Data Processing Using Thermo Freestyle Software with NIST MSMS Search for Compound Identifications

Setting Up NIST Search and Freestyle Parameters

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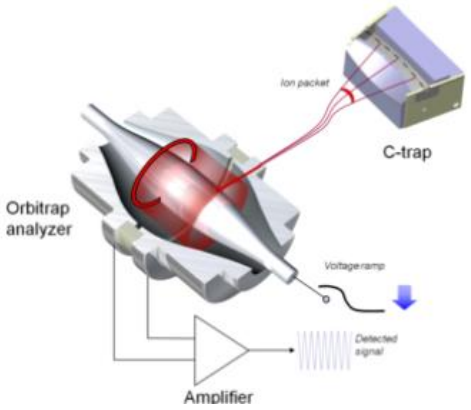
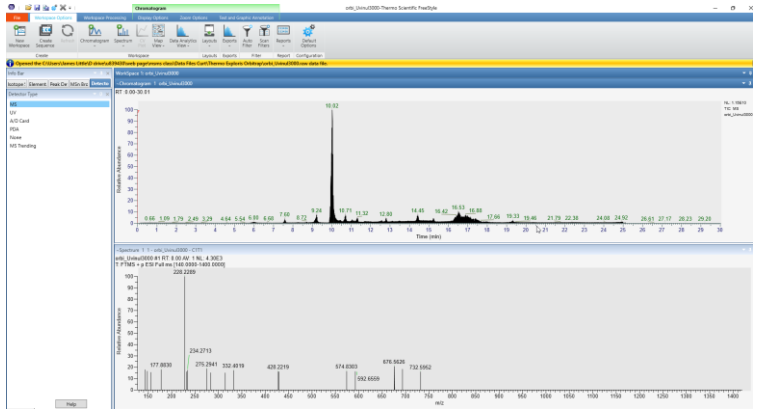
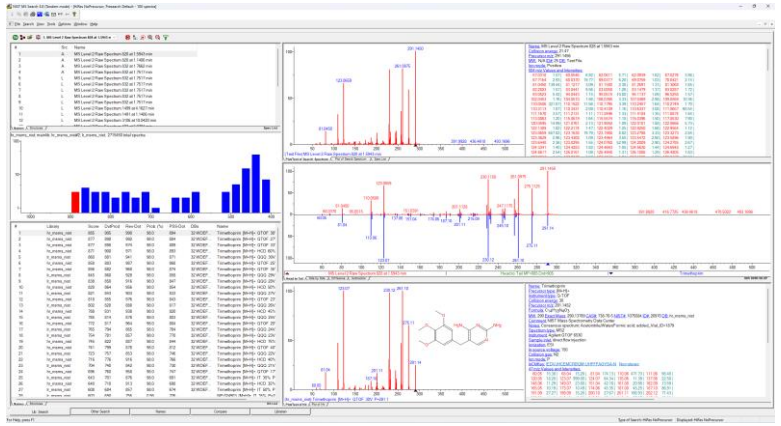
Thermo Orbitrap MSMS



Thermo Freestyle



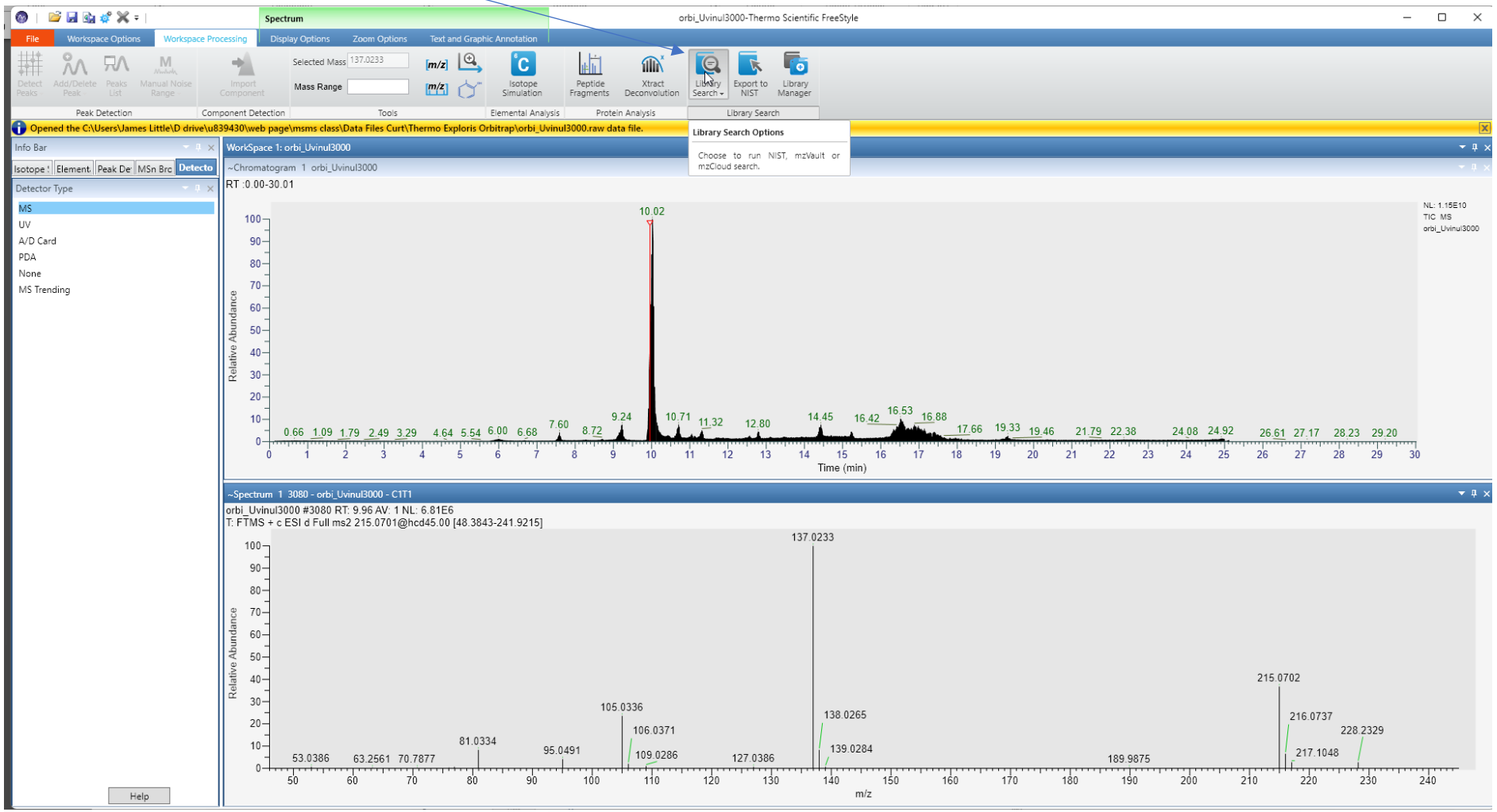
NIST MSMS Search



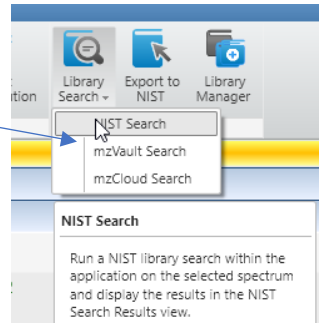
- Screenshots shown in ***this*** handout for settings
- These screenshots also discuss types of searches
- More information found on my website in “Advanced NIST MSMS Search Course”

Important Initial Settings in Freestyle and NIST Search

- Several important settings determined the mass accuracy of ions exported from Freestyle and then imported into NIST MS search
- Normally the precursor ion and all spectrum ions should show places to the right of the decimal
- First open a file in Freestyle and display either positive or negative MS2 spectrum
- Be sure to select the MS2 box by clicking on its window bar to make it active
- This will make the Library Search Icon in Workspace Processing be active
- Select it to start an internal NIST library search

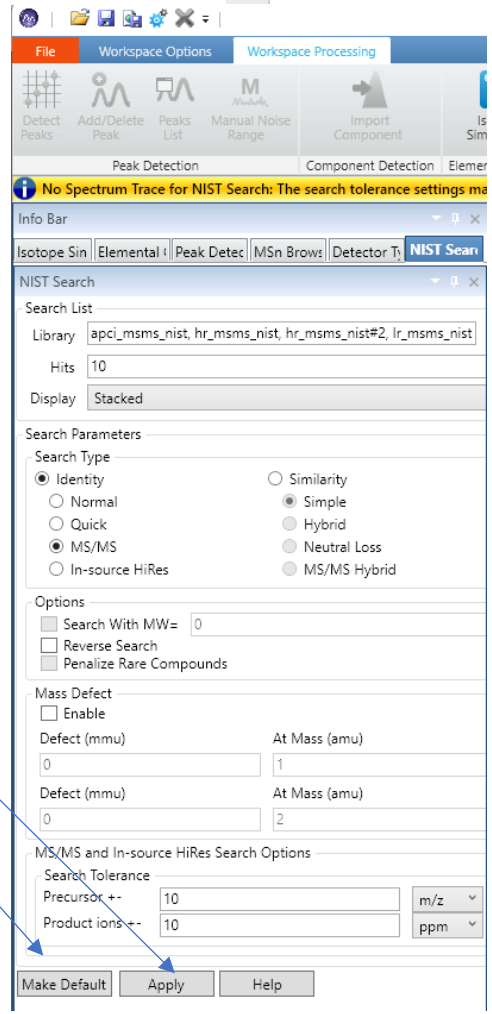


- Select the NIST Search under Library Search Icon
- This allows you to see the parameters in the ***internal library search***
- It ***also determines*** what is exported to NIST
- When first installed, it will probably have Normal radio button selected
- Change to MS/MS
- Then ***very important*** to click “Make Default” at bottom of window
- ***Then*** “Apply”
- This will export 4 digits to the right of decimal for *m/z* values



1

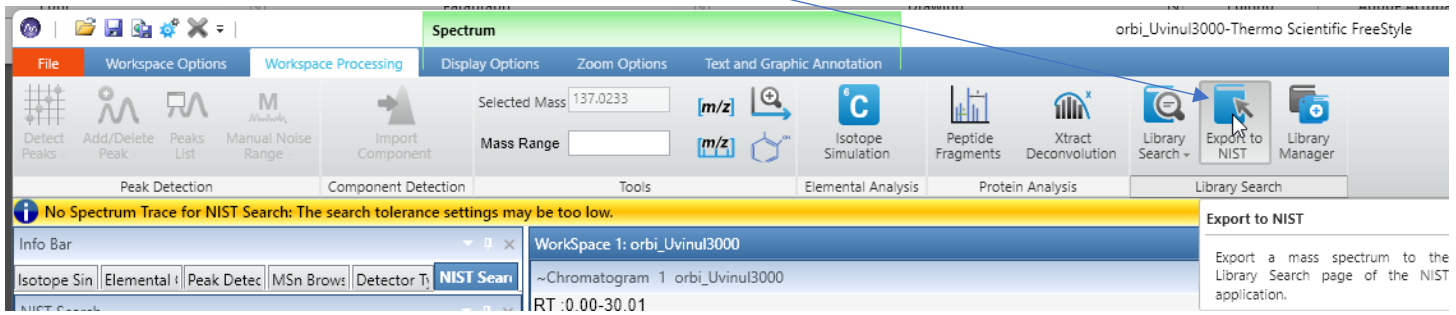
2



- Then after “Make Default” and “Apply” steps
- Close the bottom two windows below the MS1 (MSMS) window
- This is where the actual results would appear if searching NIST library within Freestyle
- Our searches will be external to Freestyle and performed in NIST MS Search program

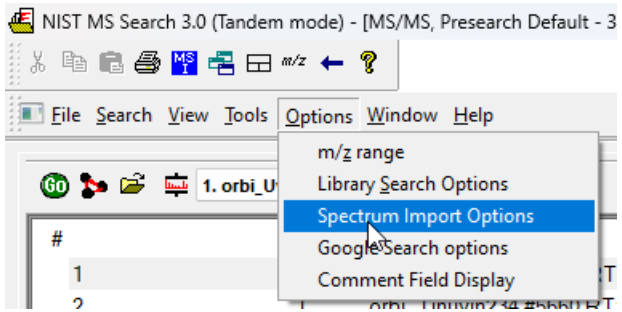
- Now you need to send an MS2 (MSMS) spectrum to the NIST search
- Select and MS2 (MSMS) spectrum by clicking on its Title bar to make the window active
- Then click on Export to NIST button in Workspace Processing Tab
- ***Remember***, accurate mass information for MS2 (MSMS) spectra is not sent with 4 significant figure to the right of decimal for averaged spectra, only single spectru!

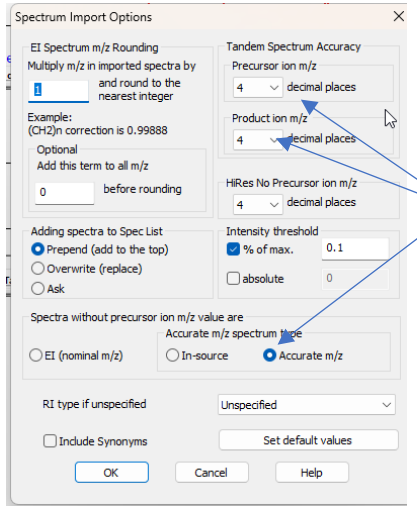
1



- This will start the external NIST MSMS Search
- After complete, click on the “Spectrum Import Options”

2



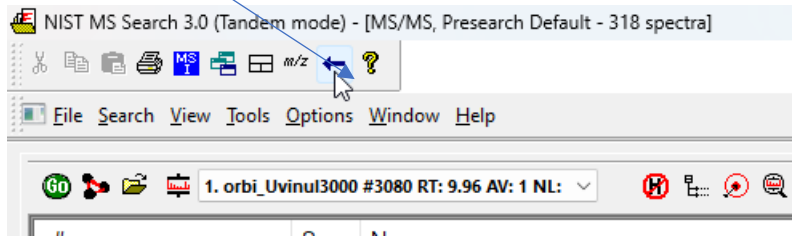


3

- Make Sure Accurate m/z Radio button is selected
- Make cure 4 decimal place for Precursot ion m/z and Product ion m/z
- Click OK to accept

4

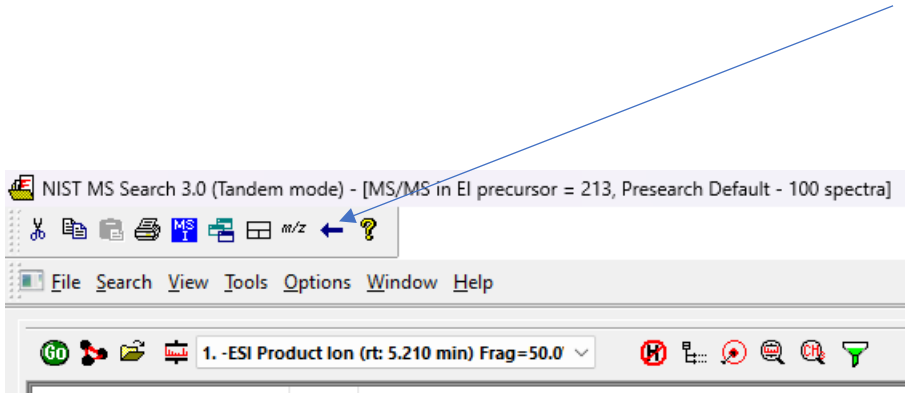
- Can return to Freestyle program by clicking on switch to caller arrow on menu bar



Typical Settings for NIST MS Search 3.0 (Tandem Mode)
Plus
Quick Start Guide Supplied by NIST for NIST23 Tandem MS Search

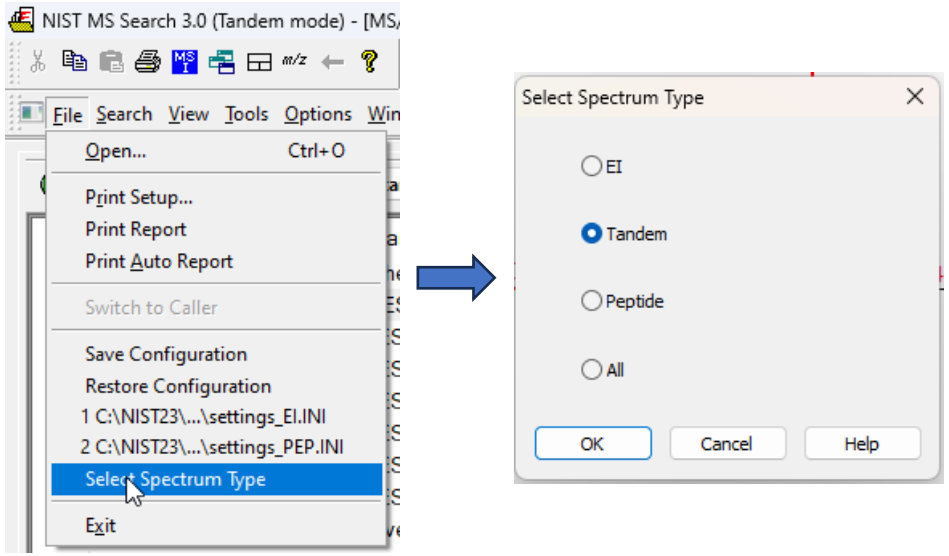
- I have supplied some screen shots with tips and suggestions for initial menu settings
- NIST also supplied a Quick Start Guide which is found attached to the bottom of my document
- Theirs labelled “Information for NIST 20 Tandem Library Users”

- To return to vendors program when processing data, click on the “Switch to Caller” Icon

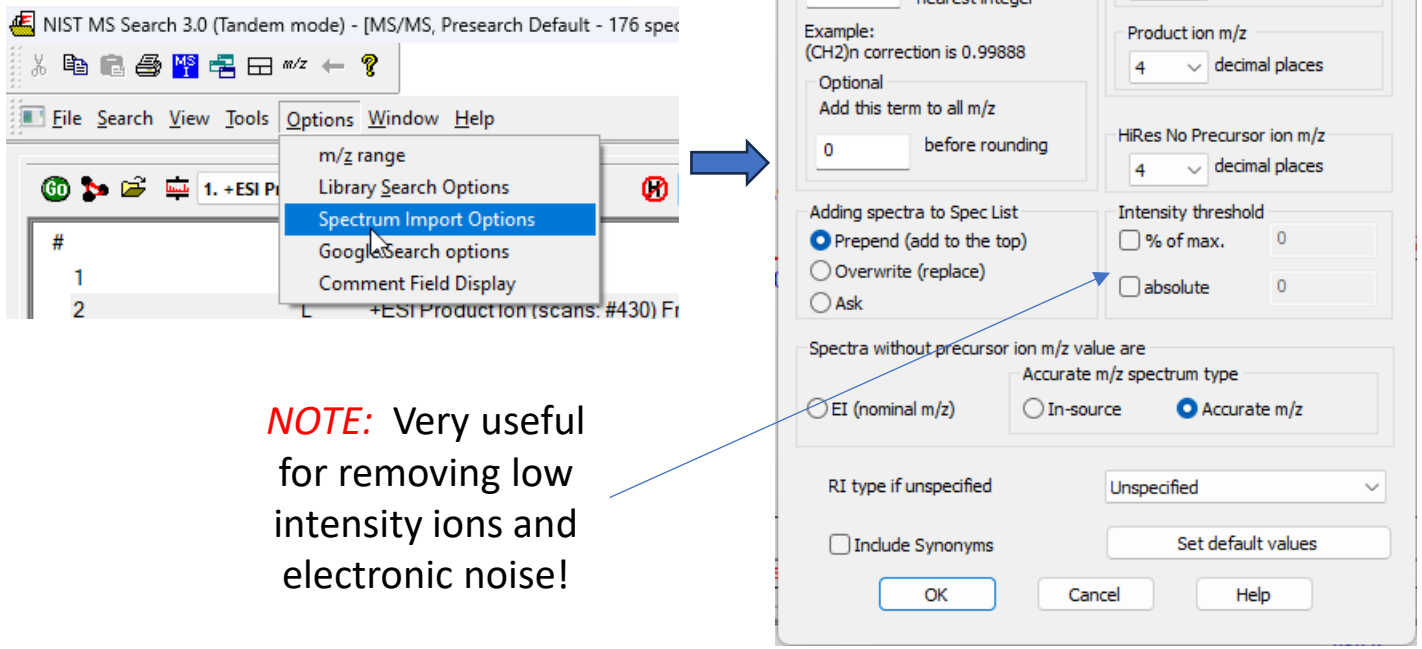


Typical Settings for NIST MS Search 3.0 (Tandem Mode)

v.3.0 NIST Search *Simplifies* Menus for Different Modes

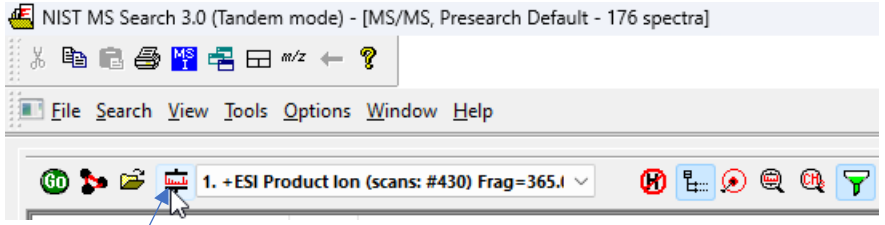


Typical Spectrum Import Options



NOTE: Very useful for removing low intensity ions and electronic noise!

Library Search Options for NIST MS Search 3.0 (Tandem Mode)



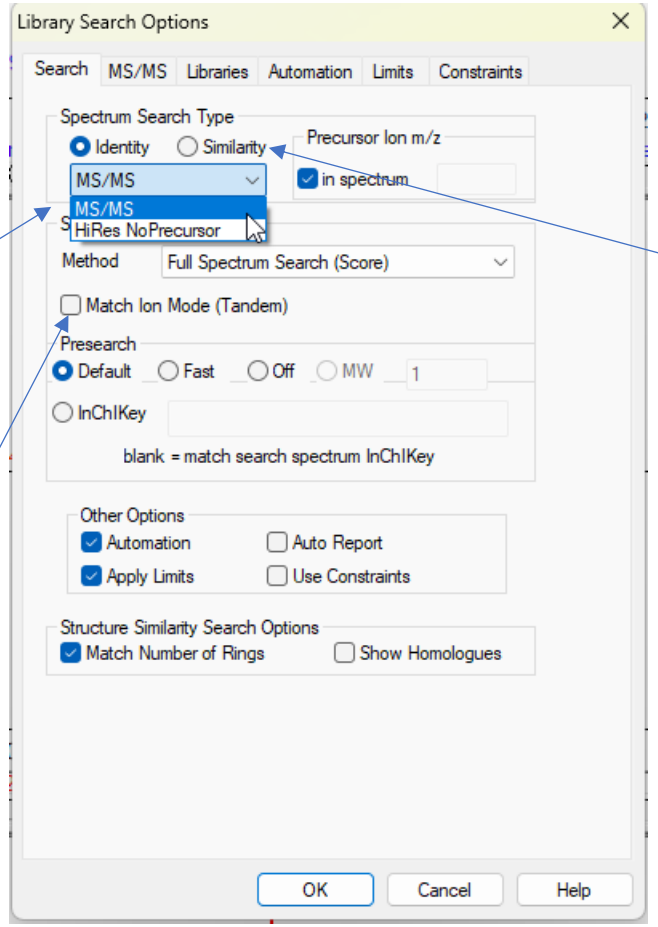
Click here

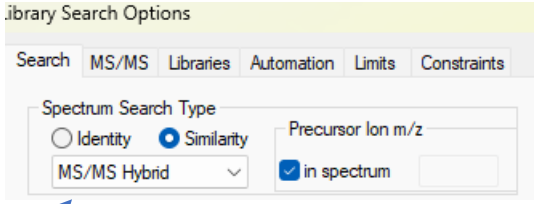
Can do Identity MS/MS which searches spectra restricting by the precursor ion and its specified *m/z* Tolerance set in MS/MS tab (this one returns fewer but likely more useful results if compound's spectrum is present in accurate mass format)

Or

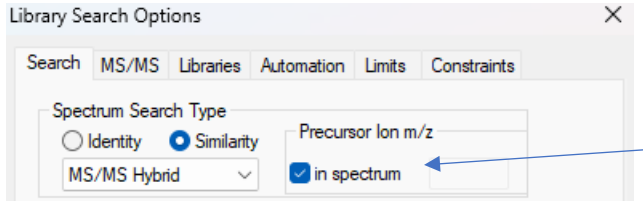
Identity/High Resolution No Precursor, just compares the overall fit of unknown spectra to reference spectra

DO NOT CHECK this box, caused some searches to fail!!

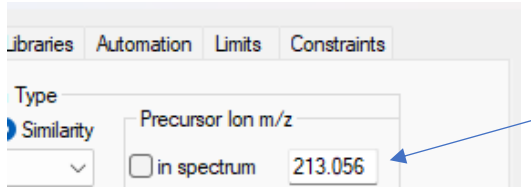




- Another type search is Similarity/Hybrid MS/MS which can be ***very*** useful if a related compound is present in the reference libraries, but the actual compound is *not* present
- See NIST MSMS ***Full*** Course for details on Hybrid Search
- The ***neg ion hybrid*** is ***not*** currently ***working***, delta mass values will be offset by 2, NIST forgot to correct for difference between M+H and M-H, thus 2



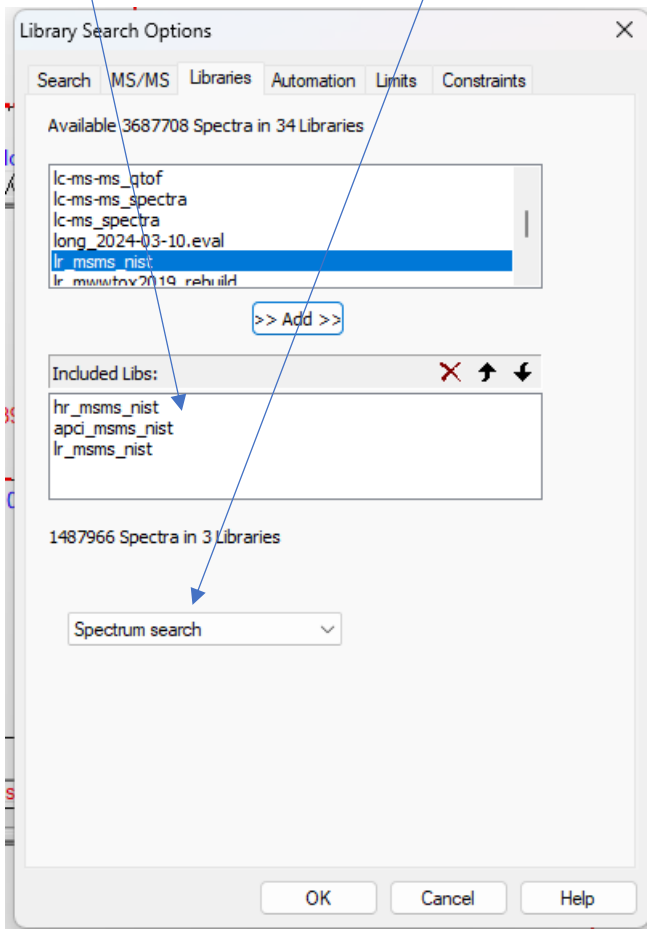
If precursor *m/z* imported correctly with spectrum, will have Precursor Ion m/z "in spectrum" selected



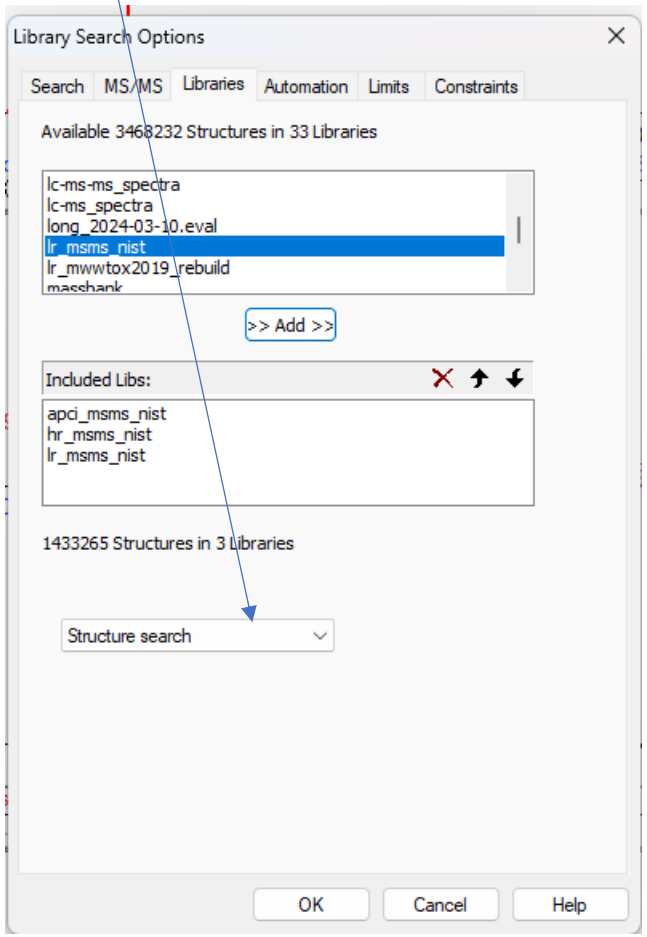
If precursor *m/z* ***not*** imported properly, user can deselect "in spectrum" and then enter value manually

Other Settings for NIST MS Search 3.0 (Tandem Mode)

Select whichever MSMS Libraries you want to search, the ***standard ones*** with NIST 2023 Software are selected below for ***spectrum search***



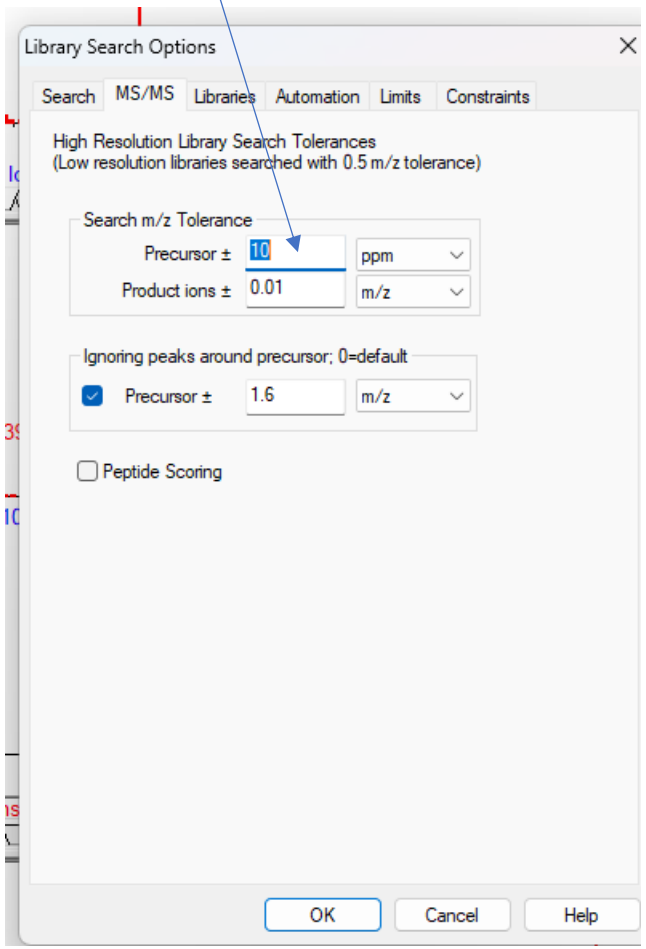
Libraries to search by Structure are also selected after toggling to ***Structure Search***

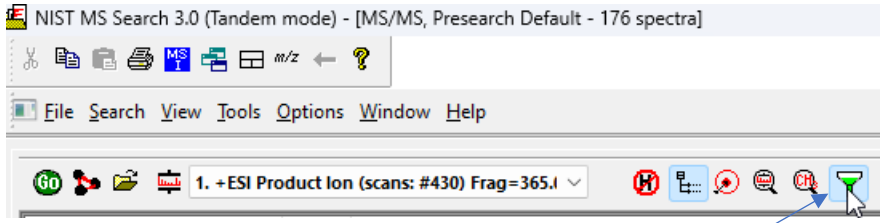


Other Settings for NIST MS Search 3.0 (Tandem Mode)

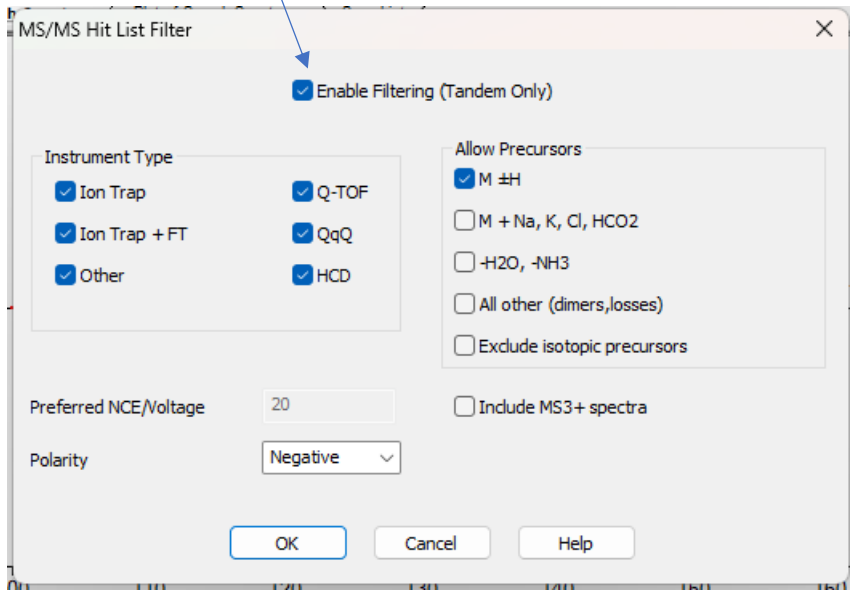
Check the settings in Library Search Options/MS/MS Tab

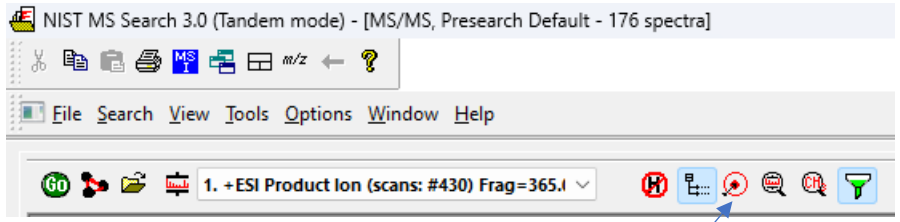
Set the tolerance for precursor *m/z* which is appropriate for your instrument





- The "funnel" settings affect the results displayed from MSMS search such as polarity of MSMS spectrum and many others if "*Enable Filtering (Tandem Only)* is selected, often best to have off when doing initial search, ***and THEN use filter AFTER search completed*** to filter results!
- Might consider ***turning off*** before next search is performed to not ***"over-filter"*** your initial results

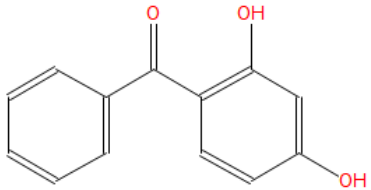




The "Best Matching" only can greatly simplify the results, removing all but the best matching spectrum using the CAS Number as a filter, ***can use after the search*** is completed to filter the results

Including or Not Including MS3+ Spectra??

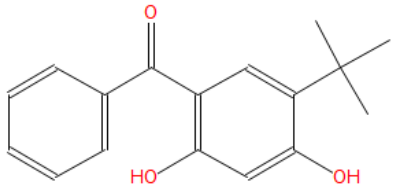
- The unfiltered results include MS3+ spectra which can be confusing and complicate the reviewing of results in the hit list
- NIST initially infuses standards with the M+H of the standard selected
- They then select certain fragment ions and fragment them again to yield MS3, and similarly MS4 ion fragments
- This is useful because the fragments are essentially a different compound which yields useful information for a related compound that might not be present in the library
- Thus, when searching the spectrum of M+H of Uvinul 3000 with MW of 214, one finds the desired spectrum for **1**, but also species such as **2** and **3**
- The MSMS spectra of fragment ions at *m/z* 215 from **2** and **3** would give very similar spectra to the M+H of **1**
- By ***selecting*** "Enable Filtering (Tandem Only)", then ***unchecking*** "Include MS3+ spectra", the ***results will be simplified*** by removing MS3+ type spectra
- The "Best matching only" will ***also remove duplicates*** from the list with the same CAS No.



1

Precursor type: [M+H]+

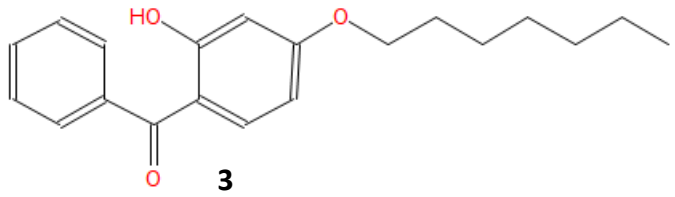
Uvinul 3000
M+H m/z 215
MW 214
MS2 Spectrum



2

Precursor type: [M+H-C4H8]+

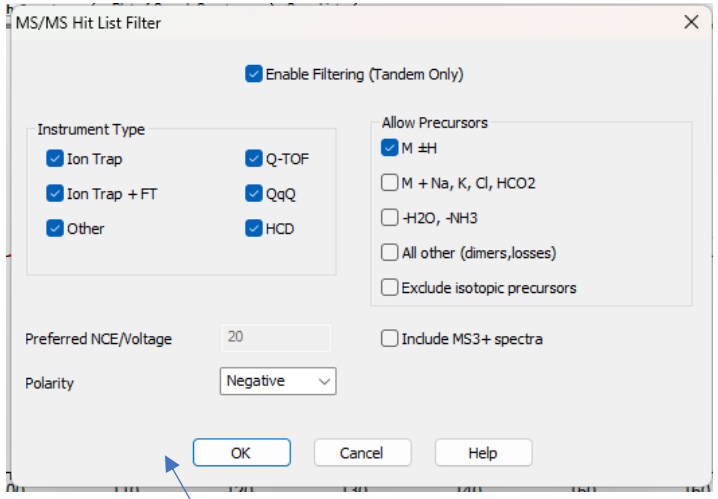
M+H m/z 271, then select m/z 215 fragment ion and fragment again,
MW 270



3

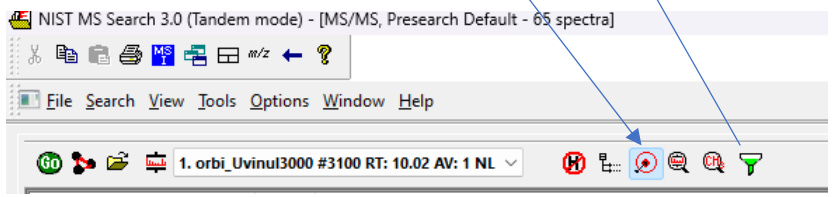
Precursor type: [M+H-C7H14]+

M+H m/z 313, then select m/z 215 fragment ion and fragment again
MW 312



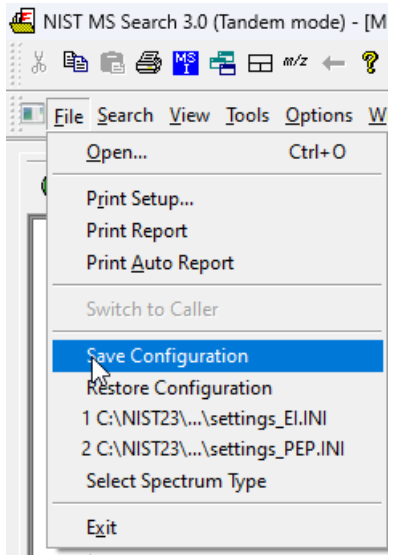
"MS/MS Hit List Filter"

"Best matching only"



Important!! Save Your Settings for Future Sessions!!

- After initially setting up search preferences, most normally do not change significantly
- *All Settings can be saved* for future searches by ***Save Configuration*** with a unique name
- User can then easily ***Restore Configuration***



Information for NIST 20 Tandem Library Users

This document describes special features of the **NIST Mass Spectral Search Program v.2.4** (NISTMS.exe) of interest to the **Tandem Library** users. General software features are presented in separate documentation.

Libraries:

There are four separate **NIST** reference libraries containing tandem spectra obtained by tandem mass spectrometry.

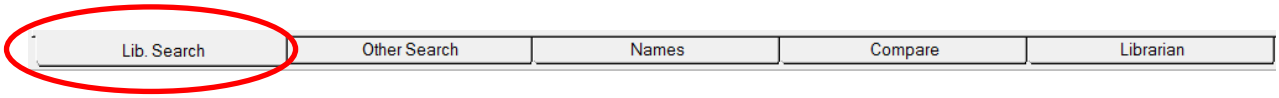
1. **hr_msms_nist** – contains all high resolution – accurate mass (HRAM) spectra of 'small molecules'
2. **lr_msms_nist** – contains all low resolution (unit mass accuracy) spectra of 'small molecules'. When searching, the fragment ion tolerance is always set to ±0.5 *m/z* units, regardless of the value set in the MS/MS search tab. The precursor ion tolerance set in the **MS/MS** tab of the **Library Search Options** dialog box is used for all libraries. Use of this library is not recommended for high resolution hybrid searches.
3. **apci_msms_nist** – contains about 4K HRAM spectra from atmospheric pressure chemical ionization (targeted for *extractables* and *leachables*)
4. **biopep_msms_nist** contains all spectra of peptides of biological interest (mostly HRAM)

Library Searching:

Library search settings are specified in the dialog box opened by selecting the fourth button in the **Button Bar** shown below:

Button Bar (at the top of the **Lib Search** tab view)

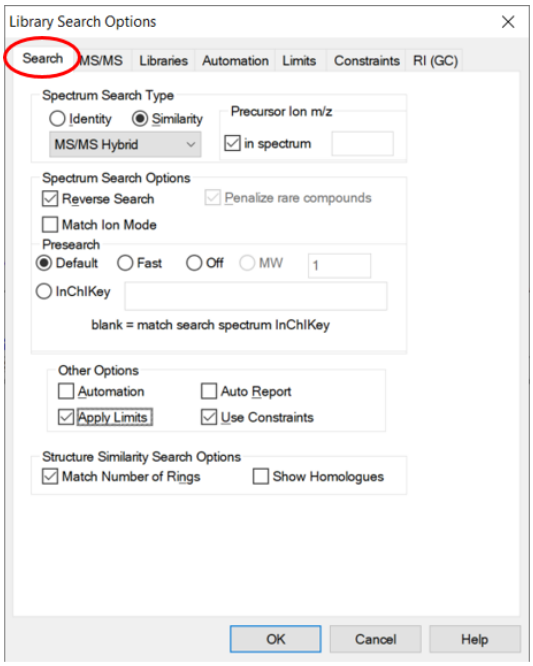
This bar appears only in when the **Lib. Search** tab view is selected (at bottom of the Program's display)

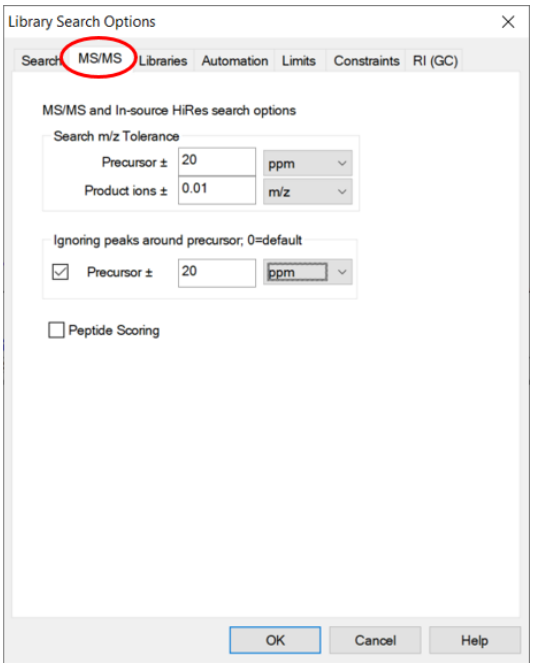


Search Tab (shown below in the **Library Search Options** dialog box) selects the type of search and relevant controls. The tandem searches are:

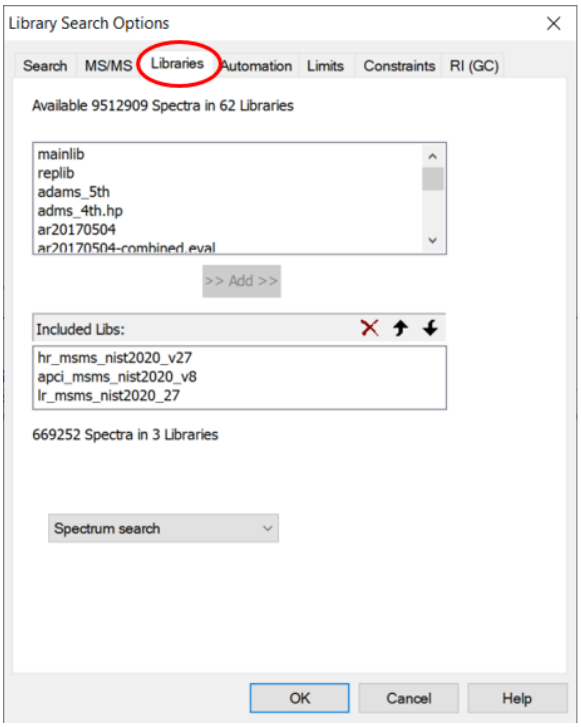
1. **Identity: MS/MS**
   Searches only library spectra whose precursor-ion's *m/z* value matches that of the query spectrum (this value should be in the **PrecursorMZ:** field of submitted spectrum or may be manually entered in the **Precursor ion *m/z*** field of the dialog box).
2. **Identity: In-source HiRes**
   Same as MS/MS search except does not use the precursor mass to constrain searches.
3. **Similarity: Hybrid MS/MS** (Shown selected in figure below)
   This finds all spectra having matching product ion and neutral loss peaks. Can aid the identification of compounds not present in the library.

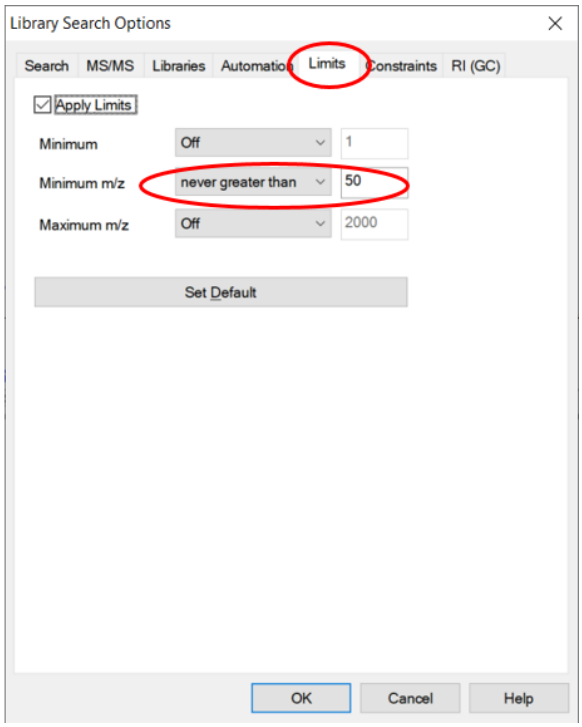
Other searches are intended for unit mass resolution electron ionization (EI) spectra as is common for GC/MS analysis using quadrupole mass analyzers. Other settings are associated with all MS libraries and are described in the main documentation.





MS/MS Tab (above right):
The precursor mass tolerance should be set to reflect the accuracy of your instrument - NIST Tandem Library spectra always have the exact mass value for the precursor ion. It is generally recommended that the product-ion tolerance be set at 0.01 *m/z* units to ensure that lower mass peaks are matched. Due to the fact that spurious peaks commonly appear near the precursor ion, a setting of 20 ppm is recommended for the **Ignoring peaks around precursor** specification. **DO NOT** select **Peptide Scoring** unless using peptide libraries.





Libraries Tab (shown above left):

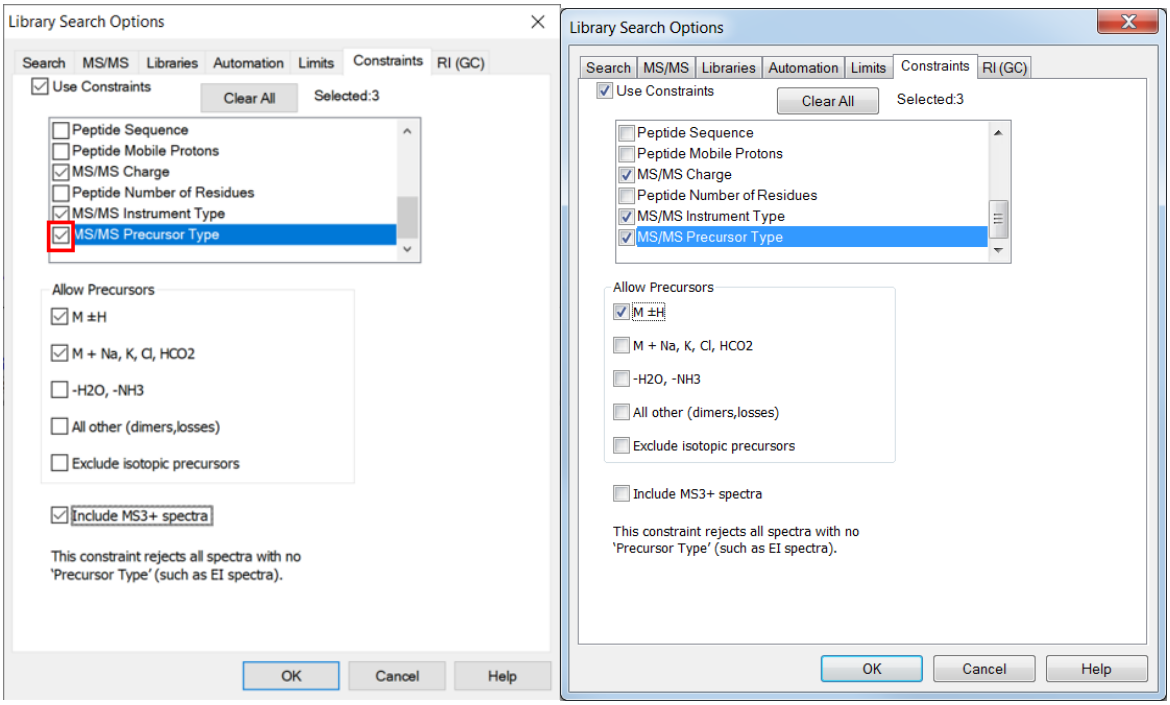
Select libraries for searching by double clicking on the library in the list in the upper window. Your libraries may have different names than shown here. Libraries with 'lr' (low-resolution) prefix are always searched using a wide mass tolerance for product ion masses. Libraries can be added by double-clicking on the name in the upper list or removed by double clicking on its name in the lower list.

Limits Tab (shown above right):

It is recommended that **Minimum *m/z*:** box be set in the **Limits** tab in the **Library Search Options** search dialog. This should correspond to the lowest *m/z* value reported by your instrument. The default is 50 *m/z*. Unless set, spectrum comparison begins at the higher of the lowest *m/z* value in the two spectra being compared. Make sure there is a check in the check-box next to the **Apply Limits** label. Selecting this check-box will also be reflected in the box with the same label in the **Search** tab of the **Library Search Options** dialog box.

Constraints tab (shown next page:

This shows a variety of filters that reduce the number of spectra in the Hit List. Three filters are of special interest for tandem library users: **MS/MS charge**, **MS/MS Instrument Type** and **MS/MS Precursor Type**. These filters have no effect on EI (GC/MS) searches. When any constraint is selected, a check is automatically placed in check-box next to **Use Constraints** label. Selecting this check-box will also be reflected in the box with the same label in the **Search** tab of the **Library Search Options** dialog box.

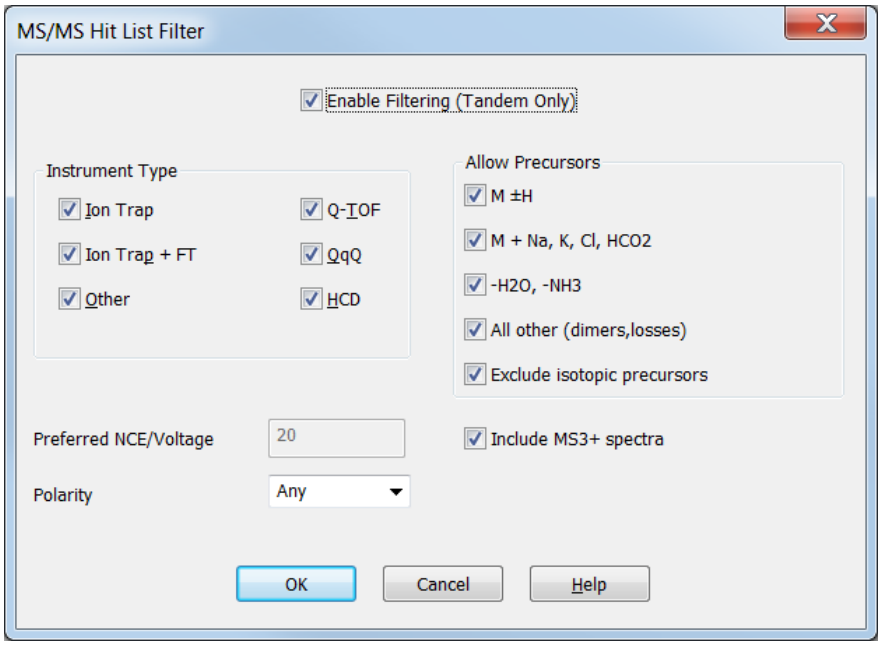


Scores (reported in the **Lib Search** tab view's **Hit List** window)

When matching two product-ion mass spectra, **Scores** are reported as well as the **Dot Product**. The **Dot Product** is a simple mathematical measure widely used in reporting spectrum similarity. An exact match is reported as 999. The **Score** is adjusted for spectra with few peaks and is intended to adjust for the reduced selectivity when matching small numbers of peaks. For a single peak spectrum for example, a match will report a 999 for the **Dot Product** and significantly lower the **Score** depending on abundance. The difference between the **Dot Product** and **Score** will reflect an estimate of the uncertainty in identification due to the small number of peaks (generally less than four). This **Score** is simply a rough measure of identification confidence and has no well-defined statistical meaning.

Hit List Filtering (rightmost button on button bar in Search tab – see below)

The **NIST Tandem Libraries** contain product-ion spectra formed over a range of energies, precursor-ion types, and fragmentation methods. This means that a search of the **Tandem Libraries** can produce many Hits generated for a single compound, which can be tedious to sort through. To facilitate this task, a filtering process is available that readily controls the types of spectra shown. This is available for both the **Lib Search** and **Other Search** tab views. The Filter is invoked by pressing the filter button at the end of the **Button Bar** shown above for the **Lib Search** tab view. (it is at the top of **Hit Lists** in the **Other Search** tab view). The filtering process may be turned on and off with the **Enable Filtering** check-box at the top of the **MS/MS Hit List Filter** dialog box (below). When the filter is enabled, the filter button will appear to be pressed. Clicking on the **OK** button in this dialog box causes the Hit List to conform to the selections made. These controls match corresponding controls in the **Constraints** section, except they do not permanently remove Hits – all original Hits can be seen by turning off the **Enable Filtering** check-box. The **Preferred NCE/Voltage** is not active for spectrum searches – it limits **Other Search** results to the value closest to the entered **Preferred** value.



Hit List Display tab: (of the **Library Search Properties** dialog box)

It is recommended that when examining Hit Lists of spectra from Tandem Libraries, three Tandem-specific additional columns be selected for display. They are **Prec. Type** (precursor-ion type) **Instr. Type** (instrument type) and **Energy**. The first, **Prec Type**, identified the ion that was selected for fragmentation, which include in-source generated ions. **Instr. Type** gives the fragmentation method Q-TOF, QqQ, HCD. Ion-trap (IT) and high-resolution Ion-trap (IT-FT). For all **Hybrid searches** the **DeltaMass** selection should also be made.

The **Library Search Properties** dialog box is displayed placing the Mouse Pointer on the Hit List window (lower left panel of the display in the **Lib. Search** tab) and pressing the Right Mouse Button (RMB); then select **Properties** from the RMB menu, the last item in the list. The **Hits List** tab is one tab in the display, as shown in the figure on the right).