“BABEŞ-BOLYAI” UNIVERSITY

CLUJ-NAPOCA

FACULTY OF PHYSICS

Development of spectroscopic methods with biomedical applications and control on some food

Summary

Scientific coordinator:

Prof. Univ. Dr. CULEA Monica

PhD Student:

CORNEA (BLEIZIFFER) Cristina Ramona

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Keywords

• Mass Spectrometry (MS), Gas Chromatography (GC), GC-MS, Quantitative Analysis

• Validation parameters, measurement uncertainty, control diagrams

• Extraction, derivatization, quantitative analysis of amino acids, antioxidant activity

• Wine varieties, seeds, plants

• Statistical methods (chemometry), clinical data

The purpose of this thesis is to determine some biologically beneficial compounds for human health, by testing and developing methods of analysis in food (wine, seeds, plants) and monitoring them.

This thesis consists of 7 chapters, the first three presenting theoretical aspects, and the next four, the results of GC-MS analysis and other spectroscopic methods of complex biological systems.

Chapter I presents general concepts of mass spectrometry, ionization modes used in mass spectrometry, mass analyzers: quadrupole analyzer and ion trap analyzer, types of detectors, and medical applications of mass spectrometry.

Chapter II presents the basic principle of gas chromatography, the columns used here, as well as their characteristic parameters. The chapter concludes with the presentation of three of the most commonly used detector types in gas chromatography: the flame ionization detector, the thermo ionic detector and the electron capture detector and the GC-MS coupling, respectively.

The gas chromatography method - mass spectrometry (GC-MS) is particularly advantageous because gas chromatography is an ideal introduction system that allows the separation of components from the biological sample and the mass spectrometer is an ideal detector, which offers the possibility of identifying them. It is an exceptional technique through the areas of investigation in which it finds its applications and because it can identify unknown structures. Compared to conventional instrumental and chemical analytical methods applicable to components in large quantities, the GC-MS technique serves to identify components in small quantities in the nanogram and picogram range.

Chapter III presents the main validation criteria that are absolutely necessary to demonstrate that the chosen analytical method is the right one. For verification of validation parameters (detection limit, quantitative determination limit, precision, accuracy, linearity, etc.), it is demonstrated that the chosen method is correct and corresponds to the intended purpose.

Chapter IV has as main objective the development of a method of analysis of amino acids and fatty acids in wines by GC-MS with isotopic dilution. Six white wines from the Blaj vineyard were compared to their characteristics and free amino acids. The amino acid content is mainly influenced by grape varieties, geographical origin and fermentation status. Amino acids are important for the fragrance and aroma of wine. All white wines in Blaj contain a large amount of proline compared to other amino acids and essential amino acids. Fatty acids have been found in very small quantities.

Chapter V has as main objective the development of a method of analysis of seed amino acids by GC-MS with isotopic dilution. The amino acid content and antioxidant

activity of some linum seed, poppy, grape, hemp, walnut, pumpkin, sesame, watermelon, chia, corn, almonds and hazelnuts used as food supplements were compared.

Chapter VI aims to develop a method of analyzing plant amino acids with beneficial effects on the human body used in medicine and food. The purpose of the investigations was to determine the differences between plants purchased in Romania with regard to the amino acids present in these plants, often used as tea or spices, quantitative studies that were compared with antioxidant activity.

Chapter VII presents the results of optimization of diagnosis from blood analysis data using statistical methods (chemometry).

IV. ANALYSIS OF WINE GRAPE AMINO ACIDS AND FATTY ACIDS BY ISOTOPIC DILUTION - GC-MS

In this experiment, the comparison of free amino acids and volatile compounds from six white wines from the Blaj vineyard was compared with two other Romanian wines, one white and the other red: Fetească Roşie from Jidvei and Black Fetească from Sâmbureşti.

Methods of qualitative and quantitative analysis of amino acids and fatty acids have been developed or adapted. The results showed the differences between the analyzed wines. With respect to volatile compounds, the main compounds were 2-phenylethanol, succinic acid monoethyl ester and 4-hydroxyphenylethanol. All white wines in Blaj contain a large amount of proline compared to other amino acids and essential amino acids. The GC-MS method has proved to be an excellent method for characterizing wine.

Experimental steps

Amino acids were extracted from 100 mL of wine passed over a column (4x40 mm) with Dowex 50W-8W ion exchange resin (H^+ form). 50 μg ^{15}N - labeled glycine, ^{15}N-Gly, was added to each sample (100 mL) as internal standard. The collected solution was dried in a stream of nitrogen at 60°C or by using a vacuum centrifuge at 60°C. The derivatization method included a carboxyl group esterification using 100 μl of butanol: acetyl chloride (4: 1 v/v) for one hour at 110°C, followed by trifluoroacetylation of the amine group using 100 μl of trifluoroacetic anhydride for 20 min at 80°C.

Extraction procedure for fatty acids: 100 ml of wine was sonicated with 0.6 ml of water/NaCl and 0.8 ml of methanol for 1 min, then mixed with 0.8 ml of chloroform and 3 min centrifuged (5800 rot/min); the lower layer was collected and the extraction was repeated

with 0.4 mL of chloroform. The lower chloroform phase containing the extracted fatty acids was then dried in a stream of nitrogen at 60°C. The lipids were transformed into fatty acid methyl esters by esterification of the carboxylic groups with 100 µl of methanol: acetyl chloride 4: 1 (v: v), 20 min, at 80°C, the derivatives were evaporated to dryness in a stream of nitrogen at 60°C and then dissolved in 500 µl of dichloromethane. 10 µg of C11: 1 was added to each sample as an internal standard for quantitative determination by GC-MS.

Volatile extraction procedure: 5 ml of wine was mixed with 1 ml solvent (mixture of ethyl acetate: hexane: dichloromethane, 5/1/1) for 2 minutes and centrifuged (5800 rpm) for 3 minutes. The supernatant was collected and 1 µl was injected into GC/MS [36].

Amino acids and fatty acids were separated and identified by a gas chromatograph (Trace GC) coupled to a quadrupole analyzer mass spectrometer (Trace DSQ Thermo Finnigan) (Fig. 4. 4). Separation was performed on a capillary column Rtx-5MS (30m x 0.25 mm, film thickness: 0.25µm).

Fig. 4. 4. GC-MS with autosampler.

The temperature program used to separate the amino acids was as follows: the oven temperature was maintained at 70°C for 2 min, then increased at a rate of 5°C/min to 110°C, then 10°C/min up to 290°C, 16°C/min to 300°C and maintained there for 3 min.

The following operating conditions of the mass spectrometer were the same for the investigated compounds: transfer line temperature: 250°C, ion source temperature: 250°C, ionization with electron impact (EI, electron energy: 70 eV) and emission current intensity was 100µA. The mass spectrometer worked in SCAN mode, in the mass range: 30-520 u.am.

Results

17 amino acids were identified and determined; the method was validated using standard amino acids in 0.1 N HCl. For standard solutions, the same derivatization method

was applied as for the amino acids extracted from the wine samples. The precision was 20% (DSR), except for arginine, cysteine and tyrosine, and the detection limit (LOD) was 10 ng for each amino acid. The order of elution of the amino acids was as follows: alanine (Ala), glycine (Gly), threonine (Thr), serine (serine), valine (Val), leucine (Leu), isoleucine (Ile), cysteine (Cys), gamma-aminobutyric acid (GABA), proline (Pro), methionine (Met), aspartic acid (Asp), ornithine (Orn), phenylalanine (Phe), tyrosine (Tyr), lysine (His), glutamic acid (Glu), histidine (His) (Fig.4.7).

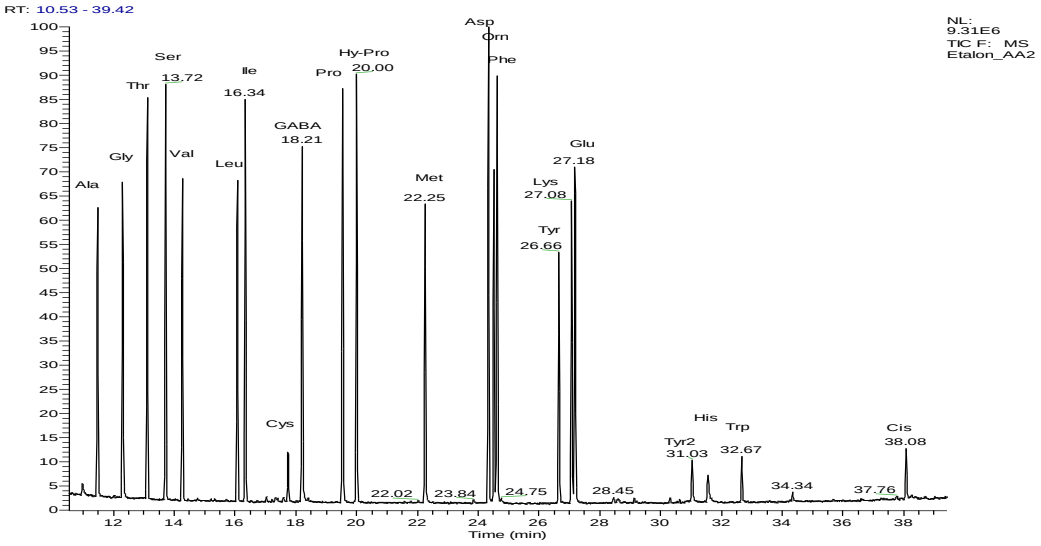

Fig. 4. 7. The chromatogram of a standard amino acid mixture

Six white wines from the Blaj vineyard (Royal Feteasca, Blasius, Neuburger, Pink Traminer, Muscat Ottonel, Selena) were studied. Volatile compounds and free amino acids were compared. The dominant amino acids identified in the wines were proline (15.7mg/ml in Blasius), glutamic acid, aspartic acid, gamma-aminobutyric acid, alanine, glycine and lysine. Proline is the main amino acid in wine samples, released in fermentation, being an intermediate product in arginine degradation [41]. High levels of proline in wine are due to the fact that yeast microorganisms do not consume this amino acid. Arginine has not been found in wine because it is consumed during yeast fermentation [16]. Total free amino acids were in the range of 10.2 mg/ml (Feteasca Regala Blaj) and 19.2 mg/ml (Traminer Rose Blaj). Essential amino acids (EAAs) ranged between 0.45mg/ml (Neuburger) and 1.44mg/ml (Rose Traminer).

The fatty acids present in wines were less than 20 μg/ml, stearic acid, palmitic acid and linoleic acid being dominant.

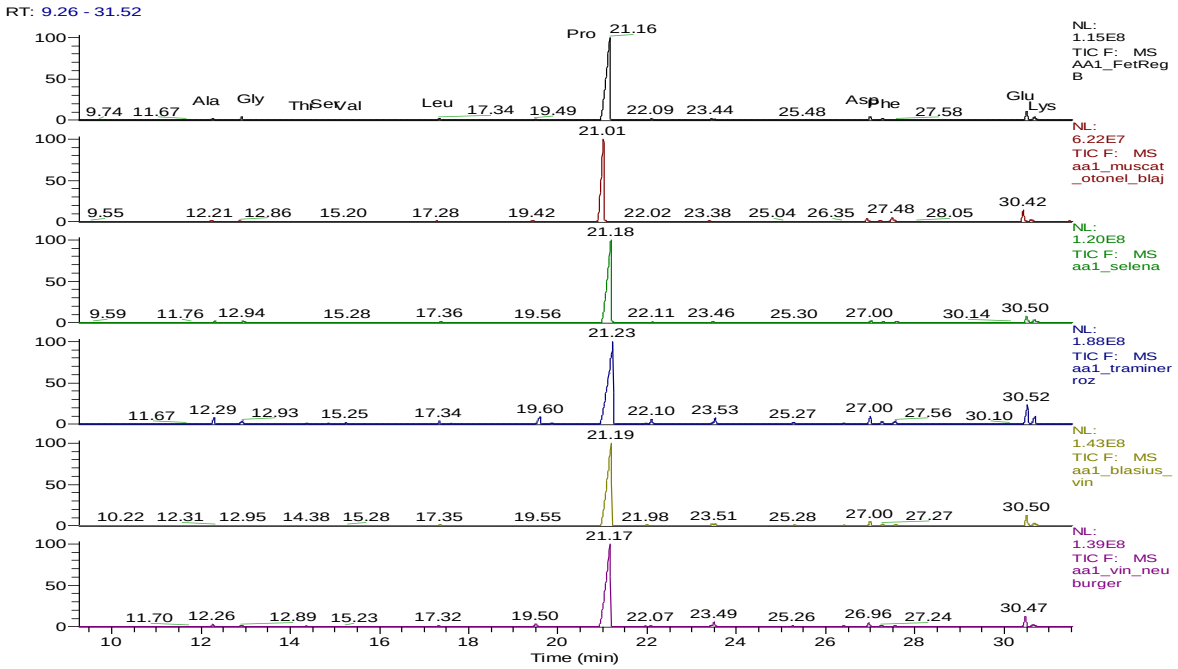

Fig. 4. 12. The comparative chromatograms of separation of amino acids

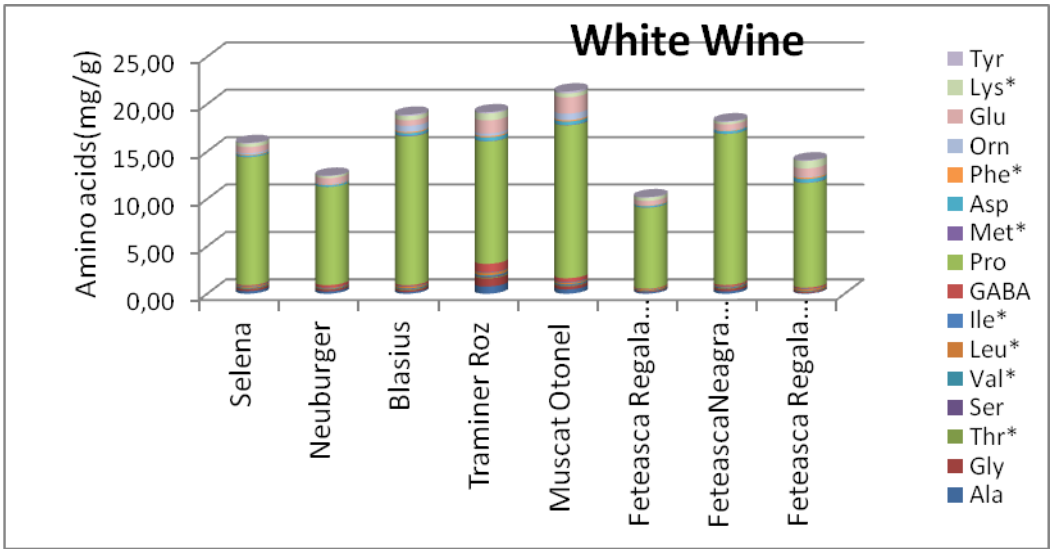

Fig. 4. 13. Free Amino Acids in Romanian Wines.

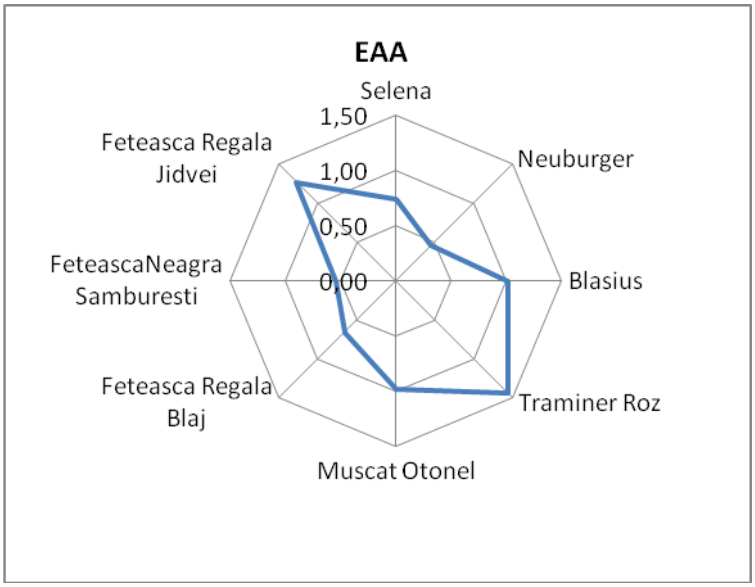

Fig. 4. 15. Comparison of essential amino acids (mg/g)

Volatile wine extracts gave very similar compounds. The main determinants were 2-phenylethanol (21.5% Otonel Muffin to 45.76% -Neuburger), succinic acid monoethyl ester (17.29% -Neuburger at 37.4% -Blasius) and 4-hydroxyphenylethanol 6.7% in Muscat Otonel to 15.37% in Feteasca Regală). [11]

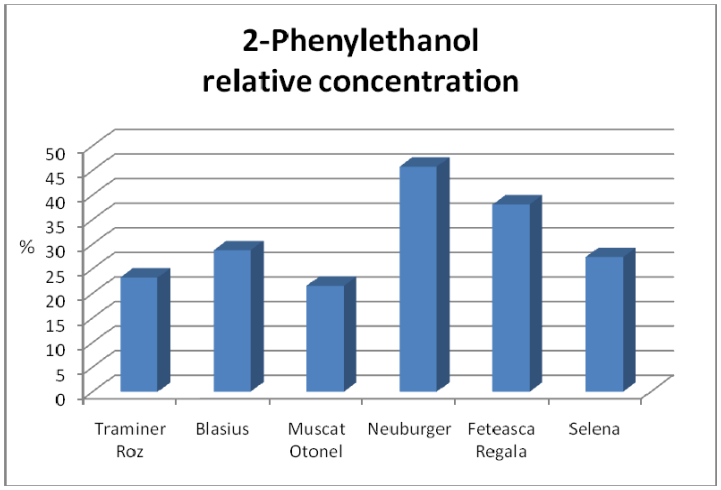

Fig. 4. 17. Relative concentration of 2-phenylethanol in different wines in the Blaj area.

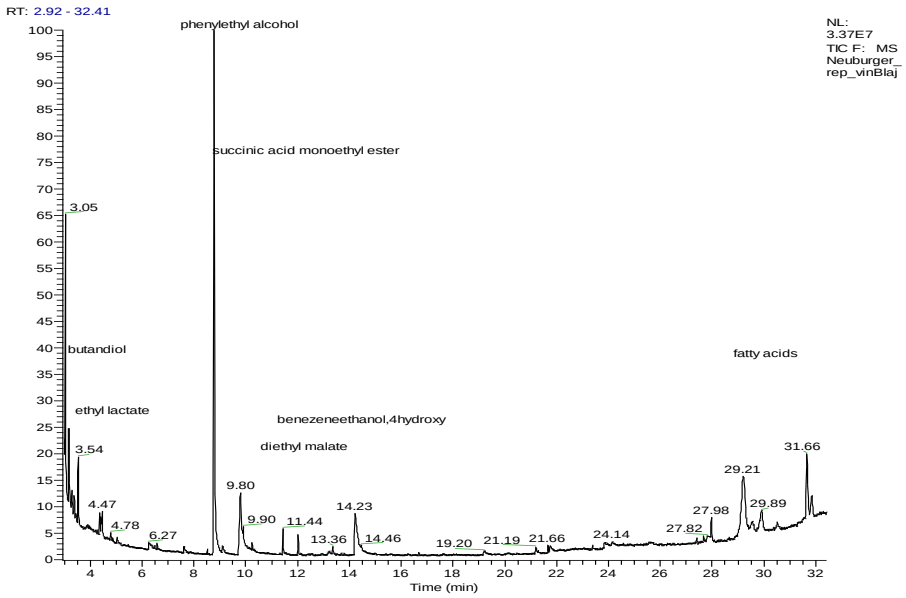

Fig. 4. 18. GC/MS separation and identification of volatile compounds from wine (Neuburger).

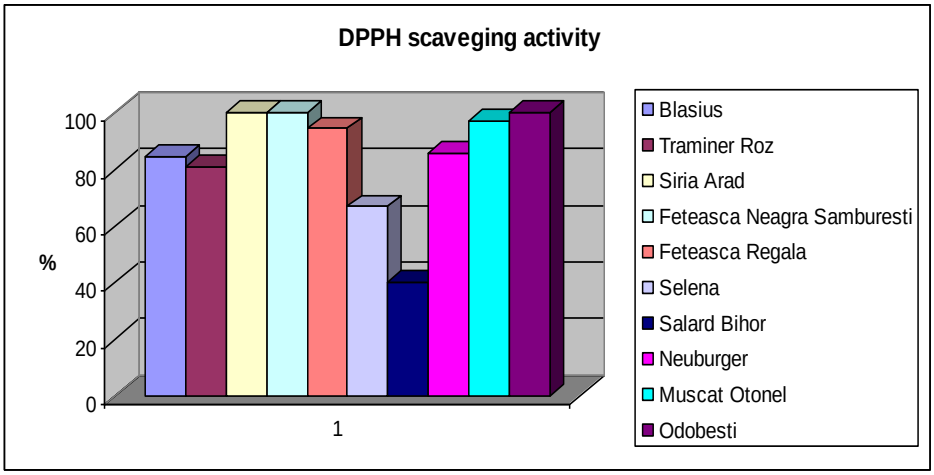

Fig. 4. 19. Antioxidant activity of the studied wines

It is known that the flavonoids found in the grapes are very healthy, but give a bitter taste to the wine [43]. That's why white wine producers do not use grape skins to change the taste of wine. Also, the flavonoid content of white wines is not as high as red wines due to the low initial amount of flavonoids from white grapes. All white wines studied have a small amount of flavonoids, the largest amount being found in Selena wine 8.96 mgQuE/L.

The antioxidant activity of the studied wines, carried out by the DPPH method (2,2 diphenyl-picryl-hydrazyl), was compared with that of some red wines; Fetească neagră from Sâmbureşti, Syria Arad Salard Bihor (homemade wine), Vin de Odobeşti (Fig 4.19). Wine-making technology influences their antioxidant capacity.

Conclusions

The analysis method developed in this experiment has good validation parameters (precision <20% DSR, LOD <10 μg/mL, 10 ng injected). Amino acids are important for the smell and aroma of wine, being involved in yeast metabolism. All white wines in Blaj contain a large amount of proline compared to other amino acids. Important essential amino acids were compared. Traminer Rose, Feteasca Regală Jidvei, Blasius, Muscat Otonel had the amount of essential amino acids higher. Fatty acids have been found in very small quantities. The analyzed samples vary both as composition, but especially in terms of the quantitative ratio of characteristic flavor components. The described extraction methods are useful for wine characterization (wine bouquet, GC fingerprint of volatile extracts) as well as for the detailed characterization of wine compounds. The GC-MS method is the most appropriate method for the qualitative and quantitative analysis of volatile organic compounds, wine bouquets and some active principles in wines.

V. ISOTOPIC DILUTION - GC-MS ANALYSIS OF SEED AMINO ACIDS

The purpose of this study was to compare the amino acid content and antioxidant activity of some seeds: linum, poppy, grape, hemp, walnut, pumpkin, sesame, watermelon, chia, corn, almonds and hazelnuts used as food supplements. The antioxidant attributes of seed extracts were evaluated using DPPH (2,2-diphenyl-picryl-hydrazyl) anti-oxidant assays that capture free radicals.

Mass Spectrometry and Gas Chromatography (GC-MS) was used for the analysis of amino acids in several selected seeds [60,62-67]. The method involves a procedure of extraction, derivatization and GC-MS analysis.

Experimental steps

100 mg of crushed seeds were extracted with 1 ml of 6% trichloroacetic acid in an ultrasonic bath for 5 minutes. The mixture was centrifuged for 5 min at 6000 rpm and the supernatant was collected for purification. 0.5 ml of supernatant and 50 μg of [^{15} N] -glycine (internal standard) were passed over a Dowex 50W-W8 column, 4 x 40 mm (activated) column. The collected solution was dried in a stream of nitrogen at 60°C by centrifugal vacuum at 60°C. 200 μl of butanol: acetyl chloride (4: 1 v/v) was used to esterify the carboxylic group for one hour at 110°C followed by acetylation of amine group using 100 μl of trifluoroacetic anhydride for 20 min at 80°C.

100 mg of the seeds were extracted with 1 ml of ethanol at 60°C in an ultrasonic bath for 15 minutes. The mixture was centrifuged at 5800 rpm and the supernatant was collected and tested for antioxidant activity. The antioxidant DPPH test was used to determine the antioxidant activity. 100 µl (10 mg/ml seed) of each extract was used.

Sample analysis was performed using a gas chromatograph coupled with a Trace DSQ quadrupole analyzer mass spectrometer (Thermo Finnigan). Separation of the compounds took place on a capillary column Rtx-5MS (nonpolar stationary phase: 5% diphenyl/95% di-methyl polysiloxane), with the following dimensions: 30m long x 0.25mm internal diameter, 0.25µm film thickness. The temperature program of the chromatographic oven was for amino acids: 70°C, holding 2 min, 5°C/min to 110°C, 10°C/min to 290°C, 16°C/min to 300°C, 3 min. The carrier gas was He 6.0 with a flow rate of 1 mL/min.

Ionization was performed by electron impact (70 eV, electron energy) and the emission current was 100µA. The transfer line temperature was set at 250°C, the injector temperature at 200°C and the ion source temperature at 250°C. 1 µL sample was injected automatically, in split mode (10: 1), using a Triplus autosampler. Mass spectrometer worked in SCAN mode, recording mass in the range: 50-500 u.am.

Results

The method developed is selective and specific. The mass spectra recorded on each chromatographic peak allow precise amino acid identification using the NIST spectra library. Also, the superposition of the components is easily highlighted. The method was validated using standard amino acids. The dominant amino acids found in the seeds were glutamic acid, aspartic acid, proline, glycine, lysine, alanine, histidine (in watermelon), and tyrosine (in linum seed). Fig. 5.1. shows Chia free amino acid separation chromatograms compared to the standard ones: (Gly: 13.53 min; Thr: 15.01 min; Val: 15.94 min; Pro: 21.7 min; Asp: 27.71 min; Glu: 31.16 min) .

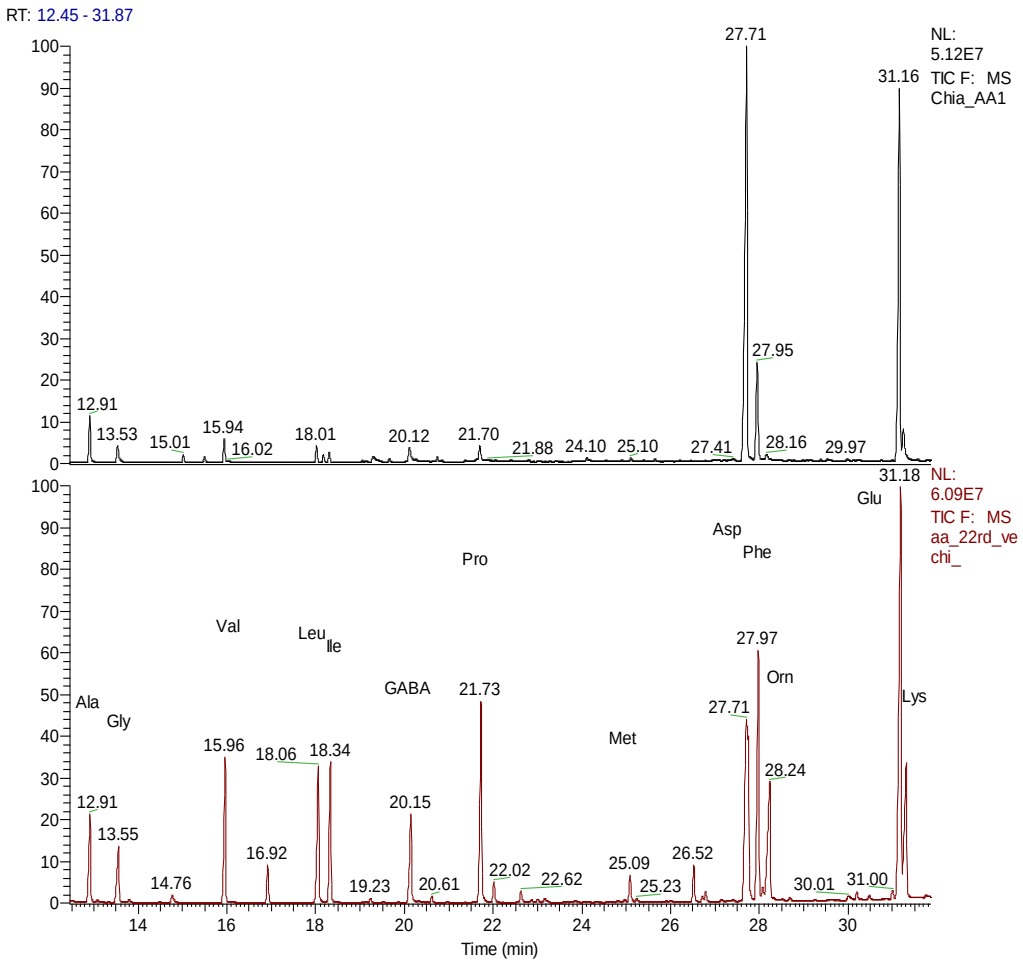

Fig. 5. 1. Chromatograms of Chia free amino acid separation: (Gly: 13.53 min; Thr: 15.01 min; Val: 15.94 min; Pro: 21.7 min; Asp: 27.71 min; Glu: 31.16 min) (up), and standard amino acids (below).

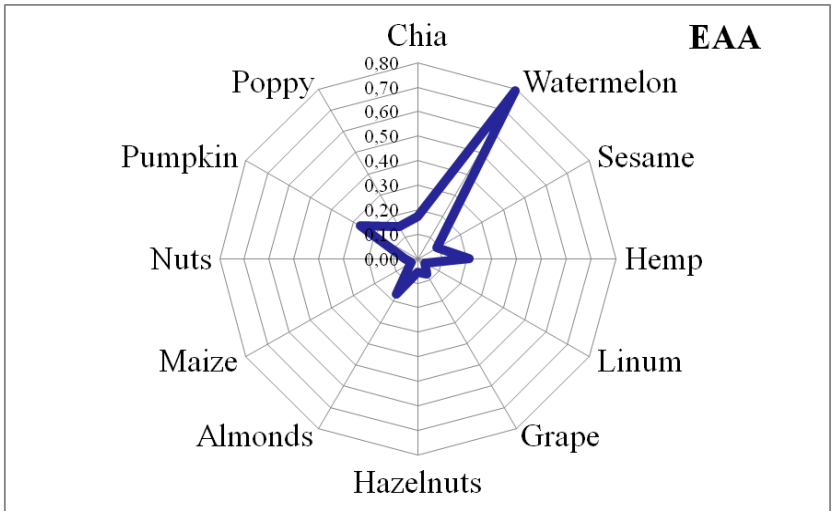

Fig. 5. 2. Comparison of essential amino acids from different seed extracts (mg/g)

All extracts exhibit antioxidant activity. The highest antioxidant activity was found to have grape seed extract, followed by nut and hemp extract, all having antioxidant activity comparable to standard antioxidants (Fig. 5. 6).

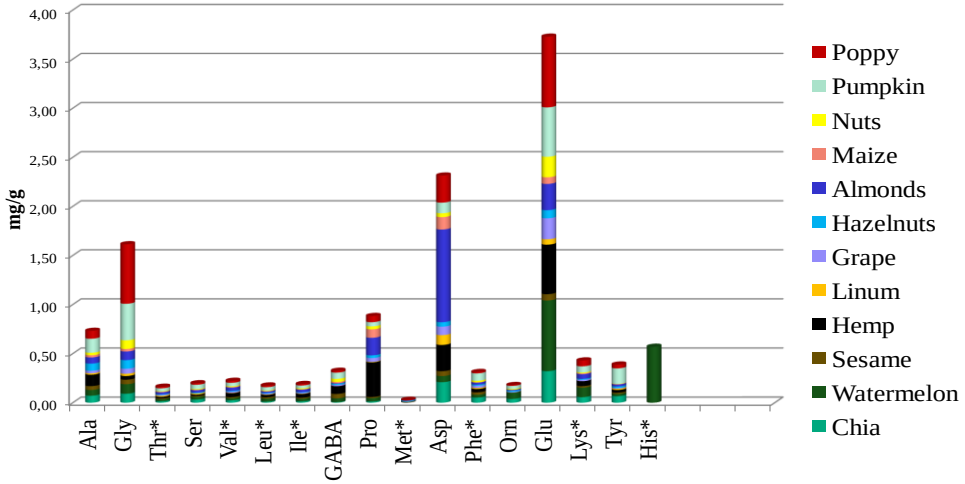

Fig. 5. 4. Free amino acids (FAA) in seed samples. Essential amino acids are marked (*)

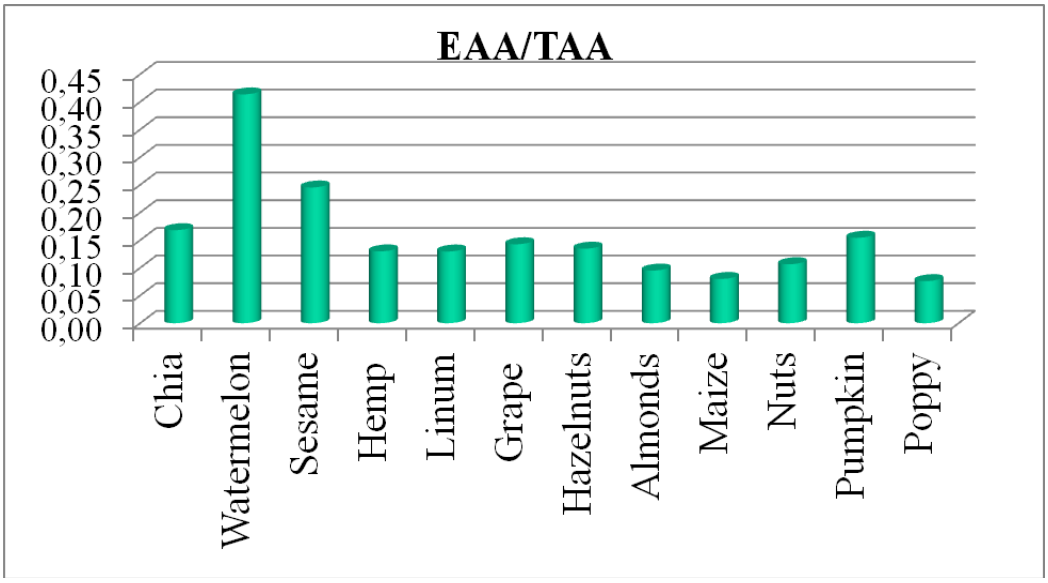

Fig. 5. 5. Total essential amino acid/total amino acid ratio in seed samples

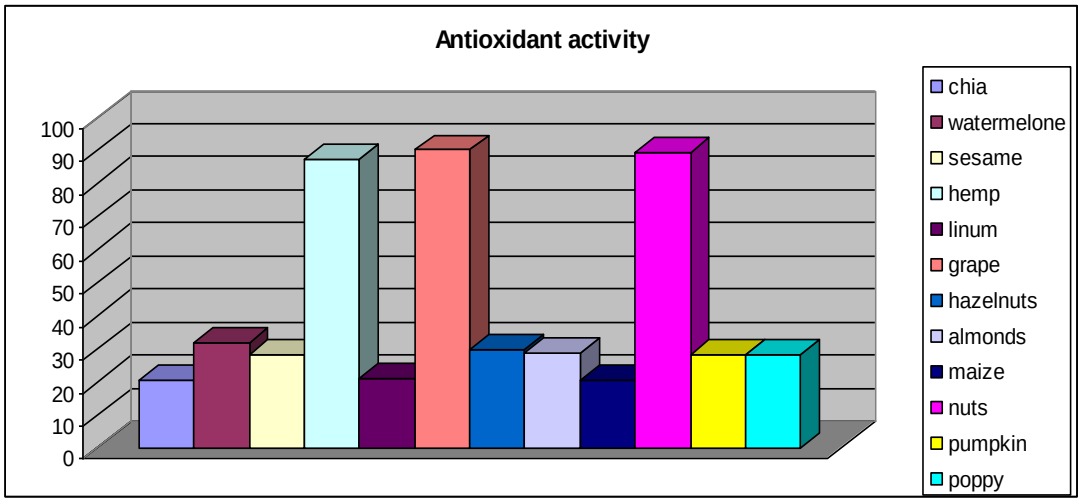

Fig. 5. 6. Antioxidant activity of studied seed samples

Conclusions

The developed isotopic dilution method has proved to be accurate and simple, useful for characterization studies of the various seed extracts studied. The method can serve to differentiate between seeds used as nutritional supplements. Significant value for essential amino acids was obtained in the case of watermelon seeds (due to the high value of histidine) followed by hemp, pumpkin, almonds, poppy and grape seeds. The greatest antioxidant activity has been shown to have grape seeds, nuts and hemp seeds. The nutritional value expressed by the amino acid content, especially essential and the antioxidant properties, has demonstrated their quality of use as nutritional supplements. The study determined the variation of free amino acids in the various samples associated with their antioxidant capacity.

VI. ISOTOPIC DILUTION - GC-MS ANALYSIS OF PLANT AMINO ACIDS

Aromatic plants are widely used in the preparation of food and fragrances, perfumes, but they are also a good source of amino acids.

The studied plants traditionally used in medicine and food have been characterized and compared in terms of volatile extracts, amino acids and antioxidant capacity. Mass Spectrometry coupled with Gas Chromatography (GC-MS) is a suitable technique for characterizing compounds in plant extracts.

The purpose of the investigations was to determine the differences between the plants purchased in Romania with regard to the amino acids present in these plants, often used as tea or spices. Also, their volatile compounds were also compared. Mass Spectrometry - Gas Chromatography Coupling (GC-MS) is a suitable technique for characterizing compounds in plant extracts [75-80]

Experimental steps

Plants: Caraway, Basil (Ocimum basilicum), Elderberry flower, Dandelion, Comfrey, Ginger, Howthorn, Lemon Verbenon, Celandine, Thyme, Artemisia, Mint (Mentha piperita), Kurry, Sage (Salvia officinalis), Rosemary and Nettle were purchased from the Botanical Garden in Târgu Mureş, Romania. All reagents and standards were purchased from Merck (Darmstadt, Germany).

For amino acid extraction, 100 mg of plant leaves were extracted with 1 ml of 6% trichloroacetic acid. The obtained mixture was centrifuged for 5 minutes at 6000 rpm and the supernatant was collected for purification. 0.5 ml of the supernatant and 50 μg of [^{15}N] - glycine (internal standard) were passed over an ion exchange resin (activated), Dowex 50W-W8 column 4 x 40 mm. The collected solution was dried in a stream of nitrogen at 60°C.

The derivatization method included an esterification of the carboxylic group using 200 μl of butanol: acetyl chloride (4: 1 v/v) for 1 hour at 110°C, followed by acetylation of the amine group using 100 μl of trifluoroacetic anhydride, for 20 minutes at 80°C.

For the extraction of volatile substances, 100 mg of crushed leaves were sonicated and extracted with 1 ml of ethanol at 60°C for 15 minutes, then centrifugated for 3 minutes. The mixture was centrifuged at 5800 rpm and the collected supernatant was filtered and injected into GC/MS.

Antioxidant Activity: 100 mg of crushed plants were extracted by sonication of 1 mL ethanol at 60°C for 15 minutes. The mixture was centrifuged at 5800 rpm and the collected supernatant was tested for antioxidant activity by the DPPH method. 100 μL (10 mg/mL plant) of each extract was used to decolorize a solution of 40 μM DPPH.

Results

We determined and compared the content of volatile substances, amino acids and antioxidant activity in medicinal herbs: cumin, basil, elder tree, dandelion, dill, ginger, hawthorn, lemon, celandine, thyme, artemisia, mint, curry, sage, rosemary, nettle. Characterization of plant extracts used extraction methods, ion exchange purification for

amino acids, derivatization steps, and mass spectrometric (GC-MS) gas chromatographic analysis.

For amino acid analysis, the methods were validated by injection of standard amino acid solutions. Samples followed the same derivatization procedure as standards. Good values for linearity, precision and detection limit were obtained [75].

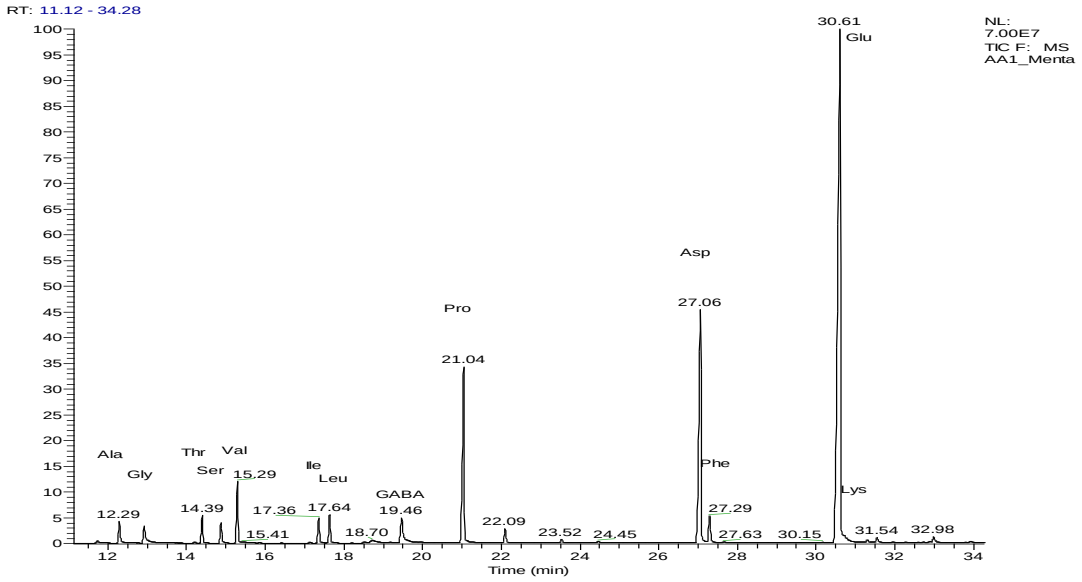

Fig. 6. 3. GC-MS separation and identification of the amino acids present in the mint extract

Figure 6. 3. shows the chromatogram of separation of the amino acids and their identifjcation in the peppermint extract. The NIST library was used to identify compounds.

The dominant amino acids found in the plants studied were proline (in mg/g, Curry (18.8), Artemisia (8.00)); glutamic acid (Menta (4.81), Dandelion (2.81), Comfrey (2.15)); acid aspartic (Nettle (3.16), Ginger, Elderberry flower (1.72), Mentha (1.28)): lysine, (Ginger (0.21), Artemisia (0.14)); glycine (Chimen 0.60), Comfrey (0.54) (Nectar (0.69), Ginger (0.37), Chimen (0.38)). The highest values for total free amino acids were observed in curry, artemisia, mentha, nettle, and celandine (> 7mg/g). Essential amino acids ranged from 0.05 mg/g in Basil to 1.70 mg/g in Dandelion (Figure 6.4).

Significant values of essential amino acids (EAA) in mg/g were obtained in the case of Dandelion (1.70) followed by Nettle (1.35), Celandine (1.27), Artemisia (1.23), Mentha (1.07), Ginger (0.87) and Lemon Verbenon (0.56). The EAA/TAA ratio was high for Elderberry flower (0.22), followed by Basil (0.19), Celandine (0.18), Nettle (0.18), Howthorn (0.17), and the lowest Ginger (0.15).

Total amino acids (FAA) ranged from Curry (20.70) to Poppy (13.47), Artemisia (12.08), Mint (9.07), Nettle (7.40), Celandine (7.03), Ginger), Lemon (4.21), Sage (4.03), Caraway (2.41), Thyme (2.26), Comfrey (1.93), Howthorn (1.04), Rosemarin (0.94) and Basil (0.26).

The method has proved to be appropriate and relatively simple to be applied to the analysis of total amino acids and may help to compare the plants.

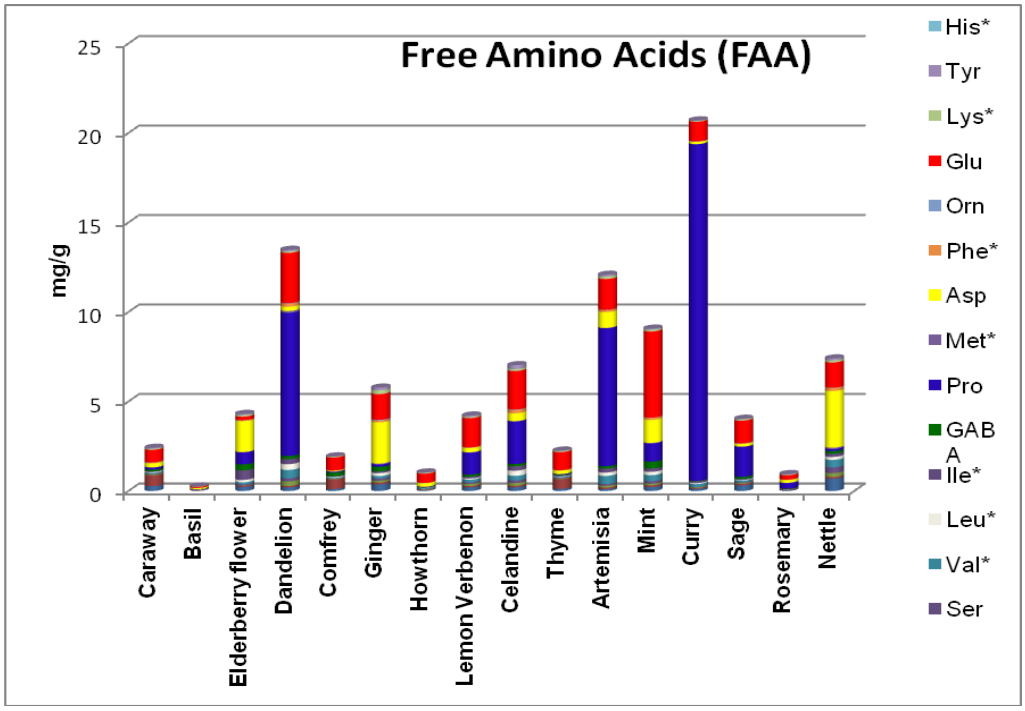

Fig. 6. 4. Comparison of free amino acids in studied plant extracts (mg/g). Essential amino acids are marked (*).

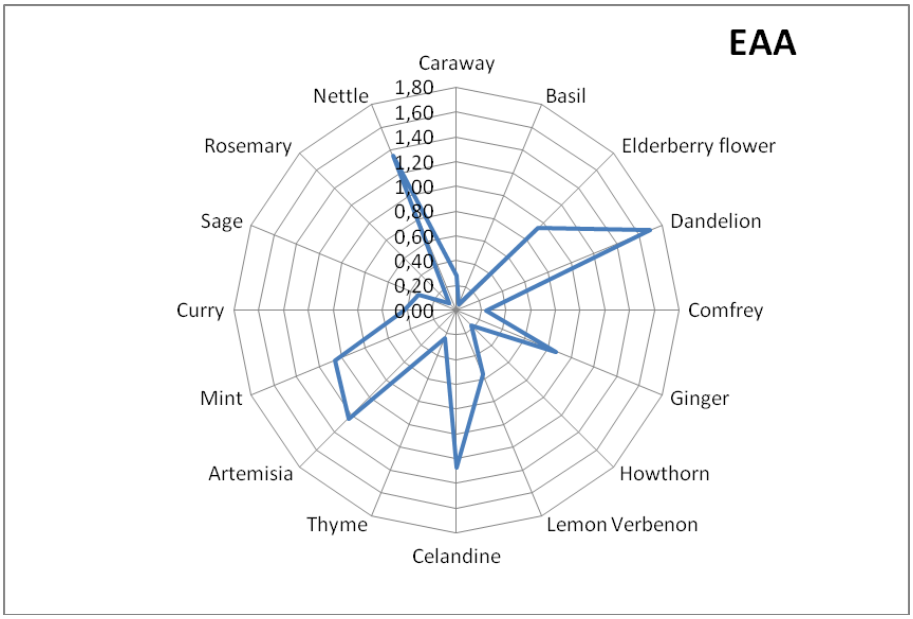

Fig. 6. 6. Comparison of essential free amino acids in plant species studied (mg/g).

Antioxidant activity was determined using the DPPH method (based on decolorizing the purple red colored 2,2-diphenyl-picryl-hydrazyl radical with absorption at anti-oxidant content at 515 nm). All plant extracts showed antioxidant activity. The largest, over 90%, was Rosemary, followed by Thyme, Howthorn, Sage, Curry. Nettle had antioxidant activity below 10%.

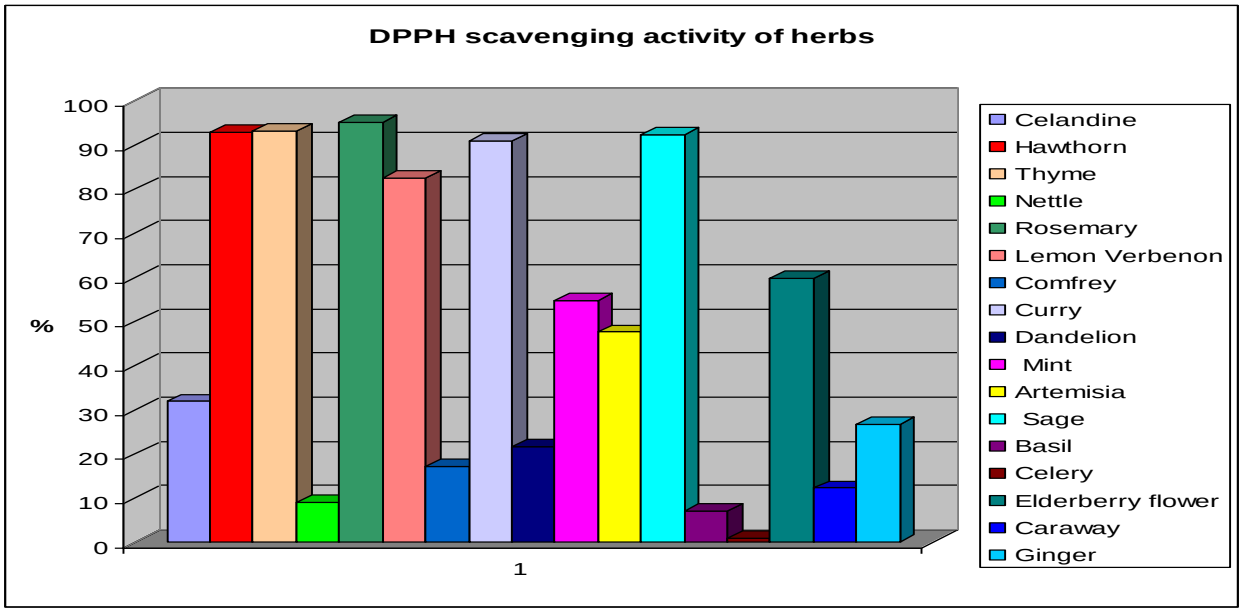

Fig. 6. 7. The antioxidant activity of plant extracts: Caraway, Basil, Elderberry flower, Dandelion, Comfrey, Ginger, Hawthorn, Lemon Verbenon, Celandine, Thyme, Artemisia, Mint, Curry, Sage, Rosemary, Nettle.

Conclusions

The GC-MS isotope dilution method is a suitable technique for determining amino acid extracts in plants. Validation parameters: Linearity, interest range, correlation coefficients, precision, were good. By isotopic dilution, using a marked internal standard, the accuracy increases, avoiding the overlapping of the analyzed compounds. The methods are useful for nutrient control and diet control. The compounds identified in the plants studied are characteristic of the fragrance or aroma of these plants.

Significant values for total free amino acids, over 7 mg/g, were obtained for Curry, Dandelion, Artemisia, Mint, Nectar and Comfrey. For essential amino acids, there were high values for Dandelion, followed by Nettle, Comfrey, Artemisia, Mint, Elderberry flower, Ginger and Lemon Verbenon. The dominant amino acids identified in the studied plants were proline, glutamic acid, aspartic acid, lysine, glycine and alanine. The highest antioxidant activity was obtained for the extracts from Rosemary, Thyme, Howthorn, Sage and Curry.

VII. STATISTICAL METHODS (CHEMOMETRY) FOR OPTIMIZATION OF DIAGNOSIS FROM BLOOD ANALYTICAL DATA

The main goal was to conduct a chemometric study of clinical data from analytical results using cluster analysis methods and main component analysis (PCA). Spectrophotometric techniques were used to obtain a diagnostic method by analyzing the blood of many patients. Similarity patterns have been found between patients and clinical data. Two classic chemometry methods, namely Cluster Analysis (CA) and Principal Component Analysis (PCA), were applied to the clinical data evaluation. These methods have been used to differentiate patients (cases) by gender and age. By analyzing the main components, the set of data was reduced to a few representative ones, and by cluster analysis, differences and inconsistencies were determined.

The subjects that were chosen are patients who have presented themselves to the Medical Analysis Laboratory in order to obtain results of some parameters of interest for the diagnosis of some diseases or for monitoring the afflictions suffered by the patient.

Experimental

Study I selected 6 men (M) and 30 women (F). The spectrophotometric method was used and the sample type: serum. Clinical analytical data are presented in Table 7.1. and Table 7.2.

Compounds investigated in human blood samples were organic compounds of clinical interest (glucose, cholesterol, triglycerides, urea, creatinine, table 7.1.), Inorganic compounds (Ca, Mg and Fe), enzymes (TGO, TGP- transaminases) and VSH (the rate of sedimentation of the red blood cells) (Table 7.2). Chemometric methods have been used to differentiate patients (cases) by gender and age [123].

Table 7. 1. Clinical data studied (mg/dl) [123].

P	Sex	Age	Glu	Chol	Trigly	Urea	Creat
1	F	65	86.5	268	145		0.95
2	F	68		260	119		
3	M	65	91.5	165	136		0.95
4	F	12	83.1			42.44	0.41
5	F	14	97	145	62	25.1	0.65
6	F	81	109.6	160	116		0.83
7	M	69	98.5	207	73		1.07
8	M	33	95	230	101	36.84	0.81
9	F	58	310	266	225		1.22
10	F	73	131.1	221	57		0.54

11	F	71	100	194	128	38	1.08
12	F	60	107	353	219	25.93	0.84
13	F	49	88	273	134		0.58
14	F	76	88.5	217	86	30.39	0.95
15	F	54	92	250	117		0.78
16	F	45	85	199	132	11.23	0.91
17	F	21	74	177	36	28.18	0.82
18	M	48	94	262	191	22	1.24
19	F	65	123	256	62	22.69	0.77
20	F	54	94	201	124	39.11	0.82
21	M	56	109	165	42	26.04	0.82
22	F	72	145	179	94		0.8
23	F	25	78		41	22.65	0.88
24	F	50	83	195	48		0.87
25	F	54	101	220	227	65.64	1.32
26	F	51	86	276	191		0.92
27	F	74	84	230	57		0.71
28	F	29	72	142	49	14.85	0.82
29	F	60	89	259	116		0.97
30	F	49	95	168	125	29.67	0.84
31	F	87	101	187			1.1
32	F	53	82	293	69		0.84
33	F	27	81	175	73	18.95	0.86
34	F	76		213	70	29.42	0.99
35	F	41	78	194	45		0.67
36	M	61	84	170	92	27.87	0.93

Biological reference range:
Glucose (Glu): 60-110 mg/dl;
Cholesterol (Chol): <200 mg/dl; high:> 240 mg/dl;
Triglycerides (triglycerides): Men (M): 40-160 mg/dl; women (F): 36-135 mg/dl;
Urea: 10-50 mg/dl;
Creatinine (created): male: 0.9-1.3 mg/dl; women: 0.6-1.1 mg/dl;

Table 7.2. Clinical data studied (mg/dl) [123].

P	Sex	Ac. uric	TGO	TGP	VSH	Ca	Mg	Fe
1	F	5.49	27	19	13			
2	F		30	38				
3	M	6.34	29	24	11			
4	F		28	14	5		2.25	87
5	F		25	17		9.27	2.11	
6	F	6.75	23	17				
7	M		32	23			2.36	98
8	M		49	66	5			
9	F	3.77	36	44	10			
10	F		20	18				

11	F		22	10	45	8.2	2.24	62
12	F		36	23	16	8.1	2.4	
13	F		24	26	6	9.57	2.35	94
14	F		27	18		9.31	2.29	
15	F	4.09	44	64	9	9.54		
16	F	4.11	23	16	13	8.63	2.19	74
17	F	3.32	30	13	5	9.17	2.18	102
18	M	11.12	53	70	5	9.49	2.32	123
19	F		28	22				
20	F		27	10	8		2.29	79
21	M	3.32	37	38				
22	F	4.66	25	27				
23	F	3.52	11	19		8.7	2.11	70
24	F	2.96	26	12	5			
25	F	7.05	27	18				138
26	F		28	22	12	8.25	2.3	87
27	F		27	16	6	8.35	2.31	69
28	F			16	5	8.94	2.25	108
29	F	4.95	22	19	5	8.79	2.46	123
30	F	3.06	34	56	15	8.93	2.27	
31	F		33	7	19	8.93	2.29	95
32	F		24	22	18	9.25	2.33	
33	F		43	46		8.73	2.06	
34	F		12	33		9.45		
35	F	3.23	15	29	5	8.86	2.56	128
36	M	5.92	57	61	18			68

Biological reference range:
Uric acid: male: 3.4-7.0 mg/dl; Women: 2.4-5.7 mg/dl;
TGO: 0-35 U/L;
TGP: male: 0-35 U/l; women: 0-36 U/I;
VSH: 2-12 mm/h Westergreen method, sample type: whole blood K3 EDTA;
Calcium (Ca): 8.6-10.3 mg/dl;
Magnesium (Mg): 1.6-2.5 mg/dl;
Iron (Fe): Children: 50-120 mg/dl; women: 60-160 mg/dL male: 80-180 mg/dl.
Another study (II):

Sudy II: There were also studied 27 men (M) and 46 women (F) of different ages at which the patient's diagnosis was considered and some analyzes in addition to the previous study, hematology analysis: WBC (Leucocytes), HGB (Hemoglobin) and PLT (Platelets).

Compounds investigated in human blood samples are of clinical interest (WBC, HGB, PLT, VSH, TGO, TGP, Table 7.3), (Ca, Mg, Fe, Urea, Creat, Uric, Glic, Col, Trig. Chemometrics were used to differentiate patients (cases) by gender and age. The patients were divided into two groups, by gender and in three groups, according to their age and three main components.

Results

The aim of the study was to find similarity patterns, both between patients and clinical trials. Two classic chemometry methods, namely cluster analysis (CA) and component analysis (PCA), were applied to the clinical data evaluation. These methods have been used to differentiate patients (cases) by gender and age. By analyzing the main components the set of data was reduced to a few representative ones, and differences and mismatches were determined by the cluster analysis [110]. Cluster analysis shows the degree of correlation through ESR (VSH), Ca, Mg, Uric acid, Creatinine, TGP, TGO, Urea, Fe, Triglyceride and Cholesterol. There are similarities between the samples from different patients. (Figures 7.1, 7.2). From the cluster analysis, a high correlation between magnesium and creatinine is observed, as other researchers have observed [114]. Calcium and uric acid also proved to be strongly correlated. There are several studies that have found a considerable percentage in patients with oxalate, a high uric acid concentration and also hypercalciuria [115]. Cluster analysis has shown that VSH is correlated with Ca, uric acid, creatinine and Mg, which make VSH a good marker for kidney disease. This was also clinically proven [116]. Very correlated are also the enzymes TGP and TGO, the enzymes of hepatic transaminases found in the metabolism of amino acids and are directly correlated with the urea concentration.

The study showed a good correlation between transaminase enzymes, urea and VSH, Ca, Mg, uric acid, creatinine. A significant correlation was obtained between Fe, triglycerides and cholesterol.

The explanation of the clusterization found is relevant and is based on the similarity between glucose level, enzyme level, hepatic function, renal function. This classification helps to optimize the clinical data for patients and to diagnose patients.

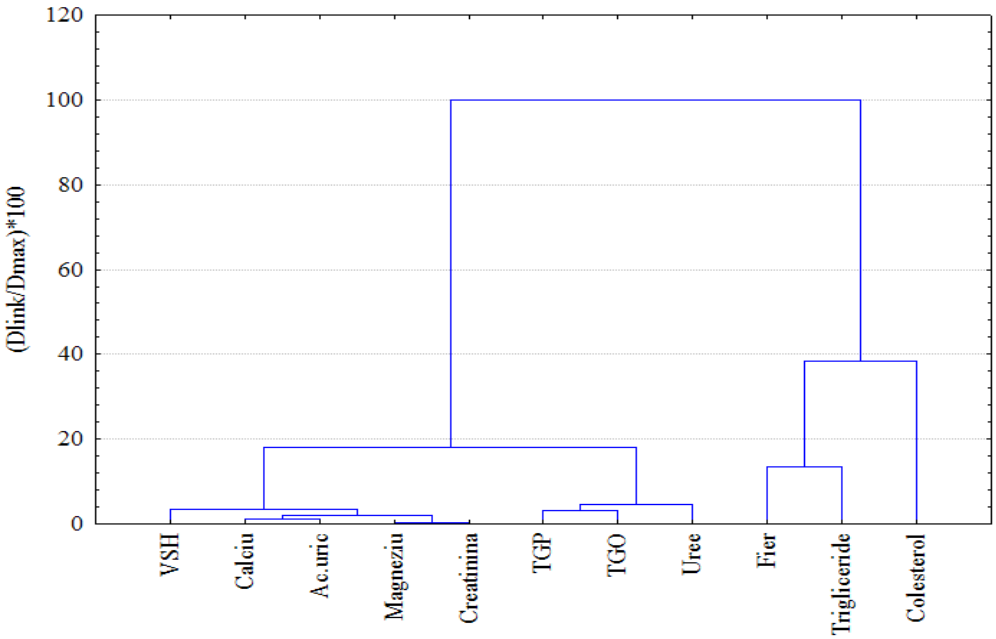

Fig. 7. 1. Dendrogram on clinical parameters considered in Study I.

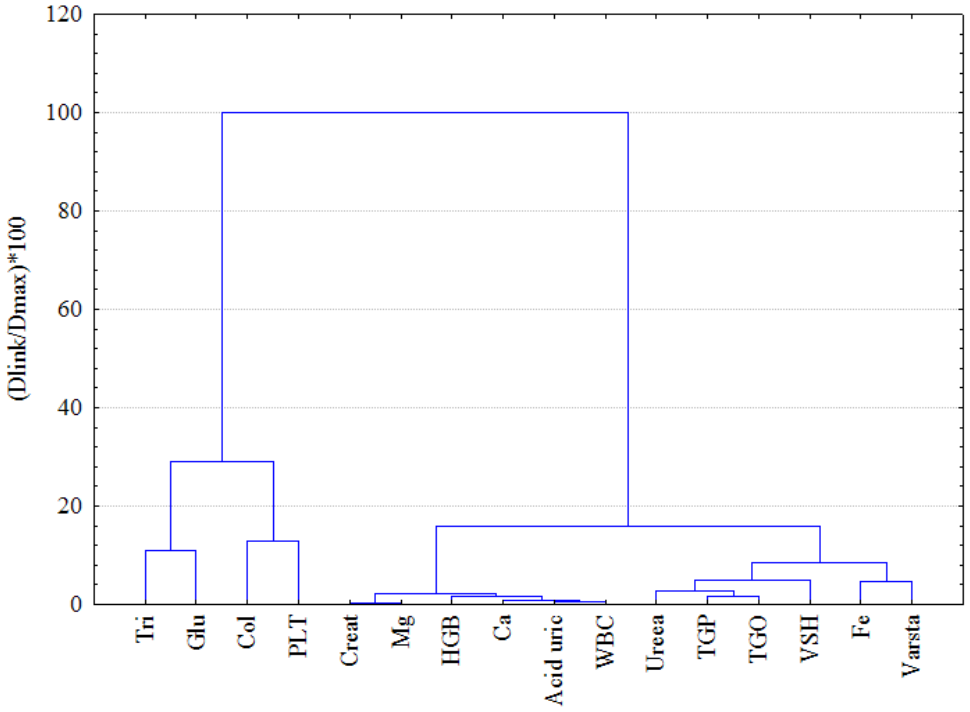

Fig. 7. 2. Dendrogram of clinical parameters considered in study II (including age of patients). There is a link between the patient's age and the amount of Fe.

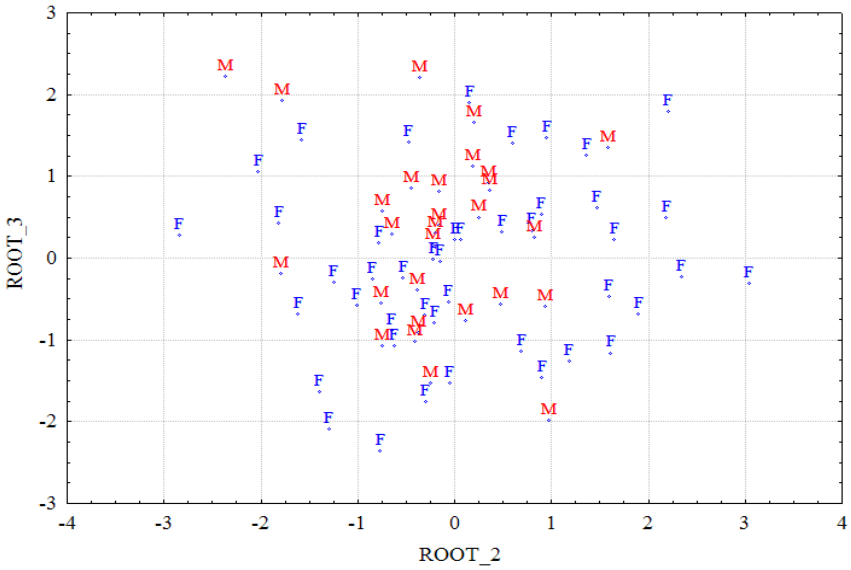

Fig. 7. 6. The graphical representation of the data dissemination relating to the sex of the patients considered in study II

There was a good correlation between the clinical parameters in the patients separated into two groups, by gender, and those divided into three groups by age; the dotted area is defined by the first three main components (Figure 7. 6 and Figure 7. 7).

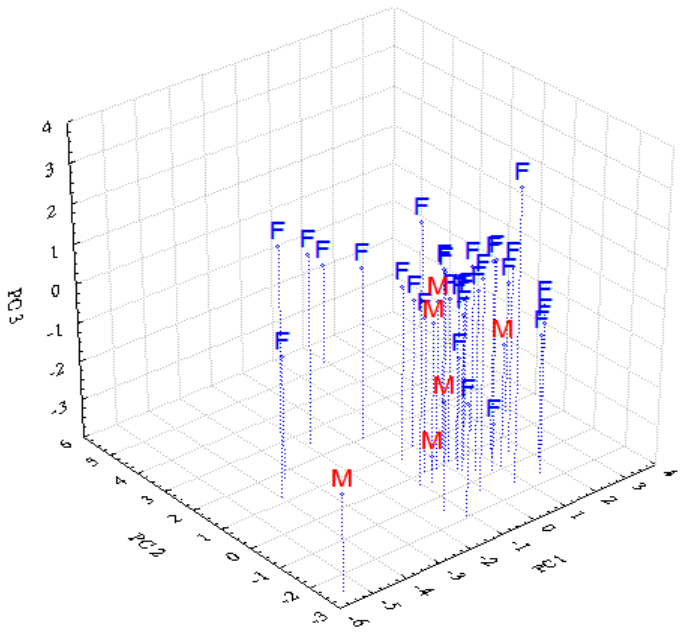

Fig. 7. 7. Graphical representation of sex data dissemination (Study I).

Conclusions

The results of our study show that there is a large correlation between some clinical parameters (Figures 7.1 and 7.2). The results confirm that clinical analyzes combined with chemometric methods are useful for diagnosis and treatment correlations and interpretations.

There was a good correlation between the clinical parameters in the patients separated into two groups, by gender, and those divided into three groups by age; dotted area defined by the first three main components (Figure 7.6 and Figure 7.7).

The PCA can extract useful information from a large number of data that can not be interpreted otherwise. This information is valuable for diagnosis and treatment [109].

Further development of the methods used may provide important insights into healthcare. Depending on the clinical analytical data, the following diseases should be studied: hepatic disorders, lipid disorders, diabetes, kidney disorders, etc. [99].

GENERAL CONCLUSIONS

Personal contributions: I believe that the development of analytical methods for applied studies in the PhD thesis represents an original contribution in the field of biomedical research and food control using spectroscopic methods. The quantitative analysis of the amino acid content in the studied foods, the essential ones, is an element of originality.

The experimental results obtained from the studies led to the following conclusions:

• Biologically active compounds beneficial for human health have been determined by developing and testing the methods of analysis in food (wine, seeds, plants) and their monitoring.

• It has been demonstrated that GC-MS is an exceptional technique through the areas of investigation in which it finds its applications and because it can identify unknown structures.

• The quantitative analysis methods used were validated, the validation parameters for linearity, precision, accuracy, detection limit, quantitative determination limit were good.

• Methods of qualitative and quantitative analysis of amino acids and fatty acids in wines have been developed or adapted. The results showed little difference between wines analyzed by comparing free amino acids, volatile compounds and antioxidant activity of six white wines from the Blaj vineyard and two wines from other vineyards. The high content of proline as compared to other amino acids is due to arginine fermentation technology. The GC-MS method has proved to be an excellent method for wine characterization, more

suitable for qualitative and quantitative analysis of volatile organic compounds, wine bouquets and active wine principles.

• We compared the amino acid content and antioxidant activity of some seeds (linum seed, poppy, grape, hemp, nut, pumpkin, sesame, watermelon, chia, corn, almonds and hazelnuts) used as food supplements. The developed isotopic dilution method was precise and simple, useful for characterization studies of the various seed extracts studied, useful for differentiating between seeds used as nutritional supplements. Significant value for essential amino acids was obtained in the case of watermelon seeds (due to the high value of histidine), hemp, pumpkin, almonds, poppy and grape seeds. The nutritional value expressed by the amino acid content and antioxidant properties has demonstrated their quality of use as nutritional supplements. Variation of free amino acids has been established in the studied samples associated with their antioxidant capacity.

• Mass Spectrometry coupled with Gas Chromatography (GC-MS) is a suitable technique for characterizing compounds in plant extracts. Aromatic plants are widely used in the preparation of food and flavorings in medicine, being a good source of amino acids. The purpose of the investigations was to determine the differences between plants purchased in Romania with regard to amino acids and antioxidant activity of plants often used as tea or spices. The dominant amino acids identified in the plants studied were proline, glutamic acid, aspartic acid, lysine, glycine and alanine. The highest antioxidant activity was obtained for the extracts from Rosemary, Thyme, Howthorn, Sage and Curry.

• Spectrophotometric techniques have been used to develop a diagnostic method for blood analysis. The main goal was to conduct a chemometric study of clinical data from analytical results using cluster analysis methods and main component analysis (PCA). Similarity models were found between patients and clinical data through Cluster Analysis (CA) and Principal Component Analysis (PCA). These methods were used to differentiate patients (cases) by gender, age and diagnosis. The study showed a good correlation between transaminase enzymes, urea and VSH, Ca, Mg, uric acid, creatinine. A significant correlation was obtained between Fe, triglycerides and cholesterol. The high correlation between certain clinical parameters confirms that clinical analyzes combined with chemometric methods are useful for diagnosis and treatment correlations and interpretations.

The results obtained in this doctoral thesis were published in 6 articles in specialized journals, 5 with ISI impact factor and one BDI, and were communicated through participation in 13 national and international events.

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