Absolute quantification of proteins: forget Amino-Acid Analysis and move to ICP/MS!

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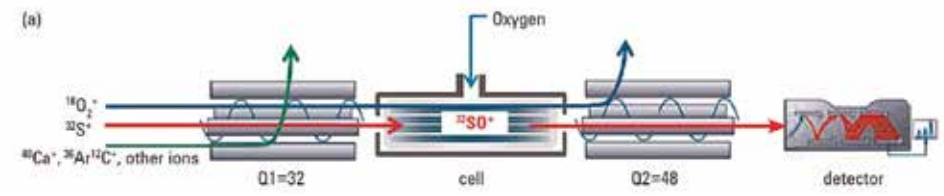
INTRODUCTION

Although drug products issued from biotechnology have now been marketed for many years, accurate protein quantification remains a challenge. Many colorimetric methods exist (Lowry, Bradford, BCA) but they all lack precision, suffer from interferences and matrix effects. Other classical methods such as peptide mapping LC-MS/MS, immunoassay, CE or UHPLC quantification of the intact protein require calibration with a highly reliable standard that is not straightforward to synthesise/purify/characterise. AAA after complete hydrolysis of the protein is probably the most commonly used method for the quantification of a single protein. There is no need of a reference protein but hydrolysis and derivatisation are time-consuming and very often result in low precision and accuracy.

In order to address the need for a suitable analytical method capable of accurately quantifying a protein without any specific reference substance, an isotope dilution ICP-MS/MS method based on sulfur determination was developed and validated, allowing very accurate determination of a single protein in solution after microwave digestion.

SULFUR DETERMINATION BY ICP/MS

Sulfur determination by ICP/MS has long been a challenge due to the high ionisation potential of this element (10.4 eV) and the interferences from polyatomic ions for all sulfur isotopes. The availability of a commercial triple quadrupole ICP/MS, such as the Agilent 8800 ICP-QQQ, allows to easily circumvent these issues, as exemplified in the following figure for the determination of $^{32}S^+$.



The fast switching capabilities of the instrument allows quasi-simultaneous determination of $^{32}S^+$ and $^{34}S^+$, making it possible to measure $^{34}S/^{32}S$ isotope ratios for all solutions injected: Q1 is set to select ions $^{32}S^+$ (*m/z* 32) or $^{34}S^+$ (*m/z* 34), and Q2 is set to select $^{32}S^{16}O^+$ (*m/z* 48) or $^{34}S^{16}O^+$ (*m/z* 50) (mass shifted due to the reaction with oxygen in the reaction cell).

MATERIALS & METHODS

Principle

After addition of $^{34}SO_4$, the protein is microwave digested with HNO_3/H_2O_2 and $^{34}S/^{32}S$ ratios are determined by ICP-MS/MS; knowing the expected Sulfur content, the protein concentration can easily be calculated with great accuracy.

No calibration necessary (isotope dilution), no reference protein needed, only a certified H_2SO_4 solution is required!

Double isotope dilution methodology

In order to improve accuracy and precision, a double isotope dilution mass spectrometry (IDMS) strategy was implemented: a known amount of a ^{34}S-enriched standard solution is added to the sample (containing mostly the natural isotope of sulfur: ^{32}S); quantification is then performed by measuring the ratio between the two isotopes. This approach also allows to compensate for response factors differences observed between ^{32}S and ^{34}S (mass discrimination).

Reagents

BSA : NIST, Standard Reference Material, 927e (67.38 ± 1.38 mg/mL)
IgG: Human Immunoglobulin, *Ph. Eur.* BRP, batch 1
^{34}S: ISOFLEX, S-34, 99 % ^{34}S
Sulfur ICP Standard: Fluka, TraceCERT, 1000 mg/mL

Equipment

All analyses were carried out with an Agilent 8800 ICP-QQQ system fitted with a standard concentric nebulizer, x-lens, Peltier-cooled double-pass Scott-type spray chamber and standard Pt interface cones. A summary of ICP/MS parameters is presented in the adjacent table. An Agilent I-AS integrated autosampler was used for sample infusion.

Parameter	Setting
RF power	1550 W
Nebulizer typer	Micromist
Nebulizer gas flow	0.25 L.min^{-1}
Dilution gas flow	0.85 L.min^{-1}
Cell gas flow rate	0.35 L.min^{-1}
Data acquisition	m/z 32 shifted to m/z 48 m/z 34 shifted to m/z 50

Digestion was performed in a Milestone UltraWAVE single reaction chamber microwave digestion system.

Sample preparation

A protein sample containing approximately 50 µg of sulfur is weighed into a disposable glass tube. 50 µg of ^{34}S are added (as $H_2{}^{34}SO_4$), followed by 2 mL of 69% HNO_3, 0.5 mL of 37% HCl and 1 mL of 30% H_2O_2. After microwave digestion, the sample is diluted to 50 mL with water. The same procedure is performed on 50 µg of NIST-traceable SO_4^{2-} reference solution for reverse IDMS *(vide supra)*.

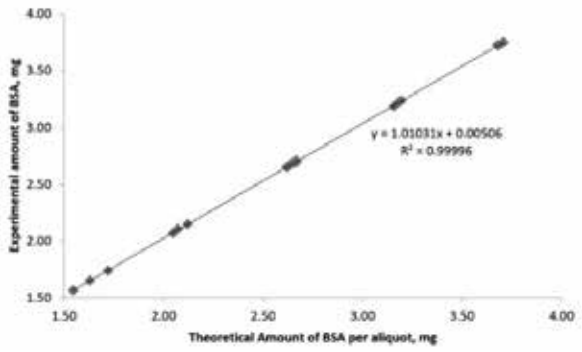
In order to take into account the possible presence of sulfur-containing species in the formulation buffer of the protein, it may be necessary to correct for this non-proteinaceous sulfur. In this case, the sample is filtered through a 3 kDa molecular-weight cut-off membrane and an aliquot of the filtrate is digested as a sample and used as a blank.

METHOD PERFORMANCE

In order to evaluate the performance of the method, Bovine Serum Albumin (BSA) was used as model compound due to its availability as a solution with a certified concentration (NIST).

Linearity/Accuracy

Linearity and accuracy of the method were evaluated by digesting different amounts of BSA standard solution (NIST SRM 927e) with a constant amount of ^{34}S added. Protein amounts corresponding to ~ 30, 40, 50, 60 and 70 µg of sulfur per sample were used. At each level, the analysis was performed in triplicate (3 independent preparations). The graph for linearity assessment is presented below. Mean recoveries ranged between 101.2 and 101.3 %. RSDs at each level ranged between 0.1 and 0.3 %. A linear relationship was obtained between the amount of BSA in the digestion tube and the amount of BSA experimentally measured (R = 1.000).



Precision

Method precision and intermediate precision were assessed for 2 samples: BSA standard solution (NIST SRM 927e) and a human Immunoglobulin solution (*Ph. Eur.* BRP).

Two analysts carried out independently 6 determinations of each sample on 2 different days. Relative standard deviations for each analyst (n = 6) ranged between 0.2 and 0.5%. The overall RSDs (n = 12) were below 0.7%. The relative differences between the means obtained by the two analysts were ≤ 0.7 %.

Sample	Analyst 1		Analyst 2		Total RSD (%)
	Average	% RSD (n = 6)	Average	% RSD (n = 6)	n = 12
NIST BSA (mg/mL)	67.28	0.41	67.50	0.52	0.6
IgG (mg Sulfur/g)	8.39	0.13	8.33	0.19	0.7

Matrix compatibility

Most Drug Products containing proteins are presented in a formulation buffer; in order to show the robustness of the method *vs.* different matrices, a BSA standard solution was digested in presence of commonly used buffers for therapeutic proteins. The formulations tested and their compositions are listed below.

Mean BSA recoveries ranged between 99.6 and 100.8% and RSDs (n=3) were all between 0.0 and 0.6%.

Matrix	Composition	Mean recovery (%)	% RSD (n = 3)
Matrix 1	0.9% NaCl	100.27	0.05
Matrix 2	PBS (phosphate buffer saline)	100.24	0.36
Matrix 3	10% sucrose	100.16	0.61
Matrix 4	0.1% polysorbate	100.26	0.57
Matrix 5	15 mg/mL glycine	99.61	0.37
Matrix 6	15 mg/mL histidine	100.76	0.23
Matrix 7	FS (worst case, mix of matrices 1 to 6)	100.33	0.21

Non-proteinaceous sulfur

Protein solutions may be contaminated by sulfur from external sources like formulation ingredients or solvents.

In order to show the capability of the method to take into account the possible presence of non-proteinaceous sulfur, a diluted sulfate solution was added to a BSA solution at a concentration corresponding to ~5% of the sulfur present in the protein. Samples were filtered through a 3 kDa molecular-weight cut-off membrane and sulfur concentration in the filtrate was measured by ICP/MS. The corrected BSA recovery for the spiked solution is 99.6%, showing that total sulfur measured can be efficiently corrected for a possible contamination by a non- protein source of sulfur.

Sample	Sulfur (µg/g)
A: unspiked BSA solution	36.50
B: spiked BSA solution	38.81
C: filtered solution	2.44
B-C	36.37 (99.6% recovery vs 36.5)

CONCLUSION

Double isotope dilution ICP-QQQ/MS is an innovative method for the absolute quantification of pure proteins. The precision and accuracy of this methodology bear no comparison to the traditional methods used in most analytical labs, such as amino acid analysis. This methodology is tolerant to most matrices commonly used for therapeutic protein formulations, widely applicable, and fast (results can be obtained within half a day of work in routine use).

The method characteristics are:

- Good sensitivity: 1 or 2 mg of protein in 5 mL is enough
- High reproducibility: RSD < 1 %
- Great accuracy: < 2 % bias
- Insensitivity to matrix effect
- Same method for any protein or peptide
- Uses only very common reagents and standards
- Fast: results obtained within half a day of lab work in routine use

It is suitable for:

- Assay of a single protein or mAb in solution
- Assay of a protein adsorbed on an adjuvant (vaccines)
- Accurate determination of a protein or peptide standard solution concentration
- Determination of molar extinction coefficient

Future developments:

Such a method can be adapted to the determination of phosphorus allowing protein phosphorylation studies and **oligonucleotides quantification.**

Simultaneous measurement of sulfur and phosphorus in the same sample would be very useful for the determination of the **P=O/P=S ratio in phosphorothioates.**