Accurate characterization of β-amyloid (Aβ40, Aβ42) standards using species-specific isotope dilution by means of HPLC-ICP-MS/MS

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Abstract
The amyloid β peptide, as one of the main components in senile plaque, represents a defining pathological feature for Alzheimer’s disease, and is therefore commonly used as a biomarker for this disease in clinical analysis. However, the selection of suitable standards is limited here, since only a few are commercially available, and these suffer from varying purity. Hence, the accurate characterization of these standards is of great importance. In this study, we developed a method for the traceable quantification of the peptide content using species-specific isotope dilution and ICP-MS/MS detection. It is based on the separation of the sulfur-containing amino acids methionine and cysteine after oxidation and hydrolysis of the peptide. Using a strong anion exchange column, both amino acids could be separated from each other, as well as from their oxidized forms and sulfate. The sulfur content was determined via ICP-MS/MS using oxygen as reaction gas. Species-specific isotope dilution was enabled by using a 34S-labeled yeast hydrolysate, containing methionine sulfone and cysteic acid with different isotopic composition. The peptide contents of Aβ standards (Aβ40,42), as well as myoglobin and lysozyme with different degrees of purity, were determined. For validation purposes, the standard reference material NIST 2389a, which contains the amino acids in a similar concentration, was subjected to the developed sample preparation and analysis method. In addition to accounting for errors during sample preparation, high levels of accuracy and precision could be obtained using this method, making it fit-for-purpose for the characterization of peptide standards.

Keywords ICP-MS · Species-specific isotope dilution · Traceability · Amyloid beta peptide · Alzheimer’s disease · Speciation analysis

Introduction

Due to the steady increase in global life expectancy [1], age-related diseases such as Alzheimer’s disease (AD), the most common form of neurodegenerative disorder [2], are becoming a growing problem. According to reports from the World Health Organization in 2018 [3], the number of deaths due to dementias has more than doubled since 2000, making it the 5th leading cause of death worldwide. AD is characterized by a gradual decline in different cognitive domains such as memory, language, or personality [4], which makes it difficult to diagnose in early stages, as these symptoms are the result of years of neurological damage [5]. Because of this impairment in basic body functions, death is ultimately caused by the vulnerability of patients to infections like pneumonia [6].

The most defining pathological features of this disease are neurofibrillary tangles and senile plaque in the hippocampus of the brain [7]. As a result, there has been great interest in research for the main component of this plaque, the β-amyloid peptide (Aβ), which consists of 38–42 amino acids and originates from the amyloid precursor protein (APP) [8]. APP, which is a transmembrane protein abundant in the central nervous system [9], is believed to be involved in cell growth and health, but the exact physiological function is not yet fully understood [10]. If excessive amounts of the Aβ peptide are produced (as in the case of AD), its normal functions are...
inhibited through aggregation and toxic oligomers are formed, which are able to cause oxidative damage to the brain and then accumulate as senile plaque [9–11].

Therefore, Aβ is widely used as biomarker for Alzheimer’s disease, alongside neurogranin, phosphorylated tau, and total tau [12]. Specifically, the ratio between the most abundant fragments Aβ40 and Aβ42 is measured in cerebrospinal fluid (CSF). While the concentration of Aβ42 changes significantly with AD (decrease because of Aβ aggregation), Aβ40 remains relatively constant in most patients, thus providing normalization for measurements [13]. Aβ40 is more abundant with reported levels of about 2–20 ng mL$^{-1}$ in CSF compared to 0.2–1 ng mL$^{-1}$ of Aβ42, which can decrease in AD patients by up to 50% [14–17]. The most important tools for quantitative measurements in CSF are ELISA assays, which employ multiple antibodies with high affinity to Aβ peptides. In addition, amyloid deposits in the brain are visualized with positron emission tomography (PET) scans using 18F-fluorodeoxyglucose, since they showed good accordance to the senile plaque found in autopsy [18].

Due to the high level of responsibility that goes with patient samples, high accuracy and specificity are basic requirements for clinical analysis [19]. Even small differences in concentration can be decisive for the final diagnosis, which is why it is important to ensure traceability of the measurements, as otherwise results are dependent on the laboratory and procedure used [19, 20]. According to the international terminology of metrology (JCGM 200:2012, Clause 2.41), traceability is defined by a “documented unbroken chain of calibrations, each contributing to the measurement uncertainty” [21]. In order to ensure equivalence of results between laboratories (ISO 17511:2020) for in vitro diagnostics, a calibration hierarchy must be established, where each measurement result can be traced back to the calibration carried out previously [22]. Measurement units, standards, or procedures can serve as a reference. In the ideal case, a primary reference measurement procedure is used in which the quantity value can be traced back to the basic units of the International System (SI) [23]. Since this is not possible for every analyte, the use of certified reference materials and reference measurements, which are accompanied by measurement uncertainties and traceability provided by an authoritative body, is more common [21, 23]. However, due to their limited availability, particularly in the area of biological standards such as proteins, manufacturers often have to resort to in-house calibration procedures [24].

Typically, protein assays based on UV absorption, Biuret methods, colorimetric dye, or fluorescence are used for characterization. Accordingly, the measurements are usually carried out in a non-traceable manner without internal standard and with poor accuracy [20]. One commonly used method is the colorimetric Bradford assay where concentrations are obtained using a species-unspecific calibrant such as albumin [25]. A better approach for the quantification of proteins and peptides is amino acid analysis using ion exchange or reversed-phase HPLC, which, however, is usually limited by UV detection in terms of accuracy, selectivity, and sensitivity [26, 27].

These limitations can be overcome by the use of amino acid–based isotope dilution liquid chromatography mass spectrometry (ID-LC-MS) for the certification of protein reference materials. The method employs either isotope-labeled ($^{13}$C or $^{15}$N) amino acids, peptides, or proteins as internal standard, mainly to account for variations in the yield of the analytes from biological samples, where complex matrices can interfere with the measurement. For accurate quantification of the protein, a complete hydrolysis of the protein into the individual amino acids is required. In this regard, Aβ peptides were for example quantified in CSF from Alzheimer’s disease patients by immunoaffinity purification and ID-LC-MS analysis [28]. In another instance, $^{13}$C-labeled Aβ peptides were used as internal standard for the quantification in human plasma using MALDI-TOF [29].

Another MS–based approach for protein quantification makes use of the advantages of inductively coupled plasma-mass spectrometry (ICP-MS) for highly sensitive element quantification. Since the majority of proteins contain sulfur via the amino acids cysteine and methionine or are phosphorylated [30, 31], proteins or peptides are hydrolyzed, and an amino acid analysis based on the sulfur/phosphor content is carried out using HPLC-ICP-MS [20]. The measurement of sulfur or phosphor allows the direct traceability to SI units [23].

Because of its capabilities to measure isotopic compositions, ICP-MS also allows the use of isotope dilution analysis (IDA), which offers high degrees of accuracy and precision for quantitative measurements and SI unit traceability [23, 32]. A recent study by Feng et al. [33] showed successful results for peptide quantification using species-unspecific isotope dilution, employing an inorganic $^{34}$S spike, which was added on-line after the column. This approach is versatile and fast, reducing sample preparation significantly and preventing precipitation. However, it is only able to correct instrumental errors during measurement.

Ideally, species-specific isotope dilution is used, with an isotopically labeled form of the examined species being added during sample preparation [34]. With this approach, systematic errors, sample loss, and transformation of the species can also be accounted for [35]. However, it must be considered that this type of isotope dilution is often limited by the availability of isotopically enriched standards. Therefore, independent synthesis is usually performed, which can be done either by adding labeled substrates to specific cell cultures and thus producing the required molecules (in vivo labeling) or via multi-step chemical synthesis [36, 37]. Both methods require complex procedures, often achieving only low yields, which is why method development has become crucial in this area.
In this work, we developed a traceable method for the characterization of protein and peptide standards using species-specific isotope dilution by means of HPLC-ICP-MS/MS. It is an extension of the quantification strategy implemented in recent works [20, 38]. For this purpose, the proteins were subjected to acid hydrolysis and oxidized in order to obtain the sulfur-containing amino acids cysteic acid and methionine sulfone, which were then separated using a strong anion exchange column and used to determine the peptide content. By employing a $^{34}\text{S}$-labeled yeast hydrolysate, errors during sample preparation and measurement could be successfully removed, leading to accurate and reproducible results.

**Experimental**

**Chemicals and reagents**

Ultrapure water (18.2 MΩ cm, ELGA Water Purification System, Purelab Ultra MK 2, UK or 18.2 MΩ cm, Milli-Q Advantage, Darmstadt, Germany) and nitric acid ($\geq 69\%$, Rotipuran Supra, Carl Roth, Karlruhe, Germany) were used in dilutions for ICP-MS measurements. The sulfur standard solution (1000 mg L$^{-1}$, H$_2$SO$_4$ in 2% HNO$_3$) was purchased from Labkings (Hilversum, The Netherlands). The $^{34}\text{S}$-enriched NaSO$_4$ with 99.89% isotopic content ($^{34}\text{S}$) and 98% chemical purity was purchased from Eurisotop (Saint-Aubin Cedex, France). The standards for lysozyme (from chicken egg white) with purities of $> 90\%$ and $> 99\%$ (VETRANAL™) and myoglobin with purities of 95–100% and $> 98\%$ (SDS-PAGE), as well as the amino acid calibrants, namely methionine, methionine sulfone, cysteine, and cysteic acid, were all purchased by Sigma-Aldrich (Steinheim, Germany). The synthetic $\beta$-amyloid standards $\beta$-Amyloid 1–40 (Ultra Pure, TFA) and $\beta$-Amyloid 1–42 (Ultra Pure, TFA) were purchased from r-Peptide (Georgia, USA). $^{34}\text{S}$-labeled yeast hydrolysates were provided by ISOtopic solutions (Vienna, Austria). For validation of the analytical method, the NIST standard reference material SRM 2389a, amino acids in 0.1 mol L$^{-1}$ hydrochloric acid (National Institute of Standards and Technology, Gaithersburg, MD, USA) was used. The ammonium acetate buffer applied as eluent for chromatography was derived from ammonium hydroxide solution (25%, Suprapur) and acetic acid (100%, Suprapur), which were purchased from Lactan (Graz, Austria).

**Sample preparation**

**Hydrolysis/oxidation of the proteins** $\beta$-Amyloid (around 1 mg, corresponding to the vial content) was mixed with 1 mL 1% NH$_4$OH. To dissolve the entire content, the solution was added via a syringe, which penetrates the membrane of the vial. After that, a homogenization step was done by sonication for 1 min. The solution was then aliquoted in 100-μL fractions and 3 aliquots of each peptide (Aβ40, Aβ42) were used, while the others were stored at 4 °C. This procedure was necessary because the peptides could not be weighed in due to electrostatic interactions inside of the vial. Myoglobin and lysozyme (10 mg of each protein) were dissolved in 2 mL of 0.9% NaCl, and 3 aliquots were taken (each 40 μL). For NIST
2389a, four aliquots of the solution (each 10 μL) were directly weighed in. Each of those fractions was spiked with 20 μL of the 34S-enriched yeast hydrolysate. Subsequently, 500 μL of performic acid was added and the samples were incubated for 15 min at 65 °C. After that, they were evaporated to dryness using a GeneVac EZ-2 centrifugal evaporator (SP Industries Inc., Warminister, USA), mixed with 1 mL of 6 M HCl and incubated again for 24 h at 100 °C. The now oxidized and hydrolyzed samples were vortexed and centrifuged at 10,000 rcf for 5 min with a Hermle Z 446K centrifuge (HERMLE Labortechnik GmbH, Wehingen, Germany). The samples were transferred to Eppendorf tubes, diluted 1:10 with the buffer (25 mM CH₃COONH₄) in HPLC vials, and were directly used for analysis.

**Reduction of the sulfur background** In order to minimize sulfur contaminations, sample preparation was carried out in an ISO class 8 clean room, with measurements being done in ISO class 7. All consumables were subjected to a cleaning procedure. They were first treated with 10% HNO₃ for 24 h, then with 1% HNO₃ for 24 h at 100 °C. The now oxidized and hydrolyzed samples were vortexed and centrifuged at 10,000 rcf for 5 min with a Hermle Z 446K centrifuge (HERMLE Labortechnik GmbH, Wehingen, Germany). The samples were transferred to Eppendorf tubes, diluted 1:10 with the buffer (25 mM CH₃COONH₄) in HPLC vials, and were directly used for analysis.

| Column                  | Atlantis T3 | Dionex™ IonPac™ AS22 |
|-------------------------|-------------|---------------------|
| Flow rate [μL min⁻¹]    | 100         | 400                 |
| Injection volume [μL]   | 10          | 10                  |
| Buffer A CH₃COONH₄, pH = 5 | 10 mM      | 20 mM CH₃COONH₄, pH = 8.0 |
| Buffer B MeOH           |             | 400 mM CH₃COONH₄, pH = 8.0 |
| Gradient                |             |                      |
| 0–1 min 100% A          |             | 0–4 min 100% A       |
| 1–5.5 min Ramp to 20% B, 80% A | 4–7 min | Ramp to 100% B       |
| 5.5–6 min 20% B, 80% A  | 7–12 min    | 100% B              |
| 6–6.05 min Ramp to 100% A | 12–12.1 min Ramp to 100% A |
| 6.05–7 min 100% A       | 12.1–16 min | 100% A              |

**Species-specific isotope dilution** In order to ensure traceability with this type of isotope dilution, each standard had to be quantified step by step. Therefore, first, the 34S spike had to be measured precisely using reverse isotope dilution. The term reverse here refers to the characterization of the spike instead of the sample. After that, the two standards of the oxidized amino acids (methionine sulfone, cysteic acid) were characterized using this spike. By
adding these standards to the $^{34}$S-labeled yeast hydrolysate, its composition could be characterized using the anion exchange column. Figure 1 provides an overview of the measurements required.

**Instrumentation**

**ICP-MS/MS** An Agilent 8800 ICP-MS/MS instrument (Agilent Technologies, Tokyo, Japan) with oxygen as reaction gas was used to determine the sulfur content. The ICP-MS parameters were tuned on a daily basis to achieve high sensitivity. The ICP-MS was equipped with a MicroMist nebulizer with a sample uptake rate of $\sim 0.25$ mL min$^{-1}$ and standard nickel cones. All sulfur isotopes were measured using oxygen as reaction gas (flow rate $= 0.32$ mL min$^{-1}$) with mass selection steps on both quadrupoles. The following isotopes were monitored with a respective integration time of 0.1 s: $^{32}$S$^{16}$O$^+$, $^{33}$S$^{16}$O$^+$, $^{34}$S$^{16}$O$^+$, and $^{36}$S$^{16}$O$^+$. The instrument was coupled to an Agilent 1260 Infinity Bio-Inert HPLC system (Agilent Technologies, Waldbronn, Germany). The Agilent MassHunter software package (Workstation Software, Version C.01.03, 2016) was used for data evaluation. The instrumental parameters for the ICP-MS measurements are summarized in Table 1.

**HPLC system** The final separation of the sulfur-containing amino acids was carried out with a Thermo Scientific Dionex$^\text{TM}$ IonPac$^\text{TM}$ AS22 anion exchange column ($6 \mu$m, $2 \times 250$ mm, Thermo Scientific Inc., MA, USA). In preliminary experiments, other columns with reversed-phase chemistry,

### Table 3

| Substance          | Retention time [min] | LOQ substance [$\mu$M] | LOQ sulfur [$\mu$g L$^{-1}$] |
|--------------------|----------------------|------------------------|------------------------------|
| Methionine         | 2.8                  | 0.04                   | 1.4                          |
| Methionine sulfone | 3.3                  | 0.05                   | 1.7                          |
| Cysteine           | 10.3                 | 0.05                   | 1.5                          |
| Cysteic acid       | 11.3                 | 0.07                   | 2.3                          |
| Sulfate            | 11.7                 | 0.3                    | 9.8                          |
specifically Atlantis T3 (3 μm, 4.6 × 150 mm, Waters), were tested, providing insufficient separation of the amino acids. The summarized HPLC parameters are shown in Table 2.

Results and discussion
Development of the amino acid separation
Reversed-phase and anion exchange chromatography were evaluated for the separation of the amino acids cysteine and methionine, their oxidized forms cysteic acid and methionine sulfone, and sulfate, according to results in the literature [20, 39]. In addition to the quantification of the peptides, optimal separation enabled to evaluate if the oxidation in the sample preparation was complete.

Using gradient elution, the separation of cysteine and methionine was possible using reversed-phase chromatography; however, they showed an overlap with their oxidized forms (see Supplementary information (ESM) Fig. S1). In addition, evaluation of cysteine was difficult due to the similar retention time of sulfate.

Therefore, anion exchange chromatography was used as an alternative. First isocratic elution was tested at different CH₃COONH₄ concentrations to evaluate the retention time of the respective substances. Methionine and methionine sulfone eluted at 20 mM CH₃COONH₄, cysteine and cysteic acid at 100 mM CH₃COONH₄, and sulfate required 400 mM CH₃COONH₄ for an acceptable retention time. Based on these results, a gradient was developed to ensure that the substances elute within the isocratic steps, as the sulfur background increases with higher salt concentration and this could have an impact on the isotope dilution (ID) measurements. The gradient started with isocratic elution at a low concentration of CH₃COONH₄ to achieve optimal separation of methionine and methionine sulfone within 4 min. Directly after the methionine sulfone peak, the concentration was rapidly increased (400 mM CH₃COONH₄) to elute the remaining substances as quickly as possible. The increase was gradual over 3 min to ensure a stable eluent for the next isocratic step. Due to cysteine having more negatively charged ions than methionine in a basic environment, it eluted later at 10.3 min. Cysteic acid and sulfate have even higher acidity and thus showed the longest retention on the column with 11.3 and 11.7 min. After the elution of all the analytes, the gradient was changed back to the starting conditions (20 mM CH₃COONH₄) and left for an additional 4 min to equilibrate the system for the next measurement. This made it possible to reliably separate the 5 compounds within a measurement time of 16 min, as can be seen in Fig. 2.

Separation of the protein/peptide standards
Using the optimized gradient, the hydrolyzed protein and peptide standards were separated by anion exchange chromatography and ICP-MS/MS detection. In addition to Aβ40 and Aβ42, the proteins myoglobin and lysozyme were measured. For Aβ40, only one peak at 3.3 min was visible (Fig. 3a), which corresponds to methionine sulfone. The amino acid thus appears to have been almost completely oxidized,
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Table 5 Measured mass fractions and recoveries for the amino acids in the standard reference material NIST 2389a. These parameters were used to compare the accuracy and precision of external calibration and species-specific isotope dilution. The certified values are shown for comparison.

| Amino acid | Certified mass fraction [mg g⁻¹] | Mass fraction [mg g⁻¹] (external calibration) | Recovery [%] | Mass fraction [mg g⁻¹] (species-specific ID) | Recovery [%] |
|------------|----------------------------------|-----------------------------------------------|--------------|-----------------------------------------------|--------------|
| Cysteine   | 0.295 ± 0.013                    | 0.195 ± 0.02 (n = 4)                            | 66.12 ± 0.06 | 0.297 ± 0.01 (n = 8)                            | 100.68 ± 1.69 |
| Methionine | 0.373 ± 0.011                    | 0.260 ± 0.02 (n = 4)                            | 69.73 ± 0.05 | 0.368 ± 0.01 (n = 8)                            | 98.66 ± 3.22  |

proving the sample preparation to be successful. A slight increase in intensity is only noticeable at 11.7 min because of the low sulfate background. Aβ42 showed an identical pattern (Fig. 3b), but the area of methionine sulfone was significantly lower despite the same concentration, leading to more noise in the chromatogram.

The chromatogram of myoglobin resembled the one of the Aβ peptides, containing only 2 methionine molecules in its sequence (Fig. 3c). Due to the small peak at 6.4 min, it can be assumed that a slight contamination with cysteine may have occurred. Lysozyme, which contains 2 methionine and 8 cysteine molecules, showed a comparatively higher sensitivity when oxidized forms (Fig. 3d). The ratio of the peak areas was in good agreement with the number of sulfur-containing amino acids.

Quantification of the peptide content by species-specific isotope dilution

Using the optimized gradient, calibration curves of the individual standards were created in a concentration range of 50–1000 μg L⁻¹ (ESM Fig. S2). Limits of quantification (LOQ) were determined by means of a blank (0.6 M HCl), calculated as the mean value of 10 measurements of the blank +10 times the standard deviation of the blank measurements. For the LOQ of sulfate, the peak areas of the blank were used. However, since no peaks were visible for the amino acids, the counts around their retention times had to be employed. Accordingly, cysteic acid showed a higher LOQ because of an elevated sulfur background at 400 mM CH₃COONH₄ (Table 3).

Species-specific isotope dilution using a ³⁴S-labeled yeast hydrolysate was chosen for the quantification, as the approach, where an isotope-enriched standard is added online to the sample, does not account for analyte losses during sample preparation.

For the calculation of the amino acid concentration using isotope dilution, Equation S1 (see ESM) was used. To determine the performance of this method, external calibration with a sulfur standard was also carried out for comparison. In a first step, the ³⁴S-labeled yeast hydrolysate was characterized using species-specific reverse isotope dilution (Fig. 4). For this purpose, a mixture with similar concentrations of the yeast hydrolysate (Fig. 4a) and the two amino acid standards methionine sulfone and cysteic acid (Fig. 4b) was created. The exact sulfur concentration of the standards was previously quantified by isotope dilution using flow injection ICP-MS as described in the procedure of Fig. 1. The yeast hydrolysate was measured a total of 10 times, and numerous peaks were visible with methionine sulfone, cysteic acid, and sulfate being the most abundant ones.

Both amino acids showed similar concentrations, whereas sulfate was present in lower concentrations (Table 4).

The isotopic abundances of sulfur were determined within the yeast (ESM Table S1). No ³²S peaks were detected, which

Table 6 Peptide content of the amyloid β peptides (40, 42) and protein standards with different grades of purity. The content was determined by comparing the measured value of the sulfur-containing amino acids with the theoretical ones (based on the amino acid sequence). External calibration was used for comparison with species-specific isotope dilution.

| Sample          | Manufacturer information | External calibration | Species-specific ID |
|-----------------|--------------------------|----------------------|--------------------|
| Myoglobin       | 95–100                   | 53.05 ± 1.02 (n = 3) | 96.44 ± 3.32 (n = 6) |
| Myoglobin (SDS-PAGE) | ≥ 98                  | 59.06 ± 3.75 (n = 3) | 100.72 ± 2.84 (n = 6) |
| Lysozyme        | ≥ 90                     | 59.29 ± 5.52 (n = 3) | 97.44 ± 0.89 (n = 6) |
| Lysozyme (VETRANAL®) | 100                   | 61.54 ± 5.33 (n = 3) | 99.98 ± 1.57 (n = 6) |
| Amyloid β 1-40  | 98                       | 49.19 ± 3.22 (n = 3) | 126.78 ± 0.05 (n = 15) |
| Amyloid β 1-42  | > 97                     | 19.99 ± 1.71 (n = 3) | 52.56 ± 0.01 (n = 15) |
was beneficial for the IDMS measurements. Only small quantities of $^{33}\text{S}$ were visible.

By adding this characterized $^{34}\text{S}$-labeled yeast hydrolysate to the samples, it was possible to accurately quantify the amino acids methionine and cysteine using species-specific isotope dilution and HPLC-ICP-MS/MS. The peptide content was determined by comparing the measured values with the theoretical ones, which were based on the amino acid sequence of the peptides.

**Validation of the developed method**

In order to validate the developed method, the standard reference material (SRM) NIST 2389a was subjected to the developed sample preparation procedure. The used SRM is an amino acid mix, containing cysteine and methionine in equimolar concentration ($\sim 2.5 \text{ mmol L}^{-1}$), and the mass fractions were determined by species-specific isotope dilution and external calibration. In the chromatogram, both amino acids showed similar peak areas (ESM Fig. S4). The mass fractions for cysteine and methionine determined by species-specific isotope dilution were in good agreement with the certified values, whereby a recovery of almost 100% was achieved for both amino acids (Table 5). External calibration in contrast provided only recoveries of 70% for both amino acids.

**Measurement of the peptide standards**

After validation, the developed method was used to quantify the peptide contents of the protein and peptide standards, as can be seen in Table 6. Since it was not possible to weigh in $\alpha\beta$ accurately, the peptide content here refers to the manufacturer’s specification (r-Peptide) of 1 mg $\alpha\beta$ per vial. In another attempt, where a different vial of $\alpha\beta$ was used, $\alpha\beta$40 was weighed in completely with the microbalance showing a value of 1.269 mg. Each of the 3 $\alpha\beta$ aliquots was measured 5 times, the protein aliquots 2 times. For external calibration, each fraction was only measured once.

Species-specific isotope dilution resulted in peptide contents similar to the information provided by the manufacturer. The differences between the various degrees of purity were visible, with the analytical versions of the protein standards showing, on average, higher peptide contents. External calibration on the other hand had on average 30–40% of the peptide content missing. Therefore, similar patterns as with NIST 2389a were seen, leading to the conclusion that there were significant losses during sample preparation, which are corrected by using species-specific isotope dilution. The peptide content of $\alpha\beta$, however, showed a significant deviation from the specifications. $\alpha\beta$40 showed values that were more than 2.4 times higher than $\alpha\beta$42. External calibration showed similar ratios between the two peptides. Since the entire content of the vial has been solved and the differences between the individual fractions were small, it can be assumed that there is a problem with the weight specifications of the manufacturer. According to r-Peptide, their $\alpha\beta$ standards are prepared by lyophilizing the corresponding peptide solution into the vial and adding 5–10% more of the product to compensate for losses during resuspension. For this purpose, the peptide solutions are characterized chromatographically using amino acid analysis. This statement could provide a good explanation for the results. With the procedure described by the manufacturer, errors in the determination of the peptide content are possible. In contrast to simple weighing, an amino acid analysis has numerous influencing factors, such as calibration, measuring conditions, or evaluation, resulting in greater uncertainty. In addition to possible losses during lyophilization, the supplement of another 5–10% product makes it impossible to estimate the exact peptide content, so it is misleading to state 1 mg on the analysis certificate.

**Conclusions**

This work successfully demonstrated the capabilities of combining species-specific isotope dilution with HPLC-ICP-MS/MS to determine the peptide content and thus the purity of protein standards. This was made possible by a novel $^{34}\text{S}$-labeled yeast hydrolysate, which contains the amino acids methionine sulfone and cysteic acid that are almost completely enriched in $^{34}\text{S}$ (98.89%). The optimized HPLC method using a strong anion exchange column provided excellent separation for the sulfur-containing amino acids, as well as their oxidized forms and sulfates. Compared with external calibration, species-specific isotope dilution was able to demonstrate significantly higher precision and accuracy, resulting in recoveries of nearly 100% for the standard reference material (NIST 2389a) and RSD values below 4%. While the purity of the protein standards could be confirmed using this method, the $\alpha\beta$ peptides showed significant deviations, which is most likely due to problems in the manufacturer’s weight specifications. Since even small differences in concentration can be decisive in clinical analysis, species-specific isotope dilution offers a good possibility for the characterization of biological standards in an accurate and traceable manner.

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**Declarations**

**Conflict of interest** The authors declare no competing interests.
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