

Study of a new derivatizing reagent that improves the analysis of amino acids by HPLC with fluorescence detection: application to hydrolyzed rape bee pollen

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Abstract A simple and sensitive method for evaluating the chemical compositions of protein amino acids, including cystine (Cys)₂ and tryptophane (Try) has been developed, based on the use of a sensitive labeling reagent 2-(11*H*-benzo[α]-carbazol-11-yl) ethyl chloroformate (BCEC-Cl) along with fluorescence detection. The chromophore of the 1,2-benzo-3,4-dihydrocarbazole-ethyl chloroformate (BCEOC-Cl) molecule was replaced with the 2-(11*H*-benzo[α]-carbazol-11-yl) ethyl functional group, yielding the sensitive fluorescence molecule BCEC-Cl. The new reagent BCEC-Cl could then be substituted for labeling reagents commonly used in amino acid derivatization. The BCEC-amino acid derivatives exhibited very high detection sensitivities, particularly in the cases of (Cys)₂ and Try, which cannot be determined using traditional labeling reagents such as 9-fluorenyl methylchloroformate (FMOC-Cl) and *ortho*-phthaldialdehyde (OPA). The fluorescence detection intensities for the BCEC derivatives were compared to those obtained when using FMOC-Cl and BCEOC-Cl as labeling reagents. The ratios $I_{\text{BCEC}}/I_{\text{BCEOC}}=1.17\text{--}3.57$, $I_{\text{BCEC}}/I_{\text{FMOC}}=1.13\text{--}8.21$, and $UV_{\text{BCEC}}/UV_{\text{BCEOC}}=1.67\text{--}4.90$ (where I is the fluorescence intensity and UV is the ultraviolet absorbance). Derivative separation was optimized on a Hypersil BDS C₁₈ column. The detection limits calculated from 1.0 pmol injections, at a

signal-to-noise ratio of 3, ranged from 7.2 fmol for Try to 8.4 fmol for (Cys)₂. Excellent linear responses were observed, with coefficients of >0.9994 . When coupled with high-performance liquid chromatography, the method established here allowed the development of a highly sensitive and specific method for the quantitative analysis of trace levels of amino acids including (Cys)₂ and Try from bee-collected pollen (bee pollen) samples.

Keywords Rape bee pollen · Amino acids · 2-(11*H*-Benzo[α]-carbazol-11-yl) ethyl chloroformate (BCEC-Cl)

Introduction

Bee pollen is a product that has great nutritional value to human beings because of the medical properties attributed to bee honey and pollen loads. Many chemical, biochemical and microbiological studies have found a wide variety of compounds, such as sugars, proteins, lipids, vitamins and flavonoids, in bee pollen [1, 2]. As a group, bees depend strongly on pollen for their sustenance, obtaining all of the proteins they require from it, together with lipids and other nutrients. Literature reports on the complete composition of protein amino acids in bee pollen are relatively poor. Protein amino acids found in bee pollen play physiologically important roles at trace levels in the regulation of a variety of physiological and biological functions. Investigations of the composition of protein amino acids in bee pollen are of equal importance. Most protein amino acids from bee pollen show neither natural absorption in the UV region nor natural fluorescence; however, easily detectable amino acid derivatives can be obtained through labeling reactions. Generally, derivative separation depends on the use of chromatographic techniques. Such techniques fre-

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quently require an initial labeling step in order to increase the volatility of the analytes in GC or GC/MS [3–5] or to enhance the sensitivity of determinations obtained using high-performance liquid chromatography (HPLC) or HPLC/MS [6–8], although it is also possible to perform the direct determination of unlabeled amino acids via contactless conductivity detection [9]. To label protein amino acids, a number of different types of photochromic labeling reagents [10–14] have been proposed, which are used in either precolumn or postcolumn mode. However, when they have been applied, a variety of shortcomings have also been reported. For example, the *ortho*-phthalaldehyde (OPA) method offers greater sensitivity [15–17], but its use is limited to primary amino acids; proline and cysteine do not react with OPA. 7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) [18] has been developed for the determination of primary and secondary amino compounds. It is reported that about 50% of the reagent itself decomposes in methanol–water solution exposed to daylight within 25 min [19]. 9-Fluorenyl methylchloroformate (FMOC-Cl) [20, 21], 1-(9-fluorenyl)-ethyl chloroformate (FLEC-Cl) [22] and 2-(9-anthryl)-ethyl chloroformate (AEOC-Cl) [23] have also been developed for the derivatization of amino acids and peptides, but serious interferences from excesses of these reagents or from reaction by-products have been observed. 6-Aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) has also been developed as a popular precolumn derivatization reagent for the determination of amino acids with satisfactory results [24, 25]. However, the fluorescent intensity of its derivatives in aqueous solution is only 10% of the intensity in pure acetonitrile solution. Thus, the detection limits for the early-eluted amino acids are usually higher than those for later ones [24]. At the same time, (Cys)₂ and Try are not determined by the AQC method due to intense fluorescence quenching.

The combination of a sensitive functional group such as chloroformate together with a strong absorption moiety would result in an attractive reagent. Several new photochromic molecules and their application to the analysis of amino compounds containing amino acids have been described in our laboratory [26–29]. Based on the photochromic characteristics of the 1,2-benzo-3,4-dihydrocarbazole moiety [29], we have synthesized a novel photochromic molecule 2-(11*H*-benzo[α]-carbazol-11-yl) ethyl chloroformate (BCEC-Cl) where the chromophore of 1,2-benzo-3,4-dihydrocarbazole was dehydrogenated by chloroanil (tetrachloro-1,4-benzoquinone, 1.05 equiv.) in dry xylene, resulting in a very sensitive photochromic molecule, BCEC-Cl. BCEC-Cl has been found to be very stable in its crystal state. The corresponding derivatives exhibited very high sensitivities, especially for the determination of (Cys)₂ and Try, which were difficult to determine sensitively by traditional labeling methods. In this study,

the optimal reaction conditions—such as buffer pH, reaction time and reagent concentration—were evaluated. The detection responses for fluorescence were compared to those obtained when FMOC-Cl [20], BCEOC-Cl [29] and AQC [30] were used as labeling reagents. Linearities and detection limits were also determined. At the same time, their application to determine the amino acid compositions of hydrolyzed rape bee pollen samples is also reported. The developed method was found to be suitable for the analysis of real samples.

Experimental section

Instrumentation

All of the HPLC system devices were from the HP 1100 series (Waldbronn, Germany), and comprised 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). The mass spectrometer from Bruker Daltonik (Bremen, Germany) was equipped with an electrospray ionization (ESI) source (dry temperature 350 °C, nebulizer, 35.00 psi, dry gas, 9.0 L/min). The mass spectrometer system was controlled by Esquire-LC NT software, version 4.1. The HPLC system was controlled by HP Chemstation software. Fluorescence excitation and emission spectra were obtained with a 650-10S fluorescence spectrophotometer (Hitachi, Tokyo, Japan). Excitation and emission bandpasses were both set to 10 nm. A pH 211C acidimeter was used to measure the pH values of the buffers (Hainan, China). Derivatives were separated on Hypersil BDS C₁₈ column (200×4.6 mm, 5 μ M, Yilite Co., Dalian, China). The mobile phase was filtered through a 0.2- μ m nylon membrane filter (Alltech, Deerfield, IL, USA).

Chemicals

Amino acid 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, USA). Borate buffer was prepared from 0.2 M boric acid solution adjusted to pH 9.0 with 4 M sodium hydroxide solution prepared from sodium hydroxide pellets. The quenching reagent was 36% acetic acid solution.

Rape bee pollen sample (No. 1) was obtained from Mengyuan City, Qinghai Province (China). Rape bee pollen sample (No. 2) was obtained from Kouerle City, Xinjiang

Province (China). The two samples were transported to the laboratory and dried at 50 °C until their weights remained constant and then stored in a sealed volumetric flask until analysis.

Preparation of standard solutions

The derivatizing reagent solution 1.0×10^{-3} mol/L was prepared by dissolving 4.1 mg 2-(11*H*-benzo[α]carbazole)-ethyl chloroformate in 10 mL of anhydrous acetonitrile prepared by distilling the dried HPLC-grade acetonitrile with P₂O₅. Individual stock solutions of the amino acids were prepared in water, and if necessary, HCl or NaOH was added until the compound dissolved. Standard amino acid solutions for HPLC analysis at individual concentrations of 5.0×10^{-5} mol/L were prepared by diluting the corresponding stock solutions (1.0×10^{-3} mol/L) of each amino acid with 0.2 M borate buffer (pH 9.0). When not in use, all standards were stored at 4 °C.

Synthesis of the derivatization reagent (BCEC-Cl)

Synthesis of 1,2-benzo-3,4-dihydrocarbazole

1,2-Benzo-3,4-dihydrocarbazole was synthesized as previously described [31]: 17 mL of hydrochloric acid (36%, 0.2 mol) and 50 mL water were mixed. The mixture was heated to 75 °C, 10.8 g of hydrazinobenzene was added successively, and the contents of the flask were rapidly heated to reflux with stirring. 14.6 g of 3,4-dihydro-1(2*H*)-naphthalenone was then added dropwise within 1 h, and the mixture was continuously heated to reflux for 1 h. After cooling, the precipitated solid was recovered by filtration, washed with water and 75% ethanol, and dried at room temperature for 48 h. The crude product was recrystallized three times from methanol (100 mL \times 3), affording a white crystal (81% yield).

*Synthesis of 11*H*-benzo[α]carbazole*

1,2-Benzo-3,4-dihydrocarbazole (8.8 g), tetrachloro-1,4-benzoquinone (1.05 equiv.) and dry xylene (100 mL) were mixed. After the mixture had been stirred for a period of 1 h at room temperature under N₂, the mixture was refluxed for 2 h under N₂. After cooling, the precipitated solid was recovered by filtration, and the tetrachlorohydroquinone was washed with NaOH (10%, w/w) and filtered off by suction. The residue was washed with deionized water until pH 7.0 was achieved, and dried for 48 h at room temperature. The crude products were recrystallized three times from ethanol (150 mL \times 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), 3436.16 (–N–H); 1626.05 ($\delta_{\text{N–H}}$), 1561.10, 1528.58 (Ph); 1460.11 (C–H); 1384.38, 1329.03 (C–H); 818.27 ($\gamma_{\text{N–H}}$), 738.68. MS: *m/z*: 218 [M+H]⁺.

*Synthesis of 2-(11*H*-benzo[α]carbazol-11-yl) ethanol*

11*H*-Benzo[α]carbazole (20 g), KOH (7.0 g), and 200 mL 2-butanone were mixed and rapidly cooled to 0 °C with ice water along 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 and 30% ethanol solution, and dried at room temperature for 48 h. The crude products were recrystallized three times from methanol (200 mL \times 3), affording 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), 3432–3163.4 (–OH); 2922.44 (Ph); 1470.80 (C–H); 1405.3, 1384.96 (C–H); 1126.54 ($\delta_{\text{C–O}}$), 810.75, 741.67. MS: *m/z*: 261.8 [M+H]⁺, *m/z*: 243.8 [MH⁺–H₂O].

*Preparation of 2-(11*H*-benzo[α]carbazol-11-yl) ethyl chloroformate (BCEC-Cl)*

To a solution containing 4.0 g solid phosgene and 100 mL dichloromethane (0 °C) in a 500-mL round-bottomed flask, a mixture of 2-(11*H*-benzo[α]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, before the solution was concentrated using a rotary evaporator. The residue was extracted four times with warm diethyl ether; the combined diethyl ether layers were concentrated in vacuum to yield a white crystal. The crude products were recrystallized twice from diethyl 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), 1769.86 (–C=O); 1469.33 (C–H); 1405.64, 1384.94 (C–H); 1147.28, 1101.35, 814.02, 741.18.

High-performance liquid chromatography

HPLC separation of BCEC derivatives was carried out on a Hypersil BDS C₁₈ column by gradient elution. Eluent A

was 30% acetonitrile containing 35 mM formic acid buffer (pH 3.5); **B** was acetonitrile–water containing 30 mM formic acid buffer (pH 3.5; 50:50; v/v); **C** was acetonitrile–water (95:5; v/v). The gradient conditions: initial = 55% A and 45% B, 15 min = 45% A and 55% B, 17 min = 28% A and 72% B, 27 min = 100% B, 37 min = 80% B and 20% C, 40–42 min = 50%B and 50% C, 50 min = 40% B and 60% C, 55 min = 100% C, followed by a wash with 100% C for 5 min and re-equilibration for 10 min at initial elution conditions. The flow rate was constant at 1.0 mL/min and the column temperature was set to 30 °C. The fluorescence excitation and emission wavelengths were set to $\lambda_{\text{ex}} = 279$ and $\lambda_{\text{em}} = 380$ nm, respectively.

Hydrolysis of amino acids from rape bee pollen samples

Rape bee pollen (4.0 mg) was placed in a 50×6 mm test tube; 6 M hydrochloric acid (100 μL) was added and the test tube was sealed. After hydrolysis at 110 °C for 24 h, the contents were evaporated to dryness with a stream of nitrogen. The precipitate was redissolved with 1.0 mL of borate buffer (pH 9.0) and filtered through a 0.2- μm nylon membrane filter. The final solution was made up to 2-mL with borate buffer (pH 9.0) and stored at 4 °C until HPLC analysis.

Derivatization procedure

The BCEC–amino acid derivatization was carried out in aqueous acetonitrile in a basic medium. Twenty to thirty microliters of aqueous amino acids were placed into a vial, and 200 μL of 0.2 M borate buffer (pH 9.0) and 20–40 μL of BCEC–Cl acetonitrile solution were then added to the vial. The solution was shaken for 3 s and allowed to stand for 3 min at room temperature. After derivatization, the mixture was extracted with hexane/ethyl acetate (10:1, v/v) to remove the excess reagent. The aqueous phase was transferred to another conical vial and 10 μL of 36% acetic acid (v/v) were added until the final pH was close to neutral (pH \approx 7.0). This procedure was easily performed using a pH paper test. Then the derivatized sample solution was injected directly into the HPLC system for analysis. The derivatization process is shown in Fig. 1.

Preparation of representative hydrophilic BCEC–glutamic acid (Glu) and hydrophobic BCEC–phenylalanine (Phe) derivatives in order to evaluate their fluorescence properties

BCEC–Glu and BCEC–Phe derivatives were, respectively, prepared by the reaction of BCEC–Cl with glutamic acid and phenylalanine, as described in the “**Experimental**”

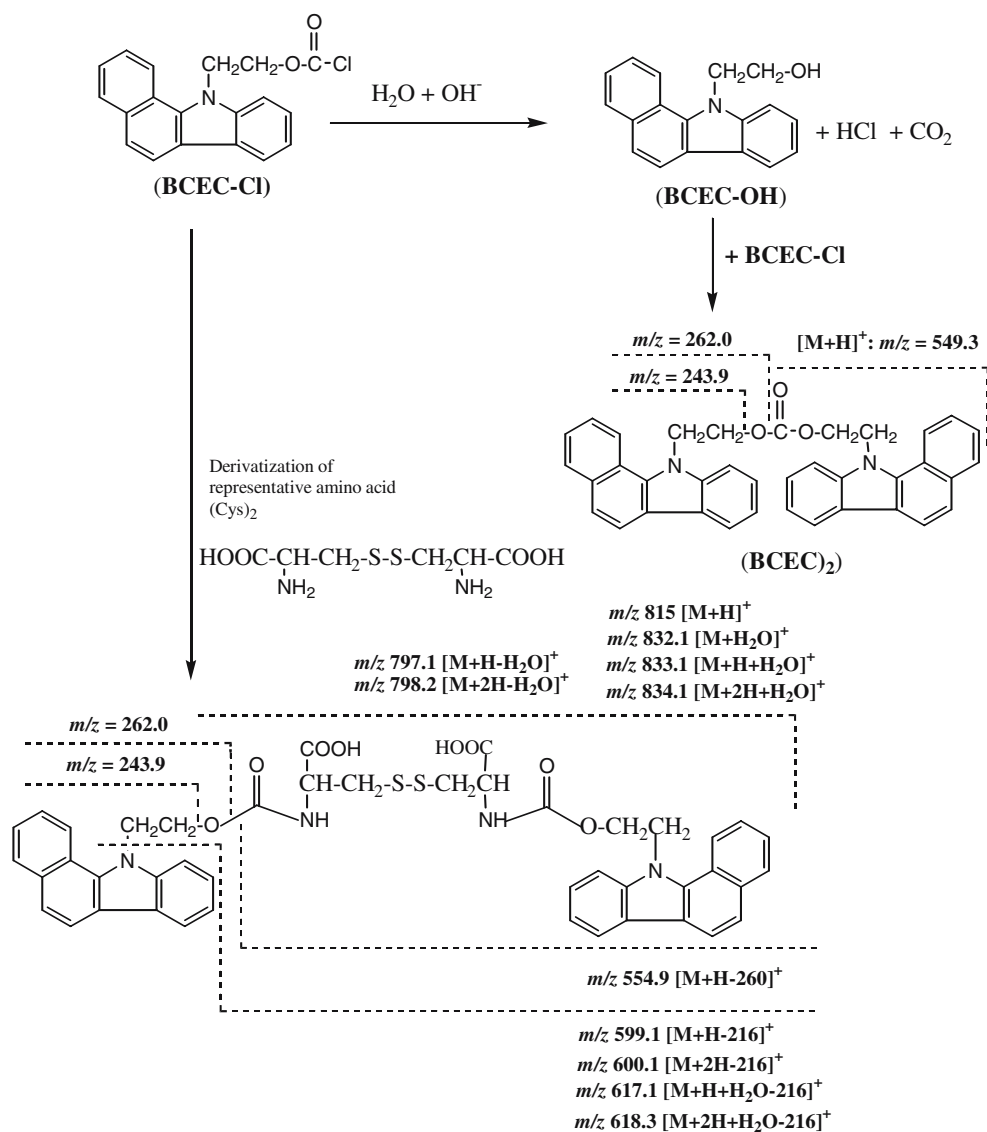
section. One milliliter of glutamic acid (1.0×10^{-3} mol/L) and 1.0 mL of phenylalanine (1.0×10^{-3} mol/L) were, respectively, added to two 5.0-mL test tubes, and then 2.0 mL of borate buffer (0.2 M) and 1.2 mL of BCEC–Cl acetonitrile solution (1.0×10^{-3} mol/L) were added to each test tube. The mixture was shaken for 3 s and allowed to stand for 3 min at room temperature. After the reaction had taken place, the mixture was extracted with hexane/ethyl acetate (10:1, v/v) to remove the excess reagent. The aqueous phase was removed and neutralized to near-neutral conditions (pH 7.0) with acetic acid (36%, v/v). The neutralized solution was, respectively, passed through a preconditioned Sep-Pak silica cartridge with 4 mL methanol and 5 mL water. The desired BCEC–Glu and BCEC–Phe were, respectively, eluted with 10 mL aqueous acetonitrile (50%, v/v) and 12 mL aqueous acetonitrile (80%, v/v). The eluted solutions were evaporated to dryness by a stream of nitrogen gas. The residue was redissolved with acetonitrile and made up to a total volume of 5.0 mL; the corresponding obtained BCEC–Glu and BCEC–Phe concentrations were 2.0×10^{-4} mol/L. The low concentrations of BCEC–Glu and BCEC–Phe used to test fluorescence properties were prepared by diluting the stock solution (2.0×10^{-4} mol/L) with the acetonitrile. When not in use, all solutions were stored at 4 °C.

Results and discussion

Ultraviolet absorption of 2-(11*H*-benzo[α]-carbazole-11-yl)-ethanol (BCEC–OH)

Benzo-carbazole derivatives are one of the most studied and important classes of photochromic molecules [32]. They exhibit interesting photochromic properties. In this study, the structure of the 2-(11*H*-benzo[α]-carbazole-11-yl)-ethanol (BCEC–OH) synthesized in our laboratory was similar to that of the benzo-carbazole derivatives. As expected, it exhibits high absorption efficiency in the UV range. In order to determine λ_{max} , the absorbance (*A*) and the molar absorption coefficient (ϵ) of BCEC–OH, 1.5×10^{-5} mol/L of each solvent solution (methanol, ethanol, dioxane, acetonitrile, and tetrahydrofuran) were prepared. The absorption wavelength of BCEC–OH was obtained over the scanning range 200–400 nm in the five solvent systems mentioned above. Maximum ultraviolet absorption responses were observed at the wavelengths of 230, 244, 253, 280 and 305 nm, respectively (the response at 230 nm was not observed for dioxane and tetrahydrofuran). The maximum ultraviolet responses did not exhibit obvious blue- or redshifts in five solvent systems. The molar absorption coefficients in five solvent systems are shown

Fig. 1 Derivatization scheme of 1,2-benzo-3,4-dihydrocarbazole-9-ethyl chloroformate (BCEC–Cl) with cystine, and mass cleavage mode of its derivatives



in Table 1. As can be seen from Table 1, the maximum absorption wavelength in methanol is $\lambda_{\text{max}} = 280$ nm, and the molar absorption coefficient (ϵ) at this wavelength is 4.65×10^4 L mol⁻¹ cm⁻¹.

Fluorescence excitation and emission

Solutions of BCEC–Glu ($3.0 \mu\text{mol L}^{-1}$, ACN/H₂O, 20:80, v/v) and BCEC–Phe ($3.0 \mu\text{mol L}^{-1}$, ACN/H₂O, 60:40, v/v) in aqueous acetonitrile were, respectively, used to obtain

Table 1 Maximum absorption and corresponding absorbance (*A*) of BCEC–OH at different wavelengths and in different solvents

Solvent	Absorbance (<i>A</i>) and absorption coefficient (ϵ)				
	230 nm <i>A</i> , ϵ ($\times 10^4$)	244 nm <i>A</i> , ϵ ($\times 10^4$)	253 nm <i>A</i> , ϵ ($\times 10^4$)	280 nm <i>A</i> , ϵ ($\times 10^4$)	305 nm <i>A</i> , ϵ ($\times 10^4$)
Methanol	0.568, 3.79	0.589, 3.93	0.659, 4.39	0.697, 4.65	0.392, 2.61
Ethanol	0.530, 3.53	0.558, 3.72	0.626, 4.17	0.671, 4.47	0.382, 2.55
Acetonitrile	0.499, 3.33	0.525, 3.50	0.589, 3.93	0.610, 4.07	0.336, 2.24
Solvent	245 nm <i>A</i> , ϵ ($\times 10^4$)	254 nm <i>A</i> , ϵ ($\times 10^4$)	259 nm <i>A</i> , ϵ ($\times 10^4$)	281 nm <i>A</i> , ϵ ($\times 10^4$)	306 nm <i>A</i> , ϵ ($\times 10^4$)
Tetrahydrofuran	0.468, 3.12	0.509, 3.39	0.491, 3.27	0.520, 3.47	0.303, 2.02
Dioxane		0.509, 3.39	0.485, 3.23	0.536, 3.57	0.282, 1.88

the maximum excitation and emission wavelengths. Fluorescence spectra of representative BCEC–Glu and BCEC–Phe derivatives showed two maximum excitation wavelengths at 280 and 300 nm, and two maximum emission wavelengths at 365 and 380 nm, respectively (data obtained using 650-10S fluorescence spectrophotometer were not corrected). The fluorescence emission intensities of the BCEC–Glu and BCEC–Phe derivatives in methanol (100%) were, respectively, 2.82% and 3.23% higher than those in acetonitrile (100%). The fluorescence intensities of the BCEC–Glu and BCEC–Phe derivatives were minimally quenched by inorganic anions (such as sulfate, nitrate, and phosphate) and organic anions (such as citrate) and divalent cations that are abundant in biological fluids. We also examined the effects of the methanol and acetonitrile concentrations on the fluorescence of each of the BCEC–Glu and BCEC–Phe derivatives. Increasing the methanol or acetonitrile content from 0 to 100% (v/v) resulted in an increase in the fluorescence intensity. A significant increase in fluorescence intensity was observed when the methanol or acetonitrile content was increased from 0 to 45% (v/v). This was probably due to the fact that BCEC–Glu and BCEC–Phe were only partially dissolved in low-concentration methanol or acetonitrile solutions. When the methanol or acetonitrile concentration was varied from 45 to 100% (v/v), slight increases in fluorescence intensity were observed. The variations in fluorescence intensity in methanol solution were, respectively, 5.6% for BCEC–Glu and 6.4% for BCEC–Phe. Similarly, the variations in fluorescence intensity in acetonitrile solution were 7.4% for BCEC–Glu and 8.3% for BCEC–Phe, respectively. The solvent polarity exerted little effect on the emission spectra. The maximum emission wavelengths in acetonitrile or methanol solutions (0–100%, v/v) exhibited no obvious blue- or redshifts.

Stabilities of the reagent (BCEC) and its derivatives

As observed, the structure of the synthesized BCEC–Cl molecule was similar to that of BCEOC–Cl and FMOC–Cl. After an anhydrous acetonitrile solution of BCEC–Cl had been stored at 4 °C in darkness for two weeks, the amino acid derivatization yields obtained with the BCEC–Cl barely changed. The corresponding neutral solution of derivatives was stored at 4 °C and at room temperature in darkness as well as in daylight for a period of two weeks, during which time they were analyzed three times. Only the disubstituted His exhibited significant breakdown; the other disubstituted derivatives, such as Tyr, Orn and Lys, were stable. In a derivatization solution that had not been neutralized (pH≈9.0), the corresponding disubstituted derivatives exhibited remarkable degradation within 12 h. When the derivatization solution was adjusted to near-

neutral conditions through the addition of acetic acid (36%, v/v) and stored at 4 °C, the derivatives were stable enough to be efficiently analyzed by HPLC analysis at least 48 h later, with the normalized peak areas varying by <2.4%. However, the disubstituted derivative of histidine exhibited significant degradation resulting in an increase of the corresponding monoderivative, which was eluted prior to the Arg.

Optimization for derivatization

Effect of BCEC concentration on derivatization

BCEC–Cl underwent the same chloroformate reaction with primary and secondary amino acids as previously reported for BCEOC and CEOC [29, 33]. Derivatization of BCEC–Cl with amino acids could be achieved within 3 min at room temperature. The effects of BCEC concentration on derivatization yield was investigated. The fluorescence intensities of the BCEC derivatives increased as the derivatization reagent concentration was increased. A constant fluorescence intensity was achieved upon the addition of a 2.0- to 3-fold molar reagent excess over the total molar amount of amino acids; increasing the reagent excess beyond this level exerted no significant effect on yields. When an excess of reagent of more than 3.0-fold over the total molar amount of amino acids was employed, the ability to distinguish between Pro and BCEC–OH decreased significantly. With as little as a 2.0-fold molar reagent excess, incomplete derivatization of the amino acids was observed, and this obviously resulted in monosubstituted derivatives such as His (m/z : 443.6) and Tyr (m/z : 469.1). Yields of 88–89% have been reported in the preparative derivatization of amino acids with FMOC–Cl [20]. Very high yields (96–98.4%) were observed when five amino acid derivatives obtained using a preconditioned Sep-Pak silica cartridge were used as reference substances. Additionally, two side reactions were also observed, which can be attributed to reagent hydrolysis; the by-products were generally 2-(11*H*-benzo[α]carbazole)ethanol (BCEC–OH) (m/z : 246.1), and bis-(2-(11*H*-benzo[α]carbazole)ethyl)-carbonate (BCEC)₂ (m/z : 549.3). The (BCEC)₂ was usually formed by the reaction of the hydrolyzed BCEC–OH with the excess reagent BCEC–Cl. The presence of (BCEC)₂ and BCEC–OH did not interfere with the separation of amino acid derivatives. This degradation could be monitored by the appearance of the monosubstituted degradation product. Assuming first-order kinetics, the half-life of dilabeled histidine was close to 28 h. However, when the derivatized solution was neutralized to pH 6.0–6.5 with a 36% acetic acid solution, the degradation could be avoided; its dilabeled derivative was very stable and could be further analyzed for 48 h at room temperature.

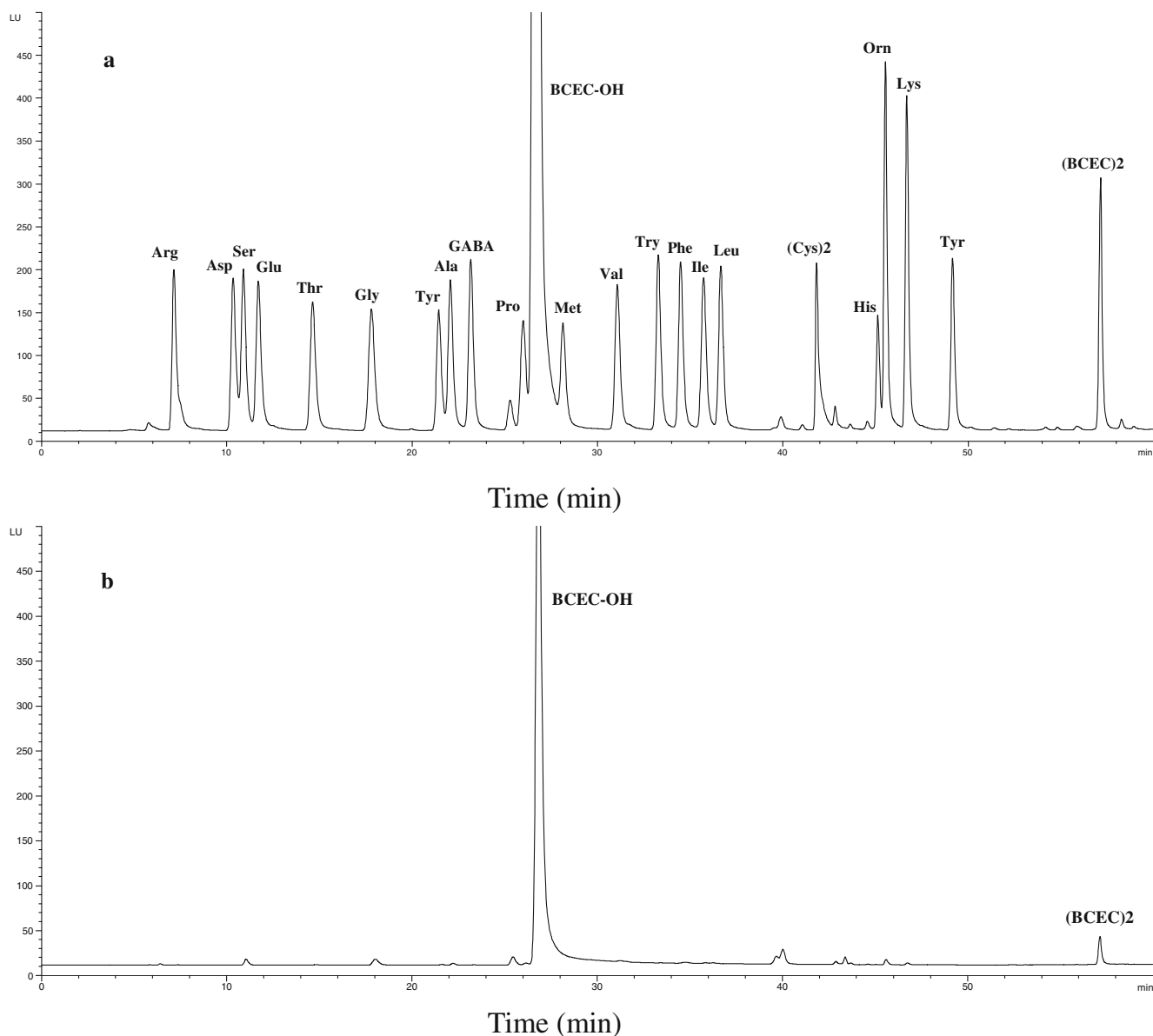


Fig. 2 a-b Optimized chromatography for amino acid standard mixtures using **a** 50 pmol injection; **b** blank experiment. Column temperature 35 °C; excitation wavelength λ_{ex} 279 nm, emission wavelength λ_{em} 380 nm; column was a 200×4.6 mm Hypersil BDS C₁₈ (5 μm); flow rate = 1.0 mL min⁻¹ Arg (arginine); Asp (aspartic acid); Ser (serine); Glu (glutamic acid); Thr (threonine); Gly (glycine);

Ala (alanine); GABA (4-amino-butyrac acid); Pro (proline); Met (methionine); Val (valine); Phe (β -phenylalanine); Trp (tryptophan); Ile (iso-leucine); Leu (nor-leucine); (Cys)₂ (cystine); His (histidine); Orn (ornithine); Lys (lysine); Tyr (tyrosine); BCEC-OH: 2-(11H-benzo[α]-carbazole)-ethanol; (BCEC)₂: bis-(2-(11H-benzo[α]-carbazole -ethyl) carbonate

The degradation procedure for the disubstituted histidine was similar to those of FMOc, BCEOC and CEOC, which have been reported previously [20, 29, 33].

Effect of pH on derivatization

Several types of basic media were evaluated for the derivatization of amino acids, including carbonate buffers, phosphate buffers and borate buffers. The results showed that borate buffers were the best choice. The effect of the

pH on derivatization yields was then investigated in the pH range 7.7–10.5 using borate buffer solution (0.2 M). The maximum derivatization yields were achieved in the pH range 8.5–9.0. All subsequent derivatization was, therefore, performed in this pH range; however, decreased responses were observed outside this range, particularly in more acidic solutions (pH < 8.5). At even higher pH values (>9.0), the disubstituted amino acid His exhibited significant hydrolysis and partially converted to its monosubstituted derivative; 45–50% of its disubstituted derivative was

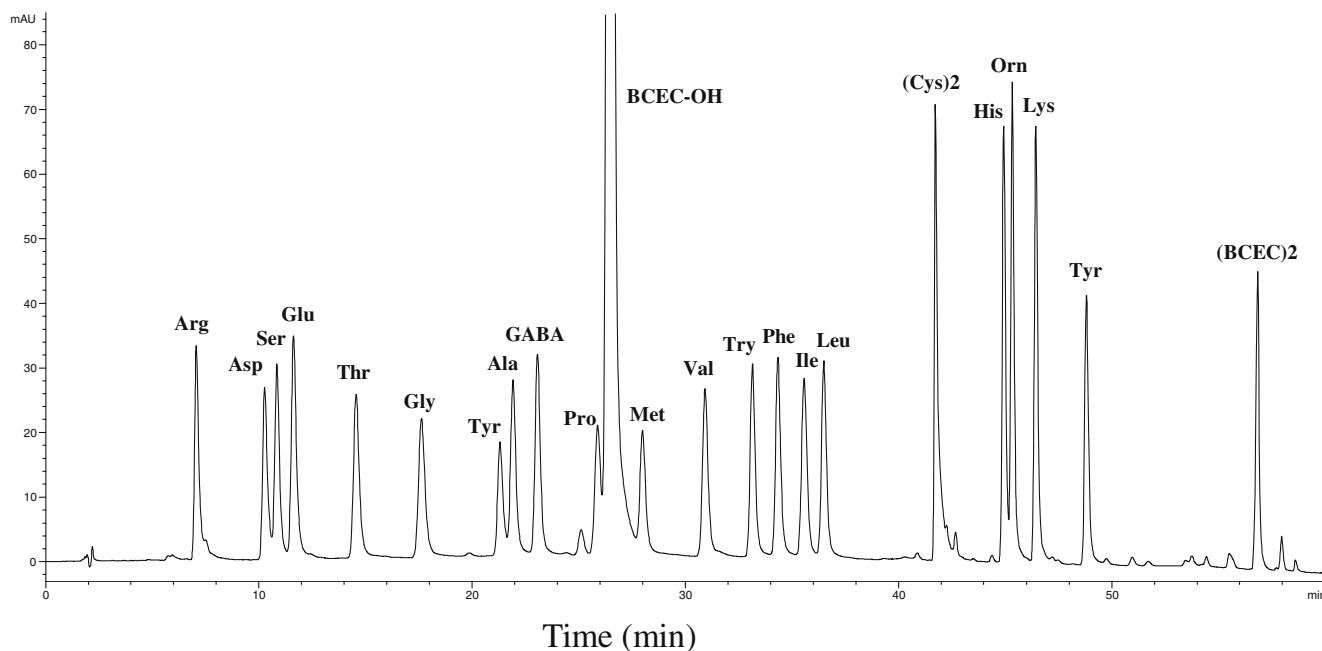


Fig. 3 Optimized chromatography of standard mixtures of amino acids, with UV detection performed at 279 nm. Chromatographic conditions and peaks are as described for Fig. 2a

lost. At pH 8.0–8.5, histidine could be measured as its disubstituted derivative. At pH > 8.5, monosubstituted histidine was initially formed and this eluted prior to the Arg. To achieve optimal derivatization yields for tyrosine, the effects of buffer pH values on derivatization were also evaluated. The results indicated that both mono- and disubstituted tyrosine derivatives were constantly obtained in the pH range of 8.0–10.0. At pH > 10.5, only the disubstituted tyrosine derivative was obtained. At pH < 7.7, only the mono-substituted tyrosine was observed. However, the use of a low pH probably also reduces the yields of derivatives of other phenolic substances that might be present. Therefore, 0.2 M borate buffer solution at pH 8.5 was chosen for all amino acid derivatizations. In order to accurately quantitate tyrosine, two linear regression equations for the derivatization of tyrosine were also obtained at pH values 7.7 and 10.5, respectively. The quantitative results for tyrosine were obtained by summing the results for the mono- and disubstituted derivatives.

HPLC separation

The resolution could be significantly affected by the pH of the mobile phase. A constant pH was not suited to the separation for all amino acid derivatives with a good resolution. A decrease in retention was observed for all amino acids at higher pH. A loss of resolution was also seen for the bands eluted early on. The pH had no effect on the retention of the product of the hydrolysis, and by choosing the pH appropriately it was easy to separate this

from the amino acids. Various pH values were tested for mobile phase A to achieve optimal separation on a Hypersil BDS C₁₈ column. The derivatized amino acid standards were usually separated under acidic conditions (pH 3.5 or even 3.3–3.5). At pH < 3.3, most of the amino acids were resolved with the exceptions of Asp and Ser (which co-eluted), Bcec–OH and Met (which co-eluted), and Ile and Leu (which partially co-eluted). In comparison with operation at pH 3.5, operation at pH 3.5–4.0 resulted in a marked decrease in retention time for most amino acid derivatives; at the same time, the pairs of Asp and Ser, Bcec–OH and Met, Pro and GABA, and Ile and Leu were completely co-eluted, respectively. However, the retention times of His, Orn, Lys, and Tyr barely changed. Moreover, Bcec–OH was eluted prior to Pro under this condition. After further runs, it was found that if the pH value of mobile phase A was adjusted to 3.5, the complete baseline resolution of all amino acid derivatives could be achieved within the shortest time along with sharp peak shapes under the proposed elution program. The separation of the standard containing 20 amino acids and the corresponding blank experiment on the Hypersil BDS C₁₈ column are shown in Fig. 2a,b.

Comparison of the responses of Bcec, BcecO and Fmoc for fluorescence and ultraviolet detection

Despite the popularity of AQC and Fmoc as precolumn methods, there have been many reports describing various shortcomings when they are applied. In general, an intense

Table 2 Comparison of relative fluorescence and UV intensities of derivatized amino acids obtained when BCEC, BCEOC and FMOC were used as labeling reagents

Amino acid	Relative FL intensity			Ratio		Relative UV intensity		Ratio UV _{BCEC} /UV _{BCEOC}
	BCEC	BCEOC	FMOC	I _{BCEC} /I _{BCEOC}	I _{BCEC} /I _{FMOC}	BCEC	BCEOC	
Arg	311.3	151.7	239.9	2.05	1.30	29.2	6.9	4.23
Asp	271.6	131.3	198.2	2.07	1.37	30.8	6.2	4.97
Ser	323.2	154.5	147.4	2.09	2.19	27.8	6.3	4.41
Glu	276.4	130.0	125.0	2.13	2.21	27.6	5.9	4.68
Thr	244.7	116.4	205.6	2.10	1.19	22.3	5.1	4.37
Gly	555.6	184.2	198.1	3.02	2.80	22.1	7.4	2.99
Cys	356.7	100.1	–b	3.57	–b	14.1	6.0	2.35
Ala	321.2	220.4	195.6	1.46	1.13	34.7	10.0	3.47
GABA	427.6	248.0	184.4	1.72	2.32	42.1	10.5	4.01
Pro	272.9	187.2	167.2	1.46	1.63	23.1	10.8	2.14
Met	328.9	177.1	86.7	1.86	3.79	21.6	4.8	4.5
Val	290.3	150.0	71.3	1.93	4.07	24.4	6.4	3.81
Try	337.1	224.3	#	1.50	–b	26.1	15.6	1.67
Phe	516.9	258.6	201.0	2.00	2.57	45.3	12.5	3.62
Ile	444.5	179.6	179.2	2.47	2.48	39.2	8.0	4.90
Leu	455.6	227.9	166.2	2.00	2.74	41.4	10.7	3.87
(Cys) ₂	458.2	156.8	55.8	2.92	8.21	39.6	10.8	3.67
His	216.1	185.2	166.2	1.17	1.30	63.3	21.4	2.96
Om	945.0	526.6	345.3	1.79	2.74	95.2	30.1	3.16
Lys	965.6	526.6	309.9	1.83	3.12	102.9	31.4	3.28
Tyr	880.7	298.8	222.4	2.95	3.96	109.8	26.8	4.10

The concentration of the derivatized amino acid was 1.0×10^{-5} mol/L; 10 μ L were injected for fluorescence detection, corresponding to an injected amount of each amino acid of 100 pmol; UV responses quoted correspond to an injected amount of each amino acid of 500 pmol
#: no fluorescence

fluorescence quenching was observed for the derivatized His, (Cys)₂ and Try when FMOC and AQC were used as labeling reagents, as previously reported [20, 30]. In this study, the UV and FL properties of the derivatized amino acids obtained when using BCEC–Cl as the labeling reagent were evaluated in detail. To perform a quantitative comparison in terms of relative UV intensities, standard solutions containing 20 amino acids were derivatized using, respectively, BCEC–Cl and BCEOC–Cl as labeling reagents (the final derivatized concentration was adjusted to 5.0×10^{-5} mol L⁻¹; 10 μ L were injected, corresponding to an injected amount of each derivatized amino acid of 500 pmol). The difference in UV intensities between BCEC derivatives and BCEOC derivatives was used to estimate the relative sensitivities. Figure 3 shows the chromatographic separation obtained using BCEC–Cl as the labeling reagent for the derivatized amino acids (corresponding injected amount 500 pmol; here, the chromatographic separation obtained using BCEOC–Cl as the labeling reagent for the derivatized amino acids is not shown) and DAD detection. Relative UV responses for the individual derivatized amino acids obtained using BCEC–Cl and BCEOC–Cl as the derivatization reagents are given in Table 2. The ratios of UV responses for the amino acids

(UV_{BCEC}/UV_{BCEOC}) ranged from 1.67 to 4.90. As apparent from Table 2, the UV responses obtained when BCEC–Cl was used as the labeling reagent for His and (Cys)₂ were basically similar to those obtained for the other amino acids (the UV responses of the derivatized amino acids obtained using FMOC–Cl as labeling reagent were not gained in this experiment; these data were reported in previous work [29]).

The relative fluorescence responses for the individual derivatized amino acids using BCEC–Cl, FMOC–Cl and BCEOC–Cl were evaluated. In order to perform a quantitative comparison with respect to the relative fluorescent intensities, standard solutions containing 20 amino acids were derivatized, respectively, using BCEC–Cl, BCEOC–Cl and FMOC–Cl as labeling reagents (the final derivatized concentration was adjusted to 1.0×10^{-5} mol L⁻¹, 10 μ L were injected, corresponding to an injected amount of each derivatized amino acid of 100 pmol). The standard amino acid derivatives were separated according to the established method described in the “Experimental” section. The detection wavelength was set to the optimal wavelength range (the BCEOC derivatives were detected at $\lambda_{ex}/\lambda_{em}$ = 333/390 nm, the FMOC derivatives were detected at $\lambda_{ex}/\lambda_{em}$ = 263/313 nm; the optimal resolution of the derivatized

Table 3 MS and MS/MS data for the derivatized amino acids

Mono-labeled amino acids	[M+H] ⁺	MS/MS
Arg	462.4	445.1, 401.0, 391.3, 428.2, 261.7, 243.9
Asp	423	402.8, 374.2, 262.0, 243.9
Ser	395	391.8, 376.0, 346.9, 261.9, 243.9
Glu	437	416.7, 388.9, 262.1, 243.9
Thr	409	387.9, 360.8, 261.9, 243.9
Gly	365	362.8, 346.0, 290.0, 282.0, 262.0, 243.9
Ala	379	358.0, 262.0, 243.9
Tyr (mono)	469.1	422.7, 390.8, 278.1, 261.9, 243.9
GABA	391.1	372.8, 261.8, 243.8
Pro	403.1	404.0, 356.9, 243.7, 261.8
Met	437.1	390.8, 342.8, 280.4, 261.7, 243.8
Val	405.0	391.6, 358.8, 261.7, 243.8
Trp	492.1	473.9, 445.9, 274.7, 261.8, 243.8
Phe	453.1	406.8, 390.8, 262.0, 243.8
Ile	419.0	372.8, 261.8, 243.8
Leu	421.0	372.8, 261.9, 243.8
Dilabeled amino acids		
(Cys) ₂	832.1 [M+H ₂ O] ⁺	815, 798.2, 797.1, 617.1, 618.3, 599.1, 600.1, 554.9
	833.1 [M+H+H ₂ O] ⁺	261.8, 243.9
	834.1 [M+2H+H ₂ O] ⁺	
His	730.3 [M+2H] ⁺	686.2, 353.3, 269.8, 243.8, 216
Orn	724.3 [M+H ₂ O] ⁺	707.1, 507.1, 490.1, 446.1, 261.8, 243.9
Lys	738.3 [M+H ₂ O] ⁺	721.2, 523.3, 486.3, 461.2, 261.8, 243.9
Tyr	774.1 [M+H ₂ O] ⁺	756.6, 539.2, 495.4, 401.3, 261.8, 243.9
(BCEC) ₂	549.3	529.1, 476.6, 432.7, 390.7, 372.8, 331.7, 262.0, 243.9

amino acids obtained using BCEOC and FMOC as labeling reagents was not adjusted any further). The results indicated that the fluorescence intensity of an individual derivatized amino acid obtained using BCEC–Cl as the derivatizing reagent was significantly enhanced. The ratios of the fluorescence responses ranged from 1.17 to 3.57 for $I_{\text{BCEC}}/I_{\text{BCEOC}}$ and from 1.13 to 8.21 for $I_{\text{BCEC}}/I_{\text{FMOC}}$ (I : relative fluorescence intensity; see Table 2). This was probably due to the fact that BCEC–Cl has a large molar absorbance, which makes it more sensitive to the derivatized amino acids than BCEOC–Cl and FMOC–Cl. The different molar absorbances can be attributed to the molecular core structure of BCEC–Cl, in which its π – π conjugation system is dramatically augmented due to the addition of the 2-(11*H*-benzo[α]carbazole)-ethyl functional group [BCEC: $\epsilon=4.60 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$ (279 nm); BCEOC: $\epsilon=2.40 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$ (320 nm); CEOC: $\epsilon=2.34 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$ (249 nm); FMOC: $\epsilon=1.7 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$ (263 nm)]. Obviously, when BCEC–Cl is used as the labeling reagent, the derivatized (Cys)₂ exhibits greater fluorescence. To perform a quantitative comparison with respect to the fluorescence responses obtained for the derivatized His and (Cys)₂ using BCEC–Cl, FMOC–Cl and BCEOC–Cl as labeling reagents, the ratios of the relative

fluorescence intensities were calculated. The results were as follows: His: $I_{\text{BCEC}}/I_{\text{BCEOC}}=1.17$, $I_{\text{BCEC}}/I_{\text{FMOC}}=1.30$; (Cys)₂: $I_{\text{BCEC}}/I_{\text{BCEOC}}=2.92$, $I_{\text{BCEC}}/I_{\text{FMOC}}=8.21$. The data obtained indicated that the fluorescent responses obtained using BCEC–Cl as the labeling reagent were greater than those of BCEOC–Cl and FMOC–Cl. In addition, intense fluorescence quenching of the derivatized tryptophane (Try) was observed in our study when FMOC and AQC were used as the labeling reagents (100 pmol Try was derivatized, no response was observed). In contrast to the results obtained using FMOC–Cl and AQC, the BCEC–Cl exhibited very high fluorescence with the derivatized Try. In summary, the BCEC–Cl reagent was found to be very sensitive and to exhibit excellent fluorescent response to the derivatized amino acids, especially to (Cys)₂ and Try, which cannot be determined using AQC and FMOC due to intense fluorescence quenching.

Identification performed via MS–ESI in positive ion detection mode

Using MS–ESI in positive ion mode, m/z values for the intense lines corresponding to protonated molecular ions [M+H]⁺ of the derivatized amino acids produced when

Table 4 Detection limits, linearities, and correlation coefficients for the derivatized amino acids (detection was via fluorescence)

Amino acid	<i>R</i>	Detection limits		
		BCEC	BCEOC ^a	AQC ^b
Arg	0.9999	15.0	12.2	119
Asp	0.9997	7.2	27.2	311
Ser	0.9996	5.9	17.2	237
Glu	0.9997	7.4	19.8	315
Thr	0.9996	8.3	12.7	162
Gly	0.9998	6.6	9.3	293
Monosubstituted Tyr	0.9997	7.2		
Ala	0.9998	6.7	10.4	155
GABA	0.9998	1.6	11.8	#
Pro	0.9996	7.4	13.1	278
Met	0.9996	15.0	13.0	74
Val	0.9996	6.1	13.6	57
Try	0.9995	7.2	9.2	38
Phe	0.9994	2.7	8.3	#
Ile	0.9994	4.0	19.4	47
Leu	0.9994	3.8	10.4	48
(Cys) ₂	0.9998	8.4	19.4	794
His	0.9998	11.7	177.6	188
Orn	0.9997	1.9	7.7	#
Lys	0.9997	1.6	6.3	112
Disubstituted Tyr	0.9996	2.3	15.8	93
		Mean detection limit: 6.6	Mean detection limit: 21.7	Mean detection limit: 195.4

^aData are from [29]; ^bdata are from [30]

BCEOC-Cl is used as the labeling reagent were obtained as described previously [29]. Similar results were observed when BCEC-Cl was used as the labeling reagent instead. Selected reaction monitoring based on the m/z $[M+H]^+ \rightarrow m/z$ 261.8 and m/z 243.8 transitions was specific for the amino acid derivatives, except for their disubstituted derivatives. In most cases, the collision-induced dissociation spectra of the disubstituted derivatives such as (Cys)₂, His, Orn, Lys and Tyr showed intense protonated ions obtained by adding one H₂O. The MS and MS/MS spectra of the (Cys)₂ were quite complex. The collision-induced dissociation spectra of (Cys)₂ usually contained intense lines corresponding to protonated ions obtained by the addition of one H₂O molecule to form mono-, di- and triprotonated ions, as follows: m/z 832.1 $[M+H_2O]^+$; m/z 833.1 $[M+H+H_2O]^+$; m/z 834.1 $[M+2H+H_2O]^+$. At the same time, the collision-induced dissociation spectra of (Cys)₂ also contained intense lines due to fragment ions resulting from the loss of one H₂O molecule as follows: m/z 797.1 $[M+H-H_2O]^+$; m/z 798.2 $[M+2H-H_2O]^+$. The other characteristic fragment ions mainly arose from the loss of

the core structural moiety (neutral fragments of m/z 216 or 260) to form specific ions as follows: m/z 617.1 $[M+H+H_2O-216]^+$; m/z 618.3 $[M+2H+H_2O-216]^+$; m/z 599.1 $[M+H-216]^+$; m/z 600.1 $[M+2H-216]^+$ and m/z 554.9 $[M+H-260]^+$; m/z 261.8 and m/z 243.9. The collision-induced dissociation spectra of Orn, Lys and Tyr contained only the lines from intense protonated ions produced by the addition of one H₂O molecule; no loss of an H₂O molecule was observed. The protonated ions were as follows: Orn: m/z 707.1 $[M+H]^+$; m/z 724.3 $[M+H_2O]^+$; m/z 507.1 $[M+H_2O-216]^+$; m/z 490.1 $[M+H-216]^+$; m/z 446.1 $[M+H-260]^+$; m/z 261.8 and m/z 243.9. Lys: m/z 721.2 $[M+H]^+$; m/z 738.3 $[M+H_2O]^+$; m/z 523.3 $[M+H_2O-216]^+$; m/z 486.3 $[M+H_2O-216]^+$; m/z 461.2 $[M+H-260]^+$; m/z 261.8 and m/z 243.9. His was an exception; its collision-induced dissociation spectra exhibited only lines from intense diprotonated ions and corresponding specific ions formed by the loss of neutral fragments, as follows: m/z 730.1 $[M+2H]^+$; m/z 514.6 $[M+2H-216]^+$ and m/z 469.3 $[M+2H-260]^+$; m/z 261.8 and m/z 243.9. There were no detectable signals from the blank water samples when this transition was used. Although other endogenous compounds presented in natural environmental samples were presumably coextracted and derivatized by BCEC-Cl, no interference was observed due to the highly specific parent mass-to-charge ratio used and the characteristic m/z values of the product ions: $[M+H]^+$ for monosubstituted derivatives or $[M+nH+H_2O]^+$ for disubstituted derivatives ($n=1-2$) \rightarrow m/z 261.8 (262) and m/z 243.9 (244) transitions. To minimize the interference, gradient elution with HPLC was selected for the separation and determination of derivatized BCEC-amino acids. In most cases, the collision-induced dissociation spectra of $[M+H]^+$ or $[M+H+H_2O]^+$ contained intense lines from fragment ions. The characteristic fragment ion at m/z 261.8 (262) derives from the cleavage of the CH₂O-CO bond, while the fragment ion at m/z 243.9 (244) arose from the cleavage of the CH₂-OCO bond. Data from the MS and MS/MS spectra for all of the derivatized amino acids are shown in Table 3.

Analytical precision, accuracy, reproducibility and recovery

The reproducibility of the derivatization was ascertained by performing out six assays on the same sample over two days (after derivatization, the amino acid concentration was 5.0×10^{-6} mol/L, 10 μ L were injected, corresponding to an injected amount of 50 pmol), with each derivatized solution injected twice. The standard deviations for the peak areas ranged between 0.48 and 2.32%, and relative standard deviations were between 2.4 and 5.2%. In addition, a derivatized standard solution containing 5.0×10^{-6} mol/L amino acids was prepared in order to examine the repeatability of the method (10 μ L were injected, $n=12$).

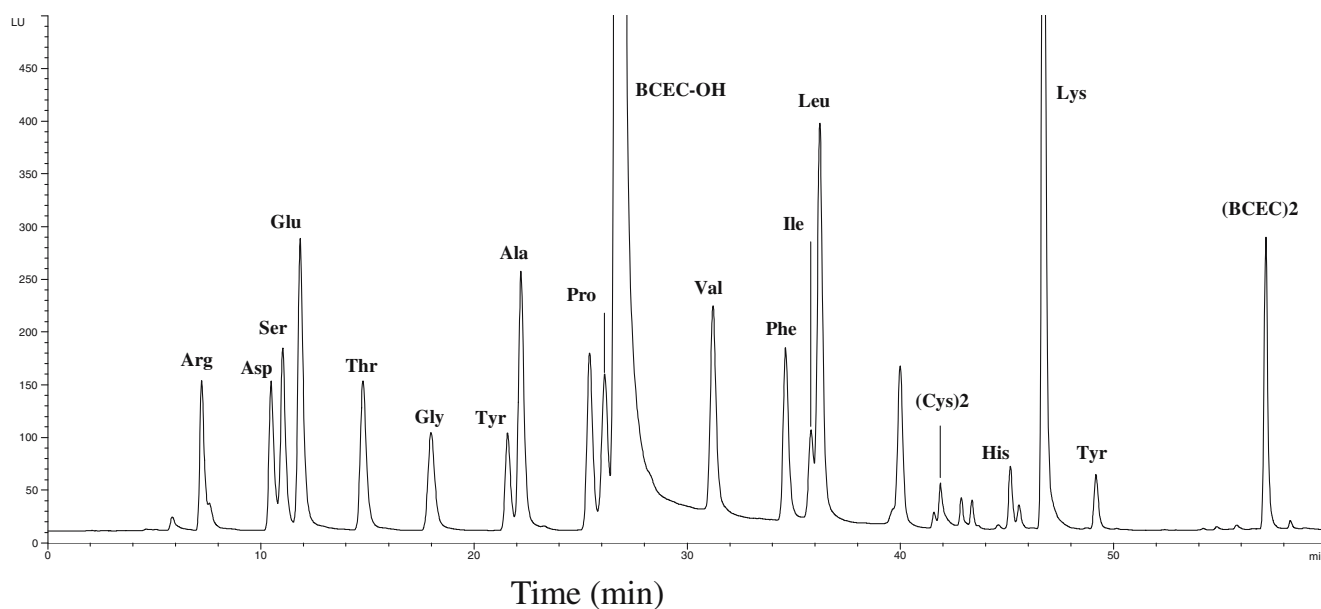


Fig. 4 Chromatogram of the amino acids from rape bee pollen, observed with fluorescence detection. Chromatographic conditions and peaks as in Fig. 2a

The relative standard deviations (RSDs) of the peak areas and retention times ranged from 0.78 to 2.08% and from 0.004 to 0.038%, respectively. To ascertain the precision and accuracy, six replicates ($n=12$) at amino acid concentrations of 1.0×10^{-7} mol/L, 1.0×10^{-6} mol/L and $1.0 \times$

10^{-5} mol/L were used to cover a wide range of concentrations (10 μ L were injected, corresponding to injected amounts of 1.0 pmol, 10.0 pmol and 100 pmol, respectively). The mean interday accuracy ranged from 94 to 105.4% for fluorescence detection, with a mean %CV of <6.5. The mean interday precision for all standards was <7.2% of the

Table 5 Composition analysis of hydrolyzed bovine serum albumin (detection was via fluorescence; the hydrolyzed sample contained 1020 ng; approximately 6.28% was analyzed by HPLC)

Amino acid	Sample (64.06 ng, 962 fmol)		
	Literature (residues/ moL)	Calculated (residues/ moL)	Error (%)
Arg	23	24.75	7.61
Asp	54	56.93	5.34
Ser	28	27.0	3.57
Glu	79	82.7	4.68
Thr	34	33.52	1.41
Gly	16	16.47	2.93
Ala	46	46.64	1.39
Pro	28	27.92	0.28
Met	4	3.67	8.25
Val	36	37.29	3.58
Phe	27	31.55	16.8
Ile	14	17.30	23.57
Leu	61	62.36	2.22
(Cys) ₂	nd	9.63	–
His	17	16.67	1.94
Lys	59	62.46	5.86
Tyr	19	19.32	1.68
Average error (%)			5.69

nd: not reported

Table 6 Compositional analysis of amino acids from hydrolyzed rape bee pollen

Amino acid	Rape bee pollen (mg/g)	
	Xinjiang	Qinghai
Arg	7.27	6.45
Asp	10.60	14.1
Ser	8.26	7.97
Glu	9.50	11.85
Thr	5.83	6.82
Gly	6.28	6.46
Ala	6.50	7.13
GABA	0.34	0.16
Pro	11.4	12.17
Met	nd	0.450
Val	6.4	9.07
Try	#	#
Phe	4.89	5.25
Ile	4.96	5.02
Leu	9.28	9.86
(Cys) ₂	nd	0.56
His	2.9	3.89
Orn	nd	nd
Lys	9.85	10.36
Tyr	3.86	4.27
Mean value:	5.41	6.09

nd: not determined or below LOQ; # not identified

expected concentration. A known amount of amino acids was added to two rape bee pollen samples. The samples were treated according to the method described in the “Experimental” section and derivatized with BCEC–Cl, and the analyses were carried out in duplicate. The experimental recoveries obtained were in the range 94–106%.

Detection limits and linearity

Detection limits are an important consideration when the components of biological matrices are analyzed, particularly when the components are present at low or trace concentrations. The calculated fluorescence detection limits from an injection of 1.0 pmol of each derivatized amino acid, for a signal-to-noise ratio of 3:1, ranged from 1.6 to 14.0 fmol. The linearity of the response of the technique to each amino acid was established over a 1000-fold concentration range by analyzing serial dilutions of the standard amino acids ranging in concentration from 5.0×10^{-9} mol/L to 5.0×10^{-6} mol/L (0.05–50 pmol of each derivatized amino acid injected). The technique gave highly linear responses to all of the amino acids over this concentration range, with correlation coefficients of >0.9994 obtained (see Table 4). Linear regression analysis was not performed for higher concentrations of amino acids.

Testing the reliability of the method with bovine serum albumin hydrolysate

A quantitative analysis of the hydrolyzed amino acids from bovine serum albumin was performed by the external standard calibration method; the results from this analysis were agreement with those obtained from the application of the internal standard method, using γ -aminobutyric acid (GABA) as an internal standard compound. Amino acid compositional data for hydrolyzed bovine serum albumin are shown in Table 5. These compositional data are in excellent agreement with those published in the literature [24]. The data were obtained from ca. 1020 ng (ca. 15.0 pmol) of hydrolyzed bovine serum albumin. Note that (Cys)₂ and Try were easily determined using the proposed method. In fact, useful compositional data could even be obtained when low or subpicomole amounts of hydrolyzed bovine serum albumin were used. Error data were calculated according to the procedure described by Strydom et al. [34], which yielded an average error of 5.69%.

Analysis of samples

The chromatogram used to analyze the amino acids from the hydrolyzed rape bee pollen sample is shown in Fig. 4. Amino acid compositional data from hydrolyzed rape bee pollen samples are shown in Table 6. The compositional

data for hydrolyzed bee pollen were compared with those reported in the literature, and excellent agreement with the data reported by González Paramás et al. [35] was obtained. ESI/MS identification of the amino acids from the hydrolyzed rape bee pollen samples indicated that the amino acids were mainly Arg, Asp, Glu, Pro, Phe, Ile, Leu, and Lys. It is a dietary requirement that all eight of the essential amino acids should be present in the human body, and seven of these amino acids—Lys, Thr, Leu, Ile, Val, Met and Phe—were identified. Try and Orn were probably not identified because they were present at concentrations below the detection limits.

Conclusions

The present paper introduced a new reagent for derivatizing amino acids, BCEC–Cl, which possesses superior properties compared to the reagents currently employed for this purpose, including rapid, convenient derivatization, and excellent sensitivity, stability and derivatization yields. The reagent BCEC–Cl exhibited excellent fluorescence properties during the quantitative analysis of amino acids, especially when determining (Cys)₂ and Try. Complete derivatization in a basic medium at room temperature took less than 3.0 min. The method described here yielded results that correlated well with those obtained when other labeling reagents (BCEOC, CEOC, and FMOC) were used for amino compound derivatization. Detection limits were in the femtomole range. LC separation of the derivatized amino acids showed good repeatability. This powerful derivatizing reagent (BCEC–Cl) is likely to find numerous applications in analytical chemistry. Current studies are aimed at exploring the derivatizations of other amine-containing compounds, such as alkylamines, catecholamines and polyamines.

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