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# *In vivo* synthesized $^{34}\text{S}$ enriched amino acid standards for species specific isotope dilution of proteins

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## Abstract

A generic quantification approach was introduced addressing the characterization of protein standards while fulfilling the principles of metrology. Traceable absolute quantification was achieved combining a proven biochemical method, i.e. protein hydrolysis followed by amino acid quantification with the concept of species specific isotope dilution analysis (IDA). The method relies on the determination of the two sulfur containing amino acids, cysteine and methionine by sulfur speciation analysis and is hence applicable to any protein containing sulfur. *In vivo* synthesis using  $^{34}\text{S}$  as sulfur source in yeast fermentations provided species specific isotopically enriched standards for IDA quantification of cysteine and methionine in the oxidized forms, methionine sulfone and cysteic acid. Reverse isotope dilution mass spectrometry (IDMS) characterization by inductively coupled plasma mass spectrometry (ICP-MS) combined to anion exchange showed that very high concentrated spike material could be produced with  $\mu\text{mol}$  amounts of proteinogenic sulfur containing amino acids per g cell dry weight. An enrichment of  $^{34}\text{S}$  to  $96.3 \pm 0.4\%$  (N=3) and  $98.5 \pm 0.4\%$  (N=3) for cysteic acid and methionine sulfone, respectively, was assessed. The established IDA method was validated for the absolute quantification of commercially available lysozyme and ceruloplasmin standards including the calculation of a total combined uncertainty budget.

## Introduction

The development of reference methods and standards of high analytical order is a topical research theme in biomedical analysis of proteins representing a significant fraction of clinically addressed biomarkers <sup>1,2</sup>. In the case of clinical analysis it is especially important to accurately quantify absolute concentrations of proteins. Indeed several directives, such as e.g. the directive 98/79/EC <sup>3</sup> on in vitro diagnostic medical devices clearly states that “the traceability of values assigned to calibrators and/or control materials must be assured through available reference measurement procedures and/or available reference materials of a higher order”. Furthermore, the standard EN ISO 17511:

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3 2003<sup>4</sup> demands reference measurement systems including reference measurement procedures for  
4 the determination of analytes in samples of human origin. It is particularly important for medical  
5 laboratory measurements concerned with both patient care and health screening to give adequately  
6 comparable, reproducible and accurate results (ISO 15193:2009).  
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9 In light of these directives, it would be highly desirable to establish methods ensuring measurement  
10 traceability, since it is required to standardize results irrespective of the measurement procedure and  
11 laboratory. As a matter of fact, up to date the concentration of most commercially available proteins  
12 and even protein standards is not assessed by methods providing traceability of values assigned to  
13 calibrants, following the principles of metrology. E.g. in most cases the purity of protein solutions is  
14 reported as SDS page- or as HPLC-purity. The given concentration is typically obtained by Bradford  
15 assays<sup>5</sup> which are calibrated against a species unspecific calibrant, i.e. in most cases albumin. As a  
16 consequence, the traceability of many proteins is maintained by reference to the first standard  
17 produced, which may no longer exist, with values assigned by consensus. This reliance on the original  
18 standard preparations causes severe problems.  
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23 In this work, we propose a traceable quantification method based on elemental speciation analysis,  
24 which could be generalized to a wide range of protein standards. The validity of elemental analysis  
25 serving as a reference method has been recognized by metrological institutions and was shown by  
26 several recent publications<sup>2</sup>. For example transferrin was accurately quantified in a serum<sup>6</sup>. Our  
27 work focused on sulfur speciation analysis as a generic absolute protein quantification method. The  
28 potential of elemental analysis addressing hetero-elements in proteins has been comprehensively  
29 reviewed<sup>7</sup>. The most evident direct targeting approach would involve quantification of intact  
30 proteins by sulfur using size exclusion chromatography and ICP-MS detection<sup>8,9</sup>. As a prerequisite,  
31 the amino acid sequence and hence the number of sulfur containing amino acids has to be known.  
32 Size exclusion chromatography is perfectly suited to separate inorganic and low molecular weight  
33 organic impurities from proteins. However, as a major drawback the size exclusion chromatography  
34 shows column recoveries depending on the protein, the column life time and the separation  
35 conditions. Accordingly, species unspecific quantification has to be corrected for column recovery  
36 and this column recovery – since it might be changing along the experiments- has to be monitored.  
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42 Due to these limitations we turned to a proven biochemical method as an alternative strategy, which  
43 is protein quantification by analyzing the amino acid content of protein samples after complete  
44 decomposition of the proteins in its constituents, the amino acids. Amino acid analysis techniques  
45 formed an integral part in almost all biochemical structure determinations in earlier days. At that  
46 time, dedicated sample preparation methods addressing the recovery and extraction efficiency of  
47 individual amino acids were developed. For the two highly reactive sulfur compounds, methionine  
48 and cysteine, a sample preparation including oxidative protection prior to hydrolysis proved to be  
49 suitable among other methods. In a recent publication<sup>10</sup> we implemented this approach, validating  
50 the procedure by external calibration using cysteic acid and methionine sulfone standards and  
51 determination by LC-ICP-MS. Applying a weak anion exchange chromatographic separation excellent  
52 column recoveries of  $95 \pm 2\%$  for cysteic acid and  $105 \pm 10\%$  for methionine sulfone standards were  
53 found. The limits of detection obtained by reaction cell technology ICP-MS were at  $1 \mu\text{M}$  level for the  
54 two investigated sulfur containing compounds. The analytical technique was robust, since LC-ICP-MS  
55 showed a long term repeatability of 1.9% for methionine sulfone and 3.5% for cysteic acid[7].  
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In this work we combine the proven biochemical method of amino acid based quantification for the first time with the concept of isotope dilution using sulfur speciation analysis. The objective was to introduce a traceable accurate quantification strategy for any protein standard containing sulfur amino acids. Moreover, we apply for the first time an *in vivo* synthesized  $^{34}\text{S}$  enriched yeast hydrolysate as internal standard for isotope dilution based quantification of proteins.

## Experimental

### Reagents and materials

The standard for lysozyme (from chicken egg white) with a purity > 90% and the amino acid calibrants, namely methionine sulfone and cysteic acid, the reagents phenol purified by redistillation,  $\geq 99\%$ , and ammonium formate  $\geq 99.995\%$  trace metals basis were all purchased from Sigma Aldrich (Steinheim, Germany). Ceruloplasmin (Human Plasma) was purchased from Athens Research and Technology (Athens, USA). Formic acid 98-100% (Suprapur<sup>®</sup>), fuming hydrochloric acid 37% for analysis (EMSURE<sup>®</sup>), sulfur ICP standard traceable to SRM from NIST  $\text{H}_2\text{SO}_4$  in  $\text{H}_2\text{O}$  1000  $\text{mg L}^{-1}$  S CertiPUR<sup>®</sup> and hydrogen peroxide solution 31% Ultrapur were purchased from Merck (Darmstadt, Germany). Acetonitrile (LC-MS grade) was obtained from Fluka (Buchs, Switzerland). The ammonium acetate buffers applied as eluent for chromatography were derived from ammonium hydroxide solution  $\geq 25\%$  in  $\text{H}_2\text{O}$  (TraceSELECT<sup>®</sup>, for trace analysis), Sigma Aldrich, Steinheim, Germany) and ammonium acetate (Fractopur<sup>®</sup>), Merck, Darmstadt, Germany.

All other reagents used throughout the study were of ultra-pure grade. Ultrapure  $\text{HNO}_3$  was prepared by double sub-boiling distillation of 65% nitric acid of p.a. grade (Merck) using a duoPUR quartz sub-boiling unit (MLS Lab Systems GmbH, Leutkirch, Germany). Ultra-pure water was used for the preparation of standards, aqueous buffers samples and model solutions by sub-boiling distillation of purified water (18.2 MU cm) obtained from an ultra clear system (SG water GmbH, Barsbüttel, Germany). The used hydrochloric acid was also subject to sub-boiling distillation in order to prevent sulfur contaminations. For calibration of amino acid analysis, the NIST standard reference material, SRM 2389a, Amino Acids in 0.1 mol/L Hydrochloric Acid, was used.

Cooled centrifugation (4 °C) was performed with a Mikro 200 R (Hettich Zentrifugen; 2424B rotor). For evaporation of the samples a Genevac EZ-2 Series Personal Evaporator was used. For incubation of the samples at 100 °C for 24 h a Heraeus T5042K oven was employed.

### Oxidation of amino acids and subsequent hydrolysis of proteins

Oxidative treatment and protein hydrolysis is described elsewhere<sup>10</sup>. In short, protein samples were oxidized by performic acid prior to hydrolysis. The performic acid derivatisation reagent was prepared by mixing 36 mL of formic acid, 4 mL of  $\text{H}_2\text{O}_2$  and 200  $\mu\text{L}$  of phenol and incubation for 30 min at room temperature until the typical yellow colour of performic acid was reached. Then, 500  $\mu\text{L}$  of performic acid for protein standards and yeast samples were added either to 10 mg of protein or 50 mg of yeast sample. The samples were mixed until they were fully covered with acid, then transferred to a water bath and incubated at 65 °C for 15 min. During this time, a homogenization step by mixing after 7 min was applied. Afterwards, the samples were cooled on ice for 5 min and evaporated to dryness. Finally, 1 mL of 6 M HCl was added and the samples were hydrolysed at 100 °C for 24 h.

### Separation and detection of oxidized amino acids by SAX-ICP-MS

The measurement of the sulfur isotopes by elemental speciation analysis was carried out on two different ICP-MS systems, namely, the Perkin Elmer Elan DRC II combined with a Perkin Elmer Series 200 HPLC system and the ICP-QQQMS in MS/MS mode (Agilent Technologies, 8800) combined with an Agilent 1200 HPLC system. For both systems oxygen was applied as reaction gas. The chromatographic separation was based on a PRP X100 strong anion exchange (SAX) column (250 mm x 2.1 mm, 5  $\mu$ m, Hamilton) applying conditions described elsewhere<sup>10</sup>. This separation employs ammonium acetate, pH 8 gradient elution with a flow rate of 0.4 mL min<sup>-1</sup> at a temperature of 20 °C. The injection volume was set to 8  $\mu$ L.

### Synthetic medium for yeast cultivation

The synthetic cultivation medium for preculture and cultivation in shake flasks contained per liter: 22 g glucose, 22 g citric acid, 3.93 g (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, 0.16 g MgCl<sub>2</sub> • 7 H<sub>2</sub>O, 0.68 g KCl, 0.027 g CaCl<sub>2</sub> • 2 H<sub>2</sub>O, 2 mL biotin stock solution (0.2 g L<sup>-1</sup>), 0.12 g Na<sub>2</sub>SO<sub>4</sub> and 4.6 mL PTM2 trace salt stock solution. The pH was set to 5.0 with KOH. The trace salt stock solution PTM2 contained (per liter): 63.3 g FeCl<sub>2</sub> • 6 H<sub>2</sub>O, 20.0 g ZnCl<sub>2</sub>, 5.77 g CuCl<sub>2</sub> • 2 H<sub>2</sub>O, 3.94 g MnCl<sub>2</sub> • 4 H<sub>2</sub>O, 0.82 g CoCl<sub>2</sub> • 6 H<sub>2</sub>O, 0.2 g Na<sub>2</sub>MoO<sub>4</sub> • 2 H<sub>2</sub>O, 0.08 g NaI, 0.02 g H<sub>3</sub>BO<sub>3</sub> and 5 mL HCl (25%).

For the labeling approach, Na<sub>2</sub>SO<sub>4</sub> in cultivation medium was replaced by enriched <sup>34</sup>S sodium sulfate (isotopic distribution: < 0.1% <sup>32</sup>S, 1.1% <sup>33</sup>S, 98.8% <sup>34</sup>S and < 0.05% <sup>36</sup>S) from Isoflex USA.

### Shake flask cultivations

Precultures (100 mL) with "S-natural abundance" medium were inoculated with *Pichia pastoris* CBS7435 wildtype strain and cultivated over night (25 °C, 180 rpm). Main cultures on "S-natural abundance" medium and medium for <sup>34</sup>S labelling (100 mL) were inoculated with washed precultures for a starting Optical density (OD) (600 nm) of 25 and then cultivated 24 h (25 °C, 180 rpm). Finally, samples were taken for yeast dry mass (YDM) determination and cells were harvested, washed twice with reverse osmosis (RO) -water (2500 relative centrifugal force (RCF); 20 °C) and stored at -20 °C until analysis.

### Sample preparation and spiking strategy

All sample preparation steps were performed gravimetrically. The spike material, i.e. the hydrolyzed <sup>34</sup>S yeast was prepared out of 1 g dry cell material. The material was split in 20 aliquots and subsequently subject to the oxidation and hydrolysis procedure and finally pooled. For the IDMS quantification (including IDMS and reverse IDMS), the cysteic acid and methionine sulfone standards as well as the protein samples were spiked with the hydrolysates of the <sup>34</sup>S enriched yeast prior to the oxidation and subsequent hydrolysis procedure. The spike levels were based on preliminary screening experiments.

### Separation of amino acids by HILIC and detection via ESI MS/MS

The separation procedure was carried out on a LC system consisting of a Thermo Scientific CTC PAL autosampler and a Thermo Scientific Accela 1250 pump. For HILIC separation of the amino acids, a Nucleodur® silica-based column (100 mm x 2.0 mm, 1.8  $\mu$ m particle size) equipped with a guard column (20 mm x 2.0 mm, 1.8  $\mu$ m particle size), both purchased from Macherey-Nagel (Düren,

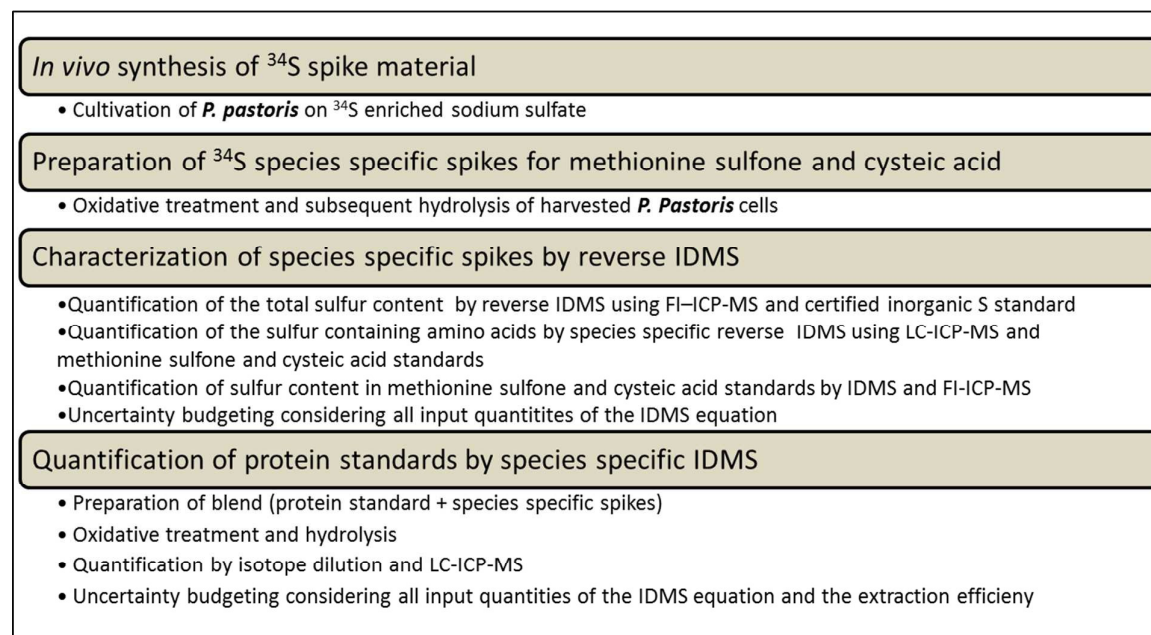
Germany), were used applying a gradient of 10 mM ammonium formate, pH 3,25, (0-100%)The column temperature was set to 40 °C. The injection volume was 5 µl. Tandem mass spectrometric analysis was carried out on a Thermo Scientific TSQ Vantage MS/MS. The capillary temperature was 370 °C and the vaporizer temperature was 400 °C. The collision gas pressure was 1.5 mTorr and the spray voltage was set to 3300 V. The elution times and SRM transitions is published elsewhere <sup>11</sup>. IDMS quantification of methionine sulfone and cysteic acid was based on the principle by Fasset and Paulson <sup>12</sup>.

### Calculation of uncertainty

The determination of combined uncertainties was based on the EURACHEM/CITAC guide "Quantifying Uncertainty in Analytical Measurement" <sup>13</sup>.

### Overview on experimental workflow

Figure 1: Experimental workflow of the introduced species specific IDMS method addressing protein quantification by the sulfur containing amino acids



## Results and discussion

### Sulfur speciation analysis

Protein quantification addressing the amino acids was considered a mature technique already decades ago. The combination with isotope dilution mass spectrometry however, was only rarely considered. Purpose of this study is the integration of an elemental speciation concept to the field of traceable absolute protein quantification, namely quantification by species specific isotope dilution via the sulfur containing amino acids. As described elsewhere <sup>10</sup>, oxidative treatment with performic acid secured a uniform oxidation of all the sulfur containing amino acids to methionine sulfone and cysteic acid, respectively during protein hydrolysis. A proof of principle study showed that



methionine could be recovered from a lysozyme standard with  $89.4 \pm 10\%$ , while cysteine showed a recovery of  $99.3 \pm 3\%$ <sup>10</sup>. These recovery figures were assessed by external calibration using methionine sulfone and cysteic acid standards. Strong anion exchange chromatography enabled separation of the amino acids, methionine, cysteine, the corresponding oxidized forms methionine sulfone, cysteic acid and sulfate. Separation of underivatized and derivatized amino acids was essential to optimize the oxidative protection during protein hydrolysis. In the following chromatography of hydrolyzed samples, no traces of native methionine or cysteine could be detected.

In the present study, sulfur speciation analysis was performed by ICP-MS detection using oxygen as reaction gas (hence measuring S as SO). Reaction cell technology was applied in single quadrupole- as well as triple quadrupole ICP-MS configuration. It has to be mentioned that the major goal was not ultimate sensitivity; accordingly no effort was put into finding the ideal LC flow regime of the LC-ICP-MS hyphenation in this regard. Both instruments revealed absolute limits of detection in the low pmol to sub-pmol range for sulfur when combined with the implemented ion exchange chromatography. The QQQ-MS approach was superior regarding detection limit by a factor of 3-4 when  $^{32}\text{S}^{16}\text{O}$  was considered, and superior by at least a factor of 30 for  $^{34}\text{S}^{16}\text{O}$  measurement. As a consequence, superior isotope ratio measurement precision was achieved measuring the  $^{34}\text{S}/^{32}\text{S}$  ratio in methionine sulfone and cysteic acid, respectively by LC-ICP-MS. As can be readily observed in Table 1, the ratios assessed by LC-ICP-MS analysis of 60  $\mu\text{M}$  methionine sulfone and cysteic acid standards revealed an excellent standard uncertainty of 0.6% (N=4). Consequently, only this approach was further considered.

**Table 1** Analytical figures of merit of LC-ICP-DRC-MS and LC-ICP-MS/MS assessed using 60  $\mu\text{M}$  standards; (sulfur measured using oxygen as reaction gas)

	LC-ICP-DRCMS		LC-ICP-MS/MS	
	LOD (3s) $\mu\text{mol L}^{-1}$	$^{32}\text{S}/^{34}\text{S}$ Standard uncertainty / % (n=4)*	LOD (3s) $\mu\text{mol L}^{-1}$	$^{32}\text{S}/^{34}\text{S}$ Standard uncertainty / % (n=4)*
Met. Sulf. $^{32}\text{S}$	0.45		0.14	
Cys. Ac. $^{32}\text{S}$	0.40		0.25	
Met. Sulf. $^{34}\text{S}$	16		0.56	
Cys. Ac. $^{34}\text{S}$	55		1.0	
Met. Sulf.		2.8		0.6
Cys. Ac.		2.5		0.6

\*Individually prepared standards

#### Characterization of *in vivo* synthesized spike material

In recent years, *in vivo* synthesized isotopically enriched standards has become an indispensable tool for metabolite profiling<sup>14</sup>. These so called targeted metabolomics strategies involve the production of fully  $^{13}\text{C}$  labelled biomass utilizing different organisms. In our group a routine was

established producing  $^{13}\text{C}$  fully labelled *P. pastoris* metabolome as internal standard<sup>15</sup>. The use of such standards demonstrated to be essential in sample preparation development and for accurate absolute quantification of metabolites<sup>16</sup>. In this work the concept of *in vivo* synthesis was transferred to  $^{34}\text{S}$  enrichment focusing on the use of the labelled proteome as spike material. As described in the experimental section  $^{34}\text{S}$  enriched *P. pastoris* was produced in cultivations with  $^{34}\text{S}$  enriched sulfate as sole sulfur source. The obtained biomass was prepared according to the previously established procedure<sup>10</sup> yielding  $^{34}\text{S}$  enriched methionine sulfone and cysteic acid.

In a first step the spike material, i.e. the hydrolyzed  $^{34}\text{S}$  yeast extract was characterized by reverse IDMS using methionine sulfone and cysteic acid standards. For this purpose the  $^{34}\text{S}$  yeast preparation was spiked with the oxidized forms of the two sulfur amino acids prior to analysis. LC-ICP-QQQ-MS measurements revealed isotopic abundances of  $^{34}\text{S}$  enriched up to  $96.3 \pm 0.4\%$  (N=3) and  $98.5 \pm 0.4\%$  (N=3) for cysteic acid and methionine sulfone, respectively. Table 2 gives the obtained concentration levels of the spike material. A total expanded uncertainty was assessed (1) traced to the concentration of methionine sulfon and cysteic acid standards as given by the certificate of analysis (based on titration versus NaOH and  $\text{HClO}_4$ ) and (2) considering their quantity as determined by their sulfur content (based on the certificate of an inorganic sulfur standard). The total sulfur determinations of the standards were carried out by reverse IDMS and flow injection analysis. Considering total sulfur, concentrations of  $103 \pm 3\%$  and  $105 \pm 3\%$  (weight %) for cysteic acid and methionine sulfone standards, respectively were assessed. The concentration obtained by titration was 99.0-101.0% for both calibrants. Accordingly, the calculated uncertainty of the spike quantification was higher in the case of tracing the standard concentration to the sulfur content. As can be readily seen in Table 2, the experimental repeatability (% RSD) of spike preparation was below the calculated total expanded uncertainties proving that the quantification procedure was under control.

The determination of the total sulfur content in the  $^{34}\text{S}$  yeast hydrolysate, again by reversed IDMS using single element sulfur standards and flow injection analysis revealed a sulfur concentration of  $5.7 \mu\text{mol g}^{-1}$  (3% RSD; n=3), which implied that the amount of sulfur recovered as proteinogenic amino acids was 62%, the rest corresponding to untransformed inorganic sulfate. The total sulfur concentration in the dry yeast material given as  $\mu\text{mol sulfur per g cell dry weight}$  was  $81 \mu\text{mol g}^{-1}$ .

**Table 2** Concentration values in prepared  $^{34}\text{S}$  spike material, i.e. yeast cell hydrolysates as determined by reversed IDMS using methionine sulfone and cysteic acid standards. The total expanded uncertainty was calculated considering for the standard concentration an uncertainty (1) deduced from the certificate of analysis and (2) deduced from the determination of the sulfur content.

	Concentration In yeast hydrolysate	Standard uncertainty	Total expanded uncertainty	Total expanded uncertainty
	$\text{nmol g}^{-1}$	% (n=3)*	% (k=2) traced to certificate of analysis	% (k=2) traced to sulfur content
<b>Methionine Sulfone</b>	1670	5	6	12



<b>Cysteic acid</b>	1820	2	5	8
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\*Individually prepared standards

### Absolute quantification of model protein

Finally, a quantification exercise was carried out addressing accurate quantification of lysozyme preparations by species specific isotope dilution. For this purpose the  $^{34}\text{S}$  yeast extract was spiked to lysozyme prior to sample preparation. As can be observed in Table 3 the resulting protein concentrations (given as nmol protein per g protein sample intake (dry weight)) considering the two amino acids, respectively are in excellent agreement. The strategy resulted in experimental repeatability expressed as standard uncertainties for  $n=3$  individually prepared samples of  $\leq 5\%$ .

**Table 3** Quantification of lysozyme by IDMS addressing the two sulfur containing amino acids by LC-ICP-QQQ-MS

Quantified via	Concentration/ nmol protein/g	Standard uncertainty/ RSD % (n = 3)	Expanded uncertainty (k=2)* / %	Concentration / weight %
<b>Met. Sulf.</b>	54280	5	13	78
<b>Cys. Ac.</b>	53820	2	14	77

\*for the calculation of the total combined uncertainty a factor E was introduced to the isotope dilution equation accounting for the extraction efficiency which is not corrected for by the ID approach. This factor was assumed to be 1, associated with a standard uncertainty of 4%.

The finally observed total combined uncertainty was 13 and 14% (coverage factor  $k=2$ ) considering the two amino acids. The budget was calculated assuming an uncertainty for the spike concentration of 5% and 2% for methionine sulfone and cysteic acid, respectively. Moreover, a factor E was introduced in the model equation otherwise given by the IDMS. The factor E, with the value of 1 accounted for the extraction efficiency, since this not corrected for by the ID approach. For the calculation, it was assumed that the extraction efficiency (and hence the factor E) was associated with an uncertainty of 4%. Both input quantities, the spike concentration and the extraction efficiency contributed to the highest degree to the calculated total expanded uncertainty. The uncertainty of the spike concentration determination was the highest contribution ranging at 40 % and the sample preparation contributed with 30%.

For cross validation the protein standard was additionally investigated by hydrolysis followed by HILIC-MS/MS analysis addressing 6 amino acids, namely alanine, leucine, arginine, lysine, proline and serin. The HILIC-MS/MS approach is described in detail elsewhere<sup>11</sup>. A lysozyme concentration of  $77 \pm 10\%$  (weight %,  $n=3$  replicate samples) was found. Evidently there is no universal hydrolysis procedure applicable to all amino acids. Hence, implementing simply acid hydrolysis with only 6M HCL as reagent resulted in a comparatively high experimental uncertainty of amino acid quantification. It should be noted that, internal standardization by  $^{13}\text{C}$  fully labelled yeast was carried out only prior to HILIC-MS/MS and not upon sample preparation. However, within the obtained uncertainties, the average concentrations of lysozyme obtained by the complementary approaches were in good agreement.

Finally, the enriched spiking material was used for IDMS quantification of ceruloplasmin. It appears from Table 4 that the biomarker was quantified with a relative standard uncertainty of 5%, disregarding which amino acid was chosen for quantification. The importance of traceable protein standards becomes clear considering the state of the art of the art of ceruloplasmin determination, a clinical relevant biomarker<sup>2,17</sup>. In this specific case poor comparability of results resulting from different immunological measurement platforms have been reported<sup>17</sup>. Accordingly, traceable protein standards for calibration would significantly improve this situation.

**Table 4:** Quantification of ceruloplasmin

Quantified via	Concentration/ nmol protein/g	Relative standard uncertainty/ RSD % (n = 3)*
Met. Sulf.	830	5
Cys. Ac.	790	5

\*Individually prepared standards

### Conclusion

Amino acid quantification based on IDMS provided a valuable tool for protein or peptide quantification. However it failed as method in complex biological samples (due to limited selectivity and sensitivity). The method offers the introduction of traceable protein standards, which are of utmost importance in clinical analysis (and the implied requirements of reference methods of higher metrological order). As a matter of fact, the use of species specific isotope dilution poses a major advantage as there is no need to produce spike material for each investigated protein when amino acids are considered for quantification. In principle, any protein can be quantified provided it is present at sufficiently high concentration with a HPLC or SDS page purity > 95% and contains at least one of the sulfur bearing amino acids. The value of such a generic approach for the production of metrological valid protein standards cannot be underestimated.

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## *In vivo* synthesis of $^{34}\text{S}$ spike material

- Cultivation of *P. pastoris* on  $^{34}\text{S}$  enriched sodium sulfate

## Preparation of $^{34}\text{S}$ species specific spikes for methionine sulfone and cysteic acid

- Oxidative treatment and subsequent hydrolysis of harvested *P. Pastoris* cells

## Characterization of species specific spikes by reverse IDMS

- Quantification of the total sulfur content by reverse IDMS using FI-ICP-MS and certified inorganic S standard
- Quantification of the sulfur containing amino acids by species specific reverse IDMS using LC-ICP-MS and methionine sulfone and cysteic acid standards
- Quantification of sulfur content in methionine sulfone and cysteic acid standards by IDMS and FI-ICP-MS
- Uncertainty budgeting considering all input quantities of the IDMS equation

## Quantification of protein standards by species specific IDMS

- Preparation of blend (protein standard + species specific spikes)
- Oxidative treatment and hydrolysis
- Quantification by isotope dilution and LC-ICP-MS
- Uncertainty budgeting considering all input quantities of the IDMS equation and the extraction efficiency