MALDI Mass Spectrometry for Quantitative Proteomics – Approaches, Scopes and Limitations

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Abstract. The determination of absolute protein amounts and the quantification of differentially expressed proteins belong to the most important goals in proteomics. Despite being one of the key technologies for the identification of proteins, the application of matrix assisted laser desorption/ionization (MALDI) mass spectrometry (MS) for quantitative analyses is hampered by several inherent factors. The goal of the present paper is to outline these difficulties but also to present some selected approaches which enable MALDI MS to be used for the quantification of biomolecules. In particular, methods for the improvement of the homogeneity of MALDI samples and the use of internal standards for the relative quantification are discussed. Strategies for *in-vivo* and *in-vitro* labelling of peptides and proteins with stable isotopes are presented. The need for guidelines for the presentation and evaluation of data as well as for bioinformatical approaches for the interpretation of quantitative data will be addressed.

1 Introduction

The goal of proteomic analyses is to describe phenotypical changes in an organism, induced by environmental factors or natural processes, on the level of proteins. The proteome itself has been defined as the quantitative image of the complete set of proteins in an organism or a cell at a distinct point of time under defined conditions [1]. Therefore, along with the identification of proteins, the gain of quantitative information about protein expression is one of the key subjects.
The classical workflow in proteome analysis involves the isolation of the proteome or a subproteome from an organism, the separation of the proteins by means of electrophoretic or chromatographic methods and the identification and quantification of the proteins. Further steps involve the characterization of the proteins, e.g. the identification of posttranslational modifications, the determination of the activity or

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the function of the proteins as well as the elucidation of protein-protein- and protein-ligand interactions. Identification, characterization and quantification can be performed either on the level of intact proteins or on the level of peptides formed by selective cleavage of the target proteins.
Quantification comes in two forms: (i) absolute and (ii) relative quantification. In case of absolute quantification, the amount or the concentration of protein e.g. in a cell or a solution is the subject of interest. Absolute quantifications can be performed by using internal standards or after measurement of a calibration curve with known amounts of the particular analyte. A typical example for an absolute quantification is the determination of biomarkers in blood plasma. In a relative quantification the amounts or concentrations of proteins within two cell states are compared. This differential analysis is the upmost frequently followed strategy of quantification in proteomic experiments. Examples include the comparison of the expression profiles between healthy and ill cells, the comparison between a wildtype and a mutant strain of an organism, the protein profile of a bacterium grown on different media or the comparison of the effect of different drugs on a cell culture.

2 MALDI Mass Spectrometry

2.1 General Principle

A concise description of the principles of MALDI MS would go far beyond the scope of this article. Briefly, the analyte is mixed with a high excess (up to more than 20.000:1 (mol:mol)) of a low molecular weight matrix. Most matrices are organic, aromatic compounds which are able to absorb the ultraviolet laser light applied in most MALDI instruments. Numberless matrices suited for the analysis of different biomolecules have been tested. The matrix-analyte-mixture is then applied on a metal plate (target) where the solvent evaporates, leaving back a matrix-analyte cocrystallite. The target with this cocrystallite is brought into the high vacuum of the mass spectrometer. Pulsed laser shots (pulse lengths in the ns range) are applied on the cocrystallite. This results both in desorption into the gas phase (plume) and in ionization of the analyte. In most cases, protons are the charge carriers leading to the formation of $[M+H]^+$ or $[M-H]^-$ analyte ions. Thus, in contrast to electrospray (ESI) MS, mainly singly charged ions are formed. The ions are accelerated in an electric field and then separated according to their mass-to-charge (m/z) ratio. The most commonly used mass analyzer for MALDI MS is the time of flight (ToF) analyzer.
Due to its (moderate) tolerance against salts, the high speed of analysis and the comparably simple spectra caused by the formation of the singly charged ions, MALDI MS is nowadays the most popular technique for the identification and characterization of proteins on the level of peptides (peptide mass fingerprint analysis).

2.2 Sample Homogeneity and Quantification in MALDI MS

The cocrystallization of the analyte with the matrix is the prerequisite for smooth ionization of biomolecules, but is on the other hand the main reason for the problems encountered with the use of MALDI MS for quantitative purposes. A major issue is the non-homogeneous distribution of the analyte in the cocrystallite. This leads to the observation that at several points of the sample no analyte signals can be found whereas at other positions strong signals can be monitored (hot-spot formation). The phenomenon of hot-spot formation cannot be predicted and is dependent on the physicochemical properties (e.g. hydrophobicity, polarity, H-bond-formation potential) of the analyte, the matrix and the solvent used for the sample preparation. The varying ion response on different positions on the sample spots leads to poor spot-to-spot and shot-to-shot reproducibilities and is therefore one of the main reasons hampering quantitative MALDI MS. Caused by the inhomogeneous distribution of the analyte, the laser energy/fluence has to be adjusted from position to position on the target, which also contributes to the nonlinear ion response. Additionally, secondary ion reactions in the plume formed after desorption/ionization, ion suppression effects observed in complex mixtures and instrumental parameters (mainly the phenomenon of detector saturation outlined below) complicate quantitative application of MALDI MS.

Despite these hampering factors, quantification by MALDI MS is possible by (i) a proper choice of the matrix used, (ii) application of optimized measurement protocols [2] (iii) the decomplexing of samples by means of separation methods (e.g. LC-MALDI-coupling), (iv) the application of internal standards, and (v) the use of methods for the improvement of sample homogeneity. The two later issues will be addressed in this article.

Ideal internal standards are substances showing a high physicochemical similarity to the analyte. Standards labelled with stable isotopes like ^{2}H, ^{13}C, ^{14}N or ^{18}O deliver best results in terms of accuracy of the quantification [2, 3]. Another strategy is the use of non-isotope labelled internal standards [4] applying structurally modified compounds. For example, peptides with high molecular similarity [5] were successfully used as internal standards for the relative quantitation of peptides.

3 Strategies for Quantitative Proteome Analysis

3.1 General Classification

Generally one can distinguish between three main strategies for quantitative proteome analysis: (i) methods for gel based quantification, (ii) intensity based quantification and (iii) the use of stable isotope labelled compounds [6]. The later strategy will be briefly reviewed in this article. It has to be mentioned, that most of the methods presented here are not restricted for quantification by MALDI MS but are also suited for studies based on ESI MS. Stable isotopes can be introduced in the analysis either by spiking of labelled internal standards as described above or by *in vivo*- or *in-vitro*-labelling.

3.2 In-vivo labelling

The technique of *in-vivo* labelling of proteins is based on the incorporation of stable isotopes by protein biosynthesis. The most commonly used technique is the so called protein metabolic labelling (Fig. 1) [7, 8]. Hereby, metabolic precursors containing stable isotopes are used to feed the organism. By cell growth and protein turnover, the stable isotopes are incorporated in all proteins, replacing after a while all unlabelled proteins. After combination of the cells, the combined proteomes are digested and analyzed together by means of mass spectrometry.

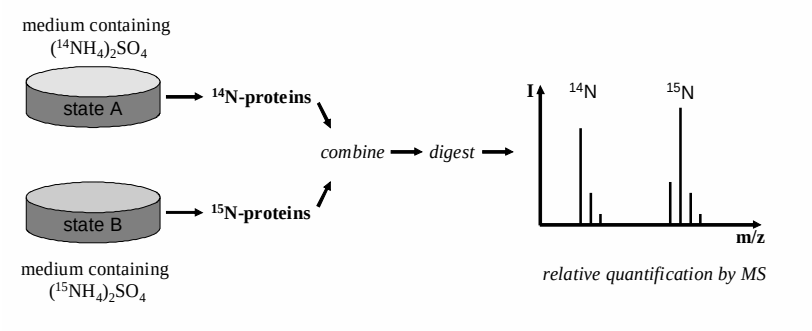

Fig. 1. Differential analysis of protein expression by protein metabolic labelling

A commonly used isotope is ^{15}N, which can be incorporated in many cases in form of inorganic salts contained in the medium. Due to higher costs, ^{13}C-containing precursors are used to some lesser extent for protein labelling. The main advantage of this method is the lack of side-reactions. The method is ideally suited for fast growing cell cultures, e.g. for bacterial strains. Unfortunately, this form of *in-vivo* labelling is difficult to apply on mammalian cells or tissues and rarely applicable in medical investigations.
A closely related strategy for the incorporation of stable isotopes by biosynthetic pathways is the SILAC (stable isotope labelling by amino acids in cell culture) method [9]. Here, the organism is fed with an isotope-labelled amino acid, which cannot be synthesized by the organism itself (essential amino acids). This method has been applied for a number of studies also on human cell cultures [6].

3.3 In-vitro labelling

The second main strategy is the incorporation of stable isotopes into peptides (after digestion of the proteins) or intact proteins either by chemical modification or enzymatically catalyzed derivatization. One can distinguish between reactions modifying (i) the N-terminus as well as lysine side chains (amino-specific labelling), (ii) the carboxy terminus as well as the side chains of glutamic and aspartic acid residues (carboxy-specific labelling) and (iii) residue specific labelling reactions. A

large number of methods for these modifications has been presented, therefore only an incomplete description of some selected methods can be given here.

3.3.1 Amino-specific labelling

A wide range of reaction principles is available for the derivatization of amino groups. The most commonly used method in proteomics is the acetylation by d_0- or d_3-acetic acid, thus leading to a light (hydrogenated) or a heavy (deuterated) derivative. The activation of the acetyl group can be achieved for example by standard N-hydroxysuccinimide (NHS) chemistry, which leads to high yields of derivatization under smooth conditions. In dependence of the number n of amino groups present in the peptides, mass differences of $\Delta m = 3n$ are introduced by this method. A special case of quantification is realized in the so called iTRAQ- (isobaric tag for relative and absolute quantification) method [10]. Here, quantification is performed on the level of MS/MS-fragments. The derivatizing reagent consists of three groups. The first is a NHS-activated amino reactive moiety, which is linked to a balance and a reporter group. The total mass of reporter and balance is 145 Da, with the reporter group being available with masses from 114 through 117 Da; the corresponding balance groups thus posses masses of 31 to 28 Da. Thus, two to a maximum of four proteomes can be derivatized with these reagents. After combination of the proteomes followed by digestion and separation, e.g. by means of liquid chromatography, equal peptides from the different proteomes are isobaric. This leads to relatively simple spectra interpretation. Upon fragmentation by MS/MS, the linkages between the reporter, the balance and the reactive group are fragmented. The resulting fragments of the reporters at m/z 114, 115, 116 and 117 can now easily be used for the relative quantification.

3.3.2 Carboxy-specific labelling

The carboxyl groups at the C-terminus and the side chains of Asp- and Glu-residues can be derivatized by esterification with d_0/d_3-methanol/hydrochloric acid [11]. This procedure, which is frequently also applied for the masking of carboxyl groups prior to IMAC (immobilized metal affinity chromatography) enrichment of phosphorylated peptides, proceeds within 2 h. A drawback is the necessity for water free conditions, which can lead to loss of peptides adsorbed at the reaction vessel during evaporation of the water.

A step involved in all proteome analyses applying the bottom-up approach is the enzyme-catalyzed site-specific hydrolysis of peptide bonds by proteases. During this reaction, water is formally added to the peptide bond. This approach can be used for relative quantification by performing the hydrolysis of cell state A in $H_2{}^{16}O$, whereas in cell state B $H_2{}^{18}O$ is used as solvent. The mass difference between the peptides formed in both states is +2 or +4 [12, 13], which can be used for relative quantification by MS. A major problem is the small mass difference resulting in case of incorporation of only one oxygen atom. This endangers a potential overlap of the signal of the heavy version of the peptide with the isotope peaks of the light version.

In order to reduce this problem, two sets of experiments can be performed, in the first incorporating ^{18}O in cell state A, in the second set in cell state B (inverse labelling).

3.3.3 Residue-specific labelling

Amongst the side chain functionalities present in the 21 proteinogenic amino acids, the thiol group in cysteine is one of the most reactive groups. In order to prevent unspecific disulfide formation out of cysteine residues during digestion of proteins, cysteine thiol groups have to be blocked prior to digestion, for example by alkylation with iodacetic acid or iodacetamide. The same chemistry can also be used for differential tagging. This principle has been realized in the so called ICAT (isotope coded affinity tag) approach [14]. The ICAT reagent consists of three main parts, the Cys-reactive group, a linker molecule, available in light or heavy form (realized by incorporation of hydrogen or deuterium) and an affinity group (biotin). This latter group is used to enrich the labelled peptides by affinity chromatography applying streptavidin or concanavallin columns. Proteome of cell state A and B can be labelled with the light or heavy version of the ICAT-reagent, respectively. After combination of both labelled proteomes followed by digestion, the labelled peptides are purified by affinity chromatography. The resulting peptides are then relative quantified in MS. Newer derivatives of the reagent replace the H/D-labelling by $^{12}C/^{13}C$-labelled linkers, thus avoiding isotope effects of deuterium in chromatographic separations. Numerous modifications of this basic principle have been presented in the literature (e.g. solid phase ICAT [15], ALICE [16]). An intrinsic problem of the method is the fact, that only peptides containing Cys-residues can be covered.

4 Data Interpretation, Dynamic Range and Accuracy

The incorporation of stable isotopes for differential quantification of protein expression profiles poses challenges for data interpretation, especially for the interpretation of mass spectra. Together with the mass shift induced by the labelling, the natural isotope patterns can be changed (Fig. 2). In case of protein metabolic labelling, the number of incorporated ^{15}N-atoms can facilitate the identification of a peptide, but the assignment and interpretation of the mass spectra can be complicated. This is especially true for the assignment of correct pairs of light and heavy peptides in complex mixtures. Commercial programs handling these problems are not yet available.

Further problems can originate from overlapping isotopes resulting from partially labelled peptides. This phenomenon can occur both in protein metabolic labelling caused by incomplete turnover of the protein pool as well as by incomplete derivatization reactions in the *in-vitro* methods. If the mass difference between the light and the heavy version of a peptide is too small, overlap of the heavy peptide signals with isotope peaks of the light version can occur, thus leading to potential quantification errors. In chemical derivatization procedures, unwanted side-reactions can additionally contribute to these problems.

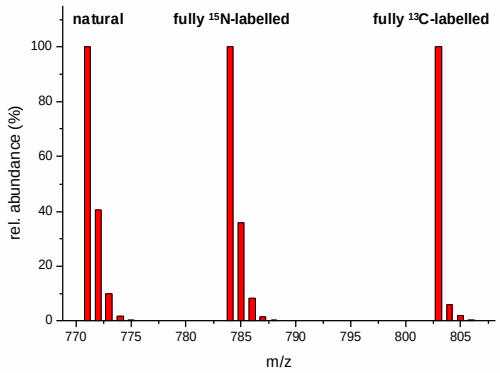

Fig. 2. Simulated isotope pattern for the non-protonated heptapeptide Leu-Arg-Arg-Ala-Ser-Leu-Gly. Natural- (left), fully ^{13}C-labelled- (middle) and fully ^{15}N-labelled isotope pattern (right)

A key parameter for the design of quantification experiments is the dynamic range which can be expected in a biological system. From mRNA-data it is known that the expression of many proteins does only change in the range between 1.5 – 5 fold, but in other cases changes can exceed easily more than 50 fold depending on the situation of the organism. For the interpretation of quantitative data in a biological environment, it is essential to bear in mind, that the amount of a protein expressed in a cell does not necessarily tell anything about both its functions and/or its activity. In addition to the effects directly related to the change of protein expression, protein activities can be triggered by posttranslational modifications, protein-protein- or protein-ligand-interactions or by the localization of the protein in a cell.

From the analytical point of view, the dynamic range of the quantification methods is of high significance. For MALDI MS, several factors originating from the ionization mechanism(s) and from instrumental setup (e.g. the kind of detector used) influence this dynamic range. In a differential analysis with a high excess of one analyte over the comparant, it is important to prevent detector saturation of the high abundant species and, on the other hand, gaining a signal above the ion threshold for the low abundant analyte. The later factor is strongly related to the limit of detection of the analytes. It is possible to enhance the signal of the low abundant species by increasing the laser energy/fluence. This in turn can lead to the saturation of the high abundant species and can further cause peak broadening and partial in-source decay of the analytes. As a rule of thumb, a range of relative signal intensities of 1:10 to 10:1 (analyte:internal standard) can be covered by standard MALDI MS.

An issue for each analytical method is the achievable accuracy. At this point it has to be mentioned, that not only the mass spectrometric quantification has to be taken into account in this discussion. In fact, the complete workflow of proteome analysis, ranging from the isolation of the proteome to data interpretation, posses numberless sources for experimental errors. Particularly critical is the performance of parallel steps, which can lead to differential errors thus preventing accurate differential analysis (Fig. 3, adapted from [6]).

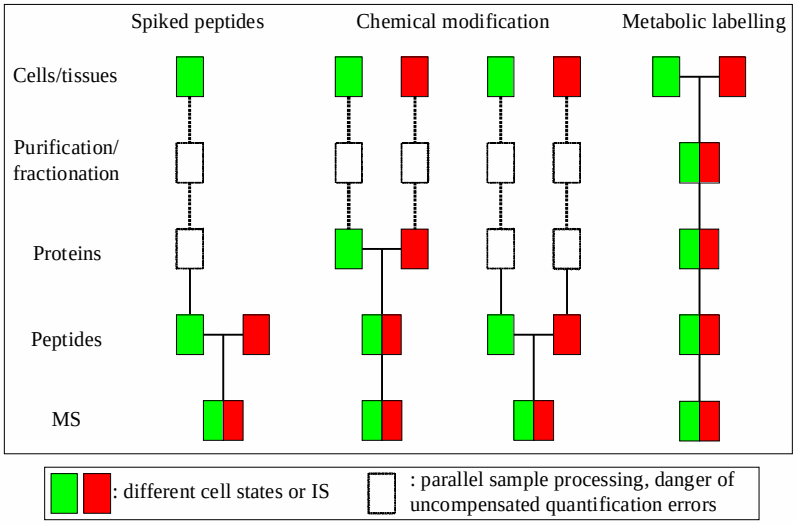

Fig. 3. Parallel processing can lead to uncompensated errors. Vertical lines represent stages where samples are pooled. Figure adapted from [6]

A wide variety of numbers annotating the accuracy of the one or the other method can be found in the literature. Unfortunately, these numbers mostly refer to a single step, e.g. for the relative quantification by MS itself, which prevents the presentation of reliable data at this point. For the relative MS quantification (both applying internal standards or by differential analysis) average errors around 5 % have been reported for most of the methods presented above.

5 Approaches for increased sample homogeneities facilitating quantitative MALDI MS

As outlined above, inhomogenous distribution of the analyte within the matrix-analyte cocrystallite is one of the key factors hampering quantitative MALDI MS. A groundbraking approach for the improvement of sample homogeneity was the introduction of the ionic liquid matrices (ILM) by Armstrong and coworkers [17]. These ILM are mixtures of classically used MALDI matrices like a-cyano-4-hydroxy-cinnamic acid (CCA) or 2,5-dihydroxy benzoic acid (DHB) with equimolar amounts of organic bases. Many of these ILM form highly viscous layers on the MALDI target. The ILM have been successfully applied for the measurement of peptides, proteins and polymers [17], oligonucleotides [18], low molecular weight compounds like amino acids and sugars [19-21] and phospholipids [22]. Due to the high sample homogeneity achievable, these matrices enable an improved relative quantification of amino acids [19], sugars [20] and peptides, small proteins and oligonucleotides [23] applying suited internal standards. In a narrow dynamic range and under defined molar matrix-to-analyte ratios these matrices can also allow a quantification of peptides without the use of internal standards [24]. Unfortunately, these narrow limits

together with the need for defined molar matrix-to-analyte ratios prevent the applicability of this approach for proteome analysis. An application of this approach lies in the functional analysis of proteins, e.g. for the determination of enzymatic activities. Nevertheless, the high sample homogeneity provided by the ionic liquid matrices may lead to a facilitated quantification by means of the methods outlined above in the future. First studies showed, that some of the ILMs can also improve qualitative analysis of protein digests thus facilitating the identification of proteins by peptide mass fingerprint analysis [25].

6 Outlook

A number of approaches for the use of MALDI MS for quantitative purposes are available. Nevertheless, further improvements both in sample homogeneity as well as in sensitivity have to be achieved in the future.
The use of MALDI MS for the analysis of intact proteins is hampered by low resolution for high molecular weight compounds resulting from adduct formation and neutral losses (water, ammonia) during ionization processes. Therefore other methods, in particular electrospray MS, will gain further interest in the future for quantification in top-down approaches [26].
As already addressed for the identification of proteins in proteomics by several initiatives (e.g. by the proteome standard initiative (PSI)), there is also an urgent need for guidelines both for the reporting of quantitative data as well as for the evaluation of the methods applied. Further, open platforms allowing the interlaboratory exchange of mass spectrometric data are urgently needed. The contribution of the suppliers of the mass spectrometers in the establishment of such platforms is necessary.
Isolated data about expression of proteins alone are not sufficient to solve biological problems. For a system based understanding of biological processes there is an ongoing need for the correlation and combination of these data with those obtained in genome, transcriptome and metabolome analysis.

7 References

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