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RESEARCH NOTE

Method Validation of 16 Types of Structural Amino Acids using an Automated Amino Acid Analyzer

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Abstract An analytical method for the simultaneous determination of 16 types of structural amino acids in infant formula using an automated amino acid analyzer was validated through tests of precision, accuracy and linearity. The automated analysis of the amino acids was performed on an ion-exchange packed column with a visible detector. The certificated reference material (CRM) 1546 from NIST was used as the test sample to determine the precision and accuracy of the analytical method. The regression analyses revealed good correlations [correlation coefficient (r^2)] that were greater than 0.99. The recovery values of the amino acids were ranged from 87.18 to 118.08%. The limits of detection and limits of quantification were less than 0.059 mg/100 g and less than 0.198 mg/100 g, respectively. The intra- and inter-day precisions were less than 14.62% in the CRM sample.

Keywords: amino acids, automated amino acid analyzer, analytical method, validation, post column reaction

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Introduction

Amino acids are biologically important organic compounds composed of amine ($-NH_2$) and carboxylic acid (-COOH) functional groups and a side-chain that is specific to each amino acid. The key elements of an amino acid are carbon, hydrogen, oxygen, and nitrogen. There are approximately 500 known amino acids (1). Amino acids are one of the most important types of natural compounds because they participate in many essential and well-known physiological processes. One of these processes is the construction of peptides and proteins, in which 20 alpha-amino acids participate (2).

Amino acids are investigated in numerous research fields. Within these research fields, there are two primary fields that analyze amino acids. One of these fields is proteomics, and the other field is protein engineering. Proteomics is the large-scale study of proteins, particularly their structures and functions. The goal of proteomics is to provide a comprehensive, quantitative description of protein expression and its changes under the influence of biological

perturbations such as disease or drug treatment (3). Protein engineering is generally understood to mean the use of site-directed or random mutagenesis to alter the properties of a protein or enzyme, which often involves modulating the substrate specificity or selectivity of an enzyme (4). These two research fields are an important component of molecular biology and are utilized to analyze structural amino acids.

The role of amino acids as the major structural and functional component of the body is also well-known. Many studies on the function of essential amino acids, non-essential amino acids, and various other peptides have been conducted. In addition, studies have been conducted on maintaining the balance between essential and non-essential amino acids, the composition of amino acids in relation to the protein nutritional quality of food groups, and the effect of protein and/or amino acid supplements on promoting of muscle strength and function (5). Consequently, the accurate analysis of amino acids is an important field of basic chemistry.

Numerous analytical methods for detecting structural amino acids have been investigated to date. The most common method for the identification and quantification of amino acids involves an HPLC system coupled to a diode-array detector (6) and a fluorescence detector (7) through pre-column derivatization. The use of an HPLC separation coupled with a mass spectrometer for detection has been reported in a series of articles (8-13). Other researchers have employed nuclear magnetic resonance (14-16) and gas chromatography coupled with mass spectroscopy to analyze amino acids (17-20).

Since the first report of ion-exchange separation followed by post-column derivatization with ninhydrin in 1958 (21), many automatic amino acid analyses have been developed and reported to this day (22-24). However, these articles did not report data with sufficient precision and accuracy using a certified reference material (CRM) in considerable amounts of time. The standard addition method for validating the precision and accuracy of the analysis of structural amino acids is only a stability test because the analysis of structural amino acids involves a hydrolysis step that uses a strong acid to disintegrate the protein into amino acids.

In this study, we focused on fully validating the post-column derivatization analytical method for the determination of structural amino acids in a CRM and in infant formula as reference samples using an automated amino acids analyzer in considerable amounts of time and to compare this method with the pre-column derivatization analytical method.

Materials and Methods

Chemicals and materials The standard solution mixture AA-S-18, which contained aspartic acid (Asp), serine (Ser), glutamic acid (Glu), glycine (Gly), histidine (His), threonine (Thr), arginine (Arg), alanine (Ala), proline (Pro), tyrosine (Tyr), valine (Val), methionine (Met), lysine (Lys), isoleucine (Ile), leucine (Leu) and phenylalanine (Phe), was purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydrochloric acid (HCl) was obtained from Junsei (Tokyo, Japan). Distilled water (DW) was purified using a Milli-Q system (Millipore, Bedford, MA, USA). The CRM 1546 sample was purchased from the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA), and the infant formula sample was purchased at a local market in Seoul, Korea. The infant formula sample was stored at room temperature, and the standard solution mixture and the CRM sample were stored in a refrigerator at 4°C. The AccQ-Tag reagent kit for pre-column derivatization method was purchased from Waters (Milford, MA, USA).

Preparation of working standard solutions One milliliter aliquots of the above standard solution were transferred into 10, 25, 50, 100, and 250 mL volumetric flasks. Then, 0.02 N HCl solutions were added to produce working solutions with five different concentrations. The concentrations of each working standard solution ranged from 0.075 mg/100 g to 4.530 mg/100 g.

Sample preparation The sample preparation method used in this study was based on the AOAC official method 994. 12. (25). To determine the amounts of the 16 types of structural amino acids, 0.3 g of the CRM sample and the infant formula sample were added to a digestion tube (solid caps with a PTFE liner, 20 mm, Supelco, Bellefonte, PA, USA). Fifteen milliliters of 6 N HCl was added to the digestion tubes, which was then vortex mixed for 1.0 min. The digestion tubes were filled with nitrogen gas to remove air and then tightly capped. The tubes were placed in a dry oven at 110°C for 22 h to hydrolyze the samples. After hydrolysis, the tubes were placed in the dark for 30 min to cool. The supernatant was carefully transferred into a 50 mL volumetric flask, pure DW was added and then the mixture was filtered through No. 2 filter paper (Advantec, Tokyo, Japan). One milliliter of the filtered solution was collected, transferred into a 10-mL volumetric flask and then reconstituted with DW Finally, the above solution was filtered through a 0.2-μm syringe filter and transferred into

a small glass vial for analysis.

To compare the results from the analysis of the structural amino acids with the AccQ-Tag pre-derivatization method in a sample of infant formula, we concurrently used Seo's analysis method (26) and the automated amino acid method presented in this study. Seo's method used same test solutions in this study and the test solutions were reacted with AQC following method of Accq-Tag (27) to analyze using an HPLC coupled with fluorescence detector.

The condition of the automated amino acid analyzer An automated amino acid analyzer (L-8900; Hitachi High-Technologies Corp., Tokyo, Japan) was used. The analytical column was a Hitachi HPLC Packed column (Ion-exchange resin, 4.6 mm i.d., 60 mm length, 3 μm particle size; Tokyo, Japan) with sulfone (SO_3^-) groups as the active exchange site, and the analytical detector was a visible detector (Hitach High-Technologies Corp.), set to measure a specific wavelengths of 570 and 440 nm for proline. Whole package and four prepared buffer solutions (Mitsubishi, Tokyo, Japan) were used as the mobile phase. The buffer solutions consisted of four different pH values (3.3, 3.2, 4.0, and 4.9). The re-generation solution and HPLC-grade water were used to clean and generate the analytical column. These two solutions were purchased from Mitsubishi (Tokyo, Japan). The aforementioned five types of buffers and water were used as a mobile phase using the gradient elution mode. The flow rate of the amino acid analyzer was 0.999 mL/min and the injection volume was 20 μL. The ninhydrin reagent, which contained sodium borohydrate and propylene glycol monomethyl ether, was purchased from Wako (Osaka, Japan).

The buffer solution that contained lithium acetate dihydrate, glacial acetic acid and propylene glycol monomethyl ether was also purchased from Wako (Osaka, Japan). These two solutions were used for the post-column reaction of analytes. During the running of the samples, the separate delivery pump for the ninhydrin reagent automatically mixes these two solutions, which are kept under nitrogen in the amino acid analyzer. The flow rate for the ninhydrin solution was 0.30 mL/min.

Method validation The validation procedures in this study were performed according to the ICH Q2B validation methodology (28).

Statistical analysis The obtained results were subjected to statistical analysis using the SPSS 13.0 for Windows (LEAD TOOLS, LEAD Technologies, Inc., 2004, Charlotte, NC, USA) software package. The comparison of the analysis results with the AccQ-Tag pre-column derivatization method was performed using a paired sample *t*-test to determine the significance (or lack thereof) of differences between the results from the AccQ-Tag pre-column derivatization method and the automated amino acid analyzer method presented in this study.

Results and Discussion

Linearity, limits of detection (LOD) and limits of quantification (LOQ) To determine the linearity of the 16 types of structural amino acids, five different concentrations (approximately 0.1, 0.3, 0.6, 1.3, and 3.3 mg/100 g) of standards were used for the structural amino acids. Each solution was injected 10 times, and the averages of the analytical results are presented in Table 1. The regression analyses revealed correlation coefficients (r^2) that ranged from 0.9994 to 0.9999, as shown in Table 1. For determining the sensitivity for the amino acids, we calculated the sensitivity values using the five previously described standard concentrations. The LOD and LOQ for the method were estimated at SD/b ratios of 3.3 and 10, where SD and b represent the standard deviation of the intercept and slope of the regression line, respectively. The LODs ranged from 0.007 to 0.059 mg/100 g and the LOQs ranged from 0.023 to 0.198 mg/100 g for all analytes.

Precision and accuracy The CRM 1546 sample purchased from the NIST was used for determining the precision and accuracy of the method. The relative standard deviations (RSDs) for the intra-day (n=2) and inter-day (n=24) repeatability and a recovery test for accuracy were performed on the CRM sample. The assay sample was calculated from

$$\%Recovery=(C_t/C_a)\times 100$$

where C_t is the calculated concentration of the CRM sample and C_a is the certified concentration of the CRM sample.

The recovery values were 87.18-118.08% for all analytes. The RSD values for the intra-day and inter-day repeatability were less than 3.01% and 14.62%, respectively. All values for the validation of this study were in accordance with the FDA guidelines for bioanalytical validation (29). The results of the precision and accuracy analyses are shown in Table 2.

Comparison of the analysis results with the AccQ-Tag pre-column derivatization method To compare the results from the analysis of the structural amino acids with the AccQ-Tag pre-derivatization method in a sample of infant formula, we concurrently used Seo's (26) analysis method and the automated amino acid method presented in this study to determine the structural amino acids. Seo's method was based on pre-column derivatization to derivate

Table 1. The linearity and sensitivity of 16 types of amino acids[1)]

Component	Linear range (mg/100 g)	r^2	LOD (mg/100 g)	LOQ (mg/100 g)
Asp	0.1331-3.3275	0.9999	0.0174	0.0581
Thr	0.1191-2.9775	0.9998	0.0107	0.0358
Ser	0.1051-2.6275	0.9999	0.0114	0.0381
Glu	0.1471-3.6775	0.9999	0.0334	0.1113
Pro	0.1151-2.8775	0.9998	0.0154	0.0514
Gly	0.0751-1.8768	0.9999	0.0070	0.0234
Ala	0.0891-2.2273	0.9998	0.0183	0.0609
Val	0.1172-2.9300	0.9997	0.0136	0.0454
Met	0.1492-3.7300	0.9999	0.0393	0.1309
Ile	0.1312-3.2800	0.9999	0.0183	0.0609
Leu	0.1312-3.2800	0.9999	0.0526	0.1753
Tyr	0.1812-4.5300	0.9997	0.0593	0.1977
Phe	0.1652-4.1300	0.9999	0.0224	0.0747
Lys	0.1462-3.6550	0.9994	0.0166	0.0554
His	0.1552-3.8800	0.9999	0.0152	0.0507
Arg	0.1742-4.3550	0.9999	0.0430	0.1433

[1)]All values were calculated using standard solution intra-day (n=10) analyses.

Table 2. The precision[1)] and accuracy[2)] for the analysis of 16 types of amino acids in the CRM sample

Component	Intra-day RSD (%)	Inter-day RSD (%)	Recovery (%)
Asp	0.69	6.67	110.51±7.26
Thr	0.64	7.00	118.08±8.14
Ser	0.71	8.35	105.56±8.69
Glu	0.55	6.30	114.94±7.15
Pro	2.15	12.55	99.30±12.18
Gly	0.70	6.90	108.98±7.41
Ala	0.73	7.88	110.26±8.57
Val	0.90	11.36	104.88±11.76
Met	1.83	14.62	91.47±13.15
Ile	1.22	8.12	106.95±8.55
Leu	1.41	3.63	115.98±4.00
Tyr	3.01	14.04	87.18±12.22
Phe	1.19	6.59	115.04±7.39
Lys	0.79	6.99	105.46±7.26
His	1.19	8.25	105.35±8.51
Arg	1.21	8.85	107.32±9.32

[1)]Amount of CRM sample=0.3 g; values represent the results of the intra-day (n=2) and inter-day (n=24) analyses.
[2)]Values represent the mean of the intra-day (n=2) and inter-day (n=24) analyses±SD.

Table 3. Comparison of the results[1)] from the AccQ-Tag pre-column derivatization method and those obtained using the automated amino acid analyzer

Component	AccQ-Tag derivatization method	Automated amino acid analyzer method
Asp	1144.14±53.96	1181.88±10.04
Thr	708.44±16.79	667.86±6.80
Ser	720.65±14.39	669.85±21.79
Glu	2448.34±222.05	2643.63±26.12
Pro	1030.96±43.43	957.65±21.24
Gly	244.99±14.42	234.61±5.04
Ala	498.42±32.45	480.02±5.60
Val	716.63±29.18	732.39±11.24
Met	290.21±19.68	232.98±17.38
Ile	666.06±28.64	693.57±19.56
Leu	1197.73±52.72	1448.67±11.49
Tyr	292.55±19.07	256.87±23.28
Phe	534.49±18.34	532.75±15.80
Lys	1027.78±49.23	1071.16±4.13
His	302.54±16.18	278.14±8.67
Arg	521.64±9.76	433.37±9.82
Total amounts[2)]	12345.60	12515.40
t-test[3)]	-0.46 (1.75)	

[1)]Values represent the mean of the intra-day (n=6) analysis±standadrd deviation (mg/100 g).
[2)]Sum of 16 types of amino acids in the sample of infant formula (mg/100 g)
[3)]Tabulated values of t at p=0.05 are shown in parentheses.

the analytes using the AccQ-Tag reagent kit. The results (intra-day, n=6) of the comparison of the structural amino acids are illustrated in Table 3. These two methods (AccQ-Tag derivatization method and the amino acid analyzer method presented in this study) exhibited no significant differences, as shown in Table 3. The AccQ-Tag derivatization method involves a complex sample pretreatment step because of a pre-column derivatization that must use various reagents, such as a derivatization reagent and buffer solutions. However, the automated amino acid analyzer method involves a simple pretreatment step and provides the same analysis results as the AccQ-tag derivatization method. Consequently, the automated amino acid analyzer method could be more conveniently applied

for the analysis of food, such as infant formula, than pre-column derivatization methods (8,26).

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