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Application of amino acid analysis using hydrophilic interaction liquid chromatography coupled with isotope dilution mass spectrometry for peptide and protein quantification

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ABSTRACT

Amino acid analysis that is based on the use of hydrophilic interaction liquid chromatography (HILIC) coupled with isotope dilution mass spectrometry (IDMS) has been developed for the accurate quantification of underivatized amino acids from hydrolyzed protein/peptide. Sufficient separation of amino acids on a zwitterion chromatography (ZIC)-HILIC column was achieved after removal of chloride ions in the hydrolyzate. The detection limits and quantification limits as concentration of the four amino acids ranged from 0.003 to 0.04 pmol μL^{-1} and from 0.01 to 0.1 pmol μL^{-1}, respectively. The analytical results for the certified reference materials, angiotensin I and bovine serum albumin (BSA), were satisfactory. Furthermore, the quantitative results by this method were compared with those by the commercially available precolumn method, derivatizd with aminoquinolylhydroxysuccinimidyl carbamate (AQC method), and better recovery and more precise data were obtained with this method.

1. Introduction

Proteomics is the analysis of the protein/peptide complement present in a cell, organ, or organism at any given time. The broad application of proteomics technology has the potential to develop both the understanding of the molecular mechanism of the disease and the discovery of new drug targets and diagnostic disease markers [1]. While most of the initial efforts in proteomics have focused on protein identification, quantitative analysis of global protein levels, termed "quantitative proteomics," is in progress and expected to be applied in the field of therapeutic intervention or prognosis and dope testing [2–5]. To achieve an accurate measurement in this approach, a reference method and/or reference material that validates the sample-handling and analytical process is necessary. Moreover, the known content of protein/peptide will enable extensive use for 'calibrating' other quantification methods such as proteomics studies, absorbance at 280 nm (A280), fluorescence, and Bradford assays.

Amino acid analysis is one of the most classical methods, and the quantification of highly purified protein/peptide has recently been proposed using this method [5,6]. In this method, the constituent amino acids released after hydrolysis from the original protein/peptide are quantified. The concentration of protein/peptide is then calculated from the concentration determined for each of the amino acids. This method allows quantification that does not depend on the kind of protein/peptide.

Under standard hydrolysis conditions, not every amino acid is completely recovered because of the effect of the protein's steric hindrance and/or the instability of the amino acid itself. Moreover, the most general amino acid analysis, which utilizes derivatization agents, such as ninhydrin [7,8] o-phthalaldehyde (OPA) [9], phenylisothiocyanate (PTC) [10,11] and aminoquinolylhydroxysuccinimidyl carbamate (AQC) [12,13] presents some disadvantages, such as derivative instability, reagent interferences [14], and few kinds of internal standards. In contrast, isotope dilution mass spectrometric amino acid analysis using isotopically labeled amino acids as internal standards is thought to have the smallest achievable uncertainties for analytical measurement [15]. Isotope-dilution mass spectrometry (IDMS) is based on the principle that stable internal standards behave in the same manner as the corresponding target (natural) compounds throughout the hydrolysis and analytical processes [16]. Thus, the recovery problems throughout these processes can be cancelled out by the use of isotope-labeled internal standards. In previous several reports about isotope dilution mass spectrometric amino acid analysis, mobile phase modifier(s) were frequently used in combination with reverse-phase chromatography [6,17,18] to improve retention and separation by the adducting hydrophilic side chain on each amino acid. However, it is conceiv-

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able that the MS sensitivity of amino acids receives interference because of the easy-ionized property of mobile phase modifier(s) itself.

In order to improve its qualitative and quantitative performances, we adopted another separation mode, the hydrophilic interaction liquid chromatography (HILIC) separation mode, which can retain polar compounds such as amino acids without any mobile phase modifier(s). Moreover, HILIC coupled with MS was expected to have the advantage of increasing MS sensitivity because of the high content of organic solvent in the mobile phase. In the present study, we established amino acid analysis using HILIC coupled with IDMS and evaluated its performances for protein/peptide quantification.

Angiotensin I and bovine serum albumin (BSA) were selected as a target protein/peptide because they are well characterized and easy molecules to handle. Both certified reference materials (National Institute of Standard and Technology, Standard Reference Material (NIST SRM) 998 for angiotensin I and NIST SRM 927d for BSA) enabled us to validate our method on the basis of its reference and certified values.

2. Experimental

2.1. Materials

2.1.1. Samples

Angiotensin I (Human) (sequence: DRVYIHPFHL, relative molecular mass (Mr): 1296.5) used was NIST SRM 998. The solution was prepared gravimetrically from lyophilized material that was dissolved in 0.1 mol L^{-1} hydrochloric acid. The amount of substance content of the angiotensin I sample solution was prepared to be approximately 50 nmol g^{-1}.

The BSA (sequence: Entrez Protein Accession Number P02769, relative molecular mass (Mr): 66432.3) used was NIST SRM 927d. The protein solution of lower concentration was gravimetrically prepared with 0.15 mol L^{-1} sodium chloride. The amount of substance content of the BSA sample solution was prepared to be approximately 15 nmol g^{-1}.

2.1.2. Reagents

The natural amino acid mixture used as the standard for angiotensin I quantification was NIST SRM 2389, which is a solution of 17 amino acids in 0.1 mol L^{-1} hydrochloric acid. The diluted solution of the mixture was gravimetrically prepared to be approximately 50 nmol g^{-1} with 0.1 mol L^{-1} hydrochloric acid, and the exact amount of substance content of each amino acid was calculated on the basis of the certified values. The natural amino acids L-alanine (Ala), L-leucine (Leu), L-phenylalanine (Phe), L-proline (Pro) and L-valine (Val), used as a standard for BSA quantification, were purchased from Fluka. The purities of the amino acids were evaluated as >99% by titration and liquid chromatography (LC) analysis for contamination with other amino acids. Each natural amino acid solution was gravimetrically prepared with 0.1 mol L^{-1} hydrochloric acid, and the natural amino acid mixture was prepared gravimetrically with the ratio of each component being almost the same molar ratio as that of the BSA. The amount of substance content of each amino acid in the mixture ranged from approximately 50–1250 nmol g^{-1}.

The following labeled amino acids used as internal standards for the IDMS method, Ala (U–$^{13}C_3$, 98%, ^{15}N, 98%), L-isoleucine (Ile) (U–$^{13}C_6$, 98%, ^{15}N, 98%), Leu (U–$^{13}C_6$, 98%, ^{15}N, 98%), Phe (U–$^{13}C_5$, 98%, ^{15}N, 98%), Pro (U–$^{13}C_5$, 98%, ^{15}N, 98%), and Val (U–$^{13}C_9$, 98%, ^{15}N, 98%), were purchased from Taiyo Nippon Sanso (Tokyo, Japan). Each of the labeled amino acids was dissolved in 0.1 mol L^{-1} hydrochloric acid, and the mixed labeled amino acid solution for angiotensin I quantification or BSA quantification was prepared with the ratio of each component at almost the same molar ratio as that of angiotensin I or BSA, respectively.

The α-aminobutyric acid and norleucine used as internal standards for the AQC method were purchased from Wako Pure Chemicals (Osaka, Japan). Each of the internal standards was dissolved in 0.1 mol L^{-1} hydrochloric acid, and the resulting solutions were combined with the amount of substance content of each component to be approximately 500 nmol g^{-1}.

Acetic acid (LC–MS grade) and hydrochloric acid (Amino Acid Auto analyzer-grade) were purchased from Wako Pure Chemicals (Osaka, Japan). Acetonitrile (LC–MS grade and high performance liquid chromatography (HPLC) grade) was purchased from Kanto Chemical (Tokyo, Japan). Ultrapure water prepared by the use of a Milli-Q water purification system (resistivity 18 MΩ cm^{-1}, Nihon Millipore Kogyo, Tokyo, Japan) was used throughout the experiments.

2.2. Preparation of calibration and sample blends

A solution of a natural amino acid mixture was used to make the calibration blend. The calibration blend was prepared gravimetrically by mixing natural and labeled amino acid mixtures. Solutions of angiotensin I and BSA were used to make a sample blend. The sample blend, which contained angiotensin I or BSA, was prepared by mixing with the labeled amino acid mixture. The final concentration of each natural and labeled amino acid in the calibration and sample blends was adjusted to be almost the same.

2.3. Hydrolysis

Aliquots of 25 μL of the blends were placed into glass sample tubes (10 mm × 75 mm Pyrex) and then completely dried by the use of the Pico Tag Work station (Waters, Milford, MA, USA) prior to hydrolysis. A set of a calibration and angiotensin I sample blend was hydrolyzed in a hydrolysis chamber (Waters, Milford, MA, USA) with gaseous 6 mol L^{-1} HCl at 130 °C for 48 h in the absence of oxygen. Another set of a calibration and BSA sample blend was hydrolyzed at 145 °C for 6 h. After the hydrolysis was completed, individual samples were evacuated to dryness and then resolved with 20 mM HCl.

2.4. Pretreatment

The hydrolyzate solution was pretreated with anion-exchange diethylaminoethyl cellulose (DEAE) resin, DE52 (Whatman, KENT, UK) to remove the chloride ions. The mixture of hydrolyzate and resin was stirred for several minutes. The supernatant was separated by centrifugation, filtrated with hydrophilic PVDF membrane unit (Nihon Millipore Kogyo, Tokyo, Japan), and then subjected to the LC–MS analysis.

2.5. Separation and detection

The HPLC system consisted of two LC-10AD_{VP} pumps, a SIL-HTc autosampler, and a CTO-10AC_{VP} column heater (Shimadzu, Kyoto, Japan). Shimadzu LC–MS solution software (ver.3) was used for data acquisition and analysis. Mobile phase A was 10 mM acetic acid, and mobile phase B was acetonitrile (LC-MS grade). Hydrolyzed samples were injected onto a zwitterion chromatography (ZIC)-HILIC column, 5 μm × 2.1 mm × 250 mm (Merck Sequant, Sweden), using an injection volume of 2 μL and eluted at a flow rate of 0.1 mL min^{-1} at 30 °C. The gradient conditions were: initial = 25% A, 75% B, 25 min = 90% A, 10% B, 55 min = 25% A, 75% B, (all segments linear) followed by reequilibration for 33 min at 25% A, 75% B.

Mass spectrometry measurements were made on an LC–MS 2010-EV (Shimadzu, Kyoto, Japan) in the selective ion monitoring (SIM) mode. The ions selected for each amino acid were as follows: m/z 90.0 for Ala, m/z 94.0 for Ala-$^{13}C_3$, ^{15}N, m/z 132.0 for Ile and Leu, m/z 139.0 for Ile-$^{13}C_6$, ^{15}N and Leu-$^{13}C_6$, ^{15}N, m/z 166.0 for Phe, m/z 176.0 for Phe-$^{13}C_9$, ^{15}N, m/z 116.0 for Pro, m/z 122.0 for Pro-$^{13}C_5$, ^{15}N, m/z 118.0 for Val, and m/z 124.0 for Val-$^{13}C_5$, ^{15}N.

2.6. Calibration

The amount of substance content of each amino acid was calculated by inserting each value into Eq. (1) below, where one-point calibration was applied because the linearity was good enough in the range of the solutions tested prior to the sample analyses.

$$Cx = Cs \times \frac{m_z}{m_{yc}} \times \frac{m_y}{m_x} \times \frac{R'_B}{R'_{BC}} \quad (1)$$

Cx is the amount of substance content of the amino acid in the sample, Cs is the amount of substance content of the amino acid in standard solution, m_z is the mass of the amino acid standard solution added to the calibration blend, m_x is the mass of the sample solution added to the sample blend, m_{yc} is the mass of the isotopically labeled standard solution added to the calibration blend, m_y is the mass of the isotopically labeled standard solution added to the sample blend, R'_B is the measured ratio (peak area of the natural amino acid/peak area of the isotopically labeled amino acid) of the sample blend, and R'_{BC} is the average measured ratio (peak area of the natural amino acid/peak area of the isotopically labeled amino acid) of the calibration blend injected before and after the sample.

Upon calculating the amount of substance content of angiotensin I, the amount of substance content of each amino acid was adopted as it was because there is one molecule of each amino acid per angiotensin I molecule.

The mass fraction of BSA was calculated by inserting each value into Eq. (2) below.

$$C\omega = Cx \times \frac{M_{rBSA}}{M_{A.A}} \quad (2)$$

$C\omega$ is the mass fraction of BSA, Cx is the amount of substance content of each amino acid, M_{rBSA} is the relative molecular mass of BSA, and $M_{A.A}$ is the number of molecules contained per BSA molecule.

The uncertainties of the property values were calculated according to the Guide to the expression of uncertainty in measurement (GUM). [19]

2.7. Amino acid analysis with the AQC method

The derivatization was performed with a commercially available derivatization kit. The hydrolyzate in 20 μL of 20 mM HCl was mixed with 20 μL of an AccQ Fluor reagent (Waters, Milford, MA, USA) after mixing with 60 μL of an AccQ Fluor Borate Buffer (0.2 M boric acid with 5 mM calcium disodium ethylenediaminetetracetic acid (EDTA), pH was adjusted to 8.8 with sodium hydroxide) (Waters, Milford, MA, USA). The derivatization reactions were carried out at 55 °C for 10 min.

The HPLC system consisted of a 2695 separation module and 2475λ fluorescent detector (Waters, Milford, MA, USA). Waters Empower software was used for data acquisition and analysis. Mobile phase A (140 mM sodium acetate, 17 mM triethylamine (TEA), pH 5.05, containing 1 mM calcium disodium EDTA) was purchased from Waters (Milford, MA, USA). Mobile phase B was acetonitrile (HPLC-grade), and Mobile phase C was ultrapure water. Hydrolyzed samples were injected onto an AccQ-Tag reverse phase column, 4 μm × 3.9 mm × 150 mm (Waters, Milford, MA, USA), using an injection volume of 10 μL and eluted at a flow rate of 1.0 mL min^{-1} at 35 °C. The gradient conditions were: initial = 100% A, 0.5 min = 99% A, 1% B, 18 min = 95% A, 5% B, 19 min = 91% A, 9% B, 29.5 min = 83% A, 17% B, 33 min = 60% B, 40% C, 41 min = 100% A (all segments linear) followed by reequilibration for 9 min at 100% A. The amino acid concentrations were calculated with the calibration curve method.

3. Results and discussion

3.1. Separation conditions

In this study, we selected ZIC-HILIC column chromatography, in which the Si stationary phase has highly polar zwitterionic functional groups of the sulfobetaine type covalently attached. Therefore, this column enables the separation of the compounds with both hydrophilic and electrostatic interaction while maintaining low-eluent ion strength.

Fig. 1 shows the selected ion chromatograms of 12 amino acids dissolved in ultrapure water, which were isocratically eluted in 25:75, 10 mM acetic acid:acetonitrile at a flow rate of 0.1 mL min^{-1}. Each amino acid represents a single peak with a regular shape. The concentration of acetonitrile was optimized on the basis of the separation between isomeric compounds, Ile and Leu, and consequently, those were nicely separated. This high resolution appears to be involved with the property of the ZIC-HILIC column, which discriminates between compounds with both hydrophilic and electrostatic interaction.

3.2. Pretreatment of a hydrolyzed sample solution using an anion exchanger

Same amino acids dissolved in 0.1 mol L^{-1} hydrochloric acid showed poor chromatographic retention, and the chromatograms of several amino acids were split into several peaks (data not shown). Therefore, it seemed difficult to inject the hydrolyzed peptide sample directly onto the ZIC-HILIC column because it contained residual hydrochloric acid from the hydrolyzate.

We first attributed the poor retention to the reduced polarity of the amino acid due to the protonation of its carboxyl group in strong acid, such as hydrochloric acid. Therefore, we added ammonia water to the solution of amino acids in 0.1 mol L^{-1} hydrochloric acid and made the solution pH-neutral. However, the retention of

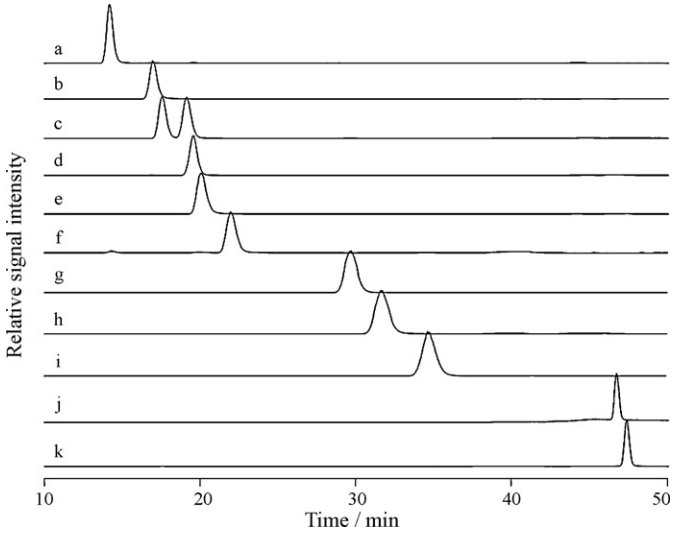

Fig. 1. Selected ion chromatograms of 10 pmol μL^{-1} each of 12 amino acids. Peak description: a, Phe; b, Met; c, (left) Leu, (right) Ile; d, Tyr; e, Pro; f, Val: g, Ala; h, Ser; i, Gly; j, His; k, Arg.

the neutralized amino acid solution was not improved (data not shown).

We then thought that the chloride ion of hydrochloric acid might diminish the retention of the amino acid by breaking the water-enriched liquid layer on the ZIC-HILIC stationary phase, binding with the positive charge of the zwitterionic functional side chain. A similar drastic change of the retention time was observed when increasing the trifluoroacetic acid (TFA) percentage in the mobile phase [20], which suggested that strong negative charges might interfere with the HILIC mechanism.

Therefore, in order to remove the chloride ion from the sample solution, we pretreated the sample using an anion exchanger. With the sample pretreated with weak anion-exchanger DE52 resin, each amino acid was nicely separated with regular single peaks (data not shown). The pH of this sample solution was neutral, and the concentration of the chloride ion was below the level of 1 mmol L^{-1} (data not shown). These results indicated that the DE52 resin could effectively remove only the chloride ion from the solution of amino acid and hydrochloric acid. As the DE52 resin is DEAE sepharose, which is only slightly charged and in a neutral condition, zwitter amino acids might not bind to this resin secondarily. The double resin of the exchange capacity was quantitatively sufficient to remove the chloride ion from the sample solution (data not shown). The retention time of each amino acid after pretreatment with the DE52 resin showed good repeatability.

3.3. Amino acid analysis coupled with stable isotope dilution mass spectrometry

Four amino acids were chosen for measurement in hydrolyzed peptide for the following reasons: (1) all four amino acids are present in the targeted peptide, angiotensin I. (2) Phe, Val and Pro are comparatively stable and easy to hydrolyze. (3) Separation of Ile from its isomer Leu is a good indicator to assess the chromatographic resolution of our method.

The detection limits (S/N = 3) and quantification limits (S/N = 10) as concentration for the four amino acids by our HPLC-electrospray ionization (ESI)-MS method were 0.008 and 0.03 pmol μL^{-1} for Ile, 0.003 and 0.01 pmol μL^{-1} for Phe, 0.006 and 0.02 pmol μL^{-1} for Pro, and 0.04 and 0.1 pmol μL^{-1} for Val. The reason why the sensitivity of Val was 15 times lower than that of Phe is attributed the high background signal noise of the ion m/z 118.0. The overall sensitivity was almost 3–7 fold higher than that of the HPLC–ESI–MS/MS method on the basis of the use of the volatile ion-pair reagent in Ref. [17].

In IDMS, isotope-labeled internal standards behave in a similar manner to the corresponding (natural) target compounds throughout the analytical process. As we expected, both the isotope-labeled amino acid and its corresponding (natural) target amino acid showed almost the same chromatogram and retention time in our separation condition (Fig. 2). Moreover, the relationship between the mass ratio and peak ratio in the calibration solution showed good linearity and repeatability of less than 1% in relative standard deviation (data not shown).

3.4. Amino acid analysis of the angiotensin I sample solution

The angiotensin I sample blends and calibration blends were hydrolyzed in 6 mol L^{-1} gaseous phase hydrochloric acid at 130 °C for 48 h in the absence of oxygen. The hydrolysis time was determined on the basis of the time course experiment reported by Burkitt [6]. Two samples were hydrolyzed on separated days, and each hydrolyzate was analyzed two or three times (within day, $n = 2$ or 3). The amount of substance content of each amino acid in the angiotensin I sample solution and its precision and corresponding uncertainties are listed in Table 1. The precision of both "within day" and "total" was satisfactory (0.20–0.88%). The analytical result of each amino acid, except for Ile, ranged from 49.1 to 50.1 nmol g^{-1} and was in good agreement with the expected value, 50.2 nmol g^{-1}, based on the reference value in the certificate. The low value of Ile might be attributed to the fact that some peptide bonds, such as Ile–Ile and Val–Val, are incompletely digested [21], which suggests that some amino acids suitable for quantification should be selected.

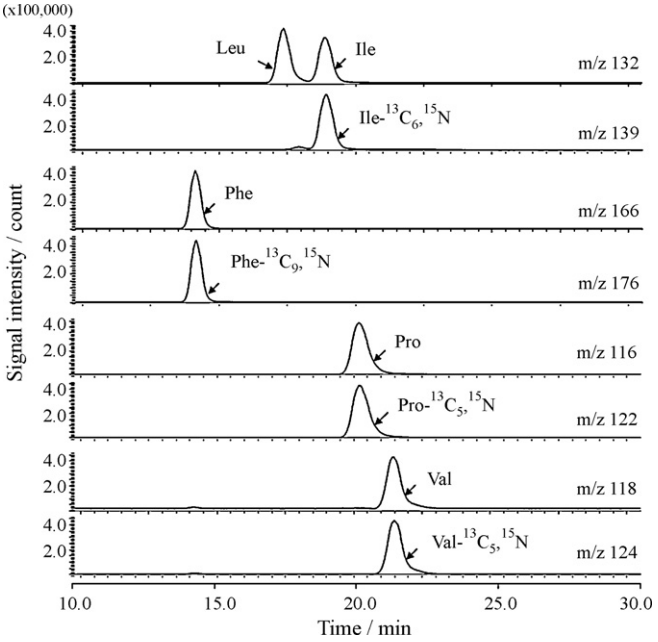

Fig. 2. Selected ion chromatograms of 25 pmol μL^{-1} each of the natural and isotope-labeled amino acids of interest.

Uncertainty was calculated according to the ISO Guide [19]. The sources of uncertainty associated with the analysis of each amino acid and their relative contributions are shown in Fig. 3. The tendency shown by every amino acid is similar in that the largest uncertainty component is associated with the certified concentration of the amino acid calibration solution (52–58%) followed by that between the hydrolysis (14–23%) and the measurement of calibration standards (8–18%). The uncertainties associated with the measurement precision and the accuracy of the balance appear to be negligible in comparison to those of others described.

Table 1
Analytical results of angiotensin I solution calculated from each amino acid determination.

		Concentration (nmol g^{-1})			
		Ile	Phe	Pro	Val
Dayl	Hydrolysis l	47.43	49.54	49.55	49.98
		47.04	49.40	49.74	50.17
Day2	Hydrolysis 2	46.69	49.18	50.31	49.57
		46.35	48.73	50.33	49.61
		46.83	48.53	50.49	49.81
Average (total)		46.9	49.1	50.1	49.8
CV(%)	Within Dayl	0.58	0.20	0.27	0.27
	Within Day2	0.53	0.68	0.20	0.26
	Total	0.86	0.88	0.82	0.51
Expanded uncert. ($k = 2$)		1.5	1.8	2.0	1.8

Table 2
A comparison of the accuracy of the IDMS and AQC methods.

	IDMS method			AQC method (AABA)[a]			AQC method(Nle)[a]		
	Mean recovery[b] (%)	Precision[c] CV (%)		Mean recovery[b] (%)	Precision[c] CV (%)		Mean recovery[b] (%)	Precision[c] CV (%)	
		Within	Between		Within	Between		Within	Between
Ile	94.6	0.04	0.86	95.2	0.37	2.26	96.0	0.03	2.18
Phe	98.2	0.17	0.57	95.2	0.39	0.15	96.2	0.05	3.93
Pro	100.8	0.33	0.64	99.2	0.52	1.59	99.9	0.86	5.79
Val	100.6	0.33	0.66	98.5	0.37	1.03	99.1	0.15	3.22

[a] α-Aminobutyric acid (AABA) and norleucine (Nle) were used as internal standards for AQC method, respectively.
[b] The recovery means the ratio of analytical results to the reference value.
[c] The precision within hydrolysis (within) and between hydrolysis (between) was calculated on the basis of three consecutive analyses for each of the three hydrolyzates.

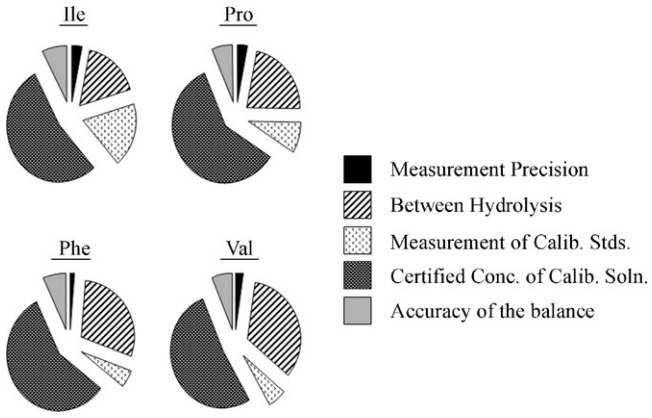

Fig. 3. Uncertainty budget for the analytical results of the angiotensin I solution calculated by each amino acid.

3.5. Accuracy of the method

To examine the accuracy of this IDMS method, the same peptide sample solution was also assayed by the AQC method using the commercially available precolumn derivatization system with α-aminobutyric acid or norleucine as the internal standards. Aliquots of sample blends or samples spiked with internal standards were hydrolyzed under the same conditions on the same day and subjected to either analytical method. Three samples for the IDMS method and two samples for the AQC method were hydrolyzed, and each hydrolyzate was analyzed three times. The quantitative data obtained by the IDMS method and the AQC method are shown in Table 2. The recovery, i.e., the ratio of analytical results to the reference value, by IDMS ranged from 94.6% to 100.8% and was higher than that of the AQC method except for Ile. Furthermore, the precision (CV%) obtained for "between hydrolysis" with the IDMS method was significantly less than that obtained with the AQC method. This suggested that better recovery and more precise data were obtained with the IDMS method with the use of stable isotopic internal standards, which behave in a similar manner chemically and physically, throughout the hydrolysis and analytical processes. Thus, developed IDMS method is superior to standard AQC method in terms of accuracy and sensitivity, although it takes about 2 times speed of analysis and price per analysis [22].

3.6. Amino acid analysis of the BSA sample solution

The diluted solution of the BSA sample solution (NIST SRM 927d) was quantified by this IDMS method. The protein hydrolysis was undertaken in 6 mol L^{-1} gaseous phase hydrochloric acid at 145 °C for 6 h. Four samples were hydrolyzed on three separate days, and each hydrolyzate was analyzed twice (within day, $n = 2$ or 4). Data were collected for five kinds of amino acid, which included Ala and Leu in addition to the same three amino acids used for angiotensin I quantification except for Ile. Based upon the known amino acid sequence for BSA, the mass fraction of BSA was calculated from the amount of substance content determined for each amino acid. The mass fractions of the BSA sample solution calculated by each amino acid, their precision, and corresponding uncertainties are listed in Table 3. The "within day" and "total" precision was satisfactory (0.04–0.99%). The analytical results between amino acids ranged from 63.67 to 65.95 g kg^{-1} and were in good agreement with each other (CV = 1.32%). The averaged value calculated for the mass fraction of BSA was 65.03 ± 2.6 g kg^{-1} (2.0%), with $k = 2$, and was in

Table 3
Analytical results of BSA solution calculated from each amino acid determination.

		Concentration (g kg^{-1})				
		Ala	Leu	Phe	Pro	Val
Dayl	Hydrolysis 1	63.72	65.37	66.03	64.70	64.30
		63.95	66.05	66.34	65.62	65.81
	Hydrolysis 2	63.63	65.03	65.22	64.69	64.67
		62.92	65.41	65.95	64.99	64.88
Day2	Hydrolysis 3	63.01	65.52	65.87	65.35	64.99
		62.57	64.93	65.50	65.31	64.42
Day3	Hydrolysis 4	65.02	65.57	66.38	65.55	65.07
		64.57	65.87	66.29	65.26	64.81
Average (total)		63.67	65.47	65.95	65.19	64.87
CV (%)	Within Day1	0.69	0.65	0.72	0.67	0.99
	Within Day2	0.50	0.64	0.40	0.04	0.62
	Within Day3	0.48	0.32	0.10	0.32	0.29
	Total	0.84	0.38	0.41	0.36	0.46
Expanded Uncert. ($k = 2$)		2.2	0.9	1.4	3.6	3.2

good agreement with the certified value, $64.27 \pm 0.81\ g\ kg^{-1}$, shown on the certificate. This result suggests that reliable protein/peptide quantification can be achieved by using IDMS and by selecting some stable amino acids in various hydrolysis conditions.

4. Conclusions

In this work, we established sensitive and reliable HPLC–ESI-MS coupled with IDMS for the amino acids of angiotensin I and BSA quantification. The analytical results of certified reference materials by this IDMS method were in good agreement with the reference value. Moreover, by comparing with the AQC method, which is one of the most widely used methods for amino acid analysis, this IDMS method demonstrated better performance than the latter one in terms of recovery and precision. In the future, we will apply this method to the development of reference materials for newly targeted protein/peptide.

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