N-Hydroxysuccinimidyl-Fluorescein-*O*-Acetate for Precolumn Fluorescence Derivatization of Amino Acids and Oligopeptides in Liquid Chromatography

Hong Wang, Jin Li, Ting-Xian Yang, and **Hua-Shan Zhang***
Department of Chemistry, Wuhan University, Wuhan 430072, China

Abstract

A new fluorescent derivatizing reagent, *N*-hydroxysuccinimidyl-fluorescein-*O*-acetate, is used for the high-performance liquid chromatographic analysis of amino acids and oligopeptides. This reagent has the advantages of high-detection sensitivity in the visible region, specifically with amino groups, mild derivatization conditions, and little interference induced. The fluorescence properties of the reagent and its derivatives with amino acids and oligopeptides are studied. The conditions of the derivatization are investigated in detail. In the mobile phase of methanol–water (42:58, v/v) containing a 10mM pH 5.0 citric acid–Na_2HPO_4 buffer, six amino acids and oligopeptides are separated in 20 min with fluorescence detection at excitation and emission wavelengths of 492 and 513 nm, respectively, with the detection limits for injected standards ranging from 0.64 to 12 fmol.

Introduction

The liquid chromatographic (LC) analysis of amino acids and peptides has been applied widely in life science. Chemical derivatizations are usually used in the determination of these substances, because most amino acids and peptides have no obvious signal for a sensitive detection. In these cases, a fluorophore, chromophore, or electrophore has to be introduced to amino acids and peptides in order to form conjugates that can be detected sensitively using corresponding detectors. Because of the high sensitivity and selectivity of the fluorescence detection, the studies on fluorescent derivatizing reagents are a subject of considerable interest.

In general, an ideal derivatizing reagent must consist of an appropriate reactive group and a strong fluorophore.

In reactions with amino compounds, *N*-hydroxysuccinimidyl ester has been proven to be an excellent reactive function. It can react with amino compounds selectively in mild conditions because it does not react with -OH and -SH groups. During reaction with amino groups, the excess of the reagents is hydrolyzed to the corresponding carboxylic acids. Recently, some reagents with the substituent of *N*-hydroxysuccinimidyl ester have been reported as the derivatizing reagents in the determination of amino acids and peptides by chromatographic methods (1–13), such as 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) (1–7), *N*-hydroxysuccinimidyl-3-indolylacetate (SIIA) (9), and *N*-hydroxysuccinimidyl-α-(9-acridine)-acetate (HSAA) (13). These kinds of reagents provide ideal candidates for the analysis of amino acids and peptides. However, the fluorescence wavelengths of the reported reagents mainly fall in the ultraviolet region similar to those of biological active substances having fluorescence, which will cause severe interference. Therefore, the research of new derivatizing reagents with visible fluorescence is preferred in reducing interference from biological active substances themselves.

Until then, the determination of trace amounts of biological active substances calls for high sensitivity at low sample concentrations, which can be solved by the introduction of a highly sensitive fluorophore. Fluorescein is a widely used fluorophore because of its convenient wavelengths for measurement in the visible region, high-extinction coefficient, and high-fluorescence quantum yield in water. Furthermore, the excitation wavelength (λ_{ex}) for fluorescein closely matches the 488-nm spectral line of the argon-ion laser often available in commercial capillary electrophoresis (CE)–laser-induced fluorescence (LIF) instruments. Fluorescein isothiocyanate (FITC) has been proposed for the analysis of amino acids and peptides (14,15). However, the chemistry of this reagent with the isothiocyanate-reactive moiety is sluggish and inefficient, involving several stages of drying under vacuum, extensive by-products, and low specificity to amino functions. It has to derivatize amino acids or peptides at a high concentration (10^{-4}M) for an effective reaction yield (16).

5-Carboxyfluorescein succinimidyl ester (CFSE) has been developed for fluorescence detection in CE with regard to the limitations of FITC (16). CFSE can derivatize nanomolar concentrations of amino acids, which represents a 1000-fold improvement compared with FITC.

In this study, a new reagent synthesized in our lab, *N*-hydroxysuccinimidyl-fluorescein-*O*-acetate (SIFA) (17), with fluorescein as the fluorophore and *N*-hydroxysuccinimidyl ester as the reactive function (similar with CFSE) was used as the precolumn derivatizing reagent for the determination of amino acids and

oligopeptides with high-performance liquid chromatography (HPLC). This reagent reacted with amino compounds specifically in mild conditions, and the excess reagent was hydrolyzed to a hydrolysis product during the derivatization procedure. In the mobile phase of methanol–water (42:58, v/v) containing a 10mM pH 5.0 citric acid–Na_2HPO_4 buffer, glutamic acid (Glu), glycyl glycyl glycine (Gly-Gly-Gly), glycyl glycine (Gly-Gly), glycine (Gly), cystine ($(Cys)_2$), and oxidized glutathione (GSSG) have been separated in 20 min with fluorescence detection at an excitation wavelength (λ_{ex}) and λ_{em} of 492 and 513 nm, respectively. The proposed method of SIFA for amino acid and oligopeptide analysis has been shown to be a promising one in terms of sensitivity and selectivity with the detection limits of 0.64–12 fmol (the signal-to-noise ratio was 3).

Experimental

Apparatus and reagents

An LC-6A HPLC (Shimadzu, Kyoto, Japan) with an RF-530 fluorescent detector (Shimadzu) and a CR-3A integrator (Shimadzu) was used. Separations were performed on a C18 column (5 µm, 250- × 4.6-mm i.d., Alltech, Deerfield, IL). Fluorescence spectra were recorded on an RF-5000 spectrofluorometer (Shimadzu). The pH values were determined by a DF-801 accurate acidimeter (Zhongshan University, Guangzhou, China).

Unless otherwise specified, all reagents were of analytical-reagent grade and all solutions were prepared from double-distilled water.

A 5mM SIFA solution was prepared with dried acetonitrile. The standard solutions of Glu, Gly-Gly-Gly, Gly-Gly, Gly, $(Cys)_2$, and GSSG were prepared by dissolving each of them in water, respectively. The citric acid–Na_2HPO_4 buffer was obtained by mixing 0.2M citric acid solution and 0.2M Na_2HPO_4 solution to the required pH value. The H_3BO_3–$Na_2B_4O_7$ buffer was prepared by mixing 0.2M H_3BO_3 solution and 0.05M $Na_2B_4O_7$ solution to the required pH value.

The mobile phase consisted of a methanol–water solution (42:58, v/v) containing the 10mM citric acid–Na_2HPO_4 buffer (pH 5.0) and was purified with a Milli-Q filtration system (Millipore, Bedford, MA).

Preparation of the SIFA derivatives with Glu, Gly-Gly-Gly, Gly-Gly, Gly, $(Cys)_2$, and GSSG

The acetonitrile solution of 2.4 g SIFA (approximately 5 mmol) was added to the amino acid or peptide saturated solution (6 mmol). After the mixture had been allowed to stand for 3 h at room temperature, it was evaporated to dryness in vacuum. The residue was recrystallized in absolute ethanol and given SIFA–Glu, SIFA–Gly-Gly-Gly, SIFA–Gly-Gly, SIFA–Gly, SIFA–$(Cys)_2$, and SIFA–GSSG in 75, 79, 73, 70, 81, and 70% yields, respectively.

Fluorescence quantum yield

The fluorescence quantum yields of fluorescein and fluorescein-*O*-acetic acid (FOAA, a hydrolyzed product of SIFA) were measured using a quinine sulfate solution in 0.05M H_2SO_4 as a standard solution according to the method described in reference 18.

Derivatization procedure

The H_3BO_3–$Na_2B_4O_7$ buffer (2.0 mL, 0.2mM, pH 8.5) and an aqueous solution (6.25–1250 µL) containing 10µM of Glu, Gly-Gly-Gly, Gly-Gly, Gly, $(Cys)_2$, and GSSG, respectively, were transferred into a 25-mL volumetric flask. To this solution, 7 mL of methanol and 1.0 mL of 5mM SIFA solution were added. After the reaction was performed at 30°C for 30 min, the whole solution was diluted to 25 mL with methanol–water (7:3, v/v).

Chromatographic method

Before analysis, the C18 column was pre-equilibrated with the mobile phase for 30 min. A 20-µL aliquot of the prepared test solvent was injected into the column and the derivatives separated at a flow rate of 1.0 mL/min with fluorescence detection at λ_{ex} =

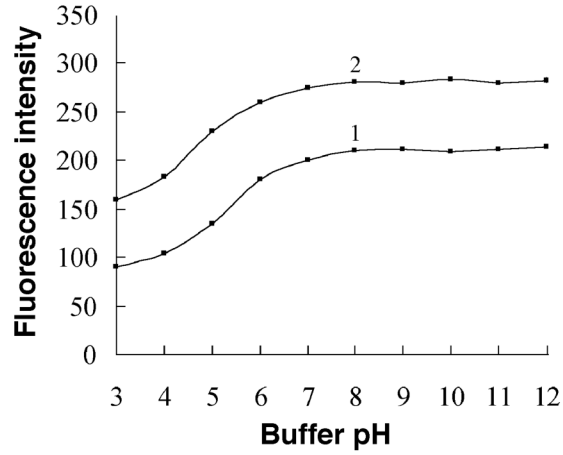

Figure 1. Effect of the buffer pH on the fluorescence intensity of FOAA, 1, and SIFA–Gly, 2.

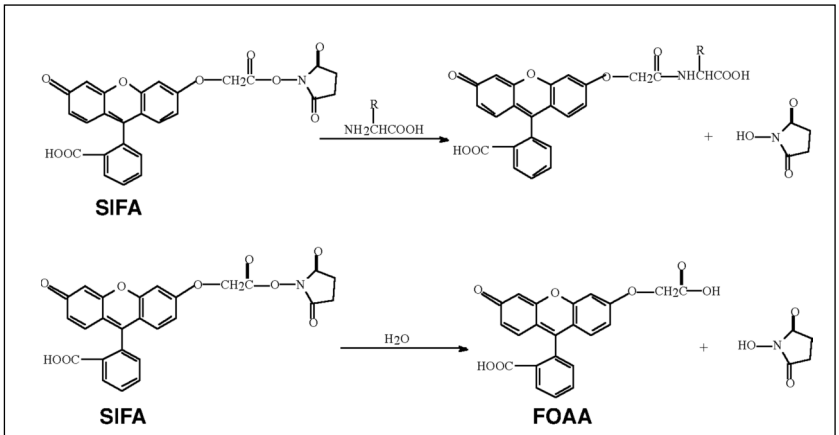

Figure 2. Scheme of the derivatization reaction and hydrolysis reaction of SIFA.

492 nm and λ_{em} = 513 nm. The peak areas were measured for quantitative calculations.

Results and Discussion

Fluorescence properties

The purpose of this study was to evaluate the feasibility of the new fluorescent derivatizing reagent SIFA in the determination of amino acids and peptides by HPLC. Glu, Gly-Gly-Gly, Gly-Gly, Gly, $(Cys)_2$, and GSSG (which can be conveniently obtained) have been used as the model analytes. Gly, diglycine, triglycine, and GSSG are very similar in their structures and chromatographic behaviors and belong to the oligopeptide group (except Gly). $(Cys)_2$ and Glu have also been chosen because of their comparability with GSSG. Furthermore, the chosen analytes have no native fluorescence, which is ideal for the fluorescence investigation. The reactivities and chromatographic behaviors of SIFA were well-expressed by using more than six amino acids and oligopeptides.

After being derivatized with SIFA, Glu, Gly-Gly-Gly, Gly-Gly, Gly, $(Cys)_2$, and GSSG exhibited similar fluorescence characteristics with that of SIFA, as indicated in reference 17. The analyte, SIFA, and FOAA had the highest fluorescence intensities (λ_{ex} = 492 nm and λ_{em} = 513 nm), which was confirmed by the maximum peak areas appearing in HPLC (also at λ_{ex} = 492 nm and λ_{em} = 513 nm).

Using an SIFA–Gly derivative and FOAA as the samples, the effect of the pH value of the 0.1M citric acid–Na_2HPO_4 buffer on the fluorescence was tested. Figure 1 demonstrates that the fluorescence intensities of the samples decreased with the decrease of the pH level as the pH decreased to levels below 8, which was in accordance with that of fluorescein. In the pH range of 8 to 12, the fluorescence had no obvious change. Moreover, the fluorescence quantum yields of FOAA and fluorescein were almost the same under identical conditions. In the 0.1M sodium hydroxide solution, they were all 0.92.

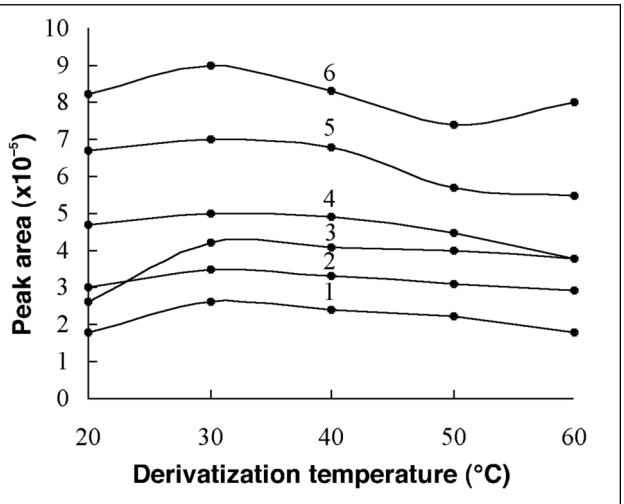

Figure 3. Effect of the derivatization temperature on the peak areas: SIFA–Gly, 1; SIFA–GSSG, 2; SIFA–Gly-Gly, 3; SIFA–Glu, 4; SIFA–Gly-Gly-Gly, 5; and SIFA–$(Cys)_2$, 6.

Separation conditions

Optimal conditions for the separation of FOAA and the SIFA derivatives were investigated. The effect of methanol content in the mobile phase on the capacity factors (k') of FOAA and the SIFA derivatives was investigated. When the methanol content was higher than 44%, the peaks of Gly-Gly-Gly, Gly-Gly, and Gly overlapped gradually, whereas when it was lower than 40%, the analysis time was longer and the peaks were broader.

The effect of the pH of the citric acid–Na_2HPO_4 buffer on the analyte retention was very complicated, and the pH range suitable for the separation was narrow because of the carboxyl groups on amino acids and peptides. In the pH range of 4.6 to 5.4, the retention times of Glu and FOAA decreased with the increase of pH and those of Gly-Gly-Gly, Gly-Gly, Gly, and $(Cys)_2$ were almost constant. The peak of FOAA overlapped with that of SIFA–$(Cys)_2$ at pH 5.2 and SIFA–GSSG at pH 4.7.

Experiments on the effect of the buffer concentration were also done. It was found that in the range of 5 to 40mM, the retention of each derivative increased slightly as the concentration increased.

According to the experiment results, a mobile phase of methanol–water (42:58, v/v) containing a 10mM citric acid–Na_2HPO_4 buffer (pH 5.0) was chosen as the optimal condition.

Derivatization conditions

There is a competition between the derivatization of SIFA with amines and the hydrolysis of SIFA itself. The reaction of SIFA with amino acids and peptides is presented in Figure 2. SIFA reacted with the analytes, and the excess was hydrolyzed to FOAA during the derivatization procedure.

The derivatization conditions were optimized based on the peak area of each derivative. The highest derivatization yield was thought to be reached when the maximum peak area appeared.

A study of the analytes' peak area value as a function of the SIFA volume (5mM) was conducted. The peak areas of the analytes reached maximum when the volume of SIFA solution was greater than 0.75 mL. The data were almost constant as the SIFA volume ranged from 0.75 to 1.25 mL.

The peak areas of SIFA derivatives increased with the increase of the pH value of the H_3BO_3–$Na_2B_4O_7$ buffer at levels less than 8.5. The largest peak areas were observed at pH 8.5.

The effect of the reaction temperature and time was investigated in the range of 20°C to 60°C. A higher temperature could accelerate the derivatization, but too high of a temperature would favor SIFA hydrolysis in the competition. From Figure 3, it could be found that the highest derivatization yields of SIFA with amino acids and peptides were obtained at 30°C. When the reaction time was longer than 25 min, the reaction proceeded completely.

Therefore, the derivatization reaction was carried out at 30°C for 30 min in a methanol–water (7:3, v/v) solution using 6.25–1250 µL sample solution containing 10µM of Glu, Gly-Gly-Gly, Gly-Gly, Gly, $(Cys)_2$, and GSSG, respectively; 1.0 mL of 5mM SIFA; and 2.0 mL of a 0.2M pH 8.5 H_3BO_3–$Na_2B_4O_7$ buffer. The total volume was 25 mL. The labeled amino acids and peptides were stable for at least 48 h.

The derivatization was performed in a solution with the volume being at least 10 mL, which was reported in the Derivatization procedure section. Under the optimized derivatization conditions the lowest derivatization concentration limits of Glu, Gly-Gly-Gly, Gly-Gly, Gly, $(Cys)_2$, and GSSG could reach 8.0, 1.8, 9.0, 15, 0.8, and 11 × 10^{-10}M, respectively, with a large excess of SIFA, which were comparable to those using CFSE as the derivatizing reagent with a CE–LIF detection system (16). The lowest derivatization concentration limits were calculated according to the detection limits obtained in this study.

Under these conditions, the excess reagent was hydrolyzed completely to FOAA because the SIFA peak in the chromatogram disappeared. The derivatization yields of the SIFA derivatives would become much larger than those obtained in the Experimental section. With an SIFA excess of at least 67 fold, the reaction was carried out in the solution that was analyzed directly after the derivatization without any loss occurring in the preparation of solid compounds. Thus, the derivatization yields given in the Experimental section could only be regarded as a reference.

Calibration ranges and detection limits

Under the optimized conditions for the separation and derivatization mentioned, a typical chromatogram was created (given in Figure 4). Glu, Gly-Gly-Gly, Gly-Gly, Gly, $(Cys)_2$, and GSSG were separated in 20 min at λ_{ex} = 492 nm and λ_{em} = 513 nm. The reproducibilities of the method were expressed as the relative standard deviation (RSD) of seven replicate results for retention times and peak areas. The RSDs of the retention times were 0.09%, 0.18%, 0.09%, 0.16%, 0.06%, and 0.06% for Glu, Gly-Gly-Gly, Gly-Gly, Gly, $(Cys)_2$, and GSSG, respectively. Those for the peak areas were 0.78%, 1.11%, 1.34%, 1.73%, 1.55%, and 1.41%, respectively. The excess reagent was hydrolyzed completely to FOAA and *N*-hydroxyl succinimide (no fluorescence) during the derivatization procedure, and the derivatization of SIFA with amino acids and oligopeptides generated few by-products. Therefore, the FOAA peak was the only one brought by the

Table I. Linear Calibration Ranges, Regression Equations, and Detection Limits of Derivatives

Derivative-SIFA	Calibration range (pmol)	Regression equation	γ	Detection limit* (fmol)
Glu	0.05–10	$y^{\dagger} = 1999 + 72437x^{\ddagger}$	0.9993	6.4
Gly-Gly-Gly	0.01–10	$y = 3735 + 86059x$	0.9998	1.42
Gly-Gly	0.07–10	$y = -690 + 55802x$	0.9993	7.2
Gly	0.2–10	$y = -4833 + 30561x$	0.9992	12
$(Cys)_2$	0.01–10	$y = -1871 + 118332x$	0.9996	0.64
GSSG	0.2–10	$y = 1425 + 43589x$	0.9988	8.0

* Signal-to-noise ratio = 3 (per 20 µL injection volume).
† *y*, arbitrary unit.
‡ *x*, pmol (per 20 µL injection volume).

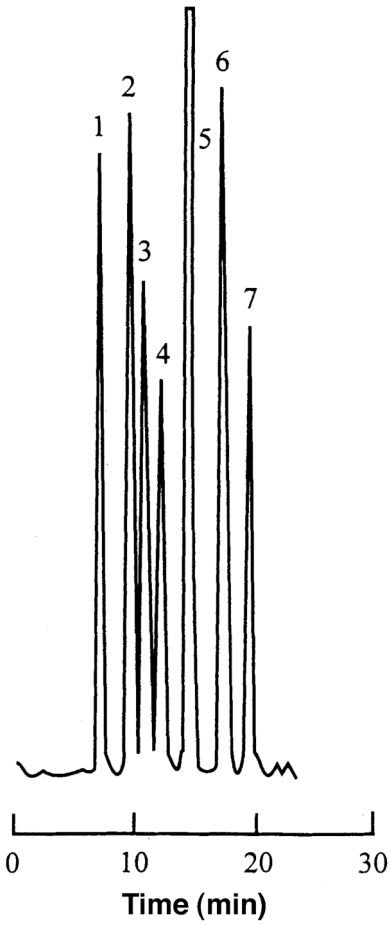

Figure 4. Typical chromatogram of FOAA and the SIFA derivatives with oligopeptides and amino acids (25°C temperature): SIFA–Glu, 1; SIFA–Gly-Gly-Gly, 2; SIFA–Gly-Gly, 3; SIFA–Gly, 4; FOAA (hydrolyzed product of SIFA), 5; SIFA–$(Cys)_2$, 6; and SIFA–GSSG, 7.

Table II. Comparison of *N*-Hydroxysuccinimidyl Esters with Different Fluorophores as Derivatizing Reagents for Amino Acids and Oligopeptides in HPLC

Reagent	λ_{ex} (nm)	λ_{em} (nm)	Mobile phase (v/v/v)	Detection limit* (fmol)	Reference
SIIA	278	355	methanol–water (20:80)	200–2500	9
HSMPA†	310	385	methanol–water–triethylamine (75:23:2)	15–68	11
FMOC-osu‡	270	385	methanol–water–triethylamine (75:23:2)	15–46	12
HSAA	385	435	methanol–water–triethylamine (38:60:2)	25–65	13
SIFA	492	513	methanol–water (42:58)	0.64–12	current study

* Signal-to-noise ratio = 3.
† HSMPA, *N*-hydroxysuccinimidyl-α-(9-phenanthrene)-acetate.
‡ FMOC-osu, 9-fluorenylmethoxy carbonyl succinimide.

SIFA labeling in the chromatogram.

The quantitative data of the derivatives of amino acids and peptides with SIFA are listed in Table I. Within-day and between-day precision was examined by using a mix-standard sample containing 0.1µM of Glu, Gly-Gly-Gly, Gly-Gly, Gly, $(Cys)_2$, and GSSG, respectively. The RSDs for within-day determination (n = 7) were 1.21%, 1.57%, 1.79%, 1.83%, 2.14%, and 1.95%, respectively. Those for between-day determination (n = 7) were 2.58%, 2.61%, 3.43%, 3.76%, 3.28%, and 4.01%, respectively. The detection limits were 0.64–12 fmol (signal-to-noise ratio of 3), which has been improved greatly in comparison with those using *N*-hydroxysuccinimidyl esters with other fluorophores as the derivatizing reagents in HPLC. The comparison of *N*-hydroxysuccinimidyl esters with different fluorophores as the derivatizing reagents for amino acids and oligopeptides in HPLC is shown in Table II.

Conclusion

A new fluorescent reagent SIFA has been synthesized for the HPLC analysis of amino acids and oligopeptides. This reagent has been proven to be a favorable precolumn derivatizing reagent with the advantages of high detection sensitivity in the visible region, specifically with amino groups, ease in synthesis, and handling and low cost of reagent. In comparison with FITC with the same fluorophore, the introduction of the reactive group of *N*-hydroxysuccinimidyl ester in SIFA leads to a considerably short derivatization time and relatively clean chromatograms. Furthermore, SIFA can derivatize nanomolar levels of amino acids, which is three orders of magnitude lower than that obtainable with FITC. Compared with AQC, the merits of SIFA include a new and simple synthesis method using cheap materials and a much higher fluorescence quantum yield. However, the peak of the hydrolysis product of SIFA is in the chromatogram, and the derivatization needs more time.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 29575206, Beijing), the Natural Science Foundation of Hubei Province (No. 99J061, Wuhan), and the Foundation of Wuhan University (Wuhan).

References

1. S.A. Cohen and D.P. Michaud. Synthesis of a fluorescent derivatizing reagent, 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate, and its application for the analysis of hydrolysate amino acids via high-performance liquid chromatography. *Anal. Biochem.* **211:** 279–87 (1993).
2. S.A. Cohen and K.M. De Antonis. Application of amino acid derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate-Analysis of feed grains, intravenous solutions and glycoproteins. *J. Chromatogr. A* **661:** 2534 (1994).
3. K.M. De Antonis, P.R. Brown, Y.-F. Cheng, and S.A. Cohen. Analysis of derivatized peptides by capillary electrophoresis. *J. Chromatogr. A* **661:** 279–85 (1994).
4. H.J. Liu. Determination of amino acids by precolumn derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate and high-performance liquid chromatography with ultraviolet detection. *J. Chromatogr. A* **670:** 59–66 (1994).
5. K.M. De Antonis, P.R. Brown, and S.A. Cohen. High-performance liquid chromatographic analysis of synthetic peptides using derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate. *Anal. Biochem.* **223:** 191–97 (1994).
6. J. Diaz, J.L. Lliberia, L. Comellas, and F. Broto-Puig. Amino acid and amino sugar determination by derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate followed by high-performance liquid chromatography and fluorescence detection. *J. Chromatogr. A* **719:** 171–79 (1996).
7. G.-D. Li, I.S. Krull, and S.A. Cohen. Electrochemical activity of 6-aminoquinolyl urea derizatives of aminl acids and peptides. Applicaton to high performance liquid chromatography with electrochemical detection. *J. Chromatogr. A* **724:** 147–57 (1996).
8. Y.-Y. Zhao, G.-L. Xu, H.-S. Zhang, and J. Li. *N*-Hydroxysuccinimidyl-α-naphthylacetate as pre-column derivatizing reagent to separate and determine peptides and their hydrolysates by reversed phase HPLC. *Chem. J. Chin. Univ.* **17:** 1044–47 (1996).
9. X. Liu, G.-L. Xu, H.-S. Zhang, and J.-K. Cheng. Analysis of peptides and amino acids by pre-column derivatization high performance liquid chromatography with fluorescence detection. *Chem. J. Chin. Univ.* **18:** 873–76 (1997).
10. Y.-Y. Zhao, H. Wang, H.-S. Zhang, G.-L. Xu, and J. Li. *N*-hydroxysuccinimidyl-3-indolylacetate as pre-column derivatizing reagent to separate and determine peptides and their hydrolysates by reversed-phase high performance liquid chromatography. *J. Liq. Chromatogr. Relat. Technol.* **21:** 1807–16 (1998).
11. J.M. You, G.H. Xie, H.E. Wang, J.X. Su, and C.L. Zhou. HPLC of amino acids and oligopeptides by pre-column fluorescence derivatization with *N*-hydroxysuccinimicyl-α-(9-phenanthrene)-acetate. *Chromatographia* **46:** 245–50 (1997).
12. J. You, X. Fan, H.E. Wang, G. Wang, J.X. Su, and C.L. Zhou. High-performance liquid chromatographic determination of amino acids and oligopeptides by pre-column fluorescence derivatization with 9-fluorenylmethoxy carbonyl succinimide. *J. Liq. Chromatogr. Relat. Technol.* **21:** 2103–15 (1998).
13. J. You, X.J. Fan, Q. Zhu, and Y. Su. Liquid chromatographic deterjination of amino acids and peptids by pre-column fluorescence dericatization with *N*-hydroxysuccinomodyl-α-(9-acridine)-acetate. *Anal. Chim. Acta* **367:** 69–79 (1998).
14. M. Koller and H. Eckert. Derivatization of peptides for their determination by chromatographic methods. *Anal. Chim. Acta* **352:** 31–59 (1997).
15. F.Q. Dang and Y. Chen. Capillary electrophoresis of fluorescein isothiocyanate label amino acids with laser induced fluorescence detection. *Sci. China Series B* **42:** 663–669 (1999).
16. S.K. Lau, F. Zaccardo, M. Little, and P. Banks. Nanomolar derivatizations with 5-carboxyfluorescein succinimicyl ester for fluorescence detection in capillary electrophoresis. *J. Chromatogr. A* **809:** 203–10 (1998).
17. H. Wang, J. Li, X. Liu, T.-X. Yang, and H.-S. Zhang. *N*-hydroxysuccinimidyl-fluorescein-*O*-acetate as a fluorescent derivatizing reagent for catecholamines in liquid chromatography. *Anal. Biochem.* **281:** 15–20 (2000).
18. T. Yoshida, Y. Moriyama, K. Nakamura, and H. Taniguchi. 6-Methoxy-2-methylsulfonylquinoline-4-carbonyl chloride as a fluorescence derivatization reagent for amines in liquid chromatography. *Analyst* **118:** 29–33 (1993).

Manuscript accepted May 14, 2001.