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High-throughput comprehensive analysis of D- and L-amino acids using ultra-high performance liquid chromatography with a circular dichroism (CD) detector and its application to food samples☆

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ABSTRACT

A rapid and comprehensive analytical method for D- and L-enantiomers of proteinogenic amino acids was developed using ultra-high performance liquid chromatography (UHPLC) equipped with a circular dichroism (CD) detector. Pre-column derivatization reagents were examined for enhanced sensitivity and selectivity for UV and CD detection: 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) was selected. The method, using a CD detector, does not require separation of optical isomers on a column to calculate the enantio ratio (*%D*) using the g-factor value and produces a simple chromatogram in comparison to other reported methods. Using this advantage, combined with UHPLC technology, analysis time for the derivatized proteinogenic amino acids was within 5.5 min. The UV detection limit was 4.9–23 pmol/injection and the CD detection limit was 11–64 pmol/injection. The method was applied to the analysis of D- and L-amino acids in food samples. D-Ala, D-Asp, D-Glu and D-Ser were detected at high concentrations in some Japanese black vinegars, fermented milks and yogurts. The results were identical to the results determined by the OPA method. We suggest the UHPLC–CD method would be useful in screening the D-amino acid content of foods and in helping to clarify the importance and reason for the presence of D-amino acids in foods.

1. Introduction

Proteinogenic amino acids, except glycine, have D- and L-isomers. It was thought that L-isomers were widespread in nature while D-isomers were considered to be rare and contained only in lower organisms, such as bacteria. Recently, the development of enantio-selective separation has provided evidence for the presence of D-amino acids in some plants, foods and mammals, and has revealed important roles of D-amino acids [1–5]. D-Amino acids have attracted attention as new biologically active substances. D-Ser [6,7] and D-Asp [8,9] were already well known for their functions in the human body and are expected to be used in medicines and dietary supplements.

D-Amino acids have been found in fermented foods, such as beer, wine, cheese, yogurt, fermented milk, vinegar and fish sauce [10–15]. In fermented foods, D-amino acids, especially D-Ala, D-Glu and D-Asp, are mostly derived from bacterial cell walls. They are also produced in foods, either biochemically by enzymatic activity or induced by heat and alkaline treatment. People take D-amino acids on a daily basis and the nutritional effects were also clarified [16].

Various analytical techniques for D- and L-amino acids had been reported using GC/MS, capillary electrokinetic chromatography (cEKC), micellar electrokinetic chromatography (MEKC), HPLC, LC/MS and an enzyme based biosensor [1,17]. In addition, many kinds of derivatization reagents and enantioselective columns for enantiomeric separation have been developed [18]. A GC/MS method, using achiral derivatization with pentafluoropropionic anhydride (PFPA) and separation on a Chirasil L-valine fused silica column, was applied to analysis of food and biological samples [19]. In the cEKC and MEKC methods, cyclodextrins (CDs) are mostly used as chiral selectors and fluorescence derivatization reagents such as NBD-F and NDA are utilized for high sensitive detection [20,21]. The CE methods are suitable for microdialysis because small sample volumes (nL–μL) can be used. LC/MS analysis has been performed using Marfey's reagent and adapted to D-Ser analysis in biological fluids [22].

Because of the simplicity, HPLC methods are widely used for the determination of amino acid enantiomers. There are two general HPLC methods for distinguishing D- and L-amino acids. The

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first is an indirect chiral method which uses chiral derivatization reagents such as OPA and chiral-thiols; the resulting diastereomers are separated on reversed phase columns [23,24]. The second is a direct chiral method in which non-labeled amino acids are separated on a chiral stationary phases using chiral crown ethers [25], teicoplanin [26] and zwitterionic structures [27], and chiral ligand-exchange stationary phase column with copper ion in the mobile phase [28]. In addition, a direct method was performed with derivatization using achiral reagents, such as NBD-F [29], and separation on a chiral stationary phase or in chiral mobile phase [30,31]. These derivatization methods mostly use fluorescent detection to achieve high sensitivity and selectivity. All these methods require advanced skills for the separation of D- and L-amino acids on columns and the length of time taken to perform the separation is over 1 h.

The CD detector enables differential absorption of left and right circularly polarized light so the abundance ratio of enantiomers can be determined [32]. Rapid analysis can be performed using the CD detector since enantiomers do not need to be separated on the column. Achiral compounds are not detected by the CD detector enabling selective detection of enantiomers. The CD detector has been used in the analysis of optically active compounds, such as drugs and pesticides [33,34]. Unfortunately, the CD detector is not sensitive enough to detect free amino acids. In this study, a CD detector was applied for the first time to HPLC analysis of D- and L-amino acids. A pre-column derivatization technique was examined for sensitive and selective detection. Using ultra-high performance liquid chromatography (UHPLC) and a circular dichroism (CD) detector, a high-throughput comprehensive analytical method for 20 proteinogenic amino acids was developed. D- And L-amino acids did not need to be separated on the column. The analysis time was 5.5 min. Our method was applied to fermented foods.

2. Experimental

2.1. Materials

Amino acids (D- and L-Ala, D- and L-Arg, D- and L-Asn, D- and L-Asp, L-Cys, L-Gln, D- and L-Glu, D- and L-His, D- and L-Ile, D- and L-Leu, D- and L-Lys, D- and L-Met, D- and L-Phe, D- and L-Pro, D- and L-Ser, D- and L-Thr, L-Tyr, D- and L-Trp, D- and L-Val, Gly) were purchased from Sigma–Aldrich (St. Louis, MO, USA). D-Cys was purchased from Fluka chemical (Milwaukee, USA). D-Gln was purchased from Wako pure chemical (Osaka, Japan). D-Tyr was purchased from Kokusan Chemical (Tokyo, Japan). NBD-F (4-fluoro-7-nitro-2,1,3-benzoxadiazole) and 2,4-dinitrofluorobenzen were obtained from Tokyo Chemical Industry (Tokyo, Japan). AccQ-Tag™ (6-aminoquinolyl-*N*-hydroxy-succinimidyl carbamate) was purchased from Waters Corporation (Milford, USA). APDS (3-aminopyridyl-*N*-hydroxysuccinimidyl carbamate) and TAHS (4-(trimethylammonium)anilyl-*N*-hydroxysuccinimidyl-carbamate iodide) were prepared as described in our previous reports [35,36]. Dansyl-chloride and *O*-phthaldialdehyde obtained from Wako pure chemical (Osaka, Japan) and Boc-L-Cys-OH obtained from Merck KGaA (Darmstadt, Germany). Acetonitrile (HPLC grade) and methanol (HPLC grade) were purchased from Junsei Chemical (Tokyo, Japan). Heptafluorobutyric acid was obtained from Tokyo Chemical Industry (Tokyo, Japan). Tetrahydrofuran (THF) (HPLC grade) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Deionized water was prepared with a Milli-Q system (Millipore, Bellerica, MA).

2.2. Food samples

Yogurts (Bifidus yogurt, Morinaga Milk Industry Co. Ltd., Tokyo, Japan and Megmilk, Nippon Milk Community Co. Ltd., Tokyo, Japan), fermented milk drinks (yakult 400, Yakult Honsha Co. Ltd., Tokyo, Japan and Pilkul, Nissin york Co. Ltd., Tokyo, Japan) and Japanese black vinegars (Sakamoto kurozu Inc., Kagoshima, Japan and Shobunsu K.K., Fukuoka, Japan) were obtained from the market.

2.3. Standard solutions

Standard solutions of free D- and L-amino acids were prepared in deionized water at a concentration of 1–2 mM and further diluted before use.

2.4. Preparation of food samples

Protein extraction: Removal of high molecular weight proteins in food samples were performed prior to amino acids extraction. For the Japanese black vinegars, each sample was loaded into a 10 kDa cut-off membrane centrifugal filter device (AmiconUltra-4, Millipore) and centrifuged at 7500 × *g* for 10 min at 4 °C. Three hundred μL of the supernatants was mixed with 2.7 mL of deionized water and 50 μL of 1 M aqueous hydrochloric acid solution. For the other samples, 10 mL of aqueous 6% sulfosalicylic acid solution was added to each 20 g of yogurt samples or 20 mL of fermented milk drinks and mixed for 30 s using a vortex mixer and centrifuged at 1200 × *g* for 15 min at 4 °C, supernatants were then collected.

Solid-phase extraction: For amino acid extraction, solid phase extraction (SPE) cartridges (Bond Elute SCX 500 mg/3 mL, Varian Inc., USA) were used. The SPE cartridge was pre-conditioned with 4 mL methanol, 4 mL 50% methanol/deionized water, and 4 mL Ten mM aqueous hydrochloric acid solution, sequentially. The deproteinized samples mentioned above (3 mL of black vinegar sample, 6 mL of yogurt and fermented milk drink sample) were loaded on the cartridge column and washed with 3 mL of deionized water. The amino acids were eluted with 3 mL of 6.7% aqueous triethylamine solution. The eluent was evaporated under reduced pressure for 3.5 h (45 °C, 0.1 Torr) and redissolved in 100 μL of deionized water before used for the following derivatization.

2.5. Derivatization procedure

To select an optimum reagent for our method, derivatized D- and L-Ala, Leu, and Phe were analyzed using NBD-F, AccQ-Tag™, dansyl-chloride, 2,4-dinitrofluorobenzen, APDS, or TAHS and the CD spectrum was monitored using the LC–CD/UV system. Each of the reagent solutions was prepared by dissolving in acetonitrile at 10 mg/mL (NBD-F, dansyl-chloride, 2,4-dinitrofluorobenzen, APDS, TAHS) or 10 mM (AccQ-Tag™). Ten μL of 1 mM standard amino acid solution and 30 μL of 200 mM sodium borate buffer (pH 8.8) was mixed, followed by addition of 10 μL of any reagent solution. After heating the mixture at 60 °C for 10 min, 150 μL of 0.1% aqueous formic acid solution was added to terminate the reaction. The aliquots were analyzed within 5 min of termination.

To monitor CD spectrum using the CD polarimeter, the derivatization method was as follows; 150 μL of 10 mM D- and L-alanine and 150 μL of 200 mM sodium borate buffer (pH 8.8) were mixed, followed by addition of 150 μL of NBD-F (10 mM). After heating the mixture at 60 °C for 1 min, 300 μL of 1% aqueous TFA solution was added to terminate the reaction. The aliquots were 20 times diluted and the final concentration of NBD derivatives of D- and L-alanine was 0.1 mM in the samples.

To validate the method and analyze food samples by LC–UV/CD analysis, 20 μL of 400 mM sodium-borate buffer (pH 9.0) and 20 μL of sample solution or standard amino acid solution were mixed.

Twenty μL of NBD-F solution (50 mM) in acetonitrile was added and heated at 60 °C for 1 min. Twenty μL of 1 M aqueous HCl solution was added to terminate the reaction and a 5 μL aliquot was analyzed within 5 min.

2.6. Apparatus

For selection of an optimum reagent, the CD spectrum was measured using a spectropolarimeter J-820 (JASCO) and a JASCO HPLC system, consisting of a pump (PU-2080 Plus), a degasser (DG-2095 Plus), a gradient unit (LC-2080-04), an autosampler (AS-2057 Plus), a UV detector (UV-2070 Plus), a CD detector (CD-2095 Plus) and a chromatography data system (ChromNAV).

For method validation and food analysis, the JASCO X-LC UHPLC system consisted of two pumps (X-LC 3185PU), a degasser (X-LC 3080DG), a mixer (X-LC 3080MX), an autosampler (X-LC 3159AS), a column oven (X-LC 3067CO), a circular dichroism detector (X-LC 3195CD) and a chromatography data system (ChromNAV).

D- and L-amino acid analysis of food samples using the OPA method was performed on an Acquity UPLC™ system (Waters, Milford, USA), consisting of a binary solvent manager, sample manager, column manager, fluorescence detector and chromatography data system (Empower).

2.7. CD spectra of derivatized amino acids and free amino acids

HPLC–CD conditions for recording CD spectra of derivatized amino acids used either NBD-F, AccQ-Tag™, dansyl-chloride, 2,4-dinitrofluorobenzen, APDS, or TAHS. Amino acids derivatized with NBD-F were separated on an ODS column Ascentis Express C18 (4.6 mm I.D. × 100 mm, 2.7 μm) (Supelco). The column was maintained at 40 °C. The injection volume was 10 μL. Mobile phase A was 0.1% TFA/deionized water and B was 0.1% TFA/acetonitrile. The stepwise gradient profile was 0–10 min, 15% mobile phase B; 10.1–20 min, 35% mobile phase B. The flow rate was maintained at 1 mL/min. Amino acids derivatized with AccQ-Tag™, APDS, or TAHS were analyzed using the same column and mobile phase as NBD-F and isocratic elution, 10% mobile phase B. The injection volume was 20 μL. Amino acids derivatized with dansyl-chloride were separated on the same column and mobile phase as NBD-F and analyzed by isocratic elution, 35% mobile phase B. The injection volume was 20 μL. Amino acids derivatized with 2,4-dinitrofluorobenzen were separated on the same column and temperature. Mobile phase A was 0.1% formic acid/deionized water and B was methanol and isocratic elution, 50% mobile phase B. The flow rate was 0.7 mL/min. The injection volume was 10 μL.

The CD spectra of free D- and L-Ala and NBD-D- and L-Ala were recorded on a JASCO J-820 spectrometer, using 1 mL quartz microcell. The samples were monitored at 1 nm band width, 200 nm/min scanning speed, 1 s of response time, 0.1 nm of data pitch, and 4 of integrated number. All data were blank subtracted, according to conventional procedures.

2.8. Chromatographic conditions for UHPLC–CD method

UHPLC conditions were as follows: amino acids derivatized with NBD-F were separated on an ODS column Ascentis Express C18 (2.1 mm I.D. × 100 mm, 2.7 μm) (Supelco). The column was maintained at 40 °C. The injection volume was 5 μL. Mobile phase A was 5 mM heptafluorobutyric acid/deionized water and B was methanol. The stepwise gradient profile was 0–2 min, 20–40% mobile phase B; 2–2.05 min, 40–55% mobile phase B; 2.05–3 min, 55–65% mobile phase B; 3–4 min, 65–70% mobile phase B; 4–5 min, 70–80% mobile phase B; 5–5.05 min, 80–20% mobile phase B; 5.06–10 min, 20% mobile phase B. The flow rate was maintained at 0.5 mL/min. UV and CD detection was carried out at 235 nm (1 V = 2 AU, 1 V = 1 deg). The peak heights of UV detection were used for quantification of amino acid concentration and g-factors calculated from UV and CD peak heights were used to determine the D, L ratio of the amino acids.

2.9. OPA analysis

Derivatization procedure was as follows. Ten μL of food sample solution or standard amino acid solution was mixed with 60 μL of 400 mM sodium-borate buffer (pH 9.0). The derivatization reagent (OPA, 10 mg and Boc-Cys-OH, 10 mg in 1 mL methanol) was prepared before use and 20 μL of the reagent was added and mixed. After 2 min, a 5 μL aliquot was subjected to the UHPLC system. OPA analysis was modified from the method reported by Hashimoto et al. [23] to shorten the analysis time. The analytical conditions were as follows: the column (Acquity UPLC BEH C18 2.1 mm I.D. × 150 mm, 1.7 μm) was maintained at 30 °C. The injection volume was 5 μL. Mobile phase A was 0.1 M sodium acetate (pH 6.0) with 7% acetonitrile and 3% THF, and mobile phase B was 0.1 M sodium acetate (pH 6.0) with 47% acetonitrile and 3% THF. The flow rate was 0.3 mL/min. The gradient profile was 0–30 min, 0–50% mobile phase B; 30–35 min, 50–80% mobile phase B; 35–35.1 min, 80–0% mobile phase B; 35.1–40 min, 0% mobile phase B. The excitation and emission wavelengths were 344 nm and 443 nm, respectively. The peak heights of fluorescent detection were used for quantification of D- and L-amino acid concentration.

3. Results and discussion

3.1. Selection of derivatization reagent

The main advantage of a CD detector is simultaneous detection of optical isomers without separation on a column. Unfortunately, it is difficult to monitor CD chromatograms of free D- and L-amino acids because they do not have a specific UV-absorbing chromophore to increase sensitivity and specificity for CD detection. To produce an appropriate UV-absorbing chromophore that exhibits circular dichroism, pre-column derivatization was applied prior to analysis. The most appropriate reagent in this study for CD detection was selected from commercially available derivatization reagents for amino groups (NBD-F, AccQ-Tag™, dansyl-chloride, 2,4-dinitrofluorobenzen) or were previously reported by our group (APDS, TAHS). Derivatization of D- and L-Ala, D- and L-Leu and D- and L-Phe, which have different side chains, was performed using the reagents above; CD and UV spectra were obtained using a CD detector combined with HPLC and the stopped flow method. The results from NBD-F, AccQ-Tag™ and dansyl-chloride are shown in Fig. 1.

To show a clear distinction between D- and L-amino acids using the CD detector, three chromophore properties were required: the first was a symmetric CD spectrum around the ellipticity maximum wavelengths. The ellipticity maximum, at 235 nm was completely symmetric after NBD-F derivatization. However, except for dansyl-Leu, AccQ-Tag™ and dansyl-chloride, derivatized amino acids did not show a mirror image around the ellipticity maximum. Symmetric ellipticity maxima were not observed after derivatization with the other tested reagents (2,4-dinitrofluorobenzen, APDS, TAHS) (data not shown).

Secondly, higher molar ellipticity (θ), shown in the vertical axis (mdeg) of the CD spectrum, was desired for more sensitive detection. Fig. 2 shows the CD spectrums of underivatized D- and L-Ala (left), and NBD-derivatized D- and L-Ala (right), measured using a CD polarimeter. Label-free D- and L-Ala showed the absolute molar ellipticity value at 210 nm was about 2 mdeg, while NBD-derivatized D- and L-Ala was 20 mdeg at 235 nm. Sensitivity

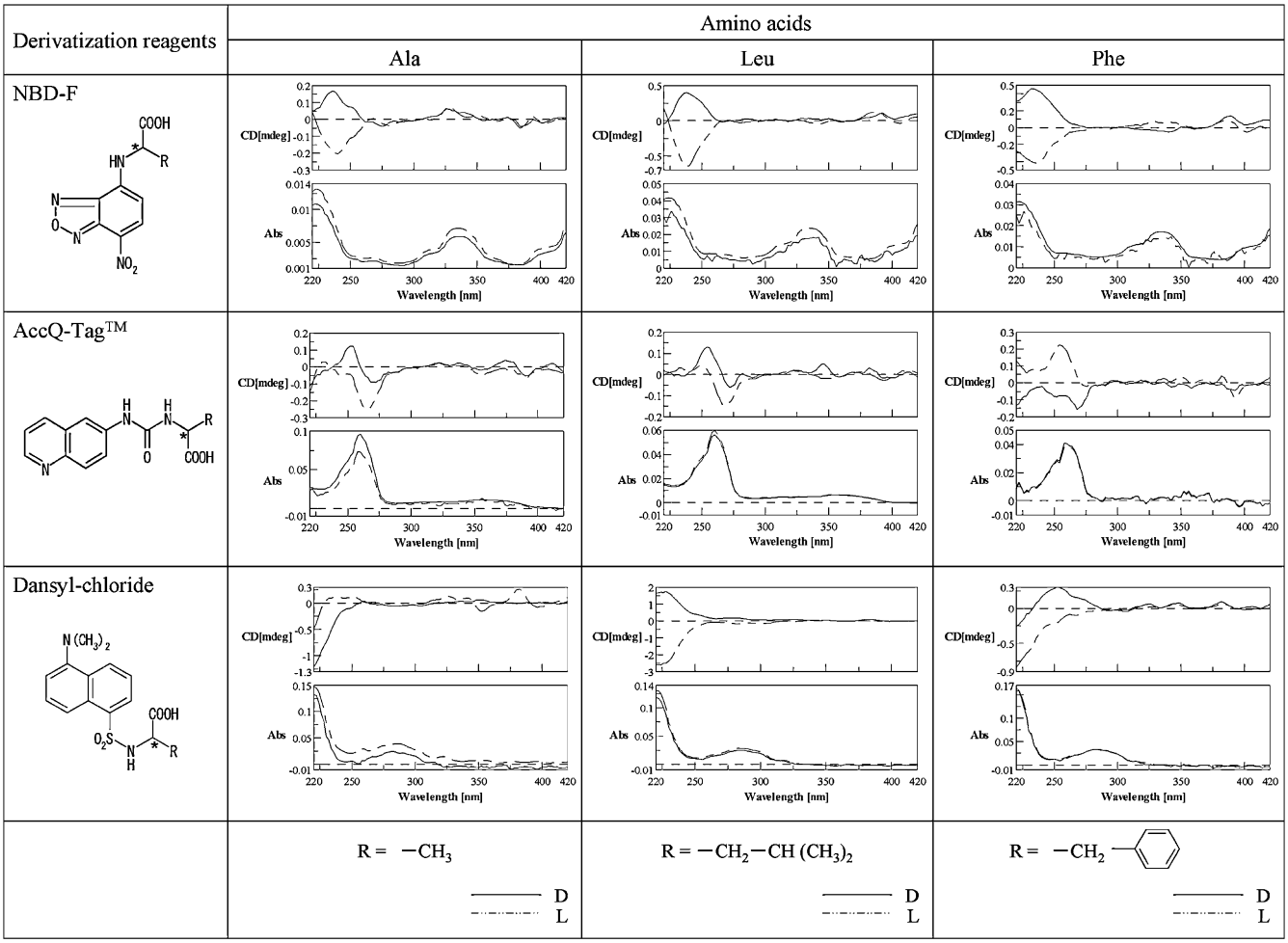

Fig. 1. CD spectra of derivatized amino acids. Amino acids (Ala, Leu, Phe) derivatized with NBD-F, AccQ-Tag™ or dansyl-chloride were separated on an ODS column; the CD and UV spectra were monitored using a CD detector combined with HPLC using the stopped flow method. CD (mdeg) is the molar ellipticity (θ). Abs is UV absorbance.

was 10 times higher in the labeled forms compared to the label-free forms.

The third important property was selectivity of the CD spectrum. Fig. 2 shows the ellipticity maximum wavelength of Ala shifted from 210 nm to 235 nm after NBD-F derivatization. This shift was effective in reducing background absorption of the mobile phase and other compounds, without altering the specific absorbing wavelength. We concluded NBD-F to be the best reagent for D- and L-amino acid detection in this study. For sensitive CD detection, it was suggested that the chromophore structure should be located close to the stereogenic center of the amino acids such as NBD-F derivatized amino acids.

It is well known that the absorption maximum wavelength of the CD spectrum is close to that of the UV absorption maximum wavelength. NBD-derivatized amino acids showed ellipticity maximum at 235 nm in the CD spectrum and absorption maximum at 225 nm in the UV spectrum. Although NBD-derivatized amino acids have a fluorescence excitation at 470 nm and emission at 530 nm,

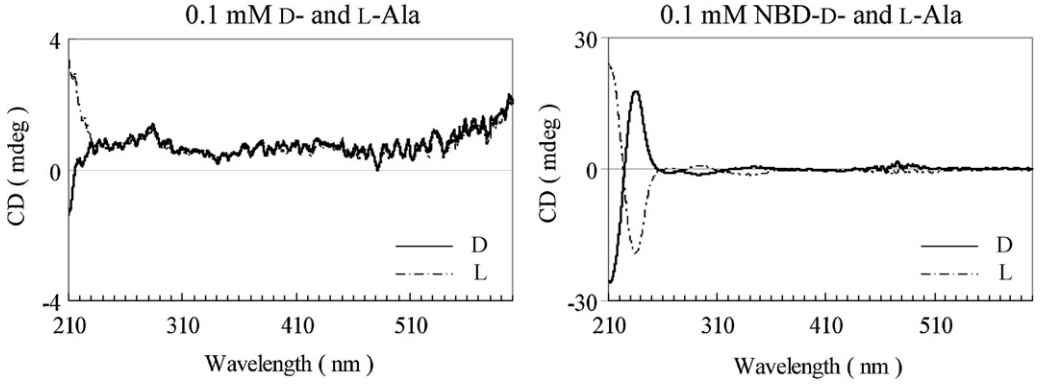

Fig. 2. CD spectra of free Ala and NBD-derivatized Ala. CD spectra of underivatized D- and L-Ala and NBD-derivatized D- and L-Ala were recorded on a JASCO J-820 spectrometer which monitored from 210 to 600 nm.

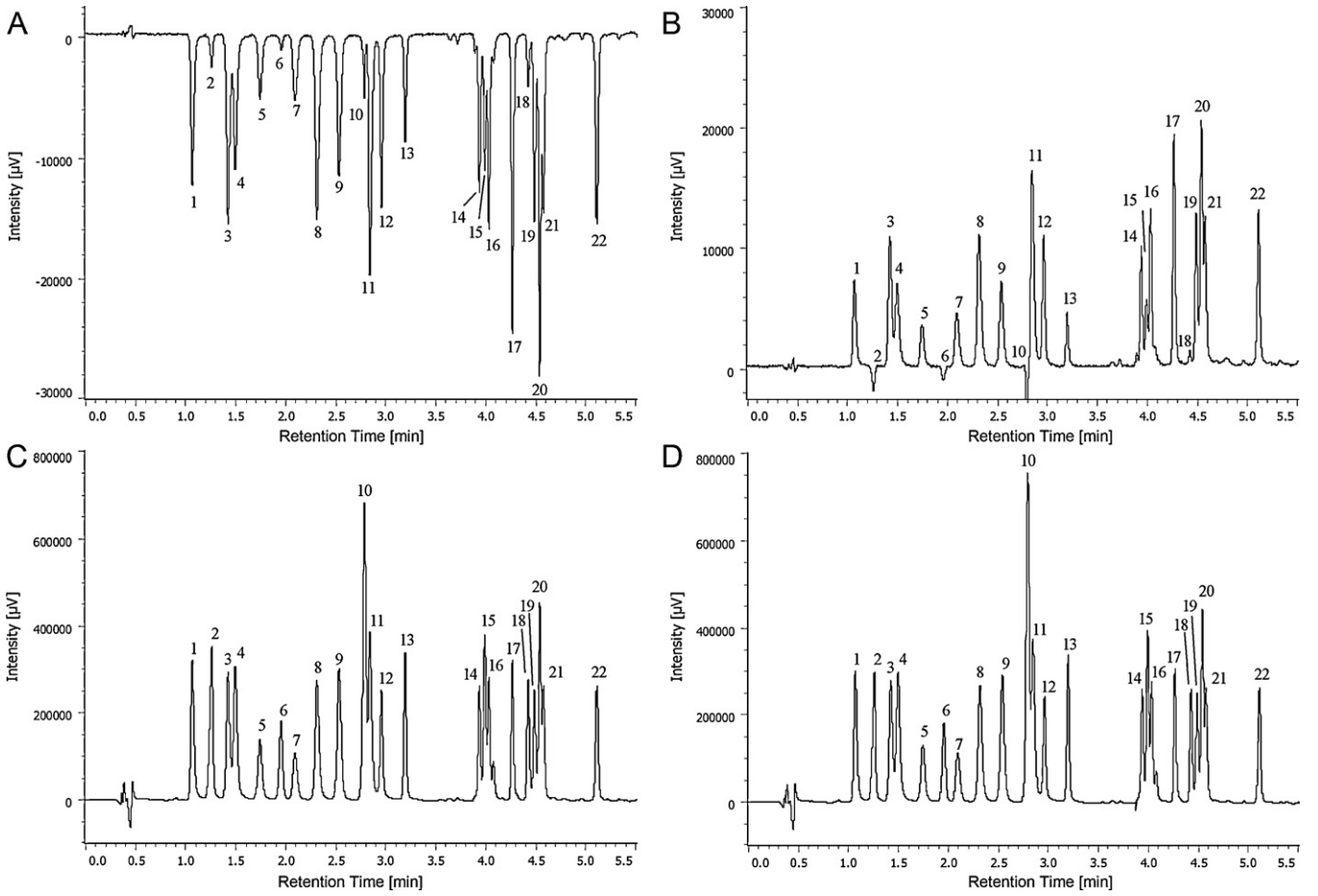

Fig. 3. CD chromatograms of NBD-D-amino acids (A) and NBD-L-amino acids (C). UV chromatogram of NBD-D-amino acids (B) and NBD-L-amino acids (D). Peak identification is as follows: (1) NBD-Asn, (2) NBD-OH, (3) NBD-Gln, (4) NBD-Ser, (5) NBD-Asp, (6) NBD-Gly, (7) NBD-His, (8) NBD-Glu, (9) NBD-Thr, (10) NBD-F, (11) NBD-Arg, (12) NBD-Ala, (13) NBD-Pro, (14) NBD-Met, (15) NBD-Lys, (16) NBD-Val, (17) NBD-Trp, (18) NBD-Cys, (19) NBD-Phe, (20) NBD-Ile, (21) NBD-Leu, (22) NBD-Tyr. HPLC conditions were as follows: 1 or 2 mM of D- and L-amino acids solvents derivatized with NBD-F were separated on an Ascentis Express C18 column (2.1 mm I.D. × 100 mm, 2.7 μm) at 40 °C. The injection volume was 5 μL. Mobile phase A was 5 mM heptafluorobutyric acid/deionized water and B was methanol. The stepwise gradient profile was 0–2 min, 20–40% mobile phase B; 2–2.05 min, 40–55% mobile phase B; 2.05–3 min, 55–65% mobile phase B; 3–4 min, 65–70% mobile phase B; 4–5 min, 70–80% mobile phase B; 5–5.05 min, 80–20% mobile phase B; 5.06–10 min, 20% mobile phase B. The flow rate was 0.5 mL/min. UV and CD detection was carried out at 235 nm (1 V = 2 AU, 1 V = 1 deg).

the CD detector could detect from 220 to 420 nm. In this study, 235 nm was the most appropriate wavelength in the detection range.

3.2. UHPLC separation

For high-speed separation, the fused-core type column, Ascentis Express C18 (2.1 mm I.D. × 100 mm, 2.7 μm) was used. The column efficiency was the same as a sub-2 μm type column and the backpressure was lower than with the sub-2 μm column. The flow rate was increased up to 0.5 mL/min and the backpressure was lower than the system pressure resistance, at 1000 bar. In the mobile phase, heptafluorobutyric acid (HFBA) and methanol were used. Optimized separation conditions are detailed in the experimental section. The CD and UV chromatograms of NBD-derivatized D- and L-amino acids are shown in Fig. 3.

In the CD chromatogram, the NBD-derivatized D-amino acids were detected as positive signals and L-amino acids were detected as negative signals, while the UV chromatogram of both NBD-derivatized D- and L-amino acids were detected as positive signals. The analysis time for 20 derivatized proteinogenic amino acids was 5.5 min and the cycle time was 10 min, including washing and equilibration.

Besides sensitivity and selectivity, the advantage of derivatization is the ability to separate amino acids on a reversed phase column: it is difficult to separate free amino acids on a reversed phase column because it is hydrophobic and retains very few

Table 1
Correlation coefficients (r^2) for the concentration of amino acids dereivatized with NBD-F.

Concentration[a] 1, 0.5, 0.2, 0.1 mM		Concentration[a] 2, 1, 0.4, 0.2 mM	
Amino acid	r^2	Amino acid	r^2
Asp	0.9957	Asn	0.9973
Gly	0.9999	Gln	0.9994
His	0.9964	Ser	0.9995
Ala	0.9994	Glu	0.9990
Pro	0.9997	Thr	0.9992
Met	0.9998	Arg	0.9960
Lys	0.9971	Ile	0.9989
Val	1.0000		
Trp	0.9994		
Cys	0.9999		
Phe	0.9999		
Leu	0.9999		
Tyr	0.9997		

[a] Concentration of amino acids before derivatization.

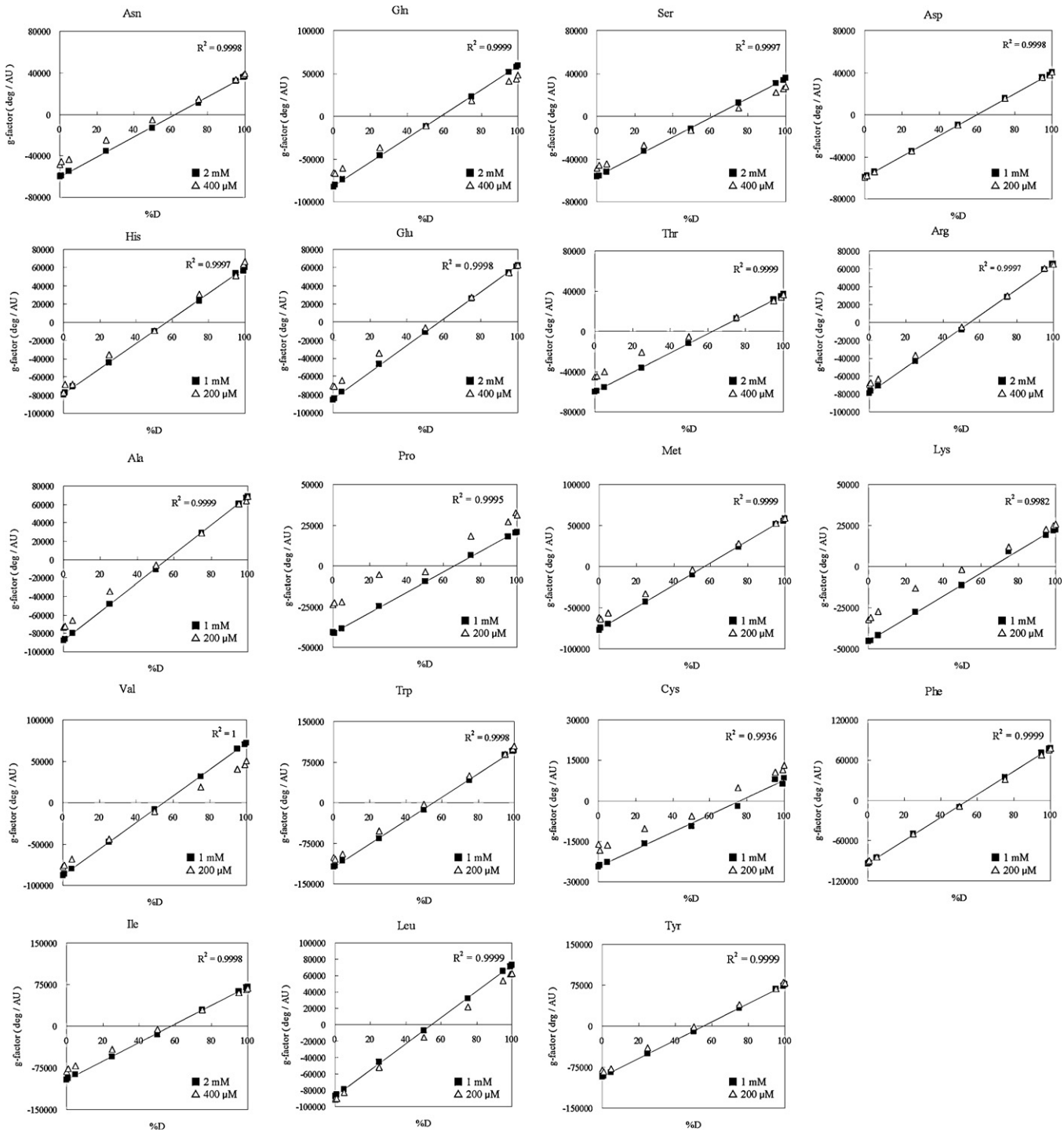

Fig. 4. Linear regression and correlation coefficients (r^2) for g-factor of amino acids derivatized with NBD-F (x = D-form ratio (%D = D/(D + L)), y = g-factor). The linearity of the g-factor was tested at 9 ratios, ranging from 0% to 100% D-amino acids (D/L ratio = 0/100, 1/99, 5/95, 25/75, 50/50, 75/25, 95/5, 99/1, 100/0). g-Factor was plotted on the graph at high concentration (1 mM or 2 mM) and low concentration (200 μM or 400 μM). The linear regression and correlation coefficients were determined at high concentration (1 mM or 2 mM).

amino acids. The derivatization technique added hydrophobicity and increased amino acid retention on the reversed phase column.

HFBA needed a long time to reach equilibration but it was effective in separating NBD-derivatized amino acids from the large peaks of NBD-OH and NBD-F. When formic acid or TFA aqueous solution was used as mobile phase A it was difficult to separate NBD-derivatized asparagine from NBD-OH. The UV absorbance of both HFBA and methanol, used as mobile phases, did not affect the detection of NBD-derivatized amino acids at wavelength 235 nm.

3.3. *Quantification of* D- *and* L-*amino acids*

Total concentration of D- and L-amino acids was determined from UV peak height. The range of linearity was tested using L-amino acids solution before derivatization at concentrations of

Table 2
Results of method validation.

Amino acids	Minutes	Retention time		CD peak height			UV peak height			g-Factor	
		RSD[a](%)		RSD[b](%)		LOD[e] (pmol/injection)	RSD[c] (%)		LOD[f] (pmol/injection)	RSD[d] (%)	
		Low	High	Low	High		Low	High		Low	High
Asn	1.06	0.21	0.12	5.30	0.75	46	2.01	0.16	12	4.03	0.58
Gln	1.42	0.20	0.14	3.75	0.66	37	3.63	0.60	16	4.31	0.72
Ser	1.49	0.19	0.14	4.91	0.71	54	1.03	0.44	15	1.81	1.01
Asp	1.74	0.20	0.15	12.87	0.84	49	2.99	0.47	14	9.51	0.98
Gly	1.95	0.17	0.13	–	–	–	1.78	0.56	11	–	–
His	2.09	0.23	0.16	11.37	1.45	57	4.18	1.62	23	3.52	1.28
Glu	2.31	0.11	0.18	4.24	0.64	38	1.08	0.24	14	8.39	0.62
Thr	2.53	0.10	0.18	7.64	0.78	54	1.48	0.24	13	5.75	0.93
Arg	2.84	0.07	0.19	4.44	0.88	29	1.72	1.16	6.1	1.89	1.53
Ala	2.95	0.07	0.17	2.68	0.52	20	1.00	0.63	7.1	4.62	0.71
Pro	3.19	0.07	0.17	6.90	1.31	34	1.32	0.36	6.2	18.07	1.46
Met	3.93	0.07	0.14	3.22	0.61	22	1.71	0.46	8.1	6.00	0.84
Lys	3.98	0.07	0.14	6.14	1.58	29	1.49	0.51	4.9	5.92	1.73
Val	4.03	0.06	0.14	3.74	0.62	19	1.61	0.44	7.7	5.62	0.59
Trp	4.26	0.07	0.14	4.01	0.58	11	2.97	0.49	6.5	2.32	0.15
Cys	4.42	0.07	0.14	18.36	2.52	64	4.29	1.05	8.1	15.83	1.72
Phe	4.48	0.07	0.14	2.94	0.73	17	1.09	0.78	8.6	5.83	0.49
Ile	4.53	0.07	0.14	1.40	0.35	21	1.31	0.53	9.0	2.59	0.49
Leu	4.57	0.07	0.14	2.29	0.60	19	1.16	0.71	8.5	4.87	0.59
Tyr	5.10	0.07	0.15	4.64	2.07	18	3.79	1.40	7.8	6.60	1.08

Asn, Gln, Ser, Glu, The, Arg, Ile are tested at 2 mM. Asp, Gly, His, Ala, Pro, Met, Lys, Val, Trp, Cys, Phe, Leu, Tyr are tested at 1 mM.
[a] RSD = Relative standard deviation of retention time (n = 6, at concentration 1 or 2 mM (High) and 100 or 200 μM (Low)).
[b] RSD = Relative standard deviation of CD peak height (n = 6, at concentration 1 or 2 mM (High) and 100 or 200 μM (Low)).
[c] RSD = Relative standard deviation of UV peak height (n = 6, at concentration 1 or 2 mM (High) and 100 or 200 μM (Low)).
[d] RSD = Relative standard deviation of g-factor (n = 6, at concentration 1 or 2 mM (High) and 100 or 200 μM (Low)).
[e] LOD = Detection limit of CD peak height (S/N = 3).
[f] LOD = Detection limit of UV peak height (S/N = 3).

1–2 mM and further diluted down to 100–200 μM. The maximum concentration was optimized for 20 amino acids, at 1 mM or 2 mM, to avoid saturation of UV peak detection. The linearity of amino acid concentrations was tested at 4 concentration levels, ranging from 100 μM to 2 mM (the concentration of amino acid solution before derivatization), using UV peak height (Table 1). The regression coefficients were above 0.99 for all amino acids.

D-Form ratio (D/(D + L)) was determined by the g-factor (= $\Delta\varepsilon/\varepsilon$) which was calculated from UV (ε) and CD ($\Delta\varepsilon$) signals [32,37,38]. CD signals from the CD detector (X-LC 3195CD) were converted to degree order: g-facor (deg/AU) was calculated as follows (g-factor = CDsig × 4/2.303 × π/180/AUsig). The g-factor was independent of the concentration of each amino acid and allowed us to determine the D-form ratio of samples at any concentration. The linearity of the g-factor was tested at 9 ratios, ranging from 0% to 100% D-amino acids (D/L ratio = 0/100, 1/99, 5/95, 25/75, 50/50, 75/25, 95/5, 99/1, 100/0) (Fig. 4). To get sufficient sensitivity, the concentration was fixed at 1 or 2 mM. The output on the chromatogram was the g-factor which was calculated automatically from CD and UV peak height. The regression coefficients were above 0.99 for all amino acids. g-Factors in low concentration of amino acids solvents (200 μM or 400 μM) were added in Fig. 4, and g-factor of most amino acids showed same value as that in high concentration (1 mM or 2 mM). However, g-factor of Pro, Lys, and Cys at low concentration were different from that in high concentration because of their low sensitivities on CD detection as shown in Fig. 3. In case of low sensitive amino acids like Pro, Lys, and Cys, the linearity should be determined in sufficient concentration.

For all amino acids tested, a specific negative signal on the CD detector was produced. It was also present in the achiral NBD-Gly peak (Fig. 3). The negative signal caused the g-factor to be plotted on a minus value, not a zero point, when the ratio of D-amino acid was 50%. It was caused by minor difference of zero-point on CD detector but it was too difficult to adjust in the true zero-point accurately. Although there was negative difference, it was no problem in the linearity and determination of all amino acids.

Table 3
D-Form ratio and amino acids concentration obtained by CD analysis and OPA analysis.

		Japanese black vinegar				Fermented milk				Yogurt			
		Sakamoto		Shobunsu		Yakult		Pilkul		Bifidus		Megmilk	
		CD	OPA	CD	OPA	CD	OPA	CD	OPA	CD	OPA	CD	OPA
Ser	%D	–	–	10%	8%	–	–	12%	18%	22%	39%	–	–
	Conc.	676	813	515	563	1.0	1.7	6.0	7.3	8.7	13.1	1.8	6.0
Asp	%D	–	–	18%	14%	39%	35%	36%	31%	46%	28%	–	–
	Conc.	258	211	361	316	7.8	6.2	30.1	25.2	6.8	1.4	4.0	0.7
Glu	%D	19%	15%	21%	16%	27%	23%	14%	11%	21%	19%	70%	69%
	Conc.	349	292	307	243	21.3	20.4	108.2	104.3	10.6	9.9	14.2	12.6
Ala	%D	28%	27%	32%	32%	52%	55%	53%	56%	52%	58%	45%	45%
	Conc.	2669	3716	1546	1833	6.1	6.5	30.8	36.8	35.0	47.1	17.4	22.0
Met	%D	9%	12%	8%	7%	–	–	–	–	–	–	–	–
	Conc.	146	217	119	113	2.4	2.4	7.7	7.5	2.8	0.6	0.0	0.1

%D = D form ratio (D/(D + L)) of amino acid (%).
Conc. = DL-amino acid concentration in food samples (μM).

3.4. Method validation

The detection limits (LOD) and the relative standard deviations (RSD) for NBD-derivatized amino acids were obtained using L-amino acids samples (Table 2). The relative standard deviations (RSD) of retention time at high concentration levels of 1 mM or 2 mM and low concentration levels of 100 μM or 200 μM were lower than 0.23% ($n = 6$). The peak height of CD, UV and g-factor at high concentration showed high precision and RSD were not larger than 2.52%. The RSD of some amino acids at low concentration were over 10% because of their low sensitivity. The detection limits (LOD) were calculated from signal to noise ratio at concentrations of 100 or 200 μM (amino acids solution before derivatization). The LODs of NBD-derivatized amino acids in CD detection ranged from 11 to 64 pmol/injection and varied according to the amino acid. The LODs in UV detection were relatively low compared with CD detection and ranged from 4.9 to 23 pmol/injection. In this method, although HFBA was not added in mobile phase B, reproducible analysis was possible. The peak height of Tyr had a tendency to decrease with time: it was stable for 30 min, and started to decrease after 40 min (data not shown).

3.5. Food analysis

Sample pretreatment was necessary to remove any contaminants as these may have interfered with derivatization and column separation. Deproteination and delipidation were performed using sulfosalicylic acid and 10 kDa cut-off filters then amino acids were extracted from other low molecular weight substrates and concentrated by cation-exchange type SPE column and evaporation under reduced pressure. The recovery rates of the typical amino acids in SPE were 78% (Ser), 52% (Asp), 65% (Glu), 80% (Ala), 78% (Met). The amino acid concentrations of Japanese black vinegar, fermented milk, and yogurt samples were adjusted to the appropriate range for CD detection through the pretreatment methods. For the extraction solvent, triethylamine was used instead of ammonia. An NBD-NH_2 peak which derived from the ammonia co-eluted and interrupted the detection of the NBD-Asp peak.

The proposed analytical method was applied to determine D- and L-amino acids in fermented foods (Japanese black vinegar, fermented milk, and yogurt). The results are shown in Table 3. Amino acid concentrations and D-form ratios measured using UHPLC were compared with measurements using the existing method (OPA method). The typical chromatograms of Japanese black vinegar (Shobunsu) were shown in Fig. 5.

In the OPA method, the relative standard deviations (RSD) of retention time were lower than 0.3% and peak heights were lower than 5% ($n = 6$). The linear range was 1–100 μM (amino acids concentration before derivatization) in this study.

The g-factor at 1% D-amino acids showed linearity but it was not possible to get same values as OPA method at lower 5% D-amino acids in food samples. Using this method, accurate identification was achieved when the ratio was greater than 5% D-amino acids.

D-Ser, D-Asp, D-Glu, D-Ala and D-Met were found in the food samples. These results were similar to that were already reported [10–15]. Especially D-Ala, D-Glu and D-Asp were included in any

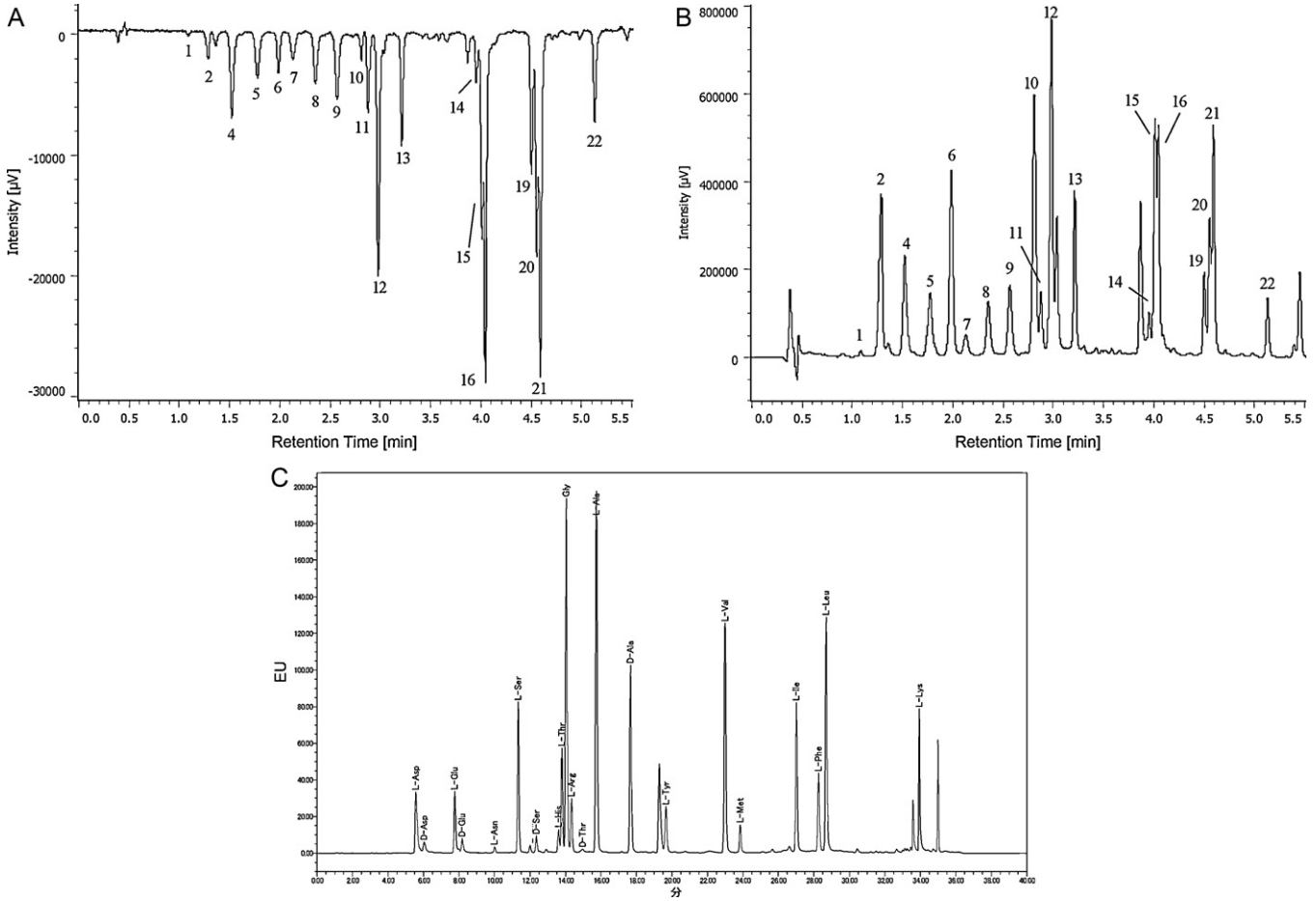

Fig. 5. CD chromatogram (A) and UV chromatogram (B) of NBD-amino acids in Japanese black vinegar (Shobunsu) using CD method. Peak identification is same as in Fig. 3. Chromatogram of amino acids derivatized with OPA in Japanese black vinegar (Shobunsu) using OPA method (C).

fermented foods and thought to be derived from bacterial cell walls. The CD and OPA methods produced the same results, except in a few cases. The *%D* value of D-Ser in Bifidus yogurt differed between the CD and OPA method. It was suggested that unknown contaminants overwrapped the OPA-D-Ser peak in the OPA method because the concentration of Ser in the OPA method was higher than in the CD method. Another significant difference was observed in the %D of D-Asp in Bifidus yogurt. The %D and concentration of Asp in the CD method was much higher than in the OPA method which may have been due to ammonia in the yogurt sample. NBD-NH_2 was co-eluted with NBD-Asp, as previously mentioned. When checked using an amino acid analyzer, the yogurt sample contained more ammonia (11 mM) than any other sample (data not shown).

4. Conclusions

A new D- and L-amino acid analysis method was developed using a CD detector. For sensitive and selective UV and CD detection, 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) was selected as the derivatization reagent. Ultra-high performance liquid chromatography (UHPLC) was coupled with a circular dichroism (CD) detector for rapid analysis. The analysis time for 20 derivatized proteinogenic amino acids was 5.5 min. The linear range was 100 μM to 1 mM or 200 μM to 2 mM which was enough to determine D-amino acids in food samples. D-Form ratio was accurately determined when it was greater than 5% D-amino acids. To determine D- and L-amino acids in fermented foods, a pretreatment method was optimized. The results of the D,L-amino acid ratios and concentrations using the CD method were the same as the OPA method. To investigate the origin of D-amino acids, the formation mechanism, nutritional effect, involvement in taste, etc., it is necessary to analyze many foods under various conditions. The developed method enabled rapid and comprehensive analysis of D- and L-amino acids and will greatly help our study of D-amino acids in the future.

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