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Total resolution of 17 DL-amino acids labelled with a fluorescent chiral reagent, *R*(−)-4-(3-isothiocyanatopyrrolidin-1-yl)-7-(*N*,*N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole, by high-performance liquid chromatography

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Abstract

Total resolution of 17 DL-amino acids after derivatization with a fluorescent chiral tagging reagent, 4-(3-isothiocyanatopyrrolidin-1-yl)-7-(*N*,*N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole [*R*(−)-DBD-PyNCS], was studied by reversed-phase liquid chromatography. The reaction of the reagent with amino acids proceeds effectively at 55°C for 20 min in the presence of 1% TEA to produce the corresponding fluorescent diastereomers (excitation at 460 nm, emission at 550 nm). Each pair of the resulting derivatives was efficiently separated with water–acetonitrile containing 1% acetic acid as the mobile phase. Peak resolution was in the range of 0.92 (DL-Arg)~9.8 (DL-Cys). Although mutual separation of some DL-amino acids was possible using the elution solvent, simultaneous resolution of 17 DL-amino acids was difficult with a single chromatographic run, even if some gradient elutions were adopted. Therefore, both gradient and isocratic elution systems were used for total resolution of the DL-amino acids. Thus, 17 DL-amino acids were well resolved by a gradient and an isocratic elution systems. The proposed derivatization and elution methods were applied to the determination of DL-amino acids in yogurt. The results showed that some of the L-amino acids, i.e., Glu, Asp, Ser, Gly, Ala, Thr, Pro, Lys, Phe and Met, were found in the methanol extracts of yogurt. On the other hand, the D-amino acids that were identified in the extracts were D-Glu, D-Asp and D-Ala, and the mean % to each L-amino acid were 11.9% (D-Glu), 27.6% (D-Asp) and 56.7% (D-Ala), respectively.

Keywords: Derivatization, LC; Diastereomer separation; Amino acids; 4-(3-Isothiocyanatopyrrolidin-1-yl)-7-(*N*,*N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole

1. Introduction

It has been firmly believed that only L-amino acids participate in vital function [1]. However, the presence of D-amino acids has been reported in biological specimens and tissues of higher animals [2,3]. The D-enantiomer(s) may have biological function, e.g., as antagonists. Resolution of amino acid enantiomers has commonly carried out by high-performance

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liquid chromatography (HPLC) either directly with a chiral stationary phase column [4–6] or indirectly after derivatization with a chiral reagent [7,8]. Indirect resolution utilizing a fluorescent chiral tagging reagent is predominant in terms of sensitivity, versatility and separatability. Although a number of fluorescent tagging reagents toward amino acids have been developed, only a few chiral reagents are successfully used for highly sensitive resolution of amino acid enantiomers. The most important reagents are chiral 1-(9-fluorenyl)ethyl chloroformate (FLEC) [9], and *o*-phthalaldehyde (OPA)/chiral thiols such as *N*-acetyl-L-cysteine [10] and *N*-acetyl-D-penicillamine [11]. Although OPA/chiral thiol method is suitable for primary amino acids, secondary amino acids such as proline are not detected directly without any treatment. Another disadvantage is the relative low stability of the resulting isoindole derivatives. On the other hand, (+)-FLEC is a excellent reagent which labels both primary and secondary amino acids, because the resulting fluorescent derivatives are fairly stable and separated well by reversed-phase chromatography. The isothiocyanate type of reagents represented by 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) [12], a chromogenic reagent, is attractive in terms of its reactivity and separatability of the derivatives by reversed-phase liquid chromatography.

We have developed a series of fluorescent chiral isothiocyanete reagents, i.e., 4-(3-isothiocyanatopyrrolidin-1-yl)- 7-(*N*,*N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole (DBD-PyNCS) and 4-(3-isothiocyanatopyrrolidin-1-yl)-7-nitro-2, 1,3-benzoxadiazole (NBD-PyNCS) [*R*(−)- and *S*(+)-enantiomers] [13,14], and have applied them to the separation of racemic amines (peptides and β-blockers, etc.) [15,16] and thiols (tiopronin and penicillamine, etc.) [17,18]. Recently, chiral sequential analyses of D-amino acid(s) containing peptides were also performed using *R*(−)-DBD-PyNCS [19,20]. As described in a previous paper [14], the separations of each pair of DL-amino acids after labelling with the reagents are possible by reversed-phase liquid chromatography. This paper describes resolution of total amino acid enantiomers labelled with *R*(−)-DBD-PyNCS and the application to the determination of DL-amino acids in yogurt.

2. Experimental

2.1. Materials and reagents

4 - (3 - Isothiocyanatopyrrolidin-1-yl) - 7 - (*N*,*N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazoles, [*R*(−)- and *S*(+)-DBD-PyNCS] were synthesized as described previously (m.p. 160–170°C, decomp.) [14]. The reagents are now commercially available from Tokyo Kasei (Tokyo, Japan). Amino acid enantiomers were obtained from Sigma (St. Louis, MO, USA). Triethylamine (TEA) and acetic acid (AcOH) were of special reagent grade (Wako Pure Chemicals, Osaka, Japan). Trifluoroacetic acid (TFA), methanol (MeOH) and acetonitrile (CH_3CN) were of HPLC grade (Wako Pure Chemicals). Deionized and distilled water was used throughout. All other chemicals were of analytical-reagent grade and were used without further purification.

2.2. On-line HPLC–ESI-MS–MS

A Hewlett-Packard HPLC 1100 series (Wilmington, DE, USA) coupled to a Finnigan MAT LCQ ion trap mass spectrometer (San Jose, CA, USA) fitted with an electrospray ionization (ESI) source was used. The separation of reaction products was carried out on an Ultron VX-ODS column (150×4.6 mm, I.D., 5 μm; Shinwa Chemicals, Kyoto, Japan) with water–acetonitrile containing 1% AcOH as a mobile phase at 1.0 ml/min. The ESI capillary temperature and capillary voltage were 275°C and 3.0 V, respectively. The source voltage and source current were 4.8 kV and 100 μA, and the tube lens offset was set at 20.0 V. All spectra were obtained in the positive mode, over a mass range of m/z 100–500, at a range of one scan every 2 s. The collision gas was helium (He), and the relative collision energy scale was set at 30.0% (1.5 eV). Product ions were scanned between 100–500, and the spectra were collected in the form of continuum data.

2.3. HPLC

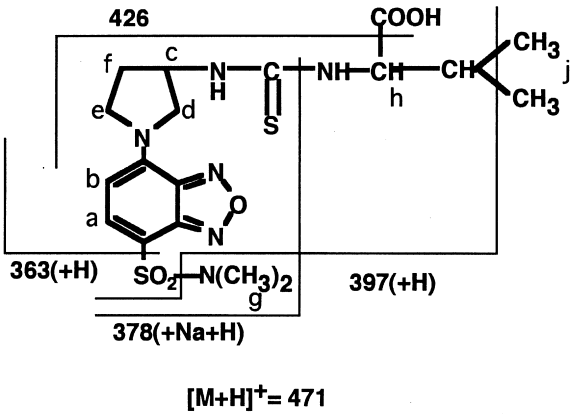
A Shimadzu HPLC system (Kyoto, Japan) consisted of two LC-10AD pumps and an SCL-10A system controller. Sample solutions were injected

with a SIL-10A$_{XL}$ auto injector (Shimadzu). The analytical column was a Wakosil-II 3C18 RS (150× 4.6 mm I.D., 3 μm) for reversed-phase chromatography. The column was maintained at 40°C with a CTO-10A column oven (Shimadzu). A Shimadzu RF-10A$_{XL}$ fluorescence monitor equipped with a 12 μl flow cell was used for the detection of the derivatives. The excitation and emission wavelengths were fixed at 460 nm and 550 nm, respectively. The peak areas obtained from the fluorescence monitor were calculated with a chromatography software Vstation (GL Sciences, Tokyo, Japan), installed in Gateway 2000 computer. All mobile phases were degassed with a Shimadzu on-line degasser (DGU-12A). The flow-rate of the eluent was 1.0 ml/min.

The retention factor (k), separation factor (α) and the resolution (R_s) were calculated according to the following equations: $k=(t_R-t_0)/t_0$, $\alpha=k_2/k_1$ and $R_s=2(t_{R2}-t_{R1})/(W_1+W_2)$, where t_R, t_{R1} and t_{R2} are the peak retention times, t_0 is due to the void volume of the column ($t_0=1.5$ min) and W_1 and W_2 are the widths of the bases formed by triangulation of the peaks.

2.4. Synthesis of the derivatives of DL-valine

To valine (35.45 mg, 0.30 mmol) and *R*(−)-DBD-PyNCS (22.25 mg, 60 μmol) dissolved in 2 ml water–acetonitrile (1:1) was added 1 ml of 3% TEA. After heating at 55°C for 60 min, the reaction solution was evaporated under reduced pressure. The residue was dissolved in acetonitrile and centrifuged at 400 *g* for 5 min and the supernatant was evaporated in vacuo. Then the residue redissolved in benzene–acetic acid (7:1) was applied to a silica gel column (150×15 mm I.D., 75~150 μm; Wako Pure Chemicals). The fluorescent fractions corresponding to DL-valine derivatives of *R*(−)-DBD-PyNCS were eluted with benzene–methanol (95:5, v/v). The fraction collected were evaporated to dryness in vacuo and lyophilized to obtain the yellow powder (yield 85%). The structure of the derivative was identified with NMR and MS spectra. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a JNM GSX 500 NMR spectrometer (Jeol, Tokyo, Japan) at 500 MHz using tetramethylsilane (0.00 ppm) as the internal standard. For describing the NMR characteristics, the following abbreviations are use: s, singlet, d, doublet and m, multiplet. NMR in DMSO-d$_6$ (ppm): 7.83 (1H, d, Jab=8.5 Hz, a), 6.22 (1H, d, Jab=8.5 Hz, b), 4.69 (1H, m, c), 3.9–4.3 (4H, m, d+e), 2.70 (6H, s, g), 2.13 (2H, m, h). MS (*m*/*z*): protonated molecular ion, 471 [M+H]$^+$; product ions obtained from MS–MS (*m*/*z*): 426, 398, 378, 363.



2.5. Derivatization procedure for DL-amino acids

A 10-μl volume of the reagent [10 m*M* *R*(−)-DBD-PyNCS] in acetonitrile and 10 μl of amino acid enantiomers (1 m*M* of each enantiomer) in acetonitrile–water (1:1) and 10 μl of acetonitrile containing 3% TEA were mixed in a 1.5-ml minivial (GL Sciences). The vials were tightly capped and heated at 55°C for 20 min utilizing a dry heat block. The reaction mixture was adequately diluted with acetonitrile and then 5 μl of the solution was injected into the column. The reagent blank without amino acids was also treated in the same manner.

2.6. Sample pretreatment of yogurt

To 1 g of yogurt obtained from the market was added 1.0 ml of methanol and rigorously mixed with a Vortex-mixer. After centrifugation at 1600 *g* for 10 min, 100 μl of the supernatant was separated and evaporated under reduced pressure. The residue was dissolved in 100 μl of water and the solution was used for derivatization. An aliquot (20 μl) of the solution was reacted with the same volumes of 36

m*M* *R*(−)-DBD-PyNCS and 3% TEA in water–acetonitrile at 55°C for 20 min. The solution (3 μl) was injected onto the column for HPLC.

3. Results and discussion

The reaction of amino acids with the fluorescent chiral tagging reagent, *R*(−)-DBD-PyNCS, proceeds in basic medium to form the corresponding diastereomers (Fig. 1). The derivative of DL-valine was synthesized in order to identify the structure. Judging from the NMR and MS spectra, it was obvious that the structure was a thiocarbamoyl derivative.

The aim of the investigation is total resolution of 17 DL-amino acids which are the usual component in the hydrolysate of peptides and proteins. The optimal derivatization conditions and the separation of some DL-amino acids were reported in a previous paper. The proposed derivatization conditions at 55°C for 20 min in aqueous acetonitrile containing 1% TEA as base catalyst were also adopted in the present research.

Initially, the separation of each pair of DL-amino acids was studied by isocratic elutions with water–acetonitrile containing 1% AcOH on a 3 μm ODS column (150×4.6 mm, I.D.). The retention factor (k), separation factor (α) and resolution (R_s) of DL-amino acid derivatives are listed in Table 1. Relatively good separation was obtained for the hydrophobic amino acids such as phenylalanine (Phe), tryptophan (Trp) and cystine (Cys). The D-amino acids, except histidine and arginine, were eluted consistently faster than those of L-enantiomers. The elution order is advantageous because L-amino acids are the dominant components in real samples. Of course, the opposite elution order was observed with the resulting derivatives of *S*(+)-DBD-PyNCS as tagging reagent. Similar separation was also observed with water–acetonitrile containing 0.1% TFA instead of 1% AcOH.

Table 1
Enantiomeric separation of amino acids after derivatization with *R*(−)-DBD-PyNCS

Amino acid	k		α	R_s	Eluent
	D-Isomer	L-Isomer			
His	6.31	5.65	1.12	2.21	a
Arg	6.94	6.66	1.04	0.92	a
Se	25.00	25.68	1.03	1.43	a
Glu	11.10	11.59	1.05	1.25	b
Asp	12.93	13.63	1.06	1.57	b
Thr	15.48	17.32	1.12	3.43	b
Ala	8.53	9.22	1.08	2.25	c
Pro	12.51	13.78	1.10	2.95	c
Cys	31.50	45.63	1.45	9.8	d
Lys	32.65	40.59	1.24	6.69	d
Tyr	4.52	4.87	1.08	1.69	e
Val	6.12	7.02	1.15	3.64	e
Met	6.36	7.08	1.12	2.83	e
Leu	9.52	11.10	1.17	4.53	e
Ile	9.96	11.72	1.18	4.85	e
Phe	10.37	12.60	1.21	5.90	e
Trp	10.43	12.89	1.24	6.32	e

Eluent: CH_3CN–water mixtures of containing 1% acetic acid. CH_3CN contents: a=20%; b=25%; c=30%; d=37%; e=40%

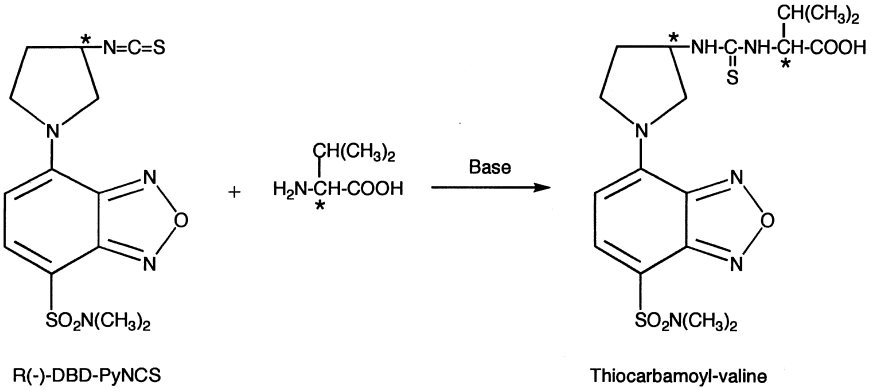


Fig. 1. Reaction of valine with *R*(−)-DBD-PyNCS. Asterisks represent chiral centers.

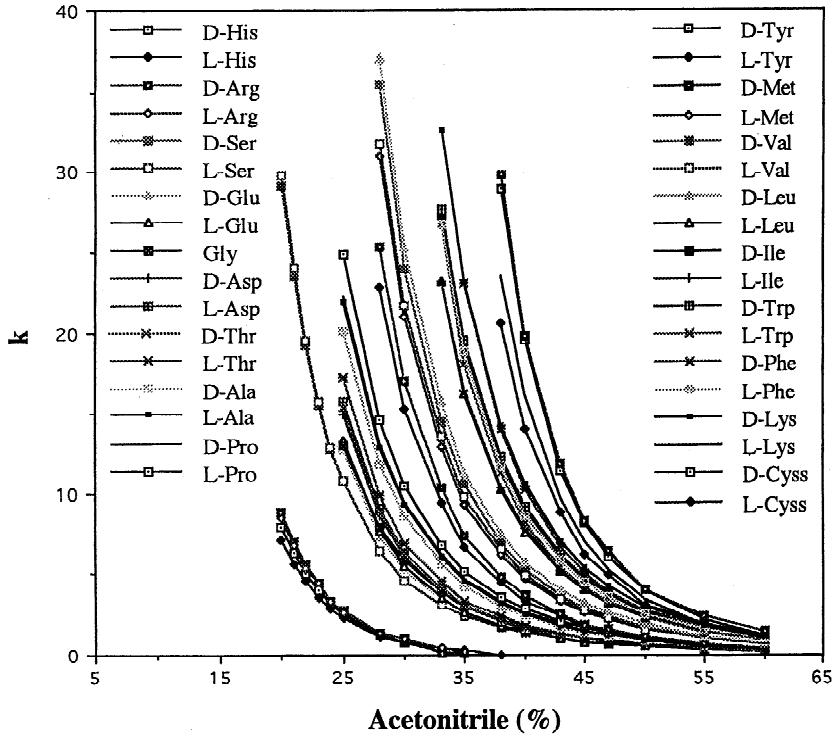


Fig. 2. Effect of acetonitrile in mobile phase on elution of various DL-amino acid derivatives. Mobile phase, water–acetonitrile containing 1% acetic acid. Other HPLC conditions as in Section 2.3.

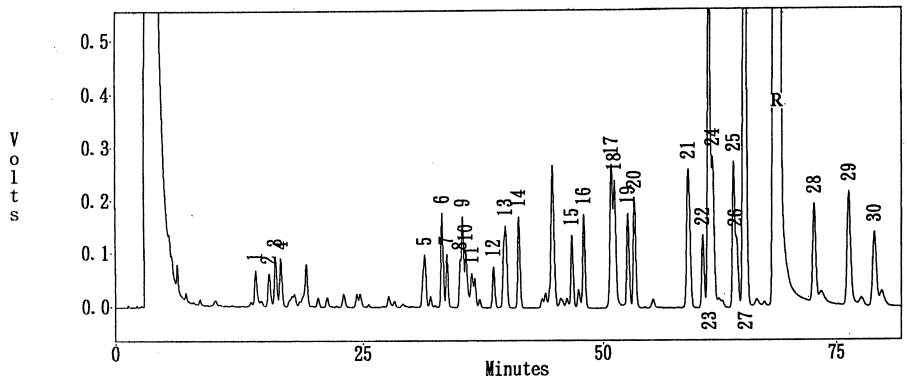


Fig. 3. Separation of 17 DL-amino acid derivatives with gradient elution. Peaks: 1=L-His; 2=D-His; 3=L-Arg; 4=D-Arg; 5=DL-Ser; 6=Gly and D-Glu; 7=L-Glu; 8=D-Asp; 9=D-Thr; 10=L-Asp; 11=L-Thr; 12=D-Ala; 13=L-Ala and D-Pro; 14=L-Pro; 15=D-Tyr; 16=L-Tyr; 17=D-Met; 18=D-Val; 19=L-Met; 20=L-Val; 21=D-Leu and D-Ile; 22=D-Trp; 23=D-Phe, L-Leu and blank peak; 24=L-Ile; 25=L-Trp; 26=L-Phe; 27=blank peak; 28=D-Lys (disubstituted derivative); 29=L-Lys (disubstituted derivative); 30=L-Cys (disubstituted derivative); R=reagent. Eluents: A, 1% AcOH in water; B, 1% AcOH in acetonitrile. Elution: an isocratic of A–B (80:20) for 10 min, a linear gradient from A–B (80:20) to A–B (70:30) for 20 min, a linear gradient from A–B (70:30) to A–B (58:42) for 30 min, and a linear gradient from A–B (58:42) to A–B (55:45) for 30 min. Other HPLC conditions as in Section 2.3.

studied next. Fig. 2 shows the correlation between retention factor (k) and acetonitrile concentration in mobile phase. As shown in Fig. 2, the approximate elution order is as follows: His, Arg, Ser, Gly, Glu, Asp, Thr, Ala, Pro, Tyr, Met, Val, Leu, Ile, Trp, Phe, Lys, Cys. Although it is possible to resolve each pair of DL-amino acids, however, the separation of all amino acids tested is difficult with the isocratic elution and mutual separation of His and Arg, of Gly and Glu, of Asp and Thr, of Ala and Pro, of Met and Val, and of Leu, Ile, Trp and Phe were observed. Consequently, it seems difficult to resolve all 17 DL-amino acids with a single isocratic run. Therefore, gradient elution method was tried for the total resolution of DL-amino acids, based upon the observation of isocratic elution. Fig. 3 shows a typical chromatogram of the best separation achieved using water–acetonitrile containing 1% AcOH as the solvent. However, some peaks, i.e., D-Ser and L-Ser (peak 5); Gly and D-Glu (peak 6); L-Ala and D-Pro (peak 13); D-Leu, D-Ile, D-Phe and blank (peak 23), were overlapped with each other. The insufficient peak separations were also observed between DL-Asp and DL-Thr (peaks 8, 9, 10 and 11). Thus, the 17 amino acids were divided into five groups according to their hydrophilicity; group I, DL-His, DL-Arg and DL-Ser, group II, Gly, DL-Glu, DL-Asp, DL-Thr, DL-Ala and DL-Pro, group III, DL-Tyr, DL-Met and DL-Val, group IV, DL-Leu, DL-Ile, DL-Trp and DL-Phe, group V, DL-Cys and DL-Lys. The separations of groups I–V with water–acetonitrile containing 1% AcOH by isocratic elution are shown in Figs. 4–8, respectively. However, the separation of some amino acids was still difficult even though group separation was adopted. Therefore, the addition of methanol to the mobile phase and replacing AcOH with TFA were tried to improve the separation. As the results, both separation of Asp and Thr and of Ala and Pro were improved using water–30% methanol in acetonitrile (72:28) containing 0.1% TFA (Fig. 9). It seems to be due to the difference of pH of the acids (pH 2.1 versus pH 2.8), and the addition of protic solvent. However, the mutual separation of DL-Leu, DL-Ile, D-Trp and D-Phe was still insufficient in this solvent system. Since the separation seems to be depending on the pH of the eluent, judging from the results of AcOH and TFA, the separation using 25 mM acetate buffer (pH 5.2), that is higher pH than 1% AcOH, was tried finally. Fig. 10 shows the separation of the racemic mixtures of Tyr, Val, Met, Leu, Ile, Trp, Phe and Lys using 25 mM acetate buffer (pH 5.2)–acetonitrile by gradient elution. As shown in Fig. 10, all eight DL-amino acids were completely separated with the buffer solution by the linear gradient elution. Thus, the derivatives of 17 DL-amino acids were separated well using both elution systems: the isocratic elution with water–30% methanol in acetonitrile (72:28) containing 0.1% TFA, and the gradient elution with 25 mM acetate buffer (pH 5.2)–acetonitrile. From these observations, two gradient and one isocratic elution systems were recommended for the resolution of 17 DL-amino acids in real samples. The gradient elution system (G-I) with water–acetonitrile containing 1% AcOH is used for a

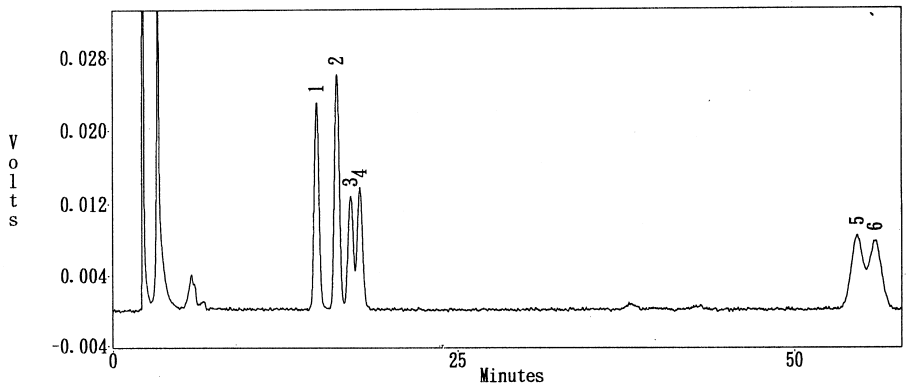


Fig. 4. Separation of DL-His, -Arg and -Ser (group I) with an isocratic elution. Peaks: 1=L-His; 2=D-His; 3=L-Arg; 4=D-Arg; 5=D-Ser; 6=L-Ser. Mobile phase: water–acetonitrile (80:20) containing 1% AcOH. Other HPLC conditions as in Section 2.3.

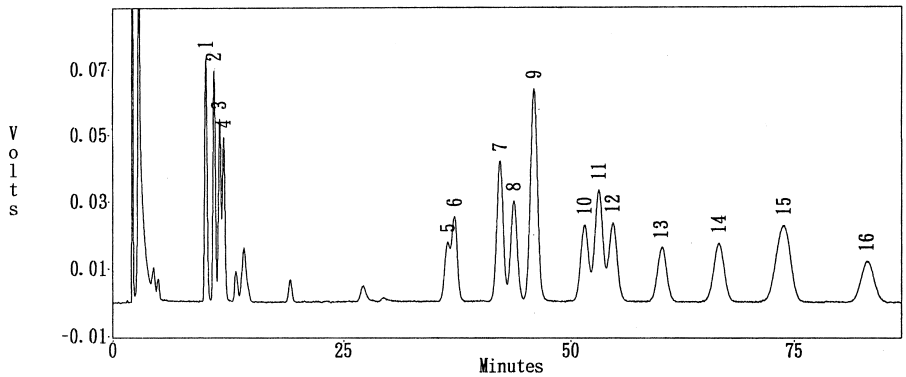


Fig. 5. Separation of DL-Glu,-Asp, -Thr, -Ala and -Pro (group II) with an isocratic elution. Peaks: 1=L-His; 2=D-His; 3=L-Arg; 4=D-Arg; 5=D-Ser; 6=L-Ser; 7=Gly; 8=D-Glu; 9=L-Glu; 10=D-Asp; 11=D-Thr; 12=L-Asp; 13=L-Thr; 14=D-Ala; 15=L-Ala and D-Pro; 16=L-Pro. Mobile phase: water–acetonitrile (78:22) containing 1% AcOH. Other HPLC conditions as in Section 2.3.

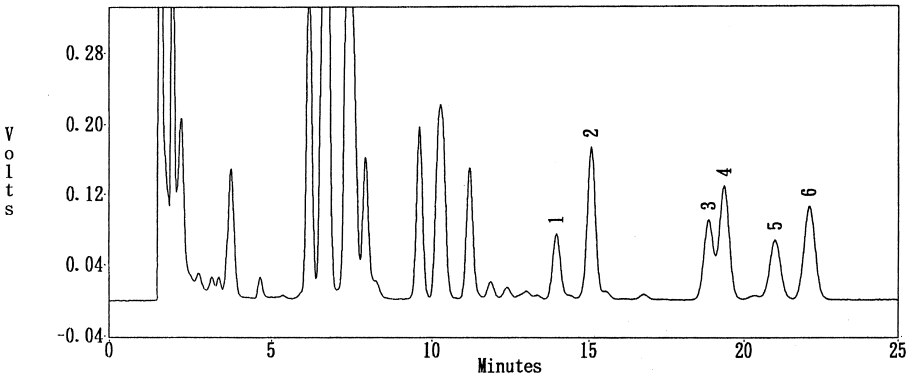


Fig. 6. Separation of DL-Tyr, -Met and -Val (group III) with an isocratic elution. Peaks: 1=D-Tyr; 2=L-Tyr; 3=D-Met; 4=D-Val; 5=L-Met; 6=L-Val. Mobile phase: water–acetonitrile (65:35) containing 1% AcOH. Other HPLC conditions as in Section 2.3.

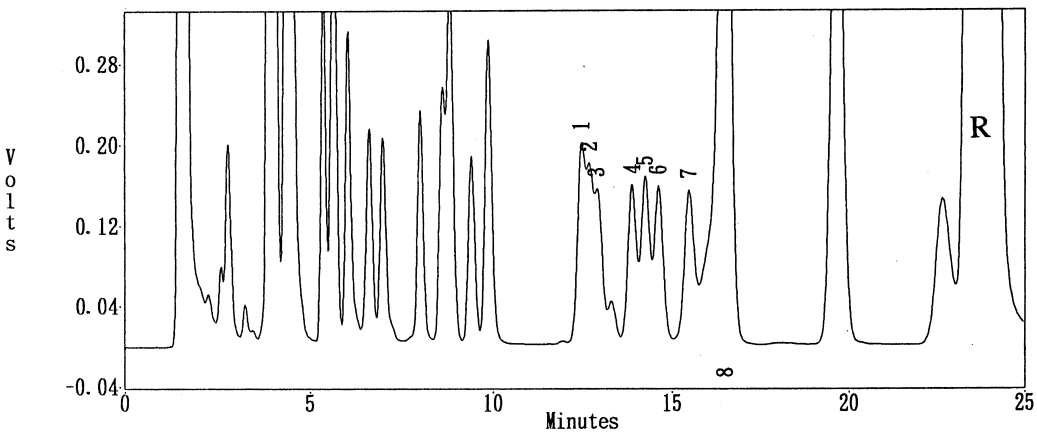


Fig. 7. Separation of DL-Leu, -Ile, -Trp and -Phe (group IV) with an isocratic elution. Peaks: 1=D-Leu; 2=D-Ile; 3=D-Trp; 4=D-Phe; 5=L-Leu; 6=L-Ile; 7=L-Trp; 8=L-Phe; R=reagent. Mobile phase: water–acetonitrile (58:42) containing 1% AcOH. Other HPLC conditions as in Section 2.3.

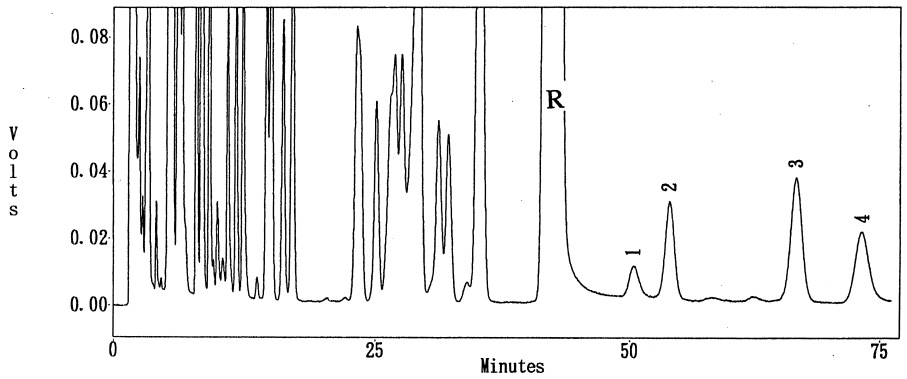


Fig. 8. Separation of DL-Cys and -Lys (group V) with an isocratic elution. Peaks: 1=D-Cys (disubstituted derivative); 2=D-Lys (disubstituted derivative); 3=L-Lys (disubstituted derivative); 4=L-Cys (disubstituted derivative); R=reagent. Mobile phase: water–acetonitrile (63:37) containing 1% AcOH. Other HPLC conditions as in Section 2.3.

preliminary indication of the species of DL-amino acids containing in sample solution (Fig. 3). Then, the species and the concentrations are exactly determined in the isocratic elution system (I-I) and the gradient elution system (G-II): I-I elution, water–30% methanol in acetonitrile (72:28) containing 0.1% TFA for the separation of relative hydrophilic amino acids (His, Arg, Ser, Gly, Glu, Asp, Thr, Ala, Pro) (Fig. 9), and the gradient elution (G-II), a linear gradient from 25 m*M* sodium acetate (pH 5.2)–acetonitrile (80:20) to 25 m*M* sodium acetate (pH 5.2)–acetonitrile (75:25) for 25 min, a linear gradient from 25 m*M* sodium acetate (pH 5.2)–acetonitrile (75:25) to 25 m*M* sodium acetate (pH 5.2)–acetonitrile (72:28) for 35 min, a linear gradient from 25 m*M* sodium acetate (pH 5.2)–acetonitrile (72:28) to 25 m*M* sodium acetate (pH 5.2)–acetonitrile (60:40) for 10 min, and then an isocratic elution of 25 m*M* sodium acetate (pH 5.2)–acetonitrile (60:40) for 10 min, for the separation of relative hydrophobic amino acids (Leu, Ile, Trp, Phe, Lys and Cys) (Fig. 10).

The proposed derivatization and separation methods were applied to the determination of DL-amino acids in food sample. The determination of DL-amino acids in yogurt is described here as an example. Almost all amino acids were of the L-enantiomer, and high concentrations of Glu, Asp, Ser, Gly, Ala,

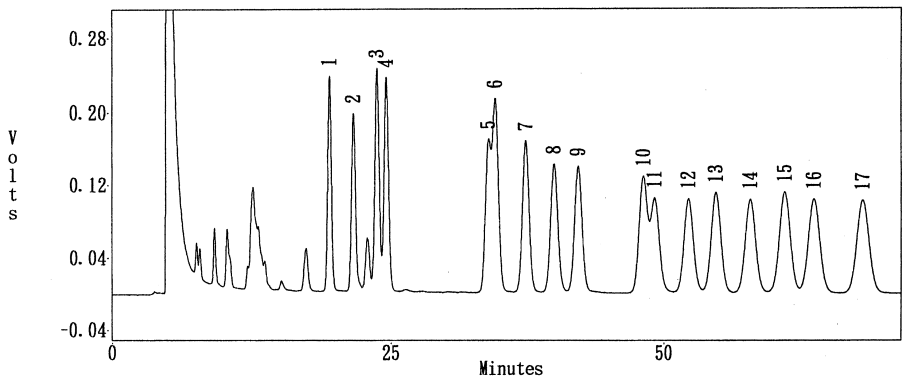


Fig. 9. Separation of hydrophilic DL-amino acids with an isocratic elution. Peaks: 1=L-His; 2=D-His; 3=L-Arg; 4=D-Arg; 5=D-Ser; 6=L-Ser; 7=Gly; 8=D-Glu; 9=L-Glu; 10=D-Thr; 11=D-Asp; 12=L-Asp; 13=L-Thr; 14=D-Ala; 15=D-Pro; 16=L-Ala and 17=L-Pro. Mobile phase: water–30% methanol in acetonitrile (72:28) containing 0.1% TFA. Other HPLC conditions as in Section 2.3.

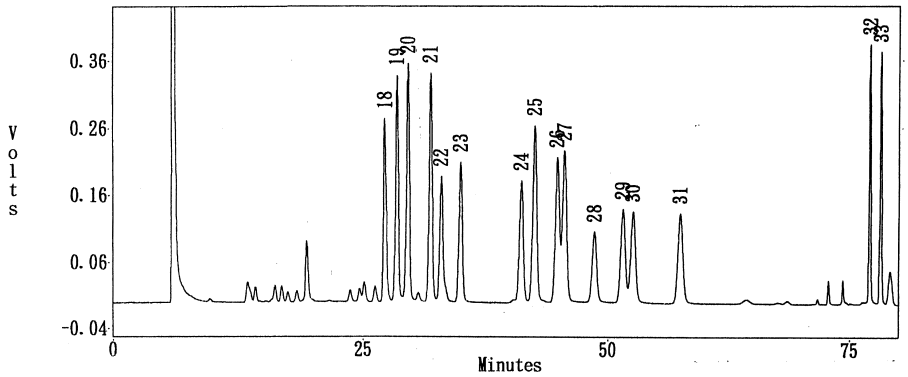


Fig. 10. Separation of hydrophobic DL-amino acids with gradient elution. Peaks: 18=D-Tyr; 19=L-Tyr; 20=D-Val; 21=L-Val; 22=D-Met; 23=L-Met; 24=D-Ile; 25=D-Leu; 26=L-Ile; 27=L-Leu; 28=D-Phe; 29=D-Trp; 30=L-Phe; 31=L-Trp; 32=D-Lys (disubstituted derivative); 33=L-Lys (disubstituted derivative). Eluents: A, 25 m*M* sodium acetate (pH 5.2); B, acetonitrile. Elution: a linear gradient from A–B (80:20) to A–B (75:25) for 25 min, a linear gradient from A–B (75:25) to A–B (72:28) for 35 min, a linear gradient from A–B (72:28) to A–B (60:40) for 10 min, and then an isocratic of A–B (60:40) for 10 min. Other HPLC conditions as in Section 2.3.

Thr, Pro, Lys, Phe, and Met appeared in the methanol extracts of yogurt. Furthermore, the peaks corresponding to the elution time of D-Glu, D-Asp and D-Ala appeared on the chromatogram together with those of L-enantiomers (Fig. 11). The chromatogram obtained from large amount injection of the sample solution was compared with that of amino acid standard solution. To make sure the existence of D-Glu, the determination was also carried out with *S*(+)-DBD-PyNCS instead of *R*(−)-DBD-PyNCS. As shown in Fig. 12, opposite peak height and elution order were observed on the chromatogram obtained from *S*(+)-DBD-PyNCS. The phenomena indicates the existence of D-Glu, D-Asp and D-Ala. The concentrations of each D-amino acid were determined by comparison with L-enantiomers. The average ratios (%) against L-amino acid, calculated from the results of *R*(−)-DBD-PyNCS and *S*(+)-DBD-PyNCS, were 11.9% (D-Glu), 27.6% (D-Asp) and 56.7% (D-Ala), respectively. The results agree with those of previous reports using OPA (D-Glu 16.4%, D-Asp 32.2% and D-Ala 62%) [21] and GC (D-Glu 53-66%, D-Asp 20-32% and D-Ala 64-68%) [22].

In conclusion, the proposed derivatization and chromatographic methods are useful for the determination of small quantities of DL-amino acids in real sample, because of good sensitivity, selectivity and

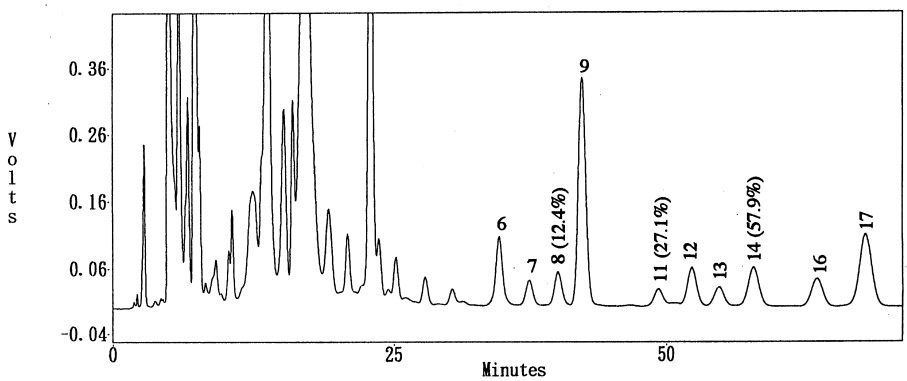


Fig. 11. Chromatogram of the derivatives obtained from the reaction of *R*(−)-DBD-PyNCS with methanol extracts of yogurt. Peaks: 6=L-Ser; 7=Gly; 8=D-Glu; 9=L-Glu; 11=D-Asp; 12=L-Asp; 13=L-Thr; 14=D-Ala; 16=L-Ala and 17=L-Pro. Mobile phase: water–30% methanol in acetonitrile (72:28) containing 0.1% TFA. Other HPLC conditions as in Section 2.3.

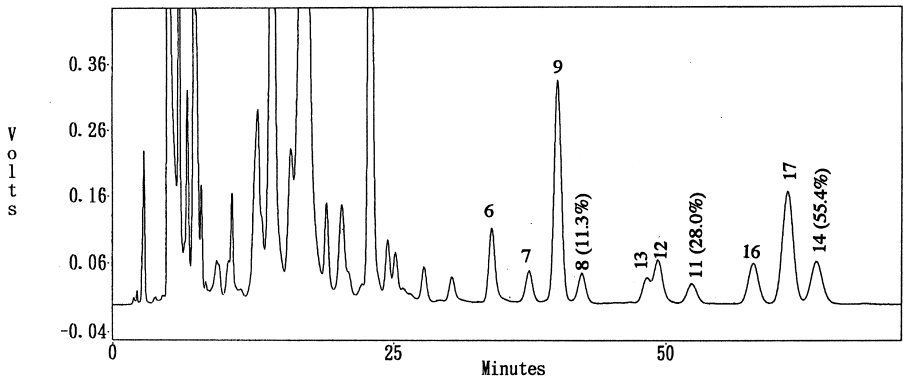


Fig. 12. Chromatogram of the derivatives obtained from the reaction of *S*(+)-DBD-PyNCS with methanol extracts of yogurt. Peaks: 6=L-Ser; 7=Gly; 8=D-Glu; 9=L-Glu; 11=D-Asp; 12=L-Asp; 13=L-Thr; 14=D-Ala; 16=L-Ala and 17=L-Pro. Mobile phase: water–30% methanol in acetonitrile (72:28) containing 0.1% TFA. Other HPLC conditions as in Section 2.3.

separatability. The sensitivity and the separatability of the derivatives seem to be similar, comparing with FLEC. However, an advantage of our reagent is less hydrophobicity than FLEC. Although the separation of FLEC derivatives is usually performed with the eluents containing relatively high concentration of tetrahydrofuran (THF), the addition of THF is not necessary for the separation of DBD-PyNCS derivatives. Thus, the derivatives are eluted with low concentration of organic solvents such as methanol and acetonitrile. Further applications using the proposed procedures are currently in progress in our laboratory.

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