DOI 10.1002/pmic.200900481 *Proteomics* 2010, *10*, 650–670

REVIEW

Peptide and protein quantification: A map of the minefield

Marc Vaudel[1], Albert Sickmann[1] and Lennart Martens[2,3]

[1] ISAS – Institute for Analytical Sciences, Dortmund, Germany
[2] Department of Medical Protein Research, VIB, Ghent, Belgium
[3] Department of Biochemistry, Ghent University, Ghent, Belgium

The increasing popularity of gel-free proteomics technologies has created a strong demand for compatible quantitative analysis methods. As a result, a plethora of different techniques has been proposed to perform gel-free quantitative analysis of proteomics samples. Each of these methods comes with certain strengths and shortcomings, and they often are dedicated to a specific purpose. This review will present a brief overview of the main methods, organized by their underlying concepts, and will discuss the issues they raise with a focus on data processing. Finally, we will list the available software that can help with the data processing from quantitative experiments. We hope that this review will thus enable researchers to find the most appropriate method available for their research objectives, and can also serve as a basis for creating a reliable data processing strategy.

Received: July 6, 2009
Revised: October 6, 2009
Accepted: October 27, 2009

Keywords:
Bioinformatics / Quantification / MS

1 Introduction

When whole-proteome investigation of biological samples became commonplace, the issue of quantitative protein analysis on these scales was raised with it. The original workhorse of proteomics analysis, 2-D PAGE [1] implicitly provided a solution, as the intensity of staining for a particular spot could be used for quantification purposes [2]. With the advent of gel-free proteomics techniques however [3], this protein separation step was largely omitted, prompting an urgent need to develop novel quantification methods that were compatible with the peptide-centric gel-free proteomics analyses. As a result, many different quantification methodologies for gel-free proteomics analyses have sprung up over the last years, all related to each other in that they exploit the signal from the mass spectrometer for quantification at the peptide level, rather than the protein staining used in 2-D PAGE.

From biomarker discovery to fundamental biological understanding, the application areas for whole-proteome quantitative information are as broad as they are interesting. Indeed, the availability of reliable quantification methods will be instrumental in further advancing the importance of proteomics in the life sciences [4]. It is important to note however that the various quantification procedures developed for proteomics provide a truly multi-disciplinary challenge where chemistry, biology, physics, informatics and engineering come together.

Although reliable protocols are typically available to carry out the quantification from the initial samples up to the measurements on the mass spectrometer, the limiting factor in an analysis pipeline today is often found at the stage of data processing [5].

Indeed, people often rely on software applications they do not fully understand or that provide precious little documentation or background information (the notorious black-box problem that pervades several aspects of data processing in high-throughput fields such as proteomics). As a result, users often fail to perceive correctly the strengths and limitations of their data processing tools, and the areas of application where they perform optimally. Many researchers

Correspondence: Marc Vaudel, ISAS – Institute for Analytical Sciences, Bunsen-Kirchhoff-Str. 11, 44139 Dortmund, Germany
E-mail: marc.vaudel@isas.de
Fax: +49-231-1392-120

Abbreviations: emPAI, exponentially modified protein abundance index; **ICPL,** isotope-coded protein labels; **PAI,** protein abundance index; **SRM,** selected reaction monitoring; **TMT,** tandem mass tag; **TOPP,** The OpenMS Proteomics Pipeline

in the field today are thus left quite bewildered by the amount of data they generate, the data processing workflow they should employ or the inability to correctly assess the validity of some of their results.

In this review we therefore aim to provide an overview of the data processing involved in the various methods, highlighting the potential pitfalls, strengths and sensitive points of the main gel-free proteomics quantification methods.

We will first give a short overview of the different protocols available today, and then proceed to explain both general and specific quantification issues. Finally we will list the various available software packages to the best of our knowledge.

2 An overview of the different methods for quantification in gel-free proteomics

The quantification of proteins in biological samples is used to answer a diverse array of questions. Typically, the information obtained falls apart in two distinct categories: quantitative comparison of two proteomes or relative quantification and quantification of the proteins in a single proteome or absolute quantification. The former will provide fold changes between samples, whereas the latter supplies actual amounts or concentrations for the proteins of interest. The applications of these methods are equally diverse, ranging from the quantitative evolution of a protein over time or in disease progression, to the direct comparison of samples, for instance before and after treatment with a compound of interest. Obviously, each of these applications comes with its own characteristics and expectations. In order to address these varied goals, a broad set of different protocols has been developed over the last few years. We will discuss these in some more detail here, and organize them according to their underlying concepts.

Quantification methods can be divided in two main families: stable isotope based and label-free methods. As the name implies, the first family uses stable isotopes to label peptides, introducing a mass difference between the labeled and unlabeled peptides in the process. The intensity signals obtained from the unlabeled and labeled peptide will then provide quantitative information in the MS spectrum. Correct processing of the information in the spectrum can then retrieve this information for downstream analysis. The second family of methods does not introduce labels, and is therefore often negatively defined as label-free quantification. These methods typically rely on chromatographic and MS data to quantify peptides in the samples. An overview of the various methods, clustered by type, is given in Fig. 1.

2.1 Stable isotope labeling methods

Labeling of peptides by stable isotopes allows the quantification of peptides to take place directly in an MS spectrum, since the isotope label is perceived by the mass spectrometer as a shift in the mass-over-charge ratio (m/z). By design, these methods are dedicated to relative quantification (labeled *versus* unlabeled or comparisons between different labels) but with the appropriate experimental design the same method can readily be used for absolute quantification. This is typically accomplished by adding a synthetic, labeled peptide at known concentration to the sample, and comparing the intensity of the unlabeled peptide to that of the synthetic, labeled peptide.

Within the family of isotope labeling methods, we can again distinguish two main types of labeling. The first category obtains quantitative information from the MS^1 (parent ion) spectra, whereas the second category requires MS^2 (daughter ion) spectra to be recorded in order to reveal the label, which is typically present in a so-called reporter ion that is only released upon fragmentation. Furthermore, a hybrid approach called multiplexing also exists, which builds upon the same labeling strategy used in the MS^1 methods, but quantifies based on the information in the MS^2 spectra. Finally, a chimeric approach can be constructed, by combining both MS^1 and MS^2 labeling methods in order to increase the total number of relatively analyzed proteomes.

2.1.1 MS^1 methods

These quantification methods are based on the differentiation of samples at the MS^1 level. Stable isotopes with

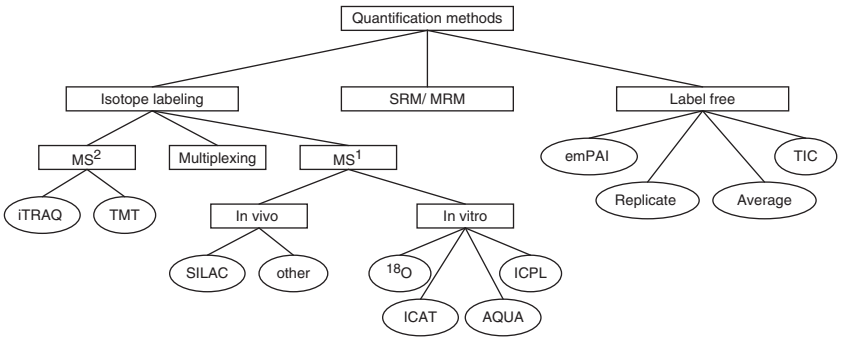

Figure 1. The most commonly used quantification strategies: Sorted according to the presence of label, then where the label discriminates the peptides and finally where the label is applied.

different masses are used to label the samples. A single peptide that is found in more than one sample will yield multiple isotopic envelopes in the MS^1 spectrum, allowing the differential quantification of the samples.

This labeling can be done either *in vivo* or *in vitro*, and this distinction in approach is used here to further subdivide the methods.

2.1.1.1 *In vivo* labeling

In vivo labeling relies on the labeling of proteins inside living cells. This can be done by feeding organisms with medium or food containing only heavy isotopes. In cell culture with labeled medium, this is convenient and economical, but the overall process can also be scaled up to allow the creation of labeled fruit flies or even mice [6]. ^{15}N is for instance used to produce labeled plants, animals as well as yeast [7]. But the quantification is often jeopardized by the isotope envelope.

An important issue common to the different metabolic methods is the possibility of directly influencing the organism by labeling it. Indeed, the change in the living environment of the cell can create metabolic stress, thus introducing differences in the proteome.

SILAC will label the proteins of a cell population in culture during growth and replication [8]. The technique makes use of the fact that mammalian cells cannot synthesize certain (so-called essential) amino acids. While building proteins, the cell will therefore have to use these amino acids as present in the culture medium. By growing one population of cells in normal non-labeled medium and a second population in medium containing an essential amino acid in only a stable isotope labeled version, the newly synthesized proteins in the former will remain mostly composed of light isotopes, whereas the latter will start incorporating the heavier, stable isotope labeled amino acid into its proteins instead. After a few replication cycles (typically five to seven) nearly all the proteins in the cells in the second culture will be labeled.

When the cultures have been labeled in this way, they can be mixed straight away. This early combination of samples means that they will be subjected to exactly the same downstream workflow throughout sample processing and analysis, thus minimizing variability [9].

2.1.1.2 *In vitro* labeling

In these labeling strategies, the label is incorporated after cell lysis, requiring a chemical or enzymatic step to couple the label to the protein or peptide. Several labels can be employed, which are listed below.

2.1.1.2.1 ^{18}O incorporation

The ^{18}O method [10] relies on the differentiation of two samples using different stable isotopes of oxygen. The heavy ^{18}O isotope is introduced into the carboxyl terminus (C-terminus) of peptides by performing the digestion in ^{18}O containing water. While often performed with trypsin, other proteases such as Lys-C and Glu-C can be used as well. The reactions involved are:

$$RC^{16}ONHR' + H_2O \rightarrow RC^{16}O^{18}O^- + H_3NR' \quad (1)$$

$$RC^{16}O^{18}O^- + H_2^{18}O \rightarrow RC^{18}O^{18}O^- + H_2^{16}O \quad (2)$$

One of the problems with this method is the occurrence of background exchange, which re-introduces ^{16}O into a previously fully labeled C-terminus. This can occur because of the slow accumulation of $H_2^{16}O$ in the digestion buffer as a result of labeling (see reaction (2) above) or more commonly through residual activity of the proteolytic enzyme after digestion is deemed complete, at which point the sample is reintroduced to buffers containing normal, mostly ^{16}O water. The result of the background exchange is an overall reduction in efficiency leading to the rise of a population of peptides labeled with only one ^{18}O, along with a very small minority of completely unlabeled peptides, which are again indistinguishable from the peptides in the unlabeled sample. The presence of the half-labeled peptides actually makes the distinction between samples much more complicated, as the isotope envelopes of the three forms start to overlap. This background exchange problem can be addressed by using a protocol that stops this process in its tracks [11].

The ^{18}O method has many advantages: the reaction is simple and uniform, every peptide will be labeled equally and the resulting 4 Da separation allows labeled peptides to be distinguished from unlabeled ones. Furthermore, the method is universal, as it can be applied to any protein sample.

2.1.1.2.2 ICAT

ICAT has been developed in 1999 [12], and employs tags that consist of three elements (see Supporting Information Fig. 1). The first of these is the reactive group, which will ensure the labeling of reduced cysteines. On the other end of the molecule is the affinity tag, which will be used to separate ICAT-labeled peptides out of a whole-lysate proteolytic digest. These two functional groups are spaced by a linker, which can be ''light'' or ''heavy'', depending on whether it contains light or heavy stable isotopes (''X'' atoms as indicated in Supporting Information Fig. 1).

The main drawbacks of ICAT are adverse side reactions and its inability to label peptides that do not contain cysteine. On the other hand, it is a well established and widely used method that does not require much post-processing. As the ''heavy'' reagent carries a linker loaded with eight deuteriums, there is a shift of 8 Da in the MS^1 spectrum between the isotope distributions of the ''light'' and ''heavy'' peptides.

2.1.1.2.3 Isotope-coded protein labels

The isotope-coded protein labels (ICPL) method relies on the same principle as ICAT of labeling the peptides with reagents of different masses. In 2004 Schmidt *et al.* employed the method to quantify proteins in a dynamic range from 0.25 to 30 with a coefficient of variation under 10% [13]. Compared with ICAT, this method has the advantage that it labels all peptides, as the reactive group targets free amines. ICAT on the other hand presents a mechanism for peptide selection through the affinity tag that ICPL does not support.

Usually ICPL reagents are used to compare two proteomes. However, taken together the four ICPL reagents shown in Supporting Information Fig. 2 allow multiplexing of up to four samples. The samples will then be distinguished by shifts of 4, 6 and 10 Da compared with the ICPL0 carrying peptide.

2.1.1.2.4 AQUA

For the AQUA method [14], the idea is once again to compare two samples at the MS^1 level. It differs from the previous methods in that quantification is here performed on a known peptide. AQUA labeling is therefore used to monitor the quantitative behaviour of a specific (set of) protein(s). The method works by synthesizing peptides originating from the target proteins using stable isotopes as labels. The synthetic peptides are then spiked into the experimental sample at known concentrations. Being chemically identical, the synthesized, labeled peptides will be indistinguishable until the MS^1 stage where a shift of a few Daltons – typically six to ten – will distinguish them from the native peptides.

One can then use the known signal from a synthetic, labeled peptide in relation to the intensity of the native peptide to quantify the protein. The quantitative investigation of proteins in low concentration is made possible through this targeted approach. Peptides that can easily be lost in the noise or that lie outside of the dynamic range of the instrument can be studied very accurately using this method.

Today, this overall strategy is particularly useful for people doing selected reaction monitoring (SRM), which implicitly targets known peptides and a set of their fragments.

2.1.2 MS^2 methods utilizing reporter ions

The reporter ion methods were successfully introduced in quantitative proteomics a few years ago. The key concept is to label the peptides in each sample with a different label molecule after protein digestion. The different labels that are used (currently up to eight can be used in a single analysis) are all designed to have exactly the same mass, but achieve this mass in different ways. Each of the labels consists of several parts, typically containing a reactive group that can bind to a peptide, a variable spacer group and a variable reporter group. The masses of the spacer and reporter balance each other out to ensure that different labels remain isobaric. As a result, a peptide occurring in different samples, and thus carrying different labels in the final mixture will still show up as a single peptide isotopic envelope in an MS^1 analysis, albeit at an increased m/z (corresponding to the constant mass increase contributed by the labels). The labels are designed to fragment very efficiently upon MS^2 analysis however, and they fragment in such a way that the reporter moiety is released. Since the reporter in each label has a unique mass, it is then easy to search the spectrum for signal at each of the expected masses. The overall benefit is of course sample multiplexing up to MS^2 spectrum, where the cleavage of the reporter group demultiplexes the different samples. Working at the MS^2 level is also beneficial in the analysis of complex samples, since the initial precursor selection inherent in MS^2 analyses will implicitly ignore a substantial part of the background signal. Finally, identification is not required in order to quantify the signal of a given precursor across samples, since it is sufficient to collect the intensities of the various reporter ions, which can be found at known masses.

The labeling itself is performed *in vitro* in this approach, which allows for a controlled incorporation of the label. A shortcoming comes from the size of the reagents; according to the law of mass action, the larger the tag, the lower the reactivity.

Since the quantification takes place in the MS^2 spectrum, it delivers (relative) peptide quantification information, which can subsequently be compiled into protein ratios after protein inference.

2.1.2.1 iTRAQ

Over the past few years, iTRAQ has become one of the most popular quantification methods in proteomics. Developed in 2004 [15] and distributed by Applied Biosystems, this reporter method allows the relative and absolute quantification at the MS^2 level of up to eight samples.

iTRAQ labeling reagents consist of three parts that will be separated during fragmentation: The peptide reactive group that reacts with primary amines, the reporter group (m/z can be one of 113, 114, 115, 116, 117, 118, 119 or 121) and the balancer group that will ensure a constant mass for the intact label molecule (see Supporting Information Fig. 3).

2.1.2.2 Tandem Mass Tag

The tandem mass tag (TMT) molecule has a structure made of four main modules [16]. The overall outline of reactive group, spacer (here called normalizer) and reporter is followed here as well, with the addition of a specific linker group to ensure good reporter cleavage upon fragmentation (see Supporting Information Fig. 4).

Different TMT types are available. The simplest form is TMT^0, which is dedicated to the testing and optimization of the method at low cost, containing no differential labeling at all. According to requirements, it is also possible to use TMT^2 to compare two different proteomes, or TMT^6 to multiplex up to six samples. The different reagents are shown in Supporting Information Fig. 4.

2.1.3 Multiplexing method

The multiplexing method also uses an isotope to separate two samples into ''heavy'' and ''light'' peptides [17]. The method differs from typical MS^1 methods in that it does not record separate MS^2 spectra for the ''light'' and ''heavy'' peptides, but a single MS^2 spectrum for both the peptides using a larger window for the precursor selection.

In the resulting fragmentation spectrum, the ''light'' and ''heavy'' MS^2 peaks are multiplexed (see Fig. 2). Both labeled and unlabeled MS^2 peaks are used for the identification. The quantification ratios are also calculated by comparing the intensities of the peaks in the MS^2 spectrum, rather than relying on the two precursor peaks in the MS^1 spectrum.

The wide precursor selection window employed in this method does not significantly decrease the quality of the MS^2 spectra; neither does the position of the ion in the window as shown in [18] where a window of m/z 10 is introduced. This m/z 10 window gave more identifications than an m/z 2.5 window, and from m/z 2.5 to 10 the intensity increased until it reached a maximum. It is however important to remember that increasing the window could decrease the selectivity of the method, which is obviously a sensitive issue when analyzing complex samples.

2.2 Label-free quantification

As the name implies, label-free quantification aims to provide quantitative information without introducing any form of labeling. Indeed, the principle is to find relevant indicators of (relative) protein abundance directly in the mass spectrometer output. Different promising methods have been developed based on different signals.

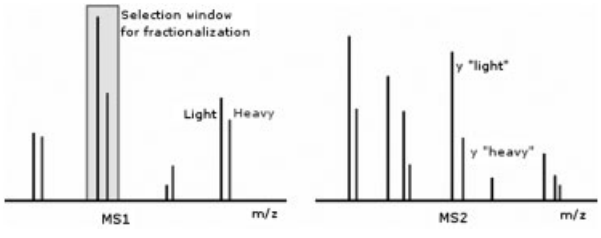

Figure 2. The multiplex strategy. A wide window is used to select the precursor and both ''light'' and ''heavy'' peptides are selected for fragmentation. The quantification is done comparing the peaks of the MS^2 spectra.

These methods present many advantages over labeled strategies. First of all, they are free and biologically simple since no additional sample preparation is required. They also support a high dynamic range compared with isotope labeling techniques [19]; differences up to 60:1 can be resolved using label-free methods, compared against 20:1 for label-based methods [20].

The important caveat for label-free methods is that the quality of the quantification will rely heavily on the quality of the data. High precision and accuracy are vital, and complex mixtures require both high-resolution mass spectrometers as well as state of the art data processing tools.

2.2.1 Spectral counting

One of the metrics used for label-free quantification is the amount of acquired spectra identified to a particular peptide. This method assumes a linear relationship between the level of sampling observed for a protein and its relative abundance. Such linearity was observed to hold over two orders of magnitude [21].

Spectral counting is of course highly dependent on peptide identification. If the number of identified peptides is too low, it is very difficult to perform proper statistical testing.

2.2.2 Protein abundance index (PAI)

The PAI metric allows an approximate quantification of proteins based on the results of peptide identification [19]. The PAI of a protein is defined as $\mathrm{PAI} = \frac{N_{\mathrm{observed}}}{N_{\mathrm{observable}}}$ with N_{observed} and $N_{\mathrm{observable}}$ the number of observed peptides and the number of observable peptides for this protein, respectively.

A revision of PAI, called exponentially modified protein abundance index or emPAI, and defined as $\mathrm{emPAI} = 10^{\mathrm{PAI}} - 1$, has been subsequently introduced as a more reliable and robust quantification indicator. A benefit of this method is that it only requires the identification results, and the data processing is correspondingly simple. Its simplicity has led to ready adoption, even in commercial tools; for instance, emPAI will be calculated by MASCOT for every database search containing more than 100 spectra.

2.2.3 TIC

The TIC, sum of the ion currents, can be measured or calculated in order to target specific ions [22]. While the length of proteins might bias spectral counting quantification, TIC-based quantification has been shown to be more accurate and to expand the dynamic range. In [20] a concentration ratio of 20:1 was measured at 20.8:1 and 17.9:1 using TIC and spectral counting, respectively. If

ratios under 20:1 are nearly identical high ratios differ dramatically. A TIC ratio of 59.3:1 gave 31.5:1 using spectral counting suggesting an increase of the dynamic range.

2.2.4 Replicate

Spectral counting, emPAI and TIC were primarily used on low-resolution mass spectrometers, where they provide a relatively simple and fast quantification. Their performance varies however, and can often prove insufficient. A higher accuracy method was found in the analysis of extracted ion chromatograms. The metric used here to represent a peptide concentration is the total intensity of the corresponding precursor measured at the MS^1 level [23] as illustrated in Fig. 3.

It can be applied to known peptides, which will be targeted in the MS^1 spectrum but also without prior peptide identification. Precursors are detected in the MS^1 spectrum by a so-called feature finder algorithm. The areas of the peaks belonging to the pattern will then be used to calculate the concentration of the peptide.

With high-resolution mass spectrometers and reproducible peptide separation, peptides from different samples that were each recorded in a separate run can be associated and quantitatively compared with each other [24].

This label-free quantification method offers a wide dynamic range as well as a high precision and accuracy. Furthermore, it can be used to compare complex samples for biomarker discovery, and works very well in the context of SRM. The biggest caveat of the method is that it relies very strongly on complex data processing algorithms.

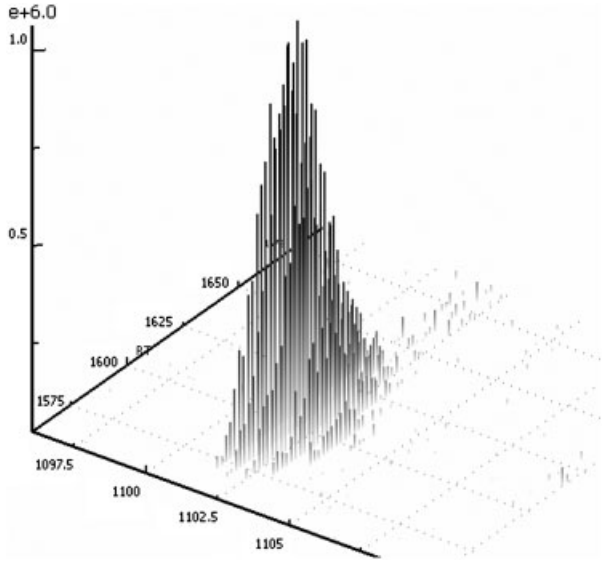

Figure 3. Precursor pattern (Intensity *versus* Retention Time and *m/z*), which will be used to estimate the amount of the corresponding peptide in label-free quantification. The data processing aim is to identify the patterns in the MS^1 spectrum and to measure their intensity.

2.2.5 Average intensity method

The average intensity method is dedicated to absolute quantification. It relies on the intensity of the three most intense tryptic peptides for a protein. Proteins can be quantified across two orders of magnitude with a CV of less than 30% [25].

A single point calibration is set for the mass spectrometer in this method to act as a reference for the absolute quantification.

2.3 SRM/multiple reaction monitoring (MRM)

SRM (the IUPAC standard term, although the now deprecated term MRM remains widely used) was widely used in the small molecule environment before being used in proteomics. It was then referred to as SIM [26].

The focus is on the targeted quantitative study of a specific set of *a priori* known proteins [27]. Selected peptides for these proteins are characterized by monitoring transitions, which are precursor-fragment ion pairs isolated by MS. A triple quadrupole mass spectrometer is typically used for these analyses, although ion trap instruments can be used as well.

Using a triple quadrupole, the width of the mass window configured in the first quadrupole as well as the collision energy have an important impact on the quality of the quantification. A larger window will provide a higher intensity but also a lower *S/N*. Reducing the mass window will decrease the overall intensity but may offer better selectivity on the transition as illustrated Fig. 4.

3 Quantification general issues

3.1 Common issues

3.1.1 Data pre-processing

Pre-processing steps can be applied to raw spectra. Since they occur at such an early stage in the overall process, these pre-processing steps can also have a disproportionally large impact on the quality of the final results.

The first step is to perform a baseline correction to find the actual zero point for the peak intensities. In this process, the overall background intensity in an *m/z* window around the peak of interest is analyzed, and the peak intensity is adjusted by subtracting the estimated background intensity. Mandatory for TOF data, it might however not be necessary for some high-resolution mass spectrometer. This tool has to be used carefully since it has a direct and dramatic impact on the ratios.

A pre-processing of the signal (typically smoothing) is often used. Reference signal processing tools like the Savitzky-Golay filter will correct for interfering noise. Smoothing can be troublesome as well however, for instance in the case of a double peak (see Fig. 5). With high-resolu-

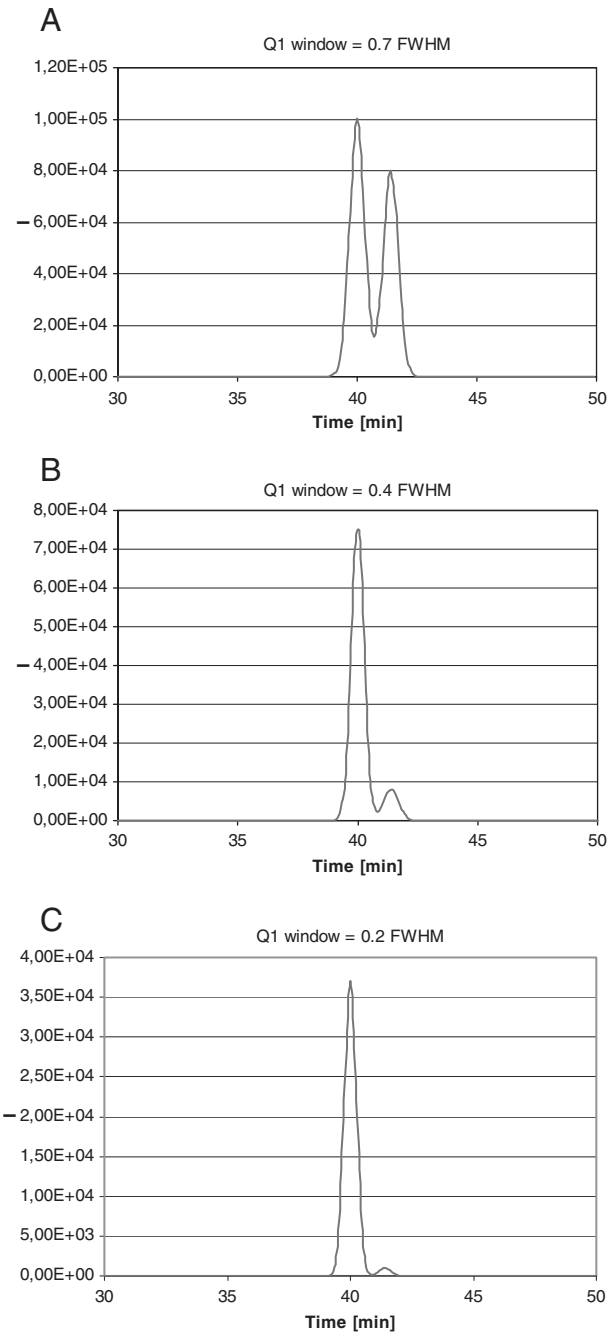

Figure 4. Selectivity in a triple quadrupole SRM experiment. While the mass window decreases (0.7 for (A), 0.4 for (B) and 0.2 for (C)) the intensity decreases (1E5 for (A), 7.5E4 for (B) and 3.7E4 for (C)) but the *S*/*N* dramatically increases (238 for (A), 676 for (B) and 25 953 for (C)). With a window of 0.7 (see (A)) the quantification is endangered by an overlap of two peaks. This problem is solved by reducing the mass window to 0.2, there is thus no more overlap (see (C)).

tion mass spectrometer this step may not be mandatory but it can remove spurious points and thus improve the quality and the speed of the processing.

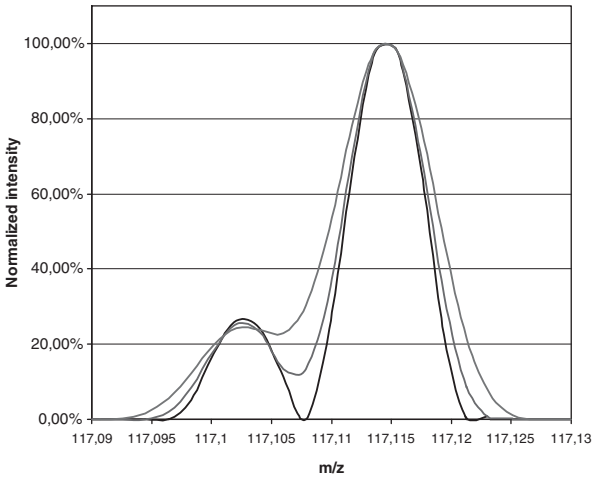

Figure 5. Double peak on iTRAQ data. With a high-resolution mass spectrometer (0.02 Da in black) the two peaks are clearly resolved. With a lower resolution (0.04 Da in grey) the accuracy of the quantification will rely on the peak picker. With an even lower resolution (0.08 Da in light grey) it is almost impossible to distinguish the peaks. If the peak picker is not able to identify accurately two intensities an important error will be carried forward throughout the quantification workflow.

Some data processing tools like a feature finder will also be more efficient if the low intensities of the spectrum are removed. By sight one can estimate a cut-off and remove the lower intensities. This step will also reduce the amount of data. However it can immediately limit the dynamic range of the downstream processing if performed too greedily.

Finally when comparing different runs one can normalize the intensities. The normalization can be done at this early step to uniform the performance of the following processing. The normalization can be done on the highest intensity detected which may not be relevant if this intensity corresponds to an over-regulated peptide.

3.1.2 A common root for problems

The cornerstone of any workflow for quantitative processing of MS data is measuring the area of peaks. The rationale is always that the detected amount of ions will somehow reflect the actual peptide concentration. Any errors made during this important step will however propagate throughout the whole calculation, placing enormous importance on the quality of this peak area metric.

Some workflows start from peak lists, regardless of the underlying performance of the peak picker algorithm employed to create these peak lists from the raw detector output. It is important to realize however that the limitations of the peak picker are carried forward throughout the rest of the analysis; *i.e.* if the peak picking occurred with poor accuracy, the final result will suffer correspondingly and will be mostly unreliable. For this reason, many more advanced

workflows work on the raw data from the instrument to be able to exert control over every step of the quantification process, especially the important first step of peak detection.

A second often-encountered pitfall in the initial steps of a quantification workflow is to ignore the surroundings of the targeted peaks. Do surrounding peaks have an effect on the targeted peaks, or are they perhaps related to these (*e.g.* ion adducts, neutral losses, isotopes)? Knowing more about the surrounding peaks can help delineate whether you are measuring noise or valuable information.

Because it is such an all-important step, we will now discuss the caveats and problems involved in peak picking in detail.

One of the first tools which is used during spectral analysis is the peak picker. It translates raw spectra into peak lists, greatly reducing the amount of data in the process [28]. The problem with quite a few peak picking algorithms is that they were originally designed to support protein or peptide identification. And for identification purposes, the peak picker primarily has to provide a precise value for the m/z of the peaks, while the intensity is allowed substantial leeway in terms of accuracy. For quantification purposes however, we really do need a precise value for the area of these peaks. We will now describe the basic steps involved in peak picking to further clarify the caveats involved in determining a peak area.

The area calculation itself can subsequently be performed by simply integrating the peak intensities. This simple method can be very sensitive to noise however.

The preferred approach to smoothing a peak is to fit and replace the peak with an ideal shape of similar area. The problem here is that it is often rather difficult to calculate the actual theoretical shape of a peak. Some peak pickers resolve this problem by simply relying on a fixed, predefined shape, but this may not fit well to the actual data. Figure 6 shows an example of poor matching between an imposed theoretical shape and a real peak shape, resulting in a substantial error in the area measurement.

As each software has its own fitting strategy, with several programs even offering multiple fitting strategies (see for instance Fig. 7), it is worth looking into the quality of fit, especially in light of the propagation of any errors made at this stage of the quantification process.

Peak fitting thus requires the use of a shape that is adapted to the actual spectra, and in practice this would be most easily achieved by using an algorithm that can adapt to the actual peak shapes found in the spectra.

Since nearly all samples will contain large dynamic range differences in protein concentrations, the peak picker must also be able to detect low intensity peaks whenever they are relevant. If the quantification method used is based on *a priori* known precursors or fragments of interest, the peak picker should take particular care of investigating and fitting peaks at the relevant m/z values concerned. Similarly, if a mass shift between two isotopic envelopes is to be detected, the peak picker should focus on correctly resolving such peak pairs or sets. Finally, label-free methods typically require the entire isotope pattern for a peak to be clearly detected and resolved in detail. Especially whenever this leads to peak detection below the S/N limit for the spectrum, peak picker performance and robustness is crucial.

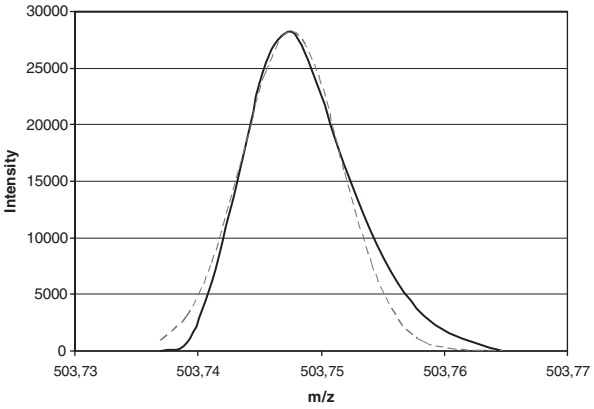

Figure 6. Fitting of a good quality peak. Since the peak is not symmetrical, fitting it with a Gaussian shape (dashed line) leads to an error of 7.2%. Actual shape fitting can often be much worse.

3.1.3 Data reduction

The actual peak picking is often followed by deisotoping and feature finding. During the overall process, the amount of data points from the original raw data is greatly reduced, prompting the moniker of "data reduction tools" for such software.

To proceed to deisotoping the distance between peaks is used to estimate the charge. This can be done using a Fourier Transform. Once the charge is known the monoisotopic mass and overall intensity is estimated fitting the peak distribution with a theoretical shape. This spectral analysis can be conducted by the Horn Mass Transform [29] as illustrated in Fig. 8.

On the retention time standpoint a peptide will provide an elution profile. Combined to the isotope distribution (see Fig. 9) it will result in a feature characterized by a certain retention time, a monoisotopic mass and an intensity. Those three parameters will represent the peptide and allow further analysis with few datapoints.

3.1.3.1 An example of free software for raw data pre-processing: open MS

The Open MS software [30], used in the The OpenMS Proteomics Pipeline (TOPP) tools [31], is a good example of a free software suite that can perform the abovementioned pre-processing steps. It is important to note that raw instrument data first has to be converted into open format xml files before data pre-processing can be applied using Open MS (See Section 3.1.4). The software features the standard signal processing methods as well as a high-performance peak picker. In Open MS, the peak picking

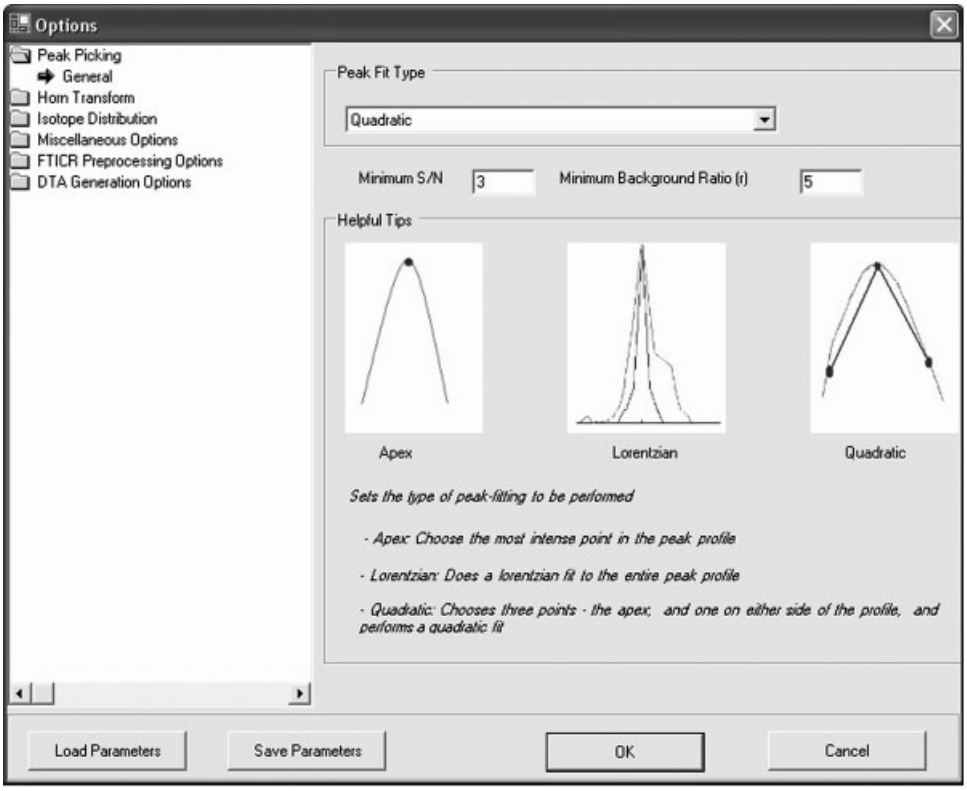

Figure 7. Peak picking option in decon2LS. The user can choose between three models to estimate the intensity of the peak. The most intense point in the peak profile, which does not take the shape of the peak into account. A fitting with a Lorentzian distribution, which will not be adapted to asymmetrical peaks. A quadratic fit, which will only rely on three points.

algorithm is wavelet-based, and is able to adapt to the actual shape of individual peaks. It can typically outperform instrument vendor-provided peak pickers by distinguishing more peaks, dramatically improving the downstream capacity for label-free quantification [32]. Thanks to the adaptive fitting shape, it provides accurate area values for the peaks, even for non-symmetrical peaks (see Fig. 10).

Similar free software exists, for instance XCMS [33] and MZmine [34].

3.1.4 Data formats

Ideally, processing of raw data files would involve the actual files recorded by the instrument. However, most of these files are proprietary, binary compressed files that are virtually impossible to process without access to the corresponding, proprietary vendor libraries [28]. It is therefore difficult to support such file formats, as each vendor's format is different, and often differences even exist between different instruments made by the same vendor. As a result, standardized, open formats have been proposed to capture the vendor data in more accessible, XML-based files. The most adopted of these are mzXML [35] and mzData, and the new consensus format recommended by the HUPO Proteomics Standards Initiative, mzML, which supersedes both of these [36].

Although a variety of freely available convertors exist that can translate vendor-specific binary files into these open, standardized files, there is an important caveat involved with the conversion process: it is often quite time consuming, and the standardized files can be extremely large.

3.1.5 Confidence level

It is advisable to maintain a confidence (or quality) indicator that starts at the raw spectrum, all the way through to the final result. Such an indicator should take into account the quality of the fitting, the properties of the peaks and all the other sources of error that can occur, depending on the quantification method.

While a fitting score can easily be set by the peak picker, a feature score can be used in label-free quantification, and an iTRAQ confidence indicator can also be set using the distribution of peptide ratios and intensities; a global workflow metric is however often missing from quantitative analyses.

The availability of such a score would make it far easier to validate the end results of the quantification by flagging suspicious data.

3.2 Quality

From the sample preparation to the quantification calculation, every single operation can introduce errors in the measurement. It is important to note however that the different steps affect the final result differently. As a result, it is vital to focus quality control strategies on certain defined parts of the analysis.

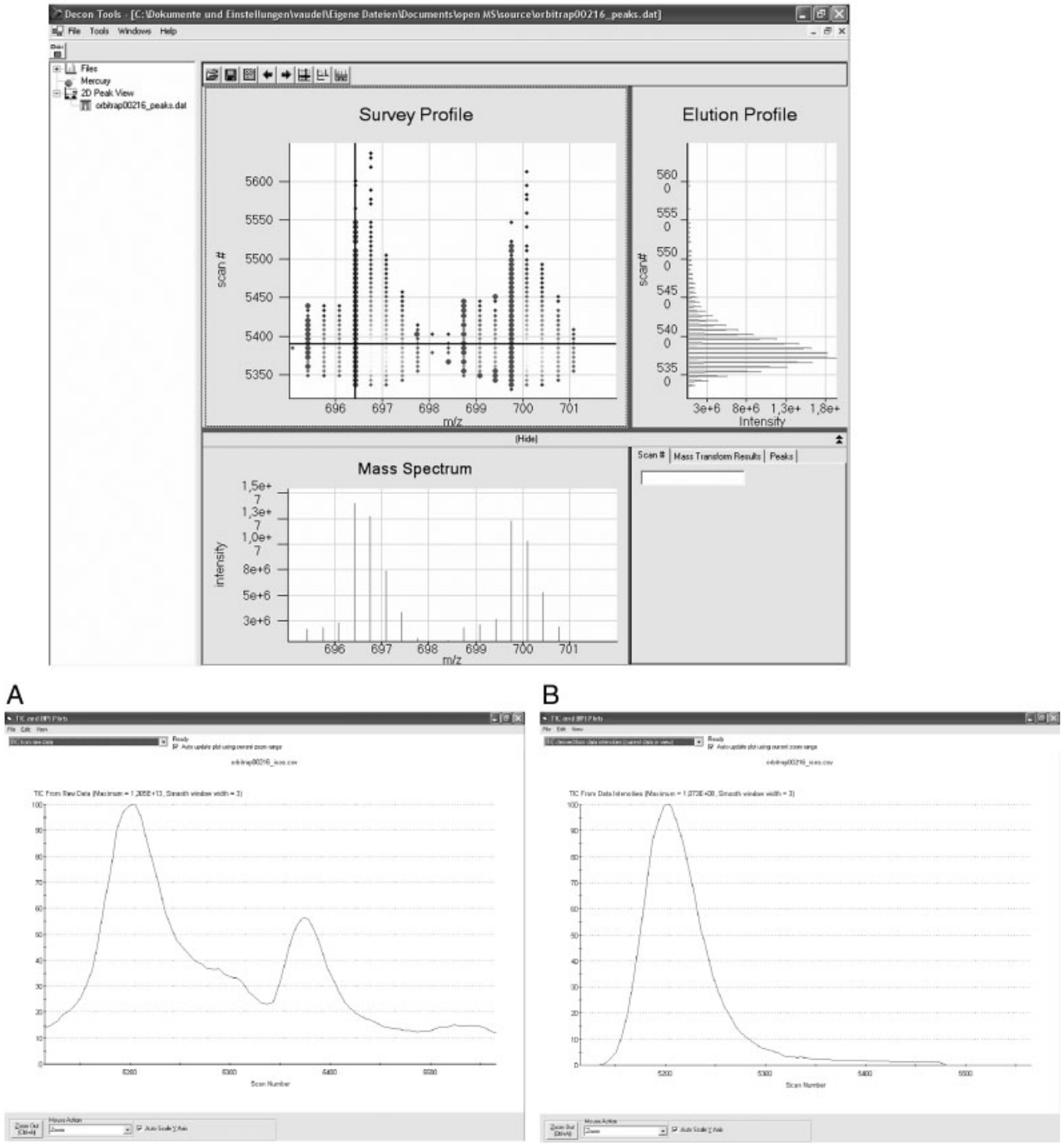

Figure 8. The isotope patterns can be deisotoped (in green) using the Horn mass transform. Here the deisotoping was conducted under Decon2LS; (A) shows the TIC of an isotopic pattern, and (B) the TIC of the deisotoped pattern, which may be used for label-free quantification.

3.2.1 Precision and reproducibility

The quantification precision and reproducibility are mainly affected by the sample processing. The subsequent ionization process, MS analysis and data processing steps all have a higher precision and reproducibility than this initial sample preparation.

A complex but sometimes neglected step is the compilation of peptide quantification results into protein ratios. This issue, which is tackled in more detail in a following section, will directly determine the ultimate quantification result. The location of the distribution of peptide ratios will give the protein ratio while the scale of this distribution provides a straightforward

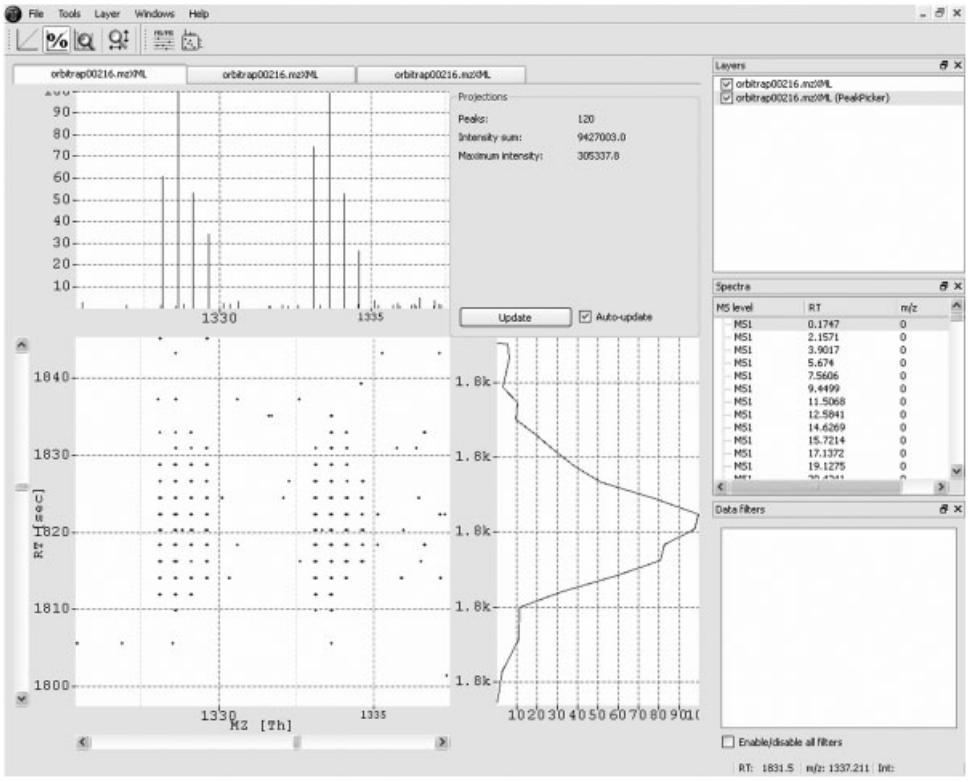

Figure 9. Isotope distribution and elution profile after peak-picking by Open MS. The identification of the isotope distribution and of the elution profile in the MS^1 map and their fitting will allow data reduction and label-free quantification.

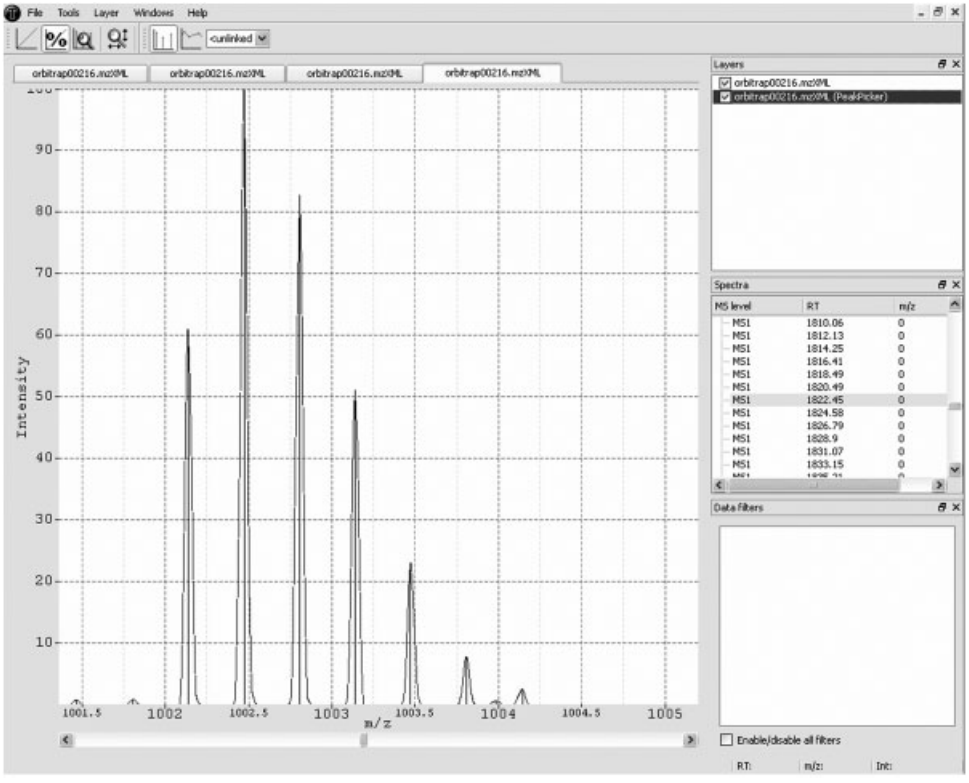

Figure 10. Peaks picked under Open MS. TOPP provides a versatile free platform for proteomics analysis. The peak picker is one of the TOPP tools. Using a wavelet transformation it accurately detects more peaks than vendor-provided peak pickers. The peaks are fitted with an adaptive shape providing a reliable basis for accurate quantification.

view on the quality of the quantification. A distribution with a small scale will be the aim of the precision improvement.

3.2.2 Accuracy

The accuracy can be affected by each of the steps. One of the main contributors to varied accuracy of the results can be found in the varying ionization and fragmentation efficiencies from one peptide to the other. Improving the uniformity of the processes is however far from trivial, and the corresponding costs will probably not outweigh the benefits for most methods. Targeted methods such as AQUA and SRM can however take these factors into account, as the choice of the target peptide is relatively open. As a result, substantial effort has recently been invested in creating prediction software that *a priori* selects optimal peptides to serve as targets [14, 27]; these are the so-called proteotypic peptides.

While the data processing following MS does not have much effect on precision since algorithms have an optimized bijective behaviour, these steps can have an impact on accuracy that is often underestimated.

Improving the quantification workflow from the raw spectra to the final results will dramatically improve the accuracy. As we have discussed in detail before, the peak picking is one of the key stages in the overall calculation.

3.2.3 Dynamic range

Accuracy and precision also depend on the dynamic range and the complexity of the sample. Compared with a mid-range reference concentration, it is very difficult to quantify very high or very low concentrations.

As detailed above, the peak picker will determine how deep you can go into the data, but it will be obvious that the more extreme the concentration difference, the lower the accuracy of the quantification [3]. This is illustrated schematically in Fig. 11. Usually relative ratios under 0.3:1 and over 10:1 suffer from a poor accuracy.

3.2.4 Error detection

Precision errors can be found using reference proteins spiked into the sample background. Once the distributions of the quantitative data are known, the difference between their known and determined abundance can be measured and the precision error can be estimated and possibly corrected for.

The accuracy error is more complicated to detect. As said before, it is recommended to use a continuously updated quality indicator to estimate the final reliability of the result.

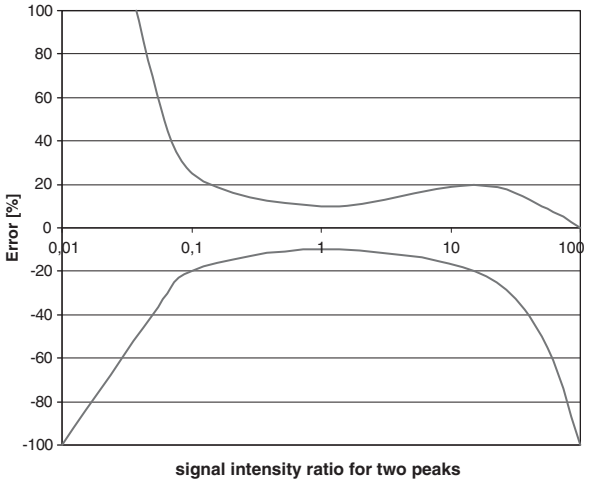

Figure 11. Intuitive representation of the quantification error as a function of the signal intensity ratio of two peaks. An ideal quantification provides the perfect result (0% error), the real measure will be found between the lines.

3.3 Protein quantification

The final goal of a quantitative proteomic analysis is the estimation of the amount of a protein in a sample. There are two main ways to do this: measuring the amount of protein directly or the more commonly used estimation of the amount of peptides, which is then used to calculate a protein quantitative value after protein inference.

3.3.1 From peptide quantification to protein quantification

In a typical scenario, multiple peptides can be identified for a set of proteins. A simple and straightforward approach to quantify a protein can then consist of grouping together all peptides that map to a protein, rejecting any obvious outliers, and calculating a mean or median value on the peptides as the protein result.

However, some of these peptides can be uniquely mapped to only one of the proteins in the set, whereas other peptides may be shared between proteins. In such a scenario the uniquely mapping peptides will be of lower abundance than the shared peptides, as the latter will report an abundance that is effectively the sum of abundances of all their precursor proteins. If the shared peptides now outnumber the unique peptides, the unique peptides will likely be considered outliers, ultimately providing us with the sum of the shared protein amounts for individual proteins. Our quantification can therefore easily have an error of 100% or more for at least one of these proteins!

One way to alleviate this issue is to preselect relevant spectra before compiling the quantitative results at the peptide level. Software such as Peptizer [37] can easily perform such filtering, selecting only uniquely mapping peptides, precursors

containing the selected modification or more generally any peptides matching to one or more predefined criteria.

Alternatively, the quantity of peptide-sharing protein groups can be assessed, rather than the quantity of individual proteins, which also provides solace from the shared peptide problem, albeit at the price of the loss of resolution [38].

3.4 Quantification and data management

The integration of a quantification tool into a laboratory information management system can greatly simplify data management and workflow automation. Rover (http://genesis.ugent.be/rover/) is an example of such a quantification tool, as it integrates perfectly into the ms_lims data management system (http://genesis.ugent.be/ms_lims/), providing a high-quality quantification package that supports different methods in a user-friendly graphical environment.

MASPECTRAS [39], a platform for management and analysis of the data also integrates quantification throw ASAPRatio.

4 Method-specific quantification issues

Different quantification methods require different types of data processing. This section will describe the main issues for each quantification method described in Section 2. Specific tools that address data processing will be listed in Section 5.

4.1 Isotope labeling quantification

Isotope labeling quantification data processing seems easier than label-free processing since it is known where the quantitative information can be found: in the labeled/unlabeled peak pairs, at the specific masses of reporter ions. High-accuracy quantification will of course rely on the general issues described above, but is further affected by a few method-specific issues.

4.1.1 MS^1 methods

The data processing in this case consists of measuring the relative intensities of the isotope patterns of the ''heavy'' and the ''light'' peptides as illustrated in Fig. 12. Difficulties may arise when such a peptide overlaps with another peptide's isotope pattern. Such overlap can easily occur between the light and heavy version of a peptide if their absolute mass difference is relatively small, they carry a higher charge and the resolution of the instrument is not optimal. The main challenge is then to identify, separate and quantify accurately both isotope distributions.

The dynamic range will be limited by the ability of the peak picker to find the isotope distribution of the peptide. In the case of extreme ratios, one of the two patterns can easily be lost in the noise or in another overlapping isotope pattern.

The main issue in the processing of ^{18}O data is the quantification of samples where background exchange has occurred, labeling certain peptides incompletely. Software such as ZoomQuant [40] has been developed to tackle this issue.

However, since the problem of the background exchange can be corrected by improving the experimental protocol, carefully obtained ^{18}O data can be processed similar to a classic precursor method where isotope patterns separated by a few Daltons have to be found.

4.1.2 MS^2 methods utilizing reporter ions

The expected output of the iTRAQ 8Plex method is seven ratios *per* spectrum, representing the relative intensities of the reporter ion peaks for the concerned peptide. There are only seven ratios, as one peak will always serve as the reference. TMT will provide up to five ratios.

Many software packages (see Fig. 13) are available to process reporter data more or less accurately, although most of these cannot process the raw data directly.

Regardless of the tool used, the most sensitive operations that are most deserving of special attention are outlined below.

The potential pitfalls in the first step, picking the peaks and determining their areas at the specific m/z for the reporter ions have already been described in the general issues above.

4.1.2.1 Isotope correction

The labeling reagents are not 100% pure to start with, so an isotope correction is to be applied on any intensities measured. In the case of iTRAQ for instance, the necessary correcting factors are provided with each purchased set of labels.

$I_0 = \begin{pmatrix} I_{113_0} \\ I_{114_0} \\ I_{115_0} \\ I_{116_0} \\ I_{117_0} \\ I_{118_0} \\ I_{119_0} \\ I_{121_0} \end{pmatrix}$ are the ''perfect'' intensities as they would be without isotopes and impurities. We however measure

$I = \begin{pmatrix} I_{113} \\ I_{114} \\ I_{115} \\ I_{116} \\ I_{117} \\ I_{118} \\ I_{119} \\ I_{121} \end{pmatrix}$. The link between I and I_0 is then given by:

$I = M.I_0$ with $M = (m_{i,j}), 1 \leq i,j \leq 8$.

The non-zero $m_{i,j}$ are provided by the label vendor. Table 1 provides these coefficients for 8-plex iTRAQ as

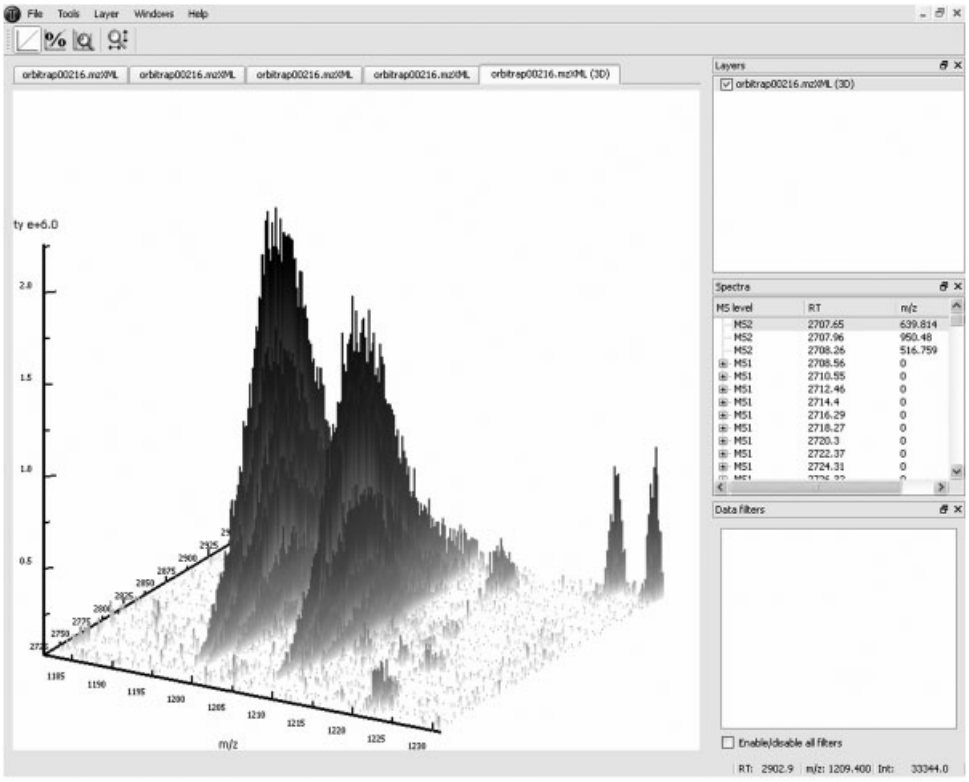

Figure 12. 3-D view of two precursor distributions. The comparison of the intensities of the features will give a relative quantification of the peptide between two proteomes. Features can be linked and compared using TOPP tools.

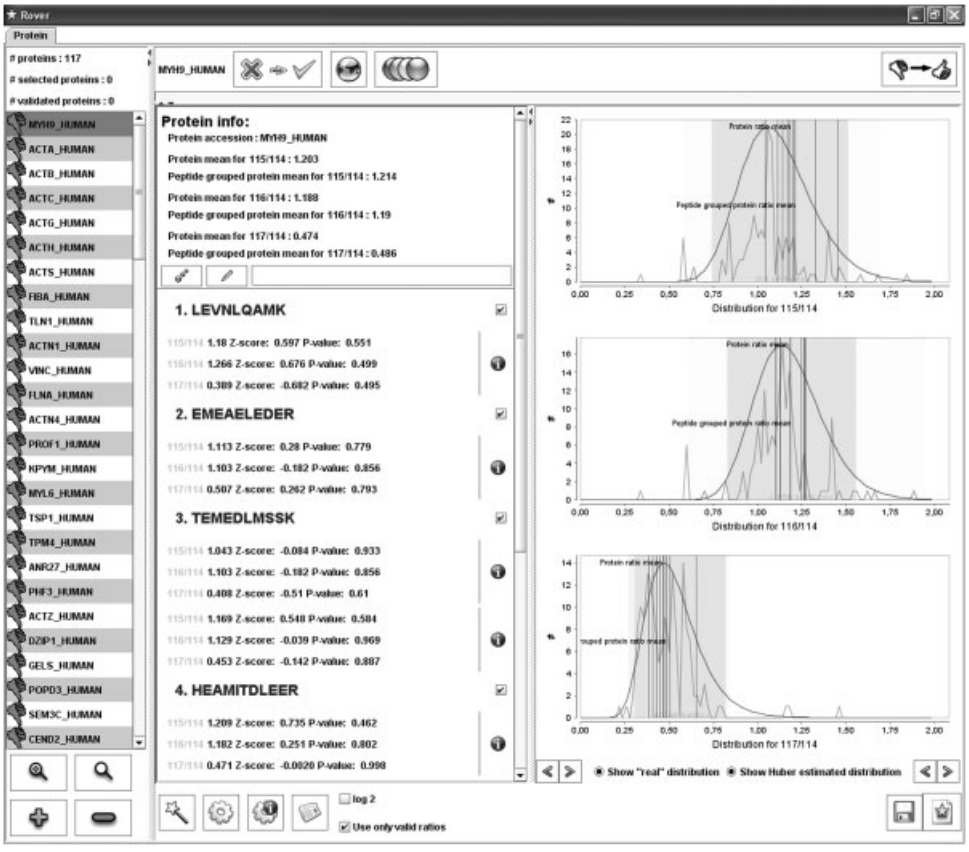

Figure 13. iTRAQ quantification using Rover. Rover provides a user-friendly interface to post-process quantitative data.

Table 1. iTRAQ 8plex isotope correction parameters

Reagent	% of −2	% of −1	% of 0	% of +1	% of +2
ITRAQ® Reagent- 8plex 113	0.00	0.00	m1.1 = 92.89	m2.1 = 6.89	m3.1 = 0.22
ITRAQ® Reagent- 8plex 114	0.00	m1.2 = 0.94	m2.2 = 93.01	m3.2 = 5.90	m4.2 = 0.16
ITRAQ® Reagent- 8plex 115	m1.3 = 0.00	m2.3 = 1.88	m3.3 = 93.12	m4.3 = 4.90	m5.3 = 0.10
ITRAQ® Reagent- 8plex 116	m2.4 = 0.00	m3.4 = 2.82	m4.4 = 93.21	m5.4 = 3.90	m6.4 = 0.07
ITRAQ® Reagent- 8plex 117	m3.5 = 0.06	m4.5 = 3.77	m5.5 = 93.29	m6.5 = 2.88	m7.5 = 0.00
ITRAQ® Reagent- 8plex 118	m4.6 = 0.09	m5.6 = 4.71	m6.6 = 93.32	m7.6 = 1.88	m8.6 = 0.00
ITRAQ® Reagent- 8plex 119	m5.7 = 0.14	m6.7 = 5.66	m7.7 = 93.34	0.87	0.00
ITRAQ® Reagent- 8plex 121	m7.8 = 0.27	7.44	m8.8 = 92.11	0.18	0.00

provided by Applied Biosystems on the 15th of May 2009, which gives:

$$M = 10^{-2}.\begin{pmatrix} 92.89 & 0.94 & 0 & 0 & 0 & 0 & 0 & 0 \\ 6.89 & 93.01 & 1.88 & 0 & 0 & 0 & 0 & 0 \\ 0.22 & 5.90 & 93.12 & 2.82 & 0.06 & 0 & 0 & 0 \\ 0 & 0.16 & 4.90 & 93.21 & 3.77 & 0.09 & 0 & 0 \\ 0 & 0 & 0.10 & 3.90 & 93.29 & 4.71 & 0.14 & 0 \\ 0 & 0 & 0 & 0.07 & 2.88 & 93.32 & 5.66 & 0 \\ 0 & 0 & 0 & 0 & 0 & 1.88 & 93.34 & 0.27 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 92.11 \end{pmatrix}$$

With M known, the correction is then performed by calculating the matrix multiplication of M^{-1} with the measured intensities matrix I.

Once properly measured and corrected, the area of the peak should correspond to the amount of the selected peptide in each sample. Dividing each of them by a chosen reference reporter intensity will provide the seven ratios.

4.1.2.2 Precision and accuracy

There is no predefined precision and accuracy standard that can be relevant to every analysis. However one is likely to encounter the following issues that affect these two quality metrics.

As mentioned above, precision is mainly influenced by sample processing. When the peak picker used is not adapted to the data, a lack of precision might also result.

Accuracy is mainly a matter of data processing. As such, the simple workflow described above for the reporter method contains a few ambushes.

The first of these is incorrect peak picking. To put this in context: is it relevant to perform an isotope correction of 1.88% if the peak picking results in errors of up to 20%?

Another issue concerns the surrounding peaks. The reporter labeling peaks are situated at precise m/z, which might overlap with the m/z of immonium ions that are generated in the fragmentation process. Furthermore, it is always prudent to assume that other unexpected ions may also interfere with the reporter ion peaks.

4.1.2.2.1 iTRAQ

There is no iTRAQ Reagent at $m/z = 120$ because this m/z corresponds to the immonium ion of phenylalanine, which is commonly observed. But the +1 isotope of this immonium ion will contribute to the reporter ion peak at $m/z = 121$ as well, which therefore has to be corrected if the mass spectrometer cannot separate the two peaks. The intensities of the phenylalanine immonium ion isotopes at m/z 120 and 121 can be theoretically calculated as 91.01 and 8.62%, respectively. This can be implemented in the matrix M seen before as:

Table 2. Immonium ions and related ions which might interfere with iTRAQ peaks

Nature	Name	Mass	Comment
Immonium ion	Phenylalanine	120	Strong
Related ion	Arginine	112	
Related ion	Histidine	121	Weak or absent
Related ion	Lysine	112	
Related ion	Tryptophan	117	
Amino acid residue	Acetylproline	112	N-terminal
Amino acid residue	Aminoethylcysteine	120	

$$M_1 = 10^{-2}.\begin{pmatrix} 92.89 & 0.94 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 6.89 & 93.01 & 1.88 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0.22 & 5.90 & 93.12 & 2.82 & 0.06 & 0 & 0 & 0 & 0 \\ 0 & 0.16 & 4.90 & 93.21 & 3.77 & 0.09 & 0 & 0 & 0 \\ 0 & 0 & 0.10 & 3.90 & 93.29 & 4.71 & 0.14 & 0 & 0 \\ 0 & 0 & 0 & 0.07 & 2.88 & 93.32 & 5.66 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 1.88 & 93.34 & 0 & 0.27 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0.87 & 91.01 & 7.44 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 8.62 & 92.11 \end{pmatrix}$$

Here a line and a column were added to take into account the phenylalanine ion and the iTRAQ correcting factors at $m/z = 120$. We can now use this revised matrix M to correct our measured intensities I as detailed above.

Other ions might interfere in the reporter ion m/z range [41], and these are presented in Table 2.

Furthermore, [41] reports of a conflicting Tyr-related ion at 117 Da, and [42] describes an additional interfering ion at 107 Da.

While the ions falling on 112 and 120 may only have an impact *via* their heavier isotopes, all other ions listed here will directly disturb the quantification process. This problem

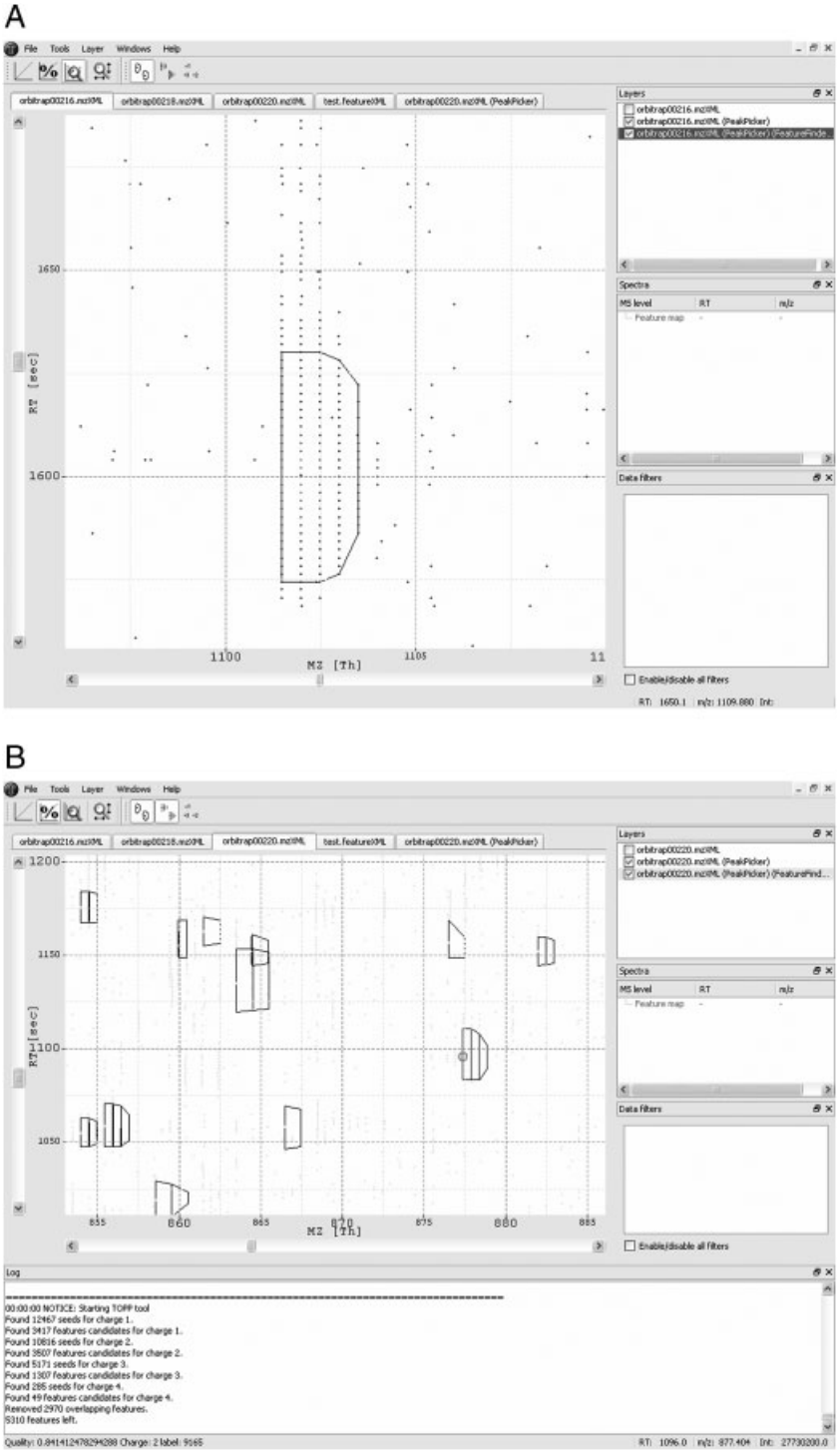

Figure 14. (A) Feature finding by Open MS, the intensities of the peaks circumscribed by the black pattern will represent the amount of this peptide. Maps of different runs can be aligned and compared. (B) Feature finding in low intensity with Open MS. Low intensity features might not be detected or noise might look like a feature. A score is given by Open MS to help the user in discriminating the results. The parameters of the features finder might be optimized to the data processed as well.

is compounded by the observation that the intensity of these ions can have the same order of magnitude as the reporter ion peaks themselves. They may sometimes even be more intense than the reporters, leading to very important errors. A typical sign of such contaminant ion interference is the occurrence of negative ratios after application of the correction factors.

4.1.2.2.2 TMT

Reporter ions in TMT have peaks situated at m/z 126 to 131. The immonium ions and related ions observed in this mass window [41, 42] are presented in Table 3. As one can easily notice, care has to be taken when processing the peaks at m/z 129, 130 and 131.

Table 3. Immonium ions and related ions which might interfere with TMT peaks

Nature	Name	Mass	Comment
Immonium ion	Arginine	129	Usually weak
Related ion	Lysine	129	Medium
Related ion	Glutamine	129	Medium
Related ion	Tryptophan	130	Weak to medium
Amino acid residue	Acetyllysine	126	

4.1.2.3 Dynamic range

As explained before, the dynamic range will depend on the peak picker's ability to detect small peaks and to pull them out of the noise. Since we know exactly where to expect the reporter ion peaks, the peak picker should take particular care at those m/z. At these positions peaks are not likely to be noise, especially if other reporter ions can be found more easily in the same spectrum, which can allow a peak picker to accept peaks under the normal S/N limit. However, it must be observed here once again that smaller peaks lead to lower accuracy.

4.1.2.4 Error detection

Error in precision will be seen when plotting the ratios as a distribution or when monitoring spiked background proteins. If a global shift can be seen in these analyses, a normalization process can correct for the systematic precision offset.

As outlined above, accuracy errors are far more complicated to detect and correct. Interfering ions might fall in the dedicated reporter m/z values. However, they may differ to some extent in exact m/z value. As illustrated in Fig. 5, depending on the resolution of both the mass spectrometer as well as the peak picker, such an interfering ion will be resolved together with the reporter ion in a single peak, both ions will be combined in a double peak or they will be perceived as two distinct peaks. A single peak will not allow error detection as the two ions are wholly collapsed and have become indistinguishable. A double peak will support error detection based on the peak shape, which can be recorded in the confidence indicator or corrected, depending on the performance and abilities of the peak picker. When resolved in two separate peaks, it should be straightforward to find the reporter ion unambiguously.

Errors due to extreme dynamic range are hard to detect and correct. But the intensity of the individual peaks may be introduced as a component of the confidence indicator, along with the quality of the fitting.

4.2 Label-free quantification

As mentioned before, label-free quantification can be much more challenging concerning data processing. The most promising method is the replicate method where isotope features are detected in MS^1 spectra.

Correct preprocessing of the data is a key issue in this method, more so than in any other approach. The software will need as many peaks of the isotope pattern as possible in order to find isotopic features, and noise should simultaneously be maximally suppressed.

4.2.1 TIC

TICs are usually reported by the instrument software. Third party software like Viper [43] that can calculate the TIC out of the raw data is available as well, however.

4.2.2 Replicate

The label-free quantification by isotope patterns is done at the MS^1 level. Mass trace and isotope patterns are detected and compiled into precursor features as illustrated in Fig. 14A. The sum of the intensities of the peaks will be proportional to the peptide concentration.

Chromatographic shifts of identical peptides between LC-MS runs may disturb the retention time stability and endanger the quantification. Efficient correction algorithms have been developed to correct for such shifts [44, 45]. However different retention times imply variation in the HPLC organic phase concentration, in turn inducing bias in the ionization efficiency. Even if the shifts can be corrected for, the relative comparison of the intensities may therefore not be meaningful. The reproducibility of the experimental conditions is therefore a key issue when dealing with label-free quantification.

Table 4. Pre-processing, peak picking and data reduction software

Software name	Input	Free?	Availability	Ref.
Open MS/TOPP	Open formats	Yes	www.openms.de	[30]
TPP	Raw data and xml files	Yes	http://tools.proteomecenter.org/wiki/index.php?title = Software:TPP	[47]
ProTrawler	Raw data and xml files	No	http://www.bioanalyte.com/software/protrawler/index.html	
Decon2LS	Raw data and xml files	Yes	http://omics.pnl.gov/software/Decon2LS.php	[48]
MASCOT distiller	Raw data and xml files	No	http://www.matrixscience.com/distiller.html	
Elucidator	Raw data	No	http://www.rosettabio.com/products/elucidator	
XCMS	Xml files	Yes	http://masspec.scripps.edu/xcms/xcms.php	[33]
mzMine	Xml files	Yes	http://mzmine.sourceforge.net/	[34]

Table 5. Quantification software sorted according to the different methods

Software name	Input	Free?	Availability	Ref
iTRAQ				
Rover	mzXML	Yes	http://genesis.ugent.be/rover/	
Open MS/ TOPP	Open formats	Yes	http://www-bs2.informatik.uni-tuebingen.de/services/OpenMS-release/html/TOPP_ITRAQAnalyzer.html	[30]
MASCOT	See website	No	http://www.matrixscience.com/	
Multi-Q	mzXML	Yes	http://ms.iis.sinica.edu.tw/Multi-Q/	[49]
8-Tracker	.mgf, .dat	Yes	http://www.cranfield.ac.uk/health/researchareas/bioinformatics/page10385.jsp	[50]
Libra	mzXML	Yes	http://tools.proteomecenter.org/wiki/index.php?title = Software:Libra	[48]
ProQuant	raw data	No	https://products.appliedbiosystems.com/ab/en/US/adirect/ab?cmd = catNavigate2&catID = 600925	
ProteinPilot	raw data	No	https://products.appliedbiosystems.com/ab/en/US/adirect/ab?cmd = catNavigate2&catID = 601680&tab = DetailInfo	
Quant	dta	Yes	http://sourceforge.net/projects/protms/	[51]
SILAC				
SilacAnalyser	Open formats	Yes	http://www-bs2.informatik.uni-tuebingen.de/services/OpenMS-release/html/TOPP_SILACAnalyzer.html	
MSQuant	Raw data	Yes	http://msquant.sourceforge.net/	[52]
MASCOT distiller	Raw data and xml files	No	http://www.matrixscience.com/distiller.html	
Warp-LC	Raw data	No	http://www.bdal.com/products/bioinformatics/warp-lc.html	
Elucidator	Raw data	No	http://www.rosettabio.com/products/elucidator	
MaxQuant	Raw data	Yes	http://www.maxquant.org/	[53]
^{18}O				
ZoomQuant	Raw data	Yes	http://proteomics.mcw.edu/zoomquant	[40]
MASCOT distiller	Raw data and xml files	No	http://www.matrixscience.com/distiller.html	
STEM	MASCOT results	Yes	http://www.ncbi.nlm.nih.gov/sites/entrez	[54]
ICAT				
MASCOT distiller	Raw data and xml files	No	http://www.matrixscience.com/distiller.html	
XPRESS	mzXML	Yes	http://tools.proteomecenter.org/wiki/index.php?title = Software:XPRESS	[55]
ASAPRatio	mzXML	Yes	http://tools.proteomecenter.org/wiki/index.php?title = Software:ASAPRatio	[56]
Elucidator	Raw data	No	http://www.rosettabio.com/products/elucidator	
ICPL				
MASCOT distiller	Raw data and xml files	No	http://www.matrixscience.com/distiller.html	
Warp-LC	Raw data	no	http://www.bdal.com/products/bioinformatics/warp-lc.html	
Multiplex				
MASCOT distiller	Raw data and xml files	No	http://www.matrixscience.com/distiller.html	
MS2Ratio			Not found	[17]
Label-free quantification				
Open MS/ TOPP	Open formats	Yes	www.openms.de	[30]
MASCOT	See website	No	http://www.matrixscience.com/	
Viper	Xml	Yes	http://omics.pnl.gov/software/VIPER.php	[43]
MSInspect	mzXML	Yes	http://proteomics.fhcrc.org/CPL/msinspect/index.html	[57]
SpecArray	mzXML	Yes	http://tools.proteomecenter.org/wiki/index.php?title = Software:SpecArray	[58]
MSight	Raw data and xml files	Yes	http://www.expasy.ch/MSight/	[59]
SuperHirn	mzXML	yes	http://tools.proteomecenter.org/wiki/index.php?title = Software:SuperHirn	[60]
PEPPeR	mzXML	Yes	http://www.broad.mit.edu/cancer/software/genepattern/	
QuantLynx	Raw data	No	http://www.waters.com/waters/nav.htm?cid = 513662	
SIEVE	Raw data	No	http://www.thermo.com/com/cda/article/general/1,,20429,00.html	
Elucidator	Raw data	No	http://www.rosettabio.com/products/elucidator	
Expressionist	mzXML	No	http://www.genedata.com	

Table 6. Quantification results post-processing software

Software name	Input	Free?	Availability
Rover	See Section 5.3	Yes	http://genesis.ugent.be/rover/

4.2.2.1 Precision and accuracy

The precision and accuracy will be affected by the pre-processing of the data and by the settings used for the feature finder. The boundaries for the different parameters of the feature can be set according to the data processed.

4.2.2.2 Dynamic range

One of the main benefits of this method is its ability to quantify proteins over a high dynamic range. The dynamic range will be limited by the complexity of the sample and the S/N of the spectrum however. Even if the performance of the software is outstanding, it might still be difficult to find features of low intensity. In such cases, a second pass analysis or double-check may be necessary as illustrated in Fig. 14B.

4.3 SRM/multiple reaction monitoring

The main issue in SRM protocols is to identify the best-suited SRM transitions (see also above). Optimal transitions correspond to the most intense fragment ions. A corresponding protocol (such as described in [27]) can also be software assisted (see above).

According to the actual quantification protocol chosen, one can refer to the issues raised above. Due to the variety of methods and instruments used the quality and dynamic range may vary a lot. However the SRM protocols are highly configurable and can be optimized to address a wide range of biological issues.

5 Software summary

This last section presents a list of software applications for quantitative proteomics, as found in the literature [46] or as used for each technique quoted above.

It is recommended that the prospective user verifies that the software is adapted to the precision of the mass spectrometer. High-precision instruments will not require the same care as low-precision mass spectrometers with regards to peak picking or the influence of surrounding peaks.

5.1 Pre-processing, peak picking, data reduction

Many data reduction software tools are available (see Table 4), some of which have already been highlighted above.

5.2 Quantification

Different tools are available, that are typically specifically designed to handle the processing of data derived from a given method (see Table 5). Since the different isotope labeling methods are often very similar from a data processing standpoint, some of the tools may adapt to other labeling methods as well.

5.3 Post-processing

The Rover software tool can be used to evaluate and post-process Consensus, msQuant, maxQuant and MASCOT distiller result files (Table 6).

6 Concluding remarks

The numerous methods developed for the quantification of gel-free proteomics provide efficient protocols that can be applied to a variety of problems. However, a few issues still limit our productivity. The proprietary vendor formats that require long conversion times into much larger open standards are one of these. Another issue is typically found in the lack of care that is often dedicated to the data processing in comparison to the sample preparation. While care in sample preparation is obviously vital, correct processing of the acquired MS data in the very first steps of quantification plays an equally important, if not more important, role in the entire process.

The data processing is often seen as secondary in the experimental plan and improvised once the data acquisition is complete. However if the processing is not prepared in detail, its costs are underestimated, or it is not fully integrated in the experimental project, the analysis step might jeopardize the whole experiment. As outlined above, driving an accurate quantification method requires avoiding many pitfalls. It is therefore worth investing up-front time into learning to navigate this ''map of the minefield''.

Overall, it is a great pleasure to see that gel-free quantification methods in the field of proteomics have reached a level of accuracy where every step has to be accomplished carefully. The major errors are due to the sample preparation and the data analysis, which could be reduced or avoided through the implementation of a set of standard tests as for instance suggested by HUPO [5]. Indeed, for many researchers, the data processing has become the limiting factor, in terms of throughput, accuracy, dynamic range or all of the above. But the increasing availability of user-

friendly and powerful tools, as well as the better understanding of the data processing by the user will hopefully soon help the community to satisfactorily address this issue, leading the way to important discoveries.

The authors have declared no conflict of interest.

7 References

[1] O'Farrell, P. H., The pre-omics era: the early days of two-dimensional gels. *Proteomics* 2008, *8*, 4842–4852.

[2] Lilley, K. S., Protein profiling using two-dimensional difference gel electrophoresis (2-D DIGE), *Curr. Protoc. Protein Sci.* 2002, *30*, 22.2.1–22.2.14.

[3] Gevaert, K., Van Damme, P., Ghesquiere, B., Impens, F. *et al.*, A la carte proteomics with an emphasis on gel-free techniques. *Proteomics* 2007, *7*, 2698–2718.

[4] Aebersold, R., Mann, M., Mass spectrometry-based proteomics. *Nature* 2003, *422*, 198–207.

[5] Bell, A., Deutsch, E., Au, C., Kearney, R. *et al.*, A HUPO test sample study reveals common problems in mass spectrometry-based proteomics. *Nat. Methods* 2009, *6*, 423–430.

[6] Beynon, R. J., Pratt, J. M., Metabolic labeling of proteins for proteomics. *Mol. Cell. Proteomics* 2005, *4*, 857–872.

[7] Hebeler, R., Oeljeklaus, S., Reidegeld, K. A., Eisenacher, M. *et al.*, Study of early leaf senescence in Arabidopsis thaliana by quantitative proteomics using reciprocal 14N/15N labeling and difference gel electrophoresis. *Mol. Cell. Proteomics* 2008, *7*, 108–120.

[8] Ong, S., Blagoev, B., Kratchmarova, I., Kristensen, D. B. *et al.*, Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Mol. Cell. Proteomics* 2002, *1*, 376–386.

[9] Ong, S., Mann, M., A practical recipe for stable isotope labeling by amino acids in cell culture (SILAC). *Nat. Protoc.* 2006, *1*, 2650–2660.

[10] Miyagi, M., Rao, K. C. S., Proteolytic 18O-labeling strategies for quantitative proteomics. *Mass Spectrom. Rev.* 2007, *26*, 121–136.

[11] Staes, A., Demol, H., Van Damme, J., Martens, L. *et al.*, Global differential non-gel proteomics by quantitative and stable labeling of tryptic peptides with oxygen-18. *J. Proteome. Res.* 2004, *3*, 786–791.

[12] Gygi, S. P., Rist, B., Gerber, S. A., Turecek, F. *et al.*, Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat. Biotechnol.* 1999, *17*, 994–999.

[13] Schmidt, A., Kellermann, J., Lottspeich, F., A novel strategy for quantitative proteomics using isotope-coded protein labels. *Proteomics* 2005, *5*, 4–15.

[14] Gerber, S. A., Rush, J., Stemman, O., Kirschner, M. W., Gygi, S. P., Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS. *Proc. Natl. Acad. Sci. USA* 2003, *100*, 6940–6945.

[15] Ross, P. L., Huang, Y. N., Marchese, J. N., Williamson, B. *et al.*, Multiplexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents. *Mol. Cell. Proteomics* 2004, *3*, 1154–1169.

[16] Thompson, A., Schafer, J., Kuhn, K., Kienle, S. *et al.*, Tandem mass tags: a novel quantification strategy for comparative analysis of complex protein mixtures by MS/MS. *Anal. Chem.* 2003, *75*, 1895–1904.

[17] Zhang, G., Neubert, T. A., Automated comparative proteomics based on multiplex tandem mass spectrometry and stable isotope labeling. *Mol. Cell. Proteomics* 2006, *5*, 401–411.

[18] Venable, J. D., Dong, M., Wohlschlegel, J., Dillin, A., Yates, J. R., Automated approach for quantitative analysis of complex peptide mixtures from tandem mass spectra. *Nat. Methods* 2004, *1*, 39–45.

[19] Ishihama, Y., Oda, Y., Tabata, T., Sato, T. *et al.*, Exponentially modified protein abundance index (emPAI) for estimation of absolute protein amount in proteomics by the number of sequenced peptides per protein. *Mol. Cell. Proteomics* 2005, *4*, 1265–1272.

[20] Asara, J. M., Christofk, H. R., Freimark, L. M., Cantley, L. C., A label-free quantification method by MS/MS TIC compared to SILAC and spectral counting in a proteomics screen. *Proteomics* 2008, *8*, 994–999.

[21] Liu, H., Sadygov, R. G., Yates, J. R., III, A model for random sampling and estimation of relative protein abundance in shotgun proteomics. *Anal. Chem.* 2004, *76*, 4193–4201.

[22] Zhang, X., Julien-David, D., Miesch, M., Raul, F. *et al.*, Quantitative analysis of beta-sitosterol oxides induced in vegetable oils by natural sunlight, artificially generated light, and irradiation. *J. Agric. Food Chem.* 2006, *54*, 5410–5415.

[23] Wang, W., Zhou, H., Lin, H., Roy, S. *et al.*, Quantification of proteins and metabolites by mass spectrometry without isotopic labeling or spiked standards. *Anal. Chem.* 2003, *75*, 4818–4826.

[24] Silva, J. C., Denny, R., Dorschel, C. A., Gorenstein, M. *et al.*, Quantitative proteomic analysis by accurate mass retention time pairs. *Anal. Chem.* 2005, *77*, 2187–2200.

[25] Silva, J. C., Gorenstein, M. V., Li, G., Vissers, J. P. C., Geromanos, S. J., Absolute quantification of proteins by LCMSE: a virtue of parallel MS acquisition. *Mol. Cell. Proteomics* 2006, *5*, 144–156.

[26] Jenden, D. J., Cho, A. K., Selected ion monitoring in pharmacology. *Biochem. Pharmacol.* 1979, *28*, 705–713.

[27] Lange, V., Picotti, P., Domon, B., Aebersold, R., Selected reaction monitoring for quantitative proteomics: a tutorial. *Mol. Syst. Biol.* 2008, *4*, 222.

[28] Martens, L., Nesvizhskii, A. I., Hermjakob, H., Adamski, M. *et al.*, Do we want our data raw? Including binary mass spectrometry data in public proteomics data repositories. *Proteomics* 2005, *5*, 3501–3505.

[29] Horn, D. M., Zubarev, R. A., McLafferty, F. W., Automated reduction and interpretation of high resolution electrospray mass spectra of large molecules. *J. Am. Soc. Mass Spectrom.* 2000, *11*, 320–332.

[30] Sturm, M., Bertsch, A., Gropl, C., Hildebrandt, A. *et al.*, OpenMS - an open-source software framework for mass spectrometry. *BMC Bioinformatics* 2008, *9*, 163.

[31] Kohlbacher, O., Reinert, K., Gropl, C., Lange, E. *et al.*, TOPP--the OpenMS proteomics pipeline. *Bioinformatics* 2007, *23*, e191–e197.

[32] Lange, E., Gropl, C., Reinert, K., Kohlbacher, O., Hildebrandt, A., High-accuracy peak picking of proteomics data using wavelet techniques. *Pac. Symp. Biocomput.* 2006, 243–254.

[33] Tautenhahn, R., Bottcher, C., Neumann, S., Highly sensitive feature detection for high resolution LC/MS. *BMC Bioinformatics* 2008, *9*, 504.

[34] Katajamaa, M., Miettinen, J., Oresic, M., MZmine: toolbox for processing and visualization of mass spectrometry based molecular profile data. *Bioinformatics* 2006, *22*, 634–636.

[35] Pedrioli, P. G. A., Eng, J. K., Hubley, R., Vogelzang, M. *et al.*, A common open representation of mass spectrometry data and its application to proteomics research. *Nat. Biotechnol.* 2004, *22*, 1459–1466.

[36] Deutsch, E., mzML: A single, unifying data format for mass spectrometer output. *Proteomics* 2008, *8*, 2776–2777.

[37] Helsens, K., Timmerman, E., Vandekerckhove, J., Gevaert, K., Martens, L., Peptizer, a tool for assessing false positive peptide identifications and manually validating selected results. *Mol. Cell. Proteomics* 2008, *7*, 2364–2372.

[38] Jin, S., Daly, D. S., Springer, D. L., Miller, J. H., The effects of shared peptides on protein quantitation in label-free proteomics by LC/MS/MS, J. *Proteome. Res.* 2008, *7*, 164–169.

[39] Hartler, J., Thallinger, G. G., Stocker, G., Sturn, A. *et al.*, MASPECTRAS: A platform for management and analysis of proteomics LC-MS/MS data. *BMC Bioinformatics* 2007, *8*, 197.

[40] Halligan, B. D., Slyper, R. Y., Twigger, S. N., Hicks, W. *et al.*, ZoomQuant: an application for the quantitation of stable isotope labeled peptides. *J. Am. Soc. Mass Spectrom.* 2005, *16*, 302–306.

[41] Falick, A., Hines, W., Medzihradszky, K., Baldwin, M., Gibson, B., Low-mass ions produced from peptides by high-energy collision-induced dissociation in tandem mass spectrometry. *Am. Soc. Mass Spectrom.* 1993, *4*, 882–893.

[42] Papayannopoulos, I., The interpretation of collision-induced dissociation tandem mass spectra of peptides. *Mass Spectrom. Rev.* 1995, *14*, 49–73.

[43] Monroe, M. E., Tolic, N., Jaitly, N., Shaw, J. L. *et al.*, VIPER: an advanced software package to support high-throughput LC-MS peptide identification. *Bioinformatics* 2007, *23*, 2021–2023.

[44] Jaitly, N., Monroe, M. E., Petyuk, V. A., Clauss, T. R. W. *et al.*, Robust algorithm for alignment of liquid chromatography-mass spectrometry analyses in an accurate mass and time tag data analysis pipeline. *Anal. Chem.* 2006, *78*, 7397–7409.

[45] Lange, E., Gropl, C., Schulz-Trieglaff, O., Leinenbach, A. *et al.*, A geometric approach for the alignment of liquid chromatography-mass spectrometry data. *Bioinformatics* 2007, *23*, i273–i281.

[46] Mueller, L. N., Brusniak, M., Mani, D. R., Aebersold, R., An assessment of software solutions for the analysis of mass spectrometry based quantitative proteomics data. *J. Proteome Res.* 2008, *7*, 51–61.

[47] Keller, A., Eng, J., Zhang, N., Li, X., Aebersold, R., A uniform proteomics MS/MS analysis platform utilizing open XML file formats. *Mol. Syst. Biol.* 2005, *1*, 2005.0017.

[48] Jaitly, N., Mayampurath, A., Littlefield, K., Adkins, J. N. *et al.*, Decon2LS: An open-source software package for automated processing and visualization of high resolution mass spectrometry data. *BMC Bioinformatics* 2009, *10*, 87.

[49] Lin, W., Hung, W., Yian, Y., Wu, K. *et al.*, Multi-Q: a fully automated tool for multiplexed protein quantitation. *J. Proteome Res.* 2006, *5*, 2328–2338.

[50] Shadforth, I. P., Dunkley, T. P. J., Lilley, K. S., Bessant, C., i-Tracker: for quantitative proteomics using iTRAQ. *BMC Genomics* 2005, *6*, 145.

[51] Boehm, A. M., Putz, S., Altenhofer, D., Sickmann, A., Falk, M., Precise protein quantification based on peptide quantification using iTRAQ. *BMC Bioinformatics* 2007, *8*, 214.

[52] Schulze, W. X., Mann, M., A novel proteomic screen for peptide-protein interactions. *J. Biol. Chem.* 2004, *279*, 10756–10764.

[53] Cox, J., Mann, M., MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat. Biotechnol.* 2008, *26*, 1367–1372.

[54] Shinkawa, T., Taoka, M., Yamauchi, Y., Ichimura, T. *et al.*, STEM: a software tool for large-scale proteomic data analyses. *J. Proteome Res.* 2005, *4*, 1826–1831.

[55] Han, D. K., Eng, J., Zhou, H., Aebersold, R., Quantitative profiling of differentiation-induced microsomal proteins using isotope-coded affinity tags and mass spectrometry. *Nat. Biotechnol.* 2001, *19*, 946–951.

[56] Li, X., Zhang, H., Ranish, J. A., Aebersold, R., Automated statistical analysis of protein abundance ratios from data generated by stable-isotope dilution and tandem mass spectrometry. *Anal. Chem.* 2003, *75*, 6648–6657.

[57] May, D., Law, W., Fitzgibbon, M., Fang, Q., McIntosh, M., Software platform for rapidly creating computational tools for mass spectrometry-based proteomics. *J. Proteome Res.* 2009, *8*, 3212–3217.

[58] Li, X., Yi, E. C., Kemp, C. J., Zhang, H., Aebersold, R., A software suite for the generation and comparison of peptide arrays from sets of data collected by liquid chromatography-mass spectrometry. *Mol. Cell. Proteomics* 2005, *4*, 1328–1340.

[59] Palagi, P. M., Walther, D., Quadroni, M., Catherinet, S. *et al.*, MSight: An image analysis software for liquid chromatography-mass spectrometry. *Proteomics* 2005, *5*, 2381–2384.

[60] Mueller, L. N., Rinner, O., Schmidt, A., Letarte, S. *et al.*, SuperHirn - a novel tool for high resolution LC-MS-based peptide/protein profiling. *Proteomics* 2007, *7*, 3470–3480.

