Application Note
Proteomics

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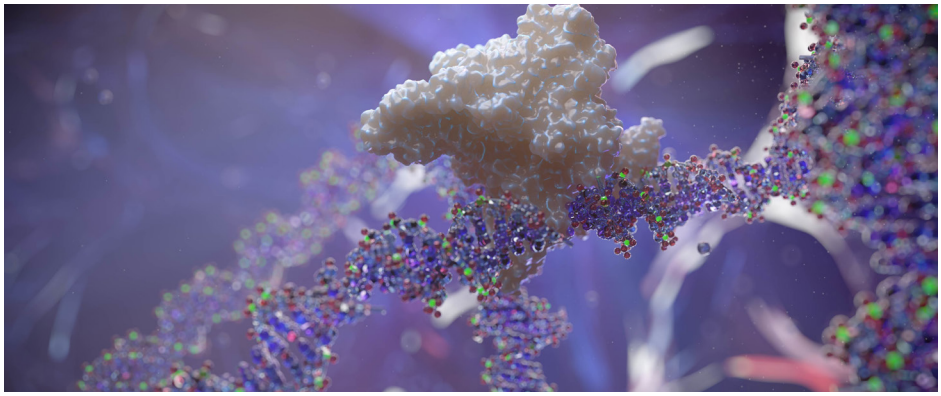
Direct and Traceable Mass Purity Certification of Protein Standards using LC-ICP-MS/MS

Absolute quantification of proteins using capillary LC coupled to Agilent 8900 Triple Quadrupole ICP-MS



Authors

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Introduction

Developments in the absolute quantification of proteins for life science and clinical research have increased the demand for better-quality calibration standards to ensure the quality of results. Existing strategies for certification of protein/peptide standards in quantitative proteomics (e.g., amino acid analysis) are indirect and labor-intensive. Therefore, there is an urgent need for novel strategies that can provide simple, direct, generic, and traceable certification of protein standards (*1*).

Sulfur (S) is present in methionine and cysteine amino acids and is therefore found in virtually all proteins. Capillary liquid chromatography (capLC) can separate the individual proteins, while triple quadrupole – or "tandem" – ICP-MS (ICP-MS/MS) can determine the protein concentrations based on the sulfur content. As the sulfur response is independent of the compound, the combination of capLC with ICP-MS/MS provides matrix- and species-independent quantification of the proteins (*2*).

Methods based on capLC-ICP-MS/MS are an interesting and effective generic solution to certify mass concentration and purity of protein standards (*3–6*). The capLC provides isolation of the specific protein species to be certified, removing any potential impurities (salts, isoforms, contaminants, etc.). The use of a total consumption nebulizer provides complete sample nebulization that is essential for biological analysis and species-independent quantification, while also assuring ICP-MS/MS compatibility with low-flow and low sample volume chromatography.

The addition of a carbon-source (CO_2 mixed with Ar gas) into the plasma ensures that the sulfur response is constant during the whole analysis. Without carbon addition, ionization enhancement would cause the S response to vary with changes in the carbon content of the organic solvent in the mobile phase. Finally, LC-ICP-MS/MS enables the use of any S-containing compound as a certification standard, such as a certified reference material (CRM) or mass-certified compound, ensuring traceability and comparability of the quantitative results.

This application note describes the procedure for the certification of several protein standards by capLC-ICP-MS/MS. For comparison purposes, quantitative results were obtained using both internal and external standardization:

- For the internal standardization method, a S-containing compound, BOC-L-methionine, was added to the sample. Internal standardization automatically corrects for any injection variability during the analysis, improving precision. However, the internal standard must meet several criteria regarding chromatographic performance, such as adequate retention factor and resolution (*4, 5*).
- For the external standardization method, a certified sulfur standard was analyzed using Flow Injection Analysis (FIA) – where the injection bypasses the column – before the chromatographic analysis of the protein standard. Using FIA removes any chromatographic requirements for the S standard, so any certified S-containing compound – such as an ICP-MS sulfate standard – can be used for the quantification, improving traceability. Studies have shown that, using an autosampler module in the capLC system, external calibration did not compromise data quality and gave equivalent precision compared to internal standardization (*6*).

Both standardization approaches allow the mass purity of the target protein to be calculated from the S response. The S response factors are correlated with the known certified S concentration of the standard (BOC-L-methionine for internal standardization using capLC or sulfate for external standardization by FIA). The measured S concentration in the target protein is converted to protein concentration based on the known sulfur:protein stoichiometry (number of cysteine and methionine amino acids and therefore S atoms).

Experimental

Reagents

An elemental sulfur ICP standard (1000 mg/L) was bought from Merck KGaA (Germany). Amino acid and protein standards, BOC-methionine-OH, bovine serum albumin (BSA), human transferrin, bovine β-casein, and cytochrome C from bovine heart were bought from Sigma-Aldrich (Steinheim, Germany). All solutions were prepared using ultrapure water. Acetonitrile (HPLC Grade) was bought from Fisher Scientific (USA), and formic acid was bought from Merck KGaA (Germany).

Instrumentation

Chromatographic separations were performed using an Agilent 1260 Infinity II HPLC fitted with a capillary pump and equipped with an autosampler module and a Spark Holland oven heating system (Mistral, The Netherlands). The reversed phase capillary column was a 150 mm × 0.3 mm BIOShellTM C4, A400, 3.4 µm particle size, 400 Å pore size, from Sigma-Aldrich (Steinheim, Germany). The LC method used mobile phases consisting of water (A) and acetonitrile (B) with 0.2% formic acid. The 1260 HPLC was connected to an Agilent 8900 Triple Quadrupole ICP-MS (ICP-QQQ) using the Agilent capillary LC interface kit (p/n G3680A).

The 8900 ICP-QQQ was fitted with an Agilent total consumption nebulizer (p/n G3280-80602) and an Agilent single-pass spray chamber (p/n G3280-80603) suitable for use with the capillary LC interface. A controlled flow of carbon dioxide (CO_2:Ar), mixed online with the option gas (O_2:Ar, Air Liquide, Madrid, Spain), was added continuously to the ICP-MS plasma to correct for any signal variation and to enhance sensitivity (*6, 7*). For maximum sensitivity, the ion lenses were optimized under the plasma conditions used for running organics. ICP-MS measurements were carried out using MS/MS mode and sulfur was measured using a mass-shift method with oxygen cell gas. Experimental conditions of the capLC-ICP-MS/MS system are given in Table 1.

Table 1. Typical capLC-ICP-QQQ operating conditions and capFIA parameters.

ICP-QQQ parameters	
RF power (W)	1550
Sampling depth (mm)	8
Carrier gas flow rate (L/min)	0.85
Makeup gas flow rate (L/min)	0
Option gas flow rate: O_2:Ar (20:80) (%) CO_2:Ar (10:90) (mL/min)	 6 30
Reaction cell gas flow O_2 (mL/min)	0.25
MS/MS mass-shift quadrupole settings (*m/z*)	Q1: 32 (S) Q2: 48 (SO)
CapLC parameters	
Flow (µL/min)	3.5
Injection volume (µL)	1
Gradient	
Time (min) 0 5 25 28 30	% mobile phase B 2 2 60 90 90
CapFIA parameters	
Flow (µL/min)	5
Injection volume (µL)	1
Mobile phase	98% A / 2% B
A: Water with 0.2% formic acid; B: Acetonitrile with 0.2% formic acid	

Results and discussion

Using capLC (internal standardization) and capFIA (external standardization) procedures described previously (*4–6*), four proteins with molecular weights ranging from 12 kDa (cytochrome C) to 66 kDa (BSA) were analyzed. Sulfur chromatograms obtained using both standardization approaches are shown in Figure 1.

Internal standardization requires that the chromatographic peaks are clearly resolved (*6*) but has the benefit that quantification of the target protein is accomplished in a single injection. By contrast, external standardization requires the capFIA (calibrant) injections to be combined with the chromatographic (target protein) analysis. However, external standardization allows the generic standard to be injected several times in a short period at the start of the measurement, providing additional statistics for calculating measurement precision and determining the response factor.

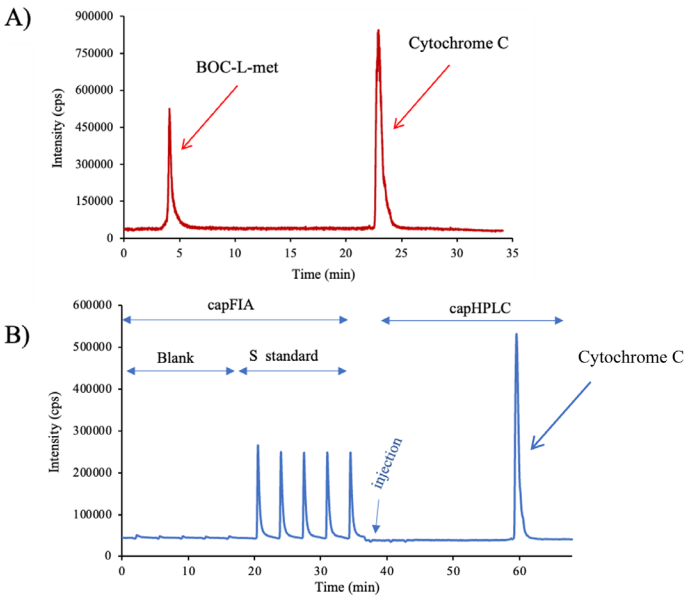

Figure 1. Cytochrome C analysis with (A) Internal standardization using certified BOC-L-methionine, ~2 µg S/mL (15 µg compound/mL), and cytochrome C, ~5.5 µg S/mL (490 µg prot/mL). (B) External standardization using certified ICP-MS SO_4^{2-} standard, 1 µg S/mL and cytochrome C, ~5 µg S/mL (360 µg prot/mL).

Representative chromatograms for both internal and external standardization acquisitions for the cytochrome C target protein are shown in Figure 1. The concentration results for cytochrome C calculated using the two calibration strategies are shown in Table 2, together with the results for the other three proteins. For the internal standardization results, the final protein concentration and standard deviation were calculated from three separate chromatographic injections. For the external standardization results, the protein concentration was calculated by averaging three chromatographic replicates. The overall standard deviation was calculated from the combination of the three chromatographic replicates and the five FIA injections made at the start of the first acquisition (n=8 in total).

As can be seen from the results in Table 2, both calibration strategies provided similar accuracy and precision for protein quantification and certification. The choice of method may depend on the requirements of the proteins being studied or the availability of standards.

Accurate protein quantification by ICP-MS without species-specific standards needs an identical response factor (nebulization and ionization) for the generic standard and the target proteins. A recent study has shown that this requirement can only be achieved when a total consumption nebulizer is used (*7*). Also, accurate quantification requires complete elution of calibrant and target proteins from the LC column. Elution efficiency can be confirmed by comparing the mass purity of the target proteins calculated using FIA (no column) and chromatographic separation. Good agreement between these values confirms that elution was complete for all the proteins analyzed (*4–6*). Protein mass purity certification of the assayed protein standards agreed with manufacturer assessed purities (theoretical values), as summarized in Table 2.

Table 2. Experimental results for mass certification of protein standards by capLC-ICP-MS/MS using absolute quantification with generic standards and both internal and external standardization. Theoretical protein purity values supplied by the manufacturer are included.

Protein	Protein Mass Purity (%)		
	Internal Standard Method	External Standard Method	Theoretical (Manufacturer) Values
Bovine Serum Albumin	99 ± 2%	97 ± 3%	≥ 98%
Transferrin	95 ± 1%	93 ± 3%	≥ 95%
β-casein	93 ± 6%	94 ± 5%	≥ 98%
Cytochrome C	92 ± 1%	96 ± 4%	≥ 95%

Conclusion

The novel capLC-ICP-MS/MS method used an Agilent 1260 Infinity II HPLC interfaced to an Agilent 8900 ICP-QQQ using an Agilent capillary LC interface kit. The Agilent capLC interface uses a total consumption nebulizer to ensure consistent response factors, while the addition of carbon (CO_2) directly into the plasma eliminated variability in S response due to the mobile phase solvent gradient. The methodology provided a direct, simple, sensitive, and generic approach for the absolute quantification of proteins by measuring their sulfur content.

The study showed that S-containing generic certified standards can be used for both internal and external calibration methods. Accurate, precise, and traceable protein quantification data was produced for four proteins using both standardization strategies.

CapLC-ICP-MS/MS is a useful and interesting technique for the certification of protein standards, providing quality assurance of quantitative data needed for MS-based biopharma, life science, or proteomic studies.

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