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Novel amino acid analysis using derivatization of multiple functional groups followed by liquid chromatography/tandem mass spectrometry

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Abstract

We have developed a novel amino acid analysis method using derivatization of multiple functional groups (amino, carboxyl, and phenolic hydroxyl groups). The amino, carboxyl, and phenolic hydroxyl groups of the amino acids were derivatized with 1-bromobutane so that the hydrophobicities and basicities of the amino acids were improved. The derivatized amino acids, including amino group-modified amino acids, could be detected with high sensitivity using liquid chromatography/tandem mass spectrometry (LC-MS/MS). In this study, 17 amino acids obtained by hydrolyzing proteins and 4 amino group-modified amino acids found in the human body (*N,N*-dimethylglycine, *N*-formyl-L-methionine, L-pyroglutamic acid, and sarcosine) were selected as target compounds. The 21 derivatized amino acids could be separated using an octadecyl-silylated silica column within 20 min and simultaneously detected. The detection limits for the 21 amino acids were 5.4–91 fmol, and the calibration curves were linear over the range of 10–100 nmol/L (r^2 > 0.9984) with good repeatability. A confirmatory experiment shoed that our proposed method could be applied to the determination of a protein certified reference material using the analysis of 12 amino acids combined with isotope dilution mass spectrometry. Furthermore, the proposed method was successfully applied to a stable isotope-coded derivatization method using 1-bromobutane and 1-bromobutane-4,4,4-d_3 for comparative analysis of amino acids in human serum.

1. Introduction

Amino acid analysis has been indispensable in a great variety of fields, such as medicine, pharmaceuticals, and agriculture, and has been used for metabolomic analysis,[1,2] investigation of amino acid metabolism disorders,[3] and quantification of proteins and peptides.[4,5] Moreover, in many fields, it is necessary that related compounds, such as amino acids and amino group-modified amino acids, are simultaneously analyzed and evaluated because these compounds are involved in many function of the human body, including protein synthesis, organ networks, and metabolic regulation of physiological states.[6] For example, the presence of *N,N*-dimethylglycine, *N*-formyl-L-methionine, L-pyroglutamic acid, and sarcosine, which are amino group-modified amino acids, has been reported to be related to various diseases.[7-12]

Many methods for amino acid analysis using liquid chromatography (LC) have been reported, including precolumn derivatization-reversed phase chromatography,[13,14] anion exchange chromatography,[15] ion-pair chromatography,[16] and hydrophilic interaction liquid chromatography.[4,5,17,18] The chromatographic separation of many kinds of amino acids in the human body can take a long time, which severely limits high throughput analysis. Therefore, LC-MS or LC-MS/MS methods have an increasingly important role in amino acid analysis because the use of these methods allows for the analysis of amino acids with structural similarities and with diverse functional groups without complex chromatographic separation. Furthermore, the combination of MS

methods with isotope dilution quantification (ID-MS) using isotope-labeled compounds as internal standards, allows for accurate quantification of amino acids in the presence of complex biological matrices. The use of isotope-labeled compounds in ID-MS can compensate for losses during sample generation, varying derivatization yields, or ion suppression in MS ion sources. Currently, the number of reported applications employing reversed-phase LC-MS for the analysis of amino acids is increasing. Although it is difficult to directly analyze highly polar amino acids using reversed-phase LC-MS, the use of derivatization methods or ion-pair reagents[19] enables the analysis of these amino acids. The chemical modification of amino acids by derivatization, which is widely used in LC-MS analyses, has the advantage of increasing the volatility and decreasing the polarity of the analyte to obtain greater sensitivity and selectivity. In addition, derivatization of the analyte can decrease matrix effects.[20] For these reasons, a number of precolumn derivatization reagents for analysis of amino acids using LC-MS have been reported, including 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC),[21-23] dansyl chloride,[24] phenylisothiocyanate (PITC),[25,26] 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA, Marfey's reagent),[27,28] 9-fluorenylmethyl chloroformate (FMOC),[29,30] and *p*-*N,N,N*-trimethylammonioanilyl *N'*-hydroxysuccinimidyl carbamate iodide (TAHS).[31] Most of these reagents only react with the amino group of amino acids. Although the derivatization reagents for the amino group are useful for increasing the volatility and decreasing the polarity of amino acids, it is difficult to apply these reagents to amino group-modified amino acids, such as *N,N*-dimethylglycine or

N-formyl- L –methionine. Conversely, a method that can be used for derivatization of both the amino and carboxylic acid groups of amino acids using alkyl chloroformate for GC-MS and LC-MS analysis was reported as a powerful tool for the simultaneous measurement of various amino acids (75 free amino acids and dipeptides).[32] However, complicated cleanup procedure was required such as liquid-liquid extraction to remove the derivatization reagent.

In this study, we have developed a novel derivatization method using 1-bromobutane as a derivatization reagent for multiple functional groups (amino, carboxyl, and phenolic hydroxyl groups) of amino acids. LC-MS/MS analysis was used to simultaneously analyze the various amino acids, including the amino group-modified amino acids. As the derivatization reagent was easily removed by evaporation, no complicated cleanup procedure, such as liquid-liquid extraction or solid-phase extraction, was required. Seventeen major amino acids, which were obtained by hydrolyzing proteins, as well as *N,N*-dimethylglycine, *N*-formyl-L-methionine, L-pyroglutamic acid, and sarcosine were selected as target compounds. The derivatized amino acids were analyzed using multiple reaction monitoring (MRM) in MS/MS analysis, and the conditions for derivatization, LC separation, and MS/MS detection were optimized using standard solutions. The sensitivity of this method was compared to that of a current commercially available derivatization method using AQC.

We applied our method to the quantification of a bovine serum albumin (BSA) certified reference material using an analysis of 12 amino acids combined with ID-MS. BSA was completely

hydrolyzed by hydrochloric acid and the hydrolyzed amino acids were analyzed. The accuracy of the present method was evaluated by comparison with the certificated value.

Furthermore, this method was applied to the stable isotope-coded derivatization method,[33] which is capable of high-throughput quantitative metabolomic profiling on a global scale using isotope-coded protein labels (ICPL).[34] It is easy to obtain an isotope labeled reagent for the present method, such as 1-bromobutane-4,4,4-d_3, because the structure of 1-bromobutane is very simple and its isotopes are commercially available from many manufacturers. As a model comparison experiment, the stable isotope-coded derivatization method using 1-bromobutane-4,4,4-d_3 and 1-bromobutane was applied to the analysis of amino acids in a human serum sample.

2. Experimental

2.1. Reagents and materials

Hydrochloric acid (HCl), phenol, acetic acid, acetonitrile, potassium carbonate, sodium sulfate, 1,4,7,10,13,16-hexaoxacyclooctadecane (18-crown-6), 1-bromoethane, 1-bromobutane, 1-bromohexane, and 1-bromooctane were purchased from Wako Pure Chemical (Japan). 1-Bromobutane-4,4,4-d_3 was purchased from C/D/N Isotopes Inc. (Canada). 6-Aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) was purchased from Waters (USA). The 17 amino acids produced by the National Metrology Institute of Japan (NMIJ) were used as

reference materials: L-alanine (Ala, NMIJ CRM 6011-a, 99.9 ± 0.2%), L-arginine (Arg, NMIJ CRM 6017-a, 99.8 ± 0.2%), L-aspartic acid (Asp, NMIJ CRM 6027-a, 99.9 ± 0.2%), L-cystine (Cys-Cys, NMIJ CRM 6025-a, 99.8 ± 0.3%), L-glutamic acid (Glu, NMIJ CRM 6026-a, 99.8 ± 0.2%), glycine (Gly, CRM 6022-a, 99.9 ± 0.2%), L-histidine (His, NMIJ CRM 6014-a, 99.9 ± 0.2%), L-isoleucine (Ile, NMIJ CRM 6013-a, 99.7 ± 0.2%), L-leucine (Leu, NMIJ CRM 6012-a, 99.9 ± 0.2%), L-lysine hydrochloride (Lys, NMIJ CRM 6018-a, 99.8 ± 0.2%), L-methionine (Met, NMIJ CRM 6023-a, 99.9 ± 0.2%), L-phenylalanine (Phe, NMIJ CRM 6014-a, 99.9 ± 0.2%), L-proline (Pro, NMIJ CRM 6016-a, 99.9 ± 0.2%), L-serine (Ser, NMIJ CRM 6021-a, 99.1 ± 0.2%), L-threonine (Thr, NMIJ CRM 6020-a, 99.9 ± 0.2%), L-tyrosine (Tyr, NMIJ CRM 6019-a, 99.9 ± 0.2%), and L-valine (Val, NMIJ CRM 6015-a, 99.8 ± 0.2%). *N,N*-dimethylglycine (DG), *N*-formyl-L-methionine (FM), L-pyroglutamic acid (PA), and sarcosine (SA) were purchased from Tokyo Chemical Industry Co., Ltd. (Japan). The isotopically labeled amino acids L-$^{13}C_3{}^{15}N$-alanine, L-$^{13}C_6{}^{15}N_4$-arginine hydrochloride, L-$^{13}C_4{}^{15}N$-aspartic acid, L-$^{13}C_5{}^{15}N$-glutamic acid, $^{13}C_2{}^{15}N$-glycine, L-$^{13}C_6{}^{15}N$-isoleucine, L-$^{13}C_6{}^{15}N$-leucine, L-$^{13}C_6{}^{15}N_2$-lysine hydrochloride, L-$^{13}C_9{}^{15}N$-phenylalanine, L-$^{13}C_5{}^{15}N$-proline, L-$^{13}C_9{}^{15}N$-tyrosine, and L-$^{13}C_5{}^{15}N$-valine were purchased from Isotech (USA). The BSA certified reference material (NIST SRM 927d) was purchased from the National Institute of Standards and Technology (NIST). Acetic acid and acetonitrile were LC-MS grade. Ultrapure water, purified using a Milli-Q gradient system (Millipore, USA), was used to prepare all aqueous solutions. Serum

samples were obtained from Jackson ImmunoResearch Labs (USA) and stored at −30 °C.

Standard amino acids mixtures for the optimization study and the quantification of protein were gravimetrically prepared by dissolving the individual natural amino acids in 0.1 M HCl before mixing.

2.2. Derivatization procedure

The standard solutions were evaporated under reduced pressure. The obtained residues were dissolved in 50 μL acetonitrile and 20 mg potassium carbonate, 20 mg sodium sulfate, 100 μL 18-crown-6 (200 mM in acetonitrile), and 20 μL 1-bromobutane were added. After the mixing, the samples were heated at 80 °C for 30 min. After reaction, the obtained solutions were dried under a N_2 stream at 80 °C and then dissolved in 100 μL water.

2.3. Instrumentation and conditions

An Agilent 1100 series HPLC (Agilent, USA) consisting of a quaternary pump, a degasser, an automatic injector, and a column oven with a Kinetex® core-shell XB-C18 (75 × 2.1 mm i.d., 2.6 μm, Phenomenex, USA) was used. Solvents A (1% acetic acid in water) and B (1% acetic acid in acetonitrile) were used as the mobile phase for gradient elution (gradient curve: 0–20 min, linear change from 1 to 75% B; 20–20.01 min, linear change from 75 to 1% B; hold at 1% B for 5 min for

column re-equilibrium; total run time, 25 min). The flow rate and the column oven temperature were 0.3 mL/min and 40 °C, respectively. The effluent from the LC column was directly introduced into the ion source of the mass spectrometer without splitting.

A Micromass Quattro Ultima tandem quadrupole mass spectrometer (Waters, USA) was operated in the positive ESI mode. The operating conditions were as follows: ESI capillary voltage, 2.0 kV; cone voltage, 5 V; source temperature, 120 °C; desolvation temperature, 400 °C; cone gas, 50 L/h; desolvation gas, 570 L/h. For the multiple reaction monitoring (MRM) mode, the entrance, collision, and exit potentials for the derivatized amino acids were 5, 0, and 5 eV, respectively. The precursor ions, product ions, and collision-induced dissociation energies (CE) for the MRM method with derivatized amino acids are shown in Table 1. Data processing was performed using MassLynx 4.1 software.

Table 1

2.4 Optimization study

Initially, the optimal derivatization reagent was determined from among 1-bromoethane, 1-bromobutane, 1-bromohexane, and 1-bromooctane. After 1-bromobutane was selected as the derivatization reagent, the optimal derivation conditions using 18-crown-6 and 1-bromobutane were investigated. The optimization tests were performed using 1 μM standard solution of six amino acids (Ala, Asp, Ile, Leu, Lys, and Tyr) with various concentrations of 18-crown-6 (100 μL; 1–400 mM)

and various volumes of 1-bromobutane (1–100 μL). A temperature of 25−80 °C and a time of 1−60 min were sufficient for reaction completion. The reaction yields of derivatization of six amino acids (Ala, Asp, Ile, Leu, Lys, and Tyr) were calculated by the MS spectra of individual amino acids.

2.5. Validation study

Standard calibration solutions with concentrations of 10, 25, 50, 75, and 100 nmol/L were prepared by diluting the standard solutions. The peak area precision in the present method was estimated from a precision study including the analytical procedures (derivatization, LC separation, and detection) using the 10 nmol/L standard solutions. The intraday precision values were assessed by performing the analysis six times on the same day. Matrix effect was calculate from the ratio of peak area of matrix sample to peak area of non-matrix sample. The matrix sample was prepared by diluting the derivatized 0.1 M amino acids 100 times with 200 mM 18-crown-6 (H_2O). The non-matrix sample was prepared by diluting the 0.1 M amino acids 100 times with H_2O.

The limit of detection (LOD) was determined as the lowest concentration for which the signal-to-noise ratio was 3.

2.6. Comparison study with AQC method

In order to examine the sensitivity of this method for LC-MS analysis, measurements using

AQC as a derivatization reagent were carried out using the same LC-MS/MS conditions as outlined in Section 2.3, except for the derivatization procedure and the MRM conditions. The derivatization procedure and MRM conditions were the same as previously reported.[22] The analytes for the comparison study were the 17 amino acids (Ala, Arg, Asp, Cys-Cys, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Tyr, and Val).

2.7 Quantification of BSA by ID-MS

Twelve amino acids (Ala, Arg, Asp, Glu, Gly, Ile, Leu, Lys, Phe, Pro, Tyr, and Val) were used for quantification of BSA by the ID-MS method. To prepare the labeled amino acid mixtures, the 12 isotopically labeled amino acids were gravimetrically dissolved in 0.1 M HCl and mixed. The sample blends were prepared gravimetrically by mixing the BSA solution with the corresponding labeled amino acid mixture. The calibration blend was prepared gravimetrically by mixing the standard amino acid mixture with the labeled amino acid mixture. The concentration of the labeled amino acids and corresponding natural amino acids in the calibration blend and sample blend were adjusted to be almost equal.

Three sets of BSA solutions were hydrolyzed for quantification. The sample and calibration blends (15 μL) were placed into individual glass tubes, and then freeze-dried under reduced pressure. Gas phase hydrolyses were conducted with 6 M HCl containing 2% phenol in the absence of oxygen.

After the hydrolysis, the samples were dried under reduced pressure. The obtained residues were derivatized following the procedure in Section 2.2. The optimal hydrolysis conditions for the quantification of BSA were investigated in the range of 110–145 °C and 3–72 h. The area ratios of the natural to isotopically labeled amino acids were measured three times by LC-MS/MS for each set of BSA solutions using the LC-MS/MS conditions described in Section 2.3. Quantitative results were obtained using a two-point calibration equation. The concentrations of BSA were calculated from the following equation (Eq. 1) from each amino acid measurement.

$$C_s = \frac{C_{std} \times M}{R_{VS} \times N} \times \left[\frac{R_{IS} - R_{IL}}{R_{IH} - R_{IL}} \times \left(R_{VH} - R_{VL} \right) + R_{VL} \right] \quad (1)$$

where C_s is the concentration of BSA in the sample solution; C_{std} is the concentration of amino acid in the standard solution; R_{VS}, R_{VL}, and R_{VH} are the natural/labeled mass ratios of the sample blend, lower calibration blend, and higher calibration blend, respectively; R_{IS}, R_{IL}, and R_{IH} are the natural/labeled measured ratio of the sample blend, lower calibration blend, and the higher calibration blend, respectively; M is the molecular weight of BSA; N is the number of each amino acid in BSA.

The precision of the hydrolysis and the MS/MS measurements were obtained by analysis of the variance. The uncertainty was calculated according to the guide to the expression of uncertainty

in measurement (GUM). The expanded uncertainty was calculated using a coverage factor ($k = 2$).

2.8. Comparative analysis study

To 200 µL of the spiked serum sample (1 µmol/L each amino acid), 800 µL of acetonitrile was added, vortexed, and centrifuged at 10000 *g* for 10 min. Two 400 µL portions of the supernatant were evaporated and derivatized with 1-bromobutane-4,4,4-d_3 and 1-bromobutane (20 µL), respectively, using the derivatization procedure, as described in Section 2.2, with appropriate derivatization reagent. After derivatization, mixing of the obtained samples was measured using a semi-microbalance and the samples were analyzed simultaneously using the LC-MS/MS procedure described in Section 2.3, with the exception of the MRM conditions. The MRM conditions used in the comparative analysis study for derivatives with 1-bromobutane-4,4,4-d_3 are shown in Table 1. Each of these samples was analyzed 5 times. The analytes used in the comparative analysis were the 17 amino acids and the amino group-modified amino acids (Ala, Arg, Asp, Cys-Cys, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Tyr, Val, DG, FM, PA, and SA).

3. Results and discussion

3.1 Optimization of the derivatization reaction

3.1.1 Derivatization reagent

Bromoalkyl compounds are known to react with amino, carboxyl, and aromatic hydroxyl groups, and this reaction, especially with carboxyl groups, is accelerated in the presence of 18-crown-6 and potassium ions.[35] Therefore, we used a bromoalkane, 18-crown-6, potassium carbonate, and sodium sulfate as reagents for derivatization. Sodium sulfate was added for dehydration of the derivatization reaction solution. Fig. 1 shows the reaction used for derivatization of amino acids in this study.

Several bromoalkanes (1-bromoethane, 1-bromobutane, 1-bromohexane, and 1-bromooctane) were examined as a derivatization reagent using standard solutions of six amino acids. The retention times of the derivatized amino acids increased with the number of carbons in the derivatization reagents, but the sensitivity of detection was almost the same with each derivatization reagent. The complete separation of the derivatives of Ile and Leu, which have same molecular weight, was not achieved with 1-bromoethane. 1-Bromoethane and 1-bromobutane could be removed by evaporation under atmospheric pressure. Considering these results, 1-bromobutane was selected as the derivatization reagent.

Fig. 1

3.1.2. Derivatization conditions and structures of the derivatives

The derivatization conditions, such as concentration of 18-crown-6, volume of 1-bromobutane, reaction time, and temperature, were investigated using standard solutions of six amino acids. The

optimal derivatization conditions were determined to be the addition of 200 mM 18-crown-6 and 20 μL 1-bromobutane to the sample and reaction for 30 min at 80 °C. The effects of reaction time (1 − 60 min) at 80 °C on the peak areas were examined. The reaction yields of derivatization of Ala, Asp, Ile, Leu, Lys, and Tyr were 84 %, 78 %, 89 %, 91 %, 69 %, and 80 %, respectively. A reaction time of over 20 min was required to obtain constant peak areas. The derivatized amino acids obtained were stable for at least 1 day in the dark at 4 °C.

The structures of the derivatized amino acids and the number of derivatization reagents attached to each amino acid molecule were confirmed by the ESI-MS and MS/MS spectra and are shown in Fig. 2 and Table 1. 1-Bromobutane can react with amino, carboxylic, and phenolic hydroxyl groups of amino acids. In most cases, each group was derivatized with only one molecule of bromobutane, but the amino group of Gly and the ε position of Lys were derivatized with two bromobutane molecules. This difference may be attributed to steric hindrance.

Fig. 2

3.2 MS/MS detection conditions

All derivatives were ionized using the positive ionization mode, and protonated ions ($[M + H]^+$) with the expected structures were found as the respective precursor ions. Fragment ions, such as $[M+H-HCOOC_4H_9]^+$ and $[M+H-C_4H_{10}]^+$, were observed with most of the amino acids. The most intense fragment ions obtained from the precursor ions under the examined collision-induced

dissociation conditions were: Ala $[M+H-HCOOC_4H_9]^+$, Arg $[M+H-HC=NHNHC_4H_9]^+$, Asp $[M+H-HCOOC_4H_9]^+$, Cys-Cys $[M+H-Cys(C_4H_9)_2]^+$, Glu $[M+H-HCOOC_4H_9]^+$, Gly $[M+H-C_4H_{10}]^+$, His $[M+H-HCOOC_4H_9]^+$, Ile $[M+H-HCOOC_4H_9]^+$, Leu $[M+H-HCOOC_4H_9]^+$, Lys $[M+H-HN(C_4H_9)_2]^+$, Met $[M+H-HCOOC_4H_9]^+$, Phe $[M+H-HCOOC_4H_9]^+$, Pro $[M+H-HCOOC_4H_9]^+$, Ser $[M+H-HCOOC_4H_9]^+$, Thr $[M+H-HCOOC_4H_9]^+$, Tyr $[M+H-HCOOC_4H_9]^+$, Val $[M+H-HCOOC_4H_9]^+$, DG $[M+H-HN(CH_3)_2]^+$, FM $[M+H-HCOOC_4H_9]^+$, PA $[M+H-HCOOC_4H_9]^+$, SA $[M+H-C_4H_{10}]^+$. The fragment patterns and MS/MS spectra of the derivatized amino acids are shown in Fig. 3. Although other fragment ions for the derivatized amino acids were observed in the MS/MS spectra, the most intense precursor–product transitions were set as the quantification transitions in MRM mode to obtain the most sensitive quantitative analysis of the amino acids. Other MS/MS detection conditions were also optimized to obtain the highest peak intensities for the derivatized amino acids.

Fig. 3

3.3 LC separation

A mixture of water and acetonitrile containing 1% acetic acid as the mobile phase for gradient elution provided satisfactory separation. The concentration of acetic acid was tested between 0.1% and 2%; the optimal concentration of acetic acid was determined to be 1%, as the maximum peak areas of all derivatized amino acids were obtained when more than 1% acetic acid was added.

Typical MRM chromatograms of the derivatized amino acids are depicted in Fig. 4. As expected, the derivatized amino acids were retained on the ODS LC column, and good peak shapes were obtained. Separation of the derivatives of Ile and Leu, which have same molecular weight, was achieved. No excess 1-bromobutane was detected, and 18-Crown-6 eluted between DG and SA (ca. 8 min) and thus there was no matrix effect in the detection of derivatized amino acids by ESI-MS/MS.

Fig. 4

3.4 Validation

Table 2 shows the coefficients of determination of the calibration curves, the intraday precision values of the peak areas, and the LODs for the derivatized amino acids. The calibration curves showed good linearity and the correlation coefficients were greater than 0.9984 for all examined concentration ranges. The relative standard deviations (RSDs) of the peak areas obtained from the intraday determinations (n = 6) of standard solutions were within 4.3%. The LODs of the amino acids on the column were between 5.4 and 91 fmol. Because the present reagent have no site to be enhanced detection such as pyridine or tertiary amine and so on, a large difference of detection limit was obtained. Therefore, all amino acids, including the amino group-modified amino acids were detected sensitively and could be quantified using the present method. Matrix effects of all

amino acid are shown in table 2. There was little matrix effect from derivatization solution when detecting all amino acid.

Table 2

3.5 Comparison with AQC method

To demonstrate the sensitivity of this method, the present method was compared with AQC method, which is a typical derivatization method for LC-MS analysis of free amino acids. Table 2 shows the LODs for the 17 amino acids analyzed by LC-MS/MS using the AQC method. Higher sensitivities were obtained using the present derivatization method for the high polarity amino acids, such as His and Ser, and the acidic amino acids, such as Asp and Glu. As the derivatized amino acids in our method had increased volatility and decreased polarity, the amino acids with higher polarities were detected with higher sensitivity owing to the improved ionization response in the ESI.

3.6 Quantification of BSA

The optimal hydrolysis conditions for the quantification of BSA were investigated. A hydrolysis time >24 h at 130 °C or a hydrolysis time of >18 h at 145 °C were required to obtain constant peak areas. Consequently, a hydrolysis time of 24 h at 130 °C was adopted for this study.

The concentration of BSA (NIST SRM 927d) obtained from the results of each amino acid, the precision between hydrolysis, the precision of the MS/MS measurements, and the combined relative standard uncertainty are shown in Table 3. Good precision was achieved in the MS/MS measurement for the ratio of natural and isotope-labeled amino acids was (≤0.28%). In comparison with the calibration curve method, the reproducibility of the amino acid analysis with hydrolysis was improved in ID-MS. The BSA concentrations determined from each amino acid measurement agreed well and the final determined concentration of 66.0 ± 2.4 g/L (k = 2) was in good agreement with the certified value (65.41 ± 0.82 g/L). These results demonstrate that this LC-MS/MS method, in conjunction with derivatization of 12 amino acids, enables an accurate and precise analysis of amino acids for the quantification of BSA.

Table 3

3.7. Comparative analysis of amino acids in 1-bromobutane and 1-bromobutane-4,4,4-d_3 samples

The proposed method was also applied to the stable isotope-coded derivatization method using 1-bromobutane and 1-bromobutane-4,4,4-d_3 for a comparative analysis study of human serum. This technique is based on differential isotopic labeling of amino acids derived from two different states with either light or heavy tags. The two samples are then mixed and analyzed with LC-MS/MS.

The quantitative analysis is performed by comparing the relative signal intensities of the light and heavy labeled peptide or amino acids in the MS spectra. In this study, the two identical samples derivatized with 1-bromobutane or 1-bromobutane-4,4,4-d_3 were mixed and analyzed simultaneously, and the obtained data is shown in Table 4. Within error, the obtained peak intensity ratios agreed with the sample preparation value for all measured amino acids. These results demonstrate that the proposed method could be applied to the difference mass tagging method for comparative studies and would be a very useful tool for the study of amino acid metabolomic analysis.

Table 4

4. Conclusions

A method to derivatize multiple functional groups (amino, carboxyl, and phenolic hydroxyl groups) on amino acids with 1-bromobutane followed by the LC-MS/MS analysis was developed. Twenty-one amino acids, including amino group-modified amino acids, were successfully analyzed by the present method. The derivatization reagent was easily removed by evaporation without complicated procedures, such as liquid-liquid extraction or solid-phase extraction. The obtained derivatives were retained on the ODS column and good separations and peak shapes were obtained for the derivatized amino acids. Positive ESI-MS/MS detection enabled highly sensitive analyses of

these derivatives and, in comparison with the AQC method, high sensitivities for the high polarity and acidic amino acids with present method. The advantages of the present method were shown in Table 5 by comparing the previous method. Furthermore, this method was applied to the quantification of a certified reference material of BSA (NIST SRM 927d) by combining ID-MS with the certified reference materials of amino acids, and the determined value was in good agreement with the certified value. These results demonstrated that this method enables the accurate and precise analysis of amino acids for the quantification of proteins. The proposed method was also successfully applied to the difference mass tagging method for a comparison study and was found to be a very useful tool for the study of metabolomic analysis. The present method, with which various amino acids can be reliably analyzed, can be applied to the studies of metabolomic analysis, amino acid metabolism disorders, and quantification of proteins and peptides.

Table 5

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Table 1. Precursor and product ions (Q1 and Q3), collision energies, and derivatization number for the MRM method with derivatized amino acids

Amino acid	Derivatization reagent	Q1 (*m/z*)	Q3 (*m/z*)	Collision energy (eV)	Derivatization reagents attached
L-alanine	1-bromobutane	202	100	22	2
	1-bromobutane-4,4,4-d_3	208	103		
L-$^{13}C_3{}^{15}N$-alanine	1-bromobutane	206	103		
L-arginine	1-bromobutane	399	301	25	4
	1-bromobutane-4,4,4-d_3	411	310		
L-$^{13}C_6{}^{15}N_4$-arginine	1-bromobutane	409	308		
L-aspartic acid	1-bromobutane	302	186	22	3
	1-bromobutane-4,4,4-d_3	311	192		
L-$^{13}C_4{}^{15}N$-aspartic acid	1-bromobutane	307	189		
L-cystine	1-bromobutane	465	232	26	4
	1-bromobutane-4,4,4-d_3	477	238		
L-glutamic acid	1-bromobutane	316	216	24	3
	1-bromobutane-4,4,4-d_3	327	222		
L-$^{13}C_5{}^{15}N$-glutamic acid	1-bromobutane	322	219		
glycine	1-bromobutane	244	188	19	3
	1-bromobutane-4,4,4-d_3	253	194		
$^{13}C_2{}^{15}N$-glycine	1-bromobutane	247	191		
L-histidine	1-bromobutane	324	222	24	3
	1-bromobutane-4,4,4-d_3	333	228		
L-isoleucine	1-bromobutane	244	142	22	2
	1-bromobutane-4,4,4-d_3	250	145		
L-$^{13}C_6{}^{15}N$-isoleucine	1-bromobutane	251	148		
L-leucine	1-bromobutane	244	142	22	2
	1-bromobutane-4,4,4-d_3	250	145		
L-$^{13}C_6{}^{15}N$-leucine	1-bromobutane	251	148		
L-lysine	1-bromobutane	371	242	19	4
	1-bromobutane-4,4,4-d_3	383	248		
L-$^{13}C_6{}^{15}N_2$-lysine	1-bromobutane	379	249		

L-methionine	1-bromobutane	262	160	18	2
	1-bromobutane-4,4,4-d_3	268	163		
L-phenylalanine	1-bromobutane	278	176	22	2
	1-bromobutane-4,4,4-d_3	284	179		
L-$^{13}C_9{}^{15}N$-phenylalanine	1-bromobutane	288	185		
L-proline	1-bromobutane	228	126	23	2
	1-bromobutane-4,4,4-d_3	234	129		
L-$^{13}C_5{}^{15}N$-proline	1-bromobutane	234	131		
L-serine	1-bromobutane	218	116	20	2
	1-bromobutane-4,4,4-d_3	224	119		
L-threonine	1-bromobutane	232	130	20	2
	1-bromobutane-4,4,4-d_3	238	133		
L-tyrosine	1-bromobutane	350	248	20	3
	1-bromobutane-4,4,4-d_3	359	254		
L-$^{13}C_9{}^{15}N$-tyrosine	1-bromobutane	360	257		
L-valine	1-bromobutane	230	128	20	2
	1-bromobutane-4,4,4-d_3	236	131		
L-$^{13}C_5{}^{15}N$-valine	1-bromobutane	236	133		
N,N-dimethylglycine	1-bromobutane	160	58	13	1
	1-bromobutane-4,4,4-d_3	163	58		
N-formyl-L-methionine	1-bromobutane	234	132	20	1
	1-bromobutane-4,4,4-d_3	237	132		
L-pyroglutamic acid	1-bromobutane	186	84	20	1
	1-bromobutane-4,4,4-d_3	189	84		
sarcosine	1-bromobutane	202	146	10	2
	1-bromobutane-4,4,4-d_3	208	149		

Table 2. Linearity of the calibration curves, repeatability, and LODs of the derivatized amino acids with bromobutane or AQC derivatization

Amino acid	Coefficients of determination (r^2)[a]	Repeatability (RSD)[b] (%, n = 6)	Detection limit[c] (fmol)		Matrix effect (average ± SD) (%, n = 3)
			C_4H_9Br	AQC	
Ala	0.9994	2.2	31	34	98.7 ± 3.0
Arg	0.9988	2.1	32	47	98.6 ± 5.4
Asp	0.9993	2.3	11	52	98.4 ± 4.0
Cys-Cys	0.9995	1.6	28	19	99.5 ± 1.9
Glu	0.9991	2.4	13	51	98.2 ± 4.8
Gly	0.9996	3.4	14	13	112.1 ± 6.0
His	0.9992	4.3	27	147	104.6 ± 2.1
Ile	0.9991	3.7	16	7.2	97.7 ± 3.7
Leu	0.9997	3.2	15	8.1	101.4 ± 5.2
Lys	0.9994	3.3	6.0	38	102.7 ± 3.4
Met	0.9999	2.6	22	15	101.2 ± 4.4
Phe	0.9999	1.5	6.5	12	102.0 ± 1.6
Pro	0.9984	1.9	8.6	17	101.7 ± 4.0
Ser	0.9999	0.9	39	330	102.3 ± 4.7
Thr	0.9998	1.8	5.4	15	105.5 ± 1.7
Tyr	0.9995	2.0	29	11	99.0 ± 1.9
Val	0.9997	1.8	25	13	96.1 ± 3.5
DG	0.9991	2.1	34	-[d]	98.4 ± 1.9

FM	0.9996	1.7	23	-[d]	102.9 ± 2.4
PA	0.9989	1.3	91	-[d]	101.2 ± 1.0
SA	0.9997	2.3	46	-[d]	104.6 ± 4.5

[a]Calibration curves in the range of 10–100 nmol/L per 5 µL injection.

[b]Relative standard deviation of the peak area for 1 µmol/L per 5 µL injection.

[c]Defined as the amount per 5 µL injection volume yielding a signal-to-noise ratio of 3.

[d]Not measured.

Table 3. BSA quantification by amino acid analysis

	Ala	Asp	Glu	Gly	Ile	Leu	Lys	Phe	Pro	Tyr	Arg	Val
Concentration (BSA, g/L)	66.0	66.8	66.6	66.9	66.4	65.9	65.0	65.1	65.2	66.8	65.7	65.6
Combined relative standard uncertainty (%)	1.04	1.54	1.42	1.05	1.00	1.11	1.54	1.78	1.28	1.22	1.69	1.79
Precision between hydrolysis samples (RSD, %)	0.42	0.65	0.47	0.23	0.60	0.19	0.34	0	0.095	0.041	0.49	0.24
Precision of the MS/MS measurements (RSD, %)	0.027	0.071	0.20	0.037	0.26	0.057	0.21	0.16	0.28	0.12	0.12	0.036
Concentration of BSA (g/L)	66.0 ± 2.4 (k = 2)											
Certified concentration value of NIST 927d (g/L)	65.41 ± 0.82 (k = 2)											

Table 4. The ratio of amino acids derivatized with 1-bromobutane to those derivatized with 1-bromobutane-4,4,4-d_3 in the mixture of two serum samples derivatized with each reagent

Amino acids	Quantification data	Sample preparation value
Ala	0.979 ± 0.0035	
Arg	0.977 ± 0.0048	
Asp	0.979 ± 0.0071	
Cys-Cys	0.979 ± 0.0062	
Glu	0.980 ± 0.0058	
Gly	0.980 ± 0.0058	
His	0.980 ± 0.0034	
Ile	0.981 ± 0.0050	
Leu	0.980 ± 0.0042	
Lys	0.980 ± 0.0045	
Met	0.979 ± 0.0047	0.978
Phe	0.980 ± 0.0032	
Pro	0.979 ± 0.0033	
Ser	0.980 ± 0.0045	
Thr	0.980 ± 0.0062	
Tyr	0.978 ± 0.0041	
Val	0.978 ± 0.0067	
DG	0.978 ± 0.0057	
FM	0.980 ± 0.0041	
PA	0.980 ± 0.0036	
SA	0.979 ± 0.0051	

Table 5. The advantages of the derivatization method using 1-bromobutane

Method	Purification	Detection limit	Target functional group
1-Bromobutane	Not necessary	6 – 91fmol	Amino group and carboxylic group
TAHS[31]	Not necessary	0.05 – 0.34 fmol	Amino group
AQC	Not necessary	7.2 – 330 fmol	Amino group
Alkyl chloroformate[32]	LLE [a]	50 – 2000 fmol	Amino group and carboxylic group

[a] Liquid-Liquid Extraction

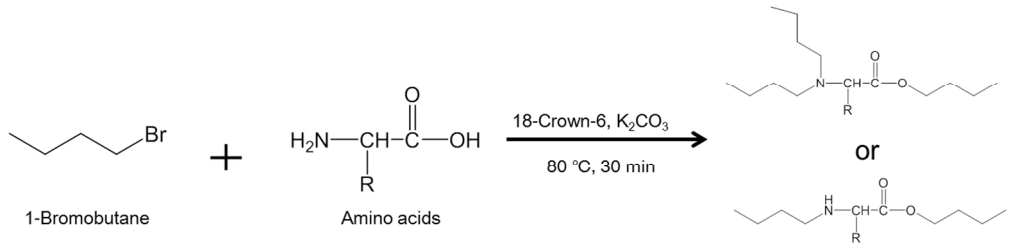
Figure Captions

Fig. 1. Amino acid derivatization reaction used in this study.

Fig. 2. The structure of the amino acids derivatized with 1-bromobutane.

Fig. 3. The MS/MS spectra of amino acids derivatized with 1-bromobutane. A; Ala, B; Arg, C; Asp, D; Cys-Cys, E; Glu, F; Gly, G; His, H; Ile, I; Leu, J; Lys, K; Met, L; Phe, M; Pro, N; Ser, O; Thr, P; Tyr, Q; Val, R; DG, S; FM, T; PA, U; SA

Fig. 4. Typical MRM chromatograms for the 100 nM standard solutions of amino acids derivatized with 1-bromobutane. Ala; *m/z* 202 → 100, Arg; *m/z* 399 → 301, Asp; *m/z* 302 → 186, Cys-Cys; *m/z* 465 → 232, Glu; *m/z* 316 → 216, Gly; *m/z* 244 → 188, His; *m/z* 324 → 222, Ile; *m/z* 244 → 142, Leu; *m/z* 244 → 142, Lys; *m/z* 371 → 242, Met; *m/z* 262 → 160, Phe; *m/z* 278 → 176, Pro; *m/z* 228 → 126, Ser; *m/z* 218 → 116, Thr; *m/z* 232 → 130, Tyr; *m/z* 350 → 248, Val; *m/z* 230 → 128, DG; *m/z* 160 → 58, FM; *m/z* 234 → 132, PA; *m/z* 186 → 84, SA; *m/z* 202 → 14



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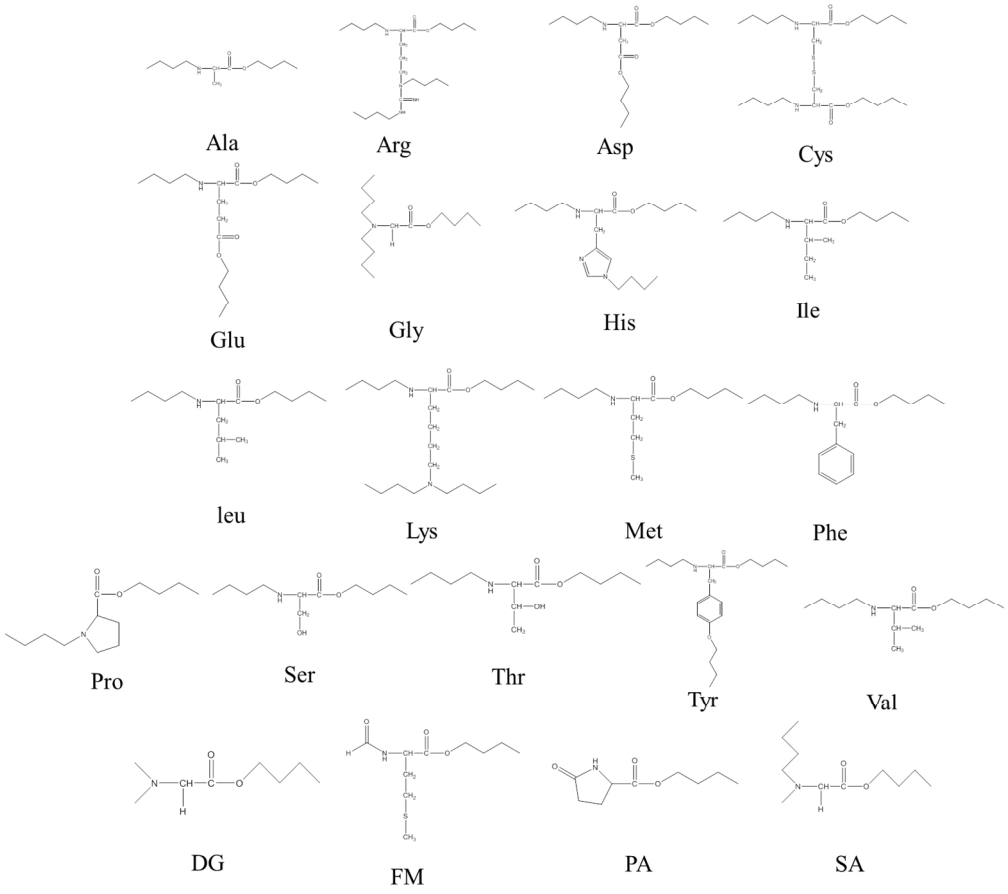

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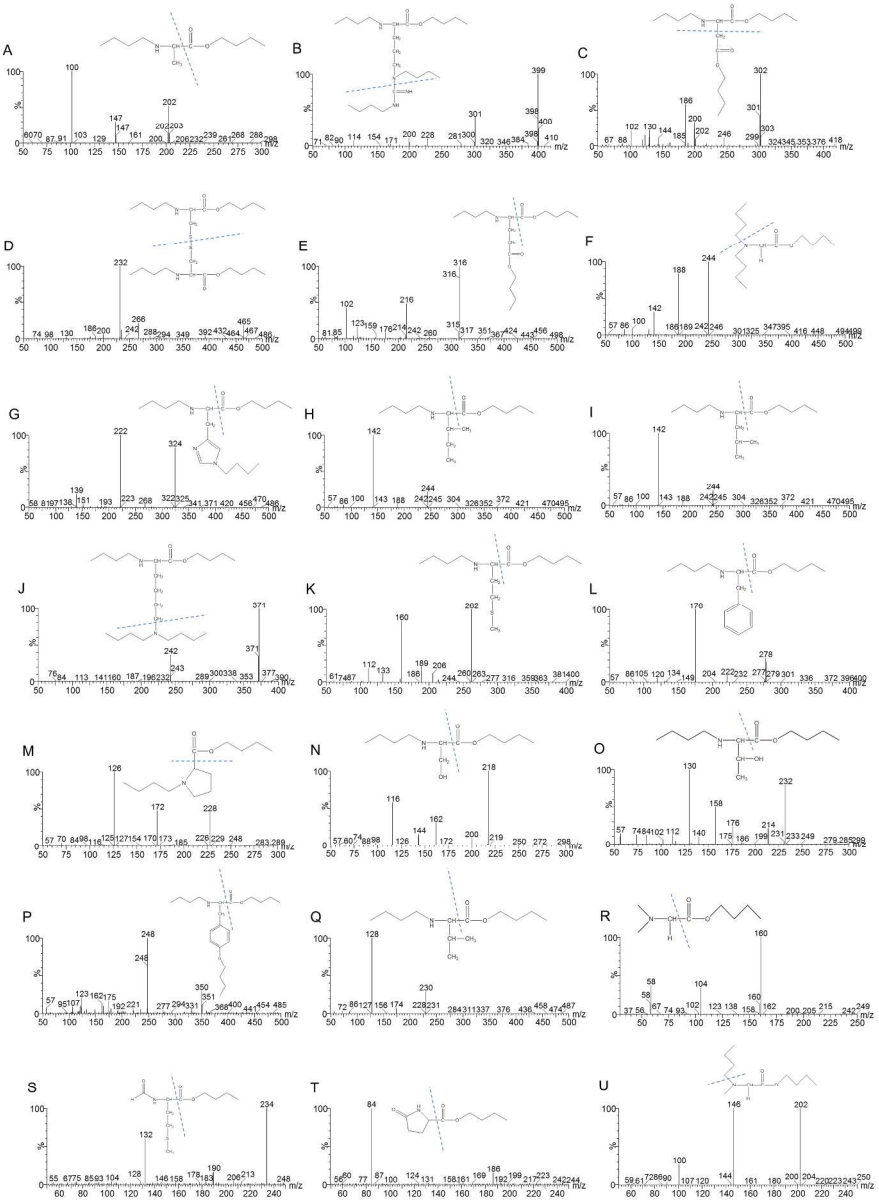

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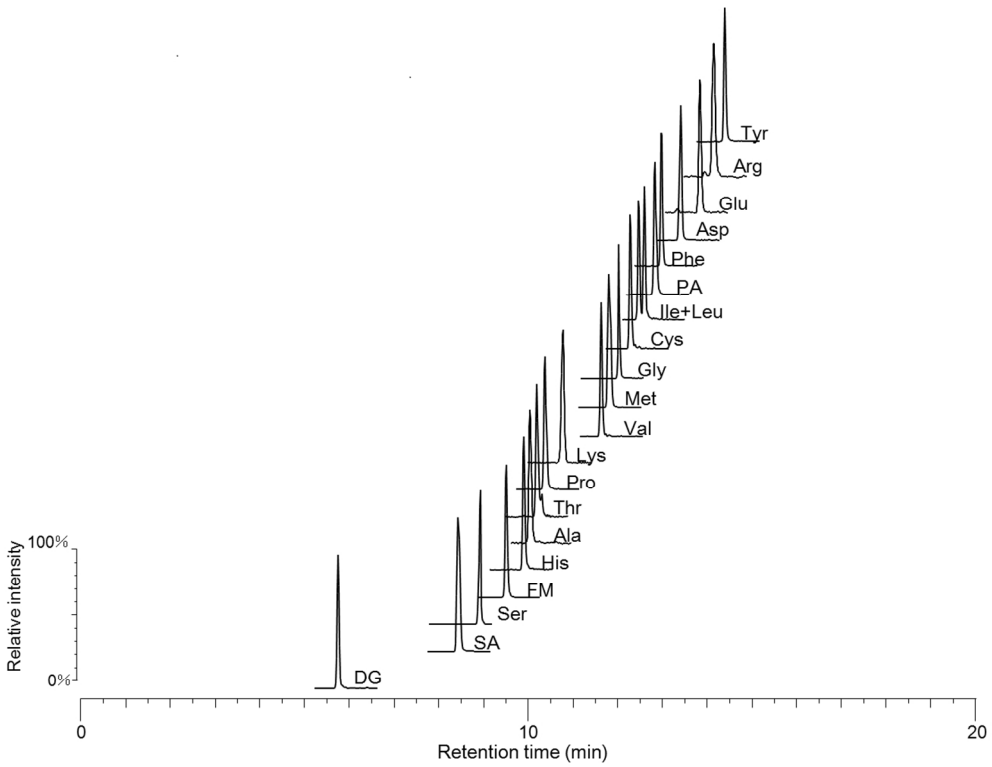

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