Mascot Distiller Hot Topics

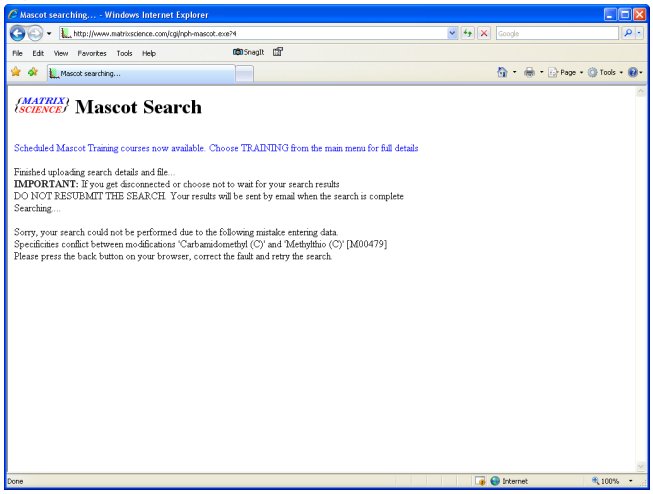
MASCOT

MATRIX SCIENCE

This presentation will touch on some aspects of using Mascot Distiller for quantitation. I would have called it Quantitation FAQ, but we already used that title for a presentation last year. That won't stop me taking the first few slides direct from the 2009 FAQ, because they concern three topics that keep coming up again and again.

I will then move on to discuss label-free quantitation, memory problems with very large, multi-file projects, the importance of including Arg-Pro conversion in a SILAC method to get best accuracy when arginine is being labelled, and automation of quantitation using Mascot Daemon

4. Why do I keep getting errors about conflicting modifications?



First of all, the three FAQ from 2009. This question usually comes up with reference to iTRAQ. A typical error message is “Specificities conflict between modifications 'Carbamidomethyl (C)' and 'Methylthio (C)' [M00479]”

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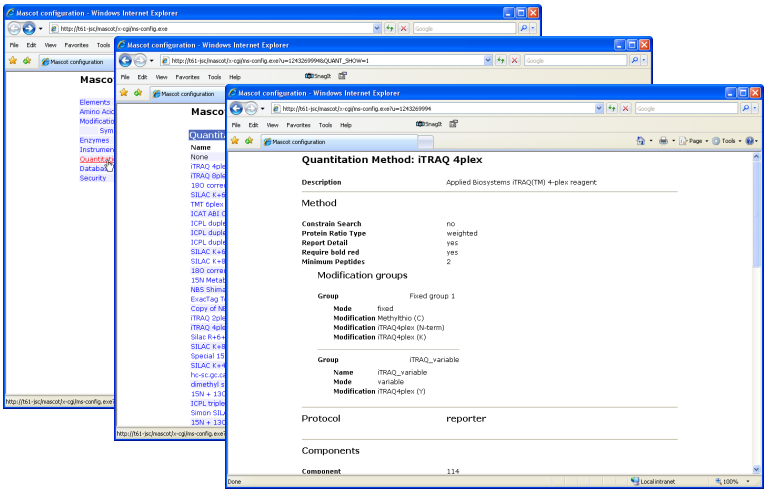

To understand this, take a look at the quantitation method. You can get a pretty printed summary from the Configuration Editor. Choose Quantitation then Print for the method of interest. This is the original iTRAQ method, that shipped with Mascot 2.2. The iTRAQ modifications are defined in the quantitation method. In this particular case, this isn't strictly necessary, but for many quantitation methods the modifications have to be defined within the method because the modifications define the components. For example, a peptide with 16O at the C-terminus is light while a peptide with 18O at the C-terminus is heavy. So, the approach we took was that the isotopic labels should be defined within the method. We also felt that specifying as much as possible in the method was a good thing in terms of standard operating procedures. The original iTRAQ method was based on the Applied Biosystems procedure that used methythio (MMTS) to block the cysteines, so we also included this modification. The problem comes when someone uses a different alkylation agent, such as iodoacetamide, and chooses carbamidomethyl in the search form.

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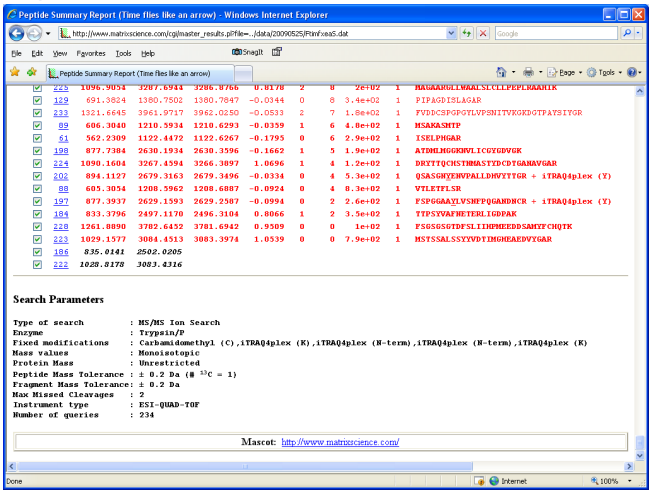
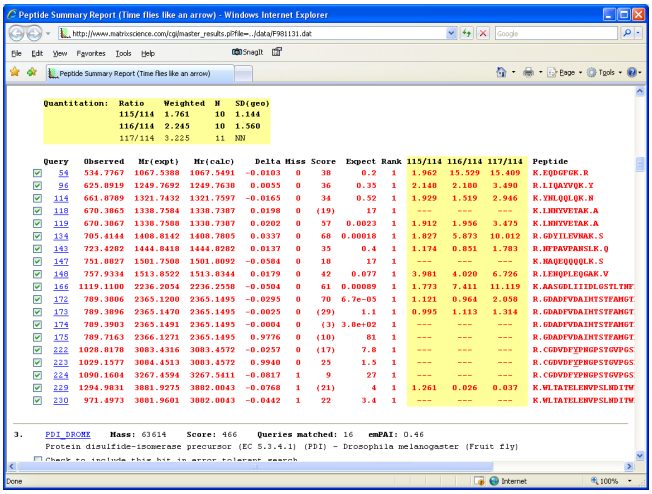


Because it became clear that few people use MMTS, we took this out of the method when we updated it. If you haven't done so already, you should grab an updated quantitation.xml file from our web site, using the link on the Distiller support page. This fixes the MMTS problem, but it doesn't stop people using a method without looking at it. I suspect that many people have search results that look like this. The iTRAQ mods are there twice, because they have been chosen in the search form even though they are already in the method. This isn't an error, because there is no conflict, but it does indicate that we haven't got the message across about embedding the important search parameters in the method. Hopefully, this FAQ will help clarify that the modifications that are essential to the method should go in the method while those that are 'optional' and will vary from experiment to experiment, such as oxidation or acetylation, should be selected in the search form.

7. Why do I get no results for certain peptide matches?



The second repeated FAQ: A Mascot quantitation method includes certain rules and quality thresholds. One of the most important is the Mascot score. If you aren't confident of the peptide match, then you can't be confident of the protein assignment for that match and wouldn't want to include it in any calculations. A cut-off, which is often used in the default methods, is that the score should be above the homology threshold. So, first thing to check is that the ions score for the match is above threshold. Here, this is the reason for most of the missing ratios. It isn't the explanation for the match to CGDVDFYPNGPSTGVPGSENVIEAVAR with a score of 25. This has been excluded by a rule in the method, that any match with iTRAQ on tyrosine should be ignored. This is because the reaction with tyrosine is slower than with lysine and the amino terminus, which means that the ratio is likely to be skewed.

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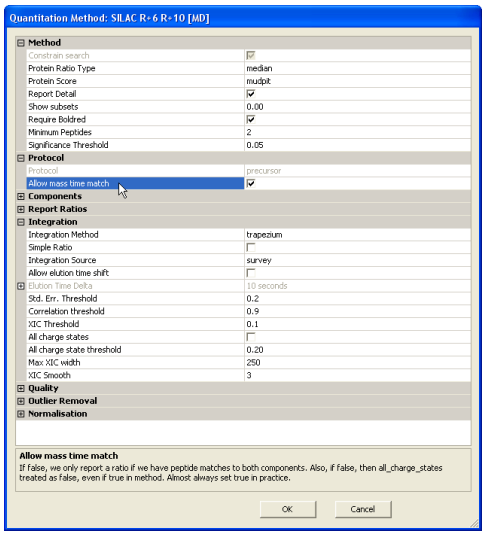

If you have less peptide matches than expected, check that ‘Allow mass time match’ is true. If this is false, then you must have peptide matches for all components before a ratio can be reported. If true, you only need a peptide match to one component. Its partner or partners will be identified from their molecular mass and elution time. This is almost always what is wanted.

10. Should I choose normalisation? If so, which type is best?

When to use normalisation

- Analysing equal total weights of protein from a complex mixture, e.g. cell lysate – YES
- Starting with equal numbers of cells – NO
- Isolating a sub-set of proteins by affinity methods – NO
- Looking at a synthetic dilution series - NO

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The final repeated FAQ concerns normalisation. The correct time to use normalisation is when you have two or more samples, each of which is supposed to contain the same total amount of a reasonably complex mixture of proteins. Some proteins may be up-regulated, others down-regulated, but the majority are unchanged. In such a case, the mean ratio is expected to be close to unity. If it is not unity, this probably reflects systematic errors, such as failure to fully label or digest a sample, or maybe the sample aliquots were not identical. By forcing the mean ratio to unity, we can eliminate these systematic errors.

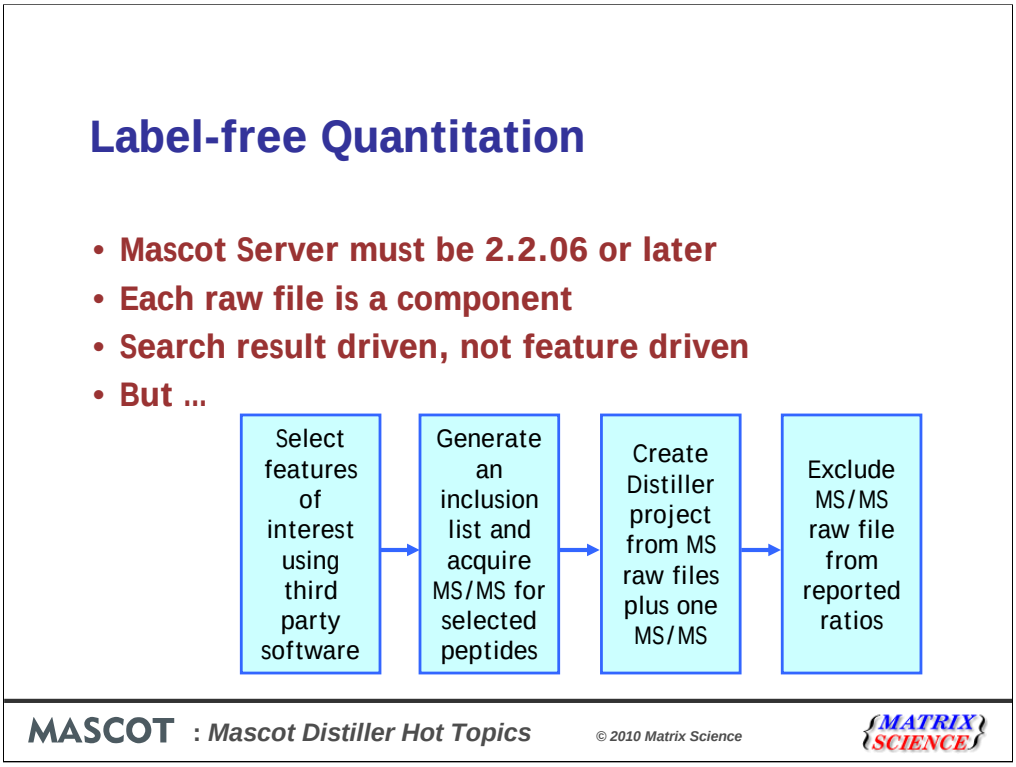
That is, normalisation forces the average or median peptide ratio to be 1. If the average or median ratio is supposed to be 1, this is the smart thing to do. In other cases, it is absolutely the wrong thing to do. For example:

•If you are analysing a standard, where the ratio is supposed to be 3:1, you wouldn't want to force it to be 1:1, would you?

•If you start with equal numbers of cells and want to see the effect of starvation on one line then normalisation would be incorrect because you expect less protein from starved cells

•If you are isolating a sub-set of proteins by affinity methods then normalisation is not appropriate

Whether you get better results using average or median depends on various factors. The safest way to choose is to run some standards (e.g. similar samples spiked with varying amounts of a known protein) and see which gives the best accuracy and precision.



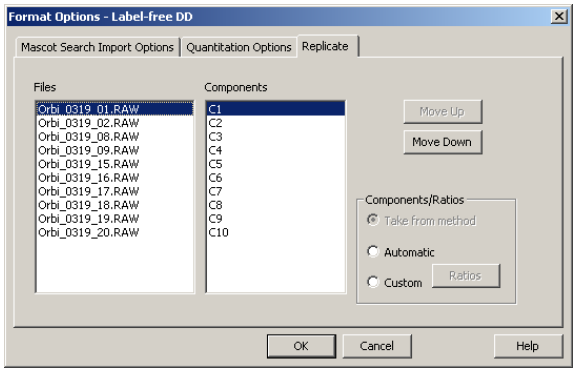
Label-free, or Replicate, was the final quantitation protocol to be added to Distiller, in version 2.3. It requires Mascot Server 2.2.06 or later.

This is quantitation based on the relative intensities of extracted ion chromatograms (XICs) for precursors in multiple analyses, aligned using mass and elution time. Each raw file is a component and the chromatography needs to be highly reproducible.

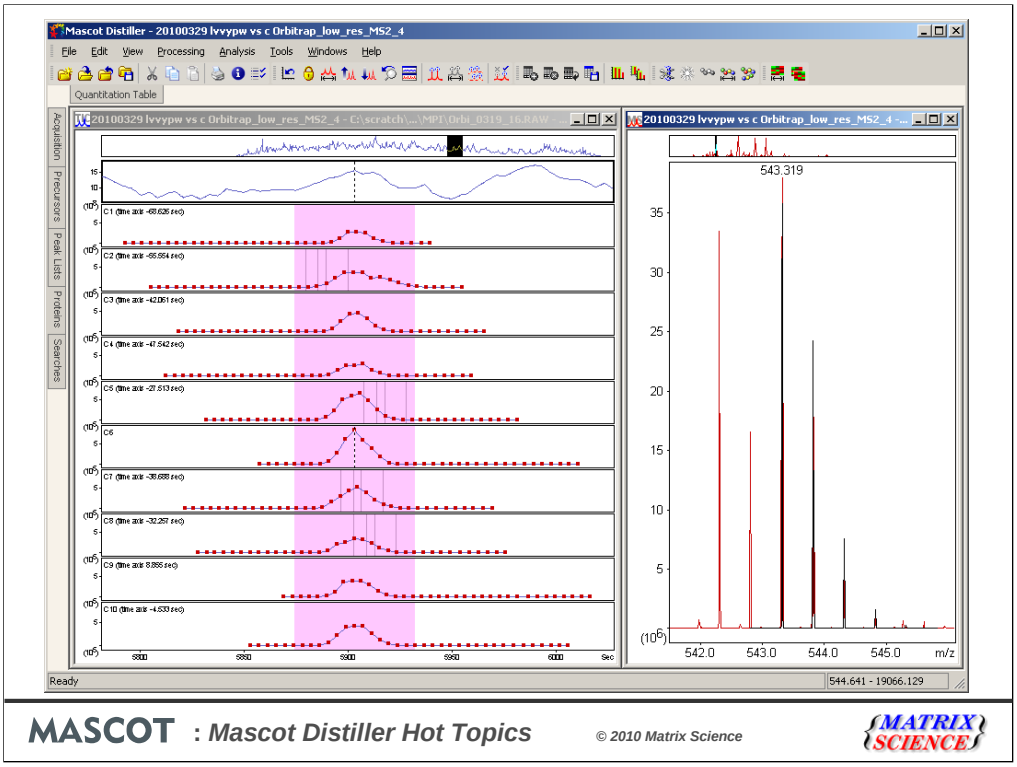
Within Distiller, quantitation is driven by the search results. That is, Distiller takes the list of peptides returned by the Mascot search and looks for the precursors in each of the survey scans. In most cases, the majority of proteins are unchanged from sample to sample. Only a small number are significantly up or down regulated. The alternative workflow is to align multiple files of MS data, identify features that are up or down regulated, generate an inclusion list for a mass spectrometer to obtain MS/MS data for these peptides, import the resulting peptide matches, then perform quantitation on just the features of interest.

Distiller doesn't provide such an interface but, if you have some third party software that can be used to generate an inclusion list, you can easily create a Distiller project containing the MS/MS data in a separate file. Distiller will happily use the MS/MS to locate the set of features to be quantified across all the MS files. Just omit the MS/MS file from the ratios to be reported.

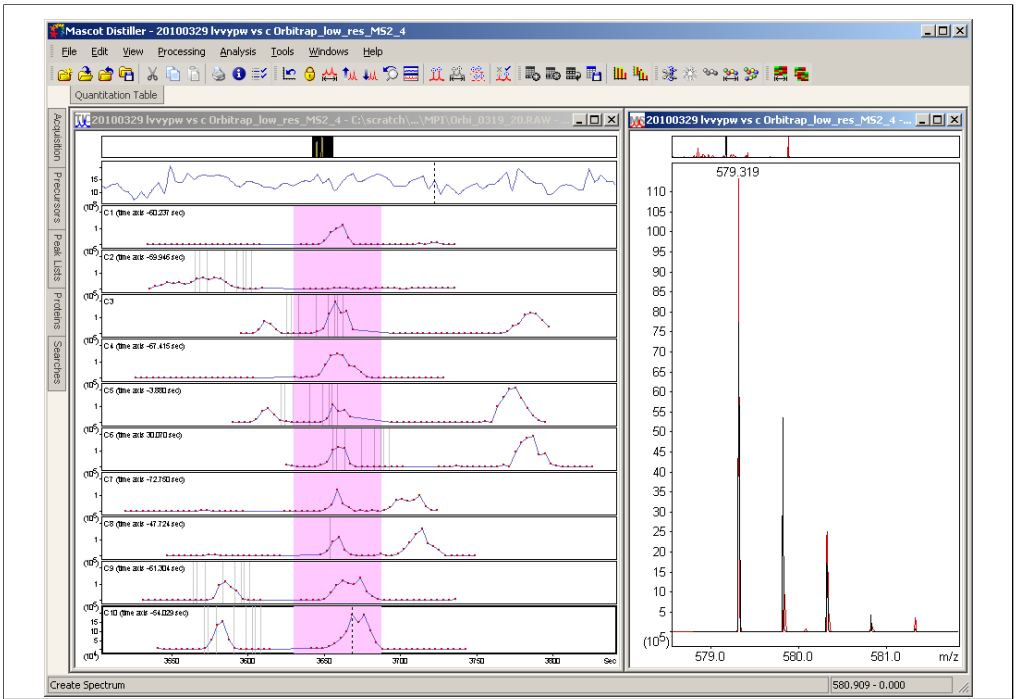
Label-free Quantitation



A replicate project is always a multi-file project, with one file for each component. You can define ratios or have them auto-generated, with one file selected as reference and all the others reported relative to it.



The tricky part of label free is time alignment of the chromatograms. Distiller does its best, and here is an example where things are working, even though the precursor region is heavily overlapped



But, here it fails. Distiller can manage small misalignments OK, but not severe misalignments or multiple XIC peaks. If alignment fails for a particular peptide, then the ratio is meaningless.

Large, Multi-file Projects

Current release of Distiller (2.3.2) cannot handle large, multi-file projects

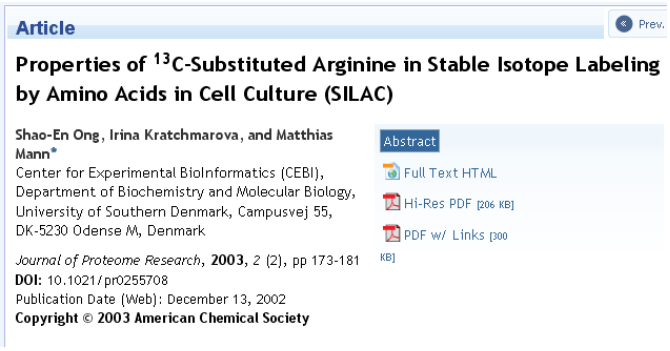
- Problem typically seen when total size of all raw files > 5 GB
- 64-bit Windows with plenty of free RAM helps, but is not a fix
- Recommend saving large projects after time-consuming steps.

Quite often, a label-free experiment will comprise a large number of raw files. Unfortunately, the current release of Distiller runs into problems with multi-file projects when the total size of all the raw files is several GB. It isn't a precise threshold – depends on size of peak list, number of peptide matches, and many other factors.

Running under 64-bit Windows with plenty of RAM helps, but is not a fix. If working with a large set of raw files, its a good idea to save the project after each time consuming step, so as not to lose the results if Distiller should crash.

Distiller is a 32-bit application and it is trying to do too much in memory. We are working hard on a fix and hope to have something later this summer

Arg-Pro Conversion



Article

Prev.

Properties of ^{13}C-Substituted Arginine in Stable Isotope Labeling by Amino Acids in Cell Culture (SILAC)

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Journal of Proteome Research, **2003**, *2* (2), pp 173-181
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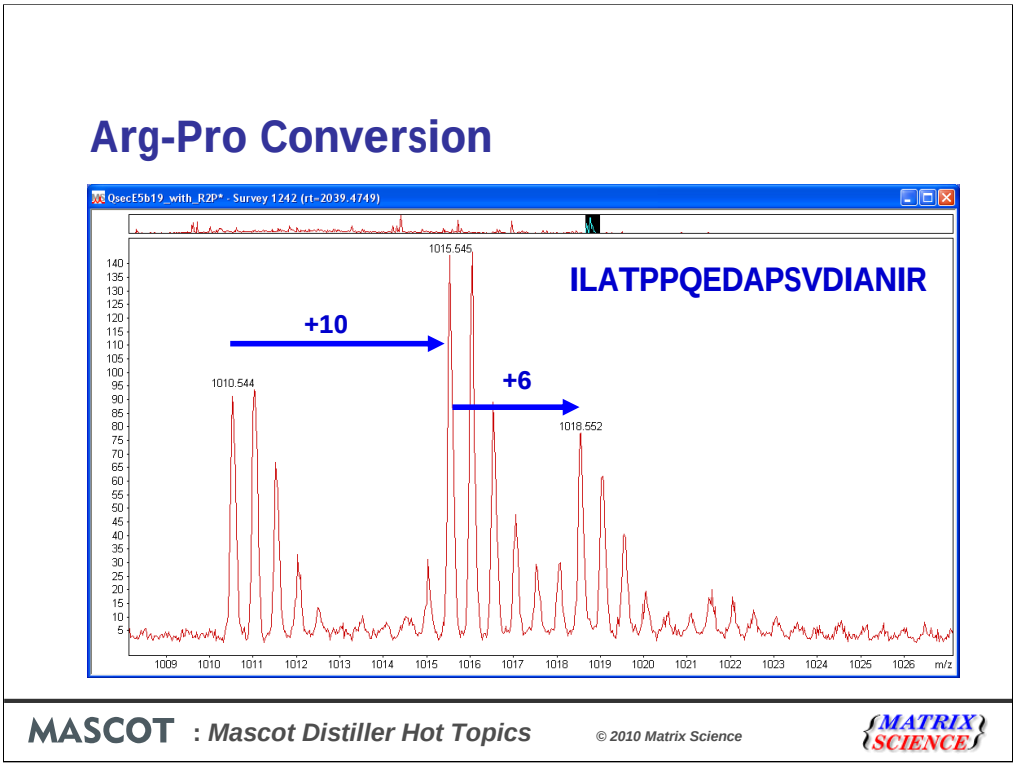
Publication Date (Web): December 13, 2002

Abstract
Full Text HTML
Hi-Res PDF [206 KB]
PDF w/ Links [300 KB]

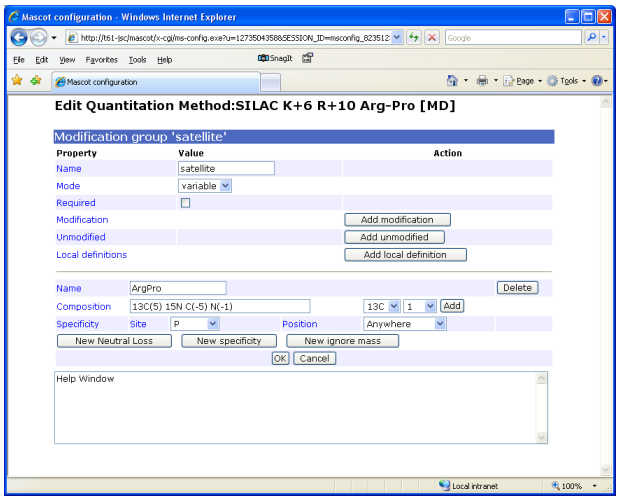
Ong reported that cells grown in media containing labelled arginine could yield peptides containing labelled proline. To obtain an accurate ratio, it becomes necessary to account for the label distributed across these additional peaks.



This can be handled in a very general way in Distiller by defining a satellite modification group. Distiller sums the intensity of any corresponding satellite peaks into the heaviest component. (This is only practical for the heaviest component. If the method includes multiple labelled components, it is not possible to de-convolute the satellite peaks for intermediate components.)

The proline label is not identical to the arginine label. For example, Arg labelled with 13C(6) converts to Pro labelled with 13C(5), while 13C(6)15N(4) converts to 13C(5)15N(1).

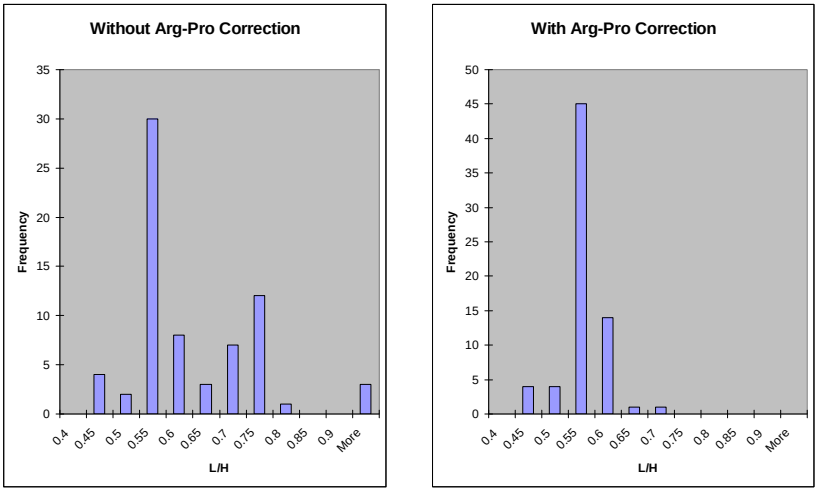
Arg-Pro Conversion



To add this to a new method, from the Component tab, select the heavy component and choose Add Satellite Group.

Usually, it will be easiest to define this as a local modification definition, within the quantitation method, as shown here

Arg-Pro Conversion



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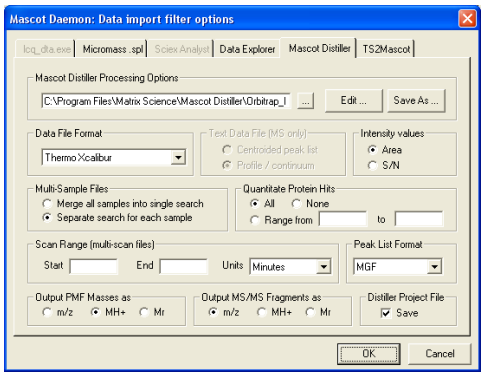
This histogram shows the improvement for a single protein. This is Orbitrap data and the labelling was K+6 and R+6. We've made a histogram of all the peptide ratios. Without a correction, we have a bi-modal distribution. The proline containing peptides give a ratio that is too high because part of the heavy intensity has been shifted to the peptide with heavy proline. When we correct for Arg-Pro conversion, the second peak pretty much disappears and we have a much narrower distribution

Automation

All steps from peak picking to quantitation can be automated using Mascot Daemon 2.3



Finally, just to mention that Mascot Daemon release 2.3 is capable of automating all stages of a quantitation experiment. You choose to quantitate all the proteins or just a range, such as hit 1 or hits 1 to 10. When quantitation is complete, Daemon saves the the results to the Distiller project file.

One limitation: Daemon can only automate single file Distiller projects, not multi-file projects. But, if you think about it, you wouldn't gain much by using Daemon for a multi-file project. Easier just to select the set of files in Distiller.