

# Interactions of Enantiomers of Aromatic Amino Acids with Highly Sulfated Cyclodextrins



The variation in  $\alpha$ -amino acid side chains and their derivatives at the  $\alpha$ -amino terminal provide an ideal model for systematic study of the structural interactions with chiral selectors. The author reports a comparative study of the interaction of aromatic amino acids with a class of highly sulfated cyclodextrins as the chiral selectors by capillary electrophoresis. The structural characterization of the highly sulfated cyclodextrins indicated a narrow range of heterogeneity in each highly sulfated cyclodextrin. The average degrees of substitution for highly sulfated  $\alpha$ -,  $\beta$ -, and  $\gamma$ -cyclodextrins were 11, 12, and 13 sulfate esters, respectively. The hydrophobic cavity of the highly sulfated cyclodextrins functions as host for the aromatic moiety of the amino acids. The author proposes that this structural arrangement in highly sulfated cyclodextrins is responsible for regiospecific interactions with enantiomers of the aromatic  $\alpha$ -amino acids.

**T**he  $\alpha$ -amino acids found in nature contain a free carboxylic acid, an amino group, and hydrogen as the common groups connected to the  $\alpha$  carbon atom. They differ from each other in the structural makeup of their side chains, which determines the fundamental characteristics related to the genetic codes. All  $\alpha$ -amino acids, except glycine, contain a chiral  $\alpha$  carbon center. A variation of  $\alpha$ -amino acid in the side chains provides an ideal model for studying structural interactions with chiral selectors.

Analyses of enantiomers of  $\alpha$ -amino acids and their derivatives by capillary electrophoresis (CE) have been reported extensively in the literature, and cyclodextrins are by far the most common chiral selectors for this application. Fanali and Bocek (1) showed that optimum resolution of tryptophan, phenylalanine, and tyrosine racemates was achieved with  $\alpha$ -cyclodextrin in an acidic buffer by CE in which the amino groups were fully protonated.

The superior resolution of aromatic  $\alpha$ -amino acid by CE was reported first by Kuhn and co-workers (2) in a study in which the chiral crown ether 18-crown-6-

tetracarboxylic acid was the chiral selector of choice. The binding cavity in 18-crown-6-tetracarboxylic acid is specific to cationic species such as the alkali, alkaline earth, and ammonium salts of the primary amine only. This binding specificity occurs on the protonated  $\alpha$ -amino group of  $\alpha$ -amino acids at the center of the crown ethers (2,3). The remaining carboxylic acid and the side chain are responsible for chiral interactions with the carboxylic acids in 18-crown-6-tetracarboxylic acid, hence the differentiation sites.

Gahm and Stalcup (4) reported the use of sulfated  $\beta$ -cyclodextrin for the chiral analysis of catechol-based  $\alpha$ -amino acids, catecholamines, and related compounds in which the CE runs were performed in an untreated fused-silica capillary with phosphate buffer (pH 3.2) using reversed polarity.

Many other varieties of cyclodextrins and their derivatives are available commercially as chiral selectors. The anionic cyclodextrins, in general, appear to be the most valuable as the charged chiral selectors because most racemic drugs are basic in nature and contain positively charged moieties (for example, protonated amine groups) at buffer pH levels lower than pH 9.0. Among all

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anionic cyclodextrins, sulfobutylether and sulfated cyclodextrins in particular received the most attention and appeared to be the most effective charged chiral selectors (4–10). However, these anionic cyclodextrins were relatively heterogeneous and provided no reproducible results as chiral reagents. Nonetheless, several researchers reported applications using different lots of chiral selectors such as sulfobutylether (11,12) and sulfated  $\beta$ -cyclodextrins (13–15) that showed excellent reproducibility in chiral analysis.

My co-workers and I recently have developed a class of highly sulfated cyclodextrins for CE-based chiral analysis (5,16). The highly sulfated  $\alpha$ -,  $\beta$ -, and  $\gamma$ -cyclodextrins were characterized by  $^{13}\text{C}$  nuclear magnetic resonance spectroscopy (NMR) with structural assignment that the sulfation occurred at the C-6 primary and C-2 secondary hydroxyls exclusively and with complete absence of substitution at the C-3 hydroxyls. Highly sulfated cyclodextrins have substantially higher degrees of sulfation (5) than most of the sulfated cyclodextrins used in previous reports (6–8). Enantiomers of neutral, basic, and acidic drugs with widely varying structures were successfully resolved by CE using highly sulfated cyclodextrins as chiral selectors (5,9,16–18) (also see [http://www.beckmancoulter.com/resourcecenter/labresources/ce/pdf/chiral38\\_alpha.pdf](http://www.beckmancoulter.com/resourcecenter/labresources/ce/pdf/chiral38_alpha.pdf)).

Perrin and colleagues (9) reported the great ability of highly sulfated cyclodextrins for rapid screening in the enantiomeric separation of a group of amino-acid derivatives, beta-blockers, and other basic drugs. Lurie and co-workers (17) reported that an extremely high resolution of 2-phenethylamine and its derivatives was obtained by CE with highly sulfated  $\gamma$ -cyclodextrin as the chiral selector. Similarly, di- or tripeptides that contained two chiral centers, including diastereomeric isomers and a large number of drugs, and few amino acids and their esters were well resolved preferentially in highly sulfated  $\beta$ - and  $\gamma$ -cyclodextrins (10).

The objective of my study is to probe the structure and function relationship of host-guest interaction with regard to their affinity and chiral resolution. In this article, I report a CE-based systematic chiral analysis using highly sulfated cyclodextrins and racemic aromatic  $\alpha$ -amino acids as the model hosts and ligands, and I attempt to provide a ligand-based structural elucidation and prediction for the selection of ideal chiral selectors.

## Experimental

**Materials:** The highly sulfated cyclodextrins were obtained as 20% (w/v) aqueous solutions from Beckman Coulter, Inc. (Fullerton, California). Racemates of aromatic  $\alpha$ -amino acids and pure enantiomers were obtained from Sigma Co. (St Louis, Missouri). All aromatic  $\alpha$ -amino acids were dissolved in 0.5 N hydrochloric acid at 20 mM and diluted to 0.5 to 1.0 mM in aqueous solution containing 0.25 mM 1,3,6,8-pyrenetetrasulfonic acid tetrasodium salt (Acros Chemicals, Pittsburgh, Pennsylvania) as the internal migration marker. The polyethylene oxide solution was 0.4% polyethylene oxide (MW 300,000) from Aldrich (Milwaukee, Wisconsin) in lithium acetate buffer (pH 4.75).

**Analysis:** CE analyses were performed on a P/ACE MDQ CE system (Beckman Coulter) equipped with a photodiode-array detector. All chiral separations were performed with 5% highly sulfated cyclodextrins in 25 mM phosphate (pH 2.5) (triethylammonium salt), unless otherwise specified. A 31.5 cm  $\times$  50  $\mu\text{m}$  fused-silica capillary was rinsed with methanol and water at 20 psi for 2 min and then rinsed with a polyethylene oxide solution at 20 psi for 4 min. Between runs, the capillary was rinsed with running buffer for 2 min at 20 psi. Samples were introduced by pressure injection at 0.3 psi for 4 s. The separation temperature was maintained at 22  $^{\circ}\text{C}$  with an applied potential of 15 kV.

## Results

**General conditions for enantiomeric separation of racemates by CE in highly sulfated cyclodextrins:** The original intent of using highly sulfated cyclodextrins for CE-based chiral separation was to resolve the neutral enantiomeric species. The sulfate moieties in highly sulfated cyclodextrins act as the charge carriers for electrophoretic separation, and the host-guest interaction would remain intact in the hydrophobic domain and independent of buffer pH. To perform enantiomeric separation of neutral chiral species, I selected a pH 2.5 phosphate buffer to reduce the electroosmotic flow. Under these conditions, neutral analytes were dragged to the anode by complexation with negatively charged highly sulfated cyclodextrins.

An internal migration reference marker — 1,3,6,8-pyrenetetrasulfonate — was added to the sample to serve as a migration reference marker for each chiral analysis using highly sulfated cyclodextrins. 1,3,6,8-Pyrenetetrasulfonate contains four sulfonic

acid residues and does not complex with highly sulfated cyclodextrins. It has a relatively high electrophoretic mobility that produces a short migration time toward the anode. In pH 2.5 phosphate buffer, the migration time of 1,3,6,8-pyrenetetrasulfonate gradually decreased on the first few runs with a freshly prepared, bare fused-silica capillary, which is indicative of residual electroosmotic flow toward the cathode.

Figure 1 summarizes the changes in migration time of 1,3,6,8-pyrenetetrasulfonate and glutethimide enantiomers (neutral analyte) in highly sulfated  $\beta$ -cyclodextrin with multiple sequential runs of a freshly prepared capillary. The gradual significant decrease in the migration time of 1,3,6,8-pyrenetetrasulfonate and glutethimide enantiomers in a freshly prepared capillary was evident after the first 10 runs. After treatment of the capillary with a polyethylene oxide solution during the 11th run, the migration time became constant and reproducible. The repeating oxyethylene units in polyethylene oxide form hydrogen bonds with silanol moieties of the silica surface at low pH that provide a stable polymeric coating and reduce the electroosmotic flow. To provide reproducible runs on a freshly prepared capillary, I washed the capillary with methanol and water and again with a polyethylene oxide solution. Rinsing the capillary with a polyethylene oxide solution is required for only the first run on daily basis. From that point forward, the capillary is reconditioned between runs only with the running buffer.

**Enantiomeric separation of aromatic  $\alpha$ -amino acids by CE in highly sulfated cyclodextrins:** All CE-based enantiomeric separations of aromatic  $\alpha$ -amino acids were performed using a 31.5 cm  $\times$  50  $\mu\text{m}$  capillary (10.5 cm to the detector), a phosphate buffer containing a 5% solution of highly sulfated cyclodextrins (pH 2.5), and an applied potential of 15 kV. Under these conditions, a 5% solution of highly sulfated cyclodextrins is equivalent to approximately 250 mequiv of fully ionized species yielding roughly 150  $\mu\text{A}$  current and producing 2.3 W of heat. An efficient cooling system is most desirable to dissipate the heat generated during the separation process.

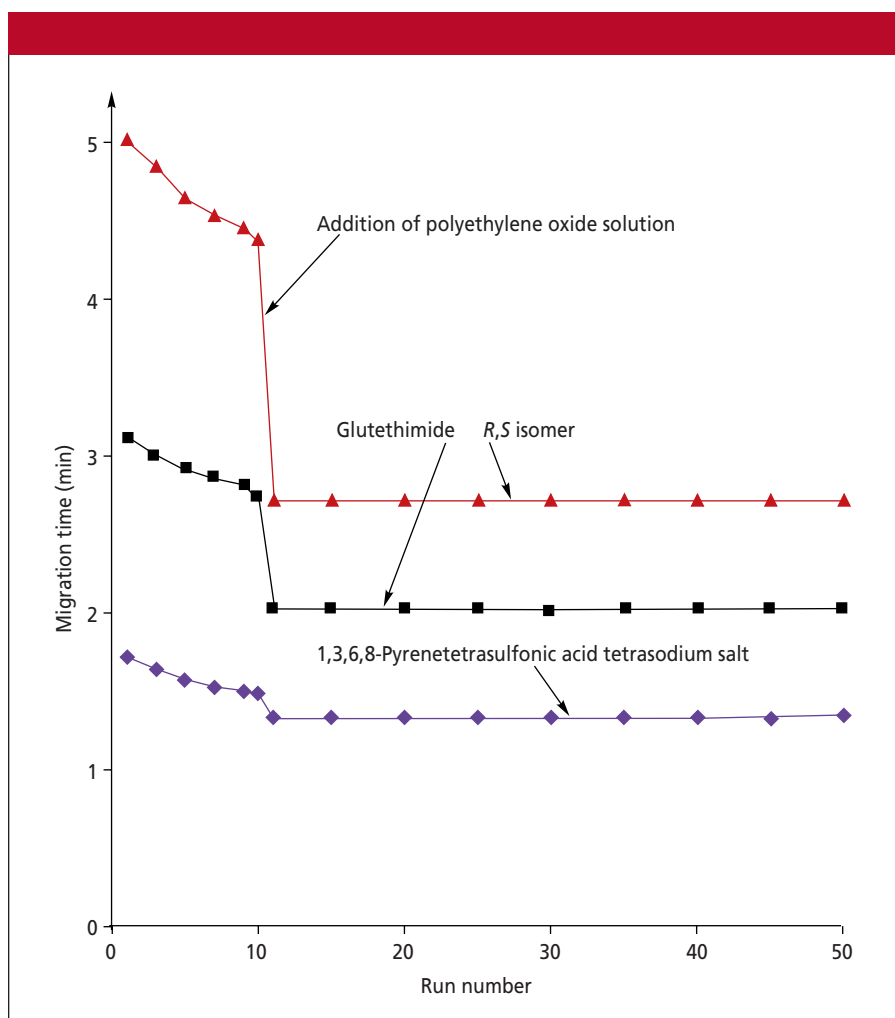
Figure 2 shows the separation of phenylalanine enantiomers by CE in highly sulfated  $\alpha$ -,  $\beta$ -, and  $\gamma$ -cyclodextrins under the standard conditions with resolutions of 3.6, 4.3, and 12.4, respectively. The migration times of the *R* isomer in highly sulfated  $\alpha$ -,  $\beta$ -, and  $\gamma$ -cyclodextrins were 4.4, 3.8, and

6.2 min, respectively. 1,3,6,8-Pyrenetetrakisulfonate served as a reference migration marker in each electropherogram for the normalization of migration time. Changing the buffer pH from 2.5 to 1.5 or 4.5 did not appear to change the resolution of racemates of phenylalanine significantly. However, the migration times of both enantiomers were shortened in pH 1.5 buffer, and those in pH 4.5 buffer were lengthened considerably (data not shown). These changes presumably were the result of the influence of buffer pH on the ionization of carboxylic acid in phenylalanine. Under the standard conditions, highly sulfated  $\gamma$ -cyclodextrin exhibited the best resolution for most aromatic  $\alpha$ -amino acids tested.

Lurie and colleagues (17) observed that highly sulfated  $\gamma$ -cyclodextrin provided unrivaled high resolution for basic drugs with phenethylamine-type structures, mostly illicit drugs such as methampheta-

mine and its analogs. Likewise, most aromatic  $\alpha$ -amino acids hold the basic skeleton of the phenethylamine.

All aromatic  $\alpha$ -amino acids exhibited a relatively strong affinity to highly sulfated  $\alpha$ -,  $\beta$ -, and  $\gamma$ -cyclodextrins in acidic buffer, as evident from their short migration times in Table I. Figure 3 shows the structures of the aromatic  $\alpha$ -amino acids. When using phenylalanine as a reference compound, phenylglycine yielded a substantially prolonged migration time in highly sulfated  $\beta$ - and  $\gamma$ -cyclodextrins, which is indicative of weaker binding in comparison to phenylalanine. Because the  $\alpha$ -amino group is fully protonated at pH 2.5 and the  $pK_a$  of  $\alpha$ -carboxylic acid is 1.83 for both phenylglycine and phenylalanine (19), the charge distribution for both species at pH 2.5 buffer should be identical. Thus, the reduced binding of phenylglycine to highly sulfated cyclodextrins presumably is due to



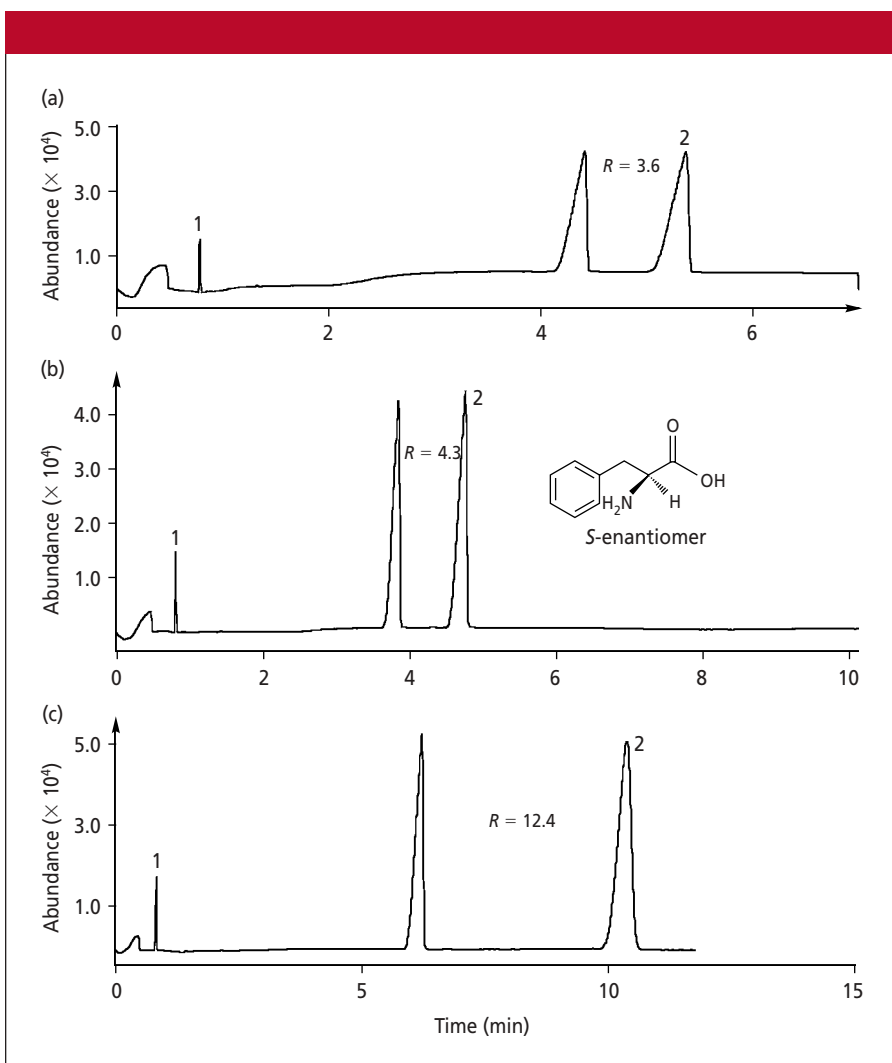
**Figure 1:** Effect of polyethylene oxide-treated capillary on the migration time of 1,3,6,8-pyrenetetrakisulfonate and enantiomers of glutethimide. Capillary: 31.5 cm  $\times$  50  $\mu$ m bare fused-silica (20 cm to the detector); 1,3,6,8-pyrenetetrakisulfonate concentration: 0.25 mM; glutethimide concentration: 0.5 mM; cyclodextrin: 5% highly sulfated  $\beta$ -cyclodextrin in 25 mM phosphate buffer; separation voltage: 15 kV (150–153  $\mu$ A).

the lack of a methylene moiety in the side chain in comparison to that of phenylalanine.

The insertion of a methylene group to the side chain of phenylalanine or an addition of a *p*-nitro group to the phenyl ring, as in homophenylalanine and *p*-nitrophenylalanine, respectively, appeared to enhance binding to highly sulfated cyclodextrins significantly when compared with the binding affinity for phenylalanine. As the side-chain aromatic moiety becomes bulkier, as in the naphthyl group in 1- and 2-naphthyl alanine and the indolyl group in tryptophan and tryptophanamide, the binding to highly sulfated  $\gamma$ -cyclodextrin is significantly stronger than that to the highly sulfated  $\alpha$ - and  $\beta$ -cyclodextrins. Presumably the hydrophobic cavity in highly sulfated  $\gamma$ -cyclodextrin can accommodate bulky groups such as indolyl and naphthyl groups

better than highly sulfated  $\beta$ - and  $\alpha$ -cyclodextrins.

Tyrosine isomers and kynurenine with hydroxyl and amino groups on the phenyl ring do not discourage binding with highly sulfated cyclodextrins nor diminish their chiral resolution in highly sulfated cyclodextrins. However, DOPA, a catechol-based side chain of an  $\alpha$ -amino acid, was well resolved in highly sulfated  $\beta$ - and  $\gamma$ -cyclodextrins but failed to complex with highly sulfated  $\alpha$ -cyclodextrin, which is indicative of the geometric constraint in highly sulfated  $\alpha$ -cyclodextrin to interact with catechol moiety. The overall differences in affinity between highly sulfated cyclodextrins and aromatic  $\alpha$ -amino acids are the interactions in and around the hydrophobic cavity of highly sulfated cyclodextrins. The indolyl and naphthyl moieties in tryptophan and naphthyl ala-



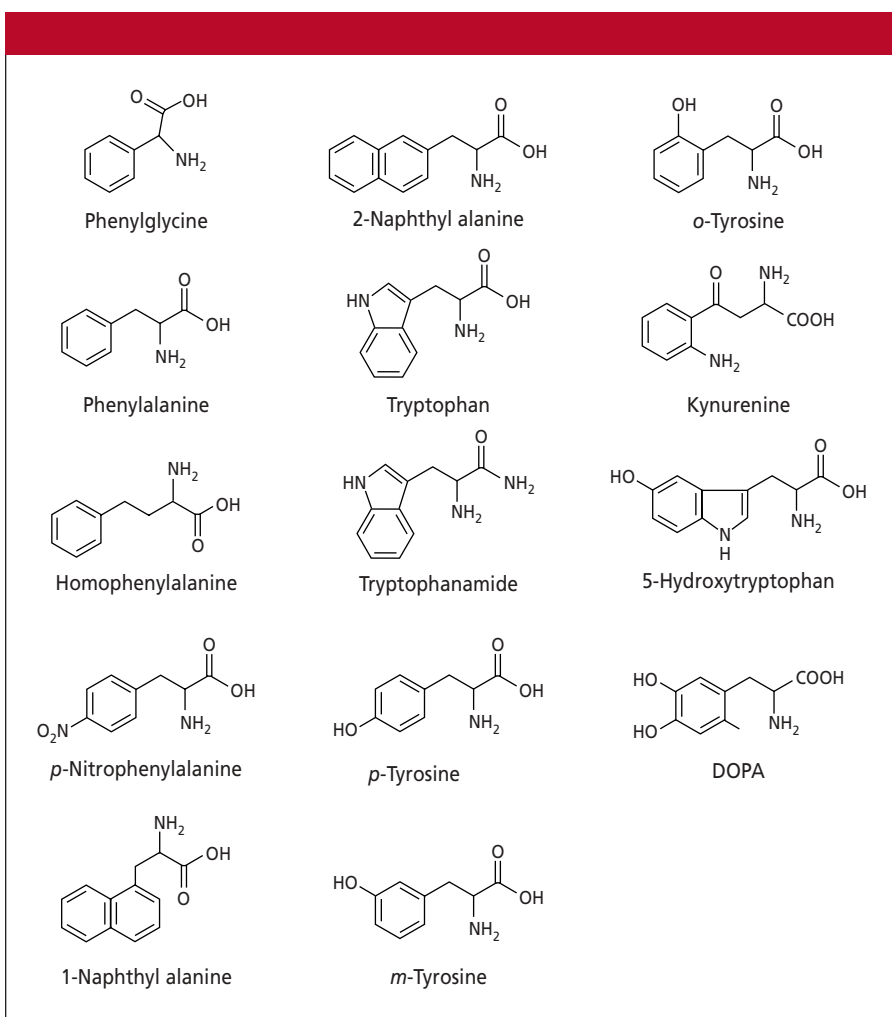
**Figure 2:** Electropherogram of phenylalanine in highly sulfated (a)  $\alpha$ -, (b)  $\beta$ -, and (c)  $\gamma$ -cyclodextrins. Capillary: 31.5 cm  $\times$  50  $\mu$ m untreated fused-silica (10.5 cm to the detector); cyclodextrins: 5% highly sulfated cyclodextrin in 25 mM phosphate buffer (pH 2.5); separation voltage: 15 kV; detection wavelength: 200 nm; injection: 0.3 psi for 4 s. Peaks: 1 = 1,3,6,8-pyrenetetrasulfonate, 2 = S-enantiomer.

**Table I: Relative migration time of first-appearing enantiomer and resolution of aromatic  $\alpha$ -amino acids in highly sulfated cyclodextrins\***

Name of $\alpha$ -Amino Acid	Highly Sulfated $\alpha$ -Cyclodextrin		Highly Sulfated $\beta$ -Cyclodextrin		Highly Sulfated $\gamma$ -Cyclodextrin	
	Resolution	Migration Time (min)	Resolution	Migration Time (min)	Resolution	Migration Time (min)
Phenylglycine	1.2	4.5	2.3	9.6	1.8	11.80
Phenylalanine	3.4	4.41	4.3	3.80	10.0	6.19
Homophenylalanine	1.1	4.10	0.7	1.50	4.4	4.03
<i>p</i> -Nitrophenylalanine	2.5	3.21	3.0	3.12	1.5	5.70
1-Naphthyl alanine	3.0	3.13	2.4	2.03	2.1	0.94
2-Naphthyl alanine	0.9	3.40	1.2	1.68	2.5	2.10
Tryptophan	0.00	4.85	3.6	3.63	10.1	2.98
Tryptophanamide	1.80	2.12	0.3	2.12	3.4	1.70
<i>p</i> -Tyrosine	3.30	4.43	3.5	3.15	5.6	6.63
<i>m</i> -Tyrosine	2.90	9.26	4.7	3.06	9.2	5.31
<i>o</i> -Tyrosine	5.30	6.98	1.5	9.23	8.4	6.70
Kynurenine	1.80	4.61	4.1	4.70	12.5	4.43
5-Hydroxytryptophan	0.00	6.12	2.0	4.40	6.6	5.07
DOPA	—	NP†	5.9	3.90	5.0	9.49

\*31 cm  $\times$  50  $\mu$ m capillary with an effective length of 10 cm.

†NP = no peak for a 20-min run time.

**Figure 3:** Structures of  $\alpha$ -amino acids.

nine bind highly sulfated  $\alpha$ -cyclodextrin significantly, and presumably only a portion of the hydrophobic moiety is necessary to interact with the hydrophobic cavity of highly sulfated  $\alpha$ -cyclodextrin.

**Proposed mechanism for the interaction of aromatic amino acids with highly sulfated cyclodextrins:** The  $^{13}\text{C}$  NMR analysis of highly sulfated cyclodextrins indicated that sulfation occurs exclusively at the C-6 primary and C-2 secondary hydroxyls, and the C-3 hydroxyls are left fully intact (16). Highly sulfated cyclodextrins exhibit a toroidal shape with the C-6 sulfate in the equatorial position at the narrower end of the truncated cone, and the wider side of the cone contains a hydrophobic pocket with the functional moieties of C-2 and C-3, both at equatorial positions. The protonated  $\alpha$ -amino group in aromatic amino acids provides the site of ionic interaction with the sulfate moieties at either the C-6 or the C-2 position of the glucopyranosyl ring of the highly sulfated cyclodextrins. The phenyl group may extend into the cavity of the interior hydrophobic pockets of highly sulfated cyclodextrins from both sides.

To provide chiral selectivity, phenylalanine might interact with the narrower end of the highly sulfated cyclodextrins at the C-6 sulfate, as the initial ionic contact and the phenyl moiety would enter the hydrophobic cavity of the highly sulfated cyclodextrins. The oxygen of the acetal linkage [in highly sulfated cyclodextrins is a rather poor hydrogen-bonding site with the  $\alpha$ -carboxylic acid of phenylalanine. From the wider side of the hydrophobic cavity of the highly sulfated cyclodextrins however, the

equatorial 2-sulfate provides the initial charge contact with protonated moiety of the  $\alpha$ -amino group, and the immediate neighboring 3-OH on the same glucopyranosyl moiety provides a hydrogen-bonding opportunity with the  $\alpha$ -carboxyl group of phenylalanine. Thus, the  $\alpha$ -carboxylic acid of the *R*-enantiomer in phenylalanine is in a favorable position to interact with the 3-OH on the same glucopyranosyl unit, and the C-2 sulfate is tied up with the protonated  $\alpha$ -amino group of the amino acids (Figure 4a).

The  $\alpha$ -carboxylic acid moiety of the *S*-enantiomer must take a longer path for hydrogen bonding with the 3-OH group of the neighboring glucopyranosyl unit because of stereochemical constraint (Figure 4b). Thus, the affinity of phenylalanine

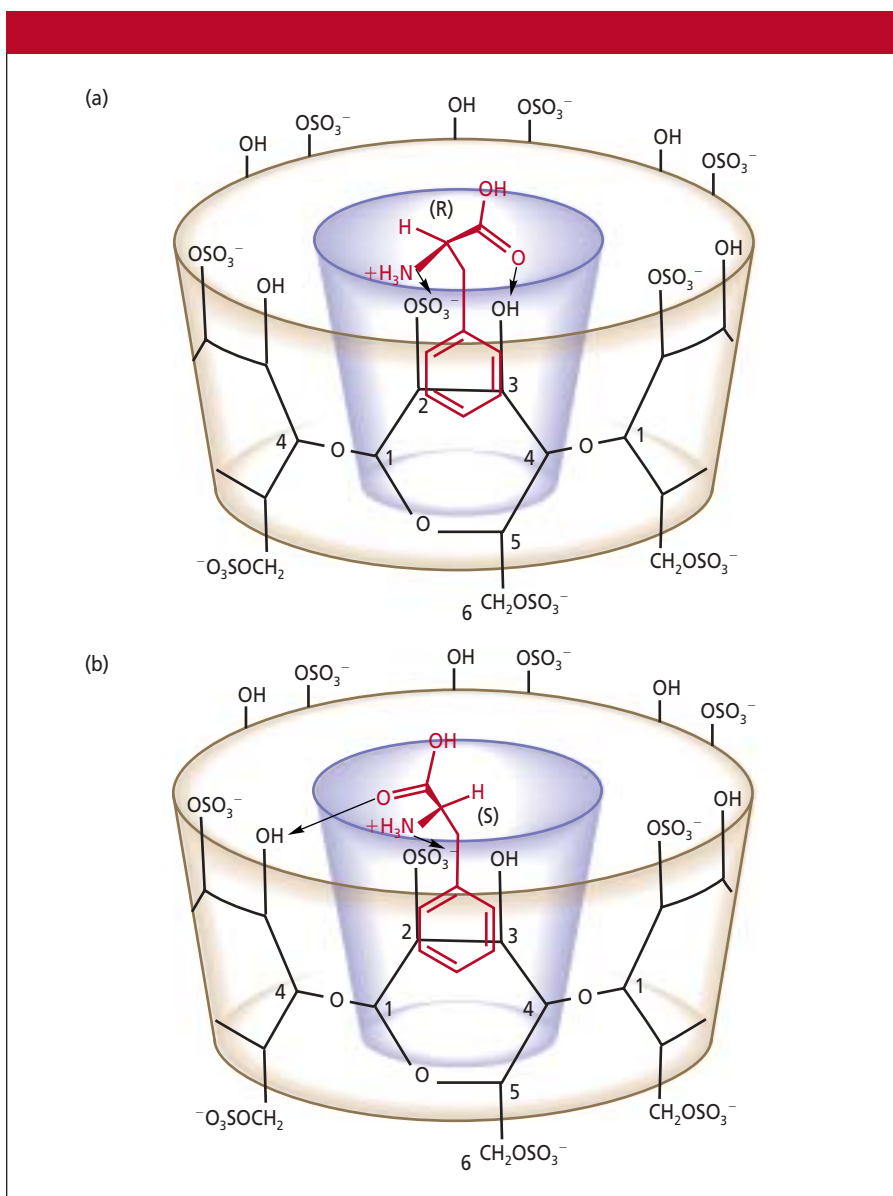
with highly sulfated cyclodextrins is more favorable to the *R*-enantiomer than the *S*-enantiomer, and the difference in affinity provides the chiral resolution.

In CE-based enantiomeric separations using highly sulfated cyclodextrins as the chiral selectors, the electrophoretic mobility of *R*-enantiomers of phenylalanine, *p*-nitrophenylalanine, tyrosine, 1- and 2-naphthyl alanine, tryptophan, 5-hydroxytryptophan, DOPA, and tryptophanamide are faster than each of their corresponding *S*-enantiomers, which is consistent with the mechanism of interaction proposed above.

### Conclusions

This article demonstrated the enantiomeric separation of racemates of aromatic  $\alpha$ -amino acids by CE using highly sulfated

cyclodextrins. Although the size and substituent of aromatic moiety in aromatic  $\alpha$ -amino acids provide a general trend of the relative binding to each highly sulfated cyclodextrin, all racemates of aromatic  $\alpha$ -amino acids in the present study were resolved in both highly sulfated  $\beta$ - and  $\gamma$ -cyclodextrins. The systematic study of the chiral analysis of enantiomers of aromatic  $\alpha$ -amino acids in highly sulfated cyclodextrins in this article provides an indication of the mechanism of host-guest interaction. The higher electrophoretic mobility of the *R*-isomer of aromatic  $\alpha$ -amino acids using highly sulfated cyclodextrins as the chiral selectors is consistent with the proposed mechanism and should be applicable to the highly sulfated cyclodextrin-based enantiomeric separation of esters of aromatic  $\alpha$ -amino acids reported by Verleysen and co-workers (10).



**Figure 4:** Proposed interaction of (a) *R*- and (b) *S*-phenylalanine with highly sulfated cyclodextrins.

### References

- (1) S. Fanali and P. Bocek, *Electrophoresis* **11**, 757–760 (1990).
- (2) R. Kuhn, F. Erni, T. Bereuter, and J. Hausler, *Anal. Chem.* **64**, 2815–2820 (1992).
- (3) K. Verleysen and P. Sandra, *Electrophoresis* **19**, 2798–2833 (1998).
- (4) K.H. Gahm and A. Stalcup, *Chirality* **8**, 316–324 (1996).
- (5) F.-T.A. Chen and R.E. Evangelista, *J. Chin. Chem. Soc.* **46**, 847–860 (1999).
- (6) A. Stalcup and K.H. Gahm, *Anal. Chem.* **68**, 1360–1368 (1996).
- (7) W. Wu and A. Stalcup, *J. Liq. Chromatogr.* **18**, 1289–1315 (1995).
- (8) J.B. Vincent, D.M. Kirby, T.V. Nguyen, and G. Vigh, *Anal. Chem.* **69**, 4419–4428 (1997).
- (9) C. Perrin, Y.V. Heyden, M. Maftouh, and D.L. Massart, *Electrophoresis* **22**, 3203–3215 (2001).
- (10) K. Verleysen, T. Van den Bosch, and P. Sandra, *Electrophoresis* **20**, 2650–2655 (1999).
- (11) S. Mayer, M. Schleimer, and V. Schurig, *J. Microcol. Sep.* **6**, 43–48 (1994).
- (12) R.J. Tait, D.O. Thompson, V.J. Stella, and V.J. Stobaugh, *Anal. Chem.* **66**, 4013–18 (1994).
- (13) L.S. Lurie, N.G. Odeneal, T.D. McKibben, and J.F. Casale, *Electrophoresis* **19**, 2918–2925 (1998).
- (14) L. Zhou, B. Johnson, C. Miller, and J.M. Wyvrat, *J. Chromatogr.* **875**, 389–401 (2000).
- (15) A. Stalcup and K.H. Gahm, *Anal. Chem.* **68**, 1360–1368 (1996).
- (16) F.-T.A. Chen, G. Shen, and R.E. Evangelista, *J. Chromatogr.* **924**, 523–532 (2001).
- (17) I.S. Lurie, N.G. Odeneal II, T.D. McKibben, and J.F. Casale, *Electrophoresis* **19**, 2918–2925 (1998).
- (18) M.C. Vescina, A.M. Fermier, and Y. Guo, *J. Chromatogr.* **973**, 187–197 (2002).
- (19) J.A. Dean in *Lange's Handbook of Chemistry* (McGraw-Hill, New York, 15th ed., 1999), p. 8.64. ■