



Title	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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1 Running title: Simultaneous determination of D- and L-amino acids

2

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

7

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16 **This work describes a method for the simultaneous determination of primary D-**
17 **and L-amino acids and secondary amino acids such as D- and L-proline. In order to**
18 **remove interferences in the simultaneous determination of primary and secondary**
19 **amines, the primary amines were derivatized with**
20 ***o*-phthalaldehyde/*N*-acetyl-L-cysteine (OPA/NAC) and subsequently with**
21 **1-(9-fluorenyl)ethyl chloroformate (FLEC) for secondary amines, in a pre-column**
22 **separation derivatization technique. These fluorescent diastereomers of the amino**
23 **acids were obtained within 3 min at room temperature and determined**
24 **simultaneously by changing wavelengths during analysis in a single eluting run in**
25 **the high-performance liquid chromatography column. This method, referred to as**
26 **the “two-step labelling method”, is effective for the simultaneous determination of**
27 **D- and L-amino acids.**

28

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

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

51 *o*-Phthalaldehyde (OPA) has been widely used for the optical resolution of
52 enantiomeric amino acids. OPA readily reacts with primary amines in an alkaline
53 medium and in the presence of chiral thiol compounds such as *N*-acetyl-L-cysteine
54 (NAC)^{2,3)} (Fig. 1A) or *N*-*tert*-butyloxycarbonyl-L-cysteine (Boc-L-Cys),^{4,5)} to give
55 highly fluorescent diastereomeric isoindole derivatives. The reaction with OPA/NAC or
56 OPA/Boc-L-Cys has been used for pre-column derivatization in reversed-phase HPLC.
57 Since OPA/chiral thiol reagents are selective toward reaction with primary amines, the
58 major drawback of OPA/thiol reagents, i.e., their inability to react with secondary
59 amines such as proline (Pro), has been overcome by adopting an derivatization step with
60 9-fluorenylmethyl chloroformate (FMOC-Cl).⁶⁾ However, it is impossible to separate
61 the enantiomeric amino acids of secondary amines by this methodology.

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

72 HPLC analysis would need to be run three times.

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

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

86

87 **Materials and Methods**

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

93

94 *Derivatization of secondary amines with FLEC after OPA treatment of primary*
95 *amines.* Pre-column derivatization of D- and L-amino acids with OPA/NAC and FLEC
96 was performed according to the methods proposed by Aswad²⁾ and Todoroki *et al.*¹¹⁾,
97 respectively, with minor modifications. For derivatization of primary amines, 10 μ L of
98 the sample solution was mixed with 65 μ L of a saturated sodium borate solution and 20
99 μ L of a mixture of 10 mg OPA and 10 mg NAC in 1 mL of methanol. After reacting for

100 1 min at room temperature, 5 μ L of the 18 mM FLEC solution in acetone was added for
101 derivatization of the secondary amines. After reacting for 2 min at room temperature,
102 the reaction mixture was injected directly into the HPLC system.

103

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

105 Chromatographic analysis was performed with a JASCO (Tokyo, Japan) HPLC
106 system consisting of a PU- 2089 quaternary gradient pump with a degasser, a CO-2065
107 column oven, an AS-2057 autosampler with a cooling system, a FP-2020 fluorescence
108 detector, and a ChromNAV data processor. The analytical column was a reversed-phase
109 ODS-80T_S (4.6 \times 250 mm) (Tosoh, Tokyo, Japan) with a guard column (3.2 \times 15 mm)
110 packed with the same resin. Elution was carried out with a mixture of solvent A (a 50
111 mM sodium acetate buffer at pH 5.6) and solvent B (methanol:solvent A = 80:20) at
112 40°C; the flow rate was 1.0 mL min⁻¹. For fluorometric detection of the eluted
113 OPA/NAC derivatives from 0 to 75 min, the excitation and emission wavelengths were
114 set at 350 and 450 nm, respectively.¹²⁾ For fluorometric detection of the eluted FLEC
115 derivatives from 75 to 90 min, these wavelengths were set at 260 and 315 nm,
116 respectively. The elution gradient was set as follows: 0–20 min, 0–20% solvent B in
117 solvent A; 20–35 min, 20–20% solvent B in solvent A; 35–50 min, 20–50% solvent B in
118 solvent A; 50–75 min, 50–65% solvent B in solvent A; and 75–90 min, 65–100%
119 solvent B in solvent A.

120

121 **Results**

122 Fig. 2 shows the chromatograms of (A) OPA/NAC and (B) FLEC derivatives of the
123 same standard amino acids. As the reagents OPA/NAC are selective toward reaction
124 with primary amines, secondary amines such as L-hydroxyproline (Hyp; 43), sarcosine
125 (Sar; 44), D- and L-Pro (45 and 46) were not detected (Fig. 2A). The unknown peak at
126 87.4 min in Fig. 2A cannot be assigned to secondary amines because this peak was also
127 detected in the case of the OPA/NAC derivatives of Milli-Q water (data not shown). All

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

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

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

154 FLEC has two types of enantiomeric isomers. When (+)-FLEC was used, D-Pro
155 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 method¹²⁾ 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⁾

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

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

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

202 The C₃ and 2D C₃ Marfey's method for amino acid analysis was reported in
203 2016.¹³ The Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide, FDAA)
204 derivatives are not fluorescent and need to be detected with a UV detector (340 nm).
205 Therefore, the traditional Marfey's method is inferior to the point of sensitivity.
206 However, C₃ and 2D C₃ Marfey's method utilizes a single quadrupole electrospray
207 ionization mass detector (ESI-MS) to overcome the weak point. Although the two-step
208 labelling method discussed in this work utilizes a fluorescence detector, a mass detector
209 or fluorescence and mass detector can certainly be coupled together.

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

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

219

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223

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268 **Figure Legends**

269

270 **Fig. 1.** Derivatization of enantiomeric amino acids using (A) OPA/NAC³⁾ and (B)
271 FLEC⁷⁾ reagents. Asterisks indicate an asymmetric carbon atom.

272

273 **Fig. 2.** (A) Chromatogram of OPA/NAC derivatives of standard free amino acids, where
274 the excitation and emission wavelengths were set at 350 and 450 nm, respectively. (B)
275 Chromatogram of (+)-FLEC derivatives of standard free amino acids, where the
276 excitation and emission wavelengths were set at 260 and 315 nm, respectively. Each
277 peak represents 10 and 5 pmol of primary and secondary amines, respectively.

278 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,
279 10. D-Gln, 11. D, L-His, 12. D-Thr, 13. Gly, 14. L-Thr, 15. L-Cit, 16. L-Arg, 17. D-Arg,
280 18. Tau, 19. β -Ala, 20. D-Ala, 21. L-Ala, 22. D-*allo*-Thr, 23. L-*allo*-Thr, 24. GABA, 25.
281 L-Tyr, 26. D-Tyr, 27. L-Val, 28. D-Met, 29. L-Met, 30. L-Trp, 31. D-Val, 32. D-Trp, 33.
282 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
283 -Lys, 41. D-Leu, 42. L-Leu, 43. L-Hyp, 44. Sar, 45. D-Pro, 46. L-Pro.

284

285 **Fig. 3.** Chromatogram of OPA/NAC and (+)-FLEC derivatives of standard free amino
286 acids. Each peak represents 10 and 5 pmol of primary and secondary amines,
287 respectively. The wavelength was changed at 75 min.

288 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,
289 10. D-Gln, 11. D, L-His, 12. D-Thr, 13. Gly, 14. L-Thr, 15. L-Cit, 16. L-Arg, 17. D-Arg,
290 18. Tau, 19. β -Ala, 20. D-Ala, 21. L-Ala, 22. D-*allo*-Thr, 23. L-*allo*-Thr, 24. GABA, 25.
291 L-Tyr, 26. D-Tyr, 27. L-Val, 28. D-Met, 29. L-Met, 30. L-Trp, 31. D-Val, 32. D-Trp, 33.
292 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
293 -Lys, 41. D-Leu, 42. L-Leu, 43. L-Hyp, 44. Sar, 45. D-Pro, 46. L-Pro.

294

295

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

300

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

304

305

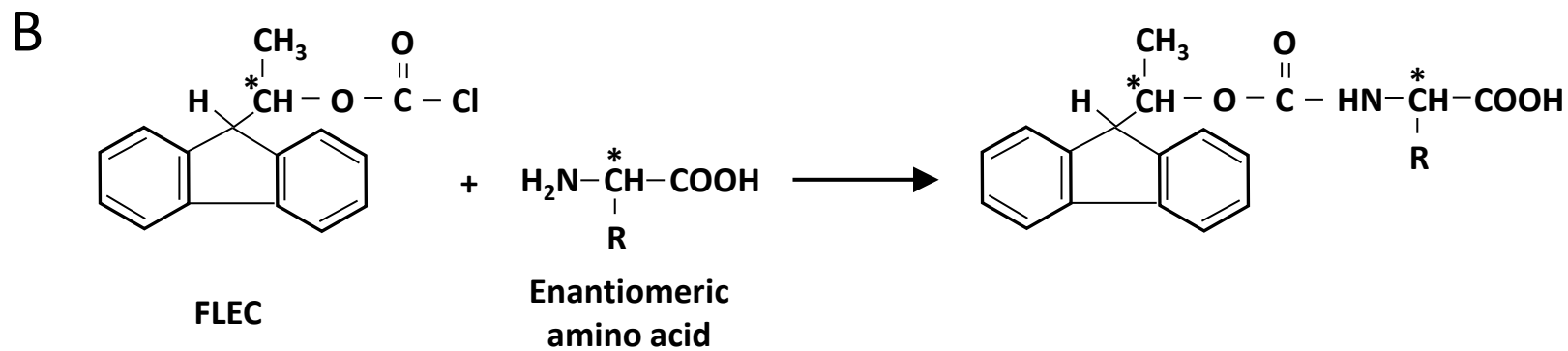
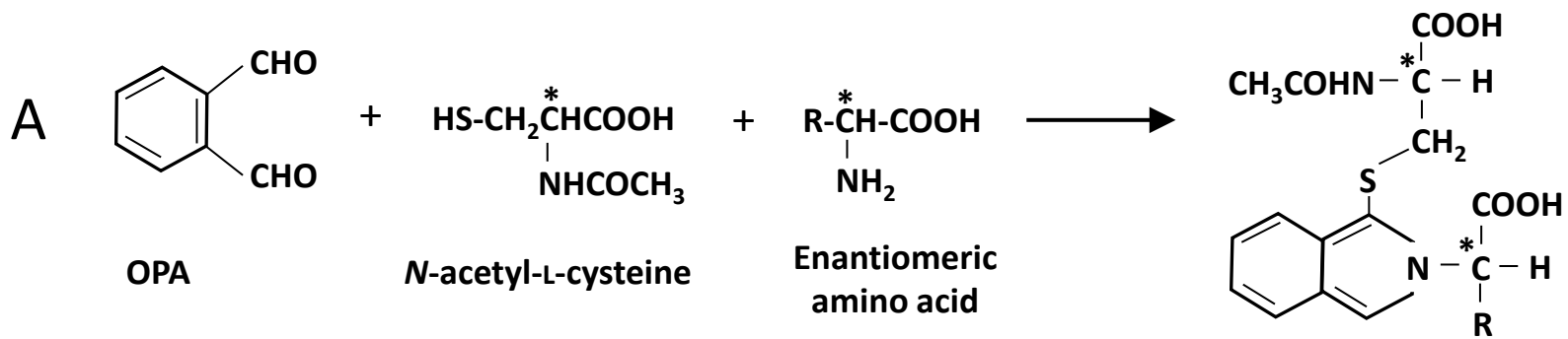


Fig. 1. Yokoyama *et al.*

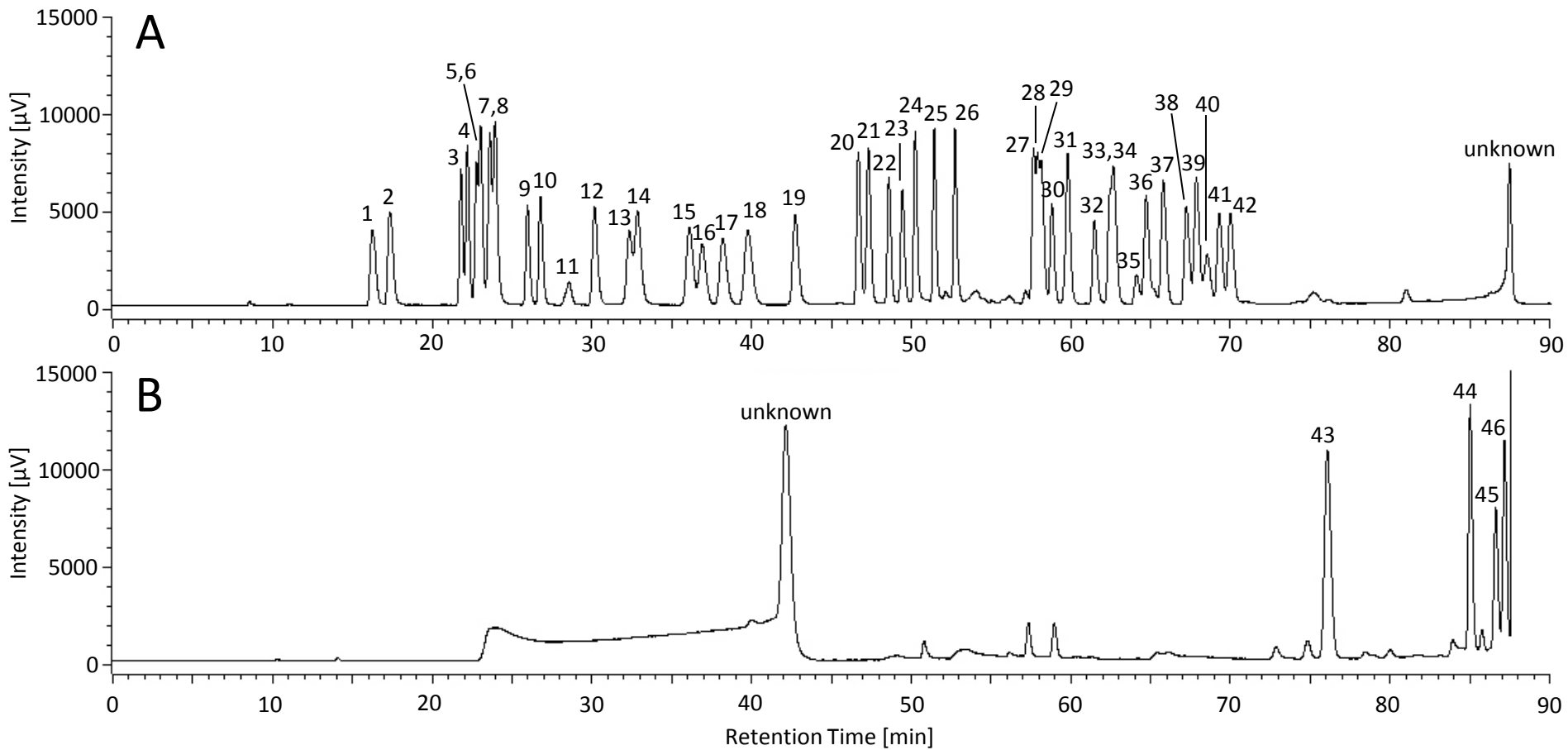


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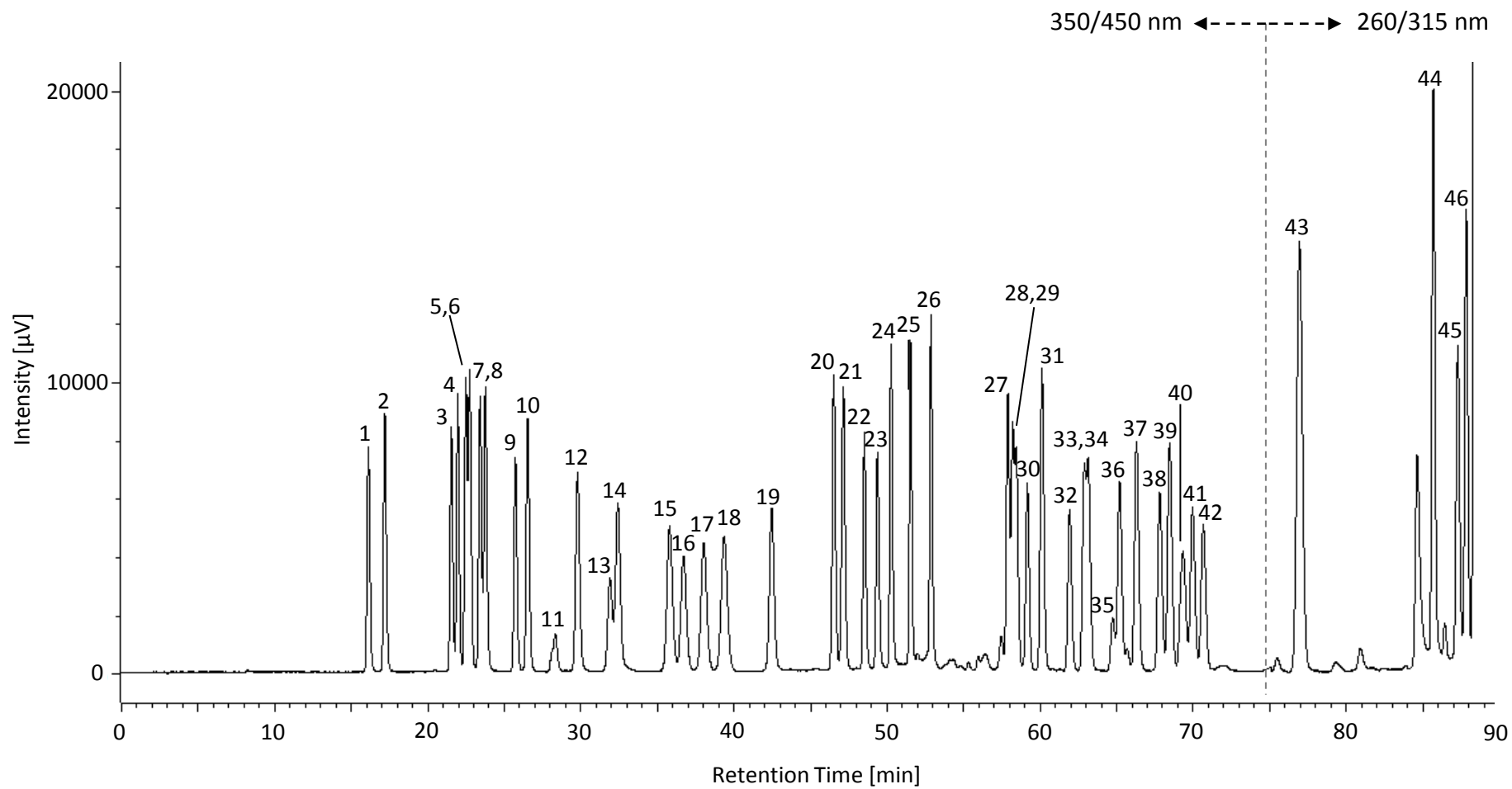


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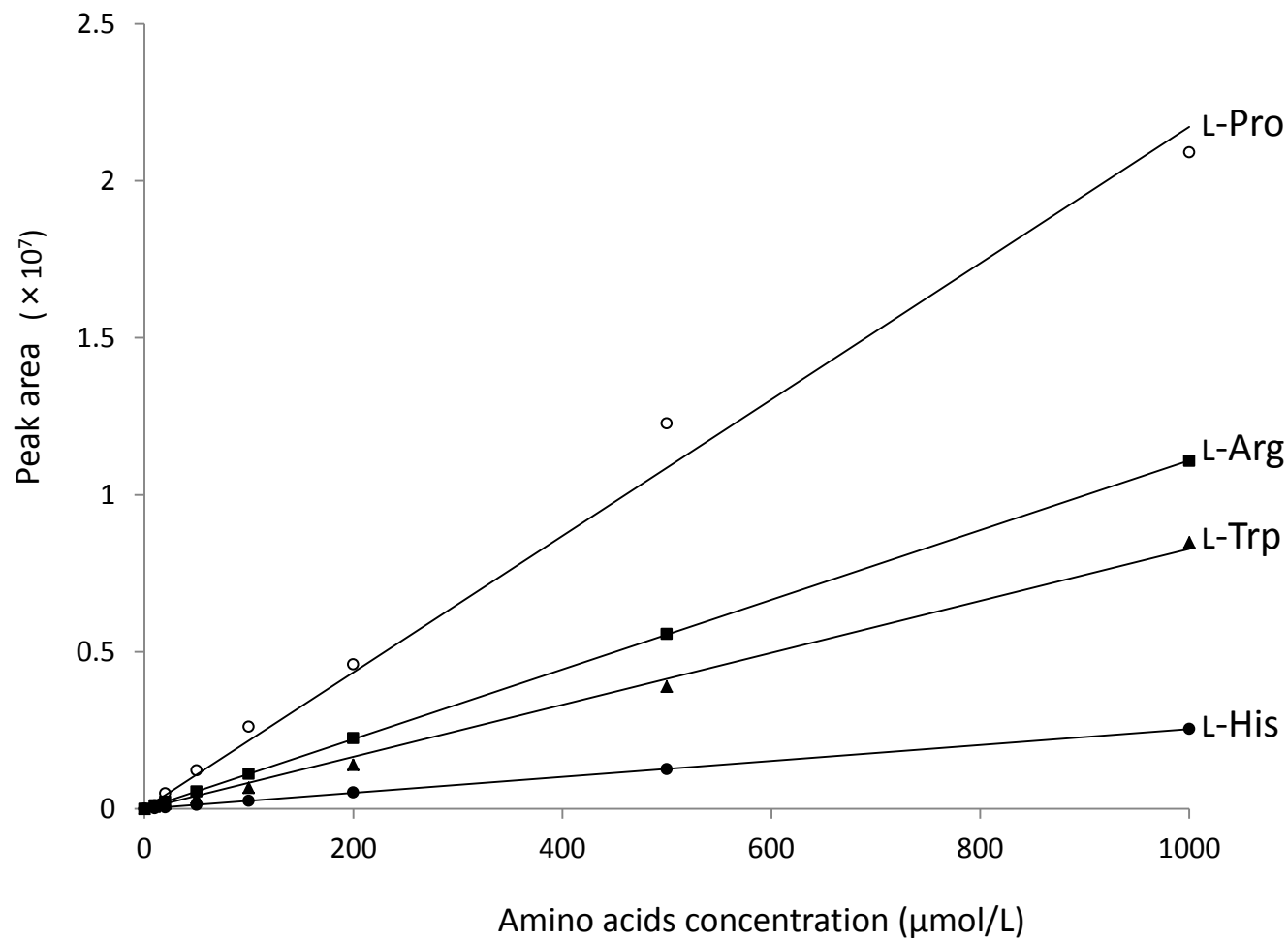


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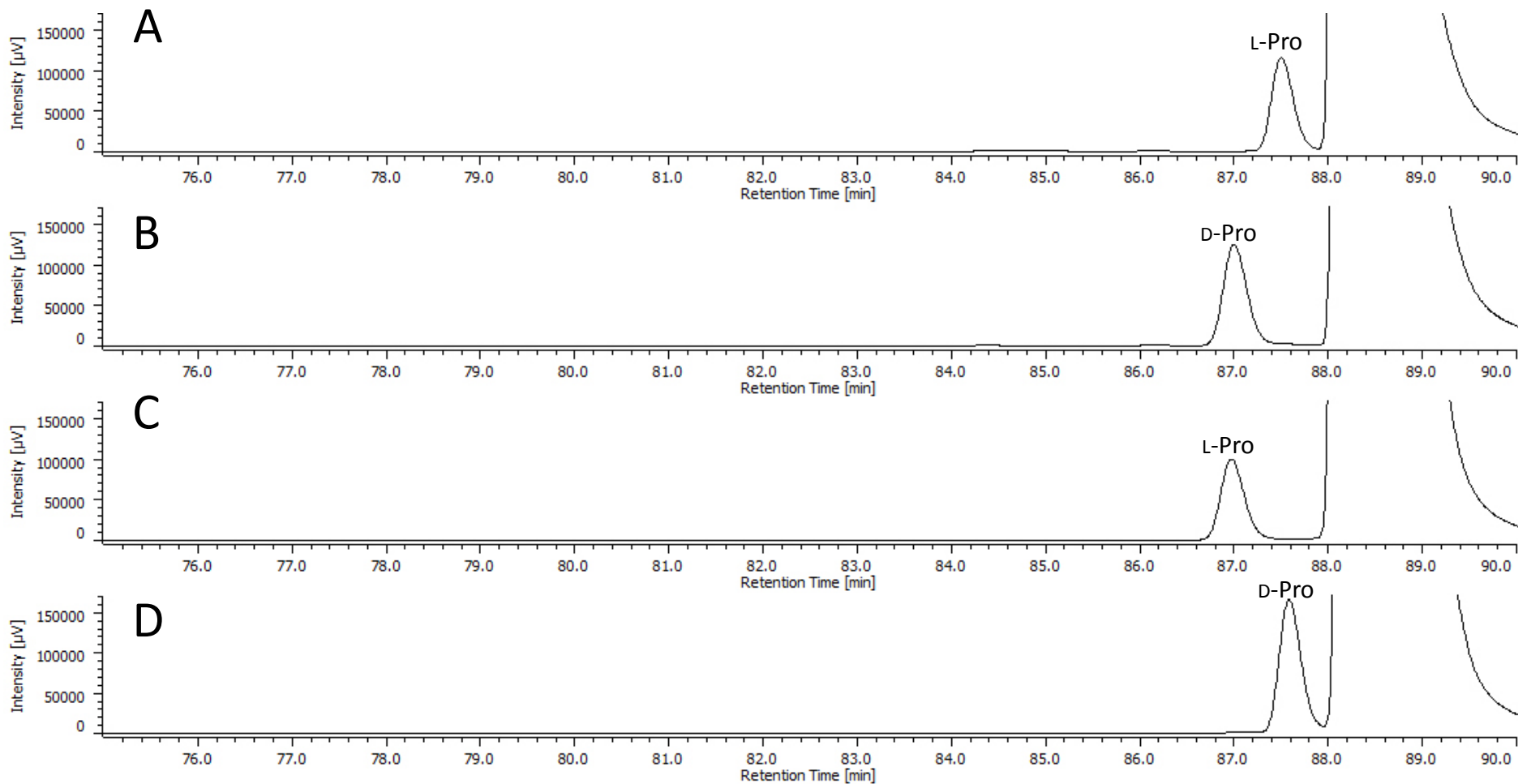


Fig. 5. Yokoyama *et al.*