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Journal of Chromatography A, 1063 (2005) 143–151
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6-Oxy-(*N*-succinimidyl acetate)-9-(2′-methoxycarbonyl)fluorescein as a new fluorescent labeling reagent for aliphatic amines in environmental and food samples using high-performance liquid chromatography

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Received 14 July 2004; received in revised form 22 November 2004; accepted 23 November 2004

Abstract

6-Oxy-(*N*-succinimidyl acetate)-9-(2′-methoxycarbonyl)fluorescein (SAMF), a new fluorescein-based amine-reactive fluorescent probe was well designed, synthesized and used as a pre-column derivatizing reagent for the determination of aliphatic amines in HPLC. It exhibited relatively pH-independent fluorescence (pH 4–9) and excellent photostability. The derivatization was performed at room temperature in 6 min. On a C_{18} column, the derivatives of SAMF with eight aliphatic amines were baseline separated in 28 min with a mobile phase of methanol–water (57:43, v/v) containing 10 mmol l^{-1} pH 5.0, H_3Cit_3–NaOH buffer. With fluorescent detection at $\lambda_{ex}/\lambda_{em} = 484/516$ nm, the detection limit could reach 2–320 fmol (signal-to-noise = 3), which was equivalent to or better than the detection limits obtained from other analytical methods of aliphatic amines. The proposed method has been applied to the determination of the aliphatic amines in environmental and food samples such as lake water, red wine, white wine, and cheese with satisfying recoveries varying from 95 to 106%.
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Keywords: 6-Oxy-(*N*-succinimidyl acetate)-9-(2′-methoxycarbonyl)fluorescein (SAMF); Aliphatic amines; Derivatization

1. Introduction

Short-chain aliphatic amines are widely used as starting materials in the manufacturing industry. Thereby, they are regular pollutants in the environment. On the other hand, as they are degradation products of organic materials such as amino acids and proteins, they also exist in biological systems and different foods (e.g. wines, cheese), which also might be contaminated by environmental aliphatic amines [1–3]. As we all know, these amines are not only irritants to human bodies, but also important middle products of nitro-amines, which are potential mutagenic and carcinogenic compounds [4]. Certain amount of aliphatic amines in the environment and food are considered as a serious risk. Thus, they are target analytes of US Environmental Protection Agency (EPA) Method 8260 and the EPA maintains an increasing interest in analytical methods for amines [5].

Analytical methods developed to determine aliphatic amines include gas chromatography (GC) [6,7], thin-layer chromatography (TLC) [8,9], spectrofluorimetry [10], high-performance liquid chromatography (HPLC) [11–14] and capillary electrophoresis (CE) [15,16]. HPLC is a rapid, reliable, sensitive and widely used method, which can be applied to the determination of aliphatic amines in environmental samples and foods such as wines, beverages, and meat.

As most of aliphatic amines have no UV absorption or fluorescence, derivatizing or labeling becomes a necessary procedure to determine them. Most of HPLC separation is carried out using UV absorption detection due to its easiness to use, however, because of its poor detection limits this method is not suitable for the detection of environmental and food samples which usually contain trace amines. Thus HPLC combined with fluorescence detection is generally

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doi:10.1016/j.chroma.2004.11.093

preferable due to its high sensitivity. Various fluorescent reagents were used for aliphatic amines labeling including 5-dimethylaminonaphthalene-l-sulfonylchloride (Dns-Cl) [17], 4-fluoro-7-nitrobenzo-2-oxa-l, 3-diazole (NBD-F) [18], *o*-phthalaldehyde (OPA) [19,20], 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) [21] and so on. These reagents have drawbacks such as short detection wavelengths, serious interference in the determination of biological samples, or bad stability of derivatives determined, which brings difficulties to the quantitative analysis.

Fluorescein is an important fluorophore for sensitive determination and other useful application such as confocal laser-scanning microscopy and flow cytometry. In spite of its pH-dependence and bad photostability, the amine-reactive fluorescein derivatives have been the most common fluorescent derivatizing reagents. In addition to its relatively high absorptivity and excellent fluorescence quantum yield, fluorescein has an excitation maximum (488 nm) that most closely matches the 488 nm spectral line of the argon-ion laser. Up till now, there are few amine-reactive fluorescein derivatives, except for fluorescein isothiocyanate (FITC) [22,23], 5 (6) carboxyfluorescein (CFSE) [24], *N*-hydroxysuccinimidyl fluorescein-*O*-acetate (SIFA) [25]. As it is well known, FITC needs high effective derivatizing concentration (1×10^{-4} mol l^{-1}), tedious reaction time [24]. Besides, a clean chromatogram can hardly be obtained because of the strong peaks of excessive FITC, fluorescein amines, and other by-products. Although using CFSE, a *N*-hydroxysuccinimidyl fluorescein active ester as a labeling reagent can overcome these drawbacks, it yields a mixture of isomers at the 5- and 6-positions of fluorescein's phenyl ring, which results in interference in separation, and single isomers are much more expensive [26]. What is more, the fluorescence of all these fluorescein-based reagents is pH-dependent which greatly limits the application. Therefore, our interest is focused on developing new *N*-hydroxysuccinimidyl fluorescein active esters that can solve these problems.

To synthesize less pH-sensitive fluorescein active esters with good photostability, single active group, good reactivity, high purity, and satisfying derivatizing efficiency, we designed and synthesized a novel fluorescent labeling reagent, 6-oxy-(*N*-succinimidyl acetate)-9-(2′-methoxycarbonyl)fluorescein (SAMF). Its fluorescence properties were studied in detail. It exhibits relatively pH-independent fluorescence (pH 4–9) and at pH 4, the fluorescence quantum yield (Φ) of SAMF derivatives is about 0.30 which is far greater than those of fluorescein and FITC derivatives ($\Phi = 0.07$). As the mobile phase of HPLC is acidic in many cases, these properties make it advantageous over FITC and other fluorescein-based dyes for HPLC. Besides, SAMF is much more photostable than either fluorescein or FITC when exposed to ordinary light (tungsten light bulb). As a derivatizing reagent, it reacted readily with amines and was successfully used as a labeling reagent to determine seven aliphatic amines with HPLC. The detection limit of ethanolamine reached 2 fmol. The proposed method has been applied to determining aliphatic amines in lake water, red wine, white wine, and cheese with recoveries varying from 95 to 106%.

2. Experimental

2.1. Apparatus

An LC-6A HPLC system (Shimadzu) with an RF-530 fluorescence detector (Shimadzu) and a CR-3 A integrator (Shimadzu) was used. Separation was performed on a Lichrosorb C_{18} column (5 μm, 200 mm × 4.6 mm i.d., Agilent, USA). A RF-5000 spectrofluorometer (Shimadzu) equipped with a 1 cm × 1 cm quartz cell was used for recording spectra. A DF-801 pH meter (Zhongshan University, China) was used. All melting points were determined with an X-4MP apparatus from Shanghai Instrument Co. (Shanghai, China). ESI–MS spectra were obtained by means of a Finnigan LQC^{Duo} instrument. ^{1}H NMR spectra were recorded on a Varian Mercury UX 300 spectrometer. FTIR spectra were obtained for the products in KBr disks by means of a Bruker (Karlsruhe, Germany) IFS48 instrument.

2.2. Chemicals and reagents

Ethanolamine (EOA), methylamine (MA), ethylamine (EA), *n*-propylamine (PrA), *n*-butylamine (BA), *n*-pentylamine (PA) and *n*-hexylamine (HA) were of analytical grade and purchased from Shanghai Chemicals Company in China. FITC was obtained from Sigma (St. Louis, MO, USA).

Unless otherwise specified, all other reagents were of analytical reagent grade. All solutions were prepared with doubly distilled water.

The stock solutions of EOA, MA, EA, PrA, BA, PA, HA were prepared by dissolving the appropriate amine in water. H_3BO_3-$Na_2B_4O_7$ buffer was prepared by mixing 0.05 mol l^{-1} $Na_2B_4O_7$ solution with 0.2 mol l^{-1} H_3BO_3 solution to the required pH value. H_3Cit_3–NaOH buffer was prepared by mixing 0.1 mol l^{-1} H_3Cit_3 solution with 0.01 mol l^{-1} NaOH solution to the required pH value.

SAMF was synthesized in our laboratory and a 2×10^{-3} mol l^{-1} solution was prepared in anhydrous acetonitrile.

2.3. Synthesis of 6-oxy-(N-succinimidyl acetate)-9-(2′-methoxycarbonyl)fluorescein

Fluorescein methyl ester was synthesized according to the literature [27]. A red solid (I) was obtained: yield 3.4 g (98%); m.p. 212–214 °C. MS *m/z*: 346 $(M)^+$. IR (KBr pellet): $\nu_{\text{—O—H}}$, 3036 cm^{-1}; $\nu_{\text{=C—H}}$, 2900 cm^{-1}; $\nu_{\text{C=O}}$, 1719 cm^{-1}; $\nu_{\text{—C=C—}}$, 1638–1604 cm^{-1}; $\nu_{\text{—C—O—C—}}$, 1281–1139 cm^{-1}.

A mixture of bromoacetic acid *tert*-butyl ester (2 g), diisopropylethylamine (5 ml) and DMF (10 ml) containing

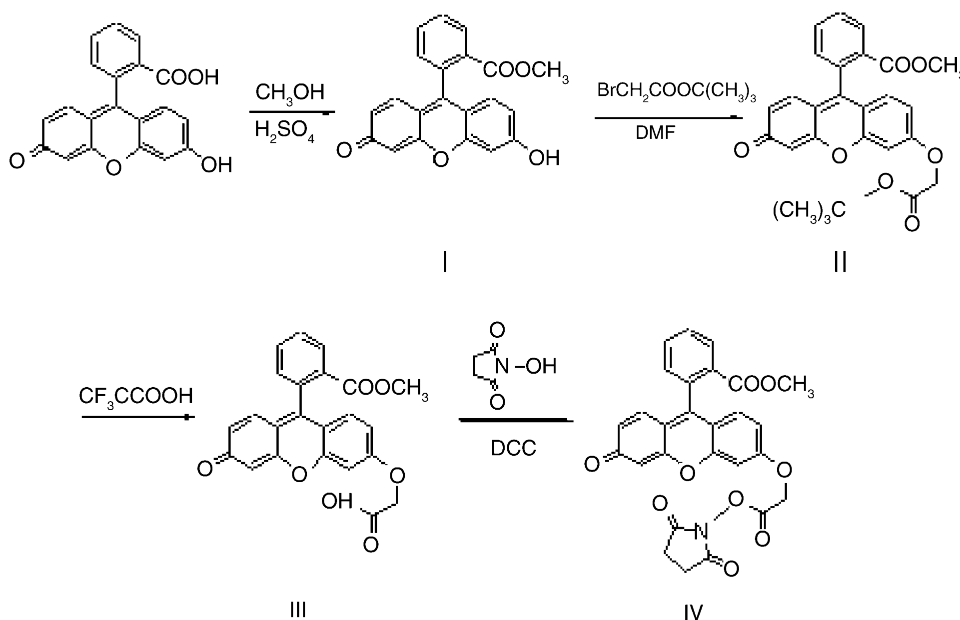

Fig. 1. The synthesis scheme of SAMF.

the above product (I) (2.8 g) were refluxed. The mixture was added to ethyl acetate and extracted with $NaHCO_3$, sodium citrate, $NaHCO_3$, and water, respectively. $MgSO_4$ was used to dry the organic phase, then the phase dissolved in diethyl ether to form crystals (II): yield 2.95 g (80%); m.p. 188–192 °C; MS *m/z*: 460 $(M)^+$. IR (KBr pellet): $\nu_{=C-H}$, 2920 cm^{-1}; $\nu_{C=O}$, 1751–1721 cm^{-1}; $\nu_{-C=C-}$, 1642–1598 cm^{-1}; ν_{C-O-C-}, 1292–1081 cm^{-1}.

The above product (II) (1.84 g) was dissolved in trifluoroacetic acid (10 ml), and the mixture was refluxed. Most part of the trifluoroacetic acid was removed, and the product was precipitated with diethyl ether. It was recrystallized from ethanol and water to give orange crystals (III): yield (76%); m.p. 220–222 °C; MS *m/z*: 404 $(M)^+$. IR (KBr pellet): ν_{-O-H}, 3432 cm^{-1}; $\nu_{=C-H}$, 2918 cm^{-1}; $\nu_{C=O}$, 1719 cm^{-1}; $\nu_{-C=C-}$, 1639–1598 cm^{-1}. ^1H NMR [deuterated dimethyl sulfoxide (DMSO)] δ 4.902 (2H, s), 6.535 (1H, s), 6.641 (1H, d), 6.671 (1H, d), 6.869 (1H, d), 6.993 (1H, d), 7.337 (1H, s), 7.510 (1H, d), 7.819 (1H, m), 7.877 (1H, m), 8.235 (1H, d), 3.640 (3H, s) (this peak was overlapped by that of deuterated DMSO, but it appeared when CD_3C1 was used as the solvent).

The above product (III) (0.8 g), *N*-hydroxysuccinimide (0.276 g), and dicyclohexylcarbodiimide (0.5 g) in anhydrous DMF (2 ml) were stirred for hours at room temperature. The mixture was filtered by a Buchner funnel to eliminate the precipitated dicyclohexylurea. Diethyl ether and light petroleum (b.p. 60–90 °C) was added to the filtrate, and the precipitation was combined. It was washed with ether and dried in vacuo: yield (80%). The product SAMF (IV) was contaminated with DMF and could not produce a sharp melting point (m.p. 158–182 °C). MS *m/z*: 501 $(M)^+$. IR (KBr pellet): $\nu_{=C-H}$, 2910 cm^{-1}; $\nu_{C=O}$, 1712 cm^{-1}; $\nu_{-C=C-}$, 1640–1597 cm^{-1}; ν_{C-N-}, 1211 cm^{-1}.

The synthesis route is given in Fig. 1.

2.4. Photostability study

Photostability study was carried out according to the literature [28]. It was performed by irradiating a 5×10^{-8} mol l^{-1} sample in anhydrous acetonitrile or 0.1 mol l^{-1} H_3BO_3-$Na_2B_4O_7$ buffer, pH 9, using a lamp with a 200 W soft white bulb (General Electric, China) positioned 10 cm from the sample flask. The flask was cooled by flowing room-temperature water. Fluorescence spectra were recorded on aliquots taken at different exposure times.

2.5. Derivatization procedure

Fig. 2 shows the competitive reactions of SAMF. To a 10 ml vial containing appropriate amount of mixed

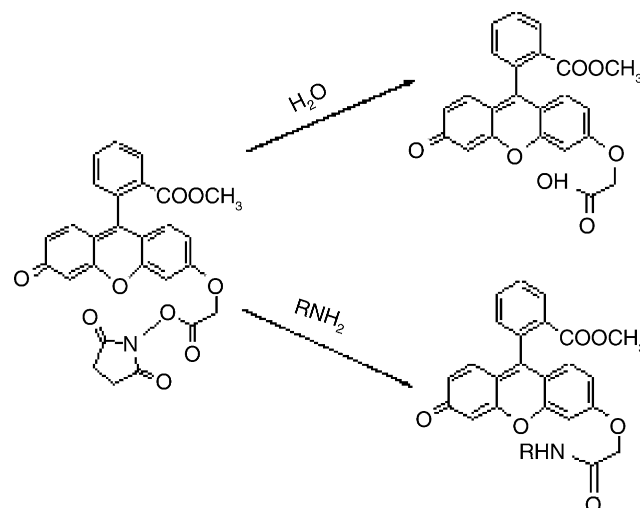

Fig. 2. The reaction of SAMF with water and aliphatic amines, respectively.

amines and 1 ml of H_3BO_3-$Na_2B_4O_7$ buffer (pH 8.0), 1×10^{-3} mol l^{-1} SAMF was added. The whole solution was diluted to the mark with water and was kept at 20 °C for 6 min.

2.6. Chromatography

Before the analysis, the C_{18} column was pre-equilibrated with the mobile phase for 30 min. A 20 μl aliquot of sample solution was injected to the chromatograph. Separation was performed at a flow rate of 0.7 ml/min and the detection wavelengths were set at $\lambda_{ex}/\lambda_{em} = 484/516$ nm.

2.7. Sample preparation

Red wine, white wine, and cheese were made in China and obtained from local supermarket. Lake water, wines were used for analysis directly after being filtered through a 0.45 μm membrane filter.

The preparation procedure for cheese was performed as described by Jana Lange and Christine Wittmann [29]. Ten grams of grated cheese were suspended in 20 ml 0.1×10^{-3} mol l^{-1} HCl and mixed in a Vortex for 5 min. Subsequent to a centrifugation step (3619 g for 30 min at room temperature), the supernatant was collected. The extraction was repeated with 20 ml of 0.1×10^{-3} mol l^{-1} HCl for three times. The supernatants were combined and stored at 4 °C to freeze most of the fat. The agglomerated fat layer was removed, and the supernatant was filtered.

One millilitre of the supernatant was added to a mixture of 0.25 ml of 5 mol l^{-1} NaOH, 0.75 g NaCl and 5 ml *n*-butanol. After shaking this mixture for 3 min and centrifugation for 10 min, the *n*-butanol layer was separated. The aqueous layer (1.25 ml) was extracted again with 5 ml of *n*-butanol. The *n*-butanol of every extraction step was transferred in another tube containing 5 ml 0.1×10^{-3} mol l^{-1} NaOH saturated with NaCl. The second centrifuge tube was shaken and centrifuged in the same way as before. An 8 ml aliquot of the butanol extract was transferred to a third tube containing 2.5 ml of 0.1 mol l^{-1} HCl and 7 ml *n*-heptane, shaken for 1 min and subsequently centrifuged. After removing the organic layer, the aqueous layer (2.5 ml) containing the amines was analyzed.

3. Results and discussion

3.1. The design strategy and fluorescence properties of SAMF

N-Hydroxysuccinimidyl active esters have good activity and selectivity for amino compounds and are employed as an excellent active group. They can react with amines in low concentration as quickly as a few minutes to form stable derivatives [24]. In particular, the excessive reagents form hydrolysates without any by-products and interference. So, *N*-hydroxysuccinimidyl active esters using fluorescein as a fluorophore are favorite choices as fluorescent labeling reagents for amines. However, fluorescein-based dyes and their conjugates have several drawbacks, including a relatively high rate of photobleaching and pH-sensitive fluorescence (pK_a ∼6.4), which restricts the selection of labeling and separation media in order to maintain high sensitivity [26]. Therefore, pH-independent probes with good photostability are desired. However, there are no fluorescein-based dyes available that completely solve these problems.

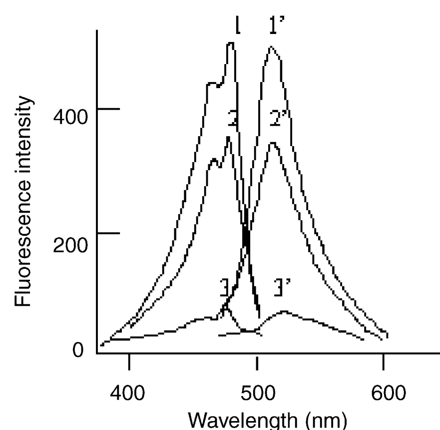

Fig. 3. Fluorescence spectra of SAMF and its derivatives.(1 and 1′) Excitation and emission spectra of hydrolyzed SAMF, respectively; (2 and 2′) excitation and emission spectra of SAMF-MA, respectively; (3 and 3′) excitation and emission spectra of SAMF in acetonitrile, respectively. Slit widths: 3 and 3 nm for excitation and emission spectra, respectively.

Generally, the 2′-carboxyl group and 6-phenolic OH are considered to playing a critical role in these properties [28]. Based on this point, we designed to protect the carboxyl group through forming ester and lock the fluorophore into a fluorescent quinoid form to exploit relatively pH-independent reagent. Besides, the functional group was introduced through conjugating it to the 6-phenolic OH.We studied the fluorescence properties of SAMF and compared it to fluorescein and FITC, one of the most popular fluorescent labeling reagents. The fluorescence spectra of hydrolyzed SAMF and SAMF derivatives are given in Fig. 3. As is expected, SAMF have stable fluorescence properties superior to those of fluorescein and FITC. Since SAMF readily hydrolyses in water to form carboxylic acid which is useless in analysis, the derivatives of methylamine with SAMF and FITC have been used as models to evaluate the fluorescence properties. As is shown in Fig. 4, SAMF derivatives are significantly more photostable than either fluorescein or FITC derivatives. FITC derivatives almost show no fluorescence when exposed to ordinary light from a 200 W bulb for about 10 h. However, the fluorescences of SAMF and its derivatives exhibit slightly change over 30 h. We also studied the fluorescence quantum yields of SAMF-MA, FITC-MA and fluorescein in the pH range of 4–9 (Table 1). Although the fluorescence of SAMF-MA in basic medium is not encouraging, the fluorescence quantum yield of SAMF-MA is 0.30, which is far greater than fluorescein and FITC-MA ($\Phi = 0.07$) at pH 4. Thus, SAMF

Table 1
The fluorescence quantum yield of fluorescein, FITC-MA, and SAMF-MA in media of different pH values

Compound	Fluorescence quantum yield[a]					
	pH = 4.0	pH = 5.0	pH = 6.0	pH = 7.0	pH = 8.0	pH = 9.0
Fluorescein	0.07	0.21	0.39	0.51	0.58	0.61
FITC-MA	0.07	0.17	0.52	0.63	0.65	0.66
SAMF-MA	0.30	0.30	0.31	0.31	0.33	0.33

[a] The fluorescence quantum yield was determined using the expression: $\Phi_u = \Phi_s \times F_u \times A_s/F_s \times A_u$ where Φ_u, Φ_s are the fluorescence quantum yield of samples and the standard solution, respectively, A_u and A_s are the areas under the emission curves of the samples and the standard, respectively, and F_u, F_s are the absorbances of the samples and the standard, respectively. Fluorescein in 0.1 mol l^{-1} NaOH was used as standard solution, $\Phi = 0.92$.

is advantageous for direct determination of samples in different environments such as various intracellular compartments, where fluorescein and FITC is almost devoid of fluorescence. In particular, as the mobile phase of HPLC is acidic in many cases, SAMF is a pre-column derivatizing reagent far superior to FITC and other fluorescein-based dyes especially for HPLC. Besides, the ester group increases the reagent's hydrophobic ability and enables it to easily penetrate the cells, which suggests cell imaging prospect.

3.2. Separation of SAMF derivatives

The parameters affecting separation were optimized. When methanol content was above 60%, the peaks of ethanolamine, methylamine, and ethylamine overlapped. If the methanol content was lower than 55%, longer analysis time was needed. When methanol content was in the range of 57–60%, the peaks could be separated on the baseline. Thus, 57:43 (v/v) was employed as the optimum methanol content.

The pH value of mobile phase was also studied. As we all know, pH 2–8 is usually used for mobile phase. In this experiment, it was found that pH above 6 or below 4 produces bad separation and reproducibility. Between pH 4–6, we also studied the effect of pH on k' in detail using H_3Cit_3–NaOH buffer. The pH value of 5.0 was employed as the optimum. As stated above, the fluorescence intensity of SAMF derivative changes little between 4 and 9 and the fluorescence quantum yield reaches 0.3 at pH 4.0 and 5.0, whereas those of fluorescein and FITC derivatives are 0.07 at pH 4.0 and about 0.2 at pH 5.0. Therefore, in the acidic medium the fluorescence reagent we synthesized can afford more sensitive determination compared to FITC.

The effect of the H_3Cit_3–NaOH buffer concentration on k' was also examined. When the concentration is lower than 5×10^{-3} mol l^{-1}, the reproducibility is poor. Between 8×10^{-3} and 12×10 mol l^{-1}, a good separation was obtained. So, 10×10^{-3} mol l^{-1} of H_3Cit_3–NaOH buffer was used in this experiment.

Under the optimum condition, the chromatogram was achieved and shown in Fig. 5. The baseline separation was obtained within 28 min.

3.3. Optimization of derivatization conditions

It has been well documented that FITC, a widely used reagent produces several by-products resulting in severe interference [24]. For succinimidyl ester labeling, the

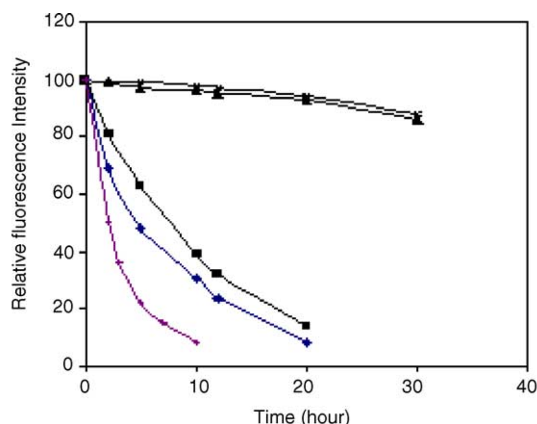

Fig. 4. Photostability of fluorescein (■), FITC (□), FITC-MA (+), SAMF (×), SAMF-MA (▲); 5×10^{-8} mol l^{-1} SAMF in anhydrous acetonitrile; 5×10^{-8} mol l^{-1} of other samples in 0.1 mol l^{-1} H_3BO_3-$Na_2B_4O_7$ buffer, pH 9; excitation at 488 nm for fluorescein, FITC, and FITC-MA, 484 nm for SAMF and SAMF-MA after aliquots were removed from solutions exposed to the radiation from a 200 W soft white bulb.

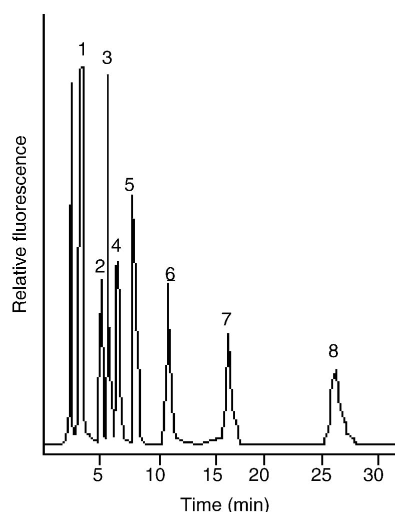

Fig. 5. Typical chromatogram of SAMF and amine derivatives. Mobile phase: methanol–water (57/43 (v/v)); flow rate: 0.7 ml/min. Standard amines concentration: 0.40 μmol/1. Peaks: (1) hydrolyzed SAMF; (2) SAMF-EOA; (3) SAMF-MA; (4) SAMF-EA; (5) SAMF-PrA; (6) SAMF-BA; (7) SAMF-PA; (8) SAMF-HA.

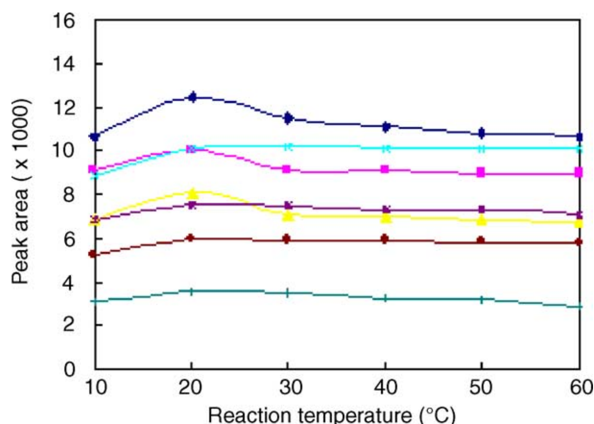

Fig. 6. Effect of reaction temperature on the peak area of amine derivatives. (♦) SAMF-EOA; (■) SAMF-MA; (▲) SAMF-EA; (×) SAMF-PrA; (*) SAMF-BA; (●) SAMF-PA; (+) SAMF-HA.

derivatizing reaction is usually performed in aqueous phase. In this case, the excessive reagents form hydrolysates without any other by-products and thus offer relatively less interference. The influence of the amount of reagent on the derivatization was investigated. As there is a competition between the derivatizing and the hydrolysis for the derivatizing method presented here, excess reagent is needed. At the same time, too much reagent leads to a strong peak of the hydrolysates and interferes with the determination. When the concentration of reagent is in the range of 8×10^{-6} mol l^{-1} and 14×10^{-6} mol l^{-1}, the peak areas of derivatives are highest and unchangeable. So, 12×10^{-6} mol l^{-1} was chosen as the optimal concentration.

The influence of various pH values on the peak areas was also studied using H_3BO_3-$Na_2B_4O_7$ buffer. It was found that the peak areas of the derivatives were almost stable at pH 7.5–9.5. At pH 8.0, different volume of buffer was further tried. One millilitre was found to be the best at pH 8.0.

High temperature could accelerate the derivatization reaction as well as the hydrolysis of the reagent. Therefore, the experiment aimed at the best temperature to achieve the best derivatization yield. Fig. 6 indicates that the effect of different temperature on the peak areas. Room temperature (20 °C) was employed.

Usually, the derivatizing is expected to be performed in a short time with satisfying efficiency. For FITC, the reaction needs 12–16 h at room temperature (20–25 °C) or 4–6 h at 40–45 °C [24,30]. The tedious reaction companied with other disadvantages demonstrates that FITC is not the best choice for derivatizing amino compounds. In this experiment, the investigation of suitable reaction time was carefully carried out at 20 °C. It was demonstrated that the reaction was completed in 5 min. To get reproducible results, the derivatization at room temperature (20 °C) for 6 min was performed.

3.4. Interference

SAMF can react readily with amino acid. To exclude the interference of amino acid, the retention time of familiar amino acids derivatives such as the derivatives of glycine, glutamic acid, cysteine, tryptophan, lysine, taurine, serine, aspartic acid, alanine was investigated. It was found that under the chosen chromatographic condition, the peaks of derivatives and the hydrolysates overlapped. The retention time of aliphatic amines was much longer than that of amino acids, therefore the amino acids have no interference with the analysis. Besides, aromatic amines and alcohols did not make any interference with the determination of aliphatic amines for the active ester exhibits no reaction activity toward aromatic amines and alcohols.

3.5. Analytical calibration

A test mixture with different concentrations of standard aliphatic amines was prepared and analyzed using the optimized derivatization procedure and separation conditions for the determination of the linearity. The lowest detectable amount of aliphatic amines was calculated as the amount of aliphatic amine that resulted in a peak three times higher than that of the baseline noise. The linear calibration ranges, regression equations, and detection limits of aliphatic amines was calculated and listed in Table 2. The correlation coefficients for these aliphatic amines are from 0.9954 to 0.9999, indicating good linearity. The R.S.D.s for the SAMF derivatives are from 0.4 to 4.1% for within-day determination ($n=6$) and from 1.5 to 4.7% for between-day determination ($n=6$). It was shown that the quantification and qualification of aliphatic amines could be well done by this method. The detection limits for the labeled amines range from 2 fmol for ethanolamine to 320 fmol for n-hexylamine,

Table 2
Linear calibration range, regression equation and detection limits of SAMF derivatives

Amines	Calibration range (μmol l^{-1})	Regression equation	R^2	R.S.D. (%, $n=6$, between-day)	R.S.D. (%, $n=6$, within-day)	Detection limit (fmol)
Ethanolamine	0.004–8	$y=122873x+7482.5$	0.9954	4.7	2.1	2
Methylamine	0.01–8	$y=95602x+7184$	0.9993	2.4	3.5	20
Ethylamine	0.04–8	$y=95268x-1537$	0.9981	1.5	0.4	80
n-Propylamine	0.08–8	$y=99230x+805.95$	0.9998	3.5	1.6	80
n-Butylamine	0.08–8	$y=100014x-3845.5$	0.9999	2.7	1.7	160
n-Pentylamine	0.16–8	$y=38356x+4983.6$	0.9981	2.6	4.1	320
n-Hexylamine	0.16–8	$y=67837x-20184$	0.9967	1.8	2.8	320

x: concentration of amine (μmol l^{-1}); y: peak area of amine derivatives.

Table 3
Analytical results of samples

Samples	Lake water[a]				Red wine[a]				White wine[a]				Cheese			
	Added (μg/1)	Founded (μg/1)	R.S.D. (%, $n = 6$)	Recovery (%)	Added (μg/1)	Founded (μg/1)	R.S.D. (%, $n = 6$)	Recover y (%)	Added (μg/1)	Founded (μg/1)	R.S.D. (%, $n = 6$)	Recovery (%)	Added (μg/1)	Founded (μg/1)	R.S.D. (%, $n = 6$)	Recovery (%)
Ethanolamine	0	0.26	2.1		0	35.4	3.4		0	10.2	0.6		0	0		
	19.5	19.4	1.4	98	19.5	55.6	4.5	103	19.5	30.3	3.5	103	19.5	20.7	2.5	106
	34.2	34.6	1.9	100	34.2	68.6	1.2	97	34.2	44.8	4.2	101	34.2	35.6	3.1	104
Methylamine	0	0.47	1.4			0			0	0.5	1.1		0	0		
	9.9	10.6	3.2	102	9.9	9.6	3.1	97	9.9	10.3	3.5	99	9.9	10.0	1.3	100
	17.4	17.6	1.6	99	17.4	16.83	4.5	97	17.4	18.2	0.6	105	17.4	18.0	2.4	104
Ethylamine	0	0			0	0			0	3.9	4.1		0	0		
	14.4	13.8	4.2	96	14.4	14.9	1.2	103	14.4	19.0	3.6	105	14.4	15.0	4.3	104
	25.2	25.5	1.5	101	25.2	25.3	2.3	100	25.2	29.8	1.4	103	25.2	26.5	2.3	105
n-Propylamine	0	0			0	5.9	0.9		0	0			0	0		
	18.9	20.1	0.9	106	18.9	23.9	3.0	95	18.9	18.7	0.7	99	18.9	18.7	1.8	99
	33.0	34.5	2.6	105	33.0	38.1	97.6	95	33.0	34.2	3.4	104	33.0	32.3	3.9	98
n-Butylamine	0	9.34	3.4		0	86.6	2.3		0	31.4	2.1		0	0		
	23.4	25.5	2.7	95	23.4	109.2	4.1	97	23.4	54.0	1.5	96	23.4	24.0	1.8	103
	40.9	51.0	3.1	102	40.9	126.7	3.3	98	40.9	74.3	1.9	105	40.9	41.3	3.3	101
n-Pentylamine	0	0			0	0			0	0			0	0		
	27.8	29.3	1.6	105	27.9	27.8	4.1	100	27.8	27.5	1.4	98	27.8	26.5	1.6	95
	48.7	46.6	4.3	96	48.7	49.4	2.8	101	48.7	50.7	2.8	104	48.7	47.5	3.5	98
n-Hexylamine	0	0			0	0			0	0			0	0		
	32.3	32.2	3.4	101	32.3	32.4	3.7	100	32.3	33.0	3.3	102	32.3	33.2	1.9	103
	56.6	54.5	1.0	96	56.6	54.9	2.1	97	56.6	53.5	2.1	95	56.6	53.8	2.5	95

[a] The concentration of the sample is 8% of the real sample.

which was equivalent to or better than the detection limits obtained from other analytical methods of aliphatic amines [2,31–33]. The derivatization limits for amines would be improved if more sensitive approaches, such as laser-induced fluorescence (LIF) detection system, were available.

3.6. Application to sample analysis

The proposed method was applied to the determination of aliphatic amines in lake water, red wine, white wine, and cheese. In order to make the concentration of analyte in linear calibration range, the lake water, red wine and white wine were diluted with water in this case. It was found that these samples have no native fluorescence at $\lambda_{ex}/\lambda_{em} = 484/516$ nm. It was reported that phenolic compounds exist in the wines [2], but they do not react with the reagent and show no fluorescence at $\lambda_{ex}/\lambda_{em} = 484/516$ nm resulting in no interference. The chromatograms of samples unspiked and spiked with standard solutions are shown in Fig. 7. The analytical results are summarized in Table 3. The recoveries ranged from 95 to 106% and the R.S.D.s from 1.5 to 4.7%.

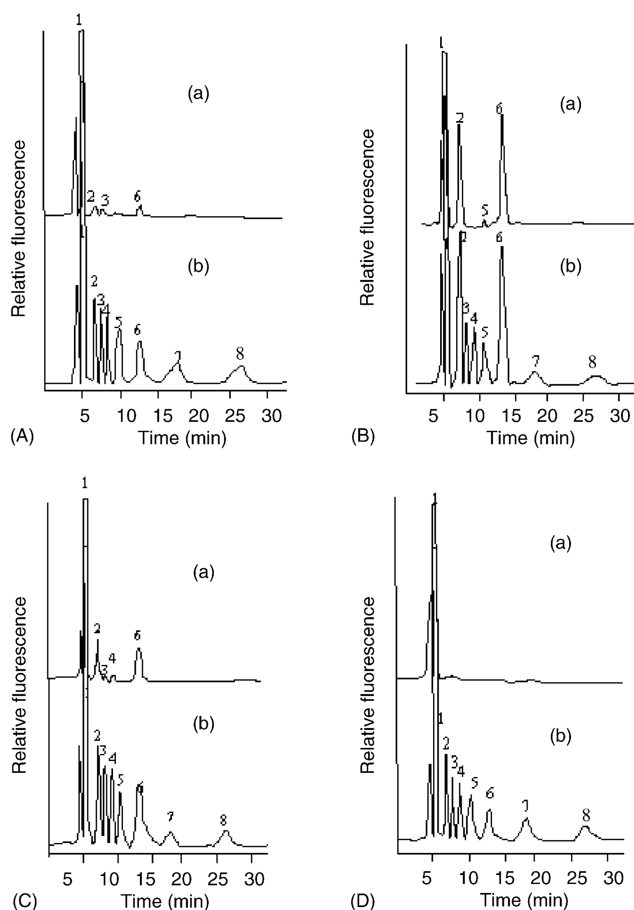

Fig. 7. Chromatograms obtained from samples. (A) Chromatograms obtained from: (a) waste water sample; (b) the same sample spiked with 0.32 μmol/l of standard amines. Chromatographic conditions as in Fig. 5. Peaks: (1) hydrolyzed SAMF; (2) SAMF-EOA; (3) SAMF-MA; (4) SAMF-EA; (5) SAMF-PrA; (6) SAMF-BA; (7) SAMF-PA; (8) SAMF-HA. (B) Chromatograms obtained from: (a) red wine sample; (b) the same sample spiked with 0.32 μmol/l of standard amines. Other conditions as in part A. (C) Chromatograms obtained from: (a) white wine sample; (b) the same sample spiked with 0.32 μmol/l of standard amines. Other conditions as in part A. (D) Chromatograms obtained from: (a) cheese sample; (b) the same sample spiked with 0.32 μmol/l of standard amines. Other conditions as in part A.

4. Conclusions

A new sensitive fluorescent labeling reagent, 6-oxy-(*N*-succinimidyl acetate)-9-(2′-methoxycarbonyl)fluorescein, was developed for the determination of aliphatic amines by HPLC. Compared to other fluorescein-based reagents, it exhibits relatively pH-independent fluorescence (pH 4–9) and excellent photo stability. SAMF reacts with amines under mild conditions, which is comparable to 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate and superior to other active esters used for amine labeling. The detection limits were 2–320 fmol with a signal-to-noise ratio of 3. The proposed method was successfully applied to determining trace aliphatic amines in environmental and food samples such as lake water, red wine, white wine, and cheese with recoveries varying from 95 to 106%. This work provides rational design strategy for the further investigations of novel and potentially useful fluorescence probes that can afford better sensitivity as well as these advantages SAMF possesses.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 29575206, Beijing, China) and the Foundation of Education Ministry of China and Chengguang Project of Wuhan.

References

[1] K. Kuwata, E. Akiyama, Y. Yamazaki, H. Yamasaki, Y. Kuge, Anal. Chem. 55 (1983) 2199.
[2] O. Busto, J. Guasch, F. Borrull, J. Chromatogr. A 718 (1995) 309.
[3] H. Kataoka, J. Chromatogr. A 733 (1996) 19.
[4] P. Simon, C. Lemacon, Anal. Chem. 59 (1987) 480.
[5] US EPA, Test Methods for Evaluating Solid Waste (SW-846), vol. 1B, third ed. US Environmental Protection Agency, Washington, DC, USA, November 1986.
[6] Y.-Y. Zhao, L.-S. Cai, Z.-Z. Jing, H. Wang, J.-X. Yu, H.-S. Zhang, J. Chromatogr. A 1021 (2003) 175.
[7] C. Aguilar, H.-G. Janssen, C.A. Cramers, J. Chromatogr. A 867 (2000) 207.
[8] L. Simon-Sarkadi, A. Kovacs, E. Mincsovics, J. Planar Chromatogr. 10 (1997) 59.
[9] S. Bardocz, T. Karsai, P. Elodi, Chromatographia 20 (1985) 23.
[10] L.-W. Cao, H. Wang, X. Liu, H.-S. Zhang, Talanta 59 (2003) 973.
[11] Y. Moliner Martinez, C. Molins Legua, P. Campins Falco, Talanta 62 (2004) 373.

[12] H. Wang, J. Li, X. Liu, H.-S. Zhang, Anal. Chim. Acta 423 (2000) 77.
[13] J. Krol, P.G. Alden, J. Morawski, P.E. Jackson, J. Chromatogr. 626 (1992) 165.
[14] Z. Loukou, A. Zotou, J. Chromatogr. A 996 (2003) 103.
[15] W.H. Matchett, W.C. Brumley, J. Liq. Chromatogr. Rel. Technol. 20 (1997) 249.
[16] I. Rodrigue, H.K. Lee, S.F.Y. Li, J. Chromatogr. A 745 (1996) 255.
[17] K. Yamada, Y. Aizawa, J. Pharmacol. Methods 9 (1983) 1.
[18] K. Imai, Y. Watanabe, Anal. Chim. Acta 130 (1981) 377.
[19] K.B. Male, J.H.T. Luong, J. Chromatogr. A 926 (2001) 309.
[20] J. Lange, K. Thomas, C. Wittmann, J. Chromatogr. B 779 (2002) 229.
[21] A. Kovacs, L. Simon-Sarkadi, K. Ganzler, J. Chromatogr. A 836 (1999) 305.
[22] S.P. Lalljie, P. Sandra, Chromatographia 40 (1995) 519.
[23] J. Mattush, K.J. Dittrich, J. Chromatgr. A 680 (1994) 279.
[24] S. Kei Lau, F. Zaccardo, M. Little, P. Banks, J. Chromatogr. A 809 (1998) 203.
[25] H. Wang, J. Li, X. Liu, T.-X. Yang, H.-S. Zhang, Anal. Biochem. 281 (2000) 15.
[26] P. Richard, Haugland, Handbook of Fluorescent Probes and Research Chemicals, ninth ed., Molecular Probes, USA, 2003, Section 1.5.
[27] M. Adamczyk, J. Grote, A.M. Jeffrey, Bioconjugate Chem. 10 (1999) 544.
[28] J. Gao, P. Wang, W.G. Roger, Anal. Chem. 74 (2002) 6379.
[29] J. Lange, K. Thomas, C. Wittmann, J. Chromatogr. B 779 (2002) 229.
[30] H. Li, H. Wang, J.-H. Chen, L.-H. Wang, H.-S. Zhang, Y. Fan, J. Chromatogr. B 788 (2003) 93.
[31] S. Meseguer Lloret, C. Molins Legua, P. Campins Falco, J. Chromatogr. A 978 (2002) 59.
[32] J. Verdu Andres, P. Campins Falco, R. Herraez Hernandez, Chromatographia 55 (2002) 129.
[33] E. Dabek-Zlotorzynska, W. Maruszak, J. Chromatogr. B 714 (1998) 77.