Enantioselective Determination of Extraterrestrial Amino Acids Using a Two-Dimensional Chiral High-Performance Liquid Chromatographic System

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Abstract
A two-dimensional chiral high-performance liquid chromatographic (2D-HPLC) system has been established for the analysis of extraterrestrial amino acids. As the targets, 8 chiral amino acids (alanine (Ala), valine (Val), 2-aminobutyric acid (2AB), norvaline (nVal), N-methylalanine (N-MeAla), isovaline (iVal), 3AB and 3-α-aminoisobutyric acid (3AIB)) and 5 non-chiral amino acids (glycine (Gly), β-Ala, γ-aminobutyric acid (GABA), sarcosine (Sar) and 2AIB) were selected. These amino acids were tagged with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F), and non-enantioselectively separated by a capillary monolithic ODS column in the first dimension. The target fractions were automatically introduced into the second dimension and further separated by Pirkle-type enantioselective columns. By using this system, the 2D-HPLC separation of 21 components in small particles of a carbonaceous chondrite (Yamato 791191, Antarctic CM2 meteorite) could be successfully performed, and all of the target amino acids were observed. The D/L ratios of the chiral molecules are almost 50/50 for all of the tested proteinogenic and non-proteinogenic amino acids.

Keywords: Enantiomer separation; Extraterrestrial amino acid; 2D-HPLC; Meteorite

1. Introduction
Amino acids are essential molecules for higher organisms on Earth. Using so-called proteinogenic L-α-amino acids, all of the proteins are biosynthesized, and the free amino acids also have diverse functions such as signal transduction, regulation of endocrine tissues and gluconeogenesis [1]. These amino acids had been believed to be synthesized chemically in primitive Earth from methane, ammonia, hydrogen and water as their chemical sources, using thunderbolts and/or geotherm as their energy sources [2]. After chemical evolution, peptides and proteins were thought to be produced from the amino acids, and these molecules are believed to be the origins of life on Earth [3,4]. These chemical syntheses are logically not stereoselective, therefore, enantiomers should be equally synthesized. Concerning the amino
acids, the vast majority has an asymmetric carbon at the α-position of the carboxylic acid, and the D- and L-forms are present. However, in the current ecological system on Earth, L-amino acids are predominantly observed in the organisms. On the other hand, the presence of D-amino acids is normally in very trace amounts [5,6], and the origin of this “homochirality” is still unclear.

In 1969, a carbonaceous chondrite fell close to the Murchison village (Victoria, Australia), and in this meteorite, large amounts of glycine (Gly), alanine (Ala) and valine (Val) were found [7]. After the discovery of the Murchison meteorite, several carbonaceous chondrites were investigated and the presence of various amino acids including non-proteinogenic ones was demonstrated [8-15]. For some of the amino acids, an excess of one enantiomer compared to the opposite isomer was also found [16-18]. Therefore, the production of amino acids in cosmic space and the proportions of their enantiomers are matters of interest. Besides the carbonaceous chondrites, more than 1,000 tons of cosmic dust is estimated to land on the Earth every year [19], and these extraterrestrial amino acids are increasingly gathering attention as the origin of amino acids and also the origin of enantiomer excess on Earth.

The aim of the present research is to establish a highly sensitive and selective analytical method for the enantioselective determination of extraterrestrial amino acids, and to clarify the presence of chiral amino acids in small cosmic sample particles. As the cosmic samples, meteorites are widely used. However, meteorites are collected on Earth, and the contamination of terrestrial amino acids is always the problem to be considered. Therefore, several missions to collect cosmic samples in the cosmic space such as the Stardust mission (collecting particles from the comet Wild 2, 2006 NASA [20-22]) and Hayabusa mission (collecting sand from the asteroid Itokawa, 2010 JAXA [23]) are continuously planned and carried out. However, the amounts of the cosmic samples collected in the extraterrestrial space are normally very low (less than 1 mg is usable), and the determination of the amino acid enantiomers is practically difficult due to the lack of sensitivity of the analytical method.

For the determination of the extraterrestrial amino acids, gas chromatographic (GC) methods using chiral stationary phases [17-18] or chiral derivatization reagents [8-10] have already been reported. High-performance liquid chromatographic (HPLC) methods using chiral derivatization reagents have also been described in the literature [15,16]. These methods are powerful tools to determine the major chiral amino acids in the cosmic samples, and the amounts of Gly, 2-aminobutyric acid (2AB), Ala enantiomers, Val enantiomers, norvaline (nVal) enantiomers, isovaline (iVal) enantiomers and some other amino acids have been reported in the carbonaceous meteorites. However, in the cosmic samples, a wide variety of amino acids including β-amino acids, α-methyl amino acids and N-methyl amino acids are present, and the determination of small amounts of amino acids is frequently interfered by unknown substances. Concerning the detection sensitivity, the detection limits of most of the already reported methods are 100 fmol - 1 pmol and more than 1 mg of cosmic samples are needed for the amino acid analysis. For the determination of trace amounts of chiral amino acids, a two-dimensional (2D) HPLC approach combining reversed-phase and enantioselective columns is useful, and widely used for the analysis of small amounts of D-amino acids in complicated biological matrices [24-27]. Therefore, in the present study, a highly selective 2D chiral HPLC method has been established focusing on the analysis of extraterrestrial amino acids, and applied to the enantioselective determination of amino acids in small particles (less than 1 mg) of the cosmic samples.

2. Experimental
2.1. Materials
Enantiomers of Ala and 2AB were obtained from Wako Pure Chemicals (Osaka, Japan). Enantiomers of Val and nVal were purchased from Nacalai Tesque (Kyoto, Japan) and Sigma (St. Louis, MO, USA), respectively. Enantiomers of Val were obtained from Nagase (Osaka, Japan), and enantiomers of 3AB, 3-aminoisobutyric acid (3AIB) and N-methylalanine (N-MeAla) were the products of Watanabe Chemical Industries (Hiroshima, Japan). Achiral amino acids (Gly, β-Ala, 2AIB, γ-aminobutyric acid (GABA) and sarcosine (Sar)) were purchased from Wako Pure Chemicals. Acetonitrile (MeCN) of HPLC grade and 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) were obtained from Nacalai Tesque and Tokyo Kasei (Tokyo, Japan), respectively. Methanol (MeOH) of HPLC grade, trifluoroacetic acid (TFA), and formic acid were purchased from Wako. Water was purified using a Milli-Q gradient A 10 system (Merek Millipore, Billerica, MA, USA). All other reagents were of the highest reagent grade and were used without further purification.

2.2. Sample preparation
Yamato 791191 meteorite was collected in 1979 on the bare ice field close to the Yamato mountain range in Antarctica. Yamato 791191 is classified as a CM2 chondrite containing 1.99% carbon [28]. The sub sample (no. 82) was obtained from the inner part at the National Institute of Polar Research. After the meteorite fragment was powdered on a clean bench, the sample powder (3.91 mg) was placed in an amino acid-free glass vial, and doubly distilled HCl (about 5.8 M, 100 μL) was added. After heating at 105°C for 20 h, the reaction mixture was dried under reduced pressure. To the residue, H2O and aqueous...
1 M NaOH was added to make a 10 μg meteorite / μL solution (pH 7). An aliquot of this solution (20 μL) was placed in a light shielded vial, and 400 mM Na-phosphate buffer (pH 9, 20 μL) was added. After adding 5 μL of 200 mM NBD-F in anhydrous MeCN, the vial was heated at 60°C for 2 min. An aqueous 2% (v/v) TFA solution (55 μL) was then added, and 10 μL of the reaction mixture was subjected to the 2D-HPLC system. All glassware used in this study were baked at 500°C for 3 h prior to use.

2.3. 2D-HPLC determination of amino acid enantiomers

The amino acids were determined by a 2D-HPLC system (NANOSPACE SI-2 series, Shiseido) combining a microbore-monolithic ODS column and narrowbore-enantioselective columns. The 2D-HPLC system consisted of 5 pumps (3101 and 3201), a 3202 degasser, an auto-sampler (NASCA), two column ovens (3004 and 3014), two high-pressure valves (3011 and 3012), a laboratory-made multi-loop valve (set of 9 loops (300 µL)) and two fluorescence detectors (3213). After the amino acids were pre-column derivatized with NBD-F as shown in the section 2.2., the amino acids were separated by a reversed-phase column as their NBD-derivatives. NBD-F is a fluorescent carboxylic acids [29]. The fractions of the target NBD-amino acids were collected on-line in the multi-loop device, and automatically introduced into the enantioselective columns representing the second dimension to separately determine the D and L forms. As the target extraterrestrial amino acids, 8 chiral amino acids (Ala, Val, 2AB, nVal, αMeAla, 2AB, Val, nVal and iVal) or containing 0.02% formic acid (for β-Ala, GABA, 3AB and 3AIB) at the flow rate of 200 μL / min. Fluorescence detection of the NBD-amino acids was carried out at 530 nm with excitation at 470 nm. All the detector responses were monitored using an EzChrom Elite system, and high-pressure valves and a multi-loop valve were controlled by a KSAA valve controlling system (Shiseido).

3. Results and discussion

3.1. Reversed-phase separation of extraterrestrial amino acids as their NBD-derivatives

In the present 2D-HPLC system, the extraterrestrial amino acids were separated by a reversed-phase column as their D plus L mixtures in the first dimension. The fractions of the target NBD-amino acids were collected on-line in the multi-loop device, and automatically introduced into the enantioselective columns representing the second dimension to separately determine the D and L forms. As the target extraterrestrial amino acids, 8 chiral amino acids (Ala, Val, 2AB, nVal, αMeAla, iVal, 3AB and 3AIB) and 5 non-chiral amino acids (Gly, β-Ala, GABA, Sar and 2AIB) were selected. The structures of these amino acids are shown in Fig. 1. In order to improve the detection sensitivity and to also have the appropriate retention on the reversed-phase column and anion-exchange based enantioselective columns, the amino acids were pre-column derivatized with NBD-F. NBD-F is a fluorescent reagent against primary and secondary amines, and by the reaction, the amino acids are derivatized to form fluorescent carboxylic acids [29]. For the reversed-phase separation of the extraterrestrial
amino acids as their NBD-derivatives, various microbore-ODS columns were tested. As a result, a capillary monolithic ODS column of 0.53 mm i.d. x 1000 mm gave better separation than the packed ODS columns and was used for this experiment. Among the 13 tested amino acids, N-MeAla and 2AB showed similar retention factors, and their separation was difficult. However, by increasing the column temperature, baseline separation of N-MeAla and 2AB could be obtained at 45°C. Therefore, the gradient elution conditions of MeCN were optimized at 45°C, and a linear gradient from aqueous 7% MeCN to 20% MeCN containing 0.05% TFA for 150 min followed by the isocratic elution using aqueous 20% MeCN containing 0.05% TFA was selected. By using these conditions, the 13 target NBD-amino acids were sufficiently separated within 180 min (Fig. 2).

3.2. Enantioselective separation of extraterrestrial amino acids as their NBD-derivatives

In the second dimension, the enantiomers of 8 chiral amino acids (Ala, Val, 2AB, nVal, iVal, 3AB and 3AIB) were separated. As the chiral stationary phases, 14 Pirkle-type columns were tested, and the structures of the chiral stationary phases of these columns are depicted in Fig. 3.

Sumichiral OA-2000 series columns have a 3,5-dinitrophenyl group and an amino acid (as a chiral selector) with the amide-type spacer. The Sumichiral OA-3000 series columns and KSAACSP-001S column have a urea-type spacer between the 3,5-dinitrophenyl group and the amino acid chiral selector. The Sumichiral 4000 series columns have 2 chiral centers. As the mobile phases, mixed solutions of MeOH and MeCN containing formic acid were used.

For each NBD-amino acid and for each Pirkle-type column tested, the concentration of the formic acid and the composition of MeOH / MeCN were selected to provide the appropriate retention. Separation factors of the NBD-amino acids are summarized in Table 1. The elution orders of the enantiomers are also shown by indicating the enantiomers that eluted faster. Concerning the α-amino acids having common structures (Ala, Val, 2AB and nVal), the enantiomers were sufficiently separated by many of the tested narrowbore Pirkle-type columns. On the other hand, the enantiomer separations of N-MeAla (N-methyl amino acid), iVal (α-methyl amino acid), 3AB and 3AIB (β-amino acids) were relatively difficult. Especially, the structural difference of the iVal enantiomers is small (only the methyl group and ethyl group were directly connected to the α-carbon), and only the Sumichiral OA-46000SS column could selectively recognize the NBD-β-iVal ((R)-2-amino-2-methylbutanoic acid) and l-iVal. However, the enantiomers of NBD-Ala and NBD-2AB were not separated by the OA-46000SS column. Therefore, the KSAACSP-001S column was selected for the enantiomer separations of Ala, Val, 2AB, nVal and N-MeAla (as their

Fig. 2. Separation of NBD-amino acids as their D plus L mixtures using a monolithic ODS column.

Fig. 3. Structures of Pirkle-type chiral stationary phases tested. OA-2000 series have an amide-type spacer between 3,5-dinitrophenyl group and an amino acid chiral selector. OA-3000 series and KSAACSP-001S have a urea-type spacer. OA-4000 series have 2 chiral centers.
Table 1. Separation factors of NBD-amino acid enantiomers obtained by various Pirkle-type chiral stationary phases.

<table>
<thead>
<tr>
<th></th>
<th>Ala</th>
<th>Val</th>
<th>2AB</th>
<th>nVal</th>
<th>N-MeAla</th>
<th>iVal</th>
<th>3AB</th>
<th>3AIB</th>
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<tbody>
<tr>
<td>OA-2000S</td>
<td>1.05 D</td>
<td>-</td>
<td>1.04 D</td>
<td>1.04 D</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>OA-1500S</td>
<td>1.15 D</td>
<td>1.14 D</td>
<td>1.18 D</td>
<td>1.15 D</td>
<td>1.05 L</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>OA-3200S</td>
<td>1.28 D</td>
<td>1.17 D</td>
<td>1.25 D</td>
<td>1.28 D</td>
<td>1.12 D</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>OA-3400S</td>
<td>1.37 D</td>
<td>1.26 D</td>
<td>1.31 D</td>
<td>1.37 D</td>
<td>1.04 L</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>OA-3300S</td>
<td>1.14 D</td>
<td>1.06 D</td>
<td>1.11 D</td>
<td>1.11 D</td>
<td>1.06 L</td>
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<tr>
<td>KSAACSP-001S</td>
<td>1.23 D</td>
<td>1.15 D</td>
<td>1.21 D</td>
<td>1.23 D</td>
<td>1.20 D</td>
<td>-</td>
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<td>-</td>
<td>1.11 D</td>
<td>1.14 L</td>
<td>1.05 L</td>
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<tr>
<td>OA-4100SR</td>
<td>1.14 D</td>
<td>1.12 D</td>
<td>1.14 D</td>
<td>1.17 D</td>
<td>-</td>
<td>-</td>
<td>1.05 L</td>
<td>-</td>
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<tr>
<td>OA-4400SS</td>
<td>1.17 L</td>
<td>1.10 L</td>
<td>1.15 L</td>
<td>1.14 L</td>
<td>-</td>
<td>-</td>
<td>1.16 L</td>
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<tr>
<td>OA-4500SR</td>
<td>-</td>
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<td>-</td>
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<td>1.03 L</td>
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<tr>
<td>OA-4600SS</td>
<td>-</td>
<td>1.05 L</td>
<td>-</td>
<td>1.07 D</td>
<td>1.18 D</td>
<td>1.05 L</td>
<td>1.27 L</td>
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<tr>
<td>OA-4700SR</td>
<td>1.26 D</td>
<td>1.24 D</td>
<td>1.25 D</td>
<td>1.34 D</td>
<td>-</td>
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<td>-</td>
<td>1.17 L</td>
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<tr>
<td>OA-4900SS</td>
<td>1.07 L</td>
<td>1.07 L</td>
<td>1.08 L</td>
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<tr>
<td>OA-4900SR</td>
<td>-</td>
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<td>-</td>
<td>1.14 D</td>
</tr>
</tbody>
</table>

Flow rate, 200 μL/min. D/L, enantiomer eluted faster.

NBD-derivatives. As shown in Table 1, the enantiomers of these 5 amino acids were nicely separated by the KSAACSP-001S column. For the separation of iVal, 3AB and 3AIB as their NBD-derivatives, the OA-4600SS column was selected.

For the enantiomer separation of the proteinogenic α-amino acids as their NBD-derivatives, various reports have already been published. Using Pirkle-type enantioselective columns, such as the Sumichiral OA-2500S and OA-3200S, a variety of NBD-proteinogenic amino acids could be separated [24-26, 30-33]. By using the quinine based anion-exchange type chiral stationary phases, enantiomers of the NBD-amino acids were also nicely separated [27,34]. In the present study, enantiomers of the NBD-amino acids having common structures (Ala, Val, 2AB and nVal) were frequently separated by using the Sumichiral OA-2000 and 3000 series columns, and these results are consistent with the previous reports. However, concerning iVal (α-methyl amino acid), structures of the enantiomers are similar, and they were not separated by the OA-2000 and 3000 series columns; the enantiomers were separated only by the OA-4600SS column. Separations of the β-amino acid enantiomers (3AB and 3AIB) were also difficult due to their weak retention to the Pirkle-type columns, however, they could be separated using weak eluting mobile phases.

3.3. Establishment of a 2D-HPLC system and validation of the method

Combining the capillary monolithic ODS column (ML-1000, 0.53 mm i.d. x 1000 mm) and narrowbore-enantioselective columns (KSAACSP-001S and OA-4600SS, 1.5 mm i.d. x 250 mm), a 2D-HPLC system was established. The flow diagram is illustrated in Fig. 4. After the reversed-phase separation of 13 extraterrestrial amino acids as their scalemic mixtures in the first dimension, peaks of the NBD-amino acids were monitored by a fluorescence detector. The fractions of the target NBD-amino acids were trapped to the multi-loop device by switching the high-pressure valve and the multi-loop valve, then further separated by the enantioselective columns representing the second dimension. In the second dimension, the fractions of NBD-tagged Gly, Sar, Ala, 2AB, N-MeAla, 2AB, Val and nVal were automatically introduced into the KSAACSP-001S column, and those of iVal, β-Ala, GABA, 3AB and 3AIB were also automatically introduced into the OA-4600SS column. By using these two enantioselective columns, the D- and L-amino acids and also the achiral amino acids were simultaneously and independently determined. The separation conditions are described in section 2.3. As shown in Fig. 5, all of the NBD-tagged extraterrestrial amino acids including 8 chiral amino acids and 5 achiral amino acids were nicely separated.

The present 2D-HPLC system was validated by the calibration line and between-run precision of the target 21 compounds (enantiomers of 8 amino acids plus 5 achiral amino acids). The results are summarized in Table 2. The calibration lines for all of the target amino acid enantiomers were linear with correlation coefficients higher than 0.999. The between-run precision was practically sufficient, and the values were around 5% for most of the amino acid enantiomers. The detection sensitivity of the present 2D-HPLC system was high, and the LOQ (limit
of quantitation) values were 2 fmol (per injection) for most of the amino acids.

Until now, several methods have been reported for the enantioselective analysis of extraterrestrial amino acids. Concerning GC, the separation of chiral amino acids as their diastereomeric derivatives (N-trifluoroacetyl-D-butyl ester) [7-11] was widely performed. The chiral stationary phase approach using Chirasil-L-Val [17,18] was also frequently used. By using these methods, several proteinogenic amino acids as well as non-proteinogenic ones were simultaneously determined. However, by the diastereomeric GC method, the enantiomers of N-MeAla, iVal and 3AB could not be separated. By the Chiralsil-L-Val method, the enantiomers of N-MeAla and 3AB were not separated. Concerning HPLC, the diastereomer formation method using o-phthaldialdehyde (OPA) plus chiral thiol (N-acetyl-L-cysteine, NAC) has been reported [15]. A time-of-flight mass spectrometer (TOF-MS) was used as the detector, and this LC-MS method is one of the most sensitive and selective approaches. However, the OPA method could not be applied to secondary amines, such as N-MeAla and Sar, and also the 2AB and 3AIB enantiomers are not separated. By the 2D chiral HPLC method established in the present study, the simultaneous determination of 13 amino acids including 8 chiral amino acids has been accomplished. The present method involves the pre-column derivatization with NBD-F, and the both primary and secondary amino acids could be the targets. For the enantiomer separation, multiple enantioselective columns could be used for the second dimension of the 2D-HPLC system, and in the present method, the enantiomers of all 8 target chiral amino acids were nicely separated using the Sumichiral OA-4600SS and KSAACP-001S columns. Concerning the sensitivity, LOQ of the GC method is about 500 fmol [35], and that of the HPLC diastereomer method is about 1 pmol [36-38]. The detection sensitivity of the LC-MS method [15] is in the fmol - sub fmol range. By the present 2D-HPLC method, LOQ for most of the chiral amino acids is 2 fmol and a highly sensitive analysis of extraterrestrial amino acids could be performed in addition to the highly selective two-dimensional analysis.

3.4. Determination of amino acids in the Yamato meteorite

By using the 2D chiral HPLC system described in section 3.3., amino acids in the Yamato 791191 meteorite were determined. The Yamato 791191 meteorite is a carbonaceous chondrite collected in 1979 in Antarctica close to the Yamato mountains. To avoid possible contamination of terrestrial amino acids on the meteorite surface, the inner fragment of meteorite was obtained by chipping and pulverized on a clean bench. After
hydrolysis using HCl, the amino acids were derivatized with NBD-F and only the reaction mixture derived from the small particles (20 µg of the meteorite) was subjected to the 2D-HPLC system. The obtained chromatograms are shown in Fig. 6. In the first dimension, peaks of the amino acids could not be clearly observed (except Gly and Ala). However, in the second dimension, most of the amino acids were nicely observed, and the chiral amino acids turned out to be almost racemic mixtures. The RSD values of the between-run precision using a meteorite sample were around 5% for all the amino acids, and the recovery values were also around 100% (although the recovery values for the α-methyl amino acids (2AIB and iVal) were relatively low (66.0-67.4%)). The amounts of amino acids in the Yamato 791191 meteorite are summarized in Table 3.

Concerning the amino acid amounts in the carbonaceous chondrite, various reports have already been published. In a famous carbonaceous chondrite, the Murchison meteorite, various amino acids were found [13,15-17], and the amounts were as follows (Gly, 26-103 nmol/g; β-Ala, 9-16 nmol/g; Ala, 14-90 nmol/g; 2AIB, 27-145 nmol/g; 2AB, 4-16 nmol/g; iVal, 21-53 nmol/g; Val, 2-23 nmol/g; nVal, 0.3-4 nmol/g). The amounts of extraterrestrial amino acids (as D plus L mixtures) obtained in the present study are consistent with these reports. Concerning the D/L ratio, an excess of the L-enantiomer is also observed in some of the reports [16,17]. However, in the present study, all of the chiral amino acids were found to be almost racemic mixtures. The inconsistency might due to the difference in the cosmic samples and/or the selectivity of the methodology, and further detailed investigations should be needed.

4. Conclusion

In the present investigation, a 2D-HPLC system has been established, and the amounts of extraterrestrial amino acids in the Yamato 791191 meteorite were determined. All of the tested amino acids were present in the meteorite, and the amounts were relatively high for some of the amino acids (higher than 10 nmol/g meteorite). Concerning the ratios of the D and L-forms, the values are almost 50/50 for all the chiral amino acids tested. Because the racemization of amino acids during acid hydrolysis is negligible [39], these results indicate that relatively large amounts of amino acids are actually present in extraterrestrial space, and their enantiomeric ratios are almost racemic (at least in the area where the Yamato 791191 meteorite had been formed). Further studies using other meteorites and also the samples collected in the cosmic space are in progress.

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