Available online at www.sciencedirect.com
SCIENCE DIRECT
Analytica Chimica Acta 565 (2006) 168–177
ANALYTICA CHIMICA ACTA
www.elsevier.com/locate/aca

2-(2-Phenyl-1*H*-phenanthro-[9,10-*d*]imidazole-1-yl)-acetic acid (PPIA) and its application for determination of amines by high performance liquid chromatography with fluorescence detection and identification with mass spectroscopy/atmospheric pressure chemical ionization

Jinmao You [a,*], Xianen Zhao [a], Yourui Suo [b], Honglun Wang [b], Yulin Li [b], Jing Sun [b]

[a] *College of Chemistry Science, Qufu Normal University, Qufu Shandong 273165, PR China*
[b] *Northwest Plateau Institute of Biology, Chinese Academy of Sciences, Xining, PR China*

Received 24 November 2005; received in revised form 14 February 2006; accepted 15 February 2006
Available online 6 March 2006

Abstract

A simple, sensitive, and mild method for the determination of amino compounds based on a condensation reaction with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC·HCl) as the dehydrant with fluorescence detection has been developed. Amines were derivatized to their acidamides with labeling reagent 2-(2-phenyl-1*H*-phenanthro-[9,10-*d*]imidazole-1-yl)-acetic acid (PPIA). Studies on derivatization conditions indicated that the coupling reaction proceeded rapidly and smoothly in the presence of a base catalyst in acetonitrile to give the corresponding sensitively fluorescent derivatives with an excitation maximum at λ_{ex} 260 nm and an emission maximum at λ_{em} 380 nm. The labeled derivatives exhibited high stability and were enough to be efficiently analyzed by high-performance liquid chromatography. Identification of derivatives was carried out by online post-column mass spectrometry (LC/APCI–MS/MS) and showed an intense protonated molecular ion corresponding *m/z* $[MH]^+$ under APCI in positive-ion mode. At the same time, the fluorescence properties of derivatives in various solvents or at different temperature were investigated. The method, in conjunction with a gradient elution, offered a baseline resolution of the common amine derivatives on a reversed-phase Eclipse XDB-C_8 column. LC separation for the derivatized amines showed good reproducibility with acetonitrile–water as mobile phase. Detection limits calculated from 0.78 pmol injection, at a signal-to-noise ratio of 3, were 3.1–18.2 fmol. The mean intra- and inter-assay precision for all amine levels were <3.85% and 2.11%, respectively. Excellent linear responses were observed with coefficients of >0.9996. The established method for the determination of aliphatic amines from real wastewater and biological samples was satisfactory.

Keywords: HPLC; Amines; 2-(2-Phenyl-1*H*-phenanthro-[9,10-*d*]imidazole-1-yl)-acetic acid (PPIA); Mass spectrometry

1. Introduction

The quantification and identification of a variety of aliphatic amines at trace amount of levels in an aqueous environment and biological tissue is very important in the field of chemical and life sciences. It is well known that most aliphatic and aromatic amines may occur as biodegradation products of organic matter like proteins, amino acids, and other nitrogen-containing organic compounds. In addition, amines are used as raw material or as intermediates in the manufacture of a wide range of industrial chemicals. Volatile amines not only have an unpleasant smell but also possess heat hazards. Moreover, they may react with nitrosating agents, leading to the formation of potentially carcinogenic *N*-nitrosaamine compounds [1–5]. Therefore, it is important to determine certain amino compounds in real environmental samples. However, analysis of amines has been traditionally difficult due to their particular physicochemical properties, i.e., high volatility and polarity, basic character, and high solubility in water. Gas chromatography is frequently used to determine amines using various derivatization reagents [6]. Other methods including enzymatic [7,8] and ion-exchange chromatographic analysis [9] have been described for the determination of amines in various matrices. These methods are usually limited due to low sensitivity.

* Corresponding author. Tel.: +86 537 4458501; fax: +86 537 4458501/4456305.
E-mail address: Jmyou6304@163.com (J. You).

0003-2670/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.aca.2006.02.029

Commonly, most amino compounds show neither natural UV absorption nor fluorescence. Therefore, fluorescence probes are extensively used in physical, chemical and biological sciences for investigating the structure and dynamics of living system [10,11]. Other techniques used to improve detection limits are micro-column LC [12] and capillary electrophoresis [13]. At present, popular methods for the determination of amino compounds are pre-column and post-column derivatization with fluorescence detection. Several common fluorescent derivatization reagents [14–25] have also been developed fully. In spite of the popularity of these pre-column methods, there have also been many reports describing various shortcomings in application, and to date no method has been shown to overcome all problems. For example, orthophthalaldehyde (OPA) method offers greater sensitivity widely, but is limited to primary amines. The (2-phthalimidyl) benzoyl chloride (PIB-Cl) method offers high sensitivity, but is not suitable to aromatic amines. At the same time, with standing of derivatized solution, more interfering peaks are observed; it is possible that PIB-Cl derivatives exhibit decomposition during analysis [26]. Although 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) is more reactive than 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) for amino compounds, data reported previously indicate that the two reagents themselves show ca. 30–50% decomposition in methanol–water solution within 25 min when exposed to daylight [27]. Recently 9-fluorenylmethyl chloroformate (FMOC) [28], 1-(9-fluorenyl)ethyl chloroformate (FLEC) [29] and 2-(9-anthyl)ethyl chloroformate (AEOC) [30] reagents have been used for the derivatization of amines, amino acids and peptides for chiral or non-chiral separation in LC or CE. These reagents result in a good UV absorption and very high sensitivity with laser-induced fluorescence detection, but current procedures with FMOC are also troublesome. For the effective derivatization, an excess reagent must be used, and derivatized solution must be extracted with pentane to remove excess of reagent [31,32] because it interferes with the separation of derivatives and is detrimental to column performance. 6-Aminoquinolyl-*N*-hydroxysuccinimidylcarbamate (AQC) is rapid and convenient derivatization reagent and yields stable derivatives with amines or amino acids. However, only 10% of the fluorescence intensity in aqueous solution relative to that in pure acetonitrile solution is observed for its derivatives. Thus, the detection limits for the early eluted amine derivatives are usually higher than those for later ones [33].

In our previous studies [34], we describe the synthesis of a number fluorescence tagging reagents and the application for the analysis of common amino compounds. In this study, the principal goal is to develop a new labeling reagent 2-(2-phenyl-1*H*-phenanthro-[9,10-*d*]-imidazole-1-yl)-acetic acid (PPIA), which use EDC·HCl as coupling reagent to label amines. PPIA has been found to be very stable in its crystal state. Reaction of PPIA with amines in acetonitrile solvent in the presence of dimethylaminopyridine catalyst at ambient temperature gives higher yields of acidamides. The introduction of a 2-phenyl-1*H*-phenanthro-[9,10-*d*]imidazole functional group in molecular core structure makes n–π conjugation system to be augmented dramatically resulting in a strong fluorescence. To the best of our knowledge, this is the first time that PPIA fluorescent probe and its application for the determination of amino compounds have been reported. In this study, the optimal derivatization conditions such as buffer pH, reaction time and solvent system are investigated. Linearity, detection limits and precision of the procedure are also determined. At the same time, applications for the determination of amines from wastewater and telencephalon of male Wistar rat have been reported. The suitability of the developed method for the analysis of actual samples is satisfactory.

2. Experimental

2.1. Instrumentation

Experiments were performed using a LC/MSD-Trap-SL electrospray ion trap liquid chromatography/mass spectrometry (1100 Series LC/MSD Trap, a complete LC/MS/MS). All the HPLC system devices were from the HP 1100 series and consisted of a vacuum degasser (model G1322A), a quaternary pump (model G1311A), an autosampler (model G1329A), a thermostated column compartment (model G1316A), a fluorescence detector (FLD) (model G1321A), and a diode array detector (DAD) (model G1315A). Ion source type, APCI (positive mode); nebulizer pressure 60 psi; dry gas temperature, 350 °C; dry gas flow, 5.0 l/min. APCI Vap temperature 450; corona current (nA) 4000 (pos); capillary voltage 3500 V. Derivatives were separated on reversed-phase Eclipse XDB-C_8 column (150 mm × 4.6 mm, 5 μm, Agilent). The HPLC system was controlled by HP Chemstation software. The mass spectrometer from Bruker Daltonik (Bremen, Germany) was equipped with an atmospheric pressure chemical ionization (APCI) source. The mass spectrometer system was controlled by Esquire-LC NT software, version 4.1. Fluorescence excitation and emission spectra were obtained at a 650-10 S fluorescence spectrophotometer (Hitachi). Excitation and emission bandpass are both set at 10 nm. The mobile phase was filtered through a 0.2-μm nylon membrane filter (Alltech, Deerfiled, IL).

2.2. Chemicals

All aliphatic amine standards were purchased from Sigma Co. (St. Louis, MO, USA). HPLC grade acetonitrile was purchased from Yucheng Chemical Reagent Co. (Shandong Province, China). Formic acid was analytical grade from Shanghai Chemical Reagent Co. (Shanghai, China). Water was purified on a Milli-Q system (Millipore, Bedford, MA). 4-Dimethylaminopyridine (DMAP) was purchased from Shanghai Chemical Reagent Co. (Shanghai, China). Male Wistar rat (250–300 g) was purchased from Jining Medical Factory (Jining, Shandong, China).

2.3. Synthesis of derivatization reagent (PPIA)

2.3.1. Synthesis of 2-phenyl-1H-phenanthro[9,10-d] imidazole

The synthesis of 2-phenyl-1*H*-phenanthro[9,10-*d*]imidazole (PPI) was carried out according to the method previously

reported [35]. 9,10-Phenanthraquinone (16 g), benzaldehyde (10 ml) and ammonium acetate (120 g) were fully mixed in 500 ml of round-bottom flask. After the addition of 300 ml glacial acetic acid, the contents were rapidly heated to 80–90 °C with vigorous stirring for 3 h. After cooling, pH of solution was adjusted to 7–8 with ammonia water. The precipitated solid was recovered by filtration, washed with water, and dried at room temperature for 48 h. The crude product was recrystallized twice from acetonitrile/DMF mixed solvent (acetonitrile/DMF 5:1, v/v) to afford a slight yellow crystal, yield 92%.

2.3.2. Synthesis of 2-(2-phenyl-1H-phenanthro[9,10-d] imidazole-1-yl)acetic acid

2- (2-Phenyl -1 *H* -phenanthro[9, 10- *d*]imidazole-1-yl)acetic acid was synthesized as follows:. 2-Phenyl-1*H*-phenanthro [9,10-*d*]imidazole (28 g), KOH (30 g) and dimethylsulfoxide (250 ml) were mixed in a 500 ml round-bottom flask and rapidly heated to 120–125 °C for 10 min with vigorous stirring. A solution of 50 ml ethyl bromoacetate in 30 ml dimethylsulfoxide was added dropwise within 2.5 h with stirring. After cooling, 300 ml water and 20 g KOH were added and heated continuously at 100 °C for 30 min. After the solution was cooled to ambient temperature, the mixture was filtrated, the result solution was neutralized to pH 4.0 with 4 mol/l HCl solution. The precipitated solid was recovered by filtration, washed successively with water, 40% ethanol solution (ethanol/water 2:3, v/v). The crude product was dried at room temperature for 48 h and recrystallized twice from acetonitrile/DMF mixed solvent (acetonitrile/DMF, 5:1, v/v) to afford a white crystal, yield 84%. m.p. > 300 °C. Found: C 78.32; H 4.58; N 7.94. Calculated: C 78.39; H 4.57; N 7.95. IR (KBr): 3110.57 (–OH, dimer), 1711.82 (C=O), 1657.09, 1612.53 (ph–C=N–), 1529.62, 1474.14, 1460.11, 1424.59 (ph), 1399.26 (C–H), 1229.85, 1111.75, 773.37, 750.7, 720.84, 703.84. *m/z* $[M+H]^+$ 353.1.

2.4. High-performance liquid chromatography

Derivatives were separated on a reversed-phase Eclipse XDB-C_8 column (150 mm × 4.6 mm, 5 μm, Agilent) by a gradient elution. Eluent A was 30% of acetonitrile consisting of 30 mM ammonium formate, pH 3.7; B was 100% of acetonitrile. Gradient conditions: initial = 100% A; 35 min = 100% B (kept for 5 min, injection 10 μL). Before injection of the next sample, the column was equilibrated with mobile phase A for 10 min. The flow rate was constant at 1.0 ml/min and the column temperature was set at 30 °C. The fluorescence excitation and emission wavelengths were set at λ_{ex} 260 nm and λ_{em} 380 nm, respectively. The detection and identification of PPIA derivatives were performed by online post-column fluorescence and MS/APCI at positive ion detection mode.

2.5. Preparation of standard solutions

The derivatizing reagent solution 2.0×10^{-3} mol/l was prepared by dissolving 17.6 mg 2-(2-phenyl-1*H*-phenanthro-[9,10-*d*]imidazole-1-yl)-acetic acid (PPIA) in 10 ml of anhydrous acetonitrile prepared by distilling the dried HPLC grade acetonitrile with P_2O_5, and this solution was added 2 ml pyridine and diluted to 25 ml with acetonitrile. Individual stock solutions of the amines were prepared in acetonitrile. The standard amines for HPLC analysis at individual concentrations of 1.0×10^{-4} mol/l were prepared by dilution the corresponding stock solutions (1.0×10^{-2} mol/l) of each amine with acetonitrile. Standard solution of 0.15 mol/l coupling reagent was prepared by dissolving 0.2876 g EDC·HCl in 10 ml of anhydrous acetonitrile. When not in use, all standards were stored at 4 °C. Standard solution of basic catalyst (0.30 mol/l) was prepared by dissolving 0.366 g of DMAP in 10 ml of acetonitrile.

2.6. Preparation of PPIA-butylamine derivative

PPIA-butylamine was prepared by the reaction of PPIA with *n*-butylamine as follows: 2-(2-Phenyl-1*H*-phenanthro-[9,10-*d*]imidazole-1-yl)-acetic acid (PPIA, 20 mmol) and *n*-butylamine (25 mmol) were dissolved in anhydrous acetonitrile (10 ml). A solution of EDC·HCl (50 mmol) and pryidine (25 mmol) in acetonitrile (10 ml) was added. The solution was kept at 40 °C for 1 h with stirring. The urea was removed by filtration, and the solution was concentrated to dryness by a rotary evaporator. The residue was recrystallized twice from methanol to give a white solid 1.8 g, and used to test the fluorescence properties.

2.7. Pretreatment of wastewater and telencephalon of male Wistar rat

(1) To a solution containing 50 ml of wastewater in 50 ml round-bottom flask, 2.0 ml hydrochloric acid (3.0 M) was added. The contents of the flask were vortexed for 2 min and filtrated. The result solution was evaporated to dryness under reduced pressure in nitrogen atmosphere. The residue was re-dissolved with 50% acetonitrile solution consisted of 0.2 M borate buffer (pH 9.0) to a total volume of 5 ml and stored at 4 °C until HPLC analysis.
(2) Male Wistar rats (about 300 g) were decapitated, and immediately taken out the whole brain and washed by ice-cooled physiological salt solution. Telencephalon was separated on a glass plate. To a hard glass tube, 1.2 ml 0.1 mol/l ice-cooled $HClO_4$ solution and the weighted telencephalon (2.000 g) were added and slurried for 20 min. The slurried solution was centrifuged (18,000 rpm for 30 min at 4 °C). The supernatant was separated and added 4 mol/l NaOH to neutralize the excess amount of $HClO_4$ to pH 6.5–7.0. The result solutions were made up to total volume of 2.0 ml with deionized water and stored at −80 °C until HPLC analysis.

2.8. Derivatization procedure

The derivatization process is shown in Fig. 1. To 50 μl aqueous of amines in a 2-ml vial were successively added 40 μl

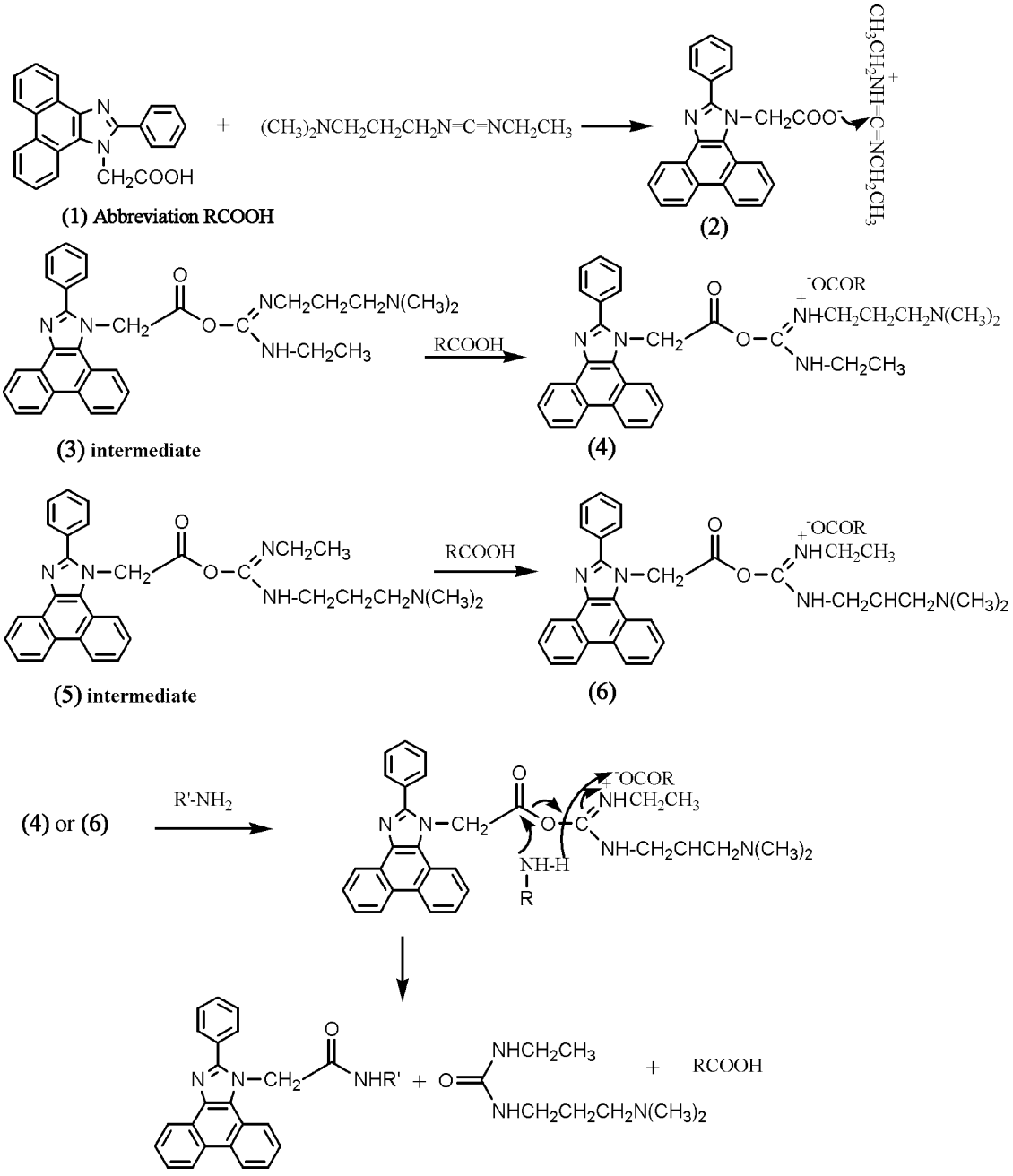

Fig. 1. Derivatization scheme of 2-(2-phenyl-1*H*-phenanthro-[9,10-*d*]imidazole-1-yl)-acetic acid (PPIA) with amines.

EDC·HCl, 120 μl derivatization reagent, and 50 μl pyridine solution, respectively (here, pyridine is a catalyst of coupling reaction). The vial was then sealed and heated at 60 °C for 15 min in a thermostatic water-bath and then left to cool at room temperature. After the addition of 400 μl acetonitrile, a 10 μl volume of the crude reaction solution was injected into the chromatograph either directly or after the following purification step. To the reaction mixture mentioned above was added a 1.0 ml volume of chloroform. The mixture was washed successively with 4 ml each of 0.2 M hydrochloric acid, water, 0.2 M sodium hydroxide and deionized water, respectively. The organic phase was separated and evaporated to dryness under a stream of nitrogen gas. Residue was redissolved in 500 μl acetonitrile, 10 μl of which was injected into the chromatograph.

2.9. Quantitative analysis

Quantitative conversion of amines from the wastewater and telencephalon of male Wistar rat to their PPIA-amine derivatives was guaranteed by using an excess of PPIA. All amines were quantified in wastewater and telencephalon of male Wistar rat using the external standard method with detection at 380 nm. The calibration curves for each PPIA-amine were obtained by linear regression plotting peak area versus concentration.

3. Results and discussion

3.1. Ultraviolet absorption of 2-(2-phenyl-1H-phenanthro-[9,10-d]imidazole-1-yl)-acetic acid

The ultraviolet absorption of 2-(2-phenyl-1*H*-phenanthro-[9,10-*d*]imidazole-1-yl)-acetic acid (PPIA) was investigated in methanol–water (1:1) solution. The absorption wavelength of PPIA was obtained with the scanning range of 200–400 nm. Maximum ultraviolet absorption responses were observed at the wavelengths of 251 and 256 nm, respectively. The molar absorption coefficients (ε) were 6.3×10^5 l/mol cm (251 nm) and 6.2×10^5 l/mol cm (256 nm), respectively.

3.2. Fluorescence excitation and emission

The solution of the fluorescence derivative of *n*-butylamine (2.0 μM) in 30 mM borate buffer/acetonitrile solution (20/80, v/v) was used to obtain the fluorescence spectra. The fluorescence spectra of PPIA-butylamine derivative showed the maximum excitation and emission wavelengths at 260 and 380 nm, respectively. No obvious blue- or red-shift in acetonitrile or methanol solution (0–100%) was observed. Fluorescence intensity of representative *n*-butylamine-derivative was minimally quenched by inorganic anions (such as sulfate, nitrate, and phosphate) and organic anions (such as citrate) and divalent cations that were abundant in biological fluids.

The relationship between the high concentration of formic acid and the fluorescence intensities of the PPIA-butylamine was investigated. In the neutral conditions, the intensity was more than 10–12 times higher than those obtained in pure formic acid solution (100%). Commonly, fluorescence (FL) responses of the derivatives decreased with increasing the acidity including acetic acid, trichloroacetic acid and trifluoroacetic acid (here, other organic acids were not further investigated). In three acetonitrile aqueous samples (50%, v/v) containing 1.0% of formic acid, trichloroacetic acid and trifluoroacetic acid (ca. 78 mM for each acid), respectively. A 90–95% of the fluorescence intensity relative to that in 50% acetonitrile aqueous was observed for the derivatized amines. It was probably due to the fact that relatively strong formic acid resulted in, at least in part, protonation with the basic nitrogen atom from molecular core structure and resulted in the quenching in fluorescence intensity. We therefore concluded that, for a more sensitive detection of the derivatives, the detection should be performed with low concentration formic acid as mobile phase. In general, experiments were performed in formic acid concentration range of 10–30 mM in which no obvious quenching was observed.

3.3. Effect of PPIA concentration on derivatization

2-(2-Phenyl-1*H*-phenanthro-[9,10-*d*]imidazole-1-yl)-acetic acid (PPIA) had the same condensation reaction with amines as do of carbazole-9-acetic acid (CRA) previously reported in our experiments [36]. The molecular structure of PPIA in which its n–π conjugation system was augmented due to increasing a phenyl-1*H*-phenanthro-[9,10-*d*]-imidazole function group that made it more sensitive with fluorescence detection relative to that of CRA (absorption coefficient of CRA $\varepsilon = 2.4 \times 10^4$ l/mol cm at 254 nm). Derivatization of PPIA with amines can be achieved within 15 min at 60 °C. The derivatives were stable for more than 48 h at room temperature. The fluorescence intensity of PPIA-derivatives increased with increasing the amounts of derivatization reagent. For all 12 amines studied, maximal yields close to 100% were observed with a four- to five-fold molar reagent excess to total molar amines, increasing the excess of reagent beyond this level had no significant effect on yields. With as little as a 3.0-fold molar excess of derivatization reagent to total molar amines, the derivatization of amines was incomplete and obviously resulted in low detection responses. To an unknown concentration of sample, complete derivatization was guaranteed by using a large excess of PPIA until constant peak intensity for detector responses. Several side reactions were also observed and they should be attributed to the reaction of reagent itself with coupling reagent EDC·HCl to form two major by-products, such as intermediate (**3**) *N*′-(3-(dimethylamino)propyl)-*N*-ethylcarbamimidoyl-2-(2-phentl-1*H*-phenanthro[9,10-*d*]-imidazol-1-yl) acetate (*m*/*z* 508.1, chromatographic peak is **A**) and intermediate (**5**) *N*′-ethyl-*N*-(3-(dimethylamino)propyl)carbamimidoyl-2-(2-phentl-1*H*-phenanthro[9,10-*d*]imidazol-1-yl) acetate (*m*/*z* 508.1, chromatographic peak is **B**). The intermediate (**3**) and (**5**) had the same mass-to-charge ratio, however, their molecular structure was different (see Fig. 1), and thus the elution order exhibited obvious difference in HPLC system. With MS/MS analysis (see Fig. 2), the intermediate (**3**) was eluted earlier than intermediate (**5**). The positive-ion mass spectra, corresponding to the separated intermediates, were quite complex. In most cases, the collision-induced dissociation spectra of *m*/*z* $[MH]^+$ produced intense fragment ions by losing small molecules. Intermediate **A** loosed small molecules as follows: **A**—*m*/*z* 508.1 $[MH]^+$; *m*/*z* 380.2, MH^+-($NHCH_2CH_3 + CH_2CH_2CH_2N(CH_3)_2$); *m*/*z* 354, MH^+-EDC; *m*/*z* 295.1, MH^+-(–CH_2COO + EDC). Intermediate **B** loosed small molecules as follows: **B**—*m*/*z* 508.1 $[MH]^+$; *m*/*z* 437.2, MH^+–$CH_2N(CH_3)_2$; *m*/*z* 354, MH^+-EDC; *m*/*z* 295.1, MH^+-(–CH_2COO + EDC). In addition, the small amount of by-products of 2-phenyl-1*H*-phenantho[9,10-*d*]imidazole (PPI, *m*/*z* 295.1) was also observed with MS analysis of the eluted components, and proved to be the molecular core structure of reagent PPIA produced by the loss of acetic acid molecule. The presence of intermediates (**3**), (**5**) (major by-products) and PPI (small amount) did not interfere with the separation of amine derivatives under the conditions proposed.

3.4. Effect of basic catalyst on derivatization

Several types of basic catalysts were tested in this study for derivatization, including triethylamine, pyridine, 4-dimethylaminopyridine (DMAP). Derivatization was carried out at 60 °C for 15 min using EDC·HCl as the coupling reagent in the presence of various base catalysts (concentration at

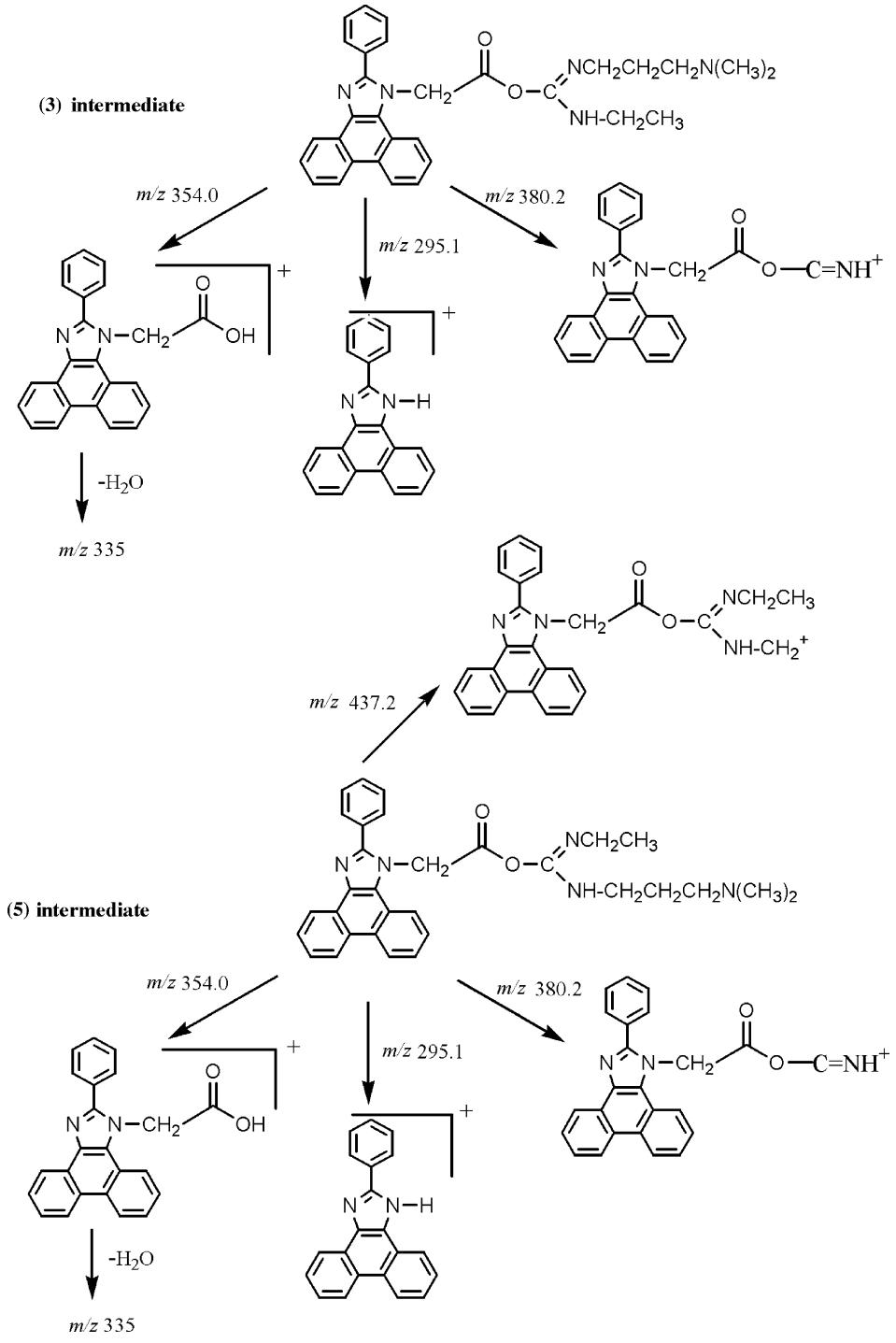

Fig. 2. Scheme of MS cleavage mode of intermediates (**3**) and (**5**) (**3** and **5** are stand for peaks A and B in chromatogram, respectively).

0.05–0.2 mol/l). Each value was an average of six runs with DMAP taken as 100%. The results showed that DMAP gave the highest detector responses. All subsequent derivatization was, therefore, performed by the use of DMAP as the basic catalyst. Further study indicated that the final DMAP concentration in derivatization solution should be >0.10 mol/l to give complete derivatization, with further increasing the concentration of DMAP in derivatized solution did not significantly increase the reaction yield.

3.5. Effects of different coupling reagent on detector responses

Dicyclohexylcarbodiimide (DCC) and EDC·HCl as reaction coupling reagents were investigated. Reactions were carried out at 60 °C for 15 min with 1.0×10^{-4} mol/l of all 12 amines using DMAP (0.1 mol/l) as the base catalyst in the presence of EDC·HCl or DCC (concentration 0.15 mol/l). Detector responses for derivatized amines using EDC·HCl as cou-

pling reagent were eight times greater than that of DCC under the derivatization conditions proposed. EDC·HCl as reaction condensation reagent proved optimal as it was freely soluble in acetonitrile [37] or dissolved in water [38]. It was observed that if coupling reagent was insufficient to obtain maximal yield, addition of more coupling reagent could reproducibly increase the yield to the maximum. In general, the % yield of the derivatization procedure for an unknown concentration sample was calculated by intergrating the peak areas reached maximum for the derivatized solutes by addition of increasing amounts of EDC·HCl. In most cases, 0.15 mol/l EDC·HCl was used for the derivatization of the analyses.

3.6. Temperature conditions and time effects

The optimum temperature and time for derivatization were investigated. The results indicated that heat had a significant effect on reaction time and yield. When tested at different temperatures over various periods of time, the reaction was completed within 10, 15, 20 and 25 min at 100, 80, 60 and 40 °C, respectively. It was found that above 80 °C the position of the equilibrium reduced the proportion of PPIA reacting with amines because of forming some by-products; while below 60 °C the rate of reaction was decreased and led to long derivatization time. Although, a clean reaction occurred at room temperature, time in excess of 24 h or more was needed for maximum responses. Therefore, most subsequent derivatization temperature selected in experiments was at 60 °C. Increasing the reagent concentration to more than 0.15 mol/l (already a large excess of reagent to amines) did not significantly alter the time and temperature needed for derivatization reaction to be completed.

3.7. Effects of solvents and heavy atom on the fluorescence of derivative

Acetonitrile, dichloromethane, ethyl acetate, chloroform and acetone were investigated as reaction co-solvents for derivatization procedure. The reactions were, respectively, carried out at 60 °C for 15 min with 1.0×10^{-4} mol/l of all 12 amines, 0.15 mol/l EDC·HCl and 0.1 mol/l DMAP. The results indicated that acetonitrile and ethyl acetate gave the best results as assessed by the detector responses. A slight decrease in detector responses in chloroform (ca. 90% response relative to that of acetonitrile) and dichloromethane solvents (ca. 80%) was observed. Acetone gave the lowest response (46%) under the conditions proposed. In general, acetonitrile was used as the reaction solvent throughout this study because of the sparing solubility of EDC·HCl in ethyl acetate and the immiscibility of ethyl acetate in the mobile phase.

The heavy atom effect on the fluorescence characteristics of representative PPIA-butylamine derivative was investigated. In this experiment, we chose four general quenchers Pb^{2+}, Ag^{+}, Hg^{2+} and Fe^{3+}. When compared with the representative PPIA-butylamine fluorescence spectrum in the presence and absence of a low concentration of heavy atom salt, no obvious red- and blue-shift in λ_{em} was observed (diagram not shown). A slightly quenching on fluorescence emission of PPIA-butylamine in the low concentrations of heavy atom (PPIA-butylamine kept at 1.0 μmol/l; heavy atom concentration 1.25–5.0 μmol/l) was observed. When heavy atom concentration was kept at 15 mmol/l in acetonitrile aqueous (50%, v/v), the relative emission intensities I/I_0 were between 0.98 and 0.84 (where I_0 and I are emission intensities observed in the spectrum of PPIA-butylamine before and after the heavy atoms existed, respectively). In general, no fluorescence quenching was observed in the presence of some heavy atoms (below 20 μmol/l). As observed, the fluorescence intensities of all 12 amine derivatives were rarely quenched by inorganic anions (such as sulfate, nitrate, and phosphate) and organic anions (such as citrate) and divalent cations that were abundant in biological fluids.

3.8. HPLC separation for derivatized amines

For the separation of derivatized amines, several mobile phase compositions were tested. They included methanol and acetonitrile, in aqueous mixtures. For the simultaneous separation of C_1–C_{12} amine derivatives, an Eclipse XDB-C_8 column was selected and eluted with (A) 30% acetonitrile containing 30 mmol/l formic acid and (B) 100% acetonitrile. The gradient elution from (A) to (B) within 40 min was used to give the best separation with the shortest retention times and the sharpest peaks. As observed, the resolution of amine derivatives can be significantly affected by pH of mobile phase. In comparison with the acidic conditions, operation at pH > 7.0 resulted in obviously increase in retention value for all derivatives. With pH 3.7, all amine derivatives were separated with a good baseline resolution (see Fig. 3).

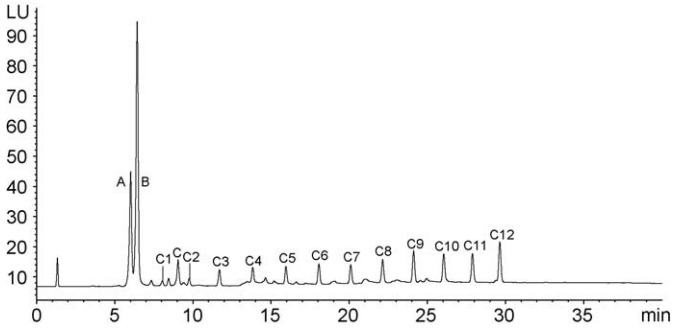

Fig. 3. Chromatogram for aliphatic amine standard mixtures with 0.78 pmol injection. Column temperature is set at 30 °C; excitation wavelength: λ_{ex} 260 nm, emission wavelength: λ_{em} 380 nm; reversed-phase Eclipse XDB-C_8 column (5 μm); flow rate = 1.0 ml/min. (1) methylamine; (2) ethylamine; (3) propylamine; (4) butylamine; (5) pentylamine; (6) hexylamine; (7) heptylamine; (8) octylamine; (9) nonylamine; (10) decylamine; (11) undecylamine; (12) dodecylamine. (A) *N′*-(3-(dimethylamino)propyl)-*N*-ethylcarbamimidoyl-2-(2-phentl-1*H*-phenanthro[9,10-*d*]-imidazol-1-yl) acetate (*m/z* 508.1); (B) (*N′*-ethyl-*N*-(3-(dimethylamino)propyl)carbamimidoyl-2-(2-phentl-1*H*-phenanthro[9,10-*d*]-imidazol-1-yl) acetate (*m/z* 508.1); (C) 2-phenyl-1*H*-phenanthro[9,10-*d*]imidazole (hydrolyzed by-product PPI; molecular core; *m/z* 295).

3.9. Identification with APCI source at positive-ion detection mode

The ionization and fragmentation of the isolated PPIA-amines was studied by mass spectrometry with atmospheric pressure chemical ionization detection in positive-ion mode. As expected, the PPIA-amines produced an intense molecular ion peak at m/z $[M+H]^+$. In most cases, the collision-induced dissociation spectra of m/z $(MH)^+$ produced intense fragment ions m/z 335 and m/z 295. The selected reaction monitoring, based on the m/z $[M+H]^+ \rightarrow m/z$ 335 and m/z 295 transition, was specific for amine derivative. There was no detectable signal from the blank water sample using this transition. Although other endogenous basic compounds present in natural environmental sample were presumably coextracted and derivatized by PPIA reagent, no interference was observed due to the highly specific parent mass-to-charge ratio and the characteristic product ions in the m/z 335 and m/z 296 transition. First, the collision-induced dissociation of molecular ion [m/z $(MH)^+$] produced parent molecule and then further lost one H_2O to produce fragment ion m/z 335. The collision-induced dissociation of fragment ion m/z 335 produced the intense fragment ion m/z 295. As observed, $I_{335}/I_{295} = 1{:}12$ (I_{335} and I_{295} were, respectively, mass spectrum ion current intensity, see Fig. 4). The characteristic fragment ions of m/z 295 (PPI, molecular core structure) came from the cleavage of N–CH_2COO bond. All molecular ions $[M+H]^+$ and corresponding fragment ions for 12 amine derivatives are shown in Table 2. To reduce the disturbance to minimum, the gradient elution with HPLC for the separation and determination of derivatized PPIA-amines was an efficient method.

Additionally, as expected, an acidic mobile phase would be useful to the ionization of PPIA-amines (for example, mobile phases containing 30 mM formic acid buffer, pH 3.7). The ionization in the gas phase can be directly affected by the acidity of solution. The mobile phase, consisting of the separated PPIA-amines and 30 mM (ca. 0.1%, v/v) formic acid, was introduced into the ionization chamber of mass spectrometer and converted to gaseous state, the ionization efficiency of PPIA-amines was significantly augmented compared to liquid phase conditions. Since ionization in the gas phase was easier than that in liquid phase. The relative intensities of molecular ion peaks in the presence and absence of 30 mM formic acid were investigated. The ratios $I/I'_{ion} = 4.7 - 6.7/1$ were observed (here, I_{ion} and I'_{ion} were, respectively, the intensities of molecular ion peaks in the presence and absence of 30 mM formic acid).

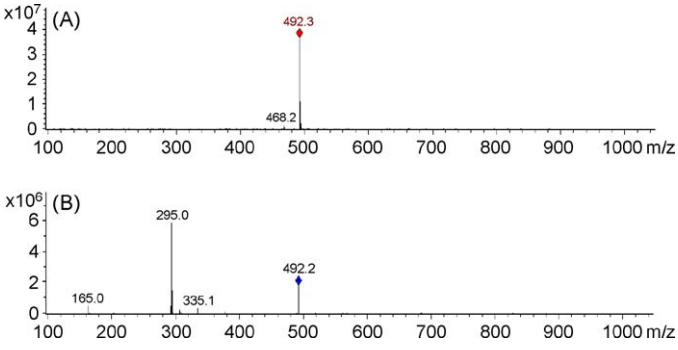

Fig. 4. The profile of ion mass spectra and scanning of the derivatized representative decylamine derivative. Typical MS chromatogram of decylamine derivative from full scanning range from 100 to 1000 amu under APCI positive-ion mode; the derivative was isolated from a reversed-phase Eclipse XDB-C_8 column, and into the on-line mass spectrometer ((A) molecular ion MS; (B) MS/MS).

3.10. Comparison of the fluorescence responses between PPIA and CRA

Relative responses for fluorescence detection for the individual derivatized amine using PPIA and CRA were investigated. To make a quantitative comparison between PPIA and CRA in terms of relative fluorescent intensity, separation of standard amine derivatives was performed. The results indicated that fluorescence intensity for individually derivatized amine using PPIA as derivatizing reagent was 2.6–3.7-fold stronger than those obtained using CRA. This was probably due to the fact that PPIA had the large molar absorbance relative to that of CRA. The difference in molar absorbance may be attributed to the PPIA molecular structure, in which its n–π conjugation system was dramatically augmented due to increasing a phenyl-1*H*-phenanthro-[9,10-*d*]imidazole functional group that made it more sensitive than that of CRA with fluorescence detection.

3.11. Analytical precision, accuracy, reproducibility and recovery

A standard consisting of 50 pmol amine derivatives was prepared to examine the method repeatability. The relative standard deviations (R.S.D.s; $n = 12$) of the peak areas and retention times were from 0.22 to 1.62% and 0.01 to 0.03%, respectively. Precision and accuracy: within-day and between-day precision for 12 amines was examined by using three identical wastewater samples, respectively, spiked with 0.05, 0.1, and 0.2 μmol/l of amines to make the low to high-range concentrations. The relative standard deviations for within-day determination ($n = 11$) were 1.70–2.79% for 12 tested amines. The mean intra- and inter-assay precision for all amine levels were <3.85% and 2.11%, respectively.

The recoveries were determined from values obtained following actual analysis of the wastewater as calculated from

Table 1
Recoveries of aliphatic amines

Amine	Recoveries (%)
C_1	86.6 ± 2.4
C_2	92.5 ± 2.3
C_3	97.7 ± 2.0
C_4	105.1 ± 2.6
C_5	102.6 ± 1.8
C_6	93.7 ± 1.6
C_7	99.9 ± 1.4
C_8	104.4 ± 2.3
C_9	89.2 ± 2.5
C_{10}	91.9 ± 1.8
C_{11}	101.3 ± 1.8
C_{12}	104.4 ± 2.3

Table 2
Linear regression equations, correlation coefficients, detection limits and mass spectral data

Amine	$Y = AX + B$	R	R.S.D.s (%)	Detection limits (fmol)	Molecular ion $[M+H]^+$	Fragment ions
C_1	$Y = 20.03X - 3.628$	0.9996	2.79	18.2	366.1	295.1, 307.1, 335.2
C_2	$Y = 25.59X + 5.556$	0.9996	2.76	16.5	380.1	295.1, 307.0, 335.2
C_3	$Y = 56.41X + 7.253$	0.9999	2.43	7.5	394.1	295.1, 307.1, 335.1
C_4	$Y = 52.42X + 14.20$	0.9996	2.36	6.8	408.2	295.1, 306.9, 335.1
C_5	$Y = 58.33X + 16.32$	0.9996	2.48	7.6	422.2	295.0, 307.1, 335.2
C_6	$Y = 65.67X + 16.25$	0.9998	1.70	5.1	436.2	295.0, 307.1, 335.0
C_7	$Y = 65.24X + 15.51$	0.9997	1.87	6.1	450.3	295.0, 307.3, 335.0
C_8	$Y = 78.75X + 20.55$	0.9997	1.86	4.8	464.3	295.1, 307.1, 336.1
C_9	$Y = 109.9X + 27.16$	0.9997	1.90	3.8	478.3	295.0, 308.0, 336.0
C_{10}	$Y = 107.5X + 22.94$	0.9998	1.84	4.5	492.3	295.0, 307.0, 335.1
C_{11}	Y=101.8X+22.75	0.9997	2.22	4.2	506.3	295.0, 307.1, 335.0
C_{12}	$Y = 141.3X + 35.28$	0.9997	2.10	3.1	520.3	295.0, 307.1, 335.0

X: injected amount (pmol); Y: peak area.

the calibration graph constructed by using the performed amine derivatives. In two identical wastewater samples, known amount of 12 above-mentioned amines were added. The samples were treated according to the method as described in text and derivatized with PPIA, and the analyses were carried out in duplicate. The experimental recoveries were in the range of 86.6–105.1% with their standard deviations in the range of 1.6–2.5 (see Table 1).

3.12. Detection limits and linearity for derivatized amines

Detection limits were an important consideration when the components of biological matrices were analyzed, particularly when they were present at low or trace concentrations. Fig. 3 showed the injection of 0.78 pmol of each derivatization amine. Based on this experiment, the calculated detection limits (at a signal-to-noise ratio = 3:1) for each derivatized amine were from 3.1 to 18.2 fmol. The linearities were established over a 4100-fold concentration range for amines with analysis of serial dilutions of the standard solution ranging from 4.88×10^{-3} to 20.0 μmol/l. All of the amines were found to give linear responses over this range, with correlation coefficients of >0.9996 (see Table 2). The linear regression analysis for higher concentrations of amines was not tested as the responses were over linearity range.

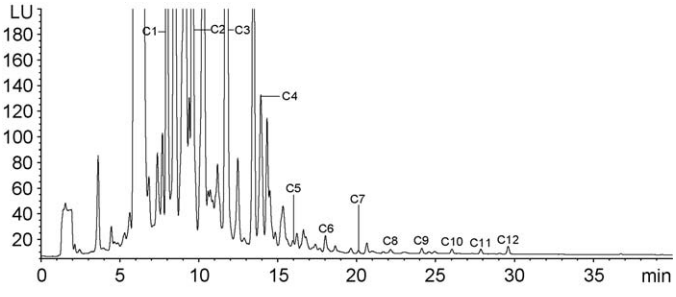

Fig. 5. Chromatogram for the separation of aliphatic amines from real wastewater derivatized with PPIA. Column temperature is set at 30 °C; excitation wavelength: λ_{ex} 260 nm, emission wavelength: λ_{em} 380 nm; Column Eclipse XDB-C_8 (5 μm); flow rate = 1.0 ml/min; peaks as Fig. 3.

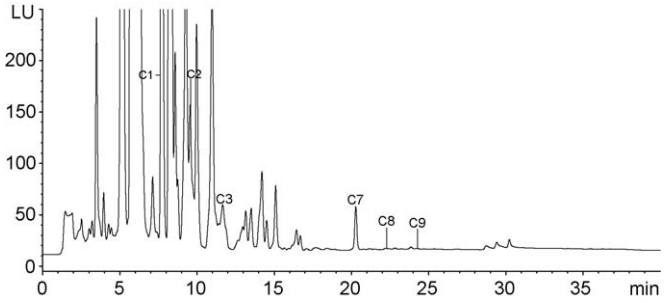

Fig. 6. Chromatogram for the separation of aliphatic amines from real telencephalon tissue of male Wistar rat. Column temperature is set at 30 °C; excitation wavelength: λ_{ex} 260 nm, emission wavelength: λ_{em} 380 nm; Column Eclipse XDB-C_8 (5 μm); flow rate = 1.0 ml/min; peaks as Fig. 3.

3.13. Analysis of samples

Chromatogram for the analysis of free amines from real wastewater and telencephalon tissue of male Wistar rat with fluorescence detection are shown in Fig. 5 (wastewater sample) and Fig. 6 (telencephalon tissue of male Wistar rat). Amine compositional data of wastewater and telencephalon tissue of male Wistar rat are shown in Table 3. As can be seen, the

Table 3
Contents of aliphatic amines from wastewater and telencephalon tissue of male Wistar rat

Amine derivatives	Waster water of paper mill (μg/l)	Telencephalon of male Wistar rat (μg/g)
C_1	8.62	6.13
C_2	9.87	2.51
C_3	7.94	0.61
C_4	6.36	Nd
C_5	1.21	Nd
C_6	7.61	Nd
C_7	1.21	0.58
C_8	2.03	0.043
C_9	1.81	0.013
C_{10}	1.52	Nd
C_{11}	2.19	Nd
C_{12}	2.94	Nd

Nd: not detectable or below detection limits (LOQ).

established method is suitable for the determination of these components from real wastewater and biological samples with satisfactory results. The facile PPIA derivatization coupled with mass spectrometry allows the development of a highly sensitive and specific method for the quantitative analysis of trace levels of amines from wastewater or natural environmental samples.

4. Conclusions

The present paper introduces a new reagent PPIA for derivatizing amines with superior properties including convenient derivatization and excellent sensitivity. The improved performance of the reagent PPIA for quantitative analysis of amines has been demonstrated in detail. One of the most attractive features of this method exhibits its simpleness for the preparation of amine derivatives. The described method shows good correlation in comparison with CRA methods used for amine derivatization. Detection limits are in the femtomole range. Current studies should further explore the derivatization of different amine-containing compounds such as alkylamines, catecholamines and polyamines. The LC separation for the derivatized amines shows good repeatability. A possible disadvantage of the proposed method is that the reagent PPIA can be only be used in the pre-column derivatization.

Acknowledgement

This work was supported by the National Science Foundation under Grant # 20075016 and 30370218.

References

[1] J. You, X.J. Fan, W.J. Lao, Q.Y. Ou, Q.C. Zhu, Talanta 48 (1999) 437.
[2] F. Sacher, S. Lenz, H. Brauch, J. Chromatogr. A. 764 (1997) 85.
[3] G.W. Schade, P.J. Crutzen, J. Atmos. Chem. 22 (1995) 319.
[4] A. Terashi, Y. Hanada, A. Kido, R. Shinohara, J. Chromatogr. 503 (1990) 369.
[5] M. Abalos, J.N. Bayona, F. Ventura, Anal. Chem. 71 (1999) 3531.
[6] D.R. Knapp, Handbook of Analytical Derivatization Reactions, Wiley Interscience, New York, USA, 1979.
[7] B. Vinet, Clin. Chem. 33 (1987) 2204.
[8] Y. Sekine, M. Suzuki, T. Takeuchi, E. Tamiya, I. Karube, Anal. Chim. Acta 280 (1993) 179.
[9] M.C. Gennaro, E. Mentasti, C. Sarzanini, V. Porta, Chromatographia 25 (1988) 117.
[10] B. Valeur, in: S.G. Schulman (Ed.), In Molecular Luminescence Spectroscopy, Part 3, Wiley, New York, 1993, p. 25.
[11] Q.Z. Zhu, F. Li, X.Q. Guo, J.G. Xu, W.Y. Li, Analyst 122 (1997) 937.
[12] M.D. Oates, J.W. Jorgenson, Anal. Chem. 61 (1989) 432.
[13] Y.F. Cheng, N.J. Docichi, Science 242 (1988) 562.
[14] Y. Yamada, Y. Aizawa, J. Pharmacol. Methods 9 (1983) 1.
[15] Y. Tsuruta, K. Kohashi, Anal. Chim. Acta 192 (1987) 309.
[16] T. Ishida, M. Yamaguchi, T. Iwata, M. Nakamura, Anal. Chim. Acta 223 (1989) 319.
[17] M. Masuda, C. Toriumi, T. santa, K. Imai, Anal. Chem. 76 (2004) 728.
[18] P. Lindroth, K. Mopper, Anal. Chem. 51 (1979) 1667.
[19] D.W. Hill, F.H. Walters, T.D. Wilson, J.D. Stuart, Anal. Chem. 51 (1979) 1338.
[20] H.G. Barth, W.E. Barber, C.H. Lochmuller, R.E. Majors, F.E. Regnier, Anal. Chem. 60 (1988) 387.
[21] R.F. Chem, C. Sctt, E. Trepman, Biochim. Biophys. Acta 576 (1979) 440.
[22] R.M. Hu, L.S. Ma, Analysis of Nitroso Compounds, Science Press, Beijing, 1980.
[23] M. Ahnoff, I. Grundevik, A. Anders, J. Fonselius, A.P. Bengt, Anal. Chem. 53 (1981) 485.
[24] Y. Watanabe, K. Imai, Anal. Biochem. 116 (1981) 471.
[25] H. Kotaniguchi, M. Kawakatsu, T. Toyo'oka, K. Imai, J. Chromatogr. Biomed. Appl. 420 (1987) 141.
[26] M.H. Zheng, C.G. Fu, H.D. Xu, Analyst 118 (1993) 269.
[27] M. Ahnoff, I. Grundevik, A. Arfwidsson, J. Fonselius, A.P. Bengt, Anal. Chem. 53 (1981) 485.
[28] A. Engström, P.E. Anderson, B. Jossfsson, D.P. William, Anal. Chem. 67 (1995) 3018.
[29] S. Einarsson, B. Josefsson, P. Moller, D. Sanchez, Anal. Chem. 59 (1987) 1191.
[30] A.J. Faulkner, H. Veening, H.D. Becker, Anal. Chem. 63 (1991) 292.
[31] D.S. Burgi, R.L. Chien, Anal. Biochem. 202 (1992) 306.
[32] C. Schwer, B. Gas, F. Lottspeich, E. Kenndler, Anal. Chem. 65 (1993) 2108.
[33] A.C. Steven, P.M. Dennis, Anal. Biochem. 211 (1993) 279.
[34] J. You, W. Zhang, Q. Zhang, L. Zhang, C. Yan, Y. Zhang, Anal. Chem. 74 (2002) 261.
[35] C.K. Frederik, S. Holger, J. Org. Chem. 67 (2002) 7185.
[36] J. You, B. Zhang, W.B. Zhang, P. Yang, Y.K. Zhang, J. Chromatogr. A 909 (2001) 171.
[37] A.I. Haj-Yehia, L.Z. Benet, J. Chromatogr. A 724 (1996) 107.
[38] Y. Mechref, Z.E. Rassi, Anal. Chem. 68 (1996) 1771.