Asian Journal of Chemistry; Vol. 24, No. 10 (2012), 4380-4384

ASIAN JOURNAL OF CHEMISTRY

www.asianjournalofchemistry.co.in

Simultaneous Determination of Amino Acids in *Hippophae rhamnoides* L. with Pre-column Derivatization by HPLC

LIMAO CAIRANG[1,2,3] and Y. RUI-SUO[1,*]

[1]Northwest Plateau Institute of Biology, Chinese Academy of Sciences, Xining, P.R. China
[2]Graduate School of the Chinese Academy of Sciences, Beijing, P.R. China
[3]Department of Chemistry Science, College of Nationality Normal, Qinghai Normal University, Xining, P.R. China

*Corresponding author: E-mail: yrsuo@nwipb.ac.cn; qh_lmcrr@sohu.com

(*Received*: 18 July 2011; *Accepted*: 2 May 2012) AJC-11393

A novel, sensitive and selective HPLC-FLC method coupled with pre-column derivatization was developed for amino acid determination in *Hippophae rhamnoides* L. fruit from Qinghai-Tibetan plateau. The method is based on the derivatization reaction of amino group with 2-[2-(dibenzocarbazole)-ethoxy]ethyl chloroformate (DBCEC) and the derivatives could be measured at 390 nm (excitation wavelength: 300 nm). Detection limits (calculated as the signal-to-noise ratio 3:1) were 12.85-51.68 mol for the labeled amino acids. Linearity was observed with coefficients > 0.9991 and the recoveries ranged from 92.7-105.4 %. Results in this research showed that the amount of essential amino acids accounted for 70.42 % of free amino acids and 22.68 % of the total amino acids, respectively, which benefits people's health through drinking the juice or eating the related products. Proline was dominating among free amino acids in samples, which plays a vital role for *Hippophae rhamnoides* L. in resisting to drought, tolerating soil salinity and lowing temperature in Qinghai-Tibetan plateau.

Key Words: Amino acids, *Hippophae rhamnoides* L., HPLC, Pre-column derivatization.

INTRODUCTION

Hippophae rhamnoides L. (sea buckthorn), a perennial bush in the genus *Hippophae*, family Elaeagnaceae, which is widely distributed throughout the temperate zone of Asia and Europe[1]. It is resistant to cold, drought, salt and alkali. The vigorous vegetative reproduction and the strong, complex root system with nitrogen-fixing nodules make it an optimal pioneer plant in soil and water conservation and reforestation of eroded areas. In Qinghai-Tibetan plateau, *Hippophae rhamnoides* L. grows mostly at an elevation of 2000-4500 m above sea level and fruits are berries of red colour and have an acid, lightly bitter taste. It has been used for the treatment of diseases in traditional medicine of various countries since ancient time[2]. For instance, it was reported from Si Bu Yi Dian in the 8th century[3] that *H. rhamnoides* L. fruits have the capacity to strengthen the spleen and the stomach and to promote blood circulation, to remove blood stasis and there are 84 prescriptions with sea buckthorn. Recent studies have shown that *H. rhamnoides* L. fruits possess immune-modulatory, antioxidant, antitumoral and hepato-protective properties containing a series of chemical compounds including amino acids, fatty acids, carotenoids, tocopherols, sterols, flavonoids, *etc*.[4]. Among these compounds, amino acids are not only the basic structural units of proteins but are also important flavour and functional compound.

The photochemistry of sea buckthorn has been extensively reported[5] and many studies of them have focused on tocopherols, flavonoids and oil. The methods for determination of chemical compounds of *H. rhamnoides* L. include preliminary chromatographic separation, GC, HPLC, GC-MS, HPLC-MS and capillary electrophoresis. Due to majority of amino acids show neither natural UV absorption nor fluorescence. Many of them cannot be accurately detected by spectrophotometeric methods, thus many chemical derivatives as an efficient means has been widely used to increase detection sensitivity and improve selectivity by means of pre-column or post-column high-performance liquid chromatography. In our previous studies, we have designed some kinds of sensitive labeling reagents for analysis of amino acids[6,7]. In this study, 2-[2-(dibenzocarbazole)-ethoxy]ethyl chloroformate (DBCEC) was employed to simultaneous determinate amino acids compounds in *H. rhamnoides* L. fruit from Qinghai-Tibetan plateau using HPLC with selective pre-column derivative, which seems to be essential for the further utilization and development of *H. rhamnoides* L. fruit from Qinghai-Tibetan plateau.

EXPERIMENTAL

Fruits of *H. rhamnoides* L. were harvested from Datong County (Qinghai, China) in September 2010 at the stage of maturity. The fruits were cleaned and then stored in polyethylene bags at -10 ºC until analysis.

All amino acid standards arginine, aspartic acid, serine, glutamic acid, threonine, glycine, alanine, 4-amino-butyric acid, proline, methionine, valine, phenylalanine, tryptophan, nor-leucine, iso-leucine, cystine, histidine, ornithine, lysine, tyrosine were purchased from Sigma Co. (St. Louis, USA). 2-[2-(Dibenzocarbazole)-ethoxy] ethyl chloroformate (DBCEC) was synthesized in our laboratory as reported earlier[8]. HPLC-grade acetonitrile was purchased from Jining Reagent Co. (Shandong, China). Formic acid was analytical grade from Shanghai Chemical Reagent Co. (Shanghai, China). Water was purified on a Milli-Q system (Millipore, Bedford, USA). Borate buffer was prepared from 0.2 mol/L boric acid solution adjusted to pH 8.5 with 4 M potassium hydroxide solution. All other reagents used were analytical grade unless otherwise stated.

HPLC instrumentation and conditions: DBCEC-amino acid derivatives analyses were performed using Agilent HP 1100 Series high-performance liquid chromatography (Agilent Technologies, USA). Derivatives were separated on Eclipse XDB-C_8 column (4.6 mm × 150 mm, 5 mm) by gradient elution. Mobile phase A was 30 % of acetonitrile consisting of 30 mM formic acid buffer (pH 3.7), B was acetonitrile-water (50:50; v/v) and C was acetonitrile-water (95:5, v/v). The flow rate was constant at 1.0 mL/min and the column temperature was set at 30 ºC. The fluorescence excitation and emission wavelengths were 300 nm of λ_{ex} and 390 nm of λ_{em}, respectively.

Extraction of amino acids: *H. rhamnoides* L. fruits were dried, crushed, ground and sieved through a 74 μm mesh size sieve. Each 5.0 g sample of *H. rhamnoides* L. fruit powder was placed into a 250 mL round-bottomed flask containing 100 mL of solvent mixture acetonitril solution (CH_3CN/H_2O 1:1, v/v), the contents of the flask were sonicated for 2 h. After that, it was filtered and transferred to another 250 mL flask. The residue was extracted twice with 80 mL deionized water and the filtrate collected was vacuum evaporated to obtain a crude extract, which was re-dissolved in 0.2 mol/L borate buffers (pH 8.5) to a total volume of 50 mL and stored at 4 ºC until analysis.

Hydroxylation: The powdered fruits (100 mg) were placed in vial, added 6 M HCl 1mL in it and then sealed. The contents were evaporated to dryness with a stream of nitrogen after hydrolysis at110 ºC for 24 h, the precipitate was re-dissolved with 2 mL of borate buffer (pH 8.5) and filtered through a 0.2 μm nylon membrane filter. The residue was washed with 50 % acetonitrile (acetonitrile/water, 1:1) 2.0 mL. The combined solution was made up to 10 mL and stored at 4 ºC until HPLC analysis.

Procedure of sample derivatization: The DBCEC-amino acids standard derivatives proceeded in acetonitrile solution (CH_3CN/H_2O 1:1, v/v) in a basic medium. 50 μL of the amino acids mixture was added in a vial, to which 100 μL of 0.2 mol/L borate buffer (pH 8.5), 150 μL of acetonitrile and 100 μL of DBCEC solution were added. The solution was shaken for 10 min and allowed to stand at 45 ºC temperature in bath for 10 min. The typical derivatives scheme of amino acids with DBCEC is shown in Fig. 1. After derivatives, 100 μL of 36 % acetic acid solution was added into the mixture until the final pH ranging in 6.0-6.5. Then the amino acid derivatives solution in samples were directly injected into the HPLC system for analysis.

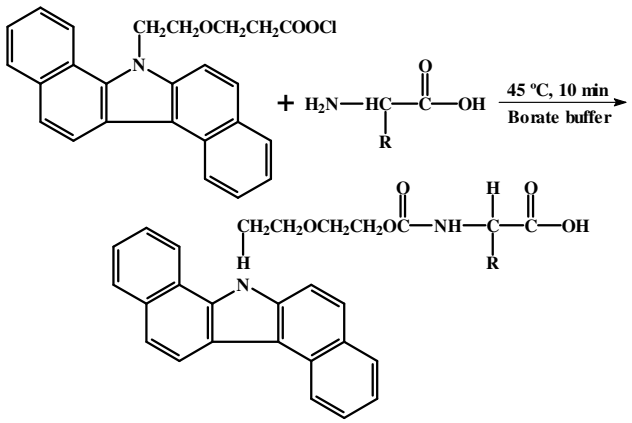

Fig. 1. Derivatization scheme of DBCEC with amino acids

RESULTS AND DISCUSSION

Derivatization: The derivatization process of DBCEC with amino acids was performed according to previous reports[9,10]. The optimum conditions for the derivatization procedure included buffer pH, DBCEC concentration and reaction time. Borate buffers were basic catalyst in the derivatization process. The maximum derivatization yields were achieved in the pH range of 8-9, outside this range, especially in more acidic solution, the responses were decreased. Therefore, 0.2 M borate buffer solution at pH 8.5 was chosen for amino acid derivatization. The DBCEC concentrations have a direct effect on fluorescence intensity in a certain range of DBCEC concentrations. The fluorescence intensity of DBCEC-derivatives increased with increasing the amounts of DBCEC in the range of 0.2-1.0 mmol/L. It was also found that further increasing DBCEC concentration over 0.5 mmol/L can shorten the reaction time required for quantitative derivation to *ca.* 3-5 min and the BDCEC-OH as the by-product in the derivatization process was obtained and to interfere the neighbour peaks of amino acid derivatives. To achieve quantitative derivatization within the shortest reaction time and the smallest DBCEC-OH peak, a DBCEC solution of 0.5 mmol/L was chosen in the subsequent experiment.

HPLC method development: Chromatographic conditions were studied in order to provide an overall optimum peak shape and reduce retention time. The mobile phase composition was investigated and obtaining good results when ammonia was used in the mobile phase. The gradient condition used for the separation of amino acid derivatives is shown in Table-1.

Validation of the method: The targeted amino acid derivatives were identified basing on direct comparison of retention times with standards. Good correlation was obtained between the retention time of standard and sample of individual

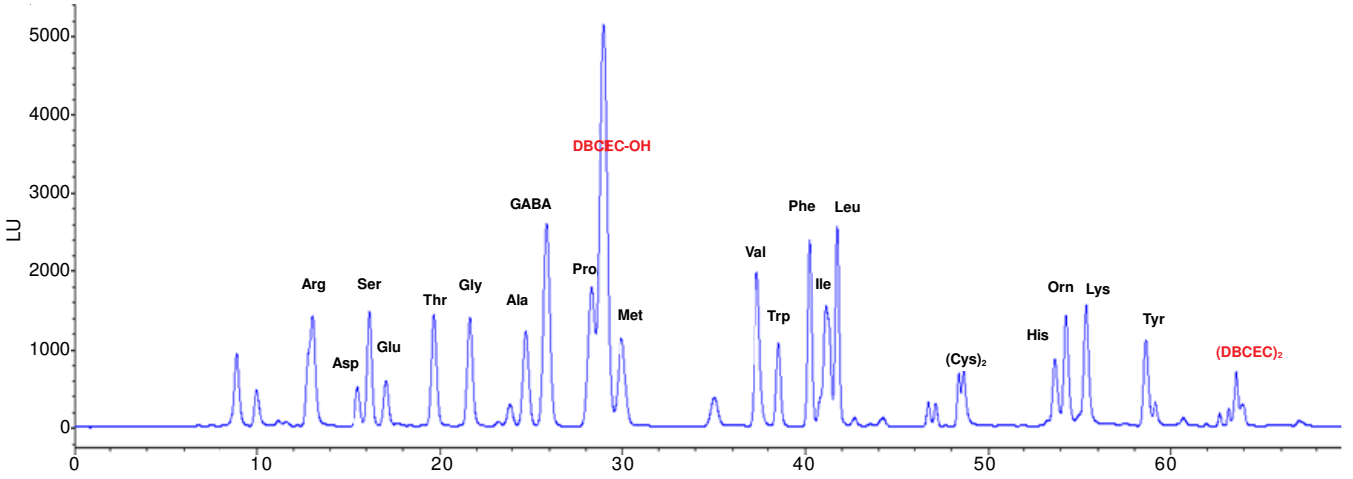

Fig. 2. Chromatogram of the standard amino acid derivatives with DBCEC

amino acid derivatives with good separation between peaks and repeatable retention times. The method was validated in terms of linearity, limit of detection, repeatability of measurement and intra-day and inter-day precision (Fig. 2).

TABLE-1
ELUENT COMPONENT AND GRADIENT PROGRAM

Time (min)	A (%)	B (%)	C (%)
0	30	70	0
20	5	95	0
40	0	35	65
50	0	30	70
60	0	0	100

Eluent A was 30 % aqueous acetonitrile solution (CH_3CN/H_2O, 30/70, v/v) consisting of 9 mL ammonia. Eluent B was 50 % aqueous acetonitrile solution (H_2O/CH_3CN, 50/50, v/v) consisting of 7.5 mL ammonia. Eluent C was 95 % aqueous acetonitrile solution (H_2O/CH_3CN, 5/95, v/v).

Linearity and limit of detection: A linear regression analysis of the calibration data was performed using the equation y = ax + b where y is the peak area, x is the concentration of amino acids b is the slope and intercept, respectively. The correlation coefficients were more than 0.9991. The detection limits, counted with signal-to-noise ratio (S/N) of 3, were in the range of 12.85-55.29 fmol (Table-2).

Precision: The standard amino acids derivatives of 5×10^{-5} mol/L were prepared for HPLC analysis. These were injected six times for 3 days. Intraday and intermediate precision was estimated using peak area. The results (Table-2) shown that the relative standard derivation of overall intra-day variations were less than 2.10 % and the relative standard derivation of inter-day variations were less than 2.19 %.

Recovery: Recovery experiment and the accuracy expressed as percentage recovery were investigated as follows: a series of concentration of 8.95×10^{-3} mg/L was immersed into an

TABLE-2
LINEAR REGRESSION EQUATIONS, CORRELATION COEFFICIENTS, MIGRATION TIME, DETECTION LIMITS OF THE METHOD, INTRA-DAY (n = 3) AND INTER-DAY PRECISION FOR PEAK AREAS (n = 6) AND RECOVERY (n=3) OF AMINO ACID DERIVATIVES

Amino acids	Regression equation	Correlation coefficients	Detection limit (t/fmol)	Migration time	Peak area RSD (%)		Recovery (%)
					Intraday	Interday	
Arg	Y = 78.96X - 67.88	0.9995	36.75	12.85	1.24	1.55	96.7
Asp	Y = 38.58X - 43.19	0.9994	29.26	15.25	0.48	1.08	98.0
Ser	Y = 57.71X - 11.64	0.9999	46.98	15.90	1.17	2.04	94.4
Glu	Y = 39.62X - 42.88	0.9994	23.08	16.79	1.21	1.03	99.2
Thr	Y = 59.90X + 1.55	0.9997	36.57	19.36	2.09	2.12	94.6
Gly	Y = 57.09X + 7.58	0.9998	20.89	21.28	1.58	0.94	98.1
Ala	Y = 66.82X + 7.32	0.9999	51.68	14.32	0.83	1.10	101.3
GABA	Y = 78.66X + 23.57	0.9994	18.61	25.43	0.59	1.52	99.5
Pro	Y = 62.05X + 9.29	0.9998	41.09	17.82	1.80	1.99	97.9
Met	Y = 9.766X - 11.03	0.9995	13.86	29.42	1.51	1.78	92.7
Val	Y = 77.58X + 15.38	0.9998	24.97	26.96	1.46	2.02	105.4
Trp	Y = 50.30X + 0.26	0.9999	15.72	38.21	1.67	2.01	90.6
Phe	Y = 83.02X + 6.98	0.9999	43.52	19.97	0.72	1.57	97.2
Ile	Y = 69.06X + 13.46	0.9998	21.33	40.88	1.21	1.06	100.8
Leu	Y = 73.62X + 14.08	0.9997	35.86	31.50	1.59	1.93	93.6
$(Cys)_2$	Y = 21.56X - 13.88	0.9991	43.94	48.95	1.87	1.77	95.2
His	Y = 40.72X - 22.83	0.9996	35.38	33.64	2.04	2.18	93.8
Orn	Y = 64.40X - 18.19	0.9996	12.85	24.21	1.29	1.04	99.9
Lys	Y = 85.28X - 8.20	0.9997	51.32	55.29	1.83	1.69	94.6
Tyr	Y = 101.52X + 23.40	0.9996	43.51	50.53	2.10	2.19	98.5

Y = AX + BX: Injected amount (fmol) Y: Peak area.

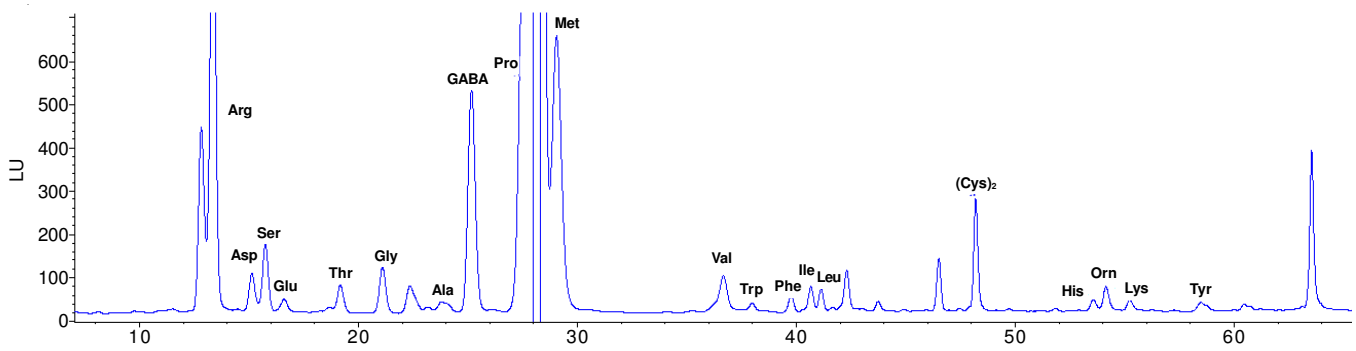

Fig. 3. Chromatogram for the analysis of free amino acid derivatives from the extracted *H. rhamnoides* L. fruit samples

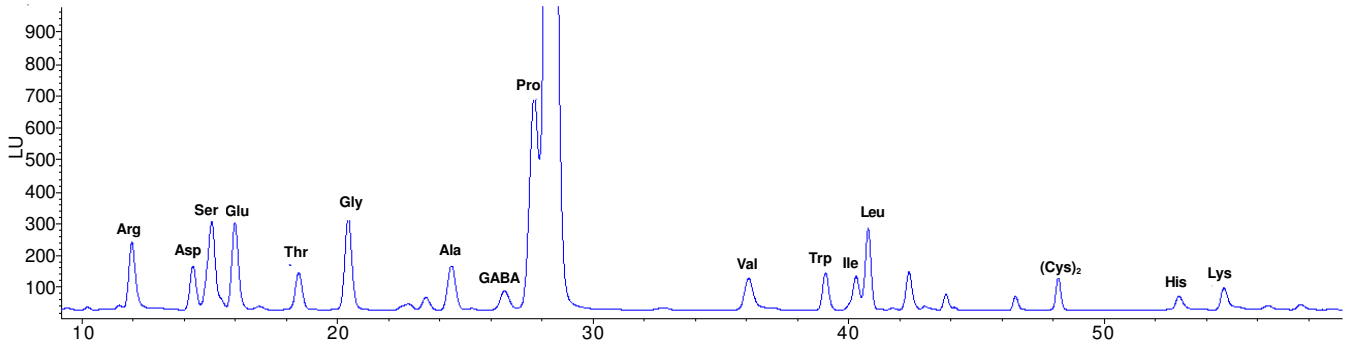

Fig. 4. Chromatogram for the analysis of total amino acids derivatives from the extracted *H. rhamnoides* L. fruit samples

acetonitrile solution of *H. rhamnoides* L. fruit and sonicated at 40 ºC for 1 h. Standard solution was added to each solution to afford a concentration of 5×10^{-5} mol/L, the solutions were then filtered and subjected to HPLC analysis in triplicate. The recovery (%) was calculated by the equation: (C3 - C2)/C1 × 100 %, where C3 was the measured concentration obtained from the extracted samples which were spiked standard; C2 was the concentration of analysis in the matrix and C1 was the added known concentration to the matrix. The results were shown in Table-2. The results shown that amino acid recoveries (n = 5) ranged between 92.7-105.4 %. This method is of high recovery.

Analysis of amino acids from *H. rhamnoides* L. fruit: Identification of amino acids was carried out by comparing retention time with the standards. Twenty amino acids derivatives were detected, all the detailed information were shown in Figs. 3 and 4. The quantitative amino-acid content was calculated in mg/g of sample. Eight essential amino acids including methionine, threonine,valine, phenylalanine, tryptophan, leucine, lysine and iso-leucine were simultaneously detected in present research by our method with good accuracy. Results showed the amount of essential amino acids range from 0.19-1.15 mg/g (free amino acids) and 0.23-2.56 mg/g (total amino acids), which accounted for *ca.* 70.42 % of the total free amino acid and 22.68 % of the total amino acid in *H. rhamnoides* L. fruit from Qinghai-Tibetan plateau, respectively. The essential amino acids were mainly part of free amino acids in *Hippophae rhamnoides* L. which benefits people's health through drinking the juice or eating the related products.

Proline (3.27 mg/g) was dominating among free amino acids in samples, which is the one of reasons why *H. rhamnoides* L. was used as an optimal pioneer plant in soil and water conservation and reforestation of eroded areas. Because proline was the physiological indicator of cold-tolerance breeding and drought resistance breeding in plants, it can stabilize the cell metabolic process and protoplasm colloid to decrease the freezing point and prevent cell dehydration in plants due to proline with strong hydrophilic amino acid. Moreover, plenty of proline will be accumulated in many plants at low temperature environment to increase cold-tolerance.

The flavour of *Hippophae rhamnoides* L. fruits was perceived as sour and astringent. Sourness and astringency correlate negatively with pleasantness in sea buckthorn. Sourness flavour of fruit was mainly due to the high acid content in *H. rhamnoides* L. But the peptides, or free amino acids, can directly contribute to taste or indirectly contribute as precursors of other taste compounds[11]. Also, they have shown to intensify, modify or mask the flavour of certain foods for some time. So, when change the flavour of sea buckthorn, the influence of amino acids need to be taken into account in the future.

The content of the free amino acids obtained by ultrasonic extraction and the total amino acids by hydrolyzation of *H. rhamnoides* L. fruits are shown in Table-3. The content of Glu, Ser, Asp and Gly are more than 4.0 mg/g in total amino acid of *H. rhamnoides* L. fruits. Compared with the reported proportion of amino acids in *H. rhamnoides* L. was different for different sources. For instance, the total essential amino acid is 22.6-49.5 % of the total amino acids in *H. rhamnoides* L. juice in Russia[12-14]. Because of the special weather conditions like the long time sunshine, 2000-4500 h and -5.7-8.5 ºC of the mean temperature in Qinghai, it is obvious that the content of the essential amino acid in *H. rhamnoides* L. fruit from Qinghai-Tibetan plateau is more than that of from Russia.

Conclusion

The high nutritional value of the *H. rhamnoides* L. fruits has resulted in an increased interest in its use as an ingredient

TABLE-3
AVERAGE CONTENT OF FREE AND TOTAL AMINO ACIDS FROM *H. rhamnoides* L. FRUIT SAMPLES (n = 3)

BCEC-amino acid derivatives	Free amino acids (mg/g)	Total amino acids(mg/g)
Arg	2.4769	1.7349
Asp	0.0200	4.6344
Ser	0.7985	6.3770
Glu	0.2437	11.1418
Thr	0.5402	2.5628
Gly	0.2129	4.7808
Ala	0.3790	0.6500
GABA	0.1881	2.4387
Pro	3.2640	1.5053
Met	0.1850	None
Val	1.1458	2.1974
Trp	0.7142	0.0693
Phe	0.4896	2.4720
Ile	0.7308	1.3224
Leu	0.6029	3.5888
(Cys)2	0.8520	2.3814
His	0.3561	1.9681
Orn	0.03248	1.8412
Lys	0.2450	0.2312
Tyr	0.0777	0.3598

in healthy foods. In this study, we not only established a simple, selective and sensitive LC method for the simultaneous determination of amino acids in *H. rhamnoides* L., results also found *H. rhamnoides* L. fruits from Qinghai-Tibetan plateau hold an extremely nutritional value and the distribution of amino acids are unique feature because its origin has special geography position.

ACKNOWLEDGEMENTS

The authors acknowledged the financial assistance from Chinese Academy of Sciences (CAS).

REFERENCES

1. V.B. Guliyev, M. Gul and A. Yildirim, *J. Chromatogr. B*, **812**, 291 (2004).
2. Z.H. Zheng, Z.H. Dong and J. Yu, Beijing University of Traditional Chinese Medicine, Xue Yuan Press, Beijing, China (1997).
3. Y.D.G.B. Yutu,, Si Bu Yi Dian (in Tibet), Tibet People Press, Tibet (1982).
4. R. Zadernowski, M. Naczk, S. Czaplicki, M. Rubinskiene and M. Szatkiewicz, *J. Am. Oil Chem. Soc.*, **82**, 175 (2005).
5. L. Hongju, M. Clara Sanuda-Pena, J. Dale Harvey-White, S. Kalra and S.A. Cohen, *J. Chromatogr. A*, **828**, 383 (1998).
6. I. Krause, A. Bockhardt, H. Neckermann, T. Henle and H. Klostermeyer, *J. Chromatogr. A*, **715**, 67 (1995).
7. R. Minocha and S. Long, *J. Chromatogr. A*, **1035**, 63 (2004).
8. J. You, C. Song, T. Yan, Z. Sun, Y. Li and Y. Suo, *Anal. Chim. Acta*, **658**, 98 (2010).
9. J. You, Y. Ming, Y. Shi, X. Zhao, Y. Suo, H. Wang and J. Sun, *Talanta*, **68**, 448 (2005).
10. J. You, L. Liu, W. Zhao, X. Zhao, Y. Suo, H. Wang and Y. Li, *Anal. Bioanal. Chem*., **387**, 2705 (2007).
11. M.A. Sentandreu, S. Stoeva, M.C. Aristoy, K. Laib, W. Voelter and E. Toldra, *J. Food Sci*., **68**, 64 (2003).
12. L.P. Solonenko, G.A. Loskutova, T.A. Druzheva and A.F. Sherstkin, *Nauka, Novosibirsk*, 16 (1991).
13. N. Kh. Mekhtiev and F.Sh. Azizov, *Izv. Akad. Nauk Az. SSR, Ser. Biol. Nauk*, **118**, 139 (1981).
14. S.M. Repyakh, A.P. Kargapol'tsev, N.A. Chuprova and G.G. Yushipitsyna, *Khim. Prir. Soedin*., **133** (1990).