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Development of a sensitive fluorescent derivatization reagent 1,2-benzo-3,4-dihydrocarbazole-9-ethyl chloroformate (BCEOC) and its application for determination of amino acids from seeds and bryophyte plants using high-performance liquid chromatography with fluorescence detection and identification with electrospray ionization mass spectrometry

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

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

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Abstract

A pre-column derivatization method for the sensitive determination of amino acids and peptides using the tagging reagent 1,2-benzo-3,4-dihydrocarbazole-9-ethyl chloroformate (BCEOC) followed by high-performance liquid chromatography with fluorescence detection has been developed. Identification of derivatives was carried out by liquid chromatography/electrospray ionization mass spectrometry (LC/ESI–MS/MS). The chromophore of 2-(9-carbazole)-ethyl chloroformate (CEOC) reagent was replaced by 1,2-benzo-3,4-dihydrocarbazole functional group, which resulted in a sensitive fluorescence derivatizing reagent BCEOC. BCEOC can easily and quickly label peptides and amino acids. Derivatives are stable enough to be efficiently analyzed by high-performance liquid chromatography. The derivatives showed an intense protonated molecular ion corresponding m/z $(M + H)^+$ under electrospray ionization (ESI) positive-ion mode with an exception being Tyr detected at negative mode. The collision-induced dissociation of protonated molecular ion formed a product at m/z 246.2 corresponding to the cleavage of C─O bond of BCEOC molecule. Studies on derivatization demonstrate excellent derivative yields over the pH 9.0–10.0. Maximal yields close to 100% are observed with a 3–4-fold molar reagent excess. Derivatives exhibit strong fluorescence and extracted derivatization solution with *n*-hexane/ethyl acetate (10:1, v/v) allows for the direct injection with no significant interference from the major fluorescent reagent degradation by-products, such as 1,2-benzo-3,4-dihydrocarbazole-9-ethanol (BDC-OH) (a major by-product), mono-1,2-benzo-3,4-dihydrocarbazole-9-ethyl carbonate (BCEOC-OH) and bis-(1,2-benzo-3,4-dihydrocarbazole-9-ethyl) carbonate $(BCEOC)_2$. In addition, the detection responses for BCEOC derivatives are compared to those obtained with previously synthesized 2-(9-carbazole)-ethyl chloroformate (CEOC) in our laboratory. The ratios $AC_{BCEOC}/AC_{CEOC} = 2.05–6.51$ for fluorescence responses are observed (here, AC is relative fluorescence response). Separation of the derivatized peptides and amino acids had been optimized on Hypersil BDS C_{18} column. Detection limits were calculated from 1.0 pmol injection at a signal-to-noise ratio of 3, and were 6.3 (Lys)-177.6 (His) fmol. The mean interday accuracy ranged from 92 to 106% for fluorescence detection with mean %CV < 7.5. The mean interday precision for all standards was <10% of the expected concentration. Excellent linear responses were observed with coefficients of >0.9999. Good compositional data could be obtained from the analysis of derivatized protein hydrolysates containing as little as 50.5 ng of sample. Therefore, the facile BCEOC derivatization coupled with mass spectrometry allowed the development of a highly sensitive and specific method for the quantitative analysis of trace levels of amino acids and peptides from biological and natural environmental samples.

Keywords: Derivatization; HPLC; Amino acids; 1,2-Benzo-3,4-dihydrocarbazole-9-ethyl chloroformate (BCEOC)

1. Introduction

Pre-column derivatization in combination with reversed-phase liquid chromatography is today the most used technique for the determination of amino acids. Recently, biologically

* Corresponding author.
E-mail address: Jmyou6304@163.com (J. You).

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active peptides containing D-amino acid have been found in eukaryotes [1]. Interest in these amino acids continues to increase as they have been implicated in physiological roles as diverse as learning, and movement as well as several diseases including Alzheimer's, Parkinson's, and Epilepsy [2]. Achieving high efficient separation of amino acids and peptides has important implications for amino acid analysis, peptide sequencing and protein structure determination. Most peptides and amino acids show neither natural UV absorption nor fluorescence. Therefore, chemical derivatization is necessary to increase detection sensitivity and improve selectivity by means of pre-column or post-column HPLC [3–5] and electrophoretic microchips [6] or CE [7–9] separation. These techniques have been used in physical, chemical and biological sciences for investigating the structure and dynamics of living systems. Although a number of different types of fluorescent tagging reagents [10–14] have been developed, a variety of shortcomings in their applications have also been reported. For example, the *o*-phthaldialdehyde (OPA) method offers greater sensitivity [15–17], but is only limited to primary amino acids. 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 the reagent itself has about 50% decomposition in methanol–water solution exposed to daylight within 25 min [19]. At the same time, a large number of fluorescent components were eluted later than the derivative itself. 9-Fluorenyl methylchloroformate (FMOC) [20,21], 1-(9-fluorenyl)-ethyl chloroformate (FLEC) [22] and 2-(9-anthryl)-ethyl chloroformate (AEOC) [23] reagents have also been developed for the derivatization of amino acids and peptides for chiral or non-chiral separation by LC or CE. Recently, 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) has also been developed as a popular pre-column derivatization reagent [24,25] for the determination of amino acids with satisfactory results. However, 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 amino acids are usually higher than those for later ones [24].

FMOC is well known as a blocking functional group in the peptide synthesis. The reagent is not optimized regarding to chromophoric properties for quantitative spectrophotometric determination. The combination of a sensitive functional group such as chloroformate together with a strong absorption moiety will result in an attractive reagent. In our previous studies [26–28], we describe the synthesis of a number fluorescence tagging reagents and the application for the analysis of common amino compounds. On the basis of the fluorescence characteristics of carbazole moiety as we previously described [29], we have synthesized a novel fluorescence reagent 1,2-benzo-3,4-dihydrocarbazole-9-ethyl chloroformate (BCEOC) that the chromophore of carbazole functional group was replaced by 1,2-benzo-3,4-dihydrocarbazole resulting in a strong fluorescence. BCEOC has been found to be very stable in its crystal state. In this study, the optimal derivatization conditions such as buffer pH, reaction time and solvent system are investigated. The detection responses for fluorescence are compared to those obtained with 2-(9-carbazole)-ethyl chloroformate (CEOC) previously synthesized in our laboratory [30]. Linearity, detection limits and precision of the procedure are also determined. At the same time, applications for the determination of peptides and amino acids from extracted pumpkin and sunflower seeds have also been reported. The suitability of the developed method for the analysis of actual samples is satisfying.

2. Experimental

2.1. Instrumentation

All the HPLC system devices were from the HP 1100 series and consisted of a vacuum degasser (model G1322A), a quaternary pump (model G1311A), an autosampler (model G1329A), a thermostated column compartment (model G1316A), a fluorescence detector (FLD) (model G1321A), and a diode array detector (DAD) (model G1315A). Mass spectrometer was equipped with an electrospray ionization (ESI) source; dry temperature, 350 °C; nebulizer, 35.00 psi; dry gas, 9.0 L/min. Derivatives were separated on Hypersil BDS C_{18} column (200 mm × 4.6 mm 5 μM, Yilite Co. Dalian, China). The HPLC system was controlled by HP Chemstation software. The mass spectrometer from Bruker Daltonik (Bremen, Germany) was equipped with an electrospray ionization (ESI) source. The mass spectrometer system was controlled by Esquire-LC NT software, version 4.1. Fluorescence excitation and emission spectra were obtained at a 650-10 S fluorescence spectrophotometer (Hitachi). Excitation and emission bandpass were 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 peptides (Gly-Gly, Gly-Gly-Gly, Gly-Gly-Gly-Gly, Gly-Gly-Gly-Gly-Gly, Gly-Gly-Gly-Gly-Gly-Gly) and 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). Pumpkin and sunflower seeds were obtained from commercial supplier (Qufu, China). Bryophyte plant (No. 1 and No. 2) was used as a gift from Biology Department of Qufu Normal University (bryophyte plants were collected from Taishan mountain and Tianshan mountain, respectively). 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.

2.3. Preparation of standard solutions

The derivatizing reagent solution 1.0×10^{-3} mol/L was prepared by dissolving 3.26 mg 1,2-benzo-3,4-dihydrocarbazole-9-ethyl chloroformate in 10 mL of anhydrous acetonitrile prepared

by distilling the dried HPLC grade acetonitrile with P_2O_5. Individual stock solutions of the amino acids were prepared in water, and if necessary, HCl or NaOH was added until the compound gets dissolved. The standard amino acids for HPLC analysis at individual concentrations of 5.0×10^{-5} mol/L were prepared by dilution the corresponding stock solutions (1.0×10^{-3} mol/L) of each amino acid with 0.2 M borate buffer (pH 9.0). Peptides standard solutions (5.0×10^{-5} mol/L) were prepared by diluting the corresponding stock solutions (1.0×10^{-3} mol/L) of each peptide with 0.2 M borate buffer (pH 9.0). When not in use, all standards were stored at 4 °C.

2.4. Synthesis of derivatization reagent (BCEOC)

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

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

2.4.2. Synthesis of 1,2-benzo-3,4-dihydrocarbazole-9-ethanol

1,2-Benzo-3,4-dihydrocarbazole-9-ethanol was synthesized by a modified method as follows: 1,2-benzo-3,4-dihydrocarbazole (25 g), KOH (20 g) and 80 mL 2-butanone were mixed and rapidly cooled to 0 °C with ice-water with vigorous stirring. A cooled mixture of oxane (7.5 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 heated to 50 °C for 1 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, 75% ethanol, and dried at room temperature for 48 h. The crude product was recrystallized three times from methanol (100 mL × 3) to afford a white crystal, yield (78%). mp 141.3–141.7 °C. Found, C 79.46, H 6.37, N 5.28; calculated, C 82.13, H 6.46, N 5.32; IR (KBr), 3347.16 (–OH); 3051.23 (ph–N–CH_2–); 2941.5, 2875.8, 2839.4 (ph); 1462.8 (C–H); 1368.7, 1345.9 (C–H); 1233, 1174, 738.7, 759.9. *m/z*: 263 (M^+), 246^+ (M–H_2O); ^{1}H NMR, ($CDCl_3$, 500 MH_2) δ: 1.68(1H, *bs*,OH), 2.87–2.97 (4H, m, CH_2CH_2), 4.09 (2H, *t*, *J* = 5.9H_2, NH_2), 4.56 (2H, *t*, *J* = 5.9H_2, CH_2O), 7.12–7.70 (8H, *m*, Ar–H).

2.5. Preparation of 1,2-benzo-3,4-dihydrocarbazolecarbazole-9-ethyl chloroformate (BCEOC)

To a solution containing 15 g solid phosgene and 100 mL dichloromethane (0 °C) in 500-mL round-bottom flask, a mixture of 1,2-benzo-3,4-dihydrocarbazole-9-ethanol (25 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 26.5 g (85.5%), mp 105–105.7 °C. Found, C 69.3, H 4.12, N 4.34, Cl 11.2; calculated, C 69.9, H 4.90, N 4.29, Cl 11.0; IR (KBr), 1772.8 (–C=O); 1461.6 (C–H); 1372.9, 1346.0 (C–H); 1202.4, 1128, 848.9, 739.1.

2.6. High-performance liquid chromatography

HPLC separation of BCEOC derivatives was carried out on Hypersil BDS C_{18} column by a gradient elution. Eluent A was 30% of acetonitrile consisting of 30 mM formic acid buffer (pH 3.7); B was acetonitrile–water (50:50, v/v); C was acetonitrile–water (95:5, v/v). During conditioning of the column and prior to injection, the mobile phase composition was 70% A and 30% B. The gradient condition used for the separation of amino acid and peptide derivatives is shown in Table 1. The flow rate was constant at 1.0 mL/min and the column temperature was set at 36 °C. The fluorescence excitation and emission wavelengths were set at λ_{ex} 335 and λ_{em}390 nm, respectively.

2.7. Method validation

The analytical method was validated to demonstrate the specificity, recovery, limit of detection (LOD), precision and accuracy of samples, respectively. Triplicate sets of standard curve and quality control samples were analyzed on 3 separate days to determine the inter- and intraday validation. The recovery was determined by comparing the content of amino acids from extracted seeds to the spiked seeds with various amounts of standard amino acids for three replicates of the high-, medium-, and low-level samples. The calibration range for amino acids and

Table 1
Chromatographic gradient conditions eluted on Hypersil BDS C_{18} column

Time (min)	A	B	C
0	70	30	0
15	55	45	0
20	2	98	0
28	2	96	2
28.3	2	88	10
30	2	83	15
40	0	80	20
50	0	20	80
55	0	5	95
57	0	0	100
65	0	0	100

(A) 30% of acetonitrile solution consisting of 30 mM formic acid buffer (pH 3.7). (B) 50% of acetonitrile. (C) 95% of acetonitrile.

peptides was established using standard solutions. The standard concentrations were from 0.0052 to 5.280 mM for amino acids and from 0.0024 to 3.00 mM for peptides, respectively (injection volume 10 μL).

2.8. Extraction of free amino acids from seeds

To a solution containing 80 mL of ethanol–water (60:40, v/v) in 250-mL round-bottom flask, 6.0 g powdered pumpkin or sunflower seeds was added, then the contents of the flask were rapidly heated to reflux with magnetic stirring for 30 min under a nitrogen atmosphere. After cooling, the water phase was transferred to another 250-mL round-bottom flask. The residue was extracted twice with 60 mL ethanol–water solution. Three millilitres of hydrochloric acid (3.0 M) was added to the combined ethanol–water solution. The mixture was concentrated in vacuum to dryness. The residue was re-dissolved with 0.2 M borate buffer (pH 9.0) to a total volume of 50 mL and stored at 4 °C until analysis.

2.9. Hydrolysis of bryophyte plants

Bryophyte plant was washed successively with 40 mL each of 0.1 M hydrochloric acid, water, 0.1 M sodium hydroxide, and deionized water. The washed bryophyte plant was dried under a stream of nitrogen. Hydrolysis of proteins from bryophte plant was performed essentially as described by Weiss et al. [32]. 13.5 mg bryophyte plant was placed in a 50 mm × 6 mm test tube; 500 μL 6 M hydrochloric acid 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 re-dissolved with 2.0 mL of borate buffer (pH 9.0) and filtered through a 0.2 μm nylon membrane filter. The residue was washed with 1.0 mL of 70% acetonitrile. The combined solution was made up to 10 mL and stored at 4 °C until HPLC analysis.

2.10. LC/ESI/MS procedure

Prior to its use, the instrument was checked to meet the sensitivity defined by the manufacturer. The FLD were calibrated and tested using the FLD diagnosis procedure of the ChemStation software for HP1100 system. The HP1100 LC/MS/MS was calibrated with electrospray ioniztion (ESI) tuning solution obtained from Agilent Technology (Palo Alto, CA). The mass spectrometer was calibrated so that mass accuracy specification and sensitivity were achieved over the entire mass range.

2.11. Derivatization procedure

The BCEOC-amino acids derivatization proceeded in a water/acetonitrile (≈1:1, v/v) solution in a basic medium. Twenty to thirty microlitres of aqueous of amino acids or peptides was added in a vial, to which 200 μL of 0.2 M borate buffer (pH 9.0) and 100–200 μL of BCEOC acetonitrile solution were then added. The solution was shaken for 1 min and allowed to stand for 10 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

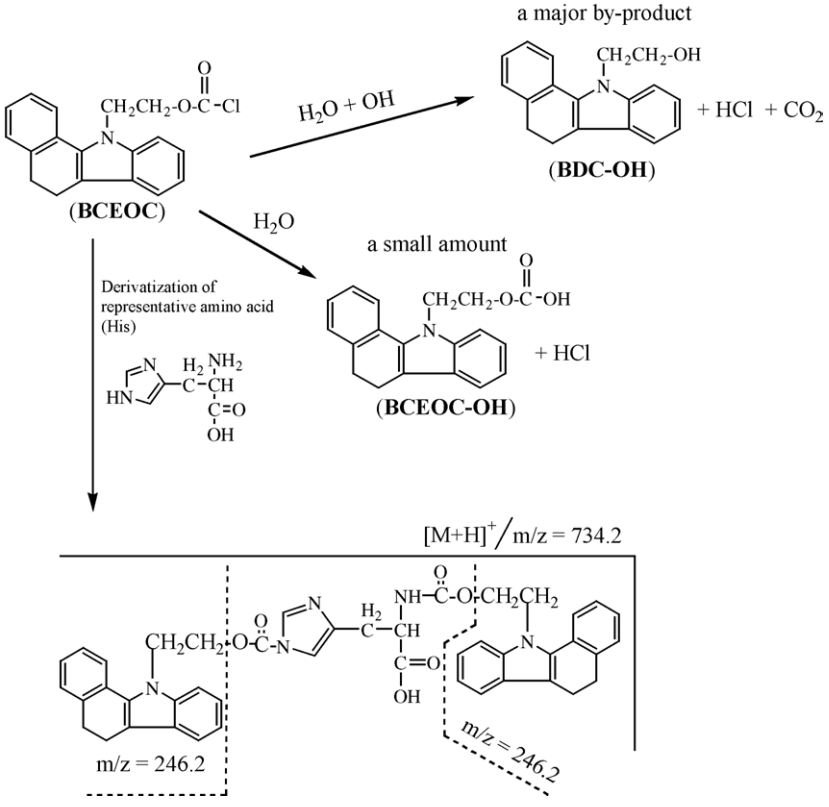

Fig. 1. Derivatization scheme of 1,2-benzo-3,4-dihydrocarbazole-9-ethyl chloroformate (BCEOC) with amines, and mass breakage mode of its derivatives.

to another conical vial and added an appropriate amount of 36% formic acid until the final pH range of 6.0–6.5. Then the derivatized sample solution was directly injected into the HPLC system for analysis. The derivatization process is shown in Fig. 1.

3. Results and discussion

The objective of this study was to develop a sensitive fluorescence reagent that can be used to analyze trace concentrations of amino compounds containing amino acids and peptides from biological or natural environmental matrices. The main challenge of the present work was to test the feasibility of labeling reagent in a variety of conditions.

3.1. Ultraviolet absorption of the 1,2-benzo-3,4-dihydrocarbazole-9-ethanol

The ultraviolet absorption of 1,2-benzo-3,4-dihydrocarbazole-9-ethanol (BDCE-OH) was investigated in methanol–water (1:1) solution. The absorption wavelength of BDCE-OH was obtained with the scanning range of 200–400 nm. Maximum ultraviolet absorption responses were observed at the wavelengths of 249 nm and 320 nm, respectively. The molar absorption coefficients (ε) were 2.54×10^4 L/mol cm (249 nm) and 2.40×10^4 L/mol cm (320 nm), respectively.

3.2. Fluorescence excitation and emission

Maximum fluorescence responses were achieved at the excitation wavelength of 333 nm and emission wavelength of 390 nm. The fluorescence emission intensity of representative Gly-derivative in methanol (100%) was 2.1% higher than that in acetonitrile (100%). The maximum excitation and emission wavelengths in acetonitrile or methanol solution (0–100%) exhibited no obvious blue- or red-shift. Fluorescence intensity of representative Gly-derivative was minimally quenched by inorganic anions (such as sulfate, nitrate, and phosphate) and organic anions (such as citrate) and divalent cations that were abundant in biological fluids. The relative fluorescence intensity of derivative changed from 139 to 132 in going from 0 to 100% acetonitrile and from 149.3 to 134.7 in going from 0 to 100% methanol, respectively. It was observed that fluorescent response in water was 6.0% higher than that in acetonitrile, and 8.6% higher than that in methanol, respectively. As expected, the detection limits (fluorescence responses) for the earlier eluted amino acids was no significant loss compared to those eluted for later ones.

3.3. Stabilities of reagent (BCEOC) and its derivatives

In this study, the structure of synthesized BCEOC in our laboratory was similar to that of CEOC, FMOC and AEOC. Anhydrous acetonitrile solution of BCEOC could be stored at 4 °C for 2 weeks, and the derivatization yields for peptides or amino acids showed no obvious difference. The corresponding derivatives were stable for normalized peak areas with an exception of histidine, which showed a remarkable degradation of its doubly labeled derivative. The labeled peptides stored at 4 °C allowed further HPLC analysis at least 2 weeks with normalized peak areas varying <2.2%.

3.4. Optimization for derivatization

3.4.1. Effect of BCEOC concentration on derivatization

1,2-Benzo-3,4-dihydrocarbazole-9-ethyl chloroformate (BCEOC) has the same chloroformate reaction with primary and secondary amino acids as do of CEOC [30], FMOC or AEOC previously reported [20,23]. Derivatization of BCEOC with amino acids can be achieved within 10 min at room temperature. The effect of BCEOC concentrations on the derivatization yields was investigated for amino acid derivatives. The fluorescence intensity of BCEOC-derivatives increased with increasing the amounts of derivatization reagent. A constant fluorescence intensity was achieved with the addition of 3–4-fold molar reagent excess to total molar amino acids, 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 amino acids was incomplete and it obviously resulted in mono-substituted derivatives such as His (*m*/*z*: 444) and Tyr (*m*/*z*: 732). Additionally, several side reactions were also observed and they should be attributed to the reagent hydrolysis, such as mono-(1,2-benzo-3,4-dihydrocarbazole-9-ethyl)-carbonate (BCEOC-OH) (*m*/*z*: 307), 1,2-benzo-3,4-dihydrocarbazole-9-ethanol (BDC-OH) (*m*/*z*: 246) and bis-(1,2-benzo-3,4-dihydrocarbazole-9-ethyl)-carbonate $(BCEOC)_2$ which formed by the reaction of hydrolysated BCEOC-OH with the excess reagent BCEOC. The presence of $(BCEOC)_2$ and BCD-OH (major by-product) did not interfere with the separation of amino acid derivatives. The interference from a small amount of BCEOC-OH could be eliminated by the extraction with hexane/ethyl acetate (10:1, v/v). The derivatized amino acids and peptides were found to be stable for more than 24 h at room temperature, except for the doubly labeled histidine, which degraded to the mono-labeled form at higher pH value (pH > 9.5). This degradation could be monitored by the appearance of the mono-substituted degradation product. Assuming first-order kinetics, the half-life of doubly labeled histidine was near 20 h. However, if the derivatized solution was neutralized to pH 6.0–6.5 with 36% acetic acid solution, degradation could be avoided, its doubly labeled derivative was very stable and can be further analyzed for 48 h at room temperature. The degradation of doubly substituted histidine was similar to that reported previously using CEOC as derivatization reagent in our study [30].

3.4.2. Effect of pH on derivatization

Several types of basic media were tested in this study for derivatization, including carbonate buffers, phosphate buffers and borate buffers. The results showed that borate buffer was found to be the best choice. The effect of pH on the derivatization reaction was then investigated with borate buffer (0.2 M) in the pH range of 8.8–10.5. The maximum derivatization yields were achieved in the 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

Table 2
LC/MS/MS for amino acid and peptide derivatives

Compound	$(M+H)^+$	Fragment ion[a]
Arg	464	447 (S), 246.2 (L)
Asp	423	405 (S), 246.2 (L)
Ser	395	246.2
Glu	437	419 (S), 246 (L)
Thr	409	246.2
Gly	365	246.2
Ala	379	246.2
GABA	393	375 (S), 246.2 (L)
Pro	405	246.2
Met	439	246.2
Val	407	246.2
Trp	494	448 (S), 246.2 (L)
Phe	455	246.2
Ile	421	246.2
Leu	421	246.2
$(Cys)_2$	–	–
His	734	690 (L), 246.2 (S)
Orn	711	299
Lys	674	666
Tyr	758	495
$(Gly)_2$	422	246.2
$(Gly)_3$	479	404, 246.2
$(Gly)_4$	536	461
$(Gly)_5$	593	518 (M), 246.2 (L)
$(Gly)_6$	650	246.2
BCEOC	307	246.2
BDC	264	246.2

[a] S, M and L stand for relative intensities; S: small; M: middle; L: large.

solution, decreased responses were observed. At further higher pH values (>10.5), the doubly substituted amino acids, such as Orn, Lys and Tyr, exhibit some hydrolysis and partially convert to their mono-substituted derivatives; there was about 20–30% loss of their doubly substituted derivatives over a 24-h period; therefore, 0.2 M borate buffer solution at pH 9.0 was chosen for amino acids and peptides derivatization. As expected, the BCEOC derivatives produced an intense molecular ion peak at m/z $[M+H]^+$ under positive mode with an exception being Tyr detected under negative mode (see Table 2). In most cases, the collision-induced dissociation spectra of m/z $[M+H]^+$ produced an intense fragment ion at m/z 264.2. As observed from Fig. 1, the characteristic fragment ion of m/z 264.2 was from the cleavage of the C–O bond of reagent molecule.

3.4.3. Selectivity of extraction solvent

To eliminate the interference from the unhydrolyzed BCEOC reagent, several types of organic extraction solvents were tested, including *n*-hexane, ethyl acetate and mixed hexane/ethyl acetate. With *n*-hexane as extraction reagent, low extraction efficiency for BCD-OH and BCEOC-OH was observed. With ethyl acetate as extraction solvent, a complete extraction of BCD-OH and BCEOC-OH could be achieved; however, about 80% of loss in detection responses for the latter eluted hydrophobic amino acid derivatives, such as His, Orn, Lys, Tyr was observed. To maintain the desirable extraction efficiency with no loss of hydrophobic amino acid derivatives, the mixed solvent of hexane and ethyl acetate (hexane/ethyl acetate = 9:1–10:1, v/v) was proved to be effective.

3.5. HPLC separation for derivatized amino acids and peptides

The resolution can be significantly affected by pH of mobile phase. The constant pH will be unable to fully suit the separation for all amino acid derivatives with a good resolution. To achieve optimal separation, the choice of pH value of mobile phase **A** was tested on Hypersil BDS C_{18} column. Separation of the derivatized amino acid standards can be accomplished at acidic condition with pH 3.7, or in more acidic mobile phase with pH 3.5–3.7. With pH < 3.5, most of the amino acids were resolved with the exception of Asp and Ser (co-eluted), BDC-OH and Met (co-eluted), Ile and Leu (partially separated). In comparison with the acidic conditions (pH3.7), operation at pH 3.7–4.0 resulted in obviously decrease in retention value for most of amino acid derivatives; at the same time, Asp and Ser, BCD-OH and Met, Pro and GABA, Ile and Leu were co-eluted, respectively. After further experiments, it was found that if the pH value of mobile phase **A** was adjusted to 3.7, a complete baseline resolution for all amino acid derivatives could be achieved within the shortest time. The separation of standard consisting of amino acids on Hypersil BDS C_{18} is shown in Fig. 2A. Complete resolution of six oligopeptide derivatives could also be obtained on Hypersil BDS C_{18} column with the same elution conditions as described in Fig. 3.

3.6. Comparison of the responses between BCEOC and CEOC for fluorescence detection

Relative responses for fluorescence detection ($\lambda_{ex}/\lambda_{em}$: 333/390 nm) for the individual derivatized amino acid were investigated. No fluorescent quenching was observed for derivatized Trp and $(Cys)_2$. But the difference in fluorescence response for His-derivative using BCEOC as derivatization agent was at least 15% lower than that of other amino acid derivatives (His/Lys = 0.15). In addition, a quenching phenomenon for Cys-derivative using BCEOC as tagging reagent was observed. This quenching phenomenon for derivatized Cys and Trp using AQC and FMOC as derivatizing reagents was also observed as previously reported [29,33]. The quenching in fluorescence response can, at least in part, be attributed to the interaction between thiohydroxy group and probe molecules. To make a quantitative comparison between BCEOC and CEOC in terms of relative fluorescent intensity, separation of standard amino acids, respectively, using BCEOC and CEOC as tagging reagent under the same conditions proposed was investigated (the chromatographic conditions for the separation of CEOC derivatives were the same as those of BCEOC derivatives). The results indicated that fluorescence intensity for individually derivatized amino acid using BCEOC as derivatizing reagent was 2.05–6.51-fold stronger than that using CEOC. This is probably due to the fact that BCEOC has the large molar absorbance, which make it more sensitive for derivatizing amino acids and peptides (BCEOC: $\varepsilon = 2.40 \times 10^4$ L/mol cm (320 nm); CEOC: $\varepsilon = 1.7 \times 10^4$ L/mol cm (249 nm)). The difference in

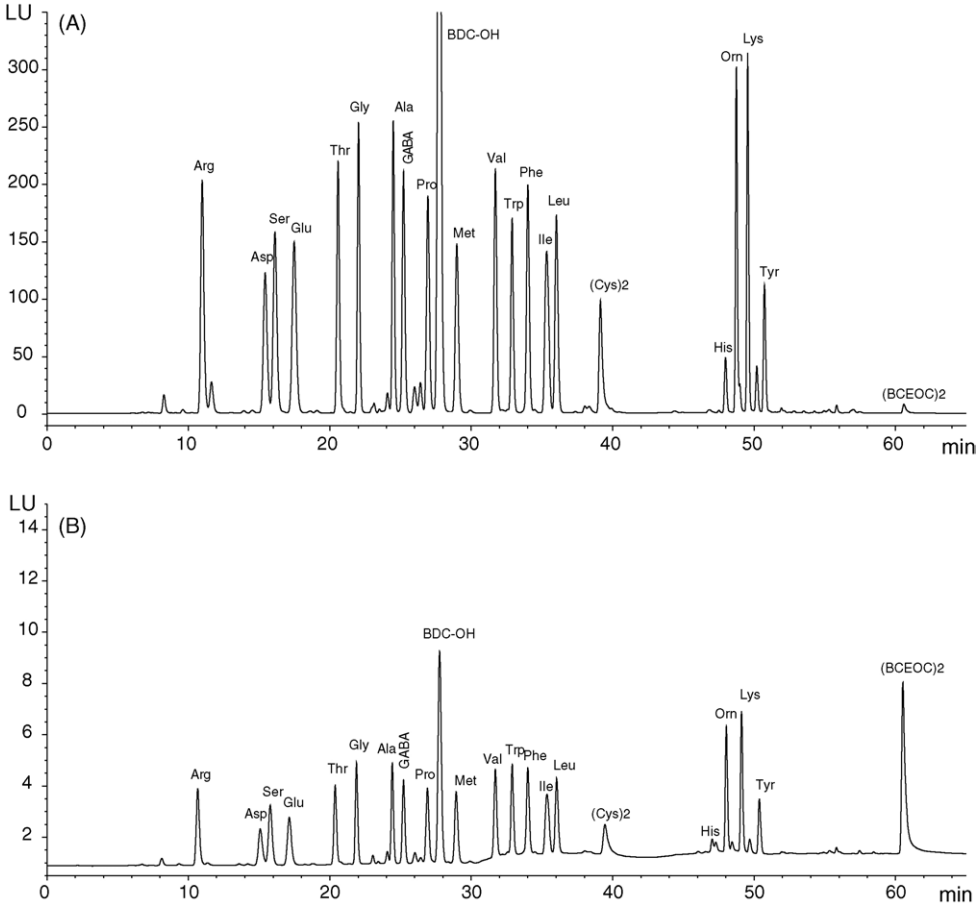

Fig. 2. Optimized chromatography for amino acid standard mixtures using: (A) 50 pmol and (B) 1.0 pmol for injection. Column temperature is set at 36 °C; excitation wavelength λ_{ex} 333 nm, emission wavelength λ_{em} 390 nm; column 200 mm × 4.6 mm Hypersil BDS C_{18} (5 μm); flow rate = 1.0 mL min^{-1}; Arg (arginine); Asp (aspartic acid); Ser (serine); Glu (glutamic acid); Thr (threonine); Gly (glycine); Ala (alanine); GABA (4-amino-butyric acid); Pro (proline); Met (methionine); Val (valine); Phe (β-phenylalanine); Trp (tyrptophan); Ile (iso-leucine); Leu (nor-Leucine); $(Cys)_2$ (cystine); His (histidine); Orn (ornithine); Lys (lysine); Tyr (tyrosine); BDC-OH (1,2-benzo-3,4-dihydrocarbazole-9-ethanol); $(BCEOC)_2$ (bis-(1,2-benzo-3,4-dihydrocarbazole-9-ethyl) carbonate).

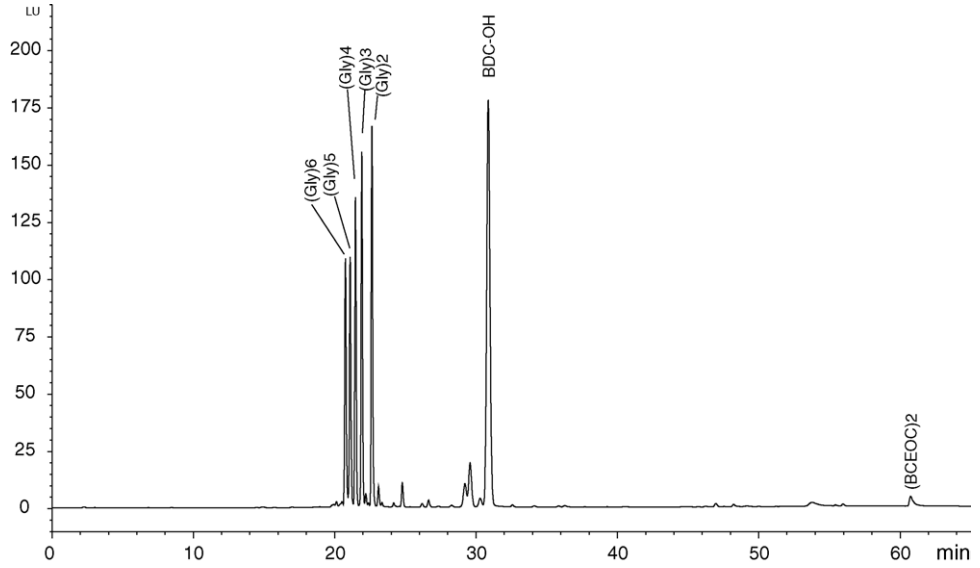

Fig. 3. Chromatogram for 38 pmol of standard peptide derivatives. Column temperature is set at 36 °C; excitation wavelength λ_{ex} 333 nm, emission wavelength λ_{em} 390 nm; column 200 mm × 4.6 mm Hypersil BDS C_{18} (5 μm); flow rate = 1.0 mL/min; chromatographic conditions as Fig. 2.

molar absorbance may be attributed to the BCEOC molecular structure, in which its n–π conjugation system is dramatically augmented due to increasing a 1,2-benzo-functional group that makes it more sensitive than that of CEOC with fluorescence detection.

3.7. Analytical precision, accuracy, reproducibility and recovery

The reproducibility of the method was ascertained by carrying out five assays on the same sample over two days; each solution was injected twice. The values of the standard deviations were between 0.44 and 0.62%. The corresponding coefficients of variation were between 3.2 and 7.0%. In addition, a standard consisting of 52.8 pmol amino acids was prepared to examine the method repeatability. The relative standard deviations (R.S.Ds.) of the peak areas and retention times are from 0.86 (Trp) to 2.26% $(Cys)_2$ and from 0.004 to 0.045%, respectively. Precision and accuracy: six replicates ($n = 6$) at 0.825, 13.2 and 52.8 pmol of amino acids were used to make the low- to high-range concentrations. The mean interday accuracy ranged from 92 to 106% for fluorescence detection with mean %CV < 7.5. The mean interday precision for all standards was <10% of the expected concentration. In two bryophyte plant samples, the known amount of 19 above-mentioned amino acids was added. The samples were derivatized with BCEOC and the analyses were carried out in duplicate. The experimental recoveries are in the range of 92–108%.

3.8. Detection limits and linearity for derivatized amino acids and oligopeptides

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. 2B showed the injection of 1.0 pmol of each derivatization amino acid. Based on this experiment, the calculated detection limits (at a signal-to-noise ratio = 3:1) for each derivatized amino acid were from 6.3 (Lys) to 177.6 (His) fmol. Similarly, 1.0 pmol each derivatized standard peptide mixture was also injected on Hypersil BDS C_{18} column (chromatogram was not shown); the calculated detection limits (at a signal-to-noise of 3:1) were from 26.5 to 34.8 fmol or lower. The linearities were established over a 2046.5-fold concentration range for amino acids and a 1250-fold concentration range for standard peptides, with analysis of serial dilutions of the standard solution ranging from 0.0052 to 5.28 mM for amino acids and 0.0024 to 3.00 mM for standard peptides. All of the amino acids and peptides were found to give linear responses over this range, with correlation coefficients of >0.9999. The linear regression analysis for higher concentrations of amino acids or peptides was not tested as the responses were over linearity range.

3.9. Composition analysis of amino acids

The quantitative analysis of hydrolyzed amino acids from bovine serum albumin by standard curve method is agreement with the internal standard method using γ-aminobutyric acid (GABA) as internal standard compound. Amino acid compositional data for hydrolyzed bovine serum albumin are shown in Table 3. There is an excellent agreement with the actual compositions published in the literature [24]. The data in Table 2 were obtained from ca. 1010 ng (ca. 15 pmol) of hydrolyzed bovine serum albumin. In fact, even with low- or sub-picomole amounts hydrolyzed bovine serum albumin, useful compositional analysis can also be obtained. Error data were calculated according to the procedure described by Strydom et al. [36] and gave the result with an average error of 6.61%. Chromatogram for the analysis of standard hydrolyzed bovine serum albumin is shown in Fig. 4.

Table 3
Composition analysis of hydrolyzed bovine serum albumin (the hydrolyzed sample contained 1010 ng; approximately 5.0% was analyzed by HPLC)

Amino acid	Sample (50.5 ng, 751 fmol)		
	Literature (residues/mol)	Calculated (residues/mol)	Error%
Arg	23	24.62	7.04
Asp	54	57.33	6.16
Ser	28	26.01	7.11
Glu	79	82.04	3.85
Thr	34	31.65	6.91
Gly	16	17.18	7.37
Ala	46	47.47	3.19
Pro	28	27.54	1.64
Met	4	4.46	11.50
Val	36	37.24	3.44
Phe	27	28.89	4.78
Ile	14	16.64	18.85
Leu	61	62.45	2.38
Cys	nd	nd	nd
His	17	15.77	7.23
Lys	59	62.28	5.56
Tyr	19	17.32	8.84
Average error (%)			6.61

nd: Not determined.

3.10. Analysis of samples

The chromatogram for the analysis of free amino acids extracted from pumpkin seeds with fluorescence detection shows in Fig. 5 (chromatogram of extracted sunflower seeds not shown). As can be seen from Fig. 5, a small amount of γ-aminobutyric acid (GABA) from pumpkin and sunflower seeds is observed. The structure identification of γ-aminobutyric acid by LC/MS/MS is shown in Fig. 6. The direct monitoring of GABA is very difficult because it is insensitive to UV–vis spectroscopic and electrochemical methods. γ-Aminobutyric acid is well known as a neurotransmitter that regulates inhibitory neurotransmission in mammalian central nervous system [34]. The contents of GABA from extra-cellular have been determined using liquid chromatography combined with pre-/post-column derivatization as previously reported [35]. In this study, the simultaneous determination of GABA and amino acids can be easily achieved using BCEOC as labeling reagent. Amino acid and GABA compositional data from extracted pumpkin seed,

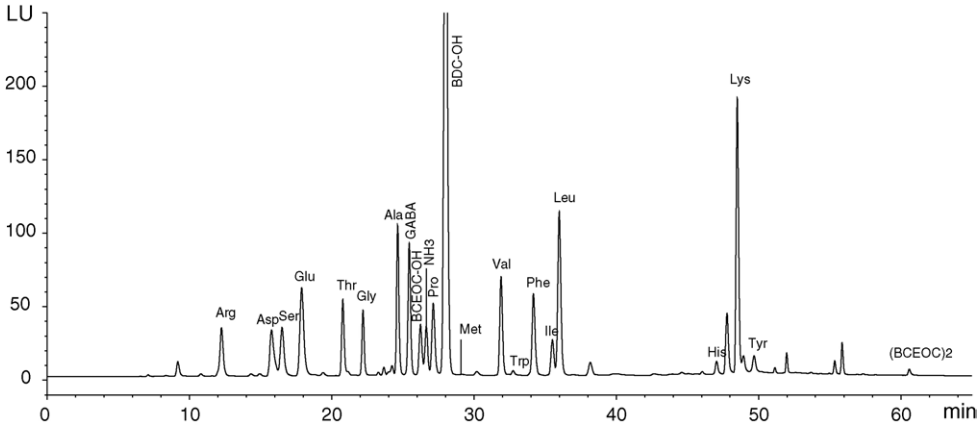

Fig. 4. Chromatographic separation of hydrolyzed amino acids derived from hydrolyzed bovine serum albumin, sample was ~1010 ng (ca. 15 pmol) hydrolyzed, 5% injected (ca. 751 fmol). Chromatographic conditions as Fig. 2; GABA (internal standard).

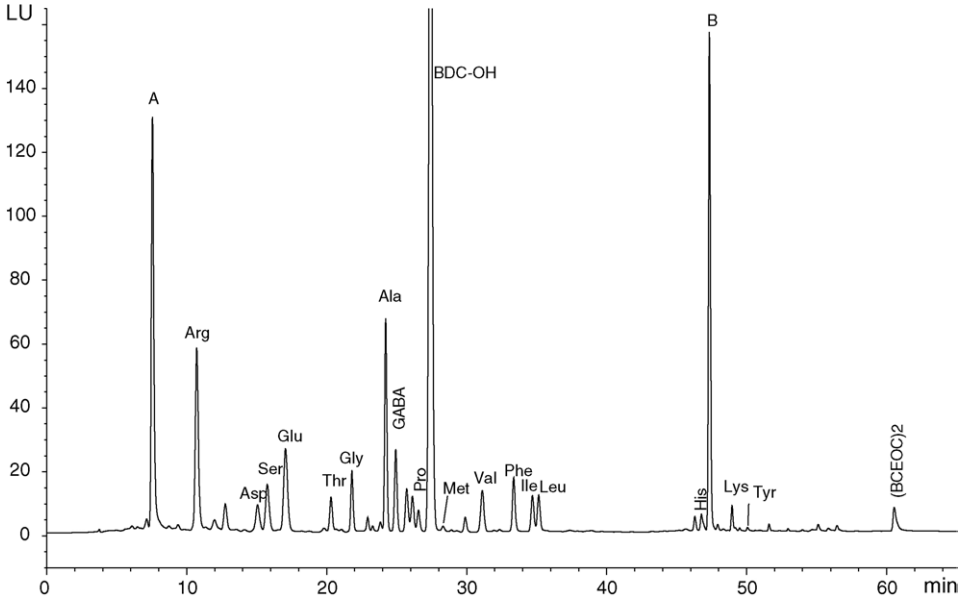

Fig. 5. Chromatogram of amino acids from extracted pumpkin seeds. Column temperature is set at 36 °C; excitation wavelength λ_{ex} 333 nm, emission wavelength λ_{em} 390 nm; column 200 mm × 4.6 mm Hypersil BDS C_{18} (5 μm); flow rate = 1.0 mL/min. Chromatographic conditions as Fig. 2. The structures of A and B were not determined.

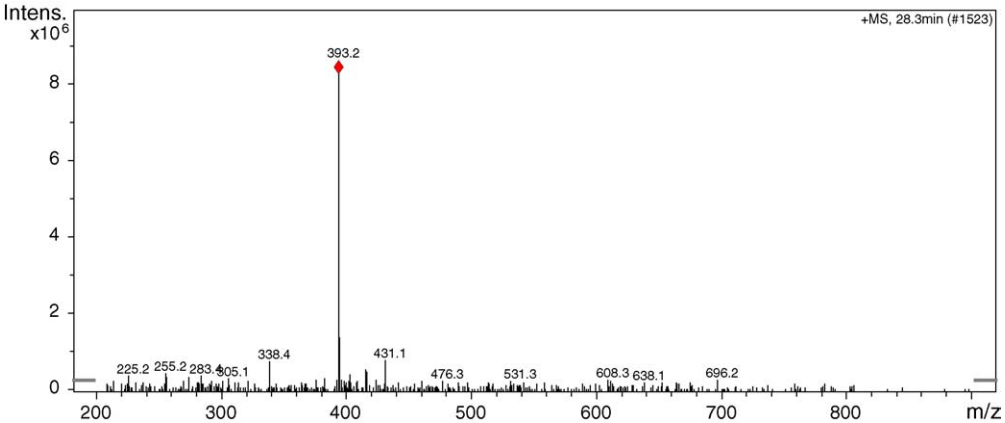

Fig. 6. The profile of molecular ion chromatogram of γ-aminobutyric acid (GABA) and scan range from 200 to 900 amu under ESI positive mode. The extracted solution of pumpkin seeds was derivatized with BCEOC and isolated from a Hypersil C_{18} column and identified by the online mass spectrometer by a split-flow valve with split ratio 3:1.

Table 4
Compositional analysis of free amino acids from extracted pumpkin and sunflower seeds, and from hydrolyzed bryophyte plant

Amino acid	Bryophyte plant (mg/g)		Sunflower seed (μg/g)	Pumpkin seed (μg/g)
	No. 1	No. 2		
Arg	5.23	22.9	167.5	458.7
Asp	38.9	29.4	292.6	107.6
Ser	14.3	9.7	60.5	94.1
Glu	29.5	20.9	153.0	268.9
Thr	14.2	8.4	29.1	58.1
Gly	13.7	9.7	27.2	50.2
Ala	16.7	11.5	138.6	206.7
GABA	1.2	1.3	66.9	108.5
Pro	10.9	7.2	45.6	33.9
Met	6.6	2.8	–	–
Val	10.8	7.2	41.5	70.5
Trp	–	–	–	–
Phe	8.0	6.4	44.1	109.1
Ile	7.0	4.9	36.7	61.9
Leu	–	–	18.2	61.6
Cys	–	–	6.7	6.3
His	–	–	–	185.2
Orn	–	–	–	6.5
Lys	3.6	4.5	21.4	23.3
Tyr	–	1.4	18.3	

sunflower seed and hydrolyzed bryophyte plants are shown in Table 4. As can be seen, the established method is suitable for the determination of these components from extracted plant seeds and hydrolyzed bryophyte plant with satisfactory results.

4. Conclusions

The present paper introduces a new reagent, BCEOC for derivatizing amino acids and peptides with superior properties to currently employed reagents, including rapid, convenient derivatization, excellent sensitivity, stability and derivatization yields. The improved performance of the reagent BCEOC for quantitative analysis of amino acid has been demonstrated in detail. One of the most attractive features of this method exhibits its simpleness for the preparation of amino acid derivatives. The described method shows good correlation in comparison with CEOC, FMOC and AQC methods used for amino compound derivatization. Detection limits are in the femtomole range. Such a developed powerful derivatizing reagent (BCEOC) is likely to find numerous applications in analytical chemistry. Current studies should further explore the derivatization of different amine-containing compounds, such as alkylamines, catecholamines and polyamines. Complete derivatization in basic medium at room temperature takes less than 10 min. The LC separation for the derivatized amino acids showed good repeatability. Reagent and its hydrolysis products did not interfere with the separation by means of the extraction treatment using hexane and ethyl acetate (hexane/ethyl acetate = 9:1–10:1; v/v) as extraction solvent. These characteristics indicated that BCEOC would be another popular and attractive derivatizing reagent for amino compounds following CEOC and FMOC.

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