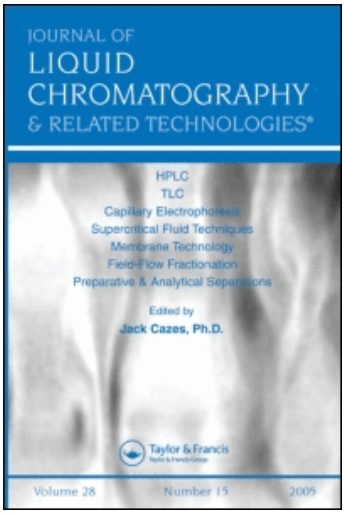

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ABSTRACT

A new derivatizing reagent, *N*-hydroxysuccinimidyl-α-naphthylacetate (SINA), has been used as a precolumn derivatizing reagent for the separation and determination of amino acids (AAs) and oligopeptides with HPLC. The spectral properties of SINA derivatives with AAs and oligopeptides have been studied. The derivatization and separation conditions of SINA derivatives with most amino acids and some oligopeptides have been investigated. With spectrophotometric detection at 280 nm, the detection limits were in the range of 0.49-2.76 pmol; with fluorescence detection at $\lambda_{ex}/\lambda_{em}$=299/338 nm, the detection limits were in the range of 19-376 fmol when the ratio of signal to noise (S/N=3) was 3.

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INTRODUCTION

The analysis of amino acids and peptides with high performance liquid chromatography has developed rapidly in the last 20 years. Because of the great polarity and the lack of intrinsically sensitive chromophores and fluorophores, a derivatization with appropriate reagents prior to analysis is necessary. Therefore, the research on suitable derivatizing reagents is always a subject of great interest in the separation and determination of amino acids and peptides.

The well-known derivatizing reagents for amino acids and peptides are *o*-phthaldialdehyde (OPA) together with various thiols,(1,2) phenyl isothiocyanate (PITC),(3,4) 5-dimethylaminonaphthalene-1-sulfonyl chloride (dansyl-Cl),(5,6) 9-fluorenylmethyl chloroformate (FMOC),(7-9) and 7-fluoro (chloro)-4-nitrobenzo-2-oxa-1, 3-diazole (NBD),(10,11) etc. Although, precolumn derivatization of amino acids with these reagents, not only enhances the detection sensitivity, but also improves the retention behavior of AAs, there are some disadvantages using these reagents. PITC derivatization is long and involves several stages of drying under vacuum.

The derivatization with FMOC is also troublesome. The excess of FMOC has to be removed by extraction with pentane or derivatized with an amine, using it as the scavenger whose derivative does not interfere. OPA does not derivatize secondary amino acids, such as Pro or Hpr. Moreover, the derivatives exhibit low stability, i.e., a few minutes. Dansyl-Cl reacts with Lys, His, Tys, and $(Cys)_2$ to form multi-derivatives, which affects the quantitative analysis of these AAs. On the other hand, its derivatives are sensitive to UV rays. NBD and its derivatives are unstable when exposed to daylight.

More recently, a derivatizing reagent, 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) has become popular in the analysis of AAs and peptides.(12-16) The advantages of this reagent are the stability of its derivatives, excellent derivative yields, and no interference from the only major by-product, 6-aminoquinoline. On account of these virtues, it is of great importance to exploit new derivatizing reagents in analogy with AQC in the derivatization of AAs and peptides.

The naphthyl group is a common fluorophore used in fluorescence detection. For example, 2-naphthoxycarbonyl chloride (NOC-Cl) and 2-(naphthylmethyl)oxycarbonyl chloride (NMOC-Cl) have been employed as analytical reagents for the precolumn derivatization of AAs, followed by the liquid chromatographic separation of the resultant derivatives.(17) In our previous studies, a new and simple route with cheap materials to synthesize *N*-hydroxysuccinimidyl-α-naphthylacetate (SINA),(18) analogous to AQC, has been proposed. In this paper, the spectral properties of SINA and its derivatives with amino acids and peptides have been studied in detail.

The derivatizing conditions and their chromatographic behaviors has also been investigated. The separation of AAs was achieved in the mobile phase of

methanol-ethyl acetate-water system with the detection limits of 0.49-2.76 pmol (S/N=3) at λ=280 nm. Using oxidized glutathione (GSSG), glycyl glycyl glycine (Gly-Gly-Gly), glycyl glycine (Gly-Gly), glycine (Gly), glutamic acid (Glu), and cystine ($(Cys)_2$) for the fluorescence determination, the detection limits were 19-376 fmol (S/N=3) at $\lambda_{ex}/\lambda_{em}$=299/338 nm.

EXPERIMENTAL

Apparatus and Reagents

An LC-6A HPLC equipment (Shimadzu) with a C_{18} column (5 μm, 250×4.6 mm i.d., Alltech, USA) followed by a RF-530 fluorescence HPLC monitor (Shimadzu) and a C-R3A chromatopac integrator (Shimadzu) was used. An LC-10 HPLC apparatus (Japan Analytical Ind. Co.) with an C_{18} column (5 μm, 150×4.6 mm i.d., Dalian Institute of Chemical physics, Chinese Academy of Science) followed by a S-3702 UV-VIS detector (AIC, Japan) and SIC chromatocorder 11 integrator (System Instruments Co., Ltd., Japan) was used. Fluorescence spectra were recorded on an RF-5000 spectrofluorometer (Shimadzu). Absorption spectra were recorded on a UV-3000 spectrophotometer (Shimadzu). pH values were determined by a DF-801 accurate acidimeter (Zhongshan University, China).

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

SINA was synthesized in our lab and its 10 mmol/L solution was prepared with dehydrated acetonitrile. The 1 mmol/L standard solutions of amino acids and oligopeptides were prepared by dissolving each of them in water, respectively. 1 mol/L acetate buffer solution was prepared by adjusting the pH value with 1 mol/L acetic acid and 1 mol/L sodium acetate solution. 0.2 mol/L borate buffer was prepared from 0.1 mol boric acid in 400 mL water, adjusted to the required pH with 5 mol/L NaOH solution and diluted to 500 mL.

The mobile phases consisted of methanol-water or methanol-ethyl acetate-water solution containing 10 mmol/L H_3cit-Na_2HPO_4 buffer or acetate buffer (pH=5.00), and were purified with a Milli-Q filtration system (Millipore, Bedford, MA).

Synthesis of *N*-Hydroxysuccinimidyl-α-naphthylacetate (SINA)

The synthesis of SINA was according to Ref. 18, which was described as follows: α-Naphthylacetic acid (NA, 5.60 g) and *N*-hydroxy succinimide (3.45 g) were dissolved in a 250 mL flask containing 150 mL of tetrahydrofuran

(THF), and a 20 mL THF solution of dicyclohexylcarbodimide (DCC, 6.18 g) was dropped slowly into it. After being stirred for 12 hr at 0°C, the mixture was filtered. SINA residue was recovered from the filtrate by the reduced-pressure distillation and recrystallized from absolute ethanol. A white crystal was obtained with a m.p. of 97-99°C. The results of elemental analysis of SINA were as follows: Calc.: C, 67.82; H, 4.28; N, 4.95%; Found: C, 67.48; H, 4.54; N, 4.59%.

Derivatization Procedure

A 2.0 mL of 0.2 mol/L pH8.00 borate buffer and 1.0 mL of 10 mmol/L SINA solution were mixed with 0.2 mL of the standard solution containing 50 μmol/L amino acids and oligopeptides, respectively. The solution was heated in a water bath at 60°C for 45 min, and diluted to 10.00 mL with de-ionized water after being cooled to room temperature.

Analysis Procedure

Before the analysis, the C_{18} column was pre-equilibrated with the mobile phase for 30 min. A 20 μL aliquot of the prepared test solution was injected into the C_{18} column and the derivatives were eluted at a flow rate of 1.0 mL/min and detected with fluorescence at $\lambda_{ex}/\lambda_{em}$=299/338 nm, or with absorbance at 280 nm. The peak areas were measured for quantitative calculations.

RESULTS AND DISCUSSION

Spectral Properties

In alkaline medium, SINA reacts with AAs and oligopeptides to give the corresponding stable amides. The maximum absorbances of these amides fall in the range of 275-282 nm. For example, the maximum absorption wavelengths of SINA-Gly, -Gly-Gly, -Gly-Gly-Gly, -GSSG, -$(Cys)_2$, -Ser, -Glu, -Tyr, -His, -Lys, -Phe, and –Trp are 280, 280, 280, 282, 278, 279, 279, 275, 282, 282, 280, and 279 nm, respectively.

Most amino acids and peptides have no or low fluorescence. After being derivatized with SINA, they exhibit similar fluorescence because of the same fluorophore, naphthyl. Figure 1 is the representative spectra of SINA in acetonitrile, SINA in water (hydrolyzed to NA), and amino acid or peptide deriva-

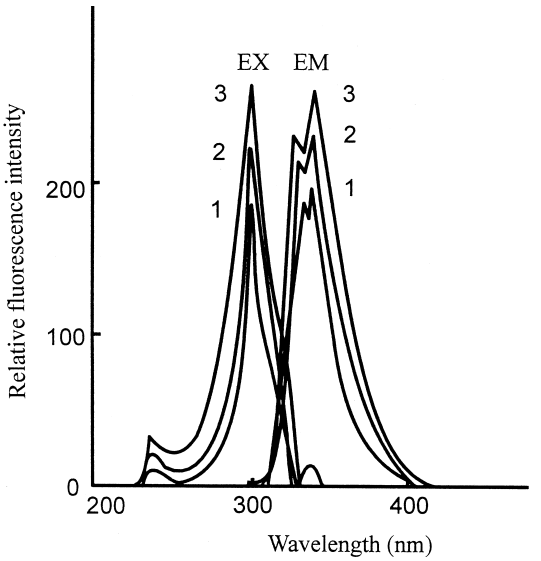

Figure 1. Fluorescence spectra of SINA and its derivatives. 1. SINA in acetonitrile (1×10^{-5} mol/L). 2. SINA-amino acid or peptide derivative (1×10^{-6} mol/L). 3. NA (1×10^{-6} mol/L); Slit width: 5 nm.

tives with SINA. In the derivative preparation, an excess analyte was used to react with SINA to ensure that no SINA was left, which was confirmed by HPLC.

In aqueous solution, the fluorescence of samples is often affected by many factors. It was found that λ_{ex} and λ_{em} of NA and SINA derivatives had no obvious change with the change of the buffer pH. The fluorescence intensity was almost constant, except for the slight decrease in strong acidic solutions. Although, the methanol content had no effect on λ_{ex} and λ_{em}, it would influence the intensity.

Using the hydrolysis product of SINA, 2-(1-napthyl)acetic acid (NA) and SINA-Gly as representatives, the effect of methanol content on the fluorescence intensities of NA and SINA derivatives was tested. Figure 2 indicated that methanol content had the similar effect on the fluorescence intensities. The variation of intensity was small with the methanol content lower than 60%. Otherwise, it dropped down sharply.

The fluorescence intensities of SINA, NA, and SINA derivatives were strongest at $\lambda_{ex}/\lambda_{em}$=299/338 nm, and the largest peak areas were obtained at the detection wavelength of $\lambda_{ex}/\lambda_{em}$=299/338 nm on HPLC.

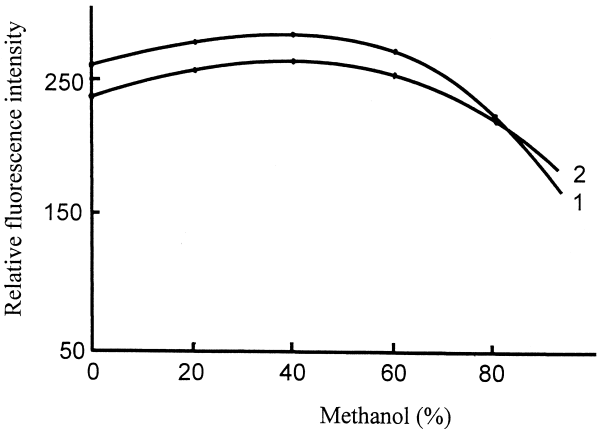

Figure 2. Effect of methanol content on the relative fluorescence intensity of NA and SINA-Gly. 1. NA 2. SINA-Gly.

Separation of SINA-AA Derivatives

The chromatographic behaviors of a mixture of amino acid derivatives were studied by isocratic elution using a C_{18} column. It was found, that in the presence of ethyl acetate, peak spreading was reduced and column efficiency improved. But, even under the optimum elution conditions, which was methanol-ethyl acetate-water (10:2:88, v/v/v) containing 10 mmol/L pH5.00 acetate buffer, only seven amino acids could be separated in one chromatographic run (Figure 3a). Just changing the contents of methanol and water in the mobile phases to mimic the gradient elution condition, the separation and determination of a large range of amino acids were performed. The chromatograms of SINA-AA derivatives, using isocratic elution with mobile phases differing in the compositions of methanol and water, are shown in Figure 3a-c, respectively.

With spectrophotometric detection at 280 nm, good resolution of SINA-AA derivatives was obtained in the mobile phase of methanol-ethyl acetate-water modified with pH 5.00 acetate buffer. Under the conditions presented in Figure 3, the linear ranges and the detection limits of these SINA-AA derivatives are given in Table 1, respectively. These experiments revealed that using SINA as the labeling reagent, the separation of all amino acids should be achieved with gradient elution. More intensive studies have not been carried out due to the lack of gradient elution device.

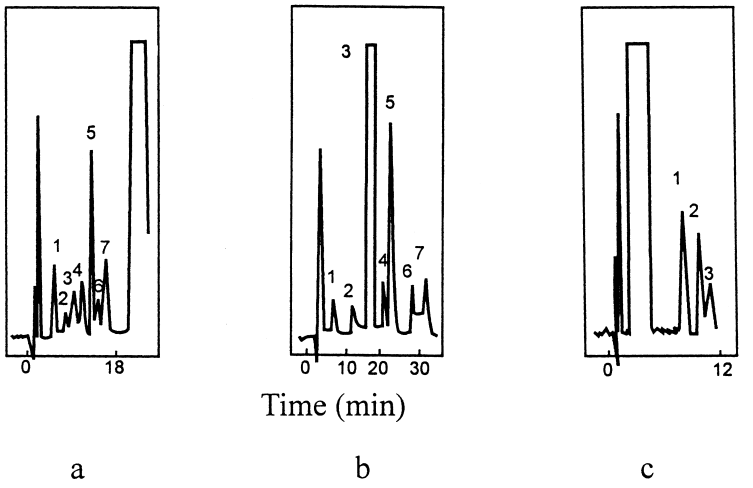

Figure 3. Chromatograms of SINA-AA derivatives using isocratic elution. Mobile phase: 10 mmol/L pH5.00 acetate buffer in variable proportion methanol-ethyl acetate-water solution; Column: C_{18} (5 μm, 150×4.6 mm i.d.); Flow rate: 1.0 mL/min; Temperature: 20°C; Detection wavelength: 280 nm. a) methanol-ethyl acetate-water: 10:2:88 (v/v/v). 1. SINA-Trp, 2. SINA-Asp, 3. SINA-Glu, 4. SINA-Ser, 5. SINA-Gly, 6. SINA-Thr, 7. SINA-Ala, 8. NA; b) methanol-ethyl acetate-water: 26:2:72 (v/v/v). 1. SINA-Cys, 2. SINA-His, 3. NA, 4. SINA-Tyr, 5. Impurity peak, 6. SINA-Met, 7. SINA-Val; c) methanol-ethyl acetate-water: 45:2:53 (v/v/v). 1. NA, 2. SINA-Ile, 3. SINA-Phe, 4. SINA-Lys.

Validation of SINA in AAs and Oligopeptides Analysis

In order to evaluate the feasibility of the new derivatizing reagent, SINA, in the determination of AAs and peptides by HPLC, Glu, Gly-Gly-Gly, Gly-Gly, Gly, $(Cys)_2$ and GSSG have been used as the model analytes. Gly, diglycine, triglycine, and GSSG are very similar in their structures and chromatographic behaviors, and belong to oligopeptides; Gly. $(Cys)_2$and Glu have also been chosen because of their comparability with GSSG. Furthermore, the chosen analytes have no native fluorescence, which are ideal for the fluorescence investigation.

SINA is an activated ester, which is readily attacked by nucleophiles. SINA will react with amino groups prior to water molecules, as the nucleophilic ability of the former is higher than the latter. Some SINA molecules will be hydrolyzed in the reaction medium of water, which affected the derivatization of amino compounds. The reaction process was shown in Figure 4.

Table 1. Linear Calibration Ranges and Detection Limits of SINA Derivatives with Amino Acids with UV-Vis Detection at 280 nm

SINA-AA	Linear Range (μmol/L)	Regression Equation (μmol/L)	γ	Detection Limit* (pmol)
Trp	5.867–19.58	y=3644+11490x	0.9981	1.17
Asp	10.91–36.36	y=−482+6741x	0.9950	2.76
Glu	8.645–28.82	y=−681+10560x	0.9981	1.96
Ser	11.42–38.06	y=1996+13870x	0.9954	1.79
Gly	13.71–45.70	y=7163+81000x	0.9971	0.49
Thr	10.07–33.58	y=375+11900x	0.9988	2.18
Ala	13.47–44.90	y=3045+21950x	0.9995	1.19
Cys	9.904–33.01	y=−53+7750x	0.9999	1.61
His	7.734–25.78	y=899+82290x	1.0000	1.66
Tyr	7.616–25.39	y=37+7289x	0.9985	2.25
Met	12.47–41.55	y=−928+6906x	0.9995	2.11
Val	13.32–44.39	y=−2802+29060x	0.9948	0.92
Ile	14.17–47.24	y=3771+24370x	0.9993	0.92
Phe	9.744–32.48	y=3025+21590x	0.9999	0.82
Lys	8.208–27.36	y=−214+6332x	0.9960	2.18

*S/N=3, per 20 μL injection volume.

The influence of variables, such as the amount of SINA, pH, and the concentration of buffer, temperature, and time of reaction on the derivatization reaction was studied.

An experiment of peak area value, as a function of SINA concentration, has been done when the concentrations of the tested amino acids and peptides were 5 μmol/L, respectively. In Figure 5, representative plots of peak value vs. derivatizing reagent concentration were shown. Maximum and constant peak areas of the derivatives were observed when SINA concentration in the reaction medium was

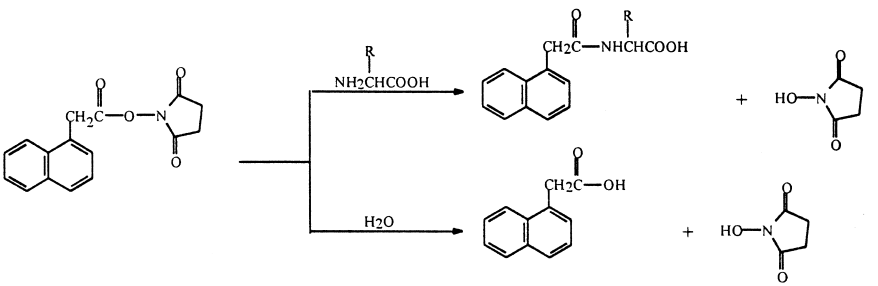

Figure 4. Scheme of the derivatization reaction and hydrolysis reaction of SINA.

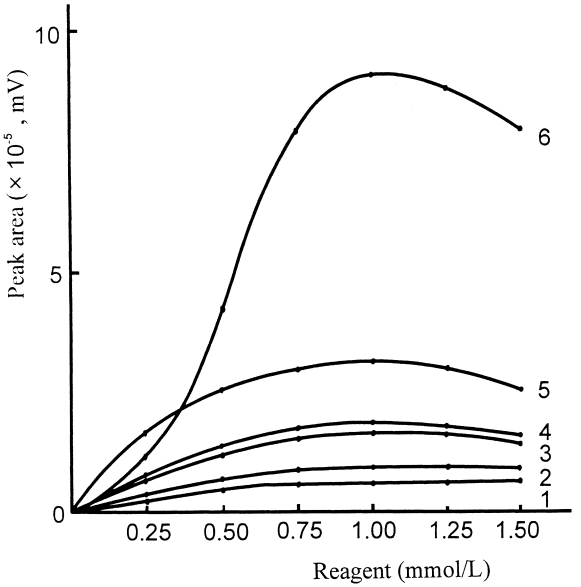

Figure 5. Effect of SINA concentration on the peak area of the derivatives. Mobile phase: methanol-water (36/64, v/v) solution containing 10 mmol/L pH5.00 H_3cit-Na_2HPO_4 buffer; H_3BO_4-$Na_2B_4O_7$ buffer: pH8.00, 0.04 mol/L; Column: C_{18} , 5 μm, 250×4.6 mm i.d.; Flow rate: 1.0 mL/min; Derivatization temperature: 60°C; Derivatization time: 45 min. 1. SINA-$(Cys)_2$ 2. SINA-Gly-Gly-Gly, 3. SINA-Gly-Gly 4. SINA-Glu 5. SINA-Gly 6. SINA-GSSG.

greater than 0.75 mmol/L. Therefore, 1.00 mmol/L was chosen as the optimal concentration of SINA with the molar excess at least 33 fold.

Borate buffer solution was used to facilitate the derivatization of GSSG, Gly-Gly-Gly, Gly-Gly, Gly, Glu, and $(Cys)_2$ with SINA. From Figure 6, it can be seen that high yields of the SINA derivatives were achieved, as the pH value was 8.00, which was used as the optimum in the reaction. The effect of the buffer concentration on the derivatization has also been investigated, and the results were shown in Figure 7. The derivatization was carried out in 0.04 mol/L borate buffer.

Effects of the heating temperature and time on the derivatization reaction, under the conditions employed, were also studied in this paper (Figure 8 and Figure 9). Maximum and constant peak areas of the corresponding peptides and amino acids were obtained at 60°C for 45 min.

The effect of methanol content on the derivative retention was studied. When the methanol content was higher than 38%, the peaks of Gly-Gly-Gly, Gly-

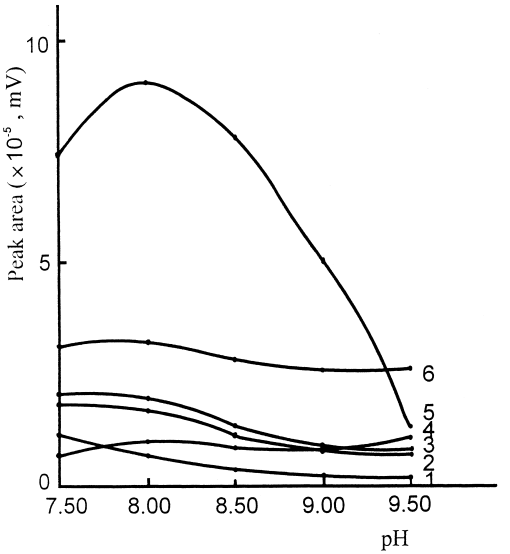

Figure 6. Effect of H_3BO_4-$Na_2B_4O_7$ buffer pH on the peak area of the derivatives. H_3BO_4-$Na_2B_4O_7$ buffer: 0.04 mol/L; SINA: 1.00 mmol/L; the other conditions are the same as those in Figure 5. 1. SINA-$(Cys)_2$ 2. SINA-Gly-Gly-Gly 3. SINA-Gly-Gly 4. SINA-Glu 5. SINA-GSSG 6. SINA-Gly.

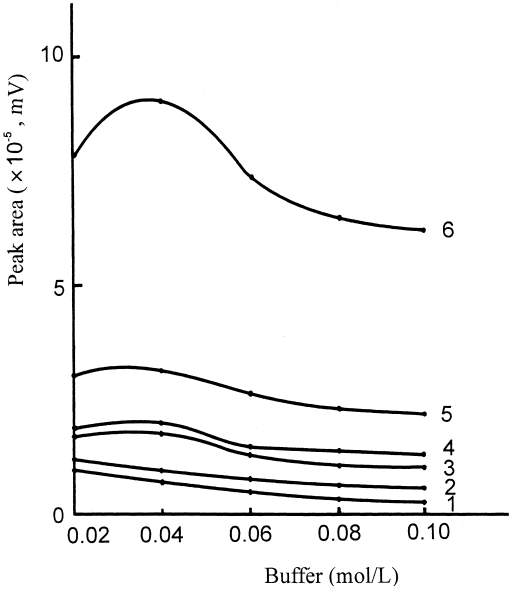

Figure 7. Effect of H_3BO_4-$Na_2B_4O_7$ buffer concentration on the peak area of the derivatives. H_3BO_4-$Na_2B_4O_7$ buffer: pH8.00; the other conditions are the same as those in Figure 6. 1. SINA-$(Cys)_2$ 2. SINA-Glu 3. SINA-Gly-Gly-Gly 4. SINA-Gly-Gly 5. SINA-Gly 6. SINA-GSSG.

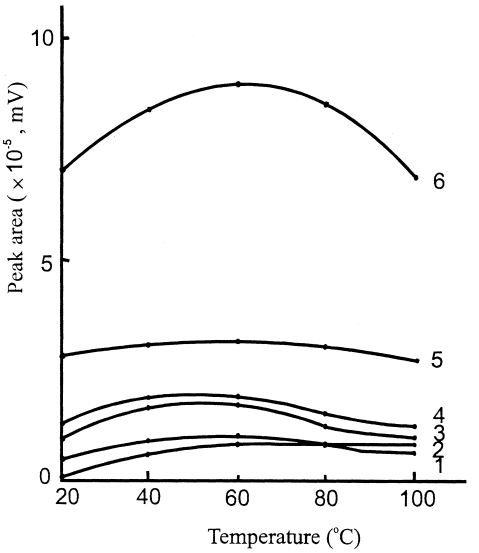

Figure 8. Effect of derivatization temperature on the peak area of the derivatives. H_3BO_4-$Na_2B_4O_7$ buffer: pH8.00, 0.04 mol/L; the other conditions are the same as those in Figure 6. 1. SINA-Glu 2. SINA-$(Cys)_2$ 3. SINA-Gly-Gly-Gly 4. SINA-Gly-Gly 5. SINA-Gly 6. SINA-GSSG.

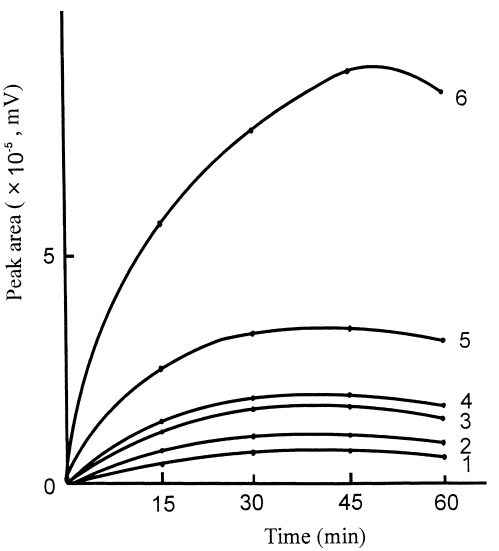

Figure 9. Effect of derivatization time on peak area of the derivatives. Derivatization temperature: 60°C; the other conditions are the same as those in Figure 6. 1. SINA-$(Cys)_2$ 2. SINA-Glu 3. SINA-Gly-Gly-Gly 4. SINA-Gly-Gly 5. SINA-Gly 6. SINA-GSSG.

Gly, and Gly overlapped gradually. While it was lower than 34%, the peak shapes were poor. Therefore, 34% aqueous methanol was regarded as the optimum in the further studies.

The effect of various buffers in the mobile phase on the derivative retention was investigated. Among them, H_3cit-Na_2HPO_4 buffer was most suitable. It is interesting to note. that the k' values of SINA-Gly-Gly-Gly, Gly-Gly, and Gly remained constant in the tested pH range (pH4.60-5.40), while that of GSSG presented a relatively complicated change. In this paper, pH5.00 buffer was used. The retention time of each derivative, prolonged slightly with the increase of the buffer concentration in the mobile phase and 10 mmol/L of the buffer, was available.

Under the optimized conditions of the derivatization and separation, a typical chromatogram of Glu, Gly-Gly-Gly, Gly-Gly, Gly, $(Cys)_2$ and GSSG derivatives with SINA was given in Figure 10. The excess SINA has been hydrolyzed to NA completely, and only one peak induced by SINA can be found in the chromatographic run within 20 min. Although, Gly-Gly and Gly-Gly-Gly all consist of Gly residues, the peak areas of their derivatives are much smaller than that of Gly-derivative. This is probably due to the different reactivities of Gly, Gly-Gly, and Gly-Gly-Gly with SINA. The derivatization yield of Gly with SINA is the highest. Meanwhile, the quantitative data of these SINA derivatives were listed in Table 2.

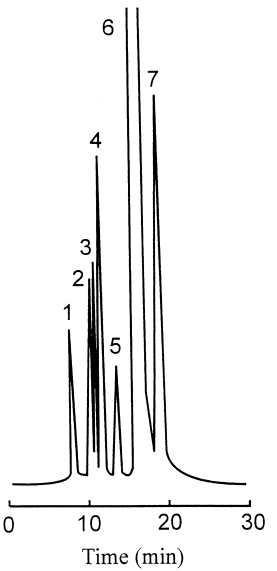

Figure 10. Chromatogram of SINA derivatives with oligopeptides and amino acids. Mobile phase: methanol-water (36/64, v/v) solution containing 10 mmol/L pH5.00 H_3cit-Na_2HPO_4 buffer. 1. SINA-Glu 2. SINA-Gly-Gly-Gly 3. SINA-Gly-Gly 4. SINA-Gly 5. SINA -$(Cys)_2$ 6. NA 7. SINA-GSSG.

Table 2. Linear Calibration Ranges, Regression Equations, and Detection Limits of SINA Derivatives with Fluorescence Detection at $\lambda_{ex}/\lambda_{em}$ = 299/338 nm

SINA-AA or Oligopeptide	Calibration Range* (pmol)	Regression Equation* (pmol)	γ	Detection Limit* (fmol)
Glu	1.250–100	y=1263+30024x	0.9999	96
Gly-Gly-Gly	0.625–100	y=438+43958x	0.9995	62
Gly-Gly	0.625–100	y=852+44981x	0.9998	59
Gly	0.625–100	y=−1079+66876x	0.9999	44
$(Cys)_2$	3.750–100	y=1234+81318x	0.9993	376
GSSG	0.312–100	y=1163+13269x	0.9994	19

*S/N=3, per 20 μL injection volume.

The within-day precision was established by repeated determinations (n=7) using a standard mixture of Glu, Gly-Gly-Gly, Gly-Gly, Gly, $(Cys)_2$ and GSSG (1 μmol/L each). The relative standard deviations did not exceed 2.83%. The between-day precision was obtained by performing the analyses (n=5) for 5 days using the same standard mixture stored in a refrigerator. The relative standard deviations did not exceed 5.41%. The recoveries, tested 5 times in synthetic samples spiked with 1 μmol/L of Glu, Gly-Gly-Gly, Gly-Gly, Gly, $(Cys)_2$ and GSSG, respectively, were 95.7-104.5%.

CONCLUSIONS

A new derivatizing reagent, *N*-hydroxysuccinimidyl-α-naphthylacetate (SINA), has been developed for the HPLC analysis of amino acids and peptides. This reagent can react with amino functions, selectively, in aqueous solution under mild conditions, and the excess reagent can be hydrolyzed promptly, which does not interfere with the determination. Compared to the reagent with a similar reactive group, 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC),(12) the advantage lies in a new and simple method to synthesize SINA using cheap materials. However, the disadvantages are the peak of the hydrolysis product of SINA in chromatogram, low fluorescence of the naphthyl group, and use of an off-line heating device. The introduction of the reactive group of N-hydroxysuccinimidyl ester leads to relatively clean chromatograms in comparison to naphthyloxycarbonyl chlorides, which carry the same fluorophor.(17)

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