One Suggested Approach

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Starting an Analysis

1. Use Leucine Enkephalin or your favorite compound to determine what mass accuracy window setting to use. For example, see the video "Module 3. Setting the Mass Error Window" on the MathSpec channel of YouTube.
2. Open a new Excel file that has at least one worksheet. Click on "Add-Ins" at the very top, and then click on the spectrum icon (upper left) to launch the Rational Numbers Add-In for Excel. (Help worksheets are available in six languages.)
3. Open the DataInput worksheet. Paste the data into the blue cells. If there are too many fragment ions to fit into the blue shaded areas, use Sheet1 to sort the data by intensity and throw out less intense ions (but keep the protonated or deprotonated molecule).
4. Choose the four parameters, modifying from the default values if needed.
5. After adding the data and setting the parameters, click on the "Transform!" command button. The lower left corner will update the progress of the analysis. Run time increases with the square of the mass. If the molecular weight is greater than 500 daltons, the analysis might take over 15 minutes, depending on the data, the parameter settings, and the computer available. At greater molecular weights, a tighter mass error window parameter may be required to reduce the number of possible formulas.
6. After the analysis is completed, save the results in a "Trusted Folder" as a macro-enabled file type (e.g. Filename.xlsm). See Installing the Rational Numbers Add-In on the MathSpec channel of YouTube for Excel Trust settings.

Results of the Partitioning

1. Up to six partitions may be found presented from highest "Partition Score" (Partition 1) to lowest (Partition 6). A partition with a higher "Partition Score" accounts for more of the more intense fragment ions than a partition with a lower score. The scores range from about 30 to 130. Scores above 100 are uncommon. If the number of subfragments cell (B31) is flagged with an orange background, this indicates that the number of subfragments is equal to the number of assigned fragments. In this case,

especially if the assigned formula of one subfragment is a subset of a second subfragment, this partition may not be meaningful.

2. The "Mass Accuracy Score" looks at the average mass error between the subfragment masses that were calculated from the data (Row 23) and masses calculated from the proposed subfragment formulas in the columns below.
3. The "Overall Accuracy Score" reduces the "Mass Accuracy Score" with a penalty based on the difference between the theoretical isotope ratio of the formula found in Column I and the isotope ratio calculated from the data (Cell A36). The formulas are sorted in descending order of "Overall Accuracy Score".
4. The alignment is given in Row 35. A yellow arrow appears in Cell I20 if there are multiple ways to align the subfragments in space. The arrow can be used to view the different possible alignments.
5. The analysis can be repeated with different parameters by returning to the DataInput worksheet, changing parameters, and clicking on "Transform!" This will overwrite the partitions that were previously found unless these partitions were renamed prior to the reanalysis. (Before rerunning the analysis, be sure to clear all filters that you may have selected on the Summary worksheet.)

Reviewing the Results – Molecular Formula

1. A molecular formula with a greater "Overall Accuracy Score" is usually more probable than a partition with a lower score. The best way to find the most likely formula is to go to the Summary worksheet and use the filters. The summary sheet is a listing of every row of every partition which includes the overall score for each given formula. Click on the filter at the top of the overall score column and unselect all. Then select the three or four highest scoring sets of formulas. Usually one or two formulas appear superior to the rest. Keep in mind that even if the same molecular formula appears in multiple partitions, that molecular formula is not more likely than a formula that only appears in a single partition but that has a higher overall score.
2. If you have very high resolution MS data, it may be very helpful to plot possible formula candidates on the isotope worksheet. Save a copy of the isotope worksheet in the Excel workbook and then plot other potential formulas on new sheets for comparison with the unknown's isotope data.
3. Use the Plaus() function (on Partition worksheets) to see how common the elemental compositions of the subfragments are in the formula candidates. If any row of elemental compositions has two subfragments with plausibility scores summing to 9 or less, the overall composition of that row is very unlikely and may be eliminated from consideration.

4. A partition having two or more subfragments of identical mass and composition is more likely that a partition with fewer identical subfragments.

Reviewing the Results – Comparing partitions while viewing a single formula

1. On the Summary worksheet use the filter in the Formula column (this is the molecular formula column) to select a candidate formula. Then go to the FragMass column and choose the filter to select the mass of the whole molecule. This is a very informative display that often shows possible relationships of the subfragments making up the whole molecule. Look for larger subfragments that may be further resolved in other partitions (pseudo MS^n). For example, Partition 2 of xemilofiban has a subfragment of 135.0800 while Partitions 1 and 6, partitioned differently, have smaller subfragments of 17.0268 and 118.0538 or 42.0208 and 93.0588. On the Summary worksheet, the formulas appear instead of the masses. C7H9N3 = NH3 + C7H7N2 or C7H9N3 = CH2N2 + C6H7N. This is also helpful because you can look at one formula at a time even if there are 50 possible formulas.

Reviewing the Results – Alignment

1. If no alignment is assigned to the first partition, this usually indicates that the molecule might have two subfragments that are identical in mass while only one subfragment (representing both) is in the partition, leading to contradictory alignments. This could also indicate that the molecule is rearranging in the mass spectrometer.
2. Apply the RDE() function to find the number of rings and double bonds for each subfragment formula. Any alignment having adjacent subfragments with an RDE = 0 can be eliminated from consideration. Those two subfragments cannot be connected.
3. Odd-electron subfragments (subfragments with non-integral values) come in pairs and should be adjacent to each other; any alignment with odd-electron subfragments that are not adjacent can be eliminated from consideration.
4. SO2 and H2SO2 subfragments may appear on the outside position of a partition based on the logic, but these subfragments are formed by extrusion and are always on the inside of the molecule.
5. Alignments that have subfragments with a maximum number of connections less than the number of

connections required for the subfragment in that alignment can be removed from consideration. For example, the formula C4H10O can have a maximum of one connection and therefore could not be in a center position where it would hold three other subfragments together. Likewise, an H2O subfragment can connect a maximum of two other subfragments. Subfragments with a negative number of hydrogens always connect two subfragments.

Reviewing the Results – Checking significant unassigned fragment ions

1. Look for "Twelves". These are two fragment ions differing in mass by 12.0000 +/- experimental error, where one fragment is assigned by the Excel Add-In and the other is not assigned. This indicates that there is an X-CH2-CH- group at the break between the two fragment ions where X is N, O, or S.
2. Look for unassigned fragment pairs may further corroborate the structure. For example, if may not be possible to assign a fragment mass of 200 daltons with a particular set of subfragments. However, if there were a subfragment of 42 (ketene) and an unassigned 158 fragment ion with a mass difference of ketene from the 200, that would tend to corroborate the results and may help assign these fragments.