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Variation in Amino Acids Composition through Pre-column Derivatisation using Phenylisothiocyanate by HPLC in Some Economically Important Less Explored Wild *Allium* Species of Western Himalayas

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ABSTRACT

Less explored *Allium* species are being used as green vegetables and as a condiment by the local inhabitant of high-altitude areas of Uttarakhand. In the present study, four economically important less explored wild *Allium* species viz. *A. auriculatum, A. ampeloprasum, A. ascalonicum* and *A. rubellum* have screened for their amino acid contents by High-performance liquid chromatography. The hydrochloric acid hydrolysate of these four species, the *Allium* amino acids were derivatised with phenylisothiocyanate resulting phenylthiocarbamyl derivatives and separated on a reverse-phase column by gradient elution with aqueous buffer and acetonitrile-water (60:40 v/v) and detected in UV region at 254 nm. The Pico-tag (3.9 × 300 mm) C_{18} column equilibrated with the solvents. The elution of all amino acid derivatives was achieved in 12 min using gradient elution by increasing concentration of aqueous buffer and acetonitrile-water. Total seventeen amino acids were present in these *Allium* species. The ratio of essential amino acids to total amino acids found 1:2.14 in *Allium auriculatum,* 1:2.35 in *Allium ampeloprasum,* 1:1.38 in *A. ascalonicum* and 1:3.44 in *Allium rubellum.* These less explored *Allium* species contained substantial amount of essential and non-essential amino acids. Among these *Allium* species, *Allium auriculatum* and *Allium rubellum* found most promising as far as essential and non-essential amino acids composition concerned.

Keywords: High-performance liquid chromatography; *Allium* species; essential and non-essential amino acid; gradient elution; reverse phase

1. INTRODUCTION

The amino acid content, their proportions and digestibility define protein's biological value. The formation of protein depends on the synthesis and expense of free amino acids. Thus the accumulation of free amino acids depends on the rate of their incorporation into proteins. The citrate, glutamate, pyruvate etc. formed in TCA cycle, undergo amination and transamination to form amino acids which then combine to form proteins[1]. The amino acid and their oxygenases play an essential role in the prevention of several diseases viz; inflammations, cancer, bacterial infections[2,3]. The essential amino acids are important, since, they are taken from food and are essential in the synthesis of proteins and are precursors in the formation of many secondary metabolites, which participate in cell signalling, gene expression and homeostasis regulation[4], protein phosphorylation, synthesis of hormones and antioxidant capacity[5,6]. The non-essential amino acids are also important for growth and maintenance, but they may form with in the body from intermediary products of carbohydrate and fat metabolism[1]. The amino acids also participate in various physiological processes such as skeletal muscle function, atrophic conditions, sarcopenia and cancer[7-10]. The amino acids are the biologically active compounds naturally occur in the food items, which affects food quality[11]. Several investigative methods have been recommended for the amino acids analysis, containing high-performance liquid chromatography (HPLC), capillary electrophoresis and gas chromatography. To analyse the quantity of amino acids through HPLC precolumn derivatisation is required, followed by separation through reversed-phase HPLC. High-performance liquid chromatography has superior resolving power due to its high-pressure separation condition, making it the most preferred chromatographic technique for the separation of biological compounds[12].

Allium is an important genus of family Amaryllidaceae, has 700 species, confined chiefly to the northern temperate and alpine zones of the world[13]. Hooker in 'Flora of British India' has recorded 31 *Allium* species from India. In the western Himalayan regions wild *Allium* species viz. *A. auriculatum, A. ampeloprasum, A. ascalonicum* and *A. rubellum* are commonly used for flavouring and garnishing dal and curries. The leaves of these plants contain an appreciable amount of carbohydrates, minerals, fat, dietary fibre and protein. Hence, they are very popular among local people. Fredotovic[14], *et al*. reported the composition of amino-acids and volatile sulfur compounds in *A. cornutum* and they compared them with the corresponding components of *A. cepa*. Due to the presence of a substantial amount of nutrients, these less explored *Allium* species can

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contribute to meet out the nutritional security of the local population of high-altitude area of western Himalayas. Since, these less explored *Allium* species of western Himalayan regions have not been studied so far. Hence, the aim of the present study was to identify the variation in amino acids composition in four *Allium* species.

2. MATERIALS AND METHODS

2.1 Collection of Plant Material

A. auriculatum Kunth*, A. ampeloprasum* L.*, A. ascalonicum* L and *A. rubellum* M. Bieb. were collected from high altitude region of Auli (Joshimath) (9000' altitude) Distt. Chamoli (Uttarakhand), India. The plant identified from the Botanical Survey of India (BSI), Dehradun (Voucher accession No. 87001 and 50017). The collected samples then were grown at Defence Institute of Bio-energy research, field station, Auli for further multiplication. The plant samples (leaves) collected in the month of May-June, at full maturity stage and dried for further studies.

2.2 Reagents

Amino acid standards (L-Aspartic acid, L-Glutamic acid, L-Serine, L-Glycine, L-Histidine, L-Arginine, L-Threonine, L-Alanine, L-Proline, L-Tyrosine, L-Valine, L-Methionine, L-Cysteine L-Isoleucine, L-Leucine, L-Phenylalanine, L-Lysine) procured from Merck chemicals, Germany. HPLC grade, the two eluants A (0.05M Ammonium acetate aqueous buffer) and B (60% acetonitrile) purchased from Water Corporation, USA.

2.3 Determination of Amino acid HPLC

2.3.1 HPLC Instrumentation

Amino acids were analysed using Water's quaternary gradient HPLC by Pico-Tag 2, Bidlingmeyer[15], *et al.* methodan integrated technique for pre-column derivatisation of amino acids using phenylisothiocyanate (PITC). Water's quaternary gradient HPLC consist of Water's 600 pump, 717 Autosampler, 996 Photodiode array detectors. The Chromatographic separation of the hydrolysates performed by the reverse-phase Pico-tag column C_{18} (3.9×300 mm) at 40 °C and wavelength of 254 nm.

2.3.2 Derivatisation Mixture

The procedure used was a modification of the method of Elkin[16], *et al.* The derivatisation mixture 1000 µL prepared by ethanol-water-PITC-TEA (7:1:1:1 v/v). The derivatisation mixture tubes were agitated and kept for 20 min at room temperature.

2.3.3 Preparation of Samples

100 mg dried leaves was dissolve in 6 ml 6N HCl. The solution digested at 120 °C for 20-24 h. After digestion test tube dried and 1ml of derivatising reagent added with vigorous shaking for 15 min. After that, the solution kept for incubation at room temperature, and 2.5 mL of hexane added to remove PITC. After shaking hexane layer was removed. 50 µL of the sample taken from the aqueous layer, and then 1500 µL diluents added, shaken properly and filtered through PTFE 13 µ membrane. Further, 25 µL of the filtrate taken for HPLC analysis.

2.3.4 Preparation of Amino Acid Standard

Amino acid standard (100 µL) (Merck chemicals, Germany) was dissolve in 400 µL derivatisation mixture. The mixture was incubated at room temperature for one hour after that 1ml of hexane added to remove PITC, shaken properly and rejected organic layer. 5 µl from aqueous layer has taken and diluted with water to 200 mL. Further, 25 µL injection volume taken for HPLC analysis[17].

2.3.5 Reverse Phase HPLC Separation Procedure

The Chromatographic separation of the hydrolysates performed by the reverse-phase Pico-tag column C_{18} (3.9×300 mm) at 40 °C and wavelength of 254 nm using a gradient elution with HPLC system (Water's quaternary gradient HPLC), the gradient elution program represented in Table 1. Water Corporation, USA, supplied Pico-Tag® Eluent A (0.05M Ammonium acetate aqueous buffer of pH 6.8) and B (acetonitrile-water 60:40 v/v)[18].

Table 1. Gradient elution used for the separation of PITC-Amino Acids

Time (min)	Flow (ml/ min)	Eluent A (%)	Eluent B (%)
-		100	0
10.00	1.00	54	46
10.50	-	0	100
11.50	-	0	100
12.50	-	0	100
20.00	-	100	0
25.00	-	100	0

*The system was run at constant temperature 40 °C. In gradient program, Eluent A was 0.05 M ammonium acetate (pH 6.8), and Eluent B was acetonitrile-water 60:40 v/v. The phosphoric acid used to adjust the pH of eluent A and B. The reverse-phase Pico-tag column was used.

3. RESULTS AND DISCUSSION

The amino acid composition of *Allium* species summarised in Table 2. The amino acids profile of *Allium* species represented in the chromatogram (Figs. 4 -8). Total seventeen amino acids were detected, and the separation of the amino acids from the samples, reasonably resolved. All essential amino acids (Fig. 1), i.e. methionine, lysine, arginine, leucine, phenylalanine, isoleucine, valine and threonine and nine non-essential amino acids (Fig. 2) found in these *Allium* species. Total amino acids found in leaves of *Allium auriculatum* was 35.36 µmol./g, *A. ampeloprasum* 6.077 µmol./g, *Allium ascalonicum* 16.626 µmol./g and in *Allium rubellum* 5.24 µmol./g on a dry basis. Out of total amino acid contents present in these species 16.455 µmol./g, 1.825 µmol./g, 6.996 µmol./g, and 1.523 µmol./g amino acids were made up of essential amino acids in the *A. auriculatum, A. ampeloprasum, A. ascalonicum* and *A. rubellum* respectively (Fig. 3). Schuphan & Schwerdtfeger[19] reported that arginine and glutamic acid found in abundant quantity, than other amino acids in *Allium cepa.*

Table 2. Quantitative Screening of Amino acids in *Allium* Species

Amino acids	Retention time (Min.)	Quantity of Amino-acid in Allium species (µmol./g) D.W.							
		Allium auriculatum		*Allium ampeloprasum*		*Allium ascalonicum*		*Allium rubellum*	
		Conc. (µmol./g)	% of TAA	Conc. (µmol./g)	% of TAA	Conc. (µmol./g)	% of TAA	Conc. (µmol./g)	% of TAA
Aspartic acid (Asp) [b]	1.613	1.650	4.679	0.788	**12.966**	0.825	4.962	0.446	8.511
Glutamic acid (Glu) [b]	1.755	1.910	5.416	0.466	**7.668**	0.075	0.451	0.051	0.973
Serine (Ser) [b]	3.362	1.135	**3.218**	0.097	1.596	0.411	2.472	0.049	0.935
Glycine (Gly) [b]	3.666	1.092	3.096	0.062	1.020	0.292	1.756	0.195	**3.721**
Histidine (His) [b]	4.044	1.206	3.420	0.197	3.241	0.358	2.153	0.265	**5.057**
Arginine (Arg) [a]	4.612	2.030	5.757	0.402	6.615	1.370	**8.240**	0.312	5.954
Threonine (Thr) [a]	4.834	1.485	**4.211**	0.074	1.217	0.166	0.998	0.030	0.512
Alanine (Ala) [b]	5.022	6.118	17.35	1.501	24.699	5.157	**31.029**	1.151	21.965
Proline (Pro) [b]	5.346	2.912	8.258	1.031	**16.965**	1.930	11.608	0.526	10.038
Tyrosine (Tyr) [b]	7.310	1.632	**4.628**	0.093	1.530	0.529	3.181	0.075	1.431
Valine (Val) [a]	7.907	4.000	11.344	0.581	9.560	2.011	**12.095**	0.486	9.274
Methionine (Met) [a]	8.319	0.566	**1.605**	0.022	0.362	0.107	0.643	0.016	0.305
Cysteine (Cys) [b]	9.096	1.150	3.261	0.025	0.411	0.052	0.312	0.957	**18.263**
Isoleucine (Ileu) [a]	9.397	2.472	**7.610**	0.258	4.245	0.907	5.455	0.208	3.969
Leucine (Leu) [a]	9.557	2.966	**8.411**	0.310	5.101	1.391	8.366	0.347	6.622
Phenylalanine (Phe) [a]	10.348	1.565	**4.438**	0.103	1.694	0.521	3.133	0.062	1.183
Lysine (Lys) [a]	11.231	1.371	**3.888**	0.067	1.102	0.524	3.151	0.064	1.221
Total	**TAA**	**35.26**		**6.077**		**16.626**		**5.24**	
	TEAA%		**46.67**		**29.87**		**42.08**		**29.10**
	TNEAA%		**53.33**		**70.13**		**57.92**		**70.90**

a *-Essential amino acids,* ***b*** *- Non- essential amino acid,* ***Conc.-*** *Concentration of Amino acid,* ***TAA*** *-Total amino acids,* ***TEAA*** *- Total essential amino acids,* ***TNEAA*** *- Total non-essential amino acids.*

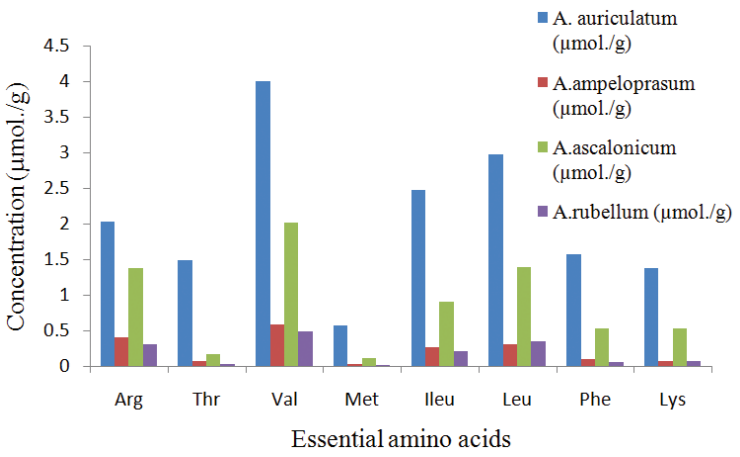

Figure 1. Essential amino acids present in the different *Allium* species.

Fredotovic[14], *et al.*, reported amino acid composition of two *Allium* species *viz. A. cornutum* and *A. cepa* extracts and observed that the most abundant amino acids in both onions were glutamic acid and arginine[14].

The ratio of essential amino acids to total amino acid is 1:2.14, i.e. almost half (46.67 %) of the total amino acid in *Allium auriculatum* consist of essential amino acids. The ratio of essential amino acids to non-essential amino acids was 1:1.14. The results also indicated that the *Allium auriculatum* is rich in alanine, valine, leucine, proline, isoleucine, arginine, glutamic acid, tyrosine and threonine. Methionine, glycine, cysteine, serine and histidine were present in lower amounts than the other amino acids. According to a study determination of amino acids in green beans by derivatisation with phenylisothiocyanate and high-performance liquid chromatography with ultraviolet detection, in green

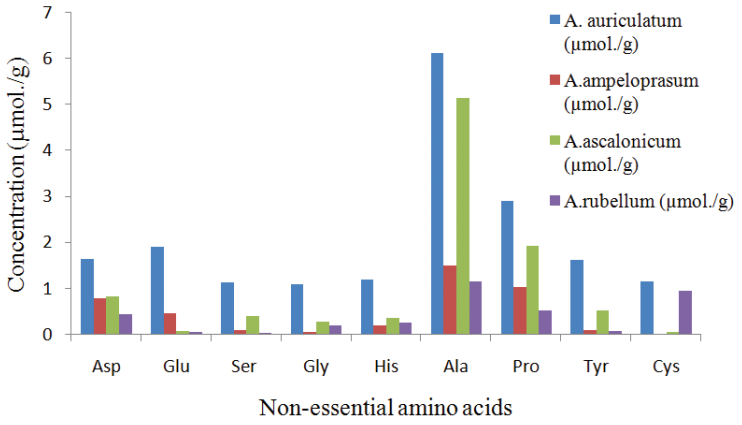

Figure 2. Non-essential amino acids present in the different *Allium* species.

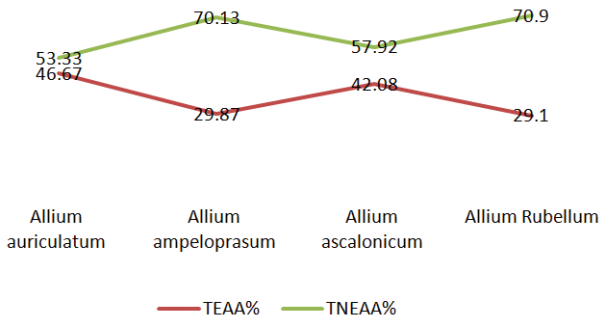

Figure 3. Total essential and non-essential amino acids present in the different *Allium* species.

beans, aspartic acid 2.44g/100g, glutamic acid 1.6g/100g and arginine 1.29g/100g were major amino acids out of seventeen amino acids analysed[20].

In *A. ampeloprasum,* the ratio of essential amino acids to total amino acid was 1: 3.35, i.e. almost one-third of total amino acids of *A. ampeloprasum* consist of essential amino acids. The result also indicated that the ratio of essential amino acids to non-essential amino acids was 1:2.35. This species was found rich in alanine, proline, aspartic acid, valine and glutamic acid, Ma[21], *et.al.* reported that chromatographic method for determination of the free amino acid contents of Chamomile flowers and observed that the alanine, proline, leucine found in abundant amount, whereas the tyrosine and methionine were present in least quantity.

The ratio of essential amino acids to the total amino acid in *Allium ascalonicum* found 1:2.38, slightly lower than half of the total amino acids made up of essential amino acids. The results also indicated that the ratio of essential amino acids to non-essential amino acids was 1:1.38. The plant was rich in alanine, valine, proline, arginine, leucine and aspartic acid. Cysteine, glutamic acid, methionine and threonine were present in lower quantities than the other amino acids.

In *Allium rubellum,* the ratio of essential amino acids to total amino acids was found 1: 3.44 and the ratio of essential amino acids to non-essential amino acids is 1:2.44. This *Allium* sp. was rich in alanine, cysteine, proline and valine. While,

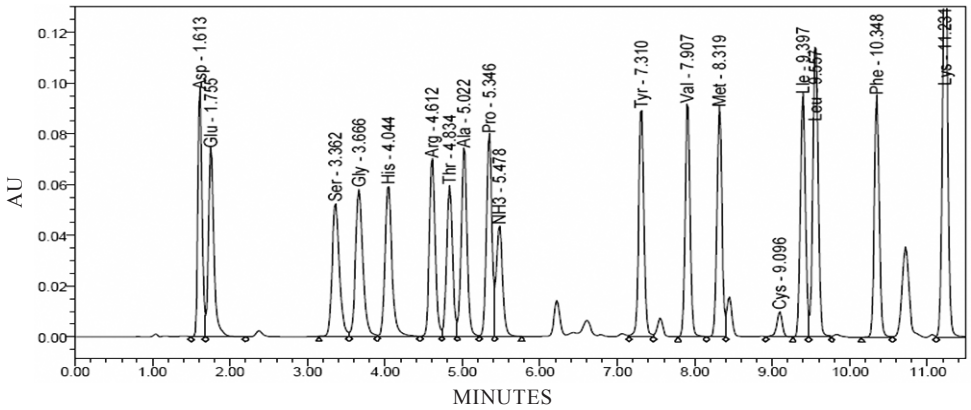

Figure 4. HPLC chromatogram of standard amino acids.

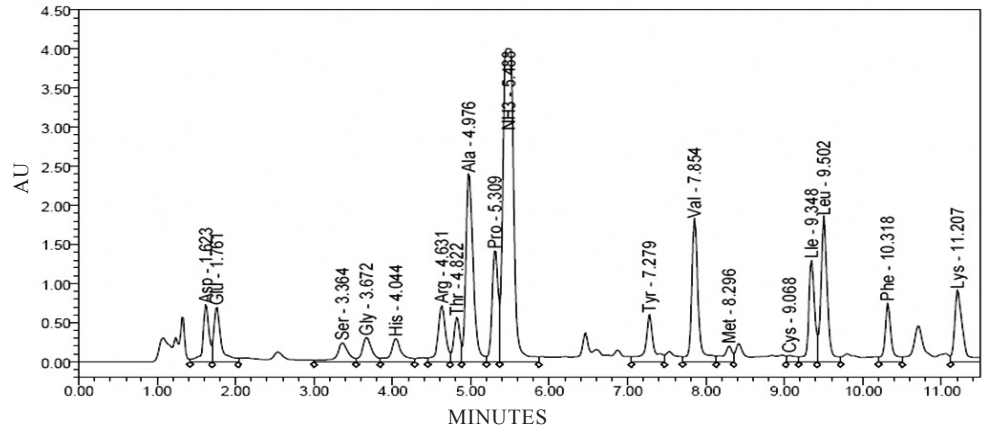

Figure 5. HPLC chromatogram of *Allium auriculatum.*

methionine, threonine, serine and glutamic acid, were present in lower amounts, in comparison to the other amino acids. Due to presence of substantial amount of nutrients, these less explored *Allium* species can contribute to meet out the nutritional security of local population and therefore recommended for more and more use. Moran-Palacio[6], *et.al.* reported that fifteen amino acids, detected in medicinal plant samples, with aspartic acid, glutamic acid, serine, glycine, alanine and leucine (43.55 nM, 44.84 nM, 29.60 nM, 58.17 nM, 43.05 nM, and 38.73 nM, respectively) presenting the highest concentrations. The chromatograms of standard amino acids and *Allium* species are represented in Figs. 4-8.

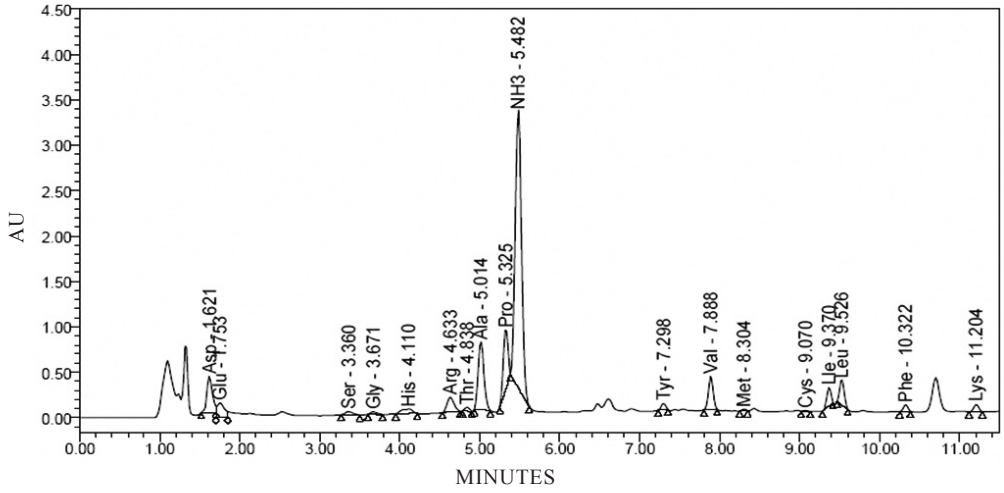

Figure 6. HPLC chromatogram of *Allium ampeloprasum.*

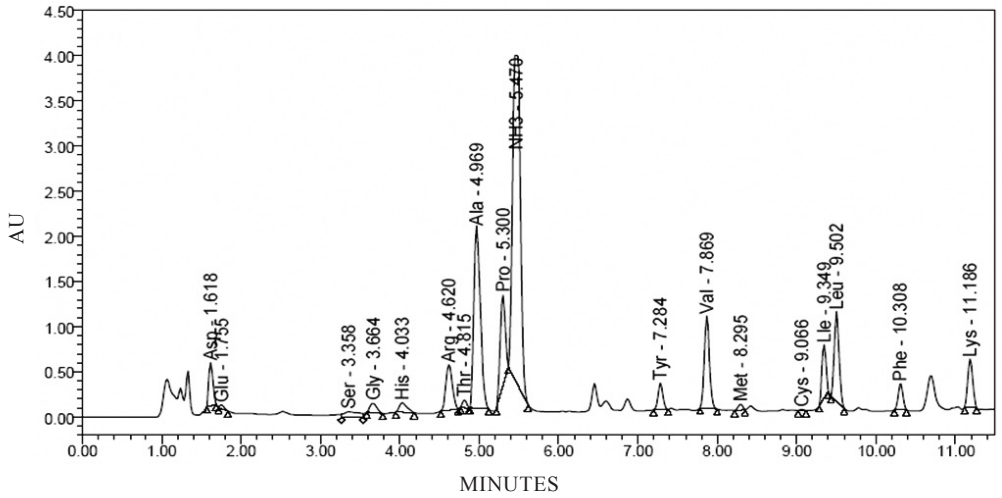

Figure 7. HPLC chromatogram of *Allium ascalonicum.*

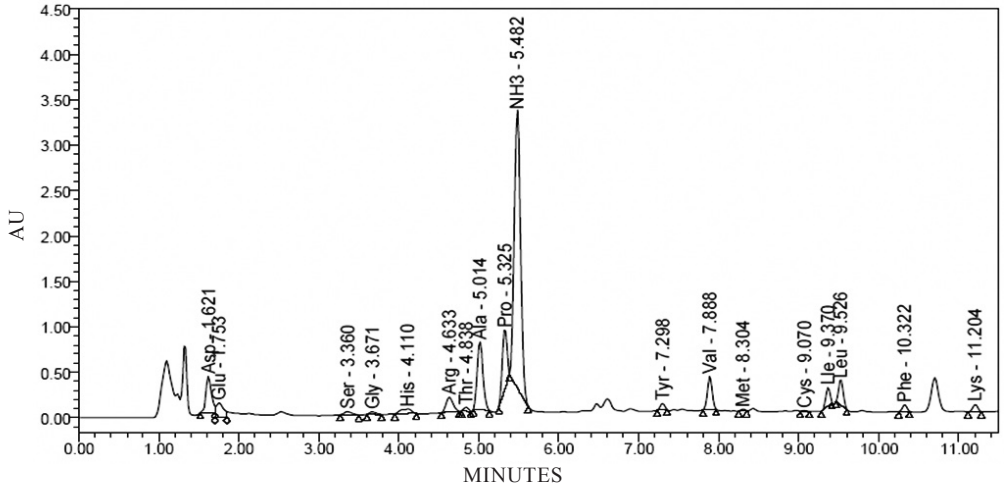

Figure 8. HPLC chromatogram of *Allium rubellum.*

4. CONCLUSION

The high-performance liquid chromatography (HPLC) method is reliable for the determining of the free amino acids in *Allium* species. The amino acids like aspartic acid, glutamic acid, arginine, threonine, proline, valine, alanine, cysteine and leucine were present in a substantial quantity in all these *Allium species.* These wild *Allium* species are also a rich source of essential amino acids and non-essential amino acids. Among four *Allium* species, *Allium auriculatum* and *Allium rubellum* found most promising as far as essential and non-essential amino acids composition concerned. These *Allium* species can be promoted for commercial scale cultivation so that these species can contribute in the nutritional security for the local population of high altitude areas as far as amino acids compositional concerned.

CONFLICT OF INTEREST

There is no conflict of interest among the authors.

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In the current study, she has provided active and continuous guidance for this work.