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8-Phenyl-(4-oxy-acetic acid *N*-hydroxysuccinimidyl ester)-4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacene as a new highly fluorescent-derivatizing reagent for aliphatic amines in disease-related samples with high-performance liquid chromatography

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Abstract

A novel fluorescent-activated ester, 8-phenyl-(4-oxy-acetic acid *N*-hydroxysuccinimidyl ester)-4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacene (TMPAB-OSu) has been designed and synthesized for amine labeling in HPLC. Being used 11 aliphatic amines as the models, the derivatization conditions were optimized. In 0.2 mol/l borate buffer (pH 8.8), amines reacted with TMPAB-OSu at 30 °C to form the derivatives in 10 min. The fluorescent quantum yield of TMPAB-OSu and its amine derivatives are high even compared with fluorescein. The separation of these amine derivatives was achieved with a C_8 column and gradient elution by using 0.1 mol/l sodium acetate buffer (pH 5.0) and methanol. With fluorescence detection at an emission wavelength of 509 nm and an excitation wavelength of 497 nm, the detection limits of aliphatic amines were 2–18 fmol, at a signal-to-noise ratio of 3:1. The proposed TMPAB-OSu-based HPLC method has been applied to the analysis of urine samples of health, hepatic and renal patients and lake water. Recoveries from different matrices are from 96 to 104%, depending on the sample investigated.

Keywords: 8-Phenyl-(4-oxy-acetic acid *N*-hydroxysuccinimidyl ester)-4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacene (TMPAB-OSu); Fluorescence labeling; liphatic amines; High-performance liquid chromatography (HPLC)

1. Introduction

Sensitivity and selectivity of sample analysis are two key problems in analytical chemistry. Usually, high selectivity and sensitivity can been achieved by combination of all kinds of separation techniques, including high-performance liquid chromatography (HPLC) [1], gas chromatography (GC) [2] or capillary electrophoresis (CE) [3] with some sensitive detection methods such as fluorescence [4], chemiluminescence [5], electrochemical [6] and mass spectrometric methods [7]. Besides, chemical derivatization [8] is also utilized to improve the sensitivity and selectivity of the above approaches. For example, fluorescent tagging is commonly used in HPLC-fluorescence detection and various new fluorescent tagging reagents have been exploited and studied for the determination of trace levels substance in complex samples.

Amino compounds are a group of important substances that widely exist in nature and have a lot of functions in biological and environmental systems, such as amino acids [9], peptides, proteins and biogenic amines [10] in organisms, short-chain aliphatic amines [11] and aromatic amines in environment. Their determination is almost a routine task for biological, medical and environmental interest. Not surprisingly, great efforts have being devoted to exploiting the more appropriate fluorescent tagging reagents for amine derivatization and developing new analytical methods with high sensitivity and selectivity.

Until now, many fluorescent-derivatizing reagents have been developed fully [12], such as *o*-phthalaldehyde (OPA) [13], 5-dimethylaminonaphthalene-l-sulfonylchloride (Dns-Cl) [14], 7-fluoro-4-nitrobenzo-2-oxa-l, 3-diazole (NBD-Cl) [15], 9-fluorenylmethyl chloroformate (FMOC-Cl) [16], 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) [17],

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etc. These reagents have some drawbacks such as instability of derivatives, reactivity with other substances, complex extraction process and short detection wavelengths.

Succinimidyl esters are among the best reagents for derivatizing with aliphatic amines, since they can form stable carboxamides and have fine reactivity. In general, they do not react with aromatic amines, phenols or alcohols [17]. In our previous studies, a series of new fluorescent labeling reagents based on *N*-hydroxysuccinimidyl ester moiety have been synthesized and used in amino compounds analysis [18–21]. Among these reagents, SIFA is regarded as the best one, but it has the inherent limitations of fluorescein fluorephore, such as photobleaching and pH-dependence. The design of SAMF has partly solved these problems through protecting 2′-carboxyl group to form ester and lock the fluorophore into a fluorescent quinoid form. However, such change makes the fluorescence quantum yield of SAMF decrease greatly at pH > 6 [21]. Therefore, we sought for other fluorophores to overcome these drawbacks more efficiently.

Difluoroboradiaza-*s*-indacene (boron-dipyrromethene, BODIPY) is regarded as an excellent fluorophore because of its high fluorescence quantum efficiency, good photostabilization, relative independence on changes in the local environment and long emission wavelength [22]. Moreover, BODIPY-activated esters have not been applied as fluorescence derivatization reagents in HPLC with fluorescence detection. Therefore, we designed and synthesized the new fluorescent probe, 8-phenyl-(4-oxy-acetic acid *N*-hydroxysuccinimidyl ester)-4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacene (TMPAB-OSu) for amine labeling. Using 11 aliphatic amines as models, the feasibility of this reagent for amine derivatization followed HPLC-fluorescence detection has been investigated. TMPAB-OSu itself and its derivatives with amines have very strong fluorescence emitting at 509 nm with the excitation wavelength of 497 nm. A baseline separation of them has been accomplished within 13 min by using gradient elution mode. Compared with other amine-tagging reagents, TMPAB-OSu offered several distinct advantages for amine determination such as higher sensitivity, excellent photostability, tolerating a wide pH range and being easy to operate.

2. Experimental

2.1. Apparatus

The HPLC system Agilent 1100 series from Agilent (Germany) used in the experiments consisted of a high-pressure gradient quatpump, an online vacuum degasser, a manual injection (20 μl), and a fluorescence detector (FLD). The system was controlled by the ChemStation software. Fluorescence detection wavelengths were set at $\lambda_{ex}/\lambda_{em}$ = 497/509 nm. Chromatographic separations were achieved by using an ZORBAX Eclipse XDB-C_8 column (150 mm × 4.6 mm, 5 μm, Agilent, USA). Fluorescence spectra were recorded on an RF-5000 spectrofluorometer (Shimadzu, Japan). The pH values of solutions were measured by using a Mettler Toledo DELTA 320 m. 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 VX 300 spectrometer. FT-IR spectra were obtained for the products in KBr disks by means of a Bruker (Karlsruhe, Germany) IFS48 instrument.

2.2. Reagents

Unless otherwise specified, all reagents were of analytical reagent grade and all solutions were stored in the dark at 4 °C; reagents produced in synthesis were conserved in desiccators; the water used in synthesis was double distilled, while it used in analysis was purified in a Milli-Q water purification system (Millipore, Molsheim, France).

Trifluoroacetic acid (TFA) and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) were purchased from Acros Organic Co. (Belgium). TMPAB-OSu was synthesized in our lab and its 2 mmol/l solution was prepared with pretreated acetonitrile (dried with P_2O_5).

Methylamine, ethylamine, *n*-propylamine, *n*-butylamine, *n*-amylamine and *n*-hexylamine were purchased from Shanghai Chemicals Reagent Co. and their standard solutions have been prepared with water in concentration of 0.1 mol/l and further diluted before being used, respectively. *n*-Heptylamine, *n*-octylamine, *n*-nonylamine, *n*-decylamine and *n*-dodecylamine were purchased from Acros Organic Co. (Belgium) and the stock solutions were prepared in concentration of 0.1 mol/l by dissolving them in ethanol.

H_3BO_3–$Na_2B_4O_7$ buffer was prepared by mixing 0.2 mol/l H_3BO_3 solution with 0.05 mol/l $Na_2B_4O_7$ solution to the required pH value. HAc–NaAc buffer was prepared by mixing 0.1 mol/l HAc solution with 0.1 mol/l NaAc solution to the required pH value.

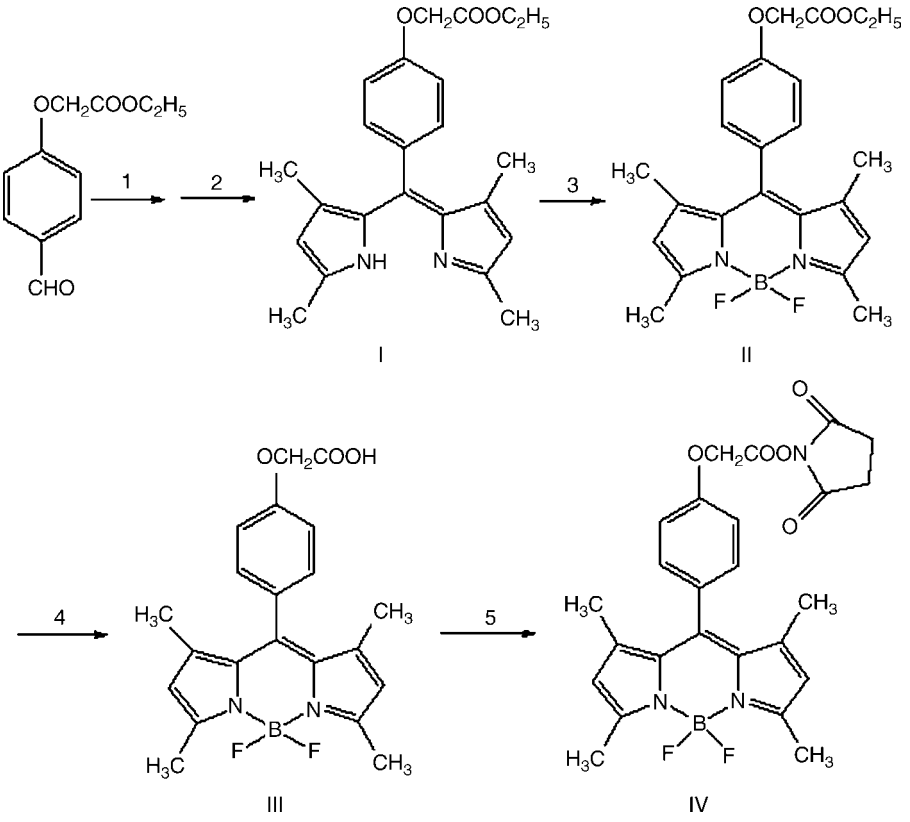
2.3. Synthesis of 8-phenyl-(4-oxy-acetic acid *N*-hydroxysuccinimidyl ester)-4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene (TMPAB-OSu)

TMPAB-OSu was synthesized from 2,4-dimethylpyrrole and the appropriate aldehyde. The synthesis route is shown in Scheme 1.

Ethyl 4-formylphenoxyacetate was synthesized according to literature [23]. The light yellow product was crystallized from ethanol, yield 84.1%; m.p. 42 °C.

2,4-Dimethylpyrrole was synthesized according to literature [24]. It must be stored in an inert atmosphere, yield 95%; b.p. 72 °C (25 mmHg).

Ethyl 4-formylphenoxyacetate (5 mmol) and 2,4-dimethylpyrrole (10 mmol) were dissolved in 250 ml of absolute CH_2Cl_2 under an Ar atmosphere. After one drop of trifluoroacetic acid (TFA) was added in, the solution was stirred at room temperature overnight. When TLC monitoring (silica; CH_2Cl_2) showed complete consumption of the aldehyde, a solution of DDQ (5 mmol) in CH_2Cl_2 was added under stirring. The reaction mixture was washed with water, dried



Scheme 1. Synthetic scheme of TMPAB-OSu, (1) 2,4-dimethylpyrrole, CF_3COOH, CH_2Cl_2; (2) DDQ, CH_2Cl_2; (3) $BF_3{\cdot}OEt_2$, Et_3N, CH_2Cl_2; (4) K_2CO_3, CH_3OH; (5) *N*-hydroxysuccinimide, DCC, DMF.

over $MgSO_4$, filtered, and evaporated. The crude compound was purified by column chromatography over aluminum oxide (CH_2Cl_2) to afford a brown powder (I) (395 mg, yield 21%).

$BF_3{\cdot}OEt_2$ (19.8 mmol) was added to the solution of (I) (1.05 mmol) and triethylamine (14.3 mmol) dissolved in absolute CH_2Cl_2 under an Ar atmosphere. Keeping stirring for 40 min, the reaction mixture was washed with water and 2 mol/l NaOH. The aqueous solution was extracted with CH_2Cl_2. The combined organic extracts were dried over Na_2SO_4, filtered, and evaporated. The crude compound was purified by silica gel chromatography (light petroleum/CH_2Cl_2) and recrystallized from $CHCl_3$/*n*-hexane to afford orange crystals (II) 326 mg, yield 73%; m.p. 205 °C; IR (KBr): ν/cm^{-1} 1754 (C=O), 1607 (C=N), 1088 (B–F), 2925 (–CH_3). MS *m*/*z*: 427 $(M)^+$. ^{1}H NMR ($CDCl_3$): δ7.19 (2H, d, J = 8.4), 7.02 (2H, d, J = 8.4), 5.98 (2H, s), 4.69 (2H, s), 4.30 (2H, quar, J = 7.5), 2.55 (6H, s), 1.42 (6H, s), 1.31 (3H, t, J = 7.5).

A solution of K_2CO_3 (1.34 mmol) was added to a methanol solution of (II) (0.67 mmol). The mixture was stirred at room temperature for 3 days. The reaction mixture was concentrated to a volume of about 20 ml under reduced pressure to remove most of methanol. The resulting mixture was diluted with 50 ml of water and extracted with 50 ml of $CHCl_3$ to remove unreated starting materials. The aqueous layer was separated and carefully acidified to pH 2–3 by dropwise addition of 0.1 mol/l HCl solution while the mixture was stirred in an ice bath. Extraction of the aqueous phase with chloroform followed by drying of the organic phase with anhydrous Na_2SO_4 and concentration under reduced pressure gave 199 mg (75%) of the desired product (III), 8-phenyl-(4-oxy-acetic acid)-4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacene (TMPAB). It was purified by silica gel column chromatography (CH_2Cl_2/acetic acid); m.p. 190 °C; IR (KBr): ν/cm^{-1} 1734 (C=O), 1609 (C=N), 1085 (B–F). MS *m*/*z*: 399 $(M)^+$. ^{1}H NMR ($CDCl_3$): δ7.18 (2H, d, J = 8.1), 7.02 (2H, d, J = 8.1), 5.95 (2H, s), 4.72 (2H, s), 2.54 (6H, s), 1.40 (6H, s).

The above product TMPAB (III) (0.5 mmol), *N*-hydroxysuccinimide (0.6 mmol), and dicyclohexylcarbodiimide (0.75 mmol) in anhydrous DMF were stirred for hours at 0 °C. 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 (IV) (TMPAB-OSu) was produced. It was washed with ether and dried in vacuo: yield (80%); m.p. 219 °C. MS *m*/*z*: 496 $(M)^+$. ^{1}H NMR ($CDCl_3$): δ7.23 (2H, d, J = 9), 7.07 (2H, d, J = 9), 5.97 (2H, s), 5.04 (2H, s), 2.88 (4H, s), 2.55 (6H, s), 1.40 (6H, s).

2.4. Measurement of the fluorescence quantum yield [25]

The fluorescence emission spectra of the sample solution (TMPAB-OSu and its derivative in ethanol, methanol or water) and the standard solution (Fluorescein in 0.1 mol/l NaOH, Φ = 0.92) were recorded at an excitation wavelength of 488 nm. The fluorescence quantum yield was determined with the expression: $\Phi_u = \Phi_s \times (D_u \times A_s)/(D_s \times A_u)$, where Φ_u, Φ_s are the flu-

orescence quantum yield of samples and the standard solution, respectively; D_u and D_s the areas under the emission curves of the samples and the standard, respectively; A_u and A_s are the absorbance of the samples and the standard, respectively. For fluorescence efficiency measurements, the concentrations of the solutions were adjusted so that the absorbance was less than 0.1, and to minimize error arising from inner filter effects.

2.5. Derivatization procedure

To a 1.0 ml vial containing appropriate amount of mixed amine solution, 0.2 ml of H_3BO_3–$Na_2B_4O_7$ buffer (pH 9.0), 11 μl of TMPAB-OSu solution (2 mmol/l), and 100 μl of acetonitrile were added. After the whole solution was diluted to 1.0 ml with water, it was incubated at 30 °C for 10 min.

2.6. Chromatographic method

Before the analysis, the C_8 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 1.0 ml/min and the detection wavelengths were set at $\lambda_{ex}/\lambda_{em}$ = 497/509 nm.

The HPLC separation of derivatives was performed in isocratic elution mode and gradient elution mode, respectively. In gradient elution mode, methanol (eluent A) and HAc–NaAc buffer (10 mmol/l, pH 5.0) (eluent B) were used as mobile phase with a linear gradient elution as follows: 0–3 min, 76% A; 3–6 min, 76–90% A; 6–13 min, 90% A. All the solvents were filtered with a 0.45 μm membrane filter.

2.7. Sample preparation

Urine sample of health and patients were collected and filtered through a 0.45 μm membrane filter, and stored in a refrigerator until analysis. Six healthy people and patient subjects were recruited from the volunteers for the studies. Each volunteer collected their appropriate urine samples with a plastic container to which hydrochloric acid (1 mol/l, 15 ml) has been previously added to prevent microbial growth and maintain amines as their water-soluble hydrochloride salts. The total urine volume was recorded and aliquots (25 ml) stored in a refrigerator until analysis, which was undertaken as soon as possible.

Lake water was used for analysis directly after being filtered through a 0.45 μm membrane filter and stored in glass bottles in a refrigerator to avoid volatilization of aliphatic amines.

3. Results and discussion

3.1. Design of highly sensitive fluorescence probe for amine

BODIPY is an excellent fluorescent chromophore, because its fluorescence is relatively insensitive to changes in the local environment [26]. The ionic charge-lack structure makes BODIPY-based compounds insensitive to pH of the solvent, and the small change in dipole moment upon excitation renders them essentially insensitive to changes in dipolarity [26,27]. Otherwise, the fluorescence properties can be tailored and tuned by a variety of different substitution patterns on the organic core as well as on the pyrrole side, so derivatives of BODIPY can emit fluorescence over a wide range from 500 to 700 nm, which will be acclimatized to all kinds of excitated lamp-house. In addition, BODIPY derivatives have visible excitation and emission wavelengths, high molar extinction coefficient, large quantum yield, good photostability, narrow emission bandwidth. Thus, major efforts have been devoted to the engineering of new BODIPY structures and the investigation of their salient physical and spectroscopic properties. It has been reported that 1,3,5,7-tetramethyl BODIPYs exhibit highest fluorescence in BODIPY analogues [22]. Therefore, 1,3,5,7-tetramethyl BODIPY has been chosen as the fluorophore in this study.

N-Hydroxysuccinimidyl ester of carboxylic acid is known to have good activity and selectivity for amino compounds and to be employed as an excellent active group. They can react with amines in low concentration to form stable derivatives under mild conditions, and the excessive reagents are hydrolyzed to corresponding carboxylic acid without any by-products and interference, which has also been substantiated in our previous studies [18–21]. Therefore, we have decided to develop 1,3,5,7-tetramethyl BODIPY-based fluorescence probes with *N*-hydroxysuccinimidyl ester as the reactive group for amino compounds in respect to their sensitivity, reactivity and selectivity. As a result, 8-phenyl-(4-oxy-acetic acid *N*-hydroxysuccinimidyl ester)-4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacene (TMPAB-OSu) has been designed and synthesized.

3.2. Fluorescence properties of TMPAB-OSu and its derivatives

The poor ultraviolet absorptivity of aliphatic amines necessitates chemical derivatization to detect small amounts. Derivatization of primary amines with TMPAB-OSu results in the formation of fluorescent substance.

The excitation and emission spectra of TMPAB-OSu in ethanol are investigated and the results were shown in Fig. 1. The results indicate that the maximal excitation and emission wavelengths are 497 and 509 nm, respectively.

TMPAB-methylamine was used as the model to investigate the fluorescence characteristics of the amine derivatives, which was prepared according to the method described in Section 2.5 by adding a great excess of methylamine to ensure that TMPAB was not existed, which was checked by HPLC. The fluorescence properties of TMPAB-methylamine derivative were examined in acetonitrile, methanol and water, which have widely used as components of the mobile phase in reversed-phase LC. The maximal excitation and emission wavelengths are almost unchanged in these organic solutions. The fluorescence quantum yield of TMPAB-methylamine derivative in acetonitrile was 0.61, which was slightly lower than in other organic solutions. The fluorescence quantum yield of TMPAB-OSu and -methylamine derivative is 0.78 in ethanol, which can match the excellence of fluorescein derivatives.

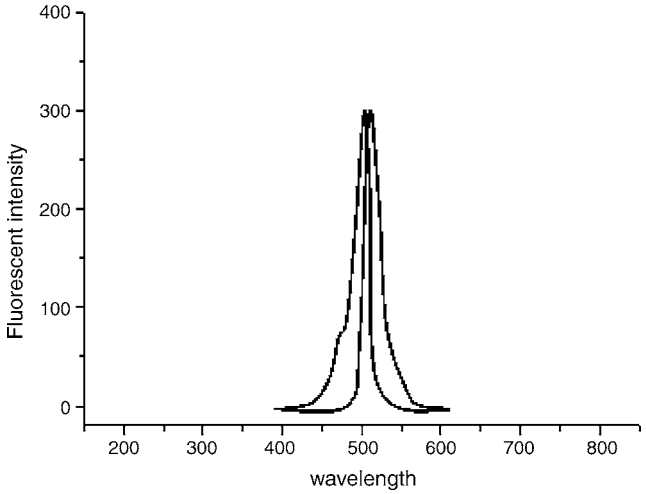


Fig. 1. Fluorescence spectra of TMPAB-OSu, CTMPAB-OSu = 10^{-7} mol/l, excitation spectrum of TMPAB-OSu at 509 nm emission wavelength; emission spectrum of TMPAB-OSu at 497 nm excitation wavelength; the slit of excitation and emission, both 3 nm.

The effect of pH on the fluorescence intensity of TMPAB-methylamine derivative was discussed. The fluorescence intensity of TMPAB-methylamine derivative is stable in buffers from pH 2 to 12, which indicates TMPAB-methylamine derivative is insensitive to pH value of the solvent.

The stability of TMPAB-OSu and its derivatives at room temperature was investigated. TMPAB-OSu in acetonitrile shows slight change on the relative fluorescent intensity when exposed to ordinary light from a 100 W bulb for about 40 h. It is photo stable. The stability of the TMPAB-OSu derivatives was investigated over 3 days. And no significant change in peak area of the derivatives was found. It appears that the TMPAB-OSu derivatives of amines are very stable, as evidenced by the fact that the derivatives showed very little degradation when analyzed by HPLC after 3 days of standing at room temperature.

Compared to fluorescein, SAMF and FITC, one of the most popular fluorescent labeling reagents, TMPAB-OSu have desired fluorescence properties superior to those of fluorescein, FITC and SAMF. In pH range of 2–12, TMPAB-OSu derivatives have almost constant fluorescence quantum yields of 0.71 in water, which are comparable to fluorescein and FITC-amine derivatives, much higher than those of SAMF derivatives. Meanwhile, TMPAB-OSu derivatives are significantly more photostable than fluorescein, FITC derivatives, as well as SAMF derivatives. Therefore, TMPAB-Osu is prospective as a precolumn derivatizing reagent for amines in terms of sensitivity and stability.

The influence of temperature and time on the fluorescence intensity of TMPAB-methylamine derivative has been studied. The fluorescence intensity was higher at lower temperature and the stability of fluorescence was better at higher temperature.

The effect of methanol on the fluorescence properties of TMPAB-methylamine was investigated in detail. In methanol–water media, the excitation wavelength and emission wavelength of TMPAB-methylamine have no obvious change with the increase of methanol content from 10 to 90% (v/v). The variation of fluorescence with methanol content was investigated. The fluorescence enhanced slightly, respectively, as methanol content changed from 10 to 40% (v/v) and was almost unchangeable with the increase of methanol content in succession.

3.3. Chromatographic separations

Since C_{18} and C_8 columns are usually used in reversed-phase liquid chromatography, the influence of the length of the bonded alkyl chain on the separations has been examined with C_{18} and C_8 stationary phases. As expected, the retention times were shorter for the C_8 column. C_8 column is weak-retained and uses water-rich mobile phases compared with C_{18} column. Even though, the content of methanol in this test also reaches to 90%. Therefore, in this paper, C_8 column was used.

At first, the separation of the TMPAB-OSu derivatives was studied in isocratic elution mode owing to its simpleness. It was found that the derivatives of TMPAB-OSu with methylamine, ethylamine, *n*-propylamine, *n*-butylamine, *n*-amylamine and *n*-hexylamine have been separated on baseline within 16 min using 76% methanol as mobile phase. The chromatogram of six aliphatic amines derivatives with TMPAB-OSu obtained in isocratic elution mode is shown in Fig. 2a.

Though the well separation of six low-chain amines has been obtained in isocratic elution mode, isocratic elution is still not satisfied for amines of interest since the detection limits are high and retention times are long for long-chain amines. In order to determine more amines sensitively and rapidly, gradient program should be added. We optimized the separation conditions as follows. The first elution step assured the separation of TMPAB-OSu derivatives. The next step was performed to reduce the time of resolution of derivatives according to the program.

The effect of the buffers on the separation has also been investigated by using phosphate and acetate buffers. In gradient elution, for high percentage of methanol, the use of phosphate concentrations greater than 0.1 mol/l occasionally gave rise to precipitation problems (most likely disodium hydrogen phosphate), thus, acetate buffers was employed. The pH value of buffer in mobile phase was studied. In this experiment, the retention time of each derivative had no obvious change as the pH value varied from 3.0 to 7.0, which have been usually used in the mobile phase of HPLC, and the peak area of each derivative also indicated that TMPAB-OSu derivatives were pH-insensitive and stable. In this experiment, pH 5.0 was been used.

The chromatogram of the aliphatic amines derivatives with TMPAB-OSu obtained in gradient elution mode is shown in Fig. 2b. The separation of the derivatives was completed within 13 min.

3.4. Optimization of derivatization conditions

For succinimidyl ester labeling, the derivatizing reaction is usually performed in aqueous phase. There is a competition between the labeling and the hydrolysis, so excess-labeling reagents should be used. The influence of the amount of reagent

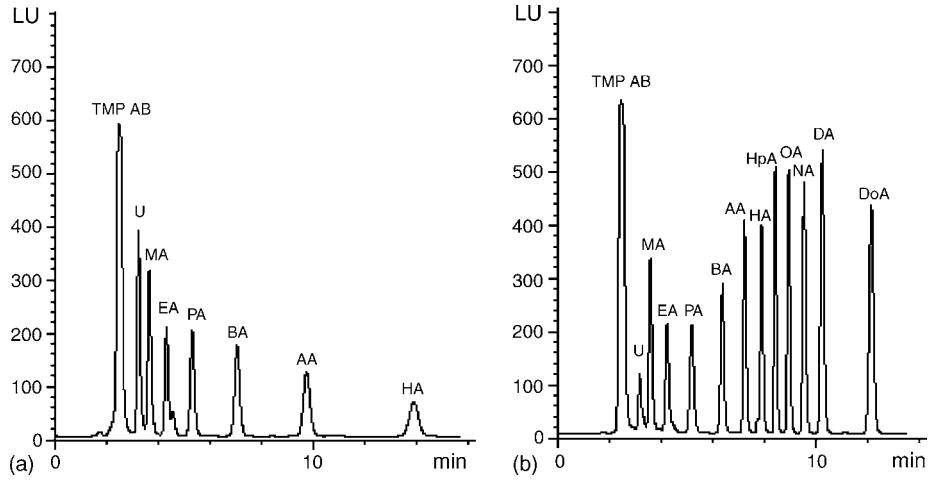


Fig. 2. Chromatograms of TMPAB and amine derivatives on column C_8: (a) by isocratic elution, mobile phase: methanol–water (76/24, v/v) and (b) by gradient elution. Gradient program is shown in Section 2.6. Detection: fluorescence excitation wavelength λ_{ex} = 497 nm, emission wavelength λ_{em} = 509 nm; flow rate: 1 ml/min; injection volume: 20 μl; standard amines concentration: 1 μmol/1; peaks: (a) TMPAB; u (unknown); MA (methylamine); EA (ethylamine); PA (*n*-propylamine); BA (*n*-butylamine); AA (*n*-amylamine); HA (*n*-hexylamine); (b) TMPAB; u (unknown); MA (methylamine); EA (ethylamine); PA (*n*-propylamine); BA (*n*-butylamine); AA (*n*-amylamine); HA (*n*-hexylamine); HpA (*n*-heptylamine); OA (*n*-octylamine); NA (*n*-nonylamine); DA (*n*-decylaimne); DoA (*n*-dodecylamine).

on the derivatization was investigated, shown in Fig. 3a. An aliquot of a mixture of the amines was reacted with various concentrations of TMPAB-OSu in derivatization procedure as described in Section 2.5. When the concentration of reagent is in the range of 22×10^{-6} to 44×10^{-6} mol/l, the peak areas of derivatives are highest and unchangeable. So, 22×10^{-6} mol/l was selected as the optimal concentration.

The reaction of TMPAB-OSu with amines was also found to be pH dependent. The influence of various pH values on the peak areas was also studied by using borate buffer. The optimum reaction pH was determined by derivatizing each of the 11 amines at pH values ranging from 7.5 to 9.2 and by measuring the fluorescence response for each eluted analyte as a function of reaction pH. From the results shown in Fig. 3b, the peak areas of the derivatives are almost stable at pH 8.5–9.0 and an optimum derivatization pH of 8.8 was selected for all subsequent experiments. Different volume of buffer was further tried. 0.2 ml was found to be the best at pH 8.8.

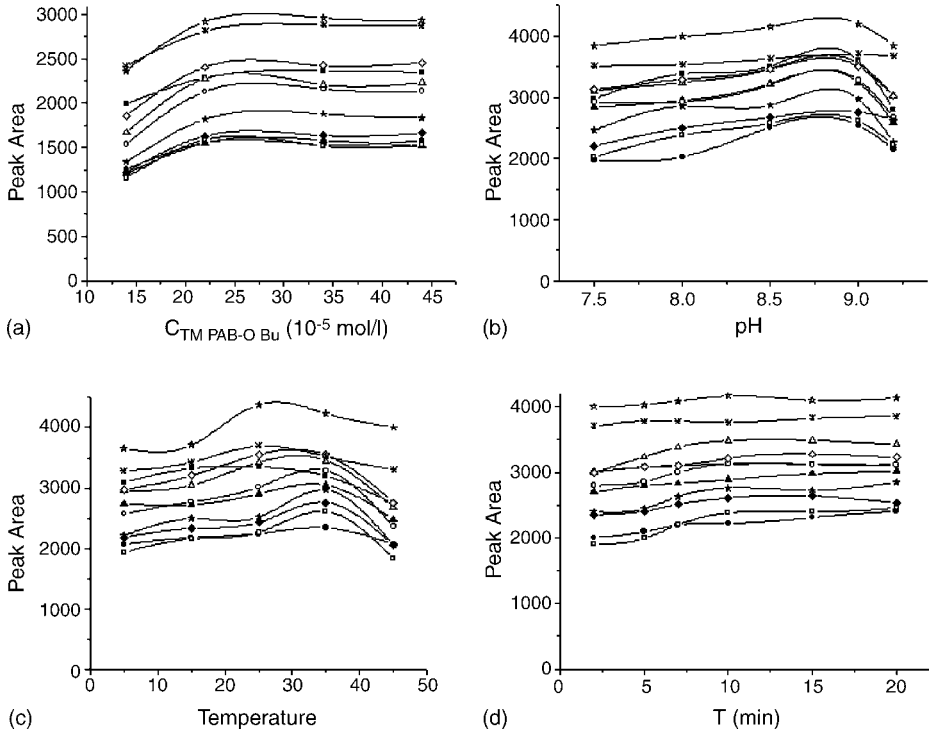


Fig. 3. Effect of (a) TMPAB-OSu concentration; (b) pH value; (c) reaction temperature; (d) reaction time on the peak area of TMPAB-amines derivatives, (■) methylamine; (●) ethylamine; (▲) *n*-propylamine; (♦) *n*-butylamine; (⋆) *n*-amylamine; (□) *n*-hexylamine; (◯) *n*-heptylamine; (△) *n*-octylamine; (◊) *n*-nonylamine; (☆) *n*-decylaimne; (*) *n*-dodecylamine.

Table 1
Linear calibration ranges, regression equations and detection limits of TMPAB-amine derivatives by isocratic elution and gradient elution

TMPAB-amine derivative	Calibration range (μmol/l)	Regression equation, Y	γ	R.S.D. (%) $n=6$, within-day	R.S.D. (%) $n=6$, between-day	Detection limit (nmol/l)[a]
Isocratic elution						
Methylamine	0.001–1.2	$3452.5X+459.7$	0.9993	2.8	3.6	0.1
Ethylamine	0.003–1.2	$2430.8X+54.2$	0.9996	2.3	3.1	0.3
n-Propylamine	0.007–1.2	$2871.4X+128.7$	0.9996	1.2	1.5	0.7
n-Butylamine	0.007–1.2	$2924.5X+86.2$	0.9991	0.8	1.1	0.7
n-Amylamine	0.009–1.2	$3009.9X+168.5$	0.9987	1.9	2.7	0.9
n-Hexylamine	0.009–1.2	$2743.1X+98.7$	0.9980	2.2	2.5	0.9
Gradient elution						
Methylamine	0.001–1.2	$3493.3X+419.3$	0.9991	2.0	1.4	0.1
Ethylamine	0.003–1.2	$2472.1X+45.4$	0.9996	1.3	3.0	0.3
n-Propylamine	0.007–1.2	$2874.1X+123.1$	0.9993	1.5	2.6	0.7
n-Butylamine	0.009–1.2	$2923.9X+89.8$	0.9992	1.8	1.9	0.9
n-Amylamine	0.007–1.2	$3018.5X+160.0$	0.9994	0.9	1.7	0.7
n-Hexylamine	0.007–1.2	$2750.5X+96.4$	0.9991	1.7	2.1	0.7
n-Heptylamine	0.003–1.2	$3423.9X+53.2$	0.9997	1.4	2.7	0.3
n-Octylamine	0.001–1.2	$3566.9X+28.5$	0.9992	2.1	3.1	0.1
n-Nonylamine	0.001–1.2	$3556.9X+6.5$	0.9998	2.3	1.9	0.1
n-Decylaimne	0.001–1.2	$4290.3X+6.5$	0.9994	2.6	2.3	0.1
n-Dodecylamine	0.001–1.2	$4351.4X+2.7$	0.9998	1.1	1.9	0.1

X: concentration of amine (μmol/l); Y: peak area of amine derivatives.
[a] $S/N=3$, per 20 μl injection volume.

Temperature is a very important factor in optimizing the derivatization rate. Therefore, the experiment aimed at the best temperature to achieve the best derivative yield has been performed. Fig. 3c indicates the effect of different temperature on the peak areas of the amine derivatives. Usually, the derivatization is expected to proceed in a short time with satisfying efficiency. In this experiment, the investigation of suitable reaction time was carefully carried out at 30 °C. It was demonstrated that the reaction was completed in 10 min in Fig. 3d. To get reproducible results, the derivatization at 30 °C for 10 min was performed.

Because TMPAB-OSu has relatively poor solubility in water, 100 μl of acetonitrile should be added to the derivatization medium to avoid the precipitation of the reagent and derivatives.

3.5. Analytical quantitation

A test mixture with different concentrations of standard aliphatic amines was prepared and analyzed by using the optimized derivatization procedure and separation conditions for the determination of aliphatic amines. The detection limits of aliphatic amines were calculated as the amounts of aliphatic amines that resulted in a peak three times higher than that of the baseline noise. By using isocratic elution, the linear calibration ranges, regression equations, and detection limits of aliphatic amines were calculated and the results were listed in Table 1. The correlation coefficients for these aliphatic amines are from 0.9980 to 0.9996. The R.S.D. for the BODIPY derivatives is from 0.8 to 2.8% for within-day determination ($n=6$) and from 1.1 to 3.6% for between-day determination ($n=6$). By using gradient elution, the linear calibration ranges, regression equations, and detection limits of aliphatic amines were calculated and the results were listed in Table 1. The correlation coefficients for these aliphatic amines are from 0.9991 to 0.9998. The R.S.D. for the BODIPY derivatives is from 0.9 to 2.6% for within-day determination ($n=6$) and from 1.4 to 3.1% for between-day determination ($n=6$). The detection limits for the labeled amines range from 2 fmol for methylamine to 18 fmol for *n*-amylamine. It was shown that the quantification of aliphatic amines could be well done with this method. The overall comparison of TMPAB-OSu with other common-used fluorescent labeling reagents for aliphatic amines in HPLC is given in Table 2. The detection limits of most reagents were higher than TMPAB-OSu, except SIFA. However, SIFA

Table 2
Comparison of the derivatization conditions and detection limit of the reagents reported for aliphatic amines

Reagent	Ex (nm)/Em (nm)	Derivative time (min)	Derivative temperature	Detection limit	Reference
OPA	340/455	1	Room temperature	2.1 pmol	[31]
FMOC-Cl	265/310	2.5	Room temperature	9 pmol	[16]
NBD-Cl	470/530	60	55	1.7 pmol	[31]
AQC	250/395	5	65	1 pmol	[32]
Dns-Cl	350/530	10	40	1.4 pmol	[31]
SIFA	488/516	30	45	85 amol	[18]
TMPAB-OSu	497/509	10	30	2 fmol	This paper

has the intrinsic limitations of fluorescein fluorephore and the labeling procedure is time consuming. Considering the detection properties (such as detection wavelength, derivatization time and temperature, and detection limits) in the determination of amines, TMPAB-OSu is more advantageous than other reagents.

3.6. Potential of TMPAB-OSu in the determination of secondary aliphatic amines with HPLC

Not all of the activated esters can react with secondary aliphatic amines to form stable derivatives [28]. Therefore, the possibility of TMPAB-OSu used in the determination of secondary aliphatic amines with HPLC has also been evaluated. The result showed that dimethylamine and dibutylamine can reacted with TMPAB-OSu to form stable derivatives in 0.2 mol/l borate buffer (pH 8.8) at 30 °C within 10 min. The separation of dimethylamine and dibutylamine derivative with TMPAB-OSu was given in Fig. 4. Further studies on the application of TMPAB-OSu in the determination of secondary aliphatic amines with HPLC are in progress.

3.7. Application to sample analysis

It is known that the urinary excretion of methylamine is enhanced in particular physiological conditions that are char-

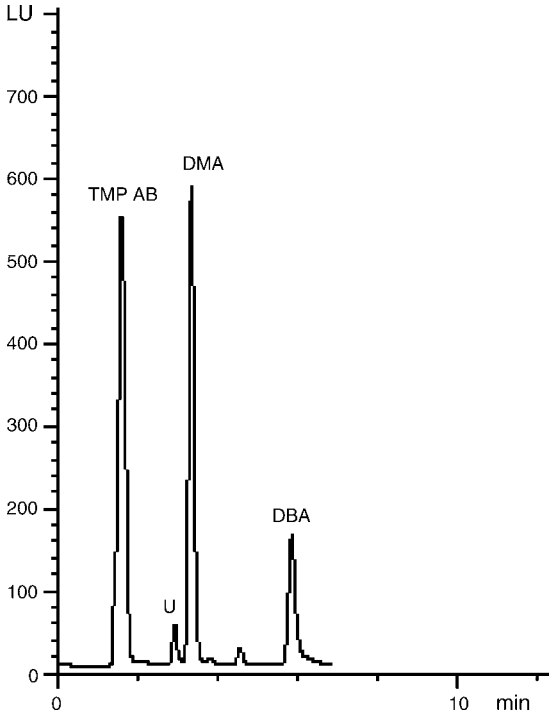


Fig. 4. Chromatograms of TMPAB and secondary aliphatic amine derivatives on column C_8. Detection: fluorescence (497/509 nm). Mobile phase: methanol–water (76/24, v/v). Flow rate: 1 ml/min. Injection volume: 20 μl. Peaks: TMPAB; u (unknown); DMA (dimethylamine); DBA (dibutylamine).

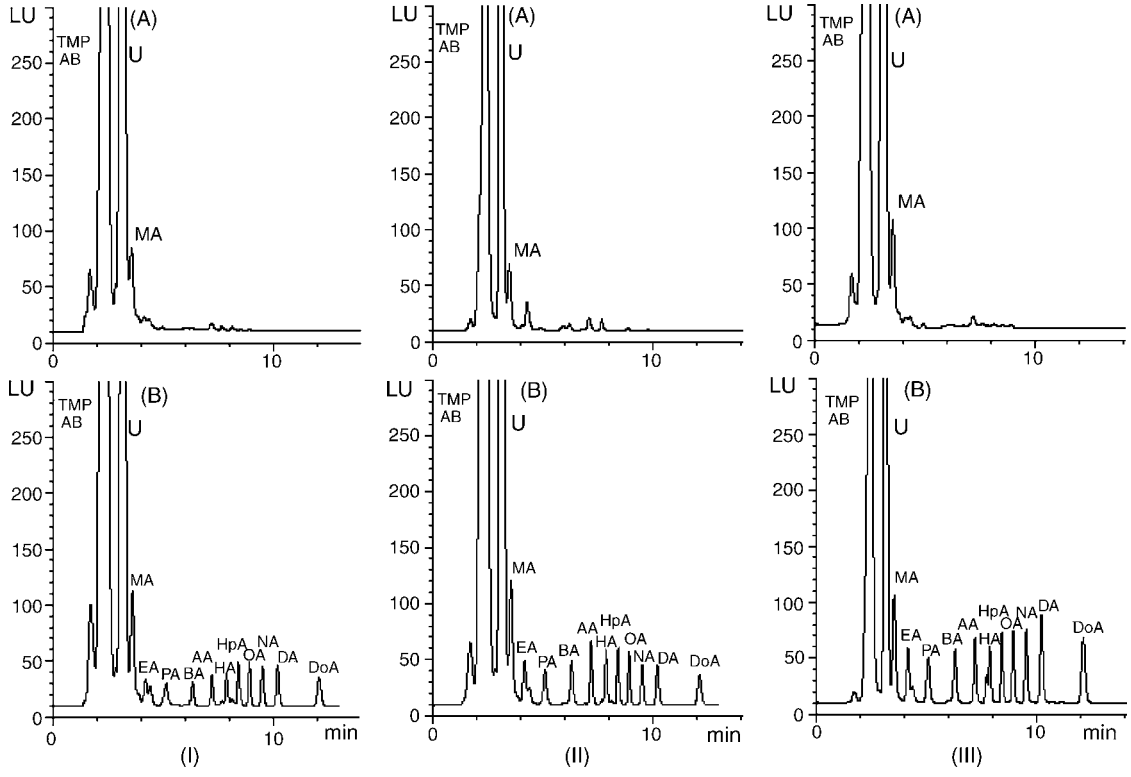


Fig. 5. Chromatograms obtained from samples. Chromatographic conditions as in Fig. 2. Peaks: TMPAB; u (unknown); MA (methylamine); EA (ethylamine); PA (*n*-propylamine); BA (*n*-butylamine); AA (*n*-amylamine); HA (*n*-hexylamine); HpA (*n*-heptylamine); OA (*n*-octylamine); NA (*n*-nonylamine); DA (*n*-decylaimne); DoA (*n*-dodecylamine). (I) Chromatograms obtained from (A) lake water; (B) the same sample spiked with 0.1 μmol/l of standard amines; (II) chromatograms obtained from (A) urine of health; (B) the same sample spiked with 0.2 μmol/l of standard amines; (III) chromatograms obtained from (A) urine of hepatic patient; (B) the same sample spiked with 0.2 μmol/l of standard amines.

Table 3
Analytical results of urine samples

Samples	Urine of health (average)				Urine of hepatic patient (average)				Urine of urine patient (average)			
	Added (μg/l)	Found (μg/l)	R.S.D. (%, $n=6$)	Recovery (%)	Added (μg/l)	Found (μg/l)	R.S.D. (%, $n=6$)	Recovery (%)	Added (μg/l)	Found (μg/l)	R.S.D. (%, $n=6$)	Recovery (%)
Methylamine	0	13.18	0.6		0	17.05	2.6		0	12.4	3.3	
	3.1	16.17	1.4	96	3.1	20.1	1.4	98	3.1	15.6	1.2	103
	6.2	19.2	1.3	97	6.2	23.4	1.3	102	6.2	18.8	1.7	103
Ethylamine	0	0			0	0			0	0		
	4.5	4.6	1.5	102	4.5	4.3	2.7	96	4.5	4.7	0.5	104
	9.0	9.4	2.6	104	9.0	8.8	0.6	98	9.0	8.7	1.1	97
n-Propylamine	0	0			0	0			0	0		
	5.9	6.1	0.9	103	5.9	5.7	1.2	97	5.9	5.8	1.8	98
	11.8	12.0	1.3	101	11.8	11.4	1.5	97	11.8	11.7	1.3	99
n-Butylamine	0	0			0	0			0	0		
	7.3	7.6	2.1	104	7.3	7.6	2.4	104	7.3	7.1	2.4	97
	14.6	14.3	2.4	98	14.6	14.5	0.9	99	14.6	14.3	1.6	98
n-Amylamine	0	0			0	0			0	0		
	8.7	8.4	0.7	96	8.7	8.5	0.7	98	8.7	8.4	1.5	97
	17.4	17.7	0.5	102	17.4	17.1	1.2	98	17.4	17.3	1.9	99
n-Hexylamine	0	0			0	0			0	0		
	10.1	10	1.1	99	10.1	10.3	1.1	102	10.1	9.9	2.0	98
	20.2	20.5	1.9	101	20.2	19.9	1.6	99	20.2	20.1	1.4	100
n-Heptylamine	0	0			0	0			0	0		
	11.5	11.9	0.8	103	11.5	11.3	1.9	98	11.5	11.1	2.8	97
	23	22.5	1.7	98	23	23.3	2.0	101	23	22.8	2.2	99
n-Octylamine	0	0			0	0			0	0		
	12.9	13.4	2.6	104	12.9	12.7	0.4	98	12.9	12.6	0.7	98
	25.8	24.9	2.1	97	25.8	25.5	1.1	99	25.8	26.1	2.9	101
n-Nonylamine	0	0			0	0			0	0		
	14.3	14.1	1.1	99	14.3	14.7	1.7	103	14.3	14.1	1.1	99
	28.6	28.1	1.3	98	28.6	28.3	0.3	99	28.6	28.5	1.8	100
N-Decylaimne	0	0			0	0			0	0		
	15.7	15.3	0.9	97	15.7	15.2	1.1	97	15.7	15.9	0.7	101
	31.4		1.4		31.4	31.1	1.9	99	31.4	31.7	0.9	101
n-Dodecylamine	0	0			0	0			0	0		
	18.5	18.7	2.5	101	18.5	18.2	3.0	98	18.5	18.1	2.1	98
	37	36.4	0.7	98	37	38	3.2	103	37	36.7	1.6	99

acterized by an increase in creatine elimination, including pregnancy, parturition and muscular exertion. It has also been reported that methylamine and other related short-chain aliphatic amines may play a significant role during hepatic and renal disease [29] and that the amine increases in hepatic or renal insufficiency is suspected of generating a variety of central or peripheral effects [30]. Therefore, the proposed method was applied to the determination of aliphatic amines in urine samples of health and patients with hepatic and renal.

Except for aliphatic amines, there are some amino acids being in existence in urine. To exclude the interference of amino acids in sample, the retention times of familiar amino acids derivatives were investigated. It was found that under the selected chromatographic condition, the peaks of amino acid derivatives and that of TMPAB overlapped. That is, the retention times of aliphatic amines were much longer than those of amino acids. Therefore, amino acids have no interference with the analysis. The chromatograms of urine samples unspiked and spiked with standard solutions are shown in Fig. 5. The analytical results are summarized in Table 3. It was found that the content of methylamine in urine samples of patients with hepatic was more than in health. No distinct increase in urine samples of patients with renal was found. The recoveries ranged from 96 to 104% and the R.S.D. from 0.3 to 3.3%.

The reliability of the proposed method was further evaluated by applying it to the determination of aliphatic amines in lake water sample. The results are given in Table 4.

The fluorescence intensity in different real samples was also studied and the result indicated that the fluorescence properties of TMPAB-OSu and -amine derivatives are insensitive to environment in accordance with the conclusion in sections before.

Table 4
Analytical results of lake water samples

Samples	Lake water			
	Added (μg/l)	Found (μg/l)	R.S.D. (%, $n=6$)	Recovery (%)
Methylamine	0	5.04	1.3	
	3.1	8.3	2.5	105
	6.2	11.1	1.1	98
Ethylamine	0	0		
	4.5	4.6	2.4	102
	9.0	8.9	1.7	99
n-Propylamine	0	0		
	5.9	6.1	0.8	103
	11.8	11.3	1.6	96
n-Butylamine	0	0		
	7.3	7.4	2.0	101
	14.6	14.3	3.2	98
n-Amylamine	0	0		
	8.7	8.4	1.4	97
	17.4	17.5	1.3	101
n-Hexylamine	0	0		
	10.1	9.9	0.9	98
	20.2	20.4	1.2	101
n-Heptylamine	0	0		
	11.5	11.3	1.1	98
	23	22.7	3.0	99
n-Octylamine	0	0		
	12.9	12.7	0.7	98
	25.8	26.1	1.9	101
n-Nonylamine	0	0		
	14.3	14.6	0.8	102
	28.6	29	2.2	101
n-Decylaimne	0	0		
	15.7	15.6	1.3	99
	31.4	32.3	0.5	103
n-Dodecylamine	0	0		
	18.5	18	1.9	97
	37	37.8	2.4	102

4. Conclusions

BODIPY is a well-known chromophore that is highly fluorescent in extensive range of pH, but it has not been utilized in fluorescence derivatization reagent to HPLC. This work was focused on the development of a new BODIPY-based fluorescence probe with *N*-hydroxysuccinimidyl ester for determination of amines in biological samples. We succeeded in developing TMPAB-OSu as a sensitive probe based on the following advantages: (1) high extinction coefficient and fluorescence quantum yield, which can improve the detection limits, (2) excellent photostability, TMPAB-OSu-derivatized aliphatic amines are stable, (3) long detection wavelengths, at which interference such as some substances that fluoresce in biological samples are avoided, (4) tolerating a wide pH range, which makes it can be more suitable for acidic environment especially compared to fluorescein-based reagents, such as FITC and SAMF [21].

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