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## Review

# Isotope dilution strategies for absolute quantitative proteomics

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### ABSTRACT

The development of mass spectrometry (MS)-based methodologies for high-throughput protein identification has generated a concomitant need for protein quantification. Numerous MS-based relative quantification methodologies have been dedicated to the extensive comparison of multiple proteomes. On the other hand, absolute quantification methodologies, which allow the determination of protein concentrations in biological samples, are generally restricted to defined sets of proteins. Depending on the selected analytical procedure, absolute quantification approaches can provide accurate and precise estimations. These analytical performances are crucial for specific applications such as the evaluation of clinical biomarker candidates.

According to bioanalytical guidelines, accurate analytical processes require internal standards and quality controls. Regarding MS-based analysis of small molecules, isotope dilution has been recognized as the reference method for internal standardization. However, protein quantification methodologies which rely on the isotope dilution principle have been implemented in the proteomic field only recently. In these approaches, the sample is spiked with defined amounts of isotope-labeled analogue(s) of specific proteolytic peptide(s) (AQUA and QconCAT strategies) or protein(s) (PSAQ strategy). In this review, we present a critical overview of these isotope dilution methodologies.

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## 1. Introduction

The combination of chromatographic separations with mass spectrometry has brought about exquisitely sensitive protein detection in complex biological samples. In addition, tandem mass spectrometry offers protein sequence information and isoform discrimination with unrivaled detection specificity. Quantitative methodologies such as SILAC, ICAT, iTRAQ or label-free approaches (reviewed in References [1,2] and [3]) have been developed to explore the dynamics of whole proteomes and generally provide a relative comparison of protein abundances between few samples. Conversely, absolute quantification methodologies aim at determining protein concentrations in biological samples, enabling the genuine comparison of data between laboratories [4]. However, to consistently reach this goal, a clear validation of the accuracy and precision of quantification methods is required to demonstrate that the estimated values are as close as possible to the real concentrations of the target proteins [5]. The isotope dilution concept, recognized since decades as the reference approach for MS-based quantification of small molecules [5], has recently been transferred to the absolute quantification of proteins in biological samples. These MS-based absolute quantification methodologies rely on the addition of defined quantities of isotope-labeled standards, which exhibit chromatographic behaviors identical to the native compounds (except for deuterium-labeled standards), but can be distinguished by their mass difference and possibly their isotopic signature [1]. These isotope dilution methods are generally targeted, i.e. focused on a restricted set of proteins. Nevertheless, ambitious projects intend to construct large-scale banks of quantification standards, which should greatly increase the number of quantifiable protein analytes [6–8]. We review here the isotope dilution-based absolute quantification strategies in proteomics, with a particular emphasis on quantification standards, in order to evaluate their impact on the accuracy and precision of measurements.

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## 2. Isotope dilution principle

Isotope-labeled internal standards are commonly used in analytical chemistry for the measurement of small molecules such as drugs [9] and hormones [10]. With regards to peptides, in 1997, Stocklin et al. developed an isotope dilution assay for determination of pro-insulin, insulin and C-peptide serum levels in healthy and diabetic patients [11,12]. They produced  $^{15}\text{N}$  and/or  $^2\text{H}$  labeled analogues of these three polypeptides in *Escherichia coli* and used them as internal standards in a “top-down” experiment consisting in MS analysis of the intact proteins. The successful detection and quantification of these polypeptides relied on a highly-efficient enrichment of targets before MS analysis. However, the sensitivity of “top-down”

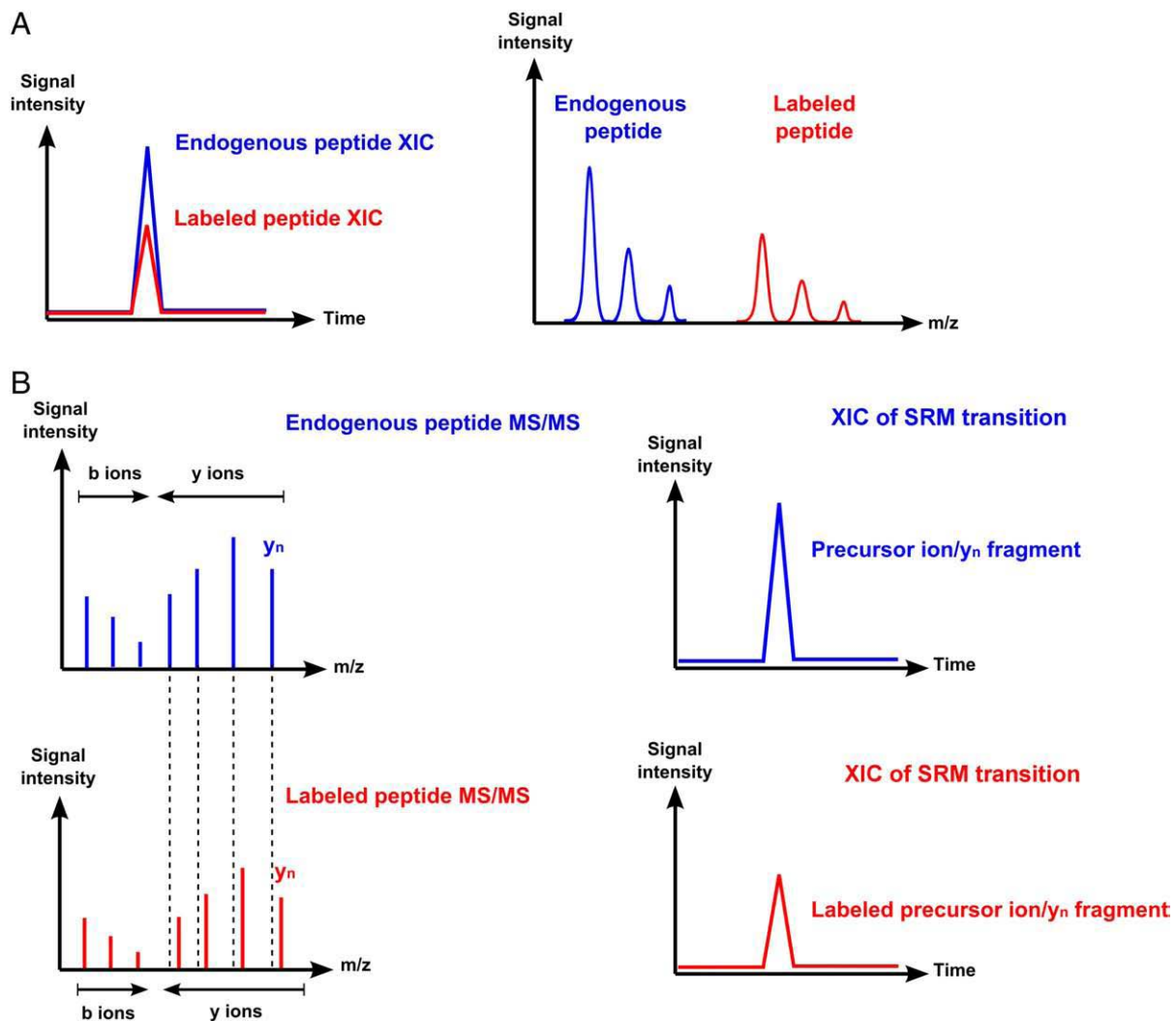
mass spectrometry methods is limited by the wide distribution of protein charge states. Thus, the detection and quantification of large proteins ( $\text{MW} > 15$  kDa) by mass spectrometry generally requires a “bottom-up” strategy in which the MS analysis is realized on peptides after digestion of the protein(s) of interest. In these cases, absolute quantification methods, which rely on isotopic dilution, require an initial analysis (experimental or predictive) to identify signature peptide(s) for each target protein(s). These signatures or so-called “proteotypic” peptides, are characterized by their sequence uniqueness in the context of a particular (predicted) proteome and their efficient detection in LC-MS [13]. Second, an internal standardization is performed with stable isotope-labeled analogs of these proteotypic peptides which are spiked into the samples in defined amount before LC-MS analysis. This standardization enables to alleviate quantification biases related to matrix effects, ionization efficiency and instrument response.

In the proteomic domain, the majority of MS methods and instrumentations are dedicated to monitor “molecular ions” (i.e. peptide ions generated by MALDI and ESI ion sources). However, an additional methodology, based on element speciation by Inductively Coupled Plasma Mass Spectrometry (ICP-MS), is worth particular mention. This approach exploits the capabilities of ICP-MS to track heteroelements (metals, semi-metals or nonmetals such as iodine, phosphorus or sulphur) to detect and quantify “heteroatom-tagged” proteins, typically metalloproteins. Absolute quantification can be achieved thanks to different isotope dilution standards: heteroatoms with altered isotopic abundances [14], heteroatom isotope-labeled peptides [15] or proteins [16]. Recently, Busto et al. demonstrated the potential of a  $^{57}\text{Fe}$  saturated protein standard for absolute quantification of transferrin isoforms in human serum [16]. In the following sections, our review will focus on conventional “molecular” MS strategies for absolute quantification. Nevertheless, comprehensive information about quantitative “elemental” MS can be found in references [17] and [18].

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## 3. Quantification modes

Quantification can be performed in the MS mode by comparing the extracted ion signal (peak height or peak area) of the isotope-labeled and the native forms of a given proteotypic peptide (Fig. 1A). Repeated analyses are required and several proteotypic peptide pairs are highly desirable in order to ensure the reliable quantification of a given target protein. In the tandem mode (MS/MS), a comparison of ion fragment signals from standard and native peptides can also be performed for quantification [19]. Finally, Selected Reaction Monitoring (SRM) experiments which harness the capabilities of triple quadrupole instruments for ion



**Fig. 1** – Mass spectrometry methodologies for absolute quantification of proteins. (1A) Absolute quantification using LC-MS. During the liquid chromatography step, the endogenous and labeled versions of a proteotypic peptide elute simultaneously. However, due to differential labeling, the isotopic contributions of the endogenous and labeled forms of the proteotypic peptide can be distinguished in the mass spectrum. Quantification is performed by comparing the extracted ion chromatogram (peak intensity or peak area) of the endogenous and the labeled forms of the proteotypic peptide. (1B) Absolute quantification using LC-SRM (Selected Reaction Monitoring). In a preliminary LC-MS/MS analysis, the most intense and characteristic transitions (pairs of precursor ion/fragment ion) are determined for a given proteotypic peptide. Because both versions of the peptide fragment identically, this mass spectrum contains the same fragments, with some of them shifted in mass due to the isotope label (the case of a peptide labeled in C-terminal is shown). In a subsequent LC-SRM experiment, the pre-determined transitions for the two versions (endogenous/labeled) of the proteotypic peptide are specifically monitored. The quantification is performed by comparing the XIC signals of related transitions corresponding to the two versions of the proteotypic peptide.

selectivity are particularly suited for quantification. These experiments consist in monitoring signals from specific fragmentation transitions (pairs of precursor-ion/fragment-ion) achieving improved signal/noise ratio, increased sensitivity and enlarged dynamic range [20] (Fig. 1B). In contrast to antibody-based detection and quantification methods which require the production of highly specific antibodies and lengthy optimizations for each target species, MS analysis offers the advantage of multiplex quantification in a single run [21]. The sensitivity and dynamic range achievability using the isotope dilution principle mainly depend on the quantification mode.

#### 4. Domains of application

MS-based targeted absolute quantification of proteins holds great promise in numerous disciplines. In the rapidly evolving field of systems biology, the consistent characterization and quantification of proteins is fundamental for the establishment and evaluation of relevant networks and models [22]. In this domain, the multiplexing capabilities and the accuracy of measurements are crucial. In biomedical research, MS-based absolute quantification is poised to fill the gap between the biomarkers' discovery and validation phases [23]. As a matter

of fact, MS-based evaluation of candidates relying on quantitative multiplexed reaction monitoring (or multiplexed SRM) experiments is presently emerging as a compelling surrogate to immunological techniques for biomarker evaluation. In the pharmaceutical industry, similar strategies could be envisioned not only to investigate the pharmacokinetic features of therapeutic proteins (tissue distribution and plasma half-life determination) but also for vaccine antigens and therapeutic proteins quality control (protein structure, purity and abundance verification) [24–27]. In the public health domain, assessment of food safety could also benefit from MS-based detection and quantification of contaminant or allergen traces [28]. We have recently demonstrated the value of MS analysis combined with the use of isotope-labeled protein standards for the identification and quantification of staphylococcal enterotoxins frequently implicated in food poisoning outbreaks [29]. In terms of national security, we have also successfully brought this methodology into play for the detection and quantification of staphylococcal enterotoxins in water and urine [7]. Finally, when coupled with immunoenrichment approaches, MS analysis coupled with isotope dilution may help to solve the challenge of ultra-high sensitive detection and accurate quantification required for anti-doping control [30,31].

## 5. Isotope-labeled quantification standards

As a general rule, isotope dilution-based quantification methods display good linearity and excellent precision regardless of the quantification standard used [7]. However, the accuracy of these methods, i.e. their ability to determine the true abundance of target proteins, may depend on the choice of standard and the overall analytical strategy. Different stable isotope-labeled quantification standards have been described. Fig. 2 depicts the three existing methods and their implementation along a typical “bottom-up” proteomics workflow: (i) AQUA standards correspond to synthetic peptides that can be spiked into the samples after the proteolysis step [32]; (ii) QconCAT concatamers are chimerical proteins composed of different proteotypic peptides from several protein targets; QconCAT standards should be added before the proteolysis step [6]; (iii) Finally, PSAQ standards are full-length proteins matching the biochemical properties of the target proteins; they can be spiked into the samples at the very beginning of the analytical process [7]. These internal standardization methods are detailed below.

### 5.1. AQUA peptides

In 1996, in an attempt to circumvent the use of immunoassays, Barr et al. [33] pioneered the application of the isotope dilution principle to MS quantification of proteins by using isotope-labeled peptide analogues. These peptide analogues were intended to measure pure apolipoprotein A-I using Fast Atom Bombardment (FAB)-MS. In 2001, Stemmann et al. built upon this strategy to quantify separase, a mitosis-regulating protein, in HeLa cell extracts [34]. By using pairs of unphosphorylated/phosphorylated isotope-labeled peptides, they showed that separase was totally phosphorylated at its

inhibitory site when cells were pharmacologically-arrested in metaphase. A couple of years later, Gerber et al. applied this strategy, under the acronym AQUA, for “Absolute Quantification”, to quantitatively determine the cell cycle-dependent phosphorylations of human separase [32]. This acronym has become the trade name under which this strategy came to be known to the proteomic community.

The commercial availability and easy use of AQUA peptides make them particularly attractive to researchers. Suppliers now propose a wide choice of sequences targeted toward particular pathways, and custom sequences are typically delivered within 2 months. Nevertheless, constraints due to chemical synthesis limit the choice of proteotypic peptides: peptides shorter than 15 amino acids should be preferred and chemically reactive residues (Tryptophane, Methionine, Cysteine...) or specific sequence patterns (Aspartate–Glycine, N-terminal Glutamine...) should be avoided. Even though production costs constantly decrease, when applied to large panels of proteins, the AQUA strategy can become expensive as AQUA peptides have to be synthesized, purified and accurately quantified one by one. Due to this technical and financial burden, proteins of interest have often been quantified with a single AQUA peptide [35,36]. Such restricted choice has to be cautious as a single peptide, even proteotypic, can correspond to different forms of the target protein (degradation products for example), especially in complex and active media such as plasma. The AQUA method is fast and straightforward to perform. AQUA standards are usually incorporated into the sample before the proteolysis step or, alternatively, right before LC-MS analysis [37]. Since the standard is added at late stages of the analytical process, the AQUA strategy is poorly compatible with sample prefractionation. Havlis et al. have shown that a simple SDS-PAGE decomplexification step before LC-MS analysis can dramatically affect the quantification accuracy of the AQUA method [38]. Moreover, since AQUA peptides are typically fully tryptic peptides, a verification of the completeness of target protein digestion is required to prevent quantification of the digested fraction only [39]. Finally, from our experience, solubilization and conservation of AQUA standards are peptide sequence-dependent and this can significantly impact measurements consistency [7,40]. We advise to store AQUA peptides at  $-80^{\circ}\text{C}$  and to carefully monitor their quality and concentration over time. To circumvent this kind of artefact, a “double exact matching isotope dilution MS” method has been proposed where the concentration of the protein is determined upon the concentration of AQUA peptides which are quantified in parallel upon amino acid standards [41].

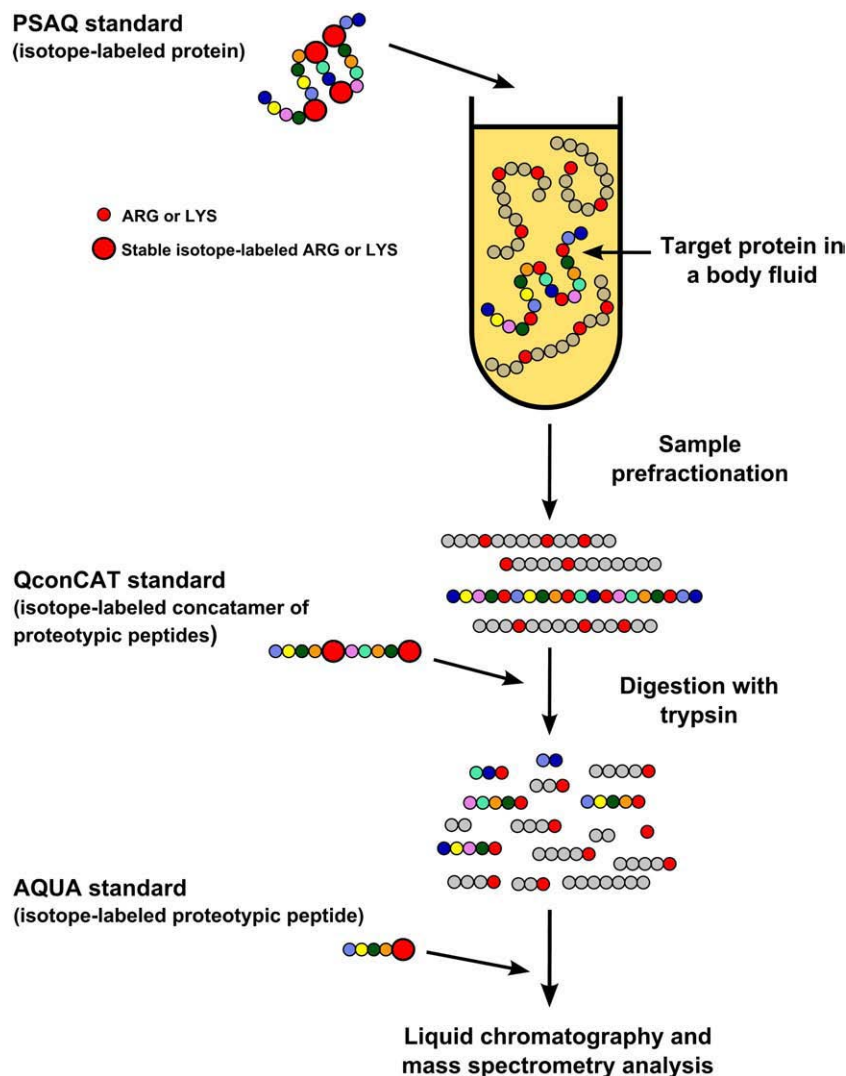
Two methodological improvements of the AQUA approach are worth particular mention. The first is the SISCAPA strategy (Stable Isotope Standards and Capture by Anti-Peptide Antibodies), in which an immunoaffinity step against a proteotypic peptide and its AQUA analog is introduced between the tryptic digestion and the LC-MS analysis [42]. As the internal standards are added prior to the immunoaffinity fractionation, they naturally take into account the recovery yield of this stage. The only remaining step that requires independent yield monitoring is thus the tryptic digestion. SISCAPA considerably increases detection sensitivity and is therefore particularly suited for the quantification of very low abundant

proteins in complex samples such as plasma. Another significant advance in AQUA strategy stemmed from the incorporation of covalent modifications such as phosphorylations into AQUA standards, opening the way to the study of enzyme regulation and signaling pathways [43].

In summary, the AQUA approach is suited for the quantification of proteins, provided that special care is exercised to assess the completeness of protein proteolysis. Moreover, if a prefractionation step precedes tryptic digestion, the recovery yield of the target protein(s) has to be evaluated. Presently, this approach is unique for the determination of phosphorylated/unphosphorylated forms of proteins [43] (Table 1).

## 5.2. QconCAT concatamers

In 2005, Beynon et al. introduced an original concept for large-scale protein absolute quantification [6] based on the synthesis and metabolic labeling of an artificial concatamer of proteotypic peptides (QconCAT for “quantification concatamer”) [44]. In contrast with AQUA peptides, QconCAT constructs are synthesized biologically, which expands the range of accessible proteotypic peptides (hydrophobic peptides, peptides with chemically reactive residues...). Peptide order as well as codon usage are crucial parameters to be optimized to minimize the occurrence of mRNA secondary structures



**Fig. 2** – Isotope-dilution strategies for targeted MS-based absolute quantification of proteins. Three types of internal standards are available for MS-based absolute quantification of a target protein in a biological sample such as a body fluid: (1) The PSAQ (“Protein Standard Absolute Quantification”) standard is an isotope-labeled version of the target protein which is directly added into the sample; (2) The QconCAT (“Quantification concatamer”) standard is a chimerical protein containing one/several isotope-labeled proteotypic peptide(s) of the target protein. This concatamer is added before the digestion step so that the standard peptide(s) is/are released in the sample; (3) The AQUA (“Absolute Quantification”) peptides are synthetic isotope-labeled copies of the target proteotypic peptides. They are generally added into the sample before LC-MS analysis.

**Table 1 – Strengths and limitations of isotope-dilution standards for MS-based absolute quantification of proteins.**

	AQUA peptides	QconCAT concatamers	PSAQ standards
Measurement accuracy	– to +++	– to +++	+++
Measurement precision	+++	+++	+++
Assessment of trypsin digestion completion	Necessary	Necessary	Needless <sup>a</sup>
Compatibility with sample prefractionation	–	–	+++ <sup>a</sup>
Discrimination for variants/isoforms	– to +	– to +	++
Study of protein complexes (partner stoichiometry)	– to +	+++	++
Quantification of post-translational modifications	+++ (Phosphorylations)	No	No

–: Poor; +: good; ++: very good; +++: excellent.  
<sup>a</sup> If the standard folds the same structure as the analyte.

and to maximize expression yield [40]. Concatamers, which can be home-made or obtained commercially, are generally added into the sample just before proteolysis. The isotope-labeled peptides are released by endoprotease cleavage and serve in LC–MS analysis as standards for target protein quantification. This methodology constitutes an ingenious and cost-effective means to increase the scale of protein quantification as 50 tryptic peptides can be included in a QconCAT construct [45]. The opportunity to include several proteotypic peptides of the same target protein in a QconCAT construct enhances the robustness of quantification [7]. For instance, Rivers et al. have successfully investigated the quantitative variations of 20 chicken skeletal muscle proteins during post-hatch growth with a single concatamer [45]. Anderson et al. have also applied this methodology to quantify major plasma proteins [46]. If target proteins display different concentration ranges (abundant vs. rare), it is possible to adjust the stoichiometry of proteotypic peptides in the QconCAT by incorporating multiple copies of selected sequences. Using such concatamers, Kito et al. have revealed the stoichiometry of the elongation factor eIF2B–eIF2 complex components in the budding yeast [47] and Nanavati et al. have recently investigated the stoichiometry of the rod photoreceptor transducin subunits [48]. Finally, one major advantage of the QconCAT strategy is that, once the QconCAT gene is cloned, the corresponding protein can be produced, labeled and quantified on demand.

Calibration of the QconCAT standard is a major concern which directly influences the accuracy of the final quantification. This calibration requires a highly purified QconCAT construct and was initially done by cysteine thiol titration [6]. However, thiol oxidation can interfere with this titration. We prefer the amino acid analysis (AAA), a reference method in bioanalysis, to accurately calibrate the standard.

In the context of staphylococcal enterotoxin quantification, we have proposed a special QconCAT design which allows the introduction of the standard before SDS-PAGE processing [7]. However, QconCAT concatamers behave in general quite poorly when combined with sample prefractionation [7]. Furthermore, as with AQUA peptides, the completeness of tryptic digestion has to be assessed for each protein target in order to achieve accurate quantification [45]. Due to their lack of folding, QconCAT constructs are typically digested at high rates. Accordingly, a differential sensitivity to proteolysis between QconCAT and target proteins has been

reported [45]. To avoid this type of bias and replicate the proteolysis yield of protein targets, Kito et al. have judiciously surrounded each proteotypic peptide in the concatamer with its native flanking sequences [47].

In summary, the QconCAT methodology possesses the main advantage of facilitating multiplex protein quantification. QconCAT concatamers are quantitative standards easily exchangeable between laboratories. Although hardly compatible with extensive sample prefractionation, QconCAT standardization is the method of choice for the determination of partner relative stoichiometry in protein complexes [48] (Table 1). “Thematic” QconCAT concatamers, targeting the enzymes of a particular metabolic pathway or enabling the control of instrument performances for peptide separation and analysis [49], also appear extremely promising.

### 5.3. PSAQ standards (full-length isotope-labeled proteins)

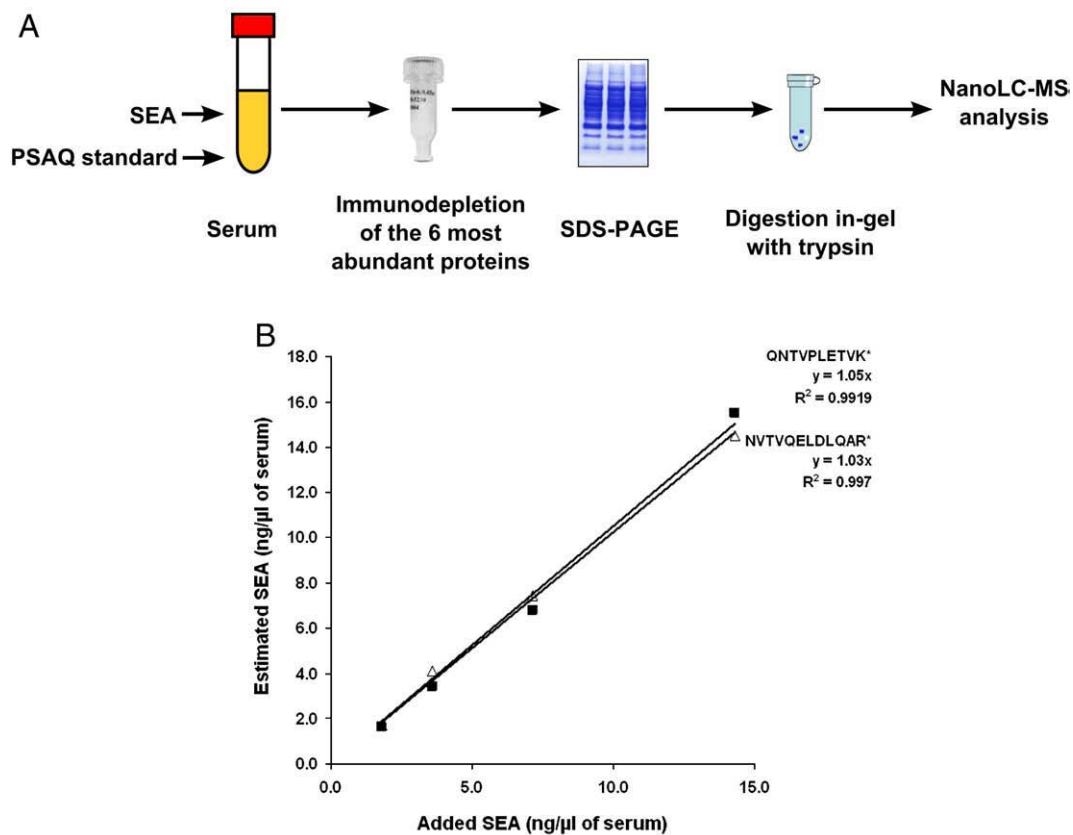
Considering the large protein dynamic range in biological samples and body fluids, sample prefractionation is often required for sensitive detection of low abundant proteins [50]. In this context, the ideal internal standard for absolute quantification of a specific protein should behave exactly like the target analyte, not only during the LC–MS analytical step, but also through all pre-analytical sample treatments. Therefore, an isotope-labeled equivalent of the full-length target protein appears to constitute the standard of choice. Such standards can be added in defined amounts at the very beginning of the analytical process. We recently provided a proof of concept of this approach by synthesizing full-length isotope-labeled staphylococcal toxins and demonstrated its advantages over existing strategies for absolute quantification of these warfare agents in water, urine and food samples [7,29]. This new quantification strategy, termed PSAQ for “Protein Standard Absolute Quantification”, was compared to AQUA and QconCAT standardizations. Our results showed that the PSAQ strategy was markedly more accurate than QconCAT and AQUA for the quantification of these toxins. This stems from two key advantages: first, PSAQ strategy prevents differences in digestion yields between the standard and the analyte, which is of particular importance since tryptic digestion is known to be rarely complete and partly unspecific [51]. This point has also been exposed in a recent report on alcohol dehydrogenase isoform quantification using an isotope-labeled protein standard [52]. Second, PSAQ is

compatible with any type of sample prefractionation provided that the “critical” biochemical properties involved in the partitioning process are shared by the recombinant standard and the natural target protein. Indeed, PSAQ was shown to be compatible with SDS-PAGE, protein hydrophobic capture [7] and immunocapture [29]. Additionally, to provide evidence for the compatibility of the PSAQ method with abundant proteins immunodepletion, we performed titration experiments of staphylococcal enterotoxin A (SEA), a major virulence factor implicated in toxic shock syndrome [53] in human serum. As SEA is not physiologically found in human serum, this fluid constitutes an analyte-free matrix, ideal to realize such a titration. Natural SEA was spiked into serum samples in increasing amounts and its PSAQ standard incorporated in defined quantity before immunodepletion of abundant serum proteins. Fig. 3A shows the detailed experimental procedure. We focused on the two most amenable proteotypic peptides for quantification. As shown in Fig. 3B, both peptides allowed the quantification of SEA with high accuracy. Furthermore, PSAQ offers the largest sequence coverage available for quantification (all detectable proteotypic peptides are considered) and consequently leads to robust quantification. Interestingly, if interferences, such as ionization competition,

prevent the detection of a proteotypic peptide in a complex matrix, PSAQ enables to switch to different reporter peptides. Along the same line, a given PSAQ standard can be used indifferently with trypsin or any other conventional endoprotease. To push this point further, it should be reminded that PSAQ standards also constitute the relevant quantification standards for “top-down” quantification trials [11,12]. Finally, thanks to the increased sequence coverage, isoforms and variants may also be distinguishable (Table 1) [29,52].

Here again, for accurate quantification, calibration of PSAQ standards is an important issue which is solved by AAA quantification of highly purified protein standards [7]. However, for any PSAQ standard, once a reliable absorption coefficient has been defined by AAA, quality controls can be routinely performed by simple UV spectrometry and SDS-PAGE.

PSAQ standards are typically produced using cell-free systems [7,54] or bacteria [52,54] and do not carry post-translational modifications. Nevertheless, numerous biomarkers of clinical interest belong to the tissue-leakage protein family [55]. Due to their intracellular origin, most of tissue-leakage biomarkers bear only labile modifications such as phosphorylations that can be easily shoved off. Regarding stable post-translational modifications such as glycosylations,



**Fig. 3 – Absolute quantification of staphylococcal enterotoxin A (SEA) in serum samples using PSAQ strategy. (3A) Experimental procedure to assess the compatibility of the PSAQ strategy with serum depletion. Serum samples were contaminated with SEA and spiked with a defined quantity of PSAQ standard (isotope-labeled SEA). The samples were depleted of the 6 most-abundant proteins using a MARS spin cartridge (Agilent Technologies) and were submitted to SDS-PAGE. Following in-gel digestion with trypsin, peptides were extracted and analyzed using nanoLC-MS. (3B) SEA quantification was derived from the extracted ion chromatograms of unlabeled/labeled peptide pairs (QNTVPLETVK and NVTVQELDLQAR peptide pairs). For both proteotypic peptide pair considered, the estimated SEA quantities in serum samples were plotted against the spiked quantities.**

they may be incorporated into PSAQ standards by means of dedicated expression systems.

Recently, the combination of a full-length isotope-labeled protein standard and Single Ion Monitoring (SIM) on a LTQ-Orbitrap, allowed the absolute quantification of minute amounts (150 attomol) of spiked maltose-binding protein in a HeLa whole cell lysate [54]. In addition, another PSAQ standard was used in this work for the determination of the per cell copy number of Grb2 (growth factor receptor-bound protein 2) in three different cell lines.

Finally, Heudi et al. have elegantly used the PSAQ strategy to measure the serum concentrations of a therapeutic monoclonal antibody [25]. Using an isotope-labeled monoclonal antibody as internal standard, they could accurately and precisely quantify the target antibody in spite of huge losses due to sample preparation (protein digestion and purification). A comparison with a reference enzyme-linked immunosorbent assay (ELISA) also revealed that the PSAQ approach allowed the determination of the total monoclonal antibody concentration present in the samples, whereas ELISA only measured the active or free monoclonal antibody fraction. This study undoubtedly emphasizes the advantages of using full-length isotope-labeled proteins for accurate quantification.

A current limitation of the PSAQ method is the cost and difficulty to produce protein standards. However, thousands of recombinant proteins have already been synthesized and purified in the framework of structural genomics. In a recent survey of structural biology initiatives [56], the overall reported production and purification success rates for bacterial and eukaryote proteins were respectively 30% and 19%. However, this field is moving fast and the so-called “Human Protein Factory” resource is reportedly able to produce individual recombinant proteins at a whole proteome scale [57]. Consequently, we propose to develop a community-based effort for the construction of large reference PSAQ standards libraries for protein biomarkers. This could extend the actual standardization initiatives of the proteomic community and would respond to demand of specific developing fields such as clinical proteomic [58].

In summary, PSAQ appears as a reference method for targeted absolute and accurate protein quantification in complex biological samples and body fluids. PSAQ already appears as the strategy of choice for MS-based evaluation of candidate protein biomarkers as well as for the pharmacokinetic study of therapeutic proteins [25].

## 6. Concluding remarks

All MS-based quantitative strategies in proteomics currently in use have demonstrated their ability to detect relevant biological differences. However, to date, the real and accurate confirmation of protein differential abundance does not typically rely on a MS-based approach. Immunoassays constitute the choice methodologies for targeted detection and quantification of proteins in complex matrices. Antibody arrays are emerging as interesting surrogates [59] but they are in their infancy and ELISA test is still considered as the reference assay. However, in numerous cases, immunological techniques display some limitations: (i) They depend on the

ability to produce antibodies; (ii) Due to a limited specificity, some antibodies often hardly discriminate tissue-specific isoforms or variants; (iii) Interferences are common when analyzing complex matrices, especially plasma; (iv) ELISA generally presents a limited quantification dynamic (2 logs) compared to MS methods such as SRM (4–5 logs). (v) In particular domains, such as the evaluation of biomarker candidates, the cost of antibody production and assay optimization can be prohibitive for further development, especially when dozens of biomarker candidates have to be evaluated [23]. In the same way, the development cost of an ELISA limits its interest for limited series of analyses [29]. In this context, we believe that MS-based targeted quantification strategies, which display an unrivaled specificity, a high sensitivity and allow multiplex absolute quantification with isotope-labeled internal standards, represent compelling alternatives to immunological approaches. On this regard, in order to gain recognition as valuable surrogates to immunological methods, MS-based absolute quantification methods should be carefully validated by titration of target proteins, preferably spiked in analyte-free matrices, and extensively evaluated for accuracy and precision [5].

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