LC-Fluorescence Detection Analysis of Amino Acids from *Stellera chamaejasme* L. Using 2-[2-(Dibenzocarbazole)-ethoxy] Ethyl Chloroformate as Labeling Reagent

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Abstract

A pre-column derivatization method for the sensitive determination of amino acids using the tagging reagent 2-[2-(dibenzocarbazole)-ethoxy] ethyl chloroformate (DBCEC) followed by liquid chromatography with fluorescence detection has been developed. Identification of DBCEC-amino acids derivatives was by liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS–MS). DBCEC can easily and quickly label amino acids, and derivatives are stable enough to be efficiently analyzed by LC. Separation of the derivatized amino acids had been optimized on Hypersil BDS C_{18} column. A perfect baseline separation for 20 amino acid derivatives was achieved with a ternary gradient elution program. The chromophore of dibenzocarbazole group, which comprise a large rigid planar structure with p–π conjugation system, resulted in a sensitive fluorescence detection for amino acid derivatives. The derivatized amino acids were detected with fluorescence detector with excitation maximum and emission maximum at 300 and 390 nm, respectively. Excellent linear responses were observed with coefficients of >0.9993, and detection limits were in the range of 0.78–5.13 fmol (signal-to-noise ratio of 3). The mean accuracy ranged from 83.4 to 98.7% for fluorescence detection. The mean inter-day precision for all standards was $<4.2\%$ of the expected concentration. Therefore, the proposed method was a highly sensitive and specific method for the quantitative analysis of amino acids from biological and natural environmental samples.

Keywords

Column liquid chromatography
Fluorescence detection
Electrospray mass spectrometry
Amino acids
Stellera chamaejasme L.

Introduction

Stellera chamaejasme L. plants, widespread in the east of Tibet in China and belonging to the family of thymelaeaceae, is a well-recognized traditional Chinese herbal medicine. It has long been used for the treatment of hydrothorax, ascites, carbuncles, cough, scrofula, bacteriosis, pulmonary tuberculosis, pneumonia and neurasthenia etc. [1]. Recently, *Stellera chamaejasme* L. plants have been found to posses obvious antitumor and antiviral activities, and may be useful in the treatment of malignant disease [2, 3]. Although many chemical components have been elucidated in the root of *Stellera chamaejasme* L. plants, such as lipophilic terpenoids, flavonoids, phenolic compounds and steroids [1, 2, 4–6], the literatures on the multiple components of amino acids in *Stellera chamaejasme* L. plants have not been elucidated in detail.

Amino acids are not only the basic structural units of proteins; they are also a source of energy and serve as precursors for the biosynthesis of neurotransmitters, porphyrins, polyamines, and nitric oxide. Further, glutamate and γ-aminobutyric acid play important roles as neurotransmitters, and carnitine is used in intracellular lipid transport.

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Quantitative analysis of amino acids is required in several fields, including clinical diagnostics of inborn errors of amino acid metabolism, biomedical research, bio-engineering, and food science. Different methods for analysis of amino acids have been developed and commercialized, such as amino acid automatic analyzer, AccQ-Tag and so on. Still, efforts to improve existing methodology with regard to robustness, reproducibility, and sensitivity are ongoing. Pre-column derivatization in combination with reversed phase LC is today the most used technique for the determination of amino acids. Most amino acids show neither natural ultraviolet absorption nor fluorescence. Therefore, chemical derivatization is necessary to increase detection sensitivity and improve selectivity by means of LC [7, 8] and CE [9, 10] separation. Although a number of different types of fluorescent tagging reagents [11–15] have been developed, a variety of shortcomings in their applications have also been reported. For example, the *o*-phthaldialdehyde (OPA) method offers greater sensitivity [16, 17], but is only limited to primary amino acids. 6-Aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) has also been developed as a popular pre-column derivatization reagent for the determination of amino acids with satisfactory results [18, 19]. 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 the later ones [18]. 9-Fluorenyl methylchloroformate (FMOC) is well known as a blocking functional group in the peptide synthesis, and have also been used for the derivatization of amino acids and peptides for LC analysis [20–22]. FMOC is not optimum regarding to chromophoric properties for quantitative spectrophotometric determination, whereas novel labeling reagents can be designed by replacing the fluorenyl with other excellent fluorescent chromophore. The combination of the active functional group of chloroformate together with a strong absorption chromophore moiety will result in an attractive reagent. Along this way, we have synthesized CEOC, BCEOC and BCEC, and their applications for the analysis of amino acids were also developed with LC-FLD [23–25]. More recently, 2-[2-(dibenzocarbazole)-ethoxy] ethyl chloroformate (DBCEC), a reagent with dibenzocarbazole as fluorescent core structure, has been synthesized and used to analyze aliphatic amines in soil samples [26]. And it has been demonstrated that the fluorescent response of DBCEC-amine derivatives were stronger than those obtained with CEOC, BECOC and BCEC. In this work, using DBCEC as pre-column labeling reagent, a method for the sensitive determination of amino acids by LC with fluorescence detection has been developed. The optimal derivatization conditions such as buffer pH, reaction time and the concentration reagent were investigated. Linearity, detection limits and precision of the procedure are also determined. At the same time, applications for the determination of amino acids from *Stellera chamaejasme* L. have also been reported. The suitability of the developed method for the analysis of actual samples is satisfactory.

Experimental

Instruments and Chemicals

Experiments were performed using Agilent 1100 Series LC/MSD Trap SL (Agilent Technologies, CA, USA). All the LC system devices 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). The mass spectrometer (model G2445D) was equipped with an electrospray ionization (ESI) source (model G1948A) The LC system was controlled by HP Chemstation software. The mass spectrometer system was controlled by Esquire-LC NT software, version 4.1.

All amino acid standards were from Sigma Co. (St. Louis, MO, USA). LC grade acetonitrile (CH_3CN) was 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). DBCEC were synthesized in authors' laboratory as described previously [26]. Borate buffer was prepared from 0.2 mol L^{-1} boric acid solution adjusted to pH 9.0 with 4 M sodium hydroxide solution. The quenching reagent was 36% acetic acid solution.

Preparation of Standard Solutions

The derivatizing reagent solution 1.0×10^{-3} mol L^{-1} was prepared by dissolving 3.26 mg DBCEC in 10 mL of anhydrous acetonitrile prepared by distilling the dried LC grade acetonitrile with P_2O_5. Individual stock solutions of the amino acids were prepared in water, and if necessary, HCl or NaOH were added until the compound dissolved. The standard amino acids for LC analysis at individual concentrations of 5.0×10^{-5} mol L^{-1} were prepared by diluting the corresponding stock solutions (1.0×10^{-3} mol L^{-1}) of each amino acid with 0.2 M borate buffer (pH 9.0). When not in use, all standards were stored at 4 °C in a refrigerator.

Preparation for Real Samples

Stellera chamaejasme L. plant samples were from the county around Qinghai Lake (Qinghai, China), and identified by Professor Changfan Zhou (Northwest Plateau Institute of Biology, Chinese Academy of Science, Xining, China). After transport to the laboratory, roots and leaves of *Stellera chamaejasme* L. were separated from each other. Leaves and roots were individually washed with tap water then deionized water, and dried at 50 °C until constant weight. All samples were then powdered with a stainless-steel mill.

Extraction of Free Amino Acids from Stellera chamaejasme L.

To a solution containing 80 mL of ethanol–water (60:40, *v*/*v*) in a 250-mL round-bottom flask, 6.0 g powdered leaves or roots were added, then the contents of the flask rapidly heated to reflux with magnetic stirring for 30 min under a nitrogen atmosphere. After cooling, the water was filtered and transferred to another 250-mL round-bottom flask. The residue was extracted twice with 60 mL ethanol–water solution. 3 mL of hydrochloric acid (3.0 mol L^{-1}) was added to the combined ethanol–water solution. The mixture was concentrated in vacuum to dryness. The residue was re-dissolved with 0.2 mol L^{-1} borate buffer (pH 9.0) to a total volume of 50 mL and stored at 4 °C until analysis.

Hydrolysis of Stellera chamaejasme L.

The powdered leaves or roots (13.5 mg) were placed in a 50 mm × 6 mm test tube; 500 μL 6 mol L^{-1} 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 (Alltech, Deerfiled, IL, USA). 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 LC analysis.

Derivatization Procedure

The DBCEC-amino acids derivatization proceeded in a water/acetonitrile (≈1:1, *v*/*v*) solution in a basic medium. The aqueous amino acids mixture (20 μL) was added in a vial, to which 200 μL of 0.2 mol L^{-1} borate buffer (pH 9.0) and 200 μL of DBCEC acetonitrile solution were added. The solution was shaken for 1 min and allowed to stand at room temperature for 10 min. After derivatization, 50 μL of 36% acetic acid solution was added into the mixture until the final pH ranging in 6.0–6.5. Then the derivatized sample solution was directly injected into the LC system for analysis.

Table 1. Gradient elution program for separation amino acids derivatives on Hypersil BDS C_{18} column

Time (min)	A (%)[a]	B (%)[b]	C (%)[c]
0	30	70	0
15	0	100	0
20	0	95	5
30	0	95	5
40	0	65	35
45	0	35	65
55	0	30	70
60	0	0	100

[a] Eluent A was 30% aqueous acetonitrile solution (H_2O/CH_3CN, 70/30, *v*/*v*) consisting of 30 mmol L^{-1} formic acid buffer (pH 3.5)
[b] Eluent B was 50% aqueous acetonitrile solution (H_2O/CH_3CN, 50/50, *v*/*v*) consisting of 30 mmol L^{-1} formic acid buffer (pH 3.5)
[c] Eluent C was 95% aqueous acetonitrile solution (H_2O/CH_3CN, 5/95, *v*/*v*)

LC-ESI-MS Conditions

DBCEC-amino acid derivatives were separated on a reversed-phase Hypersil BDS C_{18} column (200 × 4.6 mm, 5 μm, Yilite Co., Dalian, China) by a gradient elution, and the eluent components and gradient program is shown in Table 1. The flow rate was constant at 1.0 mL min^{-1} and column temperature was set at 35 °C. The maximum excitation and emission wavelengths were set at λ_{ex} 300 nm and λ_{em} 390 nm. The mobile phase was filtered through a 0.2-μm Nylon membrane filter. Chromatographic peaks were identified by spiking the working standard with each individual amino acid in turn, and simultaneously confirmed by mass spectrometry. Ion source conditions: ESI in positive ion detection mode; nebulizer pressure 35 psi; dry gas temperature 350 °C; dry gas, 9.0 L min^{-1} capillary voltage 3,500 V.

Results and Discussions

Optimization for Derivatization

DBCEC has the same chloroformate reaction with primary and secondary amino acids at room temperature as FMOC, CEOC, BCEOC or BCEC previously reported [22–25]. In this work, the optimal conditions for the derivatization of DBCEC with amino acids were investigated, including buffer pH, reaction time and concentration reagent. Also, borate buffers were used as basic catalyst of the derivatization reaction, and the effect of buffer pH on the derivatization reaction was investigated with buffer pH varying in the range of 8.5–10.5 (borate buffers were prepared by adding NaOH to 0.2 mol L^{-1} boric acid to obtain a desired pH value). The maximum derivatization yields were achieved in the pH range of 9.0–10.0. However outside this range, particularly in more acidic solution, the decreased responses were observed. At higher pH values (>10.5), the amino acid derivatives exhibited a significant loss of fluorescent response relative to those obtained in buffer of pH 9.0. Therefore, 0.2 M borate buffer solution at pH 9.0 was chosen for amino acid derivatization.

With borate solution at pH 9.0 as buffer and 10 min as reaction time, the effect of DBCEC concentration on the derivatization yields of amino acids was investigated in the range of 0.2–1 mmol L^{-1}. The fluorescence intensity of DBCEC-derivatives increased with increasing the amounts of derivatization reagent. Constant fluorescence intensity was achieved with the concentration of DBCEC increasing to 0.5 mmol L^{-1}, but further increasing DBCEC concentration beyond this level had no significant effect on yields. It was also found that further increasing DBCEC concentration over 0.5 mmol L^{-1} can shorten the reaction time required for quantitative derivation to about 3–5 min, however, the corresponding increase of the 2-[2-(dibenzocarbazole)-ethoxy] ethanol (DBCEC-OH) peak (the major by-product of the derivatization process, see Fig. 1) would

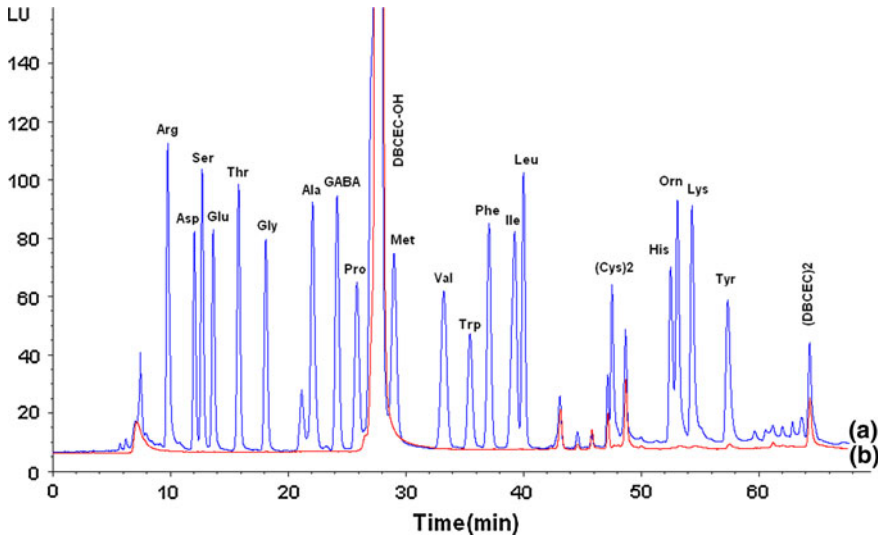

Fig. 1. Chromatograms for standard amino acid derivatives with a 20 pmol injection (**a**) and derivatization blank (**b**) Chromatographic conditions: Hypersil BDS C_{18} column (200 mm × 4.6 mm, 5 µm), column temperature 35 °C; flow rate 1.0 mL min^{-1}; excitation and emission: $\lambda_{ex}/\lambda_{em}$ = 300/390 nm; corresponding injected amount 20 pmol Peaks are labeled with abbreviations for all amino acids: **Arg** = Arginine; **Asp** = Aspartic acid; **Ser** = Serine; **Glu** = Glutamic acid; **Thr** = Threonine; **Gly** = Glycine; **Ala** = Alanine; **GABA** = 4-Aminobutanoic acid; **Pro** = Proline; **Met** = Methionine; **Val** = Valine; **Trp** = Tryptophan; **Phe** = Phenylalanine; **Ile** = isoleucine; **Leu** = Leucine; **$(Cys)_2$** = Cystine-Cystine; **His** = Histidine; **Orn** = Ornithine; **Lys** = Lysine; **Tyr** = Tyrosine; **DBCEC-OH** = 2-[2-(dibenzocarbazole)-ethoxy] ethanol; **$(DBCEC)_2$** = bis-(2-[2-(dibenzocarbazole)-ethoxy] ethyl)-carbonate

Table 2. The retention time and ESI-MS–MS data (positive mode) for DBCEC-amino acids drivatives

DBCEC-amino acid derivatives	Retention time (min)	$[M + H]^+$ or/and $[M + Na]^+$	MS–MS data
Arg	9.793	556.8; 578.7	294.8; 338.7; 356.7; 540.0
Asp	11.654	515.6	294.7; 338.7; 356.6; 470.3; 497.5
Ser	12.395	487.6	294.7; 338.6; 356.7; 442.8; 458.7
Glu	13.231	529.6; 551.7	294.7; 338.8; 356.7; 483.5; 511.5
Thr	15.434	501.7; 523.7	294.7; 338.7; 356.6; 455.6; 483.7
Gly	17.483	457.6; 479.8	294.7; 338.8; 356.6; 411.7; 439.6
Ala	21.401	471.7; 493.7	294.7; 338.8; 356.6; 425.5; 452.6
GABA	23.570	485.6; 507.7	294.7; 338.7; 356.7; 467.3
Pro	25.170	497.5	294.6; 339.1; 356.7; 451.6
DBCEC-OH	26.767	356.7	294.7
Met	27.913	531.5	294.7; 338.8; 356.6; 494.7
Val	32.092	499.6	294.6; 338.6; 356.6; 452.6
Trp	34.402	586.4	294.8; 338.7; 356.6; 540.6; 568.1
Phe	36.163	547.5; 569.6	294.7; 356.6; 501.6
Ile	38.566	513.5; 535.7	294.7; 338.5; 356.6;467.5; 495.7
Leu	39.344	513.5; 535.7	294.6; 338.5; 356.5; 467.6; 495.7
(Cys)2	47.210	1003.4	294.6; 338.7; 356,6; 457.5; 503.5; 457.6
His	51.734	918.6	284.6; 338.5; 356.5
Orn	52.304	894.7	294.7; 356.4; 451.5; 540.4; 713.2; 805.4; 877.4
Lys	53.458	909.1	294.6; 356.4; 465.6; 554.4; 598.7
Tyr	56.163	944.5	294.6; 338.5; 356.5
$(DBCEC)_2$	63.820	754.8; 775.5	294.7; 338.5; 470.3; 737.4

interfere the neighbor peaks of amino acid derivatives. With the concentration of DBCEC lower than 0.5 mmol L^{-1}, the derivatization of amino acids was incomplete within 10 min; however quantitative derivatization of most amino acids can also be achieved by prolonging the reaction time to 20–30 min. To achieve quantitative derivatization within the shortest reaction time and the smallest DBCEC-OH peak, a DBCEC solution of 0.5 mmol L^{-1} was chosen in the subsequent experiment.

LC Separation and MS Identification for Amino Acids Derivatives

The baseline separation for 20 amino acid derivatives was achieved on a Hypersil BDS C_{18} column under the gradient elution as described in "Experimental", and the chromatograms for 20 amino acid standard derivatives was shown in Fig. 1. To achieve the optimal separation, the choice of mobile phase pH value was tested on a Hypersil BDS C_{18} column with formic ammonium buffers in the range of 3.0–6.0. It was found that decreasing the mobile phase pH resulted in an obvious increase in retention value for most amino acid derivatives, and the resolution of critical pairs significantly depended on the pH of mobile phases. Satisfactory resolution for all the derivatized amino acid standards can be accomplished with acidic mobile phase pH 3.5. With pH > 4.0, most of the amino acids derivatives were resolved with the exception of methionine; the methionine derivative was embedded in the peak of DBCEC-OH which aborted the identification and quantification of this analyte. As mobile phase pH decreased from 4.0 to 3.5, the retention of amino acid derivatives increased obviously, while the retention of DBCEC-OH remained unchanged, thus methionine gradually came from the broad peak of DBCEC-OH; however, the resolution of leucine and isoleucine underwent a decrease from 1.6 to 1.0 at the same time. With the mobile phase pH further decreasing from 3.5 to 3.0, the resolution of leucine and isoleucine subjected a further decrease which was not acceptable for quantification analysis. Therefore, the subsequent chromatographic separation was carried out with the mobile phase pH 3.5.

It was found that several by-products, such as DBCEC-OH (m/z: 357), bis-(2-[2-(dibenzocarbazole)-ethoxy] ethyl)-carbonate $(DBCEC)_2$ (m/z: 755), were brought by the hydrolysis of the DBCEC during the derivatization procedure. Also some impurities from the DBCEC

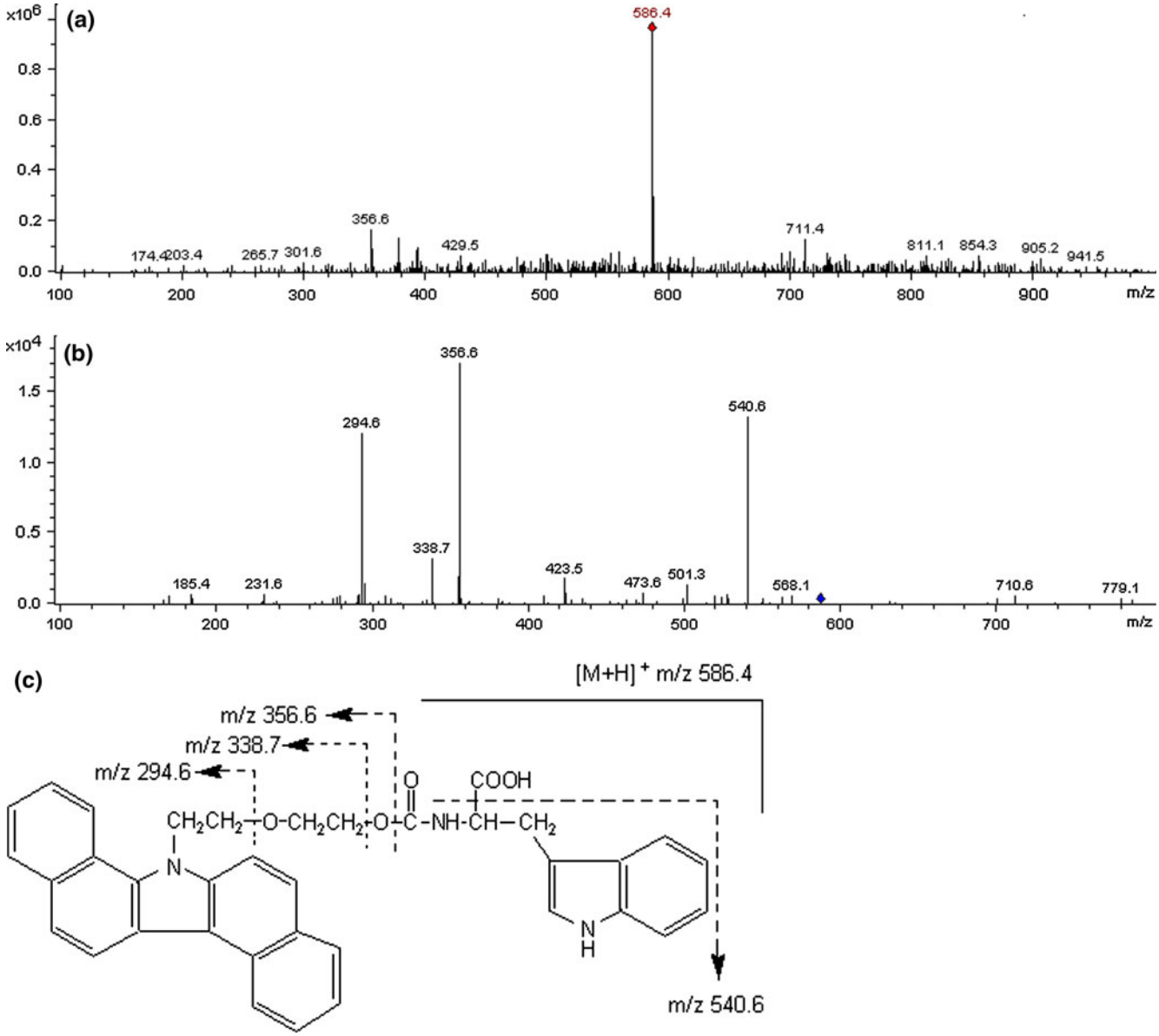

Fig. 2. The representative MS (**a**), MS–MS (**b**) and cleavage mode (**c**) for DBCEC-tryptophan derivatives

reagent were observed. To achieve a satisfactory resolution for most amino acid derivatives and minimize the interference of impurities and by-products, a slow changing gradient elution program was adopted. As can be seen from Fig. 1, the presence of DBCEC-OH, $(DBCEC)_2$ and impurities did not interfere with the separation of amino acid derivatives under the proposed chromatographic conditions.

Chromatographic peaks were identified by spiking the working standard with each individual amino acid in turn, and simultaneously confirmed by online post-column ESI-MS in positive mode. Data from the MS and MS–MS spectra for all of the derivatized amino acids are shown in Table 2. As expected, the amino acid derivatives exhibited intense quasi-molecular ion peak in the positive mode with the acidic formic ammonium buffer used in mobile phases, and abundant fragment ions at *m/z* 295, *m/z* 339 and *m/z* 357 were also observed in MS–MS. The characteristic fragment ion at *m/z* 295 derives from the cleavage of the CH2CH2–OCH2CH2O bond; the fragment ion at *m/z* 339 arose from the cleavage of the CH2CH2–OCO bond; and the fragment ions at *m/z* 357 resulted from the cleavage of the CH2CH2O–CO bond. In addition, for most DBCEC-amino acid derivatives, the ions of $[MH - 46]^+$ and $[MH - 18]^+$ can also be observed in the MS–MS profiles, which were formed by losing COOH or H_2O from the corresponding $[M + H]^+$. The representative MS profiles and cleavage mode for DBCEC-tryptophan are shown in Fig. 2. The selected reaction monitoring based on the *m/z* $[M + H]^+ \rightarrow$ *m/z* 295, *m/z* 339 and *m/z* 357 transitions was specific for the amino acid derivatives.

Table 3. Linear regression equations, detection limits and quantification limits for amino acids

Amino acids	$Y = AX + B$[a]	Correlation coefficient r	LOQ (fmol)	LOD (fmol)	LODs in literatures									
					OPA [17] mg L^{-1} (pmol)	NDA [27] fmol	Dabsyl-Cl [14] pmol	Dansyl-Cl [15] pmol	AQC [19] μM (pmol)	PITC [28] pmol μL^{-1} (pmol)	FMOC [20] pmol	CEOC [23] fmol	BCEOC [24] fmol	BCEC [25] fmol
Arg	$Y = 78.96X - 67.88$	0.9995	9.79	2.94	2.62 (76)	60	0.33	<1	0.107 (0.53)	0.11 (5.5)	2.26	12.9	12.2	15.0
Asp	$Y = 38.58X - 43.19$	0.9994	7.72	2.32	0.71 (26)	125	0.46	<2	0.066 (0.33)	0.17 (8.5)	3.83	4.3	27.2	7.21
Ser	$Y = 57.71X - 11.64$	0.9999	11.9	3.57	2.30 (110)	100	0.12	<1	0.053 (0.27)	0.14 (7.0)	1.90	4.3	17.2	5.95
Glu	$Y = 39.62X - 42.88$	0.9994	2.60	0.78	1.41 (48)	105	0.51	<1	0.057 (0.29)	0.16 (8.0)	2.23	17.6	19.8	7.35
Thr	$Y = 59.90X + 1.55$	0.9997	6.91	2.07	1.96 (84)	140	0.21	<1	0.108 (0.54)	0.12 (6.0)	2.30	17.5	12.7	8.33
Gly	$Y = 57.09X + 7.58$	0.9998	24.3	7.29	3.75 (250)	100	0.26	<1	0.046 (0.23)	0.15 (7.5)	NA	13.5	9.3	6.57
Ala	$Y = 66.82X + 7.32$	0.9999	5.25	1.58	3.00 (168)	110	0.17	NA	0.123 (0.62)	0.13 (6.5)	1.66	24.8	10.4	6.29
GABA	$Y = 78.66X + 23.57$	0.9994	3.12	0.94	4.89 (238)	NA	0.26	<1	NA	NA	NA	NA	11.8	1.59
Pro	$Y = 62.05X + 9.29$	0.9998	3.37	1.01	NA	NA	0.29	<1	0.183 (0.92)	0.09 (4.5)	1.68	33.2	13.1	7.35
Met	$Y = 9.766X - 11.03$	0.9995	3.05	0.92	6.37 (214)	95	0.10	<2	NA	0.10 (5.0)	2.98	39.8	13.0	15.0
Val	$Y = 77.58X + 15.38$	0.9998	4.86	1.46	2.87 (124)	110	0.52	<1	0.018 (0.09)	0.10 (5.0)	3.50	19.2	13.6	6.05
Trp	$Y = 50.30X + 0.26$	0.9999	4.53	1.36	1.10 (27)	85	NA	<1	NA	NA	NA	35.5	9.2	7.21
Phe	$Y = 83.02X + 6.98$	0.9999	4.68	1.40	2.39 (74)	95	0.21	<1	NA	0.11 (5.5)	1.76	16.7	8.3	2.68
Ile	$Y = 69.06X + 13.46$	0.9998	7.29	2.19	1.59 (60)	110	0.41	<1	0.181 (0.91)	0.10 (5.0)	1.82	18.6	19.4	4.03
Leu	$Y = 73.62X + 14.08$	0.9997	17.1	5.13	2.21 (83)	95	0.23	<1	0.016 (0.08)	0.10 (5.0)	1.44	16.4	10.4	3.83
(Cys)2	$Y = 21.56X - 13.88$	0.9993	6.25	1.88	NA	NA	NA	NA	NA	0.02 (1.0)	NA	126	19.4	8.4
His	$Y = 40.72X - 22.83$	0.9996	16.6	4.98	8.57 (276)	125	0.30	<10	NA	0.16 (8.0)	NA	18.6	177.6	11.7
Orn	$Y = 64.40X - 18.19$	0.9996	4.23	1.27	NA	NA	0.40	<1	NA	NA	1.10	5.7	7.7	1.87
Lys	$Y = 85.28X - 8.20$	0.9997	3.75	1.13	8.26 (283)	NA	0.28	<1	0.367 (1.86)	0.08 (4.0)	0.56	4.0	6.3	1.56
Tyr	$Y = 101.52X + 23.40$	0.9996	2.98	0.89	1.79 (49)	90	0.26	<1	0.031 (0.16)	0.10 (5.0)	2.40	5.3	15.8	2.34

NA not available

[a] X: injection amount (pmol); Y: peak area

Table 4. Precision values and accuracy values for the derivatization procedure and the whole method

DBCEC-amino acid derivatives	Precision value				Accuracy value			
	Instrumental precision[a] RSD (%) ($n = 3$)	Derivatization precision[a] RSD (%)		Method precision[b] RSD (%) ($n = 3$)	Derivatization recovery (%)[a]		Method recovery (%)[b]	
		Intraday ($n = 3$)	Interday ($n = 8$)		Average recovery	RSD ($n = 3$)	Recovery	RSD ($n = 3$)
Arg	0.7	1.3	2.0	3.3	95.6	3.6	83.4	2.7
Asp	0.6	1.4	1.7	2.6	97.1	2.9	92.3	2.1
Ser	0.5	1.3	1.9	2.9	93.4	2.9	87.3	2.3
Glu	0.5	1.5	2.1	3.1	97.5	3.0	88.6	1.9
Thr	0.8	1.3	2.4	2.9	95.7	3.1	92.0	2.1
Gly	0.6	1.2	2.2	2.3	97.3	2.7	95.6	1.8
Ala	0.7	1.4	1.9	2.2	96.8	2.8	95.7	2.2
GABA	0.6	1.2	1.6	3.9	101.6	3.3	97.4	3.2
Pro	0.8	1.0	1.7	2.0	98.0	2.9	98.7	3.0
Met	0.8	1.6	2.4	3.6	103.1	3.5	92.0	3.4
Val	0.7	1.4	1.8	2.2	96.3	2.6	85.5	2.5
Trp	0.6	1.1	1.7	[c]	98.4	2.7	[c]	
Phe	0.7	1.3	1.9	2.3	96.2	2.9	91.3	2.1
Ile	0.9	1.5	2.5	3.2	105.3	3.1	89.1	2.9
Leu	0.7	1.1	1.9	2.4	98.6	2.7	96.3	2.3
(Cys)2	0.7	1.7	2.5	[c]	104.1	3.2	[c]	
His	0.7	2.0	2.3	3.4	94.8	3.4	84.6	2.8
Orn	0.6	1.3	1.8	4.2	104.6	2.9	97.2	2.9
Lys	0.4	1.4	1.9	2.4	98.3	3.2	94.1	2.3
Tyr	0.6	2.4	2.6	3.7	92.3	3.8	84.8	3.1

[a] Estimated with standards
[b] Estimated with *Stellera chamaejasme* L. leaves powders
[c] Not investigated (analytes were destroyed during acid hydrolysis)

Analytical Parameters

The validation parameters studied included response linearity, detection limits (LOD), quantification limits (LOQ), precision and accuracy for the derivatization process and the whole method.

Linearity, Detection Limits and Quantification Limits

Linearity was tested by the analysis of standards containing 0.025, 0.05, 0.5, 2.5, 5 and 25 μmol L^{-1} each amino acid derivatized according to the procedure described before. Linearity data were calculated by examining the correlation coefficient of linear regression line for the response versus concentration of amino acid. The results (regression equations and correlation coefficients) obtained are reported in Table 3. All of the derivatized amino acids were found to give excellent linear responses in this range with a correlation coefficient >0.9993 or higher. The calculated LODs with fluorescence detection (at a signal-to-noise ratio of 3:1) were from 0.78 to 5.13 fmol, and LOQ (at a signal-to-noise ratio of 10:1) values in the range between 2.60 and 17.1 fmol. DBCEC provides lower LOD values than other derivatizing agents used in UV or fluorescence detection, such as naphthalene-2,3-dicarboxaldehyde (NDA) [27], phenylisothiocyanate (PITC) [28], Dabsyl-Cl [14], Dansyl-Cl [15], OPA [17], AQC [19], FMOC [20], CEOC [23], BCEOC [24] and BCEC [25], which permit us to determine lower content than methods involving other reagents.

Precision

The results of instrumental, derivatization procedure and method precision, expressed as coefficients of variation, are reported in Table 4. Instrumental precision was calculated from three injections of one derivatized standard (0.01 mmol L^{-1}). Average coefficients of variation in the range from 0.4 to 0.8% were obtained. The precision of the derivatization procedure was checked from injections of a standard that had been derivatized eight times over 3 days (inter-day 1.7–2.6%) and three times on 1 day (intra-day 1.1–2.4%). The precision of the method was finally estimated by applying the whole procedure to three aliquots of *Stellera chamaejasme* L. root powders, variation coefficients in the range of 2.2–4.2% were obtained. The results indicated that the described method was precise enough for routine analysis of amino acids from various biological or environmental samples.

Accuracy

The accuracy of the derivatization step was evaluated by analyzing standards in triplicate at three levels (1, 10 and 50 pmol), and comparing the analytical results to the known added value. The mean recoveries of amino acids from derivatization step were in the range of 92.3 to 105.3% (see Table 4). To evaluate the accuracy of the method, the recovery experiments were also conducted with real samples. Known amounts of each amino acid were added to the *Stellera chamaejasme* L. sample at a close concentration level with original

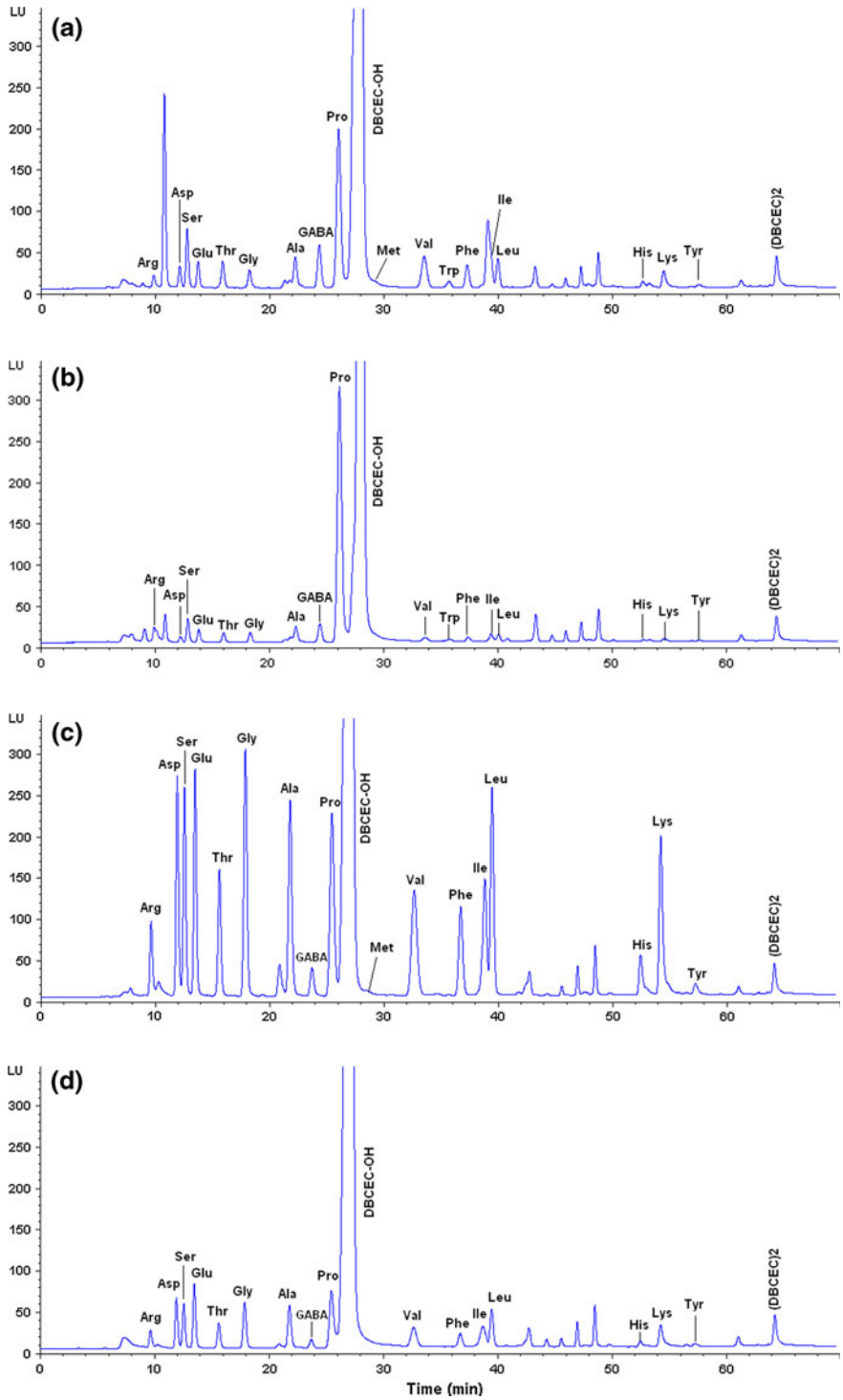

Fig. 3. Chromatograms of amino acids from *Stellera chamaejasme* L. (**a**) free amino acids profile in leaves; (**b**) free amino acid profile in roots; (**c**) total amino acid profile in leaves; (**d**) total amino acid profile in roots (the chromatographic conditions and peak symbols are as in Fig. 1)

value, and the spiked samples subjected the whole procedure, including acidic hydrolysis, derivatization and injection. The recoveries were calculated based on the formula of (measured value−endogenous value)/added value × 100. All analyses were carried out in triplicate, and the results are listed in Table 4. The results show that the recoveries of all amino acid ranged between 83.4 and 98.7% and the RSD values fell within 1.8–3.4%. Such results further demonstrated that this method is precise and practical for the analysis of amino acids from *Stellera chamaejasme* L. samples.

Application of the Method for Determination of Amino Acid from Real Samples

The proposed method was used to analyze the amino acids from *Stellera chamaejasme* L. samples, and the obtained chromatograms are shown in Fig. 3. Amino acid compositional data from *Stellera chamaejasme* L. samples are shown in Table 5. The results indicated that proline was the most abundant free amino acid in both leaves and roots samples. In the acid hydrolysis samples, the amino acids were mainly glutamic acid, aspartic acid, proline, leucine, srrine, lysine, glycine, alanine, valine, threonine, phenylalanine and isoleucine, and the total contents of each amino acid in leaves were 3.04–7.08 times higher than those in roots (average 5.16 times). These data would be of great value for all-round exploiture of the source of *Stellera chamaejasme* L.

Conclusion

The present paper introduces a new LC-FLD method for derivatizing amino acids with DBCEC as labeling reagent, which has superior properties to currently popular reagents, including rapid, convenient derivatization, excellent sensitivity, stability and derivatization yields. The improved performance of the reagent DBCEC for quantitative analysis of amino acid has been demonstrated in detail. This method exhibits its simpleness for the preparation of amino acid derivatives, and complete derivatization in basic medium at room temperature takes <10 min. The described method shows excellent linear responses with coefficients of >0.9993, and detection limits were in the range of 0.78–5.13 fmol (signal-to-noise ratio of 3). The LC separation for the derivatized amino acids showed good repeatability and

precision. These characteristics indicated that BCEOC would be another popular and attractive derivatizing reagent for amino compounds following FMOC, CEOC, BCEOC and BCEC.

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Table 5. Average content of free and total amino acids in *Stellera chamaejasme* L. samples ($n = 3$)

Amino acids	Free amino acids (mg g^{-1})		Total amino acids (mg g^{-1})	
	Leaves	Roots	Leaves	Roots
Arg	0.1360	0.1139	2.89	0.81
Asp	0.2968	0.0948	11.69	2.70
Ser	0.4156	0.1663	6.20	1.31
Glu	0.4210	0.2169	13.90	3.91
Thr	0.2561	0.0879	4.60	0.86
Gly	0.1233	0.0594	6.16	1.12
Ala	0.2309	0.1154	5.38	1.17
GABA	0.3070	0.1293	0.85	0.21
Pro	1.921	NQ	8.66	2.84
Met	0.2912	ND	0.55	ND
Val	0.3739	0.0390	5.40	0.94
Trp	0.1658	0.0462	0.36	ND
Phe	0.2534	0.0542	4.25	0.60
Ile	NQ	0.0640	4.89	0.78
Leu	0.2482	0.0576	7.62	1.12
His	0.1381	0.0611	3.48	0.47
Orn	0.0362	0.0158	0.10	ND
Lys	0.1833	0.0368	6.29	0.97
Tyr	0.0362	0.0062	0.53	0.13

ND not detection or below the LOQ; NQ not quantification for serious interference

References

1. Jiang ZH, Tanaka T, Sakamoto T, Kouno I, Duan JA, Zhou RH (2002) Chem Pharm Bull 50:137–139. doi:10.1248/cpb.50.137
2. Yoshida M, Feng WJ, Saijo N, Ikekawa T (1996) Int J Cancer 66:268–270. doi:10.1002/(SICI)1097-0215(19960410)66:2<268:AID-IJC22>3.0.CO;2-7
3. Fan J (2007) J Clin Res 24:2001–2003. doi:CNKI:SUN:HNYZ.0.2007-11-075
4. Zheng B, Hu H (2006) J Northeast For Univ 34:59–60. doi:cnki:ISSN:1000-5382.0.2006-04-021 (in Chineses)
5. Feng B, Gong X, Li S, Jiang G, Pei Y, Wang Y (2008) Chin J Chin Mater Med 33:403–405. doi:CNKI:SUN:ZGZY.0.2008-04-015 (in Chineses)
6. Feng BM, Pei YH, Hua HM (2004) Chin Chem Lett 15:61–62. doi:cnki:ISSN:1001-8417.0.2004-01-020
7. Gatti R, Gioia MG, Andreatta P, Pentassuglia G (2004) J Pharm Biomed Anal 35(2):339–348. doi:10.1016/S0731-7085(03)00584-3
8. Dong MW, Gant JR (1985) J Chromatogr A 327:17–25. doi:10.1016/S0021-9673(01)81635-6
9. Hsieh MM, Chen SM (2007) Talanta 73(2):326–331. doi:10.1016/j.talanta.2007.03.049
10. Denoroy L, Parrot S, Renaud L, Renaud B, Zimmer L (2008) J Chromatogr A 1205(1–2):144–149. doi:10.1016/j.chroma.2008.07.043
11. Manica DP, Lapos JA, Jones AD, Ewing AG (2003) Anal Biochem 322(1):68–78. doi:10.1016/j.ab.2003.07.002
12. Aito-Inoue M, Ohtsuki K, NakamurA Y, Park EY, Iwai K, Morimatsu F, Sato K (2006) J Agric Food Chem 54:5261–5266. doi:10.1021/jf060531s
13. Poboży E, Czarkowska W, Trojanowicz M (2006) J Biochem Biophys Methods 67(1):37–47. doi:10.1016/j.jbbm.2006.01.001
14. Krause I, Bockhardt A, Neckermann H, Henle T, Klostermeyer H (1995) J Chromatogr A 715:67–79. doi:10.1016/0021-9673(95)00578-B
15. Minocha R, Long S (2004) J Chromatogr A 1035:63–73. doi:10.1016/j.chroma.2004.02.026
16. Hanczkó R, Jámbor A, Perl A, Molnár-Perl I (2007) J Chromatogr A 1163:25–42. doi:10.1016/j.chroma.2007.06.013
17. Pereira V, Pontes M, Câmara JS, Marques JC (2008) J Chromatogr A 1189:435–443. doi:10.1016/j.chroma.2007.12.014
18. Cohen SA, Michaud DP (1993) Anal Biochem 211(2):279–287. doi:10.1006/abio.1993.1270
19. Bosch L, Alegría A, Farré R (2006) J Chromatogr B 831:176–183. doi:10.1016/j.jchromb.2005.12.002
20. Lozanov V, Benkova B, Mateva L, Petrov S, Popov E, Slavov C, Mitev V (2007) J Chromatogr B 860:92–97. doi:10.1016/j.jchromb.2007.10.020
21. López-Cervantes J, Sánchez-Machado DI, Rosas-Rodríguez JA (2006) J Chromatogr A 1105:106–110. doi:10.1016/j.chroma.2005.08.040
22. Kőrös Á, Varga ZS, Molnár-Perl I (2008) J Chromatogr A 1203:146–152. doi:10.1016/j.chroma.2008.07.035
23. You J, Shan Y, Zhen L, Zhang L, Zhang Y (2003) Anal Biochem 313(1):17–27. doi:10.1016/S0003-2697(02)00398-6
24. You J, Ming Y, Shi Y, Zhao X, Suo Y, Wang H, Li Y, Sun J (2005) Talanta 68(2):448–458. doi:10.1016/j.talanta.2005.09.019
25. You J, Liu L, Zhao W, Zhao X, Suo Y, Wang H, Li Y (2007) Anal Bioanal Chem 387(8):2705–2718. doi:10.1007/s00216-007-1155-9
26. You J, Song C, Yan T, Sun Z, Li Y, Suo Y (2010) Anal Chim Acta 658(1):98–105. doi:10.1016/j.aca.2009.10.061
27. Montigny PD, Stobaugh JF, Givens RS, Carlson RG, Srinivasachar K, Sternson LA, Higuchi T (1987) Anal Chem 59(8):1096–1101. doi:10.1021/ac00135a007
28. Campanella L, Crescentini G, Avino P (1999) J Chromatogr A 833:137–145. doi:10.1016/S0021-9673(98)01023-1