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Enantiomeric and diastereomeric separation of di- and tripeptides by capillary electrophoresis

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Abstract

Chiral resolution of a series of di- and tripeptides has been attempted by indirect and direct methods. For the indirect method, the analytes were derivatized with a reagent of high chiral purity, (−)-1-(9-fluorenyl)ethyl chloroformate, and the diastereomers produced were subsequently separated in a micellar electrokinetic capillary chromatography (MEKC) system. For the direct method, the analytes were derivatized with 9-fluorenylmethyl chloroformate and separated by MEKC using γ-cyclodextrin (CD) or β-CD as chiral selectors. After optimization, 9 peptides were separated when using the indirect method. Using the direct method, 9 peptides were separated with γ-CD as the chiral selector, and two other peptides were separated with β-CD. Complete separation of two peptides having two chiral centers, Leu–Ala and Leu–Leu, was obtained by the indirect method and Ala–Leu was separated by the direct method. The benefits of using organic modifiers to enhance enantioselectivity and separation efficiencies in the direct and indirect method were demonstrated and discussed. High separation efficiencies were obtained for both methods, plate numbers were in the range of 10^6 plates/m. Separation times were ca. 10 min using the indirect method and ca. 17 min using the direct method. It seems that the two methods are complementary.

Keywords: Peptides; Enantiomer saparation; Diastereomer separation; Derivatization, electrophoresis

1. Introduction

Currently there is an increasing interest in biologically active peptides e.g., for pharmaceutical purposes, and methods for simple and sensitive determination of the enantiomeric purity of such peptides are, in many cases, desirable. Chiral separation of small peptides has been performed on GC using a chiral stationary phase [1]. The application of HPLC for optical resolution of di- and tripeptides has been reported in a number of papers [2–8]. Different types of chiral selectors have been employed in HPLC. These include ligand-exchange types [2,3], Pirkle types [4,5], cyclodextrins [6,7], a crown ether [8] and teicoplanin [9]. Further, TLC separation of dipeptides, using vancomycin as chiral selector has been reported [10]. During recent years, CE has been employed for chiral separations. The merits of CE in this context are mainly due to its high inherent separation efficiency, thus utilizing the selectivities of the separation systems optimally. Moreover, the CE technique is less expensive, e.g., concerning columns and chiral selectors. In general, methods and chiral selectors developed for HPLC have thus been transferred to the CE techniques. It should be noted, however, that drawing analogies to CE is not

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always appropriate [11]. The first application of CE for chiral separation of small peptides was by Tran et al. who utilized the indirect separation method, diastereomers were thus first formed and separation was performed on a non-chiral system. Four peaks were separated with Ala–Ala and a partial separation of Ala–Ala–Ala was also demonstrated [12]. Direct separation, using a crown ether as the chiral selector, has recently been demonstrated by Schmid and Gübitz [13] and by Kuhn et al. [14,15]. Enantioseparation of a number of di- and tripeptides having one or two stereogenic centers was reported. The present authors have demonstrated enantiomeric separation of 15 di- and tripeptides in less than 12 min in CE when using vancomycin as the chiral selector [16].

Zukowski et al. [7] reported promising results concerning the application of HPLC for enantioseparation of some di- and tripeptides. After a derivatization with 9-fluorenylmethyl chloroformate (FMOC), the peptides were chirally separated using cyclodextrin bonded phases. In earlier work, the present authors showed enantioseparation of 20 FMOC-amino acids in CE using cyclodextrins (CDs) and vancomycin as chiral selectors [17,18]. In the present work, we report the chiral separation of di- and tripeptides in MEKC using cyclodextrins as chiral selectors. Further, a comparison is made between the separation of a given set of analytes when applying the indirect method, that is, after the formation of diastereomers, and the direct method. Optimization of the separation conditions was done by means of factorial design, earlier demonstrated, by the present authors [16–20] and Rogan et al. [21], as an excellent approach for the optimization in chiral CE. Further, optimization in MEKC was recently reviewed [22].

2. Experimental

2.1. Chemicals and reagents

The peptides DL-Ala–Ala, Ala–Gly, Ala–Gly–Gly, Ala–Leu, Gly–Ala, Gly–Asn, Gly–Asp, Gly–Leu, Gly–Met, Gly–Phe, Gly–Val, Leu–Ala, Leu–Gly, Leu–Gly–Gly, Leu–Leu were obtained from Sigma (St. Louis, MO, USA). The dipeptide Arg–Gly was obtained from Ferring (Malmö, Sweden). The chiral selectors β-cyclodextrin (β-CD) and γ-cyclodextrin (γ-CD) were provided from Sigma. Sodium dodecylsulfate (SDS) was obtained from Fluka (Buchs, Switzerland). The derivatization reagent, 9-fluorenylmethyl chloroformate (FMOC) was obtained from Fluka, and (+)- and (−)-1-(9-fluorenyl)ethyl chloroformate (FLEC) were provided from Eka Nobel (Bohus, Sweden). Other chemicals used in this work were of analytical grade.

2.2. Apparatus

CE was carried out using a Prince (Emmen, Netherlands) capillary electrophoresis instrument with a high-voltage supply (0–30 kV). On-column detection was done with a UV detector (254 nm), CV4 (ISCO, Lincoln, NE, USA). The temperature of the capillary was maintained at 25°C. Data were collected by means of an ELDS 900 laboratory data system (Chromatography Data System, Kungshög, Sweden). Statistical experimental design for the optimization experiments was done in Codex (Sum IT System, Sollentuna, Sweden).

Fused-silica capillaries [68 cm (46 cm to detector window)×25 μm I.D.] were obtained from Polymicro Technologies (Phoenix, AZ, USA). New capillaries were first pre-treated by flushing with 0.2 *M* NaOH for 1 h and then with water for 10 min. Between runs, the capillary was flushed with 0.2 *M* NaOH for 5 min, followed by water rinsing for 5 min, then with running buffer for 5 min. Samples were introduced by pressure (50 mbar, 0.35 min). All buffer concentrations are given as they were before the addition of 2-propanol (IPA).

2.3. Derivatization

The derivatization procedures of dipeptides with FMOC and FLEC were the same as for amino acid derivatization [19]. The concentrations of the samples were in the range of 100–200 μ*M*.

3. Results and discussion

Optimization of the separation conditions was done by the use of factorial design. In our opinion,

this is the most rapid and effective method to find optimal conditions [16–20]. Furthermore, the method gives information concerning possible interactions between variables, an increased understanding of the separation system is thereby gained. The factorial design is normally preceded by a series of scouting experiments. This is to find conditions under which a separation begins to emerge, and this serves as a starting point for the optimization. In the present work, we benefit from a previous optimization study on the chiral separation of amino acids derivatized by FLEC or FMOC [19]. Since peptides are closely related to amino acids, starting conditions for the factorial design were easily found.

3.1. Indirect separation of FLEC-peptides

3.1.1. Optimization

For diastereomeric separation of FLEC-amino acids pH and SDS concentration were found to be key variables affecting the separation. The optimization showed that high pH led to high resolution. The use of organic modifiers, e.g., acetonitrile, in general improved the separation of late eluting amino acids, but the separation of early peaks (less hydrophobic analytes) were somewhat deteriorated [19]. Considering that FLEC-peptides are more hydrophobic than FLEC-amino acids, the optimization in the present work included the organic modifier, 2-propanol and SDS concentrations while the pH was kept at 9.20. The optimization of resolution and efficiency are shown in Fig. 1, and data for four peptides are given in Table 1. The SDS concentrations giving optimal resolution were related to the hydrophobicity of the peptide derivatives where hydrophobicity increased according to: Ala–Gly< Ala–Gly–Gly<Leu–Gly<Leu–Gly–Gly. It was thus found that optimal SDS concentration decreased with increasing hydrophobicity of the analyte. Considering the efficiency, optimal SDS concentration decreased somewhat with increasing analyte hydrophobicity. Optimal 2-propanol concentration was largely the same in all cases. A buffer containing 15 m*M* SDS and 15% 2-propanol was selected for separation of the peptides. The separation of the test analytes under this condition is shown in Fig. 2. Chiral resolution of peptides having two chiral

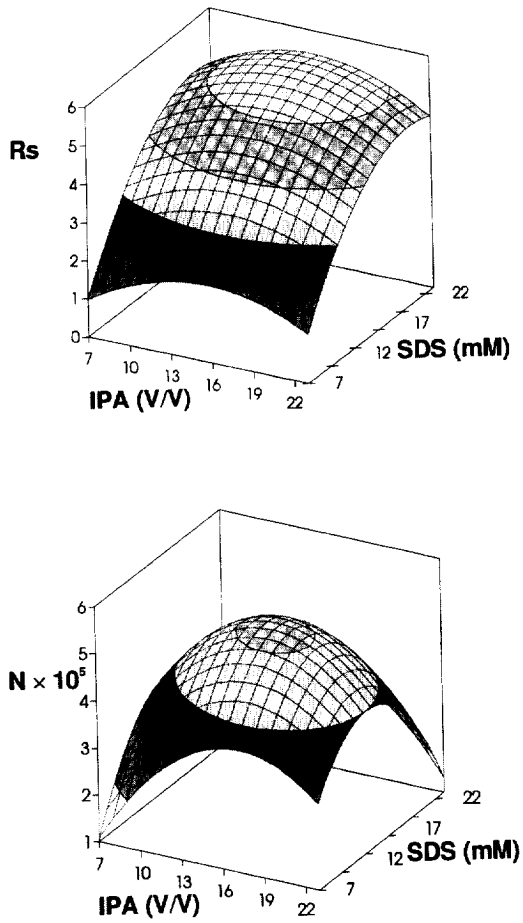

Fig. 1. Optimization of FLEC–Ala–Gly–Gly, resolution (R_s) and efficiency (N) as a function of SDS and 2-propanol concentration. Separation capillary, total 68 cm (46 cm to detector window)×25 μm I.D.; background electrolyte, 40 m*M* borate (pH 9.20), applied voltage, 30 kV; temperature, 25°C.

centers is shown in Fig. 3. In total 8 peptides were separated under the same conditions, Table 2.

Chiral selectivity was not observed for Arg–Gly with the buffer reported in Fig. 2. The migration time was much longer for this peptide than for the other test analytes, thus indicating stronger hydrophobicity. When the SDS concentration was decreased to 10 m*M*, chiral separation was achieved in buffers containing an organic modifier. Selectivity was thus found with tetrahydrofuran (THF), acetonitrile and 2-propanol, but not for methanol. The effects on resolution for a number of different modifiers are shown in Fig. 4. The best results were obtained with

Table 1
Optimal SDS and IPA concentrations for 4 FLEC-peptides

FLEC-peptides	Resolution (R_s)		Efficiency (N)	
	SDS (mM)	IPA (%, v/v)	SDS (mM)	IPA (%, v/v)
Ala–Gly	26.7	15.8	18.3	15.8
Leu–Gly	16.2	15.9	23.8	7.9
Ala–Gly–Gly	20.5	14.8	14.5	15.8
Leu–Gly–Gly	15.5	16.2	22.1	15.6

Conditions: background electrolyte, 40 mM borate (pH 9.20); separation capillary, 68 cm (46 cm to detector window)×25 μm I.D.; applied voltage, 25 kV; temperature, 25°C.

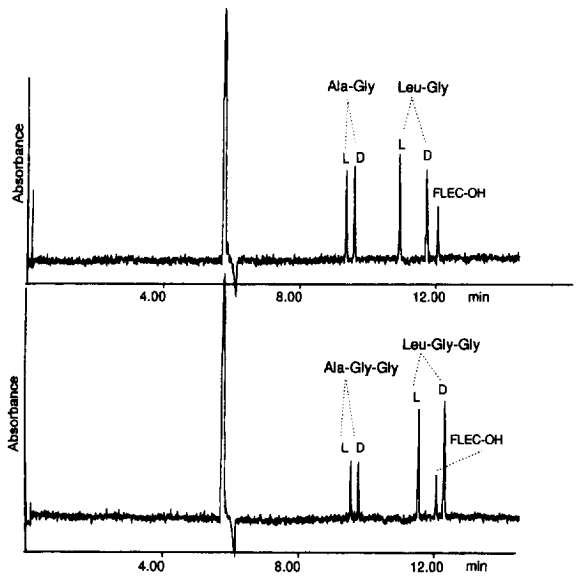

Fig. 2. Chromatogram, UV detection, of four FLEC-(−)-di- and tripeptides. Background electrolyte, 40 mM borate (pH 9.20), 15 mM SDS, 15% 2-propanol (v/v); applied voltage, 25 kV, 3.6 μA; other conditions as in Fig. 1.

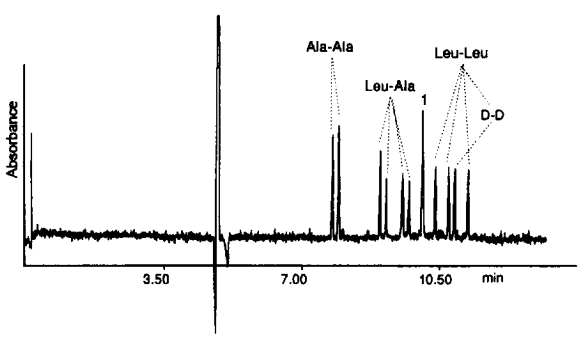

Fig. 3. Chromatogram, UV detection, of three FLEC-(−)-dipeptides with two chiral centres. 30 kV, 4.7 μA; other separation conditions as in Fig. 2. 1=FLEC-OH.

15–18% 2-propanol. This indicates that the selectivity is mainly dependent on the hydrophobicity of the diastereomers. The more hydrophobic the diastereomer, the lower the required SDS concentration. The selectivity observed for Arg–Gly in the presence of less polar organic modifiers was probably due to the effect of the solvent on micelle formation. It is known that the presence of an organic modifier can result in a change of the critical micelle concentration (CMC), micelle shape and aggregation number [23,24]. Further, the separation of the peptides Ala–Gly and Ala–Gly–Gly, and of Leu–Gly and Leu–Gly–Gly was quite similar, Fig. 2. This suggests that the selectivity is dominated by the FLEC moiety and the amino acid directly attached to FLEC. An increase in the number of Gly units does not affect the selectivity to any larger extent, it only results in a small increase in migration times.

The efficiency in micellar electrokinetic capillary chromatography (MEKC) is affected by several factors such as longitudinal diffusion, dispersion emanating from sorption/desorption kinetics (micelle polydispersivity), inter-micellar mass transfer, radial temperature effect on electrophoretic velocity, electrophoretic dispersion of the micelles and adsorption [25]. Terabe and coworkers suggested that the longitudinal diffusion is the most significant cause of band broadening in MEKC, as is often the case in capillary zone electrophoresis [26,27]. In contrast, Sepaniak and Cole showed that mass transfer and thermal gradients, to a large extent, contribute to dispersion in MEKC [25]. This observation was supported by Davies [28]. In the system investigated herein, the presence of 15% 2-propanol led to a considerable improvement in separation efficiency for most of the examined peptides, Table 2. Evidently, the presence of 2-propanol, as suggested by

Table 2
Data for diastereomeric separation of peptides derivatized with (−)-FLEC

FLEC-(−) peptides	(a) 0% (v/v) IPA				(b) 15% (v/v) IPA			
	t_1 (min)	t_2 (min)	R_s	$N/m\times10^5$	t_1 (min)	t_2 (min)	R_s	$N/m\times10^5$
Ala–Ala	4.72	4.82	3.30	10.1	9.28	9.46	2.55	6.30
Ala–Gly	4.81	4.94	3.62	6.65	9.36	9.60	4.10	9.35
Ala–Gly–Gly	9.54	9.76	4.20	8.39	5.20	5.32	3.60	11.6
Ala–Leu	5.62	5.74	4.05	11.3	11.00(1)	11.05(2)	<0.5(1,2)	1.82(1,2)
					11.20(3)		9.96(2,3)	4.68(2,3)
Leu–Ala	5.97(1)	6.05(2)	2.18(1,2)	8.60(1,2)	10.76(1)	10.95(2)	3.24(1,2)	13.1(1,2)
	6.17(3)	6.24(4)	3.08(2,3)	8.80(2,3)	11.45(3)	11.65(4)	7.22(2,3)	10.1(2,3)
			1.70(3,4)	8.00(3,4)			2.69(3,4)	9.01(3,4)
Leu–Gly	6.15	6.34	3.93	5.98	10.94	11.74	11.0	8.72
Leu–Gly–Gly	6.64	6.61	2.37	5.15	11.54	12.30	11.0	10.4
Leu–Leu	6.36(1)	6.51(2)	2.99(1,2)	5.76(1,2)	12.34(1)	12.76(2)	6.34(1,2)	13.1(1,2)
	6.61(3)	6.69(4)	2.05(2,3)	6.76(2,3)	12.93(3)	13.34(4)	2.74(2,3)	13.1(2,3)
			1.81(3,4)	5.92(3,4)			6.01(3,4)	13.6(3,4)
Arg–Gly[c]	13.24	–	0	4.63	20.24[c]	20.67	3.11	6.75

Conditions: (a) buffer, 40 m*M* borate, pH 9.20, 15 m*M* SDS; 25 kV; current, 5.4 μA. (b) 2-propanol (IPA) (15%,v/v) added to buffer (a); current, 3.6 μA; (c) separation capillary, 56 cm (40 cm to detector window)×25 μm I.D.; background electrolyte, 20 m*M* borate–15 m*M* phosphate (pH 9.20), 15% (v/v) 2-propanol; applied voltage, 30 kV; 5.0 μA; temperature, 25°C.

Balchunas and Sepaniak [29], may speed up mass transfer of hydrophobic solutes with the micelles. This result further demonstrates the advantages of using organic buffer modifiers for the separation of hydrophobic compounds. The results may also indicate that band broadening in MEKC is dependent on the nature of the analytes and the applied separation conditions.

Finally, it should be noted that a necessary condition for the successful application of the indirect method is that the intermolecular distance between the chiral centers of the derivatization reagent and of the analyte must not be too long [30,31]. Thus, it was found that the indirect method failed to give resolution of peptides having Gly as the first amino acid.

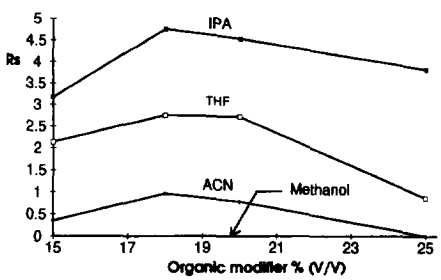

Fig. 4. Effects of organic modifiers on diastereomeric separation of FLEC–Arg–Gly. Separation capillary, 56 cm (40 cm to detector window)×25 μm I.D.; background electrolyte, 20 m*M* borate–15 m*M* phosphate (pH 9.20); applied voltage, 30 kV; temperature, 25°C.

3.2. Direct separation of FMOC-peptides

3.2.1. Optimization

Scouting experiments were performed using a 40 m*M* phosphate buffer at pH 7.50, a potential of 25 kV and different combinations of buffer additives. A pH of 7.50 was earlier found to be suitable for direct separations [17]. Test substances were Gly–Ala, Ala–Gly and Gly–Phe. Evaluated buffer additive combinations were: 12 m*M* β-CD+40 m*M* SDS; 12 m*M* β-CD+40 m*M* SDS+15% IPA; 12 m*M* β-CD; 12 m*M* β-CD+15% IPA; 12 m*M* γ-CD+40 m*M* SDS; 12 m*M* γ-CD+40 m*M* SDS+15% IPA. The best selectivities were obtained with the last set of additives, and this served as a basis for the optimization. In the optimization, SDS was varied between 15 and 44 m*M*, γ-CD was varied between 4 and 14 m*M* and the 2-propanol concentration was at 15%. In earlier work, it was demonstrated that the presence of 15% 2-propanol resulted in enantioselectivity for most FMOC and AEOC amino acids [17,20]. Two peptides, Gly–Ala and Gly–Phe, differing in hydro-

phobicity were used as test analytes. For Gly–Ala, optimal resolution was at 46 m*M* SDS and 14 m*M* γ-CD and optimal plate number was at 28 m*M* SDS and 10 m*M* γ-CD. For Gly–Phe, optimal resolution was at 65 m*M* SDS and 22 m*M* γ-CD and optimal plate number was at 29 m*M* SDS and 12 m*M* γ-CD. The results of the optimization are shown in Fig. 5. As shown in Fig. 5, the enantioselectivity was more influenced by SDS than by γ-CD and the selectivity was enhanced with increasing SDS concentration. However, an optimal efficiency was obtained at a concentration of 30 m*M* SDS. For the separation of a wide range of peptides in the shortest time, a buffer containing 40 m*M* phosphate at pH 7.50, 40 m*M* SDS, 12 m*M* γ-CD and 15% 2-propanol was selected. The SDS concentration chosen is relatively low as compared to the optimal values, but only baseline separation is needed and it is desirable to achieve this in the shortest possible time.

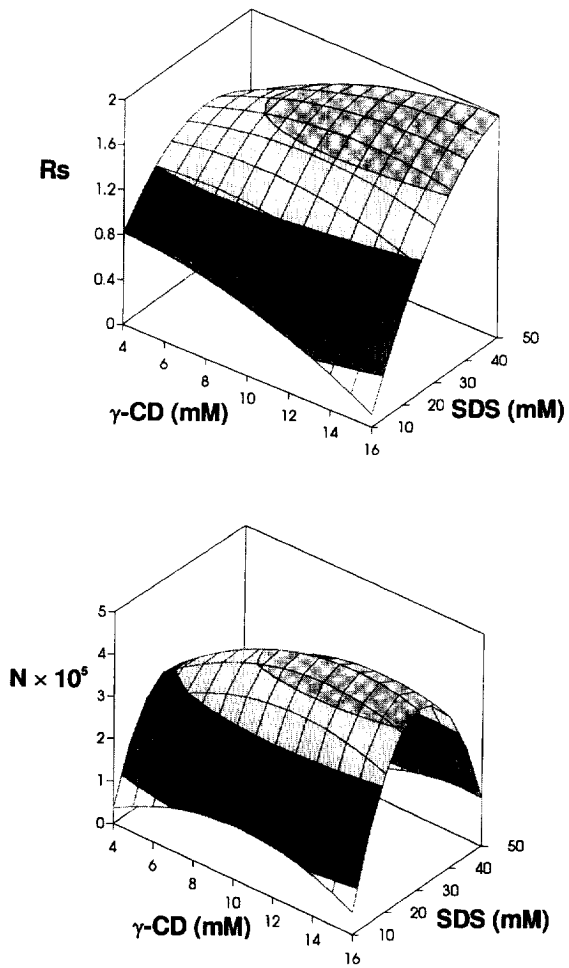

Fig. 5. Optimization of FMOC–Ala–Gly–Gly, resolution (R_s) and efficiency (N) as a function of SDS and γ-CD concentration. Separation capillary, 68 cm (46 cm to detector window)×25 μm I.D.; background electrolyte, 40 m*M* phosphate (pH 7.50), 15% (v/v); applied voltage, 30 kV; temperature, 25°C.

In addition, the effects of increasing the concentration of β-CD to 30 m*M* were examined. Separations were not improved but migration times were prolonged. This is in accordance with earlier results obtained with similar systems but with 2-(9-anthryl)ethyl chloroformate (AEOC)-amino acids as analytes [20]. Comparing the results obtained with β- and γ-cyclodextrins as selectors, the γ-CD showed better selectivity than β-CD. This result was different from the observed chiral recognition of FMOC-amino acids where β-CD was found to be more effective than γ-CD. This difference may be due to the fact that γ-CD has a relatively large cavity and thus is more suitable for the formation of complexes with FMOC-peptides.

In the present system, the analytes having the strongest complexation with CD and the lowest hydrophobicity are eluted first. It was found that the application of β-CD as chiral selector resulted in longer migration times than when γ-CD was employed, Table 3. The obvious explanation for this observation is the lower degree of complexation with β-CD, a consequence of the smaller cavity of this selector.

3.2.2. Effect of modifier type on selectivity

The effects of type of organic modifier on the resolution were examined for a series of peptides, Fig. 6. The highest resolutions were obtained with 2-propanol, but *n*-propanol was also found useful. The difference in selectivity obtained with these two solvents is further illustrated in Fig. 7. It was observed that the presence of an organic modifier was a prerequisite for enantioselectivity for all examined peptides, Fig. 6. Enantioseparation of FMOC-peptides on bonded cyclodextrin phases in HPLC was earlier studied by Zukowski et al. [7], in that work, enantioselectivity was not found when aqueous mobile phases were applied. The presence of organic modifiers may facilitate the enantioselective inclusion of FMOC derivatives within the CD

Table 3
Data for chiral separation of di- and tripeptides with cyclodextrins as chiral selector

FMOC-peptides	γ-CD				β-CD			
	t_1 (min)	t_2 (min)	R_s	$N/m\times10^5$	t_1 (min)	t_2 (min)	R_s	$N/m\times10^5$
Ala–Ala	12.14	12.40	2.30	6.93	16.95	17.06	0.74	2.70
Ala–Gly	12.23	12.31	1.03	9.29	17.14	–	<0.5	1.71
Ala–Gly–Gly	12.97	13.05	1.01	10.6	16.80	–	0	8.43
Ala–Leu	13.46(1)	14.07(2)	8.30(1,2)	12.6(1,2)	22.10	22.26	1.03	7.60
	14.26(3)	14.50(4)	2.53(2,3)	11.7(2,3)				
			2.48(3,4)	10.7(3,4)				
Gly–Ala	12.52	12.64	1.62	11.6	17.11	17.21	1.17	7.25
Gly–Asn	12.99	13.07	1.10	9.93	–	–	–	–
Gly–Asp	13.68	13.88	2.15	8.06	18.57	18.72	1.06	5.92
Gly–Leu	15.22	16.34	3.33	8.04	23.91	24.08	0.93	6.51
Gly–Met	13.53	13.70	2.23	12.0	–	–	–	–
Gly–Phe	15.99	16.39	1.88	9.65	23.86	24.03	1.05	7.89
Gly–Val	13.62	13.79	2.00	9.28	18.73	–	0	4.84
Leu–Ala	14.56(1)	14.74(2)	2.24(1,2)	12.0(1,2)	22.05(1)	22.42(2)	1.77(1,2)	4.50(1,2)
	14.93(3)		2.25(2,3)	10.5(2,3)	22.60(3)		0.76(2,3)	3.10(2,3)
Leu–Gly	15.73	–	<0.5	3.24	23.91	24.15	1.52	7.26
Leu–Gly–Gly	16.95		<0.5	2.83	23.87	24.11	1.50	8.27
Leu–Leu	17.51(1)	17.64(2)	1.06(1,2)	8.25(1,2)	27.45	28.04	2.74	5.86
	17.92(3)		2.41(2,3)	10.3(2,3)				

Conditions: buffer, 40 m*M* phosphate, pH 7.50, 40 m*M* SDS, 12 m*M* γ-CD, 15% (v/v) 2-propanol, 25 kV, 10 μA.

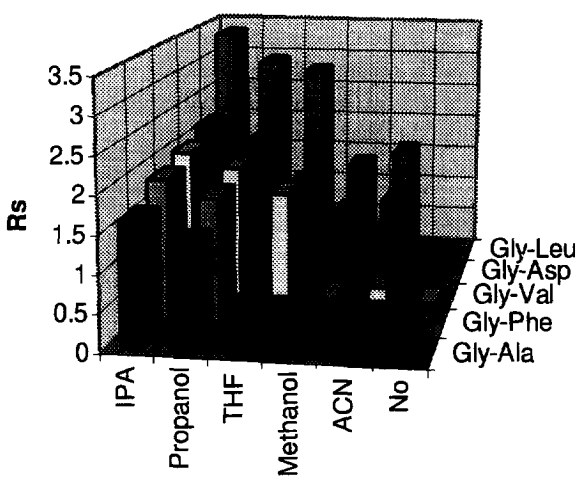

Fig. 6. Effects of organic modifiers on enantiomeric separation of FMOC-dipeptides. Background electrolyte, 40 m*M* phosphate (pH 7.50), 40 m*M* SDS, 12 m*M* γ-CD, 15% (v/v) of each organic modifier; other conditions as in Fig. 5.

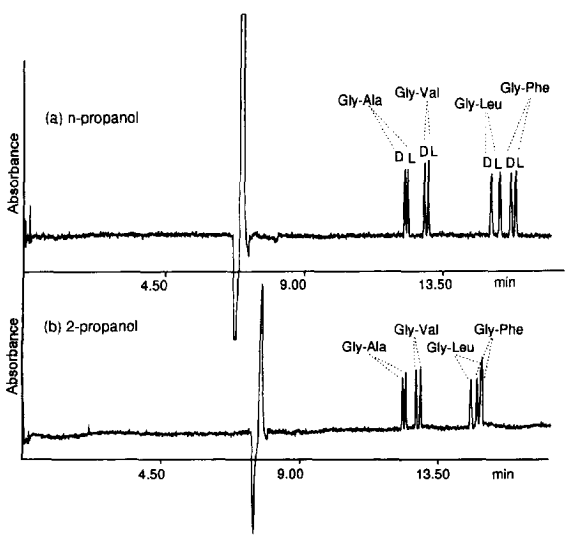

Fig. 7. Chromatogram, UV detection, of four FMOC-dipeptides, using *n*-propanol (a) and 2-propanol (b) as modifiers, other conditions as in Fig. 6. (a) and (b) have the same enantiomeric elution order.

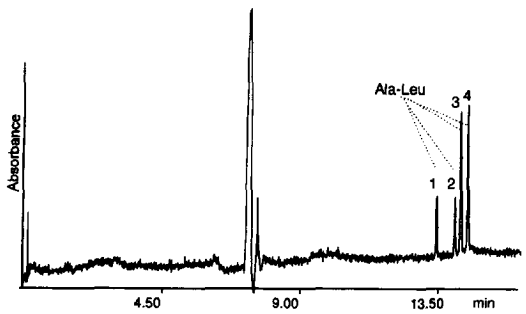

Fig. 8. Chromatogram, UV detection, of FMOC–Ala–Leu with two chiral centres; 15% 2-propanol (v/v), 25 kV, 10 μA; other separation conditions as in Fig. 6.

cavity. It has been suggested that organic modifiers decrease the rigidity of the cavity because the solvation of the CD modify the CD conformation from a lower entropy (aqueous) state to a higher entropy (organic modifier) [32].

3.2.3. Peptide separation

Separation data are given in Table 3. In total 9 FMOC-peptides were baseline separated and 6 were partially separated with γ-CD as the chiral selector. Two of the partially separated peptides, Leu–Gly and Leu–Gly–Gly, could be baseline separated when using β-CD as the chiral selector. The two chiral selectors resulted in the same enantiomeric elution order, D-form being first eluted. Chiral separation of a peptide having two chiral centers is shown in Fig. 8. Due to lack of standards, the enantiomeric elution orders could not be established in Figs. 3 and 8.

4. Conclusions

On the basis of the present results, a comparison of the performance of the indirect and the direct method for the analysis of chiral compounds can be done. First, optimization is more straightforward in the indirect method than in the direct method where the concentration of the chiral selector is an additional factor to be optimized. However, this is not a major problem when using factorial design for optimization.

Second, the derivatization reagents used for the indirect method must have a very high chiral purity, or if not, the concentration of the impurity must be known in order to facilitate a correction for this [33]. Further, chirally pure derivatization reagents are more costly than non-chiral ones.

Third, in general, chiral selectors are good for chiral separation only, that is, the chiral analytes tend to be eluted in a relatively narrow range, thus making separations of mixtures difficult using the direct method. However, with the use of SDS, the analytes are not only chirally separated but they are also separated according to their hydrophobicity thus facilitating the separation of mixtures.

Fourth, the main advantage of CE as compared with HPLC for chiral separation is the high plate numbers which makes separation possible at low α values. Often, plate numbers obtained with the indirect method are higher than those obtained with the direct method [19]. The obvious reason for this is the relatively slow kinetics of the formation of the diastereomeric complexes. However, in the present work, the application of γ-CD as chiral selector resulted in plate numbers in the same range as obtained with the indirect method, 10^6 plates/m, cf. Tables 2 and 3. The analysis times are still shorter for the indirect method, but the differences are smaller than those earlier reported [19].

Fifth, an inherent limitation of the indirect method is the requirement that the chiral center of the derivatization reagent and that of the analyte must be in rather close proximity if separation is to be achieved in a non-chiral system. Thus, peptides beginning with glycine could not be separated when applying the indirect method. Considering all factors, it seems that the two methods are complementary.

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