

Determination of Amino Acid Composition in the *Harpagophytum procumbens* Root

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ABSTRACT: The amino acid composition of the roots of *Harpagophytum procumbens* was investigated by the method of high performance liquid chromatography (HPLC) with preliminary derivatization. Sixteen free and thirteen bound amino acids were quantitatively determined. The content of protein-bound amino acids was calculated.

Key words: amino acids, *Harpagophytum procumbens*, high performance liquid chromatography.

INTRODUCTION

Traditional African plant *Harpagophytum procumbens* (Devil's claw) of the Pedaliaceae family is used to treat joints and spinal disorders, diseases of gastrointestinal tract, liver, kidneys, and bladder for many years. Roots of *H. procumbens* contain a large number of biologically active substances such as: iridoid glycosides (mainly harpagosides, harpagide and procumbide), sugars (tetrasaccharides, stachyose), triterpenoids (oleanolic, ursolic acids), phytosterols (β -sitosterol), aromatic acids (caffeic, cinnamic, chlorogenic), flavonoids (luteolin, kaempferol). Iridoid glycosides, which exert anti-inflammatory and analgesic effects, are considered to be the main active substances of the plant.¹

Analysis of the research results showed that the active components of the *H. procumbens* extract suppress the inflammation by reducing the release and synthesis cyclooxygenase (COX) enzyme involved in the inflammatory cascade. The blockade of TNF- α , IL-1 β , and IL-6 synthesis simultaneously inhibits the production of matrix metalloproteinases in chondrocytes that are involved in degeneration of cartilage tissue. The published results of the previous

study indicate that the iridoid glycosides (harpagoside and harpagide) shown on figure 1 are highly selective COX-2 inhibitors with the similar binding power to classical non-steroidal anti-inflammatory drugs.^{2,3}

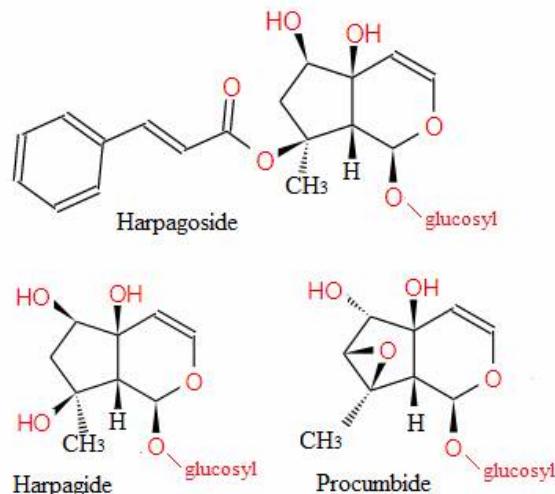


Figure 1. Iridoid glycosides of devil's claw

Currently, preparations based on the *H. procumbens* extract are considered as a safe and economically justified alternative to chemically synthesized symptom-modifying drugs.⁴

One of the promising directions in modern medicine and pharmacy is a development of co-

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formulated herbal preparation for the treatment and prophylaxis of musculoskeletal diseases. The synergetic effect of active substances allows to provide the necessary pharmacological action and reduce the effective dose of active substances, eliminating the risk of undesirable side effects. Therefore, we developed a co-formulated drug preparation in the form of soft gelatin capsules containing the *H. procumbens* extract and chondroprotectors (glucosamine sulfate and chondroitin sulfate).^{5,6}

In this research, we studied the amino acid composition of *H. procumbens* in order to expand the information about the chemical composition of raw materials and its further use for standardization of raw materials, intermediate products and the resulting dosage form.

Nowadays, liquid chromatography (HPLC) is the most common method for the amino acids determination.⁷ The absence of chromophore groups in most amino acid molecules requires the derivatization stage for their determination using fluorescence detector. The most common methods of HPLC determination of amino acids are cation-exchange chromatography with post-column derivatization and reverse-phase chromatography with pre-column derivatization. In pre-column derivatization, o-phthalic aldehyde (OPA), phenylisothiocyanate, dansyl chloride, 9-fluorenylmethoxycarbonyl chloride (FMOC) are most often used.⁸⁻¹⁰

MATERIALS AND METHODS

Preparation of standard solutions

Amino acid standards (10 to 1 nmol/μl). Solutions of 16 amino acids in five concentrations were collected from Agilent for calibration curves. Each 1 ml ampoule of standards 5061-3334 was divided into 100 μl portions in conical vial inserts.

Derivatization reagents. Borate buffers, OPA and FMOC solutions were supplied by Agilent. These reagents were transferred from their container into an autosampler vial.

FMOC reagent. FMOC, contained in a 1 ml ampule, was divided into 10 aliquots (100 μl each). Each aliquot was put into a vial with a vial insert. The vials were capped and stored at 4°C. Under these storage conditions, the aliquoted solutions are stable for 7-10 days.

OPA reagent. OPA, contained in a 1 mL ampule, was divided into 10 aliquots (100 μl each). Each aliquot was put into a vial with a vial insert. The vials were capped and stored at 4°C. Under these storage conditions, the aliquoted solutions are stable for 7-10 days.

Preparation of sample solutions

Analysis of free amino acids in the roots of *H. procumbens*. 0.148 g of a powdered raw material (passed through a sieve with a mesh size 355 μm) was placed in a vial, 4 ml of 0.1 N hydrochloric acid solution was added and kept in an ultrasonic bath at 80°C for 3 hrs. Then, 0.5 ml aliquot of a centrifuged extract was evaporated on a rotary evaporator, washed three times with distilled water in order to remove hydrochloric acid. The dry residue was resuspended in 0.5 ml of distilled water and filtered through regenerated cellulose (RC) membrane filters (13 mm i.d., 20 μl).

Test solution of total amino acids in the roots of *H. procumbens*. 0.043 g of a powdered raw material (passed through a sieve with a mesh size 355 μm) was placed in a vial, then 1 ml of 6 N hydrochloric acid solution was added and thermostated at 110°C. Hydrolysis was carried out for 24 hours. To remove hydrochloric acid, an aliquot of centrifuged hydrolysate was evaporated on a rotary evaporator. The dry residue was resuspended in 0.5 ml of distilled water and filtered through RC-membrane filters (13 mm i.d., 20 μl).

After that, 0.5 ml of centrifuged hydrolysate was evaporated on a rotary evaporator. The resulting pellet was resuspended in 2.5 ml of distilled water and filtered through RC-membrane filters (13 mm i.d., 20 μl) into vials.

The hydrolyzed samples or solutions of standard amino acid mixture were automatically derivatized

with OPA and FMOC by programming the autosampler.

Chromatographic experiments were performed on Agilent 1200 (Agilent technologies, USA) liquid chromatograph. Separation of amino acids in the investigated samples was achieved on a Zorbax AAA column (150 mm × 4.6 mm × 3 μm). Mobile phase A was 0.040 mole buffer solution of sodium hydrogen phosphate adjusted to pH 7.8 ± 0.05 with orthophosphoric acid; mobile phase B - acetonitrile : methanol:water (45 : 45 : 10, v/v/v). The separation of the amino acids in investigated samples was performed in a gradient elution mode with a constant flow rate 1.5 ml/min. Temperature of the column thermostat was at 40°C. Excitation (Ex) and emission (Em) wavelength were 340 nm and 450 nm, correspondingly. Gradient program is shown in table 1.

Identification of amino acids was carried out by comparing the amino acids retention times (RT) in sample with their RT in standards (Agilent 5061-3334). The content of bound amino acids was determined by subtracting the content of free amino acids from their total content.

Validation of HPLC method for the determination of amino acids. Validation of the

above mentioned method was performed according to the following characteristics: specificity, linearity, accuracy, precision, intermediate precision and robustness in a certain range of concentrations.

Table 1. Gradient conditions

Time (min)	% B
0.0	2
0.35	2
13.4	57
13.5	100
15.7	100
15.8	2
18.0	2
20.0	end

RESULTS AND DISCUSSION

The specificity study of the present method was performed by a comparison of the retention time (RT) of five different concentrations of the standard mixture of amino acids (Table 2).

As can be seen from table. 2, the relative standard deviation (RSD) for RT values of solutions for the standard sample of the amino acid mixture was from 0.01 to 0.09%. This demonstrates the specificity of the method. The chromatogram of a standard mixture of amino acids is shown in figure 2.

Table 2. The retention time for different concentration of amino acids in standard mixture (n = 5)

№	Amino acids	Concentration, nmol/cm ³					Average	RSD / %
		10	25	100	250	1000		
1	L-Aspartic	2.528	2.529	2.528	2.531	2.534	2.530	0.09
2	L-Glutamic	4.871	4.872	4.874	4.867	4.868	4.870	0.05
3	L-Serine	7.358	7.354	7.363	7.361	7.363	7.360	0.05
4	L-Histidine	8.206	8.208	8.202	8.204	8.209	8.206	0.03
5	Glycine	8.557	8.568	8.564	8.562	8.567	8.564	0.05
6	L-Threonine	8.728	8.734	8.733	8.739	8.737	8.734	0.04
7	L-Arginine	9.385	9.389	9.391	9.393	9.39	9.390	0.03
8	L-Alanine	9.946	9.949	9.951	9.958	9.956	9.952	0.04
9	L-Tyrosine	10.997	10.992	10.994	10.996	10.998	10.995	0.02
10	L-Valine	12.887	12.889	12.883	12.887	12.881	12.885	0.02
11	L-Methionine	13.039	13.036	13.038	13.031	13.033	13.035	0.02
12	L-Phenylalanine	14.185	14.187	14.184	14.182	14.186	14.185	0.01
13	L-Isoleucine	14.381	14.385	14.388	14.383	14.385	14.384	0.02
14	L-Leucine	14.975	14.978	14.979	14.971	14.973	14.975	0.02
15	L-Lysine	15.252	15.261	15.253	15.259	15.254	15.256	0.02
16	L-Proline	18.717	18.709	18.713	18.719	18.725	18.717	0.03

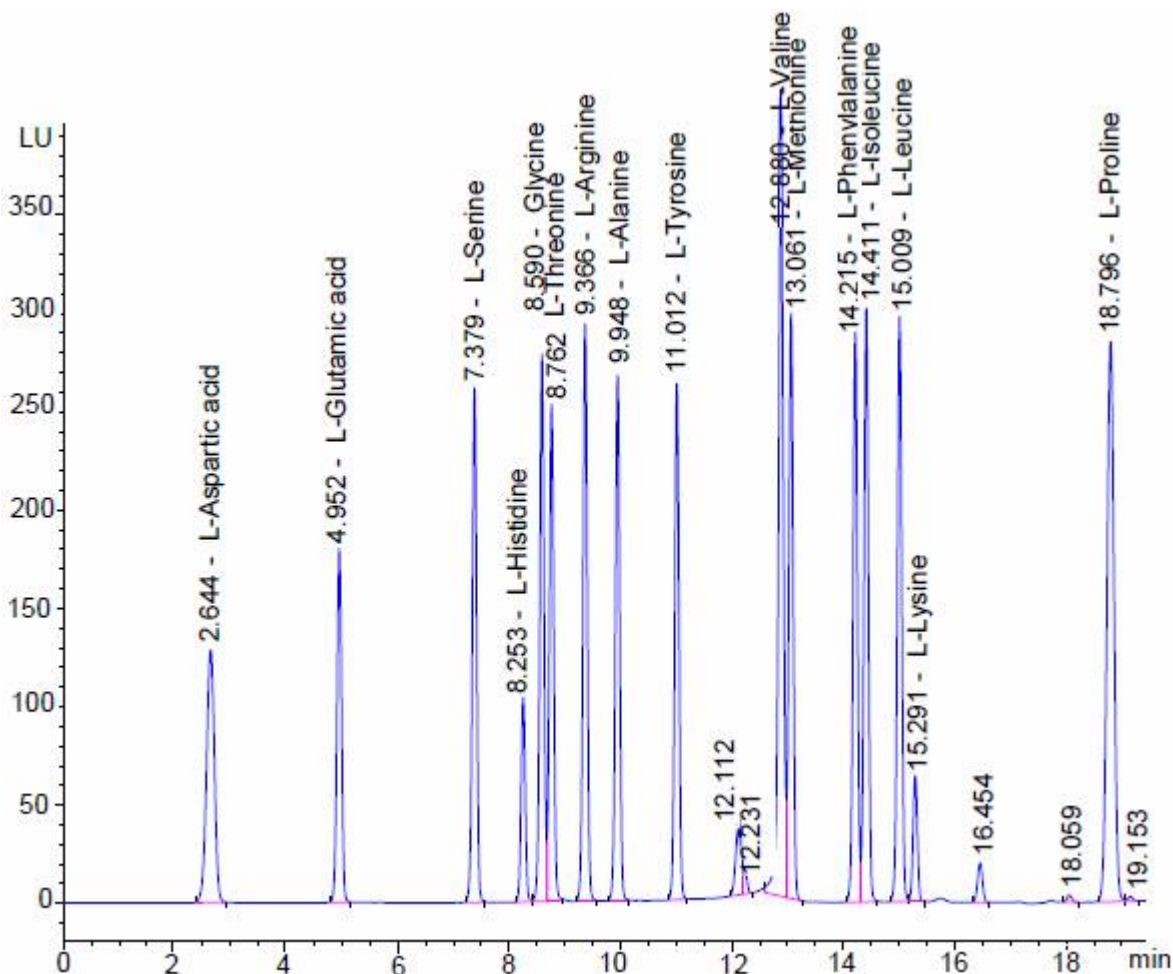


Figure 2. Chromatogram of the standard amino acid mixture.

As a result of the study, 6 free and 13 bound amino acids were identified in the roots of *H. procumbens*. The results of amino acid analysis of the investigated raw material: retention time and quantitative content of amino acids ($\mu\text{g}/\text{mg}$) on dry basis ($n = 5$) are given in table 3.

Obtained experimental data indicate that the roots of *H. procumbens* contain a fairly low content of free amino acids ($2.21 \mu\text{g}/\text{mg}$), and main content is represented by bound amino acids ($12.15 \mu\text{g}/\text{mg}$) i.e., the quantitative content of amino acids after total hydrolysis increased by more than 6 times. The highest content is characterized by L-Aspartic, L-Glutamic and L-Proline. Amino acids such as L-Histidine, L-Arginine and L-Methionine present only

in the free state. The chromatogram of the free and total amino acids of the studied sample is shown in figures 3 and 4, respectively.

Biological activity of the identified amino acids is well-known: L-Aspartic acid provides the conversion of carbohydrates into muscle energy. Therefore it is used as additives for athletes during periods of increased physical activity. It also increases the activity of the immune system, raises endurance and retains the ability to work. L-Arginine is a building block of many proteins and actively participates in the regulation of the body metabolism; activates regeneration processes in the post-traumatic period during healing of fractures, healing of trophic ulcers; participates in the processes of collagen

formation. L-Proline is one of the major amino acids building material of the body, bones, tendons.¹¹⁻¹³ used for collagen synthesis. Collagen is the main

Table 3. The quantitative composition of amino acid in *H. procumbens* roots (n=5)

No.	Amino acids	Free amino acids		Total amino acids		Bound amino acids
		RT [min]	Amount [mkg/mg]	RT [min]	Amount [mkg/mg]	Amount [mkg/mg]
1	L-Aspartic	2.533	0.26±0.01	2.54	1.53±0.01	1.26±0.04
2	L-Glutamic	4.87	0.21±0.03	4.869	2.97±0.04	2.76±0.01
3	L-Serine	7.361	0.03±0.01	7.351	1.01±0.03	0.98±0.03
4	L-Histidine	8.208	0.00	8.208	0.00	0.00
5	Glycine	8.562	0.00	8.561	0.70±0.01	0.70±0.01
6	L-Threonine	8.733	0.00	8.733	0.66±0.03	0.66±0.01
7	L-Arginine	9.391	1.55±0.01	9.373	1.55±0.02	0.00
8	L-Alanine	9.951	0.19±0.04	9.924	0.85±0.01	0.66±0.04
9	L-Tyrosine	10.991	0.00	10.995	0.35±0.01	0.35±0.01
10	L-Valine	12.88	0.03±0.02	12.853	0.74±0.02	0.71±0.02
11	L-Methionine	13.031	0.00	13.031	0.00	0.00
12	L-Phenylalanine	14.184	0.00	14.191	0.71±0.03	0.71±0.05
13	L-Isoleucine	14.38	0.00	14.386	0.64±0.04	0.64±0.01
14	L-Leucine	14.978	0.00	14.983	0.96±0.01	0.96±0.02
15	L-Lysine	15.252	0.00	15.256	0.73±0.03	0.73±0.01
16	L-Proline	18.717	0.00	18.718	1.03±0.01	1.03±0.04
Sum of amino acids			2.21		14.43	12.15

Note - RSD is not more than 2.0%, range for confidence interval: ± 0.01 - 0.5.

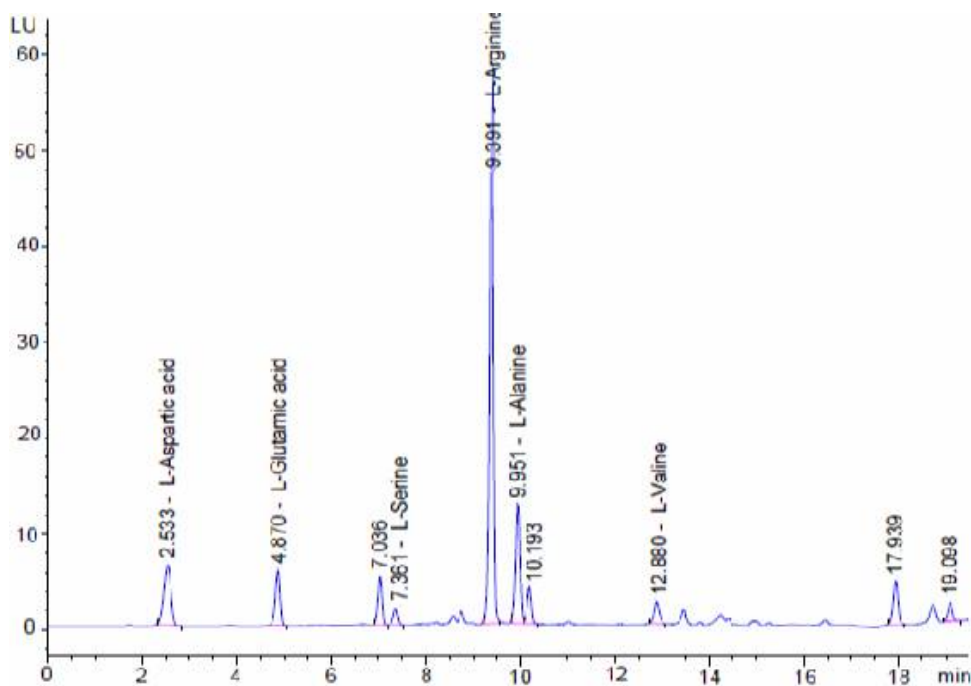


Figure 3. Chromatogram (HPLC) of the free amino acids in the roots of *Harpagophytum procumbens*.

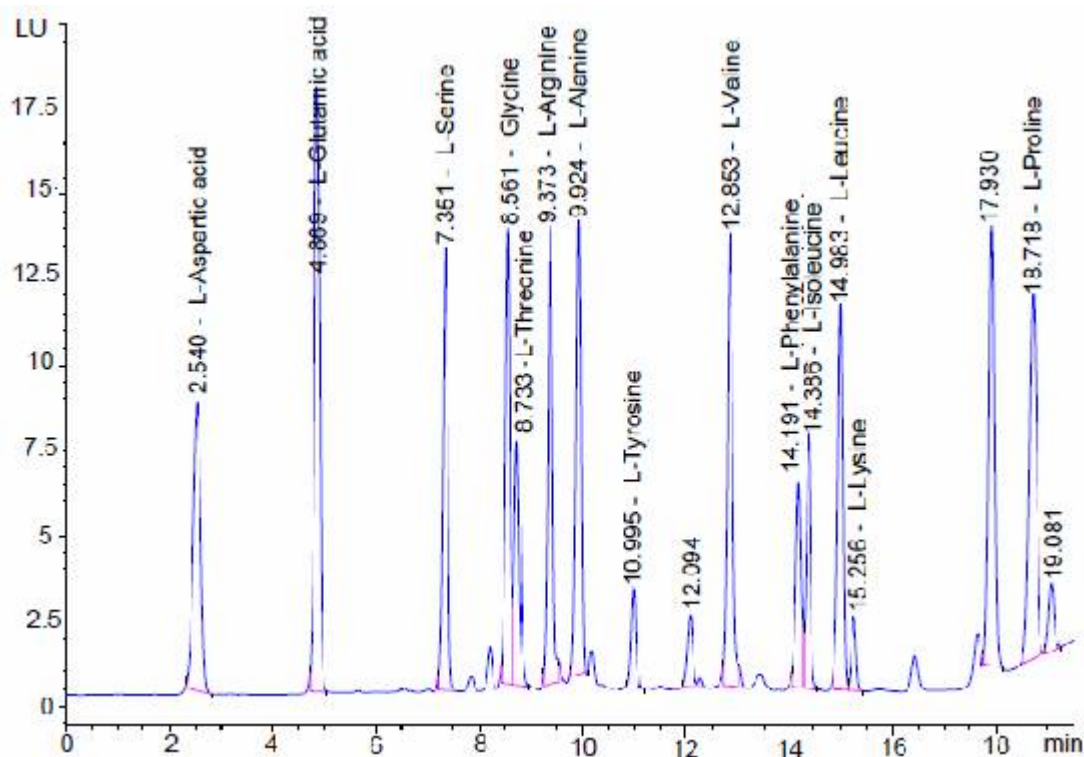


Fig. 4. Chromatogram (HPLC) of the total amino acids in the roots of *Harpagophytum procumbens*.

CONCLUSIONS

The qualitative composition and quantitative content of free, bound and total amino acids were determined in the roots of *H. procumbens* using the high-performance liquid chromatography method. 16 free and 13 bound amino acids have been identified. Main components of free amino acids are L-Arginine and L-Aspartic. The major part of the bound amino acids amount is L-Aspartic, L-Glutamic and L-Proline and their content on dry matter is 1.26, 2.76, 1.03 $\mu\text{g}/\text{mg}$, respectively. The quantitative content of amino acids after complete hydrolysis increases 6.5 times.

The obtained results of the determination of amino acid composition in the roots of *H. procumbens* indicate the possibility of application of the investigated raw material as active substance for herbal preparation to treat diseases that are caused by a violation of the collagen synthesis in the human body, in particular arthroses, dermatoses and various forms of periodontal disease.

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