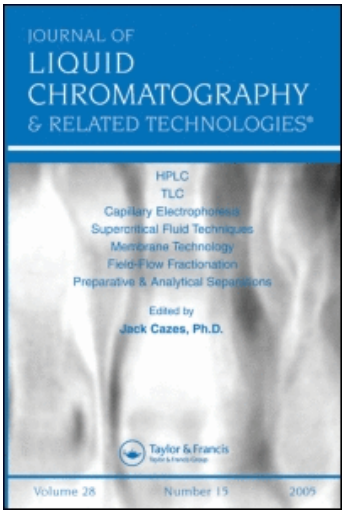

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

HPLC Enantiomeric Resolution of Phenyl Isothiocyanated Amino Acids on Teicoplanin-Bonded Phase Using an Acetonitrile-Based Mobile Phase: A Structural Consideration

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Online publication date: 12 February 2003

To cite this Article Chen, Shushi(2003) 'HPLC Enantiomeric Resolution of Phenyl Isothiocyanated Amino Acids on Teicoplanin-Bonded Phase Using an Acetonitrile-Based Mobile Phase: A Structural Consideration', Journal of Liquid Chromatography & Related Technologies, 26: 20, 3475 — 3495
To link to this Article: DOI: 10.1081/JLC-120025603
URL: http://dx.doi.org/10.1081/JLC-120025603

JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES®
Vol. 26, No. 20, pp. 3475–3495, 2003

HPLC Enantiomeric Resolution of Phenyl Isothiocyanated Amino Acids on Teicoplanin-Bonded Phase Using an Acetonitrile-Based Mobile Phase: A Structural Consideration

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ABSTRACT

A variety of α-amino acids are enantioresolved on a teicoplanin bonded chiral phase, using the acetonitrile-based mobile phase after their pre-column derivatization with phenyl isothiocyanate (PHES) in alkaline medium. The resolution is considered to be much better, as compared to that for a given amino acid in *N*-benzoylated or *N*-carbobenzyloxylated form under the same chromatographic conditions, and found reversed in the elution order as the amino acid is benzyl isothiocyanated. The resolution is sensitive to the size of the analyte, and enhanced due to the re-location of the hydrogen receptor site from sulfur to nitrogen on the

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DOI: 10.1081/JLC-120025603
1082-6076 (Print); 1520-572X (Online)

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isothiocyanyl fragment of the derivatizing reagent, which in turn alters the selectivity. The stereogenic center of analyte nearing the aromatic moiety of the tagging reagent is important as well. The resolution is either not observed, or unsatisfactory, in the reversed- or normal phase mode for most of the phenyl isothiocyanated amino acids examined in this study. Finally, the enantiomer of amino acids is found resistant to racemizeation after being phenyl isothiocyanated at room temperature.

Key Words: Teicoplanin; Phenyl isothiocyanate; Amino acid; Enantioresolution.

INTRODUCTION

A combination of utilizing a proper chiral column[1–15] and modifying the additives in the mobile phase[16–18] has been a common approach for enhancing the resolution of enantiomers in the native form. For those difficult to be resolved in the native form, chemical derivatization with an electrophilic tagging reagent prior to chromatography[19–22] is usually an alternative. Basically, these approaches can be employed to obtain or improve enantioresolution independently, or complementally, to one another.[16–22] For example, poor resolution for enantiomers on a specific chiral column usually can be improved by changing the structure of enantiomers through chemical derivatization, or by modifying the composition of mobile phase before switching to another chiral column for different enantioselectivity. Altering the enantioselectivity through modifying the chiral selector is considered to be tedious, costly, and impractical.

During the past decade, a tremendous number of LC chiral stationary phases (CSPs) have been developed and commercialized to meet the need in resolving a variety of enantiomers.[23–32] However, they tend to be derived from few classes of compounds. Most chiral selectors are based on amino acids (native or derivatized),[23–32] proteins,[26,27] cyclodextrins (native or derivatized),[28,29] derivatized linear or branched carbohydrates (e.g., amylose or cellulose),[30,31] and recently developed macrocyclic antibiotics.[32] Recently macrocyclic antibiotics have been used as novel chiral selectors in LC, TLC, CE, and foam flotation, etc.[33–43] Compounds, particularly oligophenolic glycopetides (e.g., vancomycin and teicoplanin),[33,38–41] have been successfully applied to resolve a variety of enantiomers that are neutral and negatively charged. These macrocyclic antibiotics are similar in structure, with molecular weight ranging from 1500 to 1900, and make remarkable LC CSPs when covalently immobilized to a silica gel support.[42] Antibiotics of the *ansa* family, such as rifamycin B and rifamycin SV are added to the mobile phase to resolve neutral and positively charged compounds in CE.[34,42,44] It is believed that the enantioresolution may be

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possible on these "multimodal" CSP's π–π complexation, hydrogen bonding, inclusion in a hydrophobic cavity, dipole stacking, steric interactions, or combinations thereof, using mobile phases known today.

In its various modifications, however, the most widely used method of *N*-terminal residue analysis seems to be one introduced in 1950 by Pehr Edman.[45–49] This is based on the reaction between an amine group and phenyl isothiocyanate (PHES) to form a substituted thiourea. Mild hydrolysis with hydrochloric acid, selectively removes *N*-terminal residue as the phenyl thiohydantoin, as shown in Fig. 1 (A). The great advantage of this method is that it leaves the rest of the peptide chain intact, so that the analysis can be repeated to sequence the peptide chain. Also, the derivatized amino acid becomes larger in size and hydrophobic enough to be eluted with the acetonitrile-based mobile phase. Due to the introduced chromophores, the detection limit is expected to be lowered. The other advantage for carrying out resolution with organic solvents (e.g., acetonitrile) as the mobile phase, is that the life span of the column can be extended.

In this report, a variety of amino acids were chemically derivatized with PHES in alkaline medium, an electrophilic tagging reagent used in protein

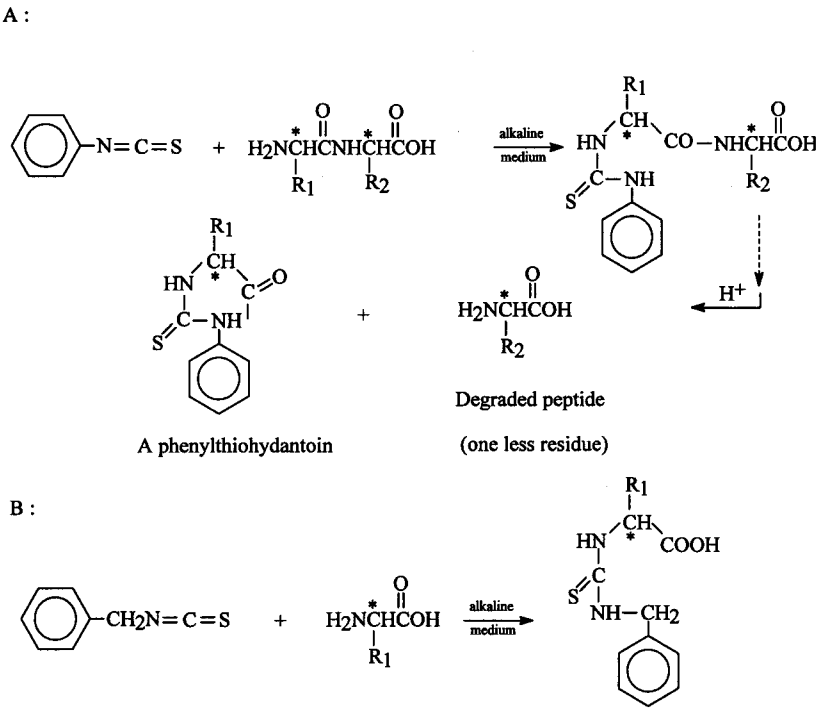

Figure 1. The simplified PHES, benzyl isothiocyanate derivatization chemistry.

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sequencing, before being enantioresolved on a teicoplanin bonded CSP using the acetonitrile-based mobile phase. Under the same chromatographic conditions, the comparison was made to benzoylated, *N*-carbobenzyloxylated and benzyl isothiocyanated amino acids, to rationalize the mechanism involved in the enantioresolution observed and enhanced in the case of phenyl isothiocyanated amino acids. The resistance to racemization of an amino acid enantiomer after being phenyl isothiocyanated at room temperature, will be demonstrated as well.

EXPERIMENTAL

Apparatus

The teicoplanin stationary phase (250×4.6 mm i.d., 5 μm particle diameter) used for all the separations carried out at ambient temperature (~28°C) and at a flow rate of 1.0 mL/min, is obtained from Advance Separation Technologies (Whippany, NJ). The HPLC system used in this study is a Hitachi model L-7100 linked to a D-2500 Chromatopac data station and a variable wavelength UV detector. The detection wavelength was set at 275 nm.

Chemicals

All chemicals used in this study were purchased from Sigma (St. Louis, MO) and Aldrich (Milwaukee, WI). All HPLC grade solvents (acetonitrile, methanol, triethylamine, glacial acetic acid, etc.) were obtained from Fisher Scientific (Pittsburgh, PA) and Merck Taiwan Ltd. (Taipei, Taiwan, ROC). Double filtered and distilled water was used in all cases.

Methods

Before being injected for HPLC separation, the purchased amino acids were first dissolved in alkaline medium (e.g., sodium carbonate solution) and then mixed with PHES (or other derivatizing reagents examined in this study) in acetonitrile for chemical derivatization, according to the procedure described previously.[50] The simplified procedure is outlined in Fig. 1. Due to the incomplete derivatization, the resulting solution contained native enantiomers and was purified through ethyl ether extraction. The ethyl ether layer was collected and further concentrated under reduced pressure before being injected for HPLC analysis.

In the racemization study, the sample once prepared was stored at room temperature for over four months, without the addition of organic modifier, till used.

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RESULTS AND DISCUSSION

The chromatographic data for the enantiomeric resolution of phenyl isothiocyanated α-amino acids, using the acetonitrile-based mobile phase, are listed in Table 1. As can be seen, most amino acids are much better than baseline resolved in the derivatized form using a single acetonitrile-based mobile phase, except for those large in size (i.e., halogenated phenylalanine). The typical chromatograms for the resolution of PHES-alanine and PHES-homoserine under the same chromatographic conditions, are shown in Fig. 2. Interestingly, the profile of these two chromatograms is found to be highly similar, indicating the enantioresolution and the retention scale are insensitive to the structural variations, as the size of analyte is small. Note that there is a hydroxyl group on homoserine for additional hydrogen bonding. When the size is further increased, as in the case of methionine, ethionine, and buthionine, the resolution, which deteriorates with the enantioselectivity α, remains almost unchanged, suggesting the kinetic part of (i.e., the adsorption/desorption rates) resolution is influenced, not the thermodynamics. However, the opposite is observed in the cases of valine/norvaline and norleucine/*tert*-leucine. As the size of the side-chain group becomes bulky enough to hinder the access for potential interactions with a chiral selector (i.e., valine and *tert*-leucine), the retention factor and the enantioselectivity decrease. The resolution even disappears in the cases of tryptophan and 5-methyltryptophan, PHES-gly-leu and PHES-gly-ala, whose stereogenic center is extended away from the PHES moiety by two carbon atoms due to glycine. As expected, the resolution of dipeptides with two stereogenic centers was unsatisfactory. All suggest that chiral recognition for the resolution on teicoplanin phase, under the elution of acetonitrile-based mobile phase, occurs in a hydrophobic pocket with the π–π interaction being the major force.

Table 1 also summarizes the chromatographic data for the enantioresolution of selected *N*-benzoylated, *N*-carbobenzyloxylated, and benzyl isothiocyanated amino acids under the elution of acetonitrile-based solvent mixtures for comparison. Figure 3(A) shows a typical chromatogram for the resolution of phenyl isothiocyanated phenylalanine, with a *D*-enantiomer first eluted at a comparable retention scale. Under the same chromatographic conditions, phenylalanine was poorly resolved, as shown in Fig. 3(B), if *N*-benzoylated. It is thought that the re-location of the hydrogen receptor site from sulfur (which is the oxygen atom of the carbonyl group in the case of *N*-benzoyl and *N*-carbobenzyloxyl reagents) to the nitrogen atom on the isothiocyanyl fragment of the derivatizing reagent, could form potential interactions to enhance the resolution based on the mechanism proposed by Pirkle.[51] Also, the sulfur atom is larger in size than oxygen atom, and expected to cause more

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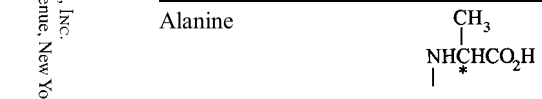
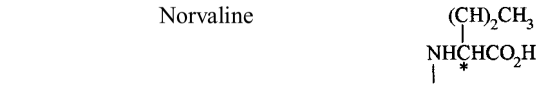
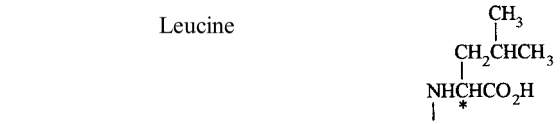
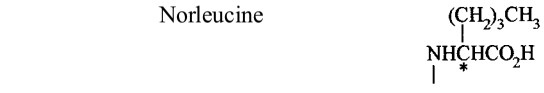
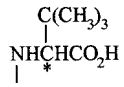
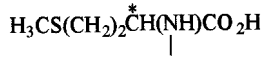
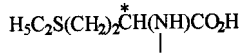
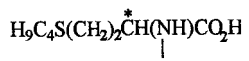
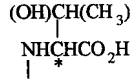
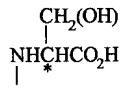
Table 1. The chromatographic data for the resolution of phenyl isothiocyanated, selected carbobenzyloxylated, *N*-benzoylated and benzyl isothiocyanated amino acids on teicoplanin CSP using the polar-organic mobile phases.

Compound	Structure	Reagent[a]	k'^{b}	α^{b}	$R_s{}^{b}$	Mobilephase[c]
Alanine		PHES	3.01	3.21	3.70	A
		CBZ	6.49	1.46	2.34	B
		BENS	3.45	2.18	4.14	A
Valine		PHES	2.06	1.17	1.12	A
			0.23	2.52	2.62	B
		CBZ	0.16	1.75	1.21	B
		BEN	2.38	1.09	0.87	A
		BENS	1.75	1.37	1.78	D
			2.26	1.36	2.00	A
Norvaline		PHES	2.22	1.97	3.20	A
			0.28	17.03	7.43	B
		CBZ	0.18	2.67	2.42	B
		BENS	2.37	2.47	5.16	A
Leucine		PHES	2.35	1.69	3.00	A
		CBZ	0.39	1.45	1.44	B
		BENS	2.34	1.71	2.82	A
Norleucine		PHES	1.94	1.72	2.77	A
		CBZ	0.22	1.82	1.25	B
		BENS	1.72	1.94	3.16	D

tert-Leucine		PHES	1.87	1.06	0.75	A
		BENS	1.62	1.19	1.03	D
			2.06	1.19	1.23	A
Methionine		PHES	2.06	1.96	3.24	A
		CBZ	0.30	2.27	3.33	B
		BENS	2.43	2.07	4.13	A
Ethionine		PHES	1.89	1.87	3.12	A
		CBZ	0.24	2.33	3.13	B
		BENS	2.09	2.01	3.76	A
Buthionine		PHES	1.57	2.01	2.61	A
		CBZ	0.24	1.50	1.35	B
		BENS	1.47	2.05	3.28	D
			1.61			
Threonine		PHES	2.19	1.72	2.47	A
		CBZ	0.29	1.70	1.02	B
		BENS	2.28	2.56	3.66	A
Serine		PHES	4.45	2.75	3.84	A
		CBZ	0.25	1.96	2.50	C
		BENS	3.84	2.38	4.71	D
			3.83	2.39	5.10	A

(*continued*)

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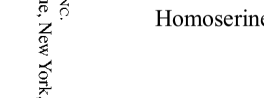
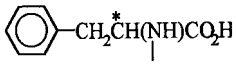
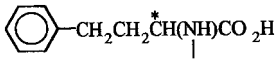
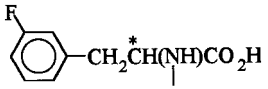
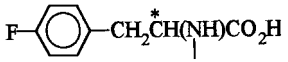
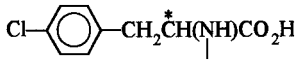
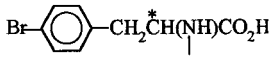
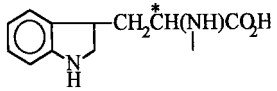
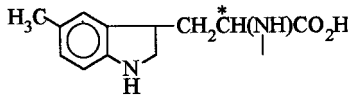
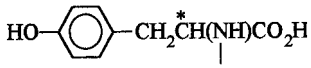
Table 1. Continued.

Compound	Structure	Reagent[a]	k'^{b}	α^{b}	R_s^{b}	Mobilephase[c]
Homoserine		PHES	3.82	2.51	3.33	A
		CBZ	5.56	1.31	1.91	C
		BENS	3.23	2.96	4.67	D
			4.32	2.85	4.37	A
Phenylalanine		PHES	2.16	1.74	3.00	A
			0.26	9.63	6.67	B
		CBZ	0.34	1.57	2.13	B
		BEN	2.39	1.13	1.01	A
		BENS	2.19	1.11	0.92	A
Homophenylalanine		PHES	1.85	1.73	2.63	A
		BENS	2.22	1.91	3.27	A
m-Fluorophenylalanine		PHES	1.58	1.16	1.11	A
			0.26	2.59	3.26	B
		CBZ	0.33	2.10	3.18	B
		BENS	1.98	1.10	0.82	A
p-Fluorophenylalanine		PHES	1.77	1.11	0.81	A
			0.31	2.32	3.10	B
		CBZ	0.35	2.38	3.30	B
		BENS	2.14	1.11	0.89	A

p-Chlorophenylalanine		PHES	1.91	1.10	1.03	A
			0.33	2.48	3.22	B
		CBZ	0.33	2.49	3.62	B
		BENS	1.88	1.12	0.90	D
			2.39	1.10	0.85	A
p-Bromophenylalanine		PHES	2.09	1.11	0.97	A
			0.35	2.46	3.00	B
		CBZ	0.45	2.25	3.15	B
		BENS	1.99	1.10	0.85	D
			2.50	1.09	0.71	A
Tryptophan		PHES	2.90	1.03	0.60	D
		CBZ	0.30	1.57	1.28	C
5-Mehtyltryptophan		PHES	2.80	1.02	0.55	D
		CBZ	0.33	1.43	2.70	B
Tyrosine		PHES	3.83	1.07	0.67	D
			4.34	1.13	1.00	A
		CBZ	0.45	1.93	2.82	B
		BENS	5.68	1.05	0.61	A
			6.01	1.05	0.60	A

(*continued*)

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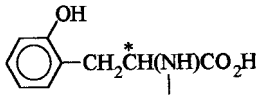
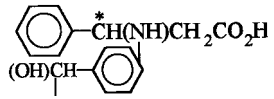
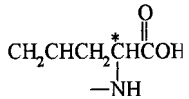
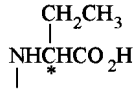
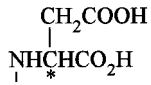
Table 1. Continued.

Compound	Structure	Reagent[a]	k'[b]	α[b]	R_s[b]	Mobilephase[c]
m-Tyrosine		PHES	4.28	1.12	0.93	A
			3.36	1.13	1.00	D
		CBZ	0.41	2.37	3.63	B
		BENS	4.26	1.09	0.74	D
			5.45	1.09	0.75	A
3-Amino-3-phenylpropionic acid		BENS	2.68	1.25	1.63	A
3-Phenylserine		BENS	1.16	1.16	0.85	D
2-Amino-4-pentenoic acid		PHES	2.01	1.48	2.19	A
		BENS	1.81	1.74	3.13	D
			2.31	1.69	3.37	A

α-Amino-*n*-butyric acid		PHES	2.45	1.46	2.28	A
		CBZ	0.26	1.74	1.67	B
		BENS	2.65	1.66	3.02	A
Aspartic acid		PHES	4.80	1.11	0.91	A

[a]PHES, CBZ, BEN and BENS stand for phenyl isothiocyanyl, *N*-carbobenzyloxyl, *N*-benzoyl and benzyl isothiocyanyl moieties, respectively.

[b]The selectivity factor, α, is equal to k_1'/k_2' and resolution factor, R_s, is equal to $2(tr_2 - tr_1)/(W_2 + W_1)$ and capacity factor, k', is equal to $(t_r - t_0)/t_0$.

[c]Mobile phase is a solvent mixture of A: 480 ACN/20 MeOH/1 HOAC/2 TEA, B: 95 MeOH/5 EE/0.4 HOAC/0.2 TEA, C: 93 MeOH/7 EE/0.4 HOAC/0.2 TEA , D: 475 ACN/25 MeOH/1 HOAC/3 TEA by volume, (v/v). The ACN, MeOH, HOAC, TEA and EE are abbreviations for acetonitrile, methanol, acetic acid, triethylamine and ethyl ether, respectively.

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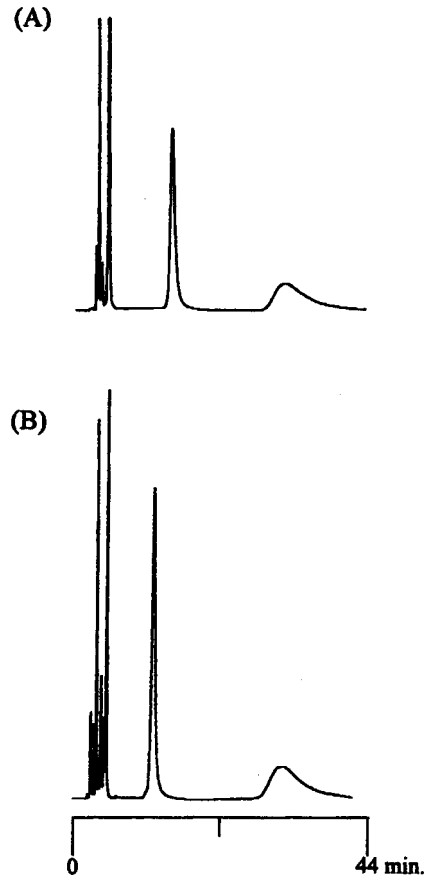

Figure 2. Chromatograms showing the enantioresolution of (A) phenyl isothiocyanated alanine and (B) phenyl isothiocyanated homoserine on teicoplanin bonded CSP, using the acetonitrile-based mobile phase of 480 ACN/20 MeOH/1 HOAC/2 TEA by volume, (v/v). As compared to homoserine, alanine is relatively small in size and lacks other functional groups, however, it is eluted at comparable retention time.

significant steric hindrance effect. Note that the repulsive interaction is as important as the attractive interaction in Pirkle-type chiral recognition model.

Unfortunately, no resolution was observed with *N*-carbobenzyloxylated phenylalanine or other derivatized amino acids examined in this study under the elution of acetonitrile-based mobile phase. It has been noted that the difference in structure between *N*-benzoyl and *N*-carbobenzyloxyl reagents is

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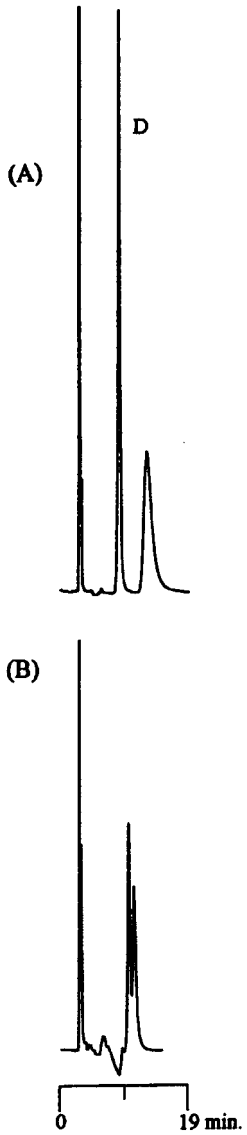

Figure 3. Chromatograms showing the enantioresolution of (A) phenyl isothiocyanated phenylalanine and (B) *N*-benzoylated phenylalanine on teicoplanin bonded CSP, using the acetonitrile-based mobile phase of 480 ACN/20 MeOH/1 HOAC/2 TEA by volume, (v/v). As can be seen, PHES-phenylalanine is better resolved with the *D*-enantiomer eluting first under the same chromatographic conditions, and is thought to be a result of the re-location of a hydrogen receptor site.

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minor except for the ether linkage. As a result, the stereogenic center of *N*-carbobenzyloxylated amino acids is relatively distant from the aromatic moiety and, thus, is believed to be disadvantageous toward the chiral recognition. This structural similarity also can be found in benzyl isothiocyanated amino acids, whose stereogenic center is away from the aromatic moiety by one carbon due to the methylene group. However, the opposite was obtained. Most benzyl isothiocyanated amino acids were better resolved, except for those with large side-chain groups (i.e., phenylalanine, halogenated phenylalanine, tryptophan, and tyrosine, etc.) and retained more strongly with the elution order reversed (i.e., phenylalanine), as compared to phenyl isothiocyanated amino acids under the elution of the acetonitrile-based mobile phase. A typical chromatogram is shown in Fig. 4. However, the resolution of *N*-carbobenzyloxylated amino acids that are larger in size (phenylalanine and its derivatives) can be dramatically improved under the elution of methanol-base mobile phase (refer to Table 1 for chromatographic data). It is believed that the interaction patterns leading to the enhanced resolution is different and is mainly due to the electrostatic forces, which is insignificant as the native amino acids are resolved in water-rich mobile phase.[36]

The racemization percentage of enantiomer of selected amino acids such as leucine, methionine, and phenylalanine was determined after the derivatization with phenyl isothiocyanate. The results are summarized in Table 2. Figure 5 shows a typical chromatogram for the elution of phenyl isothiocyanated *D*-phenylalanine, prepared and stored at room temperature for over four months without the addition of organic modifier. The racemization percentage in this particular case was calculated to be 1.28% based on the peak area and considered to be resistant to the racemization. Note that the chromatogram appears to be more complicated than the one in Fig. 2, indicating possible decomposition. The decomposition was observed to be much more severe in the case of benzyl isothiocyanated amino acids prepared at the same time period. The peak of the analyte was barely observed except for those of the decomposed components.

The attempt has been made to resolve amino alcohols in derivatized form, whose structure is highly similar to that of the resolved amino acids. A typical example is the PHES-2-amino-3-methyl-1-butanol. As compared to PHES-valine, the only difference in structure is the hydoxyl group, instead of carboxyl group in PHES-valine. The resolution of PHES-2-amino-3-methyl-1-butanol turns out to be a failure, indicating the role of carboxyl groups on the analytes is essential toward a successful resolution. The other factor that affects the enantioresolution is the position of amino group on the analyte skeleton. This can be seen in the resolution of PHES-alanine. However, the resolution disappeared in PHES-3-aminoisobutyric acid under the same chromatographic conditions. These two analytes are highly similar in struc-

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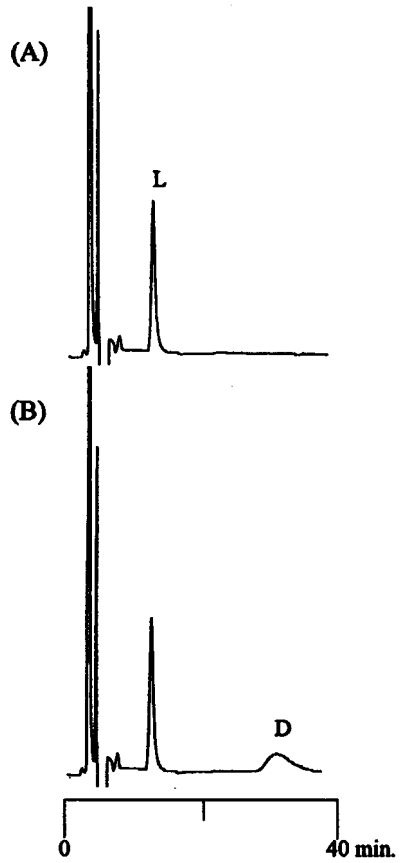

Figure 4. Chromatograms showing the elution profile of benzyl isothiocyanated *L*-homoserine (A) and the enantioresolution of benzyl isothiocyanated homoserine (B) on teicoplanin bonded CSP, using the acetonitrile-based mobile phase of 475 ACN/25 MeOH/1 HOAC/3 TEA by volume, (v/v). Note that the profile of the chromatogram is highly similar to that for the resolution of the phenyl isothiocyanated homoserine. Also, the retention scale is larger.

ture, except for the position of the amino group on the skeleton. It has been determined that the amino group nearing the stereogenic center of the analyte is essential as far as a successful resolution is concerned. The other example is the comparison of PHES-α-amino-*n*-butyric acid to the PHES-β-amino-*n*-butyric acid. Under the same chromatographic conditions, only the PHES-α-amino-*n*-butyric acid was resolved.

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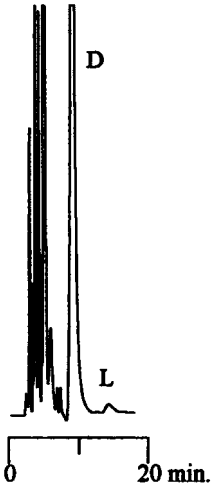

Figure 5. Chromatogram showing the elution of phenyl isothiocyanated *D*-phenylalanine prepared over four months ago, using the acetonitrile-based mobile phase of 480 ACN/20 MeOH/1 HOAC/2 TEA by volume, (v/v). As can be seen, the chromatogram appears to be more complicated indicating the possible decomposition. Also, the racemization is observed and calculated to be 1.27% of *L*-phenylalanine based on the peak area in this particular case.

Table 2. The racemization percentage of optically active enantiomer of leucine, methionine and phenylalanine at room temperature after the derivatization with PHES.

Compound[a]	%L or %D[b]	S.D.[c]	n[d]
L-Leu	1.44	0.08	4
L-Met	<0.5	—	3
D-Phe	1.22	0.10	5

[a]The analyte was derivatized according to the procedure described in the Experimental section and stored at room temperature for over four months till used in the racemization study.
[b]The racemization percentage is calculated based on the peak area.
[c]The standard deviation of n separate analyses.
[d]The number of separate analyses.

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CONCLUSION

The resolution of a variety of α-amino acids has been demonstrated on a teicoplanin bonded chiral phase, using the acetonitrile-based mobile phase after their pre-column derivatization with PHES in alkaline medium. The resolution is considered to be much better, as compared to that for a given amino acid in *N*-benzoylated or *N*-carbobenzyloxylated form under the same chromatographic conditions. The resolution is sensitive to the size effect, and thought to be enhanced due to the re-location of the hydrogen receptor site from sulfur to nitrogen on the isothiocyanyl fragment of derivatizing reagent, which in turn changes the enantioselectivity. The elution order for the resolution of phenylalanine in phenyl isothiocyanated form can be reversed, if it is benzyl isothiocyanated under the same chromatographic conditions. Also, phenyl isothiocyanated amino acids are considered to be resistant to the racemization during a time period of over four months. These are important in the optical purity determination, which requires the enantiomer to be measured quantitatively to elute first and be resistant to the racemization. Also, the carboxyl group and the amino group nearing the stereogenic center of the analyte are essential toward a successful resolution. Finally, the advantage for carrying out resolution with a polar-organic mobile phase is that the life span of the column can be extended, as there is no hydrolysis in the absence of water.

ACKNOWLEDGMENTS

Support of this work by the National Science Council of Taiwan (NSC 91-2113-M-415-005) and National Chiayi University is gratefully acknowledged.

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Received June 3, 2003
Accepted July 17, 2003
Manuscript 6174

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