Certification of Protein Biomarker Standards using Element MS and Generic Standards: Application to Human Cytokines

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Highlights:

- Direct certification of protein standards concentration (mass purity) using ICP-MS and sulfate solution as generic standard.
- Parallel detection with ICP-MS and ESI-MS to quantify and identify intact proteoforms.
- Mass purity certification values obtained were significantly lower than the protein purity values provided by manufacturer using SDS-PAGE and HPLC.
- Purity control between batches showed significant differences in mass purity and presence of impurities and/or degradation products.

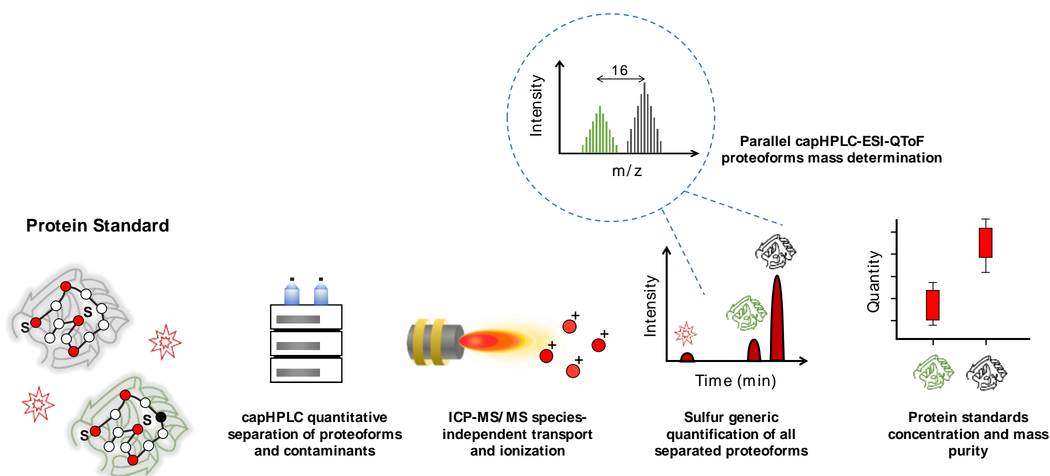
Abstract:

The availability of protein standards and methods for their characterization, quantification, and purity assessment are currently a bottleneck in absolute quantitative proteomics. In this work, we introduce an absolute quantitative analytical strategy based on ICP-MS sulfur detection that uses sulfate as generic standard to quantify and certify the mass purity of protein standards. The methodology combines capillary chromatographic separation with parallel detection with ICP-MS and ESI-MS to determine proteoforms concentration and identity, respectively. The workability of the methodology was demonstrated using recombinant human cytokine standards IP-10 and Flt3L (2 batches), which are relevant biomarkers for carcinoma or inflammatory diseases. Every key factor (transport efficiency, column recovery, signal stability and internal standard suitability) was taken into account and certified BSA standard was used as quality control for validation purposes. Protein quantification values and resulting mass purity certification of IP-10 and one batch of Flt3L were very high (100 and 86%, respectively). Lower mass purity obtained for another batch of Flt3L (<70%) concurred with the finding of significant proteoforms resulted from oxidation processes as observed by parallel ESI-MS.

Keywords:

Protein biomarkers, Protein standards, Mass purity certification, Cytokines, Mass Spectrometry, Liquid Chromatography.

Graphical abstract:



1. INTRODUCTION

The determination of protein quantities in fields like pharmacology, biochemistry, or biomedicine is essential for biomarker and drug target discovery and validation, the study of protein interactions and their pathways, clinical diagnosis and understanding of diseases, etc [1]. Protein quantification is predominantly approached through the comparison of protein levels between samples and/or to a reference state. These relative methods usually rely on the use of isotopic tags that differ between the samples and are introduced either metabolically (e.g., SILAC) or chemically (e.g., isotope-coded affinity tag, ICAT) [2]. Protein relative quantification is however insufficient in those studies that require the accurate determination of absolute protein levels in (bio)systems.

Mass spectrometric measurement of protein concentration uses protein standards as calibration references, added to the sample at known concentration. For this purpose, there are several standardization approaches in absolute quantitative proteomics, most of which use stable isotope labeled counterparts of the target protein at both the peptide digest (e.g., AQUA) and intact protein level (e.g., PSAQ) [1]. These standards are specific for each target analyte, given the non-quantitative nature of the electrospray ionization sources commonly used [3]. Therefore, quantification studies require from available -or synthesizable- standards for each target proteic species, which must be appropriately purified, characterized, and whose concentration and mass purity must be accurately assessed.

The availability of these standards is greatly challenging. On the one hand, because of the difficulty to synthesize standards for each analyte, particularly at protein level. The production and purification of proteins in most cases involves expensive and complex methods e.g., DNA recombinant technology and cloning processes [4]. The cloning process could also lead to the presence of impurities that should be removed in the following purification step (Figure 1). These steps of production, purification, storage, and handling of the final product therefore limit the feasibility of the produced protein as quantification standard [5]. It must also be considered the stability of protein standards because their degradation over time would likely impact the accuracy of the quantification.

On the other hand, the determination of the standard concentration and mass purity presents several difficulties as well. There are some sources of error to be considered that otherwise might affect the quality of the final quantitative measurement. The certification of protein standards requires firstly the determination of total protein quantity. This is commonly carried out with approaches like amino acid analysis (AAA), which are accurate but cumbersome and more appropriate for peptide quantification rather than intact proteins [6]. They require breaking the protein down to amino acids, a process that becomes more challenging the bigger the protein,

requiring extreme hydrolysis conditions of pH, temperature, and time. Moreover, hydrolysis efficiency and amino acid recovery, despite quantitative (>80%), show variability depending on the amino acid and the hydrolysis strategy [7], hence conditioning the quantification accuracy. Alternative methods for protein quantification are colorimetric and spectroscopic approaches like Lowry assay or Bradford method. They are based on spectroscopic detection of the sample after the protein reacts with metal ions (Cu) or dyes, respectively [8]. They use standard proteins like BSA for calibration, hence depending on the protein amino acid sequence, different behavior and reactivity of the proteins may lead to errors in the protein concentration estimation of over one order of magnitude in some cases [9,10]. Protein standard purity can then be assessed relative to weighted sample or using simple but indirect methods like SDS-PAGE or LC-UV-Vis (Figure 1). The combination of protein content (mass) and chromatographic/electrophoretic purity (signal ratio of target protein over the total signal) leads to the sought mass purity of the target protein standard.

Inductively coupled plasma mass spectrometry (ICP-MS) has recently turned up as an interesting alternative for protein standards certification. ICP-MS impact and applicability in life sciences fields like proteomics in the last decade has risen substantially because of the introduction of ICP tandem mass spectrometry (ICP-MS/MS) in 2012. This configuration has resulted in a spectacular decrease of the limit of detection for non-metals, especially for S and P [11]. Protein quantification can then be achieved through the ICP-MS quantitative detection of their constituent sulfur [12], once its amino acid sequence is known i.e., the sulfur to protein molar ratio. Moreover, with ICP-MS, any sulfur-containing compound of certified concentration (e.g., inorganic sulfate or small organic compounds) can be used as generic quantification standard [13]. ICP-MS great potential as robust quantitative method for the generic, direct (one single analysis) and accurate certification of intact protein standards has been already envisaged [14] but not demonstrated yet with protein biomarkers.

One of such biomarkers that requires from improved methods for standards certification are cytokines. They are a group of proteins secreted by immune cells that are involved in physiological and pathological processes like immune responses, cell signaling, cellular proliferation and apoptosis [15]. The interferon γ-induced protein C-X-C motif chemokine 10 (IP-10) and Fms-like tyrosine kinase 3 ligand (Flt3L) are two cytokines whose expression levels have been associated to several biological processes such as inflammatory response and have also been reported as biomarkers of disease progression like cancer or inflammatory processes [16,17]. The unquestionable biological importance of cytokines results in the availability of commercial standards, produced by recombinant DNA technology [18]. Despite protein purity of these commercial standards is usually provided, protein mass is not certified, hence their applicability

in quantitative studies (e.g., study of their role in autoimmune disorders or their applicability as disease biomarkers) is clearly constricted [5].

Here, we report the application of capillary liquid chromatography (capHPLC) with ICP-MS/MS detection for the mass purity certification of IP-10 and Flt3L recombinant cytokines with a direct and generic method without resorting to specific or isotopically labeled standards. The combination with parallel electrospray detection would provide complementary valuable information on the identification of potential impurities present in the cytokine products.

2. MATERIALS AND METHODS

2.1. Reagents and materials

Standards of Recombinant Human Fms-related tyrosine kinase 3 ligand Lot# 061945 (Flt3L-A) and Lot# 091945 (Flt3L-B) and Recombinant Human Gamma-Interferon Inducible Protein 10 (IP-10) Lot# 121039A were purchased from PreproTech (Cranbury, USA). Pure Bovine Serum Albumin (BSA, Uniprot P02769) standard was purchased from Sigma-Aldrich (Steinheim, Germany). ICP-MS Sulfur Standard was purchased from Merck (Darmstadt, Germany). Ultrapure water was produced by a Purelab Flex system (ELGA LabWater, UK). Acetonitrile (AcN) HPLC grade was purchased from Fischer Scientific (USA) and Formic Acid was purchased from Acros Organics (Germany).

2.2. capHPLC-ICP-MS/MS intact protein analysis

Sulfur capHPLC-ICP-MS/MS analysis was performed in a reverse phase chromatography gradient (0 min – 10% B, 20 min – 60% B, 23 min – 60% B) with a flow of 4 µL min^{-1}. Chromatographic separation was performed in a capillary HPLC system (capHPLC) Agilent 1260 Infinity Series (Agilent Technologies, Waldbronn, Germany) with a reverse phase capHPLC BIOShell™ A400 C4, 3.4 µm, 150 mm x 0.3 mm (Merck KGaA, Darmstadt, Germany), kept at controlled temperature of 80ºC with a column oven Spark Holland (Mistral, The Netherlands). The chromatographic mobile phases consisted of ultrapure water (A) and acetonitrile (B) both with 0.2% of formic acid. Column connections were made of Fused Silica peeks from Agilent Technologies (Santa Clara, USA). The ICP-MS/MS detection was performed in an Agilent 8800 Triple Quad ICP-MS (Tokyo, Japan), and a Total Consumption Nebulizer (Santa Clara, USA) was used as capillary HPLC interface. Optional gas was mixed with 50 mL min^{-1} Ar:CO_2 (90:10) gas mixture (Air Liquide, Paris, France), using a mass flow controller from Bronkhorst (Mistral, Netherlands) as described by Calderón Celis *et al.* [14]. The argon carrier gas flow for ICP-MS analysis was 0.85 L min^{-1}, and Ar:O_2 (Air Liquide, Paris, France) optional gas flow was 0.16 L min^{-1}. The MS/MS analysis used 0.25 mL min^{-1} O_2 (Air liquide, Paris, France) as reaction cell gas, to form SO^+ (m/z 48) and filter S polyatomic interferences of m/z 32 in Q2. See S.I. for detailed description of operational parameters.

Sulfate and protein standards were injected at a sulfur concentration of 1-2 mg L^{-1}, in 1 µL of injected volume. Sulfate standard and the samples were injected in Flow Injection Analysis (FIA) prior to their chromatographic analysis to carry out protein quantification and chromatographic recovery assessment, respectively.

2.3. capHPLC-ESI-QToF intact protein analysis

Molecular mass spectrometry analyses were performed with a mass spectrometer ESI-QTOF Impact II (Bruker Daltonics, Bremen, Germany). Analytes were ionized by heated electrospray ionization operating in the positive ionization mode with the following settings: capillary voltage 4000 V, dry temperature 220ºC, mass interval 300-3000 m/z, spectra rate 1 Hz, dry gas 8 L min^{-1}. Chromatographic capHPLC conditions were the same as in capHPLC-ICP-MS/MS analysis.

3. RESULTS AND DISCUSSION

3.1. Development and validation of the method for protein standards certification

ICP-MS elemental signal is species-independent, meaning that all sulfur-containing species in the sample need to be separated before ICP-MS detection. The methodology proposed for protein standards certification is based on the quantification of its constituent sulfur with ICP-MS. The developed methodology used capillary reversed-phase liquid chromatography to separate the protein analytes from any potentially present impurity or contamination during synthesis, purification and/or storage processes.

Correlation of protein's sulfur signal into sulfur quantity requires the use of a quantification standard as reference. The advantage of ICP-MS is that elemental (sulfur) response factor can be made species-independent hence any well-characterized and of certified concentration sulfur-containing standard can be used. Therefore, in a single chromatographic analysis, correlation of sulfur chromatographic peak areas of analyte and standard directly results in the quantification of sulfur concentration (Figure 2). This methodology is nonetheless based on the fact that signal response factor (sulfur signal per unit of concentration) is equal for both analyte and standard. This response factor has been proved conditioned by the efficiency of transport and ionization processes, and the chromatographic gradient used, so they must be considered when developing a generic methodology for protein quantification and particularly protein standards certification.

Firstly, it has been recently demonstrated that conventional ICP-MS nebulizers (working at the high µL min^{-1} level) show different nebulization efficiency for S-containing compounds and biomolecules, which depends on biomolecule size, structure, and hydrophobicity. In contrast, Total Consumption Nebulizers (working at the very low µL min^{-1} level) proved complete nebulization efficiency for each and every biomolecule [19]. Secondly, sulfur response factor

must be kept constant along the chromatographic analysis, in this case, the acetonitrile gradient. It is known that carbon content variations in the mobile phases during chromatographic gradients induce several physicochemical processes in the ICP plasma that result in differences in the ionization efficiency of elements with high ionization potential like S [20]. This effect is commonly corrected using isotope dilution analysis (IDA) for quantification [21]. The constant addition of isotopically enriched sulfur as standard corrects ionization variations by correlating natural/enriched sulfur. Despite its accuracy, IDA requires more complex instrumental configuration besides isotopically labeled standards and it is not applicable to biologically important monoisotopic elements like P or I. It has been recently demonstrated that the controlled addition of a carbon-containing gas to the plasma counteracts this effect so that ICP-MS elemental response factor is maintained constant along a chromatographic gradient [14,20]. Finally, it is worth remarking that in HPLC analysis, non-specific interactions between analyte species and the stationary phase could lead to their incomplete elution from the column [22]. This effect is significantly pronounced with hydrophobic compounds and high-molecular weight species like proteins [23]. Consequently, the determined quantity of the protein would be just a fraction of the total amount present in the sample, leading to underestimation of the protein concentration. This incomplete recovery must be characterized and determined so that an adequate correction could be done to the quantification calculations. This fact is a clear limitation when addressing the certification of protein standards, since another standard of the protein would be required, inquiring into a vicious cycle. Additionally, recovery correction factors could lead to biased results as they could change along the column life. Interestingly, it has been observed that the use of core-shelled particles as column packing instead of traditional fully-porous provided quantitative chromatographic recoveries for a wide range of intact proteins [24].

The developed methodology takes into consideration all commented issues to achieve protein absolute quantification with quantitative chromatographic recovery, complete nebulization efficiency and transport, and constant signal response factor along the analysis (optimal instrumental settings are described in experimental section). In order to validate the methodology for protein standards certification, well-characterized and certified commercial Bovine Serum Albumin (BSA) standard was used as quality control. BSA chromatographic column recovery was calculated by comparing the sulfur peak area of the chromatographic analysis (capHPLC-ICP-MS/MS) and a direct flow injection analysis (FIA-ICP-MS/MS). The total amount (area) of sulfur eluting from the column (S^{i}_{HPLC}) in the different S-containing species corresponds to the total amount of sulfur in the sample (S_{FIA}), determined from a FIA injection where the sample is directly injected into the ICP without passing through a HPLC column ($S_{FIA} = \sum S^{i}_{HPLC}$). The ratio (($S_{HPLC}$/ S_{FIA}) x 100) provides the column recovery value, which in the case of the BSA protein was 101 ± 1%, as can be seen in Table 1. This quantitative recovery meant that protein

quantification could be directly carried out without requiring specific standards to compute any incomplete recovery. Indeed, when carrying out the quantification of BSA using inorganic sulfate as generic standard, the concentration value obtained was 0.95 ± 0.03 g BSA per g of sample. This mass purity value of 95% was in complete concordance with reference protein content provided by the supplier (≥ 96%).

3.2. Characterization and mass purity certification of IP-10

The synthesis, isolation, and purification processes of -recombinant- protein standards might result in presence of impurities or derivate compounds (Figure 1). Their presence can jeopardize the accurate assessment of the protein purity and their subsequent use as quantification standards in biomedicine. The assessment of the developed methodology for the quantification and mass purity certification of protein standards was done using recombinant human cytokines IP-10 and Flt3L as proof of concept. In the case of recombinant human IP-10, the purchased standard is a protein of 8646.29 Da that contains 4 cysteines and 1 methionine (i.e., 5 sulfur atoms) in its amino acid sequence.

The quantitative analysis of IP-10 with capHPLC-ICP/MS showed a clean chromatographic profile in which there was a single sulfur peak eluting (peak 1) at around 13 min (Figure 3A). There must be pointed out the presence of a minor shoulder in the tail of the peak though (peak 2). Besides these main peak and shoulder, there seems to be no other proteic contaminants or impurities that contain sulfur in the sample. When considering the total area of sulfur in the chromatographic analysis (peaks 1 + 2), the chromatographic recovery calculated was 95 ± 3%.

To confirm that the main chromatographic peak is the IP-10 protein and assess the identity of the species eluting the peak shoulder, capHPLC-ESI-QToF analysis of the sample was carried out. The use of the same chromatographic conditions for both ICP-MS/MS and ESI-TOF analysis enabled overlapping of chromatograms and correlation of elemental (quantitative) and molecular (identity) information obtained, respectively. Peak 1 was identified as a protein with molecular mass of 8642.63 Da (Figure 3B), which matches very well to the mass of IP-10 (40 ppm mass error). The species eluting in peak 2 also showed the same molecular weight, so that this species might represent a structural proteoform of the protein that could result in the slight shift of the chromatographic retention time.

Quantification of IP-10 with capHPLC-ICP-MS/MS provided a quantitative value of 0.97 and 0.06 grams of protein per gram of sample for both peak 1 and 2, respectively (Table 1). The overall protein quantification resulted in a mass purity value for IP-10 of 103 ± 5%. Commercial protein purity, assessed by SDS-PAGE and HPLC was ≥ 98%. It seems thus clear that all the starting material of the IP-10 corresponded to protein content in this case.

3.3. Characterization and mass purity certification of Flt3L

Protein standard of recombinant cytokine Flt3L-A was certified with capHPLC-ICP-MS/MS. This protein is a cytokine of 17610.91 KDa that contains 8 sulfur atoms (2 methionines and 6 cysteines). Sulfur profile showed three peaks (1-3) eluting between 18 and 21 min (Figure 4A), differing from the expected single peak of the pure protein, yet the overall chromatographic recovery was also quantitative in this case (100 ± 1%, Table 1). The areas of the three peaks were significant enough to not consider any of them minor impurities in the sample.

To assess the identity of the additional peaks, capHPLC-ESI-QToF analysis was carried out. The three peaks observed in Flt3L-A with ICP-MS were likewise detected in ESI-TOF (Figure 4B). The three peaks corresponded to proteins with different molecular weights, and there was a correlation between lower retention time and greater mass. The most retained species (peak 3), which was the most abundant in ICP-MS, with a molecular weight of 17610.90 Da (Figure 4C), correspond to the theoretical mass of the commercial protein (15 ppm mass error). Molecular weight values of the species eluting in peaks 2 and 1 were 17626.91 Da and 17642.90 Da, respectively. These mass differences perfectly match the atomic mass of one oxygen (peak 2) and two oxygens (peak 1), which seems to suggest that both species correspond to oxidation forms of Flt3L. Notably, it is known that protein oxidation is a process that increases the polarity of the protein, reducing its retention time in reverse phase chromatography [25]. The elution order observed (first the di-oxidized, then the mono-oxidized, and finally the intact Flt3L) is consistent with this assumption.

Considering that the three species corresponded proteoforms of Flt3L, and they contain the same number of sulfur atoms, the concentration determined with ICP-MS was translated into 0.39 grams of protein Flt3L, 0.19 grams of mono-oxidized Flt3L, and 0.11 grams of di-oxidized Flt3L per gram of sample. Global protein mass purity of the sample was below 70% (Table 1) which, in contrast to IP-10 results, was significantly lower than the value of protein purity provided by manufacturer obtained by SDS-PAGE and HPLC (≥ 98%). This finding pinpoints towards the presence of non-accounted contaminants or impurities, considering that no other sulfur -proteic-species were observed in the chromatogram.

Analysis of a different batch of cytokine Flt3L (Flt3L-B) was carried out afterwards in order to evaluate the consistency of protein oxidation and low protein mass purity among batches. Chromatographic analyses of Flt3L-B both in ICP-MS and ESI-MS (inserts to Figures 4A and 4B, respectively) showed a major peak corresponding to Flt3L (peak 3) and only a minor peak (peak 2) corresponding to the mono-oxidized form of Flt3L (17610.90 and 17626.91 Da, respectively). It must be remarked that both Flt3L batches were prepared, and proteins were put in solution following the same procedure. Column recovery was again quantitative (103 ± 3%). Determined protein concentration with ICP-MS was 0.72 grams of Flt3L per gram of sample, and

0.14 grams of the mono-oxidized form. The global protein mass purity (86 ± 5%) was higher in this case, and closer to the protein purity provided by the manufacturer.

Notably, the Flt3L batch with the lower mass purity (A) corresponded as well to the one in which higher proportion of protein oxidized forms were observed. In contrast, batch B showed higher protein mass purity, with a major species corresponding to -the intact “non-oxidized”- Flt3L. On the other hand, despite IP-10 also contained oxidizable amino acids (e.g., methionine), no oxidized form at all was observed.

4. CONCLUSIONS

Certification of intact protein standards is a difficult and complex process because of the limitations of the available techniques and the lack of reference standards/materials. It is undoubtedly necessary to develop new and better methodologies to carry out the absolute quantification and purity assessment of protein standards in an easy, generic, and direct way. The proposed methodology uses the power of ICP-MS detection for the direct and generic quantification of proteic sulfur and consequently to provide the protein concentration and mass purity. Notably, it also enables the determination of the concentration of the multiple proteoforms or impurities likely present in the sample during the same analysis. In particular, such potential of ICP-MS for protein standards certification, which had been so far evaluated just in well characterized commercial standards like BSA, is herein demonstrated for the first time for target biomarker standards, generated by DNA recombinant technology.

The potential of combining ESI-MS and ICP-MS detection with the same chromatographic analysis is demonstrated with the simultaneous quantification and characterization of different proteoforms and/or byproducts in the problem standard samples. This leads to a better understanding of the assayed standard composition and is of utmost importance for the quality control of the protein process production and the control of differences between batches, critical in protein production (specially for recombinant proteins due to the complexity of the cloning process). This is demonstrated with the low and irreproducible mass purity obtained for Flt3L batches, whose use may imply inaccurate quantitative results in biological applications. Therefore, results shown here demonstrate the potential of capHPLC-ICP-MS/MS for direct mass purity certification of proteic products and a very useful approach for quality control in subsequent steps of protein production like handling or storage.

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TABLES

Table 1. Chromatographic recovery and mass purity values obtained for Cytokine standards IP-10, FLT3-LG (batches A and B), and commercial BSA used as quality control. Individual values corresponding to every peak observed in the corresponding capHPLC-ICP-MS/MS chromatograms are also given. Uncertainty corresponds to one standard deviation (n=3).

Protein	Peak	Chromatographic recovery	Protein mass purity	Reference value*
BSA		101 ± 1%	95 ± 3%	> 96 %
IP-10		95 ± 3%	103 ± 5%	>98 %
	1		*97 ± 4%*	
	2		*6 ± 1 %*	
Flt3L-A		100 ± 1%	69 ± 2%	>98 %
	1		*11 ± 1%*	
	2		*19 ± 1 %*	
	3		*39 ± 1%*	
Flt3L-B		103 ± 3%	86 ± 5%	>98 %
	1		-	
	2		*14 ± 1%*	
	3		*72 ± 4%*	

*Protein purity provided by manufacturer.

FIGURES

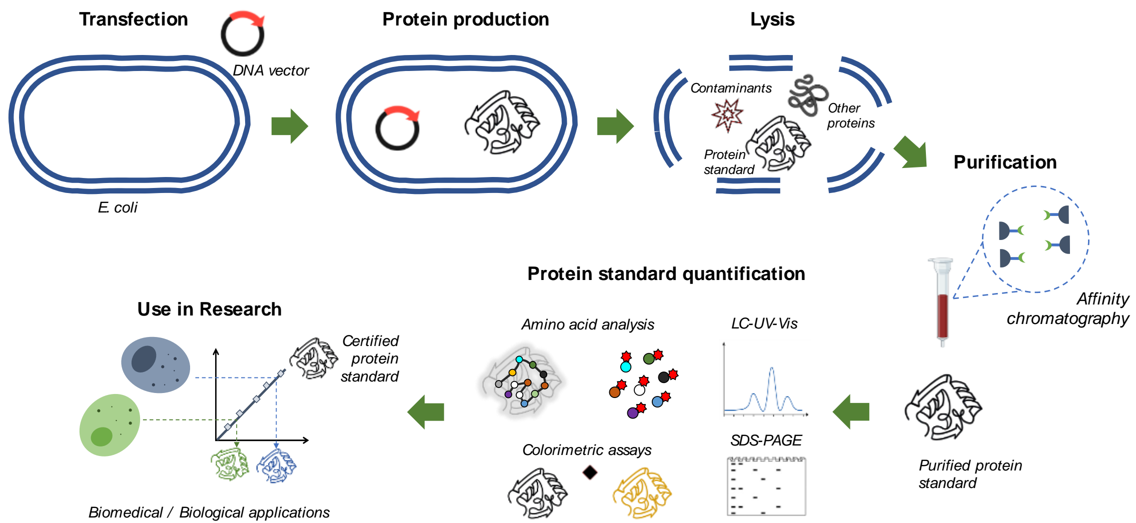

Figure 1. Schematic overview of the production, characterization, and use of protein standards in quantitative research. Recombinant proteins are produced transfecting into a host, generally E. coli, the sequence of the desired protein using a DNA vector (plasmid). The host is cultivated, and the protein is produced by the common molecular machinery of the bacteria. After cell lysis, the target protein is separated from other proteins and/or contaminants by different strategies like affinity chromatography, which uses affinity tags e.g., His-tag. The purity of the recombinant protein is obtained by LC-UV-Vis and SDS-PAGE. Such protein purity can be also combined with the protein content obtained by AAA or colorimetric assays to produce the desired certification of the protein mass purity. Certified standards can then be used as analytical calibrants or references in biological studies to determine absolute protein quantities in biological systems.

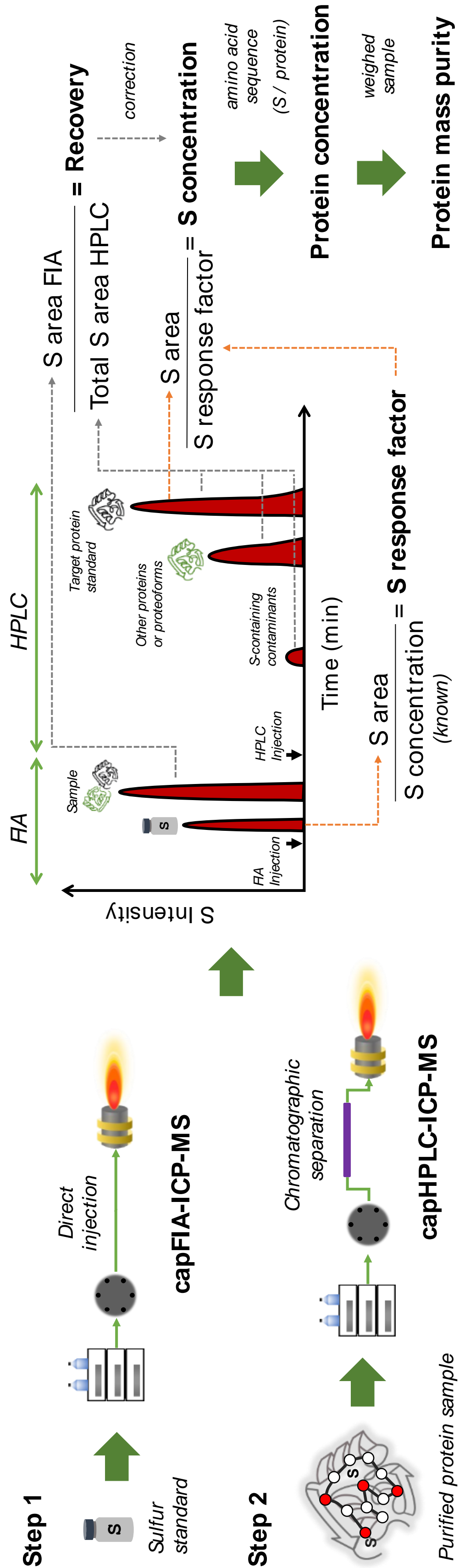

Figure 2. Protein mass purity determination in a direct and single analysis using ICP-MS and sulfate as generic standard. Step 1: via flow injection analysis (FIA), a certified and pure sulfur-

containing compound is injected to determine the sulfur response factor by correlating the peak area with the injected sulfur concentration. Step 2: the sample is injected to the chromatographic column. The sulfur peak area corresponding to the protein is transformed into sulfur concentration using the previously calculated response factor and considering the assessed chromatographic recovery. That sulfur concentration is translated into protein mass concentration given that the molar ratio sulfur/protein is known (that is, the number of cysteines and methionines in the amino acid sequence), and finally into protein mass purity in the original sample.

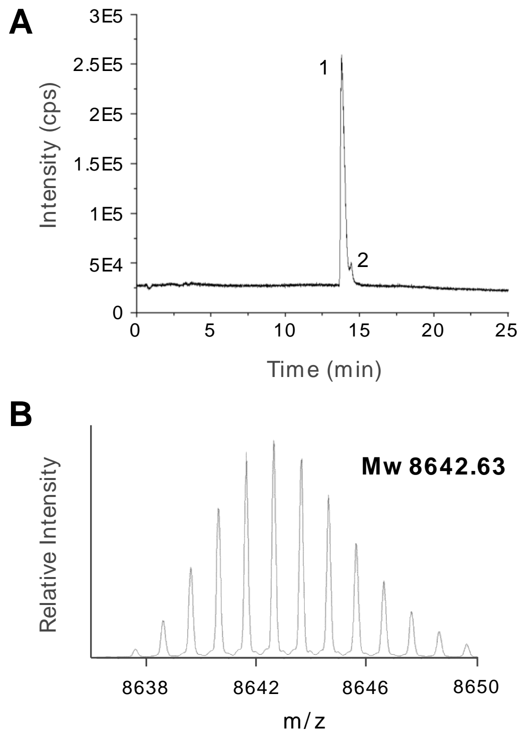

Figure 3. (A) capHPLC-ICP-MS/MS sulfur chromatogram of IP-10 cytokine recombinant standard. (B) Deconvoluted mass spectra of the intact protein observed in both peaks 1 and 2, obtained from the capHPLC-ESI-QToF analysis.

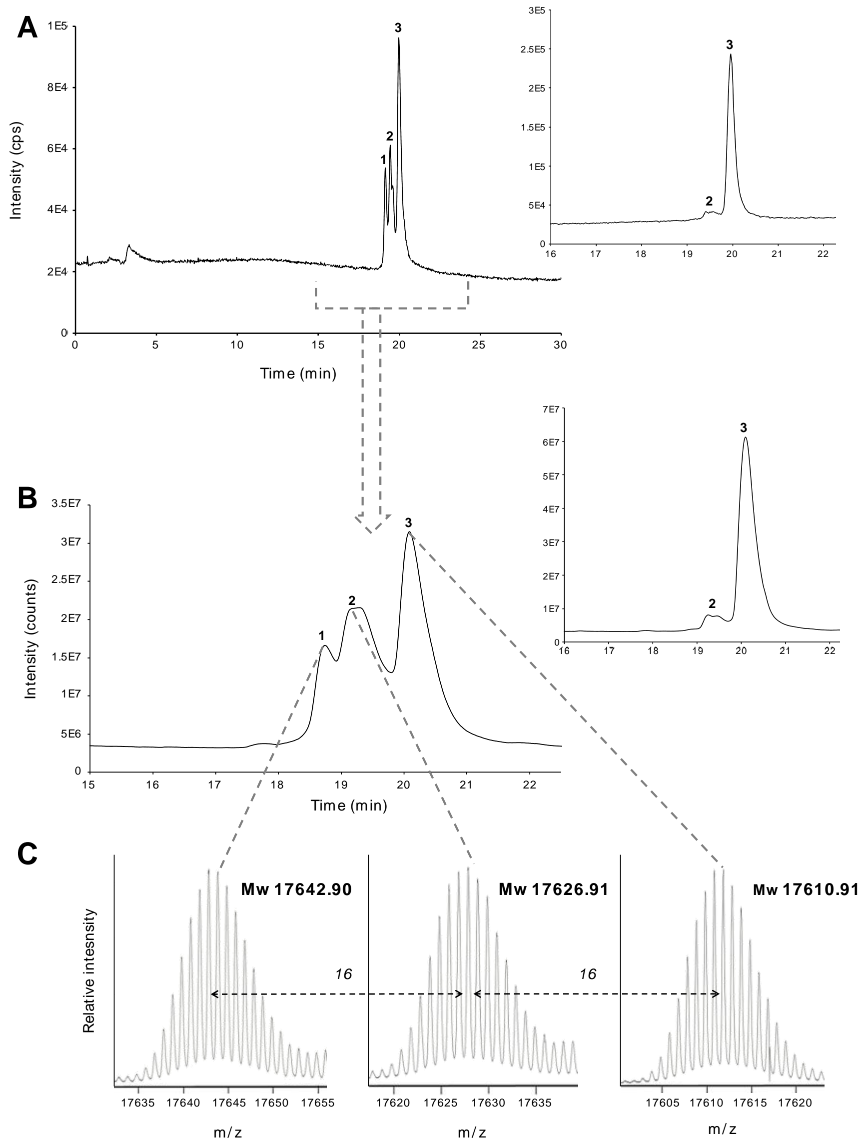

Figure 4. (A) capHPLC-ICP-MS/MS sulfur mass chromatogram of Flt3L-A and Flt3L-B (insert). (B) capHPLC-ESI-QToF TIC of Flt3L-A and Flt3L-B (insert). (C) Deconvoluted mass spectra of intact of peaks 1-3.