

# Application of 2-(11*H*-benzo[*a*]carbazol-11-yl) Ethyl Carbonochloridate as a Precolumn Derivatization Reagent of Amino Acid by High Performance Liquid Chromatography with Fluorescence Detection

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**Abstract:** On a reversed-phase Hypersil BDS C18 (200 mm × 4.6 mm, 5 μm) column, 20 amino acids, which were derivatized using 2-(11*H*-benzo[*a*]carbazol-11-yl) ethyl carbonochloridate (BCEC-Cl) as precolumn derivatization reagent, were separated in conjunction with a gradient elution. Optimum derivatization was obtained by reacting amino acids with BCEC-Cl at room temperature for 5 min in the presence of sodium borate catalyst in acetonitrile solvent. The fluorescence excitation and emission wavelengths were 279 and 380 nm, respectively. The identification of amino acid derivatives from hydrolyzed bovine serum albumin and bee pollen was carried out by postcolumn mass spectrometry with electrospray ion (ESI) source in positive ion mode. Linear correlation coefficients of the amino acid derivatives are > 0.9993, and detection limits (at signal to noise of 3:1) are 1.49–19.74 fmol for the labeled amino acids.

**Key Words:** High performance liquid chromatography/mass spectrometry; Fluorescence detection; Precolumn derivatization; Amino acids; Bee pollen; 2-(11*H*-benzo[*a*]carbazol-11-yl) Ethyl carbonochloridate (BCEC-Cl)

## 1 Introduction

Amino acids are important component of living organisms. Most of them show neither natural UV absorption nor fluorescence; thus, their detection at trace levels using absorptiometry is fairly difficult. To increase detection sensitivity, the techniques of fluorescence derivatization are commonly used. Although a number of different types of fluorescent labeling reagents, such as *o*-phthalaldehyde (OPA)<sup>[1–4]</sup>, 7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole NBD-Cl<sup>[5]</sup>, 9-Fluorenyl methylchloroformate FMOC<sup>[6,7]</sup>, 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC)<sup>[8,9]</sup> were developed, numerous shortcomings also were reported in their application. For example, the OPA was only limited to primary amino acids and the detection limit is very high.

NBD-Cl had poor stability, which underwent about 25% decomposition in methanol-water solution when exposed to daylight within 25 min. FMOC reagents were also developed for the derivatization of amino acids and peptides. However, the excess FMOC reagent should be removed by an extraction treatment with pentane as extraction solvent, and sometimes resulted in some loss of their hydrophobic derivatives. 6-Aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) was developed as a popular precolumn derivatization reagent for the determination of amino acids with satisfactory results<sup>[7,8]</sup>. However, only 10% of the fluorescent intensity in aqueous solution compared with that in pure acetonitrile solution was observed for its derivatives. Thus, the detection limits for the early-eluted amino acid derivatives were usually higher than those for the later-eluted amino acid derivatives.

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On the basis of the fluorescence reagent of 2-(9-Carbazole)-ethyl chloroformate CEOC<sup>[10]</sup> and 1,2-benzo-3,4-dihydro-carbazole-9-ethyl chloroformate BCEOC<sup>[11]</sup>, a novel fluorescence labeling reagent, 2-(11*H*-benzo[*a*]carbazol-11-yl) ethyl carbonochloridate (BCEC-Cl), was synthesized as precolumn derivatization reagent and mildly with amino acids in acetonitrile solvent in the presence of sodium borate catalyst for 5 min and yielded stable fluorescent derivatives with derivatization yields close to 100%. The derivatized solution could be injected directly for chromatographic analysis without pretreatment. Separation of the derivatized amino acids exhibited a good baseline resolution in combination with a gradient elution adopting acetonitrile/water on a reversed-phase Hypersil BDS C<sub>18</sub> column. By adopted ESI source ion positive mode and identification by on-line mass, the quick and exact analysis of amino acid of hydrolyzed bovine serum albumin and rape bee pollen was achieved.

## 2 Experimental

### 2.1 Instruments and reagents

Experiments were performed using an LC/MSD-Trap-SL electrospray ion trap liquid chromatography/mass spectrometer (1100 Series LC/MSD Trap). All the HPLC system devices were from the Agilent 1100 series and contained a vacuum degasser, a quaternary pump, a thermostated column compartment, a fluorescence detector (FLD), an autosampler, and an electrospray ionization (ESI). Twenty standard amino acids samples (Sigma, USA), spectroscopic pure acetonitrile (Merck, Germany), HPLC grade acetonitrile purchased from Yucheng Chemical Reagent Co., China, were used in this study. Others like acetic acid were all analytically pure. Formic acid was of analytical grade and was purchased from Shanghai Chemical Reagent Co., China.

Water was purified on a Milli-Q system (Millipore, Bedford, MA, USA). Derivatives were separated on Hypersil BDS C<sub>18</sub> column (200 mm × 4.6 mm × 5 μm, Yilite Co., Dalian, China). The HPLC system was controlled using HP Chemstation software.

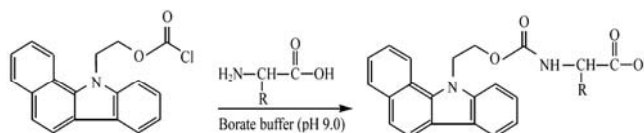
### 2.2 Experimental methods

#### 2.2.1 Preparation of standard solutions

Individual stock solutions ( $1.0 \times 10^{-2}$  M) of amino acids were prepared in water, and if necessary, 6 M HCl or 6 M NaOH was added until the solutions were dissolved. The standard amino acids for HPLC analysis at individual concentrations of  $1.0 \times 10^{-4}$  M were prepared by diluting the corresponding stock solutions ( $1.0 \times 10^{-2}$  M) of each amino acid with borate buffer (0.1 M). The derivatizing reagent solution ( $5.0 \times 10^{-3}$  M) was prepared by dissolving 16.2 mg of 2-(11*H*-benzo[*a*]carbazol-11-yl) ethyl carbonochloridate (BCEC-Cl) in 10 ml of spectroscopic pure acetonitrile. When not in use, all standards were stored at 4 °C.

#### 2.2.2 Derivatization

The BCEC-amino acids derivatization was carried out in water/acetonitrile solution in a basic medium. Fifty microlitre of amino acids was added into a 2.0-ml vial in which 100 μl acetonitrile, 100 μl borate buffer (pH 8.5) of 0.1 M, and 40 μl of BCEC-Cl acetonitrile solution were added in turn. The solution was shaken for 1 min and allowed to stand for 5 min at room temperature. After derivatization, 20 μl of 50% formic acid was added into the solution until the final pH was 5.0–7.0. Finally, the derivatized sample solution was diluted for one time then injected into the HPLC system for analysis. The derivatization chromatogram is shown as follows:



#### 2.2.3 HPLC and MS conditions

HPLC separation of BCEC-Cl derivatives was carried out on Hypersil BDS C<sub>18</sub> column by gradient elution. Mobile phase A was 30% (v/v) acetonitrile containing 35 mmol formic acid buffer (pH 3.7), B was acetonitrile-water 50% (v/v) containing 30 mmol formic acid, and C was acetonitrile-water (95:5, v/v). The flow rate was constant at  $1.0 \text{ ml min}^{-1}$ , and the column temperature was set at 30 °C. The fluorescence excitation and emission wavelengths were 279 nm of  $\lambda_{\text{ex}}$  and 380 nm of  $\lambda_{\text{em}}$ , respectively. The gradient conditions that were used for the separation of amino acids derivatives are shown in Table 1.

Table 1 Gradient elution program

Time (min)	Mobile phase A (%)	Mobile phase B (%)	Mobile phase C (%)	Time (min)	Mobile phase A (%)	Mobile phase B (%)	Mobile phase C (%)
0	55	45	0	40	0	50	50
15	45	55	0	42	0	50	50
17	28	72	0	50	0	40	60
27	0	100	0	55	0	0	100
37	0	80	20	60	0	0	100

Mass spectrometric conditions are as follows: electrospray ionization source (ESI under positive-ion mode), nebulizer pressure, 0.241 MPa, dry gas temperature of 350 °C, dry gas flow of 9.0 l min<sup>-1</sup> and capillary voltage of 3500 V<sup>[12,13]</sup>.

#### 2.2.4 Comparison of ultraviolet and fluorescence absorption

(1) Comparison of ultraviolet and fluorescence absorption. BCEC-Cl was dehydrogenated by chloroanil (tetrachloro-1,4-benzoquinone) on the base of BCEOC-Cl. Compared with these two compounds, the BCEC-Cl core structure exhibited a large conjugation system. BCEC-OH showed five main absorption bands in methanol. The maximum absorption (nm) and molar absorption coefficients in methanol (l M<sup>-1</sup> cm<sup>-1</sup>) were 230 nm (3.80 × 10<sup>4</sup>), 244 nm (3.90 × 10<sup>4</sup>), 253 nm (4.40 × 10<sup>4</sup>), 279 nm (4.60 × 10<sup>4</sup>), and 305 nm (2.60 × 10<sup>4</sup>), respectively. BCEOC-OH displayed four main absorption bands in the ultraviolet region. The maximum absorption (nm) and molar absorption coefficients (l M<sup>-1</sup> cm<sup>-1</sup>) were 215 nm (2.0 × 10<sup>4</sup>), 217 nm (1.95 × 10<sup>4</sup>), 249 nm (1.95 × 10<sup>4</sup>), and 321 nm (2.0 × 10<sup>4</sup>), respectively. The data showed that the absorption band at 321 nm for BCEOC-Cl molecule exhibited a blue shift when a C=C double bond was added. The other corresponding absorption bands were obviously red shift. The absorption band red shifted from 249 nm of BCEOC-OH to 279 nm after dehydrogenation. Molar absorption coefficients augmented 2.36 times. (2) Comparison of fluorescence absorption. The previous study<sup>[11]</sup> showed that the maximum fluorescence excitation and emission wavelength of BCEOC-OH in methanol solution were 333 and 365 nm, respectively. In this study, BCEC-OH showed two maximum fluorescence excitations, 279 and 300 nm, and two maximum fluorescence emissions, 365 and 380 nm. The comparison of the excitation intensity showed that the intensity in 279 nm was about 1.32-fold stronger than that in 300 nm. As for emission intensity, the intensity in 380 nm was about 1.21-fold stronger than that in 365 nm. Hence, λ<sub>ex</sub>/λ<sub>em</sub> = 279/380 were chosen as the excitation and emission wavelengths. Relative sensitivities for fluorescence detection for the individual derivatized amino acid using BCEC-Cl, BCEOC-Cl, and as labeling reagents were investigated. The results indicated that the detection responses of three reagents to 20 amino acids exhibited obvious differences. The results are shown in Table 3. The data showed that the ratio of BCEC-Cl to amines compounds displayed more intensity fluorescence.  $I_{\text{BCEC-Cl}}/I_{\text{BCEOC-Cl}} = 1.46\text{--}2.95$ ; (I, relative fluorescence intensity under the same condition).

#### 2.2.5 Synthesis of 2-(11H-benzo[a]carbazol-11-yl) ethyl carbonochloridate

According to the published report<sup>[14]</sup>, 2-(11H-benzo[a]carbazol-11-yl) ethyl carbonochloridate was synthesized.

### 3 Results and discussion

#### 3.1 Optimum derivatization conditions

The effect of pH on the derivatization reaction was investigated using borate buffer (0.1 M). When pH < 7.0, derivatization reaction did not occur, when it increased, the derivatization showed better results. The maximum derivatization yields were obtained at pH 8.5. At further higher pH values (> 9.0), the doubly substituted amino acids, such as Orn, Lys, and Tyr, exhibited hydrolysis to a certain degree and partially converted to their monosubstituted derivatives. Therefore, all subsequent derivatization reactions were performed at pH 8.5. Though two peaks of tyr, we can conclude them separately.

Derivatization of BCEC-Cl with amino acids could be achieved within 5 min at room temperature. The effect of BCEC-Cl concentrations on the derivatization yields was investigated for amino-acid derivatives. The fluorescence intensity of BCEC-derivatives increased with increasing the amounts of derivatization reagent. The constant fluorescence intensity was obtained with the addition of 2.5-fold to 4-fold molar reagent excess to total molar amino acids. However, increasing the excess of reagent beyond this level had no significant effect on yields. After derivatization, the derivatized solution was neutralized to pH 5.0–7.0 by adding 20 μl acetic acid solution of 50%, as a result of which, degradation of some doubly substituted amino acid derivatives (particularly His) could be effectively avoided.

#### 3.2 Optimum separation conditions

To achieve optimal separation, the choice of pH value of mobile phase A was tested on Hypersil BDS C<sub>18</sub> column. Separation of the derivatized amino acid standards could be accomplished at acidic condition with pH 3.7–3.75. With pH < 3.5, most of the amino acids were separated, with the exception of Asp and Ser (coeluted), BCEC-OH and Met (co-eluted), Ile and Leu (partially separated). In comparison with the acidic conditions (pH 3.7–3.75), operation at pH > 3.8 resulted in an obvious decrease in retention value for most of the amino acid derivatives. At the same time, Asp and Ser, BCEC-OH and Met, Pro and GABA, Ile and Leu were coeluted. 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.

#### 3.3 Chromatographic separation and MS identification

Twenty amino acid derivatives were separated with a good baseline resolution on Hypersil BDS C<sub>18</sub> column in 60 min, the chromatogram were shown in Fig 1.

The identification of amino acid derivatives was carried out by on-line postcolumn mass spectrometry with ESI in positive-ion mode. MS cleavage mode of the representative (Cys)<sub>2</sub>: cystine derivative is shown in Fig.2, and MS data for all amino acid derivatives is shown in Table 2.

### 3.4 Linear regression equations, detection limits, and reproducibility

On the basis of the optimum derivatization conditions, the calibration graphs were drawn with peak area *Y*-axis and amino

acid concentration as *X*-axis (pmol, corresponding injected amount from 100 pmol to 50 fmol). Linear regression equations, correlation coefficients, and detection limits for all amino acid derivatives are shown in Table 2.

All amino acids were found to yield excellent linear responses over this range with correlation coefficients of > 0.9993. The calculated detection limits of each amino acid (at a signal-to-noise ratio of 3:1) were from 1.49 to 19.74 fmol. Under the same chromatographic conditions, a standard solution consisting of 50 pmol of each amino acid was prepared by injecting 6 times for the examination of the reproducibility of the method. The relative standard deviations (RSDs) of the peak areas are less than 0.05% and retention times are less than 1.6%. The results are shown in Table 2.

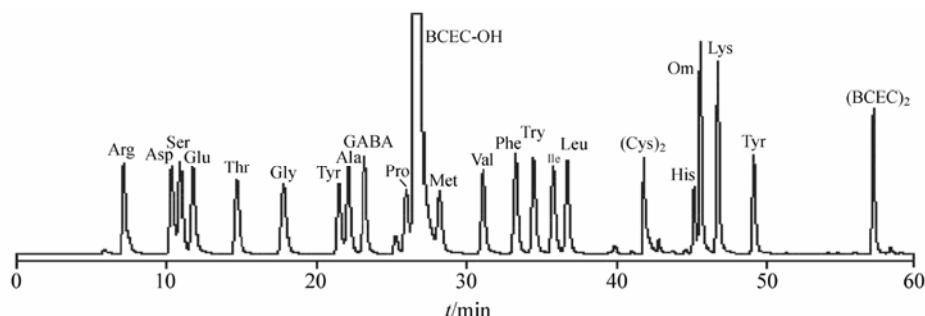


Fig.1 Chromatogram for 50 pmol of amino acid standards derivatized with BCEC-Cl

Hypersil BDS C<sub>18</sub> column (200 mm × 4.6mm × 5 μm, Dalian, China); column temperature is set at 30 °C; flow rate = 1.0 ml min<sup>-1</sup>; excitation wavelength λ<sub>ex</sub> = 279 nm; emission wavelength λ<sub>em</sub> = 380 nm; 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: b-phenylalanine; Try: tryptophan; Ile: isoleucine; Leu: nor-Leucine; (Cys)<sub>2</sub>: cystine; His: histidine; Orn: ornithine; Lys: lysine; Tyr: tyrosine; BCEC-OH: 2-(11H-benzo[a]-carbazol-11-yl)-ethanol), (BCEC)<sub>2</sub>: bis-(2-(11H-benzo[a]carbazol-11-yl)ethyl) chloroformate

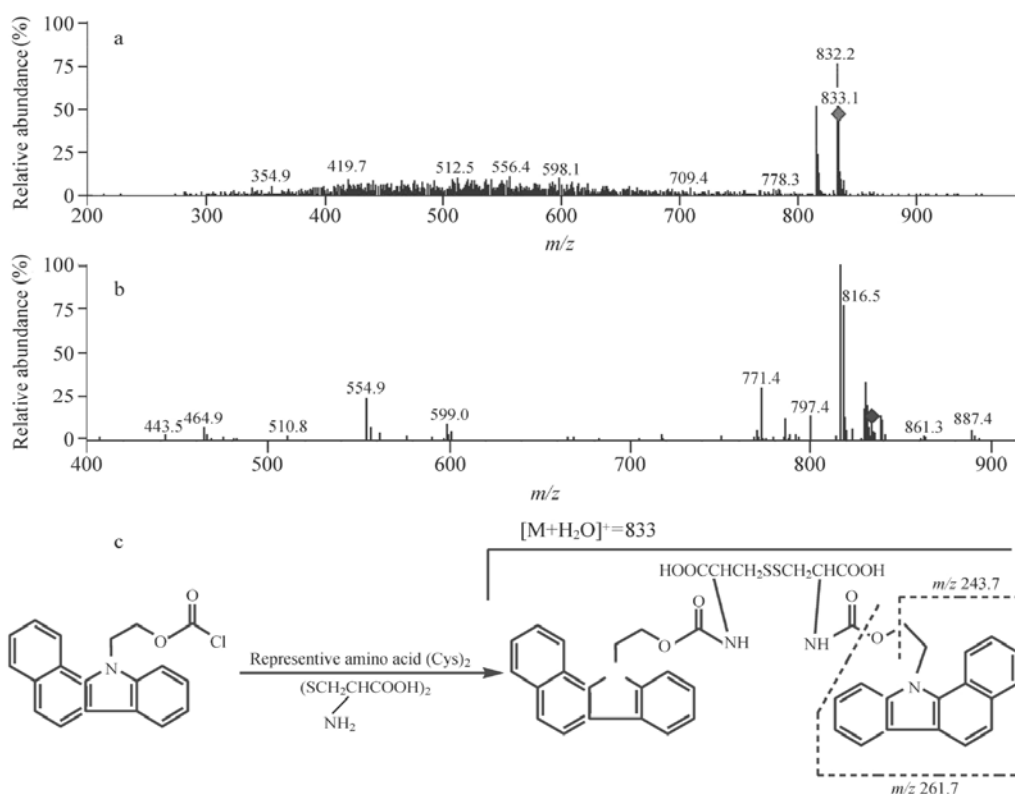


Fig.2 MS spectrum (a), MS/MS spectrum (b), and mass cleavage mode(c) for representative cystine derivatives

Table 2 Linear regression equations, correlation coefficients, detection limits, MS data and reproducibility for retention time and peak area ( $n = 6$ ) of amino acid derivatives

Amino acid	$Y = AX + B$	$R$	Detection limits (fmol)	MS $[M + H]^+$	Retention time RSD (%)	Peak area RSD (%)
Arg	$Y = 67.73X + 27.35$	0.9999	15.0	462.4	0.008	1.22
Asp	$Y = 61.36X + 31.57$	0.9997	7.21	420.1	0.014	1.25
Ser	$Y = 67.24X + 42.21$	0.9996	5.95	393.4	0.012	1.18
Glu	$Y = 70.10X + 25.15$	0.9997	7.35	435.2	0.014	1.34
Thr	$Y = 61.07X + 36.84$	0.9996	8.33	407.2	0.005	1.12
Gly	$Y = 67.45X + 28.42$	0.9998	6.57	363.2	0.006	1.23
Tyr	$Y = 50.16X + 29.43$	0.9997	6.69	469.1	0.008	1.15
Ala	$Y = 66.61X + 35.20$	0.9998	6.29	377.1	0.006	1.31
GABA	$Y = 73.70X + 35.34$	0.9998	1.49	391.1	0.006	1.26
Pro	$Y = 48.90X + 30.91$	0.9996	7.35	403.1	0.005	1.04
Met	$Y = 59.55X - 13.72$	0.9996	15.0	437.1	0.008	1.44
Val	$Y = 66.04X + 37.28$	0.9996	6.05	405.0	0.007	1.12
Try	$Y = 71.32X + 46.02$	0.9995	7.21	492.1	0.005	0.84
Phe	$Y = 69.29X + 61.93$	0.9994	2.68	453.1	0.008	0.95
Ile	$Y = 66.89X + 45.51$	0.9994	4.03	419.0	0.006	1.38
Leu	$Y = 67.83X + 42.51$	0.9994	3.83	419.0	0.007	1.04
(Cys) <sub>2</sub>	$Y = 57.52X + 30.74$	0.9998	19.74	833.0	0.042	1.29
His	$Y = 31.98X + 16.10$	0.9998	11.70	730.3	0.019	1.55
Orn	$Y = 108.41X + 58.52$	0.9997	1.87	724.2	0.012	1.33
Lys	$Y = 105.24X + 45.11$	0.9998	1.56	738.4	0.008	1.44
Tyr	$Y = 58.71X + 33.59$	0.9996	2.34	774.1	0.007	1.46

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

### 3.5 Analysis of practical samples

#### 3.5.1 Hydrolyzation of bovine serum albumin sample

A total of 4.2 mg bovine serum albumin was weighed and put into a 2-ml vial. Then, 1 ml HCl (6 M) was added. The solution mentioned above was kept 110 °C to hydrolyse for 24 h, and then dissolved in 1 ml with 0.1 M borate buffer after dried by nitrogen. The corresponding concentration was 4.2 g l<sup>-1</sup>, and the lower (0.01 g l<sup>-1</sup>) concentration of bovine serum albumin was obtained by diluting the corresponding stock solutions (4.2 g l<sup>-1</sup>).

In hydrolyzed bovine serum albumin sample, a known amount of the amino acids was added. The samples were derivatized with BCEC-Cl, and the analyses were carried out in duplicate. The experimental recoveries are shown in Table 3.

#### 3.5.2 Chromatographic analysis of hydrolyzed bovine serum albumin

According to the optimum experimental conditions described above, the hydrolyzed bovine serum albumin sample was derivatized with BCEC-Cl and analyzed by HPLC. The calculated contents of amino acids from hydrolyzed bovine serum albumin sample are shown in Table 3. The results obtained in the proposed method were in good agreement with the previously reported results<sup>[15]</sup>.

#### 3.5.3 Hydrolysis of amino acids from rape bee pollen samples

Rape bee pollen (50.0 mg) was placed in a 2-ml Vial; 6 M hydrochloric acid (1 ml) was added and the Vial was sealed. After hydrolyzed at 110 °C for 24 h, the contents were evaporated to dryness using a stream of nitrogen. The precipitate was redissolved with 2.0 ml of borate buffer (pH 9.0) and filtered through a 0.2- $\mu$ m nylon membrane filter. Then, another 2-ml borate buffer was added to wash the residual parts. The final solution was made up to 4 ml using borate buffer (pH 9.0) and stored at 4 °C until HPLC analysis. The relative concentration is 12.5 g l<sup>-1</sup>. During derivation, 150 l borate buffer (pH 8.5), 50  $\mu$ l acetonitrile, 10  $\mu$ l extracted solvent, and 90  $\mu$ l reagent ( $5 \times 10^{-3}$  M) were added, and then the solution was derivatized at 40 °C for 5 min. Finally, 20  $\mu$ l acetic acid/water solution (50%, v/v) was added and then diluted by 680  $\mu$ l 80% acetonitrile/water (v/v). Ten microliter of the above solution was injected to analysis, and the results of Xinjiang rape bee pollen and Qinghai rape bee pollen were shown in Table 4.

A sensitive method was developed for the determination of

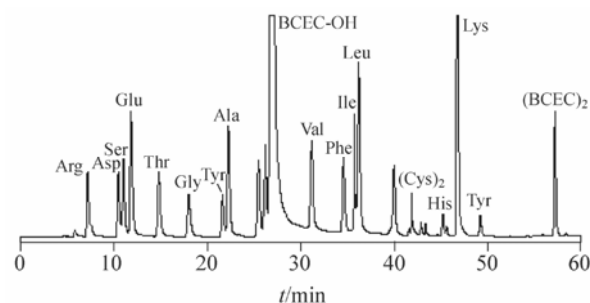


Fig.3 Chromatogram of amino acids from hydrolyzed bovine serum albumin

Table 3 Comparison of fluorescence intensity of BCEC-Cl and BCEOC derivatives, amino acids content and recovery of bovine serum albumin

Amino acids	Ratio of fluorescence intensity of BCEC-Cl/BCEOC derivatives	Recoveries (%)	Content of amino acids from bovine serum albumin			
			Literature values <sup>[15]</sup>		Calculated	
			Content of amino acids (g g <sup>-1</sup> )	Residues (mol)	Content of amino acids (g g <sup>-1</sup> )	Residues (mol)
Arg	1.97	105	0.0644	23	0.0693	24.75
Asp	2.05	102	0.0960	54	0.1012	56.93
Ser	2.07	94	0.0563	28	0.0543	27.00
Glu	2.09	98	0.0234	79	0.0245	82.70
Thr	2.13	96	0.0720	34	0.0710	33.52
Gly	2.10	104	0.0270	16	0.0278	16.47
Ala	3.02	110	0.0789	46	0.0800	46.64
GABA	1.72	106	0.0000	0	0.0000	0.00
Pro	1.46	105	0.0700	28	0.0698	27.92
Met	1.86	102	0.0000	4	0.0000	0.00
Val	1.93	96	0.0698	36	0.0723	37.29
Try	1.50	98	0.0089	0	0.0000	0.00
Phe	2.00	96	0.0961	27	0.1123	31.55
Ile	2.47	102	0.0300	14	0.0371	17.30
Leu	2.00	106	0.1745	61	0.1783	62.36
(Cys) <sub>2</sub>	1.92	98	0.031	0	0.0319	9.63
His	2.54	96	0.1453	17	0.1425	16.67
Orn	1.79	92	0	0	0.0000	0.00
Lys	1.83	97	0.1844	59	0.1952	62.46
Tyr	2.95	96	0.0979	19	0.0996	19.32

Table 4 Contents of amino acids from hydrolyzed Xinjiang Rape bee pollen and Qinghai Rape bee pollen

Amino acid	Xinjiang Rape bee pollen	Qinghai Rape bee pollen	Amino acid	Xinjiang Rape bee pollen	Qinghai Rape bee pollen
Arg	0.00727	0.00645	Val	0.00640	0.00907
Asp	0.01060	0.01410	Try	0.00000	0.00000
Ser	0.00826	0.00797	Phe	0.00489	0.00525
Glu	0.00950	0.01185	Ile	0.00496	0.00502
Thr	0.00583	0.00682	Leu	0.00928	0.00986
Gly	0.00628	0.00646	(Cys) <sub>2</sub>	0.00000	0.00056
Ala	0.00655	0.00714	His	0.00299	0.00389
GABA	0.00034	0.00016	Orn	0.00000	0.00000
Pro	0.01142	0.01217	Lys	0.00985	0.01036
Met	0.00000	0.00045	Tyr	0.00386	0.00427

amino acids using BCEC-Cl as precolumn derivatization reagent. Compared with BCEOC, BCEC-Cl show more sensitivity to amino acids, BCEC-Cl could rapidly and smoothly react with amino acids at room temperature to give sensitive, stable derivatives with derivatization yields close to 100%. The proposed method was sensitive and reproducible for the determination of amino acids from hydrolyzed bovine serum albumin sample with satisfactory results<sup>[15]</sup>. The amino acids in Xinjiang and Qinghai rape bee pollen were analyzed. This method can partly replace some of amino acid analyzer.

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