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Quantitative proteomics in biological research

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Proteomics has enabled the direct investigation of biological material, at first through the analysis of individual proteins, then of lysates from cell cultures, and finally of extracts from tissues and biopsies from entire organisms. Its latest manifestation – quantitative proteomics – allows deeper insight into biological systems. This article reviews the different methods used to extract quantitative information from mass spectra. It follows the technical developments aimed toward global proteomics, the attempt to characterize every expressed protein in a cell by at least one peptide. When applications of the technology are discussed, the focus is placed on yeast biology. In particular, differential quantitative proteomics, the comparison between an experiment and its control, is very discriminating for proteins involved in the process being studied. When trying to understand biological processes on a molecular level, differential quantitative proteomics tends to give a clearer picture than global transcription analyses. As a result, MS has become an even more indispensable tool for biochemically motivated biological research.

Keywords:

Affinity chromatography / Cell biology / Functional proteomics / MS / Proteomics methods / Protein–protein interaction

1. Introduction

There is a variety of different techniques used in quantitative biology in addition to mass spectrometry, including 2-D gel electrophoresis, fluorescence microscopy and Elisa assays, among others. This review article focusses exclusively on mass spectrometric techniques due to the breadth in molecular characterization of biological specimens. The basis of its broad application in biological research was the discovery of two soft ionization techniques in 1988/89, the Matrix Assisted Laser Desorption (MALDI) and the electrospray technique [1, 2]. With the progress of mass spectrometric techniques in sequencing proteins, the wide use in biology began [3-5]. Research projects evolved in parallel to the improving technology. First, individual proteins were identified on very low levels [6, 7], then protein complexes were characterized in a single experiment and finally, entire organelles and tissues were analyzed [8-11]. Progress towards one of the

longer-term goals, the characterization of close to all cellular proteins by at least one peptide, was made [12, 13]; however, the complete characterization of a proteome including all modifications might never be achievable because of its complexity in the form of splice variants, varying secondary modifications, the presence of variable degradation products and on a proteomic scale, relevant non-specific cleavage by the enzymes used for digestion [14].

The relationship between biological research and technical progress in proteomics is reciprocal. The biological research context determines the significance of a mass spectrometric analysis. But it is the level of protein characterization techniques that influences the design of meaningful biological experiments. Early in the proteomic development, immunoaffinity purifications of interacting proteins became a key experiment for rapidly finding proteins relevant for specific pathways. This approach was much more effective than genetic screening methods. When it was possible to characterize entire protein complexes in one experiment, systematic but still highly selective protein complex purifications made the functional network of protein complexes of a cell visible [8, 15].

Quantitative proteomics has to be seen in this context - the ability to systematically retrieve the relative amounts of all identified proteins in the sample allows new types of meaningful experiments in molecular biology to be undertaken [16, 17]. With a quantitative analysis, the characterization of protein complexes can be extended to complexes that can not

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Abbreviations: EGF, epidermal growth factor; MRM, multiple reaction monitoring; SRM, selected reaction monitoring

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be enriched to high levels of purity. Simply by comparing the enriched fraction with a control, protein complex members can be identified based on their quantitative traces [18].

By enabling analysis of temporal changes, quantitative proteomics brings a new quality to mass spectrometry-based molecular research. In the first attempts to reveal the functional organization of proteins within a cell, protein complexes were seen mostly as static entities. However, biological processes are defined by dynamic changes in protein complex composition, location and covalent modification of some of their members. Quantitative proteomics can enable visualization of these changes when it is possible to synchronize a sufficient number of cells [16, 18].

There is good reason to expect that every mass spectrometric investigation in biology should be quantitative. The analysis has become so sensitive that the relevance of identified proteins can not be deduced simply by their presence. Significance can only be attributed to a quantitative difference between experiment and control, as in all other experiments in cell biology.

Will the quantitative description of nearly every protein within a cell bring a new qualitative dimension to biological research? The sequencing of entire genomes allows inter-genomic comparisons and observations on the level of genomes as such. The systematic characterization of protein complexes has allowed networks of protein complexes to be generated and the inter-organism comparisons of these networks has revealed evolutionary changes [8, 19, 20]. Holistic views do not abolish more focussed research - they add another dimension and allow other types of observations [21, 22]. This is why it is likely that the close-to-complete characterization of the expressed proteome (in the sense that every protein is represented by at least one peptide) will bring an additional level of observation to cell biology [23]. The system as such becomes the target of the investigation - its reaction to external stimuli and change over time [24]. It is noteworthy that there are mechanisms that act upon the entire proteome, such as microRNA-based regulation of gene expression [25-27]. At least for these mechanisms, a global view of the entire expressed proteome reveals patterns and provides explanations otherwise missed.

There are alternative techniques to mass spectrometry-based proteomics to observe the proteome or a proteome equivalent. Translated mRNA regions can be accessed by using ribosomal profiling [28]. The ribosome-protected mRNA regions are prepared by nuclease digestion, sequenced and quantified using deep sequencing and sequence counting. Ribosomal profiling does not really measure the abundance of proteins, but rather the current translational activity. A correlation coefficient R^2 of 0.60 between the translational activity

and the absolute protein amount has been found [28]. This correlation, however, is not high enough if the intention of the proteomic experiment is to find mechanistic explanations of biological processes. On the other hand, translational profiling combined with a cell-type specific purification of ribosomes can have unique advantages in the characterization of complex tissues [29, 30]. Proteomic study of the central nervous system, for example, requires the enrichment of the cell type of interest typically via fluorescence-activated cell sorting or laser-capture micro-dissection. Such techniques are disruptive and might therefore lead to distorted results. This can be avoided when using ribosomal profiling. With bacterial artificial chromosomes (BACs), it is possible to express a tagged component of the ribosome under the control of a cell-type specific promoter [29, 30]. The tagged protein is used to affinity purify the ribosomes and the translated mRNA. In this way, the expression pattern of a particular cell type of the central nervous system has been studied [29, 30]. There is no corresponding technique in proteomics that would not require cell sorting of some kind.

2. Quantifying mass spectrometric measurements

There are two different kinds of mass spectrometric signals that can be used to obtain quantitative information: the signal of an intact ion in the mass spectrum - a MS signal - or the signal of one or several of the ion's fragments - a tandem MS signal. Both types of signals have their specific advantages and disadvantages. Using the intact ion signal has the advantages that often several independent spectra are available to determine a molecular ratio and that the ion intensities are high. However, in particular for low level measurements, background ions can limit the precision and for complex proteomic mixtures, other overlapping ion signals can distort the measurement. Using fragment ion intensities has the advantage that background ions in the mass spectrum interfere much less (see figure 1) [31]. However fragment ion signals are often of such a low intensity that the ion statistics can limit the precision of the measurement and in most cases there is only one spectrum available to determine a signal ratio. The fragment ion used for quantitation can be specific for the molecule quantified, as in MRM scans (see below and figure 2). In this case, overlapping precursor ions do not disturb the measurement. If the fragment ion is non-specific, as with iTRAQ (see below, figure 3 and table 1), overlapping precursors disturb the measurement in the same way as for intact molecular ion-based quantifications.

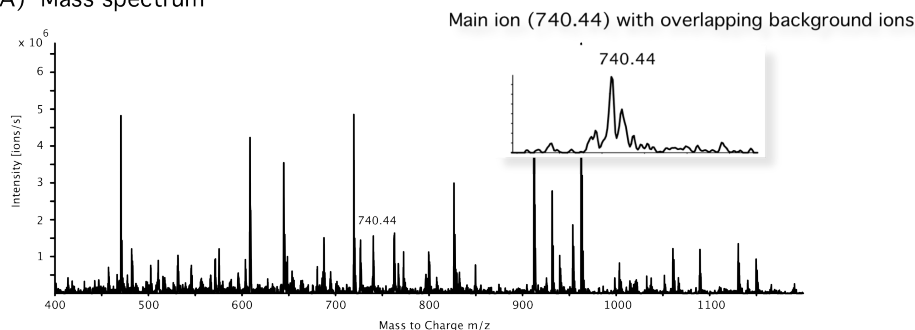
Quantities determined by mass spectrometry are always based on signal comparisons. There are two different types of references to which the measurements are related: isotopic references and global references. In the case of isotopic ref-

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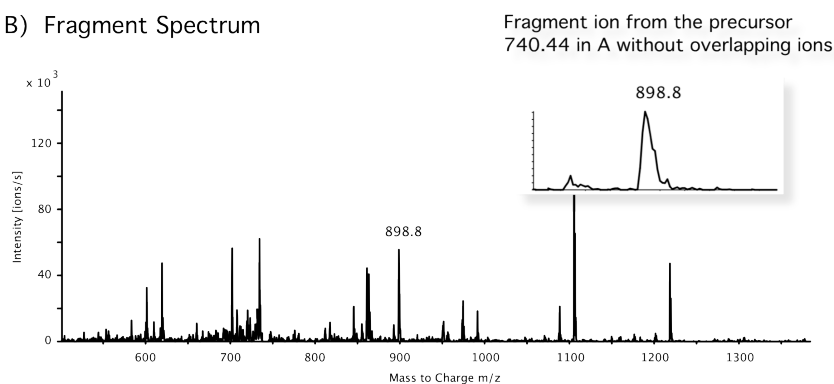
erences, the measurement of each peptide is related to an isotopically-labeled peptide of the same molecular structure. This quantification method is called isotope label-based quantification. When global references are used, all measurements are related to a set of molecules that are chemically

different from the quantified ions. This method is called label-free or direct quantification [32]. Absolute concentration measurements can be achieved if the concentration of the reference molecule is known (see for instance AQUA, table 1) [33].

A) Mass spectrum



B) Fragment Spectrum



C) Ion Chromatogram

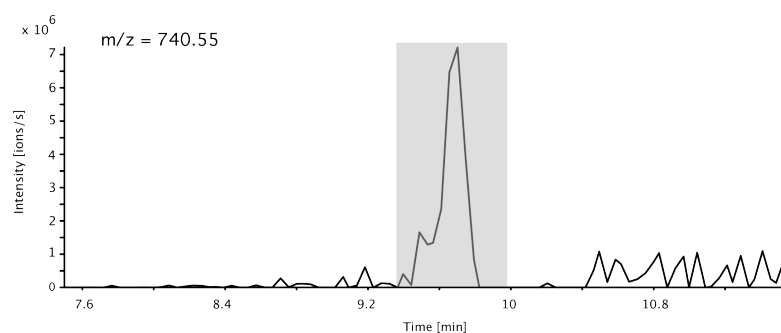


Figure 1 The three different spectral sources of quantitative information, a mass spectrum (A), a fragment spectrum (B) and an ion chromatogram (C). The spectrum A shows that a peak in a mass spectrum can show some overlap with other ions. This limits the correctness the intensity readouts of the peptides. However, if the peptide is fragmented, individual intense fragment peaks can represent exclusively the peptide without the influence of other background ions (spectrum B). This is one of the reasons why selected reaction monitoring is used to quantify low level molecules. The ion chromatogram (C) is reconstructed from a series of primary spectra (A) covering the entire elution time of the peptide. The ion chromatogram is integrated when quantifying the peptide directly without the use of isotopic labels.

2.1 Consequences of the ionization process on the quantification process

The most basic quantification is achieved when the signal intensity in the spectrometer directly corresponds to the molecular concentration in the sample. However for mass spectrometric measurements, this is in general not the case since molecules need to be transferred into the vacuum system

and ionized before they can be measured. These processes are very different for MALDI and electrospray ionizations.

2.1.1 MALDI process - Quantifications

In a MALDI mass spectrum there is often a large discrepancy between the ion intensities and the analyte concentration on the target. The most important effect responsible for the non-quantitative representation of molecules in a MALDI spectrum is the variable ionization efficiency. Ionization of peptides

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occurs via proton transfer from less basic molecules, predominantly from the acidic matrix. The number of protons available for ionizing compounds is limited. If there is a substance with a very high proton affinity it will pull protons away from other analyte molecules. The result is a pronounced ion suppression effect [1, 34, 35]. The presence of one type of ion has a significant influence on the intensity of other ions. This is why quantitative measurements with a MALDI instrument for peptides below a mass of about 3 kDa should always be done by comparing signals between an analyte and

a standard of very similar if not identical chemical structure in the same sample. Molecules of identical chemical composition would undergo the same proton transfer reactions and their intensity ratio reflects their relative abundance on the target. Proteins or peptides larger than 3 kDa have by themselves such a high proton affinity in the gas phase that it is less likely that they are suppressed by other even more basic molecules.

Triple Quadrupole

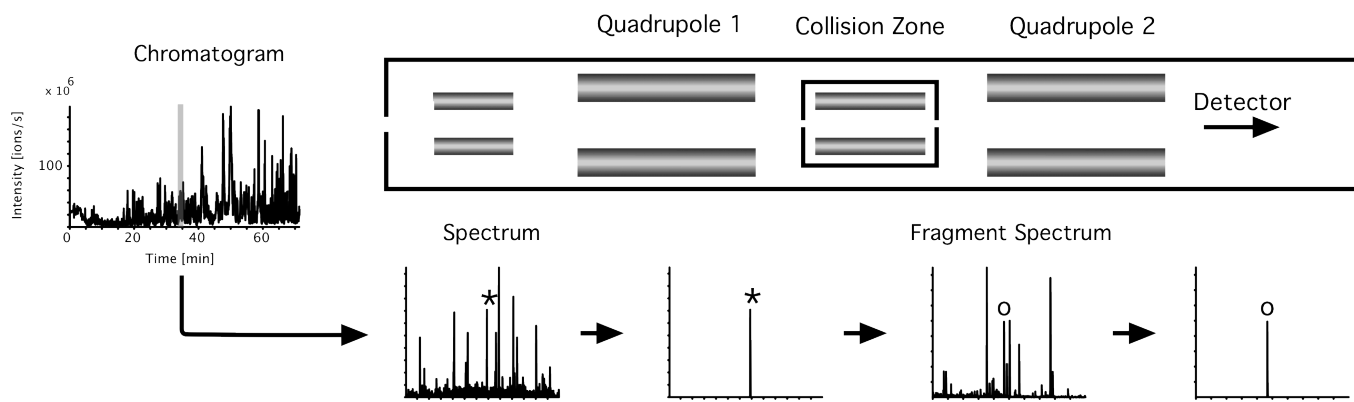


Figure 2 Selected Reaction Monitoring (SRM) or Multiple Reaction Monitoring (MRM) scan. In a standard data-dependent experiment mass spectra are acquired, an abundant peak is selected so that only these ions pass the first mass filter and are fragmented in the collision cell. An entire fragment spectrum is recorded using a second mass separating spectrometer. In a preprogrammed SRM or MRM scan the first mass filter is fixed onto a specific mass. Only ions with this m/z value reach the collision zone and are fragmented. The second mass filter is fixed to an expected m/z value. If this type of fragment is generated, the detector records a signal. The double requirement in m/z of the intact ion and of a generated fragment is the basis of the specificity of SRM or MRM scans. In a real experiment a triple quadrupole mass spectrometer can be programmed to scan for a set of m/z values at specific retention times (scheduled MRM). To every m/z value several fragment masses can be linked. As such, more than a hundred different molecules can be observed and quantified in the course of an LC run. The unique advantage of this scan method is that overlapping ions in the mass spectrum do not contribute to the signal at the detector when they don't produce the same fragment masses. This extends the dynamic range and allows precise quantification of low level molecules.

2.1.2 Electrospray process - Quantifications

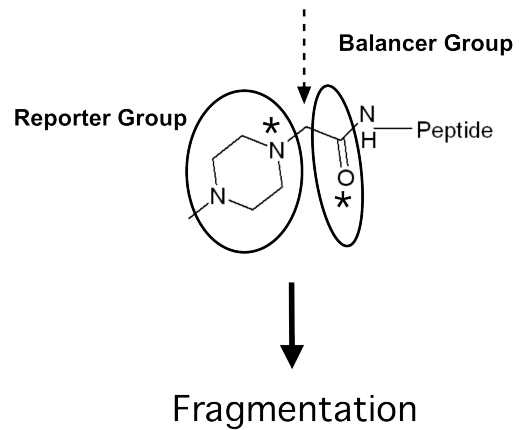
For an electrospray measurement, the most important parameter that determines whether ion intensities reflect molecular concentrations is how the spray is operated. If the spray is unsteady, ion intensities become irregular and do not reflect molecular concentrations. For stable electrospray ion sources, the spectral intensities correspond to analyte concentrations very well if the flow rate is on the order of 100 nL/minute or lower. For high flow rates above 1 $\mu\text{L}/\text{minute}$, hydrophobic molecules have a higher desolvation efficiency than hydrophilic ones and ion suppression effects can be pronounced [36] and thus the presence of hydrophobic molecules can prevent hydrophilic molecules from being detected.

The ionization mechanism of molecules larger than about 500 Da is responsible for this flow rate-dependent ion suppres-

sion. The desolvated ions that are required for the mass spectrometric analysis are generated exclusively from droplets that are smaller than 1 μm in diameter by a desolvation process in the transition between the atmospheric and the vacuum part of the mass spectrometer. For a high flow rate electrospray source, the majority of droplets generated directly by the spraying process are larger than 1 μm . Solvent evaporates from the droplets in flight but the charge is retained. This leads to such a high charge concentration that finally each droplet becomes a small electro-sprayer by itself and emits a series of much smaller droplets via the Taylor Cone mechanism which are the source of analytical ions [37]. These secondary droplets carry the majority of the available charge but a much smaller percentage of the total liquid. The remaining residual droplets do not contribute to analytical ions. Secondary droplets originate predominately from the surface of primary droplets [38, 39]. Hydrophobic molecules

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which have a high preference for the surface of primary droplets will be concentrated into the small secondary droplets, whereas hydrophilic molecules remain preferentially in the passive residual droplets. If the flow rate is well below 1 $\mu\text{l}/\text{minute}$, primary droplets are already so small that they generate the analytical ions directly. This means all molecules in the sample contribute equally to the ion beam and no ion suppression takes place [36]. Model calculations supported by experimental observations show that at a flow rate of about 20 nL/minute , absolutely no ion suppression or preferential desolvation of molecules takes place [36, 37, 40]. This flow rate-dependent desolvation effect is the reason there are two co-existing ways to quantify molecules with electrospray: relative quantitations with internal isotopic standards, or quantitations using the entire measured ion signal (the ion volume), without reference to a chemically identical isotopically-labeled molecule [32, 41-43].



Four reporter groups with different isotopes

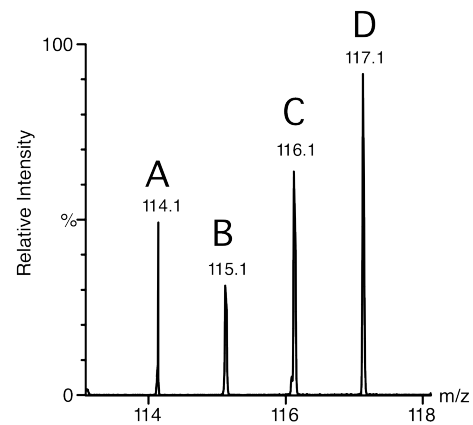


Figure 3 Quantification with the iTRAQ reagent. One of the specific advantages of the iTRAQ method is its ability to quantify several samples in a single LC-tandem MS run. The iTRAQ ligand is chemically linked to the N-terminus of peptides. It consists of two parts, the reporter group and the balancer group. The reporter group exists in four (and for a more recent variant in eight) different forms with different masses. The balancer group compensates the different weights so that all tags in all four forms have the same mass. The asterisk sign * in the figure indicates the elements that are present as different isotopes in the four forms. The four samples are each labeled with a different tag and then mixed. As such, every peptide peak in the spectrum contains contributions from the four samples. When such a peak is selected for fragmentation, the two groups fall apart as indicated with the dashed arrow and reveal the relative contributions of the four samples in the fragment spectrum.

2.2 Obtaining protein concentrations

For proteomic experiments, the amount of proteins has to be deduced from the measured peptide concentrations. There are two major factors which can lead to false measurements; firstly, the digest of proteins is often incomplete and secondly, a specific peptide might have been generated from several different proteins. There are ways to alleviate these problems but often attention has to be paid to specific experimental details [44]. One way to deal with the incomplete digestion of proteins by enzymes is to average the quantities

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of the three most abundant peptides of every protein [32]. It is often an acceptable assumption that some parts of the protein can be digested completely so that the most abundant peptides reflect the protein concentration. However, there are certainly cases where this assumption is wrong, for instance for small integral membrane proteins. Mixing unlabeled and isotopically-labeled proteins and digesting them together in one sample is the most reliable solution if isotopically-labeled reference material can be obtained [43].

Since all mass spectrometric quantifications are relative quantifications, attention has to be paid to when the standard is added, since losses of material can only be compensated thereafter. Complex biological experiments often involve protein enrichment procedures. If these enrichments are not quantitatively reproducible, protein-based standards must be used that undergo the same losses as the proteins of interest. To avoid choosing peptides for quantitation that were generated from several different proteins, sequence databanks have to be studied [44]. It is possible to choose so-called proteotypic peptides, which are peptides generated with high efficiency by digestion and that specify a single protein exclusively [45]. Databases are currently being set up that re-

fect these proteotypic peptides [46, 47]. For yeast, this database is based on extensive experimental data [46, 48]. The intention is to generate such a database for human proteins as well, even though this is a more complex endeavor because of the extent of alternative splicing [49].

3. Quantification methods

3.1 Isotope-based quantifications

Peptides of a control sample are isotopically labeled and quantitative information is derived by comparing the intensities of unlabelled to isotopically-labeled peptides. There is a large variety of isotope-based quantification protocols and the more popular are outlined in table 1.

3.2 Label-free quantifications

One of the early methods to determine relative protein abundance was the spectral counting method [50]. This method is based on the observation that the frequency with which ion trap mass spectrometers fragment a peptide is correlated to its quantity. In a strict sense, this is not a quantification method since no signal strength is used for deriving a

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Method	Description	Advantages	Disadvantages	Recommendation
<p>1) Isotope-based quantifications</p> <p>1. Absolute quantification by adding isotope labeled peptides (AQUA)</p>	<p>A peptide of interest is synthesized in an isotopically-labeled form and added to the protein digest at a known concentration. The concentration of the targeted peptide is determined by a signal comparison between the labeled and the unlabeled form or by comparing the intensity of the same fragment in a multiple reaction monitoring (MRM) experiment [33, 89].</p>	<p>It is an absolute quantification method.</p>	<p>For every peptide quantified an isotopically-labeled synthetic peptide of known concentration and good purity has to be obtained, which is expensive. No compensation for sample losses. The quantification is based on only one or two peptides per protein.</p>	<p>Use only in very targeted and very limited quantification experiments or as a singular control for less precise but generally applicable quantification regimes.</p>
<p>2. Absolute quantification by isotope labeled protein standards (PSAQ)</p>	<p>Proteins synthesized in a cell-free system and labeled with [¹³C₆, ¹⁵N₂] L-lysine and [¹³C₆, ¹⁵N₄] L-arginine are used as internal standards [73].</p>	<p>It is an absolute quantification method. Losses due to sample preparation or inhomogeneous protein digestion are compensated.</p>	<p>For every protein quantified, a standard protein has to be obtained.</p>	<p>Use only in very targeted and very limited quantification experiments or as a singular control for less precise but generally applicable quantification regimes.</p>
<p>3. Stable isotope labeling by amino acids in cell culture (SILAC)</p>	<p>A control group of cells is grown in medium where one or several amino acids are replaced by an isotopically labeled form [43]. Equal amounts of cellular material of the experimental and control group are mixed before any enrichment technique is performed. Protein ratios are determined by measuring the ratios of labeled to unlabeled peptide within any spectrum.</p>	<p>Quantifies changes occurring in the biological system Applicable for screening experiments Largely automated read out Any kind of sample preparation can be used Can be used as an absolute quantification method when labeled proteins at a known concentration are added (see above, 2.) [73, 80] Improved accuracy and sensitivity in combination with spiked protein standards and selected ion monitoring down to a level of 150 attomole (10,000 copies/cell in the cited experiment) [80]</p>	<p>Easily applicable only to cell cultures Can be expensive when used on large scale - but cheaper than any other method of this precision</p>	<p>Method of choice for cell culture-based experiments</p>

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Method	Description	Advantages	Disadvantages	Recommendation
4. Relative quantification by peptide based labeling				
a) ¹⁸ O labeling	A control group of proteins is digested in H ₂ ¹⁸ O with the effect that every cleaved peptide incorporates one ¹⁸ O isotope at its C-terminus. The labeled peptides are mixed with proteins digested in unlabelled water [86-88]	The labeling reaction is always complete The chemical reaction is simple	The mass shift of only 2 Da leads to partially overlapping isotopic envelopes between sample and control peptides Double incorporation of ¹⁸ O can occur	Use in simple small-scale experiments for a rapid quantitative measurement of few proteins
b) Dimethyl labeling	All primary amines, N-termini and lysines, of peptides are converted into di-methylamines introducing a variety of isotopes [76, 77].	The introduced mass shift is larger than 2 Da. The reaction is generally complete. Multiplexing of samples is possible.	The number of isotopes is sequence dependent which makes automatic readout difficult Basic groups are eliminated with the consequence that the charge states of peptides is changed	Use in simple small scale experiments for a rapid quantitative measurement of few proteins when multiplexing is required
5. Tag-based isotope labeling	Isotopes are introduced by linking a tag to proteins or peptides	Can be used for biological material from any source	For linking the tag quantitatively to its target residue, relatively extreme chemical conditions have to be chosen. The consequence is that undesired side reactions can occur	Use only for samples not generated from cell cultures
a) Isotope-coded protein labels (ICPL)	All free amino groups are labeled by N-nicotinoyloxy-succinimide in light or heavy form after the proteins have been reduced and alkylated [84]	The labeling reaction runs to completion Protein-based separation techniques can be used. No proteins or peptides are lost	Peptides are relatively large after tryptic digestion because all lysines are blocked The number of labels in a peptide is sequence dependent which complicates automatic read-outs	Use only if protein separation methods are to be used or iTRAQ is cost prohibitive

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Method	Description	Advantages	Disadvantages	Recommendation
<p>b) Isobaric Tags for Relative and Absolute Quantification (iTRAQ)</p>	<p>The iTRAQ tag is linked to the N-terminus of all peptides [31]. It is available in up to eight different forms which are all isobaric [82, 83]. The different isotopic forms become visible in the peptide fragment spectrum when the balancer group of the tag is fragmented off from its reporter group (figure 3)</p>	<p>The tagging reaction is complete without major side reactions. Up to eight different samples can be mixed (multiplexing). Automatic read out is simple since the reporter ions have a sequence independent fixed mass</p>	<p>Only fragmented peptides can be quantified Can become expensive in large-scale experiments</p>	<p>Use in large-scale, non-cell culture-based experiments when sample throughput and not cost is the major limitation.</p>
<p>ii) Label-free, direct quantifications</p>	<p>The total ion count of peptides in an electrospray experiment is used to derive its quantity using appropriate normalizations [32, 42]. Global instead of peptide-specific standards are used to normalize the measurements [32, 55, 81].</p>	<p>No chemical derivatization required No special cell culture medium required</p>	<p>Limited to electrospray investigations The lack of peptide specific normalization standards renders the measurement less robust to variable losses in sample preparations, variations in ionization efficiency or spray stability and generally increases the error margin. Quantities derived from only a single peptide might be unreliable</p>	<p>Method of choice for screening experiments only if all of the following criteria are met: - appropriate software available - stable electrospray ion source - stable chromatographic system - some experience with this method available before it is used on important projects - either sample preparation is on a quantitative level reproducible or variable losses can be compensated by an appropriate normalization - important findings will be confirmed by followup experiments</p>

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quantitative value, but rather a correlation-based estimate. Mass spectrometers should be operated in a mode to reduce the number of times a peptide is reselected for fragmentation to optimize the coverage of the expressed proteome. The alternative to spectral counting is to use the total ion intensity of the peptides. As software able to extract this total intensity becomes more available, the spectral counting method should no longer need to be used.

Label-free quantification is a very attractive method since it requires only minimal modification of the sample or experiment and is therefore the most compatible method for biological experiments (see table 1). To quantify peptides, their integrated electrospray ion signal is used (see figure 1) [32, 42]. An entire chromatographic run is normalized by using global internal standards instead of peptide-specific ones. To render direct quantification, robust attention has to be paid to the appropriate normalizations. Two levels of normalization can be considered: a technical normalization aimed at compensating long-term changes of the LC - electrospray - mass spectrometer system, and a sample-oriented normalization that focusses on the measured protein amounts within the biological system studied. A technical normalization can be a protein added before digestion or a set of peptides of known concentration whose elution times cover large parts of the HPLC gradient time. A sample-oriented normalization can be a protein whose concentration is assumed to be unaffected by the experimental conditions. Another possibility is to normalize the sum of all ion intensities which corresponds to the total amount of protein injected onto the HPLC column. An unbiased normalization can be obtained when comparing two samples. A normalization factor for abundance ratios of peptides can be obtained by calculating all intensity ratios of identical peptides and normalizing their median, the average change of all the peptides, to one.[42].

The error of the quantitative measurement will always be larger than the error determined with technical replicates measured with the same biological sample. The error depends on the sample complexity, the stability of the HPLC and the flow rate of the electrospray ion source. In an early study, the error was reported to be on the order of 25% for direct quantifications [42]. This is acceptable when considering that most biological effects manifest themselves by changes in abundance of more than 100% [63]. When building computer models that simulate biological systems, such an error margin is too large to use the data as a starting point for a dynamic process simulation.

3.3 Specific mass spectrometric scanning modes used for quantification

3.3.1 Multiple reaction monitoring (MRM) or selected reaction monitoring (SRM) scans

MRM scanning is a mass spectrometric acquisition method that allows very selective detection and quantification of targeted molecules in very complex mixtures (see figure 2 and table 2) [51, 52]. Its unique advantage is that molecules can be detected even when their signal is masked by other ions in the mass spectrum. A specific ion is detected by filtering all incoming ions for the m/z value of the targeted molecule and one or several of the expected fragment masses. Only molecules that comply with these two criteria, having the expected m/z value and producing fragments of the expected mass, will be detected. The disadvantage of this scanning mode is that it has to be established for every new targeted molecule. The database of proteotypic peptides will assist in this task. This database records peptides that uniquely identify proteins and some of their fragment masses that allow their specific detection with MRM scans. MRM scans can be very sensitive. In yeast, proteins can be detected and reliably quantified down to a level of 50 copies per cell (Aebersold, R., personal communication).

3.3.2 Parallel fragmentation (MSE)

Whereas MRM scans filter the data and record only the ions of interest, the MSE mode favors the opposite approach. All precursors and all fragments from all precursors are detected quasi simultaneously because the mass spectrometer switches continuously between fragmentation mode and MS mode (see figure 4 and table 2) [32, 53, 54]. All incoming precursors are fragmented in fragmentation mode without selecting an individual one. Fragments are related to their specific precursors by their identical time profile. This global fragmentation renders it currently the most complete analytical tool for proteomic investigations but it limits its sensitivity for individual low level molecules. In conventional tandem MS mode low level molecules can be better analyzed because once they are known the mass spectrometer can focus onto them and acquire their fragment spectrum for a long time period. This improves the detection sensitivity. Since the data set in MSE mode is close to complete, it can be interrogated for selected ions of any nature after the acquisition is completed in an MRM-like fashion [55].

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Method	Description	Advantages	Disadvantages	Recommendation
<p>III) Specific mass spectrometric scanning modes used for quantifications</p> <p>1. Multiple Reaction Monitoring (MRM) or Selected Reaction Monitoring (SRM)</p>	<p>MRM is a specific scanning method on mass spectrometers with two separate mass filters such as triple quadrupole machines (see figure 2) [51, 52]. MRM scans are used for the targeted detection and quantification of specific, well known ions. A MRM scan defines a set of precursor m/z values which it will search at specific retention times. For every m/z value the scan defines one or several expected fragment masses. Only ions meeting the set criteria for the intact molecule and its fragments will be detected (figure 2)</p>	<p>High specificity and sensitivity in the detection of targeted ions in a background of very complex mixtures</p> <p>The use of specific fragment ions often allows reliable quantification even in very complex mixtures</p>	<p>Can only be used in targeted mode (not a proteomic screening method)</p> <p>Every new ion requires establishment of its detection mode</p>	<p>Method of choice for the quantitative follow up of well-known molecules if their detection in a very complex mixture is limiting</p>
<p>2. Parallel fragmentation (MS^E)</p>	<p>MS^E is a scanning method on quadrupole time of flight instruments where all incoming precursors are fragmented in parallel. Fragment - precursor relationships are established retrospectively based on their identical time profiles (see figure 4) [32, 55, 81]. Quantification is achieved via direct, label-free quantification</p>	<p>The most global acquisition method; all precursors and all their fragments are covered</p> <p>Extensive post-acquisition data analysis possible as a MRM - like analysis [55]</p>	<p>Extensive post-acquisition data processing required</p> <p>No direct, manual data interpretation possible</p> <p>All associated software from data acquisition and data processing to protein identification and quantification has to come from the same supplier</p> <p>Primarily direct quantitation is used (not isotope-based quantitation)</p> <p>Smaller sensitivity for tandem MS analysis of low level precursors in comparison to a focussed conventional tandem MS experiment</p>	<p>Method of choice for proteomic screening investigations using direct quantitation (see above) if the following conditions are met:</p> <ul style="list-style-type: none"> - confidence in the ability to maintain an ultra high pressure chromatographic system (UPLC) - confidence in the ability to use direct quantitation in the experiments (see above)

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3.4 Comments on the use of statistical data evaluation in quantitative proteomics

The requirement for an explicit statistical evaluation of experimental data depends on the system studied. The focus of this review has been the measurement of relative or absolute protein concentration in a biological system using mass spectrometric tools. This measurement is based on signal

intensities. Generally each signal contains so many ions that a very large number of individual observations are the basis of the measurement. In this case a statistical evaluation is reduced to the average value and its standard deviation; for isotope-based quantifications this is around 10%, while for direct quantifications it can be up to 25% (see above).

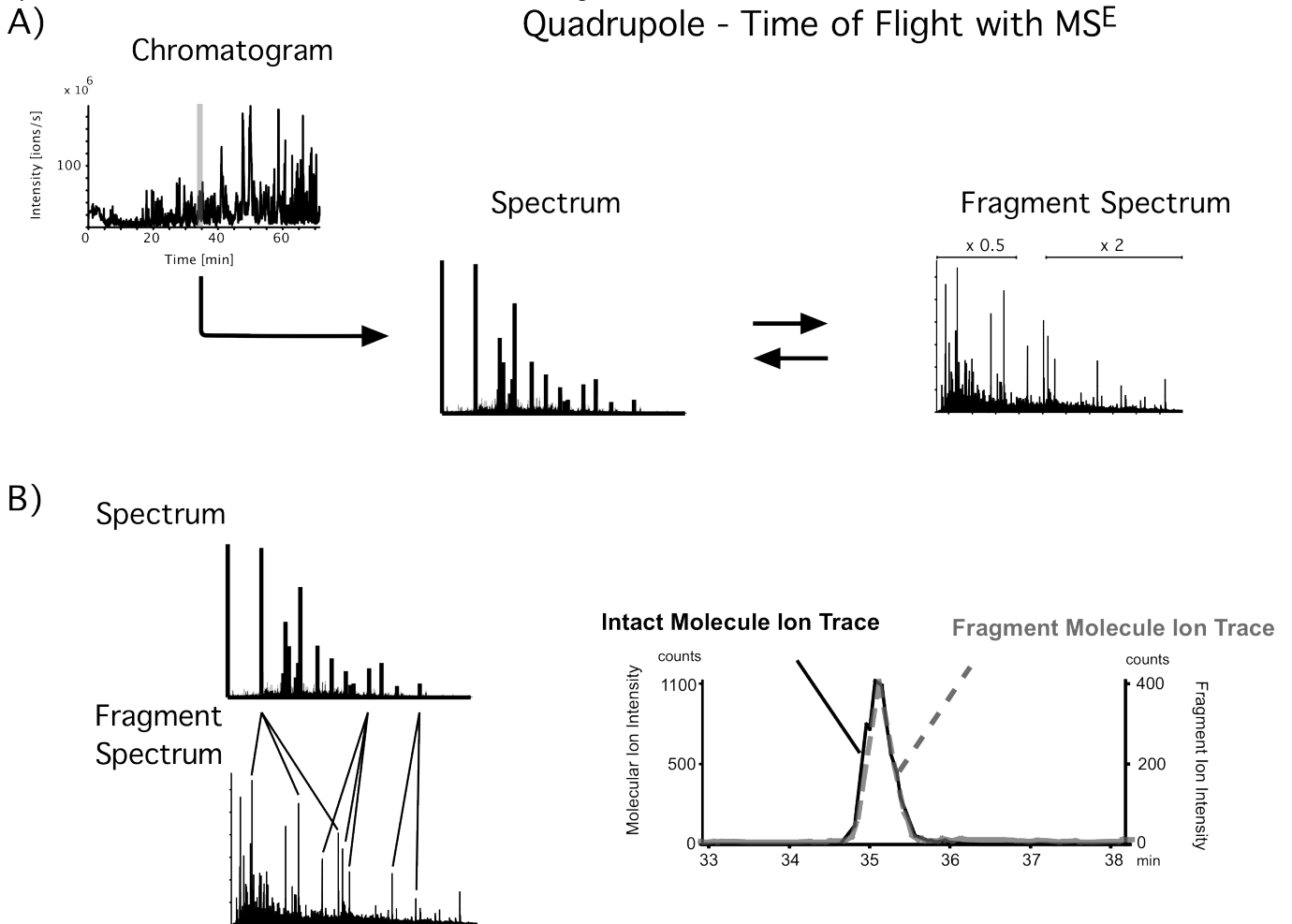


Figure 4 MSE scan. An MSE scan is the most complete mass spectrometric acquisition mode and records the maximum number of ions. Instead of selecting individual molecules for fragmentation the mass spectrometer switches continuously between MS and tandem MS (A). All fragments from all precursors are recorded simultaneously. The assignment of fragments to their precursor ions is done retrospectively by time-course correlation (B). All precursors are eluted from an HPLC column and show a time profile - their ion chromatogram. Fragments generated from a specific precursor show the same time profile. Together with a well-resolved chromatographic separation, fragments can be assigned to their respective precursors. Once the experiment is finished the data set can be interrogated for the presence of any specific ion that produced a particular set of fragment ions (pseudo - MRM scan). This software-based interrogation is not as specific as a real MRM scan. The assignment to their ion of origin might fail for fragments that are generated from several precursors simultaneously. However, the higher mass resolution of time of flight mass spectrometers probably compensates for this lack of clarity in most practical cases.

Often the scientific question is whether a protein is a member of a protein complex in a specific cell culture studied. The affinity to a bait protein measured by quantitative proteomics is an indirect, derived result [56]. Within single biological experiments, statistical methods are used to determine whether the affinity measurements of the protein in question are sig-

nificantly different from other proteins with low or no affinity. If the cell type is stable and two or three experimental replicates generate essentially the same result, it might not be necessary to use elaborate statistical data evaluation techniques. This can often be the case in yeast. When the measurement has only a limited reproducibility, statistical evalua-

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tion tools need to be used. This is typically the case when complex traits or mechanisms are observed in a highly-developed organism. In principle, this is not different from any other biological experiment of this type.

4. Applications

Yeast plays an important role as a model organism when applying new proteomic methods to biological questions. The systematic large-scale characterization of protein complexes is an example. It revealed the first functional organization of a reasonable part of the proteome beyond genomic analysis [8, 19, 20, 57, 58]. Critical for such research, either as a large-scale effort or in a more focussed way, is how the protein complexes are purified and characterized. After the success of the tandem affinity purification (TAP)-based protein complex enrichment in yeast, its principles could be successfully transferred to higher eukaryotes [15]. A further improvement was brought by quantitative mass spectrometry [18, 59-61]. Affinity purifications to a bait (antibody, protein, peptide, RNA or DNA molecule) are compared to an appropriate control in silico using the quantitative results from the mass spectrometric analysis. This allows enriched proteins to be filtered out from an overwhelming background of co-purified proteins. The technique was used to find the genuine protein components of a large RNA polymerase II pre-initiation complex in yeast and to analyze the compositional changes of protein complexes containing the transcription factor Ste12 in different states of the cell [18, 59]. In human cells it was used to explore the epidermal growth factor (EGF) receptor pathway by a comparative affinity enrichment to the SH2 domain of Grb2 in stimulated compared to unstimulated cells. The SH2 domain binds specifically to the activated, phosphorylated form of EGF. Amongst the 228 detected proteins, 28 were enriched following stimulation. Many proteins known to be involved in the EGF receptor pathway including Shc, Grb2 or AP-2 were identified in addition to 5 novel proteins not formerly associated with activated epidermal growth factor. Their close association to EGF was confirmed by confocal microscopy and FRET [61]. The relevance of using quantitative mass spectrometry to distinguish between true and false interactors is best demonstrated by the identification of Tfb5 as an essential component of the yeast RNA polymerase II pre-initiation complex [62]. A mutation in its human ortholog is responsible for one of the forms of trichothiodystrophy disorder [63]. Tfb5 was identified as a potential component of the RNA polymerase II pre-initiation complex based on only a two-fold increase compared to background proteins.

One of the standard techniques to purify protein complexes is to use an antibody against one of the components. Unlike

tag-based approaches, an antibody affinity purification does not require any further modification of the biological material. The disadvantages are that the column eluate often contains, in addition to true interactors, abundant antibody fragments, proteins binding nonspecifically to the hydrophobic column material, and proteins to which the antibody has cross-reactivity. All of these interfering proteins can be successfully filtered out when using quantitative mass spectrometry with an appropriate control. A good control sample can be obtained, for example, from a cell line whose endogenous target protein is suppressed by RNAi. Such a control allows not only nonspecifically-interacting proteins to be eliminated, but also proteins with cross-reactivity to the antibody (see figure 5) [56]. Interaction partners of β -catenin and Cbl, a protein involved in receptor-mediated endocytosis, were identified successfully using this method. In the case of β -catenin, 3 true interactors were filtered out from 140 identified proteins. Amongst the excluded proteins was γ -catenin, which likely cross-reacted to the β -catenin antibody [56].

The analytical capacity of mass spectrometers allows systems even larger than protein complexes to be studied. A new approach based on investigating the quantitative protein profiles has been used to overcome limitations in organelle purification [64]. In this method, cellular organelles are first separated using density gradient centrifugation. Various fractions are analyzed with the quantitative protein traces displayed against their location in the gradient. Organelles are characterized by the presence of specific marker proteins, and other proteins that show a similar profile are likely colocalized to the same organelle. Individual candidates can be confirmed by fluorescence tagging and light microscopy. The advantages of this technique are that several organelles can be characterized in parallel and that it is not necessary to purify them to homogeneity. Using this approach, novel proteins of the yeast peroxisome were found [65]. The protein content of human centrosomes was also successfully mapped [66]. When it is possible to purify a particular organelle, its dynamic changes under different experimental conditions can also be studied. For example, the composition of the nucleolus was analyzed under the influence of two different translation inhibitors and a proteasome inhibitor [67]. These experiments highlight a general concept for studying complex systems. The functionality of larger protein ensembles, like organelles or interwoven pathways, can often be better understood when the system is disturbed in a defined way and the cellular changes are observed more globally. Efforts towards characterizing every expressed protein by at least one peptide have to be seen in this context. The impressive ability to filter out relevant proteins by their quantitative traces might render a complete proteome analysis useful

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in many cases. Recently, close-to-complete proteome coverage was achieved by comparing haploid and diploid yeast [12]. 97.3% of the characterized proteins changed their expression level by less than 50% and only 192 proteins changed significantly. The top ten proteins were components of the pheromone pathway or transcriptional targets of pheromone signaling; pheromones are required in haploid yeast for mating and are absent from its diploid form.

It is still a considerable effort to characterize an entire proteome. To enable faster, targeted access to certain proteins without requiring affinity purifications, selected reaction monitoring (SRM or multiple reaction monitoring, MRM) scans can be used [45]. An SRM scan can very specifically detect and quantify a targeted peptide. If these peptides are proteotypic peptides (unique representatives of a particular protein) the protein is quantified. Recently a database of 1500 validated MRM transitions of proteotypic proteins from yeast has been published [48]. Algorithms have also been made available to predict proteotypic peptides in proteins from other organisms [46].

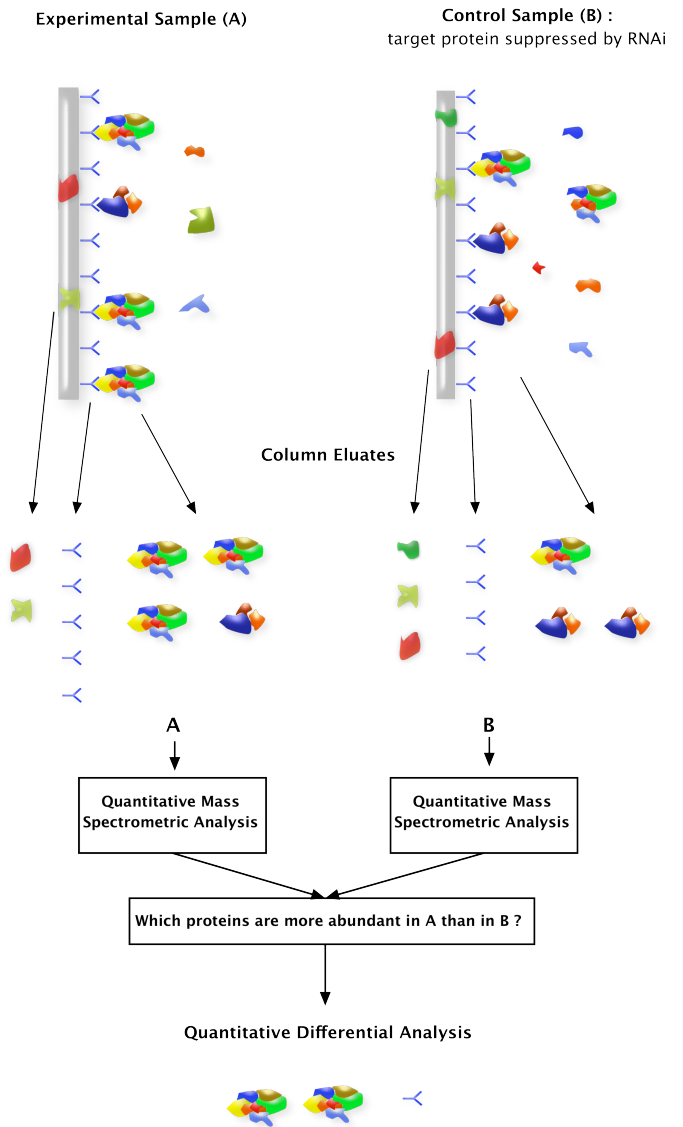


Figure 5 Immunoprecipitation of protein complexes with differential quantitative mass spectrometry. A protein complex is purified using an immobilised antibody against one of its components. The same type of column is used for the control. In the control experiment, the targeted protein has been suppressed by RNAi. Proteins eluted from the columns are identified and quantified. Only proteins that are more abundant in the experimental sample than in the control are considered to be members of the protein complex. In this way even proteins cross-reacting to the antibody can be filtered out [56]. In this example, the large protein complex assembled around the target protein (the yellow protein) is eluted in larger quantities from the experimental column (A) than from the column exposed to the sample where the targeted protein has been suppressed (B). In the control (B) the smaller protein complex of the cross-reacting protein is of higher abundance in the eluate. By comparing mass spectrometric data of the two eluates, proteins belonging to the targeted protein complex can be recognized because they are more abundant in the eluate from the experimental column.

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For systematically characterizing secondary modifications like phosphorylation, modification-specific enrichment techniques are required. Phosphorylated peptides can be enriched with strong cation exchange chromatography and metal affinity purifications such as IMAC or TiO₂. This technique was used to investigate the response of haploid yeast cells to pheromone stimulation [68]; of 700 sequenced phosphopeptides, 139 were differentially regulated upon pheromone exposure and related to downstream processes like growth stimulation, transcriptional regulation and cell cycle control. Even though not all phosphorylated peptides present in the cell can be detected, such an experiment gives a good view of the complexity of cellular processes initiated by pheromone stimulation. The experiences gained in this yeast experiment opened the way to its application in human cells. The processes caused by exposure to epithelial growth factor (EGF) in HeLa cells were followed over time [16, 69]; 6600 different phosphorylation sites on 2244 proteins were detected, quantified and mapped over 5 time points covering 20 minutes after stimulation. Following the quantitative phosphorylation pattern over time allowed the grouping of the phosphorylation sites into 6 classes. Proteins whose phosphorylation level increased were grouped into early, intermediate, late and terminal responders. Proteins whose phosphorylation level decreased were divided into early and late negative responders. An interesting result of this study was that two phosphorylation sites in the same protein can be regulated differently, demonstrating that a single protein can have multiple roles in signaling cascades. This classification together with assigning the identified proteins to different pathways using ontology databases gave a good functional overview of global cellular activity induced by EGF stimulation.

4. Concluding remarks

The introduction of quantitative mass spectrometry has led to significant research in molecular biology, mostly based on biochemical experiments in cell cultures. Interaction screens of individual peptides, sophisticated protein complex characterization, the parallel analysis of cellular organelles, the comparative observation of entire proteomes and a global mapping of signaling cascades have been achieved. In all of these experiments it was possible to filter out relevant proteins from a large set of identified ones by their quantitative signature using appropriate controls. It is this specificity that supports the expectation that the direct observation of proteomes generates clearer results than monitoring mRNA levels. For obtaining functional insight it is necessary to design experiments appropriately. Detailed follow-up experiments on individual proteins are often required. But the level to which

quantitative proteomics can see through the myriad of proteins and identify relevant ones is impressive. So far, the expansion of this research has been hampered by the limited availability of software that is able to analyze large amounts of primary data, but with companies focussing on quantitative proteomics and scientists making their programs publicly available, the context becomes more favorable to do this kind of research in biological laboratories [70-72].

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