Assessment of the potential and limitations of elemental mass spectrometry in Life Sciences for absolute quantification of biomolecules using generic standards

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ABSTRACT: ICP-MS has been widely used in Life Sciences for the absolute quantification of biomolecules without specific standards assuming the same response for generic compounds including complex biomolecules. However, contradictory results have been published on this regard. We present the first critical statistical comparison of the ICP-MS response factors obtained for fourteen different relevant S-containing biomolecules (three peptides, four proteins, one amino acid, two cofactors, three PEGs derivatives and sulfate standard), covering a wide range of hydrophobicity and molecular size. Two regular flow nebulizers and a total consumption nebulizer (TCN) were tested. ICP-MS response factors were determined though calibration curves and isotope dilution (IDA) was used to normalize results. No statistical differences have been found for low-MW biocompounds, PEGs and non-hydrophobic peptides using any of the nebulizers tested. Interestingly, while statistical differences were still found negligible (96-104%) for the proteins and hydrophobic peptide using the TCN, significantly lower response factors (87-40%) were obtained using regular flow nebulizers. Such differential behavior seems to be related mostly to hydrophobicity and partially to molecular weight. Findings were validated using IDA in intact and digested BSA solutions using TCN (98 and 100%, respectively) and the concentric nebulizer (73 and 97%, respectively). Additionally, in the case of a phosphoprotein, results were corroborated using the P trace in parallel to the S trace used along the manuscript. This work seems to suggest that ICP-MS operated with regular nebulizers can offer absolute quantification using generic standards for most biomolecules except proteins and hydrophobic peptides.

Absolute quantification of biologically relevant molecules (proteins, DNA, etc.) is essential in Life Sciences research.[1] There are several clinical, scientific or technological areas that directly benefit from such absolute data.[2–4] First, determination of exact molar biomolecule concentrations is not only critical for biomarker discovery but for disease diagnosis, determination of optimum drug delivery and monitoring during treatment and recovery. Second it allows chemical biologists to relate concentrations of relevant biomolecules (i. e. proteins, nucleic acids, lipids), which improves understanding of the dynamic processes and interactions controlling metabolism.[1] Determination of the absolute concentration of individual components is also critical to assess the stoichiometric ratios either in multiprotein complexes or signaling networks.[5]

Most common approaches nowadays for biomolecules absolute quantification are based on molecular MS and require the use of specifically designed standardization approaches to provide absolute quantification,[4,6] being mostly limited by the availability of the corresponding biomolecules standards. Of course, trueness of the results obtained will depend critically on the quality of such standards and their mass purity certification. Therefore, analytical approaches able to provide absolute quantification using nonspecific standards could play a critical role in the improvement of results validation and the development of quality assurance procedures.[7]

In this context, it is clear nowadays that ICP-MS is gathering momentum in Life Sciences research, especially for its potential to provide absolute quantification of biomolecules on the sole condition that an ICP-detectable element (any except C, H, N O and F) is present or artificially linked.[6,8,9] In this line, hybrid LC-MS configuration with both elemental (ICP) and molecular (ESI) MS detection in parallel has been already successfully applied to identify and quantify target peptides and proteins[8,10] and other biomolecules (e. g. metabolites in urine or antioxidants in water extracts of oyster mushrooms)[11,12] of interest in biological samples without specific standards. The elemental quantification achieved using ICP-MS can be easily translated into biomolecule absolute quantities provided the molar ratio element/molecule (stoichiometry) previously determined using molecular MS. Interestingly, such great potential relies on the assumption that ICP-MS response is species independent, which is still an intriguing and controversial issue. In fact, it has not been established yet under which operational conditions simple and generic element-containing standards can be used to quantify in absolute terms biomolecules containing such element. In this regard, although nebulization and transport in ICP-MS analysis are low efficient processes (typically <10%), it is extensively assumed to they are similar for any compound, regardless of whether it is a small molecule or a large chemical species.[13] Notably, if the element response factor obtained for different element-containing compounds is proved to differ significantly, the species-independent quantification capabilities of ICP-MS would be seriously compromised. Different studies have been carried out so far focusing on low molecular

weight molecules, particularly organometallic compounds of As, Hg, Sn, I and Se, and slight differences in the corresponding ICP-MS response factors were found.[14,15] depending on the volatility of the species[16,17] and the sample introduction system used.[15]

There is however no established evidence for any difference in ICP-MS response factors for non-volatile species of the same element, which could include medium and large biomolecules. In fact, Svantesson *et al.*[13] and Anan *et al.*[18] found almost identical response factors when using ICP-MS equipped with a concentric nebulizer for the analysis of cyanocobalamin (1.34 kDa) and cobalt standards and for different Se-containing compounds (selenate, selenomethionine and trimethylselenonium), respectively. Pereira *et al.*[19] found species independent ^{31}P signal from different phosphorous-containing species including phosphopeptides (1.54kDa) when using a total consumption micronebulizer. On the other hand, Guo *et al.*[20] found species independent signal after labelling small proteins (< 15kDa) with MeHg and using another nearly total consumption nebulizer (DIHEN) as well. Unfortunately, larger analytes were not analyzed at those times mostly because sulfur detection (that is present in many polymers and the vast majority of proteins) using regular quadrupole instruments suffer from severe polyatomic interferences. The use of state of the art tandem ICP-MS/MS opens now the sensitive and free of interference analysis of sulfur and in turn, of many proteins and polymers.[21–23] In fact, the use of ICP-MS in absolute biomolecule quantification approaches has increased exponentially in the last 10 years.[24,25] Surprisingly, there is no critical report so far focused on the ICP-MS species-independent response for such large non-volatile biomolecules, especially using regular nebulizers. However, most of the ICP-MS applications in proteomics make use of online isotope dilution (online IDA) as quantification strategy,[26–29] assuming same response factor for the protein species and the, generally inorganic, isotopic tracer added continuously.

The aim of this work is to critically evaluate whether ICP-MS can provide species-independent response for a wide range of biomolecules (all they contain S in their structure), which would enable their absolute quantification without specific standards. Different nebulization systems, operational conditions and wide concentration ranges will be tested. Corresponding response factors obtained for different biologically relevant compounds, such as peptides, proteins, small biomolecules and biocompatible ligands will be statistically compared following stringent procedures.

EXPERIMENTAL SECTION

Reagents and materials. Pure standards β-casein, bovine serum albumin (BSA), cytochrome C, S-(5′-Adenosyl)-L-methionine chloride dihydrochloride (SAM), L-methionine, biotin, Adrenocorticotropic Hormone (ACTH) fragment 1-17 human, [Lys^8]-Vasopressin, and Neurokinin A were all purchased from Sigma (Steihem, Germany) and intact monoclonal antibody (mAb) Mass Check Standard from Waters (Milford, MS, USA). Thiolated methoxy PEG, 1kDa, linear 20kDa and branched 20kDa, were purchased from Laysan Bio Inc (AL, USA). Ammonium acetate and Tris-HCl were purchased from Sigma. Sulfur and Phosphorous ICP standards (1000 mg mL^{-1}) were purchased form Merk (Damstadt, Germany). Solid isotopically enriched ^{34}S (the corresponding isotopically abundances were ^{32}S: 3.01 ± 0.03%, ^{33}S: 1.24 ± 0.02% and ^{34}S: 95.75 ± 0.13%) was purchased from Isoflex USA (San Francisco, USA). All the solutions tested as well as the ^{34}S-spike solution were prepared in ultrapure water.

Instrumentation. ICP-MS system consists on a triple quadrupole 8900 ICP-MS/MS (Agilent, Tokyo, Japan). Flow Injection (FI) analysis using regular flow nebulizers were carried out using a 6 port valve system 9725 (Rheodyne, CA, USA) with a 20 µL peek sample loop and PEEK tubing connections (Sigma bio-inert tubing 0.254 mm i.d.). Regular flow nebulizers employed were a concentric standard MicroMist nebulizer (Agilent, CA, USA) and a PFA X-Flow nebulizer (Savillex, MN, USA) and both were coupled to a double-pass spray chamber. For capillary flow analysis, a capillary HPLC 1260 Infinity equipped with an autosampler (Agilent Technologies, Waldbronn, Germany) was used employing fused silica PEEK tubing (25 µm i.d.). Total consumption nebulizer (TCN) selected was a Capillary LC interface from Agilent (CA, USA). It was used as interface for the capillary HPLC-ICP-MS/MS and for the FI analyses. Online IDA flow rates were provided by a peristaltic pump minipuls 3 (Gilson, France) and a syringe pump system kdScientific (Holliston, MS, USA) for regular and capillary flow analyses, respectively.

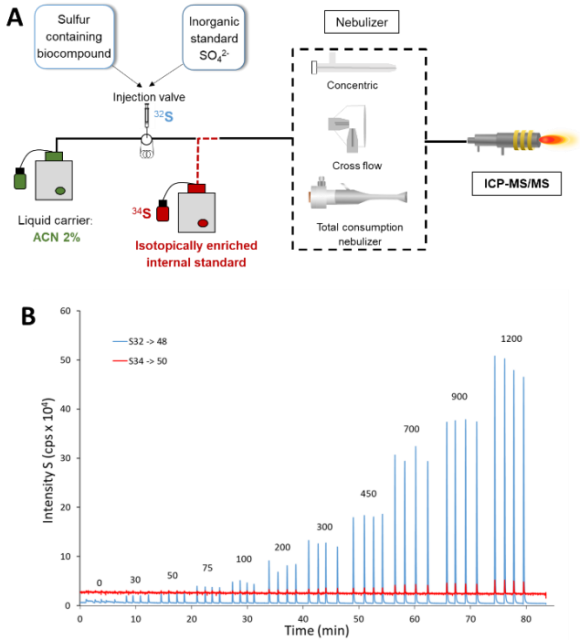

Figure 1. A) Instrumental configuration used for species-independent ICP-MS signal evaluation with the three nebulizers tested. B) Fiagram obtained for the S inorganic standard using concentric nebulizer. Blue line corresponds to the ^{32}S signal coming from natural S standard and red line corresponds to the ^{34}S signal coming from the isotopically enriched solution continuously added (online isotope dilution). Individual concentration values of the different injections (n=4) are also given (ppb S).

Procedures. A scheme of the instrumental set up is given in Figure 1A. Flow injection (FI) analysis mode was selected to neglect the influence of the S background, always observed in ICP-MS and typically associated with the reagents (O-rings, Argon gas and plastic ware) and to increase analysis throughput. Influence of the species structure was studied by direct comparison of the sulfur response factors obtained for the S-containing biomolecules tested with that of an inorganic certified sulfate standard. Fiagram obtained for such S inorganic

standard using the concentric nebulizer as an example is shown in Figure 1B.

Calibration curves, where the concentration experimentally obtained using online isotope dilution was plotted against the theoretical concentration, were built for the compounds tested and the inorganic standard used as reference. Slopes were then statistically compared with Student's t-test (95% confidence level) using the three different nebulizers that were operated under standard conditions given in Tables S1-S2 in the supporting information. A detailed description of the statistical treatment is included in the Supporting information as well. Briefly, we carried out a preliminary screening of the similarity of the population variances by the Fisher-Snedecor's F-test, following Andrade *et al.* guidelines.[30] Once this condition was evaluated, the two-tail Student's t-test at 95% confidence level could be applied to compare the slopes of regression. Then, t-values were calculated, following again Andrade *et al.* guidelines,[30] based on the equality or difference of the population variances. Additionally, the statistical treatment was carried out using the Software Statgraphics Centurion XVI (available from Statgraphics.net) providing the same results. In the case of the complementary P measurements in the phosphorylated protein β-casein, experimental concentrations were obtained using external calibration with a certified inorganic standard as they could not be obtained using online isotope dilution as previously described for the S measurements.

RESULTS AND DISCUSSION

The compounds selected for the assessment included, on the one hand, three low-MW biologically relevant compounds: a proteinogenic amino acid like L-Methionine, and two cofactors, S-Adenosylmethionine (SAM) and biotin, which are involved in methylation reactions[31] and in redox chemistry,[32] respectively. Three peptide standards of different size covering a wide range of hydrophobicity (% of hydrophobic residues present in the corresponding amino acid sequence)[33] were selected, including Adrenocorticotropic Hormone ACTH (2.1kDa, 29% hydrophobicity), Vasopressin (1.1kDa, 22%) and Neurokinin A (1.1kDa, 40%). Four purified protein standards of different nature, covering a wide range of molecular weights and structures, were also chosen (Cyt C ~ 13kDa, β-casein, ~ 24kDa, BSA, ~ 66kDa and intact monoclonal Antibody-mAb, ~ 145kDa). Finally, three thiolated Polyethylene Glycol (mPEG-SH) derivatives, one of 1kDa and two of 20kDa (linear and branched) that are extensively used in biological studies to improve water solubility and biocompatibility of molecules like nanoparticles and drugs, were selected as well.[34] Compounds structures are illustrated in Figure S1. All these compounds contain sulfur in their structure (also present in many other biologically relevant biomolecules such as oligonucleotides, vitamins and secondary metabolites),[32,35] enabling their detection using ICP-MS/MS.[22,36] Since biomolecule concentration could play a role in the response factors obtained, calibration graphs (n=10) covering more than one order of magnitude (from ca. 40 to ca. 1300 ppb S) were built to check for such concentration-dependent effects. Four replicates were performed for each calibration point. Notably, despite the great number of FI peaks processed, none of them was found to be an out layer and therefore all of them were considered.

There are several relevant factors that must be taken into account in order to make a fair and critical comparison between the response factors provided by the different nebulization systems. We needed first to assure the absence of any other S-containing species in the standard solutions prepared directly from the original corresponding solid materials in order to compare directly the sulfur signal obtained from the different species under study. This is particularly relevant for the protein standards that are obtained through relatively complex purification processes. Every stock solution was analyzed with capillary reversed phase core-shell columns (operational conditions are given in Table S3), which are able to provide quantitative recoveries even for the selected protein species.[37] Chromatograms obtained are given in Figure S2. As can be seen negligible signals (≤4% total S area) corresponding to low MW impurities containing sulfur were observed for all the standard solutions. In the case of β-casein and the 1kDa-PEG species, small S peaks (≤6%) were observed eluting very close to the main species. These impurities likely correspond to isoforms[37] and therefore, do not affect results interpretation as the nebulization behavior is expected to be very similar for the different isoform species. Once chromatographic purity was proved, total sulfur content was accurately and precisely determined using isotope dilution analysis (IDA). For that purpose, stock solutions of every compound standard were acid-digested to convert the corresponding S species into sulfate and then spiked with an isotopic tracer of sulfur ($^{34}SO_4^{2-}$). The obtained S content could be then used to compute the mass purity of every biomolecule standard under study. Complete set of results are given in Table S4. As can be seen, mass purities obtained for the low molecular weight compounds and mPEGs were very close to 100% and matched pretty well with the purity values available from the supplier. In contrast mass purity obtained for peptide standards was lower than 100%. It is worth noting that, purity values for peptide standards provided by the manufacturer included peptide purity (i.e. detection of concomitant species by HPLC-UV) together with peptide mass content (i.e. quantitative information of the total amino acid content carried out using Amino Acid Analysis, the reference method), which allowed us to validate our results experimentally obtained by acid-digestion and IDA of the peptide solutions. As can be seen in Table S4, mass purity values obtained for Vasopressin, ACTH and Neurokinin (83±1%, 70±1% and 77±2%, respectively) matched very well with those provided by manufacturer (83%, 69% and 80%, respectively). In the case of the protein standards, mass purity values obtained for Cyt C, β-casein and BSA (89±2%, 90±2% and 91±1%, respectively) are slightly lower than the protein purities provided by the manufacturer (>95%, >98% and >96%, respectively) and obtained using electrophoresis. Please note that such protein purity values provided by the suppliers only refers to the presence of other proteins as contaminants and do not take into account other contaminants such as salts or other minor reagents likely present in the solid materials. Finally in the case of the mAb mass purity obtained was 76±2%. Notably, supplier did not provide any protein purity in this case. Such sulfur mass purities were used to obtain the theoretical S concentration of the different solutions prepared for each compound under study.

Plasma stability could also affect the accuracy of this critical assessment mostly due to signal drifts and matrix effects. Therefore, we needed to correct for the instrumental sensitivity variations likely occurring both intra- and inter-calibrations of the different compounds. Possible influence of the organic moiety of the biomolecule should also be corrected. For that

purpose, a constant flow of a $^{34}SO_4^{2-}$ spike solution was mixed online with the FI flow (see Figure 1B) to serve as the ideal internal standard.[6] In that way the intensity ratio of both isotopes, the natural ^{32}S signal coming from the different compounds and the ^{34}S signal continuously coming from the isotopically enriched solution, was measured ($R_m^{32/34}$) as a function of time. We resorted then to the well-developed mathematical treatment of the online IDA to produce normalized peak areas that could be latter safely used in the calibration plots.[38] Of course, such normalized areas correspond to the mass of sulfur determined in the different FI peaks that were then translated into concentration after consideration of the different injection volumes used, as shown in equation 1.

$$Conc_{exp}^{IDA} = \frac{[fcd]_{sp}^{34}}{V_{inj}} \left(\frac{Aw_s}{Aw_{sp}}\right)\left(\frac{A_{sp}^{34}}{A_s^{32}}\right)\int_{t_1}^{t_2} \frac{R_m^{32/34} - R_{sp}^{32/34}}{1 - R_m^{32/34} R_s^{34/32}} \quad (1)$$

where d is the density of the spike (^{34}S) solution, Aw_s and Aw_{sp} correspond to the atomic weight of the natural and isotopic sulfur, respectively; and A_{sp}^{34} and A_s^{32} correspond to the abundance of isotopes ^{34}S and ^{32}S in the spike and sample (natural). $R_{sp}^{32/34}$ and $R_s^{34/32}$ are the isotope ratios in the spike and sample (natural), respectively. Finally, t_1 and t_2 are the initial and final times of the corresponding FI peak.

Such experimental concentrations obtained by online IDA ($Conc_{exp}^{IDA}$) were plotted against the theoretical concentrations used. Therefore a slope of 1 means that the experimental concentration obtained is identical to the theoretical concentration along the calibration range and therefore nebulization efficiency of the target species is identical to that of the isotopically enriched sulfate used for the corresponding quantification. Obviously, such normalization procedure allowed direct and safe comparison of the slopes of the different calibrations plots, even those obtained using different nebulizers just by changing flow (f) and concentration (c) of the ^{34}S-tracer and the injection volume (V_{inj}) of the sample.

It is noteworthy that calibration plots of the different biomolecules were always normalized by the continuous ^{34}S signal of the spike solution (see eq 1 and Figure 1B), which is actually pure inorganic sulfur (sulfate), in order to produce the experimental concentration values by online isotope dilution. Therefore, the individual slopes obtained for each different biomolecule already indicate their relative response factor with regards to the simple inorganic species. As expected, the slope obtained for the natural inorganic standard (sulfate) using the different nebulizers was always very close to one and could be used as reference in each case.

Critical comparison of ICP-MS response factors using calibration graphs. Calibration curves for the S inorganic standard and the different S-containing biomolecules were built using FI analysis and the different nebulization systems tested (concentric, cross-flow and total consumption) in order to carry out a critical statistical comparison of the ICP-MS response factors. Every calibration point was injected four times (n=4). The average experimental concentrations obtained using eq 1 (online IDA) for every individual calibration point (n=10) and their corresponding uncertainties were plotted against the theoretical concentrations for each of the compounds under study (n=14). Calibrations obtained are shown in Figure 2A, 2B and 2C for the TCN, concentric and cross-flow nebulizers, respectively. Individual slopes and intercept values with their corresponding standard deviations, regression coefficients and the statistical values at 95% confidence level are given in Tables S5-S7.

Regarding TCN, precision associated to each individual calibration point of the different species was adequate ranging from 0.5 to 15% with an average value of 5.0% RSD. As can be clearly seen in Figure 2A calibration slopes obtained for every compound using the TCN are very similar, ranging from 0.958±0.020 to 1.043±0.036 for β-casein and Cytochrome C, respectively.

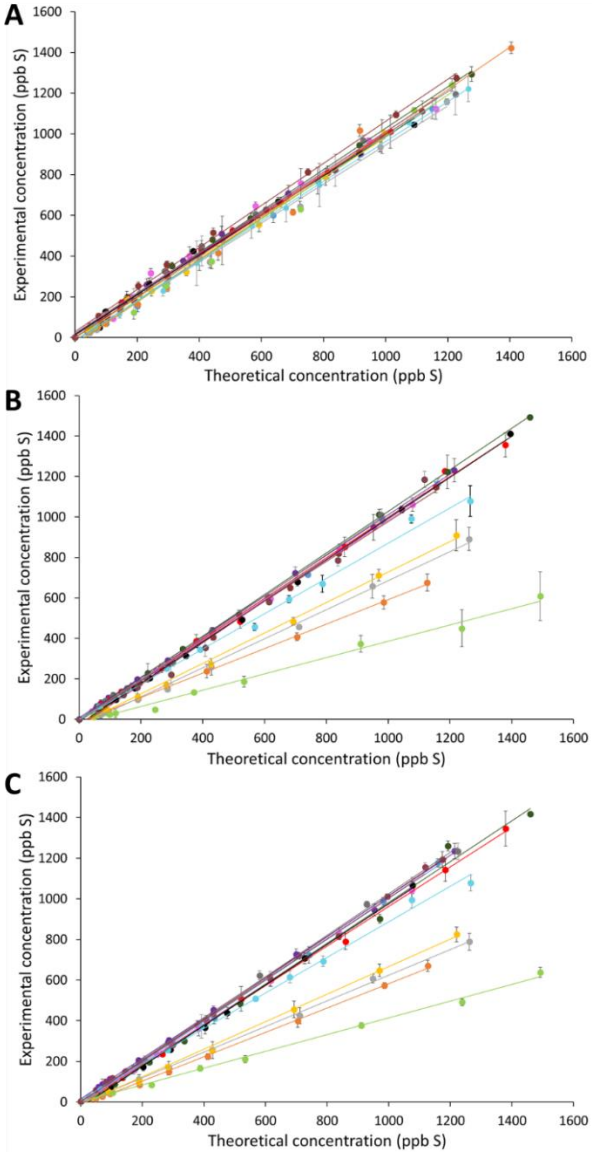

Figure 2. Calibration curves obtained for every single compound under study using A) TCN, B) concentric and C) cross flow nebulizers. Uncertainty bars correspond to 95% confidence level (2SD, n=4 replicates). Color code: Sulfate (blue), methionine (brown), SAM (red), Biotin (violet), m-PEG-SH 1kDa (pink), m-PEG-SH 20kDa linear (dark green), m-PEG-SH 20kDa branched (black), Cytochrome C (orange), β-casein (grey), BSA (yellow), mAb (light green), ACTH (garnet), Vasopressin (dark grey) and Neurokinin A (pale blue).

In fact, average value for the 14 individual slopes was 0.998. Corresponding slopes uncertainties were all below 3.5% RSD and regression coefficients (r^2) ranged from 0.991-0.999. Two tailed t-tests carried out at 95% confidence level

demonstrated that calibration slopes for every biological compound were statistically equal to the certified inorganic natural sulfate standard used as reference. In fact, as shown in Figure S3, a "multi-species" generic calibration plot containing every calibration point (n=10) of each of the fourteen compounds under study (total n=140) can be built resulting in an excellent correlation between experimental and theoretical concentrations (y=0.997x+1.069) with excellent linearity (r^2 = 0.993).

The most striking conclusion to emerge from this experiment is that full species-independent response can be obtained when using TCN coupled to ICP-MS. In fact, it demonstrates not only that the nebulization efficiency is identical for the 14 different compounds tested, but also that, as expected, ionization efficiency in the plasma is fully independent from the species. Notably, complete dissolution of every biomolecule under the conditions assayed and adequate certification of their content (mass purity) are proven as well. These statements are of paramount importance since the same ICP-MS instrument and biomolecule solutions were used in the assessment of the other two regular flow nebulizers and therefore any difference observed in the response factors of the biomolecules under study will be exclusively ascribed to differences in the nebulization efficiencies.

Regarding concentric and cross-flow nebulizers, precision associated to each individual calibration point of the different species was also found to be adequate with average values of 5.1 and 4.4% RSD, respectively. Figures 2B-2C show the calibration plots obtained for every biomolecule under study using the standard concentric and cross flow nebulizers, respectively. All the statistical parameters related are given in Tables S6 and S7. Corresponding slopes uncertainties were again adequate, below 3.5% and 2.4 % RSD, and regression coefficients (r^2) ranged from 0.990-0.999 and 0.996-0.999 for concentric and cross flow nebulizers, respectively. As can be seen, calibration slopes obtained for the low MW compounds (inorganic standard, methionine, biotin, and SAM) were very similar for both nebulizers and close to 1. Interestingly, this behavior was also observed for the more complex PEG compounds, both for the 1kDa and for the 20kDa (linear and branched) derivatives, vasopressin and ACTH peptides. Average slope values for those nine species were 1.008 and 1.001 for concentric and cross flow nebulizers, respectively. In fact, after statistical treatment, slopes of the low MW compounds, PEG derivatives and barely hydrophobic peptides (vasopressin and ACTH) were proved to be statistically equal (see Tables S6-S7) to that of the inorganic standard.

In contrast, significant differences in the ICP-MS response factors (calibration slopes) were observed for every protein tested as clearly shown in Figures 2B-2C. Actually in these cases the two-tail Student's t-tests at 95% confidence level demonstrated that proteins´ calibration slopes were statistically different (and significantly lower) than that of the corresponding inorganic standard, both for the concentric and cross-flow nebulizers (Tables S6 and S7, respectively). For small and medium size proteins (i.e. Cytochrome C, β-casein and BSA) slopes obtained ranged from 0.603 to 0.752 and from 0.599 to 0.678 for concentric and cross flow nebulizers, respectively. In the case of the larger and complex intact antibody (mAb) the slope fell to 0.403 and 0.412, respectively. Interestingly, the highly hydrophobic peptide (neurokinin A) exhibited behavior that is intermediate between the proteins and the group comprising low MW compounds, PEG derivatives and barely hydrophobic peptides. The slope values observed were slightly lower than 1 (i.e. 0.871 and 0.874 for concentric cross flow nebulizers) as shown in Figures 2B-2C. This difference is

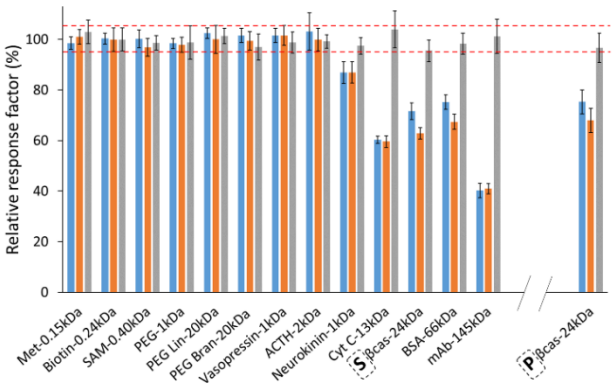

Figure 3. Relative response factors (% ratio of the calibration slopes of the individual species divided by that of the corresponding inorganic standard, see Tables S5-S7) obtained using the concentric (blue), cross flow (orange) and TCN (grey) nebulizers for all the biomolecules under study. Uncertainty was propagated taken into account the uncertainty associated to the slopes of the biomolecules and the corresponding inorganic standard. Error bars correspond to such propagated uncertainty at the 95% confidence level (2SD).

however significant enough to make such slopes statistically different (and significantly lower) than that of the corresponding inorganic standard. This set of results offer compelling evidence for the species-dependent response of ICP-MS when using regular flow nebulizers at least for the four proteins and the hydrophobic peptide tested in this study.

In order to make a global comparison of the differential behaviors observed for each biomolecule and using the 3 nebulizers, Figure 3 summarizes the relative response factors obtained by direct comparison (% ratio) of the calibration slopes of the individual biomolecules with the corresponding slope of the natural inorganic standard. If the relative response factor obtained is close to 100% means that such species behaves identically than the inorganic natural standard using the corresponding specific nebulizer. All values obtained with the TCN and those obtained using standard nebulizers for methionine, biotin, SAM, PEG derivatives and peptides (Vasopressin and ACTH) perfectly matched the response factor of the S inorganic standard at the 95% confidence level (95-105%, dotted red lines in Figure 3). However, as previously highlighted in Figures 2B-2C, relative response factors obtained for the proteins tested and the Neurokinin peptide using the standard nebulizers are significantly lower. Interestingly Figure 3 seems to suggest that the differential behavior using such regular flow nebulizers is not directly depending on the molecular weight of the compounds under study. Relative response factors obtained for the PEG derivatives, even for those in the same size range (20kDa) than proteins, were close to 100%. In fact, each and every PEG derivatives tested with the three nebulizers provided relative response factors ranging from 97±5% and 103±2%, in spite that they have very different size (1 and 20kDa) and structure (linear and branched). In contrast, it seems clear that hydrophobicity is a primary reason behind the differential behavior observed for regular flow nebulizers. In fact the most hydrophobic peptide, neurokinin (40% of hydrophobic amino acid residues present)[33] provided slightly lower, but still statistically different, relative response factors

(87±4% for both nebulizers). However, the ACTH, which is almost twice the size of neurokinin (2.1 and 1.1kDa, respectively) but considerably less hydrophobic (29%), provided relative response factors close to 100% (103±7% and 100±4%, respectively). In this line, a small protein such as Cyt C (13kDa) provided already significantly lower relative response factors (60±2% for both nebulizers). Bigger proteins such as β-casein and BSA provided similar but still slightly higher response factors for both concentric (72±3% and 75±3%, respectively) and cross-flow (63±2% and 67±3%, respectively) nebulizers. However, the significantly bigger monoclonal Ab (mAb) provided much lower response factors (40±3% and 41±2%, respectively). Notably, both nebulizers followed the same trend in spite that are based in different principles and are produced by different companies.. In fact, same results are expected using other nebulizers with similar principles and characteristics. It is also important to highlight here the % of hydrophobic residues[33] in the proteins under study that are 31%, 54%, 38% and 38% for Cyt C, β-casein, BSA and mAb, respectively. Therefore it seems that hydrophobicity plays an important, but no definitive, role in the nebulization efficiency observed using the regular nebulizers. In fact, it appears that the size of the compound might be relevant as well when comparing biomolecules with similar hydrophobicity. Actually, when comparing the neurokinin peptide with the proteins BSA and mAb (all of them with around 40% of hydrophobic residues), it can be clearly observed in Figure 3 that the relative response factors decreases as the size increases both for concentric (87±4, 75±3 and 40±3, respectively) and cross-flow nebulizers (87±4, 67±3 and 41±2, respectively). Of course, the presence of posttranslational modifications and prosthetic groups (cofactors) in the proteins could also be important factors. In the particular case of Cyt C is must be taken into account the extra hydrophobicity brought by the highly hydrophobic Heme group.[39] This could be the reason of the significantly low relative response factors (60±2% for both nebulizers) observed for this protein in spite of its relative small size and low % of hydrophobic residues (31%). Also in this line, the incorporation of up to 5 hydrophilic phosphate groups in the phosphorylated protein β-casein could explain the relatively high relative response factors (72±3 and 63±2, modified Figure 3) observed in spite of its very high percentage of hydrophobic residues (54%). It is worth stressing here that the experimental design of this work enables us to focus the critical comparison on the differences between the biomolecules tested and the enriched ^{34}S inorganic tracer spiked. In fact only these two species are present in the solution during nebulization and transport, which allows us to study their differential behavior exclusively without being affected by other factors, such as the presence of a complex matrix.[40] In fact, our experimental design would resemble the eluent of a chromatographic column where the different biomolecule species elute more or less isolated and can be quantified by online IDA or by applying the corresponding response factor obtained previously using simple generic standards.

Evaluation of different operational conditions. Furthermore, we wanted to find out whether the differential ICP-MS response factors observed for proteins using regular flow nebulizers remained under different operating conditions. We selected the concentric nebulizer and BSA as protein model, which presents an intermediate behavior (See Figures 2-3) and it is typically used as reference protein in absolute quantification workflows.[3] In this case, since the number of analyses to be performed was much lower, we selected bulk isotope dilution analysis as alternative quantification strategy. The BSA solution (containing natural S in its amino acid chain) was spiked with a known amount of the isotopically enriched inorganic standard ($^{34}SO_4^{2-}$). In that way, the target $^{32}S/^{34}S$ isotope ratio was measured in the spiked protein solutions and directly translated into concentration using conventional IDA. Corresponding blanks were also quantified and subtracted. Relative response factors were then computed by comparison (ratio, %) of the experimental concentration obtained with the theoretical calculated concentration. Different Argon carrier gas flows (Figure S4) were tested while keeping constant the liquid flow rate ($400\mu Lmin^{-1}$). Different liquid flow rates while keeping constant the Argon carrier gas flow ($1.1Lmin^{-1}$, Figure S5) were assayed as well. In both cases, no statistical differences were observed at the 95% confidence level. In fact, precision as good as 5% and 2% RSD (n=6) between the different conditions assayed, respectively, were obtained. Interestingly, the mean relative response factor obtained for BSA (71±4% and 72±1 % in Figures S4 and S5, respectively), was very similar to that obtained by the slope comparison (75±3 %, Figure 3) of the individual BSA and inorganic S standard solutions using the FI approach with online isotope dilution, what somehow internally validates the results obtained so far. Another operating parameter that could affect response factors of proteins could be the temperature of the spray chamber. The same BSA solution spiked with the ^{34}S tracer used in the previous experiment, was analyzed using the concentric nebulizer coupled to a double-pass spray chamber at four different temperatures (-5, 2, 10 and 20 ºC). Results are given in Figure S6. Once again no statistical discrepancies were observed being the mean relative response factor identical (73±2%) to those obtained previously (Figure 3 and Figures S4-S5).

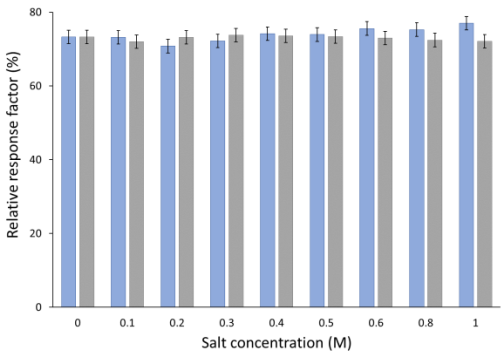

Figure 4. Relative response factors for BSA (experimental concentration obtained by IDA divided by the theoretical concentration) obtained in two different salt solutions (ammonium acetate, blue and Tris-HCl, grey) at different concentrations (0-1M) using the concentric nebulizer. Error bars correspond to 95% confidence level (2SD, n=10 replicates).

Finally we focused on the assessment of the possible impact of the salt type and its concentration on the relative response factor obtained for the same BSA solution. Increasing salt concentrations (from 0.1 to 1 M) were added to the same BSA solution spiked with the ^{34}S tracer used before. Two volatile buffer salts, ammonium acetate and Tris-HCl (pH 7.4) where selected, as they are typically used when coupling size exclusion chromatography (SEC) and ion exchange chromatography (IEC) to ICP-MS for protein analysis.[41,42] Results are given in Figure 4. Again relative response factor remained unaltered at the 95% confidence level along the concentration

range of both salt types. In fact, precision obtained between the relative response factors obtained at the nine concentration levels was excellent both for ammonium acetate (73±4%) and Tris (73±1%). Such relative response factors matched very well again with those obtained for BSA so far using the concentric nebulizer under different operational conditions (Figures S4-S6) and protein concentration levels (Figure 3).

Interestingly, the experiments carried out using the isotopically enriched S inorganic standard spiked to the BSA solution containing the natural S in its structure (Figures 2-4 and S4-S6), confirm that isotopic equilibration is necessary to obtain accurate quantification using isotope dilution analysis. Of course, although such isotopic equilibration is achieved in the plasma, the different nebulization efficiencies between the enriched inorganic standard and the biomolecule when using regular nebulizers led to biased results. To prove further this assumption, the same BSA solution spiked with the enriched inorganic standard was analyzed before and after being subjected to acidic digestion. Importantly, the same intact protein and digested protein solutions were analyzed using the concentric and TCN nebulizers. Quantification was performed using conventional IDA and again relative response factors were computed as the ratio (%) between the experimentally obtained and the theoretical BSA concentrations. When analyzing the intact protein solution with the concentric nebulizer, significantly lower IDA results were obtained being the relative response factor (73±1%) very similar to those obtained before (Figures 3-4 and S4-S6). In contrast, when acidic digestion of the same spiked protein solution is carried out prior to the isotope ratio measurement, that is to say when the natural S originally present in the protein amino acid chain has been previously converted into inorganic sulfate, the relative response factor rises up to 97±2%. On the other hand, no statistical differences in the relative response factor were obtained using the TCN for the intact (98±1%) and acid digested (100±2%) solutions. This experiment is consistent with the previous results (Figure 3) and demonstrates once again that nebulization efficiency using TCN is complete regardless of the nature of molecule containing the element and therefore that it is able to provide full species-independent response in ICP-MS for any kind of biomolecule, including proteins.

Simultaneous P and S monitoring in protein β-casein. In order to ultimately demonstrate that the differential behavior observed in regular flow nebulizers for the proteins is independent of the ICP-detectable element used to monitor the protein and it is exclusively due to species-dependent nebulization efficiency, we compared the relative response factor of β-casein obtained previously using the S present (as it is the case of the other proteins under study) and the P present in its phosphorylation sites. Note that β-casein has extensively used in phosphorylation studies as model protein. First, we assured the absence of any other P-containing species in the standard solution prepared directly from the original corresponding solid material using again capillary chromatography (operational conditions and chromatogram obtained are given in Table S3 and Figure S2). More than 99% of total P area corresponded to the target protein β-casein and its minor isoforms. Once chromatographic purity was proved, total phosphorus content was determined. For that purpose, the stock β-casein solution was acid-digested to convert the corresponding P species into phosphate, which was then determined by external calibration using a phosphate certified standard. In that way, the theoretical P concentration could be determined in the different calibration points that were subsequently measured using FI (four replicates per calibration point) and the three nebulizers under study to build the corresponding calibration graphs. Of course, since P is monoisotopic we had to resort in this case to external calibration using a certified phosphate standard. The average P experimental concentrations obtained for every individual calibration point (n=10) and their corresponding uncertainties were plotted against the theoretical concentrations for every nebulizer under study. Individual slopes and the intercept values with the corresponding uncertainties and regression coefficients are given in Table S8. As can be seen, calibration slope obtained using the TCN was again close to 1 (0.967) and significantly higher than those obtained using the concentric and cross flow nebulizers (0.753 and 0.680, respectively). In fact, relative response factors previously obtained for the β-casein protein using S (96±4, 72±3 and 63±2) match well with those obtained using P (97±6, 75±5 and 68±5) for TCN, concentric and cross flow, respectively as shown in Figure 3.

CONCLUSIONS

The capability of ICP-MS for the long-sought absolute quantification of biomolecules without the need of specific standards depends entirely on its potential to provide species-independent response. This work offers for the first time compelling evidence of the differential response factor obtained using ICP-MS with standard nebulization systems for proteins and hydrophobic peptide with regards to other simple biological relevant molecules such as amino acids, cofactors, PEG-derivatives, non-hydrophobic peptides and inorganic salts, all them containing the same ICP-detectable element present in the proteins (sulfur). Notably since the use of a total consumption nebulizer led to identical response factors for all the 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.

Interestingly it seems that while hydrophobicity is a principal reason for the different response factors obtained for the various biomolecules using regular flow nebulizers, the contribution of molecular weight is not so relevant. However, it has been observed that the magnitude of such differential behavior among species of similar hydrophobicity seems to depend on the molecular weight. The fact that there is excellent comparability between the different quantification strategies performed (i.e. FI with online IDA, bulk IDA of protein solutions spiked with the enriched isotope and comparison of the results obtained for protein solutions before and after acidic digestion) gives confidence as to the robustness of the results obtained.

Although this work has made use of the proteinaceous S present in the Cysteine and Methionine residues of proteins, our conclusions could extend to the P or Se present in phosphoproteins and selenoproteins, respectively and of course to any metal (Fe, Cu, Zn, Mn, V) coordinated (i. e., metalloproteins of coenzymes) to the target protein. This implies that species-independent quantification of intact proteins using ICP-MS cannot be carried out unless a TCN is used. Unfortunately, this is not the case of a wide range of applications

where size exclusion chromatography (SEC) and HPLC is coupled to ICP-MS using regular flow nebulizers and protein quantification is carried out using inorganic standards or online IDA.[42,43] To make matters worse such differential behavior appears to be unpredictable. Therefore, although the use of another element-containing protein as generic standard would provide better absolute quantification results, it should be demonstrated first that both, the standard and the target proteins, provide the same element response factor. It must be pointed out however that the use of isotopically enriched proteins (e.g. proteins coordinated with isotopic enriched metals or containing enriched S or Se in their amino acids) as internal standards could well correct for this effect.[27,44]

A profound impact of this work is expected in many scientific areas where absolute biomolecule quantification is performed using ICP-MS, especially in the proteomics, metallomics and clinical fields.[6,8] We still believe that the potential of ICP-MS for absolute protein quantification is enormous but the experimental workflow must be carefully designed, starting with the nebulization system and quantification strategy. The present findings might help to suggest several courses of actions to face this analytical challenge.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.
Instrumental conditions, biomolecule structures, mass purity assessment, chromatograms, detailed statistics, relative response factors for different conditions, P calibration curves (PDF).

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