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Determination of amines using 2-(11*H*-benzo[a]carbazol-11-yl) ethyl chloroformate (BCEC-Cl) as labeling reagent by HPLC with fluorescence detection and identification with APCI/MS

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Abstract

A pre-column derivatization method for the sensitive determination of amines using a labeling reagent 2-(11*H*-benzo[a]-carbazol-11-yl) ethyl chloroformate (BCEC-Cl) followed by high-performance liquid chromatography with fluorescence detection has been developed. Identification of derivatives was carried out by LC/APCI/MS in positive-ion mode. The chromophore of 1,2-benzo-3,4-dihydrocarbazole-9-ethyl chloroformate (BCEOC-Cl) reagent was replaced by 2-(11*H*-benzo[a]-carbazol-11-yl) ethyl functional group, which resulted in a sensitive fluorescence derivatizing reagent BCEC-Cl. BCEC-Cl could easily and quickly label amines. Derivatives were stable enough to be efficiently analyzed by HPLC and showed an intense protonated molecular ion corresponding *m/z* $[M+H]^+$ under APCI/MS in positive-ion mode. The collision-induced dissociation of the protonated molecular ion formed characteristic fragment ions at *m/z* 261.8 and *m/z* 243.8 corresponding to the cleavages of CH_2O–CO and CH_2–OCO bonds. Studies on derivatization demonstrated excellent derivative yields over the pH 9.0–10.0. Maximal yields close to 100% were observed with three- to four-fold molar reagent excess. In addition, the detection responses for BCEC-derivatives were compared to those obtained using 1,2-benzo-3,4-dihydrocarbazole-9-ethyl chloroformate (BCEOC-Cl) and 9-fluorenyl methylchloroformate (FMOC-Cl) as labeling reagents. The ratios $I_{BCEC}/I_{BCEOC} = 1.94–2.17$ and $I_{BCEC}/I_{FMOC} = 1.04–2.19$ for fluorescent (FL) responses (here, *I* was relative fluorescence intensity). Separation of the derivatized amines had been optimized on reversed-phase Eclipse XDB-C_8 column. Detection limits calculated from 0.50 pmol injection, at a signal-to-noise ratio of 3, were 1.77–14.4 fmol. The relative standard deviations for within-day determination ($n = 11$) were 1.84–2.89% for the tested amines. The mean intra- and inter-assay precision for all amines levels were <3.64% and 2.52%, respectively. The mean recoveries ranged from 96.6% to 107.1% with their standard deviations in the range of 0.8–2.7. Excellent linear responses were observed with coefficients of >0.9996.

Keywords: Derivatization; HPLC; Amines; 2-(11*H*-benzo[a]-carbazol-11-yl) ethyl chloroformate (BCEC-Cl)

1. Introduction

Pre-column derivatization in conjunction with reversed-phase liquid chromatography is the most used technique for the determination of amines. Various methods for the separation, identification and determination of amines by high-performance liquid chromatography have been published throughout during the last decade or so [1]. Achieving high efficient determination of them, fluorescence probes are extensively used in chemical and biological sciences for investigating the compositions of interesting environmental and biological samples [2,3]. 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. Volatile amines not only have an unpleasant smell but also possess heat hazards. Moreover, they may react with nitrosating reagents, leading to the formation of potentially carcinogenic *N*-nitrosamine compounds [4–8]. Therefore, it is important to determine certain amino compounds in real environmental sam-

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ples. 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 [9]. Other methods including enzymatic [10,11] and ion-exchange chromatographic detection [12] have been described for the determination of amines in various matrices. These methods are usually limited due to low sensitivity.

At present, popular methods for the determination of amino compounds are pre-column and post-column derivatization with fluorescence detection. Various fluorescent reagents were used for amines labeling including orthophthalaldehyde (OPA) [12,13], 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-F) [14], 9-fluorenylmethyl chloroformate (FMOC) [15], 1-(9-fluorenyl)ethyl chloroformate (FLEC) [16] and 2-(9-anthyl)ethyl chloroformate (AEOC) [17], 6-aminoquinolyl-*N*-hydroxysuccinimidyl-carbamate (AQC) [18] and so on. These reagents have also been reported some various shortcomings in their application, such as short detection wavelengths, poor stability and serious interference for the determination of real biological samples.

In our previous studies [19], we described the synthesis of 1,2-benzo-3,4-dihydrocarbazole-9-ethyl chloroformate (BCEOC-Cl) and its application for the analysis of common amino compounds. On the basis of the fluorescence characteristics of 1,2-benzo-3,4-dihydrocarbazole moiety [19,20], we have synthesized a novel fluorescence reagent 2-(11*H*-benzo[a]-carbazol-11-yl) ethyl chloroformate (BCEC-Cl) that the chromophore of 1,2-benzo-3,4-dihydrocarbazole-9-ethyl functional group is dehydrogenated by chloroanil (tetrachloro-1,4-benzoquinone, 1.05 equiv.) in dry xylene with refluxing resulting in a sensitive fluorescence reagent. BCEC-Cl has been found to be very stable in its crystal state, and exhibits relatively pH-independent fluorescence (pH 4.0–9.0) and high molar absorbance. BCEC-Cl reacts readily with amines and is successfully used as a pre-column labeling reagent for the determination of amines in combination with HPLC. 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. To the best of our knowledge, this is the first time that BCEC-Cl fluorescent probe and its applications for the determination of amines have been reported. The suitability of the developed method for the analysis of amines in food (shrimp catsup) samples was 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 contained 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 (in 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 × 4.6 mm i.d., 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. Gradient elution (A: 30% acetonitrile consisting of 0.2 mmol/l ammonium formate, pH 3.5; B: 100% acetonitrile) was selected for the separation of amine derivatives. 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). Borate buffer was prepared from 0.2 mol/l boric acid solution adjusted to pH 9.0 using 4 mol/l sodium hydroxide solution prepared from sodium hydroxide pellets. The quenching reagent was 36% acetic acid solution. The benzoic acid derivatives of aliphatic amines, which prepared by the reaction of benzoyl chloride with each amine (C1–C12) used to evaluate the reliability of the method, were used as a gift from Organic Laboratory (College of Chemistry Science, Qufu Normal University).

2.3. Synthesis of derivatization reagent (BCEC-Cl)

2.3.1. Synthesis of 1,2-benzo-3,4-dihydrocarbazole and 11H-benzo[a]carbazole

1,2-Benzo-3,4-dihydrocarbazole was synthesized according to the method as previously described in our experiment [20]. 11*H*-Benzo[α]carbazole was synthesized according to the method as previously described [21] with a little modification as follows: 1,2-benzo-3,4-dihydrocarbazole (8.8 g), chloranil (tetrachloro-1,4-benzoquinone) (1.05 equiv.) and dry xylene (100 ml) were mixed. After the mixture was stirred for a period of 1 h at room temperature, the mixture was then refluxed for 2 h under N_2. After cooling the precipitated solid was recovered by filtration, the tetrachlorohydroquinone was washed with NaOH (10%, w/v) and filtered off by suction. The residue was washed with deionized water until pH 7.0, and dried for 48 h at room temperature. The crude product was recrystallized three times from ethanol (150 ml × 3) to afford a white crystal, yield (79.4%). m.p. 235.7–236.1 °C. Found: C 88.24, H 5.10, N 6.47; calculated: C 88.47, H 5.07, N 6.45; IR (KBr),

ν (cm^{-1}): 3436.16 (–N–H); 1626.05 (δ_{N-H}), 1561.10, 1528.58 (Ph); 1460.11 (C–H); 1384.38, 1329.03 (C–H); 818.27(γ_{N-H}), 738.68. MS: *m/z*: 218 $[M+H]^+$.

2.3.2. Synthesis of 2-(11H-benzo[a]-carbazol-11-yl) ethanol

11*H*-benzo[a]carbazole (20 g), KOH (7.0 g) and 200 ml 2-butanone were mixed and rapidly cooled to 0 °C with ice-water with vigorous stirring. A cooled mixture of epoxyethane (6.2 g) in 50 ml of 2-butanone solution was added dropwise within 1 h. The contents were kept at ambient temperature for another 2 h with stirring. The solution was then heated to 50 °C for 2 h and concentrated by a rotary evaporator. After cooling, the residue was transferred into 200 ml of ice-water with vigorous stirring for 0.5 h, the precipitated solid was recovered by filtration, washed with water, 30% ethanol solution, and dried at room temperature for 48 h. The crude product was recrystallized three times from methanol (200 ml × 3) to afford a white crystal, yield (79.4%). m.p. 104.5–106.1 °C. Found: C 82.84, H 5.68, N 5.14; calculated: C 82.76, H 5.75, N 5.36; IR (KBr), ν (cm^{-1}): 3432–3163.4 (–OH); 2922.44 (Ph); 1470.80 (C–H); 1405.3, 1384.96 (C–H); 1126.54 (δ_{C-O}), 810.75, 741.67. MS: *m/z*: 261.8 $[M+H]^+$, *m/z*: 243.8 $[MH^+ - H_2O]$.

2.3.3. Preparation of 2-(11H-benzo[a]-carbazol-11-yl)ethyl chloroformate (BCEC-Cl)

To a solution containing 4.0 g solid phosgene and 100 ml dichloromethane (0 °C) in 500 ml round-bottom flask, a mixture of 2-(11*H*-benzo[a]-carbazol-11-yl) ethanol (5.0 g) and pyridine (2 g catalyst) in 150 ml dichloromethane solution was added dropwise within 2 h with stirring. After stirring at 0 °C for 4 h, the contents were kept at ambient temperature for another 6 h period with vigorous stirring, the solution was then concentrated by a rotary evaporator. The residue was extracted four times with warm ether; the combined ether layers were concentrated in vacuum to yield a white crystal. The crude products were recrystallized twice from ether to give the white crystal 3.47 g (70.0%), m.p. 138.3–139.1 °C. Found: C 70.52, H 4.24, N 4.30, Cl 10.84; calculated: C 70.48, H 4.33, N 4.33, Cl 10.97; IR (KBr), ν (cm^{-1}): 1769.86 (–C=O); 1469.33 (C=H); 1405.64, 1384.94 (C–H); 1147.28, 1101.35, 814.02, 741.18.

2.4. High-performance liquid chromatography

Eluent A was 30% of acetonitrile consisting of 20 mmol/l formic acid/ammonia buffer (pH 3.5); B was acetonitrile (100%). Derivatives were separated on a reversed-phase Eclipse XDB-C_8 column (150 × 4.6 mm i.d., 5 μm, Agilent) in conjunction with a gradient elution. Gradient conditions: initial = 70% A and 30% B; 35 min = 100% B (kept for 5 min). Before injection of the next sample, the column was equilibrated with the initial elution condition for 5 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} 279 and λ_{em} 380 nm, respectively. The detection and identification of BCEC-derivatives was performed by online post-column fluorescence and APCI/MS in positive-ion mode.

2.5. Preparation of standard solutions

The derivatization reagent solution 2.5×10^{-3} mol/l was prepared by dissolving 8.10 mg BCEC-Cl in 10 ml of anhydrous acetonitrile prepared by distilling the dried HPLC grade acetonitrile with P_2O_5. Individual stock solutions of the amines were prepared in acetonitrile. The standard amines for HPLC analysis at individual concentrations of 5.0×10^{-5} mol/l were prepared by dilution the corresponding stock solutions (5.0×10^{-3} mol/l) of each amine with acetonitrile. When not in use, all standards were stored at 4 °C.

2.6. Extraction of amines from shrimp catsup, soil and waste water samples

2.6.1. Extraction of amines from shrimp catsup

The shrimp catsup (5.0 g) was treated either with 25 ml cooled trichloroacetic acid solution (5%, w/v) or with 25 ml cooled $HClO_4$ solution (1.0 M, with 100 mg/L EDTA). After ultrasonication for 30 min, the suspension was centrifuged at 3500 × *g* for 15 min to separate the proteins. The supernatant solutions were then filtered through 2 μm Millipore filters. Before derivatization the solutions, the supernatant solution (1.0 ml) was removed and adjusted to pH 6.0–7.0 using NaOH solution (1.0 M) and made up to 1.5 ml with acetonitrile until HPLC analysis.

2.6.2. Pretreatment of waste water samples

To a solution containing 100 ml of wastewater in 200 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 at 60 °C under reduced pressure in nitrogen gas 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.

2.6.3. Pretreatment of soil samples

To a solution containing 50 ml of trichloromethane in 200 ml round-bottom flask, 100 g soil was added. The contents of the flask were vortexed for 2 min and ultrasonicated for 10 min and filtrated. The extraction procedure was carried out twice, the resulting solutions were combined and added 2.0 ml hydrochloric acid (3.0 M) and evaporated to dryness at 60 °C under reduced pressure in nitrogen gas 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.

2.7. Derivatization procedure

The BCEC-amine derivatization was carried out in aqueous acetonitrile in a basic medium. A 100 μl aqueous of amines was added in a vial, to which 150 μl borate buffer (0.2 mol/l, pH 9.0) and 50 μl BCEC-Cl acetonitrile solution were then added. The

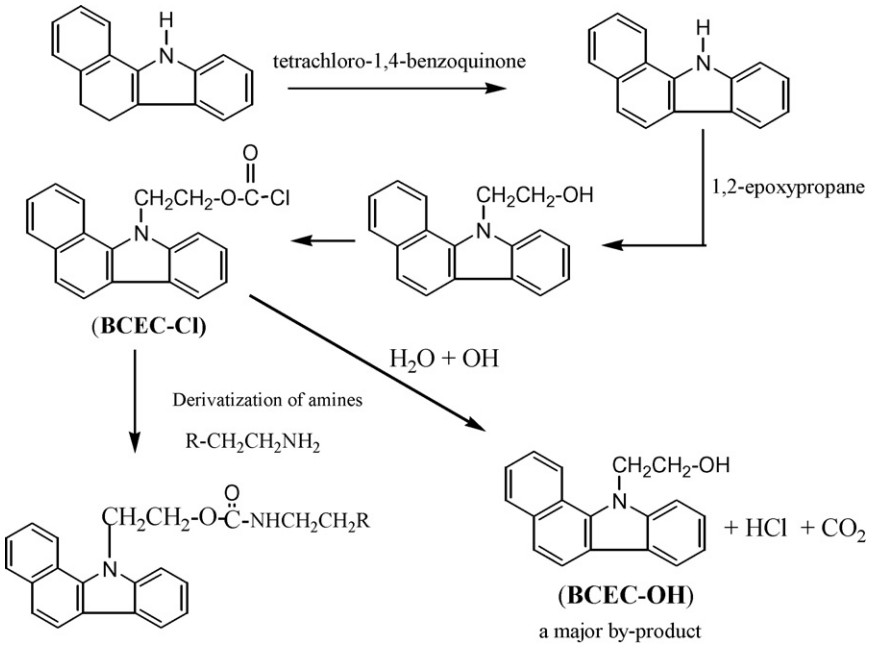

Fig. 1. Derivatization scheme of 2-(11*H*-benzo[α]-carbazol-11-yl) ethyl chloroformate (BCEC-Cl) with amines.

solution was shaken for 0.5 min and allowed to place at room temperature for 3 min, a 10 μl formic acid (36%, w/w) was then added until the final pH range of 6.0–6.5. The derivatized sample was directly injected into the HPLC system. The derivatization process is shown in Fig. 1.

3. Results and discussion

3.1. Ultraviolet absorption of 2-(11H-benzo[a]carbazole-11-yl) ethanol (BCEC-OH)

Benzo-carbazole derivatives are one of the most studied and important classes of photochromic molecules. They exhibit interesting photochromic properties. In previous works, we described the UV properties of 1,2-benzo-3,4-dihydrocarbazole-9-ethanol (BDCE-OH) [19]. BDCE-OH showed high absorption efficiency in the UV range and exhibited two main absorption bands in the 200–400 nm ranges. For the determination of λ_{max} and molar absorption coefficients (ε) of BCEC-OH, 1.5×10^{-5} mol/l solvent solutions (methanol, ethanol, dioxane, acetonitrile, and tetrahydrofuran) were used. The ultraviolet absorption of BCEC-OH was investigated in five solvent systems. Maximum ultraviolet absorption responses were observed at the wavelengths of 230, 244, 253, 279 and 305 nm, respectively (here, the wavelength at 230 nm was not showed for dioxane and tetrahydrofuran solvents, see Fig. 2). Maximum ultraviolet responses did not exhibit obviously blue- or red-shift in four solvent systems. The molar absorption coefficients in methanol (ε) were 3.80×10^4 L mol^{-1} cm^{-1} (230 nm), 3.90×10^4 L mol^{-1} cm^{-1} (244 nm), 4.4×10^4 L mol^{-1} cm^{-1} (253 nm), 4.60×10^4 L mol^{-1} cm^{-1} (279 nm), and 2.6×10^4 L mol^{-1} cm^{-1} (305 nm), respectively. With the concentrations of methanol, ethanol, dioxane, acetonitrile and tetrahydrofuran >50% (v/v), the absorption intensities and maximum wavelengths kept basically constant. When the concentrations of five solvent systems were <50% (v/v), no clear changes for the maximum absorption wavelengths were observed, however, the absorption intensities decreased obviously with decreasing the solvent concentration.

3.2. Fluorescence excitation and emission

The solution of BCEC-butylamine derivative (3.0×10^{-6} mol/l) in 0.2 mol/l borate buffer/acetonitrile (20:80, v/v) was used to obtain the maximum excitation and emission wavelengths. The fluorescence spectra of BCEC-butylamine derivative showed two maximum excitation wavelengths at 279 and 300 nm, and two maximum emission wavelengths at 365 and 380 nm, respectively (data obtained using 650-10S fluorescence spectrophotometer were not corrected). No obvious

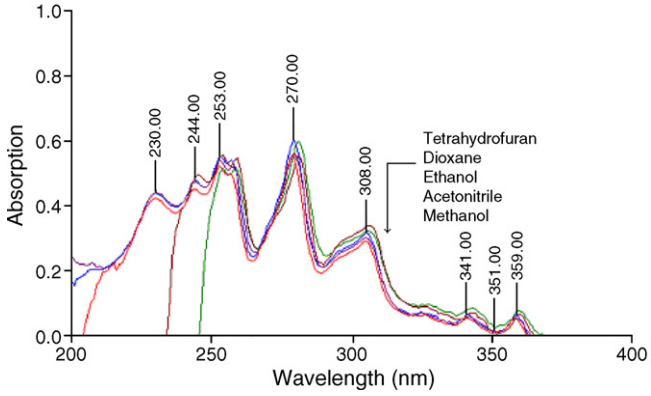

Fig. 2. Ultraviolet absorption (UV) of BCEC-OH in various solvent systems. The concentration of BCEC-OH in each solvent system was 1.5×10^{-5} M. The maximum absorption at 279 nm: ethanol ≈ dioxane > tetrahydrofuran ≈ acetonitrile ≈ methanol.

blue- or red-shift in acetonitrile or methanol solution (0–100%) were 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.

3.3. Optimization for derivatization

3.3.1. Effect of BCEC-Cl concentration on derivatization

BCEC-Cl has the same chloroformate reaction with primary and secondary amino compounds as do of BCEOC-Cl and CEOC-Cl as our previously reported [19,22]. Comparing with the core structure of BCEC-Cl molecule, the π–π conjugation system of BCEC-Cl is greatly augmented due to two hydrogen atoms in indole ring were dehydrogenated with tetrachloro-1,4-benzoquinone in xylene. In our previously reported [19], maximum ultraviolet absorption bands of BCEOC-derivatives were observed at the wavelengths of 249 and 320 nm, respectively. The molar absorption coefficients (ε) were 2.54×10^4 L mol^{-1} cm^{-1} (249 nm) and 2.40×10^4 L mol^{-1} cm^{-1} (320 nm), respectively. After dehydrogenation, the maximum excitation wavelength of BCEC-derivatives exhibited markedly red-shift, the maximum absorption band shifted from 249 to 279 nm with the absorbance index (ε) changing from 2.54×10^4 to 4.6×10^4 L mol^{-1} cm^{-1}. Contrarily, the absorption at the wavelength of 320 nm exhibited visibly blue-shift, the absorption band shifted from 320 to 305 nm with the absorbance index (ε) changing from 2.40×10^4 L mol^{-1} cm^{-1} (320 nm) to 2.60×10^4 L mol^{-1} cm^{-1} (305 nm). The absorption intensity at the wavelength of 279 nm was clearly enhanced. It was of great importance to perform a sensitively fluorescent detection with the excitation wavelength at λ_{ex} 279 nm. Undoubtedly, it was more high efficiency for fluorescence detection relative to that of BCEOC-Cl reagent.

Derivatization of BCEC-Cl with amines could be accomplished within 3 min at room temperature. The fluorescence intensities of the tested standard amines decreased with increasing the reaction times, it was probably due to the fact that the BCEC-amine derivatives rapidly resulted in the decomposition in high basic medium. Therefore, the derivatization solution should immediately be neutralized to pH 6.0–6.5 with 36% acetic acid solution when derivatization was accomplished. The fluorescence intensity of BCEC-derivatives increased with increasing the amounts of derivatization reagent. A constant fluorescence intensity was achieved with the addition of three- to four-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 2.0-fold molar excess of derivatization reagent, the derivatization of amines was incomplete and it obviously resulted in low detection responses. Two side reactions were also observed, the by-products were, respectively, 2-(11*H*-benzo[a]carbazole) ethanol (BCEC-OH) (MS: *m/z*: 261.8; MS/MS: *m/z*: 243.8 ($[M+H]^+$–H_2O)) and bis-(2-(11*H*-benzo[a]carbazole)-ethyl)-carbonate $(BCEC)_2$ (MS: *m/z* 549 $[M+H]^+$; MS/MS: *m/z* 243.8). The formation of the by-products should be attributed to the reagent hydrolysis. At the same time, the side reactions were compared to those obtained using BCEOC and CEOC as labeling reagents previously synthesized in our laboratory [19,22], the results indicated that the derivatization of BCEC-Cl with amines also formed di-substituted by-product bis-(2-(11*H*-benzo[a]carbazole)-ethyl)-carbonate $(BCEC)_2$ which formed by the reaction of the hydrolysated BCEC-OH with the excess reagent BCEC-Cl. The presence of BCEC-OH and $(BCEC)_2$ did not interfere with the separation of amine derivatives. The derivatized amines were found to be stable for more than 48 h at room temperature when the derivatization solution was neutralized to pH 6.0–6.5 with 36% acetic acid solution (w/w).

3.3.2. Effect of pH on derivatization

Several types of basic media were tested for BCEC-Cl derivatization, including carbonate buffers, phosphate buffers and borate buffers. The results showed that borate buffer was found to be the best choice. The effects of pH on the derivatization reaction were then investigated with borate buffers (0.2 mol/l) in the 8.8–10.5 pH ranges. The maximum derivatization yields were achieved in pH range of 9.0–10.0. All subsequent derivatization was, therefore, performed in this pH range, however outside this range, particularly in more acidic solution, decreased responses were observed. At further higher pH values (>10.5), the derivatives exhibit some hydrolysis and partially convert to their BCEC-OH forms, therefore, 0.2 mol/l borate buffer solution at pH 9.0 was chosen for amine derivatization.

3.4. 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 C1–C12 amine derivatives, a reversed-phase Eclipse XDB-C_8 column was selected and eluted with (A) 30% acetonitrile containing 20 mmol/l formic acid and (B) 100% acetonitrile. The gradient elution from 70% A + 30% B to 100% B within 35 min was used to give the best separation with the shortest retention times and the sharpest peaks. As observed, to achieve optimal separation, pH value of mobile phase A can be significantly affected the resolution of all amine derivatives. Separation of the derivatized amine standards can be accomplished at acidic condition with pH 3.5. In comparison with the acidic conditions (pH 3.5), operation at pH > 7.0 resulted in obviously increase in retention value for most amine derivatives. Subsequently, all separation was carried out in pH 3.5, the blank experiment was shown in Fig. 3A. The complete separation for the derivatized amine standards was showed in Fig. 3B.

3.5. Comparison of responses of BCEC-Cl, BCEOC-Cl and FMOC-Cl for fluorescence detection

Relative responses for fluorescence detection for the individual derivatized amine using BCEC-Cl, FMOC-Cl [15] and BCEOC-Cl [19] were evaluated, respectively. To make a quantitative comparison with respect to relatively fluores-

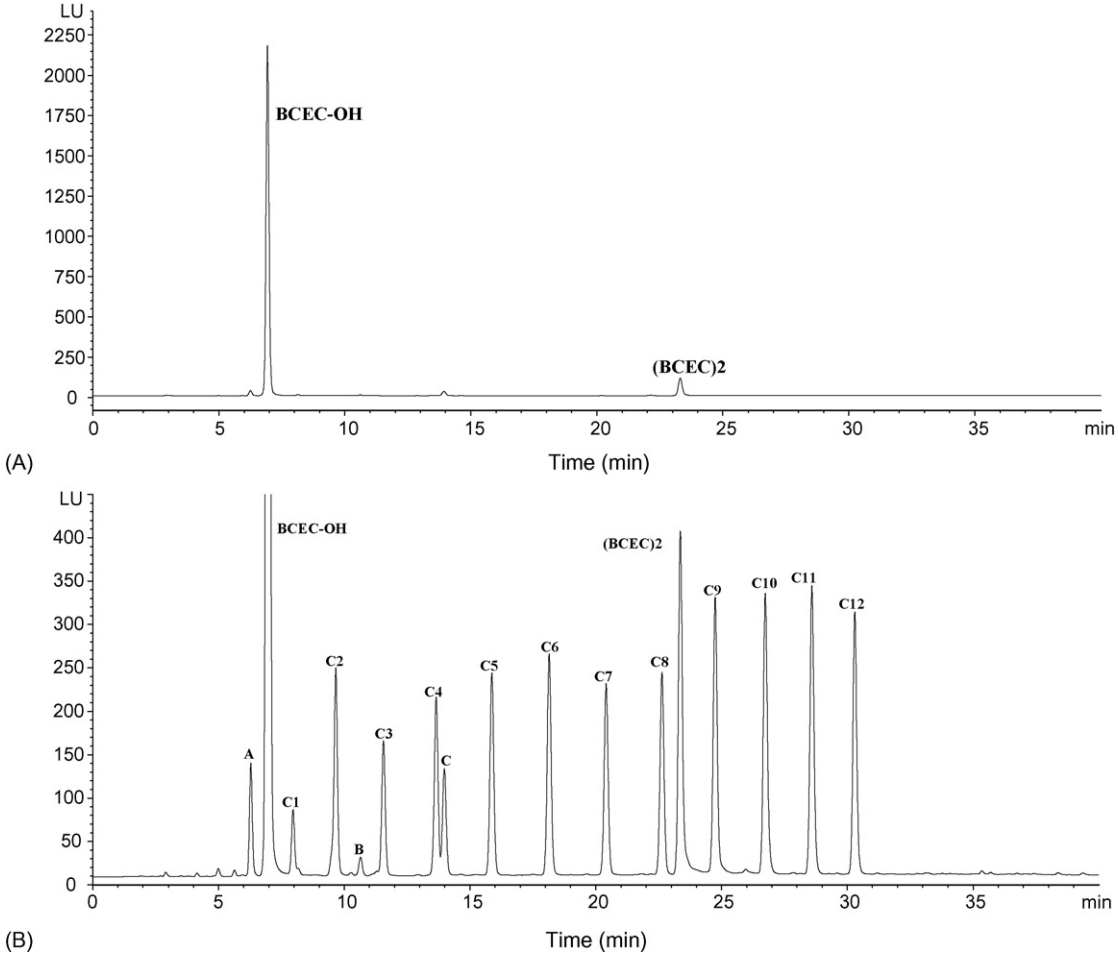

Fig. 3. Chromatogram for standard aliphatic amines derivatized with BCEC-Cl (injected amount 20 pmol). Column temperature is set at 30 °C; excitation wavelength λ_{ex} 279 nm, emission wavelength: λ_{em} 380 nm; reversed-phase Eclipse XDB-C_8 column (5 μm); flow rate = 1.0 ml min^{-1}. C1: methylamine; C2: ethylamine; C3: propylamine; C4: butylamine; C5: pentylamine; C6: hexylamine; C7: heptylamine; C8: octylamine; C9: nonylamine; C10: decylamine; C11: undecylamine; C12: dodecylamine A (unidentified); B (allylamine); BCEC-OH (2-(11*H*-benzo[a]carbazol-11-yl)ethanol) (top (A): reagent blank; bottom (B): the separation of derivatized standard amines).

cent intensities, a standard solution containing C1–C12 amines was derivatized, respectively, using BCEC-Cl, BCEOC-Cl and FMOC-Cl as labeling reagents (final derivatized concentration was adjusted to 2.0×10^{-7} mol/l, 10 μl injection, corresponding injected amount for each derivatized amine 20 pmol). The separation of standard amine derivatives was performed according to the established method as described in Section 2. The detection wavelength was set at the optimal wavelength ranges (here, BCEOC-derivatives detected at $\lambda_{ex}/\lambda_{em}$ = 333/390 nm, FMOC derivatives detected at $\lambda_{ex}/\lambda_{em}$ = 263/313 nm, the optimal resolution for the derivatized amines using BCEOC and FMOC as labeling reagent was not further adjusted). The results indicated that fluorescence intensity for individually derivatized amine using BCEC-Cl as derivatizing reagent exhibited obviously enhancement. The ratios for the fluorescence responses, I_{BCEC}/I_{BCEOC} = 1.94–2.17 and I_{BCEC}/I_{FMOC} = 1.04–2.19 (see Table 1). This was probably due to the fact that BCEC-Cl had the largest molar absorbance, which made it more sensitive for derivatizing amines relative to that of BCEOC-Cl and FMOC-Cl. The difference in molar absorbance should be attributed to the BCEC-Cl molecular core structure, in

Table 1
Comparison of relative fluorescence intensities of amine derivatives using BCEC, BCEOC and FMOC as labeling reagents (derivatized amine concentration 2.0×10^{-7} M; 10 ml injection; corresponding injected amount for each amine at 20 pmol)

Amines	Relative fluorescence intensity			Ratio	
	BCEC	BCEOC	FMOC	I_{BCEC}/I_{BCEOC}	I_{BCEC}/I_{FMOC}
Methylamine	79.1	37.1	36.2	2.13	2.19
Ethylamine	218.2	107.1	113.3	1.94	1.92
Propylamine	136.3	65.8	73.9	2.07	1.84
Butylamine	175.6	83.9	100.2	2.09	1.75
Pentylamine	192.7	92.7	115.4	2.08	1.67
Hexylamine	204.1	97.2	127.3	2.10	1.60
Heptylamine	173.3	81.7	112.8	2.12	1.54
Octylamine	175.6	81.9	123.1	2.14	1.43
Nonylamine	233.6	107.3	177.1	2.17	1.32
Decylamine	213.8	99.8	171.7	2.14	1.18
Undecylamine	205.4	102.6	195.4	2.00	1.05
Dodecylamine	189.0	91.6	181.0	2.06	1.04

which its π–π conjugation system was dramatically augmented due to increasing a 2-(11*H*-benzo[a]carbazole) ethyl functional group.

3.6. Identification with APCI/MS in positive-ion detection mode

In this study, it was found that the collision-induced dissociation of underivatized free amines with APCI/MS analysis did not generate an intense stable molecular ion and corresponding fragment ions that could be used for specific detection of amino compounds. In view of this shortcoming, derivatization of amines to specific enhancement mass spectrometric detection has been extensively employed. To enhance detection sensitivity, we sought to introduce a highly ionizable functional group into the labeling reagent molecule. The introduction of a 2-(11*H*-benzo[a]carbazole) ethyl functional group in labeling reagent molecule, which bore a weak basic nitrogen atom in molecular core structure, should enhance the ionization of BCEC-amines significantly.

As expected, the derivatized amines using BCEC-Cl as labeling reagent also exhibit excellent ionizable efficiency. Derivatives show intense protonated molecular ion corresponding *m/z* $[M+H]^+$ in positive-ion mode. Ion current intensities for derivatized amines are compared to those obtained using BCEOC-Cl as labeling reagent (here, FMOC-amines does not show ion current signals under APCI/MS in positive-ion mode because the molecular core structure of FMOC-amines do not contain an ionizable basic nitrogen to provide a site that can be accepted a $[H]^+$ to form corresponding stable ion current in MS ion chamber). The ratios $IC_{BCEC}/IC_{BCEOC} = 1.94–2.17$ for the C1–C12 amine derivatives. Although BCEC-amine derivatives exhibit high fluorescent efficiency, it gives relatively low the ionization capability relative to that of BCEOC-amine derivatives. This may be attributed to the molecular core structure of BCEC molecule, in which its π–π conjugation system is augmented and results in lowly ionizable nitrogen. However, it is not influence on the APCI/MS identification for the derivatized amines.

The selected reaction monitoring, based on the *m/z* $[M+H]^+ \rightarrow$ *m/z* 261.8 and *m/z* 243.8 transitions, was specific for amine derivatives. 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 BCEC-Cl reagent, no interference was observed due to the highly specific parent mass-to-charge ratio and the characteristic product ions in the *m/z* $[M+H]^+ \rightarrow$ *m/z* 243.8 and *m/z* 243.8 transitions. To reduce the disturbance to minimum, the gradient elution with HPLC for the separation and determination of derivatized BCEC-amines was an efficient method. In most cases, the collision-induced dissociation spectra of *m/z* $[M+H]^+$ produced intense fragment ion at *m/z* 261.8 and *m/z* 243.8. The characteristic fragment ion at *m/z* 261.8 comes from the cleavage of CH_2O–CO band and the fragment ion at *m/z* 243.8 comes from the cleavage of CH_2–OCO band. The MS and MS/MS spectra of representative C12-amine derivative are shown in Fig. 4A and B. The cleavage mode is shown in Fig. 4C.

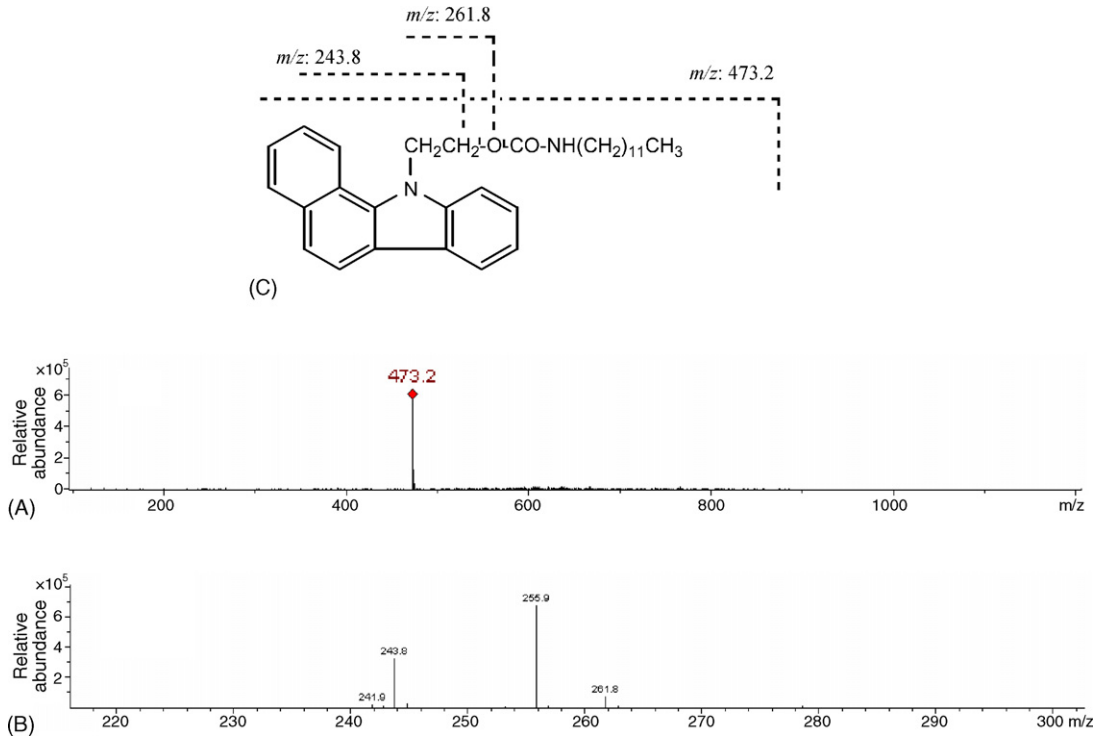

Fig. 4. The profile of cleavage mode and ion mass spectra for the scanning of the derivatized C12-amine derivative. Typical MS chromatogram of C12-amine derivative from full scanning range from 200 to 1000 amu under APCI in positive-ion mode; C12-amine derivative was isolated from a reversed-phase Eclipse XDB-C_8 column, and into the on-line mass spectrometer (A: molecular ion MS; B: MS/MS; C: cleavage mode).

3.7. Analytical precision, accuracy, reproducibility and recovery

A standard containing 50 pmol BCEC-amine derivative was prepared to examine the method repeatability. The relative standard deviations (R.S.D.; $n = 12$) of the peak areas and retention times were from 0.85% to 1.29% and 0.019% to 0.079%, respectively. Precision and accuracy: within-day and between-day precision for 12 amines was examined by using three identical shrimp catsup samples, respectively, spiked with 0.05×10^{-6}, 0.1×10^{-6}, and 0.2×10^{-6} mol/l of amines to make the low to high-range concentrations. The relative standard deviations for within-day determination ($n = 11$) were 1.84–2.89% for the tested amines. The mean intra- and inter-assay precision for all amines levels were <3.64% and 2.52%, respectively.

The recoveries were determined from values obtained following actual analysis of the shrimp catsup as calculated from the calibration graph constructed by using the performed amine derivatives. In two shrimp catsup samples, the known amount of twelve above-mentioned amines was added. The samples were treated according to the method as described in text and derivatized with BCEC-Cl, and analyses were carried out in duplicate. The experimental recoveries are in the range of 96.6–107.1% with their standard deviations in the range of 0.8–2.7.

3.8. 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. 5 showed the injection of 0.50 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 1.77 to 14.4 fmol. The linearities were established over a 2000-fold concentration range for amines with analysis of serial dilutions of the standard solution ranging from 2.5×10^{-9} to 5.0×10^{-6} mol/l (corresponding injected amounts of amines from 50 to 0.025 pmol). All of the amines were found to give linear responses over this range, with correlation coefficients of >0.9996. The linear regression analysis for higher concentrations of amines was not tested due to all peaks with large overrun. The linear regression equations, correlation coefficients and detection limits are showed in Table 2.

Table 2
Linear regression equations, correlation coefficients and detection limits

Amine	$Y = AX + B$, X: injected amount (pmol); Y: peak area	R	R.S.D.s (%)	Detection limits (fmol)
C_1	$Y = 26.56X + 4.07$	0.9998	2.79	14.4
C_2	$Y = 95.53X + 29.39$	0.9999	2.76	5.75
C_3	$Y = 61.28X + 5.82$	0.9999	2.43	6.27
C_4	$Y = 84.47X + 12.04$	0.9999	2.36	3.28
C_5	$Y = 98.14X + 12.97$	0.9999	2.48	4.30
C_6	$Y = 109.38X + 15.97$	0.9999	1.70	3.63
C_7	$Y = 95.64X + 16.10$	0.9999	1.87	4.90
C_8	$Y = 100.79X + 19.81$	0.9998	1.86	3.45
C_9	$Y = 144.22X + 37.45$	0.9997	1.90	2.02
C_{10}	$Y = 142.25X + 51.33$	0.9996	1.84	2.30
C_{11}	$Y = 134.58X + 80.55$	0.9996	2.22	1.92
C_{12}	$Y = 115.87X + 82.30$	0.9996	2.10	1.77

3.9. Comparison of derivatization conditions and detection limits of the reagents reported for aliphatic amines

Compared to AQC [23], FMOC [24], NBD-Cl [25], and OPA/NAC [26], several of the most popular fluorescent labeling reagents, BCEC-Cl exhibits desired fluorescence properties superior to those of AQC, FMOC, NBD-Cl, and OPA/NAC. The fluorescence excitation and emission wavelengths are 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

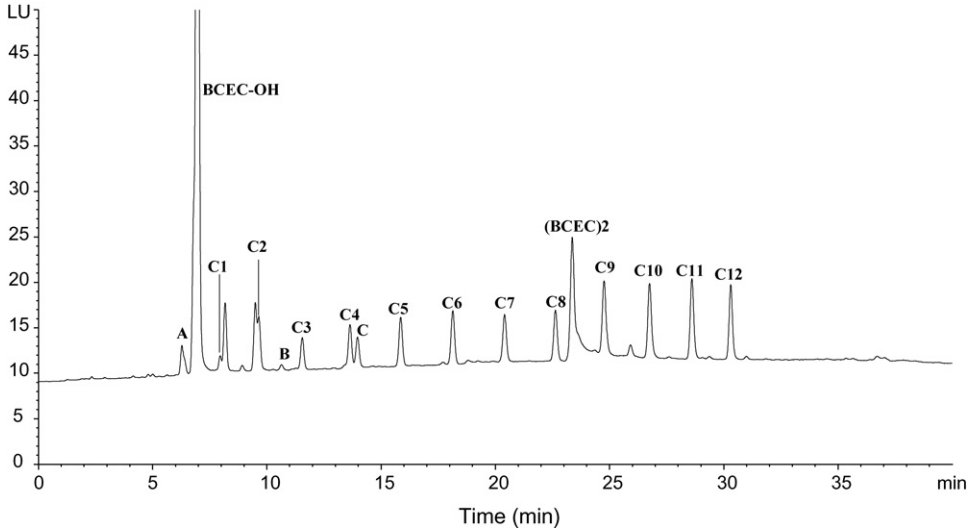

Fig. 5. Chromatogram for aliphatic amine standard mixtures with 0.5 pmol injection. Column temperature is set at 30 °C; excitation wavelength λ_{ex} 279 nm, emission wavelength λ_{em}380 nm; column: reversed-phase eclipse XDB-C_8 column (5 μm); flow rate = 1.0 ml min^{-1}; peaks as B.

Table 3
Comparison of the derivatization conditions and detection limits of the reagents reported for aliphatic amines

Reagents	BCEC-Cl	FMOC-Cl	OPA/NAC	NBD-Cl	AQC	BCEOC-Cl
$\lambda_{ex}/\lambda_{em}$	279/380	265/310	340/455	470/530	250/390	333/390
Derivatization time (min)	2–3	2–3	1–1.5	60	5	2–3
Derivatization temperature (°C)	Room temperature	Room temperature	Room temperature	55	65	Room temperature
Amines	Detection limits					
	fmol	μg/l	μg/ml	fmol	pmol	fmol
C_1	14.4	0.5	0.002	–	6.45	29.77
C_2	5.75	0.25	0.015	–	3.33	37.68
C_3	6.27	0.5	0.020	–	1.69	25.56
C_4	3.28	1	0.030	170	1.36	18.65
C_5	4.30	5	0.040	–	0.58	27.95
C_6	3.63	2.5	–	400	0.49	35.58
C_7	4.90	–	–	220	–	38.82
C_8	3.45	–	–	–	–	32.26
C_9	2.02	–	–	–	–	26.40
C_{10}	2.30	–	–	300	–	21.72
C_{11}	1.92	–	–	320	–	36.57
C_{12}	1.77	–	–	340	–	28.80
References	This paper	[23]	[24]	[25]	[26]	[28]

–: Data are not reported or not determined.

in these organic solutions. In the pH range of 9.0–10.0, the derivatization of BCEC-Cl with aliphatic amines can be accomplished within 3–4 min under room temperature and gives the almost theoretic yields of derivatives in aqueous acetonitrile, which are comparable to FMOC and OPA derivatives. The derivatization conditions are much milder than those of AQC and NBD-Cl derivatives, which are carried out at 55–65 °C. Although AQC has been developed as a popular pre-column derivatization reagent for the determination of amines and amino acids with satisfactory results, only 10% of the fluorescent intensity in aqueous solution compared to that in pure acetonitrile solution is observed for its derivatives. Thus, the detection limits for the early-eluted derivatives are usually higher than those for later ones [18]. As observed, BCEC-derivatives are more photostable than OPA and NBD derivatives. With NBD-Cl derivatization, data reported previously indicated that showed ca. 30–50% decomposition in methanol–water within 25 min when exposed to daylight, at the same time, the derivatization of NBD-Cl with amines was time consuming [27]. Therefore, BCEC-Cl is prospective significance as a pre-column derivatizing reagent for amines in terms of sensitivity and stability. It was shown that the quantification of aliphatic amines could be well done with the established method. The overall comparison of BCEC-Cl with other common-used fluorescent labeling reagents for aliphatic amines in HPLC is given in Table 3. The detection limits of most reagents are higher than BCEC-Cl. In view of the detection properties (such as wavelength, derivatization time and temperature, and detection limits) for the determination of amines, BCEC-Cl is more advantageous than other reagents. Current studies should further explore the derivatization of different amine-containing compounds such as secondary aliphatic amines and polyamines.

Table 4
Standard compositional analysis of hydrolyzed samples

Actual, (%) molar number		Sample		Sample	
		254.4 ng (~total 100 pmol)	R.S.D. (%) ($n=6$)	0.7632 ng (~total 300 fmol)	R.S.D. (%) ($n=6$)
Standard mixture of C1–C12 amine-derivatives of benzoyl chloride					
C1	8.33	8.54	0.9	8.23	1.2
C2	8.33	8.32	0.8	8.20	1.3
C3	8.33	8.20	1.0	8.24	1.1
C4	8.33	8.10	0.8	8.31	1.4
C5	8.33	8.67	1.1	8.27	1.2
C6	8.33	8.54	1.2	8.56	1.0
C7	8.33	8.35	0.8	8.43	1.3
C8	8.33	8.32	0.6	8.40	0.9
C9	8.33	8.43	0.9	8.32	1.1
C10	8.33	8.54	1.0	8.34	0.9
C11	8.33	8.44	0.9	8.43	0.8
C12	8.33	8.50	1.2	8.42	1.1

Table 5
Contents of aliphatic amines from real sample and recoveries

Samples, amines	Shrimp catsup				Soil				Waste water			
	Added (ng/g)	Found (ng/g)	R.S.D. (%, $n=6$)	Recovery (%)	Added (ng/g)	Found (ng/g)	R.S.D. (%, $n=6$)	Recovery (%)	Added (ng/g)	Found (ng/g)	R.S.D. (%, $n=6$)	Recovery (%)
C1	0	9095.2	1.1		0	1.1	1.4		0	0.2	2.3	
	100	9202.3	2.1	107.1	100	102.2	1.3	101.1	100	100.8	2.4	100.6
C2	0	124.8	2.0		0	0.3	2.2		0	0.1	2.0	
	100	227.8	1.8	103.0	100	101.1	2.3	100.8	100	99.6	2.1	99.5
C3	0	#			0	0.06	2.4		0	0.08	2.0	
	100	100.4	2.3	100.4	100	100.1	2.1	100.0	100	99.2	2.2	99.1
C4	0	#			0	0.2	2.4		0	#		
	100	100.3	2.7	100.3	100	100.3	2.2	100.1	100	99.6	2.0	99.6
C5	0	66.8	2.10		0	#			0	0.3	2.6	
	100	172.2	1.89	105.4	100	100.2	0.9	100.2	100	100.4	2.0	100.1
C6	0	7.2	1.84		0	0.1			0	4.8	1.6	
	100	103.8	1.95	96.6	100	101.1	0.9	101.1	100	105.1	1.4	100.3
C7	0	3.0	2.36			#			0	#		
	100	101.8	2.38	98.8					100	100.2	0.9	100.2
C8	0	#				#			0	#		
	100	103.2	2.70	103.2					100	100.1	0.8	100.1
C9	0	3.4	2.26			#			0	0.06	2.1	
	100	104.2	2.31	100.8					100	100.1	2.3	100.0
C10	0	2.7	2.40			#			0	0.06	2.2	
	100	101.6	2.56	98.9					100	99.9	2.1	99.8
C11	0	0.5	2.34			#			0	0.02	1.8	
	100	101.0	2.24	100.5					100	100.1	2.0	100.1
C12	0	#				#			0	0.03	2.1	
	100	100.5	2.47	100.5					100	100.1	2.0	100.1

#: Not detection or below LOQ.

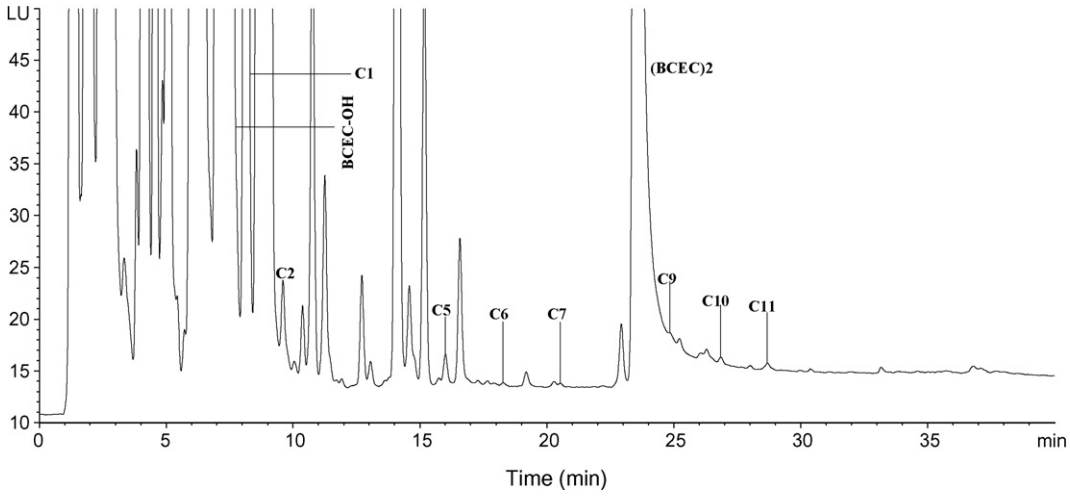

Fig. 6. Chromatogram for the separation of aliphatic amines from shrimp catsup sample derivatized with BCEC-Cl. Column temperature is set at 30 °C; excitation wavelength λ_{ex} 279 nm, emission wavelength: λ_{em} 380 nm; column eclipse XDB-C_8 (5 μm); flow rate = 1.0 ml min^{-1}; peaks as B.

3.10. Standard composition analysis of amine compounds

The reliability of the proposed method was further evaluated by applying it to the determination of standard hydrolyzed amine compounds from benzoic acid derivatives of C1–C12 amines prepared by the reaction of benzoyl chloride with each amine (C1–C12). The standard mixture of benzoic acid derivatives of C1–C12 amines were mixed at an equivalent molar amount of each derivative and dissolved in acetonitrile to give the final total concentration of 1.0×10^{-7} mol/l. The hydrolyzed procedure was carried out according to the literature [19]. The final total hydrolyzed concentration is 1.0×10^{-8} mol/l. The quantitative analysis with standard cure method is agreement with the internal standard method using γ-aminobutyric acid (GABA) as internal standard compound. Amine compositional data for hydrolyzed standard benzoic acid derivatives of C1–C12 amines are shown in Table 4. There is an excellent agreement with the actual compositions. The data in Table 4 were obtained from ca. 254.4 ng (ca. 100 pmol) of hydrolyzed benzoic acid derivatives of C1–C12 amines. In fact, even with low- or sub-picomole amounts hydrolyzed benzoic acid derivatives of C1–C12 amines (ca. 300 fmol), useful compositional analysis can also be obtained. With the analysis of 254.4 ng sample, error data were given with an average error of 0.8–1.2%. With the analysis of 0.7632 ng sample, the slightly high error data were observed with an average error of 0.9–1.4%. Chromatogram for the analysis of standard hydrolyzed benzoic acid derivatives of C1–C12 amines is not shown.

3.11. Analysis of samples

Chromatogram for the analysis of free amines from shrimp catsup sample with fluorescence detection shows in Fig. 6 (chromatograms for soil and waste water samples are not shown) Amine compositional data of shrimp catsup, soil and wastewater samples are shown in Table 5. As can be seen, the established method is suitable for the determination of amine composition from shrimp catsup with satisfactory results. Except for aliphatic amines, there are some amino acids being in existence in shrimp catsup sample. To exclude the interference of amino acids in sample, the retention times of familiar amino acid derivatives were investigated. It was found that under the selected chromatographic condition, the peaks of amino acid derivatives and that of BCEC-OH 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 facile BCEC-Cl derivatization coupled with mass spectrometry allowed the development of a highly sensitive and specific method for the quantitative analysis of trace levels of amines from food or natural environmental samples.

4. Conclusions

A new sensitive fluorescent labeling reagent, 2-(11*H*-benzo[a]carbazole) ethyl chloroformate (BCEC-Cl) was developed for the determination of amines by HPLC. The absorption intensity of BCEC-Cl reagent at the wavelength of 279 nm was clearly enhanced. Compared to amine derivatives obtained using BCEOC-Cl and FMOC-Cl as labeling reagents, BCEC-amine derivatives show excellent fluorescence properties, it exhibits relatively high fluorescence efficiency. The improved reagent 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. It takes less than 3 min to perform derivatization under mild conditions. The described method shows good correlation in comparison with BCEOC-Cl and FMOC-Cl 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 aromatic amines and polyamines.

Acknowledgments

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