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# Certification of Amyloid-Beta (Aβ) Certified Reference Materials by Amino Acid-Based Isotope Dilution High-Performance Liquid Chromatography Mass Spectrometry and Sulfur-Based High-Performance Liquid Chromatography Isotope Dilution Inductively Coupled Plasma Mass Spectrometry

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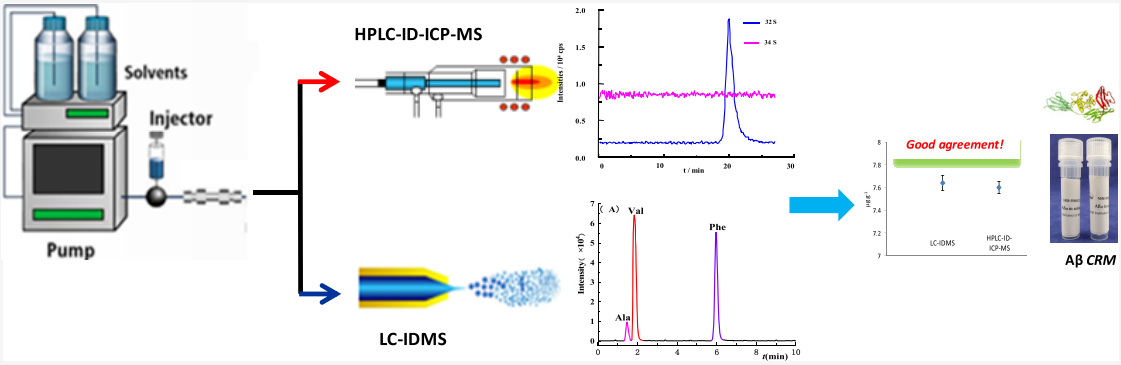


**ABSTRACT:** The use of amyloid-beta (Aβ) biomarkers could contribute to an early diagnosis of Alzheimer's disease (AD); however, there are still large variations among results from different assays. This variability can be overcome by standardization of those assays through the use of certified reference materials (CRMs) and the establishment of a traceability chain. In this study, $A\beta_{40}$ (GBW09874) and $A\beta_{42}$ (GBW09875) solution CRMs with the certified values and uncertainties of 7.58 ± 0.30 and 7.62 ± 0.30 μg $g^{-1}$ were developed with high-purity Aβ as raw materials. For the first time, isotope dilution high-performance liquid chromatography mass spectrometry (ID-LC-MS) and high-performance liquid chromatography isotope dilution inductively coupled plasma mass spectrometry (HPLC-ID-ICP-MS) strategies were employed to certify the candidate Aβ solution CRMs. The two candidate CRMs showed good homogeneity, and good stability was also demonstrated for at least 5 days at −20 °C and 14 months at −70 °C. These CRMs are primarily intended to be used for value assignment to secondary calibrators or CRMs with a clinical matrix, which will help in early diagnosis of AD.

Due to the changing demographics of our society, the impact of neurodegenerative disorders, predominantly Alzheimer's disease (AD), increases rapidly. Also, pathogenesis and early diagnosis of AD have attracted widespread attention.[1−3] Numerous clinical studies have shown that abnormal levels of Aβ in cerebrospinal fluid (CSF), blood, and brain tissues are closely related to the progression of AD,[4,5] and Aβ has become one of the most important biomarkers for studying AD. Moreover, the detection of Aβ in human serum or plasma has the potential to become a promising way to early detection of AD.[5] Aβ is produced from the amyloid precursor protein by β-secretase and γ-secretase that mainly generate amyloidogenic peptides with 90% $A\beta_{40}$ and 10% $A\beta_{42}$.[5] In the diagnosis of AD by Aβ markers, since $A\beta_{40}$ levels in CSF are relatively unchanged in AD compared to controls, low concentration of $A\beta_{42}$ might be false positive, and high concentration might be false negative. Therefore, accurate measurement of both $A\beta_{42}$ and $A\beta_{40}$ would improve early diagnostic accuracy of AD. However, the straightforward utility of Aβ markers has been partly hampered by the variability in measurement results obtained when using assays from different manufacturers.

Equivalent results among various measurement procedures are crucial in providing global comparability in diagnosis,

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treatment, and pathological disease monitoring.[6] Metrological traceability to available high-order reference materials or procedures of all assays in diagnostic tests was required by the European In Vitro Diagnostic (IVD) Directive.[7] To establish metrological traceability of Aβ measurement procedures in clinical laboratories, the use of reference materials is regarded as a crucial prerequisite.[7,8] Literature reported that quantitative methods for Aβ in clinics included enzyme-linked immunosorbent assay,[9] an electrochemical method,[10] magnetic bead labeling combined with positron emission tomography,[11] and capillary electrophoresis.[12] Some methods among them are fast, sensitive, and selective to some extent and have potential in Aβ detection of AD samples. However, because these methods cannot be traceable to SI units and suitable CRMs were not available, the analysis results between different methods and calibration reagents are quite inconsistent.[4] Currently, although there have been reports of reference methods of $A\beta_{42}$ in human CSF,[13,14] there have been no studies of reference procedures and CRMs of pure Aβ. Since the pure Aβ solution CRMs are on the top of the traceability chain in ISO 17511[15] and could be used for value assignment to clinical matrix CRMs, it is urgent to develop absolute quantitative measurement procedures and CRMs of high-purity Aβ CRMs. Therefore, the development of SI-traceable CRMs could help to establish Aβ measurement traceability and a reliable global Aβ diagnostic cutoff value, which will improve the consistency of Aβ clinical assays on diagnosis of Alzheimer's disease (AD).

At present, amino acid (AA)-based isotope dilution liquid chromatography mass spectrometry (ID-LC-MS) is more frequently considered to be a primary method for the certification of pure-protein RMs.[16−18] In the amino acid-based strategy, more than two amino acids (AAs) released from proteins are selected to achieve the quantification of proteins. Prior to either sample preparation or ID-LC-MS analysis, the selected AAs typically labeled with $^{13}C$ or $^{15}N$ (isotope-labeled AA counterparts) were used as internal standards. The addition of an isotope-labeled counterpart into samples is performed in order to be free from unknown factors that are caused by random errors (recovery of a targeted measurand during sample preparation, a mass shift via a chemical modification, variation in efficacy during ESI-MS, and so on). Among those errors, the use of an isotope-labeled counterpart is mainly used to compensate the variation in the yield of a targeted measurand from matrix samples. After quantification of corresponding amino acids, the protein quantity is determined based on the stoichiometric presence of AAs in the protein. However, the accuracy of the AA-based approach depends on the efficiency of protein hydrolysis into individual AAs.

Recently, more and more attention was paid to another protein quantification approach based on sulfur measurement, which is by taking advantage of the stoichiometric presence of sulfur in proteins containing methionine (Met) and cysteine (Cys).[19] By accurate measurement of sulfur only from the analyte and converting it into a protein amount using the stoichiometric relationship, this strategy enables SI-traceable protein quantification with several advantages. On the one hand, the difficulties in optimizing hydrolysis conditions and complexity in finding target amino acids can be avoided. Only complete separation conditions of target proteins and impurities needed to be optimized in sulfur-based analysis.[20,21] On the other hand, by measuring sulfur in protein analytes, the measurement results could be traceable to SI units more directly compared to the AA-based approach. Moreover, since the determination of sulfur has much smaller uncertainty than that of the AA determination, the sulfur-based method can minimize the uncertainty obviously.[22] However, despite the excellent advantages of ICP-MS for elemental quantification in proteins, accurate protein quantification is still difficult because of the shortage of suitable matrix-matched CRMs. Unlike an external calibration approach, an isotope dilution mass spectrometry (IDMS) method based on the measurement of isotope ratios could compensate for signal drifts, matrix effects, and analyte losses in the analysis.[19] A sulfur spike could be continuously mixed with the eluate from HPLC, and the $^{32}S/^{34}S$ ratio was measured by ICP-MS (online species-unspecific spiking). By this online HPLC-ID-ICP-MS approach, the sulfur concentration in the target protein could be accurately measured. This approach was successfully demonstrated in some international comparison[23] recently. However, its application on CRM certification has rarely been reported up to now.

In this study, two pure Aβ solution CRMs ($A\beta_{40}$ and $A\beta_{42}$) were developed. After identification and qualitative analysis of the candidate raw materials, amino acid-based and online sulfur-based isotope dilution strategies were employed together for the first time to certify the Aβ CRMs. In the amino acid-based approach, the AA hydrolysis conditions were optimized. In the online sulfur-based isotope dilution approach, the separation conditions of Aβ suitable for ICP-MS and the influence of the species-unspecific $^{34}S$ spike on the $^{32}S/^{34}S$ ratio were investigated. Good agreement was achieved between the two approaches. The homogeneity and stability of the candidate CRMs were confirmed by AA-based ID-LC-MS. In addition, the expanded uncertainties were evaluated by combining uncertainty contributions including certification procedures ($u_{char}$), homogeneity ($u_{bb}$), long-term stability ($u_{lts}$), and short-term stability ($u_{sts}$). The candidate CRMs are mainly used as calibrators for value assignment to a secondary calibrator or a secondary RM with a clinical matrix (human CSF or blood), which will improve the concordance of measurement results between different Aβ analytical platforms.

## EXPERIMENTAL SECTION

**Instrumentation.** The structural identification of Aβ raw materials was investigated by UPLC coupled quadrupole time-of-flight mass spectrometry (UPLC q-TOF MS, Synapt G2, Waters, USA). A DIONEX ICS-5000 SP ion chromatography system (Thermo Scientific, USA) with an IonPac AS19 IC column (250 × 4 mm) was used for trifluoroacetate ($TFA^-$) determination. The concentration of amino acids was measured by high-performance liquid chromatography (HPLC, Agilent 1200, USA) tandem triple-quadrupole mass spectrometry (Agilent 6410, USA) equipped with a KINETEX C18 (150 × 2 mm) analytical column and an electrospray ion source. The HPLC system (Shimadzu, Japan) with an LC-30 AD pump, an SPD-20A UV detector, and a TSK-gel G3000SW (350 × 7.8 mm) column was used for Aβ separation in the sulfur-based approach. A high-resolution (HR)-ICP-MS instrument (Thermo Fisher Scientific, USA) was operated at medium resolution ($m/\Delta m$ = 4000) in sulfur measurement experiments. The optimum instrumental parameters used for triple-quadrupole MS and HR-ICP-MS are given in Table S1. A Toledo electronic balance (Mettler, Switzerland) was used


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for sample weighing, and purified water (Milli-Q system, Germany) was used in all the experiments.

**Reagents and Standards.** The A$\beta$ raw materials were custom-synthesized by GL Biochem Co., Ltd. (Shanghai, China). The standard material sodium trifluoroacetate of high purity (>98%) was purchased from Sigma-Aldrich (USA), and the purity was further identified by ion chromatography. The CRMs of alanine (99.4%, GBW(E)100054), valine (99.4%, GBW(E)100055), phenylalanine (99.9%, GBW(E)100061), sulfur isotopic CRM solution (GBW(E)082519), and ICP-MS tuning solution (GBW(E)130242) were from the National Institute of Metrology (NIM, China). The candidate recombinant insulin CRM sample was obtained from the Korea Research Institute of Standards and Sciences (KRISS, Korea). The enriched sulfur isotope solution with a concentration of 328.81 ± 0.33 $\mu$g g$^{-1}$ and a $^{32}S/^{34}S$ isotope ratio of 0.01417 ± 0.00007 was from NIM, China. Sulfur isotopic CRM solution with a natural abundance (GBW(E)-082519) was used for mass bias correction in the sulfur determination. A Be, In, and Bi solution (GBW(E)130242) (1 ng g$^{-1}$) was used in the HR-ICP-MS tuning procedure. The labeled alanine ($^{13}C^3$, 99%; $^{15}N$, 99%), valine ($^{13}C^5$, 98%; $^{15}N$, 98%), and phenylalanine ($^{13}C^9$, 99%; $^{15}N$, 99%) were from the Cambridge Isotope Laboratory (CIL, USA) and were diluted to 10 $\mu$g g$^{-1}$ for use. Ammonium formate, ammonium acetate, formic acid, acetonitrile, sodium dihydrogen phosphate ($NaH_2PO_4$), and disodium hydrogen phosphate ($Na_2HPO_4$) were from Fisher Scientific (USA). Hydrochloric acid was from Beijing Chemical Reagent Co., Ltd. (Beijing, China).

**Preparation of Candidate A$\beta$ CRMs.** In the sample preparation of the candidate A$\beta$ CRMs, raw A$\beta_{40}$ and A$\beta_{42}$ materials were taken out from −20 °C and equilibrated at room temperature for 1 h. A certain amount of raw A$\beta$ materials was accurately weighted in a beaker, and purified water was added. The A$\beta$ solutions in the beaker were stirred for 1 h under 200 rpm. The CRM solution was subpackaged with 1.5 mL sterile microvials with screw caps. Each vial was filled with 1 mL of CRM solution and stored at −70 °C immediately after subpackaging. The sample preparation procedure is as shown in Figure 1.

**Structural Analysis and HPLC Analysis.** UPLC q-TOF MS was used for structural identification of A$\beta_{40}$ and A$\beta_{42}$ raw materials. The A$\beta_{40}$ and A$\beta_{42}$ samples were prepared in triplicate for structural analysis. In the UPLC q-TOF MS analysis, the A$\beta$ candidate solution was first separated by UPLC with an ACQUITY UPLC BEH C18 column (100 × 2.1 mm, 1.7 $\mu$m). Purified water with 0.1% formic acid (v/v) was used as eluent A, and acetonitrile with 0.1% formic acid (v/v) was used as eluent B. The flow rate was 0.25 mL min$^{-1}$, and the injection volume was 10 $\mu$L. The LC gradient was set as follows: 10% eluent B from initial to 3 min then increased to 99% at 25 min then held for 5 min and returned to 10% at 30.1 min. The MS instrument was operated in the positive ion mode. The spray voltage of the ESI source was set at +3.0 kV, and the temperature was set at 100 °C. The temperature of the carrier gas ($N_2$) was 300 °C, and the flow rate was 500 L h$^{-1}$. The parent ion of A$\beta_{40}$ and A$\beta_{42}$ was set at $m/z$ of 1083.50 (+4 charge state) and 1129.40 (+4 charge state) separately. The fragment ions were analyzed with a collision-induced dissociation (CID) energy of 30 eV, and the mass range was set at $m/z$ of 50−2000.

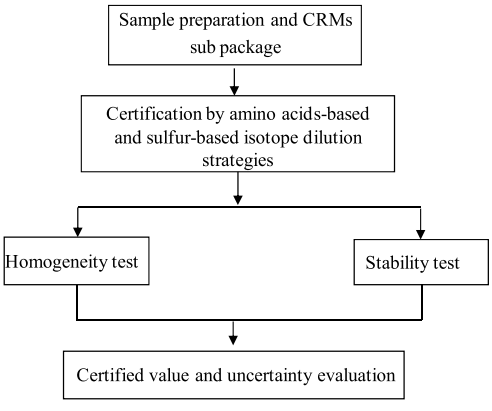


**Figure 1.** Preparation procedure of the candidate A$\beta$ solution CRMs.

Size-exclusion chromatography (SEC) was used for qualitative purity assessment of the A$\beta_{40}$ and A$\beta_{42}$ raw materials. Three independent samples were prepared for HPLC analysis. A$\beta$ solution (2.0 g) was weighed, dried in vacuum to remove the solvent, and then dissolved with ultrapure water to 200 $\mu$L. A TSK-gel G3000SWxl column (300 × 7.8 mm) column was used for separation of A$\beta_{40}$ and A$\beta_{42}$. The injection volume was 10 $\mu$L, and the flow rate was 0.6 mL min$^{-1}$ with isocratic elution. The mobile phase was aqueous solution containing 20 mmol of $Na_2HPO_4$ and $NaH_2PO_4$ buffer (64:36, v/v).

**Determination of TFA$^-$ and Metal Ions.** In the determination of TFA$^-$, the external calibration was performed by using a one-point calibration method with a known concentration of sodium trifluoroacetate as close as possible to the expected concentration in the A$\beta$ sample. The injection volume was 25 $\mu$L, and the flow rate was 1.0 mL min$^{-1}$ with isocratic elution. The mobile phase was aqueous solution containing 15 mM NaOH. Based on three independent measurements, the mass fraction of the TFA$^-$ impurity was then calculated. The metal ion impurities from Li to U in the A$\beta$ raw material were semiquantified by the semiquantification software inherited in ICP-MS (Agilent 7700x, USA).

**Hydrolysis of the A$\beta$ Sample.** Seven independent blend samples were prepared for A$\beta_{40}$ and A$\beta_{42}$ CRMs separately. A weighed 0.5 g A$\beta$ CRM sample was mixed with a certain amount of isotope-labeled alanine (ala), valine (val), and phenylalanine (phe) in a glass ampoule vessel in proportions to give a theoretical 1:1 mole ratio. The mixture was dried under vacuum to remove the solvent. Then, 0.5 mL of HCl (6 mol L$^{-1}$) was added for hydrolysis. Nitrogen was introduced to remove oxygen and sealed. The solution was hydrolyzed in an oven at 110 °C and vortexed once every 6 h. After hydrolysis for 24 h, it was taken out and dried under nitrogen. Then, the sample was reconstituted with aqueous solution containing 0.1% HCl and filtered through a 0.22 $\mu$m filter for ID-LC-MS analysis. To investigate the optimal hydrolysis conditions, different reagent amounts and hydrolysis time (with 6 mol L$^{-1}$ HCl as a reagent) were studied by using A$\beta_{40}$ as an example.

**ID-LC-MS Measurement.** A hydrolysis sample (10 $\mu$L) was injected to a KINETEX C18 column with a flow rate of 0.2 L min$^{-1}$ and eluted at 20 °C. The column was eluted with isocratic elution ($v_A/v_B$ = 9:1) of 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). Triple-quadrupole mass spectrometry was used for amino acid analysis under a selected reaction monitoring mode. The blends of the hydrolyzed A$\beta$ solutions and known concentrations of the labeled amino acid diluents were


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detected by Agilent 6410 mass spectrometry equipped with an ESI source. A multiple reaction monitoring mode was adopted, and the transitions of Ala (90→44), L-Ala (94→47.1), Val (118→72.1), L-Val (124→77), Phe (166→120), and L-Phe (176→129) were monitored for quantification. The ratio of the labeled amino acids to the natural amino acids' peak area was obtained, and the mole of the selected three amino acids in Aβ solutions was calculated by the relationship between the peak area and the amino acid mole in the blend according to equation S1. Furthermore, according to the relationship between the numbers of each amino acid in the Aβ sample, the concentration of $A\beta_{40}$ or $A\beta_{42}$ could be obtained in equation S2.

**Determination of the $^{32}S/^{34}S$ Ratio in Inorganic and Protein Matrix Solutions.** Standard sulfur solution with a natural abundance (GBW(E)082519) and enriched inorganic $^{34}S$ spike and the HPLC mobile phase as a solvent were gravimetrically mixed to obtain a series of solutions with the $^{32}S/^{34}S$ ratios of 0.7, 1.0, and 1.3. Similarly, the other series of solutions with the same $^{32}S/^{34}S$ ratios were also prepared by mixing an insulin sample with enriched inorganic $^{34}S$ spike solution. The insulin sample was an international comparison sample (CCQM-K151/P191).[23] The concentrations and uncertainties of sulfur in the insulin sample were confirmed to be 113.7 ± 1.0 μg $g^{-1}$, and the $^{32}S/^{34}S$ ratio was 22.35184 ± 0.01118. The measured sulfur concentration was in good agreement with that of the calculated stoichiometric proportion of the insulin. The corresponding $^{32}S/^{34}S$ ratios in inorganic and protein matrix solutions were identical through accurate gravimetric weighting. The $^{32}S/^{34}S$ ratios of the two series of mixtures were analyzed by HR-ICP-MS without sample digestion.

**HPLC-ID-ICP-MS Measurement.** Seven independent units were prepared for sulfur analysis in $A\beta_{40}$ and $A\beta_{42}$ CRMs separately. The separation conditions of HPLC were the same as that in the HPLC qualitative experiment. In the determination of $^{32}S/^{34}S$, mass bias corrections were conducted using GBW(E)082519. The enriched $^{34}S$ spike solution with suitable concentration was continuously mixed with the eluate from HPLC via a three-way connection. The isotope ratio of $^{32}S/^{34}S$ was measured online by HR-ICP-MS, which was directly traceable to SI units. Then, a chromatogram of isotope ratios was converted into a chromatogram of mass flow (mass vs. time) according to isotope dilution equation S3, and the mass flow rate was integrated by equation S4 to obtain the mass of sulfur corresponding to each chromatographic peak.[24]

**Homogeneity and Stability Tests.** The homogeneity and stability of Aβ candidate CRMs were tested by AA-based ID-LC-MS. In the homogeneity test, 10 units were selected randomly of each Aβ candidate CRM, and two samples were tested independently from each unit. The analysis procedures were arranged in a randomized order and completed within the shortest time to minimize instrumental drifts. The sample homogeneity was evaluated by a one-way analysis of variance (ANOVA). Homogeneity would be considered to be satisfied if the $F$ test value was smaller than that of the critical values at a 95% confidence interval.

In stability tests, short-term and long-term stabilities were both checked. For the short-term stability study, three units were kept at −20 °C for five days, and the stabilities of the two Aβ candidate CRMs were monitored. The long-term stability was checked throughout the storage period at −70 °C over 14 months. Five independent units were analyzed at 3, 6, 9, and 14 months. The short-term and long-term stabilities of the candidate CRMs were evaluated using regression analysis as recommended in ISO Guide 35:2006.[25] Moreover, to explore the intact stability of Aβ candidate CRMs, the retention time and sensitivity of Aβ peaks were monitored by SEC, and the structural integrity was checked by UPLC q-TOF MS over 14 months.

## RESULTS AND DISCUSSION

**Structural Characterization of Candidate Aβ Materials.** The identification of $A\beta_{40}$ and $A\beta_{42}$ raw materials were assessed by UPLC q-TOF MS. Figure S1 shows the full mass spectrum of $A\beta_{42}$ ($A\beta_{42}$ as an example). The observed masses of the protonated parent ions of $A\beta_{42}$ including $[M + 3H]^{3+}$, $[M + 4H]^{4+}$, and $[M + 5H]^{5+}$ were $m/z$ of 1505.52, 1129.40, and 903.52, respectively, which were identical to the theoretical monoisotopic masses of $A\beta_{42}$ ($m/z$ of 4514.6). Figure S2 shows the series of $b$ and $y$ fragment ions of $[M + 4H]^{4+}$ ($m/z$ of 1129.40), which covered most of the sequence of $A\beta_{42}$. In this study, the two Aβ raw materials were custom-synthesized from a professional peptide company. The synthesis process was in strict accordance with the theoretical amino acid sequence of Aβ, and the identified molecular weight was consistent with its theoretical one. Therefore, the UPLC q-TOF MS results indicate that the amino acid sequence of this candidate material is identical to $A\beta_{42}$. With the same experimental procedures, the amino acid sequence of raw $A\beta_{40}$ was also identified.

**Impurity Assessment by HPLC.** To assess the HPLC purity of raw materials, seven individual $A\beta_{40}$ and $A\beta_{42}$ units were selected for SEC analysis. Figure S3 shows the typical chromatograms of $A\beta_{40}$ and $A\beta_{42}$. The peaks of $A\beta_{40}$ and $A\beta_{42}$ were clearly separated by SEC. A small peak was noticed in both figures at a retention time of 4.6 min, and another obvious peak at 11.2 min was observed in the chromatogram of $A\beta_{42}$. The purities of $A\beta_{40}$ and $A\beta_{42}$ raw materials were 97.74 and 96.93% by using chromatogram peak integration. However, the impurities in the chromatogram only included trace peptides; several artificial impurities such as adducts of peptides and water generated during the ionization process were not included. For the $A\beta_{40}$ and $A\beta_{42}$ raw materials of this work, the counterion contents including $TFA^-$ and other metal ions were 15.06 and 14.82%. Moreover, moisture was also another main impurity in peptides, which was usually from 5 to 8%. As a result, the content of nonpeptide impurities that cannot be recognized by HPLC accounted for approximately 20% of the total sample mass. Therefore, the purity calculated from HPLC integration was not comprehensive, and other characterization techniques need to be employed.

**Optimization of Hydrolysis Conditions.** The accuracy of the AA-based results depends on the completeness of protein hydrolysis into individual amino acids.[22] The most critical step in amino acid analysis is chemical hydrolysis. Several factors affect the efficiency of the reaction, such as the hydrolysis reagent with additives, temperature, and time. Since HCl is a common acid hydrolysis reagent employed in literature for a variety of hydrolysis procedures,[26] 6 moL $L^{-1}$ HCl was selected as a hydrolysis reagent in this work. Also, factors including volume of the reagent and hydrolysis time were investigated during the optimization ($A\beta_{40}$ CRM as the exemplary sample). The normalized area ratios of Ala/L-Ala,


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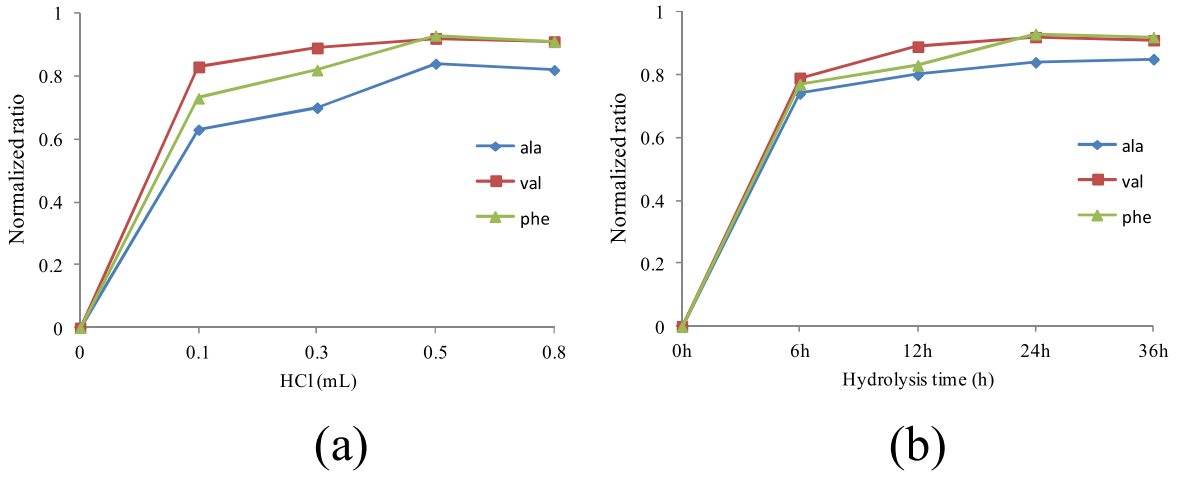


**Figure 2.** With 6 moL $L^{-1}$ HCl as a hydrolysis reagent. (a) Optimization of the HCl amount (normalized area ratios of Ala/L-Ala, Val/L-Val, and Phe/L-Phe versus the reagent amount are plotted). (b) Optimization of hydrolysis time (normalized area ratios of Ala/L-Ala, Val/L-Val, and Phe/L-Phe versus time are plotted).

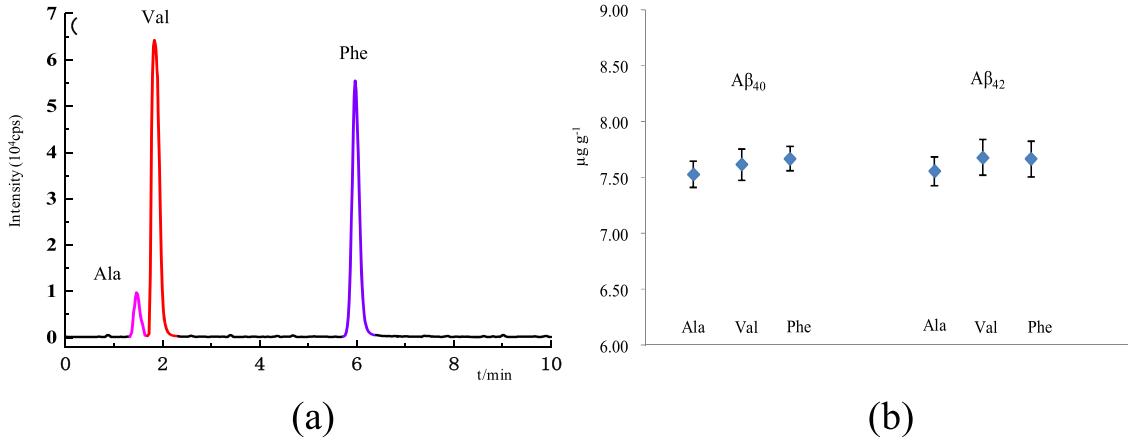


**Figure 3.** (a) LC-MS chromatogram of selected amino acids of unlabeled A$\beta_{40}$ CRM solution. (b) Certification results of A$\beta_{40}$ and A$\beta_{42}$ candidate CRMs by ID-LC-MS. Data represent the measurement result and corresponding SD of each amino acid.

Val/L-Val, and Phe/L-Phe versus the reagent amount and time are plotted in Figure 2.

Figure 2 shows the optimization of hydrolysis conditions. When 6 moL $L^{-1}$ HCl was added with 0.5 mL, the peak area ratio of all the three amino acids reached a maximum value (Figure 2a). For the hydrolysis time in Figure 2b, increasing tendencies were observed of all the three amino acids. However, it was concluded that 36 h hydrolysis time did not result in significant enhanced release of amino acids, and therefore, 24 h appeared to be enough for full completeness. As a result, the condition of 6 moL $L^{-1}$ HCl with 0.5 mL and 24 h duration was confirmed to be the optimum hydrolysis conditions.

**Certification Results of Candidate A$\beta$ CRMs by AA-Based ID-LC-MS.** In the AA-based isotope dilution approach, amino acids including Ala, Val, Leu, Ile, Pro, and Phe are candidate amino acids for the determination of proteins. However, Leu and Ile are chemical isomers that are difficult in chromatograph separation, and Pro does not exist in A$\beta$. Therefore, in this study, Ala, Val, and Phe were selected as the targeted amino acids, and isotope labeling AAs (L-Ala, L-Val, and L-Phe) were used as internal standards to minimize influences from drifts and fluctuations in ID-LC-MS analysis. The standard amino acids undergoing the same treatment as the samples were used as calibrants. Figure 3a shows the LC-MS chromatogram of unlabeled A$\beta_{40}$ CRM solution, and all the three AAs were completely separated and eluted within 10 min without interference.

The concentrations of A$\beta_{40}$ and A$\beta_{42}$ from each amino acid were calculated by equations S1 and S2. As shown in Figure 3b, although the hydrolysis efficiency of various AAs was usually different,[27] the A$\beta$ concentrations calculated from Ala, Val, and Phe were in agreement based on the stoichiometric presence of corresponding AAs in the protein. The A$\beta_{40}$ quantitative concentrations and standard deviations (SD) calculated from the three AAs were 7.53 ± 0. 12, 7.62 ± 0.14, and 7.67 ± 0.11 $\mu$g $g^{-1}$, respectively, and the RSD was within 1.84%. The A$\beta_{42}$ concentrations and SD calculated from the three AAs were 7.56 ± 0.13, 7.68 ± 0.16, and 7.67 ± 0.16 $\mu$g $g^{-1}$, respectively, and the RSD was within 2.08%. The limits of detection (LODs) of amino acids were defined as the concentrations providing signal-to-noise ratios of three, and the LODs of A$\beta$ were calculated from the stoichiometric presence of AAs in the protein. The LODs of A$\beta_{40}$ and A$\beta_{42}$ were calculated to be 140 ng $g^{-1}$ in this study.

The standard uncertainty of A$\beta_{40}$ (as an example) by using AA-based ID-LC-MS included standard deviation of the mean from Ala, Val, and Phe (SD/$\sqrt{7}$ of 0.60, 0.72, and 0.53%,


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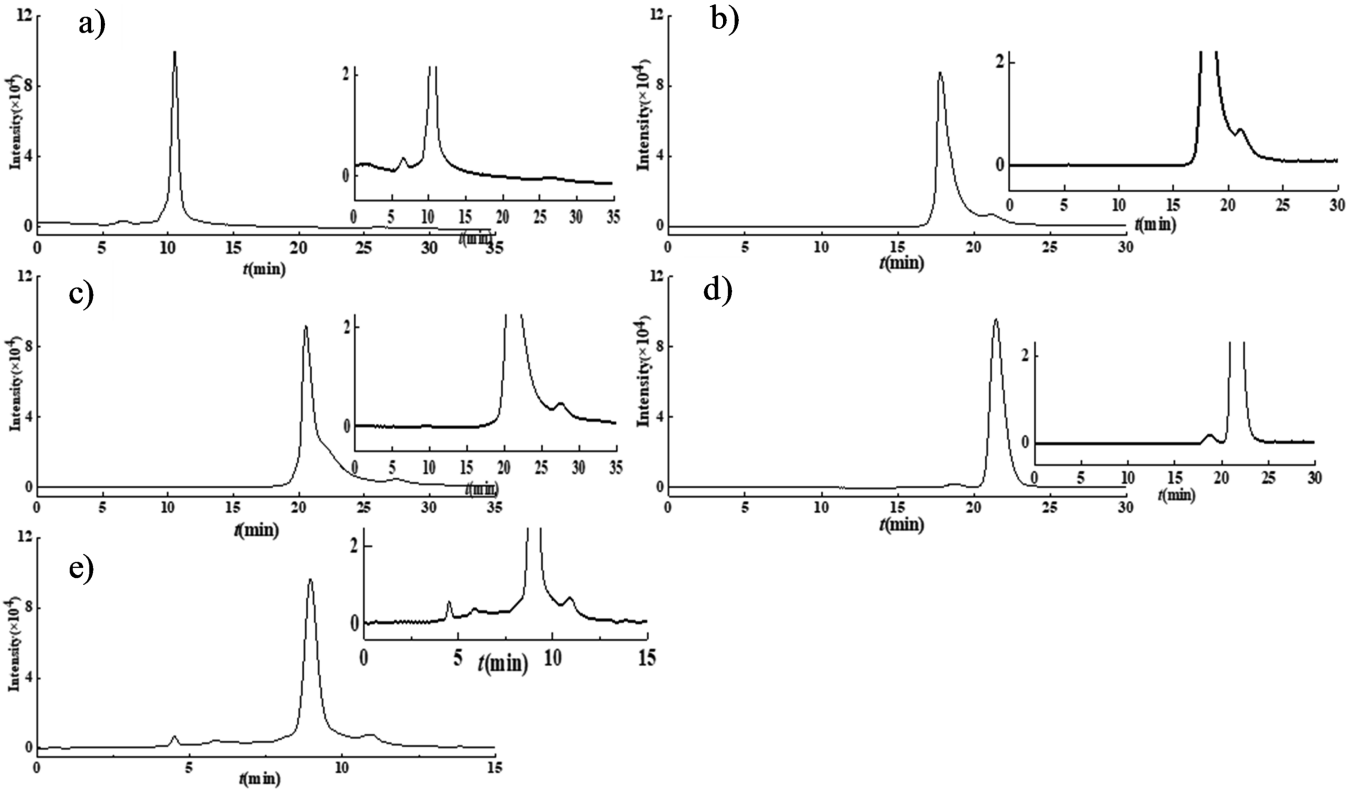


**Figure 4.** Liquid chromatogram of A$\beta_{42}$ with different mobile phases: (a) ammonium formate, (b) ammonium acetate, (c) disodium hydrogen phosphate, (d) sodium dihydrogen phosphate, and (e) $NaH_2PO_4/Na_2HPO_4$ v/v = 64:36.

respectively), the uncertainty of hydrolysis efficiency (1.0%), uncertainties from weighing (0.36%), the uncertainty from each standard amino acid CRM (0.75−0.80%). The standard uncertainty of the AA-based approach was calculated from all these contributions according to EURACHEM.[28] Similarly, the standard uncertainty of A$\beta_{42}$ was also evaluated in the same way. In this study, the mean values from the results of the three AAs were taken as the measurement values by the ID-LC-MS method, which were 7.60 and 7.64 $\mu g\ g^{-1}$ of A$\beta_{40}$ and A$\beta_{42}$, respectively. And the relative standard uncertainties of A$\beta_{40}$ and A$\beta_{42}$ by using the AA-based ID-LC-MS strategy were 2.01 and 2.19%, respectively.

**Certification Results of Candidate A$\beta$ CRMs by Sulfur-Based HPLC-ID-ICP-MS.** In the characterization of A$\beta$ raw materials, SEC interfaced with ICP-MS can be used to differentiate small-molecule impurities containing sulfur atoms from the target protein. This strategy enables SI-traceable protein quantification by determining the accurate amount of sulfur only from the analyte and converting it into a protein amount according to the stoichiometric relationship. To obtain optimum separation conditions of SEC, the mobile phase including ammonium formate, ammonium acetate, disodium hydrogen phosphate, and sodium dihydrogen phosphate was investigated at 220 nm UV absorption (A$\beta_{42}$ as the example in Figure 4).

In Figure 4a,d, the impurity peak after A$\beta$ was not eluted, and it was also not fully separated in Figure 4b. Although the impurity in Figure 4c was completely separated, the elution time was much longer. On the basis of the mobile phase in Figure 4c, mixed $NaH_2PO_4$ and $Na_2HPO_4$ solution (64:36, v/v) (Figure 4e) was confirmed to obtain optimal separation. The separation condition of A$\beta_{40}$ was the same with that of A$\beta_{42}$.

Following the separation of A$\beta$, the enriched $^{34}S$ spike solution was continuously mixed with the eluate from SEC. The isotope ratio of $^{32}S/^{34}S$ was measured online by HR-ICP-MS, and the concentration of sulfur in A$\beta$ was calculated by equations S3 and S4. In the online isotope dilution equation, the mass flow ($d_{sp}$:$f_{sp}$) was determined by weighing the mass flow of spike solution transmitted by a spiking pump at a flow rate of 0.6 mL $min^{-1}$ for 1 min.[20] Seven replicates were conducted to calculate the average $d_{sp}$:$f_{sp}$. In this study, to guarantee the mixing efficiency of the A$\beta$ elute and enriched $^{34}S$ solution, the flow rate of the spike was set to be 0.6 mL $min^{-1}$ (same with that of the SEC flow rate). The corresponding peak integration in the mass flow chromatogram provided the absolute sulfur content in the A$\beta$ sample. According to the injection volume and the sulfur stoichiometric ratio, the concentration of A$\beta$ could be calculated. The intensities of $^{32}S$ and $^{34}S$ measured by HR-ICP-MS are illustrated in Figure S4 (A$\beta_{40}$ as an example). The isotope ratio ($R_m$) in the mixture was the only measured parameter, which was less affected by matrix effects and instrument drifts. In this work, the sulfur concentrations and SD of A$\beta_{40}$ and A$\beta_{42}$ were calculated to be 5353 ± 37 and 5371 ± 38 $\mu g\ g^{-1}$, and the corresponding A$\beta_{40}$ and A$\beta_{42}$ concentrations and SD were 7.55 ± 0.06 and 7.60 ± 0.06 $\mu g\ g^{-1}$. The LODs of A$\beta$ were calculated from the stoichiometric presence of sulfur in the protein. The LODs of A$\beta_{40}$ and A$\beta_{42}$ by the sulfur-based approach were calculated to be 18 ng $g^{-1}$, which was almost 10 times lower than that of the AA-based approach.

As a definitive method for approaching chemical traceability, the sulfur-based IDMS operation could be completely


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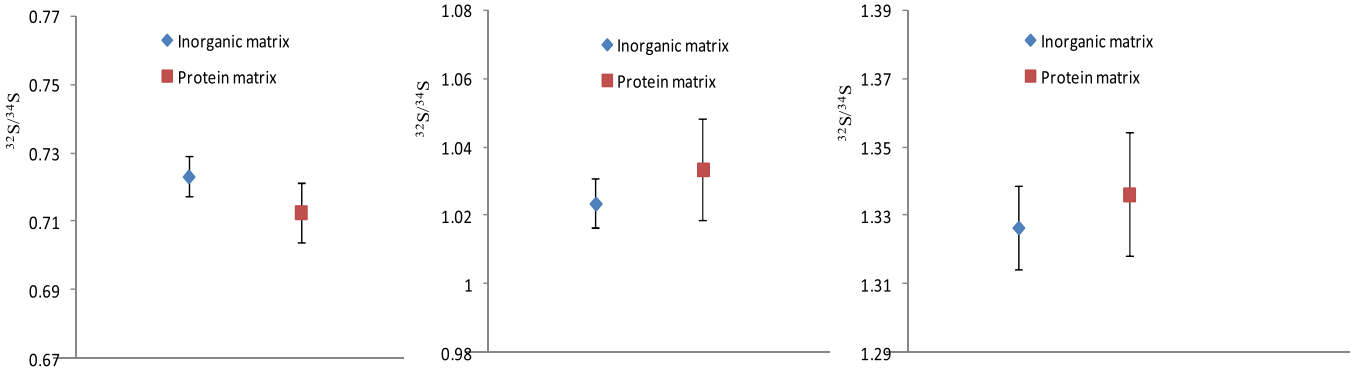


**Figure 5.** $^{32}S/^{34}S$ ratios in species-specific and species-unspecific solutions. The inorganic matrix sample is a mixture of the inorganic $^{34}S$ spike and sulfur standard with natural abundance. The protein matrix sample is a mixture of the inorganic $^{34}S$ spike and insulin. Solutions with three ratios are prepared.

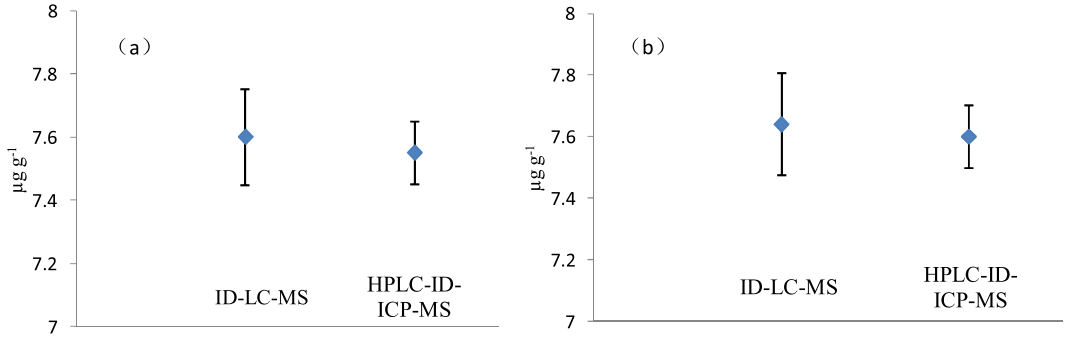


**Figure 6.** Comparison of (a) A$\beta_{40}$ and (b) A$\beta_{42}$ by using LC-IDMS and HPLC-ID-ICP-MS. Error bars represent standard uncertainties associated with the certification process.

described and understood, and complete uncertainty contributions could be evaluated in terms of SI units. Uncertainty evaluation was completed according to the EURACHEM Guideline,[28] Guide to Expression of Uncertainty in Measurement of ISO/BIPM,[29] and IDMS equations. In the online species-unspecific IDMS approach, compared to the traditional solution-based IDMS method, contributions from the $R_m$ determination and the calculation of $f_{sp}$ were two dominating sources of total uncertainty budget. Moreover, since the species-unspecific IDMS approach cannot compensate for any analyte loss during sample preparation and separation, it must be noted that the contributions during sample preparation ($u_{loss}$) and chromatographic separation ($u_{sep}$) should be included. The representatives and detailed uncertainty budget of A$\beta_{40}$ are shown in Table S2. In this work, the relative standard uncertainties of A$\beta_{40}$ and A$\beta_{42}$ by using HPLC-ID-ICP-MS were 1.32 and 1.35%, respectively.

**Investigation of Sulfur-Based Species-Unspecific Isotope Dilution.** Species-specific ID-ICP-MS employing peptides and proteins labeled with enriched elemental isotopes would have the advantage of correcting for all analyte losses after isotope equilibration. Nevertheless, sulfur in the peptide was inherent in the amino acid molecules and not complexed with the molecules. Therefore, such species-specific standards have not been synthesized and applied so far. In this study, the inorganic $^{34}S$ spike was mixed with the peptide elution after HPLC (species-unspecific approach); the different sulfur species in the mixture may possibly affect the $^{32}S/^{34}S$ ratio measurement. To investigate the influence of sulfur-based species-unspecific isotope dilution, the $^{32}S/^{34}S$ ratios in inorganic and protein matrix solutions were measured. For the protein matrix solutions, sulfur from insulin and the isotope spike was with different species. Since the prepared ratios of $^{32}S/^{34}S$ in inorganic and protein matrix solutions were the same, the discrepancy of the measured $^{32}S/^{34}S$ ratios in the two series of solutions would be attributed to the species variability. In Figure 5, although with different sulfur species, the $^{32}S/^{34}S$ ratios in inorganic and protein matrix solutions showed no significance at the ratios of 0.7, 1.0, and 1.3. However, it should be noticed that the standard deviation of $^{32}S/^{34}S$ ratios in species-unspecific solution (protein matrix) was larger than that in species-specific solution (inorganic matrix).

Strictly speaking, in species-unspecific isotope dilution, complete equilibration between the isotopes of sulfur in A$\beta$ molecules and the isotope spike cannot be achieved. However, in the ICP-MS plasma with a temperature of more than 7000 K, all biological molecules are broken down into atoms, irrespective of their chemical forms. Therefore, ICP-MS detection is virtually species-independent, and thus, sulfur isotope ratio measurement should not depend on the molecular environment in the sample.[30] Even so, in Figure 5, the standard deviation of $^{32}S/^{34}S$ ratios in the protein matrix was a little bit larger, which was possibly because of the different ionization efficiencies of sulfur with different species.

**Comparison of Characterization Results by AA-Based and Sulfur-Based Approaches.** In this study, the concentrations of A$\beta_{40}$ and A$\beta_{42}$ candidate CRMs were certified by two IDMS approaches. The results of AA-based and sulfur-based approaches are shown in Figure 6. As shown in Figure 6, since the measurement results of both A$\beta_{40}$ and A$\beta_{42}$ were in good agreement, the average value of AA-based and sulfur-based methods was used as the certified value, and the


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combined uncertainty associated with the certification process was calculated from the standard uncertainty of the two approaches. The concentrations and standard uncertainties of A$\beta_{40}$ and A$\beta_{42}$ candidate CRMs associated with certification were 7.58 ± 0.13 and 7.62 ± 0.14 $\mu$g g$^{-1}$.

It was also worth noting that the results of A$\beta_{40}$ and A$\beta_{42}$ based on the AA approach were slightly higher than those of based on sulfur. The possible reason for this tendency was that the target amino acids not only existed in A$\beta_{40}$ and A$\beta_{42}$ but also existed in the impurity peptides, which was one of the main limitations of the AA-based approach. In this work, although the hydrolysis conditions for A$\beta_{40}$ and A$\beta_{42}$ were optimized, the quantitative results of different AAs still have slight deviations. To guarantee the accuracy, the result of the AA-based measurement usually took the average value of the selected amino acids, and the uncertainty would be a bit larger. For the sulfur-based strategy, the measurement result of sulfur could be directly traceable to SI units, and the measurement uncertainty for the element was also better than that for amino acids.

**Homogeneity and Stability Results.** No significant heterogeneity is found in Table 1, and the $F$ test values of

**Table 1. Homogeneity and Stability Uncertainties of Candidate A$\beta$ CRMs**

| CRMs | $F$ value | $u_{bb}$ (%) | $u_{sts}$ (%) | $u_{lts}$ (%) | observation |
|---|---|---|---|---|---|
| A$\beta_{40}$ | 1.70 | 0.66 | 0.40 | 0.53 | no obvious differences of stability tests |
| A$\beta_{42}$ | 1.59 | 0.52 | 0.39 | 0.39 | |

A$\beta_{40}$ and A$\beta_{42}$ were 1.70 and 1.59, respectively. Since the $F$ values were smaller than the $F$ critical value (3.02 at the 95% confidence interval with 9 degrees of freedom), the candidate A$\beta_{40}$ and A$\beta_{42}$ CRMs were homogeneous enough. The uncertainty contributions from sample inhomogeneity ($u_{bb}$) were calculated using eq 1[25] and are listed in Table 1, which were 0.66 and 0.52%.

$$u_{bb} = \sqrt{(CV_b - CV_w)/2} \quad (1)$$

where $CV_b$ and $CV_w$ refer to the within variance and between variance of units that were derived from duplicate analysis of the 20 samples.

Short-term stability of the candidate A$\beta$ CRMs was monitored for five days at −20 °C. This approach mainly simulates the temperature condition of dry ice during the sample transportation process. Long-term stability of the candidate A$\beta$ CRMs during storage was investigated in 14 months of storage at −70 °C. Student's $t$ test according to ISO guide 35 was employed to evaluate the significance of short-term and long-term stabilities. The uncertainties from short-term ($u_{sts}$) and long-term stability ($u_{lts}$) were calculated according to ISO guide 35.[25] Stability results are shown in Table 1, and the long-term stability would be further monitored at regular intervals.

Most of the peptide were very stable at low temperature. In the stability test of A$\beta$ CRMs, except for ID-LC-MS, SEC and UPLC q-TOF MS methods were also employed to monitor the intact stability. No obvious retention time and sensitivity changes were observed of the A$\beta$ chromatogram, and the parent/fragment ion spectrum of UPLC q-TOF MS did not also change significantly. Moreover, the HPLC-ID-ICP-MS method was also employed during the 14 months of storage. Since this method did not involve amino acid hydrolysis, the consistent HPLC-ID-ICP-MS results proved the structural integrity of A$\beta$ peptides. Therefore, the intact stability of A$\beta$ CRMs was also good enough during −70 °C storage.

**Certified Values and Uncertainty Evaluation.** In this work, the two A$\beta$ CRMs were certified by ID-LC-MS and HPLC-ID-ICP-MS in accordance with ISO Guide 35. Of the two IDMS strategies, the AA-based LC-IDMS strategy was more frequently adopted as a primary method for the quantification of pure-protein CRMs. Also, the measurement of sulfur in the HPLC-ID-ICP-MS strategy could be directly traceable to SI units with a smaller uncertainty and shorter traceability chain. Therefore, in this work, a more robust model by both of the two IDMS approaches was employed in the certification of A$\beta$ CRMs. Since good consistency was achieved between the two IDMS strategies, the mean value was built based on this agreement, and the uncertainties were calculated according to GUM[29] and ISO Guide 35:2006,[25] which were associated with the certification process, inhomogeneity, and instability. The combined standard uncertainty ($u_{CRM}$) was calculated by eq 2, and the expanded uncertainty was calculated by eq 3

$$u_{CRM} = \sqrt{u_{char}^2 + u_{bb}^2 + u_{sts}^2 + u_{lts}^2} \quad (2)$$

$$U = k \times u_{CRM} \ (k = 2.0) \quad (3)$$

where $u_{char}$ is the uncertainty associated with the certification process by AA-based and sulfur-based approaches; $u_{bb}$ is the uncertainty associated with the between-bottle inhomogeneity; $u_{sts}$ and $u_{lts}$ are the uncertainty associated with the short- and long-term instability; $U$ is the expanded uncertainty; $k$ is the coverage factor.

In this study, the standard uncertainties of A$\beta_{40}$ and A$\beta_{42}$ CRMs associated with certification were 0.13 and 0.14 $\mu$g g$^{-1}$ respectively, and the contributions from inhomogeneity and stability are shown in Table 1. The certified values and expanded uncertainties ($U$) of candidate A$\beta_{40}$ and A$\beta_{42}$ CRMs are 7.58 ± 0.30 and 7.62 ± 0.30 $\mu$g g$^{-1}$.

## CONCLUSIONS

In this study, the development of two A$\beta$ solution CRMs was presented. Amino acid-based and sulfur-based IDMS strategies were adopted in the certification process. The CRMs were confirmed to be sufficiently homogeneous and stable. The expanded uncertainty was evaluated by combining contributions including analytical methods, between-bottle homogeneity, and stability, which was found to be approximately 4.0%. The CRMs are primarily intended for use as calibrators for value assignment to a secondary calibrator or a secondary RM with a clinical matrix. More importantly, the certification layout of this study will establish a new way for the certification of high-purity protein reference materials.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.0c02381.

(Table S1) Operating conditions of tandem triple-quadrupole MS and HR-ICP-MS; (Table S2) typical uncertainty contributions for measurement of candidate A$\beta_{40}$ CRMs by HPLC-ID-ICP-MS; (Equations S1 and S2) formula for calculation of A$\beta$ concentration by ID-



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LC-MS; (Equations S3 and S4) formula for calculation of A$\beta$ concentration by HPLC-ID-ICP-MS; (Figure S1) full mass spectrum of the A$\beta_{42}$ candidate raw material; (Figure S2) tandem mass spectrum of $[M + 4H]^{4+}$ ($m/z$ of 1129.40) of A$\beta_{42}$; (Figure S3) chromatogram of (a) A$\beta_{40}$ and (b) A$\beta_{42}$ from size-exclusion chromatography; (Figure S4) HPLC-ID-ICP-MS mass spectrum of $^{34}S$ and $^{32}S$ intensity of candidate A$\beta_{40}$ CRMs (PDF)

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### Notes

The authors declare no competing financial interest.

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