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Absolute quantification of proteins using element mass spectrometry and generic standards

Alicia Jiménez Nosti, Laura Cid Barrio, Francisco Calderón Celis *, Ana Soldado, Jorge Ruiz Encinar *

Department of Physical and Analytical Chemistry, University of Oviedo, Oviedo, Spain

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ABSTRACT

Elemental mass spectrometry is a powerful analytical technique widely established in inorganic analysis. However, despite its quantitative capabilities, it is not yet fully integrated or considered in Life Sciences fields like proteomics. Whereas it is true that ICP-MS has suffered from several instrumental and analytical limitations that have hindered its applicability in protein analysis, significant developments during the last decades have turned ICP-MS into an interesting and, in our opinion, a powerful tool to consider for accurate protein quantification without recourse to specific protein standards. Herein we will try to discuss how these traditional limitations in ICP-MS have been overcome, what further improvements are yet necessary (some of which are shared with MS-based proteomics platforms) and enlighten some of the already existing and potential applications of ICP-MS in absolute quantitative proteomics.

Significance: ICP-MS has the potential to become a complementary tool to help molecular mass spectrometry cope with existing limitations, especially those related to standardization and accuracy, in the absolute proteomics field. It can provide absolute quantification of diverse proteoforms using a single generic compound containing sulfur and/or another target element (e.g., phosphorous). Moreover, its applications in quantitative proteomics are no longer limited to protein standards certification or quantification of simple or purified mixtures. Interestingly, absolute quantification of proteins using ICP-MS is favored when carried out at the intact level, making it very compatible with top-down proteomics approaches. Recent instrumental and methodological advances enable synergic combination of ICP-MS with stablished LC-MS proteomics methodologies, setting the basis for its implementation in quantitative proteomics workflows.

1. Quantitative proteomics

The comprehensive characterization of the proteome in a biological system for its study and understanding is incontestably relevant. Proteins play a pivotal role in a non-ending list of biological processes acting, for instance, as regulatory, control, enzymatic, or maintenance factors. Nonetheless, the proteome is dynamic, and on many occasions, changes in the concentration of different target proteins is a driving factor in controlling cellular functions. Thereby the determination of the identities of the proteins associated to the system under study must be complimented with the determination of total and individual protein levels.

In a biological system, proteins also interact with other molecules, either other proteins, or molecules like mRNA, lipids, or metabolites [1]. Variations in protein levels can be produced by or induce changes in these molecules, hence in regulatory networks, molecular pathways, or even cellular metabolism, because enzymatic abundances are controlled and determine metabolic fluxes. Determination of protein quantities is therefore necessary to characterize these dynamic cellular processes and understand their association with protein levels. In fact, the correlation of variation in protein levels to disease-associated processes highlights the relevance of protein quantification given the potential of proteins to be used as biomarkers for disease diagnosis or treatment.

Protein abundance cannot be inferred from mRNA levels though, there are other factors apart from gene expression regulation that influence protein levels, such as protein turnover, alternative RNA splicing, or post-translational modifications (PTMs). These processes result in changes of protein numbers, as well the formation of proteoforms with different biological functions [2,3].

Quantitative proteomics has mostly been based on the comparison of

* Corresponding authors.
E-mail addresses: calderonfrancisco@uniovi.es (F. Calderón Celis), ruizjorge@uniovi.es (J.R. Encinar).

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a proteome of the sample(s) under study related to a reference state [4], providing information in the form of protein fold-changes. However, it has become evident that the relative ratio determination of proteoforms has limited applicability. This is why paradigm in quantitative proteomics is no longer relative but absolute, driven by the improvement of instrumentation, protocols and data treatment tools and the growing requirements of the amount and quality of data acquired. Nowadays, absolute levels of proteins are increasingly required in the study of degradation rates or PTMs levels; stoichiometries in protein complexes; biomarker discovery; enzyme kinetics; and drug delivery [5]. Furthermore, multi-omics platforms, currently under development and aiming to understand and correlate large-scale (quantitative) genomic, transcriptomic, metabolomic and proteomic data to study biological systems, also demand protein absolute quantities.

2. Absolute quantification of proteins with mass spectrometry

2.1. Mass spectrometry at the peptide or intact protein level

Mass spectrometry is the technique of reference in proteomics given its great performance in protein analysis in terms of sensitivity, reproducibility, or versatility, among others. Nevertheless, despite the excellent capabilities of LC-MS approaches in protein characterization and identification even at the level of full proteomes [6], protein quantification is significantly more challenging. First and foremost, electrospray mass spectrometry is inherently non-quantitative, because ionization efficiency is affected by sample matrix, as well as by the molecular weight and charge state of the proteins [7].

Variability in ionization efficiency is, however, mitigated in the analysis of peptides. The enzymatic digestion of proteins prior to the analysis results in samples comprised of smaller compounds with homogeneous size distribution, and usually less than five charged states. Consequently, mass spectrometry proteomics has traditionally been biased towards the identification and quantification of proteins through their peptide digests (*bottom-up*). To do so, these approaches require the detection of a unique peptide that corresponds unequivocally to a protein. Absolute quantification strategies need the production and use of stable isotopically labeled (SIL) homologous of such peptides at known concentration [8]. The correlation of intensities in the LC-MS analysis of the target peptide and its SIL homologous results in the determination of the peptide absolute quantities, which could then be translated into protein concentration assuming a one-to-one peptide-to-protein ratio. Unfortunately, this is not always the case due to the lack of efficiency in the digestion process. Besides, the loss of modifications during the peptide preparation and the fact that many proteoforms share homologous sequence regions, can finally result in misleading quantitative results and loss of information on chemical modifications in the proteoforms [7]. Consequently, characterization and quantification of proteoforms is severely limited in *bottom-up* approaches, highlighting the need to address protein quantification with MS at the intact protein level (*top-down*).

Top-down approaches forgo enzymatic digestion, avoiding errors related to digestion efficiency, simplifying sample preparation, and preserving structural integrity of the proteoforms, including PTMs. However, it cannot be obviated that *bottom-up* approaches have not been the method of reference in MS-based proteomics without reason. Although *top-down* MS has become the preferred strategy for proteoforms characterization, there are still several factors that considerably limit its applicability in quantitative proteomics. On the one hand, the development of new sample preparation protocols for intact protein analysis. In this context, proteome diversity and dynamism result in great protein variability within a proteome, demanding for adequate, sample- and MS-compatible protocols for protein solubility, extraction, enrichment, precipitation, or filtration, to improve MS *top-down* proteomics performance [9,10]. On the other hand, proteoforms analysis with LC-MS is conditioned by both lack of resolution in the chromatographic separation and poor detection with the mass spectrometers due to the complexity of the measurement of large molecules that results in low S/N ratio [11], and/or the low abundance of target proteoforms, whitchitch is exacerbated when several species co-elute. Data analysis is also a limiting factor due to the complexity and overlapping of MS and fragmented MS2 data, which is again accentuated by chromatographic co-elution. This results in the need for improved separation resolution and higher resolving power and sensitivity at the chromatographic and mass spectrometric levels, respectively.

2.2. Absolute quantification strategies in top-down proteomics

As already mentioned, mass spectrometry is inherently non-quantitative, hence any variation in protein structure, conformation or composition, as well as compounds co-elution (resulting in competition for the available charges) or differences in sample matrix at the time of ionization, can result in very different ionization efficiencies. Moreover, because electrospray MS ionization depends on molar concentration rather than mass concentration, S/N is inversely proportional to proteoform mass, resulting in lower ionization efficiency in high-molecular weight proteoforms. Such limitations are even more accentuated when doing quantification analysis despite the recent developments in the analysis of intact proteins. Lack of reproducibility, low S/N ratio, proteoforms co-elution, etc. are a significant wall to overcome in the way to achieve robust and reliable absolute quantitative strategies in *top-down* proteomics.

Current absolute quantitative strategies in *top-down* MS proteomics can be divided into labeled and *label-free*. Like peptide approaches, stable isotope-labeled (SIL) protein homologue can be used in MS *top-down* quantification as quantification standard. Absolute quantification is carried out through the MS ratio of the target protein and its SIL homologue [5]. Although quantification with these approaches is accurate and precise, the requirement of the synthesis and characterization of each SIL standard for each target protein seems unpractical, especially at the large-scale proteome level. Therefore, these quantification approaches are limited to the quantification of few predefined proteins. In contrast, the so-called *label-free* approaches have become the trend in large-scale quantitative proteomics. They are based on the correlation of spectral counting along several LC-MS runs aligned with software computation. From statistical analysis of the compared features, differences in protein abundances between two different states can be inferred.

Absolute quantification with *label-free* MS can be addressed based on the interpolation of each protein MS intensity with a model created with protein standards, assuming direct correlation between intensity vs concentration or between signals of target and standard proteins, assuming in turn that MS signal ratio reflects protein abundance [12,13]. It must be noted that this data model created, and the quantitative reference used, will define the quality of the quantitative results acquired. Furthermore, other parameters such as the variability in sample preparation, retention time shifts, loss of signals occurrence between replicates, differences in ionization efficiency between proteins that are not considered in the statistical models and the method linear range, will have to be addressed and corrected [14]. Some of instrumental issues in *label-free* quantification can be solved with the use of internal or SIL standards to control sample preparation, LC-MS performance, and improve the quality of the quantitative data [5,14,15].

It seems thus clear that although one of the preferred ways to go in MS absolute quantitative proteomics are *top-down* approaches, analytical platforms and methodologies are yet unable to provide reproducible, accurate and precise data in non-specific proteomic studies. Despite further instrumental and methodological improvements, non-quantitative nature of electrospray MS can just be mitigated or in some cases corrected, but not changed. Limitations in MS quantitative proteomics could be alternatively overcome by combining MS-based proteoforms identification and characterization with other

standardization or quantification approaches.

In this regard, inductively coupled plasma (ICP)-MS has shown great potential to be considered for integration in quantitative proteomics workflows due to its versatility, quantitative capabilities and easiness to combine with molecular MS, especially after recent instrumental and methodological advances [16]. Hereafter we will describe the role and current applicability of ICP-MS in protein analysis and the instrumental and methodological advances that have recently occurred, and need yet to occur, for implementing and integrating ICP-MS in MS-based absolute quantitative proteomics workflows.

3. ICP-MS, the missing gear in MS-based absolute quantitative proteomics?

3.1. Sulfur is the way to generic quantification

ICP-MS is a mass spectrometry-based technique in which the ionization occurs in an atmospheric pressure high-temperature argon plasma. The molecules are converted into atoms which are ionized, and the signal obtained corresponds to the m/z of said atoms. Operated under optimum conditions, this signal can be made independent on the molecule itself. Thus, ICP-MS signal of the detected element present in molecules of different size, conformation or nature shall be the same when being in equal elemental mass concentration. Unfortunately, the main constituent elements of proteins (C, H, N, O) cannot be adequately measured with ICP-MS due to their high ionization potentials and their enormous background signals, because ionization takes place at atmospheric pressure and this implies that concentration in the plasma of elements forming the gases molecules (N_2, O_2, CO_2, H_2O) present in the environment is extremely high.

As can be seen in Fig. 1, protein quantification with ICP-MS can be addressed through the determination of any ICP-detectable element associated with the protein, such as (i) coordinated metallic elements (Zn, Fe, Cu, etc.) in metalloproteins; (ii) elements present or bonded to special amino acid residues (Se-Met, Se-Cys); (iii) elements present in chemical modifications of the protein (P, I, As), (iv) elements present in complexes or chelating tags directly bonded to the aminoacid sequence; or (v) elements present in nanostructures or chelators, bonded to antibodies that are in turn immunotagged to the protein. In all these cases, applicability is conditioned to the presence of such elements, or the specificity and performance of tagging processes though. Therefore, none of them can be considered as a generic strategy that can be used to quantify in absolute terms all the proteins present in a sample, much less at the proteome level.

Nonetheless, when instrumental developments in ICP-MS (commented in the following section) enabled highly sensitive determination of sulfur [17], the paradigm changed. Most of proteins and polypeptides contain at least one methionine or cysteine, hence they contain sulfur atoms within their structure. Consequently, sulfur can be used as generic target to quantify all proteins present in a sample. The species-independent character of ICP-MS implies that the sulfur response obtained for all the proteoforms in the sample chromatographically separated, will be proportional exclusively to their sulfur mass concentration. It must be highlighted though that ICP-MS provides just elemental quantification. To translate elemental quantities into protein abundance, the stoichiometry sulfur-to-protein, i.e., the number of Met and Cys in the amino acid sequence of the protein, is essential. Thereby ICP-MS needs to be combined with MS protein identification and sequencing to achieve absolute protein quantification when the identity of the target protein is not known beforehand.

Besides being the cornerstone of the generic applicability of ICP-MS in absolute protein quantification, sulfur quantification is also at the basis for the characterization of chemical modifications or proteins complexes or conjugates. Since, ICP-MS is multi-elemental detector, any ICP-detectable element present in the chemical moiety of the modification under study can be simultaneously quantified together with sulfur. Therefore, target element-to-protein stoichiometry, which previously required the translation of the ICP-MS sulfur quantification into protein quantification [18], can be directly determined nowadays through the determination of the element-to-sulfur molar ratio in the corresponding chromatographic peak of the LC-ICP-MS analysis. The high accuracy and precision of ICP-MS quantification can be applied to

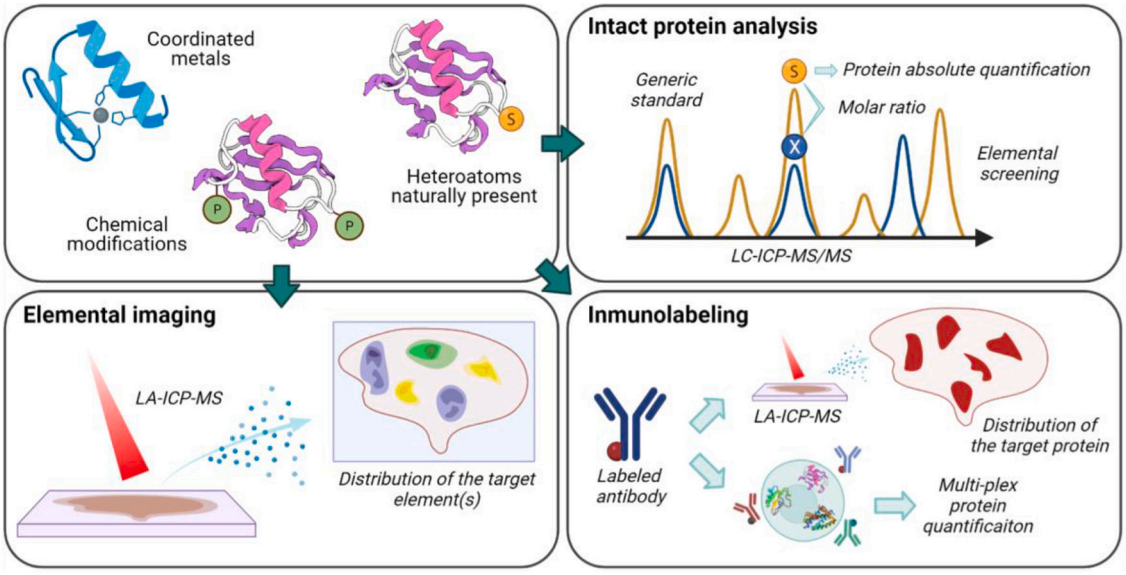


Fig. 1. Schematic of ICP-MS-based approaches in quantitative proteomics. ICP-MS protein quantification can be addressed through elements covalently bonded present in the amino acid sequence like S (Cys, Met); elements present in functional groups added to the protein with chemical modifications like P (phosphorylation); or metallic elements coordinated to the protein. Absolute quantification of intact proteins can be carried out with LC coupled with ICP-MS/MS, through the detection of naturally present sulfur in proteins, using a generic standard injected before the analysis. This analysis also allows to quantify the stoichiometry of one or more other elements (X) present in the protein (e.g., phosphorylation degree), using a generic standard for each of the target elements. When the sample is solid, like a biological tissue, quantitative distribution of metals detectable with ICP-MS can be done by coupling with laser ablation. Similarly, target quantification of certain proteins can be addressed by means of using specific antibodies labeled with a tag that contains an ICP-MS-detectable element. Adapted from [16].

the evaluation of small spatial or temporal changes in such elemental molar ratio, i.e., in the chemical modification (PTM), complexation or conjugation stoichiometry.

3.2. *Absolute quantification of proteins with ICP-MS*

Protein absolute quantification with ICP-MS requires standardization to correlate sulfur signal into concentration. Advantage of ICP-MS-based quantification respect to ESI-MS is that ICP-MS ionization is

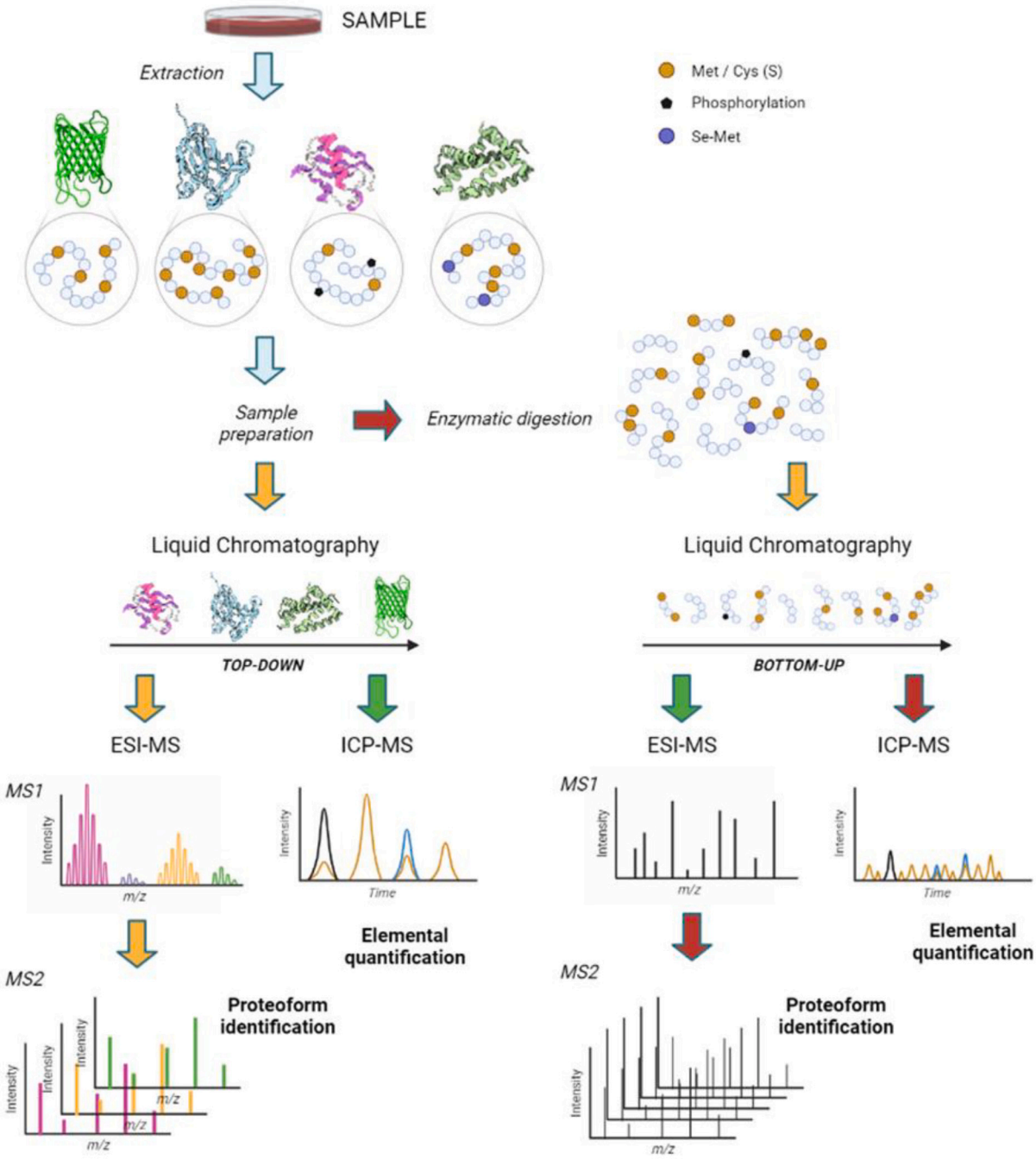


Fig. 2. Schematic workflows of *top-down* and *bottom-up* absolute quantitative proteomics combining elemental and molecular mass spectrometry. Arrow colour is a comparison indicative (favored in green, disfavored in red, and improvable in yellow) between both approaches. 1) Enzymatic digestion is a clear limitation in *bottom-up* approaches because efficiency of the digestion will compromise quality of quantitative results. 2) In both approaches, liquid chromatography step has its downsides. Whereas in *top-down* the separation of big molecules could result in non-quantitative chromatographic recovery, requiring for its evaluation and optimization; the higher number of compounds in *bottom-up* has higher demand from chromatographic resolution. Of course, chromatographic resolution is an issue to address in both MS types but far more critical for ICP-MS. 3) ESI-MS S/N ratio decreases with the molecular weight of the compounds; this way ionization efficiency is higher for peptides than proteins. 4) This fact contrasts with ICP-MS, because it is a mass concentration detector, so that sensitivity is lower in the analysis case of peptide digests because the ICP-detectable elements of the protein (i.e. S, P) are distributed among different species. 5). In the case of MS2 analysis, *top-down* approaches are conditioned by the need of high-end instrumentation capable of fragmenting intact proteins. On the other hand, the higher complexity of the analysis in *bottom-up* due to the bigger number of co-eluting compounds analyzed results in a more complex analysis and data management. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

species-independent. ICP-MS response is directly proportional to the mass concentration of the detected element, and independent of the protein concentration, structure, charge state or molecular weight. As a consequence, sulfur concentration corresponding to a protein or a mixture of proteins, can be determined using just one single generic standard that contains sulfur at known concentration (e.g., inorganic sulfur, small organic compounds) (see Fig. 1).

Elemental isotopes detection is doable with ICP-MS. In the case of study of those elements that have more than one stable isotope, as it is the case of sulfur, quantification can be addressed with isotope dilution analysis (IDA) using a single non-specific isotopically enriched standard (e.g., ^{34}S-enriched sulfate) that is added continuously post-column [19]. Quantification is then carried out based on the ^{32}S (natural, present in target protein) to ^{34}S (enriched, present in the standard) isotope ratio measured. Of course, the measurement of such isotope ratio implies the correction of any signal sensitivity variation as the enriched isotope acts as the ideal internal standard. In the case of monoisotopic elements (P, As, I, etc.), isotope dilution analysis is not feasible and internal or external standardization approaches making use of non-specific compounds containing the target element are required [20]. These approaches have been traditionally more limited than IDA in terms of precision, robustness, and are more influenced by matrix and signal instabilities.

Protein absolute quantification with ICP-MS can be carried out at both peptide and intact protein level, being potentially compatible with *bottom-up* and *top-down* MS platforms (Fig. 2). However, ICP-MS actual applicability is severely limited in *bottom-up*, hence the low implementation or consideration of the technique in traditional quantitative proteomics so far. ICP-MS *bottom-up* protein quantification is based on the quantification of the resulting sulfur-containing peptide and correlating peptide to protein concentration assuming complete enzymatic digestion [21]. As such, protein quantification is conditioned to the presence of any of its constituent peptides that contain sulfur (or another ICP-detectable element). If the peptides carrying those elements were not observed or were loss during sample preparation or enzymatic digestion, protein quantification would not possible. In contrast to MS-based quantitative proteomics, chromatographic resolution is essential to obtain high quality quantitative results. Whereas ESI-MS detection has a second resolution level arising from the different *m/z* of co-eluting compounds, ICP-MS cannot discriminate sulfur atoms from different co-eluting compounds. Of course, resolution requirement becomes more challenging when analyzing tryptic digest than intact proteins. Another limitation in ICP-MS peptide quantification is the distribution of the detectable elements of the protein among several species. Given that ICP-MS is a mass concentration detector, the higher the number of elements detected in the compound, the higher the sensitivity. In peptide digests, sulfur atoms of the protein are split in several peptides, hence their S/N ratio is lower than if the intact protein, containing all the elements, would be detected.

Parallel to MS quantitative proteomics, recent instrumental and methodological developments in ICP-MS have shifted its focus towards intact protein level (*top-down*) absolute quantification. ICP-MS-based intact absolute protein quantification offers several advantages in terms of sensitivity (higher number of detected atoms), resolution (a smaller number of species to be separated) and characterization of modifications of the protein that carry other detectable elements (PTMs), compared to *bottom-up* approaches (Fig. 2). Of course, as it is the case with *top-down* MS quantitative proteomics, there are several factors to consider for assuring quality of quantitative results, like protein-column unspecific interactions that hinder complete chromatographic elution of proteins, or the separation to baseline level of all proteins to be quantified, particularly in the case of complex samples with tens or hundreds of proteins. Large-scale quantitative proteomics is still not achievable with ICP-MS, but likewise to *top-down* proteomics, developments in terms of LC performance, combining enhanced LC, multi-mode and/or multi-dimensional LC could mitigate these limitations.

4. Instrumental and methodological advances in ICP-MS: does it meet the requisites for quantitative proteomics?

Several instrumental and analytical issues have been faced over time in order to turn LC-ICP-MS into a viable alternative for absolute protein quantification. These include interference-free sulfur determination, protein chromatographic recovery and resolution and correction of the effect of matrix and analyte nature in transport and ionization processes. They all will be described in the following sections.

4.1. Tandem mass analyzers for sulfur spectral interferences correction

ICP-MS analyzers produce elemental ions and serve as ion guides and filters, removing not only any unwanted ions of different *m/z* than the target one but any other polyatomic ion (interference) that shares the same m/z as well. In this sense, there are several non-metallic elements of huge interest in bioanalysis, including sulfur, phosphorus and selenium whose ICP-MS detection has been severely hindered because of their high ionization potential and especially for being highly interfered. This resulted in very poor sensitivity. To reduce these interferences, several analyzer systems were developed (collision/reaction cells, sector-field, etc.). However, detection limits (LOD) traditionally achieved using such approaches were still insufficient for biological applications.

In 2012 tandem mass analyzers were introduced into ICP instruments [17], bringing with them a more efficient removal of spectral interferences in the detection of high interfered elements such sulfur, phosphorous or selenium. Tandem ICP-MS/MS instruments are equipped with a collision cell located between two quadrupole mass analyzers providing two individual mass selection steps. This brings a considerable control over the ion/molecule chemistry inside the reaction cell that allows to discriminate better between ions and polyatomic ions (and even isotopes of different elements) of the same *m/z*. In the specific case of S, first quadrupole (Q1) works as 1 amu window band-pass mass filter selecting targeted S isotope (i.e., ^{32}S) and its polyatomic isobaric interferences. Much higher reaction rate with O_2 in the cell separates sulfur ions and polyatomic interferences by shifting them to new product ion masses. This leads to interference-free detection S after setting the second quadrupole (Q2) at 16 mass units higher than Q1, 48 ($^{32}SO^+$). This is the second specificity level where polyatomic interferences at nominal masses of S ($^{16}O_2^+$, $^{15}N^{17}O^+$, $^{15}N^{16}OH^+$, $^{14}N^{18}O^+$) are removed. Notably, setting Q1 at *m/z* 32 ensures that all the product ions analyzed in Q2 are derived from the selected precursor ion exclusively because ions and polyatomics likely interfering product ions at m/z 48 ($^{48}Ti^+$, $^{36}Ar^{12}C^+$, $^{31}P^{16}OH^+$, $^{31}P^{17}O^+$, $^{48}Ca^+$) have been previously removed leading to very low background levels.

Interestingly, versatility of the cells with the use of different gases and combination of gases [22], makes it possible to further improve S/N in the analysis of one or more biologically interesting elements simultaneously during quantitative analysis of proteins. Such efficient interference removal has resulted in the lowest detection limits ever reported for the LC-ICP-MS analysis of S- and P-containing species (low fmol range) [17].

4.2. Species-independent transport and nebulization processes

ICP-MS signal is widely assumed as species and matrix independent. In fact, despite sample nebulization and transport efficiency in ICP-MS is around 10% [23], this low efficiency is assumed likewise for all and any compound independently of their size or chemical nature. This fact has been controversial when quantifying with non-specific standards, particularly in the case of large molecules like proteins. However, existing limitations so far in sulfur analysis by standard ICP-MS prevented to confidently evaluate this issue in intact protein analysis. Of course, if the elemental response factor obtained for proteins and generic standards is proved to differ in determined conditions, species-

independent calibration in ICP-MS could be seriously compromised.

Based on this premise, Cid et al. carried out a critical comparison between S response factors for 14 different relevant S-containing biomolecules (three peptides, four proteins, one amino acid, two cofactors, three polyethylene glycol (PEG) derivatives, and sulfate standard), covering a wide range of hydrophobicity and molecular size. Two regular flow nebulizers and a nano/micro-flow total consumption nebulizer (TCN) were tested [24]. ICP-MS response factors for S in each compound were calculated with calibration curves. Furthermore, online IDA was used to normalize signals, minimize signal drifts and matrix effects. No statistical differences were found for low-molecular-weight biocompounds, PEGs, and non-hydrophobic peptides using any of the nebulizers tested. However, while statistical differences were still found negligible (96–104%) for the proteins (cytochrome C, β-casein, bovine serum albumin and monoclonal antibody of 12.5, 23.5, 66 and 145 kDa, respectively) and the hydrophobic peptide using the TCN, significantly lower response factors (87–40%) were obtained using regular flow nebulizers in comparison to those obtained for the inorganic sulfur standard used as reference, as shown in Fig. 3. This differential behavior was ascribed mostly to the chemical nature of the target biomolecule (i.e., hydrophobicity), which is related to the amino acid composition and potential presence of PTMs, and partially influenced by molecular weight. Notably, since the use of a TCN led to identical response factors for all species under study, reasons behind the differential behavior obtained using the regular flow nebulizers, such as discrepancies in the ionization efficiency, protein solubility, or errors in the certification of the concentration of the biomolecules solutions used, could be ruled out leaving nothing but species-dependent nebulization efficiency as the sole cause. Results obtained clearly showed that species-independent quantification of intact proteins using ICP-MS cannot be carried out unless a TCN is used.

Interestingly, this differential behavior observed for proteins using regular flow nebulizers was demonstrated to be independent of the ICP-MS detectable element used to monitor the protein species. As an example, in the case of the phosphorylated protein β-casein, statistically identical behavior was observed when monitoring both sulfur and phosphorous (see Fig. 3).

4.3. *Matrix-independent ionization processes: carbon effect along chromatographic gradients*

ICP-MS absolute quantification of proteins with generic standards requires constant response factor in sulfur detection along the whole analysis unless IDA is used. That way, protein concentration can be inferred from the S concentration determined using the S response factor obtained for the generic S-containing species that elutes elsewhere. Nevertheless, the physicochemical processes occurring in the plasma during RP or HILIC chromatographic analysis, due to the significant change of the carbon concentration along gradients of organic solvents, result in great changes in the sulfur response factor. The magnitude of this change varies with the amount of carbon and has been demonstrated to be element dependent [20].

Therefore, correction of such signal variations must be procured for ICP-MS absolute protein quantification without specific standards. Several strategies have been developed with this purpose [20,25], being the use of a post-column flow of an enriched isotope of the same heteroatom which is being monitored the most established one. However, such online isotope dilution analysis (IDA) requires for an enriched isotope for each target element, which is sometimes very expensive, and of course it is forbidden for interesting monoisotopic elements (P, As, I).

Alternatively, direct addition of carbon-containing gases (methane and carbon dioxide) to the plasma ICP has been recently explored as a means of attaining negligible elemental signal variations during HPLC gradients for the studied non-metallic elements, all of them relevant and potentially bound to protein (S, P, As, I and Se) [26,27]. Notably, such universal approach can be simultaneously applied to every element under study (including monoisotopics) opening the door to the assessment of the stoichiometries element (P, Se) to protein (S). In fact, not only signal variations during HPLC gradients were corrected, but also more than 2-fold S/N ratio enhancement was achieved for S in

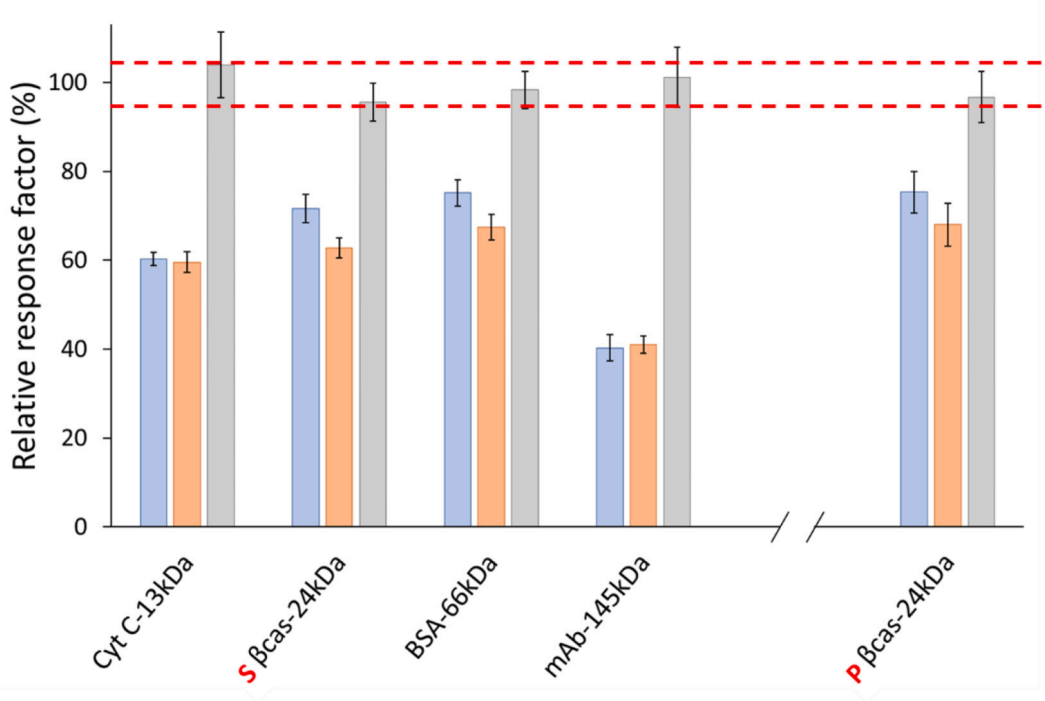


Fig. 3. Relative response factors (%, ratio between response factor of the individual proteins and the inorganic S standard) obtained for protein species using regular flow nebulizers, a concentric (blue) and a cross flow (orange), and a total consumption nebulizer (grey). Adapted from [24]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

comparison to the IDA-based approach.

4.4. Enhanced chromatography for intact proteins analysis

Loss of molecular information during ICP-MS atomization and ionization processes demands for previous chromatographic isolation to provide accurate quantification of all the proteoforms in the sample. In this regard, adequation of LC-ICP-MS to *top-down* quantitative proteomics has faced two main challenges. On the one hand, selection and optimization of the instrumentation and methodologies to be fully compatible with ESI-MS. On the other hand, improvement of chromatographic for intact (native) proteins in terms of column recovery and separation efficiency.

Typical ICP-MS sample introduction systems were not suitable to capillary and nano LC, traditionally used in ESI-based proteomics workflows. The capillary HPLC techniques were incompatible with ICP-MS because of the flow rates being 100–1000 times lower than those (0.5–1 mL min^{-1}) required by conventional nebulizers. Also, the large volume of the most-commonly used spray chambers resulted in significant washout times and peak broadening. Last but not least, methanol or acetonitrile added to the mobile phase in reversed-phase and HILIC modes negatively affects the ICP stability leading to carbon effect (see previous section), dramatic decreases in signal intensity (even plasma extinction) and carbon deposition on the cones. For this purpose, systems to remove the solvent vapor based on membrane desolvators or cooled spray chambers were developed [28], but unfortunately resulted in peak broadening and thus in loss of chromatographic resolution. Therefore, it was necessary to develop a dedicated interface between capillary HPLC and ICP-MS based on a high-efficiency low-sample-consumption zero-dead volume sample introduction system. The introduction of micro nebulizers thus enabled the analysis of lower sample volumes, to hold better chromatographic resolution, enhanced LC performance in the ICP-MS analysis of biological samples and allowed the direct introduction of high concentrations of organic solvents (up to 100%) without adverse effects [29]. At the same time, it opened the door to the dual elemental and molecular MS detection in HPLC analysis of proteins [30].

It is well established that the protein mass eluted from a chromatographic column is usually not quantitative due to protein non-specific interactions with the stationary phase on column separations [31]. This is especially relevant if quantification is accomplished directly from the chromatographic peak without any SIL standard, as the column recovery factor will have a direct influence on the calculated protein concentration. Therefore, it is necessary either to calculate column recovery and ensure its reproducibility along time or to demonstrate that chromatographic conditions ensure quantitative recovery from the column for any protein species. Of course, the first option demands for specific standards. In contrast, the fulfilment of the latter premise hence implies that recovery does not have to be calculated for the individual protein species under analysis, enabling a more generic applicability of the methodology. In this context, instrumental developments in LC column packing composition and chemistry have significantly increased LC performance for large molecules chromatographic analysis. Particularly relevant is the use of core-shell particles that consist of a solid silica core surrounded by a porous shell [32]. Such new core-shell column packing has been demonstrated to provide quantitative recoveries for both mixtures of protein standards and real venom samples (containing 25–40 protein species) without resorting to specific standards [33,34]. It is interesting to note here that the species independent response and signal stability along gradient elution allows easy computation of the protein recovery even for complex samples by direct comparison of their flow injection (FIA) and chromatographic analyses. In FIA, the samples are injected into a flowing carrier stream under conditions where no HPLC separation occurred, and the whole protein set is detected as a single transient signal of S at the detector. The total sulfur mass computed in the mass flow FIAgram and the mass flow chromatogram (sum of individual peak areas) can thus be directly compared for column recovery computation.

Despite these developments, ICP-MS based quantification approaches are still constrained to simple protein mixtures where full baseline resolution is still achievable. In this regard, it is evident that the universal and generic capLC-ICP-MS/MS approach described herein will profit from ongoing and future efforts and chromatographic developments in the *top-down* proteomics field [35,36].

5. ICP-MS applications in quantitative proteomics

The developments of new instrumental and methodological tools in LC-ICP-MS described above have implied a turning over in its traditional fields of application moving towards Life Sciences (including proteomics) with a lot of possibilities to be explored and exploited.

5.1. Certification of protein standards

As previously mentioned, absolute quantification of proteins with molecular MS requires standardization, either in labeled strategies that make use of specific synthetic labeled protein analogous or in label-free approaches to control sample preparation and analysis variability. Therefore, to assure high-quality quantitative results, the standards need to be adequately characterized in terms of composition and concentration. Most common strategies to assess the concentration of peptide and protein standards (e.g. amino acid analysis, AAA) or chemiluminiscent nitrogen detection, CNL), are nonetheless hindered by poor selectivity, specificity and robustness [5].

LC-ICP-MS turns out as an interesting alternative for the certification of protein standards, given its capacity to quantify the target compound using any certified reference that contains the detected element present in the compound as standard for quantification. That way absolute quantitative results obtained are directly traceable to a simple certified generic standard. There are several works proving capabilities of ICP-MS for the absolute quantification of intact proteins through containing elements like phosphorous or coordinated metals [37,38], and even through the introduction of labels containing ICP-detectable elements [39]. However, these strategies are very specific for some protein families or are conditioned by reactivity efficiency; hence they cannot be considered generic strategies for protein certification. In contrast, protein quantification using ICP-MS sulfur analysis can be considered so, because cysteine and methionine residues are naturally present and their accumulative abundance in proteins is high (around 5%).

In this context, once adequate chromatography with complete intact protein recovery and total consumption nebulizers are selected, and sensitivity changes along chromatographic gradients corrected, mass purity certification (protein mass per mass of sample) of protein standards becomes feasible. Internal or external calibration strategies can be carried out. In the case of internal standardization, a S-containing compound is spiked to the protein sample. However, selection of the adequate standard, though generic, must follow some chromatographic criteria like separation resolution and chromatographic retention. In the case of external standardization, replicates of a sulfur generic standard are injected separately by capillary Flow Injection Analysis (capFIA) to compute the S response factor that will be later applied in the sample analysis. In this case, any S-containing compound can be used, even small inorganic certified compounds, like sulfate solution. Table 1 collects the results obtained for three protein standards, Bovine Serum Albumin, Cytochrome C and Transferrin [27]. As can be seen, excellent agreement with the values provided by the manufacturer were obtained. Of course, as element response factor is obtained in external calibration from replicate injections, every individual analysis comes with associated uncertainty. Therefore, more reliable combined uncertainty for the final sample analysis could be obtained. Surprisingly, such combined uncertainty (~ 3% RSD) is not significantly higher than uncertainty from internal standardization (~ 1–2% RSD).

Table 1
Certification of mass purity for different protein standards using LC-ICP-MS absolute quantification, generic standards, and different quantification strategies. Uncertainty corresponds to 1 standard deviation.

Protein	Quantification strategy		Theoretical value*
	Internal calibration	External calibration	
Cyt C	92 ± 1	96 ± 4	≥ 95%
BSA	99 ± 2	97 ± 3	≥ 98%
Transferrin	95 ± 1	93 ± 3	≥ 95%

* Value provided by manufacturer.

It seems clear that the application of ICP-MS in proteomics with the greatest potential is the certification of protein standards. In fact, the proved capability to quantify protein mass purity for a series of standards, even when in presence of other by side proteins or proteoforms can be extremely helpful in biopharmaceutical research (e.g., antibody certification). Of course, it can be also useful to improve quality of standards used for quantification in MS-based quantitative proteomics. In fact, using this application as starting point, standardization platforms, quantitative references or even mathematical models could be improved in label-free proteomics by combination with single protein or total protein content determination with ICP-MS.

5.2. Application to real sample analysis: the case of snake proteomics (venomics)

The applicability of LC-ICP-MS in quantitative protein analysis has been further validated with its integration in proteomics studies for simple proteomes characterization and quantification. That was the case of snake venom proteomes quantification (quantitative *venomics*). Particularity of snake venoms is that they are relatively simple as usually less than 50 toxins have been characterized per venom, and they are mostly comprised by low-molecular weight toxins (≤ 50 KDa), such as finger toxins, phospholipases, or metalloproteinases [40].

The most extended methodology in quantitative venomics is the relative determination of toxin families through HPLC-UV, by measuring the absorbance wavelength of the peptide bond, 215–220 nm. Hence the % of total peptide bond concentration in the peak provides a relative amount of toxins by weight venom [41]. This strategy is nonetheless conditioned by the likely UV absorbance contribution of some amino acid residues besides being limited to provide relative quantification of toxins by family. Incorporation of LC-ICP-MS/MS sulfur-based absolute quantification of proteoforms using a single non-specific standard, combined with parallel MS analysis (thanks to compatible capLC conditions) for characterization and identification of the venom proteoforms, resulted in the absolute quantitative characterization of the tens of proteoforms present in snake venoms (Fig. 4).

ICP-MS use in quantitative venomics was firstly validated with the quantification of toxins present in *Naja mossambica* model characterized venom [33]. Sulfur absolute quantification was achieved using ^{34}S-IDA with internal standardization, assuring quantitative overall protein recovery from the LC system. Micro-LC elution profiles of ICP-MS and ESI-QToF could be overlapped to identify constituent toxins in the venom, resulting in the identification and absolute quantification (μmol protein / g venom) of 27 proteoforms. This quantitative strategy was used later for proteomes quantitative characterization of snakes *Naja nigricollis*, *Micropechis ikaheka* and *Pseudechis papuanus* [34].

Newly developed strategy based on the addition of a C-containing gas to the plasma resulted in enhanced absolute quantification of venom proteins without isotopically enriched standards [27]. Recent combination of LC-ICP-MS/MS quantification with *top-down* identification of proteoforms in venom provided similar results that other obtained using established quantitative strategies for the quantitative characterization of proteomes of three *Walterinnesia* snakes [42]. Future direction leads clearly to methodological and instrumentation improvements of chromatographic performance to allow separation and absolute quantification of hundreds of proteoforms.

5.3. Quantification of post-translational modifications

There are several protein modifications that imply the incorporation into the protein of a chemical group carrying an ICP-detectable element. That is the case of phosphorylation, one of the most preponderant and well-studied PTMs in proteins [43]. Phosphorylation is a dynamic process and phosphorylation degree of the protein (number of phosphates groups per protein molecule) is variable. Therefore, understanding and characterization of metabolic pathways and processes in which phosphorylation is involved require locating phosphorylation sites and accurate and precise determination of the phosphorylation degree, and the small variations it can suffer [44].

Quantitative characterization of protein phosphorylation by

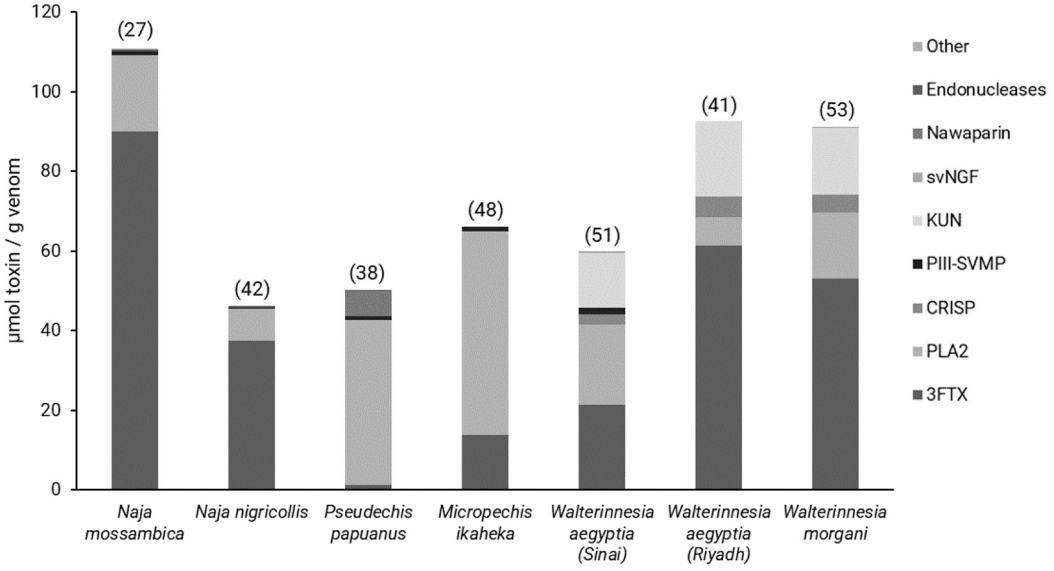


Fig. 4. Results obtained from the absolute quantification of toxins in snake venoms, plotting proteins mass concentration grouped by protein families. The number in brackets corresponds to the total number of proteoforms both identified and quantified in each sample. Results adapted from [34,42].

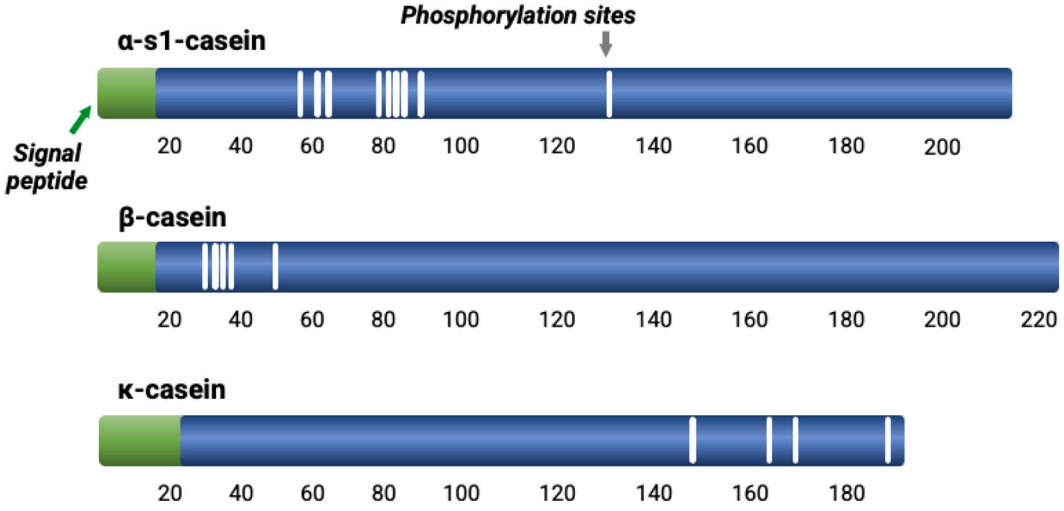
molecular MS is not an easy task mostly due to ionization suppression effects of phosphorylated species, especially intact phosphoproteins, and loss of PTMs information due to incomplete sequence coverage in *bottom-up* strategies. Therefore, quantitative phosphoproteomics urgently demand from strategies able to provide accurate and precise determination of phosphorylation degree. In this regard, selective P determination by ICP-MS established as an interesting alternative [45,46]. However, the approach was partially limited as the mass of protein was required beforehand to relate the mass of P quantified to the mass of protein present. Simultaneous quantitative determination of phosphorous and sulfur with ICP-MS, has proved useful to overcome this challenge. By means of using one non-specific standard for each target element, it is possible to determine molar ratios P/S in each protein LC peak from the corresponding P/S peak area ratio. Given that the stoichiometry S/protein is known from the amino acid sequence, P/S molar ratio is easily converted into P/protein molar ratio. That is, phosphorylation degree.

Accurate and precise determination of intact protein phosphorylation degree with the capLC-ICP-MS/MS approach has been proved for the case of casein model proteins, with external calibration using a mixture of inorganic S and P as quantification standards [27]. Results are shown in Fig. 5. Interestingly, despite their low concentration level, P/S molar ratio could be computed for the peaks corresponded to the trace isoforms impurities present, α-s1-casein and κ-casein. Results are in good agreement with the theoretical values and precision ranged from 3% to 6–10%, for the target (β-casein) and impurities, respectively. Interestingly, phosphoproteins are in low concentration levels in real samples and their analysis is often hindered by highly abundant proteins, so that enrichment procedures are typically required. Nevertheless, in this case, direct quantification of these intact phosphorylated proteins present at trace levels was achieved (detection limit of 0.7 fmol of intact phosphoprotein). It is worth noting that this strategy can be extended beyond phosphorylation to other protein chemical modifications that carry these non-metal elements like As, Se, or I.

6. Concluding remarks and perspective

Instrumental and methodological developments in the last decades have made LC-ICP-MS a powerful tool for absolute quantification of proteins, overcoming most of its critical limitations in intact protein analysis. Enhanced capabilities in sulfur detection with ICP-MS/MS have boosted its compatibility with standard platforms, being already integrated in some proteomic methodologies, as it is the case of venomics, using it as complementary detector for LC intact protein separation together with ESI. However, the universal and generic nature of ICP-MS signal for protein quantification comes with the cost of stringent chromatographic requirement of protein isolation at the time of the ICP-MS detection in order to obtain accurate quantitative results for either high and low abundance proteins.

Some interesting ICP-MS applications nowadays in proteomics include the certification of standard proteins concentration and mass purity, or the quantitative determination of PTMs with high accuracy and spatial and temporal resolution. Furthermore, because of ICP-MS quantification applicability to simple and relatively complex protein



	Theoretical P / Protein ratio[a]	P / Protein ratio determined with ICP-MS	Calculated Phosphorylation Degree (%)
α-s1-casein	1.3-1.7	1.6 ± 0.1	94.1 ± 5.8
β-casein	4-5	4.60 ± 0.14	92.0 ± 2.8
κ-casein	0.25	0.29 ± 0.03	116 ± 12

Fig. 5. Up) Amino acid sequence representation of bovine casein isoforms α-s1, β y κ, highlighting the position of sites that can be phosphorylated (phosphorylation of all sites corresponds to phosphorylation degree of 100%). Down) table summarizing results obtained on quantification of phosphorylation degree with HPLC-ICP-MS using generic standards with reviewed instrumental and methodological developments [27]. [a]Theoretical P/protein ratios corresponds to those provided from the manufacturer for the analyzed sample.

mixtures, this single and/or overall protein determination could be further considered to enhance quantification strategies and standardization in Stable-Isotope Labelling and label-free quantitative proteomics.

To this day, ICP-MS quantitative proteomics is yet not fully matured, and improved separation and MS performance requires still from further development. In this regard, for instance, improvements in chromatography shall lead to the quantification of larger number of species, at some point even at the level of complex proteomes. The basis has been already set with the proved quantitative characterization of simple proteomes containing around 50 intact protein species combining ESI-MS and ICP-MS detection. In fact, together with improvements both at the level of LC resolution and fragmentation with ESI-MS, we believe that combination of both strategies could eventually result in a powerful platform for the quantitative characterization of complex proteomes.

Notes

The authors declare no competing financial interest.

Declaration of competing interest

None.

Data availability

No

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