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ANALYSIS OF FREE AMINO ACIDS FROM PLANT EXTRACTS BY CHROMATOGRAPHIC METHODS

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Dedicated to Professor Sorin Mager
on the occasion of his 75th birthday

ABSTRACT. Three modern techniques: thin-layer chromatography (TLC), high performance liquid chromatography (HPLC), and gas chromatography-mass spectrometry (GC-MS) have been used for separation, identification and quantitative determination of free amino acids from *Equisetum arvense* and *Ocimum basilicum*. The results of this research explain the utilization of *Equisetum arvense* and *Ocimum basilicum* leaf extracts in phytopharmaceutical and cosmetic products.

Keywords: plant extract; chromatographic methods; separation; identification; quantitative determination

1. Introduction

Natural compounds offer without doubt the richest resources of chemical diversity. In the last decade the pharmaceutical and cosmetic industries have been extensively using medicinal plant. Isolation, identification and quantitative determination of the active compounds from plant extracts are some of the oldest fields for the application of chromatographic methods for studying the structure-activity relationship.

Chromatographic methods allow separation and identification of biological active compounds such as: amino acids, peptides, flavones, sugars, tannins, organic acids etc., their metabolites and intermediaries which do not display biological activity [1].

Thin-layer chromatography (TLC) is advantageous in separation and identification of amino acids from plant extracts because multiple samples can be analyzed simultaneously in a short time, with low detection limits. A variety of adsorbents such as silica gel [2], modified silica gel [3, 4], polyamide [5], alumina and cellulose [6] can be used for one- or two-dimensional separation of

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amino acids. The most frequently used mobile phase systems are n-butanol-acetic acid-water, phenol-water, and n-butanol-acetic acid-acetone-water.

Separation of amino acids can be achieved by high performance liquid chromatography (HPLC) on C-18 stationary phase using mixtures of acetonitrile-acetate, phosphate, and citrate or borate buffer as mobile phases [7, 8].

The detection has been done under UV light if the adsorbent had a fluorescent indicator or after derivatization with ninhydrine, dansyl chloride, phtalic anhydride, dimethylamino azobenzene isothiocyanate (DABITC), etc [9, 10].

The best results for the quantitative determination of amino acids have been obtained using the gas chromatography-mass spectrometry (GC-MS) technique [11, 12].

The aim of this paper is the analysis of free amino acids from *Equisetum arvense* and *Ocimum basilicum* leaf extracts using TLC, HPLC, and GC-MS techniques.

Equisetum arvense is an excellent astringent genito-urinary system. It is useful mild diuretic and it is use in the treatment of kidney and bladder problems, cystitis, urethritis, prostate disease and internal bleedings such as urinary bleeds and stomach ulcer [13]. *Equisetum* has been found to ease the pain of rheumatism and stimulate the healing of chilblains. It is restorative to damage pulmonary tissue after pulmonary tuberculosis and other lung disease. In cosmetic, it is good for splitting nails and lifeless hair [14].

Ocimum basilicum is use as a culinary and medicinal herb. It acts principally on the digestive and nervous systems: easing flatulence, stomach cramps, colic and indigestion, poor digestion, nausea, gastro-enteritis, in treatment of feverish (especially colds and influenza), migraine, insomnia, depression and exhaustion [13]. Externally, it is use to treat acne, loss of smell, insect stings, snake bites and skin imperfections [14].

2.Experimental

2.1. Materials

Standard amino acids, $Na_3P(W_3O_{10})_4$ and trifluoroacetic acid were obtained from Merck (Darmstadt, Germany). Acetyl chloride and ion exchange resins Amberlite IR 120H and Dowex 50W-X8 were from Fluka (Buchs, Switzerland). ^{15}N-glycine (Gly 98.9%) was obtained from ITIM (Cluj-Napoca). Thin-layer chromatographic separation of amino acids was achieved on 20x20 cm, 0.1 mm thick cellulose plates CEL 300-10UV254 (Macherey Nagel). All other chemicals obtained from Comchim Bucharest were analytical grade.

2.2. Extraction of Free Amino Acids

The isolation of amino acids can be done, from dry plants, using different extraction methods, such as: extraction with 5% NaCl solution, 75% ethanol,

0.25% NaOH, 0.25M HCl, metasiliconic acid or a CH_3COOH-HCl-H_2O (18:1:1 v/v/v) mixture [12].

In our experiments the isolation of amino acids from dry plant has been done using two different extraction methods. For TLC and HPLC experiments 0.5g dry plants were extracted in 10mL 1% HCl solution. Then a 10% $Na_3P(W_3O_{10})_4$ solution was used for removing proteins from the extract by precipitation. After centrifugation the clear solution was passed through an ion exchange Amberlite IR120H column eluted with 40mL ammonia solution. The obtained solution was evaporated to dryness and the residue was redissolved in 1mL aqueous solution 30% (v/v) iso-propanol. For GC-MS experiments 0.5g dry plant were extracted in ethanol. The amino acids were purified on a Dowex 50W-X8 exchange resins, in a 2x40 mm column and eluted with 3M ammonia solution.

2.3. TLC

Aliquot (1μL) of standard solutions of 17 essential amino acids (1ng/mL) and the extract solutions were applied as spots to the cellulose plates with a micropipette.

The separation and identification of the free amino acids from the standard samples and extracts were achieved by bidimensional TLC (2D-TLC). The compositions of mobile phase were optimized by “Prisma” method. The plate was eluted on the first direction with n-butanol-acetone (35:35, v/v) and acetic acid-water (7:23, v/v) prepared and mixed (1:1, v/v) before elution. The second mobile phase was methanol-water-pyridine (80:20:5, v/v/v). The elutions have been done in unsaturated N-chamber and the elution distance was 18 cm. After the elution the plates were dried in hot air. The detection has been done by spraying the plates with a 0.5% ninhydrine solution in ethanol then dried at 110^0C for 10-15 min. The identification of the amino acids was achieved by comparing the R_f values and the colors of the spots.

2.4. HPLC

The HPLC analysis amino acids from standard solution and from plant extracts were converted into their phenyl-thiocarbamilic derivatives.

The standard mixtures, the plant extracts (10μL) were analyzed by HPLC using a HPLC apparatus (Merck Hitachi D-2000) with a Spherisol 5 ODS-2 column (250 mm x 4.6 mm i.d.) and a mixture of 0.15M sodium acetate (pH 6.5)-acetonitrile, concentration gradient 5-22%, 1 mL/min, temperature 55^0C, and UV detection at 254 nm. The identification of derivatized amino acids was achieved by comparing the retention time values.

2.5. GC-MS

The amino acids were transformed into N-trifluoroacetil n-butyl ester to increase their volatility. Asparagine and glutamine were transformed into aspartic acid and glutamic acid, respectively. Hystidine and arginine were difficult to analyze by gas chromatography. [^{15}N]-glycine was used as the internal standard (10 μg/mL).

2.5.1. Derivatization

The amino acids were derivatized using two step derivatization procedure. The dry samples were esterified with 0.5mL distilled butanol-acetyl chloride (4:1, v/v) for 1h at 110^{0}C. The excess reagent was removed by bubbling nitrogen through the mixture. The amino group was acetylated with a 200μL mixture of trifluoroacetic anhydride (TFAA)-methylene chloride (1:1, v/v) at 60^{0}C for 30 min. After cooling, the excess reagent was removed by bubbling nitrogen then 1mL ethyl acetate was added.

2.5.2. GC-MS

A trace DSQ ThermoFinnigan quadrupol mass spectrometer coupled with a Trace GC was used. The amino acids derivatives were separated on Rtx-5MS capillary column, 30m x 0.25mm, 0.25μm film-thickness, using a temperature program from 50^{0}C to 300^{0}C (3 min) at 10^{0}C/min. The following conditions were followed: transfer line temperature 250^{0}C, injector temperature 200^{0}C, and ion source temperature 250^{0}C, splitter 10:1. Electron energy was 70eV and emission current 100μA. The flow rate of helium was 2mL/min. 40μg of each samples was injected into the column.

The identification of amino acids was carried out by comparing the MS spectra with those from 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 from glycine.

3.Results and Discussion

3.1. Separation and Identification of Free Amino Acids by TLC

The R_f values of standards amino acids eluted with two mobile phase systems are presented in Table 1. Some of these amino acids were separated using the first mobile phase. The second mobile phase system could separate the amino acids that are unseparated by the first one. Therefore, the bidimensional elution is recommended.

Table 1.
The R_f values of standard amino acids.

Amino acid	**R_{f1} BuOH-Acetone-HAc-H_2O (35 : 35 : 7 : 23, v/v)**	**R_{f2} MeOH-H_2O-Py (80 : 20 : 5, v/v)**
Glutamine (Glutamic acid)	0.77	0.68
Alanine	0.44	0.72
Proline	0.42	0.53
Tyrosine	0.33	0.14
Lysine	0.56	0.73
Phenylalanine	0.83	0.82
i-Leucine	0.85	0.92
Histidine	0.72	0.60
Serine	0.53	0.57
Valine	0.51	0.68
Asparagine (aspartic acid)	0.24	0.36
Methionine	0.81	0.78
Glycine	0.38	0.30
Arginine	0.50	0.43
Leucine	0.88	0.89
Threonine	0.51	0.68

The chromatographic results of bidimensional TLC analysis of studied plant extracts (Table 2) show the presence of glutamine, alanine, proline, glycine, lysine, phenylalanine, iso-leucine, asparagine, serine, threonine, methionine, valine and leucine in both *Equisetum arvense* and *Ocimum basilicum* extracts.

Table 2.
The R_f values of amino acids from plant extracts

Amino acid	***Equisetum arvense***		***Ocimum basilicum***	
	R_{f1}	**R_{f2}**	**R_{f1}**	**R_{f2}**
Glutamine	0.75	0.67	0.73	0.66
Alanine	0.43	0.73	0.42	0.74
Proline	0.41	0.55	0.42	0.54
Glycine	0.31	0.13	0.32	0.13
Lysine	0.55	0.74	0.54	0.73
Phenylalanine	0.82	0.84	0.83	0.83
i-Leucine	0.84	0.94	0.84	0.93
Asparagine	0.31	0.29	0.30	0.30
Serine	0.54	0.58	0.55	0.59
Threonine	0.52	0.70	0.51	0.70
Methionine	0.79	0.79	0.80	0.79
Valine	0.50	0.43	0.50	0.42
Leucine	0.86	0.91	0.87	0.90

The identification was made on the basis of R_f values. The intensity of spot colors is only informative because it is different for different amino acids due to different quantity of compounds or probably to differences between the sensitivity of the reaction with ninhydrine.

3.2. Separation and Identification of Free Amino Acids by HPLC

The separation of standard amino acids is presented in Figure 1 and HPLC separations of plant extracts are presented in Figure 2.

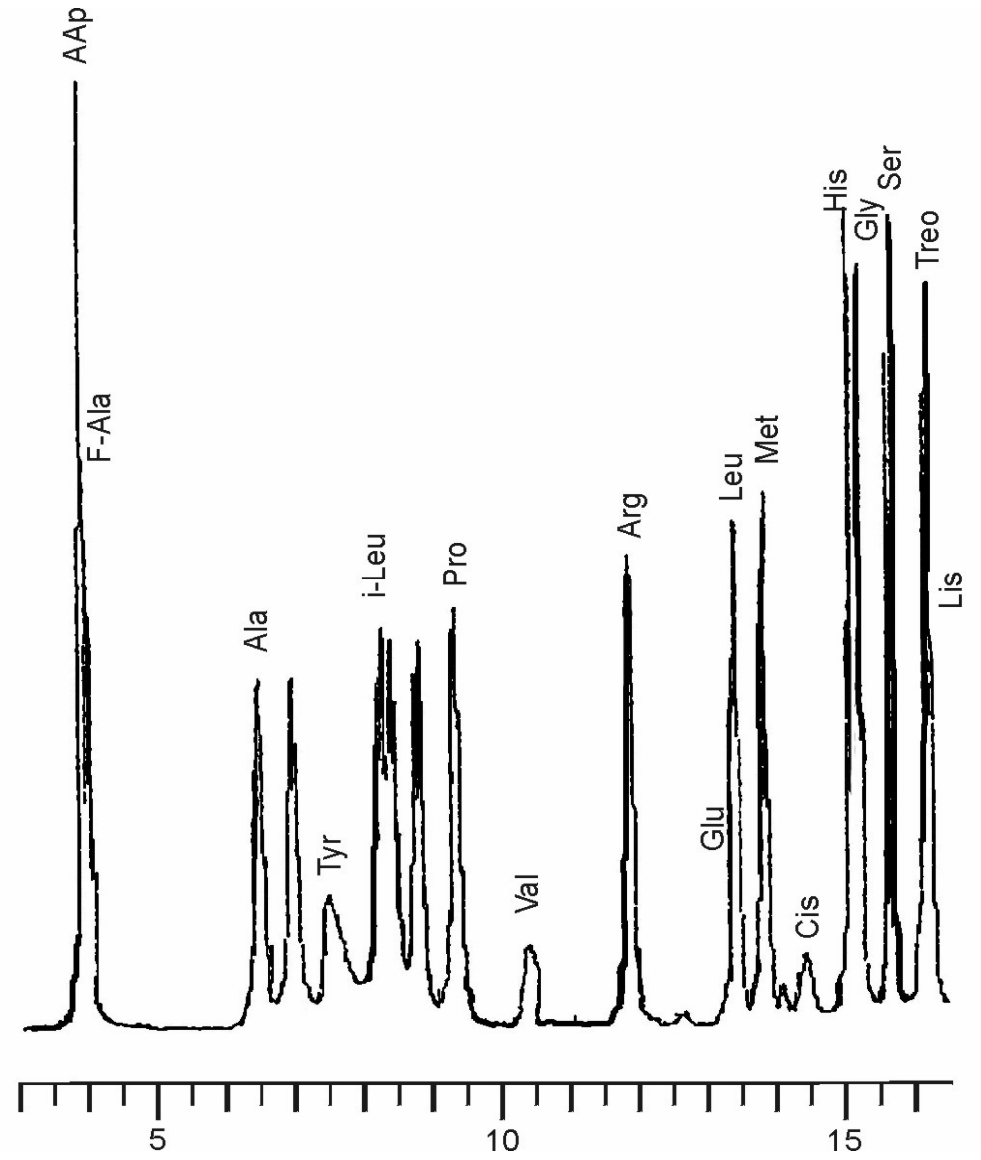

Figure 1.
The HPLC chromatogram of standard amino acids.

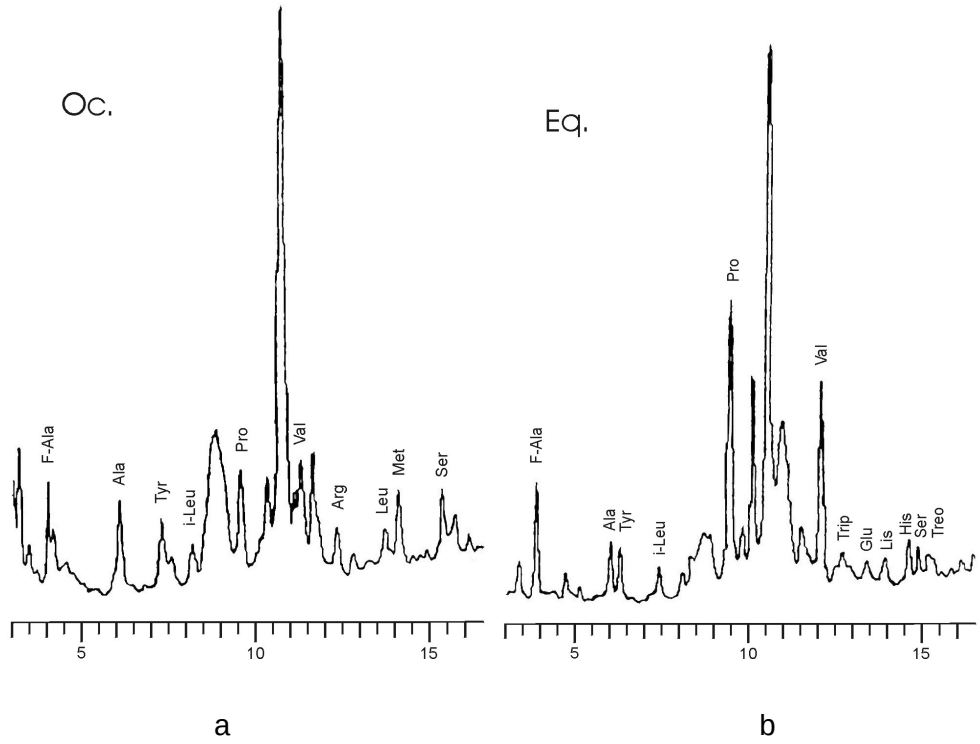

Figure 2.
The HPLC chromatograms of: a – *Ocimum basilicum*; b – *Equisetum arvense*

These figures show that the amino acids present are those identified by TLC: glutamine, alanine, proline, lysine, phenylalanine, iso-leucine, serine, threonine and valine in the *Equisetum arvense* extract and alanine, proline, phenylalanine, iso-leucine, serine, methionine, valine, and leucine in the *Ocimum basilicum* extract.

3.3. Separation, Identification and Quantitative Determination of Free Amino Acids by GC-MS

The results of quantitative determination are presented in Table 3. By GC-MS the presence of the same amino acids are confirmed. Glutamine, alanine, proline, glycine, lysine, phenylalanine, iso-leucine, asparagine, serine, threonine, methionine, valine, leucine, histidine, and tyrosine are the free amino acids from *Equisetum arvense* and *Ocimum basilicum* extracts determined by this method.

Table 3.
The GC results of quantitative determination of amino acid from plants extracts

Amino acid	**t_R**	**F**	***Equisetum arvense***		***Ocimum basilicum***	
			A (mm^2)	**C (μg/g)**	**A (mm^2)**	**C (μg/g)**
Alanine	12.75	0.77	1.04	8.65	0.96	8.27
Glycine	13.40	0.80	0.90	7.56	0.87	7.32
Threonine	14.81	0.20	0.05	1.65	0.11	3.51
Serine	15.28	0.20	0.09	3.01	0.38	12.82
Valine	15.76	0.84	0.89	7.09	1.20	9.56
Leucine	17.82	0.80	1.04	8.69	0.91	7.58
i-Leucine	18.11	0.80	0.49	4.12	0.37	3.06
Tyrosine	19.90	0.95	0.55	3.85	0.27	1.90
Proline	21.54	0.84	1.15	9.09	1.24	9.81
Metionine	24.91	0.39	0.08	1.36	0.45	7.69
Asparagine	27.73	0.88	1.07	8.07	2.35	17.84
Phenilalanine	27.90	0.82	1.30	10.53	2.38	19.34
Glutamine	30.90	1.00	2.07	13.77	2.81	18.75
Lysine	30.96	0.43	0.68	10.47	0.69	10.75
Histidine	35.93	0.51	0.07	0.98	0.23	3.02

4. Conclusions

The chromatographic methods allow the separation, the identification, and the quantitative determination of free amino acids from *Equisetum arvense* and *Ocimum basilicum* extracts. The important amino acids from *Equisetum arvense* and *Ocimum basilicum* are phenylalanine, asparagine, and lysine, which have an essential role in protein syntheses and in tissue regeneration, even if their concentration are small.

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