

observation that matrix effects are the dominant factor in peptide detectability, and that there is a linear range for good peptide quantitation.<sup>[136]</sup>

Usually, one tries to inject the same total amount of protein. Conceptually, this would work if there were only small changes between samples, but how well would it work if there were changes in abundant proteins? One solution is to use internal reference standards for alignment or for normalizing the different runs.<sup>[137,138]</sup>

Mass-spectrometry based quantitation poses significant statistical challenges. Because of the high cost per analysis, and the often limited amounts of sample, there are very few studies that have addressed the issues of biological variability (samples from different patients or animals), and technical variability (the same biological sample, split and processed independently), and experimental variability (different analyses of the same processed sample). Notable exceptions are the papers by Gan *et al.* for iTRAQ<sup>[139]</sup> and a recent paper by Li *et al.*<sup>[140]</sup> on label-free quantitation, where the authors developed a method to determine statistical significance and false positives using AMT-based label-free data. A 'false positive' is defined as a misassignment of differential expression. These authors also discuss the challenges of performing statistics on label-free analysis, as well as the added difficulties of performing statistical analysis on peptide-based quantitation data in general, because different numbers of measurements are made on different proteins, which is not the case for microarray data. A fold-change cutoff was not found to be sufficient – an additional statistical test, performed at the peptide level, was found to be necessary.<sup>[141]</sup> This method, however, was still not sufficient to determine the false discovery rate (FDR) and statistical significance of relative expression data from label-free experiments. Although the authors recognize that often only one analysis per biological sample is normally performed, they found that the minimum number of analyses required obtaining these statistics was two LC/MS analyses (i.e. two experimental replicates), spiked with the same level of <sup>15</sup>N-labeled internal standard. By quantitating the labeled and unlabeled sample separately, they were able to produce four possible pairings. Three parameters were used to determine differential expression: fold-change, the *t*-test or Wilcoxon ranksum test, and a minimum number of permuted statistical pairings (MPSPs). Using the internal standard as the control, an unlabeled protein found to be differentially labeled was considered to be a positive, while the labeled internal standard found to be differentially labeled, was considered to be a false positive. Interestingly, at a confidence level of 95%, a critical fold-change was found below which there was a drop in the number of positives, while the number of false positives stayed constant which was dependent on the number of analyses (MPSPs). This critical fold-change was 2.75, 2.5, 2.5 and 2.0 for MPSPs of 1, 2, 3 and 4, respectively, which corresponded to FDRs of 22, 15, 8.7 and 4.2%.<sup>[140]</sup>

### Metabolic versus non-metabolic labeling

Chemical labeling can be applied to any source of biological material. Metabolic labeling using SILAC can be used for cell culture, but it is not effective for autotrophic organisms such as plants or bacteria. For these autotrophic organisms, <sup>15</sup>N labeling is preferred. SILAC typically works well for mammalian cell lines, which do not synthesize all of the amino acids, and so incorporation of the labeled amino acids from the growth medium is more comprehensive.<sup>[47]</sup>

SILAC has been used for one study involving plant cell culture (*A. thaliana*), and although the average incorporation of <sup>13</sup>C<sub>6</sub> arginine

was only 75%, it allowed the study of differential expression of glutathione S-transferase in response to sialic acid treatment.<sup>[55]</sup> To our knowledge, this is the only report about the use of SILAC in plant proteomics. In contrast, virtually complete labeling (95% and higher) of proteins in both suspension cultures and entire plants of *A. thaliana* has been attained using <sup>15</sup>N isotopes.<sup>[55]</sup>

There have been a few reports on stable isotope labeled protein quantitation in 'unusual' organisms. *Drosophila* was also the first multicellular model organism subjected to labeling with <sup>15</sup>N – Heck and collaborators labeled *D. melanogaster* and *C. elegans* with <sup>15</sup>N.<sup>[58]</sup> Proteomic studies in *Drosophila* are rare, and only a few quantitative proteomics studies have been performed. Aebersold and co-workers have used 4-plex iTRAQ and protein phosphatase treatment for specific substrates in *Drosophila* cell lines.<sup>[142]</sup> Our laboratory was involved in an iTRAQ study on *Leishmania*, in which 21% of the proteome was identified and quantified over seven timepoints.<sup>[143]</sup> Siuzdak and co-workers used stable isotope labeling to monitor the expression kinetics of viral proteins, changes in the expression levels of cellular proteins, and fluctuations in metabolites in response to Flock House Virus (FHV) viral infection.<sup>[144]</sup>

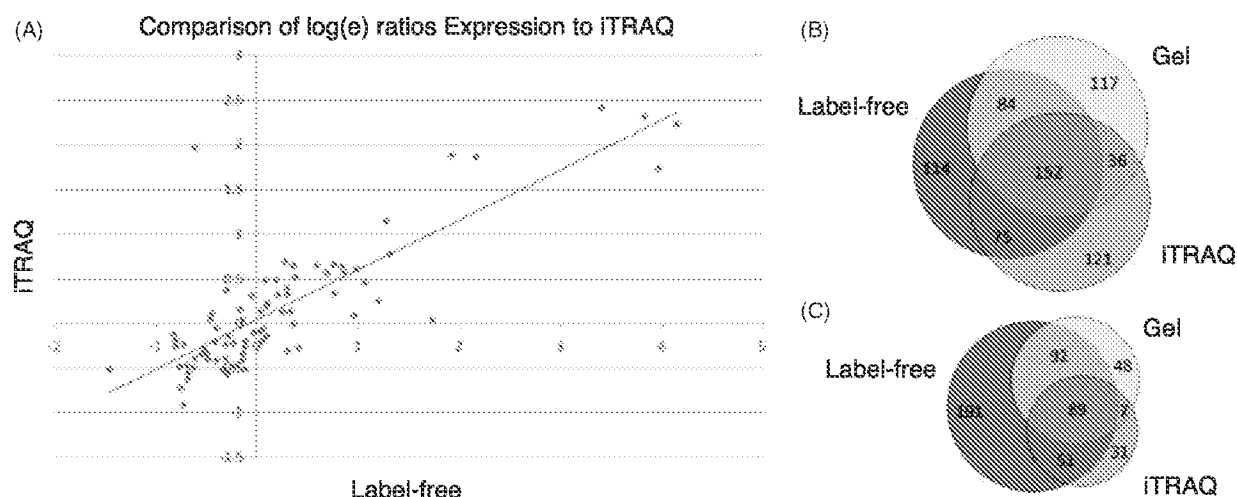
Yates and collaborators applied the <sup>15</sup>N metabolic labeling technique to *Rattus norvegicus* by feeding them a <sup>15</sup>N-enriched diet. The strategy was employed to generate internal standards to quantify proteins in mammalian tissues. This work provided the proof-of-principle that metabolic labeling of whole organisms is feasible in mammals, as had already been demonstrated for worms and flies, and opened up new possibilities for similar applications.

Mann and co-workers have established protocols for the SILAC-labeling of mice.<sup>[145]</sup> Labeling of whole animals is based on a special diet containing either the natural or the <sup>13</sup>C<sub>6</sub>-substituted version of lysine. Labeling was carried out over four generations, with no effect on development, growth or behavior. Full incorporation of SILAC amino acids was achieved for all organs in the F2 generation animals. However, metabolic labeling strategies for animals are often impractical, due to the high cost of the diet and the long time required for labeling (full incorporation is typically not achieved in the first or even the second generation of animals).

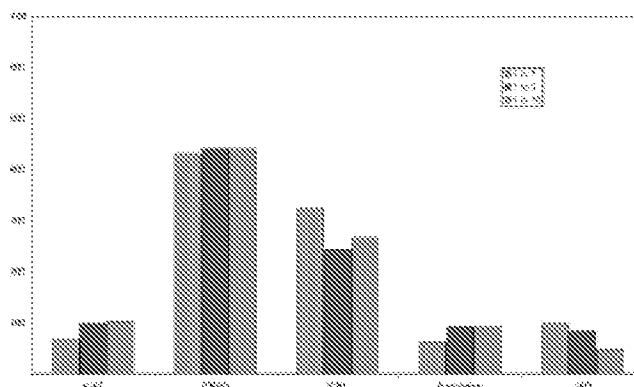
Because of the high cost of isotopically labeled materials, metabolic labeling studies tend to be used for pathway determination. iTRAQ and other chemical-based isotopic-labeling methods are used for biomarker discovery, and MRM methods are used for biomarker verification or validation.

Label-free methods for biomarker discovery are currently receiving a lot of attention, because of their simplicity and low cost. However, the lack of labeled internal standards makes them susceptible to suppression effects from other components in the sample. Of course, at the 'discovery stage' one cannot add internal standards because one does not yet know what standards you will need. This conundrum reflects the current status of protein quantitation.

Also, at this point, there is no single method that will identify and quantitate all of the proteins in the sample – different techniques will find different proteins. Several studies illustrate this point. In the first example, a study of insect salivary gland extracts, the iTRAQ technique identified 43 proteins not observed using in the LC/MS/MS analysis of salivary gland extracts from insects of the same age. This result is consistent with the previous observations that better fragmentation is obtained using this technology, giving more peptides per protein and allowing the identification of less-abundant proteins. iTRAQ labeling led to the identification of 78 proteins, 30 of which were not identified by a standard LC/MS/MS



**Figure 16.** A). The correlation between the iTRAQ results and the label-free results, B). Venn diagram of proteins identified by the three techniques. C). Venn diagram of proteins identified by the three techniques, requiring at least 2 peptides for an identification. Reprinted from [146], with permission.



**Figure 17.** The total number of high-confidence proteins identified by MASCOT in each of the five labeling experiments. MS/MS data were searched against an *E. coli* database. The 1:25 ratio gave anomalous results for the ICPL labeling, so the 1:25 ratio is not included.

analysis, illustrating the value of using both two technologies in parallel for maximum proteome coverage.

### Comparison of methods

In a very recent comparison of iTRAQ, label-free (ion accounting), and gelC by the Patel *et al.*,<sup>[146]</sup> the expression ratios were higher for the label-free analyses than for iTRAQ, as had been noted in other studies (note the slope of the line). The Venn diagram in Fig. 16 shows the number of proteins identified by the three techniques (including identifications based on a single peptide). It is clear from these studies that, at this point, there is no one technique that can quantitate – or even detect – every protein.

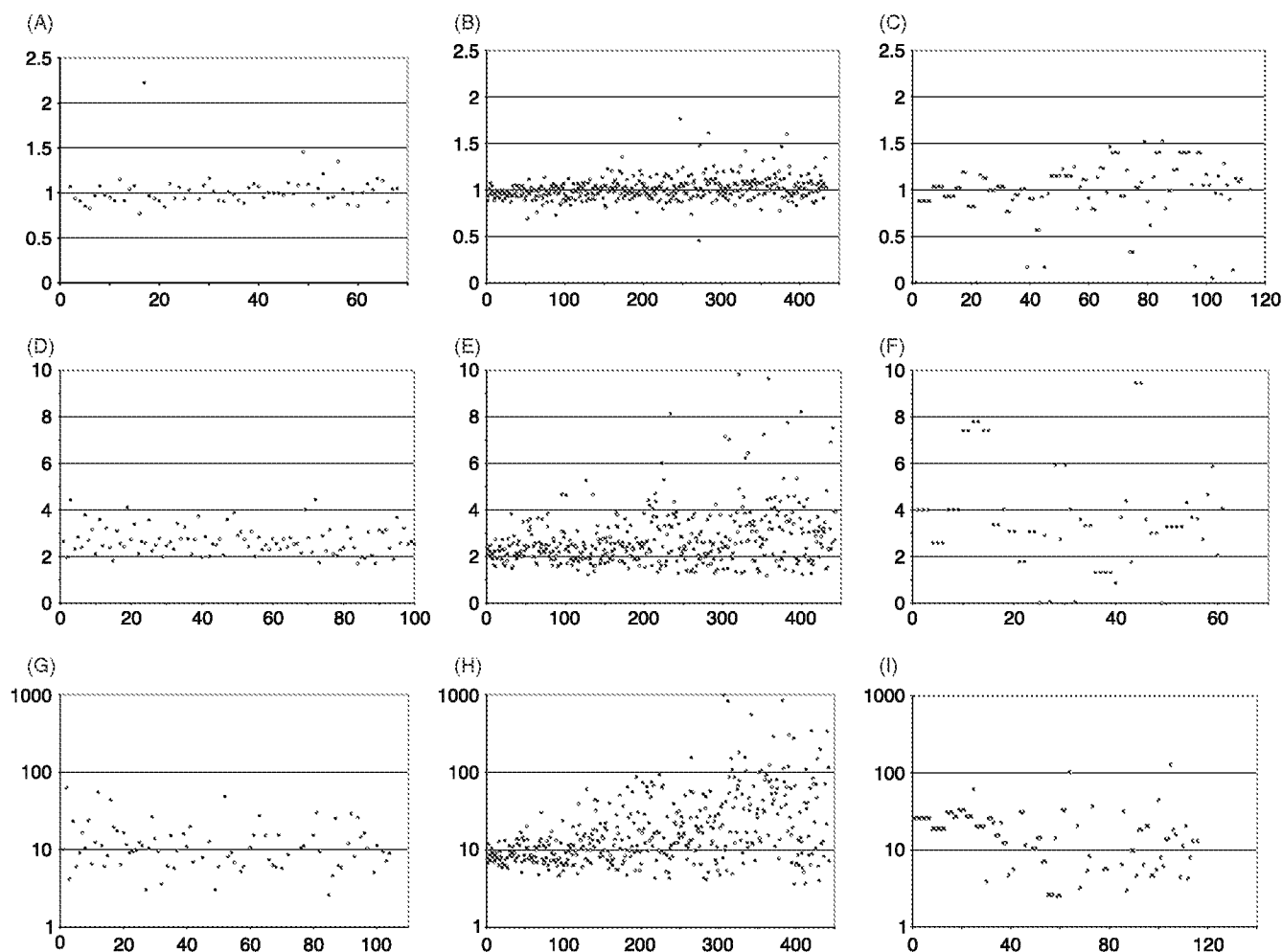
In a recent study in our laboratory, five of the most common labeling techniques – ICPL, cICAT (cleavable ICAT), iTRAQ, <sup>18</sup>O, and acetylation – were compared on an *E. coli* tryptic digest to determine the method that identifies the highest number of proteins and provides the most accurate quantitation. In this study, the highest number of proteins was identified with the iTRAQ labeling system, followed by ICPL. The peptides in these two methods, however, were separated by 2D-LC unlike the other experiments which were done using 1D-LC, thus demonstrating the advantages for prefractionation of peptides in complex samples. The three other labeling systems (<sup>18</sup>O, acetylation, and

cICAT) resulted in approximately the same number of protein identifications (Fig. 17).

Peptides labeled at 1:1 and 1:3 ratios with cICAT, iTRAQ and acetylation were quantified with reasonable accuracy. However, only the highest-confidence proteins in iTRAQ-labeled samples resulted in an acceptable amount of variation when labeled at a ratio of 1:10 (Fig. 18). We were unable to analyze the <sup>18</sup>O and ICPL data as we could not find or modify any of our software to accept these labels with the QStar data files. The variation observed in these experiments clearly demonstrates the need for both technical and biological replicates.

The advantages and disadvantages of each procedure, as found in our study, are compared in Table 1.

There have been several other comparisons of different label and label-free methods, where the same samples were analyzed through various quantitation techniques. The results of these comparisons are shown in given in the table in the Supporting Information. In iTRAQ, where the protein identification is done on the same set of labeled peptides as the quantitation, there is an inverse relationship between the confidence of the identification and the number of proteins on which quantitative data can be obtained. In a set of ten experiments on the reproducibility of iTRAQ analyses, Gan *et al.* found that allowing a ±50% change in expression ratio between biological replicates



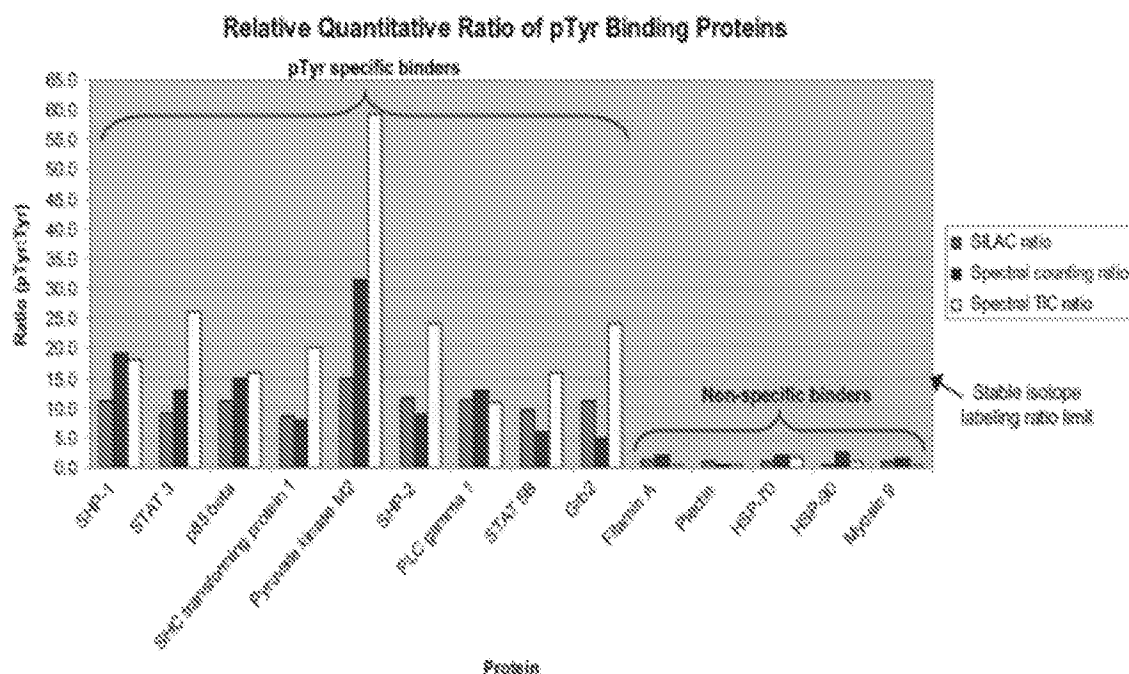
**Figure 18.** The experimental average ratio of iCAT (A), iTRAQ (B), and acetyl (C) labeled *E. coli* proteins at 1 : 1 ratios were calculated by ProteinPilot (iCAT, iTRAQ) and MSQUANT (acetyl). The experimental average ratio of iCAT (D), iTRAQ (E) and acetyl (F) labeled *E. coli* proteins at 1 : 3 ratios were calculated by ProteinPilot (iCAT, iTRAQ) and MSQUANT (acetyl). The experimental average ratio of iCAT (G), iTRAQ (H) and acetyl (I) labeled *E. coli* proteins at 1 : 10 ratios were calculated by ProteinPilot (iCAT, iTRAQ) and MSQUANT (acetyl).

resulted in 88% protein coverage, allowing a  $\pm 30\%$  change in expression ratio between *technical* replicates resulted in 95% protein coverage, with only a  $\pm 0.1\%$  variance coming from the MS.<sup>[138]</sup> Liu *et al.*<sup>[98]</sup> found that the correlation of abundance with the number of spectra observed, was better than that based on % sequence coverage or the number of peptides identified per protein. In a comparison of spectral counting *versus* peptide ion intensities, Xia *et al.*<sup>[147]</sup> found that spectral counting gave better agreement with the true protein ratios. A recent comparison of studies using the spectral counting and ion intensity-based methods of label-free quantitation, with respect to dynamic range of quantitation and dynamic range of protein detection was done by Wong *et al.*<sup>[148]</sup> Both methods were able to detect changes in protein levels of approximately 2.5. However, machine learning methods and methods using peptide ion intensity were computationally more difficult. This study concluded, however, that these label-free approaches were complementary, and recommended using both for increased confidence in the results.

Some label-free methods, however, were found to underestimate expression ratios if the true ratios were  $> 2.5$ .<sup>[149]</sup> One reason that has been proposed for the lower range of ratios

produced by peak area intensity measurements compared to spectral counting is the restriction by different software packages on the number of peptides required for a protein to be considered 'detected'.<sup>[99]</sup> A requirement for a larger number of peptides per protein discriminates against lower-abundance proteins, thus removing the larger expression differences. The larger the difference in abundance ratios, the more reliably this difference could be detected through label-free techniques. The study by Liu *et al.* reported that the *number* of spectra produced was a reliable indication of expression ratio if the concentration difference was  $> 5$ .<sup>[98]</sup> Other factors include the size of the protein, the number of tryptic cleavage sites and the amount of protein that can be loaded onto a capillary LC column.<sup>[149]</sup>

The challenge of comparing quantitation methods is shown in Fig. 19.<sup>[125]</sup> In this study, three different methods were compared, and all three gave different expression ratios – which one is correct? In this particular study, the authors were able to experimentally confirm that the spectral TIC method (using the average MS/MS TIC) was correct, and they attributed the lower ratios obtained from SILAC and spectral counting to compression. However, most studies are performed without this validation



**Figure 19.** Relative expression ratios of pTyrosine-binding proteins using three different quantitation methods. Reprinted from Ref. [125] with permission.

Table 1. Comparison of five quantitation methods on an <i>E. coli</i> tryptic digest					
	cICAT	ITRAQ	ICPL	<sup>18</sup> O	Acetylation
Advantages	Complex samples are simplified  MS-based quantitation can reduce costly analysis time	Large number of peptides per protein identified increase confidence and quantitation statistics  Multiplex with 4/8 labels	Short digest time  Lysis buffer contains guanidine which is excellent for solubilization  MS-based quantitation can reduce costly analysis time	Guanidine can be used for solubilization  MS-based quantitation can reduce costly analysis time	Inexpensive  Short preparation time  MS-based quantitation can reduce costly analysis time
Disadvantages	Very few peptides identified per protein leads to low confidence and poor statistics  Not all proteins contain cysteines	Fractionation required  MS/MS quantitation is costly in terms of MS time  Expensive	Software available only for certain instruments  Requires fractionation  Expensive	Long incubation/digestion times  Pooled sample is only stable for 1 h  Availability of software for data analysis	Requires fractionation  Availability of software for data analysis  Acetic anhydride is a controlled substance

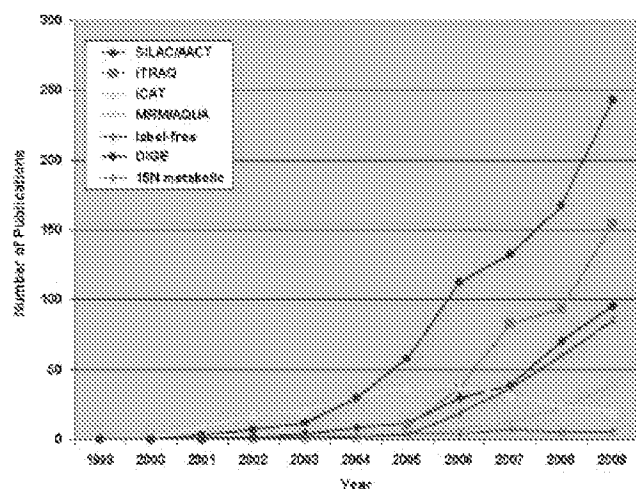
of true protein concentrations. Clearly, more work needs to be done to validate different quantitation methods using mixtures of proteins with known concentration ratios.

Although there is general agreement that comparison with a labeled standard peptide is still the 'gold standard',<sup>[98,112]</sup> it is not a global technique. However, many studies have concluded that the *trends* observed in label-free experiments are valid (i.e. overexpression *versus* underexpression) even if the actual observed expression ratios may be compressed.<sup>[149]</sup>

## Software Considerations

Before you start a project, it is important to be certain that the software 'matches' the label you are trying to use. Certain software packages, for example, can only handle certain types of labeled amino acids for SILAC. Certain companies make proprietary labels, which may only be able to be analyzed using their own software. However, there seems to be a welcome change toward 'openness' now, and iTRAQ-based quantitation, for example, can be analyzed using an increasing number of instruments and software.





**Figure 20.** Trends in mass-spectrometry-based quantitation. Publications per year, based on a keyword search in SciFinder Scholar<sup>SM</sup>. Note that comparisons over time are probably more accurate than comparisons between techniques, due to the difficulties in finding keywords to catch every reference. The number of 2009 publications was extrapolated from the number published by August 2009.

Mascot,<sup>[94]</sup> for example, can perform quantitation of a variety of 'precursor' methods (where the quantitation based on shifts in the molecular weight), as well as TMT and iTRAQ-type labels where the quantitation is based on MS/MS reporter ions.<sup>[150]</sup> However, additional software packages, such as Mascot Distiller may be required. They specifically note that for AB MALDI-TOF/TOF data, there is a special software available (TS2Mascot) that should be used because the standard GPS explorer Mascot data is de-isotoped. <sup>15</sup>N-labeling may pose a particular problem because every amino acid will get labeled, but new software (QuantiSpec) has recently been written to enable interpretation and quantitation of <sup>15</sup>N-labeled mass spectra.<sup>[151]</sup> In general, it is still prudent to make certain to select a label that your data-processing software can handle.

Multifunctional software packages are being developed to handle data from label-free and stable-labeled samples, and from a variety of instrument platforms. These include the ProteinQuant Suite,<sup>[130]</sup> developed by the Novotny group, Census software<sup>[152]</sup> developed by the Yates group, the PatternLab software<sup>[153]</sup> also

developed by the Yates group for normalizing spectral count data, and the Corra software developed by the Aebersold group, which in addition, produces protein interaction networks from the differentially expression data.<sup>[154,155]</sup> MS-Biomarker Discovery Platform (MS-BID),<sup>[156]</sup> from the Aebersold group, is designed for determining peptides that discriminate between treatment groups. Markerview software (Applied Biosystems) is also designed to facilitate detection of biomarker peptides that correlate with treatment.<sup>[157]</sup>

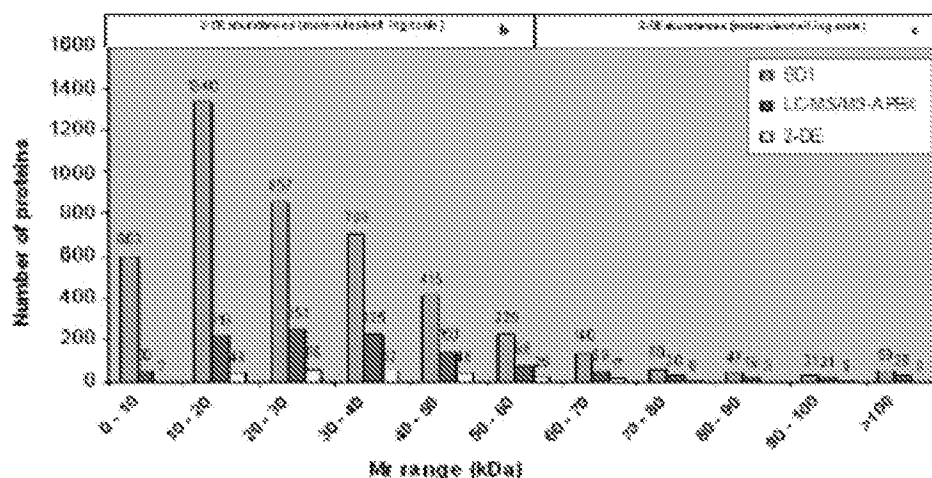
## Conclusions

It would have been nice to be able to end this article with a recommendation for a single method. However, as is clear from the above data and discussion, there is really no one single method that will solve all of the analytical problems associated with protein quantitation. This is partly because 'quantitation' means so many different things – global or targeted, absolute or relative.

The ICPL and iTRAQ methods (from our study) and the ion-accounting label-free method (from the Patel study) seemed to identify and quantify significantly more proteins than the other methods in these studies. It should be remembered, however, that the variability of the enzymatic digestion step can affect all of the chemical labeling techniques and the label-free methods by leading to analytical variability. We and others are actively exploring solutions to this problem, including the use of microwave digestion, detergents, pressure, and a variety of solvents, chaotropic agents and denaturants.<sup>[158]</sup> We are confident, therefore, that this problem will be able to be solved (or at least reduced) in the near future. Another significant source of variability comes from depletion steps. This variability, however, can be reduced by the stringent use of well-developed SOPs.

Label-free methods are based on less-rigorous mass spectrometry, with more reliance on bioinformatics and separation techniques. Factors that have to be considered when selecting a method are the number of treatments, the cost of the experiment, the complexity of the sample, the biological source of the sample and whether the experiment will be done in cell culture. These ultimately will be the determining factors in choosing the appropriate quantitation method.

For this review, we used SciFinder Scholar<sup>[159]</sup> to count the number of publications per year using these various types of



**Figure 21.** Distribution of proteins predicted from the *Shigella dysenteriae* genome (SD1, blue), and found by either 2D gel electrophoresis (2-DE, yellow)

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