Quantitation in Mass-Spectrometry-Based Proteomics

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label-free quantitation, stable isotope labeling, reciprocal labeling, statistical anaylsis, mass spectrometry, proteomics

Abstract

Mass-spectrometry-based proteomics, the large-scale analysis of proteins by mass spectrometry, has emerged as a new technology over the last decade and become routine in many plant biology laboratories. While early work consisted merely of listing proteins identified in a given organ or under different conditions of interest, there is a growing need to apply comparative and quantitative proteomics strategies toward gaining novel insights into functional aspects of plant proteins and their dynamics. However, during the transition from qualitative to quantitative protein analysis, the potential and challenges will be tightly coupled. Several strategies for differential proteomics that involve stable isotopes or label-free comparisons and their statistical assessment are possible, each having specific strengths and limitations. Furthermore, incomplete proteome coverage and restricted dynamic range still impose the strongest limitations to data throughput and precise quantitative analysis. This review gives an overview of the current state of the art in differential proteomics and possible strategies in data processing.

Contents

INTRODUCTION

Proteomics is the science of large-scale analysis of proteins. In a general sense, it applies to any large-scale analysis of protein mixtures, often without a priori knowledge of the identity of proteins in the samples. In the past decades, mass-spectrometry (MS)-based proteomics has emerged as a potent technology that allows the analysis and identification of proteins in high throughput. With the completion of genome sequencing projects of a variety of organisms across all kingdoms of life, the identification of proteins based on their peptide fragmentation patterns in tandem-mass spectrometric experiments has become an almost automated task. The success of protein mass spectrometry (MS) has been boosted by the development of soft protein ionization methods, such as electrospray ionization (ESI) or matrix-assisted laser desorption ionization (MALDI). This achievement was acknowledged in 2002 with the award of the Nobel Prize in chemistry to John Fenn and Koichi Tanaka. However, without the information derived from various full-genome sequencing projects, and without efficient algorithms for peptide sequence determination from fragmentation spectra (21, 91), proteomic experiments would be a great deal more difficult today. Recent and ongoing improvements of the mass analyzer and fragmentation technology (43, 66, 128) have further contributed to making mass-spectrometry-based proteomics a now widely used technology.

Until about a decade ago, proteomics was largely a qualitative discipline. Typical proteomic experiments resulted in lists of proteins identified in a given tissue or protein complex without any further information about abundance, distributions, or stoichiometry. In contrast, quantitative strategies have become widely used for the analysis of gene expression by microarrays or quantitative PCR, and in plants especially, the power of large-scale genomics has produced novel insights into many aspects of development and physiology. However, enzymatic reactions and signaling pathways ultimately depend on the activity of proteins. Protein amount is regulated by protein synthesis and degradation, and this may be independent of transcriptional control (92). In addition, posttranslational modifications, isoforms, and splice variants are not captured by the mere analysis of transcript abundances. Modern proteomic tools provide the technical framework for the analysis of the proteome complexity. Protein mixtures today can routinely be characterized in terms of proteins present in the sample, but in order to allow biological interpretation, quantitative analyses are necessary.

The field of quantitative mass-spectrometry-based proteomics is still under

MS: mass spectrometry, mass spectrometer

fast development; new and better instrumentation is being developed almost on a yearly basis. Most of the early developments in applications of quantitative mass-spectrometry-based proteomics were driven by research on yeast and in mammalian cell lines (8, 9, 103). Quantitative proteomic approaches have also helped in the characterization of protein complexes (2, 3, 98) and in defining true interaction partners to a given bait protein over background proteins (8, 104). In plant physiology, though, mass-spectrometry-based proteomics is no longer used only as a descriptive tool. Instead, well-designed quantitative proteomics have been applied to various aspects of organelle biology and growth regulation and signaling. Encouraging pioneer studies of specific subproteomes in plants have revealed candidate proteins that are phosphorylated specifically under different stress conditions (7, 73, 75) or during a light–dark cycle (96). Protein abundance changes were monitored in response to heat shock (86) or during leaf senescence (38), and protein turnover of photosynthetic proteins was monitored using pulse-chase labeling in combination with protein mass spectrometry (74). Organelle proteomes were characterized either by analysis of fractions of separated proteins in a sucrose density gradient (19, 20, 101), or by focused analysis of specific purified subproteomes, such as chloroplasts (53, 63, 89, 90, 96), or plasma membranes and their microdomains (50, 71). Ultimately, quantitative proteomics methods now contribute to the framework of techniques available to study regulatory processes in plants in relation to the whole plant parameters, such as growth or development (92, 94, 108).

STRATEGIES FOR DIFFERENTIAL PROTEOMICS

Most experimental designs in quantitative proteomics aim at the comparison of a stressed or disturbed status to an undisturbed reference sample. Over the last decade, several technologies and workflows have been presented that leave the biologist with an often confusing choice of strategies that all have particular advantages and disadvantages within their contexts. However, the choice of quantitation method is less important than the practical experience with the method resulting in good technical reproducibility (111). In addition, biological variation must be considered in a sufficiently replicated experimental design, and this in combination with cost considerations may well influence the choice of strategy. In proteomic experiments, sample amount is often the limiting factor since there is no amplification step like the polymerase chain reaction in microarray experiments. Thus, enrichment and purification of subproteomes often are key steps before actual comparison of protein abundances is carried out. Further, the difficulty of obtaining sufficient amounts of sample may also influence the choice for specific quantitative strategies. **Table 1** summarizes the characteristic features of the different quantitative methods discussed below.

Gel-Based Quantitation

Two-dimensional (2D) gels were initially considered the most suitable method to visualize the differences between protein samples derived from different conditions or tissues. Complex protein mixtures could be resolved efficiently, and detection of differences in band or spot intensity seemed intuitive. Today, it is possible to visualize over 10,000 spots corresponding to over 1000 proteins on single 2D gels (29). However, in traditional 2D gel experiments, protein separation and differential analysis are not coupled directly with identification of the protein underlying a given spot. In many cases, individual spots may still consist of more than one protein, but this can be recognized only if differential spots are actually excised and analyzed by mass spectrometry (35). With the development of peptide sequencing technology by mass spectrometry in the mid-1990s (123), separation and quantitative analysis by 2D gels could be coupled with protein identification. In this combination, 2D-gel-based quantitation is still attractive and has been applied in the past to

Table 1 Overview of the technical parameters of the different workflows for quantitative proteomics

	Dynamic range	Proteome coverage	Quantitative accuracy	Nature of quantitation	Number of samples to compare	Quantification level
Metabolic labeling						
^{15}N	1–2	Medium	Precise (<10% rsd) (114)	Relative	2	MS
SILAC	1–2	Medium	Precise (<10% rsd) (82)	Relative	2 or 3	MS
Chemical labeling						
ICAT	1–2	Poor	Precise (<10% rsd) (36)	Relative	2	MS
ITRAQ, TMT	2	Medium	Medium (10–30%) (6)	Relative	2–8	MS2
Standard peptide	2	Poor (There are few target proteins that can be selected efficiently in a single LC-MS/MS experiment.)	Precise	Relative/ Absolute[1]	2–many	MS or MS2
Label-free						
Ion intensities (PCP)	3	Good	Medium (10–30% rsd) (3)	Relative	Many (depends on reproducibility of chromatography)	MS
Spectrum count	3	Good	Poor (>30% rsd)	Relative	Within sample/many samples	MS2
Derived indices (APEX, emPAI)	3 or 4	Good	Poor (>30% rsd)	Relative/ Absolute[2]	Within sample/many samples	MS
Gels						
2D gels	1 to 4, depending on dye	Medium	Medium (10–30% rsd) (29)	Relative/ Absolute[1]	Many (depends on reproducibility of gels)	Image analysis

[1]Absolute quantitation is possible only through relative comparison to a spiked "known" standard.
[2]Absolute quantitation is possible only through empirical features and back-calculation using the molecular weight of the protein and total protein amount in the sample.

a variety of questions in plant biology, ranging from stress response analysis (97) to characterization of cell types or organelles (53, 64, 89).

In quantitative studies, accurate reproducibility of 2D gels is often a limitation. Perhaps as a consequence, the major advances in gel-based quantitation have occurred when using fluorescent dyes to label different protein samples that were then separated on the same gel (112). This so-called DIGE technology has improved quantitative accuracy of 2D gels significantly (**Figure 1*a***). The DIGE approach has been successfully applied to study phosphorylation responses in plant plasma membrane

DIGE: difference in gel electrophoresis

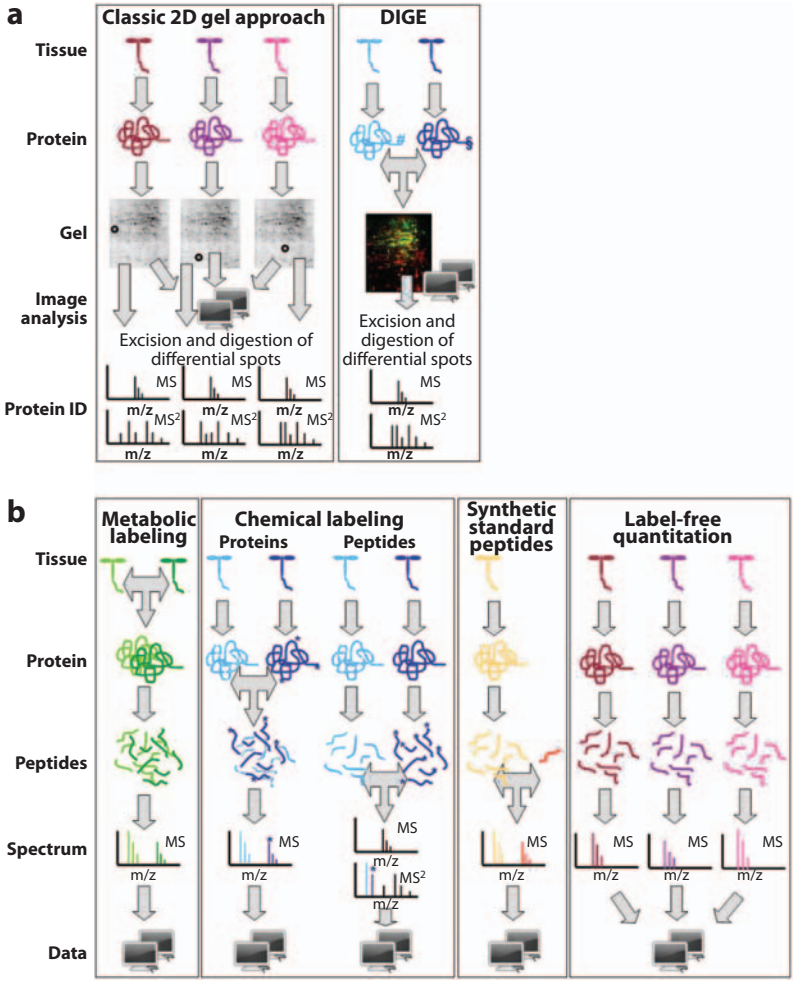

Figure 1

Overview of different strategies in quantitative proteomics. (*a*) Gel-based quantitative approaches. In classic two-dimensional gel experiments, spots with differential intensities from different gels are analyzed by mass spectrometry. The DIGE (difference in gel electrophoresis) approach allows separation of proteins from two different samples on the same gel. (*b*) Mass-spectrometry-based approaches. Metabolic labeling allows very early mixing of samples. Chemical labeling introduces a label on either protein or peptide level, and quantification can be done on survey scans or on fragment scans. Synthetic standard peptides are spiked into the sample at known concentrations. Label-free quantitation allows comparisons of multiple samples analyzed in parallel.

proteins upon brassinosteroid treatment (17) or to compare light and dark adapted proteomes of chloroplast thylakoid lumen (31). Recent advances in using the DIGE technology in combination with blue native gel electrophoresis (rather than isoelectric focusing) as a first-dimension separation offer new perspectives and applications, especially with regard to the comparative and structural study of protein complexes or the assignment of complexes to subcellular fractions (39).

Mass-Spectrometry-Based Quantitation

Protein mass spectrometry using LC-MS/MS is not quantitative as such becasue of different physical and chemical properties of different tryptic peptides. Differences in charge state, peptide length, amino acid composition, or posttranslational modifications result in great differences in ion intensities for the peptides, even when they belong to the same protein. Thus, for accurate quantitation using ion intensities, comparisons between different samples can only be done based on the same peptide mass-to-charge ratios (m/z) that were acquired under the same general conditions during LC-MS/MS experiments. Thus, all mass-spectrometry-based quantitative methods are necessarily relative comparisons between two or more samples, and comparative quantitation is feasible only when careful experimental design and suitable data analysis strategies are employed (42). A number of such comparative strategies have become increasingly popular and can be categorized as either stable-isotope-labeling approaches or label-free approaches (**Figure 1*b***).

Stable-isotope-labeling strategies that rely on survey scan quantitation typically provide relative standard deviations (rsd) below 10% (36, 82) (**Table 1**). Quantitation without stable isotopes, but instead based on peak intensities or extracted ion chromatograms in separate LC-MS/MS runs, can usually provide quantitative accuracies within 30% (3). In contrast, the quantitative precision of label-free approaches based on spectral counting or derived indices can be as low as 50% rsd (77).

LC-MS/MS: liquid chromatography coupled tandem mass spectrometry

Mass-to-charge ratio (m/z): two particles with the same m/z move in the same path in a vacuum when subjected to the same electric and magnetic fields. Mass spectrometers detect not the real mass of an ion but its m/z. The unit of mass-to-charge ratio is the Thomson (Th)

Survey scan: mass spectrometric detection of all ions within a selected mass-to-charge range. The survey scan is also called a MS full scan

rsd: relative standard deviation

Extracted ion chromatogram: the ion intensity of a given ion integrated over the chromatographic timescale. Thus, this value represents the peak volume

Fragment ion scan: mass spectrometric detection of all ions that were produced upon fragmentation of a peptide ion. The fragment ion scan is also called an MS2 scan or MS/MS scan

LABEL-FREE TECHNIQUES

Differential proteomic studies employing label-free quantitation compare two or more samples based on the ion intensities of identical peptides or based on the number of acquired spectra for each protein. Ideally, samples for label-free comparisons are run consecutively on the same LC-MS/MS setup to avoid variations in ion intensities due to differences in the system setup (column properties, temperatures), and thereby allow precise reproduction of retention times.

Label-free approaches are inexpensive; they can be applied to any biological material; and the proteome coverage of quantified proteins is high because basically every protein that is identified by one or more peptide spectra can be quantified. In addition to these advantages, the complexity of the sample is not increased by the mixing of different proteomes. Label-free methods therefore usually have a high analytical depth and dynamic range, giving this method an advantage when large, global protein changes between treatments are expected. However, especially with spectrum count, the advantage of high proteome coverage may come at the cost of relatively poor precision (77).

Protein-Based Methods: Spectrum Count and Derived Indices

The protein-based methods allow relative quantitation of protein amounts within the same sample as well as between samples, and they are very fast and easy to perform. In addition, basically unlimited numbers of samples can be compared. The spectrum count methods use the number of peptide-identifying spectra assigned to each protein as a quantitative measure (61). The rationale behind this quantitation method is that more abundant peptides and proteins are sampled more often in fragment ion scans than are low abundance peptides or proteins. Obviously, the outcome of spectrum counting depends on the settings of

data-dependent acquisition on the mass spectrometer. In particular, the linear range for quantitation and the number of proteins to be quantified are influenced by different settings for dynamic exclusion (116); the optimal settings will depend on sample complexity. The most significant disadvantage of spectrum counting is that it behaves very poorly with proteins of low abundance and few spectra. The accuracy of the spectrum count method, especially for low abundance proteins, suffers from the fact that each spectrum is scored with the value "1" independently of its ion intensities. To overcome this problem, an approach has been suggested that uses the average of the total ion count from all fragment spectra that identify a protein as a quantitative measure. Thereby, the linear dynamic range for quantitation can be vastly exceeded (4).

The empirical relationship between the number of spectra or peptides identified for a given protein and overall protein abundance in the sample has been used as a basis to calculate the absolute concentration of each protein within the sample. The exponentially modified protein abundance index (emPAI) is calculated from the number of observed spectra for each protein divided by the number of possibly observable peptides, a fraction that has been described as a protein abundance index (PAI) (95). PAI is then used as an exponent to the base 10, resulting in an exponentially modified value, the so-called emPAI index (46). A very similar approach was taken to calculate the absolute protein expression (APEX) index (62). The estimated protein concentrations calculated by emPAI indices have correlated very well with the protein concentrations calculated from enzymatic activities (92). The indices emPAI and APEX are thus derived measures of absolute protein abundance in a given sample based on the analytical features in mass spectrometric analysis. Their predictive value is certainly at least as good as quantitation based on standard protein staining.

The APEX index has been used to generate a protein abundance index of the *Arabidopsis* proteome (5) and a protein abundance map of chloroplast proteins (131). The emPAI index has been applied to calculate protein abundances of metabolic enzymes and of ribosomal proteins in a day–night cycle (92). Spectrum count has been used to study drought stress response in root nodules of *Medicago* (58) and, among other methods, in comparison of protein abundance in mesophyll and bundle sheath chloroplasts (63, 64). In combination with high mass accuracy precursor alignment, spectrum count helped to identify variant-specific proteome changes among potato varieties (41).

Peptide-Based Methods: Ion Intensities and Protein Correlation Profiling

Peptide-based approaches use averaged, normalized ion intensities of each identified peptide ion species as a basis for quantitation. The height or volume of a peak with a given m/z is a measure of the number of ions of that particular mass detected within a given time interval. This process of determining the peak volume is referred to as ion extraction and results in a so-called extracted ion chromatogram of a given ion species. Such extracted ion chromatograms can be produced for each m/z across all LC-MS/MS runs within an experiment, and the resulting peak volumes can then be compared quantitatively.

Due to differences in ionization efficiency of different peptide species, only the same ion species can be compared between different samples. This fact significantly complicates the computational effort necessary to use total ion abundance as a comparative measure. One major problem in typical MS/MS experiments is that the parent ion survey scans are interrupted by the fragment ion scan events (MS/MS), which results in discontinuous coverage of the peptide ion peaks. Depending on the fragmentation duty cycle time of the instrument, this results in more or fewer data points acquired across the elution time of each ion peak. Therefore, for each instrument and for different sample complexities, the right balance between acquisition of survey and fragment spectra has to

be experimentally determined. While frequent fragment ion scans (MS2) are necessary to allow extensive peptide sequencing and identification of as many peptides and proteins as possible in complex mixtures, multiple sampling points of the chromatographic peak by survey scans (MS) are required for a robust quantitative reading of ion intensities. Better quantitative accuracy will inevitably come at the cost of poorer proteome coverage, and vice versa.

Since ion intensities can be compared between two samples only if the exact same ion species are being used, and since two different LC-MS/MS experiments of the same complex protein extract sample usually have an overlap of approximately 60% on the peptide level, this method may result in very poor coverage of common ions between two samples. This problem is even more apparent if more than two samples are being compared. To overcome these drawbacks, a method called protein correlation profiling has been developed that aligns the total ion chromatograms of different samples (**Figure 2*a***). Typically, ion species for which fragment spectra (and thus peptide sequence identification) have been obtained are correlated based on their chromatographic retention times (**Figure 2*b***). Using the information from the retention time correlation, peaks that were fragmented in only one of the samples (**Figure 2*c***) can be identified in the survey scan spectra of the other samples, though no fragment spectrum (**Figure 2*d***) is available. Thus, based on retention time and accurate mass, extracted ion chromatograms from both samples can be used for quantitative comparisons (**Figure 2*e***). This method increases the number of proteins for quantification by up to 40%. The main disadvantage of the protein correlation profiling method is that the computational process of ion chromatogram extraction, alignment of different chromatographic profiles, and insertion of respective "missing" ion chromatograms is significantly more complicated and usually requires a high degree of manual inspection and verification compared to simple spectrum counting.

With the development of modern high-precision mass spectrometers, the label-free quantitation has become a very appealing alternative as better mass accuracy increases the reliability of mapping peptides across samples due to the more narrow mass-to-charge window that defines each peptide peak (41). However, good reproducibility of the retention time values over different LC-MS/MS runs remains crucial for precision in label-free quantitation using peptide ion intensities.

A thorough evaluation of the different label-free peptide-based methods revealed that if at least four spectra per protein were used for quantitation, sample-to-sample variation was less than twofold and protein ratios were well within 95% confidence limits (77). Removal of very abundant proteins from the sample increased the reproducibility and linearity (115). In general, comparisons of spectral count methods to quantitation methods based on peptide ion intensity reveal that both methods are well suitable to distinguish protein abundance changes of approximately twofold. Spectral counting was able to detect more proteins that undergo changes in protein abundance (greater coverage), while quantitation based on peptide ion intensities resulted in higher accuracy of the estimated protein ratios (77). Several recent studies have shown that both methods of label-free quantitation are complementary (120), thus the choice between the two methods may be based on the experience of the experimenting group (124).

Profiling methods based on ion intensity were first applied to define the proteome of the human centrosome (3), then extended to carry out whole-organelle protein profiles on mouse liver (24), and used recently to characterize mouse liver peroxisomes (122). Label-free peptide quantitation in combination with statistical profiling methods made it possible to define protein-protein interactions in pull-down experiments over background proteins (98). In plant biology, protein correlation profiling has been applied to study phosphorylation time profiles in *Arabidopsis* seedlings in response to sucrose treatment (73).

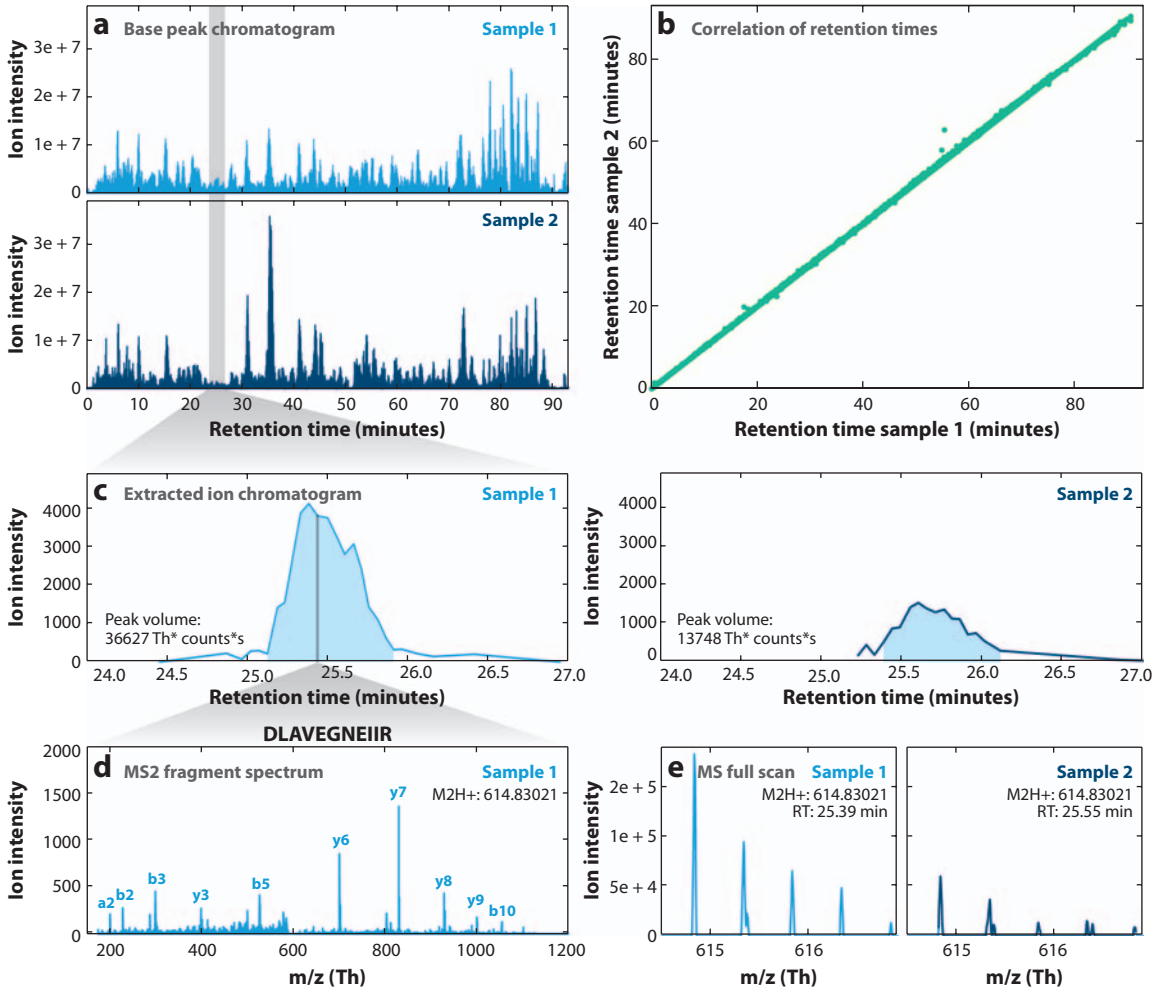

Figure 2

Protein correlation profiling. (*a*) Alignment of base peak chromatograms of two or more samples. (*b*) Pairwise correlation of retention times of common peptide ions with MS/MS fragment spectra between two samples. For comparison of multiple samples, one sample is the common base for pairwise comparisons. (*c*) Extracted ion chromatograms (XICs) from an ion with fragment spectrum (*left*) and based on retention time correlation (*right*). The peak volumes are used for quantitation. (*d*) Fragment spectrum of the peptide ion from sample 1 used for peptide identification. (*e*) Survey scan ions from both samples. Abbreviation: Th, Thomson (i.e., the unit of mass-to-charge ratio).

STABLE ISOTOPE LABELING

Stable isotope labeling approaches are based on the fact that a peptide labeled with stable isotopes differs from the unlabeled peptide only in terms of its mass but exhibits the same chemical properties during chromatography. The label can be introduced at various steps during sample preparation. In metabolic labeling, the label is introduced to the whole cell or organism through the growth medium, while in chemical labeling it is added to proteins or tryptic peptides through a chemical reaction (**Figure 1*b***). Synthetic labeled standard peptides are added to the extract after tryptic digest. The limitation of protein quantification in complex samples by stable-isotope-based methods mainly lies in signal interferences caused by co-eluting

SILAC: stable isotope labeling in cell cultures

components of similar mass. Therefore, the most efficient way to optimize the quantitative analysis is to decrease sample complexity by increasing chromatrographic gradient times or through biochemical fractionation prior to LC-MS/MS analysis.

In proteomics, [^{15}N]-labelling was first used in bacteria to study protein phosphorylation (76). The high-throughput quantitative protein analysis based on metabolic labeling was established using stable-isotope-labeled essential amino acids in mammalian cell cultures (SILAC) (81). This method has since been applied to various aspects of mammalian signaling biology, protein-protein interactions (8, 104, 105), protein dynamics (9, 78), the effect of micro-RNA expression on global protein levels (106), and the interaction of small molecules with proteins (84). SILAC also works well in bacteria or specific yeast strains in which auxotrophy for the labeled amino acid has been created (15, 33). In plants, SILAC has only yielded label incorporation of approximately 70% (34), which is not satisfying for many global proteomics applications. The only organism of the plant kingdom that has been efficiently SILAC labeled using auxotrophic mutants is *Chlamydomonas* (70).

However, as autotrophic organisms all plants can very easily be labeled metabolically through feeding of labeled inorganic compounds in the form of [^{15}N]-nitrogen-containing salts, as first demonstrated in NMR studies (45). For proteomics applications, full [^{15}N]-labeling in multicellular organisms was first done in *Drosophila* and *Caenorhabditis elegans* by feeding the animals with [^{15}N]-labeled yeast or bacteria (55). The labeling of plant cell cultures with [^{15}N]-nitrogen for large-scale proteomic analysis was demonstrated independently (22, 57), and the general data analysis workflow has been established (85).

The process of quantification in stable-isotope methods is based on extracted ion chromatograms of survey scans containing the pair of labeled (heavy) and unlabeled (light) peptide isoforms (**Figure 3*a*,*b***). Since the physicochemical properties of the peptides do not change due to the isotope label, heavy and light forms usually co-elute off the chromatographic gradient. Typically, a peak is 10 to 30 seconds wide (250 nL min^{-1}, 75 μm ID, 10 cm C18 column). Depending on the operating parameters of the mass spectrometer and instrument type, one survey scan may be collected every 1 to 5 seconds, allowing the sampling of several MS full scan spectra across the time course of the eluting peak. Each of these MS spectra is a single observation of the peptide pair, and averaging the ratios determined from several such MS spectra gives the ratio and standard deviation for each peptide. There are two ways of calculating an abundance ratio between heavy and light forms based on the respective extracted ion chromatograms. In one method, the ratio between heavy and light forms is calculated at each of the survey scan events across the ion peak, and the individual ratios are then averaged to a peptide ratio (**Figure 3*a***). In this case, each measured ion will have an average ratio and a standard deviation. In the alternative method, the ratio is formed between the peak volumes across the total extracted ion chromatogram for the heavy and the light form of the peptide (**Figure 3*b***). This method of calculation is less affected by slight shifts in chromatographic elution between heavy and light peaks.

Due to a large number of incorporated labeled atoms in the peptide sequence, more isobaric amino acid forms are generated, leading to higher ambiguities in the sequence matching process (72). Furthermore, isotope clusters of [^{15}N]-labeled peptides are broader, and this is especially true for longer peptides. Since most quantitation algorithms use the first isotope for quantitation, the accuracy with which the peptides are quantified also depends on peptide length and amino acid composition (30). However, approaches that take into account differences in isotope envelopes of heavy and light peptide isoforms have been described (118). In general, the dynamic range across which accurate quantitation is possible using [^{15}N]-labeling covers one to two orders of magnitude, depending on instrumentation (114). This means that changes

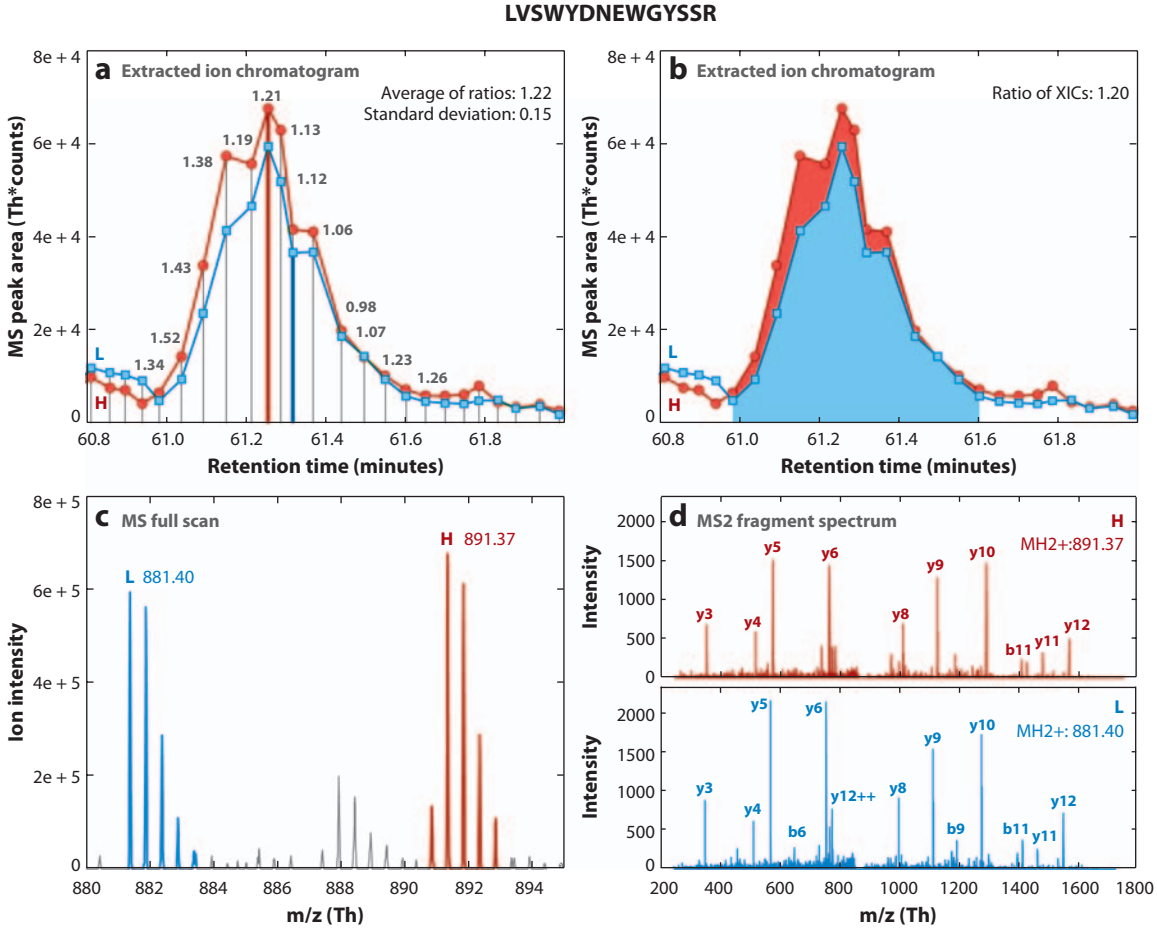

Figure 3

Metabolic labeling using [^{15}N]. (*a*) Extracted ion chromatogram (XIC) of co-eluting heavy and light peptide forms. Calculation of the average peptide [^{15}N]/[^{14}N] ratio from the average of ratios of different survey scans across the eluting peak. (*b*) Extracted ion chromatogram of co-eluting heavy and light peptide forms. Calculation of the average peptide [^{15}N]/[^{14}N] ratio from the ratio of peak volumes. (*c*) MS survey scan at the peak apex, including light and heavy form of the peptide ion. Mass difference between heavy and light form depends on number of nitrogen atoms in the peptide sequence. (*d*). Fragment spectra of the heavy and light form of the peptide. Note that in all cases both forms are fragmented. Although rather inexpensive and efficient to implement in plants, the labeling strategy with [^{15}N] has several drawbacks: The number of atoms incorporated in each tryptic peptide depends on peptide length and amino acid composition, which makes the matching process more difficult. Usually, only those pairs of labeled and unlabeled isoforms can be used for quantitation, for which a peptide sequence was assigned at least to one of the pairs (*c*). The nonfragmented heavy or light partner must then be found according to the predicted mass difference, which depends on the number of nitrogen atoms and thus the peptide sequence of the fragmented peak (22). In only a few cases are both heavy and light forms identified by tandem mass spectra (*d*).

smaller or greater than tenfold are often beyond the linear range and will be over- or underestimated. A thorough assessment of the use of full versus partial labeling revealed that in general both strategies are comparable with regard to dynamic range and accuracy. While partial labeling is more challenging with respect to automated identification of labeled and unlabeled peptide pairs, it allowed quantification of more peptides across the whole dynamic range (44).

ICAT: isotope-coded affinity tag

LC: liquid chromatography

Fragment ion: an ion that occurs after fragmentation of a peptide ion. Fragment ions are detected during the tandem mass spectometry scan (MS/MS or MS2)

Precursor ion: the ion of a peptide that was selected for fragmentation in the mass spectrometer. Precursor ions are detected during the survey scan (MS)

AQUA: absolute quantification using synthetic standard peptides

CHEMICAL LABELING

Chemical labeling essentially works the same way as described for the metabolic [^{15}N]-labeling, except that the label is introduced to isolated proteins or peptides by a chemical reaction, e.g., with sulfhydryl groups or amine groups, or through acetylation or esterification of amino acid residues (83). The isotope label can also be introduced to the peptide chain during the enzymatic reaction of the tryptic digestion by the addition of $H_2[^{18}O]$ to the peptide cleavage sites (126). Quantitation is based on the full scans, and in contrast to [^{15}N] labeling, the mass shift between heavy and light peptides is constant. However, it should be noted that for unambiguous quantitation, the mass difference between heavy and light form should be at least 4 Da in order to clearly distinguish the isotopomer clusters of the heavy and light forms of the peptide. Since the isotopomer cluster becomes larger with increasing mass of the peptide, small labels, e.g., [^{18}O] introduced by a tryptic digest in $H_2[^{18}O]$, become limiting for larger peptides.

A commonly used chemical isotope label is the isotope-coded affinity tag (ICAT), which binds to sulfhydryl groups of cysteine residues (36). It is a useful tool to study oxidation or reduction status of proteins, but since cysteine is not a very abundant amino acid, the number of peptides that can be tagged and quantified by sulfhydryl labels is rather low.

ISOBARIC MASS TAGS

Isobaric mass tagging (110) differs from the strategy described above in that addition of the mass tags initially produces labeled peptides of the same total mass that co-elute in liquid chromatography. Only upon peptide fragmentation can the different mass tags be distinguished. As each tag adds the same total mass to a given peptide, each peptide species produces only a single peak during liquid chromatography, even when two or more samples are mixed (**Figure 4*a***). Thus, there also will be only one peak in the survey MS scan, and only a single m/z will be isolated for fragmentation (**Figure 4*b***). The different mass tags separate upon fragmentation (**Figure 4*c***). The fragments are in the low mass range, which usually is not covered by typical peptide fragment ions. The intensity ratio of the different reporter ions is used as a quantitative readout (**Figure 4*d***).

Quantitation for isobaric mass tagging is based on the fragment spectra rather than on the survey scans. Thus, quantitative accuracy depends on the isolation width of precursor ions for fragmentation, since all ions isolated in that window will contribute to fragments in the reporter ion mass ranges. It is also significant that often in fragment scans only a single fragment spectrum per peptide is available, whereas in quantitation based on survey scans, usually several data points across the eluting peptide peak are sampled.

iTRAQ (99) and TMT (110) are commercially available isobaric mass tags that are introduced to the proteins of interest at the level of tryptic peptides. iTRAQ has been widely used in plant proteomics to study phosphoproteomic responses of elicitor treatment by comparing several time points post-treatment (48, 75). Protein degradation in chloroplasts (100) and developmentally induced changes in chloroplast proteomes (53) were studied. Chloroplast proteomes in different cell types were compared in maize (64) and *Brassica* (129). In an elegant study, organelle proteomes (19) and the proteomes of different endomembranes (101) were defined by differential mass tagging of sequential fractions across continuous sucrose gradients and assignment of protein profiles to profiles of known marker proteins.

STANDARD PEPTIDES

The use of stable-isotope-labeled standard peptides was first described in 1983 (18). However, only recently has increasing analytical throughput on modern tandem mass spectra made possible the large-scale use of synthetic isotope-labeled peptides as a standard for absolute quantification (AQUA) (26). In combination with multiple selected reaction monitoring

(SRM) on a triplequad mass spectrometer, such targeted analyses of specific proteins across a wide range of samples are very efficient. The combination of information on retention time, peptide mass, and fragment ion mass gives high specificity to the particular target peptide, and due to very low noise levels in the SRM spectra, the linear range for quantitation is extended up to five orders of magnitude (52).

If known concentrations of the labeled standard peptide are added to the sample, the concentration of the native peptide in the sample can be calculated. However, the amount of protein in an experiment determined by AQUA may not reflect the true expression levels of this protein in the tissue, because sample preparation steps may lead to losses or enrichments that are not addressed by the AQUA technique.

In plant proteomics, the standard peptides have been used to monitor abundance changes of different isoforms of sucrose phosphate synthase in different tissues of *Arabidopsis* (59) and in *Medicago* root nodules during drought stress (119). Target sites for phosphorylation in isoforms of trehalose phosphate synthase were studied using standard peptides in in vitro kinase assays (28).

CHALLENGES IN DATA PROCESSING

No matter the choice of quantitative method, quantitative proteomic data are typically very complex and often of variable quality. The main

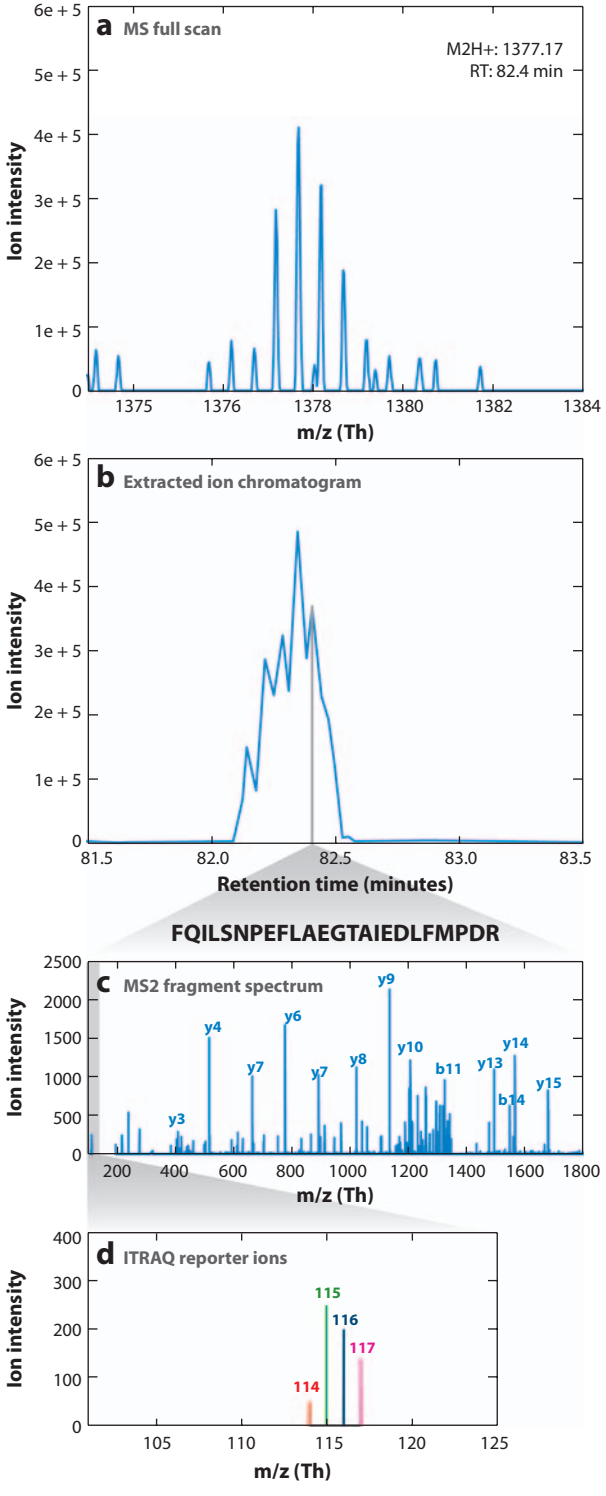

Figure 4

Isobaric mass tags. (*a*) Survey scan of the parent peptide ion. (*b*) Extracted ion chromatogram (XIC) of the precursor peptide ion that combines all different tagged forms in one single mass. (*c*) Fragment spectrum of the parent peptide ion that allows sequence identification and that produces characteristic reporter ions for quantification in the low mass region (*gray field*). (*d*) Close-up of the low mass region of the fragment spectrum. Intensities of the reporter ion of different mass are indicative of the relative abundance of the peptide in the different samples.

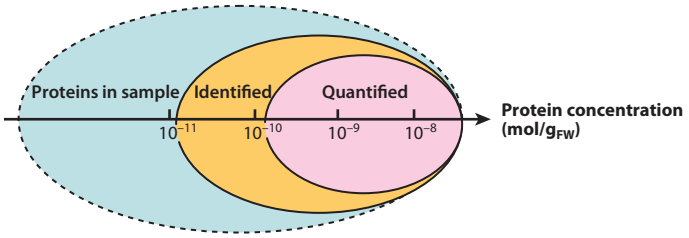

Figure 5

Proteome coverage for quantitation. Of all the proteins in the sample, only a subset is being identified in LC-MS/MS (liquid-chromatography coupled tandem mass spectrometry) experiments. Out of the identified proteins, another subset is suitable for quantitation. Usually, the higher abundance proteins are covered by identification and quantification.

challenge stems from incomplete data, since even today's most advanced mass spectrometers cannot sample and fragment every peptide ion present in complex samples (**Figure 5**). As a consequence, only a subset of peptides and proteins present in a sample can be identified (1). Furthermore, due to several quality issues (see below), the fraction of identified peptides and proteins that can actually be used for quantitation is even smaller (**Figure 5**). Therefore, careful experimental design involving steps of protein separation, enrichment, and purification is essential for successful interpretation of proteomic datasets.

From Spectra to Quantitative Information

The workflow in quantitative approaches based on mass spectrometry requires extraction of quantitative information either from survey scan or fragment spectra, as well as qualitative information for peptide identification from fragment spectra. Most important, manual validation of peptide identity and quantitation is required. During manual validation, it is possible to review sequence assignment to spectra and to assess the quality of the quantitative data with regard to signal-to-noise level, presence of interfering peaks, or isotope label incorporation. In many cases, the identification process and the quantitation process are carried out independently and are linked later at the level of individual spectra.

While advanced algorithms for protein identification have been available for quite some time [Sequest (21), Mascot (91), X!Tandem (14), Omssa (25), InsPect (109)], the development of robust workflows and algorithms to extract quantitative information from multidimensional proteomics experiments based on mass spectrometry has just begun (**Table 2**). Generic formats such as mzData, mzXML, or pepXML allow independent use of software algorithms to manipulate data without compatibility constraints. A thorough review of different freely available software for analysis of mass spectrometry data can be found elsewhere (68).

Intensity ratios of good-quality spectra are usually averaged to yield abundance ratios for a peptide, and peptide abundance ratios are averaged to obtain protein abundance ratios. The assignment of peptides to individual proteins requires special care, since many peptide sequences can match more than one protein. For accurate quantitation, it is therefore important to consider only those peptides in the quantitation that are unique to a particular protein, so-called proteotypic peptides. This is important since different protein isoforms can be differentially regulated, and this can result in peptide ratios of conserved peptides deviating from peptide ratios of proteotypic peptides (83).

At the stage of experimental planning, as well as during extraction of primary data, the following points must be considered in relation to the nature of the biological question asked and the quantitation method used.

(*a*) In general, the quantitative accuracy of peptide ratios is affected by several factors. On the technical side, instrument resolution, sensitivity, and scan speed, as well as liquid chromatography (peak width), have significant influence on the quality of the quantitation. The use of high resolution and high mass accuracy instruments certainly will increase the confidence in protein identification, and it will also increase quantitative

Table 2 Quantitation software that is free to use but may require specific input formats

Software	Website	Features	Format requirements	Reference
ASAP Ratio	**http://tools.proteomecenter.org/wiki/index.php?title=Software:ASAPRatio**	^{15}N labeling, ICAT, label-free; possibility to self-define mass tags	mzXML, pepXML, DTAselect	60
AYUMS	**www.csml.org/ayums/**	SILAC	Mascot output, converted file from Waters	102
Census	**http://fields.scripps.edu/census/index.php**	^{15}N labeling, ICAT, ITRAQ, label-free; possibility to self-define mass tags	mzXML, pepXML, DTAselect	87
i-Tracker	**www.cranfield.ac.uk/health/researchareas/bioinformatics/page6801.jsp**	iTRAQ	peak list files (*.dta; *.mgf)	107
jTraqX	**http://sourceforge.net/projects/protms/**	iTRAQ	Mascot output	69
MaxQUANT	**www.maxquant.org**	SILAC	Mascot output, instrument raw files (Thermo)	12, 13
MRMer	**http://proteomics.fhcrc.org/CPL/MRMer.html**	mSRM	mzXML	65
MSQuant	**http://msquant.sourceforge.net**	^{15}N labeling, ICAT, label-free; possibility to self-define mass tags	Mascot output in htm format, instrument raw files (Thermo, ABI, Waters)	67
XPRESS	**http://tools.proteomecenter.org/wiki/index.php?title=Software:XPRESS**	ICAT, ^{15}N labeling	mzXML, pepXML, DTAselect	37

confidence by facilitating more narrow isolation widths and less peak interferences on full scans (79, 80, 130).

(*b*) Quantitative accuracy at the peptide level depends on the number of data points available across the eluting peak. More mass spectrometry spectra provide increased confidence in the quantitative values obtained. This applies to all quantitation methods based on peptide ion intensities.

(*c*) Ion intensities greatly influence the quantitative accuracy of single peptides (**Figure 6*a***). Peptides with high ion intensities are more accurately quantified, while peptides with low ion intensities show a much greater variation of ratios. Although the majority of ratios from all quantified peptides is within the expected range in a sample 1:1 mixture, the outliers in a real biological experiment are usually considered to be the interesting candidates and must therefore be carefully validated.

(*d*) Further problems arise from overlap of unrelated signals with the isotope clusters of either member of stable-isotope peptide pairs, or with peaks to be compared in label-free protein correlation profiling. This can lead to the contribution of the unrelated peak to the peptide ratio. This problem is less apparent if instruments with high resolution can be used for quantitation of complex samples.

(*e*) Isotopic envelopes of the unlabeled and labeled peptide forms may overlap depending on the size of the peptide. This problem depends on the mass difference of the incorporated stable-isotope label used. Larger peptides tend to have greater overlap, and smaller mass differences between heavy and light labels tend to create greater overlap. Label-free quantitation is not affected.

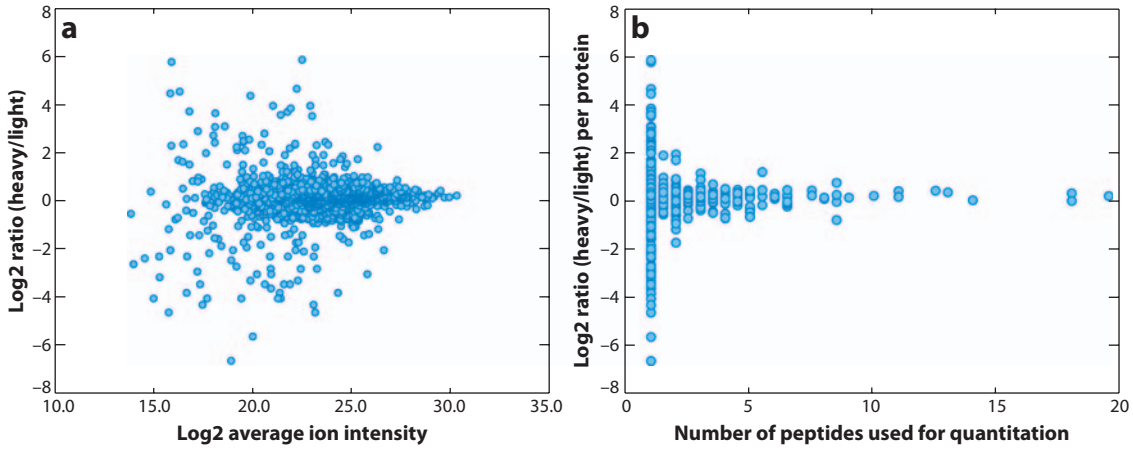

Figure 6

Quantitative accuracy in peptide ratios and protein ratios in a 1:1 mixture of labeled and unlabeled proteins. (*a*) Magnitude versus amplitude plot of log2 values of ratios versus log2 values of average ion intensities. (*b*) Protein ratios depend on the number of peptides contributing to the average ratio. Single peptide proteins require careful interpretation.

(*f*) Chromatographic separation of the heavy and light peptide pairs in stable-isotope-labeling approaches can lead to distinct ionization conditions for each member of the peptide pair, meaning that ion intensities cannot be compared from the same survey scan spectrum. Rather, peak areas must be determined for each partner separately, and the ratio of these summed peak areas of extracted ion chromatograms must be calculated. Isobaric mass tags, for which quantitation is done on the fragment ion scan, are not affected by chromatographic separation.

(*g*) Using spectrum count data can lead to zero counts of a given protein in one sample, but it might be detected in another sample. This makes calculation of a fold change impossible and leads to datasets with missing values. Similarly, in stable-isotope labeling, if only one partner of the peptide pair is detected (i.e., only the labeled form or only the unlabeled form), no ratio can be calculated and the actual abundance level of the single partner cannot be accurately determined.

(*h*) The accuracy of the protein ratio is primarily dependent on good quantitation at the peptide level but is also influenced by the number of quantified peptides per protein and the number of proteotypic peptides for each protein. Protein ratios calculated from more than one contributing peptide are more accurate than protein ratios based on the presence of only one peptide (**Figure 6*b***). Although the majority of proteins are not affected by this problem, the proteins quantified based on a single peptide must be validated especially thoroughly, as chances of incorrect quantitation are considerably high.

To overcome some of these problems, especially in the case of stable-isotope labeling, reciprocal experimental designs have been proposed that distinugish treatment effects from both labeling effects and biological variation. Reciprocal experiments involving metabolic [^{15}N]-labeling were first used to identify differentially regulated candidate proteins, prior to sequence assignment, through database search (117). In addition, a detailed statistical procedure describing identification of

treatment-responsive proteins versus biological variance of two proteomes has been developed (51). Reciprocal design of [^{15}N]-labeling experiments has been implemented in phosphoproteomic studies of elicitor treatment (7), in definition of plant sterol-rich domains (50), and in studies of proteome changes in leaf senescence (38).

Statistical Analysis

Experiments involving quantitative proteomics typically aim at either the analysis of changes in protein abundance between a set of treated samples compared to a control condition or at the definition of protein complexes or specific subproteomes compared to background proteins. The aim of the data analysis procedures is, in most cases, the definition of "deviating" proteins that ultimately are considered as "responsive candidates" in the biological context under investigation. When protein complexes or organelle fractions are characterized, large numbers of proteins with similar quantitative behavior are considered as members of the same complex or subproteome compared to other proteins with different quantitative behavior (3, 19, 20, 24).

The first step in primary data processing is usually normalization, which is a very critical step in quantitative experiments. It corrects for technical effects, such as sample mixing errors, incomplete isotope incorporation, or differences in ionization between independent LC-MS/MS experiments, and thus influences the result. Total ion counts, total number of spectra, or the average ratios of the most abundant proteins are often used as basis for normalization, relying on the assumption that the majority of proteins will remain unchanged and can thus be used for normalization. Ideally, in stable-isotope-labeling strategies, a control mixture of labeled and unlabeled untreated samples is used to determine the biological variation of ratios and mixing errors (51). Log-transformation of ratios is a common step taken to harmonize the variances of ratios. The log transformed data are subsequently used in exploratory plots showing the average log abundance on the *x*-axis and the log fold change between conditions on the *y*-axis (**Figure 6*a***). Using such plots, linear and nonlinear biases in the data can be identified, and thus normalization procedures can be benchmarked.

TREATING CONTINUOUS DATA

Relatively simple algorithms, e.g., scaling the individual log intensity values using the global median value or the average obtained from a subset of proteins (55), sometimes provide enough normalization. Other potentially more powerful methods, such as quantile normalization (10, 40), variance stabilization (54), or a "spectral index" combining different features of each data point, have been benchmarked for label-free proteomics (56, 32).

Alternatively, data can be normalized by applying a locally weighted scatter plot smoothing procedure (data plotted as in **Figure 6*a***; 125), thus efficiently removing biases that are dependent on measurement values like ion intensities. However, systematic bias coming from analysis order (56) or the data itself (49) should be minimized by the experimental design (42).

When the data sets are sufficiently comparable after normalization, the next step is to detect changes between the different conditions. The data sets can be analyzed using statistical tests such as the student's t-test or the Wilcoxon rank sum test (7). Tests taking the experimental confidence about the quantitation into account should provide more statistical power for small sample sizes (51). In cases where there are more than two groups to compare, it is usually preferable to use ANOVA type analyses, many of which can be performed using the freely available R software.

TREATING DISCRETE DATA

Spectral count data is reminiscent of data arising from the serial analysis of gene expression (SAGE), and thus statistical methods and concepts developed for SAGE can be used.

However, proteomics-specific problems have to be accounted for, e.g. that the number of observable peptides is not the same for all proteins or that not all peptides may be detected with the same confidence. In the case of continuous data, such factors can be accounted for by weighting the influence of peptide ions by their confidence (12, 60).

Methods that allow the direct assessment of count data and have been used in the proteomics field include the goodness-of-fit (G-test), Fisher's exact test, and the AC test, which all performed quite similarly (127). The G-test was extended to cover more than two conditions (127). Because these tests inherently take into account the number of spectra acquired in total per run, no normalization is required for this parameter.

Usually, extending beyond these count-based statistical approaches requires normalization that tries specifically to minimize the technical effects of spectral count data. Methods that take into account the detection confidence, protein length, or the number of observable peptides (e.g., the emPAI and APEX values discussed above) are suitable. Using these derived indices yields values that, when contrasted between experiments, behave quite like the data shown in **Figure 6*a***. The standard deviation is dependent on the protein abundance, and this behavior can be taken into account explicitly for statistical analysis when using R (88).

Alternatively, more sophisticated models that consider potentially biasing factors such as protein length and general count abundance can be applied (11).

GENERAL ISSUES

To date, the biggest challenge in comparative proteomics is to account for missing values due to incomplete proteome coverage in peptide fragmentation. The mostly random sequencing of peptides by the mass spectrometer results in not every peptide being fragmented in every sample. Especially low abundance peptides and peptides with low ionization efficiency will most likely be fragmented in a few out of several samples compared, resulting in missing quantitative values. However, statistical methods often require complete datasets. A common workaround is to estimate missing values, but that influences statistical evaluation. Eliminating incomplete datasets from the analysis strongly reduces the number of proteins that can be statistically assessed. Improving peptide separation and fractionation and optimizing chromatography influence the quantitative coverage (23).

Finally, no matter how the data is generated, modern proteomics techniques generate large amounts of data from just a single experiment, and thus multiple statistical tests must be performed. This necessitates correcting p-values for multiple testing using family-wise error rate methods or false discovery rate approaches. The latter type of correction method is often preferable when dealing with proteomics data.

INTERPRETATION OF THE RESULTS

When multiple measurements have been obtained from biological samples, insights can be gained by subjecting the data to more specialized analysis and visualization procedures. Clustering the data by treatment often reveals global trends or may help in characterizing mutants. A plethora of clustering algorithms is available; however, either hierarchical (5, 42) or k-means clustering (98) is often used, perhaps because many software tools support these algorithms. Proteins can also be grouped by clustering according to their behavior in multiple experiments or in a time course. Often, either the groups of proteins in a cluster or all differentially expressed proteins are categorized (5, 58, 71) and statistically assessed for the enrichment of biological categories. Many tools are available to the plant community for these analyses, including the GO slim categorization by TAIR; the Classification SuperViewer (93) for *Arabidopsis*; or PageMan (113), which is available for many plant species.

Commonly used tools for projecting data into a 2D space include principal component

analysis (47), which tries to preserve the maximal variance in a low-dimensional space, and independent component analysis (58), which attempts to identify independent components in the dataset. The projected data frequently provides visual separation of conditions such as stressed and unstressed plants, mutant genotypes, or different treatments (121). In cases where multiple replicates from several different plant samples are available, proteins specific for a certain condition/tissue can be identified by inspection of the data or by using machine learning (5). These methods help in assessing new protein functions and aid in their classification.

PERSPECTIVES

Although proteomics has been able for some time to obtain a relatively complete view of quantitative changes in subproteomes such as organelles, the proteomics datasets are usually much smaller than microarray datasets and tend to be biased against lower abundance proteins. Global abundance measurements in *Saccharomyces cerevisiae* have revealed a bell-shaped distribution of proteins spanning approximately six orders of magnitude in abundance (27), while only approximately three to four orders of magnitude can be covered by modern LC-MS/MS methods in complex samples (16). Most notably, the robust and repeated identification of low abundance proteins across replicates is still far from being routine. The major limitation in quantitative proteomics thus lies in the incomplete proteome coverage between different samples, with the result that many experiments result in datasets with missing values.

These limitations pose significant challenges for the researcher, as only with useful proteome fractionation and intelligent strategies for enrichment of protein targets can the coverage of the relevant proteome be maximized. On the technical side, improvement of mass spectrometer scan speed and sensitivity, as well as new developments in chromatographic separation, will likely contribute to deeper proteome coverage in future.

Despite these limitations, differential proteomics techniques are used increasingly throughout plant biology. Currently, most studies employ rather straightforward pairwise comparisons of tissue types or treatments, but more complex experimental designs will emerge. The challenge in the large-scale, quantitative proteomics experiments will lie in the application of data-mining strategies to plant biology. Well-designed experiments and focused hypotheses, in combination with high-quality mass spectrometry, are likely to contribute significantly to our understanding of protein function in plant growth and development—both at the level of global protein profiling and in-depth, targeted protein analysis.

SUMMARY POINTS

1. Comparative proteomic approaches involving stable-isotope-labeled quantitation have the potential for the highest precision and lowest relative standard deviations. However, proteome coverage may be compromised due to higher sample complexity.
2. Label-free quantitative strategies allow the comparison of a large number of samples or conditions at rather low cost. This strategy is of particular interest for expected large differences, since quantitative precision is lower compared with stable-isotope approaches.
3. Quantitation based on full scan techniques allows precise statistics across multiple scans of the chromatographic peak of each peptide ion.
4. Quantitation based on fragment ions usually has low noise levels. Often, however, only one scan per peptide is available.

5. Quantitation of a protein gains precision with the number of peptides contributing to the quantitation. Each protein quantitation based on a single peptide requires thorough verification.
6. Statistical treatment of the data is necessary and, despite the complexity of the data, many tools are available for data normalization and detection of differential expression.

FUTURE ISSUES

1. A need exists for improvement of proteome coverage due to faster instrumentation and routine implementation of peptide and protein fractionation techniques during sample preparation. We will need to formalize quality standards for quantitative analysis within the proteomic community.
2. Good experimental design around specific biological hypotheses will be key to our functional understanding of protein function in regulatory processes of plant growth and development.
3. Further development of statistical methods and data-mining workflows in combination with modeling approaches are necessary for better biological interpretation of large-scale proteomics datasets.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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