Running title: Simultaneous determination of D- and L-amino acids

A method for the simultaneous determination of primary and secondary D- and L-amino acids by reversed-phase high-performance liquid chromatography using pre-column derivatization with two-step labelling method

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This work describes a method for the simultaneous determination of primary D- and L-amino acids and secondary amino acids such as D- and L-proline. In order to remove interferences in the simultaneous determination of primary and secondary amines, the primary amines were derivatized with *o*-phthalaldehyde/*N*-acetyl-L-cysteine (OPA/NAC) and subsequently with 1-(9-fluorenyl)ethyl chloroformate (FLEC) for secondary amines, in a pre-column separation derivatization technique. These fluorescent diastereomers of the amino acids were obtained within 3 min at room temperature and determined simultaneously by changing wavelengths during analysis in a single eluting run in the high-performance liquid chromatography column. This method, referred to as the "two-step labelling method", is effective for the simultaneous determination of D- and L-amino acids.

Key words: D-amino acid; D-proline; *o*-phthalaldehyde (OPA); *N*-acetyl- L-cysteine (NAC); 1-(9-fluorenyl)ethyl chloroformate (FLEC)

D-Amino acids are rarer in nature in comparison with L-amino acids. However, in recent years, several free D-amino acids have been found in many organisms, and their biochemical and physiological functions have garnered increasing research interest.[1)] A general method for the determination of D-amino acids is based on pre-column derivatization, which allows the separation of amino acid derivatives on a reversed-phase high-performance liquid chromatography (HPLC) column in a short time with high sensitivity and simple instrumentation.

o-Phthalaldehyde (OPA) has been widely used for the optical resolution of enantiomeric amino acids. OPA readily reacts with primary amines in an alkaline medium and in the presence of chiral thiol compounds such as *N*-acetyl-L-cysteine (NAC) [2,3)] (Fig. 1A) or *N*-*tert*.-butyloxycarbonyl-L-cysteine (Boc- L-Cys),[4,5)] to give highly fluorescent diastereomeric isoindole derivatives. The reaction with OPA/NAC or OPA/Boc-L-Cys has been used for pre-column derivatization in reversed-phase HPLC. Since OPA/chiral thiol reagents are selective toward reaction with primary amines, the major drawback of OPA/thiol reagents, i.e., their inability to react with secondary amines such as proline (Pro), has been overcome by adopting an derivatization step with 9-fluorenylmethyl chloroformate (FMOC-Cl).[6)] However, it is impossible to separate the enantiomeric amino acids of secondary amines by this methodology.

Einarsson *et al*.[7)] have reported the separation of (+)-1-(9-fluorenyl)ethyl chloroformate (FLEC) derivatives of D- and L-amino acids (Fig. 1B) by using reversed-phase HPLC. The advantages of this approach include high stability of the FLEC derivatives of chiral amino acids, highly sensitive fluorescence detection, and simple derivatization procedure. In addition, FLEC can react with both primary and secondary amines. This method has also been applied to the determination of free D- and L-amino acid in tissues of crustaceans.[8)] However, the HPLC separation of FLEC derivatives of enantiomeric amino acids requires three kinds of gradient solutions and three separate runs for complete analysis of proteinogenic amino acids. Therefore, for the complete separation of all proteinogenic D- and L-amino acids containing Pro, the

HPLC analysis would need to be run three times.

Recently, a two-dimensional HPLC system combining a microbore monolithic octadecylsilyl (ODS) column and a narrow bore enantioselective column has been established for sensitive and simultaneous analysis of hydrophilic amino acid enantiomers.[9] Such a 2D-HPLC method is especially useful for the determination of small amounts of D-amino acids. However, the flow diagram of the method is very complicated and this method is limited for the analysis of hydrophilic amino acids such as serine and aspartate analogues. When Pro and their analogues need to be analyzed, another 2D-HPLC method is used.[10]

These disadvantages demand the development of a new method. The aim of this work is to develop a new method for the simultaneous determination of both primary and secondary D- and L-amino acids (especially D- and L-Pro) by sequential application of the OPA/chiral thiol and FLEC derivatization steps, designated as the “two-step labelling method.”

Materials and Methods

Chemicals. All D- and L-amino acids and related compounds, and solvents for HPLC were purchased from Wako Pure Chemical (Osaka, Japan). FLEC was obtained from Sigma-Aldrich (St. Louis, MO, USA). All chemicals were of analytical grade purity. Water for the experiments was obtained by purifying distilled water using a Simplicity UV (Merck Millipore, Billerica, MA, USA) system.

Derivatization of secondary amines with FLEC after OPA treatment of primary amines. Pre-column derivatization of D- and L-amino acids with OPA/NAC and FLEC was performed according to the methods proposed by Aswad [2] and Todoroki *et al.*[11], respectively, with minor modifications. For derivatization of primary amines, 10 μL of the sample solution was mixed with 65 μL of a saturated sodium borate solution and 20 μL of a mixture of 10 mg OPA and 10 mg NAC in 1 mL of methanol. After reacting for

1 min at room temperature, 5 μL of the 18 mM FLEC solution in acetone was added for derivatization of the secondary amines. After reacting for 2 min at room temperature, the reaction mixture was injected directly into the HPLC system.

Separation of OPA/NAC and FLEC derivatives of amino acids with HPLC.

Chromatographic analysis was performed with a JASCO (Tokyo, Japan) HPLC system consisting of a PU- 2089 quaternary gradient pump with a degasser, a CO-2065 column oven, an AS-2057 autosampler with a cooling system, a FP-2020 fluorescence detector, and a ChromNAV data processor. The analytical column was a reversed-phase ODS-80T_S (4.6 × 250 mm) (Tosoh, Tokyo, Japan) with a guard column (3.2 × 15 mm) packed with the same resin. Elution was carried out with a mixture of solvent A (a 50 mM sodium acetate buffer at pH 5.6) and solvent B (methanol:solvent A = 80:20) at 40°C; the flow rate was 1.0 mL min^{-1}. For fluorometric detection of the eluted OPA/NAC derivatives from 0 to 75 min, the excitation and emission wavelengths were set at 350 and 450 nm, respectively.[12)] For fluorometric detection of the eluted FLEC derivatives from 75 to 90 min, these wavelengths were set at 260 and 315 nm, respectively. The elution gradient was set as follows: 0–20 min, 0–20% solvent B in solvent A; 20–35 min, 20–20% solvent B in solvent A; 35–50 min, 20–50% solvent B in solvent A; 50–75 min, 50–65% solvent B in solvent A; and 75–90 min, 65–100% solvent B in solvent A.

Results

Fig. 2 shows the chromatograms of (A) OPA/NAC and (B) FLEC derivatives of the same standard amino acids. As the reagents OPA/NAC are selective toward reaction with primary amines, secondary amines such as L-hydroxyproline (Hyp; 43), sarcosine (Sar; 44), D- and L-Pro (45 and 46) were not detected (Fig. 2A). The unknown peak at 87.4 min in Fig. 2A cannot be assigned to secondary amines because this peak was also detected in the case of the OPA/NAC derivatives of Milli-Q water (data not shown). All

primary amines were eluted within 72 min. Since all primary amines were already derivatized with OPA/NAC, their FLEC derivatives were not produced (Fig. 2B). The unknown peak at 42.2 min of Fig. 2B could not be attributed to either primary or secondary amines, because the peak was also detected in the case of the FLEC derivatives of Milli-Q water (data not shown). The FLEC derivatives of secondary amines were detected after 75 min.

In order to verify the possibility of simultaneous determination of primary and secondary amines by a single fluorescent detector, the wavelength at 75 min for the OPA/NAC derivatives was changed to one corresponding to FLEC derivatives (Fig. 3). Following this procedure, most of the D- and L-forms of primary and secondary amines could be detected simultaneously with clear separation, although histidine (His) and lysine were not separated into D- and L-enantiomers (Fig. 3). Moreover, primary amines except proteinogenic amino acids such as L-citrulline (Cit), taurine, β-alanine, D- and L-*allo*-Thr, γ-aminobutyric acid (GABA), L-ornithine (Orn), and D- and L-*allo*-isoleucine, and secondary amines such as L-Hyp and Sar could be simultaneously determined (Fig. 3). Cysteine (Cys) could not be detected either because of its incomplete reaction with OPA/NAC or a very low fluorescence intensity of its derivative.

Arginine (Arg), His, Pro, and tryptophan (Trp) contain a secondary amine unit. It was next confirmed that both OPA/NAC and FLEC derivatization for secondary amine unit could be used simultaneously without interference. Because FLEC reacts with both primary and secondary amines, Arg, His, and Trp could possibly react with FLEC after treatment with the OPA/NAC. However, there was no difference in the retention times for the derivatized amino acids, and the peak area of the amino acids increased in proportion to their concentration (Fig. 4). Similar results were obtained for Pro, a secondary amine (Fig. 4).

FLEC has two types of enantiomeric isomers. When (+)-FLEC was used, D-Pro eluted first (Fig. 5B), followed by L-Pro (Fig. 5A). However, when (−)-FLEC was used,

156 L-Pro eluted first (Fig. 5C), and was followed by D-Pro (Fig. 5D).

157

158 **Discussion**

159 The present study has established an HPLC method for the simultaneous
160 determination of primary amines (D- and L-amino acid) and secondary amines
161 (especially D- and L-Pro). This two-step labelling method using OPA/NAC and FLEC is
162 a very simple derivatization method. The ODS-80T_S column is typically the first choice
163 for reversed-phase HPLC. The hydrophobicity of OPA derivatives is less than that of
164 FLEC derivatives and therefore, OPA derivatives elute before FLEC derivatives. Among
165 all primary amino acids, L-leucine was eluted last (within 72 min, Fig. 2A and 3) and is
166 followed by L-Hyp, which is eluted first among the secondary amines after 75 min (Fig.
167 2B, 3). Hence, when the wavelengths at 350/450 nm for OPA derivatives were changed
168 to 260/315 nm for FLEC derivatives at 75 min, simultaneous determination was made
169 possible with a single fluorescence detector.

170 OPA was found to be a superior fluorescence derivatization reagent and formed
171 derivatives of many D- and L-proteinogenic amino acids. Primary amines were fully
172 derivatized with OPA/NAC before FLEC treatment (Fig. 2B). A quantitative
173 determination of primary amines could be accurately performed in spite of the
174 coexistence of OPA/NAC and FLEC, because the chromatograms of the standard amino
175 acids obtained by following the reported conventional method[12] and the two-step
176 labelling method developed in this work, are nearly identical (data not shown). These
177 results suggest that OPA/NAC and FLEC could be used without interference for
178 primary amines.

179 Since FLEC reacts with both primary and secondary amines, separation of all amino
180 acids using only FLEC would be an ideal scenario. However, it is very difficult to
181 achieve such a separation because the hydrolysis products of FLEC show fluorescence
182 and give prominent peaks in the chromatogram. The unknown peak in Fig. 2B likely
183 represents a hydrolysis product of FLEC.[7]

Amino acids possessing primary and secondary amine moieties such as Arg, His, and Trp reacted with OPA/NAC first and did not react with FLEC. There were no peaks in the chromatogram at 260/315 nm. Although the reason for this trend is unclear, it can be speculated that while these amino acids reacted with OPA/NAC, their secondary amine functionalities may not react with FLEC. The peak area of these amino acids increased in proportion to their concentration (Fig. 4), supporting the accuracy of this analysis method.

The limit of quantitation (LOQ) of most primary amines lay in the range of 0.1–0.25 μM, but the LOQ for His, D- and L-methionine, D-phenylalanine and L-Orn were 1, 2, 2, and 0.5 μM, respectively (data not shown). The LOQ values of secondary amines (except D-Pro) were 0.05 μM, but for D-Pro, it was 0.125 μM (data not shown). These results suggest that the detection sensitivity of FLEC derivatives (except D-Pro) are high compared to OPA/NAC derivatives.

Both (+)- and (−)-FLEC were found to be effective for the detection of secondary amines, although the elution or retention times of D- and L-Pro were reversed (Fig. 5). Despite this reversal, the use of either (+)- or (−)-FLEC was effective. Furthermore, regardless of either enantiomer, the elution of other secondary amines such as L-Hyp and Sar was detected at the same retention time (data not shown).

The C_3 and 2D C_3 Marfey's method for amino acid analysis was reported in 2016.[13] The Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5- L-alanine amide, FDAA) derivatives are not fluorescenct and need to be detected with a UV detector (340 nm). Therefore, the traditional Marfey's method is inferior to the point of sensitivity. However, C3 and 2D C_3 Marfey's method utilizes a single quadrupole electrospray ionization mass detector (ESI-MS) to overcome the weak point. Although the two-step labelling method discussed in this work utilizes a fluorescence detector, a mass detector or fluorescence and mass detector can certainly be coupled together.

In conclusion, a two-step labelling method has been developed whereby the derivatization and determination steps of amino acids are simplified. For instance, the

fluorescent diastereomers of the amino acids were obtained within 3 min at room temperature. Furthermore, the gradient solution used in this method is easy to prepare as only 50 mM sodium phosphate and methanol are required; i.e., this method required two types of gradient solutions and a single eluting run for the analysis of proteinogenic D- and L-amino acids. Overall, the present method is expected to be useful for comprehensive determination of proteinogenic D- and L-amino acids containing Pro and other non-proteinogenic amino acids that exist in different organisms.

Acknowledgments

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Figure Legends

Fig. 1. Derivatization of enantiomeric amino acids using (A) OPA/NAC[3)] and (B) FLEC[7)] reagents. Asterisks indicate an asymmetric carbon atom.

Fig. 2. (A) Chromatogram of OPA/NAC derivatives of standard free amino acids, where the excitation and emission wavelengths were set at 350 and 450 nm, respectively. (B) Chromatogram of (+)-FLEC derivatives of standard free amino acids, where the excitation and emission wavelengths were set at 260 and 315 nm, respectively. Each peak represents 10 and 5 pmol of primary and secondary amines, respectively.
1. D-Asp, 2. L-Asp, 3. L-Asn, 4. D-Asn, 5. D-Ser, 6. L-Ser, 7. L-Glu, 8. D-Glu, 9. L-Gln, 10. D-Gln, 11. D, L-His, 12. D-Thr, 13. Gly, 14. L-Thr, 15. L-Cit, 16. L-Arg, 17. D-Arg, 18. Tau, 19. *β*-Ala, 20. D-Ala, 21. L-Ala, 22. D-*allo*-Thr, 23. L-*allo*-Thr, 24. GABA, 25. L-Tyr, 26. D-Tyr, 27. L-Val, 28. D-Met, 29. L-Met, 30. L-Trp, 31. D-Val, 32. D-Trp, 33. D-Phe, 34. L-Phe, 35. L-Orn, 36. L-Ile, 37. L-*allo*-Ile, 38. D-Ile, 39. D-*allo*-Ile, 40. D, L -Lys, 41. D-Leu, 42. L-Leu, 43. L-Hyp, 44. Sar, 45. D-Pro, 46. L-Pro.

Fig. 3. Chromatogram of OPA/NAC and (+)-FLEC derivatives of standard free amino acids. Each peak represents 10 and 5 pmol of primary and secondary amines, respectively. The wavelength was changed at 75 min.
1. D-Asp, 2. L-Asp, 3. L-Asn, 4. D-Asn, 5. D-Ser, 6. L-Ser, 7. L-Glu, 8. D-Glu, 9. L-Gln, 10. D-Gln, 11. D, L-His, 12. D-Thr, 13. Gly, 14. L-Thr, 15. L-Cit, 16. L-Arg, 17. D-Arg, 18. Tau, 19. *β*-Ala, 20. D-Ala, 21. L-Ala, 22. D-*allo*-Thr, 23. L-*allo*-Thr, 24. GABA, 25. L-Tyr, 26. D-Tyr, 27. L-Val, 28. D-Met, 29. L-Met, 30. L-Trp, 31. D-Val, 32. D-Trp, 33. D-Phe, 34. L-Phe, 35. L-Orn, 36. L-Ile, 37. L-*allo*-Ile, 38. D-Ile, 39. D-*allo*-Ile, 40. D, L -Lys, 41. D-Leu, 42. L-Leu, 43. L-Hyp, 44. Sar, 45. D-Pro, 46. L-Pro.

Fig. 4. Calibration curves of Arg, His, Pro, and Trp, which contain a secondary amine moieties. The peak area of the amino acids increases in proportion to their concentration. After each amino acid was derivatized following the two-step labelling method, 10 μL was injected into the HPLC.

Fig. 5. Chromatogram of (A, B) (+)-FLEC and (C, D) (−)-FLEC derivatives of L- and D-Pro, when the excitation and emission wavelengths were 260 and 315 nm, respectively. Each peak represents 50 pmol.

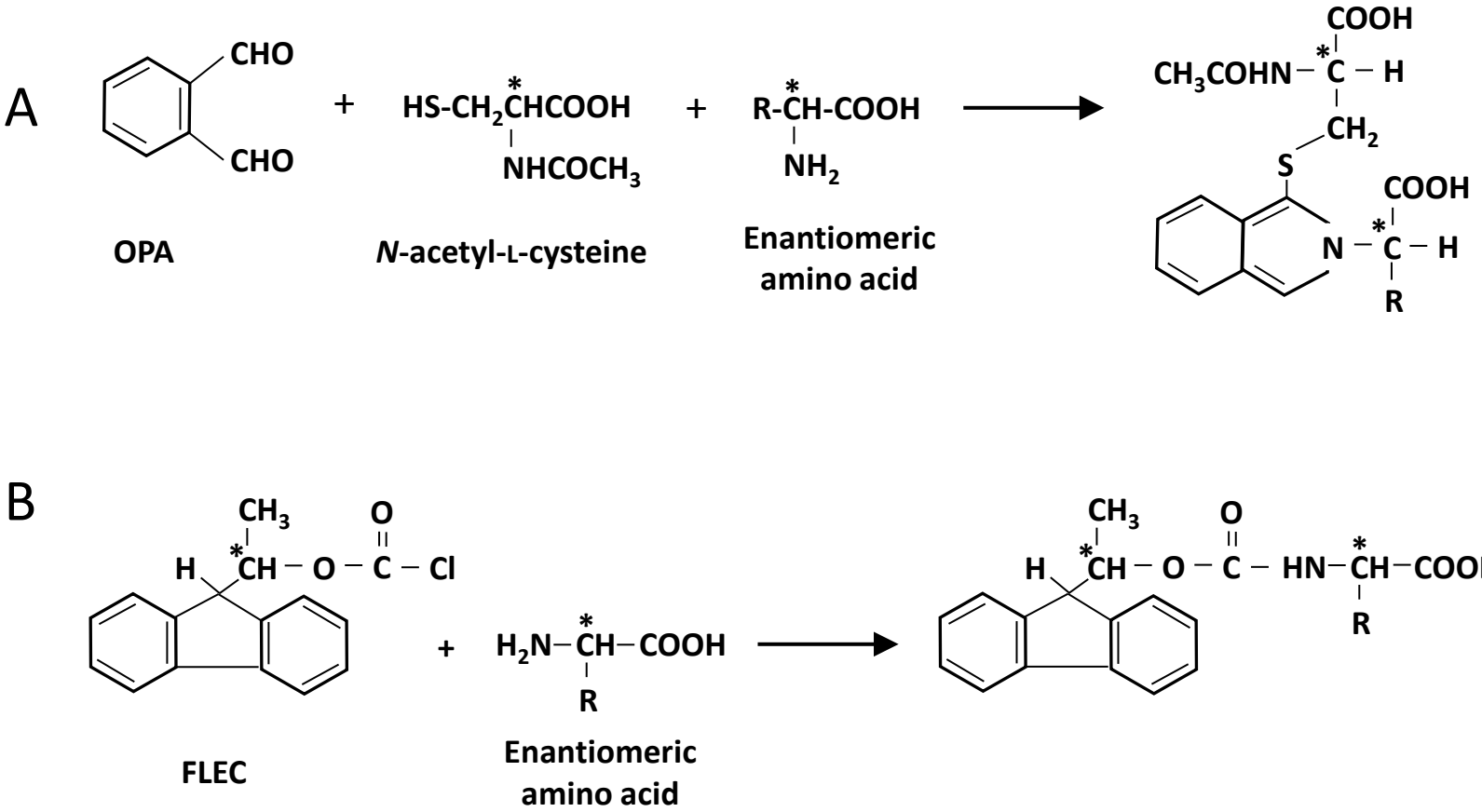
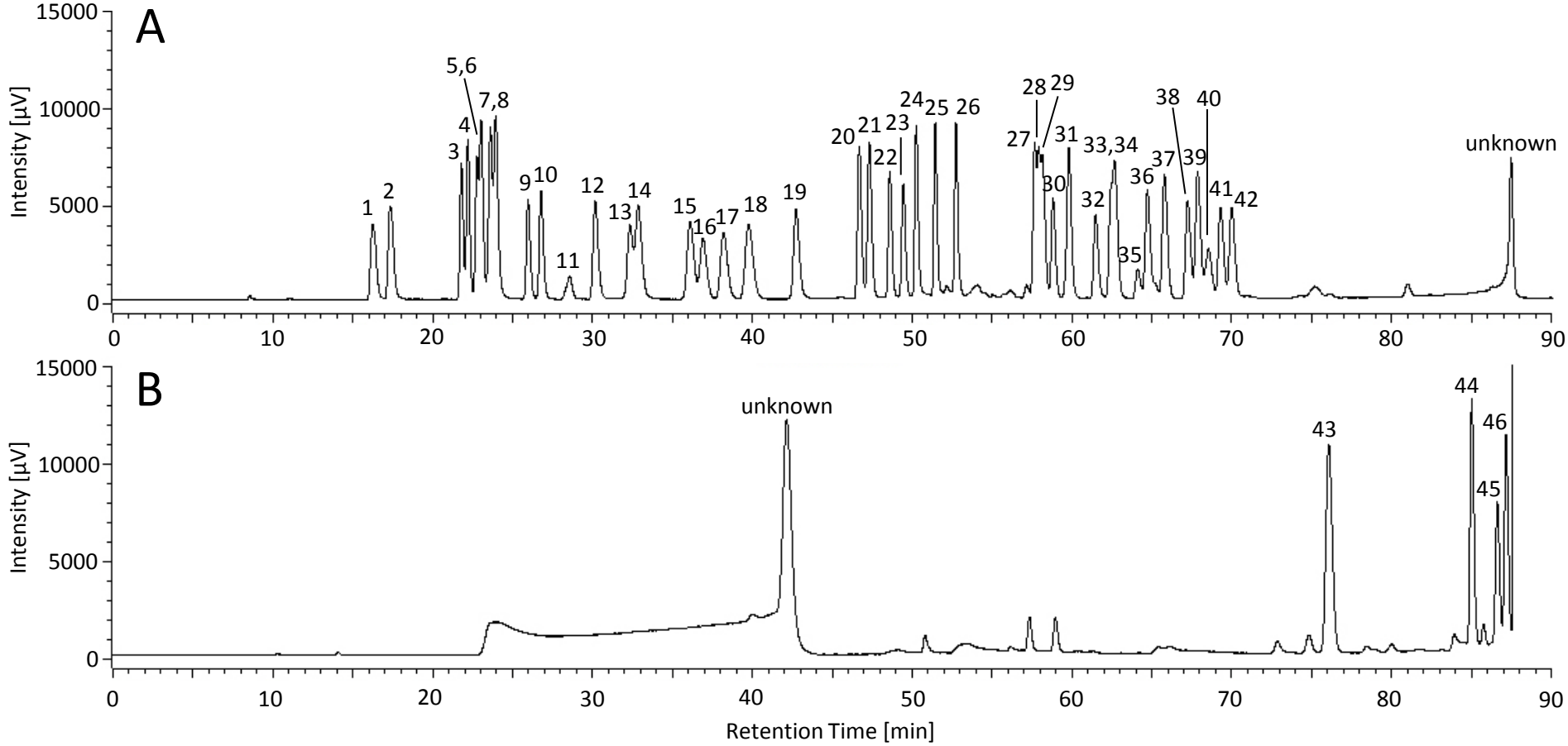


Fig. 1. Yokoyama *et al.*



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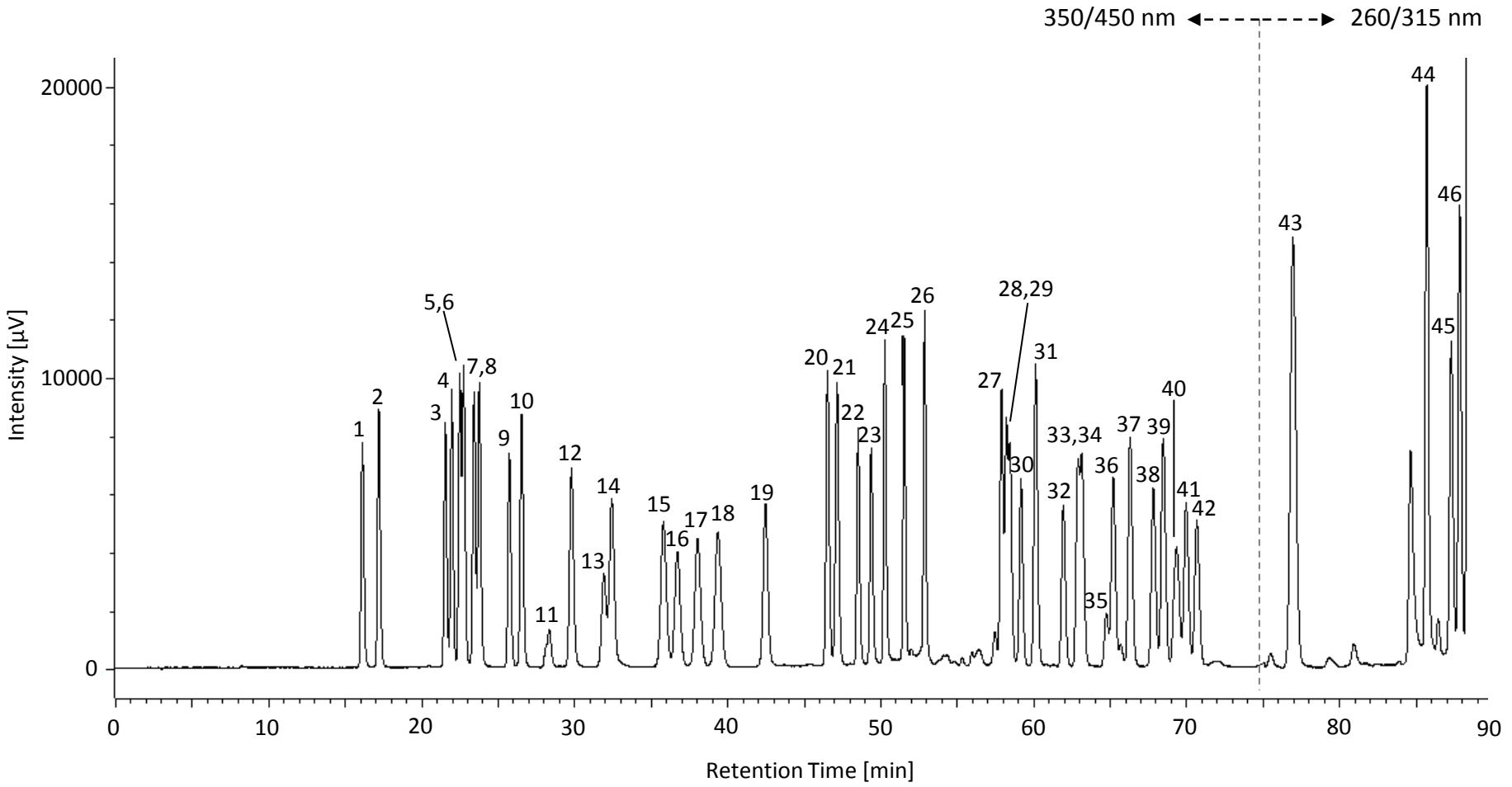

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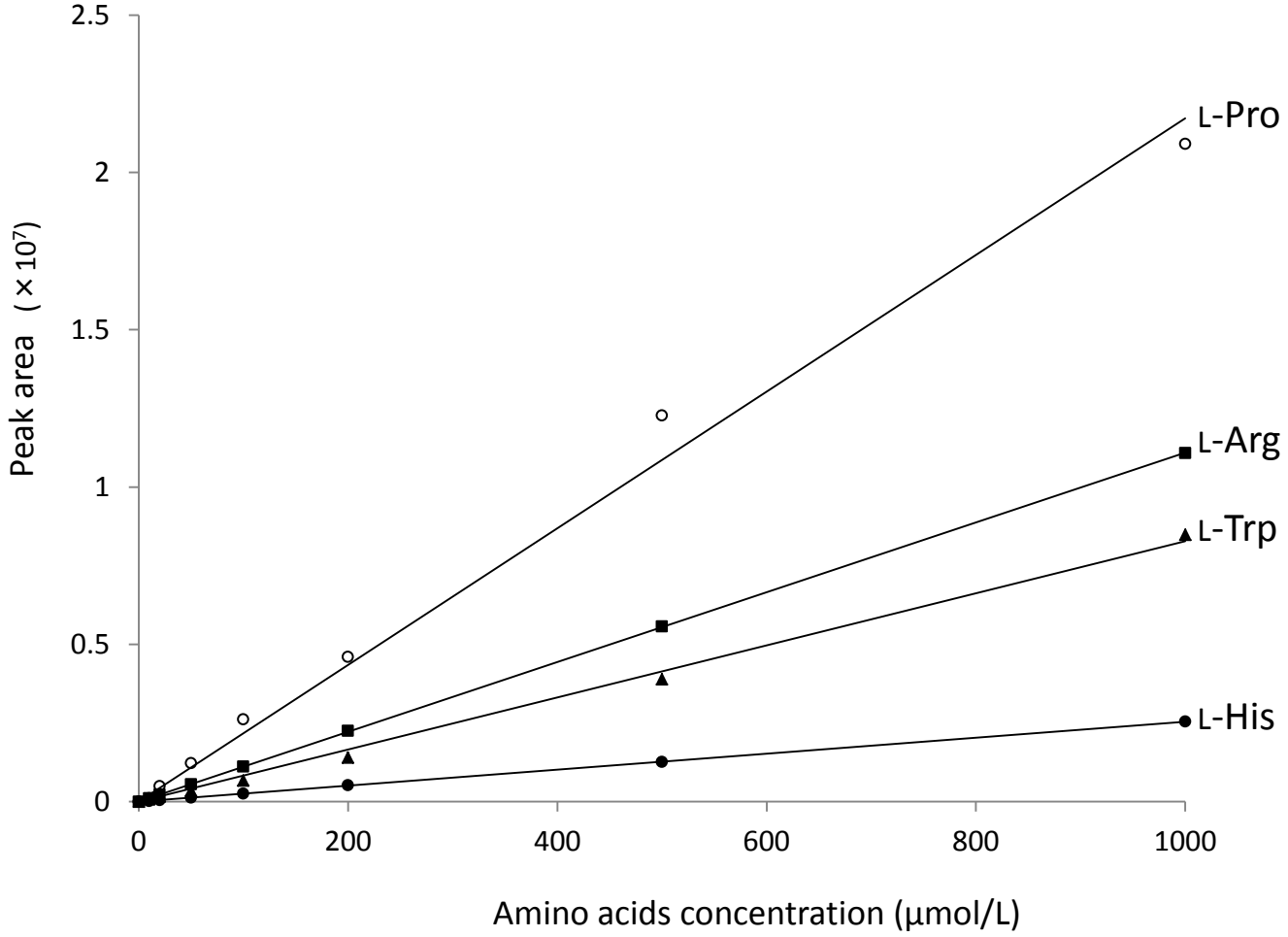

Fig. 4. Yokoyama *et al.*

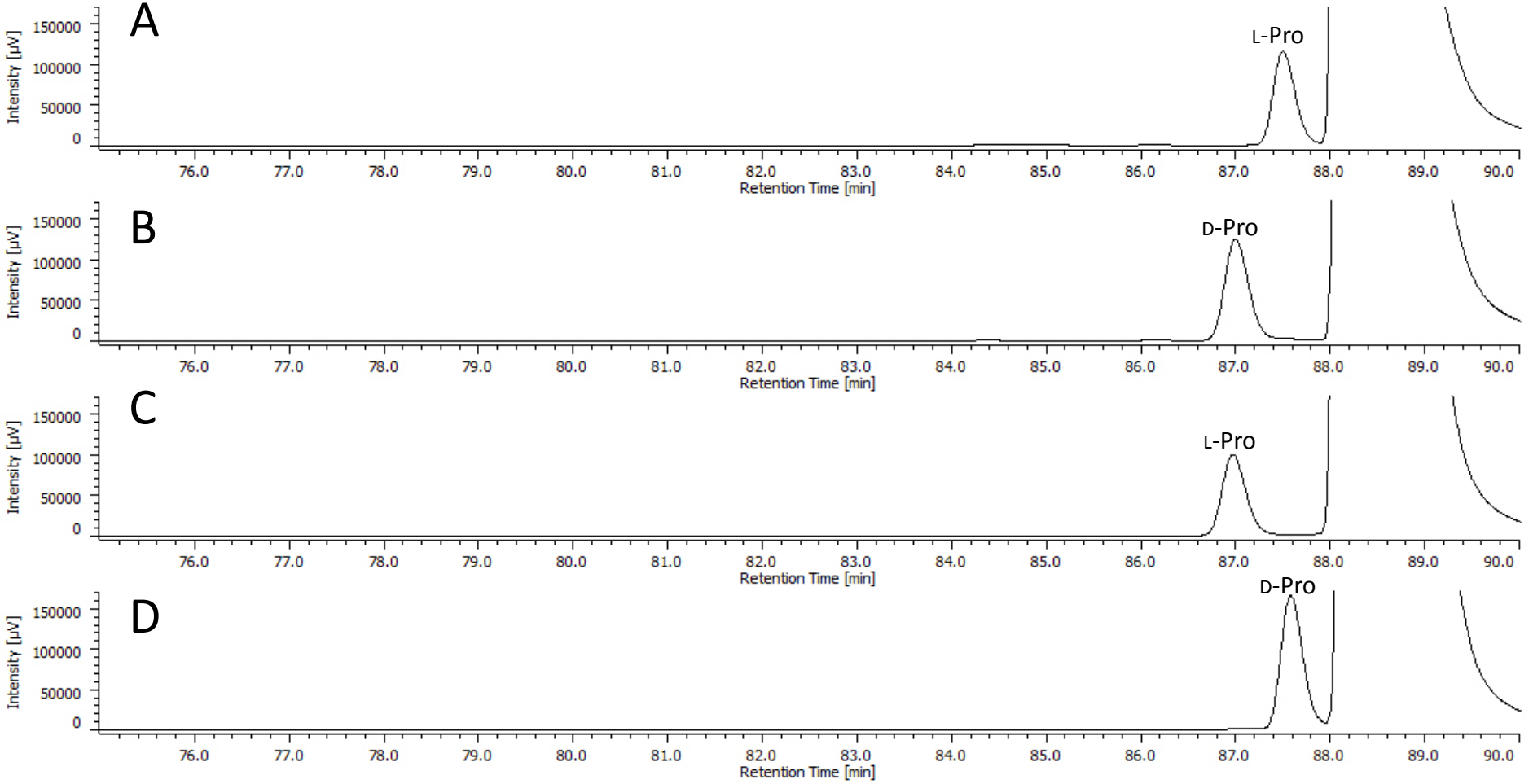

Fig. 5. Yokoyama *et al.*