** it is often seen that besides mono-isotopic peaks, the result list will also contain ^{13}C isotope peaks from the same component. This is to be expected as the basic criteria will also be valid for the ^{13}C isotope pair. Check this option if you want to remove the ^{13}C isotopes from the result table. The check is made on three scans near the top of the peak.
   Attention: if both peaks from the pair have ^{13}C isotopes the hit will be removed. If the first m/z value is found to be a ^{13}C isotope, but the second not, the hit is also removed. However, if the first m/z value is not a ^{13}C isotope, but the second is, the hit will not be removed. Default settings are to check this filter.

   An example of a false positive hit is the pair 584.107 / 587.113, shown in Figure 9. It will be clear that the hit relates to ^{13}C isotopes in both cases. The mono-isotopic peaks relate to the real hit. It might also be possible that the hit relates to more complex situations in which the peaks are non-monoisotopic.

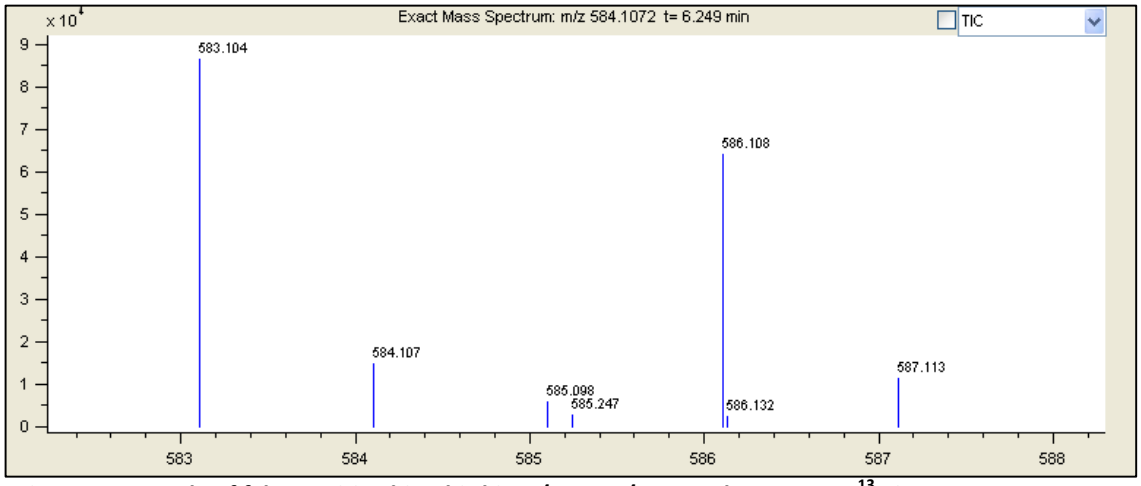


Figure 9: example of false positive hit. This hit m/z 584.1/587.1 relates to two ^{13}C isotopes.

5. **Check ^{13}C presence:** this filter is not part of the default settings, but it can be used in cases that, even after post-processing, still quite a few hits are found. Basically this filter checks that ^{13}C isotope peaks are present for both the light and heavy isotope. If both peaks do not have a ^{13}C isotope the hit will be deleted. At low intensities it is often seen that ^{13}C isotope peaks are lacking. So, somehow this filter is intensity related. The presence of ^{13}C isotope peaks is determined from an average of three scans.

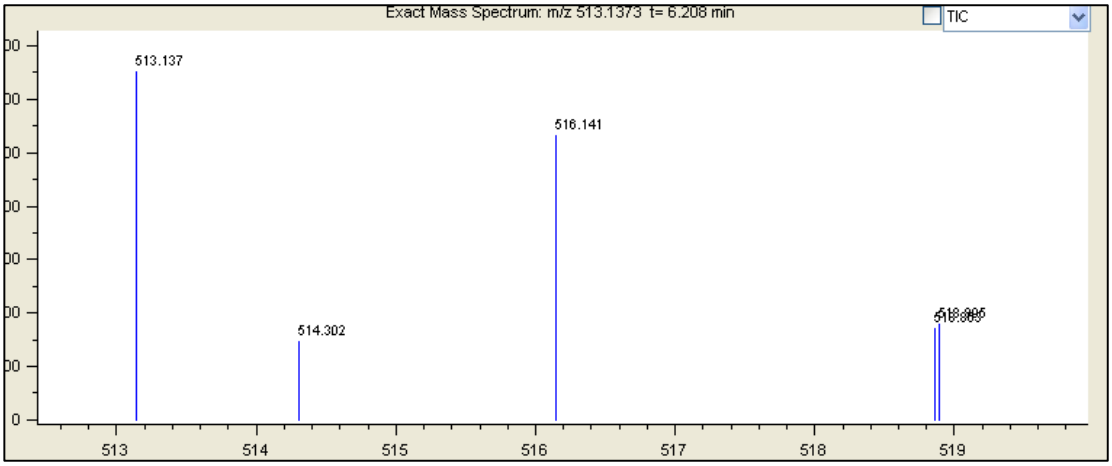


Figure 10: example of a false positive hit 513.137 / 516.141. Both mono isotopic peaks do not have a ^{13}C isotope peak. The peak at 514.302 does not fit the accuracy limit for being the ^{13}C isotope of 513.137

6. **Check 2+ ions:** it will be checked that hits are not originating from charge 2+ ions. If the user is interested in selectively finding charge 2+ ions, IPF II can be set to search for these ions. In the case of a GSH 2+ adduct search the user should apply an m/z difference of 1.5019 Da. Some of the filters need to be changed for checking false positives in 2+ mode. The check is made on three scans near the top of the peak.

7. **Checking Cl isotopes:** if the parent contains Cl, the GSH adducts will probably also contain Cl. The isotope pattern will be a little bit more complex. Beside false positives originating from ^{13}C isotopes, we will observe pairs of ^{37}Cl isotopes in the hit list. It will be checked if the typical chlorine pattern is present. These heavy isotope Cl peaks will be deleted from the analysis. The check is made on three scans near the top of the peak.

8. **Check Correlation of Isotopes:** the isotope pairs of real hits should co-elute. The correlation coefficient (CC) is used to determine if and to what extend isotopes co-elute and are similar in shape. The CC needs to be calculated from a specific time range using accurate extracted EICs. As a default a value of +/- 0.3 minutes is used. Please check the width of your peaks and use a value of about 2 times this width. The correlation threshold is set to a

default value of 0.7. Peaks having CCs smaller than 0.7 will be deleted from the result table. You will also have to check that the specified delta m/z value is equal to the one used in IPF II.

9. **Intensity Threshold:** if you want to specify a new intensity threshold set the value in absolute counts. All hits smaller than the specified intensity will be deleted.

10. **Set to 0.1%:** use this button to set an intensity threshold of 0.1% compared to the largest **hit in the result table**. It might be that the threshold level is now more related to real GSH adducts compared to the original IPF II run.

11. **Remove broad peaks:** for every peak in the table the Full Width at Halve Height will be determined (FWHH). You can specify a maximum FWHH in scans. Peaks broader than this value will be deleted.

12. **Select mass range to use:** enter the minimum and maximum m/z values to be applied. The table will be filtered to include only m/z values inside the specified region. This filter can also be applied by using the IPeaks Editor.

13. **Select time range to use:** enter the time limits to be used. Peaks outside these limits will be removed. This filter can also be applied by using the IPeaks Editor.

Running High Resolution Post Processing:

Press the *Run button*. After checking all the items, a Summary Report will be created as shown in Figure 11. It shows the sequential number of hits remaining after processing each of the filters. The Post Processing filter adequately removes a large part of the initials hits. One of the filters was not applied during this run, the ^{13}C Presence Check. Had we applied this filter, 3 additional False Positives would have been found, after which a total of 21 hits remain. As will be shown later, this filter can also be run afterwards by selecting the command from the *IPeaks Isotopes Menu*.

Sort the result table on Peak Height by pressing the *sort PH button*. The sorted result table is shown in Table 1.

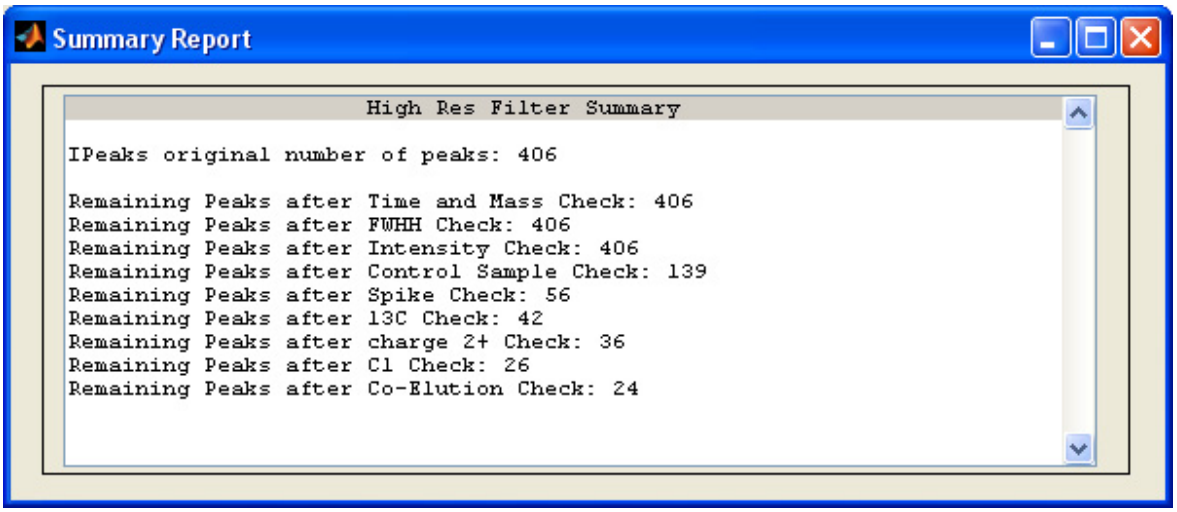


Figure 11: Filter Summary Report showing remaining number of hits after each filter operation.

Table Description:

The Result Table has the following fields: **Pair** – peak ID number of light and heavy isotope, **m/z** – the accurate mass values of the light and heavy isotope, **Ratio** – the area ratio between Light and Heavy EIC, **PH** – Peak Height at retention time, **% PH** - relative Peak Height compared to largest hit from the list, **%Area** – Relative Area compared to largest area in the list, **Pbase** – measure of peak width (in scans) near the baseline, **FWHH** – Full Width Halve Height of peak in scans, **Smooth** - calculated correlation coefficient between light and heavy EIC (co-elution measure), **S/N ratio** – Signal to Noise ratio based on a nominal EIC extraction.

Before using the additional tools for identification, you can check the table directly on a number of common characteristics. As can be seen, the largest adduct is the expected GSH adduct of Clozapine (m/z 632.206). If the table is **sorted on retention time**, we can more easily see that a number of different hits **co-elute.** Co-eluting peaks should be checked with care as these can originate from fragmentation or additions in the MS instrument. However, the co-elution pattern can also be a **confirmation for being a real hit**. For m/z 632.206 at RT=11.34 minutes we find the following co-eluting peaks:

Pair 2: m/z 503 tR=11.38 min. Loss of Gamma Glutamate, 31% compared to m/z 632
Pair 7: m/z 446 tR=11.34 min. ???, 2.4% compared to m/z 632
Pair 14: m/z 686 tR=11.34 min. ???, Area ratio only 0.83 (no Chlorine), 1.3% compared to m/z 632

Small co-eluting peaks compared to the largest peak might be difficult to identify. IPeaks has a number of tools to check whether these peaks are the result of adduct formation (Na^+, K^+, loss of gamma glutamate, contain Cl, or check other user defined mass differences).

For checking co-eluting peaks, IPeaks has a feature to automatically cluster co-eluting peaks into groups (clusters). This algorithm can be run from *Menu > Task > Cluster Peaks*. Currently this algorithm will group similar co-eluting peaks (having similar shape, based on correlation) into groups based on nominal EICs. For a more detailed overview of clustering see the manual Chapter 4.6 (MPeaks – Advanced Tasks).

The loss of gamma glutamate (-129.04) can also be observed for some other co-eluting pairs, e.g. pair 4 and 11, 17 and 18. It appears that in total 6 hits are due to the loss of gamma glutamate.

Table 1: Peak Height Sorted Result Table after Post Processing

Pair	m/z	Ratio	tR	PH	%PH	%Area	Pbase	FWHH	Smooth	S/N Ratio
1	632.20599	1.136	11.34	3021142.5	100.00	100.00	12	4	0.999	627.5
1	635.20941	1.136	11.34	2614334.8	100.00	100.00	12	4	0.999	603.4
2	503.16342	1.200	11.38	944993.0	31.28	42.23	10	5	0.997	123.8
2	506.16699	1.200	11.38	737856.6	31.28	42.23	10	5	0.997	89.3
3	632.20642	0.957	10.71	107254.9	3.55	4.23	6	4	0.995	22.3
3	635.21002	0.957	10.71	99604.9	3.55	4.23	6	4	0.995	21.2
4	618.19043	1.189	10.79	97535.6	3.23	3.25	5	4	0.993	20.6
4	621.19415	1.189	10.79	79640.3	3.23	3.25	5	4	0.993	23.5
5	550.11658	1.274	13.92	94287.5	3.12	3.66	5	5	0.987	9.8
5	553.12024	1.274	13.92	84220.4	3.12	3.66	5	5	0.987	12.2
6	503.16382	1.088	10.71	78307.4	2.59	2.63	5	4	0.994	8.6
6	506.16708	1.088	10.71	64167.7	2.59	2.63	5	4	0.994	8.1
7	446.10489	1.188	11.34	71555.7	2.37	2.66	5	5	0.943	7.5
7	449.10928	1.188	11.34	46512.2	2.37	2.66	5	5	0.943	13.7
8	650.21613	1.106	11.12	62960.3	2.08	2.86	6	5	0.981	18.6
8	653.21985	1.106	11.12	60631.2	2.08	2.86	6	5	0.981	22.8
9	662.21667	1.292	10.52	59164.5	1.96	3.35	7	6	0.994	10.4
9	665.21918	1.292	10.52	45000.7	1.96	3.35	7	6	0.994	12.6
10	626.24005	1.342	8.04	54542.1	1.81	1.79	4	4	0.980	8.7
10	629.24359	1.342	8.04	35170.4	1.81	1.79	4	4	0.980	8.8
11	489.14774	1.180	10.79	47747.0	1.58	1.79	4	5	0.978	1.9
11	492.15182	1.180	10.79	37911.6	1.58	1.79	4	5	0.978	8.1
12	660.20154	1.043	8.66	42020.4	1.39	3.35	11	7	0.934	9.1
12	663.20441	1.043	8.66	35200.8	1.39	3.35	11	7	0.934	10.2
13	632.20612	0.983	13.72	39278.9	1.30	1.68	5	5	0.850	7.4
13	635.20966	0.983	13.72	45468.5	1.30	1.68	5	5	0.850	13.8
14	686.12799	0.830	11.34	38004.9	1.26	1.42	4	5	0.913	10.8
14	689.12732	0.830	11.34	35677.2	1.26	1.42	4	5	0.913	5.7
15	580.12695	1.420	14.47	36394.2	1.20	1.37	4	5	0.968	11.6
15	583.13025	1.420	14.47	28274.2	1.20	1.37	4	5	0.968	7.1
16	520.17566	1.097	14.86	25072.9	0.83	0.86	3	5	0.817	1.0
16	523.18018	1.097	14.86	21359.2	0.83	0.86	3	5	0.817	5.3
17	598.24518	1.493	7.05	22724.2	0.75	0.84	4	5	0.957	52.0
17	601.24841	1.493	7.05	11210.8	0.75	0.84	4	5	0.957	2.3
18	469.20245	1.347	7.02	21436.4	0.71	0.94	4	5	0.998	5.6
18	472.20605	1.347	7.02	16494.2	0.71	0.94	4	5	0.998	5.1
19	441.09006	1.463	11.50	20239.4	0.67	0.59	3	4	0.933	3.5
19	444.09433	1.463	11.50	11329.1	0.67	0.59	3	4	0.933	0.2
20	497.19766	1.445	8.04	18149.3	0.60	0.48	2	4	0.942	3.8
20	500.20129	1.445	8.04	13189.8	0.60	0.48	2	4	0.942	2.2
21	529.13214	1.456	3.35	17295.3	0.57	1.40	8	10	0.881	3.8
21	532.13617	1.456	3.35	7882.0	0.57	1.40	8	10	0.881	1.7
22	531.15863	1.385	8.66	16846.6	0.56	1.29	7	9	0.905	4.2
22	534.16125	1.385	8.66	12641.7	0.56	1.29	7	9	0.905	0.4
23	650.21704	0.959	8.27	16348.4	0.54	0.59	3	5	0.907	3.7
23	653.22052	0.959	8.27	15035.5	0.54	0.59	3	5	0.907	4.8
24	680.22632	1.017	14.06	14095.0	0.47	0.43	3	5	0.982	1.0
24	683.22998	1.017	14.06	16251.4	0.47	0.43	3	5	0.982	3.8

Additional Post Processing Filters Tools:

The *IPeaks Isotopes Menu* contains all the items that are relevant to reactive metabolite detection using the procedures described in this document. Figure 12 lists all the items that can be selected from the *Isotopes Menu*:

The items in red relate to the two major processing algorithms:

- IPF II
- High Resolution Post Processing

The items in yellow are all individual filters that can be run from the *IPeaks Isotopes* menu directly. These are effectively all present in the High Resolution Post processing algorithm. If necessary or preferred, they can also be run individually from the *IPeaks Isotopes menu:*

- Check Peak Ratios: recalculate peak ratios based on Peak Height (single scan), not Peak Area
- Check/Plot Delta m/z: plots the m/z difference between light and heavy isotope peak in a control chart
- Check Single Spikes: spike checking
- Check M-1 Isotope: general procedure to check if at certain mass difference from the selected peak another peak is present. Can be used to detect if a peak is or has a ^{13}C Isotope, has a charge 2+ peak, adduct, etc.
- Check Correlation: used to calculate correlation coefficient (measure of co-elution) between light and heavy isotope.
- Check Control Sample: check if peaks in the table are also present in a user defined control sample.

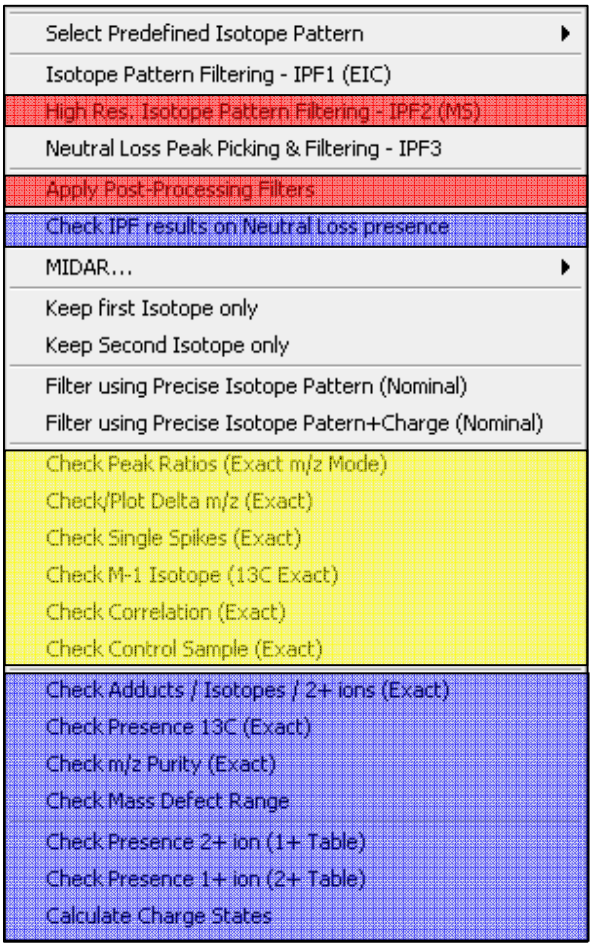


Figure 12: The IPeaks Isotopes Menu containing major items related to Reactive MetaboliteDetection

The **purple/blue items** are advanced filters or identification checks not present in the High Resolution Post Processing algorithm (except from the ^{13}C presence check). These features are explained below:

Attention: If you want to remove any added info (comment field) during the next operations, press the *Delete Info button* on the far right of the Icon Toolbar or select *Options > Delete Info Field.*

- **Check Presence of ^{13}C Isotopes**
  If too many hits are found you can apply the following restrictive rule: a peak should have a ^{13}C isotope peak for both the light and heavy isotope. For very low intensity peaks ^{13}C isotopes are often missing, as we are on the

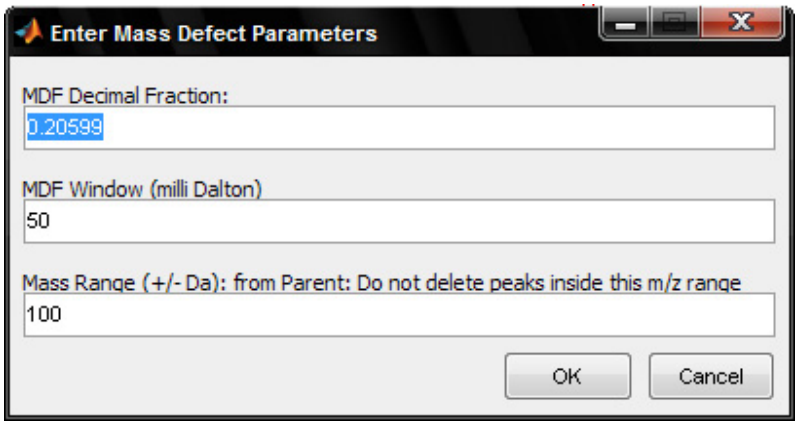
edge of detection. The ^{13}C Isotope Presence Check can be made directly in the High Resolution Post Processing module or can be run afterwards. Here we run it from the IPeaks menu. Press Menu > Isotopes > Check Presence 13C (exact). You will be asked to set the accuracy and the number of neighboring scans near the top of the peak. Finally you are asked to delete or label the peaks for which no ^{13}C isotope was found. If you select delete, only pairs in which the ^{13}C isotopes are lacking for both the light and heavy peak will be removed. In this case select Label.
From the info added to the end of the result table one can see that pairs 7, 14 and 19 have no ^{13}C peaks present. You can delete them manually by pressing the *Del* key, or run the check again and select *Delete* during the last question. In general this feature can be used if many hits are found and the very small ones are not very important. If this is not the case you should check these peaks manually (can they be identified etc.)?

Pair	m/z	Ratio	tR	PH	%PH	%Area	13C Present()
5	553.12024	1.27	13.92	84220.4	3.12	3.66	
6	503.16382	1.09	10.71	78307.4	2.59	2.63	
6	506.16708	1.09	10.71	64167.7	2.59	2.63	
7	446.10489	1.19	11.34	71555.7	2.37	2.66	No
7	449.10928	1.19	11.34	46512.2	2.37	2.66	No
8	650.21613	1.11	11.12	62960.3	2.08	2.86	
8	653.21985	1.11	11.12	60631.2	2.08	2.86	

Figure 13: Part of the result table after checking and labeling on the presence of ^{13}C isotopes

- **Check m/z Purity**
  The m/z Purity check is used to check if a larger interfering peak is present in between the light and heavy isotope mass peaks. It does not necessarily mean that the hit is a false positive. If needed the user is encouraged to further examine the pattern **in the Browser.** This is most easily done by copying the hit to the Browser. 
  Press the *Copy to Browser button from the Icon Toolbar*. The program will switch from IPeaks to the Browser and both the mass spectrum and EIC will be copied. You can then make accurate overlay plots by clicking on the m/z peaks in the top window of the Browser and see whether the interfering peak is perfectly co-eluting or not. A perfectly co-eluting interfering peak might be a false positive hit. For Clozapine pair 8 (650/653) has an interfering peak (652). However it turns out that the m/z 652 is not co-eluting, it should not be removed.

- **Check Mass Defect Range**
  The Mass Defect Check is based on the GSH adduct of the Parent. Please first select the m/z 632/635 hit. It will be checked if entries in the result table are outside a mass defect window of 50 milli Dalton compared to the m/z value of the parent's adduct and outside a absolute m/z range of +/- 100 Dalton.
  Select this feature from the Isotopes menu. You will get the following window.



The MDF Decimal Fraction is taken directly from the selected peak in the table, in this case the adduct of the Parent (m/z 632.20599 = 327.14 (Clozapine) +305.06(GSH). If needed you can change the default values for the MDF window (50 milli Da) or the Mass Range (100 Da). Only peaks outside the mass defect window and outside the specified mass range will be deleted or labeled. It is probably best to use labeling when asked. Four hits

appear to be outside the ranges: Pair 7, 11, 19 and 21 (see Figure 14). You can delete these manually if needed or rerun the algorithm and automatically delete them.

Pair	m/z	Ratio	tR	PH	%PH	%Area	MDF ()
17	601.24841	1.49	7.05	11210.8	0.75	0.84	
18	469.20245	1.35	7.02	21436.4	0.71	0.94	
18	472.20605	1.35	7.02	16494.2	0.71	0.94	
19	441.09006	1.46	11.50	20239.4	0.67	0.59	Out-MDF
19	444.09433	1.46	11.50	11329.1	0.67	0.59	Out-MDF
20	497.19766	1.45	8.04	18149.3	0.60	0.48	
20	500.20129	1.45	8.04	13189.8	0.60	0.48	
21	529.13214	1.46	3.35	17295.3	0.57	1.40	Out-MDF
21	532.13617	1.46	3.35	7882.0	0.57	1.40	Out-MDF
22	531.15863	1.38	8.66	16846.6	0.56	1.29	
22	534.16125	1.38	8.66	12641.7	0.56	1.29	

Figure 14: Part of the result table after checking for peaks outside the Mass Defect Window of 50 mDa and Range (+/- 100 Da from m/z 632). Analysis based on Parent+GSH.

To remove the displayed information from the table press the Delete Info/Comment icon. This will give a clear info field for the next procedure.

- **Check Adducts / Isotopes and presence of Charge 2+ ions**

This option runs a general but powerful algorithm to quickly check all mass spectra of all hits in the table for having a specified mass difference larger in intensity compared to the peak in the table. Basically the algorithm is used to check the presence of isotopes and adducts (co-eluting peaks). The algorithm will check three neighboring scans near the top of each peak. The GUI has a number of predefined checks but the user can also specify a new mass difference to be used. In general you can check if a peak **is or has** an adduct with a certain mass difference.
Checks can be made to see if:

1. The present hit in the table is due to the loss of gamma glutamate, which means that a peak should be present at the specified m/z value +129.04 Da. This peak should be larger than 25% compared to the hit being investigated. You can also check if the current peak has a loss of 129.04. This information supports that the peak is a real hit.
2. A hit has a Chlorine Isotope Pattern.
3. A hit **is or has** a Na+ or K+ adduct peak
4. If a peaks is a ^{13}C isotope (normally these peaks are already removed during the Post Processing run).
5. A current peak has an adduct or isotope peak at a user-defined mass difference.
6. The current peak/hit also exists in charge 2+ mode. This feature is especially useful for GSH trapping assays.

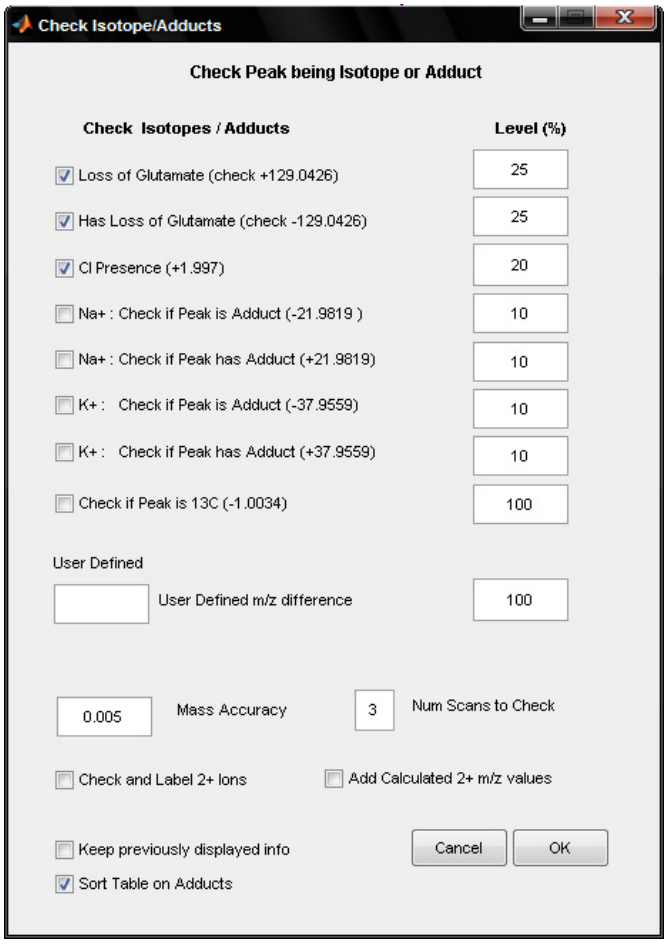


Figure 15: The Check Adducts / Isotopes GUI

All checks can be performed simultaneously. To run the check press *Isotopes > Check Isotopes / Adducts / 2+ Ions (exact).* The following window (figure 15) will open. As a default, the GUI will open with both checks on Gamma

Glutamate and Chlorine activated. Other choices can be activated as well. After the algorithms are run the result table will be sorted on peaks scoring on positive checks. If wanted, you can also decide to leave the starting table un-sorted. In this case, unmark the option *Sort Table on Adducts.*
If the table contained information/comments from a previous search, you will have the option to keep or delete the previous displayed information. The new info will be added to the existing information.
The "Level Fields" in Figure 15 defines the percentage that the "Adduct" must be larger compared to the peak from the result table. If smaller it will not be marked. Example: if the peak in the table has a mass of 500.10, and the user wants to check whether this hit is a Na+ adduct, the algorithm will search and check the value of m/z 478.21. If the intensity is larger than 10% compared to the peak in the table, the algorithm will mark the hit in the table as being a peak that is probably the Na+ adduct (Info: M-22). The information text displayed contains the position of the search location checked in the current mass spectrum. In the above example the entries will be marked with M-22.

The result for the default run is partly displayed in Table 2. Sorting will be based on peaks having a positive score. The order of sorting reflects the order in which the checks have been performed. Table 2 was sorted on retention time, which makes it easier to link adducts or losses as peaks should co-elute.

Entries in the table scoring positive on M+129 all have a peak at m/z +129.04 Dalton. This means that the entries can be identified as peaks that have lost gamma glutamate (Comment Field Glu-). Entries showing M-129 are reactive metabolites for which the mass spectrum also shows a peak at M-129.04. This confirms that the peak is probably a real hit. By sorting on retention time, the hits that are linked can be easily identified.
The other check was made to see if a peak has a Chlorine signature. All hits in the table for which this pattern was detected are marked (Cl, M+2). It appears that the majority of the hits in Table 2 have a Chlorine isotope pattern. If the parent drug contains Cl, the presence of the Chlorine pattern is a strong feature in the confirmation of real hits.

Table 2: Result Table after check on gamma glutamate and presence of chlorine

Pair	m/z	Ratio	tR	PH	%PH	%Area	Glu-	Glu+	Cl	()
1	469.20245	1.35	7.02	21436.4	0.71	0.94	M+129			
1	472.20605	1.35	7.02	16494.2	0.71	0.94	M+129			
2	598.24518	1.49	7.05	22724.2	0.75	0.84		M-129		
2	601.24841	1.49	7.05	11210.8	0.75	0.84		M-129		
3	626.24005	1.34	8.04	54542.1	1.81	1.79		M-129		
3	629.24359	1.34	8.04	35170.4	1.81	1.79		M-129		
4	497.19766	1.45	8.04	18149.3	0.60	0.48	M+129			
4	500.20129	1.45	8.04	13189.8	0.60	0.48	M+129			
5	650.21704	0.96	8.27	16348.4	0.54	0.59		M-129	M+2	
5	653.22052	0.96	8.27	15035.5	0.54	0.59		M-129	M+2	
6	531.15863	1.38	8.66	16846.6	0.56	1.29	M+129	M-129	M+2	
6	534.16125	1.38	8.66	12641.7	0.56	1.29	M+129		M+2	
7	660.20154	1.04	8.66	42020.4	1.39	3.35		M-129	M+2	
7	663.20441	1.04	8.66	35200.8	1.39	3.35		M-129	M+2	
8	662.21667	1.29	10.52	59164.5	1.96	3.35			M+2	
8	665.21918	1.29	10.52	45000.7	1.96	3.35			M+2	
9	632.20642	0.96	10.71	107254.9	3.55	4.23		M-129	M+2	
9	635.21002	0.96	10.71	99604.9	3.55	4.23		M-129	M+2	
10	503.16382	1.09	10.71	78307.4	2.59	2.63	M+129		M+2	
10	506.16708	1.09	10.71	64167.7	2.59	2.63	M+129		M+2	
11	618.19043	1.19	10.79	97535.6	3.23	3.25		M-129	M+2	
11	621.19415	1.19	10.79	79640.3	3.23	3.25		M-129	M+2	
12	489.14774	1.18	10.79	47747.0	1.58	1.79	M+129		M+2	
12	492.15182	1.18	10.79	37911.6	1.58	1.79	M+129		M+2	
13	650.21613	1.11	11.12	62960.3	2.08	2.86		M-129	M+2	
13	653.21985	1.11	11.12	60631.2	2.08	2.86			M+2	
14	632.20599	1.14	11.34	3021142.5	100.00	100.00		M-129	M+2	
14	635.20941	1.14	11.34	2614334.8	100.00	100.00		M-129	M+2	
15	446.10489	1.19	11.34	71555.7	2.37	2.66				
15	449.10928	1.19	11.34	46512.2	2.37	2.66			M+2	
16	686.12799	0.83	11.34	38004.9	1.26	1.42				
16	689.12732	0.83	11.34	35677.2	1.26	1.42				

It appears that a few hits are identified by the loss of gamma glutamate, but don't have a chlorine pattern (pair 2 and 3 from table 2). In this case we have indirectly verified some real hits not containing chlorine by checking the 129 gamma glutamate loss.

The checking of adducts can also be done graphically by reconstruction of the high resolution TIC. All peaks in the reconstruction will be marked with the actual m/z value. Marking is done at the retention time and peak height of all peaks found in the table. Co-eluting peaks (adducts) are easily detected in this way by checking the respective retention times and m/z values (Figure 17). The high resolution TIC reconstruction of will be described at the end of the chapter.

- **Check Presence of 2+ ions**
  For Clozapine if appears that many of the hits are also present as charge 2+ ions. Furthermore it is seen that these charge 2+ ions often have 10 times larger intensities compared to charge 1+ ions. The isotopes menu has an algorithm to check if entries in the current table also exist as charge 2+ ions. If found, it will report the intensity ratio between the two charge states. The presence of high intensity 2+ ions confirms the peak being a real hit.

  From the Menu select: *Isotopes > Check Presence Charge 2+ ions (1+ Table).* This means that the current table is a result of a default GSH check with an m/z difference of 3.0037. Had we run the IPF II algorithm directly for charge 2+ ions (delta m/z 1.5018) we would have to select *Check Presence 1+ ions (2+ Table).*
  If needed you can leave the already displayed information in the table, otherwise start with a clean comment field.

  For the current result table we will run the charge 2+ check using the *Check Adducts / Isotopes module*. You can activate a charge 2+ check next to the other checks (loss of gamma glutamate and the chlorine check). The result for a charge 2+ check is shown in Table 3. To better view the table, all peaks related to the loss of 129 have been deleted from the table. These peaks turned out to have no charge 2+ ions as it seem that the charge 2+ state only appears for peaks that have the intact GSH moiety.

  The charge 2+ check adds the following fields to the top of the table: **2+, m/z and I 2+/1+.**
  The **2+** field is a marker that the charge 2+ ion is detected in the current mass spectrum. If the line is empty, the charge 2+ ion was not detected. The **m/z field** contains the calculated charge 2+ m/z value. Might be that the user directly wants to check these peaks in the Browser. The last field **I 2+/1+** is the calculated intensity ratio between the charge 2+ and 1+ ion. It appears that for a large number of hits the charge 2+ ions are 10 to 20 times stronger.

  Some of the peaks that have no chlorine show a high intensity charge 2+ ion. E.g. m/z 598/601, m/z 626/629, m/z 686/689 and m/z 680/683. During the ^{13}C presence check it was concluded that the m/z 686/689 did not have ^{13}C isotopes. However, a three times higher intensity was observed for the charge 2+ ion (343.56/345.07). It now appears that the charge 2+ ion has both the ^{13}C isotope pattern and also shows the typical chlorine pattern. In this case the metabolite is easily detected in 2+ mode, but more difficult in 1+ mode.

  Whenever the parent drug has a tendency to form charge 2+ GSH adducts, the above check seems to be a strong confirmation tool for peaks being real hits. It this is the case the user should definitely also run the IPF II algorithm directly in charge 2+ mode, because the above procedure **only confirms** ions in charge 2+ mode. Low intensity reactive metabolites could have been missed in the default run (1+ Mode).
  For other types of trapping agents like KCN and MOA, the charge verification tool is probably not so useful.

Table 3: Result Table after check on loss of gamma glutamate, chlorine and charge 2+ ions. The peaks related to loss of gamma glutamate have been removed. The last column displays the ratio between the peak height intensities from a charge 2+ peak compared to the charge 1+ ion. It appears that many hits have much higher intensities for their charge 2+ ions.

Pair	m/z	Ratio	tR	PH	%PH	%Area	Glu	Cl	2+	m/z	I 2+/
1	650.21704	0.96	8.27	16348.4	0.54	0.59		M+2	M2+	325.612	24.7
1	653.22052	0.96	8.27	15035.5	0.54	0.59		M+2	M2+	327.114	20.1
2	660.20154	1.04	8.66	42020.4	1.39	3.35		M+2	M2+	330.604	13.6
2	663.20441	1.04	8.66	35200.8	1.39	3.35		M+2	M2+	332.106	15.1
3	662.21667	1.29	10.52	59164.5	1.96	3.35		M+2	M2+	331.612	4.4
3	665.21918	1.29	10.52	45000.7	1.96	3.35		M+2	M2+	333.113	4.8
4	632.20642	0.96	10.71	107254.9	3.55	4.23		M+2	M2+	316.607	18.9
4	635.21002	0.96	10.71	99604.9	3.55	4.23		M+2	M2+	318.109	19.0
5	618.19043	1.19	10.79	97535.6	3.23	3.25		M+2	M2+	309.599	20.9
5	621.19415	1.19	10.79	79640.3	3.23	3.25		M+2	M2+	311.101	24.2
6	650.21613	1.11	11.12	62960.3	2.08	2.86		M+2	M2+	325.612	18.6
6	653.21985	1.11	11.12	60631.2	2.08	2.86		M+2	M2+	327.114	19.2
7	446.10489	1.19	11.34	71555.7	2.37	2.66				223.556	0.0
7	449.10928	1.19	11.34	46512.2	2.37	2.66		M+2		225.058	0.0
8	632.20599	1.14	11.34	3021142.5	100.00	100.00		M+2	M2+	316.607	11.7
8	635.20941	1.14	11.34	2614334.8	100.00	100.00		M+2	M2+	318.108	13.4
9	632.20612	0.98	13.72	39278.9	1.30	1.68		M+2	M2+	316.607	13.3
9	635.20966	0.98	13.72	45468.5	1.30	1.68			M2+	318.108	10.2
10	550.11658	1.27	13.92	94287.5	3.12	3.66		M+2		275.562	0.0
10	553.12024	1.27	13.92	84220.4	3.12	3.66		M+2		277.064	0.0
11	580.12695	1.42	14.47	36394.2	1.20	1.37		M+2	M2+	290.567	5.7
11	583.13025	1.42	14.47	28274.2	1.20	1.37		M+2	M2+	292.069	4.9
12	529.13214	1.46	3.35	17295.3	0.57	1.40				265.070	0.0
12	532.13617	1.46	3.35	7882.0	0.57	1.40				266.572	0.0
13	598.24518	1.49	7.05	22724.2	0.75	0.84			M2+	299.626	13.9
13	601.24841	1.49	7.05	11210.8	0.75	0.84			M2+	301.128	24.0
14	626.24005	1.34	8.04	54542.1	1.81	1.79			M2+	313.624	12.0
14	629.24359	1.34	8.04	35170.4	1.81	1.79			M2+	315.125	14.5
15	686.12799	0.83	11.34	38004.9	1.26	1.42			M2+	343.568	3.4
15	689.12732	0.83	11.34	35677.2	1.26	1.42			M2+	345.067	2.6
16	441.09006	1.46	11.50	20239.4	0.67	0.59				221.049	0.0
16	444.09433	1.46	11.50	11329.1	0.67	0.59				222.551	0.0
17	680.22632	1.02	14.06	14095.0	0.47	0.43			M2+	340.617	8.4
17	683.22998	1.02	14.06	16251.4	0.47	0.43			M2+	342.119	6.9

- **Calculate Charge States**
  This option is a general IPeaks feature to calculate charge states based on the isotopic ^{13}C distribution. The algorithm used is a combination of the Patterson algorithm and Cross Correlation. It can be used for high resolution instruments from charge 1+ to 8+.

Identification of Reactive Metabolites:

The identification of possible Reactive Metabolites from known (expected) metabolic pathways is an important issue and can be done in MsXelerator from MPeaks or IPeaks. Identification can be done both in nominal and exact mass mode. The user can add or delete reactions from the predefined prediction lists using the List Editor. For a general overview on Metabolite Identification see the manual, Chapter 4.3.

Identification is based on so-called prediction lists. These lists contain expected mass differences (from the parent) due to e.g. oxidation, reduction, demethylation etc. MsXelerator uses a pre-defined list of about 60 common reactions. These lists were compiled from comparison of different MetID software products and a literature search of the most common reactions (P. Jacobs, Schering-Plough, 2008). Part of the list is shown in Table 4. It displays the accurate relative mass difference, the description and the molecular change.

Based on the Parent, or in the case of Reactive Metabolite Profiling the GSH adduct of the Parent, IPeaks can be used to check all entries in the result table for a possible match with expected transformations.

Table 4: Default Differential Prediction list for Metabolite Identification.

m/z	Modification/Info	Reaction
-42.04700	[-42.047] depropylation	-C3H6
-42.01060	[-42.011] deacetylation	-C2H2O
-33.96228	[-33.962] -HCL on GSH Adduct	-HCL +2H
-29.97420	[-29.974] N-reduction [nitro group]	+H2 -O2
-28.03130	[-28.031] 2 x demethylation	-C2H4
-26.01570	[-26.016] oxidation + deacetylation	-C2H2
-16.03130	[-16.031] demethylation + dehydrogenation	-CH4
-15.99490	[-15.995] -O	-O
-14.01560	[-14.016] demethylation	-CH2
-13.97930	[-13.979] hetero oxide reduction + hydrogenation	+H2 -O
-12.03640	[-12.036] oxidation + 2 x demethylation	+O -C2H4
-12.00000	[-12.000] demethylation + hydrogenation	-C
-2.01570	[-2.016] dehydrogenation	-H2
-0.03640	[-0.036] oxidation + demethylation + dehydrogenation	+O -CH4
+0.00000	[+0.000] parent	---
+0.98400	[+0.984] oxidative deamination	+O -HN
+1.97930	[+1.979] oxidation + demethylation	+O -CH2
+2.01570	[+2.016] hydrogenation	+H2
+4.03130	[+4.031] 2 x hydrogenation	+H4
+13.97920	[+13.979] oxidation + dehydrogenation	+O -H2
+14.01560	[+14.016] methylation	+CH2

The first step is to select the parent from the result table. In this case, sort the table on Peak Height and select the Clozapine GSH Adduct m/z 632/635 entry as the “Parent”. The algorithm will check all possible modifications relative to the m/z 632. 205 peak.

To start the identification select from the Menu: *Identification > Find Metabolite/Degradation Peaks*. You may also directly click on the **Meta** button for the Icon Toolbar. The Identification GUI will be shown (Figure 16). For a full description read the manual.

The m/z of the “Parent” is directly taken from the active entry in the result table. If the result table does not contain the GSH Adduct of the Parent, you can enter the expected m/z value manually.
The search options are selected for positive ionization mode and the Metabolite ID list. Furthermore, the search will be performed in accurate mass mode. The maximum allowed difference between calculated and measured m/z value can be expressed in ppm. Default the algorithm uses 15 ppm.

Optionally, you can mark the Apply Chaining check box. ID Chaining is a new feature in MsXelerator. The program performs a first pass run to find possible metabolites in the list. If positive ID’s are found, the program runs the Identification procedure a second time, but now it will use the actual m/z value of the identified metabolites from the first run.
Example: suppose that, compared to the selected parent, the algorithm finds a demethylation product (delta m/z -14.016 > m/z 618.19). Then, in the next pass, the identification check is made, but now using m/z 618.19 as the “Parent”.
Chaining is basically a procedure to extend the search space.

After the ID check, results are sorted. Identified entries are placed at the top of the result table. Table 5 shows the result table with Identification results added to the end of the table. The header also shows the m/z value used as “Parent”.

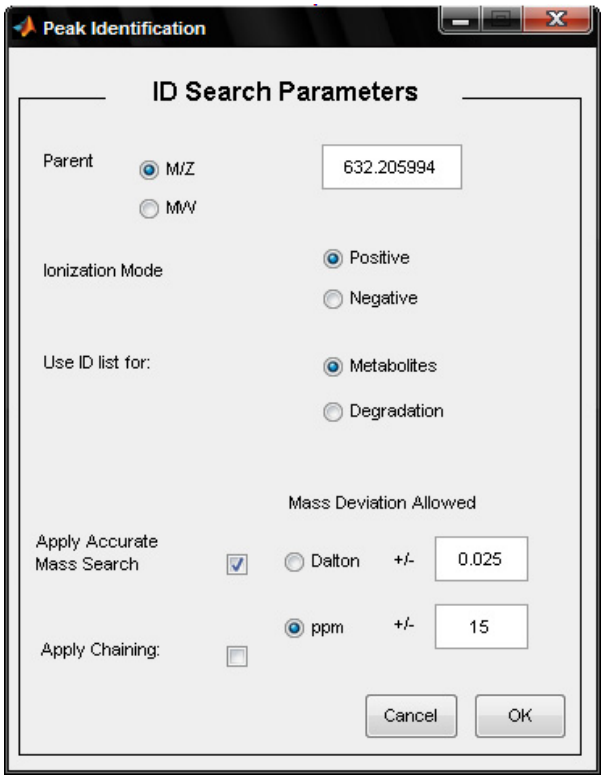


Figure 16: Metabolite ID GUI

Table 5: IPeaks result table with possible (reactive) metabolites identified

Pair	m/z	Ratio	tR	PH	%PH	%Area	PPM	Metabolite ID m/z(632.206)
1	632.20599	1.14	11.34	3021142.5	100.00	100.00	-0.001	[+0.000] parent
1	635.20941	1.14	11.34	2614334.8	100.00	100.00		
2	503.16342	1.20	11.38	944993.0	31.28	42.23	0.055	[-129.043] loss of gamma glutamate (GSH)
2	506.16699	1.20	11.38	737856.6	31.28	42.23		
3	632.20642	0.96	10.71	107254.9	3.55	4.23	0.675	[+0.000] parent
3	635.21002	0.96	10.71	99604.9	3.55	4.23		
4	618.19043	1.19	10.79	97535.6	3.23	3.25	0.058	[-14.016] demethylation
4	621.19415	1.19	10.79	79640.3	3.23	3.25		
5	503.16382	1.09	10.71	78307.4	2.59	2.63	0.843	[-129.043] loss of gamma glutamate (GSH)
5	506.16708	1.09	10.71	64167.7	2.59	2.63		
6	650.21613	1.11	11.12	62960.3	2.08	2.86	-0.721	[+18.011] hydrolysis
6	653.21985	1.11	11.12	60631.2	2.08	2.86		
7	662.21667	1.29	10.52	59164.5	1.96	3.35	0.122	[+30.011] methylation + oxidation
7	665.21918	1.29	10.52	45000.7	1.96	3.35		
8	632.20612	0.98	13.72	39278.9	1.30	1.68	0.193	[+0.000] parent
8	635.20966	0.98	13.72	45468.5	1.30	1.68		
9	598.24518	1.49	7.05	22724.2	0.75	0.84	2.448	[-33.962] -HCL on GSH Adduct
9	601.24841	1.49	7.05	11210.8	0.75	0.84		
10	650.21704	0.96	8.27	16348.4	0.54	0.59	0.687	[+18.011] hydrolysis

The PPM value, shown before the expected mass differences, is the calculated mass difference between the actual measured peak and the calculated m/z value for the Metabolite (all relative to the m/z of the parent 632.206). As you can see all identified hits have very small deviations.

In total 10 pairs can be identified directly. If chaining was applied, additionally four entries can be identified. When chaining is used, the ID is probably displayed further downwards in the table. Table 6 shows part of the table that includes one of the peaks identified from chaining.

The second entry from table 6, identifies the 598/601 pair as the loss of HCL from the GSH Adduct. Chaining will now use this entry as the so-called “Parent” and the ID run will search for possible hits based on the 598/601 pair. As you can see the first entry is the result from chaining. The ID is the loss of gamma glutamate (-129) from the 598 peak. The information shows that the result is due to chaining with the m/z 598 peak.

Table 6: ID results including Chaining

2	469.20245	1.35	7.02	21436.4	0.71	0.94	-0.266 Chain m/z: 598.245[-129.043] loss of gamma glut
2	472.20605	1.35	7.02	16494.2	0.71	0.94	
3	598.24518	1.49	7.05	22724.2	0.75	0.84	2.448 [-33.962] -HCL on GSH Adduct
3	601.24841	1.49	7.05	11210.8	0.75	0.84	

Storing selected peaks to a fixed IPeaks Container

Storing tables can be done at any moment by selecting the save button from the IPeaks menu or by *choosing File > Save Table.* You will be asked to enter a comment so that stored tables can be recognized when loading from disk.

However, IPeaks also has the possibility to store **selected peaks** to a special container named **IPeaks Store.** Entries selected can be added to this table. Storing to the IPeaks Store Table, provides a way to save intermediate results to a special table. E.g. the user can decide to store all peaks that can be identified, first from the adduct search and next from the Identification search. The IPeaks Store table can be loaded and edited when needed.
Currently, peaks that are added will not be checked if they already are present in the IPeaks Store table.
To use the IPeaks Store, select *Store Selection* from the task buttons below the IPeaks Table.

Reconstruction of the Accurate Local TIC

For presentation purposes and reporting, it is useful to reconstruct the accurate TIC based on the entries in the IPeaks table. Since the reconstructed profile will contain the positions and all m/z values of the hits, co-eluting peaks can be made visible.

To reconstruct the TIC, select *Menu > Task > Recalculate Accurate Local TIC.* You will get a message that the reconstruction will be based on the first peak in each pair. Peaks can be labeled with their m/z value if needed. The accuracy of reconstruction will be based on the resolution set in IPeaks. Current value is a window of 0.005 Da. In the next question you will have to enter the expected width of the peaks. In this case a value of 5 scans was used. This value specifies the width used for local extraction of each accurate EIC. The risk of using a too wide width is that additional peaks might pop-up in the reconstruction. Finally you will be asked if the reconstructed TIC should replace the normal TIC.

Figure 17 displays a zoomed part of the locally reconstructed TIC. Peaks are labeled with them/z value at their respective retention times and the label (arrow) is positioned at the peak height of each peak. From Figure 17, the hits and the co-eluting peaks due to loss of gamma glutamate (-129) are evident. Clicking on a peak in the figure will mark the nearest peak in the table.

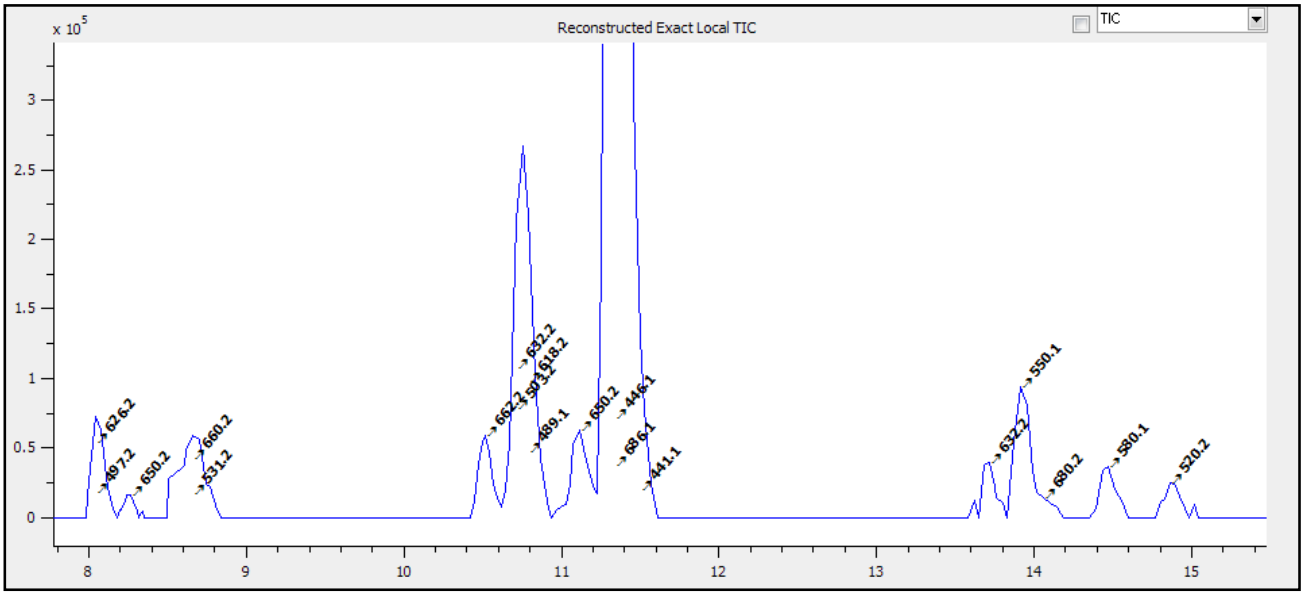


Figure 17: Reconstructed Accurate Local TIC

Post-Processing: Checking Multiple Control Samples

In general, the IPF application for detection of reactive metabolites is based on a single sample and a control. However, if you have more than one control or additional samples from other drugs, you might want to use these during the High Resolution Post-Processing run. The algorithm has the possibility to check multiple control samples in one run. The prerequisite of using multiple control samples is that the samples should be run close after each other, so that the chromatography is more or less comparable (to be tuned with the time window parameter).

The Clozapine sample was run in series with Diclofenac and Imipramine. The last two samples can be used as additional control samples in the High Resolution Post Processing run for Clozapine. To add multiple control samples during post processing, use Browse and select to add the samples to the control list. You might want to uncheck all other filters and to use labeling for output. In this case you can see which peaks are also present in both other samples.

Results:

It appears that entry 19 from table 1, m/z 441.09 is also present in the Diclofenac and Imipramine sample. This is also true for entry 21, m/z 529.13. Entry 16, m/z 520.17 and entry 7, m/z 446.10 are present in the Diclofenac sample, not in Imipramine. These 4 hits could not be identified using the Identification Prediction list, were not present in the Adducts list, did not contain chlorine and did not form charge 2+ ions. Furthermore, when using the Mass Defect check it appears that 3 out of 4 hits did not pass the Mass Defect Filter.

Conclusion: if possible try to include additional controls or samples. If due to changes in the chromatography peaks are less well aligned, you need to use a wider time window in the control check. The default value is 0.3 minutes. It can be changed to any value the user thinks is applicable.

Checking IPF results using MS^2 Neutral Loss Scans

The results from Table 2 show that quite some number of peaks easily split of gamma glutamate. This results in co-eluting peaks having a mass 129 Da lower compared to the mass of the metabolite. It is well know that Neutral Loss scanning using the loss of 129 Da is a general procedure to detect reactive metabolites in GSH assays. The neutral loss scan is based on detection of certain masses in the MS^2 spectrum that show the expected difference from the precursor ion.

The processing of neutral loss scans is handled by the **IPF III** algorithm. It can be used next to the full scan methods IPF I and IPF II. In IPeaks the user has the possibility to quickly create a table from all MS^2 precursor ions that show the expected neutral loss. The retention time of the precursor ions are linked to the full scan retention times. This makes direct linking with results from IPF I and IPF II easier. Results from IPF-I can be linked, compared or merged with results from the Neutral Loss procedure (IPF III).

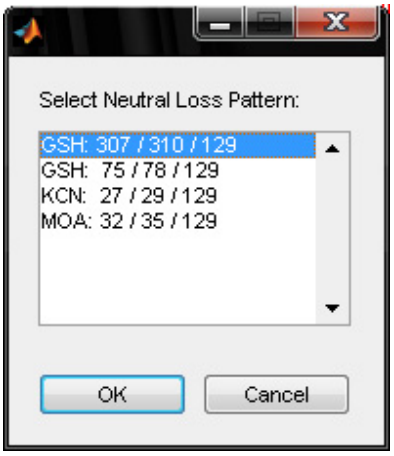
This tutorial will demonstrate how to create so-called "Neutral Loss" tables and how they can be used in co-operation with the full scan methods (IPF-I and IPF-II). All graphical tools and algorithms in IPeaks can also handle the Neutral Loss Table results (e.g. Identification, Post Processing etc.).

Construction and Loading a Neutral Loss Table:

The construction of a Neutral Loss Table consists of a series of steps:

- Specifying the neutral loss (NL) ions to be checked (IPF III currently handles three NL ions)
- Setting the mass accuracy
- Specify the intensity of the NL ions (absolute intensity or relative)
- Remove redundant MS^2 scans (belonging to same peak)
- Remove MS^2 scans that don't include the correct Neutral Loss ion
- Optionally, recalculate the exact precursor mass from the full scan. Xcalibur rounds the precursor ions to 2 decimals. Depending on the accuracy needed, the user can recalculate the exact m/z values.

All items related to Neutral Loss data processing are available from the IPeaks Neutral Loss Menu.

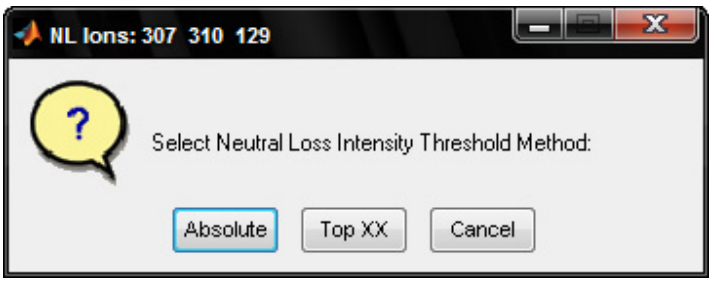


Step1: Construction of a Neutral Loss Table: from the Neutral Loss Menu *select > Load/Create MSMS Neutral Loss Table*. If the table was created in a previous session, you will be asked if it should be loaded from disk. We will create a new table, so select No. What follows, is a series of questions to create the NL Table.

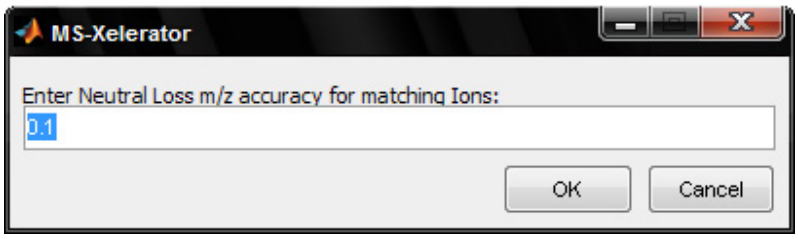
First, you will be asked to specify three Neutral Loss ions. IPeaks uses a predefined list (GSH, KCN, MOA), but you can also enter the Neutral Loss m/z values manually. In this case select *List*. The following window to the right will be shown.

Select the appropriate NL ions to be used for the sample. For Clozapine we will select the first line. The MS^2 scans will be searched for neutral losses of 307, 310 (labeled) and 129. Press *OK*.

Next you will get a question how to determine the NL ions from the MS^2 scan. You may set an absolute intensity value, but the default procedure is to check that the specified Neutral Loss ions should be in the top 5 largest ions of the MS^2 scan. You can specify a different number for the Top XX check. Select *Top XX* and *5* in the next question.

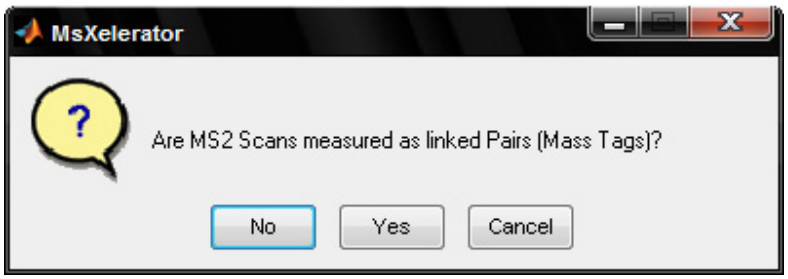


Next you will get a question regarding the mass accuracy to be used.



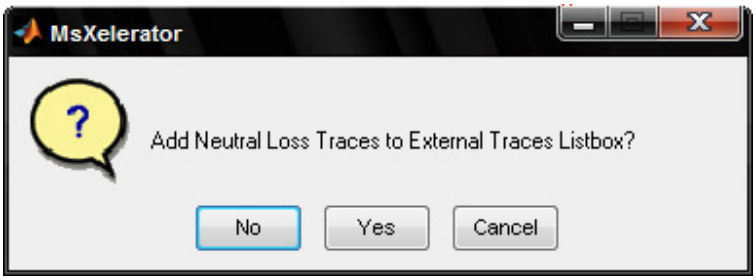
The value is necessary to determine the allowed difference between calculated mass of the Neutral Loss and the measured mass from the MS^2 spectrum. For Orbitrap data you can accept the value of 0.1 Da. For low resolution instruments (LTQ) you might consider to select a value of 0.5 Da. For Clozapine accept the default value and Press *OK*.

All 584 MS^2 scans are now read and checked on the presence of the Neutral Loss ions occurring in the top 5 intensities (given the specified accuracy). Next you will get a question if MS^2 scans are measured as linked pairs.

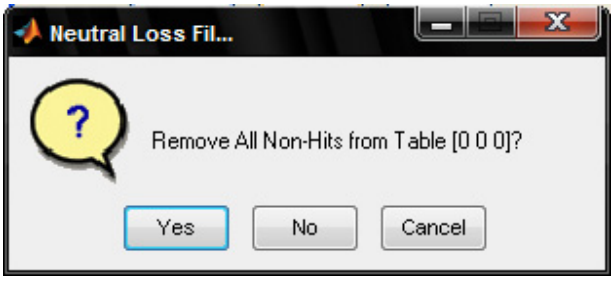


If, in the data acquisition procedure, it was specified that the MS^2 scan should be performed on both **the light and heavy isotope ion**, then the MS^2 scans are linked as pairs. If this is the case, select *Yes*, otherwise select *No*. Paired MS^2 scans will be listed as pairs in the NL table. For the Clozapine example, no paired scans were measured, so select *No*.

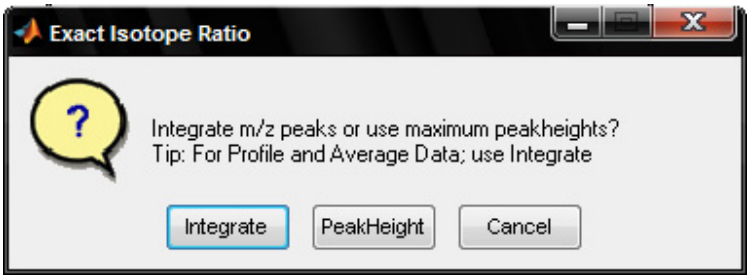
Next you will get the following question below. For all tree NL ions a so-called neutral Loss chromatogram will be constructed. These can be regarded like a TIC or BPC. If you select *yes*, the three NL chromatograms are added to the External List Box and can be used in overlay plotting together with the EICs from the table. Neutral Loss Chromatograms can also be plotted to a separate window afterwards. This option is available from the Neutral Loss Menu, *Plot Neutral Loss Mass Chromatogram.*



A total of 584 MS^2 scans are present. Of course you only want to see precursor ions for which a Neutral Loss ion was detected. Therefore, in the question below, select *Yes.* If you specify *No*, all precursor ions will be loaded into the table. If none of the NL ions was detected the line shows 0 0 0, otherwise a one will be placed at the corresponding NL ion.

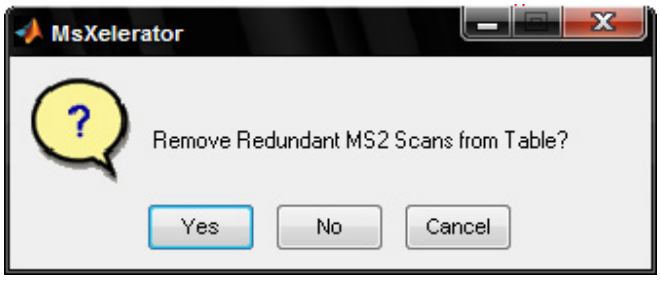


The Neutral Loss table will be constructed with the precursor ions for which the correct Neutral Losses could be detected. In the NL table we will however use the retention times from the Full Scan data. Therefore we will have to recalculate the intensity at the correct retention time. Based on a difference between centroided and profile data, we can use different methods to calculate the intensity. For these data in centroided mode, select Peak Height.



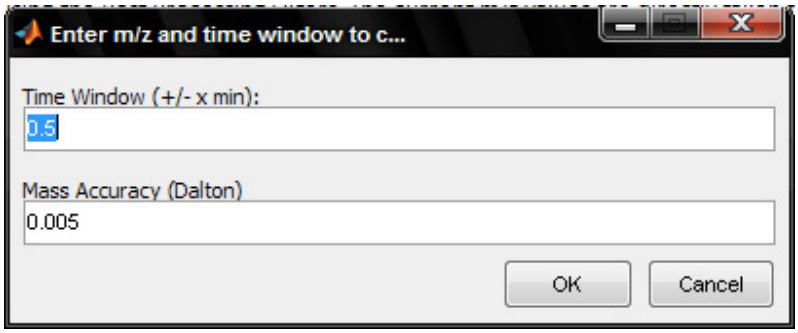
Quite a few MS^2 scans will be related to the same precursor, especially if peaks are broad. This means that a number of MS^2 scans are redundant and don't have to be loaded into the table. MsXelerator has the possibility to remove all redundant MS^2 scans. This makes the table much more compact. From the group of similar MS^2 scans it will only keep the one having the largest intensity.

So select *Yes*, when the question below pops up.



To determine redundant MS^2 scans the algorithm needs to know two parameters: the time window and mass accuracy. It will be checked if scans are similar if they elute in the same time window and have comparable mass values. The time window is set by default to a value of 0.5 minutes. The m/z accuracy is taken from the Mass Accuracy used by IPeaks. For Orbitrap data you can use of value of 0.05 Da.

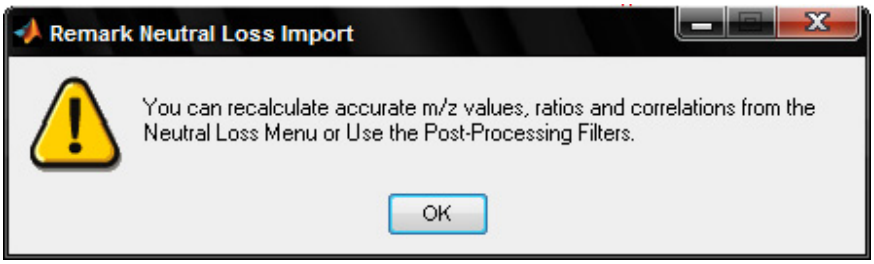
Remark: During the construction of the Neutral Loss table the results based on all MS^2 scans are automatically saved to disk. The full NL table can always be reloaded from disk. You can always remove redundant scans afterwards. The redundancy check is available from the Neutral Loss menu.



Finally you will get a remark that the accurate m/z values, ratios and correlations can be recalculated using the Neutral Loss Menu Tools or by using the Post Processing Filters. The current m/z values are directly taken from the information present in the MS^2 scan headers. **The accuracy found in the header of the MS^2 scan if often less due to rounding. You are advised to recalculate exact m/z values.**

It is suggested to directly perform the recalculation of correct accurate m/z values. In this way, plotting accurate EICs can be done using the correct m/z values and at very high resolution (0.005 Da, for Orbitrap). You can recalculate the accurate m/z values by selecting: *Menu > Neutral Loss > Recalc. exact m/z values and ratios*. Ratios will only be calculated if paired entries are found.

Example: for the largest GSH Adduct the MS^2 precursor has a reported mass of 632.21. The real Full Scan m/z value is however 632.206. This is a difference of 6 ppm. Such a large difference can influence further data processing like Identification etc.



The final NL table (recalculated m/z values) consist of 40 entries and part of this table is shown if Figure 18. Sorting is based on scan number (retention time). At this moment, all Ratio values are set to zero and also the Corr1 values that relates to the Correlation between light and heavy isotope. These can be calculated as will be shown on the next page. The comment field shows the score on the different Neutral Loss Ions (307 310 and 129). A value one means that the NL ion was detected in the MS^2 spectrum. Finally some information is shown if for the entry a MS3 scan was measured.

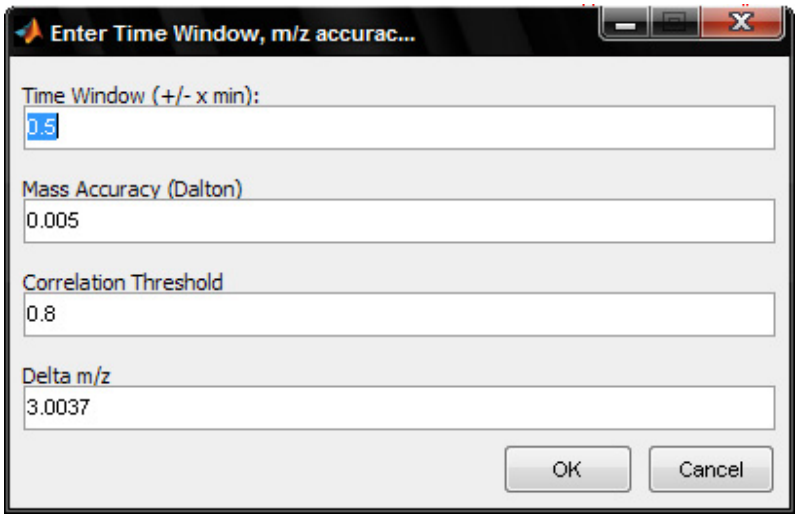
You can save this table as your starting result, before entering the Post Processing routines.

Table 7: Neutral Loss Table (40 entries). The table only contains precursor ions that have a positive score on the Neutral Loss check. Additionally, all redundant precursor ions have been removed and the table was recalculated to accurate m/z values based on full scan data.

Pair	m/z	Ratio	tR	PH	%PH	Corrl	NL	307/310/129(MS3)	
1	616.16412	0.00	2.52	80081.9	2.65	0.000	0	0	1 MS3
2	614.16357	0.00	2.57	33230.5	1.10	0.000	0	0	1 MS3
3	529.13153	0.00	3.40	15409.0	0.51	0.000	0	0	1
4	416.11313	0.00	4.60	52568.3	1.74	0.000	0	0	1 MS3
5	641.19135	0.00	4.96	44431.3	1.47	0.000	0	0	1 MS3
6	583.10394	0.00	6.25	86967.4	2.88	0.000	0	0	1 MS3
7	445.13919	0.00	6.73	15714.6	0.52	0.000	0	0	1 MS3
8	436.34283	0.00	7.30	24644.2	0.82	0.000	1	0	0
9	452.16052	0.00	7.57	29939.1	0.99	0.000	0	0	1 MS3
10	626.24005	0.00	8.04	54542.1	1.81	0.000	0	0	1 MS3
11	650.21704	0.00	8.27	16348.4	0.54	0.000	0	0	1 MS3
12	660.20154	0.00	8.66	42020.4	1.39	0.000	0	0	1 MS3
13	585.28955	0.00	10.35	48118.6	1.59	0.000	0	0	1
14	632.20642	0.00	10.71	107254.9	3.55	0.000	0	0	1 MS3
15	618.19043	0.00	10.79	97535.6	3.23	0.000	0	0	1 MS3

The Neutral Loss Menu in IPeaks contains some useful tools to operate on NL tables. The user can recalculate exact mass values, add the second heavy isotope to the table and perform a ratio analysis. You can also make use of all the features from the Task Menu.

However, it is much easier to perform a number of important tasks simultaneously using the Post Processing algorithm. From the Isotopes Menu select**: *Apply Post Processing Filters.*** It will be recognized that the current table is a Neutral Loss table. Therefore you will get two questions: you will be asked if accurate m/z values need to be recalculated. Select *Yes*, if not done before. And you will be asked if the second heavy isotope should be added to the table. Select *Yes*. In this way the ratios between light and heavy isotope will be calculated and the heavy isotope peak will be added to the table. Regarding the second question you will get the following window.



The information is needed to search for the second isotope and to do a shape analysis (correlation) between both isotopes. Default a value of 0.5 minutes will be used to correlate both isotopes and the m/z accuracy is taken from IPeaks. The correlation Threshold is not import here, as it will also be set in the Post Processing Algorithm. Important is that the correct Delta m/z value is entered. The value is taken directly from IPeaks Delta m/z Edit Box. You should use the **correct high resolution Delta m/z value** belonging to the labeling assay. Press *OK*.

The program will convert the Neutral Loss table to accurate m/z values and add the second heavy isotope. Also the ratios will be recalculated. Please remember that the ratio will be taken based on the retention time of the precursor ion (single scan). As we are still dealing with MS^2 precursors, this retention time does not necessarily have to be the peak maximum.

After this, the Post Processing window will pop-up and you can proceed as described on Page 9.
After Post Processing the table contains only 10 entries, shown in Table 8. During Post Processing, the results were compared to the control sample. Spikes, ^{13}C isotopes, charge 2+ ions and Cl isotopes have been removed and the accurate correlation was checked.

Table 8: Final Neutral Loss Table after Post Processing.

Pair	m/z	Ratio	tR	PH	%PH	Corr1	Corr2	S/N	FWHH
1	529.13153	1.81	3.40	15409.0	0.51	0.880	0.865	3.2	0
1	532.13523	1.81	3.40	8503.9	0.28	0.880	0.865	0.0	0
2	616.16412	1.81	2.52	80081.9	2.65	0.988	0.988	13.4	0
2	619.16782	1.81	2.52	44129.9	1.46	0.988	0.988	0.0	0
3	618.19043	1.22	10.79	97535.6	3.23	0.993	0.889	20.6	0
3	621.19413	1.22	10.79	79640.3	2.64	0.993	0.889	0.0	0
4	626.24005	1.55	8.04	54542.1	1.81	0.980	0.719	8.7	0
4	629.24375	1.55	8.04	35170.4	1.16	0.980	0.719	0.0	0
5	632.20599	1.16	11.34	3021142.5	100.00	0.999	0.997	627.5	0
5	635.20969	1.16	11.34	2614334.8	86.53	0.999	0.997	0.0	0
6	632.20642	1.08	10.71	107254.9	3.55	0.995	0.761	22.3	0
6	635.21012	1.08	10.71	99604.9	3.30	0.995	0.761	0.0	0
7	632.20679	1.59	13.68	37980.9	1.26	0.852	0.768	7.1	0
7	635.21049	1.59	13.68	23903.5	0.79	0.852	0.768	0.0	0
8	650.21613	1.04	11.12	62960.3	2.08	0.981	0.719	18.6	0
8	653.21983	1.04	11.12	60631.2	2.01	0.981	0.719	0.0	0
9	650.21704	1.09	8.27	16348.4	0.54	0.907	0.644	3.7	0
9	653.22074	1.09	8.27	15035.5	0.50	0.907	0.644	0.0	0
10	660.20154	1.19	8.66	42020.4	1.39	0.934	0.899	9.1	0
10	663.20524	1.19	8.66	35200.8	1.17	0.934	0.899	0.0	0

Creating an EIC – MS –MS^2 Overview Plot:

To create a summary overview plot for reporting, you can plot in one view the light and heavy EIC overlay, add the MS spectrum and the MS^2 spectrum (with the detected NL ion labeled in color). An example for the third entry in the table is shown in Figure 18. Create the Overview Plot by pressing the MS^2 Icon on the Toolbar, or use *Neutral Loss > Plot MS2 Overview.*

The plot was created by selecting the *Plot to Separate Window* option. Due to the fact that the m/z values have been recalculated, you will get a question on this topic. Press *Yes* and select the default search parameters. From the plot you can see the nicely co-eluting peaks plotted in high resolution, the correct mass difference in the MS plot and the MS^2 spectrum with the Neutral Loss of 129 plotted in purple (m/z 489.15 = 618.19 – 129.04).

Based on the Final Result Table you can plot the accurate reconstructed TIC as was discussed on Page 23, Figure 17.
You can also Plot the original Neutral Loss Chromatogram and mark the entries from the current table. In Figure 19, the original Neutral Loss Chromatogram for NL 129 is plotted by selecting, *Menu > Neutral Loss > Plot NL Mass Chromatogram*. The intensities are taken from the MS^2 Neutral Loss ions.

The peaks labeled in the plot relate to the remaining entries in the Table. You can see that the Post Processing cleared many ions which were probably also found in the control sample. Compare this result with the accurate TIC from IPF II (Figure 17).

The Overview Plot is also available for IPF I and IPF II. The Neutral Loss result file must be present on disk. If not, you will be asked to create it.

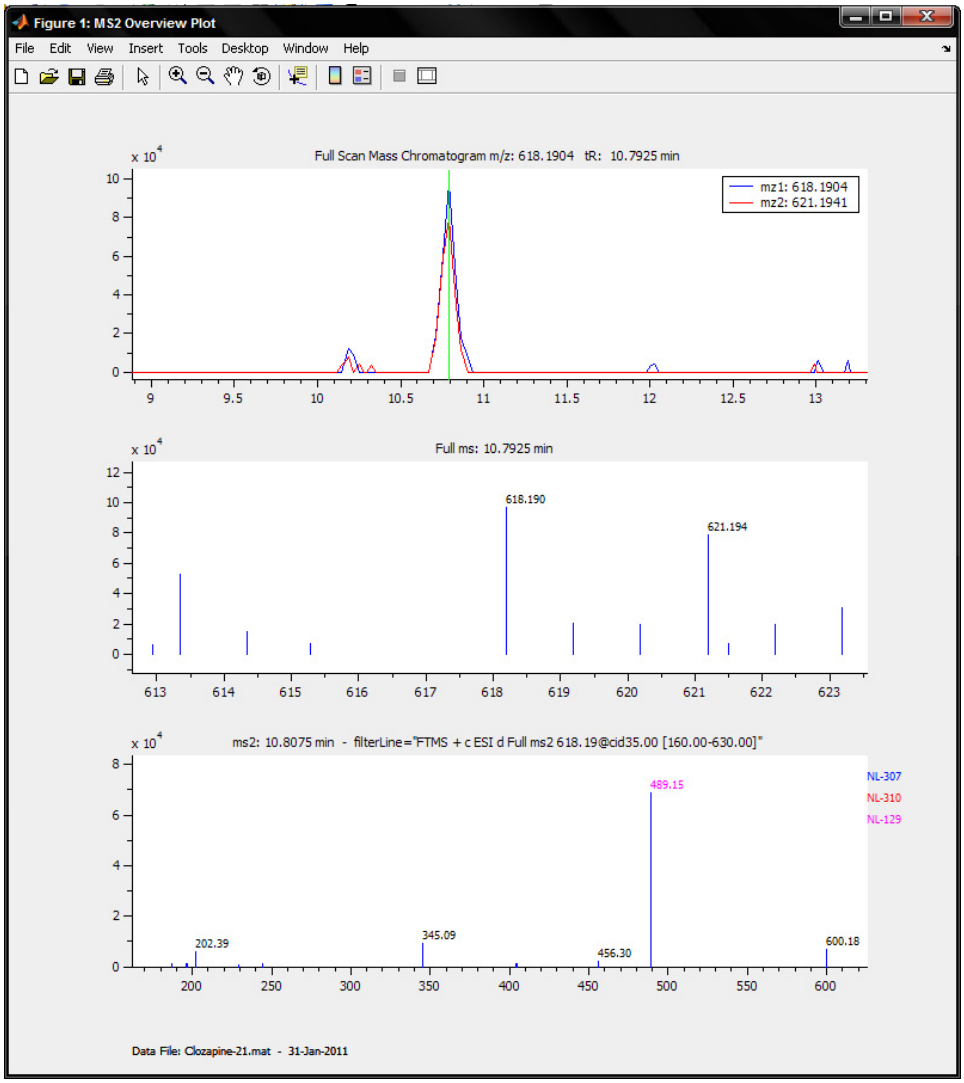


Figure 18: IPF Overview Plot showing light and heavy EIC in overlay, the MS spectrum and the MS^2 spectrum with the detected NL ion marked (m/z 489.15).

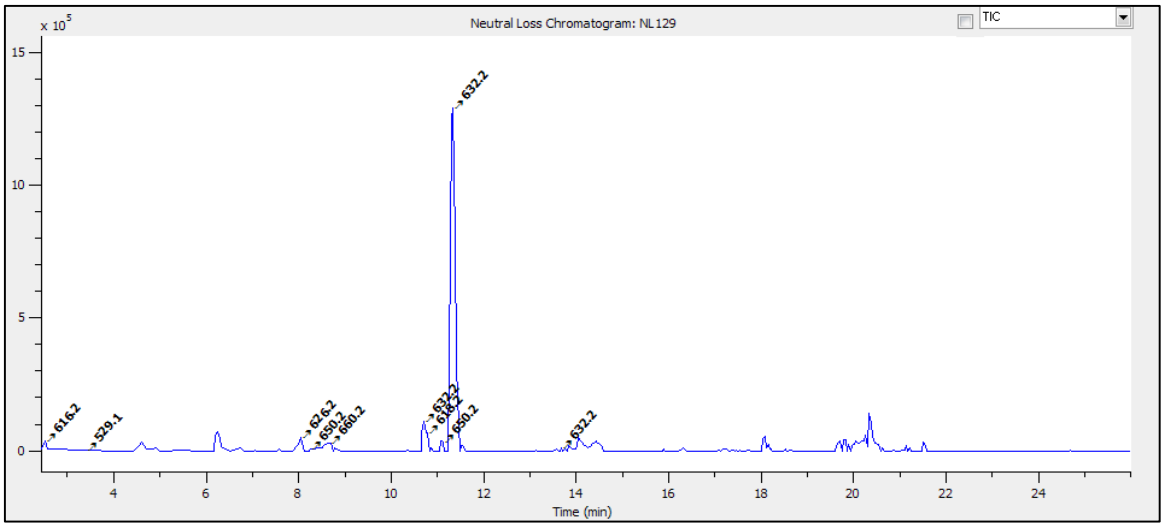


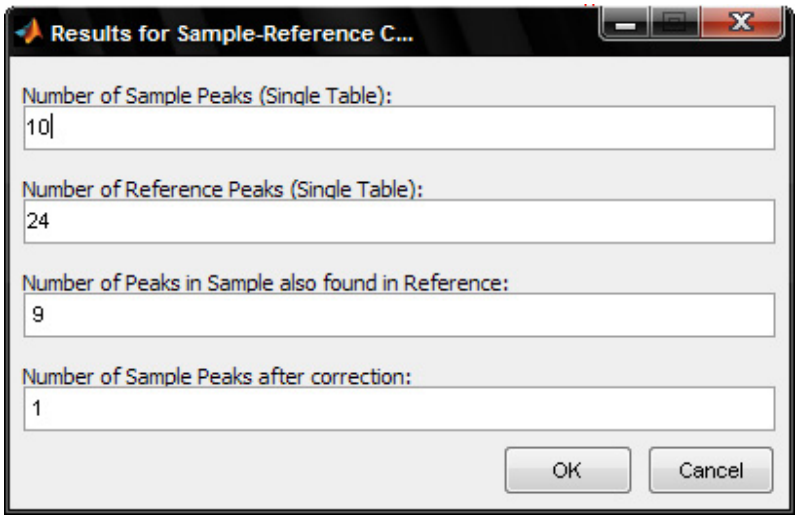
Figure 19: Neutral Loss 129 Chromatogram. Only entries present in the NL table are marked.

Comparing High Resolution IPF II results with Neutral Loss (IPF III) results /Merging Tables:

IPeaks has the possibility to compare two result tables and to show which peaks are different. Based on the current active Neutral Loss table with 10 entries we will check which of these peaks were also found in the Final IPF II result.

To compare the tables select: *Task > Find Differences between 2 Tables*. In this case the tables belong to the same sample, but you also have the possibility to compare tables from different samples. Press *Yes*.
You will get a warning to use a somewhat wider time window if one of the tables is a Neutral Loss Table, because the peak retention time does not have to be the peak maximum. For now use 0.5 minutes and a Peak Difference factor of 5.0

You will get a summary overview for the comparison. The number of peaks in both tables will be reported. The third line reports the number of peaks in the current sample that are also present in the table to be compared. 9 peaks are the same and only one entrance was not found in the IPF II table.



The table will be sorted based on the results above. Peaks that are equal are marked as “Reference Peak”. The last peak in the table is not present in the IPF II result table. The reason seems to be the high ratio between light and heavy isotope. The current NL table was not checked for ratios limits to fall in the range 0.7 to 1.5.

Table 9: Comparison of Neutral Loss Table and IPF II Result Table. Equal peaks are marked.

Pair	m/z	Ratio	tR	PH	%PH	Corrl	Reference Info()
3	629.24375	1.55	8.04	35170.4	1.16	0.980	Ref. Peak
4	632.20599	1.16	11.34	3021142.5	100.00	0.999	Ref. Peak
4	635.20969	1.16	11.34	2614334.8	86.53	0.999	Ref. Peak
5	632.20642	1.08	10.71	107254.9	3.55	0.995	Ref. Peak
5	635.21012	1.08	10.71	99604.9	3.30	0.995	Ref. Peak
6	632.20679	1.59	13.68	37980.9	1.26	0.852	Ref. Peak
6	635.21049	1.59	13.68	23903.5	0.79	0.852	Ref. Peak
7	650.21613	1.04	11.12	62960.3	2.08	0.981	Ref. Peak
7	653.21983	1.04	11.12	60631.2	2.01	0.981	Ref. Peak
8	650.21704	1.09	8.27	16348.4	0.54	0.907	Ref. Peak
8	653.22074	1.09	8.27	15035.5	0.50	0.907	Ref. Peak
9	660.20154	1.19	8.66	42020.4	1.39	0.934	Ref. Peak
9	663.20524	1.19	8.66	35200.8	1.17	0.934	Ref. Peak
10	616.16412	1.81	2.52	80081.9	2.65	0.988	
10	619.16782	1.81	2.52	44129.9	1.46	0.988	

Of course you can also perform the reversed operation; for this you should first load the IPF II Table from disk.
The same type of summary will be reported. However, as the IPF II table contains more entries you can now easily see which are in common and which are different.

Based on the loaded IPF II table you can also add the Neutral Loss info for the three selected NL ions. You don't need to have a saved NL table, but the construction of the full MS^2 NL result file must have been performed. Select from the menu: *Isotopes > Check IPF results on Neutral Loss Presence.* The result table will be sorted and marked with the NL ion scores, as displayed in Table 10.

Table 10: IPF II result table with Neutral Loss Ions and their presence or absence marked. The current entries all have a NL 129 loss detected in the MS^2 scan.

Pair	m/z	Ratio	tR	PH	%PH	%Area	NL 307/310/129()		
1	529.13214	1.46	3.35	17295.3	0.57	1.40	0	0	1
1	532.13617	1.46	3.35	7882.0	0.57	1.40			
2	626.24005	1.34	8.04	54542.1	1.81	1.79	0	0	1
2	629.24359	1.34	8.04	35170.4	1.81	1.79			
3	650.21704	0.96	8.27	16348.4	0.54	0.59	0	0	1
3	653.22052	0.96	8.27	15035.5	0.54	0.59			
4	660.20154	1.04	8.66	42020.4	1.39	3.35	0	0	1
4	663.20441	1.04	8.66	35200.8	1.39	3.35			

Merging Two Result Tables:

To merge two result tables and to add only the unique peaks of the second table to the first table, use Merging. From the task menu select *> Merge two result tables.*

When asked to mark both tables, select *Yes*. In this way you can see which result belonged to which table. Next you can remove redundant ions. It is no so useful to have all equal peaks in the combined table. So press *Yes* and select the appropriate time window and accuracy. Only unique peaks from the Neutral Loss Table will be added (Table 11). It is seen that one additional peak has been added, the m/z 616.16/619.16 entry for which an explanation has been given.

In conclusion: IPF II did not miss any peaks compared to the IPF III Neutral Loss procedure. You need to take some extra time in trying to explain the identity of the additional detected peaks. If too many peaks are found, you can always set an intensity threshold as a percentage of the largest peak in the table. A limit of 1% compared to the largest reactive metabolite found seems useful, but setting the actual threshold level is left to the user.

Table 11: IPF II table merged with Neutral Loss Table. Tables can be distinguished by the Table Type comment. Only unique peaks have been added to the IPF II table (entry 25).

23	583.13025	1.42	14.47	28274.2	1.20	0.968	IPF II
24	520.17566	1.10	14.86	25072.9	0.83	0.817	IPF II
24	523.18018	1.10	14.86	21359.2	0.83	0.817	IPF II
25	616.16412	1.81	2.52	80081.9	2.65	0.988	FSNL
25	619.16782	1.81	2.52	44129.9	1.46	0.988	FSNL

Summary Overview Isotope Pattern Matching for Detection of Reactive Metabolites:

- **Estimate Peak width, Intensity and Ratio using the Browser**
- **Run IPF II, followed by High Resolution Post Processing**
- **Check loss of Gamma Glutamate, Cl, Na, and K using the Isotope/Adduct Tool**
- **Check charge 2+ ions if applicable**
- **Perform Metabolite ID based on GSH Adduct of the Parent**
- **Check Neutral Loss 129 and others from MS^2 scans (IPF III)**
- **Add unique results from IPF III to IPF II table by merging**