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Article

Sensitive Determination of Taurine, γ-Aminobutyric Acid and Ornithine in Wolfberry Fruit and Cortex Lycii by HPLC with Fluorescence Detection and Online Mass Spectrometry Identification

Xiangming Chen[1,2,3], Jinmao You[1,4], Yourui Suo[1*] and Baolei Fan[1,3]

[1]Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Xining, China, [2]College of Pharmacy, Binzhou Medical University, Yantai, China, [3]University of Chinese Academy of Sciences, Beijing, China, and [4]The Key Laboratory of Life-Organic Analysis of Shandong Province, Qufu Normal University, Qufu, China

*Author to whom correspondence should be addressed. Email: yrsuo@nwipb.cas.cn

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A new, simple and highly sensitive method for the determination of taurine, γ-aminobutyric acid and ornithine using high-performance liquid chromatography (HPLC) with fluorescence detection is described. Three non-protein amino acids were derivatized by a novel precolumn derivatization reagent 2-[2-(dibenzocarbazol)-ethoxy]ethyl chloroformate before injected. Optimum derivatization was obtained at 40°C for 5 min in the presence of sodium borate buffer (pH 9.0). Derivatives were sufficiently stable to be efficiently analyzed by HPLC without pretreatment. On a reversed-phase Hypersil BDS C8 column, the amino acids were separated in conjunction with a gradient elution with a good baseline resolution. The identification of derivatives was carried out by online postcolumn mass spectrometry with an electrospray ionization source in positive ion mode. Excellent linear responses were observed with the correlation coefficients of >0.9996, and instrument detection limits (at a signal to noise of 3 : 1) were in the range of 0.30–0.33 nmol/L. The proposed method is sensitive and reproducible for the precise determination of the amino acids from wolfberry fruit and cortex lycii samples.

Introduction

Taurine (Tau), γ-aminobutyric acid (GABA) and ornithine (Orn) are three non-protein amino acids that are not incorporated into proteins, and they are found in all kinds of animal and plant tissues and play very important roles at trace levels in the regulation of a variety of physiological and biological functions. Tau is an important nutrient substance for human being, especially for infants; it can obviously promote the growth and development of the nervous system (1, 2). Tau has protective effects against arteriosclerosis (3), oxidant lung injury (4), deleterious effects of various drugs such as gentamicin (5), it also can protect animal's heart from injury (6) and treat diabetic nephropathy (7). GABA is widely distributed in plants and animal bodies. It is one of the most important neurotransmitter in the brain (8). GABA plays important physiological function including activating glucose metabolism in the brain (9) and promoting growth hormone secretion (10). It also can reduce blood pressure (11), prevent arteriosclerosis (12) and delay senescence (13). Orn exists in multiple tissues and cells of organism; it is an intermediate product of arginine's metabolic in liver (14). Orn can stimulate the pituitary to secrete growth hormone and then promote the synthesis of protein and the catabolism of sugar and fat.

Quantitative analysis of these three non-protein amino acids is required in many fields, including biology, agriculture, medicine and so on. Gas chromatography (15, 16) has been used for the determination of amino acids, but this method is very time consuming and requires a good sample manipulation. The most common analytical method for the determination of amino acids is high-performance liquid chromatography (HPLC) (17, 18). However, most amino acids show neither natural absorption in the visible or UV regions nor fluorescence, so it is very difficult to apply spectrometric techniques to the determination of amino acids at trace level. To overcome this difficulty, derivatization of the analytes by fluorescent labeling reagent, which can emit strong fluorescence by laser excitation, has been widely adopted because HPLC with fluorescence detection has higher sensitivity for the trace determination. These labeling reagents commonly include 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) (19, 20), *o*-phthaldialdehyde (OPA) (21, 22), phenyl isothiocyanate ester (23), 9-fluorenyl methyl chloroformate (24, 25),4-(5,6-dimethoxy-2-phthalimidinyl)-2-methoxyphenylsulfonyl chloride (26) and so on. They were widely used for the derivatization and determination of amino acids in food, chemical and biological sciences. A series of disadvantages in their applications has been reported.

In the present work, a novel fluorescence reagent 2-[2-(dibenzocarbazol)-ethoxy]ethyl chloroformate (DBCEC) was utilized as precolumn labeling reagent to develop a method for the determination of Tau, GABA and Orn. DBCEC has similar structure and chemical activity to 2-(9-carbazole) ethyl chloroformate (27), 1,2-benzo-3,4-dihydrocarbazole-9-ethyl chloroformate (BCEOC) (28) and 2-(11H-benzo[a]carbazole-11-yl) ethyl chloroformate (29), so the reaction of DBCEC with three non-protein amino acids was performed according to previously tested conditions with small modification. Optimum derivatization was obtained at 40°C for 5 min in the presence of sodium borate buffer (pH 9.0). Compared with the previous method, DBCEC can react with amino acids under mild conditions in shorter time; derivatives were sufficiently stable to be efficiently analyzed in 25 min. The main advantage of the method is the lower LODs that permit us to determine lower content than methods involving other reagents such as AQC and OPA. The established method has been successfully applied to the analysis of three non-protein amino acids in wolfberry fruit and cortex lycii. Wolfberry fruit is the fruit of *Lycium chinense*, and cortex lycii is the root bark of *Lycium chinense*. Both traditional Chinese

medicines that can regulate blood sugar level and reduce blood pression. Wolfberry fruit can also protect liver and promote the regeneration of liver cells.

Experimental

Materials and reagents

Three non-protein amino acid standards including Tau, GABA and Orn were purchased from Sigma Corporation. HPLC grade acetonitrile (CH_3CN) was purchased from Yucheng Chemical Reagent Co., Ltd (Shandong Province, China). Other reagents such as phosphoric acid and triethylamine were all analytically pure. Pure water was prepared by Milli-Q super pure water system. DBCEC was synthesized in You's Laboratory (30). Wolfberry fruit and cortex lycii were collected from Qaidam Basin (Qinghai, China).

Instrumentation

Experiments were carried out using Agilent 1100 series HPLC/mass spectrometry. The HPLC system was equipped with an online vacuum degasser (model G1322A), a quaternary pump (model G1311A), an autosampler (model G1329A), a thermostatted column compartment (model G1316A) and a fluorescence detector (model G1321A). Derivatives were separated on a Hypersil BDS C_8 column (200 × 4.6 mm, 5 μm; Dalian Elite Analytical Instruments Co., Ltd, Dalian, China). The mass spectrometer (MSD Trap-SL, model G2445D) from Bruker Daltonik (Bremen, Germany) consisted of an electrospray ionization (ESI) source in positive ion mode. The HPLC–MS system was controlled by Agilent ChemStation software (version B.01.01).

Preparation of standard solutions

The individual standard stock solutions (1.0×10^{-2} mol/L) of three non-protein amino acids were prepared in water. The mixed standard solutions for HPLC analysis were prepared by diluting the stock solutions with water. The DBCEC solution (5.0×10^{-2} mol/L) was prepared by dissolving 186.8 mg DBCEC in 10 mL acetonitrile. The corresponding low concentration of derivatization reagent solution (1.0×10^{-3} mol/L) was obtained by diluting the stock solution with acetonitrile. All solutions were stored at 4°C in a refrigerator until HPLC analysis.

Derivatization procedure

In a 2.0-mL vial, 100 μL mixed standard non-protein amino acids solution or 100 μL extraction solution from samples, 200 μL 0.2 mol/L sodium borate buffer (pH 9.0) and 210 μL DBCEC were added. The vial was bathed in water at 40°C for 5 min after sealing. Then, 100 μL 33% acetic acid and 390 μL acetonitrile were added to neutralize and dilute the solution. Finally, a 10 μL volume of diluted solution was injected directly into the chromatography system. The derivatization scheme of typical GABA with DBCEC is shown in Figure 1.

Extraction of non-protein amino acids from wolfberry fruit and cortex lycii samples

In a 50-mL round-bottom flask, 2.0 g of the powdered sample (wolfberry fruit or cortex lycii) and 10 mL of water were added. The flask was then immersed in an ultrasonic water bath, and the sample was sonicated for 30 min. The sample was allowed to incubate at room temperature for 30 min. The water was filtered and transferred into a 25-mL glass volumetric flask. The residue was extracted again according to the above operation. The resulting water layer was collected and transferred into the same volumetric flask. Then, water was added into the volumetric flask to graduation line. The extracted solution was stored at 4°C until HPLC analysis.

HPLC and MS conditions

Non-protein amine acid derivatives were separated on a reversed-phase Hypersil BDS C8 column (200 × 4.6 mm i.d., 5 μm) in conjunction with a gradient elution. Eluent A was water that contained 0.2% triethylamine (adjust pH to 4.0 with H_3PO_4), B was 100% acetonitrile and gradient conditions are initial = 60% A + 40% B and 30 min = 100% B (kept for 10 min). The flow rate was constant at 1.0 mL/min, and the column temperature was set at 30°C. The fluorescence excitation and emission wavelengths were set at $\lambda_{ex} = 300$ nm and $\lambda_{em} = 395$ nm. Chromatographic peaks were identified by spiking the working standard with each individual non-protein amine acid in turn and simultaneously confirmed by mass spectrometry. Ion source conditions are as follows: ESI in positive ion detection mode, nebulizer pressure 35 psi, dry gas temperature 350°C, dry gas 9.0 L/min and capillary voltage 3,500 V.

Quantification

Quantitative analysis was carried out by a series of injections of target compounds in the concentration range from 32 fmol to 100 pmol. A calibration curve was constructed for each compound by plotting peak area versus concentration, and all target

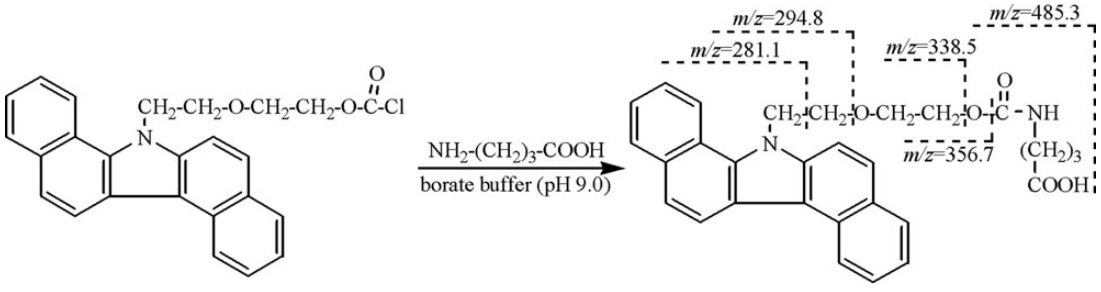

Figure 1. Derivatization scheme of DBCEC with GABA and cleavage mode for DBCEC–GABA derivative.

compounds from the extracted sample were measured using the external standard curve.

Results

Optimization for derivatization

To obtain the highest yield for the derivatization of three non-protein amino acids with DBCEC, the optimal conditions including buffer pH, reaction time and concentration reagent were investigated. Borate buffer was used as basic catalyst for the derivatization reaction according to a previous study. Various buffers (pH 8.0–11.0) were tested to investigate the effect of buffer pH on the derivatization action. The result showed that the derivatization yields were highest in the pH range of 9.0–9.5. In more basic solution (pH > 10.0), the decreased responses and more by-product peaks were observed. At lower pH values (pH < 9.0), the three amino acid derivatives showed a significant loss of peak height relative to those achieved in buffer of pH 9.0 or 9.5. Therefore, 0.2 mol/L borate buffer solution at pH 9.0 was applied in all the subsequent experiments.

The effects of DBCEC concentrations on derivatization were investigated in detail to ensure the sufficient reaction of the three amino acids. The results indicated that constant fluorescence intensity was obtained when 7-fold molar excess to total molar amino acids, further increasing DBCEC concentration beyond this level had no significant effect on fluorescence intensity. Furthermore, more DBCEC would generate more 2-[2-(dibenzocarbazole)-ethoxy]ethanol (the major by-product of the derivatization process) and other by-products that may interfere with the separation of amino acids derivatives. Therefore, 7-fold molar reagent excess was chosen and the derivatization action was completed in 10 min.

Chromatographic separation and MS identification

The baseline separation for three non-protein amino acid derivatives was achieved on a Hypersil BDS C8 column under the gradient elution as described in the 'Experimental' section, and the corresponding chromatograms are shown in Figure 2. To get the optimal separation, different mobile phase pH values were tested with buffers in the range of 3.0–6.0. The results showed that lower pH would increase the retention time and improve peak shape; however, lower pH would damage the stationary phase of a chromatographic column. Therefore, 4.0 was chosen as the mobile phase pH.

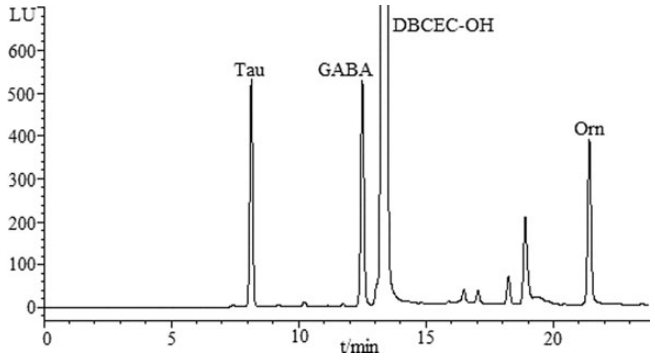

Figure 2. Chromatogram for standard non-protein amino acids derivatized with DBCEC. Peak: Tau, taurine; GABA, γ-aminobutyric acid; Orn, ornithine; DBCEC-OH, 2-[2-(dibenzocarbazole)-ethoxy]ethanol.

The structural identification of the three non-protein amino acid derivatives was carried out by online MS with an ESI source in positive ion mode. The MS and MS/MS spectra are shown in Figure 3, and the cleavage mode of representative GABA derivative is shown in Figure 1. The molecular ions of derivatives are listed in Table I. All derivatives showed intense molecular ion peaks at $[M + H]^+$ ions. The collision-induced dissociation spectra (MS/MS) of molecular ions produced intense and stable fragment ions at m/z 281.1,294.8, 338.5 and 356.7 (Figure 3b), which were specific fragment ions for DBCEC-labeled non-protein amino acid derivatives.

Linearity, detection limits and quantification limits

The linearity was established by the analysis of three non-protein amino acid standards derivatized according to the procedure described previously. The injected amount varied from 0.32 to 100 μmol/L. The linear regression equations are shown in Table I. All these three non-protein amino acid derivatives gave excellent linear response over this range, with a correlation coefficient of >0.9996. The calculated instrument detection limit (IDL) with fluorescence detection (at a signal-to-noise ratio of 3 : 1) was <0.33 nmol/L. The method detection limits (MDLs) for wolfberry fruit and cortex lycii samples were <0.81 and <0.95, respectively, and the method quantification limits (MQLs) for them were <2.7 and <3.2, respectively (see Table II). DBCEC provides lower detection limit values than other derivatizing agents used in UV or fluorescence detection, such as AQC and OPA (see Table II).

Analytical precision and recovery

To investigate instrumental precision, one derivatized standard solution including 1 pmol of three amino acids was analyzed six times. The relative standard deviation of peak area was in the range of 2.3–3.7% (see Table III). The analytical precision of the method was calculated from injections of a real sample that had been extracted and analyzed six times over 3 days and six times on 1 day. The interday precision was in the range of 4.2–5.7% for wolfberry fruit and cortex lycii samples, and the intraday precision was in the range of 2.8–3.9%. The results demonstrated that the assay was reproducible and reliable for the quantification of the three non-protein amino acids in samples.

The recovery experiments were carried out with real samples by spiking the same level amino acid standards into two samples. The extraction and derivatization for two samples were the same as described previously. The analyses were carried out in triplicate. The recoveries were calculated based on the formula $(x_1 - x_0)/c \times 100$, where x_0 is the concentration of three non-protein amino acids in the samples, x_1 is the measured concentration obtained from the extracted solutions spiked standards and c is the added known concentration to the sample from which the contents of three non-protein amino acids had been determined. The experimental recoveries obtained were ranged from 92.1 to 95.6% for wolfberry fruit and 96.1 to 102% for cortex lycii (Table IV).

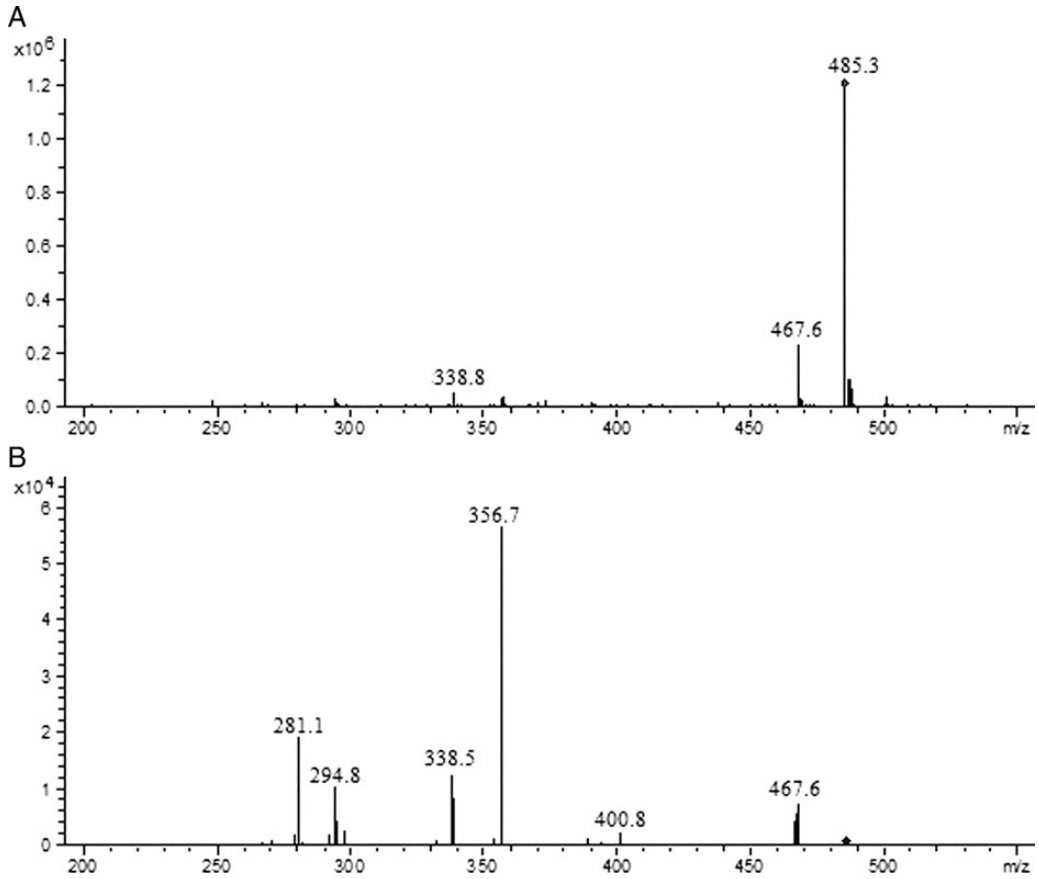

Figure 3. MS (A) and MS/MS (B) for the representative DBCEC–GABA derivative.

Table I
Linear Regression Equations, Correlation Coefficients, Linearity Range and Mass Spectral Data for Three Non-Protein Amino Acid Derivatives

Non-protein amino acids	$Y = AX + B$	R	Linearity range (μmol/L)	MS data
Tau	$Y = 66.7X + 0.15$	0.9999	0.32–100	507.2
GABA	$Y = 67.2X - 3.77$	0.9999	0.32–100	485.3
Orn	$Y = 90.4X - 5.74$	0.9997	0.32–100	894.1

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

Table II
Detection Limits and Quantification Limits for Three Non-Protein Amino Acid Derivatives (nmol/L)

Non-protein amino acids	IDL	Wolfberry fruit		Cortex lycii		Detection limits in the literature	
		MDL	MQL	MDL	MQL	IDL for AQC (19)	MDL for OPA (21)
Tau	0.30	0.71	2.4	0.77	2.6	9.36	1.4
GABA	0.33	0.68	2.3	0.74	2.5	22.24	2
Orn	0.32	0.81	2.7	0.95	3.2	18.67	NA

NA, not available.

Table III
The Precision of Three Non-Protein Amino Acid Derivatives

Non-protein amino acids	Instrumental precision	Intraday ($n = 6$)		Interday ($n = 6$)	
		Wolfberry fruit	Cortex lycii	Wolfberry fruit	Cortex lycii
Tau	2.3	2.8	3.1	4.4	4.2
GABA	3.7	3.5	3.9	4.8	5.7
Orn	3.5	3.1	3.6	5.2	5.0

Advantage of the Method

The main merits of this method include a simple derivatization procedure, a mild reaction condition and a lower detection limit than AQC and OPA (Table V). Amino acids can completely react with DBCEC at 40°C in 5 min. The reaction temperature and time of DBCEC were lower and shorter than those of AQC with amino acids. Derivatization of OPA with amino acids can be performed under the condition of room temperature and shorter time; however, OPA derivatization solution must be freshly prepared every 9 days (22), because OPA was not stable enough. In this work, the derivatization solution could be analyzed directly without being treated. Three derivatives were separated in conjunction with a gradient elution and determined by fluorescence and MS detection. MS data can afford information of molecular structure, so the qualitative analysis of every derivative was more credible by MS detection than by retention time. The detection limits (at a signal-to-noise ratio of 3 : 1) of amino acid derivatized by DBCEC are lower than by AQC or OPA. As shown in Table II, the IDLs for Tau, GABA and Orn derivatized by DBCEC

Table IV
Concentration of Three Non-Protein Amino Acids in Samples and Recoveries (μg/g)

Non-protein amino acids	Wolfberry fruit	Added amount	Found	Recovery (%)	Cortex lycii	Added amount	Found	Recovery (%)
Tau	11.3	10	20.5	92.1	17.5	20	36.7	96.1
GABA	24.1	25	48.0	95.6	29.4	30	58.5	97.2
Orn	3.21	5.0	7.93	94.4	1.62	1.5	3.15	102

Table V
Comparison of the Proposed Method with Reported Methods for Amino Acids

Labeling reagents	Analytes	Method	Derivatization conditions	Injection volume (μL)	Detection limits (nmol/L)
AQC (19)	27 amino acids (including Tau, GABA and Orn)	HPLC–FLC/UV; $\lambda_{ex} = 250$ nm, $\lambda_{em} = 395$ nm, $\lambda_{UV} = 248$ nm	55°C, 10 min	10	3.00–34.44
OPA (21)	Aspartate, glutamate, Tau and GABA	HPLC–FLC; $\lambda_{ex} = 340$ nm, $\lambda_{em} = 455$ nm	Room temperature, 1.5 min	20	1.4–3.2
DBCEC (this work)	Tau, GABA and Orn	HPLC–FLC-MS; $\lambda_{ex} = 300$ nm, $\lambda_{em} = 395$ nm	40°C, 5 min	10	0.68–0.95

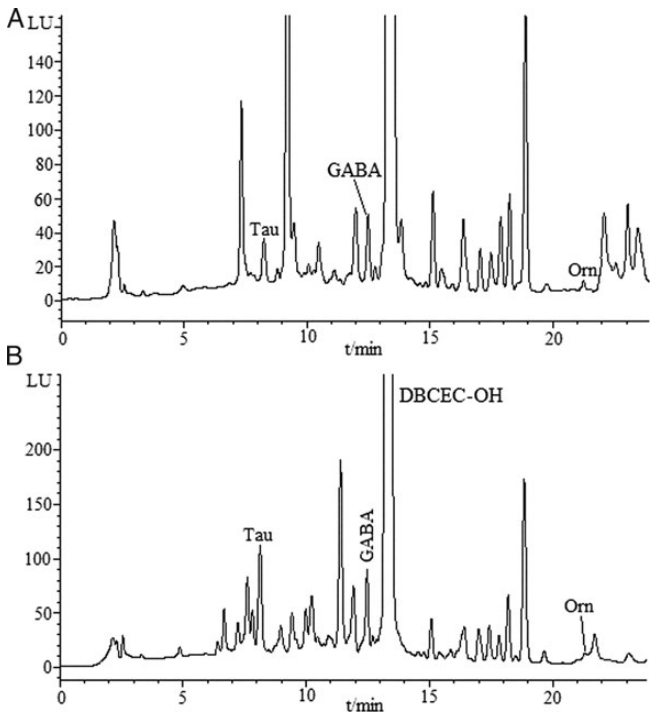

Figure 4. Chromatogram of three non-protein amino acids from wolfberry fruit (A) and cortex lycii (B) samples.

are 0.30, 0.33 and 0.32 nmol/L, respectively, and MDLs are in the range of 0.68–0.95 nmol/L. For AQC, IDLs are 9.36, 22.24 and 18.67 nmol/L, respectively (19). For OPA, MDLs are 1.4 (Tau) and 2 (GABA) nmol/L (21). Therefore, the lower detection limits of DBCEC permit us to determine lower content than the methods involving other reagents such as AQC and OPA.

Application

The developed method was utilized to analyze the three non-protein amino acids in wolfberry fruit and cortex lycii samples. The obtained chromatograms are shown in Figure 4. The compositional data of three non-protein amino acid derivatives obtained from wolfberry fruit and cortex lycii samples are shown in Table IV. It is clear that the established method is suitable for the determination of these components from wolfberry fruit and cortex lycii samples with satisfactory results, and other amino acids in samples did not interfere with the separation of the three non-protein amino acids. The results indicated that three non-protein amino acids were found in both samples, and the content in the cortex lycii sample was higher than that in the wolfberry fruit sample. In this study, we have described the pharmacological effects of three non-protein amino acids, and further studies are needed to investigate the relationship between these ingredients and pharmacological effects of wolfberry fruit and cortex lycii.

Discussion

A common problem in HPLC analysis is peak tailing, which is usually caused by the intermolecular attraction between the acidic silanol in the stationary phase's structure and a positively charged analyte (31). This type of tailing can be found when the analyte contains an amine group. Although the amine group of amino acid was derivatized by DBCEC in this work, it can still be retained by the stationary phase. To overcome this obstacle, 0.2% triethylamine was added into the mobile phase. The amine group of triethylamine in mobile phase would occupy the silanol in the stationary phase's structure, which could decrease the intermolecular attraction between the silanol and analyte, and then tailing would be improved. Another factor that causes tailing is the ionization of the carboxyl group. To inhibit the ionization of the carboxyl group of amine acid, phosphoric acid was added into the mobile phase to adjust the pH of mobile phase to <7.0. Different mobile phase pH values were tested with buffers in the range of 3.0–6.0. The results showed that when the pH of a mobile phase was 4.0, the peak shape of the analyte was satisfactory.

Conclusion

A simple and highly sensitive method to determine three non-protein amino acids was developed utilizing DBCEC as the pre-column derivatization reagent followed by HPLC with fluorescence detection and online mass spectrometric identification. The derivatization reaction was carried out under mild

conditions. The described method shows good correlation and low detection limits. We applied the method to analyze the three non-protein amino acids from wolfberry fruit and cortex lycii samples with satisfied results. Current studies will further explore the derivatization of other amino acids and other amino compounds.

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