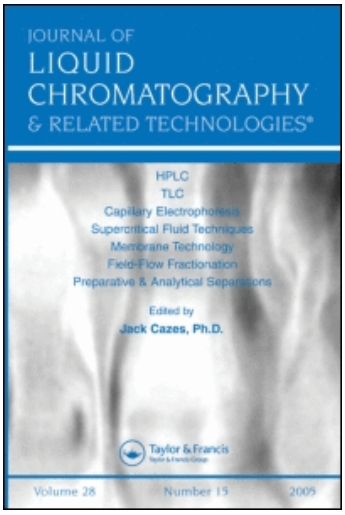

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

High-Performance Liquid Chromatographic Determination of Amino Acids and Oligopeptides by Pre-column Fluorescence Derivatization with 9-Fluorenyl-methoxy Carbonyl Succinimide

Jinmao You[a]; Xingjun Fan[a]; Hong En Wang[a]; Guojun Wang[a]; Ji Xin Su[b]; Chang Li Zhou[b]
[a] Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences, Lanzhou, PR, China [b] Department of Environmental Engineering, Shandong University, Jinan, PR, China

To cite this Article You, Jinmao , Fan, Xingjun , Wang, Hong En , Wang, Guojun , Su, Ji Xin and Zhou, Chang Li(1998) 'High-Performance Liquid Chromatographic Determination of Amino Acids and Oligopeptides by Pre-column Fluorescence Derivatization with 9-Fluorenyl-methoxy Carbonyl Succinimide', Journal of Liquid Chromatography & Related Technologies, 21: 14, 2103 — 2115
To link to this Article: DOI: 10.1080/10826079808006611
URL: http://dx.doi.org/10.1080/10826079808006611

J. LIQ. CHROM. & REL. TECHNOL., 21(14), 2103–2115 (1998)

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF AMINO ACIDS AND OLIGOPEPTIDES BY PRE-COLUMN FLUORESCENCE DERIVATIZATION WITH 9-FLUORENYL-METHOXY CARBONYL SUCCINIMIDE

Jinmao You,[1,*] Xingjun Fan,[1] HongEn Wang,[1]
Guojun Wang,[1] Ji Xin Su,[2] ChangLi Zhou[2]

[1] Lanzhou Institute of Chemical Physics
Chinese Academy of Sciences
Lanzhou 730000, P. R. China

[2] Department of Environmental Engineering
Shandong University
Jinan 250100, P. R. China

ABSTRACT

A sensitive high-performance chromatographic method for the detection of amino acids and oligopeptides with pre-column fluorescence derivatization has been developed. Glycine, glycylglycine, triglycine, glutathion, glutamic acid, and cysteine were separated on a reversed-phase C_{18} column with methanol-water-triethylamine as eluent; the derivatization and the chromatographic conditions were optimized. The six derivatives were eluted in 20 min with good reproducibility. The relative standard deviations (n=6) at an analytical concentration of 2 x 10^{-6} M are less than 4.5%. The detection limits (signal-to-noise ratio=3) for the six derivatives are in the range of 15-46 fmol.

INTRODUCTION

Recently, separations and quantitative determinations of amino acids and some oligopeptides by means of automatic analyzers and new fluorescent reagents, which are used as pre-column or post-column derivatization, are developing intensively. The separations and determinations of amino acids and some oligopeptides with RP-HPLC are still an active field; the development in this field was summarized by S. Nakani[1] and C. Schonic.[2] Some oligopeptides may be determined on a reversed-phase column with the gradient elution technique by RP-HPLC at the range of 210-225nm in spite of low-detection sensitivity, however, a great part of oligopeptides and amino acids do not have ultraviolet absorption. In order to raise the detection sensitivity and improve the selectivity, the general methods are derived with derivatization reagents.

Phenyl isothiocyanate,[3] benzoyl chloride,[4] 4-nitrobenzoyl chloride,[5] and 3,5-dinitrobenzoyl chloride[6] are well known derivatization reagents for the determination of amino compounds by high-performance liquid chromatography (HPLC) with spectro-photometric detection, but the selectivity and the sensitivity are low. On the other hand, several fluorescent derivatization reagents such as 5-dimethyl-aminonaphthalene-1-sulfonylchloride(dansyl-Cl),[7] phthalimidylbenzoyl chloride,[8] 3,4-dihydro-6,7-dimethoxy-4-methyl-3-oxoquinoxaline-2-rbonylchloride,[9] 7-ethyl aminocoumarin-3-carbonyl fluoride,[10] o-phthaldialdehyde (OPA),[11-14] 3-chloro-7-nitrobenzofurazan, and 4-(2-phthalimidyl)benzoyl chloride (PIB-Cl)[15] have also been developed for the determination of amino compounds. Many of these reagents suffer from incomplete reactions, highly fluorescent hydrolysates or reagent toxicity. Recently O. U. Keli et al. developed an improved HPLC method for the separation of amino acids derivatized with 9-fluorenylmethyl chloroformate (Fmoc).[16-18] Current procedures using derivatization with Fmoc are also troublesome. An excess of reagent must be used to provide effective derivatization. Furthermore, histidine and tyrosine form mixtures of monosubstituted and disubstituted Fmoc derivatives, and the excess Fmoc is necessary to remove prior to chromatography[19-21] because it interferes with the separation of the amino acid derivatives and is detrimental to column performance. This problem has been overcome by adding quenching reagent.

This method yielded monosubstituted amino acid derivatives for the common protein amino acids, including histidine and tyrosine. In fact, the common derivatization process, Fmoc reacts not only with amino group ($-NH_2$), but also with alcohols, phenols, thiols, and active secondary amine. The selectivity of Fmoc is relatively low.

In order to improve the selectivity and keep the high detection sensitivity, we synthesized 9-Fluorenylmethoxy carbonyl Succinimide (Fmoc-Osu), which is used as a pre-column fluorescent derivatization reagent for the determination of amino acids and oligopeptides. It is found that amino acids and oligopeptides derivatized with Fmoc-Osu are stable, and not susceptible to major matrix interference reactions. Furthermore, the precolumn derivatization process developed does not require solvent extraction steps to remove excess derivatization reagent prior to chromatography. At the same time, Fmoc-Osu has high selectivity for amino acids and some oligopeptides and reacts only with amino group(-NH_2). The derivatization and the chromatographic conditions were optimized by a series of experiments. The RP-HPLC separations of amino acid and oligopeptide derivatives were performed on a Spherisorb C_{18} column with CH_3OH-H_2O-Et_3N (75:23:2 v/v/v) as eluent at a flow rate of 0.9mL/min. The fluorescence intensity at 385nm (excitation at 270nm) was monitored.

EXPERIMENTAL

Apparatus

A model 655 liquid chromatography equipped with an 650-10S fluorescence spectrophotometer(Hitachi), a pheodyne 7125 injection valve (USA), a 655 proportioning valve, and a 644-61 intergrater (Hitachi) were used in experiments. Fluorescence emission spectra was also obtained on a 650-10S fluorescence spectrophotometer. The derivatives of amino acids and oligopeptides were separated on a 200 x 4.6mm Spherisorb-C_{18} column (5μm) obtained from Dalian Institute of Chemical Physics, the Chinese Academy of Sciences. A paratherm U_2 electronic water bath (Germany) was used to control the column temperature. All mobile phase solutions were treated by supersonic waves for 15 min prior to use.

Reagents

Methanol and triethylamine were analytical grade purchased from Jining Chemical Reagent Co. (China), and distilled prior to use. Doubly distilled water was used throughout. All solvents used for RP-HPLC were filtered through fiber glass filters, 0.45μm. Dicyclohexylcarbodiimide (DCC) was purchased from Zibo Chemical Factory (China). Glycine, glycylglycine(Gly-Gly), glutamic acid, triglycine(Gly-Gly-Gly), L-cysteine, glutathion were all supplied by Department of Biology, Qufu Normal University(Qufu, Shandong,

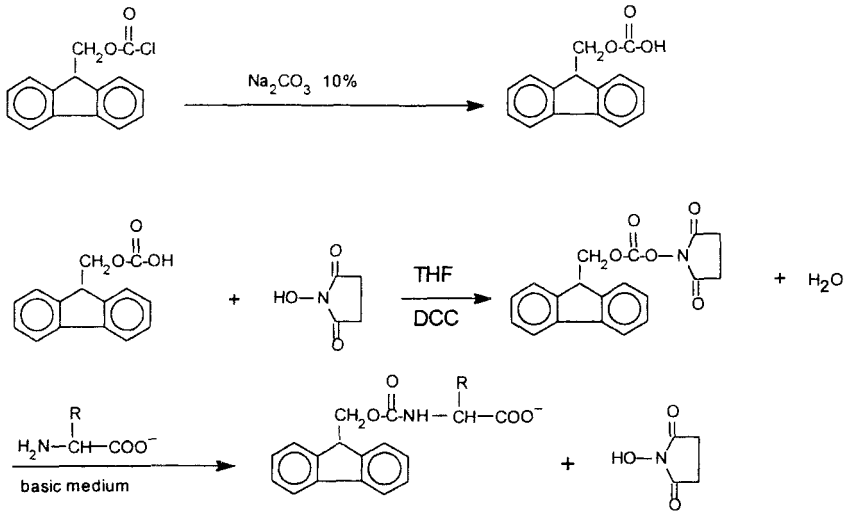

Figure 1. The derivatization reaction process.

China). 9-Fluorenylmethoxycarbonyl Chloride was purchased from Shanghai Dong Feng Biochemical Technology Company, Shanghai Institute of Biochemistry, Chinese Academy of Sciences. 9-Fluorenylmethoxy carbonyl Succinimide (Fmoc-Osu) was synthesized by means of the previous method.[22] The main reaction process was shown in Figure 1.

Stock Solution

A 5.0×10^{-3} M of Fmoc-Osu solution was prepared by dissolving 0.1595 g Fmoc-Osu in 100 mL acetonitrile. 0.898×10^{-3} M glutathion, 1.005×10^{-3} M triglycine, 1.002×10^{-3} M glycylglycine, 0.698×10^{-3} M glutamic acid, 0.416×10^{-3} Mcysteine, and 1.146×10^{-3} M glycine solutions were prepared by dissolving corresponding components in acetonitrile, respectively.

Preparation of amino acid and oligopeptide derivatives

An appropriate volume of amino acid or oligopeptide stock solution, prepared above, was added into a 25 mL volumetric flask, then 5mL of 0.2 M boric acid-sodium tetraborate buffer (pH 8.5) and 3.5 mL of 5.0×10^{-3} M Fmoc-Ous acetonitrile solution were added. The content was shaken for approximately 0.5min to ensure good mixing, then heated for 30min in a

water-bath at 60°C. After cooling, the solution was diluted with doubly distilled water to 25 mL in 25-mL volumetric flask and treated by supersonic wave for 5 min to remove oxygen bubbles.

Chromatographic Method

The HPLC separations of amino acid or oligopeptide derivatives were performed on a Spherisorb C_{18} column with methanol-water-triethylmine (75:23:2 v/v/v) as eluent at a flow rate of 0.9 mL/min. The temperature was kept at 25°C with an isothermal water-bath. The fluorescence intensity at 318 nm (excitation at 270 nm) was monitored.

Effect of Solvent Polarity on the Fluorescence Intensity of Derivatives

Glycylglycine derivative (5μM) was dissolved in various mobile phase systems. The fluorescence emission spectra were recorded with an excitation wavelength of 313 nm in a 1 cm Quartz cell on an 650-10S spectrofluorimeter. Fluorescence efficiencies were obtained by comparison with Fmoc-OH as standard ($\phi_{n'}$=1.0). The ϕ_n value for a given derivative was calculated according to the equation

$$\phi_n/\phi_{n'} = I_n (1\text{-}10^{-A'}) / I_{n'} (1\text{-}10^{-A})$$

where ϕ_n and $\phi_{n'}$ are the fluorescence quantum efficiencies for the given derivatives and the standard. A and A′ are the absorbance of the derivatives and the standard solutions (determined by UV). I_n and $I_{n'}$ are the areas under the emission curves of the derivative and the standard, respectively. Quantum efficiencies were measured in 100% acetonitrile, in 75% acetonitrile, and in 75% methanol solutions, respectively.

RESULTS AND DISCUSSION

Choice of Labeling Reagent

The enhancement of detection limits in RP-HPLC separations of amino acids and oligopeptides through the use of fluorescent detection is an important analytical goal. One of the critical aspects of developing the tools and techniques required to achieve this goal is the selection of the optimum

fluorescent labeling agent. The ideal labeling reagent would be one which keeps the high detection sensitivity and is not susceptible to major matrix interference reactions. This derivating agent selected does not require solvent extraction steps to remove excess derivatization reagent prior to chromatography. In addition, the amino acids such as histidine and tyrosine reacting with the derivating agent selected, should not form the mixtures of monosubstituted and disubstituted derivatives. Based on this theory, the fluorescent labeling reagent of Fmoc-Osu was selected in this experiment for separation of amino acids and oligopeptides.

Stability of Fluorescent Reagent (Fmoc-Osu)

3.0 mL of 0.2 M boric acid-sodium tetraborate buffer (pH8.5) and 3.5mL of 5.0 x 10^{-3} M Fmoc-Osu were added into a 25mL volumetric flask. After fixed intervals, an aliquot of the solution was injected onto the column. The extent of decomposition was determined from the ratio of the peak height for Fmoc-Osu and its hydrolyzate(Fmoc-OH) before and after heating. The derivatization reagent exhibited less than 8% decomposition at 60°C for 30 min.

Stability of Fmoc-Osu Derivatives

Several vials of each derivative (5-10 μM) in mobile phase component ($CH_3OH/H_2O/Et_3N$, 75:23:2, pH 7.0, adjusted with AcOH) were heated in a water-bath at 40°C for 1 hr. After fixed intervals, a vial was removed and an aliquot of the solution injected onto the column. The extent of decomposition was determined from the ratio of the peak height for the derivatives before and after heating. The derivatives exhibited less than 6% decomposition at 40°C for 1 hr. Comparable results were observed in 75% methanol solution at 40°C. No decomposition was found at pH 7.0 at room temperature in 1 hr.

Fluorescence Properties of Derivatives

The maximum wavelengths of fluorescence excitation and emission of the derivatives are 270 and 318 nm, respectively. The effects of solvent on the fluorescence spectra and intensities of derivatives are shown in Table 1. The fluorescence intensity in acetonitrile was 6% stronger than in methanol. The emission wavelength in acetonitrile was shorter than that in methanol-water. In acetonitrile-water, solutions caused a red shift of emission wavelength and a decrease in fluorescence intensity. An increase of triethylamine content in methanol-water solution caused a blue shift of the emission wavelength and an

Table 1

Effect of Solvent Polarity on Fluorescence Properties (Fmoc-Glycylglycine)

Solvent	Maximum Wavelent Excitation	Emission	Relative Fluorescence Intensity (n=5)	Fmoc-Osu ϕ_n
CH_3OH	270	313	1.00	0.35*
CH_3OH-H_2O				
90 + 10	270	314	0.99	
80 + 20	270	315	0.98	
75 + 25	270	316	0.96**	0.42*
70 + 30	270	318	0.94	
CH_3CN	270	310	1.17	0.41*
CH_3CN-H_2O				
90 + 10	270	312	1.12	
80 + 20	270	314	1.06	
75 + 25	270	315	1.02**	0.45*
CH_3OH-H_2O-Et_3N				
75 + 24.5 + 0.5	270	318	0.95	
75 + 24 + 1	270	316	0.96	
75 + 23 + 2	270	315	0.97	
75 + 23 + 3	270	315	0.99	

* Relative ϕ_n= 1.0 for Fmoc-OH in acetonitrile.

** Difference in fluorescence intensity = (1.02-0.96)/0.96 ≈ 6.0.

increase in fluorescence intensity. There is only a 6% difference in the fluorescence intensity between 75% of acetonitrile solution and 75% methanol solution , but the cost of methanol system is relatively low, so methanol-water-triethylamine system was used as mobile phase component.

Derivatives show little change in fluorescence quantum efficiencies with increasing of solvent polarity. The ϕ_n value for glycylcine derivative changes from 0.41 to 0.45 in going from 100% to 75% acetonitrile. The ϕ_n value for glycylcine derivative changes from 0.35 to 0.42 in going from 100% to 75% methanol.

In addition, the Fmoc-Osu derivatives of amino acids and peptides exhibited higher fluorescence emission intensity in neutral and alkaline solution (pH 5-9) than in acidic solution.

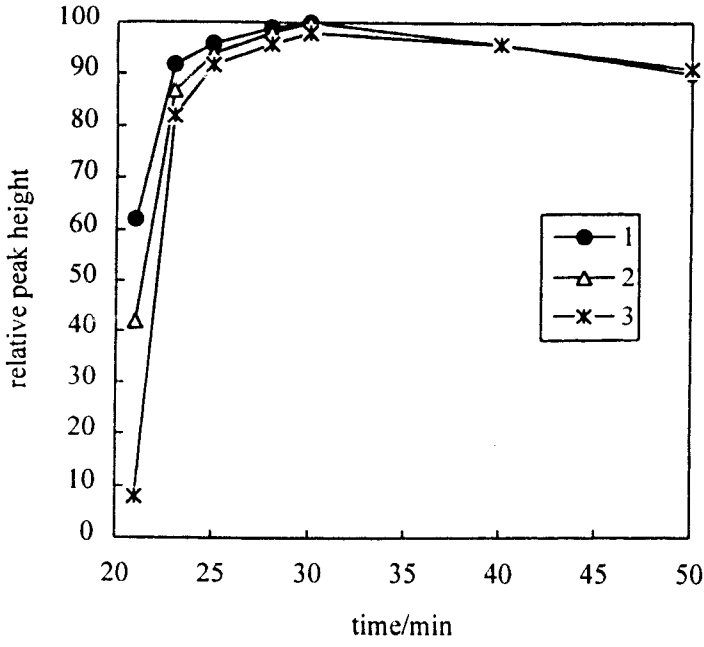

Figure 2. Reaction time courses of Fmoc-Osu with glycine, glycylglycine, and glutathione (reduced form). Reaction system composition: 5 mL of 0.2 mol/L boric acid/sodium tetraborate buffer (pH 8.5), 3.5 mL of 5.0 x 10^{-3} mol/L Fmoc-Osu, and 2.0 mL corresponding components stock solution. 1. Glycine; 2. Glycylglycine; 3. Glutathione.

Fluorescence Derivatization Condition

The optimum fluorescence derivatization condition has been investigated. The reaction of Fmoc-Osu with amino acids and oligopeptides was compared with the relative peak height of derivatives by various intervals.

The course for the formation of the labelled derivative (Figure 2) was determined by HPLC with fluorescence detection when 5 ml of 0.2 mol/L boric acid-sodium tetraborate buffer (pH 8.5), 3.5 mL of 5.0 x 10^{-3} M Fmoc-Osu solution, and 1-2 mL stock solution of amino acids and oligopeptides, prepared above, were added into a 25 mL volumetric flask. The production of the labelled amino acids and oligopeptides by reaction with Fmoc-Osu at 60°C reached a maximum after 30 min.

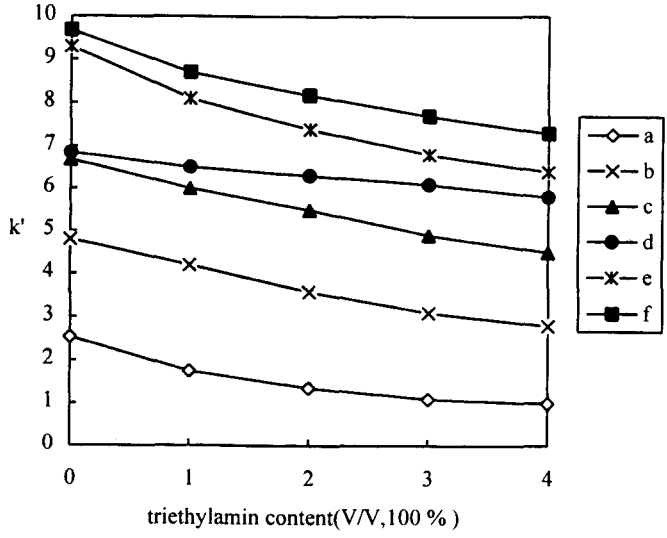

Figure 3. Effect of triethylamine content in mobile phase on k′ value. a) Glycine; b) Glycylglycine; c) Glutamic acid; d) Triglycine; e) Cysteine; f) Glutathione (reduced form).

Effect of Triethylamine Concentration on Retention Value

The effects of triethylamine concentration on the retention time of solutes were investigated. The results show that the k′ values of solutes decrease with the increasing of triethylamine content, the peak spreading is reduced, and column efficiency improved; but the resolution of components is also reduced. It is believed that these variations are from interaction of the solutes molecules with residual silanol groups on the silica surface. The nitrogen atoms in triethylamine molecules can easily form coordination bonds with oxygen atoms of residual hydroxyl groups on the surface of stationary phase. Thus the amount of residual acidic sites on the surface decrease with the amount of adding of triethylamine. This process can efficiently reduce the non-specifical sorption of solutes. The use of chromatographic eluent containing triethylamine gave a good reproducibility for the qualitative and the quantitative analysis of the derivatives. The optimum separation was obtained with 2% (v/v) of triethylamine when methanol concentration was kept to 75% (v/v). The effect of triethylamine concentration on retention value is shown in Figure 3.

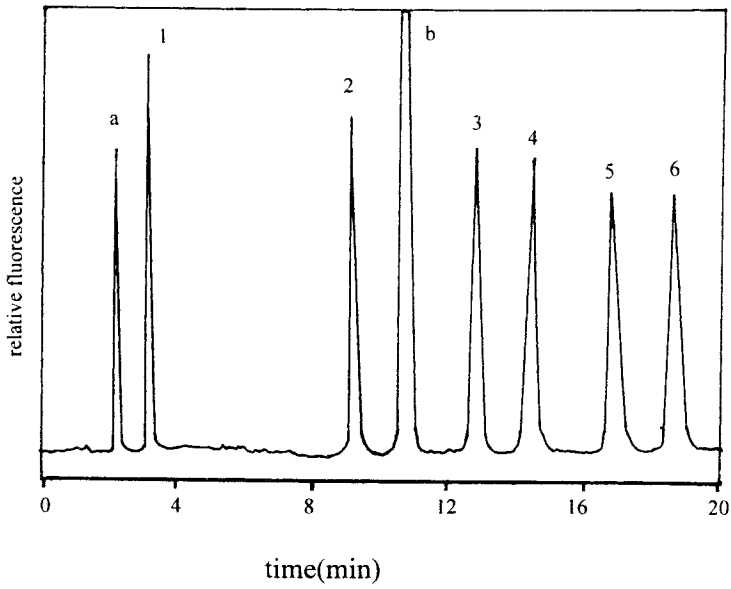

Figure 4. HPLC separation of Fmoc-Osu derivatives. Eluent: methanol : water : triethylamine (75:23:2 v/v/v; pH 7.0, adjusted with 0.1 mol/L AcOH); Peaks: a) Fmoc-OH; b) Fmoc-Osu; 1. Glycine; 2. Glycylglycine; 3. Glutamic acid; 4. Triglycine; 5. Cysteine; 6. Glutathione.

Table 2

Regression Analysis of Calibrtion Graphs and Other Quantitative Data for the Fmoc-Osu Derivatives

Compound	Linear Range nmol/mL	Calibration Graph*	Correlation Coefficient	RSD^{+} %	Detection Limit^{++} fmol
Glycine	0.12-210	y=0.330+0.165x	0.999	4.3	34
Glycylglycine $(GLY)_2$	0.12-205	y=0.312+0.158x	0.999	4.1	29
Glutamic Acid	0.08-240	y=0.186+0.246x	0.999	3.0	18
Triglycine $(GLY)_3$	0.16-200	6=0.402+0.287x	0.999	3.2	40
Cysteine	0.10-260	6=201+0.314x	0.999	4.4	15
Glutathion	0.23-240	y=0.817+0.418x	0.999	3.8	46

* y in cm x in nmol/mL; $^{+}$ relative standard deviation (n=6); $^{++}$ signal-to-noise=3.

HPLC Separation of Derivatives

The HPLC separation of a mixture of amino acid and oligopeptide derivatives was carried out by using a reversed-phase Spherisorb-C_{18} column. The elution order at pH 7.0 was shown in Figure 4.

At acidic pH, the hydrolysate (Fmoc-OH) elutes after the amino acids and oligopeptides (chromatogram was neglected). The line regression equations and the detection limits (S/N=3) were calculated from peak heights and listed in Table 2.

CONCLUSIONS

The Fmoc-Osu was developed as a high selectivity fluorescence labelling reagent for indirect determination of amino acids and oligopeptides. As expected, it is a high selective and stable fluorescence derivatization reagent, which forms single stable derivatives of amino acids and oligopeptides. The method is highly reproducible, and applicable to the analysis of amino acids in different matrices. At the same time, this method described here is simple and used to the determination of amino acids or oligopeptides with satisfactory results.

ACKNOWLEDGMENTS

We thank Dr. Bin. Zhao (Department of Chemistry, Qufu Normal University) for assistance with part of this work. We also thank Professor Yumei Wu (Department of Chemistry, Qufu Normal University) for checking and approving this paper.

REFERENCES

1. S. Nakatani, T. Kitamuta., T. Yamasak, Toso Kenkyu Hokoku, **34**, 3 (1) (1990).

2. C. Schoneich., S. K. Kwok, G. S. Wilson, Anal Chem., **65**, 67 R (1993).

3. P. Furst, L. Pollack, T. A. Graser, H. Godel, P. Stehle, J. Chromatogr., **499** 557 (1990).

4. F. A. Fitzpatrick, S. Siggia, Anal. Chem., **45**, 2270 (1973).

5. W. H. Newsome, L. G. Panopio, J. Agric. Food Chem., **26**, 638 (1978).

6. Y. Suzuki, N. Tsuchiya, Bunseki Kagaku, **30**, 240 (1981).

7. Y. Yamada, Y. Aizawa, J. Pharmacol. Methods, **9**, 1 (1983).

8. Y. Tsuruta, K. Kohashi, Anal. Chim. Acta, **192,** 309 (1987).

9. J. Ishida, M. Yamaguchi, T. Iwata, M. Nakamura, Anal. Chim. Acta, **223,** 319 (1989).

10. H. Fujino, S. Goya, Yakugaku Zasshi, **110**, 693 (1990).

11. M. Roth, Anal. Chem., **43**, 880 (1971).

12. P. Lindroth, K. Mopper, Anal. Chem., **51**, 1667 (1979).

13. D. W. Hill, F. H. Walters, T. D. Wilson, J. D. Stuart, Anal. Chem., **51**, 1338 (1979).

14. R. F. Chem, C. Scott, E. Trepman, Biochim. Biophys. Acta, **576**, 440 (1979).

15. Minghui Zheng , Chengguang Fu, Hongda Xu, Analyst, **118**, 269 (1993).

16. P. A. Haynes, D. Sheumack, J. Kibby, J. W. Redmond, J. Chromatogr., **540**, 177-185 (1991).

17. P. A. Haynes, D. Sheumack, L. G. Greig, J. Kibby, J. W. Redmond, J. Chromatogr., **588**, 107 (1991).

18. K. OU, M. K. Wilkins, J. X. Yan, A. A. Gooley, Y. Fung D. Sheumack, K. L. Williams, J. Chromatogr. A, **723**, 219-225 (1996).

19. S. A. Cohen, D. J. Strydom, Anal. Biochem., **174**, 1 (1988).

20. P. Furst, L. Pollack, T. A. Graser, H. Godel, P. Stehle, J. Chromatogr., **499**, 557 (1990).

21. R. Schuster, J. Chromatogr., **431**, 271 (1988).

22. Zhao Yuan-yuan, Xu Guo-Liang, Zhang Hua-Shan, Li Jun, Chem. J. Chinese Univ., **17**, 1044-1047 (1996).

Received June 6, 1997
Accepted June 30, 1997
Manuscript 4489