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Separation, identification and quantitative determination of free amino acids from plant extracts

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Abstract

This research presents the results obtained from the separation, identification and quantitative determination of free amino acids from *Gingko biloba* and *Hedera helix* leaf extracts, using three modern techniques: thin layer chromatography (TLC), high performance liquid chromatography (HPLC), and gas chromatography-mass spectrometry. The presence of the determined amino acids explains the utilisation of *G. biloba* and *H. helix* leaf extracts in cosmetic and pharmaceutical products.

Keywords: Plant extract; Chromatographic methods; Separation identification; Quantitative determination

1. Introduction

In the last decade the pharmaceutical and cosmetic industries have been extensively using plant extracts. The isolation, identification and quantitative determination of the active components from plants with biological activity are interesting for studying the structure–activity relationship.

The search for bioactive materials can be assisted by chromatographic methods which allow the localisation of some active compounds such as: tannin, sugars, peptides, organic acids, flavones, amino acids etc. These methods also allow for the identification of metabolites and their intermediaries which do not display biological activity [1,2].

Thin layer chromatography (TLC) is often used for the separation and identification of amino acids from plants. This method has many advantages such as: multiple samples can be analysed simultaneously, the short time required, and the low detection limits [3,4]. As stationary phases silica gel [5], modified silica gel [6] or polyamides [7] can be used and the most frequently used mobile phase systems are: *n*-butanol–acetic acid–water, phenol–water, or *n*-butanol–acetic acid–acetone–water.

Reversed phase liquid chromatography based on C18 as a stationary phase which used an acetonitrile–buffer based on acetate [8,9], phosphate [7,10,11], citrate [12] or borate [13,14] as a mobile phase can be also used. Detection has been done either by fluorescent detection after derivatization

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Table 1
The R_f values of amino acids from plant extracts

No.	Amino acid	R_f		Intensity[a]	
		G. biloba	*H. helix*	*G. biloba*	*H. helix*
1	Asparagine	0.22	0.22	++	+++
2	Glutamine	0.77	—	++	—
3	Serine	—	—	—	—
4	Glycine	0.33	0.33	++	++
5	Histidine	—	—	—	—
6	Arginine	—	—	—	—
7	Threonine	—	—	—	—
8	Alanine	0.46	—	+	—
9	Proline	0.51	0.51	+++	+++
10	Tyrosine	0.36	0.36	+	++
11	Valine	—	0.63	—	++
12	Metionine	—	—	—	—
13	Cysteine	—	—	—	—
14	I-leucine	0.30	0.30	++	+
15	Leucine	0.29	0.29	++	+
16	Phenilalanine	0.72	0.72	++	++
17	Lysine	—	—	—	—

[a] +++, high intensity; ++, medium intensity; +, low intensity; —, lack of amino acid.

with phtalic anhydride [10,14], dansyl chloride [7,13,15] or by UV detection [16,17]. Some amino acids can be transformed into hydroxamic compounds and then they can be detected by spraying with 0.1 M $FeCl_3$ solution in 0.1 M HCl [18].

The best results in the quantitative determination of amino acids have been obtained using gas chromatography-mass spectrometry (GC-MS) [19–23].

This paper presents the results of sample analysis by TLC, high performance liquid chromatography (HPLC) and GC-MS, of *Gingko biloba* (Gingkoaceae) and *Hedera helix* (Araliaceae). The amino acid content of these plants have not been determined yet.

2. Experimental

2.1. Extraction of free amino acids

The isolation of amino acids can be carried out by different extraction methods on dry material. Previous researches mention such methods as: extraction with 5% NaCl solution, 75% ethyl alcohol, 0.25% NaOH [5], 0.25 M HCl [19], metasiliconic acid [20] or a $CH_3COOH–HCl–H_2O$ (18:1:1 v/v/v) mixture [7].

In our experiments 0.5 g dry plant was extracted in 10 ml 1% HCl solution. Then a $Na_3P(W_3O_{10})_4$ solution was used for removing the proteins from the extract by precipitation. After centrifugation the solution was passed through an ion exchange Amberlite IR 120H column. The column was eluted with 40 ml 10% ammonia solution. The solution obtained was evaporated to dryness and the residue was redissolved in 1 ml aqueous solution 30% (v/v) iso-propanol.

2.2. TLC

We used 20 cm × 20 cm cellulose plates (CEL 300-10 UV 254, Macherey-Nagel) 0.1 mm thick. The standard solutions of the seventeen essential amino acids (1 mg ml^{-1}) and the extract solutions were applied with a micropipette.

The separation and identification of the free amino acids from the extracts was achieved by TLC with double elution. The elution distance

was 18 cm, in unsaturated *N* chamber using an *n*-butanol–acetone–acetic acid–water (35:35:7:23 v/v/v) mixture [10] as the mobile phase. After the first elution the plates were dried in hot air, then eluted in the same direction, along the same distance and with the same solvent mixture. The detection was carried out by spraying the plates with a ninhydrine solution in *n*-butanol–acetone (1:1 v/v) then dried at 105–110°C for 10–15 min. The identification of the amino acids was achieved by comparing the R_f values and the colours.

2.3. HPLC

Both the amino acids from standard solutions and from plant extracts were transformed in phenyl–tiocarbamilic derivatives.

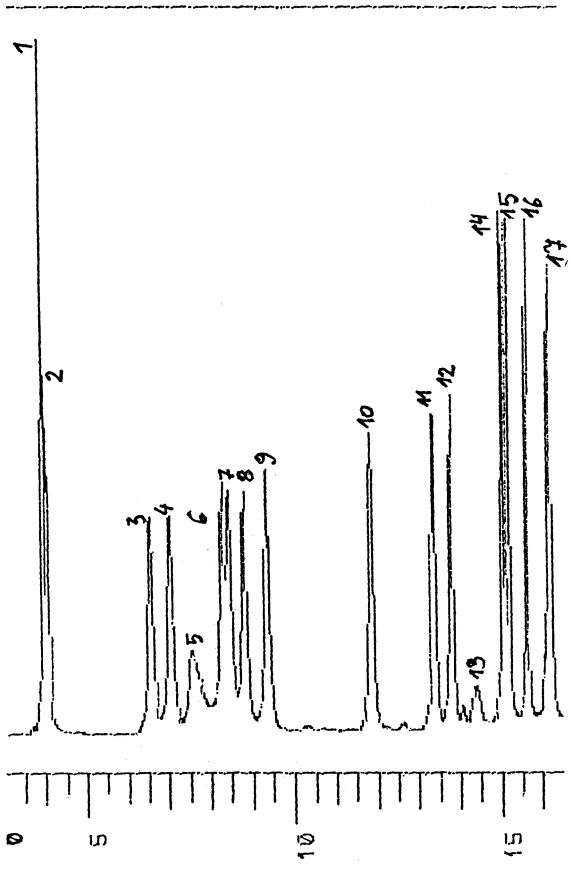

Fig. 1. The HPLC separation of standard amino acid mixtures.

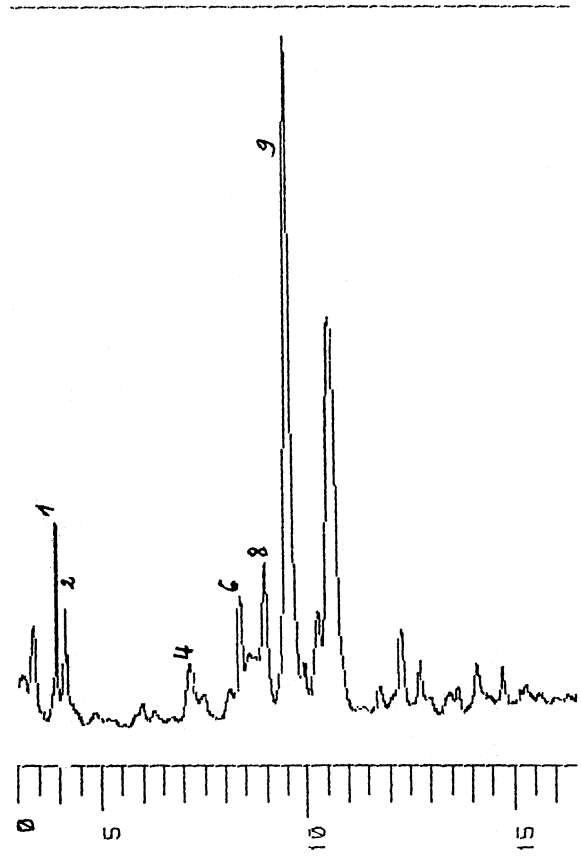

Fig. 2. The HPLC separation of *G. biloba* extract.

The standard mixtures and the plant extracts were analysed by HPLC using a chromatograph (HP 1090 with DAD) with a Lichrospher ODS 100-5 column (250 mm × 4.6 mm i.d.) and a mixture of 0.15 M sodium acetate (pH 6.5)–acetonitrile, concentration gradient 7–20%, 1 ml min^{-1}, temperature 55°C, and UV detection at 254 nm.

2.4. GC-MS

The amino acids were transformed into *N*-trifluoroacetil *n*-butyl esters [24–27] to increase their volatility. Asparagine and glutamine were transformed into aspartic acid and glutamic acid, respectively [20,21]. Hystidine and arginine were difficult to analyse by gas chromatography.

The standard amino acids and trifluoracetic anhydride were obtained from Merck (Darmstadt, Germania) and acetyl chloride was obtained from Fluka (Buchs, Switzerland). Glycine (15 N) was obtained from ITIM (Cluj-Napoca, Romania). Glycine (15 N) was used as the internal standard (10 μg 1 ml^{-1}).

2.4.1. Derivatization

The amino acids were derivatized in two steps in screw-cap tubes. The dry samples were esterified with 0.5 ml esterification reagents (distilled butanol–acetyl chloride (4:1 v/v)) for 1 h at 110°C. The excess reagent was removed by bubbling nitrogen through the mixture. The amino group was acetylated with a 200 μl mixture of trifluoroacetic anhydride (TFAA)–methylene chloride (1:1 v/v) at 60°C for 30 min [22,23]. After cooling, the excess reagent was removed under nitrogen flow and 1 ml ethyl acetate was added.

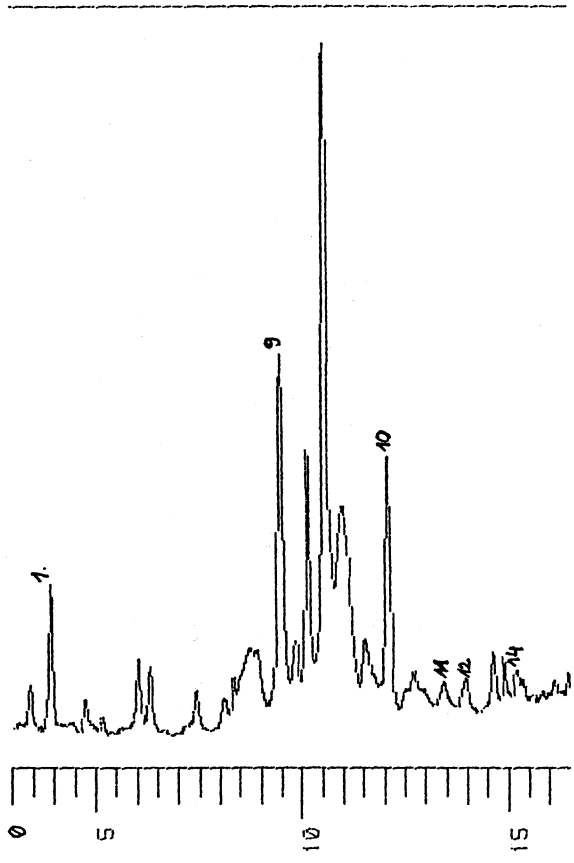

Fig. 3. The HPLC separation of *H. helix* extract.

Table 2
The results of quantitative determination of amino acids from plant extracts

Amino acid	Area (mm^2)	*f*	μg g^{-1}
G. biloba			
Alanine	0.55	0.70	88.63 ± 2
Glycine	0.90	0.34	225.91 ± 4
Leucine	0.96	0.88	95.71 ± 2
I-leucine	1.76	0.80	193.43 ± 3
Proline	27.73	0.74	3289.03 ± 10
Aspartic acid	2.26	0.62	322.08 ± 4
Phenilalanine	1.72	1.00	151.18 ± 3
Tyrosine	0.62	0.64	85.15 ± 2
Glutamic acid	3.02	0.64	416.65 ± 5
H. helix			
Glycine	0.85	0.34	155.91 ± 3
Valine	1.20	0.68	154.23 ± 3
Leucine	1.01	0.88	100.31 ± 2
I-leucine	0.74	0.80	80.84 ± 2
Proline	20.67	0.74	2441.15 ± 10
Aspartic acid	12.42	0.62	1891.68 ± 9
Phenilalanine	1.56	1.00	136.34 ± 3
Tyrosine	1.19	0.64	195.24 ± 4

2.4.2. GC-MS

A Hewlett-Packard (Palo Alto, CA) 5972A GC/MSD combination equipped with an HP-5890 gas chromatograph was used. The electron energies used were 120 and 230 eV, respectively. The electron emission used was 300 μA and the ion source temperature was 200°C. A 30 m × 0.32 mm, 0.25 μm film-thickness Durabond DB-5 (J&W Scientific, Rancho Cordova, CA) fused-silica capillary column was used with helium as the carrier gas. The temperature was programmed from 80 to 250°C at 10°C min^{-1}; the flow rate was ~2 ml min^{-1}. Approximately 40 μg of each samples was injected into the column.

The identification of amino acids was achieved by comparing the MS spectra with those from the Wiley spectra library.

The quantitative determination of glycine was performed by selected ion monitoring (SIM). The following peaks were monitored for quantitative analyses: 154, 155 m/z for glycine.

3. Results and discussion

3.1. Separation and identification of free amino acids by TLC

The R_f values of amino acids from plant extracts are presented in Table 1. Asparagine, glutamine glycine, alanine, proline, tyrosine, iso-leucine, leucine and phenylalanine are presented in the *G. biloba* extract and asparagine, glycine, proline, tyrosine, valine, iso-leucine, leucine and phenylalanine are presented in the *H. helix* extract.

3.2. Separation and identification of free amino acids by HPLC

The separation of standard amino acids is presented in Fig. 1 and the separation of the plant extracts is presented in Figs. 2 and 3. These figures show that the amino acids present are those identified by TLC: asparagine, glutamine, glycine, arginine, alanine and proline in the *G. biloba* extract and asparagine, proline, tyrosine, valine metionine and iso-leucine in the *H. helix* extract.

3.3. Separation, identification and quantitative determination of free amino acids by GC-MS

The results of the quantitative determination are presented in Table 2. By this method the presence of the same amino acids are confirmed. The quantity of free amino acids contained in the *G. biloba* and *H. helix* extract are alanine, glycine, leucine, iso-leucine, proline asparagine, phenylalanine, tyrosine, glutamic acid and glycine, valine, leucine, iso-leucine, proline, asparagine, phenylalanine and tyrosine, respectively.

4. Conclusions

The chromatographic methods allow the separation, the identification, and quantitative determination of free amino acids from plant extracts of *G. biloba* and *H. helix*. The most important amino acid is proline, which has an important role in protein syntheses and in tissue regeneration.

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