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Review

Isotope dilution mass spectrometry for absolute quantification in proteomics: Concepts and strategies

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ABSTRACT

Isotope dilution mass spectrometry is a reference technique for quantitative analysis, given that it combines the sensitivity and selectivity of MS instruments with the precision and accuracy associated with the use of internal standards. Isotope-labeled proteins are the optimal internal standards for quantitative proteomics as they closely mimic the behavior of their natural counterparts during the analytical process. A major complication of isotope dilution mass spectrometry proteomics is the technical difficulty of obtaining these internal standards, especially in studies where a high number of proteins have to be quantified simultaneously. In this paper, we review some of the characteristics of the isotope dilution mass spectrometry approach, its benefits in terms of reliability and quality control in targeted proteomic analysis and the different strategies developed for its application in proteomics.

Contents

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1. Introduction

Quality control procedures such as those related to method validation and data traceability are well implemented in clinical, environmental and drug development laboratories [1–11]. With the development of new protein biomarkers for approval by regulatory agencies, there is the need to develop analytical methods to provide protein absolute quantitative data of controlled accuracy and precision [12–14]. Isotope dilution mass spectrometry (IDMS) is the optimum analytical technique to provide reliable MS-derived absolute quantitative data [15–17]. IDMS involves the addition to the sample of an isotopically labeled compound, the internal standard (IS), followed by the simultaneous determination of both the analyte and IS by mass spectrometry. IS measurements are used to neutralize changes in the analytical performance over time and between laboratories affecting the analyte values. Typically, quantification-oriented LC-MS methods use selected MRM (multiple-reaction monitoring) transitions to obtain optimum selectivity, sensitivity and precision in the detection and quantification of analytes and internal standards [12,18].

In the past, quantification of larger proteins using IDMS has been limited by a combination of technical problems that have complicated the retrieval of accurate and reproducible absolute quantitative concentration values. The technological achievements of the last decade have solved many of these limitations, and it is currently feasible to use IDMS for the absolute quantification of a number of proteins in biological samples [19–22]. Problems still exist, however, for its application on proteome wide experiments, namely the availability of standards for each of the proteins analyzed and the confinement of the approach to targeted experiments. In addition, the process of synthesizing and purifying isotopically labeled proteins for their use as IS can be expensive both in terms of economic costs and of method development time [13,15,23]. As a consequence, along the years, several alternative strategies have been developed for protein quantification. These approaches include methods which have been reported to produce absolute quantification data of reasonably good precision without the use of internal standard normalization [24] as well as other well-known approaches using isotopic labelling (e.g. ICAT, iTRAQ, TMT, SILAC) which have been extensively used over the last 15 years to quantify relative differences between a limited number of samples [25–29]. For example, Zhang et al. [24] performed a reevaluation of the raw data from an inter-calibration exercise [30] and calculated that the variability without IS still remained around the acceptable limit for a quantitative method (CV 20–30%, the CV of the original study was ca. 10%). The authors concluded that absolute quantification without IS provided cost-effective alternatives to IDMS for many multiplexed MRM LC-MS applications [24]. Along the same line, a growing number of reports are presenting label-free approaches as viable alternatives to isotopic labeling for relative quantitation [28,31–39].

Quantification without IS has however important drawbacks. Any LC-MS-based analytical method contains a number of critical points that may lead to biased results, such as LC column deterioration, changes in MS response factor over time, low analyte recovery, matrix effects, and human errors [12,30]. Without IS, the number of failed runs related to unpredictable events is liable to increase dramatically, depending on the long-term stability of the analytical setup [42]. These problems are well-recognized in most fields that have been using MS for quantitative purposes for decades [1,8,42–47]. The absence of IS must therefore be compensated by implementing procedures to minimize possible sources of errors, with consequent costs in terms of time and performance. Even if these problems are conveniently considered and neutralized, possible matrix effects can produce biases (systematic or random) that cannot be detected nor compensated without IS [12,48,49].

Thus, in comparison to other approaches, IDMS methods hold the potential to provide absolute quantification data of the highest precision and of controlled accuracy. The characteristics of IDMS quantification have made this approach the basis for many Definitive and Reference methods in Clinical Chemistry. The correct application of the IDMS implies that quantification results will be traceable to a common (i.e. international) reference standard, a condition which allows the comparison of the results from an unlimited number of experiments as well as inter-laboratory/inter-method comparison [40]. In this respect, the implementation of quality control procedures that ensure the accuracy and precision of the quantitative data is a basic requirement for successful data comparison [41].

2. General concepts in quantitative mass spectrometry

The comparison of different datasets cannot be reliably performed unless there is the certainty that varying laboratory conditions (different analysis date, laboratory, instrumental setup and reagents) as well as variability in the sample matrix have not introduced biases into the quantitative data [5,41,50]. To achieve this goal, both calibration standards and analytical procedures are required to be technically appropriate and validated [41,51] (Fig. 1).

The calibration standards are samples of controlled composition that allow the transformation of the intensity

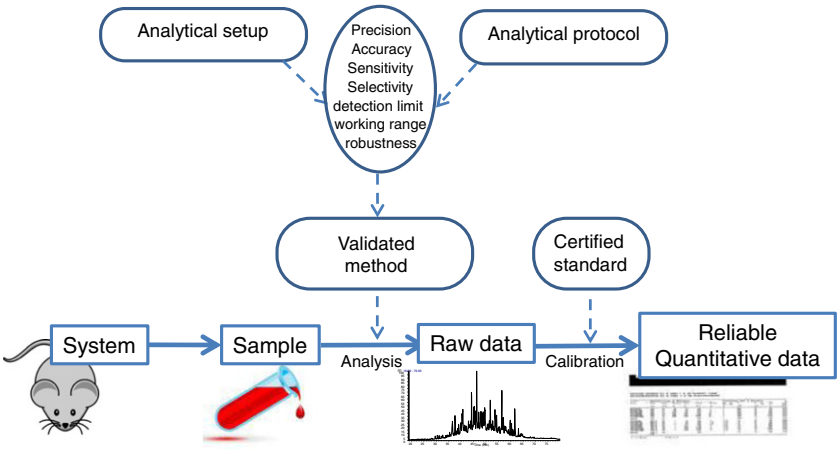

Fig. 1 – Steps involved in the quantitative analysis of biological samples. To ensure the accuracy and precision of the quantitative data, the performance of the analytical setup and protocols must be validated. This includes determination of parameters such as precision, accuracy, sensitivity, detection and quantitation limits, and working range. The use of certified standards for calibration is also essential to ensure accuracy.

signal into absolute concentration values (Fig. 1). Optimally, calibration standards should be samples of concentration certified by dedicated organizations (such as the National Institute of Standard and Technology or the Institute for Reference Materials and Measurements) [5,10,50]. In the absence of these reference materials, the use of spiked samples with pure analyte standards (of controlled purity) is an alternative to obtain reliable calibration standards [5].

The validation of an analytical protocol is a procedure that ensures the reliability of the results produced by the method within the defined range of applicability [5,10,11]. This validation process should always be considered as an essential part of the method setup [11,41] (Fig. 1). The endpoints of a validation process are estimates of parameters such as the precision, accuracy, sensitivity, detection and quantification limits, and selectivity of the analytical method under defined conditions [5,10]. Optimally, these parameters should meet the requirements previously established to address specific technical or scientific questions [51].

2.1. *Precision*

The precision of a method refers to the variability observed between independent quantitative values obtained under defined conditions. Precision is measured by performing repeated analyses of the same test sample under the same conditions (repeatability), or under varying conditions (reproducibility) such as, different analytical runs, laboratories, and analysts. The precision of a method is usually expressed in terms of standard deviation (σ) normalized by the mean value of a series of measurements (μ), and expressed as the coefficient of variation (CV = σ/μ). It is important to stress that precision refers to the dispersion of results and not to their accuracy [52].

2.2. *Accuracy*

When the repeatability of an analytical method is demonstrated, the results obtained under the same conditions may be compared. However, the comparability of results obtained in different conditions requires the determination of the accuracy of the analytical method. However, the comparability of results obtained in different conditions is difficult to demonstrate unless the accuracy of the analytical methods is demonstrated. The accuracy of a method refers to the agreement between the result of a measurement and the true value of the measure [52], and it is estimated by calculating the difference between the true value and the measured value.

Ideally, accuracy is ensured by using a national or international reference standard, which defines the true value, to calibrate the analytical method (Fig. 1) [5,50]. The traceability of the analytical results to common primary standards allows for the reliable and accurate comparison of results obtained in different conditions, independently of their origin or the method used to produce them [3,50]. Traceability in this context is defined as *the property of a result or measurement whereby it can be related to appropriate standards, generally international or national standards, through an unbroken chain of comparisons* [52]. In practice, the concept of traceability involves many aspects of the analytical procedure related with the assurance of the quality of the analytical results such as the selection of adequate references and standards or the characterization of all possible factors of variability. In clinical chemistry, traceability is a requirement for the assessment of threshold values used in the diagnosis of disease, avoiding potential misclassification of disease risk, or the administration of inappropriate intervention strategies or therapeutics for the patient [53]. Calibrators of all commercial clinical tests marketed within the European Union must demonstrate traceability to higher-order standards, if available [54]. Ideally, the highest order (primary) standard in the traceability chain is defined using a primary method, such as “exact matching” isotope dilution mass spectrometry [55].

2.3. *Sensitivity, limit of blank and limit of detection*

According to IUPAC [52], the sensitivity of a method refers to the change in response of a method produced by a concentration

change in the analyte, i.e., the slope of the analytical calibration curve in Fig. 2. According to this, a method has good sensitivity when a small change in analyte concentration produces a large signal change (high response factor). This official definition of sensitivity is in conflict with other definitions that relate sensitivity to the limit of detection (LOD) of a method [52,56].

The LOD can be defined as the lowest analyte concentration that the analytical procedure can reliably differentiate from background noise [57]. The definition of LOD is closely related to that of the limit of blank (LOB), that is, the highest apparent concentration measured from a blank sample devoid of analyte [58].

The simplest method for LOD estimation is based on the replicate measurement of a blank sample. LOD is then set to the mean value of the measurements plus 2 to 10 standard deviations [58,59]-. The weakness of this approach is that it does not take into account the particular response of the analyte. Alternatively, the LOD can be estimated from the measured average and standard deviation of the LOB (μ_B and σ_B, respectively) and the standard deviation obtained for samples spiked with a small amount of analyte (σ_S). Assuming a Gaussian distribution of the data and a 95% confidence interval [58,60]:

$$\mathrm{LOD} = \mu_B + 1.645\sigma_B + 1.645\sigma_S$$

This estimated LOD should be confirmed experimentally by adding a known amount of standard to the sample and confirming that the signal recovered can be differentiated from the blank [58].

A frequent situation in the analysis of biological samples is the presence of an endogenous non-zero analyte level in the blank samples. Blank samples can then be prepared by immunoaffinity depletion or enzymatic degradation of the endogenous compounds, or using samples from individuals negative for the target analyte [60]. Alternatively LOD can be determined by monitoring the response of isotope-labeled analogs of the analyte. Whiteaker et al. used this approach in a multiplexed SISCAPA-MS analysis to eliminate the interference from endogenous amounts of the target proteins and to determine detection limits and range of response of the method [59]. In these cases, it is also possible to determine the amount of the endogenous compound using the standard addition method. This method consists on adding variable amounts of analyte to different aliquots of a sample, and determining the LC-MS signal for each standard addition. A calibration curve is then calculated and the endogenous analyte can be inferred by the zero intercept of the regression line [61].

2.4. *Lower limit of quantification (LLOQ)*

The LLOQ of a method is defined as the concentration value below which stated acceptable precision and accuracy cannot be ensured [52,58]. As for LOD, there are several different methods for the determination of LLOQ [57,62]. LLOQ can be determined on the basis of experimental precision and accuracy data. A common approach is to determine the precision obtained in the quantification of the analyte at different concentration levels. Then, LLOQ is set at the concentration where a predefined precision is obtained (i.e. CV = 20%) [58]. Another approach is based on the determination of the signal-to-noise ratio (S/N) where signal is defined as the height of the analyte peak and noise as the amplitude of the baseline around the analyte peak. For LLOQ, S/N usually is required to be greater than 10 [57]. LLOQ can also be estimated by calculating, using a calibration curve, the concentration that would produce a signal corresponding to n times the standard deviation of the baseline noise in a blank sample [57,62]. Also in this case, a common value for n is 10. Still another common approach to

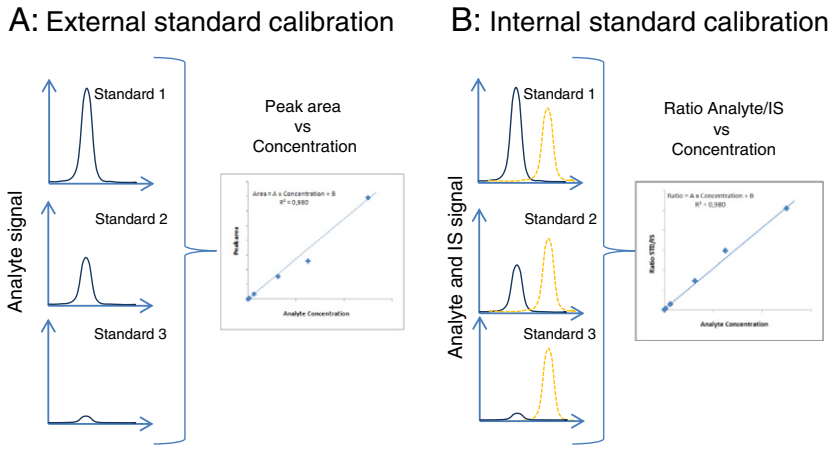

Fig. 2 – Scheme of LC-MS calibration using external standard (left) and internal standard (right). External standard calibration makes use of a series of calibration standard samples with different analyte concentration to build a calibration curve. External calibration correlates analyte signal intensity (blue traces) in the calibration standards with quantitative values in absolute units. In internal standard calibration, a calibration standard, the IS (red traces), is added in the same amount to the different calibration standard samples (and to the unknown samples). Internal standard calibration correlates analyte to IS signal ratio with quantitative values, normalizing the analyte response with that of the IS. Note that, in IDMS, the signals of the analyte and the IS would co-elute in the LC-MS chromatogram.

report LLOQ is setting its value as a multiple of the LOD value (i.e. 3 fold) [61]. The use of instrumental signal-to-noise ratios for LLOQ calculation ignores the variability associated with sample processing [63]. This is especially relevant in trace analysis (such as in proteomics), where quantitative recoveries can be difficult to achieve [21,22,64]. For this reason, protocols and guidelines published by regulatory agencies recommend the first method described above, based on experimental precision of the analyte measurements, for LLOQ determination [2,4,60].

According to these definitions, a sensitive method does not necessarily have a low LOD or LLOQ. This is the case for many quantitative methods based on enzymatic reactions, which typically produce sigmoidal calibration curves with good response factors only for relatively small concentration ranges. Outside these optimal concentration ranges, the signal change is relatively insensitive to concentration variation (low sensitivity). The LLOQ of enzymatic methods is therefore the concentration threshold below which the method becomes insensitive to concentration change.

2.5. *Selectivity*

The selectivity of an analytical method refers to the extent to which other substances interfere with the determination of the analyte [52]. The results provided by an analytical method with good selectivity should not be affected by variability in the biological matrix (e.g., individual plasma samples, anticoagulant used to stabilize the sample). It is obvious that the detector should be insensitive to other compounds present in the sample in order to achieve good selectivity. However, even with a very selective detector such as LC-MS/MS, analytical output can be indirectly affected by changes in sample matrix via influencing detector response, analyte recovery, analyte stability, or the performance of the instrumental setup [12,27,48].

3. Isotopic dilution mass spectrometry and analytical quality control

The signal intensity provided by LC-MS systems depends on multiple factors, some of which are difficult to identify and control (i.e., analyte structure, LC flow, solvent composition and gradient, MS internal conditions and adjustment, spray conditions). Therefore, the response factor of an LC-MS system (defined as the signal intensity measured by unit of analyte mass) is highly variable over time and between LC-MS systems. As a consequence, the LC-MS signal cannot be directly translated into mass or concentration values. To provide reliable absolute quantitative data, these methods require the use of calibration standards and quality controls (QC).

3.1. *Calibration*

The main methods of choice for calibration in LC-MS are external and internal calibration (Fig. 2). Methods based on external calibration (Fig. 2A) determine absolute quantitative data by direct comparison of the LC-MS signal intensity of the analyte in the sample with that obtained for calibration standards in separate analyses. The accuracy and precision of the quantitative data obtained using external calibration is based on the assumption that LC-MS response is the same for the analytes in the calibration and in the problem samples. In LC-MS analysis, a typical source of error with external calibration is due to the matrix effect [12,42,48,49]. The matrix effect is defined as changes in the response factor of an LC-MS system caused by the interfering compounds in the sample extract [49]. The matrix effect can be greatly reduced by incorporating appropriate sample cleanup procedures before instrumental analysis [48]. However, a common situation in trace analysis is that complete elimination of the matrix effect is not possible, or else that it requires complex treatments that make the analytical procedure impractical. Therefore, methods based on external standard calibration should only be used in situations in which these errors are expected to be low during sample preparation and instrumental analysis.

The precision of a quantitative measurement is optimized by the use of an IS for method calibration (Fig. 2B) [12,15,16,65]. Internal calibration is based on the addition of a constant quantity of labeled standard to all analyzed standards and samples. During the analysis, both the analyte and the IS signals are monitored, with the ratio between their intensities being proportional to the concentration of the analyte. In optimal conditions, the ratios measured will be independent of the variability over time, in the response factor of the MS instruments, matrix effects, errors in solution volume, and variable analyte recovery [12,42,48,49]. For this, three main requirements should be met. First, the IS should have identical physicochemical characteristics as the analyte because it compensates for all possible losses during sample isolation and all other steps of the analytical process [17]. Second, the samples should be devoid of this compound to avoid deviations in the measurement of the IS signal. The third condition is that the signal of the IS and the analyte must be resolved to minimize biases in the measurement of their signal ratios [17]. The best approximation to these requirements is the isotope dilution method (ID), which uses an isotopically-labeled form of the analyte as IS. Isotope-labeled analogs should be preferred to structural or chemical analogs as internal standards [17,66]. The latter may have small differences in their physicochemical properties that could result, for example, in differential recovery during sample processing and produce significant quantification errors [12]. Deuterated forms of a compound are also not perfect IS because there is a small separation of the deuterium analog IS from their endogenous forms during chromatography. Thus, both deuterated and chemical analogs, can show slight differences in chromatography between IS and the analyte that can result in differential suppression or enhancement of ionization (matrix effect) and affect the quality of the analytical data [12,66].

Addition of IS in the initial steps of the sample preparation is essential because it eliminates procedural errors and allows quantitative results to be independent of analyte recovery (Fig. 4). Optimally, IS should be added to the sample before extraction, allowing an adequate equilibration of the standard and the sample analyte to reduce the effect of variable-extraction recoveries [15]. Even in this case, the

internal standard may not correct for possible recovery problems of analyte molecules that are irreversibly adsorbed or absorbed into the sample matrix [15].

IDMS methods used for the analysis of trace organic compounds in complex matrices typically yield results with uncertainty and deviation values below 20%. In cases where high precision and accuracy are called for, quantitative error can be reduced to values below 2% using the double-exact matching procedure [67]. This is a two-step analytical process. The first step involves a crude initial estimation of analyte concentration with a relatively high error using a normal IDMS procedure. This crude estimate is used to produce a standard of similar concentration to the sample. The error is reduced in a second analysis by comparing the analyte/IS ratios of the sample and the standard [55]. This procedure is commonly used in the certification of reference materials. These approaches require strict control of all error sources that affect the precision and accuracy of the measurement, such as sample instability, isotopic impurity, standard impurity, and measurement uncertainties [67,68].

3.2. *Quality control*

Quality control procedures are required to ensure that the results obtained in an analytical run meet the expected requirements of precision and accuracy. A common procedure for quality control involves the analysis of a combination of calibration samples and quality control (QC) samples. QC samples are processed and analyzed together with the samples of unknown concentration to be measured (Fig. 3). QC and calibration samples are independent sets of samples of known composition (optimally reference materials). The calibration samples are used to generate a calibration curve that correlates intensity ratios (analyte/IS) and analyte concentration values (Fig. 3, middle). The concentration of the QC samples is experimentally calculated using this calibration curve and compared with the known, expected value (Fig. 3, top). The error obtained in the measurement of the QC samples is used to determine whether or not the quantitative data obtained in the measurements of samples meet the established acceptance criteria (Fig. 3, bottom). In regulated environments, QC values are reported with the quantitative results to demonstrate their validity [2,4]. This validation procedure assumes equal analytical performance for standards and unknown samples. Therefore, the problem of adequacy of the standards as a model to quantify real samples is usually addressed during method development and validation [2,4].

A second procedure to control the validity of quantitative measurements is the use of a surrogate analyte (SA) [69–71]. Typically the SA is an isotopically labeled compound that can be added to the sample together with the internal standard. The LC-MS method is designed to measure the signal intensity for the three compounds, that is, the analyte, the IS, and the SA. The IS is used to quantify both the analyte and the SA. Since IS and SA are added always with the same mass ratio, measured deviations from this expected ratio provide an estimate of the deviation in the quantification of the analyte. Therefore, the purpose of SA is to monitor method performance within each measurement [72], and is not used to calculate analyte concentration [69]. This SA strategy should not be confused with the concept of surrogate peptides as proxies for the quantification of protein often used in protein quantification [73]. Quality Control using surrogate analytes is commonly used in environmental analysis to calculate analyte recovery [70,71], but as far as we know there are no reports on its use in proteomics.

4. Targeted quantitative protein analysis

The high molecular mass of proteins represents a challenge for most MS systems that give optimum response in the limited range of 100–2000 m/z. On the other hand, the loss in fragmentation efficiency with the increase in molecular mass has been classically a limitation for intact protein sequencing [74,75]. Recently, developments on Fourier-Transform-based high resolution MS have made the analysis of intact protein isoforms using top-down approaches possible [74,76]. For example, Kelleher's group have demonstrated the increasing ability of these approaches to identify and characterize tens/hundreds of proteins and to provide extensive coverage of specific biological networks [77,78].

Despite these advances, top-down studies usually focus on protein structure confirmation or PTM description and are limited to samples with simple protein composition [76,79–81]. One of the major difficulties found in this respect is the fact that proteins are very heterogeneous, with different solvent requirements for solubility and maintenance of their natural structure. It is therefore technically difficult to design a protocol of universal validity to quantify intact proteins [79].

Currently, technical problems related to processing intact proteins are commonly addressed by hydrolyzing proteins to smaller peptide sequences, followed by quantification of the released peptides (bottom-up proteomics). The trade-offs of this approach are both the increased complexity of peptide digests relative to the original sample and the problem of inferring protein quantitative values from the experimental peptide-centric data [82]. The use of proteolytic enzymes to quantify proteins using IDMS was first proposed in 1996 by Barr et al. [83]. These authors synthesized deuterium-labeled peptides, corresponding to their native counterparts formed by proteolysis, to quantify the apolipoprotein A-1 content of a reference standard, with good precision (CV < 4%).

The use of tryptic peptides to quantify proteins requires the selection of peptides ensuring reliability of the quantitative output. These so-called proteotypic peptides should meet several requirements needed for quantitative purposes, which are 1) to be detectable and to provide a good response factor; 2) to be selective of the target protein (unique peptide); and 3) to be chemically stable (i.e. containing no methionine or cysteine) [84–86]. Another property desirable for proteotypic peptides is not to be affected by single amino acid polymorphisms, PTMs, or alternative splicing [85]. Selection of peptides from parts of the protein subject to sequential variability will exclude the quantitative results from all other protein variants. This specificity can be used to quantify the active center of the protein and exclude isoforms of low biological activity from the analysis.

A number of bioinformatics tools have been developed to address the problem of proteotypic-peptide selection [87].

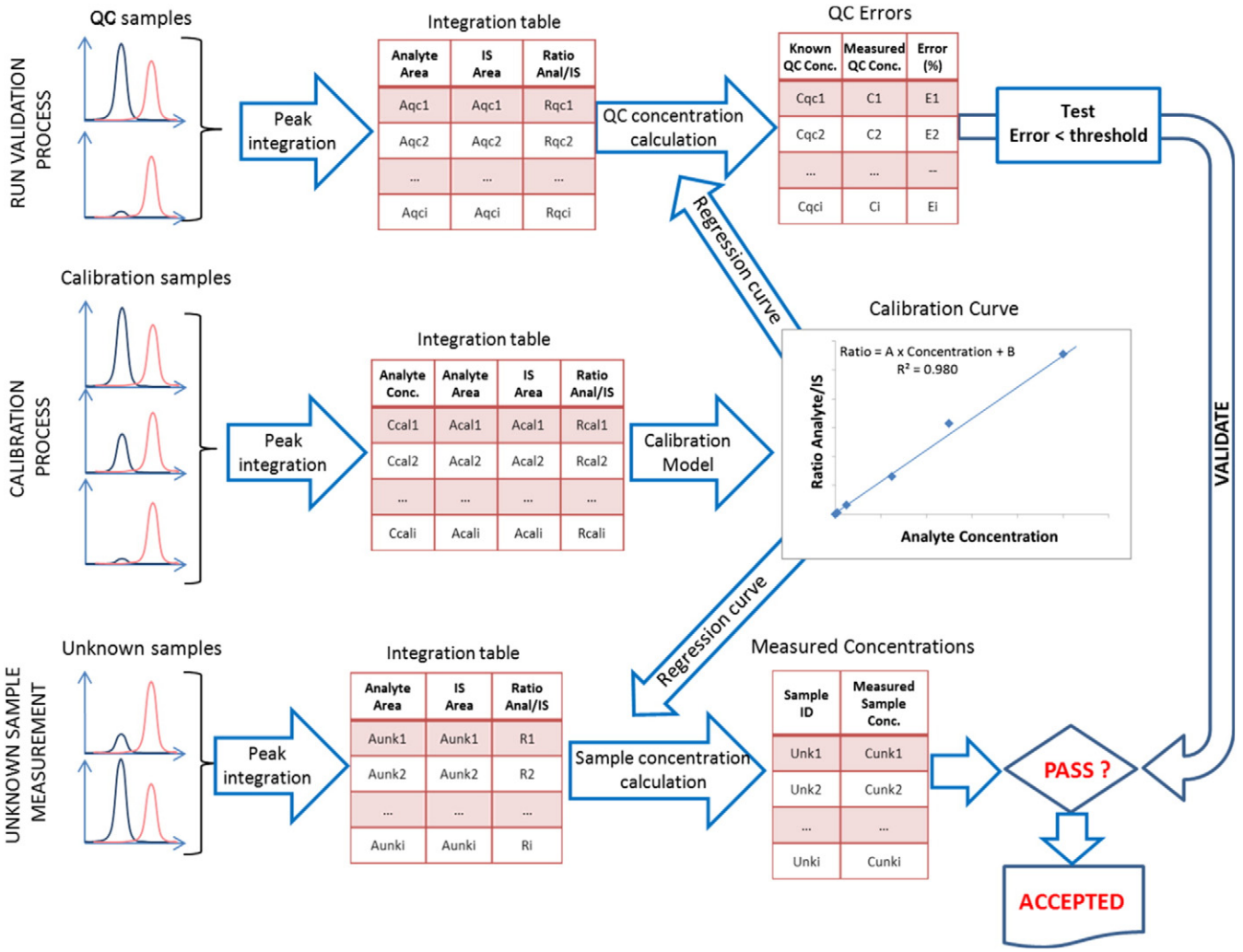

Fig. 3 – The quantification of analytes in a set of samples involves three steps: Calibration of the system using calibration samples (middle), a Run Validation based on quality controls to determine if the full set will be accepted (top) and the sample analysis itself (bottom). Calculation of the analyte concentration in QC and unknown samples is carried out using the calibration curve.

Some of these tools rely on the intrinsic physicochemical properties of the peptides to predict their potential as proteotypic [86,88]. Other tools make use of available experimental MS data of peptides. This involves the collection and validation of MS/MS spectra and their storage in public databases from which potential proteotypic peptides can be selected [84,89–92]. This approach benefits from the vast amount of experimental data obtained by the scientific community, but is of limited use in the case of less studied proteomes [85].

In practice, these bioinformatics tools have certain limitations when setting up a quantitative method for specific protein targets. The optimum instrumental conditions of triple quadrupole instruments used for sensitive targeted analysis strongly depend on the peptide sequence, charge of the precursor ion, and the instrument used. Good sensitivity can therefore only be ensured after careful instrument optimization with standards [93]. Furthermore, the "detectability" of a peptide strongly depends on the chromatographic setup [88]. Thus, there is some uncertainty surrounding the applicability of predictors calibrated using different instrumental setups. Bioinformatics tools are of use in situations where protein detection has to be achieved without the assistance of a standard. In situations where analyte-containing samples and standards are available, the selection in real conditions of the optimum peptide for protein quantification provides higher-quality results [93].

4.1. *Methodological aspects*

The need to select and validate reliable biomarkers among the high number of candidates produced in discovery studies has boosted development of new sensitive and reliable quantitative methods for the simultaneous analysis of many proteins using IDMS methods [26,87,94,95]. Nowadays, methods based on IDMS can quantify proteins in biological samples with LLOQ at the low ng/mL or pg/mL concentration level with good precision (CV < 20%, Table 1). Typical analytical protocols include an extraction step followed by purification/fractionation at the protein/peptide level, trypsin digestion,

Table 1 – MRM-based publications reporting information about accuracy and precision of protein quantitative values.

Year	Reference	Sample matrix	Analyte	IDMS Standard	Sample processing	LLOQ protein	Protein recovery	Precision (CV%)	Accuracy assessment
2007	Keshnishian et al. [73]	Plasma	6 proteins	Peptides	Depletion + SCX	1–10 ng/mL	50–100%	<15%	NA
2008	Heudi et al. [64]	Serum	Antibody	1 Protein	SPE	5000 ng/mL	14%	<20%	92–100 %
2009	Kuzyk et al. [133]	Plasma	45 proteins	Peptides	SPE	16–168000 amol on column	NA	5–25%	NA
2009[a]	Addona et al. [30]	Plasma	7 proteins (2 μg/mL)	Peptides	SPE	300–17000 ng/mL	0–93%	10–50%	NA
2011[b]	Zhang et al. [24]	Plasma	7 proteins (2 μg/mL)	No IS 1 peptide	SPE	–	NA	20–30%	–
2011	Tang et al. [134]	Serum	Multiplexed biomarker validation	No IS	Depletion + Gel fractionation	0.2 ng/mL	NA	12–32%	NA
2011	Chenau et al. [139]	Milk and soil	*Bacillus anthracis* spores	Peptides	Immunocapture	7000 spores/mg soil	NA	3–5%	3–5 %
2012	Domansky et al. [93]	Plasma	67 proteins	Peptides	SPE	5–1000 ng/mL	NA	<20%	Up to 100-fold-change depending on peptide
2012[a]	Kuhn et al. [21]	Plasma	8 proteins S100B	Peptides 1 Protein	SISCAPA	<1 ng/mL	NA	11–14% <5%	44–54% 95–101%
2012	Chen et al. [135]	Urine	63 proteins	Peptides	Ultrafiltration	0.5–12000 ng/mL	NA	<50%	NA
2012	Huillet et al. [14]	Serum	Troponin I, LDH-B, CKMB, Myoglobin	Proteins	Depletion + Gel fractionation	500 ng/mL	NA	8%	65-118%
			Troponin I		Immunoenrichment	5.5 ng/mL		3%	95%
2012	Hoofnagle et al. [22]	Plasma	6 Apolipoproteins	Peptides 1 Protein	Ultracentrifugation of HDL	–	NA	<11,5% <12.8%	NA
2012	Yu et al. [136]	Serum	Transferrin	Peptide	SPE	500 ng/mL	83–88%,	5–9%	5.4 % bias from QC
2013	Rezeli et al. [137]	Plasma	11 proteins	Peptides	Deplection	4–500 amol on column	–	3%	–
2013	Simicevic et al. [138]	Mouse pre-adipocytes	9 proteins	Peptides Proteins	SDS-PAGE	–	0.5–93%	<30%	–

[a] Multilaboratory intercomparison studies for which accuracy and precision were obtained considering all participant laboratories.
[b] A reevaluation of the raw data of the multilaboratory study reported by Addona et al.

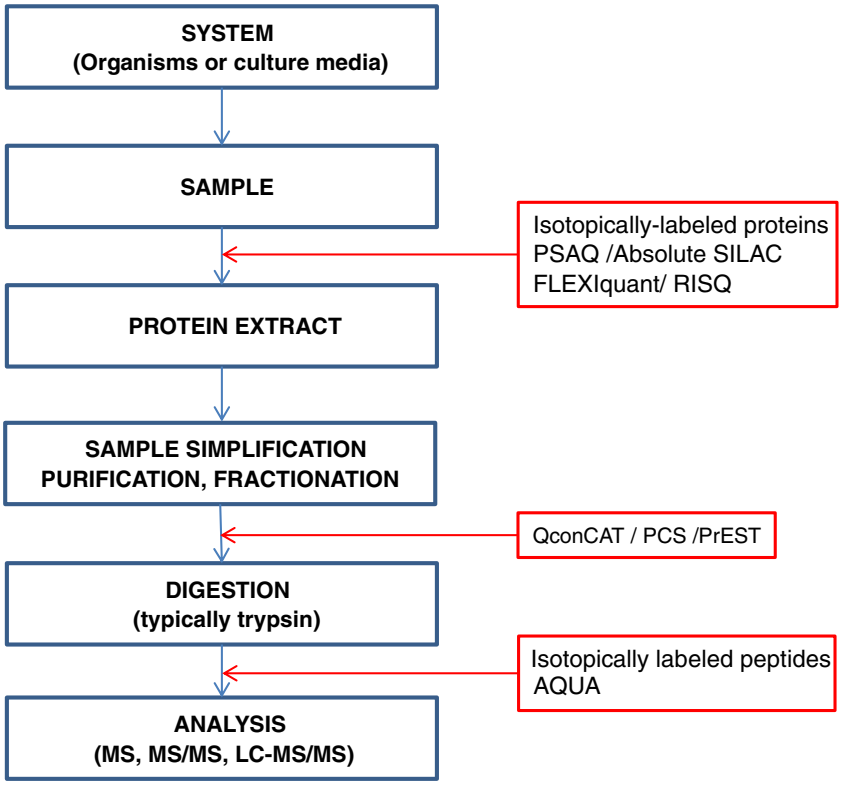

Fig. 4 – Generic proteomic analytical workflow and points of addition of different IS for IDMS. Protein IS should be added as soon as possible in the analytical process as procedural biases produced before the addition of the IS are not corrected for. QconCAT concatamers and other similar constructs are polypeptide sequences different from the natural target proteins. These polypeptides can be loss or show different recovery than the target proteins during sample prefractionation or purification and, thus, they should be added after these steps.

and instrumental analysis (Fig. 4). The sensitivity and selectivity of these analytical methods depend on both the use of appropriate sample preparation procedure and on LC-MS performance. [24,26,94,95].

4.1.1. *Sample preparation*

Since plasma (or serum) is the most commonly used biological sample in clinical applications, efforts to quantify plasma proteins have led to a constant improvement on plasma-oriented preparation methods [95]. Protein-concentration values in plasma range from about 40 mg/mL to pg/mL values (10–11 orders of magnitude). The analytical complexity involved in the quantification of plasma proteins dramatically increases with decreasing concentration levels. Initial quantitative approaches without sample fractionation produced methods able to quantify the most abundant proteins down to sub-μgram/mL concentration level [30,96]. Procedures to reduce sample complexity have been shown to reduce detection limits down to ng/mL level [30,64,96] or even pg/mL level [61].

Immunodepletion of the most abundant protein in plasma samples was among the first techniques used for sample-complexity reduction [96,97]. A number of immunodepletion systems are commercially available and their stability and reproducibility has been demonstrated [98]. Methods based on immunodepletion combined with fractionation using SDS gel electrophoresis, SCX, or SEC chromatography reported LLOQ values within the concentration ranges of 10–100 ng/mL [61,73,97,99].

A more specific approach for analyte enrichment, SISCAPA (Stable Isotope Standards and Capture by Anti-Peptide Antibodies), was introduced by Anderson et al. in 2004 [100]. SISCAPA uses immobilized anti-peptide polyclonal rabbit antibodies to capture and subsequently elute the target peptides and the labeled internal standards for MS quantification. This approach provided an average 120-fold enrichment of the antigen peptides relative to other non-antigen peptides and a LLOQ of 40 ng/mL [100]. SISCAPA, however, depends on individual antibodies for each target peptide resulting in a relatively high reagent cost for MRM assay development [18]. On the other hand, the LOD and LOQ of the assay can be affected by a low capture efficiency of the antibody or by possible antibody saturation effects due to cross-reactivity with peptide epitopes from other highly abundant proteins in the sample [21].

Despite these drawbacks, the remarkable sensitivity and specificity of this analytical approach and its potential for high-throughput analyses have stimulated intense technical development. The throughput and multiplexing capability of the method described by Anderson was enhanced by using a magnetic-bead-based platform that enabled automated analysis using 96-well plates [59,101]. This new platform was reported to provide signal enhancements of three orders of

magnitude with good precision and LOQ at the low ng/mL level [101]. In 2010, Whiteaker et al. [59] described an automated magnetic-bead-based platform for high-throughput sample processing, which was implemented in a multiplexed SISCAPA assay (nine target peptides in one assay). Good method precision (CV < 15%) was reported at the ng/mL level with 10 μL of plasma, and the LOQ could be further reduced to 50–100 pg/mL with 1 mL of plasma [59]. Schoenherr et al. [102] described how the specificity and affinity of SISCAPA-based methods could be further enhanced by using monoclonal antibodies, and designed a platform for automated screening of these antibodies.

4.1.2. *LC-MS instrumentation*

Recent advances in MS technology have also played a major role in the constant improvement in sensitivity, reproducibility, mass resolution, duty cycle, and ease of use of the reported analytical methods [18]. New analyzers and new hyphenated MS/MS systems with enhanced resolving capabilities (e.g. hybrid triple quadrupole/linear ion trap mass spectrometer) have reduced the interferences and background noise of LC-MS analysis with the consequent improvements in selectivity of the analytical methods [18]. In addition, nano-LC systems are, today, reliable systems to provide flow-rates in the sub-microliter range and have become the reference for quantitative proteomic studies [18].

These instrumental improvements have allowed researches to address analytical challenges of increasing complexity. For example, Domanski et al. [93], recently reported the multiplexed quantification by IDMS of 135 peptides in plasma samples to validate 67 putative cardiovascular disease biomarkers. In spite of minimizing the sample cleanup to a simple SPE extraction, most of the reported protein LLOQ values ranged from 5 to 1000 ng/mL. In this work, the authors used a high-flow uHPLC system coupled to a triple quadrupole ESI-MS system through an interface optimized for the 400 μL/min LC flow-rate (Agilent 6490 QQQ mass spectrometer with funnel interface and the Jet Stream technology). This setup gave a robust system that provided quantitative measurements of high reproducibility (CV < 10% for most of the peptides) and a broad linear range (from 10^3 to 10^5 in most cases). Similar LLOQ values were reported by Fortin et al. [103] for the analysis of the prostate-specific antigen in non-depleted human sera using a hybrid triple quadrupole/linear ion trap mass spectrometer (QTRAP 5500 system, ABSciex). The capability of this system to perform quantification in MS^3 mode provided the reduced background noise and selectivity required for the analysis [103].

4.2. *Internal standards for targeted protein quantification*

4.2.1. *Isotopically labeled synthetic peptides*

The use of labeled peptides as internal standards for protein quantification has been shown to be a convenient approach capable of generating reproducible results [21,61,73,93,96,97,99]. An example of this is AQUA (Absolute QUAntification) peptides, commercially available isotopically labeled synthetic peptides that typically incorporate ^{15}N and ^{13}C. These peptides, which are added to the extracts after enzymatic digestion, have been extensively used as internal standards in MRM approaches since 2003 [104]. Inter-laboratory comparison tests have additionally demonstrated that results produced by different laboratories using labeled peptides as IS are similar, provided that they use the same materials and methods [21,30]. In these tests, specific differences between laboratories were explained by linearity problems caused by cross signals between IS and analyte, or by chromatographic column degradation [30].

However, IDMS with labeled peptides has the problem that the IS is a different molecule to the analyte. It is not until after the cleavage process that the IS has the same physicochemical behavior as the peptide cleaved from the protein. Therefore, labeled peptides do not correct for systematic or accidental problems in sample processing during crucial parts of the analysis, these being protein extraction, sample purification, and trypsin digestion (Fig. 4). In fact, incomplete trypsin digestion is regularly mentioned in the literature, and its effect can be very important in the validation and quantification of protein standards [28,64,105,106]. Additionally, a complex matrix such as plasma or serum further reduces the recovery of tryptic peptides and aggravates the problems of process recovery and related accuracy issues [30,64].

A number of reports detail problems in the recovery of proteins before IS addition. For example, Keshishian et al. [73] reported protein recoveries ranging from 30% to 100%. Protein losses occurred at the purification steps (depletion by immunoaffinity followed by SCX). Remarkably, the recoveries were reproducible and independent of the protein concentration. Besides, Heudi et al. [64] reported recoveries of 28% in the analysis of specific antibodies in plasma. In this case, analyte loss was attributed to incomplete tryptic digestion. More recently, Domanski et al. [93] reported huge discrepancies (up to 100-fold differences) in protein concentrations estimated with different proteotypic peptides, indicating that accuracy of the quantitative results was compromised by systematic recovery problems of proteotypic peptides. Very remarkably, the results obtained with different peptides had a high degree of correlation (R > 0.9 in most cases), indicating good reproducibility in the recoveries [93]. It is worth observing that the above-mentioned studies coincide in the finding that, in spite of describing major accuracy problems caused by low-to-very-low recovery, method precision was not compromised (Table 1). This finding is rather unexpected since, without IS normalization, low analyte recoveries are often accompanied by low reproducibility as the resulting quantitative data is more affected by relatively small changes in analyte recovery between samples.

4.2.2. *Synthetic proteins with concatenated proteotypic peptides (QConcat/PCS/PolySIS)*

QconCAT (Quantification concatamer) and PolySIS technologies were described for the first time in 2005 [97,107,108] as efficient methods for the simultaneous absolute quantification of tens of proteins. The task of synthetizing and quantifying the high number of labeled peptides required for these analyses is facilitated by engineering and producing a synthetic protein containing all peptides selected as internal standards (the Quantification concatamer).

The synthetic protein is produced by concatenation of the coding sequences of the peptides which are inserted into a bacterial expression vector, generating a designer QconCAT

construct. QconCAT proteins are expressed in *E. coli* [107,108] or cell-free transcription systems [97] in [$^{13}C_6$]-Arg/Lys-containing medium. The synthetic protein can then be purified using a His-tag and quantified.

The number of published reports using QconCAT technology is still rather limited, and focuses on method development aspects [106–108,110–114] and specific applications where a large number of proteins are quantified simultaneously [111,115–122]. A comparison of AQUA and QconCAT strategies was reported by Aebersold et al. [110]. The study concluded that neither of the two methods is clearly superior for the production of labeled standards. The QconCAT technology has been used to assess the completeness of proteolysis by trypsin [106]. This study highlighted that there are kinetically favored routes of proteolysis that produce missed-cleaved tryptic peptides that cannot be further digested. There are also amino acid sequences that strongly influence the kinetics of tryptic digestion and can produce incomplete digestion rates. These limitations in protein digestion may produce biases in quantitative label-free approaches, and the need to implement methods for evaluating the completeness of trypsin efficiency is proposed [106]. Carrol et al. [117] reported the simultaneous quantification of 17 proteins of the Glycolytic Pathway in yeast, involving proteins that are expressed in concentration ranges between 10 and 14000 molecules per cell. In addition to this, Ding et al. [119] used the QconCAT technology to determine the stoichiometry of the Cohesin complex. The PCS (Peptide-Concatenated Standard) is a technology similar to QconCAT that was used to measure the stoichiometry of the eIF2B–eIFB complex with high precision [109]. Johnson et al. [120] quantified the stoichiometry of phosphorylation for a protein kinase at multiple predefined sites. Probably the most ambitious project that makes use of QconCAT is the COPY (Census Of the Proteome of Yeast) project [116]. This project is aimed at measuring the absolute quantity of at least 4000 proteins in the yeast Saccharomyces cerevisiae. To this end over 120 QconCATs have been designed, built and expressed, providing over 6000 Q-peptides to quantify a major fraction of the yeast proteome.

The QconCAT protein, however, is not an analog of the target proteins and, despite it is added to the sample before the digestion process, it can show different digestion rates than the natural proteins [110] and cannot correct for possible procedural biases produced during protein extraction and purification (Fig. 4).

4.2.3. *Isotopically labeled proteins*

Labeled proteins are the IS with physicochemical characteristics closer to those of their corresponding natural counterparts and, therefore, fulfill the expected requirements of a reliable internal standard for IDMS analysis [13] (Fig. 4). The analytical characteristics of these standards have shown their value in applications where reliability in the absolute quantification is likely to be mandatory [13,87], such as quantification of enterotoxins [123,124], antibodies of pharmaceutical interest [64], cancer biomarkers [14], and reference standards [125].

The first studies using isotopically-labeled proteins as IS for protein quantification were published in 2007 and several variations of the strategy were reported during the following years (PSAQ, Absolute SILAC, FLEXIquant, RISQ) [19,20,126,125]. Like QconCAT constructions, isotopically-labeled proteins are produced in expression systems (E. coli strains auxotrophic for K and R for absolute SILAC or cell free systems for PSAQ, FLEXIquant and RISQ) which are supplemented with the corresponding isotopically-labeled amino acids. Protein labeling at K and R also ensures that all tryptic peptides produced in the digestion step will be labeled.

An important point in the preparation of internal protein standards is their accurate quantification before addition to the sample. Several approaches differ significantly in the solution to this problem. PSAQ and Absolute SILAC include a His-tag to the expressed protein, and the standard is purified with Ni-affinity chromatography [19,20,127]. The His-tag can be conserved on the molecule or eliminated using a specific hydrolytic enzyme [128]. Quantification of the purified internal standard is then performed using amino acid analysis. RISQ incorporate both selenomethionine and a His-tag to the expressed protein [125]. The internal standard is purified, the His-tag eliminated, and the quantification is performed by ICP-MS. FLEXIquant uses the protein without purification, and the concentration is measured with AQUA peptides after purification and tryptic digestion of the standard mixture [126].

The outcome resulting from the analysis of a sample spiked with a protein that contains several K/R units is a series of ratios between the light/heavy isotopes of the tryptic peptides. Calculation of the protein concentration in the original sample can be performed directly from these ratios, and analytical uncertainty can be assessed from the observed variability between peptides [126,129]. Ideally, these ratios should be comparable for all peptides. Any deviation of individual peptides from the global ratio can be interpreted as proof of the existence of protein isoforms or protein post-translational modifications involving these sequences [125–127,130]. Theoretically, these PTMs can be identified and quantified with good precision and accuracy, and isotopically labeled proteins have already been used to quantify phosphorylation sites to determine mechanisms of multi-kinase substrate phosphorylation [130].

Although labeled proteins are the gold standards for accurate and reliable quantification, there are limitations that are worth noting. First, synthesis of isotopically labeled proteins requires facilities that are not available to many laboratories. This problem is aggravated in situations where multiple proteins are targeted as the dependence on individual internal standards for each protein results in a relatively high reagent cost for assay development. Also, practical problems related to the addition of multiple IS products to the sample may limit the number of targeted proteins in a single analysis [117]. On the other hand, standard proteins expressed in heterologous systems may not fold to the same structure or carry the same post-translational modifications than their original counterpart. Such differences may alter the stoichiometry between standard and analyte for some of the resulting tryptic peptides. [125,131]. Another important drawback is that, PSAQ and related technologies can only be applied to soluble proteins [132].

To overcome some of these limitations, Mann and cols recently developed a SILAC-based approach that makes use of PRotein Epitope Signature Tags (PrESTs) as the source of stable-isotope-labeled peptide standards for absolute

quantification of proteins [132]. PrEST libraries were originally designed in the Human Protein Atlas Project for the large scale production of protein specific antibodies and the Mann approach takes advantage of this established technology. A PrEST is a sequence of about 100 amino acids derived from the natural target protein that is chosen for minimal similarity to other protein sequences and that can be expressed in *E. coli*. Although the PrEST-based approach restricts the choice of quantification peptides to the epitope region of the target protein, this strategy has the advantage of having available representative sequences for ca. 98 % of the human proteome [132].

5. Conclusion

IDMS is a method of reference for highly precise, accurate, sensitive and selective targeted quantitative analysis. As long as quantitative proteomics gains new application niches and the field matures, stricter requirements for reliable quantitative measurements can be expected in areas where the provided data is likely to be critical. In such cases, some form of IDMS will probably be required.

Unfortunately, quantitative proteome analysis is much more complex than equivalent situations in analytical or bioanalytical chemistry, such as in multi-trace environmental analysis where the number of targeted components is limited to a few dozen low molecular-weight compounds. Although reliable quantitative results are currently possible by using similar targeted strategies for a limited set of proteins, full proteome quantification using IDMS approaches still appears to be a major challenge in proteomics.

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REFERENCES

[1] Bowers G, Fassett J, White E. Isotope dilution mass spectrometry and the national reference system. Anal Chem 1993;65:475–9.
[2] U.S. Department of Health and Human Services, Food and Drug Administration. Guidance for Industry: Bioanalytical Method Validation. Accessed at http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070107.pdf; 1999.
[3] Eurachem/CITAC Working Group. Traceability in chemical measurement. A guide to achieving comparable results in chemical measurement. http://www.measurementuncertainty.org/; 2003. [EURACHEM/CITAC].
[4] European Medicines Agency. Guideline on bioanalytical method validation. Accessed at www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf; 2011.
[5] Green JM. Peer reviewed: a practical guide to analytical method validation. Anal Chem 1996;68:305A–9A.
[6] Kaiser P, Akerboom T, Ohlendorf R, Reinauer H. Liquid chromatography–isotope dilution–mass spectrometry as a new basis for the reference measurement procedure for hemoglobin A1c determination. Clin Chem 2010;56:750–4.
[7] Kinumi T, Goto M, Eyama S, Kato M, Kasama T, Takatsu A. Development of SI-traceable C-peptide certified reference material NMIJ CRM 6901-a using isotope-dilution mass spectrometry-based amino acid analyses. Anal Bioanal Chem 2012;404:13–21.
[8] Phinney CS, Murphy KE, Welch MJ, Ellerbe PM, Long SE, Pratt KW, et al. Definitive method certification of clinical analytes in lyophilized human serum: NIST Standard Reference Material (SRM) 909b. Fresenius J Anal Chem 1998;361:71–80.
[9] Quevauviller P, Maier EA. Interlaboratory Studies and Certified Reference Materials for Environmental Analysis: The Bcr Approach. Elsevier; 1999.
[10] Ritter N, Hayes T, Dougherty J. Analytical laboratory quality: Part II. Analytical method validation. J Biomol Tech 2001;12:11–5.
[11] Trullols E, Ruisánchez I, Rius FX. Validation of qualitative analytical methods. TrAC Trends Anal Chem 2004;23:137–45.
[12] Ciccimaro E, Blair IA. Stable-isotope dilution LC-MS for quantitative biomarker analysis. Bioanalysis 2010;2:311–41.
[13] Brun V, Masselon C, Garin J, Dupuis A. Isotope dilution strategies for absolute quantitative proteomics. J Proteomics 2009;72:740–9.
[14] Huillet C, Adrait A, Lebert D, Picard G, Trauchessec M, Louwagie M, et al. Accurate quantification of cardiovascular biomarkers in serum using Protein Standard Absolute Quantification (PSAQ™) and selected reaction monitoring. Mol Cell Proteomics 2012;11 [M111.008235].
[15] Sargent M, Harrington C, Harte R. Guidelines for Achieving High Accuracy in Isotope Dilution Mass Spectrometry (IDMS). Cambridge: The Royal Society of Chemistry; 2002.
[16] Meija J, Mester Z. Paradigms in isotope dilution mass spectrometry for elemental speciation analysis. Anal Chim Acta 2008;607:115–25.
[17] Heumann KG. Isotope dilution mass spectrometry of inorganic and organic substances. Fresenius' Z Anal Chem 1986;325:661–6.
[18] Shi T, Su D, Liu T, Tang K, Camp DG, Qian W-J, et al. Advancing the sensitivity of selected reaction monitoring-based targeted quantitative proteomics. Proteomics 2012;12:1074–92.
[19] Brun V, Dupuis A, Adrait A, Marcellin M, Thomas D, Court M, et al. Isotope-labeled protein standards toward absolute quantitative proteomics. Mol Cell Proteomics 2007;6:2139–49.
[20] Hanke S, Besir H, Oesterhelt D, Mann M. Absolute SILAC for accurate quantitation of proteins in complex mixtures down to the attomole level. J Proteome Res 2008;7:1118–30.
[21] Kuhn E, Whiteaker JR, Mani DR, Jackson AM, Zhao L, Pope ME, et al. Interlaboratory evaluation of automated, multiplexed peptide immunoaffinity enrichment coupled to multiple reaction monitoring mass spectrometry for quantifying proteins in plasma. Mol Cell Proteomics 2012;11.
[22] Hoofnagle AN, Becker JO, Oda MN, Cavigiolio G, Mayer P, Vaisar T. Multiple-reaction monitoring–mass spectrometric assays can accurately measure the relative protein abundance in complex mixtures. Clin Chem 2012;58:777–81.
[23] Yao X. Derivatization or not: a choice in quantitative proteomics. Anal Chem 2011;83:4427–39.
[24] Zhang H, Liu Q, Zimmerman LJ, Ham A-JL, Slebos RJC, Rahman J, et al. Methods for peptide and protein quantitation by liquid chromatography-multiple reaction monitoring mass spectrometry. Mol Cell Proteomics 2011;10.

[25] Ong S-E, Foster LJ, Mann M. Mass spectrometric-based approaches in quantitative proteomics. Methods 2003;29:124–30.
[26] Elliott MH, Smith DS, Parker CE, Borchers C. Current trends in quantitative proteomics. J Mass Spectrom 2009;44:1637–60.
[27] Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R. Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. Nat Biotechnol 1999;17:994–9.
[28] Evans C, Noirel J, Ow S, Salim M, Pereira-Medrano A, Couto N, et al. An insight into iTRAQ: where do we stand now? Anal Bioanal Chem 2012:1–17.
[29] Schulze WX, Usadel B. Quantitation in mass-spectrometry-based proteomics. Annu Rev Plant Biol 2010;61:491–516.
[30] Addona TA, Abbatiello SE, Schilling B, Skates SJ, Mani DR, Bunk DM, et al. Multi-site assessment of the precision and reproducibility of multiple reaction monitoring-based measurements of proteins in plasma. Nat Biotechnol 2009;27:633–41.
[31] Asara JM, Christofk HR, Freimark LM, Cantley LC. A label-free quantification method by MS/MS TIC compared to SILAC and spectral counting in a proteomics screen. Proteomics 2008;8:994–9.
[32] Neilson KA, Ali NA, Muralidharan S, Mirzaei M, Mariani M, Assadourian G, et al. Less label, more free: approaches in label-free quantitative mass spectrometry. Proteomics 2011;11:535–53.
[33] Wang H, Alvarez S, Hicks LM. Comprehensive comparison of iTRAQ and label-free LC-based quantitative proteomics approaches using two *Chlamydomonas reinhardtii* strains of interest for biofuels engineering. J Proteome Res 2011;11:487–501.
[34] Merl J, Ueffing M, Hauck SM, von Toerne C. Direct comparison of MS-based label-free and SILAC quantitative proteome profiling strategies in primary retinal Müller cells. Proteomics 2012;12:1902–11.
[35] Trinh HV, Grossmann J, Gehrig P, Roschitzki B, Schlapbach R, Greber UF, et al. iTRAQ-based and label-free proteomics approaches for studies of human adenovirus infections. Int J Proteomics 2013;2013:16. http://dx.doi.org/10.1155/2013/581862 [Article ID 581862].
[36] Mosley AL, Sardiu ME, Pattenden SG, Workman JL, Florens L, Washburn MP. Highly reproducible label free quantitative proteomic analysis of RNA polymerase complexes. Mol Cell Proteomics 2011;10:1–13.
[37] Li Z, Adams RM, Chourey K, Hurst GB, Hettich RL, Pan C. Systematic comparison of label-free, metabolic labeling, and isobaric chemical labeling for quantitative proteomics on LTQ orbitrap velos. J Proteome Res 2011;11:1582–90.
[38] Collier TS, Randall SM, Sarkar P, Rao BM, Dean RA, Muddiman DC. Comparison of stable-isotope labeling with amino acids in cell culture and spectral counting for relative quantification of protein expression. Rapid Commun Mass Spectrom 2011;25:2524–32.
[39] Patel VJ, Thalassinos K, Slade SE, Connolly JB, Crombie A, Murrell JC, et al. A comparison of labeling and label-free mass spectrometry-based proteomics approaches. J Proteome Res 2009;8:3752–9.
[40] Whiteaker JR, Lin C, Kennedy J, Hou L, Trute M, Sokal I, et al. A targeted proteomics-based pipeline for verification of biomarkers in plasma. Nat Biotechnol 2011;29:625–34.
[41] Valcárcel M, Ríos A. Traceability in chemical measurements for the end users. TrAC Trends Anal Chem 1999;18:570–6.
[42] Tan A, Hussain S, Musuku A, Massé R. Internal standard response variations during incurred sample analysis by LC-MS/MS: case by case trouble-shooting. J Chromatogr B Analyt Technol Biomed Life Sci 2009;877:3201–9.
[43] Lowenthal M, Yen J, Bunk D, Phinney K. Certification of NIST standard reference material 2389a, amino acids in 0.1 mol/L HCl—quantification by ID LC-MS/MS. Anal Bioanal Chem 2010;397:511–9.
[44] Freisleben A, Brudny-Klöppel M, Mulder H, de Vries R, de Zwart M, Timmerman P. Blood stability testing: European Bioanalysis Forum view on current challenges for regulated bioanalysis. Bioanalysis 2011;3:1333–6.
[45] Bunk DM, Lowenthal MS. Isotope Dilution Liquid Chromatography-Tandem Mass Spectrometry for Quantitative Amino Acid Analysis. In: Alterman MA, Hunziker P, Walker JM, editors. Amino Acid Analysis. Humana Press; 2012. p. 29–38.
[46] Clémens S, Monperrus M, Donard OFX, Amouroux D, Guérin T. Mercury speciation in seafood using isotope dilution analysis: a review. Talanta 2012;89:12–20.
[47] Popp M, Hann S, Koellensperger G. Environmental application of elemental speciation analysis based on liquid or gas chromatography hyphenated to inductively coupled plasma mass spectrometry—A review. Anal Chim Acta 2010;668:114–29.
[48] Dams R, Huestis MA, Lambert WE, Murphy CM. Matrix effect in bio-analysis of illicit drugs with LC-MS/MS: influence of ionization type, sample preparation, and biofluid. J Am Soc Mass Spectrom 2003;14:1290–4.
[49] Buhrman DL, Price PI, Rudewicz PJ. Quantitation of SR 27417 in human plasma using electrospray liquid chromatography-tandem mass spectrometry: a study of ion suppression. J Am Soc Mass Spectrom 1996;7:1099–105.
[50] Panteghini M. Traceability, Reference Systems and Result Comparability. Clin Biochem Rev 2007;28:97–104.
[51] Taverniers I, De Loose M, Van Bockstaele E. Trends in quality in the analytical laboratory. II. Analytical method validation and quality assurance. TrAC Trends Anal Chem 2004;23:535–52.
[52] IUPAC. Compendium of chemical terminology: gold book. IUPAC; 2012.
[53] Pritchard C, Quaglia M, Mussell C, Burkitt WI, Parkes H, O'Connor G. Fully Traceable Absolute Protein Quantification of Somatropin That Allows Independent Comparison of Somatropin Standards. Clin Chem 2009;55:1984–90.
[54] Directive 98/79/EC of the European Parliament and of the Council of 27 October 1998 on in vitro diagnostic medical devices. Off J Eur Communities 1998;L331:1–37.
[55] Milton MJT, Wielgosz RI. Uncertainty in SI-traceable measurements of amount of substance by isotope dilution mass spectrometry. Metrologia 2000;37:199–206.
[56] Ekins R, Edwards P. Point On the meaning of sensitivity. Clin Chem 1997;43:1824–31.
[57] Peters FT, Maurer HH. Bioanalytical method validation and its implications for forensic and clinical toxicology—A review. Accred Qual Assur 2002;7:441–9.
[58] Armbruster DA, Pry T. Limit of Blank, Limit of Detection and Limit of Quantitation. Clin Biochem Rev 2008;29:S49–52.
[59] Whiteaker JR, Zhao L, Anderson L, Paulovich AG. An Automated and Multiplexed Method for High Throughput Peptide Immunoaffinity Enrichment and Multiple Reaction Monitoring Mass Spectrometry-based Quantification of Protein Biomarkers. Mol Cell Proteomics 2010;9:184–96.
[60] Clinical and Laboratory Standards Institute. Protocols for Determination of Limits of Detection and Limits of Quantitation; Approved Guideline. CLSI document EP17. Wayne, PA USA: CLS; 2004.
[61] Keshishian H, Addona T, Burgess M, Mani DR, Shi X, Kuhn E, et al. Quantification of Cardiovascular Biomarkers in Patient Plasma by Targeted Mass Spectrometry and Stable Isotope Dilution. Mol Cell Proteomics 2009;8:2339–49.
[62] International Conference on Harmonization of Technical Requirements For Registration of Pharmaceutical for

Human Use. Validation of Analytical Procedures: Text and Methodology; 1996.
[63] Quevauviller P, Maier EA, Griepink B. Quality assurance for environmental analysis. Quality Assurance for Environmental Analysis Method Evaluation within the Measurements and Testing Programme (BCR), vol. 17. Elsevier; 1995 1–25.
[64] Heudi O, Barteau S, Zimmer D, Schmidt J, Bill K, Lehmann N, et al. Towards Absolute Quantification of Therapeutic Monoclonal Antibody in Serum by LC-MS/MS Using Isotope-Labeled Antibody Standard and Protein Cleavage Isotope Dilution Mass Spectrometry. Anal Chem 2008;80:4200–7.
[65] Tomaiuolo M, Vecchione G, Margaglione M, Pisanelli D, Grandone E. Stable-isotope dilution LC–ESI-MS/MS techniques for the quantification of total homocysteine in human plasma. J Chromatogr B 2009;877:3292–9.
[66] Tan A, Boudreau N, Lévesque A. Internal standards for Quantitative LC-MS bioanalysis. Lc-Ms in Drug Bioanalysis. New York: Springer; 2012.
[67] Henrion A. Reduction of systematic errors in quantitative analysis by isotope dilution mass spectrometry (IDMS): An iterative method. Fresenius J Anal Chem 1994;350:657–8.
[68] Joint Committee for Guides in Metrology, Bureau International des Poids et Mesures. Evaluation of measurement data — Guide to the expression of uncertainty in measurement. Accessed from http://www.bipm.org/; 2008.
[69] Thompson M, Ellison SLR, Fajgelj A, Willetts P, Wood R. Harmonised guidelines for the use of recovery information in analytical measurement. IUPAC; 1996 [Available at http://eurachem.org/images/stories/Guides/pdf/].
[70] US Environmental Protection Agency. Environmental Measurement: Glossary of Terms. http://www.epa.gov/fem/pdfs/Env_Measurement_Glossary_Final_Jan_2010.pdf; 2010.
[71] EM 200-1-100. Guidance for evaluating performance-based chemical data. Chapter 13 Surrogates. U.S. Army Corps of Engineers (USACE); 2005 [Available at http://www.environmental.usace.army.mil/guide_chem.htm].
[72] Office of the Science Advisor, US EPA. Forum on Environmental Measurements. http://www.epa.gov/fem/; 2013.
[73] Keshishian H, Addona T, Burgess M, Kuhn E, Carr SA. Quantitative, Multiplexed Assays for Low Abundance Proteins in Plasma by Targeted Mass Spectrometry and Stable Isotope Dilution. Mol Cell Proteomics 2007;6:2212–29.
[74] Parks BA, Jiang L, Thomas PM, Wenger CD, Roth MJ, Boyne MT, et al. Top-Down Proteomics on a Chromatographic Time Scale Using Linear Ion Trap Fourier Transform Hybrid Mass Spectrometers. Anal Chem 2007;79:7984–91.
[75] Patrie SM, Ferguson JT, Robinson DE, Whipple D, Rother M, Metcalf WW, et al. Top Down Mass Spectrometry of <60-kDa Proteins from Methanosarcina acetivorans Using Quadrupole FTMS with Automated Octopole Collisionally Activated Dissociation. Mol Cell Proteomics 2006;5:14–25.
[76] Cui W, Rohrs HW, Gross ML. Top-Down Mass Spectrometry: Recent Developments, Applications and Perspectives. Analyst 2011;136:3854–64.
[77] Catherman AD, Li M, Tran JC, Durbin KR, Compton PD, Early BP, et al. Top Down Proteomics of Human Membrane Proteins from Enriched Mitochondrial Fractions. Anal Chem 2013;85:1880–8.
[78] Kellie JF, Catherman AD, Durbin KR, Tran JC, Tipton JD, Norris JL, et al. Robust Analysis of the Yeast Proteome under 50 kDa by Molecular-Mass-Based Fractionation and Top-Down Mass Spectrometry. Anal Chem 2012;84:209–15.
[79] Zhou H, Ning Z, Starr AE, Abu-Farha M, Figeys D. Advancements in Top-Down Proteomics. Anal Chem 2012;84:720–34.
[80] Armirotti A, Damonte G. Achievements and perspectives of top-down proteomics. Proteomics 2010;10:3566–76.
[81] Smith LM, Kelleher NL, Proteomics TC for TD. Proteoform: a single term describing protein complexity. Nat Methods 2013;10:186–7.
[82] Chen C-H (Winston). Review of a current role of mass spectrometry for proteome research. Anal Chim Acta 2008;624:16–36.
[83] Barr JR, Maggio VL, Patterson DG, Cooper GR, Henderson LO, Turner WE, et al. Isotope dilution–mass spectrometric quantification of specific proteins: model application with apolipoprotein A-I. Clin Chem 1996;42:1676–82.
[84] Craig R, Cortens JP, Beavis RC. The use of proteotypic peptide libraries for protein identification. Rapid Commun Mass Spectrom 2005;19:1844–50.
[85] Kuster B, Schirle M, Mallick P, Aebersold R. Scoring proteomes with proteotypic peptide probes. Nat Rev Mol Cell Biol 2005;6:577–83.
[86] Alves G, Ogurtsov AY, Yu Y-K. Assigning statistical significance to proteotypic peptides via database searches. J Proteomics 2011;74:199–211.
[87] Boja ES, Rodriguez H. Mass spectrometry-based targeted quantitative proteomics: Achieving sensitive and reproducible detection of proteins. Proteomics 2012;12:1093–110.
[88] Mallick P, Schirle M, Chen SS, Flory MR, Lee H, Martin D, et al. Computational prediction of proteotypic peptides for quantitative proteomics. Nat Biotechnol 2007;25:125–31.
[89] Picotti P, Lam H, Campbell D, Deutsch E, Mirzaei H, Ranish J, et al. A database of validated assays for the targeted mass spectrometric analysis of the S. cerevisiae proteome. Nat Methods 2008;5:913–4.
[90] Yates JR, Morgan SF, Gatlin CL, Griffin PR, Eng JK. Method To Compare Collision-Induced Dissociation Spectra of Peptides: Potential for Library Searching and Subtractive Analysis. Anal Chem 1998;70:3557–65.
[91] Frewen BE, Merrihew GE, Wu CC, Noble WS, MacCoss MJ. Analysis of Peptide MS/MS Spectra from Large-Scale Proteomics Experiments Using Spectrum Libraries. Anal Chem 2006;78:5678–84.
[92] Lam H, Deutsch EW, Eddes JS, Eng JK, King N, Stein SE, et al. Development and validation of a spectral library searching method for peptide identification from MS/MS. Proteomics 2007;7:655–67.
[93] Domanski D, Percy AJ, Yang J, Chambers AG, Hill JS, Freue GVC, et al. MRM-based multiplexed quantitation of 67 putative cardiovascular disease biomarkers in human plasma. Proteomics 2012;12:1222–43.
[94] Pan S, Aebersold R, Chen R, Rush J, Goodlett DR, McIntosh MW, et al. Mass Spectrometry Based Targeted Protein Quantification: Methods and Applications. J Proteome Res 2008;8:787–97.
[95] Simpson KL, Whetton AD, Dive C. Quantitative mass spectrometry-based techniques for clinical use: Biomarker identification and quantification. J Chromatogr B 2009;877:1240–9.
[96] Kuhn E, Wu J, Karl J, Liao H, Zolg W, Guild B. Quantification of C-reactive protein in the serum of patients with rheumatoid arthritis using multiple reaction monitoring mass spectrometry and 13C-labeled peptide standards. Proteomics 2004;4:1175–86.
[97] Anderson L, Hunter CL. Quantitative Mass Spectrometric Multiple Reaction Monitoring Assays for Major Plasma Proteins. Mol Cell Proteomics 2006;5:573–88.
[98] Björhall K, Miliotis T, Davidsson P. Comparison of different depletion strategies for improved resolution in proteomic analysis of human serum samples. Proteomics 2005;5:307–17.
[99] Barnidge DR, Dratz EA, Martin T, Bonilla LE, Moran LB, Lindall A. Absolute Quantification of the G Protein-Coupled Receptor Rhodopsin by LC/MS/MS Using Proteolysis Product

Peptides and Synthetic Peptide Standards. Anal Chem 2003;75:445–51.
[100] Anderson NL, Anderson NG, Haines LR, Hardie DB, Olafson RW, Pearson TW. Mass Spectrometric Quantitation of Peptides and Proteins Using Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA). J Proteome Res 2004;3:235–44.
[101] Whiteaker JR, Zhao L, Zhang HY, Feng L-C, Piening BD, Anderson L, et al. Antibody-based enrichment of peptides on magnetic beads for mass spectrometry-based quantification of serum biomarkers. Anal Biochem 2007;362:44–54.
[102] Schoenherr RM, Zhao L, Whiteaker JR, Feng L-C, Li L, Liu L, et al. Automated screening of monoclonal antibodies for SISCAPA assays using a magnetic bead processor and liquid chromatography-selected reaction monitoring-mass spectrometry. J Immunol Methods 2010;353:49–61.
[103] Fortin T, Salvador A, Charrier JP, Lenz C, Bettsworth F, Lacoux X, et al. Multiple Reaction Monitoring Cubed for Protein Quantification at the Low Nanogram/Milliliter Level in Nondepleted Human Serum. Anal Chem 2009;81:9343–52.
[104] Gerber SA, Rush J, Stemman O, Kirschner MW, Gygi SP. Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS. Proc Natl Acad Sci U S A 2003;100:6940–5.
[105] Barnidge DR, Hall GD, Stocker JL, Muddiman DC. Evaluation of a cleavable stable isotope labeled synthetic peptide for absolute protein quantification using LC-MS/MS. J Proteome Res 2004;3:658–61.
[106] Brownridge P, Beynon RJ. The importance of the digest: Proteolysis and absolute quantification in proteomics. Methods 2011;54:351–60.
[107] Beynon RJ, Doherty MK, Pratt JM, Gaskell SJ. Multiplexed absolute quantification in proteomics using artificial QCAT proteins of concatenated signature peptides. Nat Methods 2005;2:587–9.
[108] Pratt JM, Simpson DM, Doherty MK, Rivers J, Gaskell SJ, Beynon RJ. Multiplexed absolute quantification for proteomics using concatenated signature peptides encoded by QconCAT genes. Nat Protoc 2006;1:1029–43.
[109] Kito K, Ota K, Fujita T, Ito T. A Synthetic Protein Approach toward Accurate Mass Spectrometric Quantification of Component Stoichiometry of Multiprotein Complexes. J Proteome Res 2007;6:792–800.
[110] Mirzaei H, McBee JK, Watts J, Aebersold R. Comparative Evaluation of Current Peptide Production Platforms Used in Absolute Quantification in Proteomics. Mol Cell Proteomics 2008;7:813–23.
[111] Johnson H, Wong S, Simpson D, Beynon R, Gaskell S. Protein quantification by selective isolation and fragmentation of isotopic pairs using FT-ICR MS. J Am Soc Mass Spectrom 2008;19:973–7.
[112] Swainston N, Jameson D, Carroll K. A QconCAT informatics pipeline for the analysis, visualization and sharing of absolute quantitative proteomics data. Proteomics 2010;11:329–33.
[113] Carroll KM, Lanucara F, Eyers CE. Chapter seven—Quantification of Proteins and Their Modifications Using QconCAT Technology. Methods in Systems Biology, vol. 500. Academic Press; 2011 113–31.
[114] Eyers CE, Lawless C, Wedge DC, Lau KW, Gaskell SJ, Hubbard SJ. CONSeQuence: Prediction of Reference Peptides for Absolute Quantitative Proteomics Using Consensus Machine Learning Approaches. Mol Cell Proteomics 2011;10.
[115] Bislev SL, Kusebauch U, Codrea MC, Beynon RJ, Harman VM, Røntved CM, et al. Quantotypic Properties of QconCAT Peptides Targeting Bovine Host Response to Streptococcus uberis. J Proteome Res 2012;11:1832–43.
[116] Brownridge P, Holman SW, Gaskell SJ, Grant CM, Harman VM, Hubbard SJ, et al. Global absolute quantification of a proteome: Challenges in the deployment of a QconCAT strategy. Proteomics 2011;11:2957–70.
[117] Carroll KM, Simpson DM, Eyers CE, Knight CG, Brownridge P, Dunn WB, et al. Absolute Quantification of the Glycolytic Pathway in Yeast: Deployment of a complete QconCAT approach. Mol Cell Proteomics 2011;10.
[118] Castro-Borges W, Simpson DM, Dowle A, Curwen RS, Thomas-Oates J, Beynon RJ, et al. Abundance of tegument surface proteins in the human blood fluke Schistosoma mansoni determined by QconCAT proteomics. J Proteomics 2011;74:1519–33.
[119] Ding C, Li Y, Kim B-J, Malovannaya A, Jung SY, Wang Y, et al. Quantitative Analysis of Cohesin Complex Stoichiometry and SMC3 Modification-Dependent Protein Interactions. J Proteome Res 2011;10:3652–9.
[120] Johnson H, Eyers C, Eyers P, Beynon R, Gaskell S. Rigorous determination of the stoichiometry of protein phosphorylation using mass spectrometry. J Am Soc Mass Spectrom 2009;20:2211–20.
[121] Rivers J, Simpson DM, Robertson DHL, Gaskell SJ, Beynon RJ. Absolute Multiplexed Quantitative Analysis of Protein Expression During Muscle Development Using QconCAT. Mol Cell Proteomics 2007;6:1416–27.
[122] Rivers J, Hughes C, McKenna T, Woolerton Y, Vissers JPC, Langridge JI, et al. Asymmetric Proteome Equalization of the Skeletal Muscle Proteome Using a Combinatorial Hexapeptide Library. PLoS One 2011;6.
[123] Dupuis A, Hennekinne J-A, Garin J, Brun V. Protein Standard Absolute Quantification (PSAQ) for improved investigation of staphylococcal food poisoning outbreaks. Proteomics 2008;8:4633–6.
[124] Adrait A, Lebert D, Trauchessec M, Dupuis A, Louwagie M, Masselon C, et al. Development of a Protein Standard Absolute Quantification (PSAQ™) assay for the quantification of Staphylococcus aureus enterotoxin A in serum. J Proteomics 2012;75:3041–9.
[125] Zinn N, Winter D, Lehmann WD. Recombinant Isotope Labeled and Selenium Quantified Proteins for Absolute Protein Quantification. Anal Chem 2010;82:2334–40.
[126] Singh S, Springer M, Steen J, Kirschner MW, Steen H. FLEXIQuant: a novel tool for the absolute quantification of proteins, and the simultaneous identification and quantification of potentially modified peptides. J Proteome Res 2009;8:2201–10.
[127] Ciccimaro E, Hanks SK, Yu KH, Blair IA. Absolute Quantification of Phosphorylation on the Kinase Activation Loop of Cellular Focal Adhesion Kinase by Stable Isotope Dilution Liquid Chromatography/Mass Spectrometry. Anal Chem 2009;81:3304–13.
[128] Lebert D, Dupuis A, Garin J, Bruley C, Brun V. Production and Use of Stable Isotope-Labeled Proteins for Absolute Quantitative Proteomics. In: Gevaert K, Vandekerckhove J, editors. Gel-Free Proteomics, vol. 753. Humana Press; 2011. p. 93–115.
[129] Quaglia M, Pritchard C, Hall Z, O'Connor G. Amine-reactive isobaric tagging reagents: Requirements for absolute quantification of proteins and peptides. Anal Biochem 2008;379:164–9.
[130] Singh SA, Winter D, Bilimoria PM, Bonni A, Steen H, Steen JA. FLEXIQinase, a mass spectrometry-based assay, to unveil multikinase mechanisms. Nat Methods 2012;9:504–8.
[131] Simpson DM, Beynon RJ. QconCATs: design and expression of concatenated protein standards for multiplexed protein quantification. Anal Bioanal Chem 2012;404:977–89.
[132] Zeiler M, Straube WL, Lundberg E, Uhlen M, Mann M. A Protein Epitope Signature Tag (PrEST) Library Allows SILAC-based Absolute Quantification and Multiplexed Determination of Protein Copy Numbers in Cell Lines. Mol Cell Proteomics 2012;11.

[133] Kuzyk MA, Smith D, Yang J, Cross TJ, Jackson AM, Hardie DB, et al. Multiple Reaction Monitoring-based, Multiplexed, Absolute Quantitation of 45 Proteins in Human Plasma. Mol Cell Proteomics 2009;8:1860–77.
[134] Tang H-Y, Beer LA, Barnhart KT, Speicher DW. Rapid Verification of Candidate Serological Biomarkers Using Gel-based, Label-free Multiple Reaction Monitoring. J Proteome Res 2011;10:4005–17.
[135] Chen Y-T, Chen H-W, Domanski D, Smith DS, Liang K-H, Wu C-C, et al. Multiplexed quantification of 63 proteins in human urine by multiple reaction monitoring-based mass spectrometry for discovery of potential bladder cancer biomarkers. J Proteomics 2012;75:3529–45.
[136] Yu Y, Xu J, Liu Y, Chen Y. Quantification of human serum transferrin using liquid chromatography–tandem mass spectrometry based targeted proteomics. J Chromatogr B 2012;902:10–5.
[137] Rezeli M, Végvári Á, Donnarumma F, Gidlöf O, Smith JG, Erlinge D, et al. Development of an MRM assay panel with application to biobank samples from patients with myocardial infarction. J Proteomics 2013;87:16–25.
[138] Simicevic J, Schmid AW, Gilardoni PA, Zoller B, Raghav SK, Krier I, et al. Absolute quantification of transcription factors during cellular differentiation using multiplexed targeted proteomics. Nat Methods 2013;10:570–6.
[139] Chenau J, Fenaille F, Ezan E, Morel N, Lamourette P, Goossens PL, et al. Sensitive Detection of Bacillus anthracis Spores by Immunocapture and Liquid Chromatography–Tandem Mass Spectrometry. Anal Chem 2011;83:8675–82.