Amino Acids (2007) 33: 97–104
DOI 10.1007/s00726-006-0423-x
Printed in The Netherlands

Amino Acids

A fluorescent electrophilic reagent, 9-fluorenone-4-carbonyl chloride (FCC), for the enantioresolution of amino acids on a teicoplanin phase under the elution of the methanol-based solvent mixture

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Received July 25, 2006
Accepted August 21, 2006
Published online October 27, 2006;

Summary. A fluorescent electrophilic reagent, 9-fluorenone-4-carbonyl chloride (FCC), is chosen to functionalize amino acids in alkaline medium before their HPLC resolution. FCC reacts with both primary and secondary amino acids to produce stable and highly fluorescent derivatives suitable for sensitive and efficient chromatographic determination and resolution on a teicoplanin chiral stationary phase (CSP) using the methanol-based solvent mixture as the mobile phase. The detection limit is in the picomole range and approximately 0.01% of the D-enantiomer in an excess of the L-enantiomer is detectable. However, the resolution is not reproducible under the elution of either the water- or the acetonitrile-based mobile phase. The increase in solubility of analyte in the mobile phase seems to be responsible. Upon comparison under the optimal chromatographic conditions, the resolution is better than that for the 9-fluorenylmethyl chloroformate (FMOC) or 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) derivatives reported previously.

Keywords: Fluorescent tagging reagent – Column liquid chromatography – Enantioresolution – Amino acids – Teicoplanin – Trace enantiomeric impurities – Enantiomeric ratio

Introduction

As widely documented in the literature, numerous pre-column reactions that enhance the sensitivity and selectivity of chromatographic amino acids determinations have been reported in the past (Chen et al., 1993; Chen, 1994, 1996, 2003a, b, 2004a, b, 2005a, b; Chen and Ward, 2004; Minocha et al., 2004; Pawlowska et al., 1993; Pirkle et al., 1994; Zukowski et al., 1993). In these studies, the electrophilic compounds that react with nucleophiles such as amines, amino acids and amino alcohols in the alkaline medium include FMOC, AQC, DNP and many isocyanates and isothiocyanates. Among them, FMOC and AQC are fluorescent and thus more useful than those reagents that are UV-absorbing when quantitatively determining trace-levels of D-enantiomers in an excess of the more common L-amino acids.

The structure of amino acid is altered upon functionalization, which in turn increases the size and the analyte's hydrophobicity due to the introduced reagent fragment. As expected, the interaction pattern between the derivatized amino acid and the chiral selector is different compared to that when amino acid is native. It is observed experimentally that the HPLC enantioselectivity for a given fluorescent derivative may not be advantageous as compared to that for an UV-absorbing one under the elution of nonaqueous polar-organic mobile phase (Chen, 2003a, b, 2005; Chen et al., 2004; Pawlowska et al., 1993). This is because the enantioselectivity among these reagents for a given amino acid after derivatization becomes diverse under the same chromatographic conditions, which indicates the chiral recognition is highly dependent on the structure of derivatives (Chen, 2003a, b, 2004a, b, 2005; Chen and Ward, 2004). Note that the resolution of amino acid in derivatized form is usually not reproducible in the reversed-phase mode since the chiral recognition mechanism is no longer the same between these two elution modes (Chen, 2005).

In this report, a fluorescent electrophilic reagent, 9-fluorenone-4-carbonyl chloride (FCC), is chosen to functionalize amino acids in alkaline medium before their HPLC resolution. The detection limit for enantiomeric purities determination is also evaluated. Factors leading to the enantioresolution on a teicoplanin chiral stationary phase (CSP) using the methanol-based mobile phase such as the

composition of mobile phase and structural variation of derivatives, etc. are examined.

Materials and methods

Equipment

The teicoplanin stationary phase (250 × 4.6 mm i.d., 5 µm particle diameter) used in all the enantiomeric 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, USA). The C_{18} column (150 × 4.6 mm i.d., 5 µm particle diameter) used for the achiral separation and purification was from Vercopak (Taiwan, ROC). The HPLC system used in this study is a Hitachi model L-7100 linked to a D-2500 Chromatopac data station. A variable wavelength UV detector is used in the detection. The UV detection wavelength is set at 266 nm in the HPLC resolution study. In the spectroscopic measurement and enantiomeric purities determination, the excitation and emission wavelengths for the fluorescence detection are set at 266 and 520 nm, respectively according to the spectra in Fig. 1. As can be seen, the fluorescence spectral properties for FCC-OH and FCC-Phe compounds are quite similar for a given excitation wavelength. However, these two compounds are well separated achirally on a C_{18} column in the reversed-phase mode. Therefore, no detection difficulty is expected.

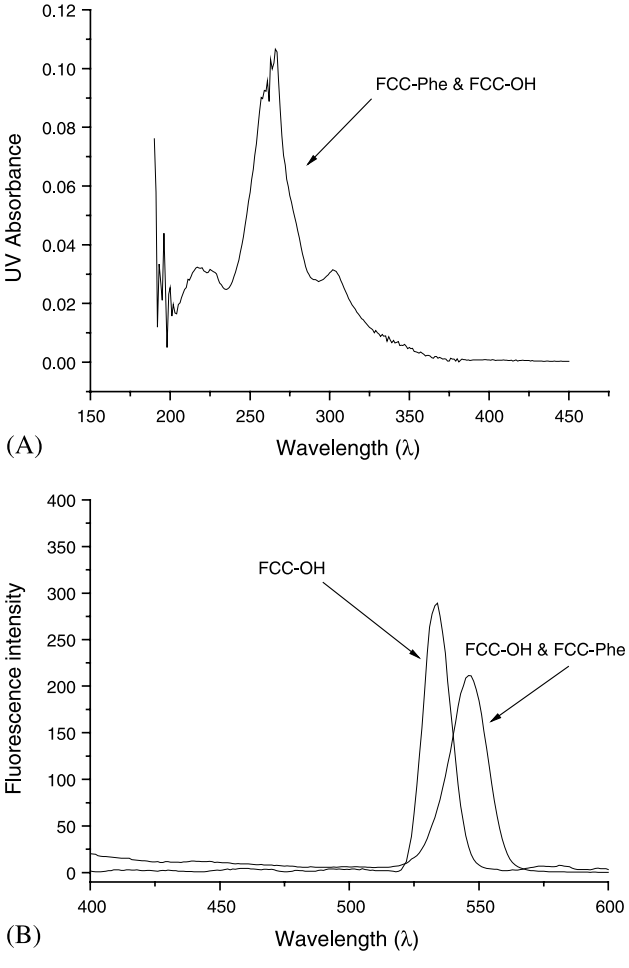


Fig. 1. The UV (**A**) and fluorescence (**B**) spectra for the mixture of hydrolyzed FCC (i.e., FCC-OH) and FCC-Phe derivatives. As can be seen, the fluorescence spectral properties for FCC-OH and FCC-Phe compounds are quite similar for a given excitation wavelength. However, these two compounds are well separated achirally on a C_{18} column in the reversed-phase mode. Therefore, no detection difficulty would be encountered

Chemicals

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

Methods

The purchased enantiomers were first dissolved in alkaline medium (e.g., sodium carbonate solution) and then mixed with FCC in acetonitrile for chemical derivatization at ~40 °C according to the procedure described previously (Chen, 1996). Wait for a few minutes before injection for HPLC resolution is made. The simplified derivtizing procedure is outlined in Fig. 2. The derivatives and the by-product formed during the hydrolyzation of FCC reagent were collected and further purified on a C_{18} column before the fluorescence measurement is performed.

The stability study is conducted by resolving the derivative at the time it was first prepared. The resolution was repeated for the same sample after a time period of three months under the same chromatographic conditions for comparison. Note that the sample was stored in the solvent mixture of buffer and acetonitrile at room temperature during the study.

Results and discussion

The chromatographic data for the enantiomeric resolution of FCC-derivatized amino acids using the methanol-based mobile phase on a teicoplanin CSP are listed in Table 1. As can be seen, most derivatives can be resolved much better than baseline using a single methanol-based mobile phase. In comparison with that for the AQC and FMOC derivatives under the optimized conditions using the polar-organic mobile phase, the selectivity factor for resolution is much larger (Pawlowska et al., 1993; Zukowski et al., 1993). Also, the variety of amino acids that can be resolved using a single mobile phase on a given CSP is richer if they are FCC-derivatized. A typical chromatogram for the resolution of FCC-pyroglutamic acid is shown in Fig. 3 for the first time. No resolution was obtained if pyroglutamic acid is either AQC- or FMOC-derivatized under the same chromatographic conditions. Besides, the resolution for many other FCC-derivatized amino acids having a polar group or sulfur atom in the side chain such as homocysteic acid, homoserine is also obtained. Interestingly, amino acid with a basic amide group in the side chain such as asparagine can be resolved as well under the same chromatographic conditions as

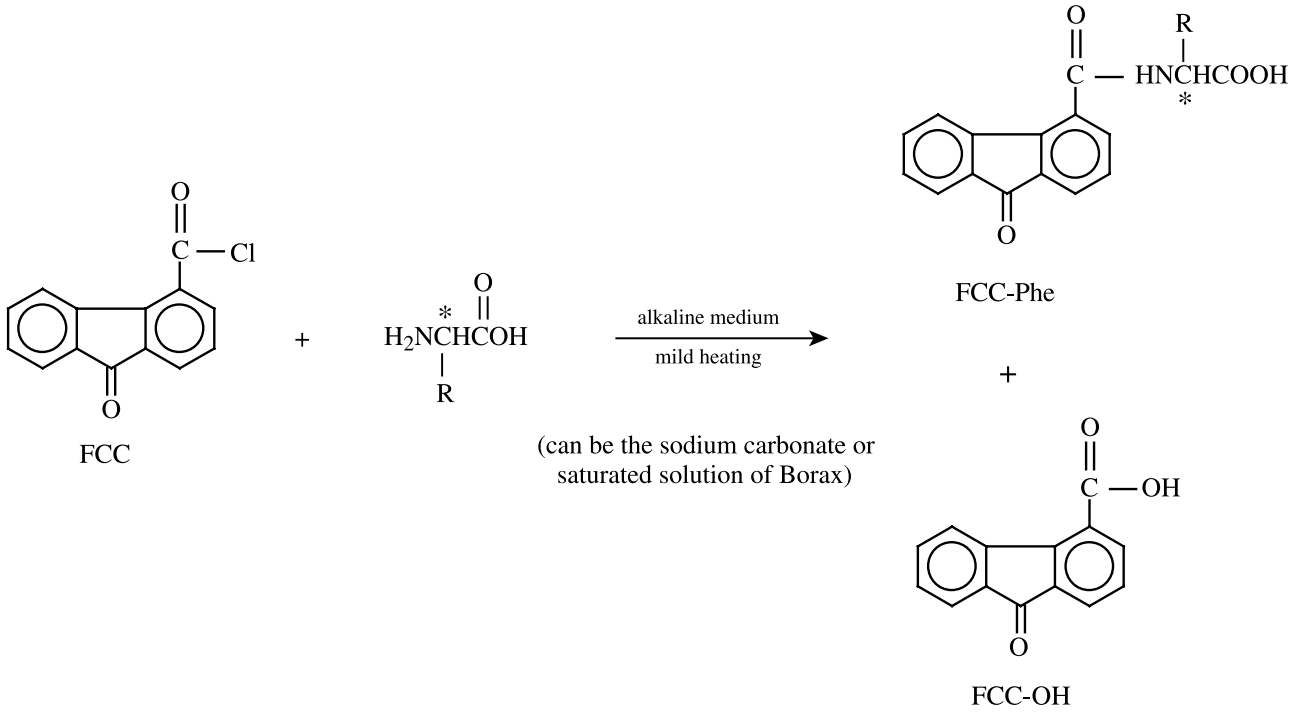


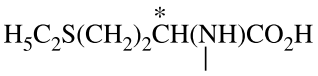
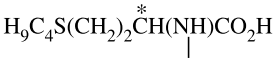
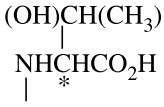
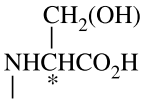
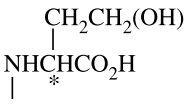
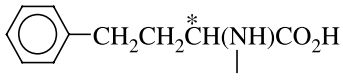
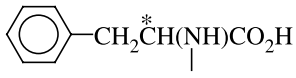
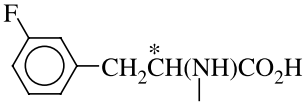
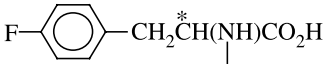
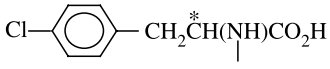
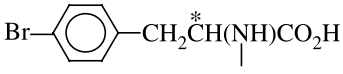
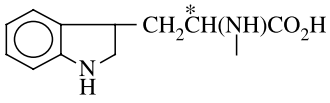
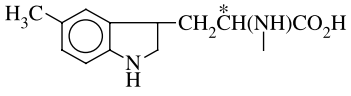
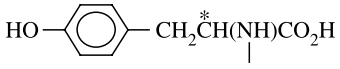
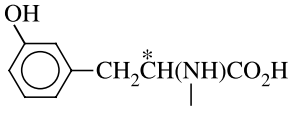
Fig. 2. Chemistry of the formation of FCC derivatives of amino acids (simplified)

Table 1. Chromatographic data for the resolution of amino acids on teicoplanin phase, using the methanol-based mobile phase after their precolumn derivatization with FCC[a]

Compound[b]	Structure	K[c]	α[c]	R_s[c]	Mobile phase[d]
Alanine*	CH_3 $NHCHCO_2H$ *	0.54	3.74	7.80	
Valine*	$CH(CH_3)_2$ $NHCHCO_2H$ *	0.26	5.32	3.62	
Norvaline	$(CH)_2CH_3$ $NHCHCO_2H$ *	0.27	9.73	6.74	
Leucine*	CH_3 CH_2CHCH_3 $NHCHCO_2H$ *	0.23 4.68	8.71 1.37	5.33 2.08	 *
Norleucine	$(CH_2)_3CH_3$ $NHCHCO_2H$ *	0.23	10.83	5.96	
Methionine*	$H_3CS(CH_2)_2\overset{*}{C}H(NH)CO_2H$	0.43 4.92	7.92 1.32	7.90 1.88	 *

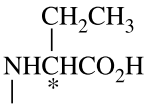
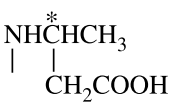
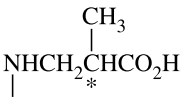
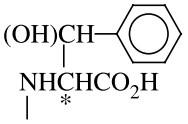
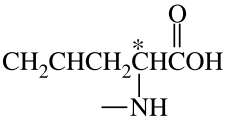
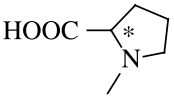
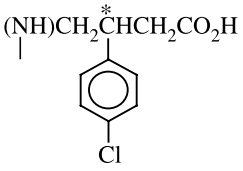
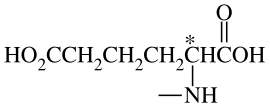
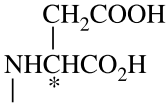
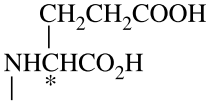
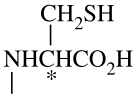
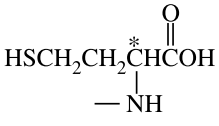
(*continued*)

Table 1 (*continued*)

Compound[b]	Structure	K[c]	α[c]	R_s[c]	Mobile phase[d]
Ethionine		0.32	10.02	6.00	
Buthionine		0.24	8.85	6.50	
Threonine*		0.48 5.28	3.43 1.24	4.37 1.01	 *
Serine*		0.57	4.41	5.47	
Homoserine		0.50	4.58	5.43	
Homophenylalanine		0.34	9.06	6.03	
Phenylalanine*		0.44 4.76	7.15 1.34	7.41 1.97	 *
m-Fluorophenylalanine		0.37	10.76	6.23	
p-Fluorophenylalanine		0.38	13.58	8.42	
p-Chlorophenylalanine		0.41	14.54	7.19	
p-Bromophenylalanine		0.47	13.19	9.55	
Tryptophan*		0.58	6.75	5.60	
5-Methyltryp-tophan		0.49	5.91	6.12	
Tyrosine*		0.46	9.12	6.22	
m-Tyrosine		0.39	7.24	6.35	
3-Amino-3-p-henylpropionic acid		0.41	1.74	2.25	

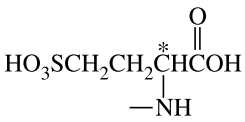
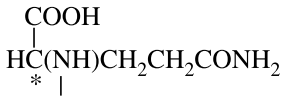
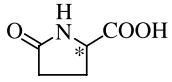
(*continued*)

Table 1 (*continued*)

Compound[b]	Structure	*K*[c]	α[c]	R_s[c]	Mobile phase[d]
α-Amino-n-butyric acid		0.33	5.30	4.24	
β-Amino-n-butyric acid		0.44	1.33	1.25	
3-Amino-iso-butyric acid		0.55	1.12	0.81	
3-Phenylse-rine		0.39	1.70	1.63	
2-Amino-4-pentenoic acid		0.32	6.71	6.89	
Proline*		0.73	1.18	1.23	
Baclofen		0.59	1.45	1.31	
α-Aminoadipic acid		1.22	2.63	3.70	
Aspartic acid*		2.06	1.16	0.94	
Glutamic acid*		1.63	1.56	2.23	
Cysteine		0.67	3.66	3.97	
Homoysteine		0.45	1.71	2.25	

(*continued*)

Table 1 (*continued*)

Compound[b]	Structure	K^c	α^c	$R_s{}^c$	Mobile phase[d]
Homocysteic acid		1.10	1.35	1.62	
Asparagine*		0.99	2.64	4.44	
Pyroglutamic acid		1.75	1.33	1.54	

[a] FCC stands for 9-fluorenone-4-carbonyl chloride with structure shown in Fig. 2

[b] Only compounds marked with asterisks are chosen in the elution order study, due to the availability of standards. The results show L-enantiomer is eluted first in all cases

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

[d] The mobile phase used in the resolution is 95 MeOH/5 EE/0.2 HOAC/0.4 TEA (except for those marked with the asterisk: 495 ACN/5 MeOH/1 HOAC/1 TEA) by volume (v/v). The ACN, MeOH, HOAC, TEA and EE are abbreviations for acetonitrile, methanol, acetic acid, triethylamine and ethyl ether, respectively

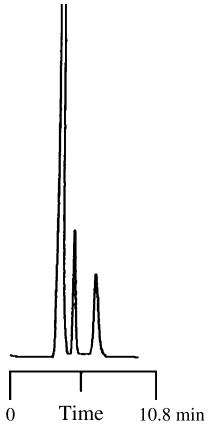


Fig. 3. Chromatogram showing the enantioresolution of FCC-pyroglutamic acid on a teicoplanin bonded chiral stationary phase using the methanol phase of 95 MeOH/5 EE/0.2 HOAC/0.4 TEA by volume (v/v). To the best of our knowledge, pyroglutamic acid is resolved in derivatized form for the first time under the elution of non-aqueous mobile phase

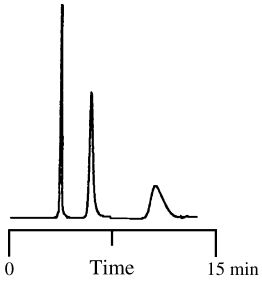


Fig. 4. Chromatogram showing the enantioresolution of FCC-Asn on a teicoplanin bonded chiral stationary phase using the methanol phase of 95 MeOH/5 EE/0.2 HOAC/0.4 TEA by volume (v/v). Note that asparagine is an amino acid with a basic amide group. Usually, this type of amino acids is not easy to resolve either in native or derivatized form with the reagents currently used on antibiotic bonded CSPs

shown in Fig. 4. Note that this type of amino acids is not easy to resolve either in native or derivatized form with the reagents currently used on antibiotic bonded CSPs. Despite the nature of side-chain group, the L-enantiomer is eluted first in cases which amino acid standards are commercially available. This is because teicoplanin is an antibiotic in nature and prefers to interact with the D-enantiomer.

The resolution was intended to reproduce under the elution of the acetonitrile-based mobile phase. Unfortunately, the results were not satisfactory. In Table 1 are listed the chromatographic data for the resolution for some of the FCC derivatives obtained under the elution of acetonitrile-based solvent mixture as the mobile phase for comparison. Typical chromatograms for the resolution of FCC-Leu using the methanol- (A) and acetonitrile-based (B) mobile phases are shown in Fig. 5. Obviously, the resolution is much better carried out with a relatively small retention time under the elution of the methanol-based mobile phase for the increase in solubility of analyte in the mobile phase (Chen, 2005). The long retention

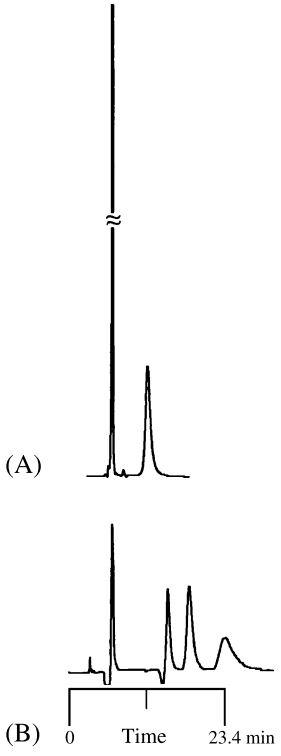


Fig. 5. Chromatograms showing the enantioresolution of FCC-Leu on teicoplanin bonded chiral stationary phase using the **A** methanol- (95 MeOH/5 EE/0.2 HOAC/0.4 TEA), **B** acetonitrile-based (495 ACN/5 MeOH/1 HOAC/1 TEA) mobile phase by volume (v/v). The chromatographic elution profile is quite different indicating the chiral recognition mechanisms involved in the resolution are different. Apparently, the resolution is better carried out using the methanol-based mobile phase

time under the elution of the acetonitrile-based mobile phase indicates the interaction between analyte and chiral selector is rather strong, however, not beneficial to the chiral recognition. It is believed that the mechanism involved in the resolution is diverse in each elution mode as mentioned in previous report except for the solubility factor (Chen, 2005).

Labeling with FCC reagent converts amino acids into derivatives with favorable properties. FCC reacts with both primary and secondary amino acids to produce stable and highly fluorescent derivatives suitable for sensitive and efficient chromatographic determination and resolution on a teicoplanin chiral stationary phase (CSP) using the methanol-based solvent mixture as the mobile phase. This is because the by-products of the derivatization reaction are eluted from the teicoplanin column close to or at the dead volume under the elution of methanol-based mobile phase. Consequently, we were able to determine the FCC amino acids at trace level and detect as little as 0.01% of D-phenylalanine in a large excess of L-enantiomer. The corresponding data are summarized in Table 2. In Table 2 also lists the previously-reported data for determination of enantiomeric purity of commercial amino acids using AQC (Pawlowska et al., 1993) and FMOC-Gly-Cl reagents (Zukowski et al., 1993) for comparison. As can be seen, the detection limit for FCC derivative is comparable.

The stability of FCC derivative was also under investigation by resolving the derivative at the time it was first prepared. The resolution for the same sample was repeated three months later under the same chromatographic conditions for comparison. Note that the sample was stored in the solvent mixture of buffer and acetonitrile at the room temperature during the study. A typical example for the resolution of FCC-Trp at the time the derivative was first prepared (bottom) and for that obtained three months after the reaction (top) is shown in Fig. 6. As can be seen, these two chromatograms look quite similar indicating the derivative is rather stable in the reaction matrix during the study. No detectable decomposition is observed.

Table 2. Comparison of the analysis of optical purity of commercial "pure" L-amino acid standards and the determination of enantiomeric ratio in the D, L mixture using three pre-column derivatizing reagents: FCC, AQC and FMOC-Gly-Cl

Name	Source	Derivatizing reagent					
		FCC[a]		AQC[b]		FMOC-Gly-Cl[b]	
		%D (SD)[c]	Stationary phase[d]	%D (SD)[c]	Stationary phase	%D (SD)[c]	Stationary phase
L-Leu	Sigma	0.054 (0.005)	Teicoplanin	0.049 (0.006)	RN-β-CD	0.045 (0.013)	γ-CD
L-Phe	Aldrich	<0.01		<0.0075		<0.0075	β-CD
D-,L-Phe	The artificial mixture	4.120 (0.060)		4.070 (0.096)		3.8994 (0.065)	

[a] FCC, 9-fluorenone-4-carbonyl chloride
[b] The data are cited from references described above for comparison
[c] SD stands for standard deviation and is determined in triplicate
[d] The mobile phase used in the determination is 95 MeOH/5 EE/0.2 HOAC/0.4 TEA by volume (v/v)

Fig. 6. Chromatograms showing the enantioresolution of FCC-Trp on teicoplanin bonded chiral stationary phase using the methanol-based mobile phase of (95 MeOH/5 EE/0.2 HOAC/0.4 TEA) by volume (v/v). The top chromatogram was obtained when the derivatization reaction was first completed and the bottom one was repeated three months after the reaction. Note that the sample was stored at room temperature during the study. The fluorescence detection was employed in both cases

Conclusion

The resolution of a variety of amino acids has been demonstrated on a teicoplanin bonded chiral phase using a single methanol-based mobile phase after their pre-column derivatization in alkaline medium with the fluorescent electrophilic reagent FCC. The resolution is better obtained as compared to that for the FMOC or AQC derivatives reported previously under the optimal chromatographic conditions. Also, the variety of amino acids can be resolved is richer if they are FCC-derivatized. The results show FCC derivatives are very stable, which is essential in quantitatively determining trace-levels of D-enantiomers in an excess of the more common L-amino acids. The other advantageous property for FCC as a fluorescent tagging reagent is that its excitation and emission wavelengths are quite different and thus expect to have minor spectroscopic interferences during the optical impurities determination at trace level.

Acknowledgments

Support of this work by the National Science Council of Taiwan and the National Chiayi University is gratefully acknowledged.

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