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CHROMATOGRAPHIC ANALYSIS OF ORGANIC ACIDS, AMINO ACIDS, AND SUGARS IN *OCIMUM AMERICANUM* L.

MARIIA SHANAIDA*, IVANNA KERNYCHNA and YURII SHANAIDA

Department of Pharmacognosy with Medical Botany, I. Horbachevsky Ternopil State Medical University, Ternopil, Ukraine

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Plants contain a complex pool of primary and secondary metabolites, which potentially can be used as herbal remedies with positive impact on human health. Plants use secondary metabolites to deter microorganisms, insects and some animals and to generate a diverse and dynamic complex of interactions from symbioses to pathogenicity (1-3). Medicinal plants are important as a source of both secondary metabolites and primary synthesis compounds. Both of them are needed for the growth and development of the plants and as a supply of chemical energy, but importantly, also can be used as medicines (4, 5).

Species of genus *Ocimum (*Lamiaceae) are used as medicinal, aromatic and ornamental plants. They are rich in chemical compounds with valuable therapeutic and prophylactic properties; many of them have a long history of uses in culinary and folk medicine (6, 7). *Ocimum americanum* (formerly known as *Ocimum canum*) includes wild species of Southeast Asia and Africa, which are now cultivated throughout the world for their essential oils or used as spices (8).

Despite being used for centuries in folk medicine, the plant is not included in any of the world renowned Pharmacopoeias. World Health Organization Monographs (9) includes only *Ocimum sanctum* (leaves); sometimes also basil seeds were subjected to the study (10). Mainly the secondary metabolites of *Ocimum americanum* (essential oils, phenolic compounds, etc.) have been studied (8, 11) with no or little attention paid to primary compounds.

The aim of our study was to analyze the contents of amino acids, organic acids and sugars in aerial parts of *Ocimum americanum*, grown in Ternopil Region, Ukraine.

EXPERIMENTAL

Plant collection

The seeds of *Ocimum americanum* were obtained from the collection of M. Hryshko National Botanical Garden, Kyiv, Ukraine, and grown in June-July of 2014, 2015 on the experimental plots in Ternopil Region, Ukraine (49.5535^{o} N, 25.5948^{o} E). The aerial parts of the plants were harvested at the flowering stage of development, dried for 6–8 days at 30-35^{o}C in shade and crushed to powder for further analysis. Voucher specimen No. LOA-110/1 for *Ocimum americanum* was deposited in the herbarium of Pharmacognosy at Medical Botany Department of I. Horbachevsky Ternopil State Medical University.

Extraction procedures

Free amino acids analysis was done as follows: 0.3 g of crushed plant material was placed in 10 mL vial with added 4 mL of 0.1 M aqueous solution of HCl and 0.2% β-mercaptoethanol and tightly sealed (12). The vial was placed in an ultrasonic bath for 1 h; the contents was centrifuged and filtered and the HCl was removed in a vacuum desiccator at 45^{o}C. Obtained dry content was placed in the analytical vial with added 200 mL of 0.8 M borate buffer (pH = 9.0), 200 mL 20 mM 9-fluoroenyl-methoxycarbonyl chloride in acetonitrile by using an automatic dispenser. After a 10 min reaction, 20 mL of 150 mM solution of amantadine hydrochloride (in 50% of the aqueous acetonitrile) was added to the vial.

* Corresponding author: e-mail: shanayda-mi@ukr.net

Table 1. Content of organic acids, amino acids, and sugars in *Ocimum americanum* L. herb

No	Retention time	Compounds	Content (mg/100 g plant material)
		Amino acids	
1	1.92	Aspartic acid (Asp)	85.6 ± 3.5
2	2.04	Glutamic acid (Glu)	121.7 ± 4.1
3	2.70	4-Hydroxyproline (Hyp)[x]	4.7 ± 0.3
4	2.89	Asparagine (Asn)	353.0 ± 11.8
5	2.99	Glutamine (Gln)	4.8 ± 0.3
6	3.12	Serine (Ser)	36.3 ± 1.5
7	3.28	Arginine (Arg)	76.3 ± 3.2
8	3.35	Glycine (Gly)	98.7 ± 4.1
9	3.44	Threonine (Thr)	53.5 ± 2.2
10	3.62	Alanine (Ala)	42.3 ± 1.7
11	3.71	Proline (Pro)	112.5 ± 3.7
12	3.77	γ-Aminobutyric acid (GABA) [x]	84.0 ± 3.5
13	4.35	Valine (Val)	21.8 ± 0.9
14	4.44	Metionyn (Met)	18.0 ± 0.8
15	4.81	Isoleucine (Ile)	4.5 ± 0.3
16	4.89	Leucine (Leu)	6.6 ± 0.4
17	5.06	Phenylalanine (Phe)	19.8 ± 0.9
18	5.48	Cystine (Cys)[x]	45.3 ± 1.8
19	6.76	Histidine (His)	17.7 ± 0.9
20	6.83	Lysine (Lis)	5.4 ± 0.3
21	8.03	Tyrosine (Tyr)	6.6 ± 0.4
		[x] marked the free amino acids (non-protein components)	
		Organic acids	
1	8.88	Dimethoxy-acetic Acid	13.2 ± 0.6
2	9.39	Oxalic acid	165.1 ± 5.5
3	11.63	Malonic acid	80.3 ± 3.3
4	12.35	Fumaric acid	7.9 ± 0.5
5	12.95	Levulinic acid	291.9 ± 9.8
6	13.43	Succinic acid	21.7 ± 0.9
7	21.71	Malic acid	88.6 ± 3.6
8	21.94	Myristic acid *	11.8 ± 0.6
9	25.86	Palmitic acid *	176.3 ± 5.9
10	26.70	Palmitoleic acid *	12.7 ± 0.6
11	29.00	Citric acid	109.7 ± 3.7
12	29.32	Stearic acid *	17.5 ± 0.8
13	29.61	Oleic acid *	18.9 ± 0.9
14	30.40	Linoleic acid *	57.9 ± 2.4
15	31.49	Linolenic acid *	103.7 ± 3.4
16	32.60	Arachidic acid *	8.4 ± 0.5
		* marked the fatty acids	
		Sugars	
1	8.18	D-Arabinose (Ara)	38.4 ± 1.6
2	8.34	D-Fucose (Fuc)	25.1 ± 1.1
3	14.31	D-Mannose (Man)	1539 ± 39.2
4	14.69	D-Glucose (Glu)	1757 ± 49.9
5	15.25	D-Galactose (Gal)	485 ±16.2
6	18.11	D-Sorbitol	IS
7	20.72	D-Fructose (Fru)	3608 ± 75.6
8	33.78	D-Sucrose	1801 ± 45.9

Analysis of fatty acids with followed detection of organic acids was done according to (13, 14) with some modifications: 50 mg of dried plant material was placed in a 2 mL vial with added 1.0 mL of the methylating agent (14% BCl_3 in methanol, Supelco 3-3033) and the internal standard (50 µg tridecane in hexane), then tightly sealed and incubated for 8 h at 65°C. The reaction mixture was centrifuged and the pellet was dissolved in 1 mL of distilled water. The preparation fatty acid methyl esters (FAMEs) was done by adding 0.2 mL of methylene chloride and incubated for 1 h with gentle shaking. The obtained extracts were used for the chromatographic analysis of methyl esters.

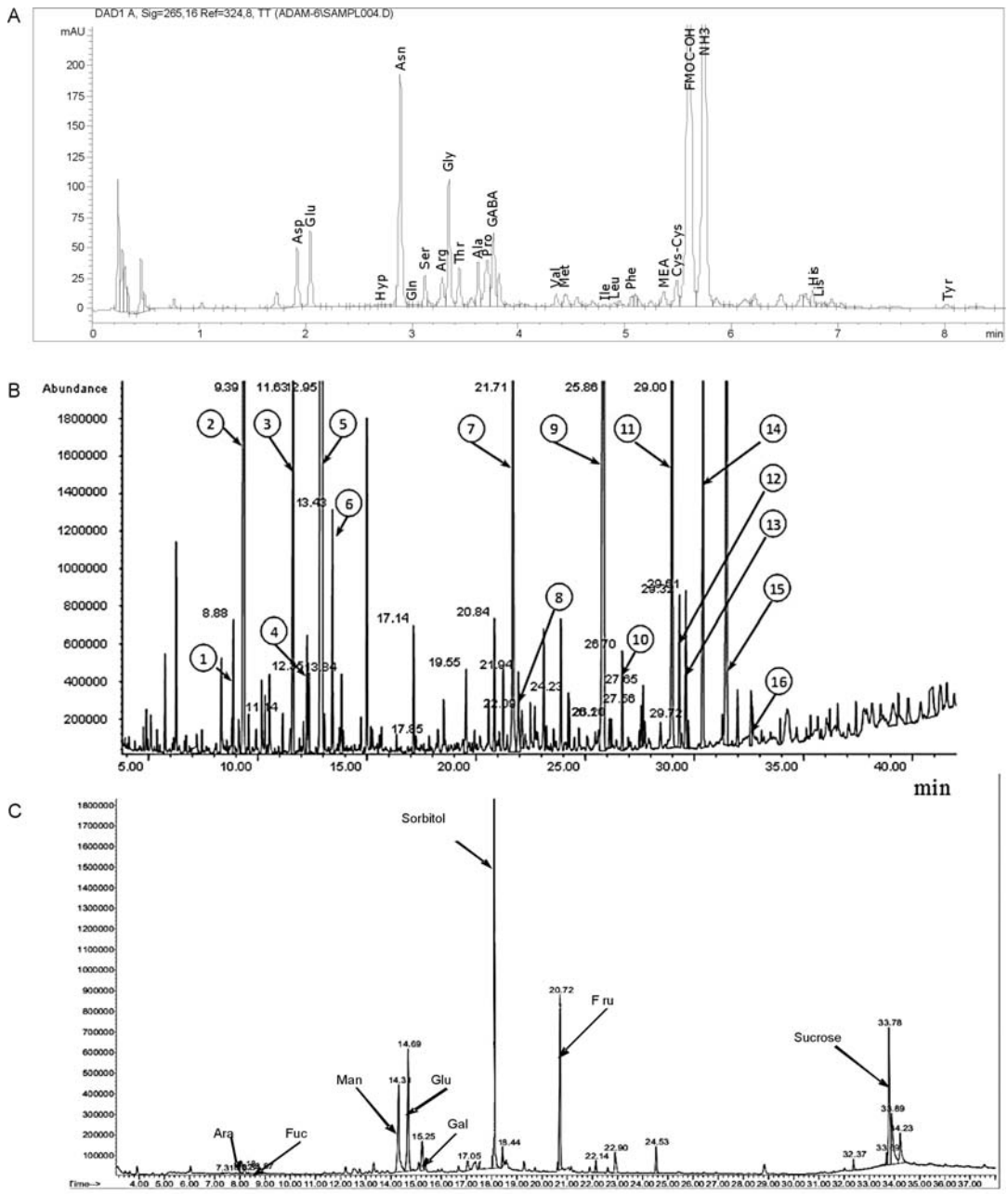

Figure 1. Chromatogram of organic acids, amino acids, and sugars in *Ocimum americanum* L. herb. A) HPLC chromatogram of amino acids; B) GC-MS chromatogram of organic acids (numbers on chromatogram that concluded in circles refer to the sequence number of organic acids in Table 1); C) GC-MS chromatogram of sugars

The extraction of sugars (water-soluble carbohydrates) was done with adding 80% ethanol and sorbitol as an internal standard. The extraction was carried out in the ultrasonic water bath at 80°C for 4 h. Extracts were dried and subjected to derivatization by 32 mg/mL of hydroxylamine hydrochloride in the mixture of pyridine/methanol (4 : 1, v/v) (15). Samples were incubated in a shaking water bath at 75°C for 25 min with following addition of 1.0 mL of acetic anhydride. After the incubation, 2.0 mL of dichloromethane was added to the mixture and the excess of the derivatization reagents was removed by double extraction with 1 M HCl and water.

Instrumentation

Analysis of the amino acids was performed by using high performance liquid chromatography (HPLC). The Agilent Technologies 1100 chromatograph with G1310A isocratic pump, G1314A variable wavelength detector, manual injector, G1316A thermostatic column compartment, G1321A fluorescence detector, G1362A refractive index detector and ZORBAX-XDB-C18 (4.6 × 50 mm) column were used.

Analysis of organic acids and water-soluble carbohydrates was done by using gas chromatography with mass spectrometric detector (GC-MS). The Agilent Technologies 6890 chromatograph with mass spectrometry detector 5973N and HP-INNOWax (0.25 mm × 30 m) column were used for the analysis.

Chromatographic conditions

The HPLC analysis of amino acids (by using Agilent Technologies 1100 chromatograph) was done by using the described conditions: solution A (0.05 M aqueous solution of sodium acetate, pH = 6.5) and solution B (0.10 M aqueous solution of sodium acetate: ACN = (23 : 22, v/v), pH = 6.5) were used as mobile phase. The supply rate was 1.5-2.0 mL/min; eluents working pressure was 220-275 kPa. Column temperature of the thermostat was 50°C, sample volume was 2 mL, the scan time was 0.5 s, the detection time was 8 min. Identification of amino acids was done according to their retention time (with using standards as a reference) at 265 nm. Identification of organic acids and sugars by GC-MS (Agilent Technologies 6890/5973N chromatograph) was done under conditions as described below: the rate of carrier gas (helium) was 1.2 mL/min; the temperature of the oven input sample was set from 50 to 250°C with 4°/min increase. Injections were performed in the split mode. The detection time was 40 min. Identifications of organic acids was done using the library of mass spectra of the reference compounds (National Institute of Standards and Technology - NIST, USA). The internal standards were used for quantitative analysis. Identification of sugars was performed by using the mixture of monosaccharides and compared to their standards (according to the NIST library). Sorbitol was used as an internal standard for the quantitative measurement of carbohydrates.

All reagents including sugars and amino acids were obtained from Sigma-Aldrich, USA and were of analytical grade purity (> 95%).

Statistical analyses

All the experiments were repeated at least six times. The data were analyzed using MathCAD 2000 Professional software (MathSoft Inc.). Differences between the means were compared by using Student's *t*-distribution test at $p < 0.05$ level.

RESULTS AND DISCUSSION

Among the all detected amino acids (Table 1, Fig. 1) of *Ocimum americanum* herb the most prevalent was asparagine (353.0 mg/100 g dry weight). It is well known that asparagine is critically required for the development and effective functioning of the human brain (16). γ-Aminobutyric acid, which was also identified (84.0 mg/100 g), is the most common inhibitory neurotransmitter in the central nervous system (17). Consequently, many of identified amino acids are important not only as components of proteins, but also as compounds of regulatory systems if they are used as dietary supplements or medicines (16, 17).

A significant number of organic acids (as identified by GC-MS) are available in *Ocimum americanum* herb (Table 1, Fig. 1). According to Table 1 of the results, among the low-molecular weight aliphatic organic acids the most abundant were oxalic, levulinic and citric. The obtained data correspond with the results of the other group of researchers studying the content of organic acids in aerial parts of species belonging to Lamiaceae family (14); both studies can be important for identification of new sources for creating herbal remedies.

It is known that organic acids can accumulate in very large quantities in fruits of many higher plants, but their amounts in leaves were rarely studied. Fatty acids are essential for producing fats as energy storage and also perform structural and signalling functions. Variations of fatty acids were previously studied in seed oil among *Ocimum* species (10). According to our study, palmitic and linolenic

acids were prevailing in *Ocimum americanum* herb (Table 1); similar results were observed in *Ocimum tenuiflorum* and *Calamintha officinalis* herb (Lamiaceae) (18, 19). Importantly, some fatty acids in *Ocimum sanctum* seed possess the anti-inflammatory activity (20).

Several water-soluble carbohydrates in the herb of *Ocimum americanum* have been identified by GC-MS (Table 1, Fig. 1). According to Table 1 of the results, the sugars, especially D-fructose, were the dominant components of the herb, which is not surprising as they are the part of the autotrophic nutrition of plants as a whole. These data are very important as the studied Lamiaceae plants can be used as an important source of different carbohydrates and also to prepare herbal medicines to help people suffering from diabetes or other disorders (21-23). Scientists in the area of medical chemistry show the possibility to obtain some pharmacologically active compounds by changing the monosaccharide scaffolds with pharmacophore groups (24).

It was shown that the administration of *Ocimum basilicum* seeds and leaves to the animal diet significantly improves their growth and increases digestive enzyme activities, probably due to the high content of primary metabolites (25). The results indicate that basil has some ability to activate the immune system of sea bream by increasing serum total protein. Another observation indicates the hypoglycemic and hypolipidemic effect of *Ocimum sanctum* leaves in diabetic rats (26). It is useful in the prevention of diabetes and other degenerative diseases and was subjected for further investigations (27).

CONCLUSIONS

The study demonstrates the composition and quantitative contents of the important groups of primary metabolites (amino acids, organic acids and water-soluble carbohydrates) in the aerial parts of *Ocimum americanum,* grown under the cultivating condition in Ukraine. Using HPLC and GC-MS as useful techniques for quantification of chemical constituents allowed us to reveal the domination of oxalic, levulinic and citric acids among carboxylic organic acids, whereas palmitic and linolenic acids were dominant among the fatty acids. The most prevalent amino acid was asparagine. D-fructose dominated among water soluble carbohydrates.

Comprehensive study of primary metabolites of *Ocimum americanum* herb was carried out for the first time. These novel results can be used in planning of pharmacological studies, primarily to determine the hypoglycemic, hypolipidemic, anti-inflammatory and sedative activities of this plant. Due to the high content of D-fructose, some amino acids and some organic acids we suggest that the plant can be used as a dietary food supplement.

Conflicts of interests

No conflicts of interests exist.

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[1]Faculty of Pharmacy, Bahauddin Zakariya University, Multan, Pakistan
[2]Faculty of Pharmacy, [3]Department of Chemistry, University of Sargodha, Sargodha, Pakistan

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