ELSEVIER

Journal of Chromatography A, 945 (2002) 147–159

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Enantioseparation of amino acids on a polysaccharide-based chiral stationary phase

Yun K. Ye*, Barbara S. Lord, Li Yin, Rodger W. Stringham

DuPont Pharmaceutical Company, Chemical Process Research and Development, Chambers Works, PRF Building (S1), Deepwater, NJ 08023-0999, USA

Received 18 September 2001; received in revised form 6 November 2001; accepted 7 November 2001

Abstract

Sulfonic acids have been shown to be more effective than the commonly used trifluoroacetic acid (TFA) in the chiral resolution of underivatized aromatic amino acids on an amylosic column. Sulfonic acid additives give a more UV transparent mobile phase, possibly allowing the detection of non-aromatic analytes. Work presented demonstrates that through the combination of sulfonic acid mobile phase additives, amine mobile phase additives and solvent modifier variations, the enantiomers of 20 of 25 probe amino acids are fully resolved, four are partially resolved with only one failing to be separated on a common amylosic column. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Enantiomer separation; Mobile phase composition; Amino acids; Sulfonic acids

1. Introduction

The chiral separation of underivatized amino acids has been achieved using chiral crown ether columns [1], antibiotic based columns [2,3] by ligand exchange [4], and recently, on an amylosic column [5,6]. Amylosic chiral stationary phases are rugged for a wide variety of chiral compounds [7–9] and due to this practicality they are available in most laboratories. With typical alcohol–alkane mobile phases, underivatized amino acids do not elute from these columns due to strong interactions between highly polar acidic and basic portions of the amino acids and the stationary phase. Incorporation of strong acid additives into the mobile phase allows the elution and chiral resolution of carboxylic acids and amino-protected amino acids [10] and underivatized phenylalanine analogs [5,6].

In recent work sulfonic acids were found to be more effective for the separation of amino acid enantiomers than the commonly used trifluoroacetic acid (TFA). Sulfonic acid additives give a more UV transparent mobile phase, which might allow the detection and separation of non-aromatic amino acids. The typical mechanism for enantioselectivity on an amylosic column invokes a interaction between aromatic groups on the stationary phase and the analyte. The work of Okamoto et al. [10] demonstrated that an analyte aromatic group is not a requirement for separation. This work explores the possibility of separating a wider variety of underiva-

*Corresponding author. Tel.: +1-856-540-4969; fax: +1-856-540-4902.

E-mail address: yun.k.ye@dupontpharma.com (Y.K. Ye).

0021-9673/02/$ – see front matter
PII: S0021-9673(01)01490-X

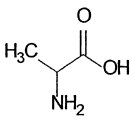
tized amino acids using polysaccharide-based amylosic chiral stationary phase and mobile phase additives. Probe analytes include all natural amino acids (except glycine) plus cystine, citrulline, norleucine, norvaline, ornithine and 3,4-dihydroxyphenylalanine. This list includes numerous small non-aromatic amino acids and amino acids with a variety of additional polar groups. Techniques developed to separate these analytes should be applicable to other small highly polar analytes as well.

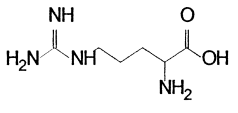
2. Experimental

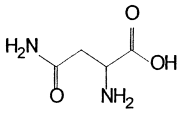
2.1. Reagents

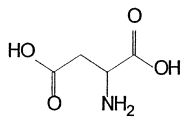
All reagents used in this study were reagent grade or better. Trifluoroacetic acid, methanesulfonic acid, ethanesulfonic acid, trifluoromethanesulfonic acid, camphorsulfonic acid and all amines were obtained from Sigma–Aldrich (St. Louis, MO). *n*-Propanesulfonic acid and *n*-butanesulfonic acid were pur-

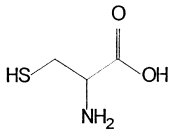
Table 1
Amino acids used in this study

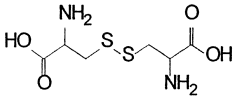

Alanine (Ala)

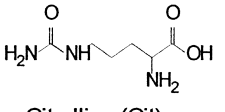

Arginine (Arg)

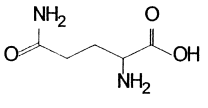

Asparagine (Asn)

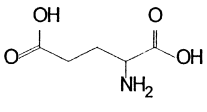

Aspartic Acid (Asp)

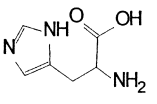

Cysteine (Cys)

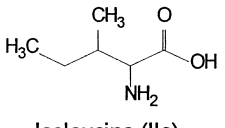

Cystine (Cyt)

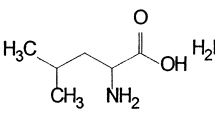

Citrulline (Cit)

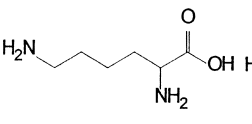

Glutamine (Gln)

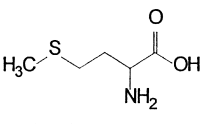

Glutamic Acid (Glu)

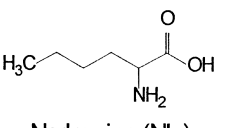

Histidine (His)

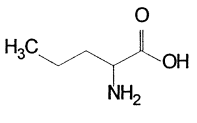

Isoleucine (Ile)

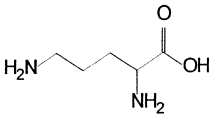

Leucine (Leu)

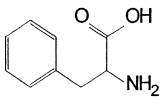

Lysine (Lys)

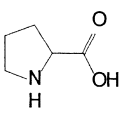

Methionine (Met)

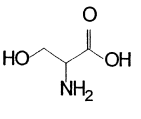

Norleucine (Nle)

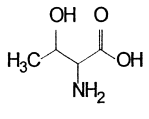

Norvaline (Nva)

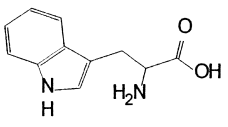

Ornithine (Orn)

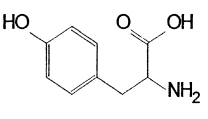

Phenylalanine (Phe)

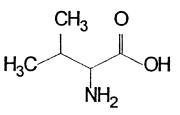

Proline (Pro)

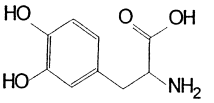

Serine (Ser)


Threonine (Thr)


Tryptophan (Try)


Tyrosine (Tyr)


Valine (Val)


3,4-dihydroxyphenylalanine (DOPA)

chased from City Chemicals (West Haven, CT), and used without further purification. HPLC grade hexane, methanol, isopropanol, *n*-propanol, *n*-butanol were purchased from EM Sciences (Gibbstown, NJ). Absolute ethanol was obtained from Aaper Alcohol and Chemicals (Shelbyville, KY).

The amino acids used in this study are listed in Table 1. All were purchased from Sigma–Aldrich (St. Louis, MO). Separate solutions of racemic mixtures and individual enantiomer of each amino acid were dissolved in 9:1 methanol–trifluoroacetic acid (v/v) at a final concentration about 5 mg/ml for non-aromatic amino acid. For aromatic amino acids and histidine, the final concentrations are about 0.5 mg/ml.

2.2. Chromatography

Chromatographic studies were performed on an HP 1100 liquid chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with vacuum degasser, quaternary pump, autosampler, thermostated-column device and a variable-wavelength UV detector. Chromatographic data were acquired and processed with computer-based HP Chemstation software. A Chiralpak AD column (250×4.6 mm, 10 μm) was purchased from Chiral Technologies (Exton, PA) and used as received. Chromatographic studies were performed at 40 °C with a 1.0 ml/min flow-rate. The mobile phase consisted of 90% (v/v) hexane and 10% (v/v) of different alcohols containing 0.2% (w/v) of acidic additive with or without 0.1% basic additives. After equilibration, 5-μl injections were made. Detection was achieved at 205 nm. Dead time was estimated from the first solvent disturbance peak. Retention factors, *k*, were calculated from $(t_R - t_0)/t_0$ where t_R is the retention time and t_0 is the dead time. Selectivity (α) was calculated as the ratio of retention factors. Resolution factors were calculated by the HP Chemstation software.

Table 2
Chromatographic results using ethanesulfonic acid in ethanol

Probe	k_1	k_2	α	R_s
Ala	1.41	1.79	1.27	3.15
Arg	4.57	4.57	1.00	0
Asn	5.29	5.50	1.04	0.5
Asp	2.51	2.51	1.00	0
Cit	3.40	3.40	1.00	0
Cysteine	1.84	2.09	1.14	1.93
Cystine	5.25	5.55	1.06	1.04
DAPA	3.68	4.92	1.34	4.41
Glu	1.56	1.76	1.13	1.64
Glutamine	4.16	4.16	1.00	0
His	3.11	3.24	1.04	0.55
Ile	1.00	1.12	1.12	1.21
Leu	0.90	1.27	1.41	3.67
Lys	3.44	3.44	1.00	0
Met	1.64	2.03	1.24	3.14
Nor-leu	0.96	1.25	1.30	3.04
Nor-Val	1.07	1.43	1.33	3.35
Orn	2.78	3.03	1.09	0.74
Phe	1.44	1.97	1.37	4.36
Pro	2.09	2.84	1.36	4.72
Ser	2.23	2.23	1.00	0
Thr	1.72	1.72	1.00	0
Trp	2.61	2.89	1.11	1.46
Tyr	2.43	4.12	1.70	7.22
Val	1.10	1.27	1.16	1.72

Conditions are described in the text. α = selectivity; R_s = resolution factor. See Table 1 for abbreviations used for amino acid probes.

3. Results and discussion

3.1. Effect of acidic additive

The 25 amino acids were chromatographed on a polysaccharide-based Chiralpak AD column with 90% hexane and 10% (v/v) ethanol and 0.2% ethanesulfonic acid (ESA) (w/v). These conditions were selected as having the broadest utility based on findings reported previously [5]. Results given in Table 2 show the success of these default conditions with nearly half of the amino acids being at least baseline resolved. Alanine, cysteine, 3,4-dihydroxyphenylalanine, leucine, glutamic acid, methionine, norleucine, norvaline, phenylalanine, proline, tyrosine and valine were well separated with these default conditions. In addition to these separations partial resolution was observed for asparagine, cystine, histidine, isoleucine, ornithine and tryptophan. No selectivity was observed for arginine, aspartic acid, citrulline, glutamine, lysine, serine or threonine.

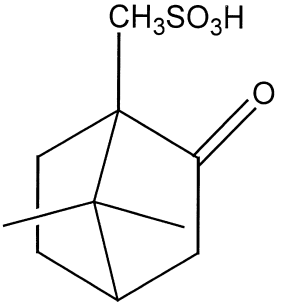


Fig. 1. Structure of camphorsulfonic acid.

In previous work [5] it was observed that different acid additives may yield different enantioselec-

Table 3
Selectivity and resolution obtained with various acidic additives in ethanol

Probe	MSA		PSA		BSA		CSA		TFMA		TFA	
	α	R_s	α	R_s	α	R_s	α	R_s	α	R_s	α	R_s
Ala	1.13	1.06	1.27	2.72	1.27	2.25	1.16	1.75	1.10	0.43	1.16	1.09
Arg	1.00	0	1.00	0	1.00	0	1.00	0	1.00	0	1.00	0
Asn	1.00	0	1.00	0	1.00	0	1.27	2.68	1.00	0	1.00	0
Asp	1.00	0	1.00	0	1.00	0	1.09	0.99	1.00	0	1.00	0
Cit	1.00	0	1.00	0	1.00	0	1.00	0	1.00	0	1.00	0
Cys	1.08	1.27	1.16	1.79	1.19	1.68	1.08	1.16	1.12	0.56	1.08	0.73
Cyt	1.00	0.00	1.17	1.83	1.21	1.52	1.15	1.35	1.00	0	neo	
DOPA	1.18	2.64	1.34	4.12	1.23	2.66	1.17	2.3	1.20	2.08	1.07	0.92
Glu	1.10	1.36	1.17	1.33	1.20	2.06	1.08	0.91	1.07	0.61	1.42	2.69
Gln	1.00	0	1.00	0	1.00	0	1.00	0	1.00	0	1.00	0
His	1.00	0	1.08	0.75	1.10	0.83	1.06	0.6	1.00	0	1.00	0
Ile	1.03	0.36	1.13	1.17	1.11	0.95	1.00	0	1.00	0	1.12	0.62
Leu	1.23	2.78	1.45	3.46	1.43	3.04	1.32	2.84	1.38	1.03	1.28	1.88
Lys	1.00	0	1.00	0	1.00	0	1.22	1.82	1.00	0	1.00	0
Met	1.15	2.34	1.28	3.20	1.29	3.13	1.13	1.82	1.14	1.01	1.13	1.6
Nle	1.17	2.16	1.34	3.04	1.36	2.91	1.22	2.06	1.13	0.84	1.21	1.55
Nva	1.17	2.02	1.34	3.24	1.37	3.02	1.24	2.71	1.11	0.63	1.21	0.98
Orn	1.00	0	1.12	0.83	1.15	1.72	1.18	1.83	1.00	0	1.05	0.31
Phe	1.23	3.23	1.41	4.20	1.40	3.74	1.20	2.52	1.24	1.56	1.23	2.98
Pro	1.22	2.81	1.41	5.33	1.44	5.15	1.32	4.42	1.18	0.75	1.20	1.77
Ser	1.00	0	1.00	0.00	1.00	0	1.00	0	1.00	0	1.00	0
Thr	1.00	0	1.08	0.48	1.08	0.66	1.00	0	1.00	0	1.00	0
Trp	1.09	1.35	1.17	1.95	1.06	0.63	1.00	0	1.13	1.11	1.00	0
Tyr	1.37	4.95	1.79	7.09	1.67	5.74	1.35	1.2	1.65	5.34	1.42	4.91
Val	1.09	0.80	1.18	1.72	1.18	1.57	1.00	0	1.00	0	1.13	0.6

Conditions are described in the text. α = selectivity; R_s = resolution factor, neo = no elution observed. MSA = methanesulfonic acid; PSA = propanesulfonic acid; BSA = butanesulfonic acid; CSA = camphorsulfonic acid; TFMA = trifluoromethanesulfonic acid; TFA = trifluoroacetic acid. See Table 1 for abbreviations used for amino acid probes.

tivities. The strength of the acid may impact selectivity. To evaluate this variable regarding the separation of amino acids, trifuoromethanesulfonic acid (TFMSA, $pK_a = -6.85$) and TFA ($pK_a = 0.67$) were substituted for ESA. While TFA generally gives poorer separation than the sulfonic acids there may be exceptions. In Table 3 it is shown that TFMSA did not offer any advantages. TFA did increase the resolution of glutamic acid from 1.64 to 2.69.

Another feature of sulfonic acidic additives that has been shown to impact enantioselectivity is the size of the alkyl side chain. The probe amino acids were chromatographed with methanesulfonic acid (MSA), butanesulfonic acid (BSA) and propanesulfonic acid (PSA) replacing ESA. MSA did not offer any improvements over ESA while PSA gave slightly enhanced selectivity for most probes relative to ESA. This enhancement was sufficient to give baseline resolution of cystine and tryptophan. The bulkier BSA also increased selectivity for most probes relative to ESA. With this additive, improved resolution of glutamic acid was obtained. Since the bulkiness of the acid additive appears to impact the enantioselectivity of amino acids in this system, an alternative camphorsulfonic acid was tested. The structure of camphorsulfonic acid (CSA) is shown in Fig. 1. The side chain of this acid consists of a bicyclic ring with a ketone functionality. When CSA is substituted for ESA dramatically different enantioselectivity is observed (Table 3). Asparagine, lysine and ornithine are fully separated with CSA as an additive compared to poor or no separation with ESA.

Since camphorsulfonic acid itself is a chiral compound, it may be expected that its use would give additional selectivity. Results listed in Table 3 were obtained with *R*-camphorsulfonic acid. When either *S*-camphorsulfonic acid or racemic camphorsulfonic acids were used as mobile phase additives, the same results were obtained as with the *R*-camphorsulfonic acid. This indicates no effect on selectivity for amino acids due to the chirality of camphorsulfonic acid. The different enantioselectivity observed in Table 3 may arise from steric hindrance introduced by the bulky bicyclic ring and possible additional hydrogen bond and dipole–dipole interactions with the keto group.

As observed previously with phenyalanine analogs retention was affected both by the strength of the additive and the size of its side chain. TF-MSA, being the strongest acid, gave the shortest retention time. TFA tended to give the longest retention times although there were several exceptions to this generalization. Retention in the alkyl sulfonic acid series decreased with the length of the side chain. This trend runs counter to the additive strength. Retention when using CSA in the mobile phase is comparable to that obtained when using ESA. Different efficiency was also observed for different acidic additives which impacts the resolution observed. Generally, the stronger acids gave better column efficiency (larger number of theoretical plates). TFA

Table 4
The best resolution of underivatized amino acids obtained on an AD column through the use of various sulfonic acid additives

Probe	R_s	Condition
Ala	3.15	ESA
Arg	0	
Asn	2.68	CSA
Asp	0.99	CSA
Cit	0	
Cys	1.93	ESA
Cyt	1.83	PSA
DOPA	4.41	ESA
Glu	2.06	BSA
Gln	0	
His	0.83	BSA
Ile	1.21	ESA
Leu	3.67	ESA
Lys	1.82	CSA
Met	3.20	PSA
Nle	3.04	ESA
Nva	3.35	ESA
Orn	1.83	CSA
Phe	4.36	ESA
Pro	5.33	PSA
Ser	0	
Thr	0.66	BSA
Trp	1.95	PSA
Tyr	7.22	ESA
Val	1.72	ESA

Conditions: ESA = 0.2% ethanesulfonic acid in ethanol–hexane (1:9, v/v); 40 °C; 1.0 ml/min. CSA = 0.2% camphorsulfonic acid in ethanol–hexane (1:9, v/v); 40 °C; 1.0 ml/min. PSA = 0.2% propanesulfonic acid in ethanol–hexane (1:9, v/v); 40 °C; 1.0 ml/min. BSA = 0.2% butanesulfonic acid in ethanol–hexane (1:9, v/v); 40 °C; 1.0 ml/min. R_s = resolution factor. See Table 1 for abbreviations used for amino acid probes.

had the poorest efficiency among the studied acids and TF-MSA the best efficiency.

Baseline separation of most of the amino acid probes was accomplished through the use of various acid additives. Arginine, citrulline, glutamine and serine have shown no separation while aspartic acid ($R_s = 0.99$ with CSA), histidine ($R_s = 0.83$ with BSA), isoleucine ($R_s = 1.21$ with ESA) and threonine ($R_s = 0.66$ with BSA) gave partial separation. Table 4 lists the best resolution obtained through the use of various acid mobile phase additive.

3.2. Effect of basic additives

Previous work [6] had shown that basic additives may enhance enantioselectivity for phenylalanine analogs as well as efficiency. Increased selectivity was attributed to a differential ability to disrupt hydrogen bonds of the enantiomer-selector complex. In several examples retention of the second eluting enantiomer was greatly increased by the presence of the amine additive suggesting formation of a barrier to modifier displacement. In this study, 13 different amine additives were tested by incorporation at the 0.1% (w/v) level in the 90:10 hexanes–ethanol (v/v) with 0.2% ESA mobile phase. Selectivity and resolution results are given in Tables 5 and 6.

The effect of amine additives was unpredictable and rarely dramatic. Selectivity was enhanced relative to the ESA for aspartic acid, citrulline, cysteine, cystine, glutamic acid, serine, threonine and lysine. Coupled with enhanced efficiency these increases in

Table 5
Selectivity obtained using various amines (0.1% w/v) in combination with 0.2% ethanesulfonic acid in ethanol–hexane (1:9, v/v)

Probe	ESA	DEA	Pro	Butyl	Amyl	Hexyl	Hept	TFEt	CyC3	CyC4	CyC5	CyC6	CyC7	CyC8
Ala	1.27	1.16	1.21	1.20	1.21	1.22	1.22	1.21	1.20	1.18	1.24	1.25	1.23	1.23
Arg	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Asn	1.04	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Asp	1.00	1.00	1.05	1.03	1.00	1.00	1.00	1.00	1.04	1.05	1.00	1.00	1.00	1.00
Cit	1.00	1.00	1.04	1.05	1.00	1.00	1.00	1.00	1.05	1.00	1.04	1.06	1.06	1.03
Cys	1.14	1.09	1.15	1.13	1.13	1.13	1.13	1.14	1.11	1.15	1.18	1.19	1.15	1.15
Cyt	1.06	1.06	1.11	1.12	1.11	1.10	1.10	1.10	1.08	1.09	1.09	1.10	1.10	1.10
DAPA	1.34	1.25	1.24	1.23	1.26	1.27	1.26	1.25	1.25	1.23	1.26	1.23	1.26	1.23
Glu	1.13	1.11	1.17	1.16	1.14	1.13	1.13	1.14	1.13	1.00	1.19	1.10	1.16	1.15
Gln	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
His	1.04	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Ile	1.12	1.08	1.11	1.09	1.10	1.11	1.12	1.10	1.05	1.09	1.12	1.10	1.10	1.11
Leu	1.41	1.31	1.31	1.36	1.32	1.32	1.33	1.31	1.28	1.31	1.30	1.31	1.34	1.32
Lys	1.00	1.00	1.00	0.93	1.00	1.00	1.00	1.00	1.00	1.04	1.00	1.00	1.00	1.00
Met	1.24	1.18	1.23	1.22	1.21	1.21	1.20	1.22	1.19	1.24	1.24	1.25	1.23	1.21
Nle	1.30	1.20	1.24	1.27	1.26	1.25	1.25	1.26	1.21	1.24	1.27	1.29	1.28	1.27
NVa	1.33	1.23	1.25	1.26	1.27	1.27	1.28	1.26	1.20	1.21	1.26	1.28	1.28	1.26
Orn	1.09	1.06	1.06	1.08	1.07	1.07	1.06	1.06	1.10	1.13	1.07	1.06	1.07	1.09
Phe	1.37	1.26	1.29	1.30	1.30	1.30	1.30	1.31	1.24	1.27	1.32	1.33	1.33	1.31
Pro	1.36	1.33	1.31	1.32	1.32	1.32	1.32	1.31	1.33	1.32	1.33	1.34	1.34	1.34
Ser	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.04	1.07	1.08	1.00	1.05
Thr	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.08	1.00	1.04	1.00	1.03
Trp	1.11	1.09	1.08	1.10	1.07	1.06	1.06	1.08	1.10	1.09	1.08	1.08	1.10	1.07
Tyr	1.70	1.56	1.50	1.57	1.54	1.56	1.56	1.57	1.51	1.48	1.52	1.54	1.62	1.55
Val	1.16	1.10	1.10	1.11	1.12	1.13	1.13	1.12	1.08	1.08	1.12	1.13	1.13	1.10

Additives: ESA = no amine; 0.2% ethanesulfonic acid in ethanol–hexane (1:9, v/v); 40 °C; 1.0 ml/min. DEA = diethylamine; Pro = *n*-propylamine; Butyl = *n*-butylamine; Amyl = amylamine; Hexyl = *n*-hexylamine; Hept = *n*-heptylamine; TFEt = trifluorethylamine; CyC3 = cyclopropylamine; CyC4 = cyclobutylamine; CyC5 = cyclopentylamine; CyC6 = cyclohexylamine; CyC7 = cycloheptylamine; CyC8 = cyclooctylamine. See Table 1 for abbreviations used for amino acid probes.

Table 6
Resolution obtained using various amines (0.1% w/v) in combination with 0.2% ethanesulfonic acid in ethanol–hexane (1:9, v/v)

Probe	ESA	DEA	Pro	Butyl	Amyl	Hexyl	Hept	TFEt	CyC3	CyC4	CyC5	CyC6	CyC7	CyC8
Ala	3.15	1.84	2.3	2.44	2.29	2.31	2.32	2.33	2.32	1.91	2.44	2.70	2.64	2.94
Arg	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Asn	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0
Asp	0	0	0.57	0.38	0	0	0	0	0.49	0.62	0	0	0	0
Cit	0	0	0.43	0.54	0	0	0	0	0.53	0	0.46	0.55	0.66	0.40
Cys	1.93	1.07	1.86	1.68	1.56	1.55	1.55	1.76	1.34	2.40	2.14	2.32	1.85	2.17
Cyt	1.04	0.61	1.21	1.29	1.24	1.09	1.08	1.21	0.68	1.15	0.96	1.25	1.03	1.40
DAPA	4.41	3.12	3.07	3.13	3.27	3.35	3.28	3.27	3.47	3.54	3.41	3.14	3.48	3.43
Glu	1.64	1.46	2.24	2.03	1.78	1.74	1.85	1.37	1.79	0.00	2.42	1.12	1.89	2.31
Gln	0	0	0	0	0	0	0	0	0	0	0	0	0	0
His	0.55	0	0	0	0	0	0	0	0	0	0	0	0	0
Ile	1.21	0.58	0.98	0.76	1.02	1.04	1.12	0.96	0.58	1.15	1.02	1.06	1.02	1.19
Leu	3.67	2.04	1.67	2.85	2.57	2.63	2.85	2.74	2.48	3.49	2.18	2.49	2.55	3.46
Lys	0	0	0	0	0	0	0	0	0	0.37	0	0	0	0
Met	3.14	2.08	2.67	2.88	2.44	2.33	2.31	2.75	2.58	3.45	2.75	3.30	2.98	3.13
Nle	3.04	1.81	2.34	2.79	2.23	2.05	2.18	2.53	2.36	3.13	2.61	2.73	2.70	2.78
NVa	3.35	2.31	2.55	2.59	2.37	2.33	2.33	2.54	2.12	2.30	2.63	3.11	2.85	3.23
Orn	0.74	0.43	0.48	0.63	0.47	0.48	0.44	0.46	0.94	0.69	0.44	0.51	0.55	0.55
Phe	4.36	3.03	3.42	3.55	3.43	3.41	3.42	3.55	3.16	3.94	4.00	3.89	3.80	4.36
Pro	4.72	4.05	3.96	3.48	4.05	4.09	4.04	3.93	3.76	4.18	4.29	4.42	3.55	4.97
Ser	0	0	0	0	0	0	0	0	0	0.52	0.87	0.93	0	0.65
Thr	0	0	0	0	0	0	0	0	0	0.80	0	0.51	0	0.46
Trp	1.46	1.25	1.06	1.32	0.83	0.73	0.68	1.03	1.33	1.42	1.15	1.16	1.37	1.06
Tyr	7.22	5.75	5.53	6.20	5.90	5.97	5.93	6.12	5.91	6.64	5.97	6.18	6.56	7.05
Val	1.72	0.85	0.96	1.3	1.23	1.31	1.31	1.3	0.89	1.13	1.26	1.48	1.45	1.48

See Table 5 for identification of amine additives. See Table 1 for abbreviations used for amino acid probes.

enantioselectivity led to improved resolution for these probes as well as for methionine. Resolution of aspartic acid, cystine and lysine remained inferior to that generated by alternative acid additives. The most significant improvements include the partial resolution of citrulline, serine and threonine not possible without amine additive. Of the amines tested, the cyclic amines appeared to have the broadest application. As a general rule retention was longer with cyclic amines than for their linear analogs. The use of amine additives results in lower sensitivity at the low wavelengths used to monitor non-aromatic amino acids. The amines used here give less background absorbance than the commonly used diethyl and triethyl amines.

Through the combination of acid and amine additives 17 of the probe amino acids have been separated (Table 7). No selectivity has been observed for only arginine and glutamine while partial separation ($R_s < 1.25$) has been obtained for aspartic acid, citrulline, histidine, isoleucine, serine and threonine.

3.3. Effect of alcohol modifiers

Changing mobile phase modifiers is the most common means of altering selectivity on polymeric stationary phases. Changes may be dramatic but are unpredictable. Four different alcohols were evaluated with acidic additives. The experiments were carried out with mobile phase consisting of 90% hexanes and 10% different alcohol (v/v) with 0.2% ESA (w/v). Results are shown in Table 8.

Isopropanol is a very widely used modifier. Substitution of isopropanol for ethanol in our default mobile phase generally decreased enantioselectivity with a few exceptions. The increase in selectivity

Table 7
Best resolution of underivatized amino acids obtained on an AD column through the use of various sulfonic acid additives or amine additives used in conjunction with ethanesulfonic acid

Probe	R_s	Condition
Ala	3.15	ESA
Arg	0	
Asn	2.68	CSA
Asp	0.99	CSA
Cit	0.66	ESA + cycloheptyl amine
Cys	2.40	ESA + cyclobutyl amine
Cyt	1.83	PSA
DOPA	4.41	ESA
Glu	2.06	BSA
Gln	0	
His	0.83	BSA
Ile	1.21	ESA
Leu	3.67	ESA
Lys	1.82	CSA
Met	3.45	ESA + cyclobutyl amine
Nle	3.13	ESA + cyclobutyl amine
Nva	3.35	ESA
Orn	1.83	CSA
Phe	4.36	ESA
Pro	5.33	PSA
Ser	0.93	ESA + cyclohexyl amine
Thr	0.80	ESA + cyclobutyl amine
Trp	1.95	PSA
Tyr	7.22	ESA
Val	1.72	ESA

Conditions: ESA = 0.2% ethanesulfonic acid in ethanol–hexane (1:9, v/v); 40 °C; 1.0 ml/min. CSA = 0.2% camphorsulfonic acid in ethanol–hexane (1:9, v/v); 40 °C; 1.0 ml/min. PSA = 0.2% propanesulfonic acid in ethanol–hexane (1:9, v/v); 40 °C; 1.0 ml/min. BSA = 0.2% butanesulfonic acid in ethanol–hexane (1:9, v/v); 40 °C; 1.0 ml/min. ESA + cycloheptyl amine = ESA conditions plus 0.1% (w/v) cycloheptylamine. ESA + cyclobutyl amine = ESA conditions plus 0.1% (w/v) cyclobutylamine. ESA + cyclohexyl amine = ESA conditions plus 0.1% (w/v) cyclohexylamine. R_s = resolution factor. See Table 1 for abbreviations used for amino acid probes.

from 1.12 to 1.14 proved sufficient to allow baseline separation of isoleucine enantiomers. Selectivity and resolution for leucine and proline were somewhat better with isopropanol while ornithine was dramatically improved (α: 1.09→1.37; R_s: 0.74→4.36). Use of *n*-propanol or *n*-butanol gave no improvements in separation relative to ethanol. To test the use of methanol, a 1:1 mix of methanol and ethanol was made to overcome the miscibility limitations of hexane. Clearly, the effect of methanol will be confounded by the presence of ethanol. Any enhancements in separation will be welcome despite this lack of clarity. The benefits derived from this mixed modifier were limited. The separation of tryptophan was improved significantly (α: 1.11→1.20; R_s: 1.46→2.43) and an already impressive separation of tyrosine was further improved (α: 1.70→1.76; R_s: 7.22→7.64).

With three modifiers containing ESA showing differential effects on chiral separations it is reasonable to believe that different acid and amine additives used with isopropanol or methanol could expand the even further the separations observed. Rather than individually examine the large number of possible mobile phase combinations we tested the combination of CSA with 1:1 methanol–ethanol mobile phase. Asparagine gave excellent separation with this combination but only marginally better than CSA in ethanol alone. Citrulline showed some separation but not as much as observed with

Table 8
Selectivity and resolution obtained with different modifier conditions

Probe	1		2		3		4		5		6	
	α	R_s	α	R_s	α	R_s	α	R_s	α	R_s	α	R_s
Ala	1.27	3.15	1.10	0.62	1.13	0.93	1.20	1.96	1.15	0.91	1.19	1.97
Arg	1.00	0	1.00	0	1.00	0	1.00	0			1.00	0
Asn	1.04	0.5	1.27	3.12	1.00	0	1.00	0	1.00	0	1.00	0
Asp	1.00	0	1.00	0.00	1.00	0	1.00	0	1.00	0	1.00	0
Cit	1.00	0	1.06	0.56	1.00	0	1.00	0			1.00	0
Cys	1.14	1.93	1.10	1.21	1.09	0.99	1.10	1.07	1.07	0.62	1.15	1.81
Cyt	1.06	1.04	1.00	0	1.00	0					1.00	0
DOPA	1.34	4.41	1.04	0.52	1.10	1.46	1.11	1.13	1.09	1.02	1.00	0
Glu	1.13	1.64	1.10	1.19	1.11	1.24	1.13	1.42	1.00	0	1.14	1.65
Gln	1.00	0	1.06	0.66	1.00	0.00	1.00	0			1.00	0
His	1.04	0.55	1.00	0	1.00	0.00	1.00	0			1.00	0
Ile	1.12	1.21	1.00	0	1.08	0.64	1.14	1.3	1.10	0.69	1.11	0.98
Leu	1.41	3.67	1.19	1.59	1.25	2.02	1.52	3.91	1.39	2.54	1.30	2.18
Lys	1.00	0	1.00	0	1.00	0	1.01	0			1.00	0
Met	1.24	3.14	1.15	1.67	1.19	2.20	1.17	1.91	1.13	1.47	1.20	2.60
Nle	1.30	3.04	1.15	1.41	1.19	1.71	1.29	2.69	1.22	1.74	1.26	2.39
Nva	1.33	3.35	1.15	0.80	1.22	1.59	1.28	2.39	1.23	1.64	1.28	2.54
Orn	1.09	0.74	1.02	0.20	1.00	0	1.37	4.36			1.00	0
Phe	1.37	4.36	1.18	1.93	1.30	3.16	1.31	3.69	1.34	3.80	1.26	3.06
Pro	1.36	4.72	1.25	3.45	1.22	2.83	1.42	5.03	1.42	3.15	1.35	3.18
Ser	1.00	0	1.12	1.36	1.00	0	1.00	0	1.00	0	1.00	0
Thr	1.00	0	1.15	1.76	1.00	0	1.00	0	1.00	0	1.00	0
Trp	1.11	1.46	1.08	1.02	1.20	2.43	1.00	0	1.09	0.94	1.00	0
Tyr	1.70	7.22	1.37	4.10	1.76	7.64	1.14	1.52	1.37	3.66	1.23	2.89
Val	1.16	1.72	1.00	0	1.15	1.03	1.17	1.68	1.09	0.81	1.12	1.18

Mobile phase condition: 1=0.2% ethanesulfonic acid in ethanol–hexane (1:9, v/v); 40 °C; 1.0 ml/min. 2=0.2% camphorsulfonic acid in ethanol–methanol–hexane (1:1:18, v/v); 40 °C; 1.0 ml/min. 3=0.2% ethanesulfonic acid in ethanol–methanol–hexane (1:1:18, v/v); 40 °C; 1.0 ml/min. 4=0.2% ethanesulfonic acid in isopropanol–hexane (1:9, v/v); 40 °C; 1.0 ml/min. 5=0.2% ethanesulfonic acid in *n*-butanol–hexane (1:9, v/v); 40 °C; 1.0 ml/min. 6=0.2% ethanesulfonic acid in *n*-propanol–hexane (1:9, v/v); 40 °C; 1.0 ml/min. α=selectivity; R_s=resolution factor. See Table 1 for abbreviations used for amino acid probes.

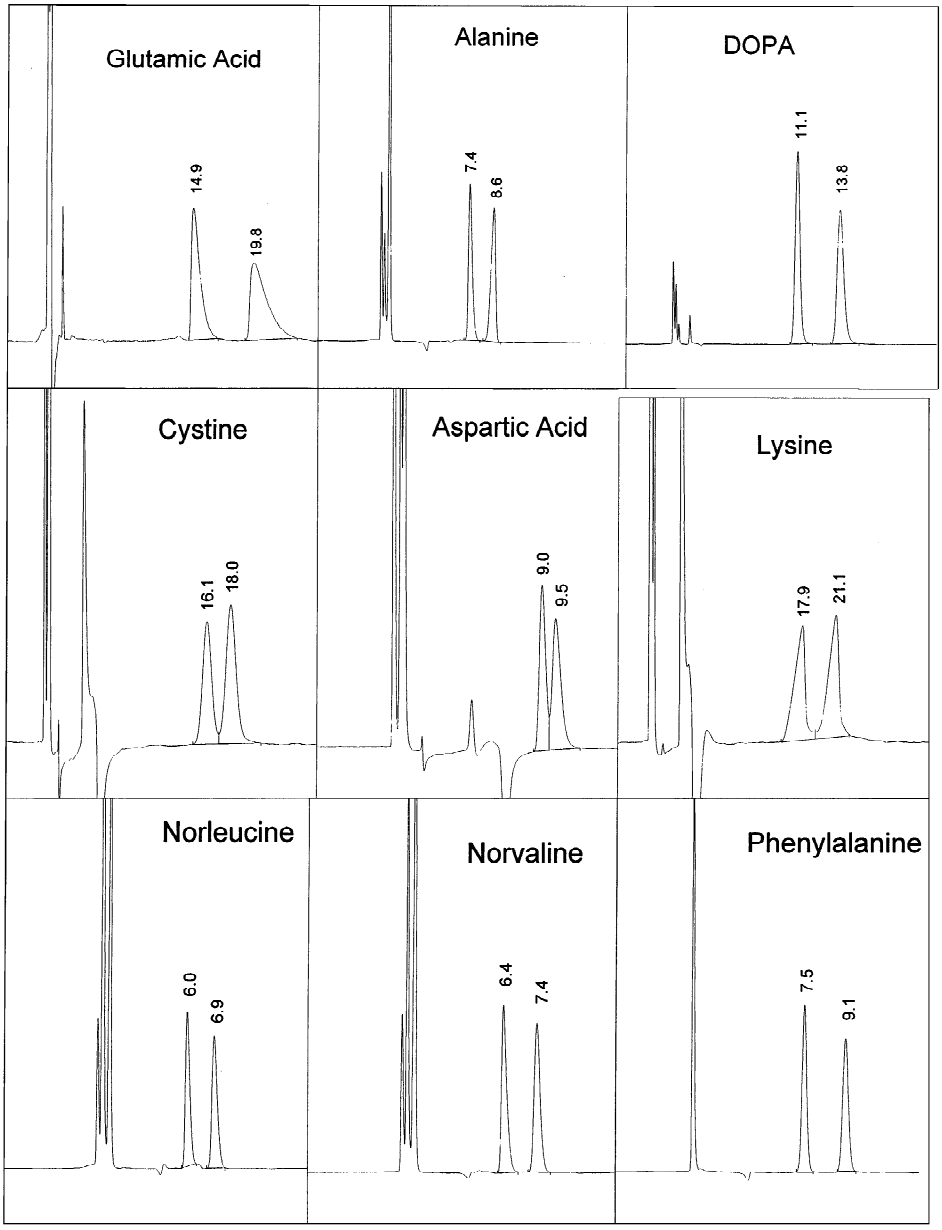


Fig. 2. Chromatograms of underivatized amino acids obtained through the use of various sulfonic acid additive, amine additive and modifier combinations. Conditions are given in Table 9. Retention times are given above each analyte peak. The (D) enantiomer elutes first in all cases.

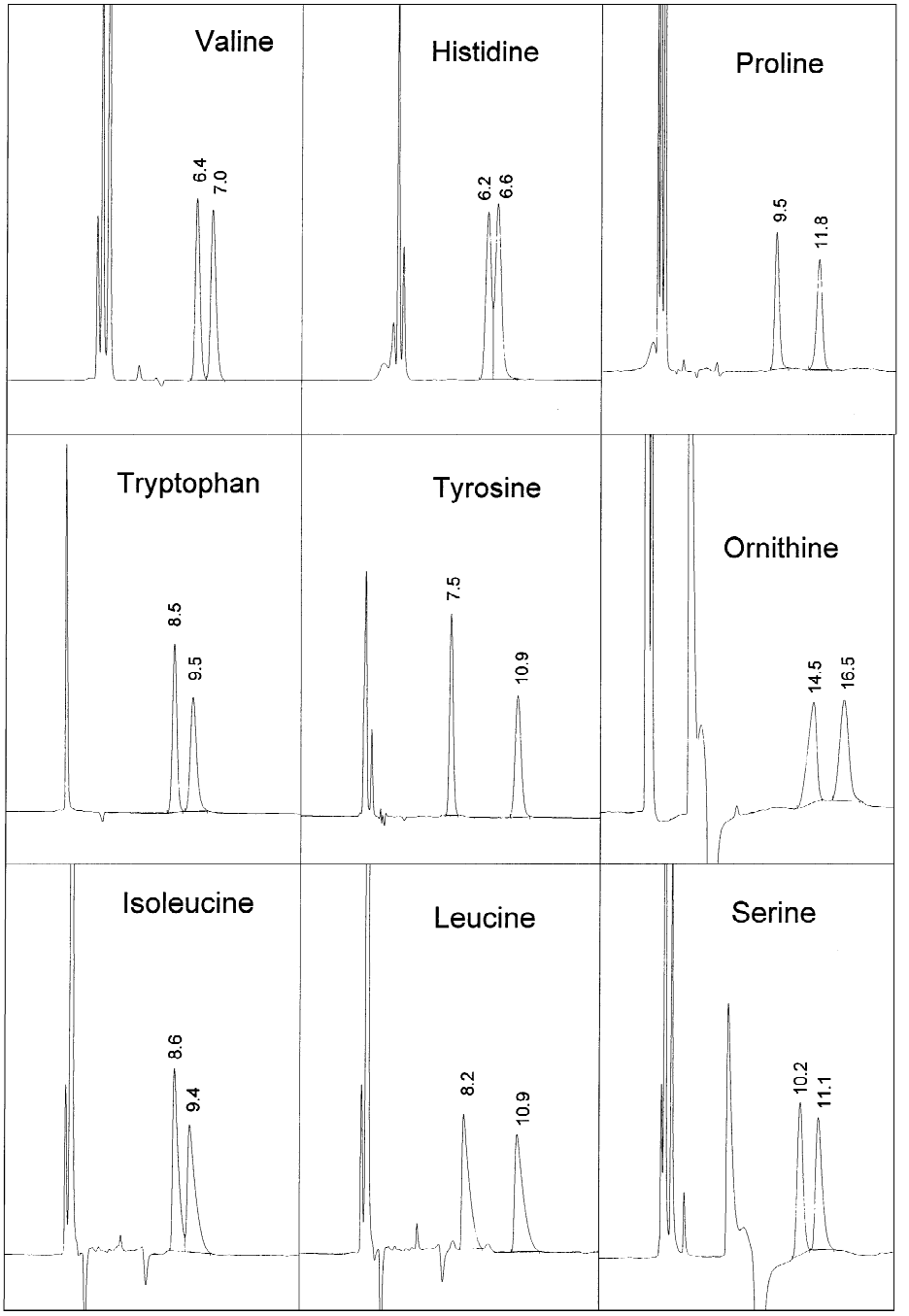


Fig. 2. (*continued*)

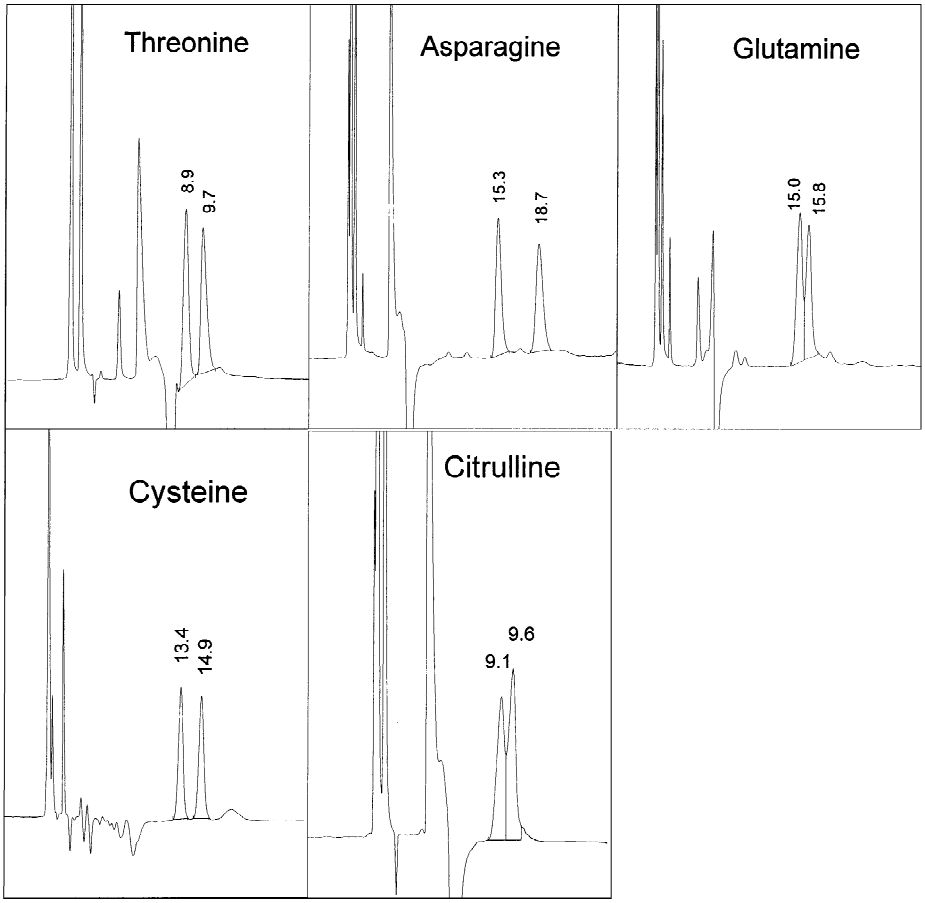


Fig. 2. (*continued*)

cycloheptylamine. Glutamine gave a partial separation which had not been previously observed. The combination of CSA with 1:1 methanol–ethanol mobile phase gave the first baseline separations of serine and threonine observed in this study.

4. Conclusions

We have observed beneficial effects of numerous mobile phase additives and changes associated with modifier. Without testing the large number of possible mobile phase combinations of acids, amines and modifiers we have fully separated 20 of the probe amino acids (Table 9 and Fig. 2). Partial separation ($R_s < 1.25$) was obtained for aspartic acid, citrulline, glutamine and histidine. Only arginine gave no selectivity. Sulfonic acids were the most useful acidic mobile phase additives with the side-chain bulk impacting selectivity. Camphorsulfonic acid proved to be a valuable additive. Amine additives were occasionally beneficial, but not as dramatically as previously observed with large phenylalanine derivatives. The small size of the non-aromatic amino acids may be a factor in the relative lack of amine effect. Use of alternate solvent modifiers also proved valuable with isopropanol and methanol (in mixtures with ethanol) being most

Table 9
Best resolution of underivatized amino acids obtained on an AD column through the use of various sulfonic acid additive, amine additive and modifier combinations

Probe	R_s	Condition
Ala	3.15	ESA
Arg	0	
Asn	3.12	CSA in 1:1 methanol–ethanol
Asp	0.99	CSA
Cit	0.66	ESA + cycloheptyl amine
Cys	2.40	ESA + cyclobutyl amine
Cyt	1.83	PSA
DOPA	4.41	ESA
Glu	2.06	BSA
Gln	0.66	CSA in 1:1 methanol–ethanol
His	0.83	BSA
Ile	1.30	ESA in isopropanol
Leu	3.91	ESA in isopropanol
Lys	1.82	CSA
Met	3.45	ESA + cyclobutyl amine
Nle	3.13	ESA + cyclobutyl amine
Nva	3.35	ESA
Orn	4.36	ESA in isopropanol
Phe	4.36	ESA
Pro	5.33	PSA
Ser	1.36	CSA in 1:1 methanol–ethanol
Thr	1.76	CSA in 1:1 methanol–ethanol
Trp	1.95	PSA
Tyr	7.64	ESA in 1:1 methanol–ethanol
Val	1.72	ESA

Conditions: ESA = 0.2% ethanesulfonic acid in ethanol–hexane (1:9, v/v); 40 °C; 1.0 ml/min. CSA = 0.2% camphorsulfonic acid in ethanol–hexane (1:9, v/v); 40 °C; 1.0 ml/min. PSA = 0.2% propanesulfonic acid in ethanol–hexane (1:9, v/v); 40 °C; 1.0 ml/min. BSA = 0.2% butanesulfonic acid in ethanol–hexane (1:9, v/v); 40 °C; 1.0 ml/min. ESA + cycloheptyl amine = ESA conditions plus 0.1% (w/v) cycloheptylamine. ESA + cyclobutyl amine = ESA conditions plus 0.1% (w/v) cyclobutylamine. CSA in 1:1 methanol–ethanol = 0.2% CSA in ethanol–methanol–hexane (1:1:18, v/v). ESA in 1:1 methanol–ethanol = 0.2% ESA in ethanol–methanol–hexane (1:1:18, v/v). ESA in isopropanol = 0.2% ethanesulfonic acid in isopropanol–hexane (1:9, v/v). R_s = resolution factor. See Table 1 for abbreviations used for amino acid probes.

useful. Despite the strength of the acids used in this study no changes in enantioselectivity or column efficiency were observed for the Chiralpak AD column in the course of this study.

Consideration of the structures of the amino acids that did not separate well does not offer insight into the failure to achieve their resolution. These amino acids have hydrogen bonding groups at the ends of side chains away from the chiral center. Hydrogen bonds between these groups and the stationary phase could dominate retention and destroy enantioselectivity. Not all such amino acids pose a problem in separations. Aspartic acid is only partially resolved ($R_s = 0.99$) while glutamic acid with one more methylene group in the side chain is well resolved ($R_s = 2.06$). Glutamine with the same methylene group is not well resolved ($R_s = 0.66$) while asparagine is very well separated ($R_s = 3.12$).

References

[1] F. Gimenez, M. Soursac, R. Farinotti, Chirality 9 (1997) 150.
[2] A. Berthod, Y. Liu, C. Bagwill, D.W. Armstrong, J. Chromatogr. A 731 (1996) 123.
[3] E. Tesarova, Z. Bosakova, V. Pacakova, J. Chromatogr. A 838 (1999) 121.
[4] G. Galaverna, R. Corradini, A. Dossena, E. Chiavaro, R. Marchelli, F. Dallavalle, G. Folesani, J. Chromatogr. A 829 (1998) 101.
[5] Y.K. Ye, R.W. Stringham, J. Chromatogr. A 927 (2001) 47.
[6] Y.K. Ye, R.W. Stringham, J. Chromatogr. A 927 (2001) 53.
[7] Y. Okamoto, M. Kawashima, K. Hatada, Chem. Lett. (1984) 739.
[8] Y. Okamoto, M. Kawashima, K. Hatada, J. Am. Chem. Soc. 106 (1984) 5357.
[9] Y. Okamoto, M. Kawashima, K. Hatada, J. Chromatogr. 363 (1986) 173.
[10] Y. Okamoto, R. Aburatani, Y. Kaida, K. Hatada, Chem. Lett. (1988) 1125.