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Enantiomeric separation of N-*tert.*-butoxycarbonyl amino acids by capillary electrophoresis using hydroxypropyl-substituted cyclodextrins

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Abstract

The separation of the enantiomers of N-*tert.*-butoxycarbonyl (N-*t*-Boc) amino acids by capillary electrophoresis employing hydroxypropyl-substituted cyclodextrins is described. The enantiomeric separation of the N-*t*-Boc derivatized amino acids studied was successfully accomplished by employing hydroxypropyl-β-cyclodextrin. The N-*t*-Boc-D,L-3-(2-naphthyl)alanine enantiomers were separated using hydroxypropyl-γ-cyclodextrin. The different forms and concentrations of cyclodextrins, and the effects of organic solvent addition to the separation buffer are investigated. In addition, the pH of the separation buffer and the effect of pH on the enantioselectivity of the N-*t*-Boc amino acids are discussed.

Keywords: Enantiomer separation; Amino acids

1. Introduction

The sequential synthesis of peptides is often performed using N-*tert.*-butoxycarbonyl (N-*t*-Boc) amino acids. It has been demonstrated that the N-*t*-Boc group serves as a protecting group during peptide synthesis because the N-*t*-Boc amino acid is resistant to racemization. The N-*t*-Boc functionality can then be easily removed following the synthesis by acid-catalyzed hydrolysis. The chiral purity of the N-*t*-Boc amino acids used throughout the synthesis is obviously of great importance to ensure chiral purity of the peptide. Oi et al. [1] demonstrated the use of amide and urea derivatives bonded to silica gel as a chiral stationary phase for the separation of N-*t*-Boc amino acid derivatives. Konishi et al. [2] described the use of an anion-binding chiral receptor based on a metalloporphyrin for enantioselective recognition of N-*t*-Boc amino acid derivatives. There have also been reports in which chiral selecting agents are used as mobile phase additives to resolve N-*t*-Boc-phenylalanine by HPLC [3,4]. The enantiomeric separation of N-*t*-Boc amino acids, and the impact that chiral impurities might have on peptide synthesis, have also been previously described by Chang et al. [5]. In that article, N-*t*-Boc amino acids were separated by HPLC using a column which had been derivatized with hydroxypropyl-β-cyclodextrin.

There have been a number of articles discussing the enantiomeric separation of amino acids using capillary electrophoresis (CE). Kuhn et al. [6] demonstrated the use of [18]-crown-6-tetracarboxylic acid as a chiral selecting agent for resolving underivatized racemic amino acids. Gassman et al. [7] described a technique known as chiral ligand exchange chromatography in which a transition metal-amino acid complex is formed; it has been proposed that a difference in the stability of this complex for

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each of the isomers produces enantioselective recognition. Gozel et al. [8] also demonstrated that chiral ligand exchange chromatography, employing a Cu^{2+}–aspartame complex as an electrolyte additive, could be used to effect an enantiomeric separation of Dansyl-amino acids. Buck and Kang [9] described a separation using micellar electrokinetic chromatography (MECC) for the resolution of OPA-derivatized amino acid enantiomers.

2. Experimental

2.1. Materials

The N-*t*-Boc amino acids discussed were purchased from Sigma (St. Louis, MO, USA). The sodium phosphate monobasic and sodium phosphate dibasic were also purchased from Sigma. The HPLC-grade methanol was purchased from Burdick and Jackson (Muskegon, MI, USA). The water used for the preparation of the separation buffers and samples was produced in-house by the Milli-Q System (Millipore, Milford, MA, USA). The fused-silica capillary was purchased from Polymicro Technologies (Phoenix, AZ, USA).

2.2. Capillary electrophoresis

The experiments discussed in this article were performed using a Beckman P/ACE Capillary Electrophoresis System (Beckman Instruments, Fullerton, CA, USA). A Beckman cartridge was fitted with a fused-silica capillary that was cut to a total length of 57 cm; the effective separation length was 50 cm, and the internal diameter was 50 μm. All separations were conducted at a field strength of 300 V/cm (17.1 kV overall), generating a current between 50 and 60 μA. The cartridge coolant temperature was maintained at 23°C. Detection was achieved by UV absorbance at 200 nm and the sample injections were made using a positive pressure of 0.5 p.s.i.; (1 p.s.i. = 6894.76 Pa) for 4 s.

A stock buffer consisting of 0.5 *M* phosphate (pH 7.0) was prepared by mixing equimolar amounts of sodium phosphate monobasic and sodium phosphate dibasic in Milli-Q water, and adjusting the pH with aqueous sodium hydroxide. The separation buffers were prepared by dissolving the hydroxypropyl-substituted cyclodextrin in the appropriate volume of the stock buffer along with methanol. For example, to prepare 50 ml of buffer containing 15% (v/v) methanol and 10 m*M* hydroxypropyl-β-cyclodextrin, 5.0 ml of the stock buffer was transferred to a 50-ml beaker, containing approximately 25 ml of distilled–deionized water (Milli-Q water, or equivalent), along with 7.5 ml of methanol; then, 750 mg of the hydroxypropyl-β-cyclodextrin was added. After the cyclodextrin was completely dissolved, the solution was transferred to a 50-ml volumetric flask, and the flask was filled to volume with Milli-Q water.

The N-*t*-Boc amino acids were prepared at a concentration of approximately 0.5 mg/ml by dissolving them first in methanol (10% of the final volume), followed by the addition of an appropriate volume of Milli-Q water. The electropherograms in Fig. 1 Fig. 2 Fig. 3 represent the enantiomeric separations of six of the N-*t*-Boc amino acids discussed.

3. Results and discussion

The data in Table 1 show the resolution factors and alpha values determined for each of the of N-*t*-Boc amino acids studied. The separation conditions were as previously described: 50 m*M* phosphate (pH 7.0), 10 m*M* hydroxypropyl-β-cyclodextrin, and 15% methanol. The separation of the N-*t*-Boc-D,L-3-(2-naphthyl)-alanine was achieved using hydroxypropyl-γ-cyclodextrin.

The resolution factors, R_s, were calculated using the following equation:

$$R_s = \frac{(t_{m2} - t_{m1})}{1/2(w_{t_{m1}} + w_{t_{m2}})} \quad (1)$$

where t_{m2} is the migration time of the second enantiomer, t_{m1} is the migration time of the first enantiomer, $w_{t_{m1}}$ is the peak width of the first enantiomer, and $w_{t_{m2}}$ is the peak width of the second enantiomer.

3.1. Effect of methanol

The addition of methanol resulted in the increased separation of enantiomeric pairs which were only

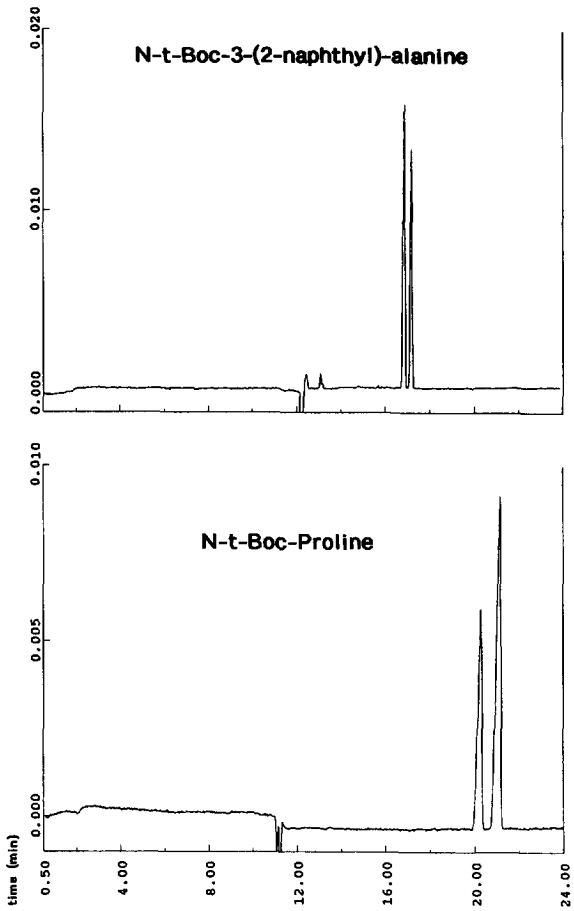

Fig. 1. Electropherograms showing the enantiomeric separation of N-*t*-Boc-3-(2-naphthyl)-alanine (top) and N-*t*-Boc-proline (bottom).

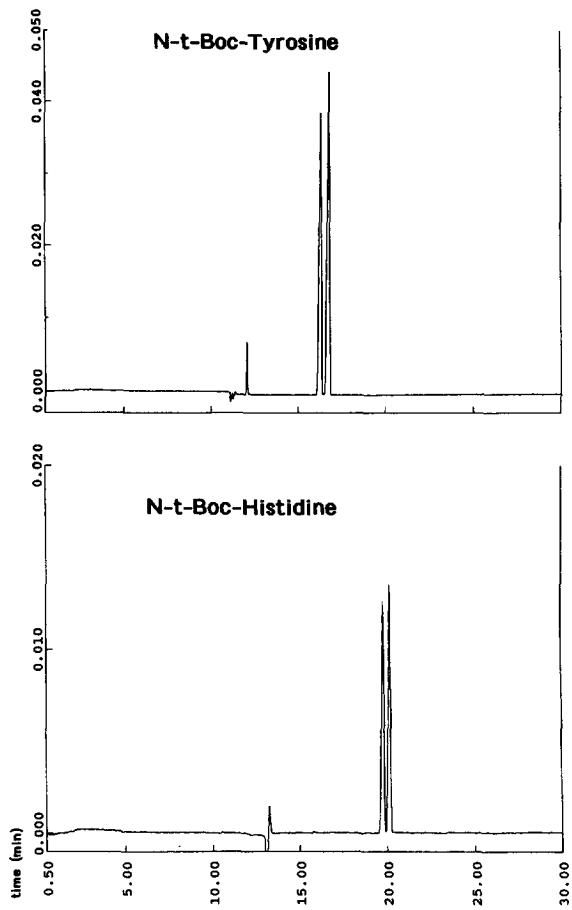

Fig. 2. Electropherograms showing the enantiomeric separation of N-*t*-Boc-tyrosine (top) and N-*t*-Boc-histidine (bottom).

partially, and in some cases, completely unresolved. The effect of organic modifiers, such as methanol, added to the background electrolyte (BGE), has been previously discussed [10–13]. The increase in resolution can be seen in Fig. 4 which shows the separation of N-*t*-Boc-D,L-histidine with increasing amounts of methanol added to the BGE. The addition of methanol is known to reduce electroosmotic flow (EOF), though the exact mechanism is not fully understood. Salomon et al. [14,15] suggests that the presence of methanol in the BGE shields charged sites on the capillary wall from each other, thereby causing an overall reduction in the zeta (ζ) potential. Additionally, a decrease in the electroosmotic flow would result from an increase in the viscosity of the separation buffer which occurs as the methanol concentration increases.

Fig. 5 shows the effect that increasing methanol concentration had on the resolution of four of the N-*t*-Boc amino acids. The resolution continued to increase as the methanol concentration increased from 5 to 25%. In fact, additional experiments indicated that the enantiomeric resolution of N-*t*-Boc-D,L-tyrosine continued to increase even as the concentration of methanol reached 40%. However, when the methanol was increased to 50%, the N-*t*-Boc-D,L-tyrosine did not migrate past the detector

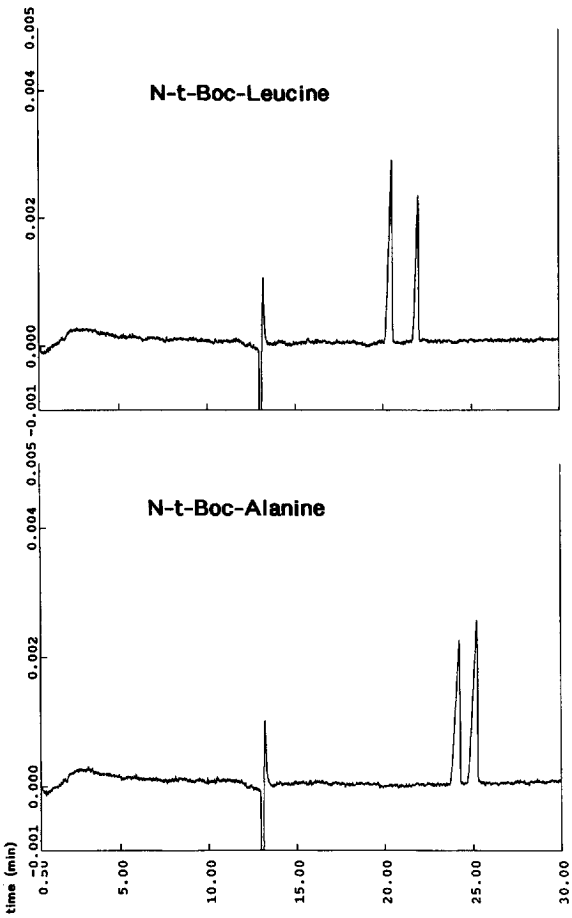

Fig. 3. Electropherograms showing the enantiomeric separation of N-*t*-Boc-leucine (top) and N-*t*-Boc-alanine (bottom).

even after 6 h due to the reduction in EOF. The following equation was used for the determination of the electroosmotic flow:

$$\mu_{eo} = \frac{l}{(t)(E)} \quad (2)$$

where l is the effective separation length, or the length of the capillary to the detection window (in cm), t is the migration time of the unretained peak (in s), and E is the field strength (in V/cm).

As seen in Fig. 6, the viscosity of a methanol–water mixture [16] indicates that the viscosity increases between 0 and 50%, but then decreases from 50 to 100%. There are probably additional factors which influence the observed reduction in μ_{eo} in addition to the change in viscosity. It is difficult to determine whether or not viscosity is the only influencing factor for the simple reason that it was not possible to operate this particular buffer system with higher levels of methanol due to the fact that the phosphate was insoluble when the methanol concentration exceeded 50%.

Table 1
The resolution (R_s) factors and alpha (α) values experimentally determined for each of the fourteen N-*tert.*-butoxycarbonyl amino acids investigated

N-*t*-Boc amino acid	α	Resolution, R_s
D,L-Alanine	1.09	1.88
D,L-Asparagine	1.05	1.53
D,L-Glutamine	1.07	2.01
D,L-Histidine	1.05	1.39
D,L-Isoleucine	1.24	4.58
D,L-Leucine	1.20	3.83
D,L-Methionine	1.14	2.81
D,L-3-(2-Naphthyl)-alanine[a]	1.07	3.20
D,L-Phenylalanine[b]	1.10	2.18
D,L-Proline	1.10	2.34
D,L-Serine	1.06	1.49
D,L-Tryptophan	1.07	1.53
D,L-Tyrosine[b]	1.09	1.55
D,L-Valine	1.23	6.13

All of the above enantiomeric pairs were separated using 50 m*M* phosphate (pH 7.0), 10 m*M* hydroxypropyl-β-cyclodextrin, and 15% methanol (v/v), except [a], which was separated using 50 m*M* phosphate (pH 7.0), 10 m*M* hydroxypropyl-γ-cyclodextrin, and 15% methanol (v/v), and [b], which was separated using 50 m*M* phosphate (pH 7.0), 10 m*M* hydroxypropyl-β-cyclodextrin, and 10% methanol (v/v).
The separation voltage was maintained at 300 V/cm (17.1 kV on a 57-cm total length capillary); detection was achieved at 200 nm; the injections were performed using 0.5 p.s.i. positive pressure for 4 s; the capillary cartridge coolant temperature was maintained at 23°C.

3.2. Effect of pH

At pH 7, the N-*t*-Boc amino acids possess a net negative charge. The pK_a of the carboxyl groups are quite low (from 1.82 for histidine to 2.34 for alanine). Therefore, when the N-*t*-Boc amino acid is incorporated into the cyclodextrin cavity, the complex migrated with a electrophoretic mobility counter to that of the electroosmotic flow vector; it is for this reason that a separation from t_0 is observed. Enantioselectivity is lost for the N-*t*-Boc-D,L-histidine below pH 7.0 due to the protonation of the amino group

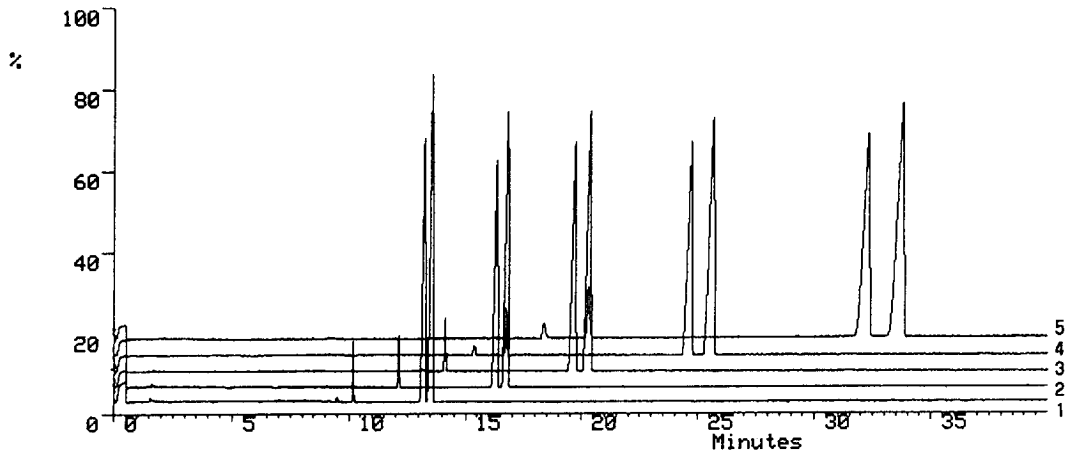

Fig. 4. The effect of increasing methanol concentration on the enantiomeric separation of N-*t*-Boc-histidine; (1) 5% methanol, (2) 10% methanol, (3) 15% methanol, (4) 20% methanol, (5) 25% methanol.

which results in the formation of a zwitterion causing the complex to migrate with t_0. The best resolution was observed at pH values between 7 and 8 for most N-*t*-Boc amino acids. Lower pH buffers did reduce electroosmotic flow which resulted in better resolution (with the exception of N-*t*-Boc-D,L-histidine), but this was not the result of a change in the interaction between the amino acid and the cyclodextrin.

The enantioselectivity may also be attributed to ionic interactions between the analyte and the cyclodextrin as described by Rawjee and co-workers [17,18]. The enantioselective behavior is consistent with the *Type II* enantiomeric separation discussed in these articles; that is, the dissociated forms of the analytes interact differently with the cyclodextrin resulting in the chiral recognition.

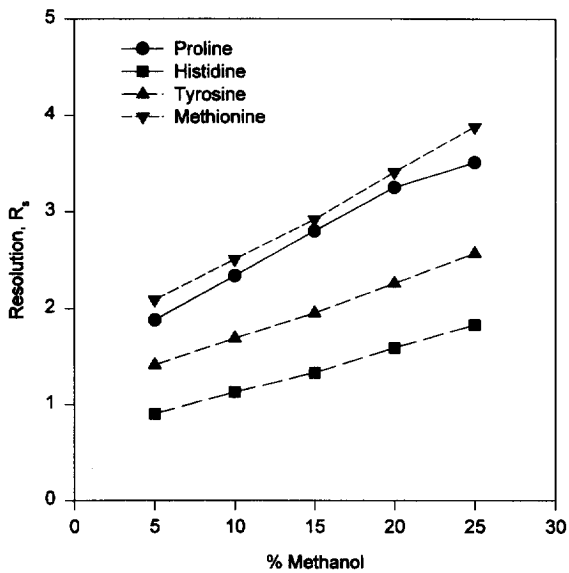

Fig. 5. The effect of increasing methanol concentration on the enantiomeric separation of four of the N-*t*-Boc amino acids investigated.

3.3. Effect of the cyclodextrin concentration

All of the N-*t*-Boc amino acids tested exhibited a chiral separation using the hydroxypropyl-β-cyclodextrin with the exception of the N-*t*-Boc-D,L-3-(2-naphthyl)-alanine enantiomers which were separated using hydroxypropyl-γ-cyclodextrin. The enantioselective recognition is believed to occur as a result of an interaction between the R-group and the hydroxypropyl group as the N-*t*-Boc group inserts itself into the more hydrophobic cyclodextrin cavity. Fig. 7 illustrates the effect of increasing cyclodextrin concentration on enantiomeric resolution. The optimum cyclodextrin concentration, for the N-*t*-Boc amino acids tested, was between 5 and 15 m*M*. Attempts to separate the N-*t*-Boc amino acids with β-cyclodextrin rather than the hydroxypropyl-substituted β-cyclodextrin proved to be unsuccessful. However, the N-*t*=Boc-D,L-3-(2-naphthyl)-alanine

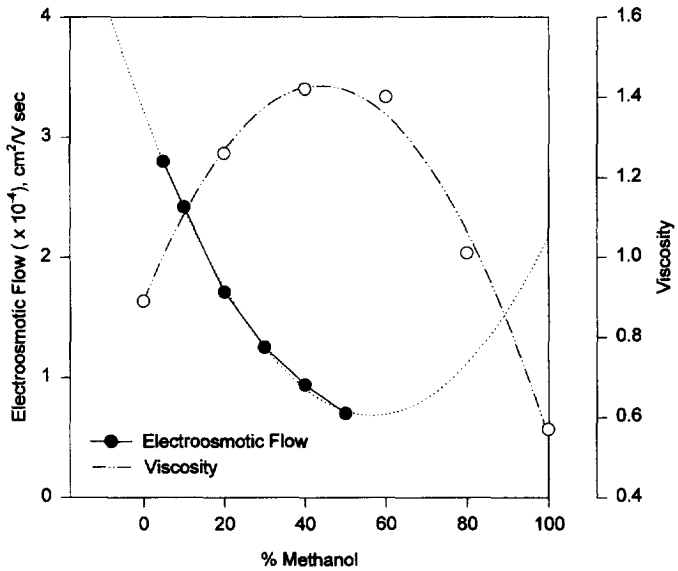

Fig. 6. The effect of increasing methanol concentration on the electroosmotic flow.

was separated with both the hydroxypropyl-substituted and the unsubstituted γ-cyclodextrin which might indicate that enantioselectivity occurred through a different mechanism. Also, since the N-*t*-Boc-D,L-3-(2-naphthyl)-alanine does not contain a carboxyl terminus, it will not possess a negatively charged functionality. This might explain why, in fact, all of the enantiomeric separations described exhibited the D-isomer first followed by the L-isomer; only the N-*t*-Boc-D,L-3-(2-naphthyl)-alanine enantiomers eluted in the reverse order. Perhaps the chiral recognition occurs due to the insertion of the naphthyl group into the cyclodextrin cavity rather than by ionic interaction between the charged side-chain and the hydroxypropyl-substituted β-cyclodextrin. Additionally, all of the N-*t*-Boc amino acid enantiomers were separated using the hydroxypropyl-substituted β-cyclodextrin with the exception of the N-*t*-Boc-D,L-3-(2-naphthyl)-alanine, which required the γ-cyclodextrin.

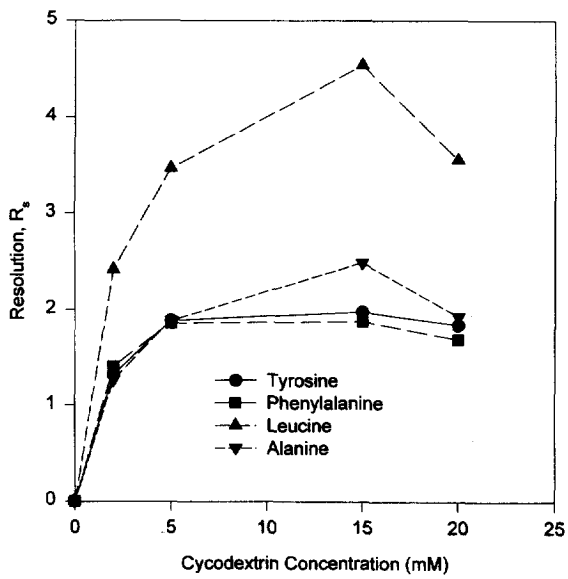

Fig. 7. The effect of increasing cyclodextrin concentration on the enantiomeric resolution.

4. Conclusion

The fact that an enantiomeric separation is accomplished for all N-*t*-Boc amino acids appears to indicate that the N-*t*-Boc is the common functionality and probably is incorporated into the cavity of the cyclodextrin. The inner cavity of the cyclodextrin is relatively hyrophobic, which serves to anchor or 'lock' the N-*tert.*-butoxycarbonyl moiety into a position in which the chiral recognition can occur

between the chiral center of the amino acid and the more hydrophilic surface of the outer ring. However, the mechanism for chiral recognition may be somewhat more complex. It appears, from those N-*t*-Boc amino acids studied, that ionic interactions between the analyte and the cyclodextrin may also play a significant role in enantioselectivity. The use of methanol to control or reduce electroosmotic flow is an interesting alternative to the more conventional route of changing the pH of the BGE, particularly in those cases in which pH may negatively effect chiral recognition. Also, the concentration of cyclodextrin needed to achieve adequate resolution is relatively low (2 to 10 m*M*). Initially, resolution appears to show improvement as the cyclodextrin concentration is increased; however, above concentrations of 15 m*M*, the apparent loss of enantiomeric resolution is observed.

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