High-Performance Liquid Chromatographic Determination of Chiral Amino Acids Using Pre-Column Derivatization with o-Phthalaldehyde and N-tert-Butyloxycarbonyl-D-cysteine and Application to Vinegar Samples

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Abstract

A reversed-phase high-performance liquid chromatographic (HPLC) method using pre-column derivatization with o-phthalaldehyde (OPA) plus N-tert-butyloxycarbonyl-D-cysteine (Boc-D-Cys) has been developed for the determination of aspartic acid (Asp), serine (Ser) and alanine (Ala) enantiomers. D-Amino acids in the real world samples are trace in most cases, and their small peaks should be eluted faster than the huge peaks of the L-forms in order to avoid overlapping. Amino acids were rapidly derivatized at room temperature with OPA plus Boc-D-Cys under simple conditions and were detected by their fluorescence. The target amino acid enantiomers were separated within 60 min on a reversed-phase column, CAPCELL PAK C18 MG II (4.6 x 200 mm), and their resolution values were higher than 2.14. The developed system was successfully validated using standard amino acids, and sufficient calibration lines ($r^2 > 0.9983$) and precision (RSD < 5.29%) results were obtained. In a Japanese traditionally fermented amber rice vinegar, all of the target D-amino acids were observed, and their %D values were 21.5 for Asp, 6.8 for Ser and 22.9 for Ala.

Keywords: Amino acids; Enantiomer separation; HPLC; Derivatization; N-tert-Butyloxycarbonyl-D-cysteine

1. Introduction

All proteinogenic amino acids, except for glycine, have a chiral carbon at the α-position, and the optical isomers, the D- and L-forms, are present. It has been believed for a long time that higher animals utilize only L-amino acids in their bodies. Along with the advances in analytical methodologies, however, various D-amino acids have been found in mammals including humans [1-3]. In addition, their distributions and physiological functions have been gradually revealed during last two or three decades. For instance, D-serine (Ser) is localized in the cerebrum and the hippocampus [4,5], and its function to regulate neurotransmission was clarified [6,7]. D-Aspartic acid (Asp) is reported to control the hormonal synthesis and secretion, such as testosterone and melatonin, in various endocrine tissues [8]. D-Alanine (Ala) is rich in the anterior pituitary gland [9] and in the islets of Langerhans of the pancreas [10], and is considered to be involved in the control of the blood glucose level. Recent studies also demonstrated the D-amino acid functions in the cosmetic area. In the skin epidermis, D-Ser is synthesized from L-Ser by serine racemase and is involved in the formation of the skin barrier [11]. D-Asp in the skin dermis has an anti-oxidant effect, and D-Ala is likely to be involved in the repair/maintenance of the basement membrane [12]. These studies clearly indicate that previously mentioned...
D-amino acids are possible novel bioactive molecules even in humans.

As the origins of D-amino acids in human bodies, biosynthesis, production by intestinal bacteria and uptake from diets are frequently reported [13]. Concerning the dietary origins, various D-amino acids have been determined in foods and beverages [14-16]. Among them, fermented products, such as cheese [17], wine [18], yogurt [19,20] and vinegar [20,21], contain relatively high levels of D-amino acids. Since the orally-administered D-amino acids were reported to be adsorbed from the intestine and distributed to various tissues [22], D-amino acids derived from diets are surely one of the key sources for intrinsic D-amino acids. Thus, the food products containing D-amino acids are expected to be beneficial for our health, and a simple analytical method applicable to various foods and beverages is required in order to evaluate the D-amino acid contents in these matrices.

For the determination of chiral amino acids in food/beverage samples, various analytical methods using gas chromatography (GC) [23], capillary electrophoresis (CE) [24] and high-performance liquid chromatography (HPLC) [13,25] have been developed. Among them, a reversed-phase HPLC method using pre-column derivatization with o-phthalaldehyde (OPA) plus N-tert-butyloxycarbonyl-L-cysteine (Boc-L-Cys) has been widely used since the 1980s [26,27]. Amino acids in sample matrices are rapidly converted to diastereomers by derivatization under relatively simple conditions, and most of the proteinogenic amino acid enantiomers can be separated by a reversed-phase column. By using OPA/Boc-L-Cys reagents, however, L-amino acids usually elute faster than the D-forms. In food/beverage samples, L-enantiomers are predominant, and their amounts are approximately 100-1000 times higher than those of the D-forms. For the determination of small amounts of D-amino acids in the presence of large amounts of L-enantiomers, the minor peaks should elute faster than the major peaks in order to avoid severe peak overlapping.

In the present study, OPA plus N-tert-butyloxycarbonyl-D-cysteine (Boc-D-Cys) reagents have been used for the derivatization of amino acids, and a reversed-phase HPLC method has been developed for the determination of the Asp, Ser and Ala enantiomers. By using Boc-D-Cys instead of Boc-L-Cys (Boc-D-Cys has an opposite chiral center, and the amino acid derivatives have opposite chemical properties) as a derivatization reagent, the retention order of the amino acid enantiomers is reversed on a reversed-phase column, and the D-amino acids elute faster than their L-forms. The established method was validated by using standard amino acids and applied to the determination of the Asp, Ser and Ala enantiomers in vinegar samples.

2. Experimental
2.1. Materials
D-Ala, L-Ala, and D-Ser were obtained from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). D-Asp, L-Asp and L-Ser were purchased from Nacalai Tesque (Kyoto, Japan). Methanol (MeOH) of HPLC grade, OPA, acetic acid, boric acid and sodium hydroxide were obtained from Fujifilm Wako Pure Chemical Corporation. Acetonitrile (MeCN) of HPLC grade was purchased from Nacalai Tesque. Boc-D-Cys and Boc-L-Cys were products of Chem-Impex International (Wood Dale, IL, USA) and Sigma-Aldrich (St. Louis, MO, USA), respectively. Water was purified using a Milli-Q Integral 3 system (Merck, Darmstadt, Germany). All other reagents were of the highest reagent grade and were used without further purification.

2.2. Sample preparation
The rice vinegar samples were purchased from a domestic provider in Japan. The traditionally fermented amber rice vinegar was obtained from Sakamoto Kurozu, Inc. (Kagoshima, Japan). The OPA reagent was prepared daily by dissolving 2 mg of OPA and 2 mg of Boc-DL-Cys in 200 μL of MeOH. To 10 μL of the vinegar sample (diluted 100 times with water), 70 μL of 400 mM sodium borate buffer (pH 9.0) and 20 μL of the OPA reagent were added. After storage at 25°C for 2 min, the reaction mixture (10 μL) was injected into the HPLC system described in Section 2.3. Derivatization of amino acids with OPA plus Boc-D-Cys was shown in Fig. 1.

![Fig. 1. Derivatization of amino acids with OPA plus Boc-D-Cys.](image-url)

2.3. HPLC system
The HPLC system consisted of a DG-4580 degasser (Jasco, Tokyo, Japan), three pumps (PU-980 and PU-2080 Plus, Jasco), a 7725i injector (IDEX, Lake Forest, IL, USA), a CO-960 column oven (Jasco), an FP-1520 fluorescence detector (Jasco) and an 807-IT integrator (Jasco). The analytical column was a CAPCELL PAK C18 MGII (4.6 mm i.d. x 200 mm, particle size 5 μm, Osaka Soda, Osaka,
Concerning the elution order, the amino acid derivatives were separated using a linear gradient column (CAPCELL PAK C18 MGII, 4.6 mm i.d. x 200 mm). As the mobile phase, 0.1 M sodium acetate buffer (pH 6.0) containing MeCN was used, and the separation conditions were tested using various concentrations of MeCN. As a result, the diastereomers of the target amino acid derivatives were separated using a linear gradient elution of 5-25% MeCN in 0.1 M sodium acetate buffer (pH 6.0) for 60 min. The obtained chromatogram is shown in Fig. 2A. The resolution values of the D- and L-amino acids were 2.14 for Asp, 4.03 for Ser and 6.18 for Ala. Regarding the elution order, the D-amino acids eluted faster than the L-forms for all the target amino acids. The chromatogram of the standard amino acids derivatized with Boc-L-Cys is shown in Fig. 2B. The retention orders of the D- and L-amino acids were reversed for all the target amino acids and the L-amino acids eluted faster.

For the validation of the present reversed-phase HPLC method, calibration lines, intra-day precision and inter-day precision were checked using standard amino acids. The detection of the amino acid derivatives with OPA plus Boc-D/L-Cys was carried out by the fluorescence emission at 443 nm with excitation at 344 nm.

### 3. Results and discussion

A reversed-phase HPLC system for the chiral amino acid analysis has been developed using the fluorescence derivatization with OPA plus Boc-D/L-Cys reagents. As the target analytes, the enantiomers of Asp, Ser and Ala were selected because their D-forms in foods and beverages are the matters of interest as functional molecules in the human body. The amino acid enantiomers were converted to their diastereomers by derivatization with OPA plus Boc-D/L-Cys, and the 6 target analytes were separated on a reversed-phase column (CAPCELL PAK C18 MGII, 4.6 mm i.d. x 200 mm). As the mobile phase, 0.1 M sodium acetate buffer (pH 6.0) containing MeCN was used, and the separation conditions were tested using various concentrations of MeCN. As a result, the diastereomers of the target amino acid derivatives were separated using a linear gradient elution of 5-25% MeCN in 0.1 M sodium acetate buffer (pH 6.0) for 60 min. The obtained chromatogram is shown in Fig. 2A. The resolution values of the D- and L-amino acids were 2.14 for Asp, 4.03 for Ser and 6.18 for Ala. Concerning the elution order, the D-amino acids eluted faster than the L-forms for all the target amino acids. The chromatogram of the standard amino acids derivatized with Boc-L-Cys is shown in Fig. 2B. The retention orders of the D- and L-amino acids were reversed for all the target amino acids and the L-amino acids eluted faster.

The method developed in the present study was applied to the determination of the Asp, Ser and Ala enantiomers in various vinegar samples. A rice vinegar from a major Japanese provider (company A) and the traditionally fermented amber rice vinegar from Sakamoto Kurozu, Inc., were analyzed. In the rice vinegar from company A, the peaks of all the target L-amino acids were observed as shown in Fig. 3. Concerning the D-forms, trace amounts of D-Asp and D-Ala were detected. On the other hand, the peaks of all the target amino acid enantiomers were

### Table 1. Method validation for the determination of Asp, Ser and Ala enantiomers.

| Amino acids | Calibration line | Precision |
|-------------|------------------|----------|
|             | Calibration range (pmol) | Equation | \( r^2 \) | Intra-day | Inter-day |
| D-Asp       | 0.25-25          | \( y = 2.28x - 0.80 \) | 0.9991 | 1.99 | 3.28 |
| L-Asp       | 1-100            | \( y = 2.78x - 5.85 \) | 0.9983 | 1.68 | 3.54 |
| D-Ser       | 0.25-25          | \( y = 4.41x - 0.66 \) | 0.9997 | 0.95 | 4.51 |
| L-Ser       | 1-100            | \( y = 4.88x - 2.27 \) | 0.9996 | 0.82 | 5.29 |
| D-Ala       | 0.25-25          | \( y = 4.69x + 2.03 \) | 0.9997 | 0.80 | 4.49 |
| L-Ala       | 1-100            | \( y = 5.60x - 2.99 \) | 0.9997 | 0.26 | 4.88 |

Equations were made where \( x \) was the injection amount of the amino acid enantiomers (pmol) and \( y \) was the peak height (mV). Injection amounts of D-amino acids were 0.25, 0.5, 2.5 and 25 pmol, and those of L-amino acids were 1, 2, 10 and 100 pmol. For the precision of the standard (\( n=3 \)), 25 pmol of D-amino acids and 100 pmol of L-amino acids were injected.

Results were summarized in Table 1. The calibration lines were constructed in the range of 0.25-25 pmol/injection for the D-forms and 1-100 pmol/injection for the L-forms. All of the calibration lines obtained for the target amino acid enantiomers were linear with correlation coefficients higher than 0.9983. Regarding the intra-day precision and inter-day precision, standard amino acid solutions containing 25 pmol of the D-forms and 100 pmol of the L-forms were used. As the result, the RSD values of the intra-day precision were 0.26-1.199% (\( n=3 \)), and those of the inter-day precision were 3.28-5.29% (3 days). These results indicated that the developed system enables the quantitative and reproducible analyses of the Asp, Ser and Ala enantiomers.
clearly observed in the traditionally fermented amber rice vinegar as shown in Fig. 4. The %D values (D/(D+L) x 100) of Asp, Ser and Ala were 21.5, 6.8 and 22.9, respectively. In order to confirm the results, the same samples were analyzed by using the OPA plus Boc-L-Cys reagents. The retention orders of the amino acid enantiomers were reversed by the OPA/Boc-L-Cys reagents, and the possible interfering compounds co-eluted with amino acids are likely to be separated. The %D values in the traditionally fermented amber rice vinegar obtained by the OPA/Boc-L-Cys derivatization were 19.0 for Asp, 5.1 for Ser and 22.5 for Ala, and these results were in good agreement with those obtained by the OPA plus Boc-d-Cys reagents.

Several analytical methods have been reported for the determination of the Asp, Ser and Ala enantiomers in vinegar samples. Reversed-phase HPLC methods using OPA plus N-acetyl-L-cysteine or Boc-L-Cys were utilized for the determination of 16 chiral amino acids in various vinegar samples [28]. A biaryl axially chiral derivatizing agent, (R)-4-nitrophényl (N-[2’-(diethylamino)-6,6’-dimethyl-[1,1’-biphenyl]-2-yl]carbamate hydrochloride, was developed for the reversed-phase HPLC system equipped with a tandem mass spectrometer (MS/MS), and 16 D-amino acids were found in the Japanese traditional amber rice vinegar [29]. Without using chiral reagents, a UPLC method equipped with a circular dichroism detector [30] and a chiral HPLC-MS/MS system [31] have been used for the analysis of all the proteinogenic amino acids in the vinegar samples. To perform a higher selective analysis, a two-dimensional (2D) HPLC system combining reversed-phase and enantioselective columns [32,33] and a 2D-HPLC-MS/MS system [34] have been developed and clarified the developmental changes of the D-amino acid concentrations during the fermentation processes. By using these analytical methods, the %D values of Asp, Ser and Ala in the rice vinegar samples were reported to be 1.3-

**Fig. 3.** Analysis of Asp, Ser and Ala enantiomers in the rice vinegar from a domestic provider.

**Fig. 4.** Analysis of Asp, Ser and Ala enantiomers in the traditionally fermented amber rice vinegar.

35.3, 0.9-21.6 and 1.1-38.3, respectively [28-30,32,34]. The D-amino acid amounts in the traditionally fermented rice vinegars seem to be higher than those in the other rice vinegars. In the present study, the Asp, Ser and Ala enantiomers were measured in two vinegar samples by the reversed-phase HPLC system using fluorescence derivatization with OPA plus Boc-d-Cys, and the obtained results were consistent with those in the previous reports.

Among the previously mentioned analytical methods for chiral amino acid analysis, the OPA derivatization (especially in combination with Boc-L-Cys) could be carried out by simple and rapid conditions (2 min at 25°C), and sufficient separations of the target amino acid enantiomers were achieved. By using Boc-L-Cys, however, the retention orders of the amino acid enantiomers (L-forms eluted faster than D-forms) sometimes cause a problem for the accurate analysis of D-amino acids. In real-world samples, the amounts of the D-amino acids are usually trace, and large amounts of the L-forms are also present in the same matrices. Thus, the huge peaks of the L-forms frequently overlap the small peaks of the D-forms eluted just after the L-forms, and disturb the precise determination. On the other hand, all the target D-amino acids eluted faster than the L-forms by using Boc-d-Cys, and the retention order is suitable for the analysis of the trace levels of D-amino acids in the real world samples. Therefore, the reversed-phase HPLC method developed in the present study using OPA plus Boc-d-Cys reagents is practically useful for the determination of chiral amino acids in food and beverage samples.

**4 Conclusion**

In the present study, a reversed-phase HPLC method with pre-column derivatization using OPA plus Boc-d-Cys has been developed for the determination of the Asp, Ser and Ala enantiomers. The retention order of the amino acid enantiomers derivatized with Boc-d-Cys (D-forms elute
faster than L-forms) is suitable for the determination of trace amounts of the D-amino acids, and is useful for the analysis of real world samples containing large amounts of the L-forms. The present system was successfully validated and was applicable to the determination of the target amino acids in vinegar samples. These results indicated that the method designed and developed in the present study is simple and practically sufficient for chiral amino acid analysis in food and beverage samples, and further applications in healthcare, pharmaceutical and medical areas are expected.

Acknowledgements
This study was partly supported by JSPS KAKENHI Grant Numbers JP19H03359 and JP19J21452. The authors appreciate KAGAMI, Inc., for their technical support.

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