Introduction

Amino acid is the general term for an organic compound that has both the amino group and the carboxyl group; amino acids play various roles as the basic constituent units of protein, nutrition components, and functional amino acids in the living body. Amino acids are frequently analyzed in many fields, such as biochemistry, food chemistry, and pharmaceutical biotechnology. For diagnostic use, changes in the concentration of amino acids in blood plasma and urine are used as an index of many diseases, such as an inborn error of amino acid metabolism, liver disease, renal disease, and endocrine disease. In addition, 17 kinds of proteinogenic amino acids are targeted for amino acid composition analysis or accurate quantification of protein/peptide. In these methods, the constituent amino acids released from the original protein/peptide through hydrolysis are quantified.

To ensure the reliability of amino acid analyses, the National Metrology Institute of Japan of the National Institute of Advanced Industrial Science and Technology (NMIJ/AIST) has developed high-purity certified reference materials (CRMs) for 17 proteinogenic amino acids. These CRMs are intended for use as primary reference materials to enable the traceable quantification of amino acids. The purity of the present CRMs was determined based on two traceable methods: nonaqueous acidimetric titration and nitrogen determination by the Kjeldahl method. Since neither method could distinguish compounds with similar structures, such as amino acid-related impurities, impurities were thoroughly quantified by combining several HPLC methods, and subtracted from the obtained purity of each method. The property value of each amino acid was calculated as a weighted mean of the corrected purities by the two methods. The uncertainty of the property value was obtained by combining measurement uncertainties of the two methods, a difference between the two methods, the uncertainty from the contribution of impurities, and the uncertainty derived from inhomogeneity. The uncertainty derived from instability was considered to be negligible based on stability monitoring of some CRMs. The certified value of each amino acid, property value with uncertainty, was given for both with or without enantiomeric separation.

Keywords Amino acids, certified reference material, purity, traceable method, titration, impurity determination

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to impurities contained in the main component. This method has an advantage in determining the purity of organic materials with a small measurement uncertainty, whereas the applicable materials are limited. It was hard to apply this method for determining the purity of amino acids due to their thermal instability. The mass balance method is becoming a de facto standard for determining the mass fraction of the main component of a high-purity organic material. In this method, i.e., the content of the related structure impurity, water content, residual solvents, and nonvolatility, is quantified and subtracted from 1.000 kg kg⁻¹ to assign the value of the mass fraction for the main component. The advantage of this method is that it can be applied, in principle, to a wide range of stable, nonvolatile compounds, and that it is capable of assigning value with a small measurement uncertainty. However, identification of all impurities is difficult, and missing the assessment of impurities will lead to a bias for the assigned value. The titration method is recommended as another approach for determining the purity of amino acids. In this method, only those compounds that react with the titer are quantified. Therefore, impurities that will bias the purity value are limited in comparison with the mass balance method. On the other hand, compounds with structures similar to the target compound cannot be distinguished by this method. Thus, the titration method cannot be used alone and should be used together with an evaluation of related compound impurities.

NMIJ/AIST has developed high-purity CRMs for 17 proteinogenic amino acids based on two traceable titration methods, the nonaqueous acidimetric titration and nitrogen determination by the Kjeldahl method. Since neither method could distinguish compounds with similar structures, we thoroughly quantified the amino acid-related impurities and subtracted the values to give the assigned value of the mass fraction for the main component. This paper describes the process for developing amino acid CRMs and discusses the measurement scheme for establishing the traceability to SI and how to calculate property values and their uncertainties using quantitative values from each analytical method.

Experimental

Preparation of materials

The candidate materials for the NMIJ CRMs of 17 proteinogenic amino acids were of specifically purified grade provided by Ajinomoto Co., Ltd. (Tokyo, Japan) or Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Two hundred to 400 glass tubes were filled with 0.5 g of a powdery type of material under an argon atmosphere, and each vial was numbered according to the bottle order and sealed in an aluminum-laminated bag by Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The tubes were stored at room temperature (15 to 25°C) in a clean desiccator until used.

Reagents

The mixture solution of 14 basic amino acid standards (γ-amino-n-butyric acid (GABA), L-α-aminocaproic acid, creatinine, ethanolamine, His, L-homocystine, ε-hydroxylysine, Lys, 1-methyl-L-histidine, 3-methyl-L-histidine, L-ornithine, and L-tryptophan) and the mixture solution of 27 acidic or neutral amino acid standards (β-alanine, Ala, L-α-aminoacidipic acid, ω-amino-n-butyric acid, DL-β-aminoisobutyric acid, L-asparagine, Asp, L-citrulline, Cystathionine, Cys-Cys, Gln, Gly, Hydroxy-L-proline, Ile, Leu, Met, Phe, o-phospho-L-lysine, o-phosphoethanolamine, Pro, Sarcosine, Ser, Taurine, Thr, Tyr, Urea, and Val) were purchased from Sigma-Aldrich Japan (Tokyo, Japan) and used to prepare the mixture solution of 41 amino acid standards. The solvent reagents, calibration standards, and GABA used as an internal standard for the analysis of amino acid-related impurities were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), as previously described by Kato et al. Isotope-labeled amino acids used as an internal standard for the LC/MS method were purchased from Taiyo Nippon Sanso (Tokyo, Japan). The calibration standards of D-amino acids for the candidate CRMs were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). AQUAMICRON AX and AQUAMICRON CXU used for Karl Fisher titration were purchased from Mitsubishi Chemical Co. (Tokyo, Japan).

The AQUAMICRON AX and AQUAMICRON CXU was obtained from NMIJ/AIST. The AQUAMICRON AX and AQUAMICRON CXU was obtained from NMIJ/AIST. The AQUAMICRON AX and AQUAMICRON CXU was obtained from NMIJ/AIST. The AQUAMICRON AX and AQUAMICRON CXU was obtained from NMIJ/AIST.

Ultrapure water prepared with a Milli-Q water purification system (resisitivity 18 Ω cm⁻¹, Nihon Millipore Kogyo, Tokyo, Japan) was used throughout the experiments.

Instrumentation

The HPLC system for the post-column derivatization with o-phthalaldehyde (the OPA-method), liquid chromatography–mass spectrometry (the LC/MS method), and pre-column derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (the AQC method) consisted of versatile devices such as LC pumps, an autosampler, a column heater, a fluorescence detector, and a mass spectrometer, as previously described by Kato et al. A post-column derivatization with ninhydrin (the NIN method) was conducted using Amino Acid Analyzer L-8900 (Hitachi High-Technologies Corp., Ibaraki, Japan). For the LC/CAD method, an Agilent 1100 HPLC system equipped with a corona charged aerosol detector purchased from Thermo Fischer Scientific Inc. K.K. (Yokohama, Japan) was used. For chiral chromatography, an Agilent 1290 HPLC system equipped with mass spectrometry, Agilent Q TOF 6500 (Agilent Technologies Japan, Tokyo, Japan) was used.

The ion chromatograph used was a DIONEX ICS-2000 with a conductivity detector and an SRS ULTRA II suppressor purchased from DIONEX (Sunnyvale, CA). The Karl Fischer Moisture Meter CA-100 with vaporizer VA-100 used for Karl Fisher titration was purchased from Mitsubishi Chemical Analytech Co., Ltd. (Mie, Japan). A Thermo Gravimetry/Differential Thermal Analyzer (TG-DTA) 6200 EXSTAR was purchased from Seiko Instruments Inc. (Chiba, Japan).

The auto titrator used for nonaqueous acidimetric titration was the AT-510 with glass electrode C-173 of Kyoto Electronics (Kyoto, Japan) or Metrohm’s 809 Titirando with the Solvotrode...
LL glass electrode of Metrohm Japan Ltd. (Tokyo, Japan). The acid digestion apparatus, KJELDATHERM KBBS, and the distillation apparatus, Vapodest 30 purchased from Gerhardt GmbH & Co. (Königswinter, Germany), were used for the Kjeldahl method. The auto titrator used was Kyoto Electronics’ AT-510 with glass electrode C-171 (Kyoto, Japan).

**HPLC methods for determining amino acid-related impurities**

Amino acid-related impurities were analyzed by combining some of the OPA method, the NIN method, the LC/MS method, the AQC method, and the LC/CAD method. As previously described by Kato et al., the candidate CRM solution was analyzed with a Shim-pack Amino-Li, 5 μm × 6.0 mm i.d. × 100 mm (Shimadzu, Kyoto, Japan) using the OPA method; an AccQ-Tag reverse-phase column, 3.9 mm i.d. × 150 mm (Waters, Milford, MA) using the AQC method; the Develosil RPAQUEOUS C30-AR-5 column, 5 mm × 2.0 mm i.d. × 150 mm (Nomura Chemicals, Aichi, Japan) using the LC/MS method. For the LC/CAD method, the column and analytical conditions were the same as those of the LC/MS method except for using 0.6 mL min⁻¹ of sheath liquid. For the NIN method, the column and solvent reagents were commercially available from Hitachi High-Technologies (Ibaraki, Japan), and the analytical conditions were those in accordance with the manufacturer’s recommendations.

Each candidate CRM solution was gravimetrically prepared with mobile phase A or Milli-Q to be approximately 2500 to 5000 mg kg⁻¹. Candidate CRMs or the amino acid products whose purities were evaluated by NMJ were used as calibration standards for amino acid-related impurities. γ-aminobutyric acid (GABA) was used as an internal standard for the OPA method and the NIN method. Isotope-labeled alanine (Ala-13C3, 15N), isoleucine (Ile-13C3, 15N), leucine (Leu-13C3, 15N), or valine (Val-13C3, 15N) was used as an internal standard for the LC/MS method. The target compound in the candidate CRM itself was used as a calibration standard for other amino acid-related impurities in the LC/CAD method.

**Chiral chromatography for D-amino acid analysis**

D-Amino acid in the candidate CRM was quantified by using chiral chromatography. Each candidate CRM solution was gravimetrically prepared with Milli-Q or 0.1 mol L⁻¹ of hydrochloric acid to be approximately 1000 to 10000 mg kg⁻¹. D-Amino acid product, whose purity was evaluated by NMJ, was used as a calibration standard. Mobile phase A was 0.1 % (v/v) acetic acid, and mobile phase B was acetonitrile or methanol. The solution of the candidate CRM was injected onto a CHIROBIOTIC™ T column, 5 μm × 4.6 mm i.d. × 250 mm (Sigma-Aldrich Japan, Tokyo, Japan), using an injection volume of 2 to 20 μL and isocratically eluted at 30°C. The mixing ratio of solvents and the flow rate of the solvent were changed depending on each candidate CRM.

**Other impurity analyses**

The water content in each candidate CRM was determined using a Karl Fischer Moisture Meter CA-100 with vaporizer VA-100. The sample size was approximately 0.5 g per measurement. AQUAMICRON AX and AQUAMICRON CXU were used as the anolyte and catholyte, respectively. The anions or cations in each candidate CRM were semi-quantified by using ion chromatography. For the determination of anions, an analytical anion exchange column, IonPac AS18 with a guard column, and 30 mmol L⁻¹ KOH as an eluent were employed. For the determination of cations, the IonPac CS12 column and 20 mmol L⁻¹ methanesulfonic acid as an eluent were used. The sample solution was prepared to be approximately 1000 to 5000 mg kg⁻¹ with ultrapure water, and then 25 μL of the sample solution was injected onto the analytical column. The concentration of chloride ion in the candidate CRM of Lys-HCl was calibrated with the standard solution of chloride ion of the JCSS (Japan Calibration Service System) purchased from Kanto Chemical Co. Inc. (Tokyo, Japan).

The mass fraction of nonvolatile impurities in each candidate CRM was determined using a TG-DTA. The sample size was approximately 10 mg per measurement.

**Nonaqueous acidimetric titration**

Accurately weighted 0.02 to 0.2 g of the candidate CRM was dissolved in 98% (w/w) formic acid, and then in acetic acid. A quantity of 0.1 mol L⁻¹ perchloric acid in acetic acid was used as a titer. For the back titration technique, accurately weighted 0.02 to 0.2 g of the candidate CRM of His or Lys-HCl was dissolved in perchloric acid in acetic acid and titrated with 0.1 mol L⁻¹ sodium acetate in hydrochloric acid. The normality of the perchloric acid solution or sodium acetate solution was determined with potassium hydrogen phthalate (NMJJ CRM 3001-a).

The purity of the amino acid by nonaqueous acidimetric titration was calculated as shown in Eq. (1),

\[
P_{AA} = \frac{(V_b - V_s) \times C_{HClO4}}{W} \times MW_{AA},
\]

where \(P_{AA}\) is the purity of the candidate CRM (kg kg⁻¹), \(V_b\) is the titration volume for the sample solution, \(V_s\) is the titration volume of the blank, \(C_{HClO4}\) is the molar concentration of perchloric acid in acetonitrile (mol L⁻¹), \(MW_{AA}\) is the molecular weight of the candidate CRM, \(W\) is the gross weight of the sample (mg), and \(W_s\) is the tare weight (mg).

**The Kjeldahl method**

Accurately weighted 0.02 to 0.2 g of each candidate CRM was transferred to a digestion test tube. Concentrated sulfuric acid and a tablet of degradation promoter, Kjetlabs CX or CT, were added, and the mixture was heated from 380 to 420°C for 1 to 2 h. The heating conditions were changed depending on each candidate CRM. The tube with the digested sample was placed in the distillation apparatus, and sodium hydroxide was added and distilled. The ammonia-containing steam was collected in boric acid. The content of ammonia was determined with 0.025 or 0.05 mol L⁻¹ sulfuric acid. The normality of the sulfuric acid was determined with sodium carbonate CRM (NMJJ CRM 3005-a). Blanks that consisted of empty digestion tubes were treated in the same manner.

The purity of each amino acid was calculated by the Kjeldahl method as shown in Eq. (2),

\[
P_{AA} = \frac{(V_b - V_s) \times C_{HClO4}}{W} \times MW_{AA},
\]

where \(P_{AA}\) is the purity of the candidate CRM (kg kg⁻¹), \(V_s\) is the titration volume for the sample solution, \(V_b\) is the titration volume of the blank, \(C_{HClO4}\) is the molar concentration of perchloric acid (mol L⁻¹), \(MW_{AA}\) is the molecular weight of the candidate CRM, \(W\) is the gross weight of the sample (mg), and \(W_s\) is the tare weight (mg).

**Homogeneity study**

Ten bottles (3 or 4 sub-samples for each bottle) of each
candidate CRM were subjected to measurement by nonaqueous acidimetric titration. An analysis of variance (ANOVA) was carried out for the results, and the obtained within-bottle mean square ($MS_{\text{within}}$) and between-bottle mean square ($MS_{\text{among}}$) were applied in Eqs. (3) and (4) to obtain the between-bottle variance ($s_{\text{within}}$) and the between-bottle variance incorporating the influence of the analytical variation ($u_{\text{b}}$), respectively.

$$s_{\text{within}} = ((MS_{\text{among}} - MS_{\text{within}})/n)^{0.5}$$

$$u_{\text{b}} = ((MS_{\text{within}}/n)^{0.5}/(MS_{\text{within}})^{0.25})$$

In Eq. (4), $MS_{\text{within}}$ is the freedom of $MS_{\text{within}}$ of each candidate CRM was basically used as the contribution of the homogeneity to the combined standard uncertainty of the property value. When the $s_{\text{within}}$ was smaller than the $u_{\text{b}}$, the $u_{\text{b}}$ was used alternatively as the contribution of the homogeneity, according to ISO Guide 35:2006.20

**Stability monitoring**

Stability monitoring of the candidate CRM was performed by quantifying amino acid-related impurities with the OPA method. At regular intervals, one vial was selected in a stratified random manner, and the amino acid-related impurities were analyzed in duplicate. The quantitative values of the amino acid-related impurities were calculated and compared with those at the point of certification. At each time point, GABA was used as the internal standard.

For some candidate CRMs that showed hygroscopic property, water content was also monitored using Karl Fischer titration (KF). At regular intervals, one vial was selected in a stratified random manner, and the water content was analyzed in singlicate.

**Results and Discussion**

**Preparation of materials**

Specifically purified-grade materials were offered as individual candidate CRMs from Ajinomoto Co., Inc. or Wako Pure Chemical Industries, Ltd., because the purity was sufficient from the viewpoint of impurities content. The numbers of the CRMs and manufacturing processes are listed in Table 1. A small amount (0.5 g) of each individual product was bottled in a glass tube in an aluminum bag in a glove box under argon gas before bottling.

**Measurement scheme for certification**

The measurement scheme for certifying amino acid CRMs is illustrated in Fig. 1. We determined the purity of our candidate CRMs based on two titration methods: nonaqueous acidimetric titration and nitrogen determination by the Kjeldahl method. Purity assay by nonaqueous acidimetric titration is based on the fact that the amino acid behaves as a weak base in acetic acid and can be titrated with perchloric acid in acetic acid. The Kjeldahl method is a method for the quantitative determination of nitrogen in a chemical substance. Purity assay by this method also utilizes titration, after the amino acid is decomposed to ammonia by acid digestion. The ammonia is titrated with sulfuric acid. The normality of perchloric acid solution and sulfuric acid was determined with potassium hydrogen phthalate (NMIJ CRM 3001-a) or sodium carbonate (NMIJ CRM 3005-a), respectively, which ensured traceability to SI.

However, these two methods have limited selectivity, and impurities with similar structures may not be distinguished. Especially, amino acid-related impurities can interfere with the purity determination, as they are potentially co-produced by the manufacturing processes of the target amino acids, such as fermentation and chemical synthesis. Therefore, the total mass fraction of these impurities could cause a purity increase with the titration method. Thus, we quantified the amino acid-related impurities and subtracted them to give the assigned value of the mass fraction for the main component. The calibration standards for determining amino acid-related impurities were the NMIJ CRM or the amino acid products whose purity was evaluated by NMIJ. Hence, the analyses of amino acid related impurities are also traceable to SI.

For the user’s convenience, we have given two certified values: the purity of the amino acid without enantiomeric separation and the purity of the L-amino acid. The purity of the L-amino acid was given by subtracting the content of the D-amino acid from the purity of the amino acid without enantiomeric separation.

**Analysis of amino acid-related impurities in the candidate CRMs**

Basically, the amino acid-related impurities contained in each candidate CRM were identified by both the post-column method, such as the OPA method or the NIN method, and the LC/MS method. In the post-column method, we identified amino acid-related impurities by comparing the retention time of each impurity with that of 41 amino acid standards. On the other hand, in the LC/MS method, we identified amino acid-related impurities by obtaining simultaneously information of both the retention time and the molecular mass for amino acid-related impurities in the analysis.

Then, amino acid-related impurities in the candidate CRM were determined using several HPLC methods. Table 2 shows the list of HPLC methods used to determine the amino acid-related impurities in each candidate CRM. To determine the amino acid-related impurities, we have already compared several kinds of amino acid analyses and concluded that the post-column method showed the best performance.20 Therefore, the OPA method was basically used to determine the identified amino acid-related impurities and the unknown impurities. For sulfur-containing amino acids such as Met and Cys-Cys, the
other post-column method, the NIN method, was alternatively used because those amino acids or their amino acid-related impurities could react with N-acetyl-L-cysteine that was contained in the o-phthaldialdehyde solution in the OPA method.20 To quantify the identified amino acid-related impurities, each candidate CRM or the amino acid products whose purity was evaluated by NMIJ were used as the calibration standard. On the other hand, unknown impurities were calibrated with GABA. As reported by Kopec et al., the amino acid produced by chemical synthesis, fermentation, or protein hydrolysis is known to contain non-amino acid-like impurities, such as sugar, carboxylic acid, and antibiotics.22 However, we thought that we only had to evaluate amino acid-related impurities because they can possibly be measured by nonaqueous acidimetric titration or the Kjeldahl method. Conversely, the post-column method, which can react with primary and secondary amines, fits well with titration methods that target the amine of amino acids.

The amino acid-related impurities detected by the post-column method were mainly confirmed by the LC/MS method. In some candidate CRMs, amino acid-related impurities were quantified by both the OPA method and the LC/MS method, and results
were in good agreement each other. For Ile or Leu which contain each other as an amino acid impurity, we could not use the LC/MS method because structural isomers have almost the same retention time, and neither was introduced into the MS instrument.20 Thus, standard addition method with the AQC method was used alternatively to confirm the result of the OPA method. For Arg or Lys-HCl, which contains unknown impurities at a level of more than 0.1 g kg⁻¹, the LC/CAD method was used to confirm the result of the OPA method because any nonvolatile or semivolatile analyte with or without a chromophore could be detected by the LC/CAD method. In any case, the results obtained by several HPLC methods were in good agreement; thus, we believe that the results of our impurity analysis are reasonable.

The amino acid-related impurities containing more than 0.1 g kg⁻¹ are listed in Table 2. In 12 of the 17 candidate CRMs, part of the determined amino acid-related impurities were the same as those listed in the impurity profile reported by Kopeck and Holzgrabe.22 All of the candidate CRMs of Ile, Phe, Val, Ser, and Glu, which contained more than two amino acid-related impurities, were produced by fermentation (Table 1). Additionally, most of the amino acid-related impurities in these candidate CRMs were compounds with similar structures in which the number or position of the methyl group of the target amino acid was changed (Table 2). This may reflect that these compounds with similar structure are easily produced by the fermentation process.

Chiral chromatography for d-amino acid analysis

The d-amino acid in the candidate CRM was analyzed by using the method described in the Experimental section. The quantitative results of the d-amino acid in all candidate CRMs are shown in Table 3. When the d-amino acid peak was detected, the authentic sample, whose purity was evaluated by NMII, was used for quantification as the calibration standard. In contrast, when the d-amino acid peak was not detected, the detection limit was calculated using the authentic sample as the calibration standard, and the detection limit was then considered as the uncertainty associated with the d-amino acid quantification. Almost all of the d-amino acid in each candidate CRM was negligible, other than 7.130 g kg⁻¹ of d-Serine in Serine.

Other impurity analyses

The water content in each candidate CRM was analyzed using the Karl Fisher titration method as described in the Experimental section. The water content in the hygroscopic amino acids, such as Lys-HCl, Arg, and Pro, was approximately 0.52, 0.49, and 0.19 g kg⁻¹, respectively. These were comparatively larger than those in other candidate CRMs with less than 0.1 g kg⁻¹ (data not shown).

Other impurities such as anions, cations, and nonvolatile impurities in the candidate CRMs were also analyzed using ion chromatography and TG/DTA as described in the Experimental section. The total mass fraction of the other determined impurity was less than 0.1 g kg⁻¹. The chloride ion included in the candidate CRM of Lys-HCl was also determined by ion chromatography, and it was confirmed that the amount of chloride ion was equal to that of Lys (data not shown).

Purity determination by nonaqueous acidimetric titration

Amino acids behave as a weak base in acetic acid; therefore, nonaqueous acidimetric titration with perchloric acid/acetic acid was attempted. The normality of the perchloric acid solution was determined with potassium hydrogen phthalate (NMIJ 3001-a). In the cases of His and Lys-HCl, the two steps of broad titration curves were observed. Then, we adopted the back titration technique, in which His or Lys-HCl dissolved with perchloric acid/acetic acid was titrated with sodium acetate/acetic acid. The normality of the sodium acetate solution was determined with potassium hydrogen phthalate (NMIJ 3001-a). Additionally, Lys-HCl has hydrochloric acid as a counter ion; thus, Lys-HCl dissolved with perchloric acid/acetic acid was heated to reflux to remove the hydrochloric acid before titration.23 Uncertainty was calculated according to EURACHEM/CITAC Guide CG424 and Guide to the Expression of Uncertainty in Measurement (GUM:2008).25 The uncertainty budget of

### Table 3 Analytical results for calculating the property value of each CRM

<table>
<thead>
<tr>
<th>CRM No.</th>
<th>Compound name</th>
<th>Obtained purity of acidimetric titration (mass fractiona/g kg⁻¹)</th>
<th>Obtained purity of Kjeldahl method (mass fraction/g kg⁻¹)</th>
<th>Sum of amino acid-related impurities (mass fraction/g kg⁻¹)</th>
<th>d-amino acid content (mass fraction/g kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRM6011-a</td>
<td>l-Alanine</td>
<td>999.1 ± 0.97</td>
<td>999.4 ± 0.79</td>
<td>ND</td>
<td>&lt;0.02^c</td>
</tr>
<tr>
<td>CRM6012-a</td>
<td>L-Leucine</td>
<td>999.2 ± 1.08</td>
<td>999.6 ± 0.66</td>
<td>0.172 ± 0.01</td>
<td>&lt;0.04^c</td>
</tr>
<tr>
<td>CRM6013-a</td>
<td>L-Isoleucine</td>
<td>1000.1 ± 0.92</td>
<td>999.0 ± 0.88</td>
<td>2.723 ± 0.13</td>
<td>&lt;0.00^c</td>
</tr>
<tr>
<td>CRM6014-a</td>
<td>L-Phenylalanine</td>
<td>1000.0 ± 0.85</td>
<td>999.3 ± 0.88</td>
<td>0.343 ± 0.04</td>
<td>0.100 ± 0.01</td>
</tr>
<tr>
<td>CRM6015-a</td>
<td>L-Valine</td>
<td>999.0 ± 0.94</td>
<td>999.3 ± 0.75</td>
<td>1.213 ± 0.03</td>
<td>&lt;0.00^c</td>
</tr>
<tr>
<td>CRM6016-a</td>
<td>L-Proline</td>
<td>999.4 ± 0.92</td>
<td>999.1 ± 0.76</td>
<td>0.049 ± 0.01</td>
<td>0.070 ± 0.01</td>
</tr>
<tr>
<td>CRM6017-a</td>
<td>L-Arginine</td>
<td>998.3 ± 0.97</td>
<td>999.0 ± 1.11</td>
<td>0.184 ± 0.04</td>
<td>&lt;0.00^c</td>
</tr>
<tr>
<td>CRM6018-a</td>
<td>L-Lysine monohydrochloride</td>
<td>998.5 ± 0.74</td>
<td>998.2 ± 1.04</td>
<td>0.172 ± 0.08</td>
<td>0.350 ± 0.03</td>
</tr>
<tr>
<td>CRM6019-a</td>
<td>L-Tyrosine</td>
<td>999.7 ± 0.99</td>
<td>998.9 ± 0.99</td>
<td>0.344 ± 0.01</td>
<td>&lt;0.10^c</td>
</tr>
<tr>
<td>CRM6020-a</td>
<td>L-Threonine</td>
<td>999.9 ± 0.86</td>
<td>999.2 ± 1.14</td>
<td>0.204 ± 0.14</td>
<td>&lt;0.10^c</td>
</tr>
<tr>
<td>CRM6021-a</td>
<td>L-Serine</td>
<td>999.9 ± 0.82</td>
<td>999.0 ± 0.91</td>
<td>1.598 ± 0.05</td>
<td>7.130 ± 0.19</td>
</tr>
<tr>
<td>CRM6022-a</td>
<td>Glycine</td>
<td>999.8 ± 1.03</td>
<td>999.1 ± 1.35</td>
<td>0.103 ± 0.00</td>
<td>0.170 ± 0.01</td>
</tr>
<tr>
<td>CRM6023-a</td>
<td>L-Methionine</td>
<td>999.6 ± 1.03</td>
<td>999.3 ± 1.59</td>
<td>0.096 ± 0.07</td>
<td>&lt;0.00^c</td>
</tr>
<tr>
<td>CRM6024-a</td>
<td>L-Histidine</td>
<td>999.3 ± 1.19</td>
<td>998.6 ± 0.68</td>
<td>0.076 ± 0.03</td>
<td>&lt;0.00^c</td>
</tr>
<tr>
<td>CRM6025-a</td>
<td>L-Cystine</td>
<td>999.4 ± 1.48</td>
<td>997.8 ± 1.18</td>
<td>0.284 ± 0.15</td>
<td>&lt;0.20^c</td>
</tr>
<tr>
<td>CRM6026-a</td>
<td>L-Glutamic acid</td>
<td>999.9 ± 1.15</td>
<td>999.0 ± 1.20</td>
<td>1.025 ± 0.02</td>
<td>0.070 ± 0.02</td>
</tr>
<tr>
<td>CRM6027-a</td>
<td>L-Aspartic acid</td>
<td>999.9 ± 0.97</td>
<td>999.1 ± 1.11</td>
<td>0.152 ± 0.00</td>
<td>&lt;0.10^c</td>
</tr>
</tbody>
</table>

a. Values listed are the means and the standard uncertainties of their measurement before impurity subtraction. b. ND means that no amino acid-related impurities was detected. c. Values with inequality sign indicate detection limit (S/N = 3).
nonaqueous acidimetric titration for Ser is shown in Table S1 (Supporting Information) as an example. The tendency shown by each candidate CRM was similar in that the largest uncertainty component was associated with both the precision of the sample measurement and that of the normality measurement. To improve the measurement precision, we optimized the sample concentration in which a clear pH change around the flexion point can be observed. Additionally, to increase the solubility of the sample, formic acid was used before dissolving with acetic acid. The uncertainty associated with the determination of the end point of the titration curve was also estimated. The estimated value, 0.0002 kg kg⁻¹, was obtained experimentally by analyzing the endpoint of the titration curve manually and calculating the variation. Acetic acid, used as a solvent in nonaqueous acidimetric titration, is known to show the volume fluctuation dependent on the temperature change. The uncertainty associated with the temperature effect on the titrant volume was estimated by the temperature change during the measurement. From these values, the combined relative standard uncertainty associated with nonaqueous acidimetric titration was optimized to be approximately less than 2.00 g kg⁻¹ for each candidate CRM, as shown in the column of quantified values by acidimetric titration in Table 3.

Purity determination using the Kjeldahl method

Amino acids are nitrogen compounds; therefore, nitrogen determination utilizing the Kjeldahl method was attempted. In this method, amino acid is decomposed to ammonia by acid digestion and then titrated with sulfuric acid. The normality of sulfuric acid was determined with sodium carbonate (NMIJ CRM 3005-a).

The Kjeldahl method has the additional steps of decomposition and distillation before titration. These steps were thought to affect the recovery of the sample. For the decomposition step, we examined the effect on the quantitative value of the decomposition temperature, the decomposition time, and the kind of degradation promoter. The maximum value of the recovery was adopted as an indicator of the optimized condition. As the degradation promoter, a tablet of Kjeltabs CX, which contains potassium sulfate and copper sulfate, was basically used. For persistent amino acids such as Lys, Ser, and Cys-Cys, a tablet of Kjeltabs CT, which contains oxidized titanium, was used alternatively. To optimize the distillation step, we examined the recovery of ammonium sulfate which can be distilled without decomposition, and confirmed that it was approximately 100%.

Since the Kjeldahl method also utilizes titration, components that were similar to those of nonaqueous acidimetric titration were considered for the uncertainty calculation. The uncertainty budget of the Kjeldahl method for Ser is shown in Table S2 (Supporting Information) as an example. The tendency shown by the Kjeldahl method was similar in that the largest uncertainty component was associated with both the precision of the sample measurement and that of the normality measurement. The uncertainty associated with the effect from decomposition and distillation was estimated empirically to be 0.0005 kg kg⁻¹. From these values, the combined relative standard uncertainty associated with the Kjeldahl method was optimized to be approximately less than 2.00 g kg⁻¹ for each candidate CRM, as shown in the column in Table 3 of the quantified values using the Kjeldahl method.

Impurity contribution to the obtained combined purity

Table 3 shows the sum of the amino acid-related impurities for each candidate CRM. The sum of the amino acid-related impurities was more than 1.000 g kg⁻¹ in some candidate CRMs, such as Ile, Val, Ser, and Glu, and less than approximately 0.300 g kg⁻¹ in other candidate CRMs. The sum of the amino acid-related impurities in each candidate CRM was low. Since the detected amino acid-related impurities were considered to be measured together in both nonaqueous acidimetric titration and the Kjeldahl method, the contribution to the mass fraction of those impurities was needed for estimation and subtraction. The impurity contribution derived from each amino acid-related impurity was calculated from Eq. (5):

\[ C_\omega = \frac{C_\lambda \times MW_{\text{target}} \times N_A}{MW_A}, \]  

where \( C_\omega \) is the mass fraction after estimating the contribution of amino acid-related impurity A to the purity, \( C_\lambda \) is the mass fraction of amino acid-related impurity A obtained from HPLC analyses, \( MW_{\text{target}} \) represents the relative molecular mass of the target amino acid, \( MW_A \) represents the relative molecular mass of amino acid-related impurity A, and \( N_A \) means the number of the amino group that can be protonated per 1 molecule of amino acid-related impurity A or the nitrogen atomicity per 1 molecule of amino acid-related impurity A. For unknown impurities, \( N_A \) was speculated by combining the data of the molecular mass of the impurity, the structure of the target amino acid, and the “Nitrogen rule”, in which it is said that a nitrogen compound having an odd nominal mass indicates that an odd number of nitrogen atoms are present, and a compound having an even nominal mass indicates that an even number of nitrogen atoms are present in the molecular ion.²⁶

Table 3 also shows the obtained purity of the nonaqueous acidimetric titration or the Kjeldahl method. All of the obtained purities by both methods were in good agreement at more than 998.0 g kg⁻¹. Slightly lower values for Arg and Lys-HCl were thought to be due to a certain level of water included in them, as mentioned before.

Then, the two corrected purities were calculated based on the analytical results of two methods after subtracting the sum of \( C_\omega \) for amino acid-related impurities. The uncertainty associated with the corrected purity was calculated using the uncertainty associated with the analytical result by each method and by the impurity analysis. The corrected purities by both methods were in agreement each other within their standard uncertainty (data not shown). This indicates that no difference in the purity values was observed between the two methods when the conditions of both methods were properly optimized.

Homogeneity and stability studies

Homogeneity testing of each candidate CRM was performed with nonaqueous acidimetric titration. The uncertainty derived from the inhomogeneity of every candidate CRM is summarized in Table 4. Lys and Pro showed slightly larger uncertainty values derived from inhomogeneity than did the others. This may reflect the fact that both amino acids contained comparatively large amounts of water, and complete homogenization before bottling was difficult. Still, the uncertainty derived from the inhomogeneity of each candidate CRM ranged from 0.010 to 0.069% and was sufficiently smaller than the uncertainty associated with the value assignment. As such, the candidate CRM was adequately homogeneous for developing the CRM.

Stability monitoring after certification was conducted for some amino acid CRMs by using the method described in the
Experimental section. The water content of hygroscopic amino acids Lys-HCl, Arg, and Pro was monitored for three years, and there were no trends for all three candidate CRMs (data not shown). The sum of the amino acid-related impurities was quantified and their trend analyses were performed over time. From these values, the certified values of our CRMs without enantiomeric separation were calculated to be the same as those with enantiomeric separation. Moreover, the property values with enantiomeric separation were calculated to be the same as those for 1 CRM (Ile) was (0.997 \pm 0.002) kg kg\(^{-1}\), (k = 2); those for 5 CRMs (Val, Arg, Lys-HCl, Ser, and Glu) were (0.998 \pm 0.002) kg kg\(^{-1}\), (k = 2); and that for 1 CRM (Ile) was (0.997 \pm 0.002) kg kg\(^{-1}\), (k = 2); and that for 1 CRM (Cys-Cys) was (0.998 \pm 0.003) kg kg\(^{-1}\), (k = 2)).

Moreover, the property values with enantiomeric separation were obtained by subtracting the D-amino acid content from the property value without enantiomeric separation. The certified values of our CRMs with enantiomeric separation are also shown in Table 5. As shown in Table 3, the D-amino acid content in almost all candidate CRMs was very small. Therefore, except for Ser, the property values of all of our CRMs with enantiomeric separation were calculated to be the same as those without enantiomeric separation. The certified value for L-Ser

Certified values and their uncertainties

The property value of each CRM was calculated as the weighted mean of two corrected purity values. The property values of the mass fraction of the candidate CRM were calculated to be 997.0 to 999.5 g kg\(^{-1}\).

The contribution of impurities to each purity value can be treated as a common denominator. Therefore, the uncertainty associated with the value assignment (\(u_{\text{char}}\)) was expressed as the following equation, Eq. (6), using the uncertainty of the analytical method before subtracting the contribution of impurities (\(u_{\text{imp}}\)), half of the difference in the purity values of the two analytical methods (\(u_{\text{nom}}\)), and the uncertainty from the contribution of impurities (\(u_{\text{inhomo}}\)).

\[
u_{\text{char}}^2 = \sum_{i=1}^{n} w_i^2 u_i^2 + u_{\text{nom}}^2 + u_{\text{inhomo}}^2
\]  

Where \(u_{\text{inhomo}}\) is the uncertainty of the value assignment, \(u(x_i)\) represents the measurement uncertainty of analytical method \(i\), \(w_i\) represents the weight of method \(i\), \(u_{\text{method}}\) means half of the difference between the purity values of the two analytical methods, and \(u_{\text{nom}}\) represents the uncertainty from the contribution of the impurities.

The combined standard uncertainty of the property value (\(u_c\)) for each candidate CRM was estimated by combining the uncertainty associated with the value assignment (\(u_{\text{char}}\)) and the inhomogeneity of the material (\(u_{\text{inhomo}}\)). The calculation of \(u_c\) was carried out using Eq. (7):

\[
u_c^2 = u_{\text{char}}^2 + u_{\text{inhomo}}^2
\]  

The \(u_c\) and its uncertainty components for each candidate CRM are summarized in Table 4. The \(u_c\) of every candidate CRM except for Cys-Cys was less than 0.1%. The slightly larger \(u_c\) for Cys-Cys was due to a larger \(u_{\text{method}}\) than for other candidate CRMs.

From these values, the certified values of our CRMs without enantiomeric separation were determined as shown in Table 5. The certified values for 10 CRMs (Ala, Leu, Phe, Pro, Tyr, Thr, Gly, Met, His, and Asp) were (0.999 \pm 0.002) kg kg\(^{-1}\), (k = 2); those for 5 CRMs (Val, Arg, Lys-HCl, Ser, and Glu) were (0.998 \pm 0.002) kg kg\(^{-1}\), (k = 2); and that for 1 CRM (Ile) was (0.997 \pm 0.002) kg kg\(^{-1}\), (k = 2); and that for 1 CRM (Cys-Cys) was (0.998 \pm 0.003) kg kg\(^{-1}\), (k = 2)).

Moreover, the property values with enantiomeric separation were obtained by subtracting the D-amino acid content from the property value without enantiomeric separation. The certified values of our CRMs with enantiomeric separation are also shown in Table 5. As shown in Table 3, the D-amino acid content in almost all candidate CRMs was very small. Therefore, except for Ser, the property values of all of our CRMs with enantiomeric separation were calculated to be the same as those without enantiomeric separation. The certified value for L-Ser...
was calculated to be (0.991 ± 0.002) kg kg⁻¹, (k = 2).

Conclusions

This paper describes the process for developing of 17 proteinogenic amino acid CRMs. The purity of the amino acids was determined by two traceable methods—nonaqueous acidimetric titration and the Kjeldahl method—both of which are thought to show little oversight of impurities. Moreover, several HPLC methods were conducted to thoroughly analyze compounds with similar structures that will interfere with the purity determination using the two methods mentioned above. The final purity for the main component was calculated by subtracting the total content of the compounds with similar structures from the analytical results of both methods. As a result, we could develop high-purity certified reference materials for 17 proteinogenic amino acids, whose purities were more than 0.997 kg kg⁻¹ with less than 0.003 kg kg⁻¹ uncertainty. The uncertainties of these CRMs are sufficiently small to be used as measurement standards for the calibration of analytical instruments or reagents and will improve the reliability of amino acid analyses.


<table>
<thead>
<tr>
<th>CRM No.</th>
<th>Compound name</th>
<th>Purity of amino acid without enantiomeric separation (mass fraction, g kg⁻¹)</th>
<th>Purity of L-amino acid (mass fraction, g kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRM6011-a</td>
<td>L-Alanine</td>
<td>999.4 ± 0.79</td>
<td>999.4 ± 0.79</td>
</tr>
<tr>
<td>CRM6012-a</td>
<td>L-Leucine</td>
<td>999.6 ± 0.66</td>
<td>999.6 ± 0.66</td>
</tr>
<tr>
<td>CRM6013-a</td>
<td>L-Isoleucine</td>
<td>999.9 ± 0.88</td>
<td>999.9 ± 0.88</td>
</tr>
<tr>
<td>CRM6014-a</td>
<td>L-Phenylalanine</td>
<td>999.3 ± 0.88</td>
<td>999.3 ± 0.88</td>
</tr>
<tr>
<td>CRM6015-a</td>
<td>L-Valine</td>
<td>999.3 ± 0.75</td>
<td>999.3 ± 0.75</td>
</tr>
<tr>
<td>CRM6016-a</td>
<td>L-Proline</td>
<td>999.1 ± 0.76</td>
<td>999.1 ± 0.76</td>
</tr>
<tr>
<td>CRM6017-a</td>
<td>L-Arginine</td>
<td>999.0 ± 1.11</td>
<td>999.0 ± 1.11</td>
</tr>
<tr>
<td>CRM6018-a</td>
<td>L-Lysine monohydrochloride</td>
<td>998.2 ± 1.04</td>
<td>998.2 ± 1.04</td>
</tr>
<tr>
<td>CRM6019-a</td>
<td>L-Tyrosine</td>
<td>998.9 ± 0.99</td>
<td>998.9 ± 0.99</td>
</tr>
<tr>
<td>CRM6020-a</td>
<td>L-Threonine</td>
<td>999.2 ± 1.14</td>
<td>999.2 ± 1.14</td>
</tr>
<tr>
<td>CRM6021-a</td>
<td>L-Serine</td>
<td>999.0 ± 0.91</td>
<td>999.0 ± 0.91</td>
</tr>
<tr>
<td>CRM6022-a</td>
<td>Glycine</td>
<td>999.1 ± 1.35</td>
<td>999.1 ± 1.35</td>
</tr>
<tr>
<td>CRM6023-a</td>
<td>L-Methionine</td>
<td>999.3 ± 1.59</td>
<td>999.3 ± 1.59</td>
</tr>
<tr>
<td>CRM6024-a</td>
<td>L-Histidine</td>
<td>998.6 ± 0.68</td>
<td>998.6 ± 0.68</td>
</tr>
<tr>
<td>CRM6025-a</td>
<td>L-Cystine</td>
<td>997.8 ± 1.18</td>
<td>997.8 ± 1.18</td>
</tr>
<tr>
<td>CRM6026-a</td>
<td>L-Glutamic acid</td>
<td>999.0 ± 1.20</td>
<td>999.0 ± 1.20</td>
</tr>
<tr>
<td>CRM6027-a</td>
<td>L-Aspartic acid</td>
<td>999.1 ± 1.11</td>
<td>999.1 ± 1.11</td>
</tr>
</tbody>
</table>

a. Values listed are the assigned values and their expanded uncertainties (k = 2).

Acknowledgements

The authors acknowledge their collaborators in the preparation of the candidate materials.

Supporting Information

The uncertainty budget of purity determination of L-Serine (CRM 6021-a) by nonaqueous acidimetric titration and that by the Kjeldahl method are shown in Table S1 and S2, respectively. The result of stability monitoring of the sum of the amino acid-related impurities in L-Proline (CRM 6016-a) is shown in Fig. S1. These materials are available free of charge on the Web at http://www.jsac.or.jp/analsci/.

References

