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RESEARCH PAPER

Dual-Sensitive Probe of 2-(Benzoacridin)ethyl-imidazole-1-carboxylate for Determination of Aliphatic Amine with Fluorescence and Online Atmospheric Pressure Chemical Ionization Mass Spectrometry Identification

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Abstract: Using three different derivatization methods to mark the amine compounds, we compared the difference of derivatization efficiency and gave the optimal method. 5-(2-Hydroxyethyl)benzoacridine reacts with coupling agent *N*,*N*'-carbonyldiimidazole (CDI) to form an activated amide intermediate 2-(benzoacridin)ethyl-imidazole-1-carboxylate (BAEIC). BAEIC, which is dual-sensitive probe, reacts preferably with amino compounds at 80 ºC in the presence of 4-dimethylaminopyridine (DMAP) catalyst in *N*,*N*-dimethylformamide (DMF) solvent to give the corresponding sensitively fluorescent derivatives with an excitation maximum at 280 nm of λ_{ex} and an emission maximum at 510 nm of λ_{em}. BAEIC-amine derivatives simultaneously exhibited high-ionization potential with percent ionization δ changing from 5.62% to 58.08% in aqueous acetonitrile and from 2.14% to 56.58% in aqueous methanol. Derivatives are not only sensitive to fluorescence but also to MS ionizable potential. The fluorescence detection limits are 0.12–0.59 ng mL^{-1} (at a signal-to-noise ratio of 3). The online Atmospheric Pressure Chemical Ionization Mass Spectrometry (APCI-MS) detection limits are 1.89–14.12 ng mL^{-1} (at a signal-to-noise ratio of 5).

Key Words: High performance liquid chromatography/mass spectrometry; Aliphatic amines; 5-(2-Hydroxyethyl)benzoacridine; 2-(Benzoacridin)ethyl-imidazole-1-carboxylate

1 Introduction

The mass spectrum and the fluorescence technology are extensively used in environmental science, toxicology, and biological sciences to investigate the structure and dynamics of living systems[1–3]. It is well known that amines in environment may occur as biodegradation products of organic matter such as proteins, amino acids, and other nitrogen containing organic compounds. Most of the amine compounds may react with nitrosating agents, leading to the formation of potentially carcinogenic *N*-nitrosamine compounds[4–8]. Therefore, it has an important meaning to determine certain amino compounds in real environmental samples. At present, gas chromatography[9] and ion-exchange chromatographic detection[10] are frequently used to determine amines. Although these methods are simple and feasible, there are usually limitations in application because of low sensitivity. Therefore, chemical derivatization is necessary to increase detection sensitivity and improve selectivity by precolumn or postcolumn high performance liquid chromatography (HPLC) derivatization methods. In spite of the diversity of these fluorescent derivatizing reagents[10–14], there are various shortcomings in application. For example,

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derivatizing with common fluorescent reagent FMOC-Cl, excessive reagent easily causes lower column efficiency and puts up weaker fluorescence to proline and cysteine derivatives, and accurate quantification is difficult.

In our previous studies[15–18], several fluorescent reagents and their applications for common amine analysis were described. These reagents exhibited very high fluorescence sensitivities; however, they exhibit relatively low-ionization potential in APCI-MS and were great disadvantage to online highly sensitive MS identification. The principal purpose of this study was to develop a novel dual-sensitive probe for derivatization of amines with fluorescence detection coupled with online sensitive MS identification. The results indicated that 2-(benzoacridin)ethyl-imidazole-1-carboxylate (BAEIC) could react rapidly with amines to give the sensitive BAEIC-amine derivatives, which not only exhibited high fluorescence but also excellent MS ionizable potential.

2 Experimental

2.1 Instruments and reagents

Agilent 1100 Series LC/MSD-Trap-SL ion trap liquid chromatography/mass spectrometry (Agilent Company, USA) was used. All the HPLC system devices were from the HP 1100 series and consisted of a quaternary pump (model G1311A), a vacuum degasser (model G1322A), a fluorescence detector (model G1321A), an autosampler (model G1329A), and APCI source. The semipreparative HPLC system was Waters Delta 600 (Waters Company, USA). F-7000 fluorescence spectrophotometer (Hitachi Company, Japan) was used. Twelve standard aliphatic amine samples were obtained from Sigma Corporation. Chromatographically pure acetonitrile was obtained from Yuwang Company, China. We prepared 5-(2-hydroxyethyl) benzoacridine (HBA) and BAEIC. Others like *N,N'*- carbonyldiimidazole (CDI) and 4-dimethylaminopyridine (DMAP) were all analytical reagent.

2.2 Preparation of stand solutions

Standard solution (0.1 M) of twelve aliphatic amines was prepared by the dilution of each amine with acetonitrile. The corresponding low concentration of mixed amines (2.0 mM) was diluted by *N,N*-dimethylformamide (DMF). The fluorescent reagent solutions, approximate 0.04 M, were prepared by dissolving 0.1156 g of HBA and 0.1532 g of BAEIC in 10 mL of DMF. Standard solutions of CDI (10 g L^{-1}) and DMAP (20 g L^{-1}) were, respectively, prepared by dissolving 0.1 g of CDI and 0.2 g of DMAP in 10 mL of DMF.

2.3 Chromatographic method and mass spectra condition

The chromatographic and spectral conditions were as follows: Akasil C_{18} column (200 mm × 4.6 mm i.d., 5 μm); mobile phase A: 30% acetonitrile (containing 20 mM, pH 3.74 ammonium/formic acid buffer); mobile phase B: 100% acetonitrile; gradient conditions: mobile phase B is changed from 10% to 100% in 30 min and then was kept in 100% for 10 min. The flow rate was constant at 1.0 mL min^{-1}, and the column temperature was set at 30 ºC. The fluorescence excitation and emission wavelengths were set at λ_{ex} = 274 and λ_{em} = 510 nm, respectively. Mass spectra conditions were as follows: atmosphere pressure chemistry ionization source, APCI (in positive mode) ion source, spray pressure of 413 kPa, dry gas flow of 5.0 L min^{-1}, dry gas temperature of 350 ºC, APCI Vap temperature of 450 ºC, capillary voltage of 3500 V, and Corona Current (nA) of 4000 (pos).

2.4 Derivatization

The derivatization procedures were carried out by three methods: A, one step; B, two steps; C, intermediate reaction.

A: To a vial, 200 μL of HBA, 200 μL of DMAP, 350 μL of CDI, and 210 of μL amines standard solution were added successively. The vial was sealed and heated at 80 ºC in water bath for 60 min, and then, a 10 μL of mixture was diluted to 200 μL with acetonitrile and injected onto the chromatograph.

B: To a vial, 200 μL of HBA, 200 μL of DMAP, and 350 of μL CDI were added successively. This mixture was allowed to react at 60 ºC for 20 min to form an amidated intermediate. Subsequently, 210 μL of amines was added and heated at 80 ºC for 60 min. A 10 μL of the mixture was diluted to 200 μL with acetonitrile and injected onto the chromatograph.

C: To a vial, 200 μL of BAEIC, 200 μL of DMAP, 350 μL of DMF, and 210 μL of amines standard solution were added successively. The vial was sealed and heated at 80 ºC in water bath for 60 min, and then, a 10 μL of mixture was diluted to 200 μL with acetonitrile and injected onto the chromatograph.

2.5 Preparation of water samples

Sewage sample was taken, and then, the acidity was adjusted to pH 3.0 with HCl. The solution was ultrasonically treated for 1 min to convert aliphatic amines to organic salts, followed by filtration. A total of 150 mL of filtrates was taken and evaporated under reduced pressure until dryness. The resulting residue was redissolved in 5 mL of aqueous acetonitrile (80%, *V*/*V*) and adjusted to alkalescence with 0.1 M of NaOH and stored at 4 ºC until analysis.

2.6 Estimation of percent ionization potential

The BAEIC core structure exhibits a rigid plane, which contains nitrogen and carbonyl oxygen atoms. It is the fact that nitrogen and carbonyl are in contraposition, which results

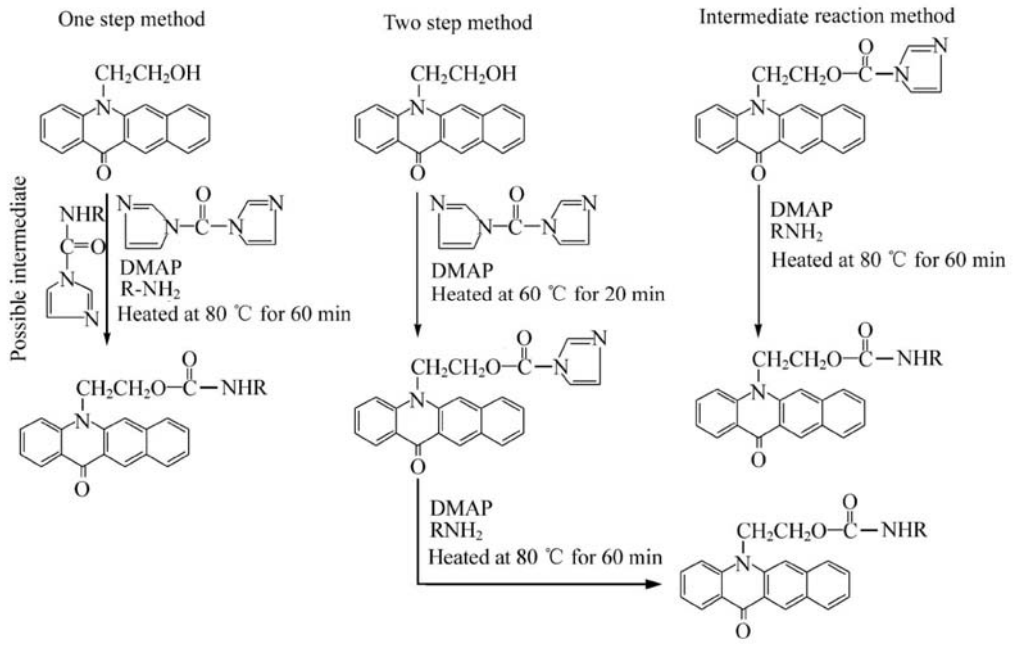

Fig.1 Derivatization scheme of methods A, B, and C

HBA, 5-(2-hydroxyethyl)benzoacridine; BAEIC, 2-(benzoacridin)ethyl- imidazole-1-carboxylate; and DMAP, 4-dimethylamiopyridine

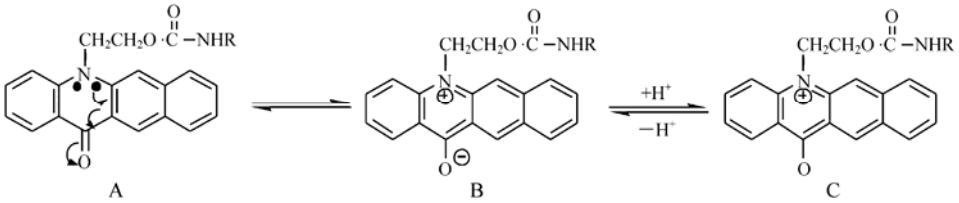

Fig.2 Isomerization mechanism of 2-(benzoacridin)ethyl- imidazole-1-carboxylate derivatives in aqueous acetonitrile

in a strong intramolecular isomerization easily. The procedure is shown in Fig.2.

Isomer **A** can quickly interconvert by the movement of a lone-pair electron from nitrogen atom to oxygen atom and results in the formation of a zwitterion **B**. The zwitterion **B** contains an phenoxide ion that provides a negative-charged oxygen atom and can be accepted a $[H]^+$ proton to form corresponding quaternary ammonium ion **C** and results in the corresponding fluorescence quenching. The quenching degree is related to the concentration of proton donor (H_2O) in system. Based on the fluorescence quenching, the ionization potential (δ) of the fluorescent molecular **A** can be easily obtained. When compound **A** is dissolved in aqueous organic system, the chemical equilibrium can be expressed as follows:

$$A + E \leftrightarrow AE \quad (1)$$

where, A is the probe, E is the proton-solvent (H_2O), and AE is the product. The quenching constant is given by

$$K = [AE]/([A]\bullet[E]) \quad (2)$$

where, [A], [E], and [AE] are the equilibrium concentrations. According to the law of conservation of mass, the percent ionization δ value can be defined as follows:

$$\delta = [AE]/[A]_0 = [AE]/([A] + [AE]) \quad (3)$$

Supposed initial concentration of unionized **A** is $[A]_0$, fluorescence intensity is F_0, the relationship between the fluorescence intensity and the concentration of **A** can be expressed as

$$F_0 = K'\varphi_f[A]_0 \quad (4)$$

where, K' is the constant depended on the relative parameters of instruments and φ_f is quantum yield of **A**. After **A** is partially ionized, the fluorescence intensity of unionized **A** is F, and in this case, the fluorescence intensity F can be expressed as:

$$F = K'\varphi_f(1 - \delta)[A]_0 \quad (5)$$

By combining Eqs. (4) and (5), we obtain

$$(F_0 - F)/F = K'\delta[A]_0/K'(1 - \delta)[A]_0 \quad (6)$$

Eq.(6) can be reorganized into

$$(F_0 - F)/F = -\delta/(1 - \delta) \quad (7)$$

By combining Eqs. (2), (3) and (7), we obtain

$$(F_0 - F)/F = K[E] \quad (8)$$

Where, [E] is the equilibrium concentration and sufficiently greater than A or AE**,** and thus, [E] ≈ C_E, C_E is the initial concentration of water in acetonitrile and expressed as molar concentration so that

$$(F_0 - F)/F = KC_E \quad (9)$$

Substituting $(F_0 - F)/F$ into Eq.(7) by Eq.(9) yields

$$\delta = KC_E/(1 + KC_E) \quad (10)$$

Thus, the percent ionization potential δ value in the presence of varying amounts of water can be calculated by Eq.(10) by combining each quenching constant K and the added molar concentration C_E of water.

3 Results and discussion

3.1 Fluorescence spectrum properties

At constant temperature, the fluorescence intensity of BAEIC-decylamine derivative decreases with the increases of the concentration of proton donor. The main reason is that the formation of quaternary ammonium ion by the protonation of probe molecular and proton donor results in the decrease in fluorescence intensity. The relative fluorescence intensity and emission wavelengths of BAEIC-decylamine in different amount of aqueous acetonitrile or methanol are shown in Table 1. The results indicate that the fluorescence intensity reduces gradually and emission wavelength presents significant red-shift following with the increase of proton donor. With acetonitrile as solvent, the red-shift is 39 nm, and with methanol as solvent, the red-shift is 13 nm.

Theoretically, the δ value versus the added volume percentage of water has a linear relationship. With aqueous acetonitrile as solvent, the percent ionization δ values versus the added volume percent of water showed a good linear relationship in the range 0–70% of water ($\delta = 2.77 + 0.2414v$, $r = 0.9921$, v: the added volume percent of water). However, the addition of progressively increasing amounts of water beyond 70% resulted in a deflective of curve. This result was also similar to the case of using aqueous methanol. In aqueous methanol, it also presented a good linear relationship in the range 0–40% of water ($\delta = 0.30 + 0.17996v$, $r = 0.9896$, v: the added volume percent of water). The estimation of percent ionization δ values revealed that the probe molecule provided the desired ionization efficiency in commonly used aqueous acetonitrile or methanol, which was usually used in LC-MS separation as mobile phase composition.

3.2 Chromatographic separation

The reaction of HBA (BAEIC) and aliphatic amines had different derivatization yield along with different catalyst, solvent, temperature, time, and the amount of CDI. In the experiment, octylamine, nonylamine, decylamine, undecylamine and dodecylamine were used as testing standard compounds to optimize the above conditions. The results indicated that the highest derivatization yield was obtained at 80 ºC in 60 min, with DMAP as catalyst and DMF as cosolvent. Under the optimized conditions, we inspected the influence of three different derivatization methods to yield. The experiments showed that method A gave the lowest fluorescence response, method C gave the highest one, and method B was a little lower than C (intensity decreased approximately 25%) (Figs.3, A, B and C). It was possible that amino functional groups were superior to hydroxyl groups from HBA molecules when reacting with CDI. This procedure probably formed another intermediate 1*H*-imidazol-1-yl-methanamide (ACDI). Subsequently, the nucleophilic substitution reaction of ACDI with HBA was a slow reaction under the reaction conditions proposed, which resulted in exceedingly low detection responses for amine derivatives. Method C gave the highest fluorescence responses; it was probably because method C was a rapid nucleophilic substitution. Although method B adopted two steps to form intermediate BAEIC first, amines reacted with the excess amount of CDI to give, in part, the intermediate ACDI as described in method A. Therefore, method B was lower than method A. Moreover, when a small quantity of water presented in derivatization solution, peak height of unreacted BAEIC was significantly reduced and the corresponding HBA and $(HBA)_2$ increased (Fig.3D).

Table 1 Fluorescence intensity of BAEIC-decylamine in presence of varying amount of water and corresponding calculated percent ionization δ values

Solvent	Acetonitrile				Methanol			
H_2O (%, *V/V*)	Emission (nm)	Relative intensity	Quenching constant K ($\times 10^{-2}$)	Percent ionization δ(%)	Emission (nm)	Relative intensity	Quenching constant K ($\times 10^{-2}$)	Percent ionization δ(%)
100	523	425	2.49	58.08	523	425	2.34	56.58
90	520	586	1.46	42.21	522	601	1.25	38.61
80	517	735	0.85	27.51	520	684	0.97	30.13
70	515	819	0.61	19.23	519	758	0.75	22.57
60	514	830	0.66	18.15	517	797	0.68	18.59
50	510	870	0.59	14.20	516	871	0.45	11.03
40	510	885	0.65	12.72	513	903	0.38	7.76
30	507	909	0.69	10.35	513	928	0.33	5.21
20	506	946	0.64	6.70	512	939	0.38	4.08
10	501	957	1.07	5.62	511	958	0.39	2.14
0	484	1014			510	979		

Final concentration of BAEIC-decylamine in each aqueous acetonitrile or methanol is kept at 1.0 μM; F_0 was measured in 100% acetonitrile or methanol. K values were calculated according to the Eq.9; δ values were calculated according to Eq.10 in each corresponding K value obtained.

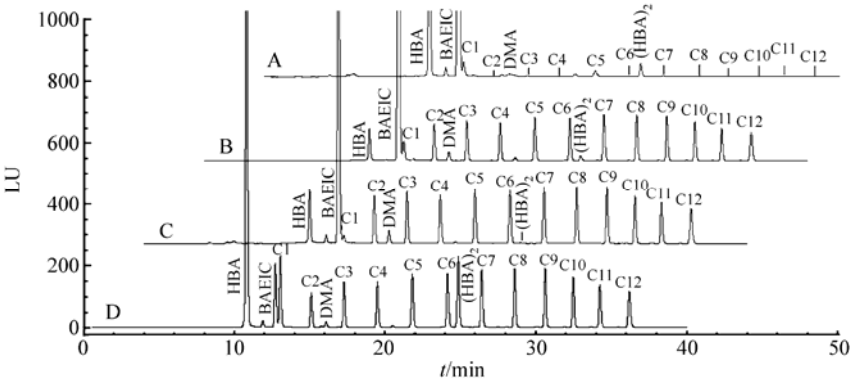

Fig.3 Chromatograms for standard aliphatic amine derivatization: A, one-step method; B, two-step method; C, intermediate reaction; and D, derivatization in the presence of a small of quantity of water

Peaks: C1, methylamine; C2, ethylamine; C3, propylamine; C4, butylamine; C5, pentylamine; C6, hexylamine; C7, heptylamine; C8, octylamine; C9, nonylamine; C10, decylamine; C11, undecylamine; C12, dodecylamine; DMY, dimethylamine; HBA, 5-(2-hydroxyethyl)benzoacridine; BAEIC, 2-(benzoacridin)ethyl-imidazole-1-carboxylate; $(HBA)_2$, bis-(2-hydroxyethyl)benzoacridine)-carbonate

This should be attributed to the reagent hydrolysis to give HBA and $(HBA)_2$ (*m/z* 605.6), which formed by the reaction of hydrolysated HBA with the excess reagent BAEIC. It should be noted that a small peak DMA (*m/z* 361.7) derived from dimethylamine is constantly observed for all derivatization. It is possible that DMF, in part, is hydrolyzed to dimethylamine.

3.3 Mass spectra identification

The MS and MS/MS data of the characteristic fragment ion peak that are produced by all BAEIC-amines derivatives are shown in Table 2. The MS, MS/MS spectra, and cleave mode of representative C10-amine derivative are shown in Fig.4.

3.4 Linear regression equations, detection limits, and reproducibility

The calibration graphs of amines compounds are drawn based on the peak area and injection amount within the limit of 21 fmol–43 pmol by derivatization procedure C. Linear regression equations, correlation coefficients, linear range, and detection limits (at a signal-to-noise ratio of 3) are shown in Table 2. A standard solution consisting of 46 pmol of each aliphatic amine is analyzed six times to determine the reproducibility of the method. Relative standard deviations of retention time and peak area are 0.052%–0.083% and 1.94%–2.49%, respectively.

3.5 Sample analysis

Chromatogram for the analysis of free amines from Yihe River in Qufu is shown in Fig.5. In the water sample, a known amount of aliphatic amine (0.5 mM) was added, and then, the mixture was treated and derivatized according to experiment method. The amount and recoveries of aliphatic amines are shown in Table 3.

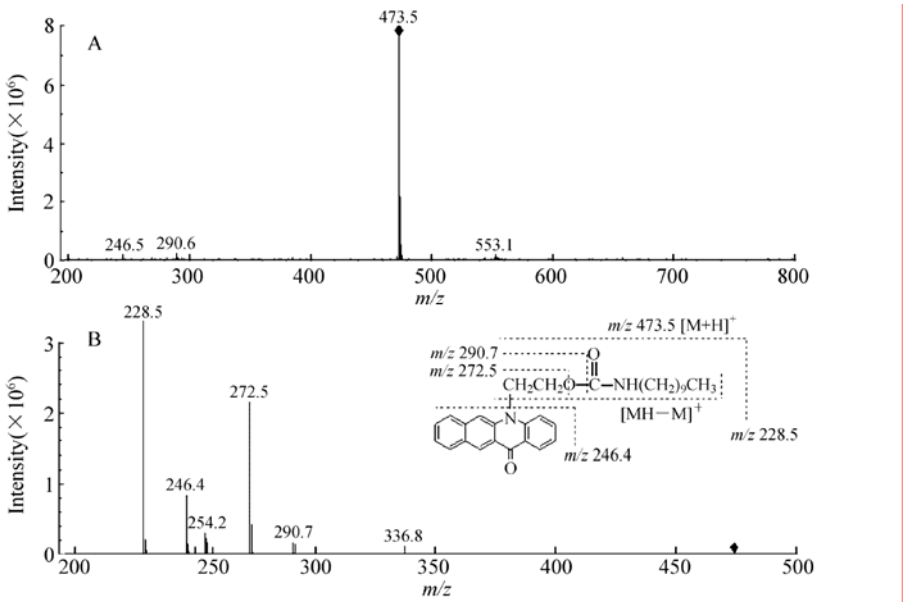

Fig.4 APCI-MS/MS spectra of representative decylamine derivative: A, MS spectra and B, MS/MS spectra

Table 2 Linear regression equations, correlation coefficients, detection limits, linear range, and MS data of amine derivatives

Amine derivatives	Regression equation	Correlation coefficient	Detection limits LOD (fmol)	Linear range (pmol)	MS $(M+H)^+$	MS/MS	APCI-MS Detection limits (fmol)
C1	$Y = 2.417270 + 6.626650X$	0.9991	79.0	0.336– 10.75	347.7	290.7, 272.6, 246.4	609.7
C2	$Y = 5.506290 + 33.09517X$	0.9992	17.4	0.084–10.75	361.7	290.7, 272.6, 246.4	544.4
C3	$Y = 2.578320 + 37.58286X$	0.9999	28.7	0.084–10.75	375.6	290.7, 272.6, 246.4	825.4
C4	$Y = 2.150190 + 35.03495X$	0.9998	28.6	0.084–10.75	389.6	290.7, 272.6, 246.4	731.5
C5	$Y = 3.742540 + 39.59094X$	0.9999	24.7	0.084–10.75	403.6	290.6, 272.5, 246.4	772.4
C6	$Y = 5.057960 + 39.26561X$	0.9998	20.3	0.168–10.75	417.5	290.5, 272.5, 246.4	726.7
C7	$Y = 12.02493 + 41.12445X$	0.9993	8.60	0.084–10.75	431.5	290.5, 272.5,246.4	708.7
C8	$Y = 2.690940 + 40.52561X$	0.9999	19.2	0.084–10.75	445.5	290.5, 272.5,246.4	795.3
C9	$Y = 6.698310 + 39.81492X$	0.9999	8.70	0.168–10.75	459.5	290.5, 272.5, 246.4	794.4
C10	$Y = 3.618940 + 33.64824X$	0.9998	23.1	0.168–10.75	473.5	290.6, 272.5, 246.4	765.6
C11	$Y = 2.522100 + 29.78792X$	0.9998	34.6	0.084–10.75	487.5	290.5, 272.5, 246.4	774.3
C12	$Y = 1.677860 + 30.71303X$	0.9999	24.7	0.168–10.75	501.6	290.3, 272.5, 246.4	763.2

Y: peak area; *X*: injected amount (pmol); linear ranges of APCI-MS were not tested.

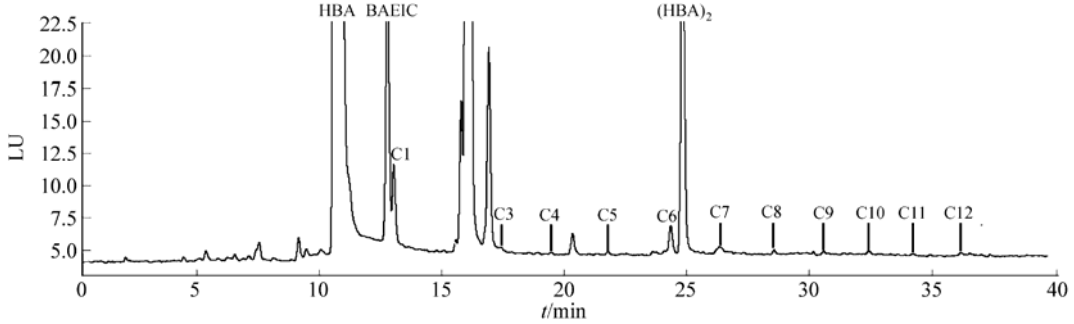

Fig.5 Chromatogram of aliphatic amine from water in Yihe River

Table 3 Content and recoveries of aliphatic amines from water in Yihe River

Amine derivatives	Amine in river (ng ml^{-1})	MS identify**	Recoveries (%)	Amine derivatives	Amine in river (ng ml^{-1})	MS identify**	Recoveries (%)
C1	2.558	Yes	99.3	C7	0.259	Yes	100.8
C2	*		100.1	C8	0.121	Yes	99.9
C3	0.075	Yes	98.5	C9	0.015		103.1
C4	0.049	Yes	101.4	C10	0.013		101.2
C5	0.067	Yes	102.2	C11	0.051	Yes	98.7
C6	0.364	Yes	103.6	C12	0.152	Yes	100.5

* C2 was not determined owing to co-eluting with uncomponent); ** component was appraised by the online mass spectrum simultaneously.

4 Conclusions

A reagent HBA and a new oxygen and nitrogen-containing, dual-sensitive probe BAEIC were used for marking amines compounds. The difference of three different derivatization methods to yield was compared. The results indicated that the reaction of intermediate BAEIC and amines had the highest derivatization yield. The obtained derivatives not only gave high fluorescence sensitivity but also exist strong MS ionizable potential. The proposed method showed wide linearity range, excellent reproducibility, and simple derivatization for the determination of aliphatic amines. The suitability of the developed method for the analysis of amines in water samples was satisfactory.

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