

Determination of amino acids content in two samples of the plant mixtures by GC-MS

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Received 22 January 2021 ♦ Accepted 17 February 2021 ♦ Published 15 March 2021

Citation: Savych A, Marchyshyn S, Harnyk M, Kudria V, Ocheretniuk A (2021) Determination of amino acids content in two samples of the plant mixtures by GC-MS. *Pharmacia* 68(1): 283–289. <https://doi.org/10.3897/pharmacia.68.e63453>

Abstract

Due to the wide range of biologically active substances, the plant mixtures can influence the development of diabetes mellitus and its complications. Amino acids attract particular attention due to their ability to stimulate insulin secretion, reduce hyperglycemia and regulate metabolic processes in patients with diabetes. The aim of this study was to investigate the content of amino acids in the plant mixture samples: 1) *Cichorium intybus* roots, *Elymus repens* rhizome, *Helichrysum arenarium* flowers, *Rosa majalis* fruits, *Zea mays* columns with stigmas, 2) *Urtica dioica* leaf, *Taraxacum officinale* roots, *Vaccinium myrtillus* leaf, *Rosa majalis* fruits, *Mentha piperita* herb, which have proven antidiabetic activity in studies *in vivo*. The amino acids were separated by validated method of gas chromatography-mass spectrometry with pre-column derivatisation. Quantitative analyses of amino acids showed that the predominant components were *L*-proline in the sample 1 and *L*-leucine and *L*-proline in the sample 2 of the plant mixtures.

Keywords

plant mixtures, amino acids, gas chromatography-mass spectrometry, diabetes mellitus

Introduction

Diabetes mellitus is one of the priority problems of the WHO, which requires immediate solution, as the epidemiological situation is alarming – the number of patients is growing rapidly each year, leading to increased disability and mortality due to the development of macro- and microangiopathies (Harding et. al. 2019; American Diabetes Association 2020). According to the official data of the International Diabetes Federation (2019), the incidence of diabetes in the world is projected to increase 1.5 times by 2030, amounting to more than 500 thousand patients. Therefore, the optimization of existing antidiabetic pharmacotherapy, search and study of new drugs for the prevention and treatment of this disease and its

complications is an important issue in modern pharmacy and medicine.

One of these areas is using phytomedicines in the form of monotherapy in the mild stages of the disease and for its prevention, and in combination with traditional therapy for more severe forms of the disease. Phytotherapy is a promising and reasonable method, as it has a number of advantages – relatively low toxicity of herbal medicines, mild pharmacological effect and the ability to use for a long time without significant side effects, the ability to combine well with synthetic drugs (Gothai et. al. 2016; Governa et. al. 2018; Savych et. al. 2019). The combinations of different medicinal plants deserve particular attention. Plant mixtures are expected to have several biologically active substances with a wide range of pharmacological actions

and a variety of mechanisms for influencing the development of diabetes and diabetic angiopathies (Oh and Jun 2014; Kooti et al. 2016; Savych et al. 2020a, 2021a, b). Therefore, in order to establish correlations between the phytochemical composition of the studied plant mixtures and its antidiabetic activity, which was studied in previous studies (Savych et al. 2020b, c, d, e, f; 2021a, b), it is advisable to conduct phytochemical analysis, in particular, amino acids as one of the most important biologically active substances in the therapy of diabetes.

Amino acids, in addition to their main function as precursors of protein synthesis, play a key role in many metabolic processes, because they have a powerful secretolytic activity – stimulate the secretion of insulin, glucagon, cortisol and insulin-like growth factor-1 (IGF-1) (Comerford and Pasin 2016). Except this, literature sources indicate the regulatory role of amino acids in the transcription and translation of genes, as well as their important function in intracellular signaling (Chen et al. 2016; Comerford and Pasin 2016). The effectiveness of amino acids in the treatment and prevention of diabetes is primarily due to their ability to stimulate insulin secretion in pancreatic β -cells, as well as increase blood glucose utilization and reduce alimentary hyperglycemia. The greatest insulinotropic effect is inherent in arginine, leucine, isoleucine, alanine and phenylalanine (Chen et al. 2016; Birech et al. 2017). In addition, amino acids have the ability to reduce muscle proteolysis and/or stimulate protein synthesis, which leads to improved protein balance in skeletal muscle and, as a result, increases the process of glucose utilization. This is an important component of antidiabetic therapy, because such patients often have a deficiency of skeletal muscle mass, which, in turn, contributes to the development of insulin resistance and progression of this disease (Comerford and Pasin 2016).

Aim of the research

Therefore, the aim of study was to investigate the content of amino acids in some plant mixtures with previously studied antidiabetic activity *in vivo* (Savych et al. 2020b, c, d, e, f).

Materials and methods (experimental part)

Plant materials

The herbal raw materials harvested from June to August 2019 in the Ternopil region and Carpathians (*Vaccinium myrtillus* leaf) (Ukraine) were used. After harvesting, the raw materials were dried, crushed and stored according to the general GACP requirements (WHO 2003). The plants were identified in the Department of Pharmacognosy with Medical Botany, I. Ya. Horbachevsky Ternopil National Medical University, Ternopil, Ukraine. The voucher specimens of herbal raw materials have been deposited in the departmental herbarium for future

records. For the study, two different plant mixtures with reliable antidiabetic activity established during pharmacological studies *in vivo* (Savych et al. 2020b, c, d, e, f) were used. The composition of the mixtures is given in Table 1.

Table 1. Composition of the plant mixtures.

Plant mixture	Plant drug component	Portion in the mixture, %	Relative ratio
Sample 1	<i>Cichorium intybus</i> roots	26.32	5
	<i>Elymus repens</i> rhizome	26.32	5
	<i>Helichrysum arenarium</i> flowers	21.05	4
	<i>Rosa majalis</i> fruits	15.79	3
	<i>Zea mays</i> columns with stigmas	10.52	2
Sample 2	<i>Urtica dioica</i> leaf	20.0	1
	<i>Taraxacum officinale</i> roots	20.0	1
	<i>Vaccinium myrtillus</i> leaf	20.0	1
	<i>Rosa majalis</i> fruits	20.0	1
	<i>Mentha piperita</i> herb	20.0	1

Chemicals and standards

All applied reagents were of analytical grade ($\geq 99\%$ purity). Standard reagents including glycine, *L*-alanine, *L*-valine, *L*-leucine, *L*-serine, *L*-threonine, *L*-isoleucine, *L*-proline, *L*-asparagine, *L*-aspartic acid, *L*-glutamic acid, *L*-methionine, *L*-cysteine, *L*-phenylalanine, *L*-glytamine, *L*-lysine, *L*-histidine, *L*-tyrosine, *L*-tryptophan were purchased from Sigma-Aldrich Chemical Co. (USA), as well as hydrochloric acid, sodium hydroxide, methanol, pyridine, methyl chloroformate, chloroform, sodium bicarbonate. Water used in the studies was produced by MilliQ Gradient water deionization system (USA).

Extraction of amino acids

For the extraction of free amino acids the samples of the herbal raw material were grinded into a powder by laboratory mill, then about 0.1 g (accurately weighed) was selected and placed into vial with 2.0 mL of 0.1 N aqueous solution of hydrochloric acid. The extractions were carried out in the ultrasonic water bath at 50 °C for 3 hours.

Extraction of bound amino acids was carried out by adding 2 mL of 6 N an aqueous solution of hydrochloric acid to 0.03 g (accurately weighed) of powdered herbal raw materials. Hydrolysis was carried out for 24 hours in a thermostat at 110 °C.

The resulting extracts were centrifuged at 3000 rpm and the supernatants were evaporated to dryness on a rotary evaporator washing three times with distilled water to remove hydrochloric acid.

Pre-column derivatisation

The dry samples of plant mixtures were dissolved in 390 μ L of 1 M sodium hydroxide, then were added 333 μ L of methanol and 67 μ L of pyridine and mixed thoroughly for 5 seconds. To the resulting mixtures was added 80 μ L of methyl chloroformate, stirred thoroughly for 60 seconds. The amino acid derivatives were extracted with 400 μ L of

chloroform followed by the addition of 400 μL of 50 mM sodium bicarbonate. The chloroform phase was used for future analysis (Vancompernelle et. al 2016).

Instrumentation and conditions of gas chromatography-mass spectrometry

The amino acids composition in the samples of the herbal raw materials was studied by gas chromatography-mass spectrometry (GC-MS) method using the Agilent Technologies (USA) system, model 6890N/5973inert (6890 gas chromatography with mass spectrometry detector 5973) and capillary column HP-5ms (30 m \times 0.25 mm \times 0.25 mm, Agilent Technologies) (Chen et. al. 2010). The evaporator temperature was 250 $^{\circ}\text{C}$, the interface temperature 280 $^{\circ}\text{C}$. The separation was performed in the mode of temperature programming – the oven temperature was initially set to 50 $^{\circ}\text{C}$, held for 4 min, then ramped at the rate of 5 $^{\circ}\text{C}/\text{min}$ to 300 $^{\circ}\text{C}$ and finally held at this temperature for 5 min. Injections of 1 μL were made in the split mode 1:50. The carrier gas flow rate through the column was 1.0 mL/min.

Identification and calculation by GC-MS

Amino acid identification was performed by comparing the retention times of amino acid standards and the presence of representative molecular and fragment ions (Table 2). The content of bound amino acids was determined by subtracting the content of free amino acids from their total content (Chen et. al. 2010).

Table 2. Conditions for chromatographic identification of amino acids.

Amino acids	t_r , min	Molecular ion, m/z	Main fragmentary ions, m/z
Glycine	14.77	147	88
L-alanine	14.85	161	102, 88
L-valine	18.56	189	146, 130, 115, 98
L-leucine	19.57	203	144, 115, 102, 88
L-serine	20.77	191	176, 144, 114, 100, 88
L-threonine	21.11	205	147, 115, 100, 88
L-isoleucine	21.31	203	144, 115, 101, 88
L-proline	21.87	187	128, 84
L-asparagine	21.97	262	146, 127, 95
L-aspartic acid	23.90	219	160, 128, 118, 101
L-glutamic acid	24.02	233	201, 174, 142, 114
L-methionine	26.86	221	147, 128, 115
L-cysteine	27.14	192	192, 176, 158, 146, 132
L-phenylalanine	29.18	237	178, 162, 146, 131, 103, 91
L-glytamine	29.74	276	141, 109, 82
L-lysine	31.90	276	244, 212, 142, 88
L-histidine	35.91	285	254, 226, 210, 194, 140, 81
L-tyrosine	37.24	296	252, 236, 220, 192, 165, 146, 121
L-tryptophan	38.91	276	130

Method validation

The method was validated for linearity, limit of detection (LOD), limit of quantitation (LOQ) and precision. Linearity was performed by injecting a series of standard solutions (0.1–10.0 mg/100 g) with a threefold derivatization

procedure and a single injection for each reference standard. The mean value and standard deviation, as well as regression analysis were calculated using Microsoft Excel software package 2016 (USA). The values for LOD and LOQ were calculated based on the data obtained during linearity testing in the low concentration range of the working in the test solution, using the following formulas: $\text{LOD} = 3.3 \cdot s / \text{Slope}$; $\text{LOQ} = 10 \cdot s / \text{Slope}$. Linearity testing was repeated with the same samples after a complete restart of the system with removal and re-installation of the column. Repeatability precision was determined by five-fold injection of the same sample in a row. For the resulting relative peak area of the quantifier ions the relative standard deviation (RSD) was calculated. To determine intra-day precision, five standard preparations of each reference standard with the same concentration were single injected and the resulting relative peak areas were used to calculate the RSD. Inter-day precision for the day of sample preparation and the two following days was specified by injecting five standard sample of each reference standard preparations once each on all three days. The RSD of the samples on that day together with the previous samples were calculated as above (Wang et. al 2020).

Results and discussion

The results of qualitative and quantitative analyses of free and bound amino acids in the plant mixtures are represented in Figures 1–4 and in Table 3.

During GC-MS analysis, 5 amino acids in free form and 14 in bound form were identified in the sample 1 of the plant mixture (Figs 1, 2); 8 amino acids in free form and 15 in bound form in the sample 2 (Figs 3, 4). The predominant amino acids in free form were L-proline, its content was 19.885 mg/g in the sample 1 and 4.794 mg/g in the sample 2 (Table 3). Proline exhibits significant hypoglycemic activity, which is due to a decrease in hepatic glucose production owing to inhibition of glycogenolysis, gluconeogenesis and glucose-6-phosphatase activity (Chen et. al. 2016). Another free amino acid with a high content in both studied plant mixtures was L-isoleucine, its content was 5.136 mg/g and 4.612 mg/g, respectively. As for the amino acids that were in the bound form, the predominant protein component was L-proline in the sample 1, its content was 13.856 mg/g. During chromatographic analysis it was found that sample 2 contains the largest amount of bound amino acid L-leucine – 8.707 mg/g (Table 3). Leucine is a branched-chain amino acid that plays an important role in controlling protein synthesis and regulating cell metabolism. One of the most important functions of leucine in diabetes is that it has the ability to stimulate insulin secretion in β -cells of the pancreas, and also acts as a source of energy for metabolic processes and an allosteric activator of glutamate dehydrogenase to enhance glutaminolysis (Birech et. al. 2017). Isoleucine, which is an isomer of leucine, does not have itself the ability to stimulate insulin synthesis, but in combination with

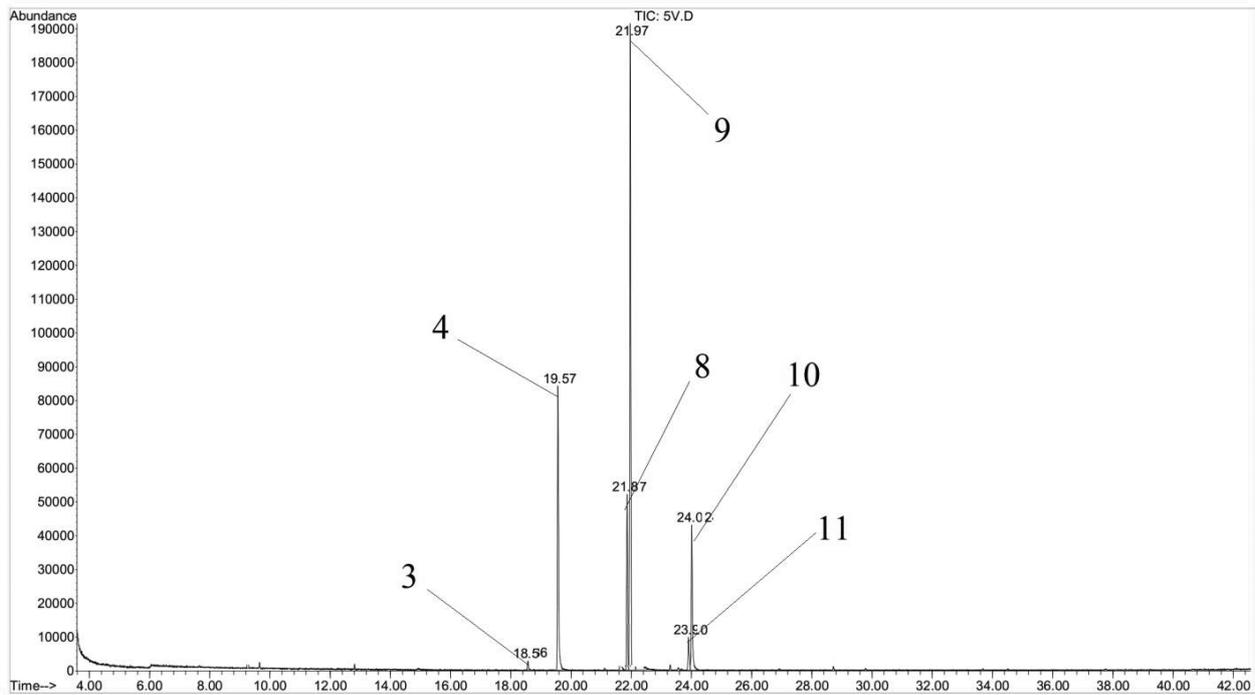


Figure 1. GC-MS chromatogram of free amino acids in the sample 1 of the plant mixture.

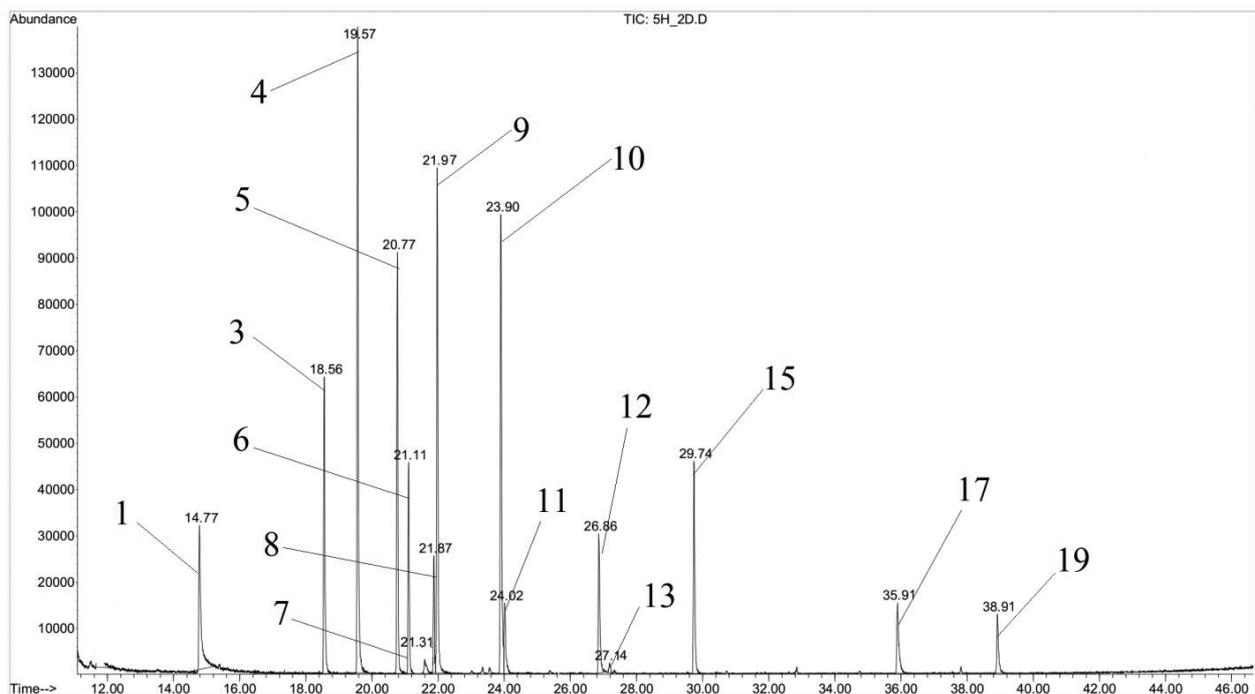


Figure 2. GC-MS chromatogram of amino acids after hydrolysis in the sample 1 of the plant mixture.

leucine, their secretolytic activity increases significantly, causing a more pronounced hypoglycemic effect (Comerford and Pasin 2016; G Birech et. al. 2017). In the next place in terms of amino acid content in the studied samples was *L*-aspartic acid in bound form, its content was 7.065 mg/g in the sample 1 and 5.772 mg/g in the sample 2. In addition, high levels of *L*-phenylalanine in bound form were found in both samples of the plant mixtures,

its content was 2.008 mg/g and 5.822 mg/g, respectively (Table 3). Phenylalanine, an aromatic amino acid that has a direct effect on the course of diabetes, in particular due to its ability to regulate carbohydrate metabolism by stimulating the release of glucan-like peptide-1 (GLP-1), which in turn enhances insulin secretion, inhibits insulin secretion, stimulates proliferation and neogenesis of β -cells of the pancreas, reduces insulin resistance (Chen

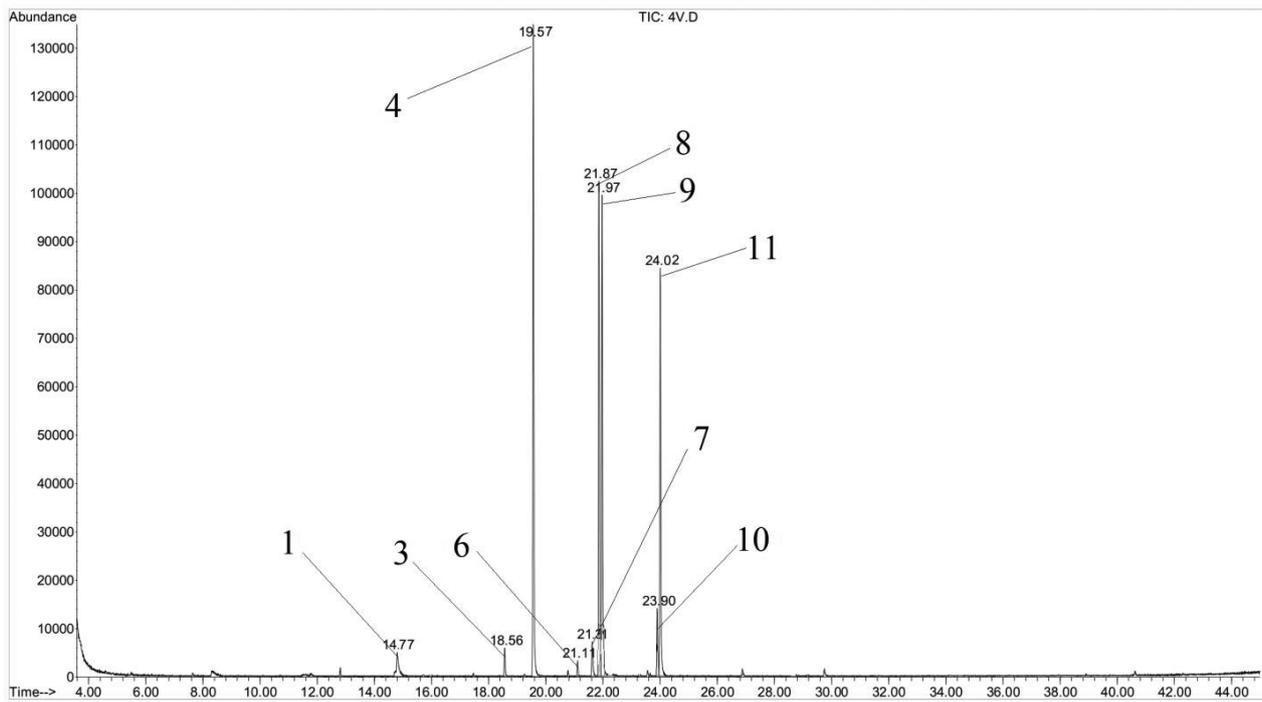


Figure 3. GC-MS chromatogram of free amino acids in the sample 2 of the plant mixture.

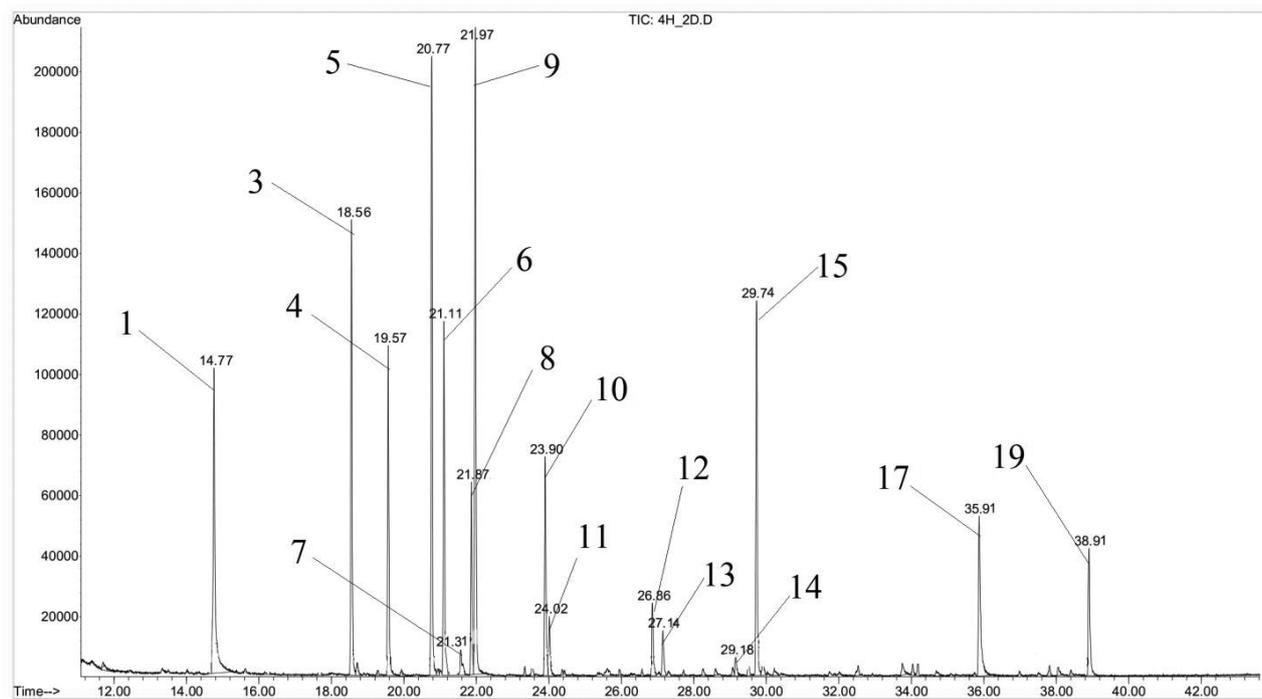


Figure 4. GC-MS chromatogram of amino acids after hydrolysis in the sample 2 of the plant mixture.

et. al. 2016). Therefore, plant amino acids, which are found in sufficient quantities in the studied plant mixture, are important for the course and treatment of diabetes.

The analytical procedure has been validated to confirm its reliability. All the peaks of reference standards showed good linearity ($R^2 > 0.98$) in a wide concentration range (0.1–10.0 mg/100 g). The results showed that the LODs and the LOQs of amino acids were in the range

of 0.01–0.07 mg/100 g and 0.02–0.20 mg/100 g, respectively, indicating that the sensitivity of the method was satisfactory. The repeatability of the subsequent derivatization and GC-measurement of five standard samples of each reference standard with the same concentration resulted in precision values for the derivatization procedure. For intra- and inter-day precision, the RSD was in a range of 1.24% to 8.10%, which is acceptable (Table 4.).

Table 3. Content of amino acid composition in the samples of the plant mixtures.

Number of peak on chromatogram	Name of amino acid	t_R , min	Content of amino acids, mg/g			
			Sample 1		Sample 2	
			Free	Bound	Free	Bound
1.	Glycine	14.77	n/d	2.066±0.12	0.559±0.01	6.147±0.05
2.	<i>L</i> -alanine	14.85	n/d	n/d	n/d	n/d
3.	<i>L</i> -valine*	18.56	0.268±0.01	2.721±0.01	0.277±0.01	6.049±0.07
4.	Nor-valine	19.57	Internal standart			
5.	<i>L</i> -leucine*	20.77	n/d	3.336±0.02	n/d	8.707±0.16
6.	<i>L</i> -serine	21.11	n/d	1.720±0.07	0.132±0.01	4.928±0.08
7.	<i>L</i> -threonine*	21.31	n/d	0.515±0.01	0.400±0.01	0.561±0.03
8.	<i>L</i> -isoleucine*	21.87	5.136±0.09	1.661±0.04	4.612±0.08	6.479±0.12
9.	<i>L</i> -proline*	21.97	19.885±0.12	13.856±0.09	4.794±0.19	4.617±0.16
10.	<i>L</i> -asparagine	23.90	0.170±0.03	0.609±0.05	0.254±0.02	0.242±0.07
11.	<i>L</i> -aspartic acid	24.02	4.592±0.05	7.065±0.06	4.070±0.03	5.772±0.11
12.	<i>L</i> -glutamic acid	26.86	n/d	1.462±0.03	n/d	1.270±0.08
13.	<i>L</i> -methionine*	27.14	n/d	0.101±0.01	n/d	0.840±0.01
14.	<i>L</i> -cysteine	29.18	n/d	n/d	n/d	0.427±0.01
15.	<i>L</i> -phenylalanine*	29.74	n/d	2.008±0.08	n/d	5.822±0.09
16.	<i>L</i> -glytamine	31.90	n/d	n/d	n/d	0.169±0.01
17.	<i>L</i> -lysine*	35.91	n/d	0.966±0.03	n/d	3.676±0.09
18.	<i>L</i> -histidine*	37.24	n/d	n/d	n/d	n/d
19.	<i>L</i> -tyrosine	38.91	n/d	0.681±0.01	n/d	2.368±0.11
20.	<i>L</i> -tryptophan	42.01	n/d	n/d	n/d	n/d

Note: 1. * – essential amino acid; 2. n/d – not detected; 3. Values are expressed as mean ± SD (n = 5).

Table 4. Results of linearity data obtained for individual amino acids after GC-MS analysis.

Amino acids	Regression Curve	R^2	LOD, mg/100 g	LOQ, mg/100 g
Glycine	$y = 95.25x + 4.308$	0.992	0.01	0.03
<i>L</i> -alanine	$y = 81.03x + 2.372$	0.996	0.01	0.04
<i>L</i> -valine	$y = 108.40x - 1.502$	0.996	0.02	0.06
<i>L</i> -leucine	$y = 44.24x + 2.285$	0.984	0.01	0.03
<i>L</i> -serine	$y = 110.90x - 0.241$	0.998	0.01	0.03
<i>L</i> -threonine	$y = 77.24x + 3.222$	0.990	0.01	0.04
<i>L</i> -isoleucine	$y = 44.24x + 2.285$	0.984	0.01	0.03
<i>L</i> -proline	$y = 124.50x + 0.359$	0.998	0.01	0.02
<i>L</i> -asparagine	$y = 80.84x + 2.885$	0.990	0.01	0.03
<i>L</i> -aspartic acid	$y = 154.40x + 2.375$	0.999	0.01	0.03
<i>L</i> -glutamic acid	$y = 65.30x + 3.934$	0.992	0.06	0.20
<i>L</i> -methionine	$y = 198.80x + 0.203$	0.999	0.01	0.03
<i>L</i> -cysteine	$y = 189.40x + 2.673$	0.994	0.01	0.03
<i>L</i> -phenylalanine	$y = 149.50x + 9.568$	0.990	0.01	0.04
<i>L</i> -glytamine	$y = 44.24x + 2.285$	0.984	0.06	0.20
<i>L</i> -lysine	$y = 127.80x + 5.598$	0.984	0.07	0.20
<i>L</i> -histidine	$y = 69.28x + 1.579$	0.992	0.03	0.10
<i>L</i> -tyrosine	$y = 124.90x + 2.897$	0.995	0.01	0.05
<i>L</i> -tryptophan	$y = 189.40x + 2.673$	0.994	0.01	0.04

Conclusion

The results of chromatographic examination indicate a sufficient content of amino acids, in particular essential, which have the ability to stimulate insulin secretion, reduce hyperglycemia and regulate metabolic processes in patients with diabetes. The predominant amino acids components were *L*-proline (19.885 mg/g in free form and

13.856 mg/g in bound form) in the sample 1 of the plant antidiabetic mixture and *L*-leucine (8.707 mg/g in bound form) and *L*-proline (4.794 mg/g in free form) in the sample 2 of the plant antidiabetic mixture, an essential amino acids with the most pronounced insulin secretolytic activity. The obtained data testify to the expediency of using the studied plant mixtures in order to optimize antidiabetic pharmacotherapy.

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