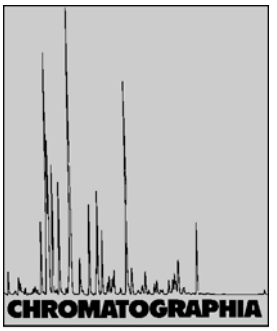
Determination of Biogenic Amines by RPHPLC with Fluorescent Detection after Derivatization with 2-(9-Carbazole)ethyl Chloroformate (CEOC)



2002, 56, 43–50

Jinmao You[1, 2] / Yukui Zhang[1*]

[1] Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian, 116011, P. R. China; E-Mail: YKZhang@ms.dicp.ac.cn
[2] Department of Chemistry, Qufu Normal University, Qufu Shandong, 273165, P. R. China

Key Words

Column liquid chromatography
Fluorescent detection
2-(9-Carbazole)ethyl chloroformate
Biogenic amines

Summary

The use of 2-(9-carbazole)ethyl chloroformate (CEOC) for pre-column derivatization of biogenic amines (BA) has been tested for the first time. The reagent reacts completely with BA within 3 min at ambient temperature in acetonitrile solution to form stable derivatives that are readily analyzed by reversed-phase HPLC. Study of the derivatization conditions revealed derivatization yields to be excellent in borate buffer over the pH range 9.0 – 10.0. Maximum yields were obtained by use of a three- to fourfold molar excess of reagent. The reaction is extremely tolerant of common buffer salts, no decrease in reaction yield is discernible in well-buffered samples. The emission maximum for the CEOC-derivatives is 360 nm (λ_{ex} = 293 nm). All the derivatives fluoresced strongly and direct injection of the reaction mixture was possible, with no significant disturbance from the major fluorescent reagent degradation by-products, 2-(9-carbazole)ethanol (CEOC-OH) and bis-(2-(9-carbazole)ethyl) carbonate $(CEOC)_2$. Separation of the derivatized BA by high-performance liquid chromatography with gradient elution was tested on a Hypersil BDS C18 column. Excellent response linearity was observed over the concentration range from 0.25 to 94.6 μmol L^{-1} for the labeled BA. Detection limits were 117 – 840 fmol at a signal-to-noise ratio of 3:1. Analysis of BA in a shrimp sauce extract was conducted to demonstrate the applicability of the technique to real sample matrixes; results were satisfactory.

Introduction

Biogenic amines, e. g. histamine, tyramine, phenylethylamine, tryptamine, cadaverine, putrescine, spermidine, and spermine, are important substances, with recognized biological activity, which can occur naturally in foods such as fish products, cheese, milk, and fermented foods [1]. The major pathway of BA formation in foods is decarboxylation of free amino acids during ripening processes or the transamination of aldehydes and ketones [2]. Among the various BA histamine, tyramine, and 2-phenylethylamine have been extensively studied, because of their high toxicity and the health risks attributed to their vasoactive and psychoactive properties. Amounts and ratios of some selected BA might serve as indicators of the quality of foods. The consumption of foods containing high concentrations of BA can cause problems, e. g. headaches, nausea, cardiac palpitations, etc., for some consumers.

The small quantities present in some foods can be difficult to determine by conventional methods. Radioimmunological [3] and enzymatic [4] methods, and chromatographic techniques such as thin-layer chromatography [5] or gas chromatography [6] have been used for BA analysis. Another method of analysis of BA is separation by ion-exchange chromatography and then pulsed amperometric detection [7]. Because most BA and amino acids have neither satisfactory absorption in the visible or ultraviolet (UV) range nor fluorescence properties, pre-column or post-column chemical derivatization is necessary to increase detection sensitivity and improve selectivity after separation by means of HPLC [8], on electrophoretic microchips [9], or by CE [10]. These techniques have been used in the physical, chemical, and biological sciences for investigating the structure and dynamics of living systems. Although several different types of fluorescent tagging reagent [11–17] have been developed, there have also been many reports describing their various shortcomings in application. For fluorescence labeling of BA suitable pre-column derivatization reagents are *o*-phthaldialdehyde (OPA) or thiols such as 3-mercaptopropionic acid (3-MPA) [18] or 2-mer-

0009-5893/00/02 43-08 $ 03.00/0

captoethanol (2-BME) [19–21]. OPA has also been widely used for the determination of amino acids with high sensitivity and selectivity [22–24], but is limited to use with primary amino compounds only. Although the instability of the OPA derivatives makes manual derivatization difficult to reproduce, satisfactory automated procedures have been developed [25]. 9-Fluorenyl methylchloroformate (FMOC) has also been widely used for the detection of BA [2] and amino acids [26, 27]. Other reagents used for fluorescence labeling of BA are, for example, 2-naphthyloxycarbonyl chloride (NOC-Cl) [28], 4-(2-phthalimidinyl)phenylsulfonyl chloride (Phisyl-Cl), and 4-(5,6-dimethoxy-2-phthalimidinyl)phenylsulfonyl chloride (DPS-Cl) [29]. 6-Aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) has also recently been developed as a popular pre-column derivatization reagent for the determination of BA [30, 31] and amino acids [32], with satisfactory results. Pre-column derivatization reagents for BA used for UV-visible detection are, for example, 3,5-dinitrobenzoyl chloride [2], dabsyl chloride [33], 5-dimethylaminonaphthalene-1-sulfonyl chloride (Dns-Cl) [34, 35], and 4-dimethylaminoazobenzene-4′-sulfonyl chloride (DABS-Cl) [36].

Although fluorescence detection is regarded as one of the most sensitive detection techniques, derivatization of the analytes of interest with an appropriate fluorophore must be accomplished to enable detection of many non-fluorescent analytes. In a previous paper [37] we described the synthesis and analytical application of carbazole-9-*N*-acetyl chloride (CRA-Cl) for the determination of amino compounds. Although its chromophore fluoresces strongly, it was somewhat difficult to prepare in the pure crystalline state and was unstable to moisture. On the basis of the fluorescence characteristics of the carbazole moiety we have synthesized a novel fluorescence reagent with the same chromophore, 2-(9-carbazole)ethyl chloroformate (CEOC). CEOC has been found to be readily accessible and very stable in its crystalline state or in acetonitrile solution. To the best of our knowledge this is the first time the CEOC fluorescent probe and its application to the determination of BA have been reported.

In this work the optimum derivatization conditions, e. g. buffer pH, reaction time, and mobile phase, were investigated and the linearity, detection limits, and precision of the whole procedure were determined. By using CEOC we have established a sensitive method for the determination of important BA such as histamine, tyramine, 2-phenylethylamine, and spermine in food. If the conditions used for elution (e. g. mobile phase composition) are carefully selected, disturbance as a result of CEOC hydrolysis (CEOC-OH) and its by-product bis-(2-(9-carbazole)ethyl) carbonate $(CEOC)_2$ can be avoided.

Experimental

Instrumentation

HPLC was performed with a Jasco PU-1580 intelligent pump (Hitachi, Tokyo, Japan) equipped with a Jasco LG-1580-04 quaternary gradient unit, a Jasco DG-1580-04 4-line degasser, a Rheodyne (USA) 7125 injection valve, a Jasco UV-1575 intelligent UV-visible detector, and a Jasco FP-1520 intelligent fluorescence detector. Data were analyzed by means of a Hitachi D-2500 chromatography integrator. Fluorescence excitation and emission spectra were obtained by means of a 650-10 S fluorescence spectrophotometer (Hitachi); excitation and emission band-pass were both 15 nm. Derivatives were separated on a 200 mm × 4.6 mm i. d., 5-μm particle, Hypersil BDS C18 column (Dalian Institute of Chemical Physics, Chinese Academy of Sciences). Before use all mobile phases were treated ultrasonically for 15 min to remove gas bubbles.

Chemicals

Amino acid and biogenic amine standards were purchased from Sigma (St Louis, MO, USA). Boric acid was analytical grade from Shanghai Chemical Reagent Co. Water was purified on a Milli-Q system (Millipore, Bedford, MA). Borate buffer was prepared from 0.2 mol L^{-1} boric acid solution adjusted to pH 9.0 with 4 mol L^{-1} sodium hydroxide solution prepared from sodium hydroxide pellets. Borate buffers of other pH were prepared similarly. The quenching reagent was acetonitrile-water-acetic acid, 20:30:50 (*v*/*v*). Acetate buffer (0.1 mol L^{-1}) was prepared by mixing sodium acetate (0.5 mol L^{-1}, 80 mL) and acetic acid (0.5 mol L^{-1}, 20 mL); this solution was diluted to 500 mL with water, the pH was adjusted to 4.0, and the solution was used to prepare the mobile phases.

Preparation of Standard Solutions

The derivatizing reagent solution (1.0×10^{-3} mol L^{-1}) was prepared by dissolving 2-(9-carbazole)ethyl chloroformate (2.62 mg) in acetonitrile (10 mL). Individual stock solutions of the amino acids were prepared in water and, if necessary, HCl or NaOH was added until the compound dissolved. Individual stock solutions of the BA were prepared in 50% acetonitrile solution containing 0.1 mol L^{-1} borate buffer (pH 9.0). Amino acid standards at individual concentrations of 5.0×10^{-5} mol L^{-1}, for HPLC analysis, were prepared by dilution of the corresponding stock solutions (1.0×10^{-2} mol L^{-1}) of each amino acid with 0.2 M borate buffer (pH 9.0). BA standard solutions (5.0×10^{-5} mol L^{-1}) were prepared by dilution of the corresponding stock solutions (1.0×10^{-2} mol L^{-1}) of each BA with 50% acetonitrile solution. Low-concentration standard solutions of amino acids or BA were all obtained by dilution of the corresponding stock solution with borate buffer or 50% acetonitrile solution. When not in use all standards were stored at 4 °C under refrigeration.

Synthesis of 2-(9-Carbazole)ethanol

2-(9-Carbazole)ethanol was synthesized by reaction of carbazole-9-yl-acetic acid with the reducing agent $LiAlH_4$, by heating under reflux in diethyl ether for 2 h. After addition of water to stop the reaction the mixture was extracted with ether. The combined ether extracts were successively washed twice with 10% Na_2CO_3 and water, dried with anhydrous Na_2SO_4, and the solvent was removed by evaporation under reduced pressure. The residue was extracted three times with warm *n*-heptane (3 × 100 mL) and the combined *n*-heptane extracts were cooled in an ice-bath. The white product was isolated by filtration and recrystallized from *n*-heptane to afford a needle-like crystals, yield 78%, m.p. 65 °C. Found, C 79.52, H 6.10, N 6.65; calculated, C 79.62, H 6.16, N 6.63; IR (KBr), 3600–3300 (OH), 1530 (Ph), 1450 (C–H), 1233, 741; *m*/*z*: 211(M^+); ^{1}H NMR, 4.11–4.20 (Ph-CH_2–), 4.64–4.67 (–CH_2), 7.32–8.28 (Ph).

Preparation of 2-(9-Carbazole)ethyl Chloroformate (CEOC)

A mixture of 2-(9-carbazole)ethanol (25 g) and pyridine (2 g, catalyst) in dichloromethane (40 mL) was added dropwise to a solution of solid phosgene (15 g) in dichloromethane (100 mL) at 0 °C in a 500-mL round-bottomed flask. After stirring at 0 °C for 4 h the mixture was kept at ambient temperature for another 6 h with vigorous stirring; the solution was then concentrated by rotary evaporation. The residue was extracted four times with warm ether and the combined ether extracts were concentrated under vacuum to yield white crystals. The crude product was re-crystallized from 1:3 (*v/v*) dichloromethane-ether to give white crystals (26.5 g, 85.5%), m.p. 83–84 °C. Found, C 68.43, H 4.62, N 5.34, Cl 13.60; calculated, C 68.83, H 4.58, N 5.35, Cl 13.57; ^{1}H NMR ($CDCl_3$), 4.30–4.42 (Ph-CH_2-), 4.74–4.78 (-CH_2), 7.22–8.13 (Ph).

Chromatography

HPLC separation of the CEOC derivatives was performed on a Hypersil BDS C18 column. A quaternary gradient was used for simultaneous separation of BA and amino acids (Table I, gradient 1) whereas a binary gradient was used for rapid separation of BA (Table I, gradient 2).

Amino Acids and BA

Mobile phase A was 20% acetonitrile in 0.02 mol L^{-1} acetate buffer (pH 4.0); mobile phase B was acetonitrile-water, 60:40 (*v/v*); mobile phase C was acetonitrile-water, 60:40 (*v/v*) containing 5 mL 0.1 mol L^{-1} borate buffer (pH 8.9); mobile phase D was acetonitrile-water, 95:5 (*v/v*).

BA

Mobile phase A was 30% acetonitrile in 0.02 mol L^{-1} acetate buffer (pH 4.0); mobile phase B was acetonitrile-water, 95:5 (*v/v*).

The gradient conditions used for separation of amino acid and BA derivatives are shown in Table I. The flow rate was constant at 1.0 mL min^{-1} and the column was at ambient temperature. The fluorescence emission wavelength was set at 360 nm (excitation was at 293 nm).

Table I. Gradient conditions used for chromatography on the Hypersil BDS C18 column.

Amino acids and BA – Gradient 1				Faster elution of BA – Gradient 2			
Time (min)	A (%)	B (%)	C (%)	D (%)	Time (min)	A (%)	B (%)
0	100	0	0	0	0	90	10
10	75	25	0	0	12	70	30
20	65	35	0	0	25	25	75
30	60	40	0	0	33	25	75
34	56	44	0	0	36	0	100
34.1	56	0	44	0	45	0	100
40	50	0	50	0			
45	40	0	60	0			
45.1	40	60	0	0			
50	0	100	0	0			
55	0	80	0	20			
60	0	70	0	30			
65	0	50	0	50			
70	0	0	0	100			
75	0	0	0	100			

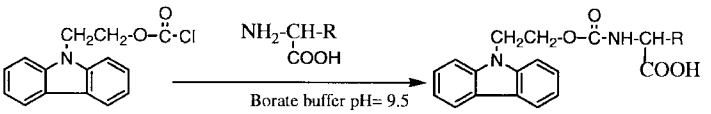


Figure 1. Derivatization of amino groups with 2-(9-carbazole)ethyl chloroformate (CEOC).

Sample Extraction

Commercial shrimp sauce (10 g) was extracted ultrasonically with trifluoroacetic acid (40% *v/v*, 20 mL) for 10 min. After centrifugation at 4000 *g* for 20 min, the supernatant solution was isolated by filtration. The residue was again extracted twice with trifluoroacetic acid (40% *v/v*, 2 × 10 mL) and the resulting extracts were combined, filtered through a 0.2-µm pore syringe filter, diluted to 50 mL with water, and stored at –20 °C until HPLC analysis.

Derivatization Procedure

An aqueous solution of amino acids and BA (50 µL) was placed in a vial and borate buffer (pH 10.0, 0.2 mol L^{-1}, 200 µL) and CEOC acetonitrile solution (100 µL) were added successively. After 5 min the reaction was stopped by addition of 20 µL quenching agent and the mixture was extracted with pentane to remove excess reagent. The extraction was repeated twice and the aqueous phase was then ready for injection. It was stable for 24 h except for the histidine and tyramine derivatives, which degraded, at least in part, to the mono-derivatized forms. If the aqueous solution of the derivatives was neutralized to pH 6.0–7.0 with quenching reagent the solution was sufficiently stable to enable HPLC analysis of all the derivatives for a further 24 h at room temperature. The derivatization process is shown in Figure 1.

Results and Discussion

Stability of CEOC and its Derivatives

When a solution of CEOC in anhydrous acetonitrile was hermetically stored at room temperature for two weeks derivatization yields for amino acids and BA were not obviously different. The stored CEOC acetonitrile solution was sufficiently stable to enable further derivatization of amino acids and BA for at least one week. The stabilities of the corresponding derivatives were also investigated by analysis of a standard containing 50 pmol of each BA and amino acid. The results indicated that HPLC analysis of the derivatives was possible for at least 24 h; if the derivatives were stored in neutral medium at 4 °C no decomposition was apparent. As expected, daylight had essentially no effect on stability.

Optimization of the Derivatization Conditions

For determination of the optimum conditions for derivatization of amino acids and BA, 0.2 mol L^{-1} borate buffers of pH between 9.0 and 10.0 were tested and the results were compared with those obtained by use of 1.0 mol L^{-1} KOH as basic catalyst to derivatize mixtures of amino acids and BA standard (100 pmol). In borate buffers at pH 9.0 precipitation of a

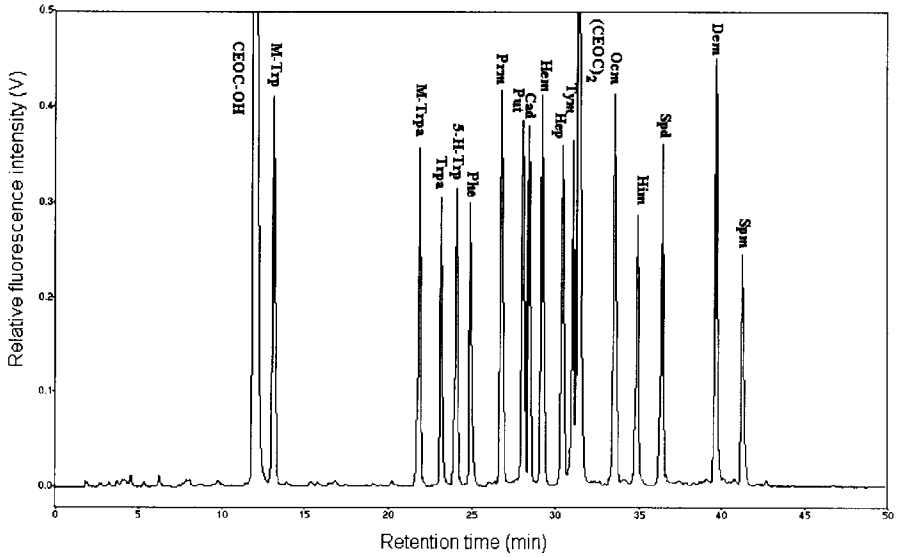


Figure 2. Chromatogram obtained from 100 pmol standard BA and some fatty amine derivatives on a 200 mm × 4.6 mm i. d., 5-μm particle, Hypersil BDS C18 column using gradient 2 from Table I. The flow rate was 1.0 mL min^{-1}, the column was at ambient temperature, the excitation wavelength was 293 nm, and the emission wavelength was 360 nm. **M-Trp** = 5-methoxytryptophan; **M-Trpa** = 5-methoxytryptamine; **Trpa** = tryptamine; **5-H-Trp** = 5-hydroxytryptophan; **Pheta** = phenylethylamine; **Prm** = 1,3-propanediamine; **Put** = putrescine; **Cad** = cadaverine; **Hem** = 1,6-hexanediamine; **Hep** = heptylamine; **Tym** = tyramine; **$(CEOC)_2$** = bis-(2-(9-carbazole)ethyl chloroformate); **Ocm** = octylamine; **Him** = histamine; **Spd** = spermidine; **Dem** = decylamine; **Spm** = spermine.

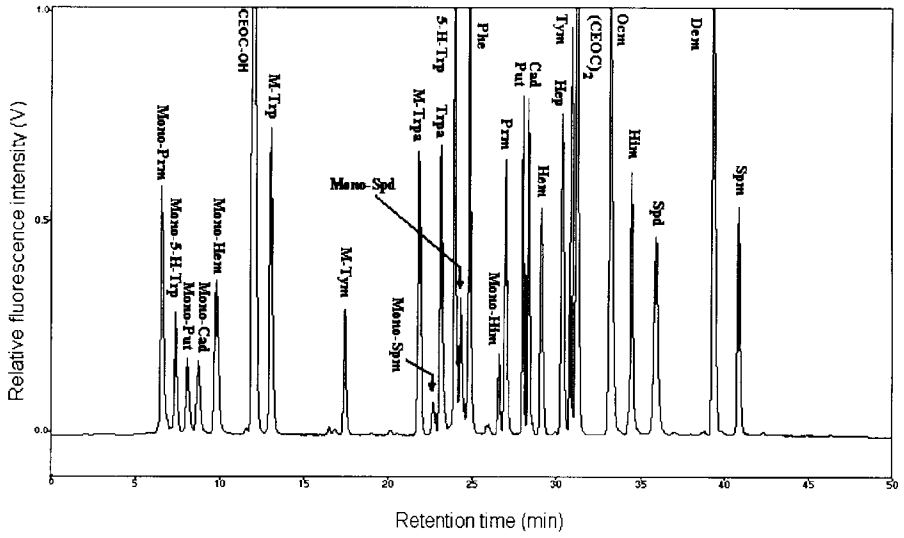


Figure 3. Chromatogram obtained from 100 pmol BA derivatives without complete derivatization on a 200 mm × 4.6 mm i. d., 5-μm particle, Hypersil BDS C18 column using gradient 2 from Table I. The flow rate was 1.0 mL min^{-1}, the column was at ambient temperature, the excitation wavelength was 293 nm, and the emission wavelength was 360 nm. **Mono-Prm** = monosubstituted propanediamine; **Mono-5-H-Trp** = monosubstituted 5-hydroxytryptophan; **Mono-Put** = monosubstituted putrescine; **Mono-Cad** = monosubstituted cadaverine; **CEOC-OH** = reagent peak; **M-Trp** = 5-methoxytryptophan; **Mono-Tym** = monosubstituted tyramine; **M-Trpa** = 5-methoxytryptamine; **Trpa** = tryptamine; **5-H-Trp** = 5-hydroxytryptophan; **Pheta** = phenylethylamine; **Mono-Him** = monosubstituted histamine; **Prm** = 1,3-propanediamine; **Put** = putrescine; **Cad** = cadaverine; **Hem** = 1,6-hexanediamine; **Hep** = heptylamine; **Tym** = tyramine; **$(CEOC)_2$** = bis-(2-(9-carbazole)ethyl chloroformate); **Ocm** = octylamine; **Him** = histamine; **Spd** = spermidine; **Dem** = decylamine; **Spm** = spermine.

white solid was observed after addition of the derivatization reagent. No precipitate was formed in borate buffers of pH 9.5–10.0 or in 1.0 mol L^{-1} KOH. Obvious decomposition of some of the derivatized amino acids and BA, e. g. His, Tyr, and tyramine, was, however, observed when 1.0 mol L^{-1} KOH was used as basic catalyst, possibly because the disubstituted derivatives were rapidly hydrolyzed by the strongly basic KOH medium. No degradation was observed in borate buffers of pH 9.5–10.0 over a 24-h period, except for His and tyramine, for which slight decomposition was observed. These experiments were usually performed with borate buffers. To achieve optimum derivatization of amino acids and BA the buffers must be within the appropriate pH range and provide adequate buffering capacity. Taking both phase-separation and buffering capacity into consideration the results indicated that 0.2 mol L^{-1} borate buffer (pH 9.5) was the optimum medium for derivatization.

The molecular structure of 2-(9-carbazole)ethyl chloroformate (CEOC) is similar to that of FMOC. It undergoes the same chloroformate reaction as FMOC or AEOC with primary and secondary amines [38]. Different derivatization times (1–10 min) and reaction temperatures (ambient temperature, 40 °C, and 60 °C) were tested to assess the optimum reaction conditions. The peak areas of the amino acid derivatives were maximum when manual shaking was performed for 2 min at ambient temperature. Those of the BA derivatives were maximum after 3–4 min. Use of higher reaction temperatures and different reaction times resulted in smaller peak areas.

For histamine, in particular, the largest peak area was observed after 3 min. Because differences between the peak areas obtained after derivatization for 3 or 4 min were very small for all other BA and amino acids, and histamine is among the most important BA, derivatization for more than 3 min at ambient temperature, with shaking, was selected. The yields were not affected within the reaction time interval 3–10 min. These results showed that the rate of derivatization of amino acids or BA with CEOC was similar to that with FMOC and AEOC [39, 40].

The effect of CEOC concentration on derivatization yield was also investigated for the derivatization of amino acids and BA. For this experiment a solution was prepared containing amino acids and BA (Arg, histamine, Ala, phenylethylamine, and tyramine); the total amine content was 250 μmol L^{-1}. The reagent concentration range investigated was from 0 to 1.5 mmol L^{-1} (final solution concentrations). The results indicated that the fluorescence intensity was constant when the concentration of derivatization agent was in three to fourfold molar excess over the amines. Although increasing the excess of

Table II. Repeatability of peak response and retention times (100 pmol injected; $n = 6$).

Amino acid/BA	Area *CV* (%)	Retention time *CV* (%)
Arg	0.46	0.12
Asp	0.58	0.11
Ser	0.67	0.14
Glu	0.54	0.10
Thr	0.60	0.16
Gly	0.53	0.13
Ala	0.61	0.11
Pro	0.47	0.15
Met	0.67	0.08
Val	0.79	0.08
Phe	0.59	0.08
Typ	0.79	0.08
Ile	0.54	0.06
Leu	0.37	0.06
Cys	0.38	0.07
His	0.49	0.07
Orn	0.43	0.06
Lys	0.54	0.06
Tyr	0.34	0.07
Histamine	0.78	0.08
Tyramine	0.64	0.12
Phenylethyl-amine	0.72	0.09

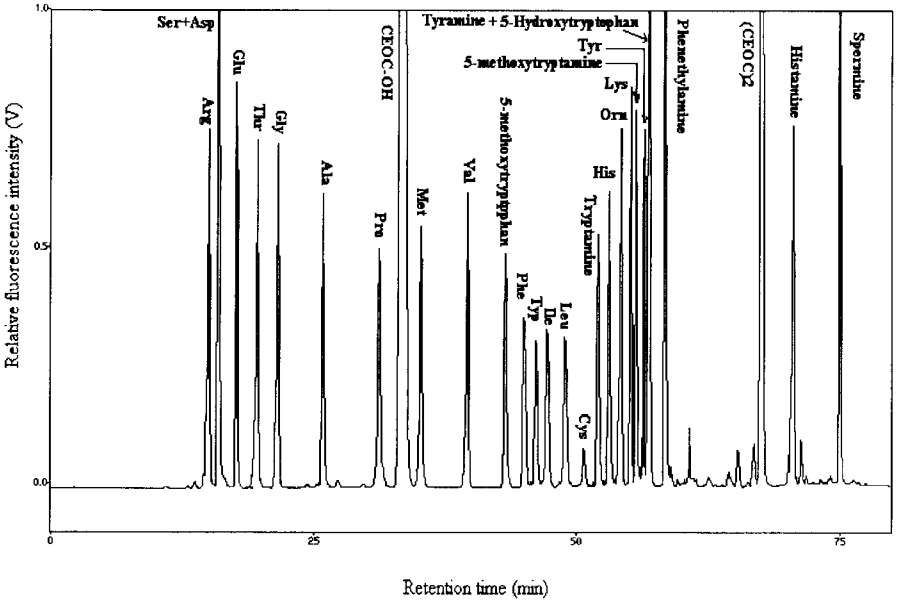


Figure 4. Chromatogram obtained from 100 pmol standard amino acids and BA derivatives on a 200 mm × 4.6 mm i. d., 5-µm particle, Hypersil BDS C18 column using gradient 1 from Table I. The flow rate was 1.0 mL min^{-1}, the column was at ambient temperature, the excitation wavelength was 293 nm, and the emission wavelength was 360 nm. **Arg** = arginine; **Asp** = aspartic acid; **Ser** = serine; **Glu** = glutamic acid; **Thr** = threonine; **Gly** = glycine; **Ala** = alanine; **Pro** = proline; **Met** = methionine; **Val** = valine; **Phe** = β-phenylalanine; **Trp** = tryptophan; **Ile** = *iso*-leucine; **Leu** = *nor*-Leucine; **Cys** = cysteine; **His** = histidine; **Orn** = ornithine; **Lys** = lysine; **Tyr** = tyrosine; **CEOC-OH** = 9-(2-carbazole)-ethanol; **$(CEOC)_2$** = bis-(2-(9-carbazole)ethyl) carbonate.

reagent beyond this level had no significant effect on yields, if higher concentrations of CEOC were used excess $(CEOC)_2$ caused interference with the chromatographic separation at the retention time of tyramine during the rapid separation of the BA. With as little as a twofold molar excess of the derivatizing agent, derivatization of the amino acids and BA was incomplete and resulted in mono-substituted derivatives of histidine, tyramine, tryptophan, and histamine, etc. A side-reaction of the reagent with its own hydrolysis product (CEOC-OH) to give the bis-(2-(9-carbazole)ethyl) carbonate $(CEOC)_2$ was also observed. Usually CEOC-OH and $(CEOC)_2$ did not interfere with the separation of the amino acid and BA derivatives.

The derivatized amino acids and BA were found to be stable for more than 24 h at room temperature, except for the doubly substituted histidine, which degraded to the monosubstituted compound at high pH. Assuming first-order kinetics the half-life of disubstituted histidine was 20 h. Degradation of disubstituted histidine could, however, be prevented by neutralizing the derivatized solution to pH 5.0–7.0 with 10% acetic acid solution. Under these conditions the disubstituted derivative was very stable when stored at 10 °C and could be analyzed within a 24-h period.

Yields of amino acids and BA in a standard mixture were evaluated by use of 0.2 mol L^{-1} borate buffer in the pH range from 6.5 to 10.5. Buffer pH had little effect on yield in the pH range 9.0 to10.0, but outside this range, particularly in more acidic solution, responses were lower. It was also found that higher pH promoted the hydrolysis of CEOC. At pH > 8.5 the CEOC also begins to react with phenol groups to form disubstituted tyrosine and tyramine. At higher pH (> 10.5), the disubstituted derivatives of tyrosine and tyramine were partially hydrolyzed to their mono-substituted derivatives-approximately 20% of their disubstituted derivatives was lost over a 24-h period. Incomplete derivatization was apparent from the appearance of several additional peaks compared with those obtained when derivatization was complete. Figure 2 shows a typical chromatogram obtained after complete derivatization of BA and amino compounds. Figure 3 shows the chromatogram obtained after incomplete derivatization of the same compounds. Complete derivatization was usually achieved when the derivatization conditions were maintained in the optimum range described above. The repeatability of peak area and retention time is shown in Table II.

Chromatographic Separation of Derivatized BA and Amino Acids

The aim of this work was to determine food-relevant BA and to investigate the simultaneous separation of BA and amino acids. To achieve optimum separation the choice of mobile-phase pH was important, especially for the simultaneous separation of amino acids and BA. The effect of mobile phase pH was tested on a Hypersil BDS C18 column. At the beginning of the gradient program a mobile phase of pH 4.0 and a more acidic mobile phase of pH 3.6–4.0 were tested. The results indicated that use of these pH conditions enabled complete separation of the first-eluting derivatized amino acids. At pH 4.0 most of the amino acids were resolved, the exceptions being Asp and Ser, and Phe and Typ. In comparison with the more acidic conditions (pH < 4.0) operation at pH 4.2–5.0 resulted in markedly reduced retention of most amino acids, with coelution of Asp and Ser, of Thr and Gly, and of Phe and Typ. Retention of the hydrophobic amino acids and BA was not obviously different. However, under more basic mobile-phase conditions elution of Arg was shifted nearer Thr in the chromatographic separation and resolution was poor (not shown). After many runs with gradient 1 (Table I) it was found that if the pH of mobile phase

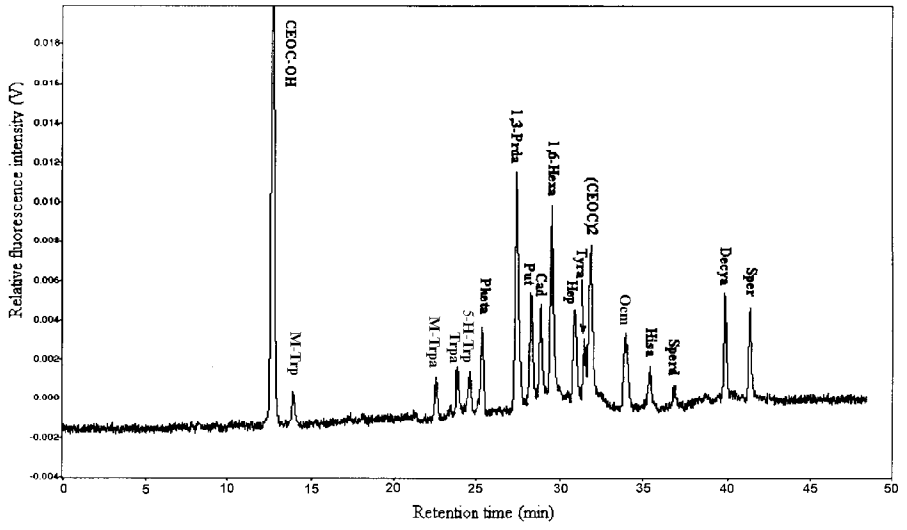


Figure 5. Chromatogram obtained from 2.5 pmol derivatized standard BA and some fatty amine derivatives. The chromatographic conditions and compound identities are the same as for Figure 2.

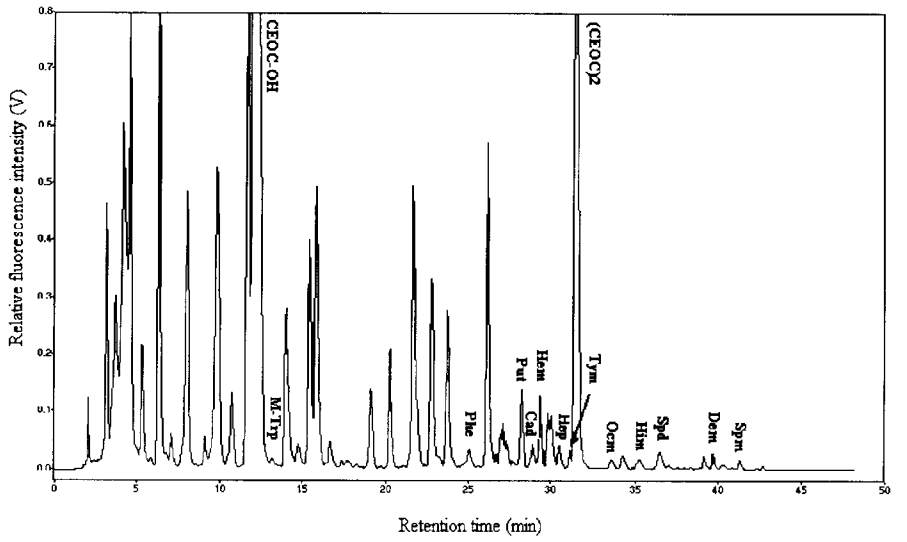


Figure 6. Chromatogram obtained from BA extracted from shrimp sauce (Sample No. 1). The chromatographic conditions are the same as for Figure 2. **M-Trp** = 5-methoxytryptophan; **Pheta** = phenylethylamine; **Put** = putrescine; **Cad** = cadaverine; **Hem** = 1,6-hexanediamine; **Hep** = heptylamine; **Tym** = tyramine; **Ocm** = octylamine; **Him** = histamine; **Spd** = spermidine; **Dem** = decylamine; **Spm** = spermine.

A was adjusted to 4.0 separation of most of the early-eluting amino acids could be achieved, the only exception being co-elution of Asp and Ser. When elution of the early-eluting amino acids was complete (after elution of Val) mobile phase B was introduced immediately and Phe and Typ were resolved with good baseline resolution. The simultaneous separation of a standard containing amino acids and some BA on Hypersil BDS C18 is shown in Figure 4. It is apparent from the figure that the derivatives of the BA and amino acids of interest are well separated in approximately 70 min. To achieve more rapid elution of the BA derivatives, elution gradient 2 (Table I) was adopted; this enabled reduction of the time required for separation of the BA derivatives to approximately 45 min (Figure 2).

Detection Limits and Linearity for Derivatized Amino Acids and BA

Detection limits are an important consideration when the components of biological matrixes are analyzed, particularly when they are present at low or trace concentrations. Figure 5 shows the chromatogram obtained after injection of 2.52 pmol of each derivatized BA and amino compound. Detection limits (signal-to-noise ratio = 3) ranging from 117 to 840 fmol were calculated for BA and the amino compounds (Table III). Analysis of serial dilutions of the standard derivatized mixture showed that the linearity of the dependence of response on concentration was excellent for concentrations ranging from 2.52 to 94.6 μmol L^{-1} for BA and from 2.50 to 40.4 μmol L^{-1} for the amino acids. Correlation coefficients were 0.9993 to 0.9999 (Table III).

Applications – Compositional Analysis of a Sample of Shrimp Sauce

The chromatogram obtained from analysis of the BA content of a fresh, unfermented, commercial shrimp sauce (denoted Sample No.1) is shown in Figure 6. The BA composition of the sauce is shown in Table IV. Fermented fish sauces are popular in Asian countries as liquid spices. A variety of fish sauces is also made from different species of fish [41]. The fermentation process is usually achieved by the action of microorganisms. A sample of a fermented shrimp sauce (denoted Sample No.2, chromatogram not shown) was found to contain very large amounts of Prm, tryptamine, Put, Cad, Him, Spd, and Spm (Table IV, Sample No. 2). The large amounts of Put, Cad, Spd, and Spm, which are usually indicators of very long storage, are also the results of serious proteolysis. In contrast, the fresh shrimp sauce, which was not fermented, contained much smaller amounts of BA (Table IV, No. 1). When a binary gradient was used for rapid separation of BA with gradient 2, M-Trpa, Trpa and 5-H-Trp are co-eluted with some amino acids, such as Met, Ile and Leu. The quantitative results of M-Trpa, Trpa and 5-H-Trp were not determined in this study. The data are in good agreement with those from previous investigations of fish sauces reported by Kirschbaum [2]. It was thus demonstrated that the method established was suitable for the determination of important BA in a variety of real samples.

Conclusions

The performance of the reagent CEOC in the quantitative analysis of BA, and the simultaneous separation of amino acids and BA, have been described in detail. This work has shown that derivatization

Table III. Detection limits, linearity, and correlation coefficients for derivatized biogenic acids and some other compounds.

Amino acid	Linearity regression, $Y = A + BX$*	R	Detection limit (fmol)
M-Trp	$Y = -41054 + 16867X$	0.9996	840
M-Trpa	$Y = 211 + 22273X$	0.9997	144
Trpa	$Y = -17620 + 26251X$	0.9997	133
5-H-Trp	$Y = 63575 + 33132X$	0.9999	117
Pheta	$Y = 112817 + 31763X$	0.9995	152
Tyra	$Y = 96753 + 32604X$	0.9997	336
Hista	$Y = 12351 + 27814X$	0.9999	623
Sperd	$Y = 478 + 5627X$	0.9989	153
Sper	$Y = 178969 + 36002X$	0.9993	140
Hep	$Y = -7666 + 16566X$	0.9999	132
1,3-Prda	$Y = -12525 + 10171X$	0.9998	510
Put	$Y = -14837 + 11046X$	0.9997	486
Cad	$Y = -13897 + 12046X$	0.9996	476
1,6-Hexa	$Y = -26872 + 24400X$	0.9999	410
Ocm	$Y = -17604 + 11227X$	0.9999	521
Decya	$Y = -17504 + 11328X$	0.9995	504

* X = amount injected (pmol), Y = peak area.

Table IV. Compositional analysis of BA and other amino compounds in commercial shrimp sauce extract.

Compound	Amount added (pmol)	Amount found (pmol)	*RSD* (%)	Shrimp sauce (amount found, mg/100g) Sample No. 1*	Sample No. 2*
M-Trp	100	101.2	1.2	2.45	3.45
M-Trpa	100	101.8	1.8	ND	ND
Trpa	100	100.6	0.6	ND	ND
5-H-Trp	100	98.8	1.2	ND	ND
Pheta	100	98.2	1.8	1.39	17.8
Tyra	100	97.6	2.3	2.49	9.4
Hista	100	93.5	6.5	0.75	11.2
Sperd	100	95.3	4.7	10.5	18.4
Sper	100	105.9	5.9	0.12	13.6
Hep	100	106.5	6.5	2.18	4.2
1,3-Prda	100	105.4	5.4	5.56	10.6
Put	100	92.9	7.1	4.19	13.4
Cad	100	100.4	0.4	1.51	11.8
1,6-Hexa	100	94.6	5.3	5.53	9.8
Ocm	100	91.4	8.6	2.57	4.67
Decya	100	97.1	2.9	3.31	7.4

The commercially produced sauce (Food Factory of Dalian, Liaoning province, China) was obtained from a shop. ND = not determined. * Sample No. 1 was not fermented; Sample No. 2 was fermented.

of BA by CEOC is rapid and quantitative. One of the most attractive features of this method is the simplicity of the preparation of derivatives of amino acids and BA. The response is highly reproducible, and results from the method correlate well with those obtained by derivatization of BA with FMOC and AQC. Detection limits are in the femtomole range. Analysis of real samples gave excellent results. Yields for derivatization of amino acids and BA were close to 100%.

These characteristics indicate that CEOC could be another popular and attractive derivatizing agent for amino compounds in addition to FMOC and AQC. Studies are currently in progress to explore the derivatization of different amine-containing compounds, e. g. aromatic amines, catecholamines, etc. Although CEOC is not available commercially, it is likely to find numerous applications in analytical chemistry. A possible disadvantage of the proposed derivatization method is that CEOC can only be used in the pre-column mode. Excess reagent can be eliminated by subsequent reaction with excess of a hydrophilic amino acid or hydroxylamine. Automated derivatization with CEOC might be possible under such conditions.

Acknowledgment

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

References

[1] Vasundhara, T.S.; Kumudavally, K.V.; Jayathiilakan, K.; Jeyashakila, R. *J. Food Sci. Technol.* **1998**, *35*, 551.
[2] Kirschbaum, J.; Rebscher, K.; Brückner, H. *J. Chromatogr. A* **2000**, *881*, 517.
[3] Eclerq, M.; Gouilloux, L. *Technol. Biol.* **1989**, *5*, 212.
[4] Gouygou, J.P.; Martin, C.; Sinquin, C.; Durand, P. *Oceanis* **1989**, *15*, 599.
[5] Beyer, C.; Van den Ende, A. *Clin. Chim. Acta* **1980**, *129*, 211.
[6] Yamamoto, S.; Itano, H.; Kataoka, H.; Makita, M. *J. Agric. Food. Chem.* **1982**, *30*, 435.
[7] Draisci, R.; Cavalli, S.; Lucentini, L.; Stacchini, A. *Chromatographia* **1993**, *35*, 584.
[8] Akira, T.; Kentaro, A.; Tomofumi, S.; Kazuhiro, I. *Anal. Chem.* **2000**, *72*, 732.
[9] Nicole, J.M.; Zhili, H.; David, N.; James, P.L. *Anal. Chem.* **2000**, *72*, 2765.
[10] Anders, E.; Per, E.A.; Björm, J.; William, D.P. *Anal. Chem.* **1995**, *67*, 3018.
[11] Blau, K.; King, G. *Handbook of Derivatives for Chromatography*, Heyden, London, **1977**.
[12] Knapp, D.R. *Handbook of Analytical Derivatization Reactions*, Wiley, New York, **1979**.
[13] Vendrell, J.; Aviles, F.X. *J. Chromatogr.* **1986**, *358*, 401.
[14] Muramoto, K.; Kamiya, H.; Kawauchi, H. *Anal. Biochem.* **1984**, *141*, 446.
[15] Hirano, H.; Wittmann-Liebold, B. *Biol. Chem. Hoppe-Seyler* **1986**, *367*, 1259.
[16] Ishida, T.; Yamaguchi, M.; Iwata, T.; Nakamura, M. *Anal. Chim. Acta* **1989**, *223*, 319.
[17] Toyo'oka, T.; Suzuki, T.; Watanabe, T.; Liu, Y.M. *Anal. Sci.* **1996**, *12*, 779.
[18] Busto, O.; Mestres, M.; Guasch, J.; Borrull, F. *Chromatographia* **1995**, *40*, 404.
[19] Petridies, K.D.; Steinhart, H. *Z. Lebensm. Unters. Forsch.* **1995**, *201*, 256.
[20] Frattini, V.; Lionetti, C. *J. Chromatogr. A* **1998**, *809*, 241.
[21] Tateda, N; Matsuhisa, K.; Hasebe, K.; Kitajima, N.; Miura, T. *J. Chromatogr. B* **1998**, *718*, 235.
[22] Lindroth, P.; Mopper, K. *Anal. Chem.* **1979**, *5*, 1667.
[23] Hill, D.W.; Walters, F.H.; Wilson, T.D.; Stuart, J.D. *Anal. Chem.* **1979**, *51*, 1338.
[24] Chem, R.F.; Scott, C.; Trepman, E. *Biochim. Biophys. Acta* **1979**, *576*, 440–445.
[25] Barth, H.G.; Barber, W.E.; Lochmuller, C.H.; Majors, R.E.; Regnier, F.E. *Anal. Chem.* **1988**, *60*, 387.
[26] Einarsson, S.; Josefsson, B.; Lagerkvist, S. *J. Chromatogr.* **1983**, *282*, 609.
[27] Einarsson, S.; Folestad, S.; Josefsson, B.; Lagerkvist, S. *Anal. Chem.* **1986**, *58*, 1638.
[28] Kirschbaum, J.; Busch, I.; Brückner, H. *Chromatographia* **1997**, *45*, 263.
[29] Inoue, H.; Kohashi, K.; Tsuruta, Y. *Anal. Chim. Acta* **1998**, *365*, 219.
[30] Kovacs, A.; Simon-Sarkadi, L.; Ganzler, K. *J. Chromatogr. A*, **1999**, *836*, 305.
[31] Busto, O.; Guasch, J.; Borrull, F. *J. Chromatogr. A* **1996**, *737*, 205.
[32] Steven, A.; Dennis, P.M.; Michaud, *Anal. Biochem.* **1993**, *211*, 279.
[33] Romero, R.; Bagur, M.G.; Sanchez-Vinas, M.; Gazquez, D. *Chromatographia* **2000**, *51*, 404.

[34] Valle, M.; Malle, P.; Bouquelet, S. *J. AOAC Int.* **1997**, *80*, 49.
[35] Moret, S.; Bortolomeozzi, R.; Lercker, G. *J. Chromatogr.* **1992**, *591*, 175.
[36] Bockhardt, A.; Krause, I.; Klostermeyer, H. *Z. Lebensm. Unters. Forsch.* **1996**, *203*, 65.
[37] Xinjun Fan; Jinmao You; Jinwu Kang; Qingyu Ou; Qingcun Zhu *Anal. Chim. Acta.* **1998**, *367*, 81.
[38] Arthur, J.F.; Hans, V.; Hans, D.B. *Anal. Chem.* **1991**, *63*, 292.
[39] Ahnoff, M.; Grundevik, I.; Arfwidsson, A.; Fonselius, J.; Bengt, A.P. *Anal. Chem.* **1981**, *53*, 485.
[40] Ghosh, P.B.; Whitehouse, M.W. *Biochem. J.* **1968**, *108*, 155.
[41] Fardiaz, D.; Markakis, P. *J. Food Sci.* **1979**, *44*, 1562.

Received: Nov 21, 2001
Revised manuscript received: Feb 27, 2002
Accepted: Mar 22, 2002