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Original article

The determination of amino acids composition of the traditional food *Potentilla anserina* L. root by high-performance liquid chromatography via fluorescent detection and mass spectrometry

Lian Xia[1,2,3] & Jinmao You[1,2*]

1 The Key Laboratory of Life-Organic Analysis, College of Chemistry Science, Qufu Normal University, Qufu Shandong, 273165, China
2 Northwest Plateau Institute of Biology, Chinese Academy of Sciences, Xining, 810001, China
3 Graduate University of the Chinese Academy of Science, Beijing, 100049, China

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Summary In this study, we developed a method of liquid chromatography with fluoresce detector (LC-FLD) and on-line mass spectrometry identification (MSI) using 2-[2-(7H-dibenzo [a, g] carbazol-7-yl)-ethoxy] ethyl chloroformate (DBCEC-Cl) as pre-column derivatisation reagent for determination of the amino acids (AAs) in *Potentilla anserina* L. Separation of the derivatised AA exhibited a good baseline resolution in combination with a gradient elution. All AA derivatives give excellent linear responses with correlation coefficients of >0.9992. The detection limits of each AA were 2.60–24.3 fmol. Eighteen AAs, involving seven essential AAs, were detected in *Potentilla anserina* L. Quantitative recoveries of the AAs from the *Potentilla anserina* L. were 84–107%, and the relative standard deviation values were $<1.45\%$. The established method in this study was sensitive and precision enough to separate and quantify AA composition of *Potentilla anserina* L. Good compositional data were obtained from the analysis of the AAs obtained from *Potentilla anserina* L. root.

Keywords Amino acids, HPLC-FLD-MSI, 2-[2-(7H-dibenzo [a, g] carbazol-7-yl)- ethoxy] ethyl chloroformate, *Potentilla anserina* L.

Introduction

The Tibetan plant *Potentilla anserina* L., a kind of perennial herb, belongs to the genus Potentilla, family Rose. Its root is also called panax fruit, longevity fruit etc among folks (The Academy of JiangXi New Medicine, 1997). *Potentilla anserina* L. widely distributed around China such as Qing-Tibet Plateau, Neimenggu, Sichuan, Xizang, Xinjiang and Yunnan Province of China, etc., but all of them differ largely from each other. In low-altitude area, its fleshy root does not grow well, while, of the root of its counterpart which grown in Qinghai province is the best in terms of rounded shape, fleshy pulp, bright red colour, high yield etc (Northwest Plateau Institute of Biology, the Chinese Academy of Sciences, 1987; Wu, 1990). Modern pharmacological studies showed that stems, leaves and roots of *Potentilla anserina* L. possess multiple bioactivities such as astringent, anti-inflammatory, antispasmodic, haemostatic and also have been used for diarrhoea, leucorrhoea, dysmenorrhoea, arthritis, cramps, kidney stones, chronic liver diseases, bleeding piles; as a mouth wash in pyorrhoea, gingivitis and sore throat (Wu,1990; Drugs control authority of Qinghai province & institute of Tibetan medicine of Qinghai province, 1996; Luodashang, 1997; Li *et al*., 2004; Zhang *et al*., 2004; Zhao *et al*., 2008a,b). However, few people derectly eat stems and leaves of *Potentilla anserina* L. because of the disagreeable taste, and the local people often use them to feed pigs, which were called *Potentilla anserina* L. pigs. For thousands of years, root of *Potentilla anserina* L. has been used frequently as a crude substance sanitarian product by food (The Academy of JiangXi New Medicine., 1997).. The most common use of *Potentilla anserina* L. root is making conjee with rice together, which is one of the principal foods for the native people. The conjee with root of *Potentilla anserina* L. both has delicious tastes of sweet, slip, flavor, etc., and is rich in nutrition for all ages of people throughout the four seasons, especially for the elderly, women, pergnant and children. Also it can be made to a kind of rice cake, a hospitality food of Tibetans, by braising with rice, and then adding butter. Now, many

*Correspondent: Fax: +86-537-4456305;
e-mail: Jmyou6304@163.com

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people cook chicken or duck arranging in pairs with *Potentilla anserina* L. to enhance taste and nutrition. It also can be fried, stewed and braised as vegetables.

The AAs of *Potentilla anserina* L. were reported as ones of the active fractions (Pi & Hu, 2007; W *et al*., 2008). Essential AAs as the major structural and functional components play an important role on human health. Recently, the researches about the possibility for utilisation of AAs as a dietary supplement for preventing or treating chronic and/or metabolic diseases has arouse large interests among scholars globally (Hiroyuki *et al*., 1995; Supski *et al*., 2009, 2010) Therefore, determination of the AAs composition of *Potentilla anserina* L. can help us to understand its active components, which are beneficial to human health, and important still to establish the qualitative standard for the further development of *Potentilla anserina* L. as edible and medicinal food. To the best of our knowledge, *Potentilla anserina* L. root contain abundant protein compared with stems and leaves. Furthermore, its AAs composition has not been evaluated systematically. It is of great importance to establish a method for analysis of the AAs composition to supply more scientific evidence for the development of *Potentilla anserina* L.

However, most of AAs show neither natural UV absorption nor fluorescence, thus their detection at trace levels using absorptiometry was fairly difficult. To overcome this shortcoming and increase detection sensitivity, the techniques of fluorescence pre-column derivatisation are commonly used. Although a number of different types of fluorescent labeling reagents have been proposed, a variety of shortcomings were also reported. For example, the *o*-phthaldialdehyde (OPA) is only used to label primary amino acids (Shuko *et al*., 1998; Tseng *et al*., 1999; Dobashi *et al*., 2002); 7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) (Ahnoff *et al*., 1981) is reported that about 50% of the reagent decomposes in methanol–water solution exposed to daylight within 25 min. 9-Fluorenyl methylchloroformate (FMOC-Cl) (Einarsson *et al*., 1983; Buratti *et al*., 2002), 1-(9-fluorenyl)-ethyl chloroformate (FLEC-Cl) (Einarsson *et al*., 1987) and 2-(9-anthryl)-ethyl chloroformate (AEOC-Cl) (Bjöklund *et al*., 1998) exhibit serious interferences from excesses of these reagents or from reaction by-products; 6-Aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) has also been reported that the fluorescent intensity of its derivatives in aqueous solution is only 10% of the intensity in pure acetonitrile solution. Thus, the detection limits for the early-eluted amino acids are usually higher than those for later ones (Cohen & Michaud, 1993; Ji, 1994; Ágnes *et al*., 1999).

In this article, a sensitive fluorescence reagent, 2-[2-(7H-dibenzo[a,g] carbazol-7-yl)-ethoxy] ethyl chloroformate (DBCEC-Cl) has been developed to label AAs. DBCEC-Cl molecule, which contained a nitrogen atom and four benzene rings, exhibited a high degree of conjugation. The DBCEC-Cl, being the derivatisation reagent for labeling AAs, could overcome the shortcomings showed by the traditional labeling reagents. The simultaneous separation and determination of twenty AAs using DBCEC-Cl as labeling reagent with HPLC was carried out successfully. The suitability of the proposed HPLC method for the analysis of free and total AAs from the Tibetan herb *Potentilla anserina* L. was satisfactory.

Materials and methods

Materials

The plant materials of the *Potentilla anserina* L. root were collected from Yushu County in August 2009 (Qinghai province, China) with random, and was identified by Changfan-Zhou who hold the post of professor in Northwest Plateau Institute of Biology, Chinese Academy of Sciences. Twenty standard AAs were obtained from Sigma Co. (St Louis, MO, USA). HPLC grade acetonitrile was obtained from Yuwang Chemical Reagent Co. (Shandong Province, China). DBCEC-Cl was prepared according to the method as our previous described (Yan *et al*., 2009). Water was purified on a Milli-Q ststem (Millipore, Bedford, MA, USA). Borate buffer was prepared from 0.2 M boric acid solution adjusted to pH 9.0 with 4 M sodium hydroxide solution prepared from sodium hydroxide pellets. All other reagents used were also of analytical grade unless otherwise stated.

Instrumentation

1100 Series LC/MSD-Trap-SL ion trap liquid chromatography/mass spectrometry (Agilent Technologies, Palo Alto, CA, USA) was used. All the HPLC system devices were from the HP 1100 series and consisted of a quaternary pump (model G1311A), a vacuum degasser (model G1322A), a fluorescence detector (FLD) (model G1321A) and a diode array detector (DAD)(model G1315A), an autosampler (model G1329A). Electrospray ionisation (ESI) source (in positive mode); Hypersil BDS C_{18} column (200 mm×4.6 mm, 5 μm, Yilite Co Dalian, China); F-7000 fluorescence spectrophotometer (Hitachi, Japan) was used to obtain fluorescence spectra. CARY 300 Bio ultraviolet obvious spectrophotometer and Carlo-Erba 1106 element analyzer were used in this study.

Extraction and preparation of AAs from *Potentilla anserina* L.

Free AAs

Tibetan herb *Potentilla anserina* L. sample was washed with water and dried under vacuum at 50 °C for 48 h, and then pulverised in a mortar. The pulverised herb

(3.0 g) were mixed with 30 mL water, and then the mixture was ultrasonic extracted for 2 h, the supernatant was collected and the residue was extracted twice with 60 mL water, After filtrating, the combined mixture was stored at −20 °C until analysis.

Hydrolysed AAs

The pulverised herb (50 mg) was placed in a 2.0-mL vial, 1.0 mL of hydrochloric acid (6 M) was added and the vial was sealed. After hydrolysis at 110 °C for 24 h, the contents were neutralised to pH 7.0 with 1.1 mL of NaOH (6 M), and then filtrated. The prepared sample was stored at −20 °C until analysis.

Preparation of standard solutions

The standard AAs for HPLC analysis at individual concentration of 1.0×10^{-2} mol L^{-1} were prepared in water, and if necessary, 6.0 mol L^{-1} HCl or 6.0 mol L^{-1} NaOH was added until the solutions were completely dissolved. The standard AAs for HPLC analysis at individual concentration of 1.0×10^{-4} mol L^{-1} were prepared by diluting the corresponding stock solutions (1.0×10^{-2} mol L^{-1}) of each AA with acetonitrile. The derivatisation reagent solution (1.0×10^{-2} mol L^{-1}) was prepared by dissolving 32.6 mg of 2-[2-(7H-dibenzo [a, g] carbazol-7-yl)- ethoxy] ethyl chloroformate (DBCEC-Cl) in 10 mL of anhydrous acetonitrile and the corresponding low concentration of reagent solution (5×10^{-3} mol L^{-1}) was prepared by diluting the corresponding stock solution (1.0×10^{-2} mol L^{-1}) with anhydrous acetonitrile.

Derivatisation procedure

The derivatisation proceeded in acetonitrile solution in a basic medium. 20 μL of standard AAs (or 40 μL of hydrolyte from the real sample) and 100 μL borate buffer (pH 9.0) were added in a 2-mL vial, to which 200 μL DBCEC-Cl and 80 μL of acetonitrile were then added. The vial was sealed and allowed to heat in water bath at 30–40 °C for 5 min. To stop reaction, 100 μL 50% acetic acid was then added until the final pH was in the range of 6.0–6.5. 50 μL derivatised solution was diluted with 300 μL acetonitrile and 50 μL water, The diluted solution (10 μL) was then injected directly into the HPLC system for analysis. The derivatisation process is shown in Fig. 1.

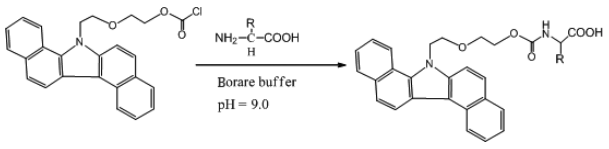

Figure 1 Derivatisation scheme of DBCEC-Cl with amino acids.

HPLC-FLD and HPLC-ESI-MSI analysis

Derivatives were separated on a reversed-phase Hypersil BDS C_{18} column (200 mm × 4.6 mm, 5 μm) in conjunction with a gradient elution. Eluent A was 30% acetonitrile (containing 30 mM ammonium/formic acid buffer, pH 3.6); B was 50% acetonitrile (containing 30 mM ammonium/formic acid buffer, pH 3.60); C was 95% acetonitrile. The flow rate was constant at 1.0 mL min^{-1} and the column temperature was set at 35 °C. The fluorescence excitation and emission wavelengths were set at λ_{ex} = 300 nm and λ_{em} = 395 nm. The gradient condition used for the separation of AA derivatives was shown in Table 1, and the run time was set 55min. MS conditions were as follows: ESI in positive mode, spray pressure of 413 kPa, dry gas flow of 5.0 L min^{-1}, dry gas temperature of 350 °C, capillary voltage of 3.5 KV, and Corona Current (nA) of 4000 (pos). The AAs quantification was carried out with HPLC-FLD, and the AA derivatives identification MS and MS–MS analysis.

Quantitative analysis of AAs

Quantitative conversion of AAs from the extract of *Potentilla anserina* L. to their DBCEC derivatives was ensured by using an excess of DBCEC-Cl. All AAs were quantified using the external standard method with detection at 395 nm. The calibration curves for each amino-acid derivative were obtained by linear regression plotting peak area (Y) versus AA injection amounts (X). All of the samples were analysed in triplicate.

Recovery test

To two identical *Potentilla anserina* L. samples, known amounts of the twenty above-mentioned AAs were added. The complete extraction and derivatisation procedures were carried out according to the described above. The total concentrations of the analytes in the spiked sample and the endogenous concentrations in the

Table 1 Gradient elution program

Time (min)	A (%)	B (%)	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

Note: A was 30% acetonitrile (containing 30 mM ammonium/formic acid buer, pH 3.6); B was 50% acetonitrile (containing 30 mM ammonium/formic acid buer, pH 3.60); C was 95% acetonitrile.

nonspiked sample were determined and used to calculate the recovery. The analyses and procedures were repeated three times.

Results and discussion

Stabilities of reagent (DBCEC-Cl) and its derivatives

The labeling reagent of DBCEC-Cl, which contained a nitrogen atom and four benzene rings with high conjugation system, showed highly hydrophobic properties. After being stored at 4 °C for 2 weeks, the solution of DBECE-Cl in anhydrous acetonitrile was used to derivatise AAs, the derivatisation yields showed no obvious difference. The derivatised AAs were found to be stable for more than 24 h at room temperature except for the double-substituted histidine, which degraded to the mono-substituted form at higher pH value (pH > 9.0). This degradation could be monitored by the appearance of the mono-substituted degradation product. However, when the derivatised solution was neutralised to pH 6.0–6.5 with 50% acetic acid solution, the degradation could be avoid. Its double-substituted derivative was very stable and can be further analysed for 48 h at room temperature.

Optimum derivatisation

Derivatisation of DBCEC-Cl with AAs can be achieved within 5 min at room temperature. The effect of DBCEC-Cl concentration on the derivatisation yields was investigated for AA derivatives. The fluorescence intensity of DBCEC-derivatives increased with increasing the amounts of derivatisation reagent. Constant fluorescence intensity was achieved with the addition of three- to fourfold molar reagent excess to total molar AAs; increasing the excess of reagent beyond this level had no significant effect on yields. With as little as a 2.5-fold molar excess of derivatisation reagent, the derivatisation of AAs was incomplete and it obviously resulted in mono-substituted derivatives such as His and Tyr. Therefore, a fourfold molar reagent excess to total molar AAs was selected.

Several types of basic media, including carbonate buffers, phosphate buffers and borate buffers, were tested to optimise derivatisation, the results showed that borate buffer was found to be the best choice. The effect of pH on the derivatisation reaction was then investigated with borate buffer (0.2 M) in the pH range of 7.0–10.0. The maximum derivatisation yields were achieved at the pH 9.0. All subsequent derivatisation were, therefore, performed in this pH value.

LC separation and MS identification

In most studies (Bruckner *et al.*, 1994; Gatti *et al.*, 2004; Schwarz *et al.*, 2005), borate or phosphate buffers were usually used to adjust the pH values of mobile phase for the separation of AA derivatives. In this study, to reduce the metal/salts pollution on MS ion chamber, formic acid/ammonia buffers were selected to control pH of mobile phase, which can significantly affect the resolution of the AA derivatives. To achieve optimal separation, the choice of pH value of mobile phase A was tested on Hypersil BDS C_{18} column. With pH < 3.5, most of the AAs were resolved with the exception of Asp and Ser (which coeluted). With pH > 3.8, DBCEC-OH and Met were coeluted, and Ile and Leu were partially coeluted. After further experiments, it was found that if the pH value of mobile phase A was adjusted to 3.7 and the flow rate was set at 1.0 mL min^{-1}, a complete baseline resolution for all AA derivatives could be achieved within the shortest time of 55 min. The separation of twenty standard AA derivatives on Hypersil BDS C_{18} is shown in Fig. 2.

The identification of AA derivatives was carried out by on-line mass spectrometry with ESI source. MS data of all AA derivatives are shown in Table S1, see

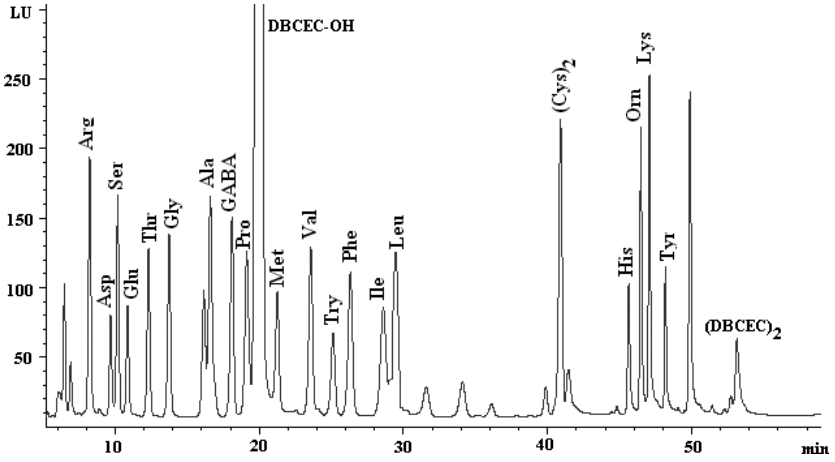

Figure 2 Chromatogram for standard amino acid derivatives. (The flow rate: 1.0 mL min^{-1}; the column temperature: 35 °C; the fluorescence excitation and emission wavelengths were set at λ_{ex} = 300 nm and λ_{em} = 395 nm. The gradient condition was as shown in Table 1

Supporting Information online. The MS, MS–MS analysis and corresponding cleavage mode for the representative BDCEC-Arginine derivative are shown in Figure S1(a–c).

As expected, the DBCEC derivatives produced an intense molecular ion peak at *m/z* $[M+H]^+$ under positive mode (Table S1). In most cases, the collision-induced dissociation spectra of *m/z* $[M+H]^+$ produced an intense fragment ion at *m/z* 294.7, *m/z* 338.7 and $[M'+CO]^+$ (M': corresponding molecular mass of the AAs). As observed from Figure S1, the characteristic fragment ions of *m/z* 294.7 and *m/z* 338.7 were from the cleavage of the NCH_2CH_2–O bond and OCH_2CH_2–O bond of reagent molecule.

Method validation

Linearity and detection limits

On the basis of the optimum derivatisation conditions, the calibration graphs were established with the peak area (*Y*) versus AA injection amounts (*X*). Injected amounts were from 50.0 fmol to 102.2 pmol with an injection volume of 10 μL (the corresponding linear range was from 5.0 to 10220 nmol L^{-1}), correlation coefficients, limit of detections (LODs) and limit of quantifications (LOQs) for all AA derivatives are shown in Table S1. All AA derivatives were found to give excellent linear responses within this range with correlation coefficients of >0.9992. The calculated LODs of each AA (at a signal-to-noise ratio of 3:1) were 2.60–24.30 fmol (0.26–2.43 nmol L^{-1}). The calculated LOQs of each AA (at a signal-to-noise ratio of 10:1) were 8.64–80.85 fmol (0.833–8.01 nmol L^{-1}). The linear regression analysis for higher concentrations of AAs was not tested as the responses were over the linearity range.

Repeatability

A standard solution consisting of 25 pmol AAs was prepared to examine the method repeatability. The relative standard deviations (RSDs.) of the peak areas and retention times were from 0.79 (Try) to 1.45% (Tyr) and from 0.004 (Met) to 0.044% $(Cys)_2$, respectively.

Accuracy and precision

Five replicates of each sample (total three samples), which were spiked into standard AAs at 0.1, 1.0 and 5.0 μmol L^{-1}, were analysed for the determination of accuracy and precision. The intra-day and inter-day accuracy and precision were measured on the same day ($n = 5$) and on the sequential 3 days ($n = 5$), respectively. Accuracies were determined as the percentage ratios of the measured concentration to the spiked concentration and the coefficients of variation (CV%) were used to report the precision. The mean intra-day accuracy ranged from 93.2 to 103.8% with the mean CV% in the range of 1.98–5.36%. The mean inter-day accuracy ranged from 91.8 to 107.4% with the mean CV % in the range of 3.51–7.94%. As a result, this experimental exhibited equilibrated accuracy by comparing with other established methods (Hou *et al*., 2006; Shi *et al*., 2006; Zhao *et al*., 2008a,b), which indicated that quantification of AA could be carried out with acceptable accuracy and precision using this method.

Comparison of sensitivity

In this study, the LODs of DBCEC-AA derivatives were compared with those of AQC and other two derivatisation reagents. The results showed that the LODs of DBCEC for AAs was lowest (Table S2), which indicated that the fluorescence detection sensitivity of DBCEC is the best. Such result may be attributed to the fact that a conjugated benzene ring was introduced to the core structure of BCEC. Additionally, MS ion current intensities for DBCEC-AAs were also compared to those obtained using BCEC-Cl and BCEOC-Cl as labelling reagent. Taken phenylalanine derivative as a representative, the ratios for the MS ion current responses were as follows: E_{DBCEC}:E_{BCEC}:E_{BCEOC} = 14.0:0.9:1.0 (E: relative ion current intensities, here, AQC-phenylalanine did not show ion current signals). Compared with BCEC and BCEOC, the modified DBCEC molecule performed excellent mass sensitivity due to the fact that a highly electronegative oxygen atom (which can also enhance water solubility of the molecule) was introduced to the DBCEC molecule by the dimeric-glycol chain.

Analysis of *Potentilla anserina* L. samples and recovery

After taking out and ice-out of the extracted AA samples, derivatisation and chromatographic separation conditions were according to the optimum conditions mentioned above, and the AA derivatives in *Potentilla anserina* L. samples were identified by chromatographic retention time with standards and on-line post column mass spectrometry. Typical chromatogram for free and total AAs with fluorescence detection is shown in Fig. 3. The contents of each AA are shown in Table 2. The results indicated that 18 AAs were contained in *Potentilla anserina* L. protein, including seven kinds of essential AAs (Lys, Leu, Ile, Thr, Val, Met and Phe), which can not be synthesised by the body and must be provided from the dietary supplement. The nutritional quality of food depends on protein quality. The balance of the essential AAs, particularly lysine has a major role. The results of the present study indicated that *Potentilla anserina* L. hydrolysate contained abundant essential AAs and high levels of lysine, threonine tryptophan. Therefore, *Potentilla anserina* L. could be used as health, high-protein food. Furthermore, as can be seen from Table 2, the content of tryptophan in free animal acid was 17.76 mg g^{-1}, whereas the total AA was 0 mg g^{-1},

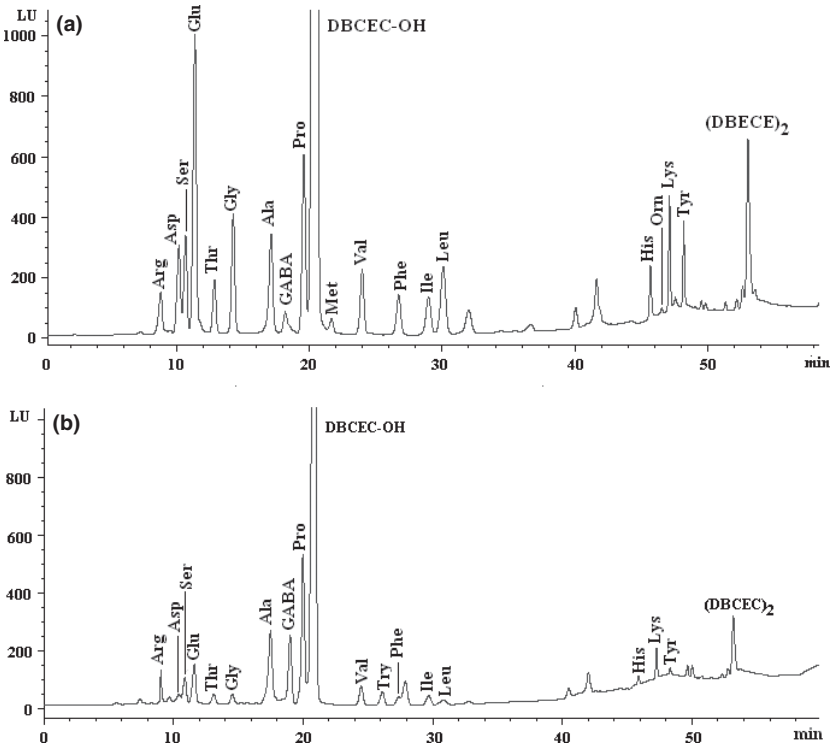

Figure 3 Chromatogram of derivatives for total and free amino acids from the Tibetan herb *Potentilla anserina* L. (a: free amino acids; b: total amino acids. Amino acid peaks and chromatographic conditions as Fig. 2)

Table 2 Compositional analysis of free and total amino acids from the Tibetan herb *Potentilla anserina* L. ($n = 3$)

	Contents (mg AAs/g dry sample)			Contents (mg AAs/g dry sample)	
Amino acids	Free amino acids	total amino acids	Amino acids	Free amino acids	total amino acids
Arg	7.54	14.48	Val	9.22	21.22
Asp	5.20	55.67	Try	12.76	0
Ser	9.88	36.21	Phe	4.42	16.63
Glu	32.80	185.00	Ile	6.86	19.50
Thr	6.59	20.53	Leu	4.99	32.87
Gly	6.29	43.39	(Cys)2	0	0
Ala	35.43	38.49	His	6.92	38.94
GABA	25.30	25.62	Orn	0	3.82
Pro	59.48	65.56	Lys	11.09	25.84
Met	0	35.66	Tyr	3.88	17.72

the reason was that the tryptophan was decomposed during the protein hydrolysis. The experimental recoveries were in the range of 84–107% (not include the tryptophan).

Conclusion

The present study provided a method for simultaneous determination of twenty AAs extracted from *Potentilla anserina* L. using DBCEC-Cl as pre-column derivatisation reagent with HPLC fluorescence detection and on-line MS identification. The method provided a rapid, sensitive, repeatable, accurate and economic alternative for the analysis of the natural AAs. The proposed method was particularly suitable for determining the AAs component from *Potentilla anserine* L. and could also be applied to routine analysis of AAs in real-life samples, such as other plant or animal protein, fruit juices, wines, etc. However, the present study showed that the *Potentilla anserina* L. contained relatively high concentration of the most deficient essential AAs (lysine, threonine, methionine and tryptophan), indicating that *Potentilla anserina* L. can be used as functional food and traditional medicine. This study is valuable for further studies and full development of *Potentilla anserina* L. Such information would facilitate the use of the *Potentilla anserine* L. in food, pharmaceutical and other technical applications, which would contribute to the sustainable use of *Potentilla anserine* L. agricultural resource.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Molecular ion (MS), MS/MS spectra, and the cleavage mode for representative Arginine derivatives.

Table S1. Linear regression equations, correlation coefficient, detection limits, mass data and repeatability for peak area and retention time of amino acid derivatives.

Table S2. The contrast of fluorescence detection limits for amino acids derivatives of the four kinds of deriverisation reagents.

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