"Babeş-Bolyai" University
Faculty of Physics

THE STUDY OF SOME BIOMOLECULES BY SPECTROSCOPIC METHODS. BIOMEDICAL APPLICATIONS

Summary

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Keywords:

Mass spectrometry, gas chromatography, GC-MS, amino acids, fatty acids, metabolomics, simultaneous extraction, derivatization method, isotopic dilution, validation, phenylketonuria.

The gas chromatography-mass spectrometry (GC-MS) coupling was chosen by most of laboratories involved in metabolomics studies. This technique implies the separation of a mixture's components on a capillary column of a gas chromatograph (the ideal separation technique) and identification using a mass spectrometer (the ideal detector).

In this thesis, the GC-MS technique was used to determine, both qualitatively and quantitatively, two classes of compounds which have a special biological significance: amino acids and fatty acids.

Besides being the basic structural units of proteins, the amino acids are energy sources and they are precursors in the biosynthesis of neurotransmitters, of porphyrins, polyamines and nitric oxide.

The fatty acids are the major constituents of lipids. The main biological functions of the lipids are: they are structural components of the cellular membranes; they are metabolic fuels and transporters of liposoluble substances. The polyunsaturated fatty acids, omega-3 and omega-6, play a very important role regarding the health and their action is similar to that of hormones.

The GC-MS coupling is a robust method, with an excellent reproducibility of retention time, which can be easily automated. This was the first technique extensively used in metabolites analysis and in diagnosis.

This thesis comprises seven chapters. The first three chapters are presenting theoretical aspects. In the following four chapters, the results of the analysis by GC-MS of complex biological systems are presented.

IV. The GC-MS and isotopic dilution analysis of Amino Acids and Fatty Acids from carp (Cyprinus Carpio) plasma fed with selenium

The purpose of this study was to develop a qualitative and quantitative analysis method of amino acids and fatty acids from carp sanguine plasma. The experimental groups were fed with a special fodder formula which contained organic selenium (Sel-Plex), while the control groups were fed with a common fodder. The amino acids concentration in plasma reflects the fodder's nutritional quality.

The experimental period lasted 159 days, in 2008 (9.06-15.11) and 200 days, in 2009 (17.04-3.11); between the two periods, the carp's winter sleep took place.

Ten carps of each group, control and experimental, 5 of Lausitz variety, and 5 of Galician variety, were captured and blood was collected in vials containing heparin.

Experimental

The amino acids and the fatty acids were extracted from 0.5 mL of plasma, on a Dowex 50W-W8 exchange resin. 10 μL of ^{15}N-Met was added to each sample, as internal

standard, before passing through the resin. 2 mL of water were used to "wash" the resin and they were collected with plasma, in glass vials. The amino acids, which were retained on the resin, were then eluted with 2 mL NH_4OH 4M. The fatty acids were extracted from the plasma and water mixture using 0.5 mL chloroform:methanol 2:1 (v:v), mixing 30 s, at room temperature.

The samples were derivatized in order to obtain fatty acids methyl esters (FAME) and amino acids n-butyl N-trifluoroacetyl esters.

The amino acids were derivatized with 200µL 3M HCl in butanol, at 100°C, for 1h. After cooling and evaporation, a trifluoroacetilation reaction was achieved, using 100µL of trifluoroacetic anhydride, at 60°C, for 20 min.

The fatty acids were derivatized with 200µL 3M HCl in methanol, at 80°C, for 20 min.

A gas chromatograph coupled with a quadrupole mass spectrometer Trace DSQ (Thermo Finnigan) was used. The capillary column Rtx-5MS had 30 m x 0.25 mm I.D. and a film thickness of 0.25µm.

The oven temperature program for separation of amino acids n-trifluoroacetyl N-butyl esters was: 50°C for 1 min, 6°C/min to 100°C, 4°C/min to 200°C, 20°C/min to 310°C, and was maintained here for 5 min.

The oven temperature program for FAME separation started from 50°C, 2 min, 8°C/min to 310°C, and was maintained here for 8 min.

Helium 5.5 was used as carrier gas, with a flow rate of 1 mL/min. The mass spectrometer was operated in the EI mode, 70 eV. The emission current was 100µA.

The qualitative analysis was carried out in the SCAN mode: 30-520 a.m.u. mass range, for amino acids, and 34-500 a.m.u, for fatty acids. The following conditions were ensured: the transfer line temperature was set at 250°C, the injector temperature was set at 200°C, for amino acids and at 250°C, for fatty acids, and the ion source temperature was set at 250°C.

One microliter of each sample was injected into the GC-MS, in the split mode (10:1) and using a TriPlus autosampler.

Results and discussion

17 amino acids were calculated and the method was validated using standards. Precision was 20% RSD, except for arginine, cysteine and tyrosine, and the limit of detection (LOD) was 1 µg/mL, for each of amino acids (1 ng injected).

The amino acids' elution order in a sample of plasma was: alanine (Ala), glycine (Gly), γ-aminobutyric acid (GABA), threonine (Thr), serine (ser), valine (Val), leucine (Leu), isoleucine (Ile), cysteine (Cys), proline (Pro), methionine (Met), aspartic acid (Asp), phenylalanine (Phe), ornitine (Orn), glutamic acid (Glu), lysine (Lys), tyrosine (Tyr) and histidine (His) (Fig. 1.)

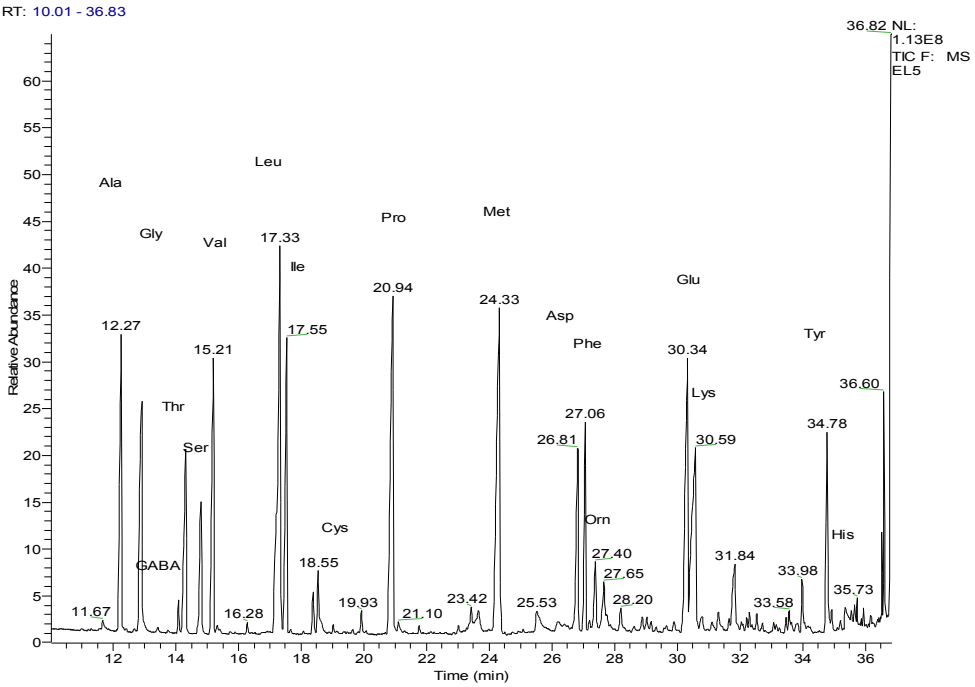


Fig.1. The representative chromatogram of amino acids from carp plasma.

The amino acids concentrations were calculated using the formulas (1) and (2).

$$F_i = \frac{A_i / A_j}{m_i / m_j} \quad (1)$$

where F_i is the response factor, m_i is the amount of compound *i*, m_j is the amount of compound $_j$ (the internal standard), A_i and A_j are the areas of the peaks *i* and *j*.

$$m_i(\mu g) = m_j(\mu g)\frac{A_i}{F_i \cdot A_j} \quad (2)$$

where m_i is the amount of compound *i*, m_j is the amount of internal standard.

The carps' feeding with organic selenium influenced the amino acids concentration. There was noticed an increase in amino acids concentrations for both varieties, Lausitz and Galician, experimental and control groups (Fig.2 and 3).

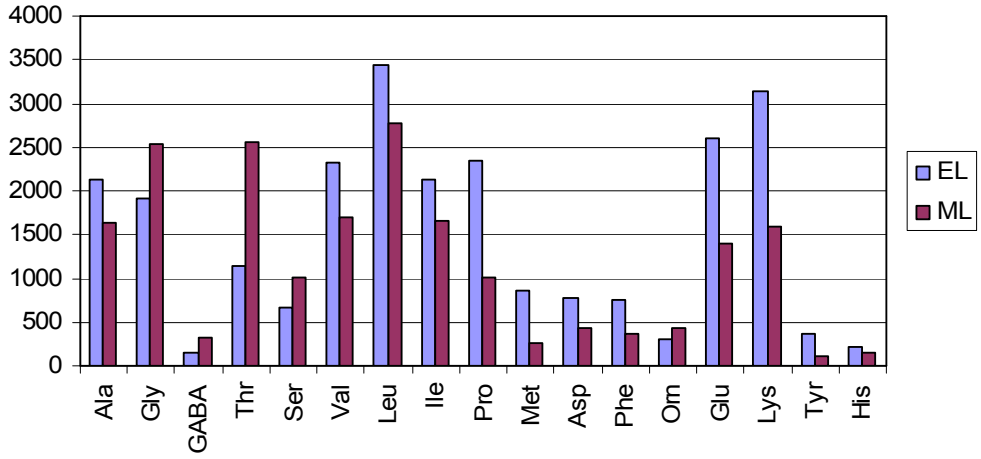


Fig.2. The amino acids (μM) from the plasma of the Lausitz carps, experimental (EL) and control group (ML).

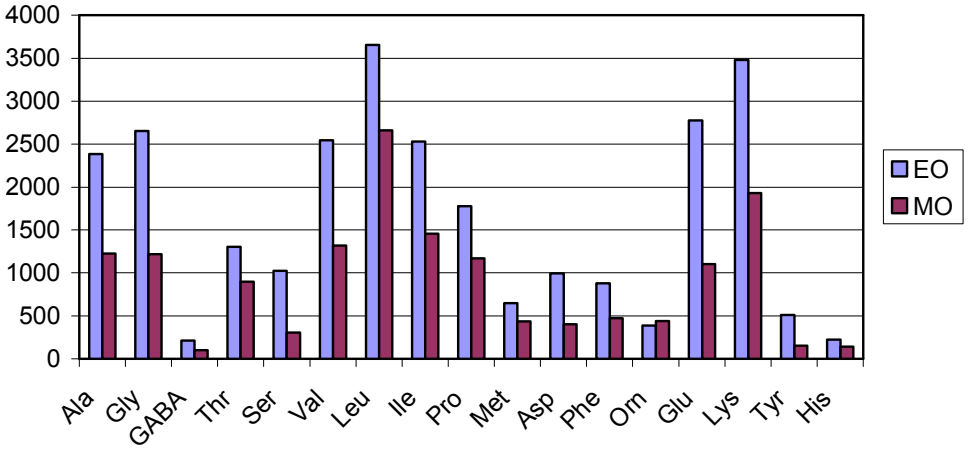


Fig.3. The amino acids (μM) from the plasma of the Galician carps, experimental (EO) and control group (MO).

There was also a positive influence of the organic Se upon the fatty acids concentration. Fig. 4 shows the representative chromatogram of the fatty acids from carp plasma.

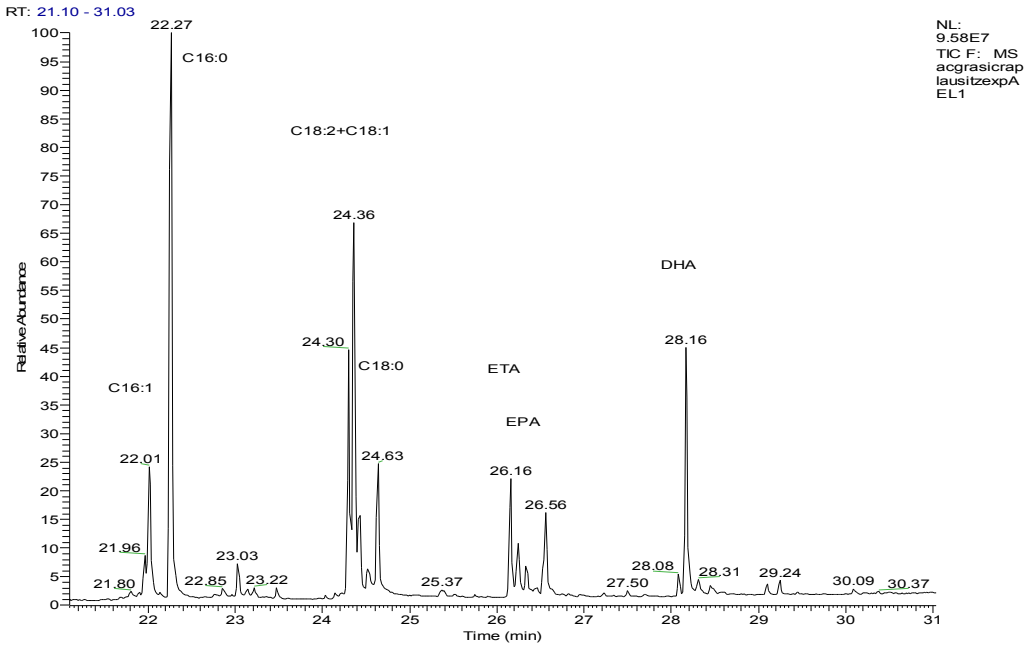


Fig. 4. FAMEs representative chromatogram of a carp plasma sample.

Using the NIST library, the following fatty acids were identified: palmitoleic acid (C16:1), palmitic acid (C16:0), linoleic acid (C18:2), oleic acid (C18:1), stearic acid (C18:0), two isomers of arachidonic acid C20:4 (5,8,11,14 eicosatetraenoic acid, ETA ω-6 and 8,11,14,17 eicosatetraenoic acid, ETA ω-3), eicosapentaenoic acid (EPA, C20:5 ω-3) and docosahexaenoic acid (DHA, C22:6 ω-3).

10 µg of C11:1 was added to each sample, before injection, as internal standard. The fatty acids concentrations were calculated using the following formula:

$$C_i\left(mass\%\right)=\frac{\frac{m_j \times A_i}{F_i \times A_j}}{\sum_{i=1}^{n}\left(\frac{A_i}{F_i}\right)} \times 100 \qquad (3)$$

where F_i is the response factor, A_i and A_j are the areas of the peaks *i* and *j*, m_j is the amount of internal standard (compound *j*).

The DHA/EPA ratio had the highest value for the experimental carps of Lausitz variety, 5.63, while the same ratio was 5.53, for the experimental carps of Galician variety. In control groups, this ratio was lower: 4.39, for Lausitz carps and 3.76, for Galician carps (Fig. 5).

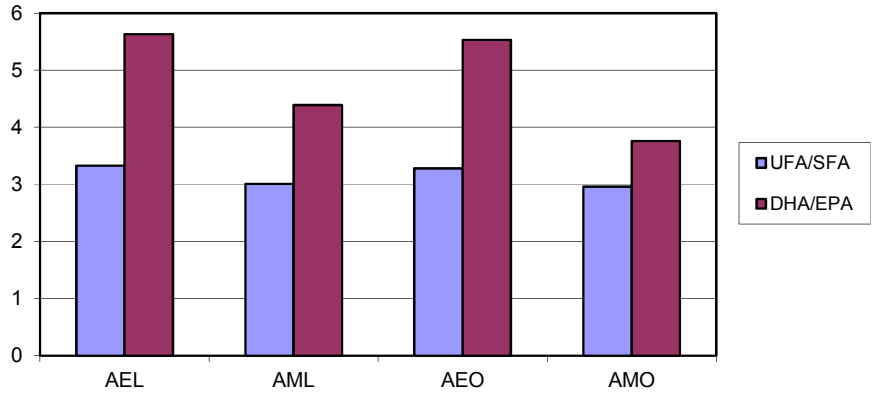


Fig. 5. Unsaturated fatty acids/saturated fatty acids (UFA/SFA) and docosahexaenoic acid/ eicosapentaenoic acid (DHA/EPA) ratios. AEL- Lausitz experimental, AEO-Galician experimental, AML and AMO-control groups, Lausitz and Galician.

Conclusions

- The analysis method developed here has good validation parameters. The precision was lower than 20% RSD, and the limit of detection (LOD), 10μg/mL (10 ng injected).
- Using an isotopic labeled internal standard allows the high precision determination of the metabolites.
- There were significant differences between the amino acids concentration for both varieties, Lausitz and Galician.
- The groups fed with organic Se had higher values of amino acids concentration in their plasma.
- The organic Se feeding determined the increase of the UFA/SFA ratio, for both varieties.
- The increased concentration of DHA in experimental carps is due to the antioxidant properties of Se.

V. The GC-MS and isotopic dilution analysis of amino acids and fatty acids from rainbow trout (Oncorhynchus Mykiss) plasma and meat. The seasonal variation

The purpose of this study was to develop a simple and reliable GC-MS method to determine the amino acids and the fatty acids in trout plasma and meat.

Fish meat is rich in proteins of high quality and contains all the essential amino acids. Besides this, fish is considered to be the most important dietary source of polyunsaturated fatty acids (PUFAs), especially of omega-3 fatty acids: eicosapentaenoic (EPA) and docosahexaenoic (DHA) fatty acids. EPA and DHA are substrates for eicosanoids, which are involved in inter- and intracellular signaling that influence the vascular, neural and immune system.

The metabolites of interest were determined from rainbow trout sanguine plasma and meat. The trout came from two different growth systems: classical (M-control) and recirculating (E-experimental). The influence of seasons and growth systems on amino acids and fatty acids concentrations was investigated.

Experimental

The plasma samples were analyzed in spring and in summer. For plasma, the same experimental steps were followed as in the previous experiment, described in Chapter IV. The separation and the identification of the compounds were performed using a Trace GC gas chromatograph coupled with a quadrupole mass spectrometer (Trace DSQ Thermo Finnigan).

Although the amino acids and fatty acids extraction was done in the same way as it has been previously described, the extraction method was different for meat.

1g of defrosted meat and 1 g of fine quartz sand were thoroughly crushed in a thick ceramic dish and homogenized with 5 ml distilled water. The homogenate was centrifuged at 5800 rpm for 5 min.

0.5 ml of the supernatant and 10 µg of ^{15}N-methionine (internal standard) were passed slowly through a Dowex 50W-W8 exchange resin, on a 2 x 40 mm column. The solution was collected in a 4 ml vial and used for fatty acids extraction. The fatty acids were extracted by adding 0.5 ml chloroform: methanol (2:1, v:v) and shaking vigorously for 30 s, at room temperature.

The amino acids purified on the exchange resin were extracted by passing 2 ml of 4 M NH_4OH through the Dowex 50W-W8 resin. The solution obtained was collected in a 4 ml vial and dried in a nitrogen flow at 60°C.

The derivatization steps were the same as those described in Chapter IV and amino acids, as butyl trifluoroacetyl esters derivatives, and fatty acids methyl esters (FAME) were obtain for GC analysis.

Results and discussion

16 amino acids were identified and their concentrations were calculated: alanine (Ala), glycine (Gly), threonine (Thr), serine (Ser), valine (Val), leucine (Leu), isoleucine (Ile), proline (Pro), hydroxiproline (Hy-Pro), methionine (Met), aspartic acid (Asp), phenylalanine (Phe), ornitine (Orn), tyrosine (Tyr), glutamic acid (Glu) and lysine (Lys) (Fig.6 and Table 1).

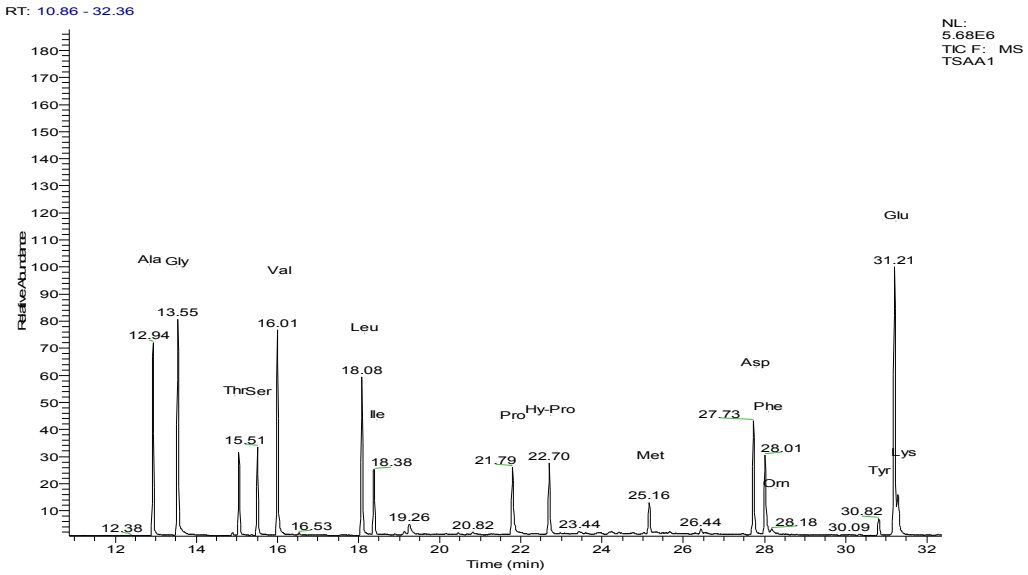


Fig. 6. The amino acids chromatogram of a trout plasma sample.

Table 1. The amino acids concentrations from trout plasma, in spring and summer.

Amino acid	Molecular weight	Retention time (min)	Concentration (µM/mL) Mean ± SD (n=10)	
			Spring	Summer
Ala	89	12.94	551.23±80.06	486.32±61.11
Gly	75	13.55	806.14±115.60	505.18±19.72
Thr*	119	15.06	265.10±27.16	208.38±16.17
Ser	105	15.51	391.32±60.41	150.70±28.74
Val*	117	16.01	854.50±74.98	750.49±30.48
Leu*	131	18.08	701.13±57.61	460.93±23.40
Ile*	131	18.37	375.14±32.02	279.62±17.06
Pro	115	21.78	393.68±21.11	460.54±25.24
Hy-Pro	131	22.68	190.10±19.62	53.47±4.92
Met*	149	25.15	242.83±13.74	182.71±5.40
Asp	133	27.72	743.95±60.65	447.63±33.02
Phe*	165	28.01	318.76±22.01	320.02±14.60
Orn	132	28.13	103.87±11.00	82.91±6.11
Tyr	181	30.82	55.54±10.17	69.17±4.03
Glu	147	31.21	1692.56±117.75	1218.23±67.53
Lys*	146	31.24	572.87±50.74	699.97±49.09

*essential amino acid

SD- standard deviation

In spring, the essential amino acids concentrations were higher than in summer, except for Phe and Lys.

After derivatization, the fatty acids became fatty acids methyl esters (FAME) and 20µg of C11:1 was added to each sample, as internal standard, before the injection into the GC.

The identified and quantitatively determined FAMEs in trout plasma samples were (Fig. 7): palmitoleic acid (C16:1), palmitic acid (C16:0), linoleic acid (C18:2), oleic acid (C18:1), stearic acid (C18:0), eicosapentaenoic acid (EPA, C20:5 ω-3) and docosahexaenoic acid (DHA, C22:6 ω-3).

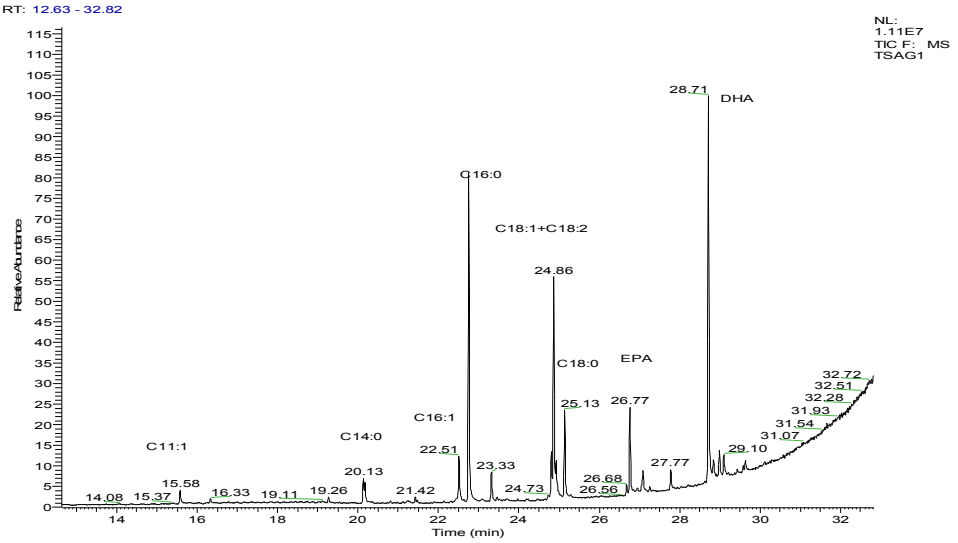


Fig.7. The FAME chromatogram of a trout plasma sample.

Table 2. The fatty acids concentrations found in trout plasma samples.

Fatty acids	Retention time (min)	Concentration (% total fatty acids) Medium (n=10)			
		Spring	Summer	Autumn	Winter
Palmitoleic acid C16:1	22.51	5.322	3.666	3.039	1.789
Palmitic acid C16:0	22.75	21.841	20.350	21.906	32.861
Linoleic acid C18:2	24.81	3.864	4.387	12.256	8.405
Oleic acid C18:1	24.86	19.855	22.592	29.455	32.578
Stearic acid C18:0	25.13	4.997	4.881	5.143	4.819
Eicosapentaenoic acid C20:5 EPA	26.77	9.830	10.036	6.029	6.281
Docosahexaenoic acid C22:6 DHA	28.71	34.324	34.088	22.174	13.267
PUFA[1]		48.02	48.51	40.46	27.95
SFA[2]		26.84	25.23	27.05	37.68
UFA[3]		73.20	74.77	72.95	62.32
UFA/SFA		2.73	2.96	2.70	1.65
ω-3/ω-6		11.43	10.06	2.30	2.33

[1]polyunsaturated fatty acids

[2]saturated fatty acids

[3]unsaturated fatty acids

Among the freshwater species, trout meat contains the highest concentration of unsaturated fatty acids.

In autumn, the fatty acids were determined in rainbow trout meat, coming from two different farming systems: classical and recirculating. Group M (control) was raised in the classical system from Fiad-Telcişor and group E (experimental), in a recirculating system located in Cluj-Napoca. The food and the frequency of feeding were identical. A total of 10 specimens from each group were sacrificed.

The limit of detection (LOD) was 10 μg/mL (10 ng injected) and the precision and accuracy were below 20%. The extraction efficiency was 21%.

Figure 8 presents the amount of fatty acids found in experimental (E) and control (C) trout meat samples.

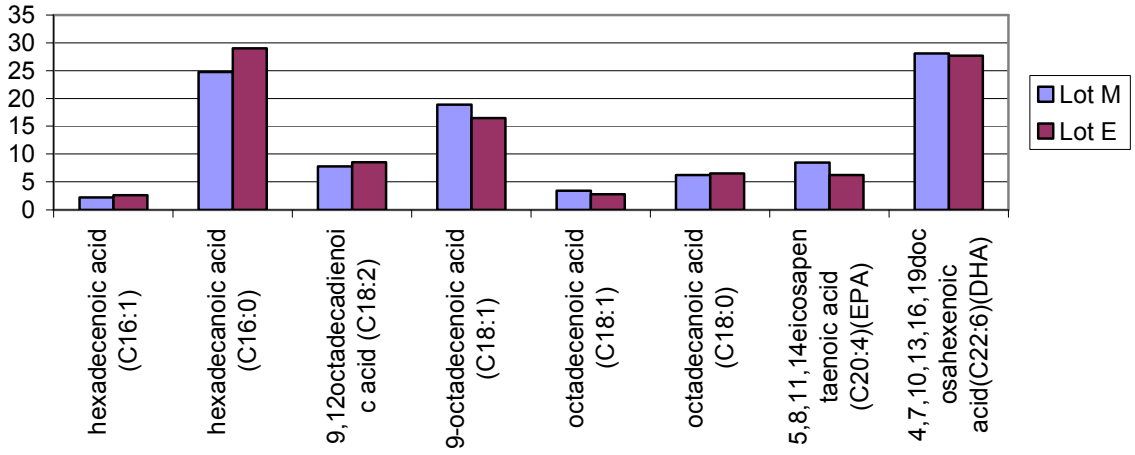


Fig.8. The fatty acids concentration (% of total fatty acids) from trout meat, control (M) and experimental (E) groups.

The trout meat from the control group is richer in unsaturated fatty acids (68.95%) than the meat of the experimental group (64.44%). Also, the trout meat from the control group contains a lower concentration of saturated fatty acids (31.04%) than that of trout from the experimental group (35.56%) and a higher fatty acid ratio ω-3/ω-6 (4.69) compared to that of trout from the experimental group (ω-3/ω-6 of 3.97).

Conclusions

- The method developed here shows good validation parameters: precision and accuracy were <20% RSD and the limit of detection (LOD), 10 μg/mL (10 ng injected).
- The amino acids and fatty acids concentrations presented seasonal variations.
- Higher concentrations of these metabolites were obtained in spring.

- In summer, the fatty acids concentrations from plasma samples decreased. This might be due to the water temperature increase and due to changing the way of nutrition.
- The meat of the control group was richer in polyunsaturated fatty acids than the meat of experimental trout, grown in recirculating system.

VI. The GC-MS and isotopic dilution analysis of amino acids and fatty acids in cold fermented salami

The purpose of this study was to establish a fast and accurate experimental protocol for the GC-MS analysis of free amino acids from cold fermented salami, using [^{15}N]-methionine as internal standard. Samples containing 0.3% glucono delta-lactone (GDL) and 0.05% sodium ascorbate (ASC), and samples containing 0.1 % sodium ascorbate were prepared. The content of free amino acids was measured at different time intervals over 45 days of processing. A simultaneous extraction procedure of free amino acids and fatty acids was established, followed by a two-step derivatization method for the GC analysis of the amino acids.

Experimental

The extraction and derivatization of the target metabolites were performed as in Chapter V, when trout meat samples were analyzed.

A gas chromatograph (Trace GC) coupled with a quadrupole mass spectrometer Trace DSQ (Thermo Finnigan) were used. The capillary column Rtx-5MS had 30 m x 0.25 mm I.D. and a film thickness of 0.25μm. The oven temperature program for the amino acids separation, as n-trifluoroacetyl N-butyl esters derivatives, was: 50^{o}C for 1 min, 6^{o}C/min to 100^{o}C, 4^{o}C/min to 200^{o}C, 20^{o}C/min to 300^{o}C, for 3 min. The oven temperature program for the fatty acids separation as fatty acids methyl esters derivatives (FAMEs) was: 50^{o}C for 1 min, 8^{o}C/min to 300^{o}C.

Helium 5.5 was used as carrier gas, with a flow rate of 1 mL/min.

The mass spectrometer was operated in the EI mode, at 70 eV. The emission current was 100μA. The qualitative analysis was carried out in the 50-500 a.m.u. mass range. The following conditions were ensured: the transfer line temperature was set at 250^{o}C, the injector temperature, at 200^{o}C and the ion source temperature, at 250^{o}C. One microliter of each sample was injected into the GC-MS using the split mode (10:1) and a TriPlus autosampler.

Results and discussion

The amino acids identified in the salami extracts were: Ala, Gly, Thr, Ser, Val, Leu, Ile, Pro, Met, Asp, Orn, Phe, Lys, Glu, His, Tyr.

According to their elution order and relative peak area, the most abundant free amino acids were: alanine (Ala), histidine (His), valine (Val) and glutamic acid (Glu).

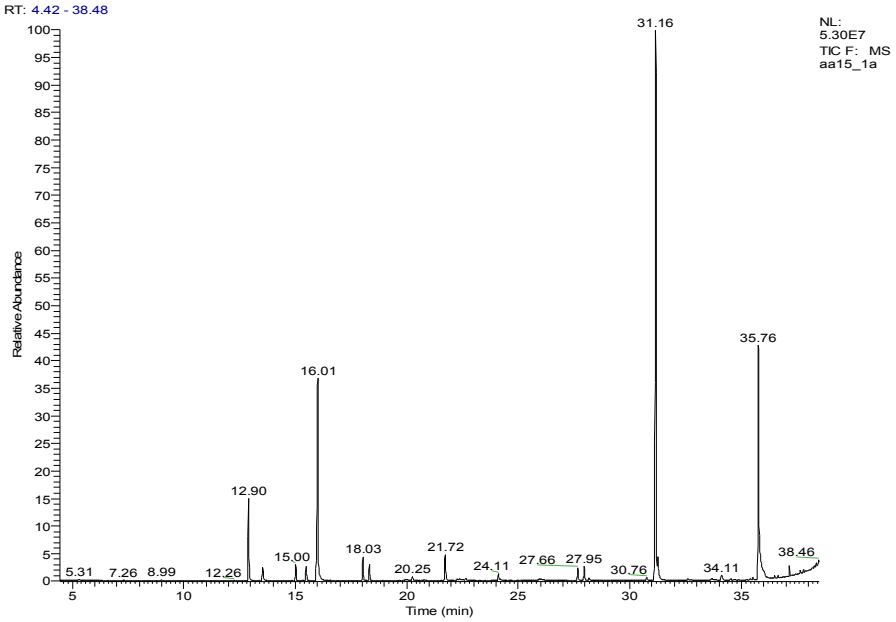


Fig. 9. The separation chromatogram of the free amino acids extracted from a salami meat sample: Ala (Rt:12.90), Gly (Rt:13.50), Thr (Rt:15.00), Val (Rt:16.01), Pro (Rt:21.72), Asp (Rt:27.66), Glu (Rt:31.16), His (Rt:35.76).

The amount of amino acids was calculated using formula 2 and the regression curves (Table 3). 10 µg of ^{15}N-Met/g were added as internal standard.

Table 3. The regression curves equation and the correlation coefficients of the standard amino acids.

Amino acid	**Regression curve**	**r**
Alanina (Ala)	y = 1.017x + 12.567	0.991
Glycine (Gly)	y = 1.0682x + 13.166	0.996
Threonine (Thr)	y = 1.1285x + 15.058	0.992
Serine (Ser)	y = 1.1041x + 12.72	0.995
Leucine (Leu)	y = 1.0044x + 16.09	0.995
Isoleucine (Ile)	y = 0.8507x + 12.37	0.994
Valine (Val)	y = 1.5124x - 13.434	0.980
Cysteine (Cys)	y = 0.1667x - 6.512	0.940
Gamma-aminobutyric acid (GABA)	y = 2.207x - 8.8747	0.980
Proline (Pro)	y = 1.0331x + 20.49	0.991
Hydroxiproline (Hy-Pro)	y = 1.3504x - 4.257	0.993
Methione (Met)	**y = 0.0355x + 0.1319**	**0.9995**
Ornitine (Orn)	y = 0.7645x + 8.7581	0.997
Phenylalanine (Phe)	y = 0.6542x + 26.844	0.978
Tyrosine (Tyr)	y = 0.3209x - 5.2564	0.973
Lysine (Lys)	y = 1.2868x - 10.384	0.978
Hysitidine (Hys)	y = 0.6916x - 10.196	0.957

Fermented food contains an abundance of amino acids. The fermentation process determines the unique taste and flavour of food.

Figures 10 and 11 show the amino acids concentration (mg/g) in salami samples prepared with GDL and without GDL.

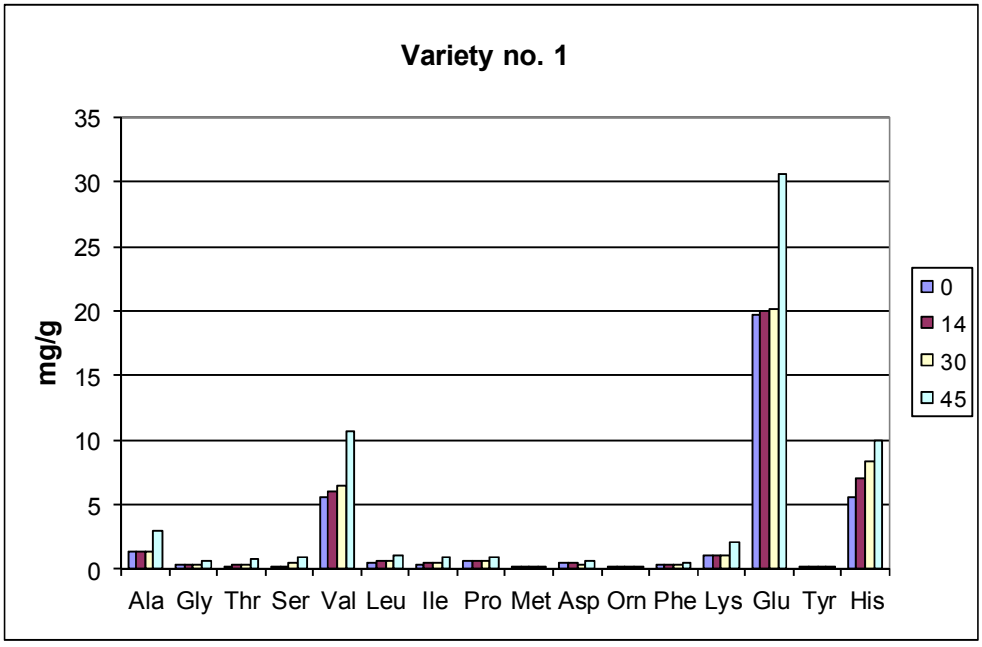


Fig.10. The amino acids variation in the samples prepared with GDL, depending on processing time: 0, 14, 30, 45 days.

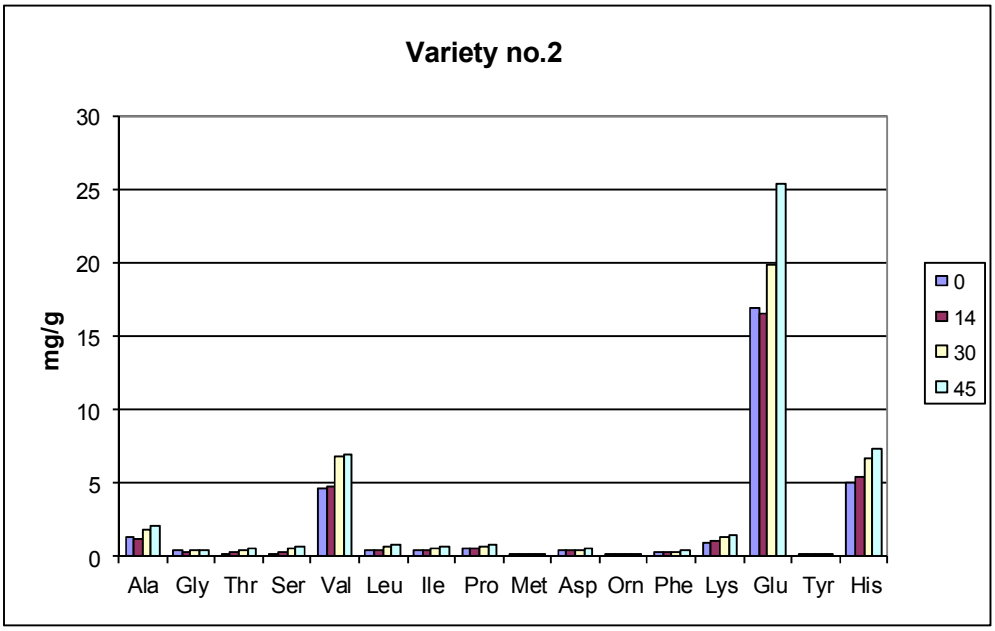


Fig.11. The amino acids variation in the samples prepared without GDL, depending on processing time: 0, 14, 30, 45 days.

The fatty acids identified in salami samples were: myristic acid (C14:0), palmitoleic acid (C16:1), palmitic acid (C16:0), linoleic acid (C18:2, ω-6), two of the oleic acid isomers (9-C18:1, 12-C18:1), stearic acid (C18:0) and arachidonic acid (C20:4, ω-6).

10 μg of C11:1 were added to each sample, before injection, as internal standard. The fatty acids concentration calculation was performed using formula 3.

Table 4. The salami samples prepared with 0.3% GDL and 0.05% ASC.

Fatty acids (%)	Processing time (days)			
	0	**14**	**30**	**45**
Myristic acid (C14:0)	0.60	0.67	0.70	0.67
Palmitoleic acid (C16:1)	1.15	1.06	1.28	1.27
Palmitic acid (C16:0)	23.01	22.11	22.22	22.41
Linoleic acid (C18:2)	12.93	12.88	13.03	13.04
Oleic acid (9-C18:1)	36.47	36.28	39.24	40.36
Oleic acid (12-C18:1)	3.64	4.59	4.33	3.42
Atearic acid (C18:0)	18.98	19.38	16.49	16.32
Arachidonic acid (C20:4)	2.85	2.62	2.18	2.15
SFA[1]	42.59	42.16	39.41	39.40
UFA[2]	57.05	57.43	60.06	60.24
MUFA[3]	41.26	41.93	44.85	45.05
EFA[4]	15.78	15.50	15.21	15.19
UFA/SFA	1.34	1.36	1.52	1.53

[1]SFA-saturated fatty acids (C14:0, C16:0, C18:0), [2]UFA-unsaturated fatty acids (C16:1, 9- and 12-C18:1, C18:2, C20:4), [3]MUFA-monounsaturated fatty acids (C16:1, 9- and 12-C18:1), [4]EFA- essential fatty acids (linoleic and arachidonic acids).

Table 5. The salami samples prepared with 0.1%ASC.

Fatty acids (%)	Processing time (days)			
	0	**14**	**30**	**45**
Myristic acid (C14:0)	0.57	0.54	0.69	0.72
Palmitoleic acid (C16:1)	0.95	1.02	1.20	1.30
Palmitic acid (C16:0)	22.31	22.31	22.81	22.84
Linoleic acid (C18:2)	13.21	14.34	14.08	13.08
Oleic acid (9-C18:1)	34.49	35.49	37.43	38.85
Oleic acid (12-C18:1)	4.68	3.92	3.35	3.49
Stearic acid (C18:0)	20.03	18.64	17.18	16.90
Arachidonic acid (C20:4)	3.50	3.46	2.96	2.42
SFA[1]	42.91	41.49	40.68	40.46
UFA[2]	56.83	58.23	59.02	59.14
MUFA[3]	40.12	40.43	41.98	43.64

EFA[4]	16.71	17.80	17.04	15.50
UFA/SFA	1.32	1.40	1.45	1.46

[1]SFA-saturated fatty acids (C14:0, C16:0, C18:0), [2]UFA-unsaturated fatty acids (C16:1, 9- and 12-C18:1, C18:2, C20:4), [3]MUFA-monounsaturated fatty acids (C16:1, 9- and 12-C18:1), [4]EFA- essential fatty acids (linoleic and arachidonic acids).

During the processing period, the UFA/SFA ratio increased, for both types of salami varieties. Among the fatty acids, the oleic acid had the highest increase in percentage of mass.

Conclusions

- The method is suitable for routine analysis, food quality control applications, metabolism studies, control of biotechnological processes, diet control and food labeling.
- The total amount of amino acids increased from 31.66 to 48.44 mg/g, in the salami samples without GDL, and from 36.67 to 63.06 mg/g, in the salami samples containing GDL.
- The amount of glutamic acid, histidine, valine and alanine increased during the biotechnological process.
- During the processing time, the salami variety with GDL had a higher growth UFA/SFA ratio than the variety without GDL.

VII. The development and optimization of some diagnostic methods

The purpose of this study was to develop a simple and rapid quantitative analysis of amino acids from dried blood spots (20μL) using the isotope dilution (ID) GC-MS technique. The method was applied to blood spot samples obtained from four patients with phenylketonuria (PKU).

In many cases where is a disturbance of amino acid metabolism, the prompt diagnosis and the rapid intervention can bring enormous benefits to infants and sometimes it can be lifesaving.

Phenylketonuria (PKU) was one of the first genetic diseases for which neonatal screening was established (1962). Initially, the Guthrie test involved the diagnosis of PKU by the restored growth of a bacterial colony when phenylalanine was present in a sample of urine or blood. The Guthrie test has now generally been replaced by GC-MS and tandem mass spectrometry.

Experimental

Whole blood samples were absorbed on filter paper. After drying at room temperature, 8 mm punch from each blood spot was transferred into a 5 mL vial. 200 μL methanol/HCl 0.1% was added to the vial and the samples were kept for 1h at 4ºC, for amino acids extraction.

To each 100 μL of extract, 0.5 μg ^{15}N-Met was added as internal standard, and the excess of reagent was removed by a nitrogen stream.

The amino acids were derivatized in a two-step reaction procedure: esterification with 100 µL butanol/acetyl chloride 4:1 (v/v), 100ºC, 30 min and acetylation with 50 µL trifluoroacetic anhydride, 100ºC, 30 min. The excess of reagent was completely removed after each step. A nitrogen stream was used at 4ºC, after acetylation. Finally, 200 µL of ethyl acetate was added and 2µL of each sample was injected into the GC/MS.

The method was validated in the range: 1 – 20 µg/mL. Table 6 presents the regression equations and the correlation coefficients of the amino acids of interest.

Table 6. Regression curve parameters

Analyte	Regression equation	Correlation coefficient
Val	y = 0.2505x – 0.2092	0.990
Leu	y = 0.0752x – 0.0771	0.978
Pro	y = 0.2023x – 0.0514	0.992
Phe	y = 0.1978x – 0.0987	0.992
Tyr	y = 0.1763x – 0.0957	0.967

The precision was lower than 5.5% (RSD%) and the accuracy lower than 6% (RSD%) (Table 7). The limit of detection was lower than 10 ng, for each amino acid injected.

Table 7. The precision and accuracy values (RSD%).

Standard	Precision RSD%	Accuracy RSD%
20µg (n=7)	5.48	10.94
30µg (n=5)	3.85	6.76

The precision was calculated using the formula:

$$RSD(\%) = \frac{SD}{\bar{x}} \cdot 100 \quad (4)$$

where SD is the standard deviation and $\bar{x}$ is the mean value.

The accuracy was calculated using the formula:

$$RSD(\%) = \frac{|V_1 - V_2|}{V_2} \cdot 100 \quad (5)$$

where V_1 is the measured value and V_2 is the true value.

The analysis was performed using a gas chromatograph (Trace GC) coupled with a quadrupole mass spectrometer (Trace DSQ Thermo Finnigan). Electron impact was the ionization mode (electron energy, 70eV, emission current, 100 µA); the transfer line temperature was 250ºC, injector temperature, 200°C, ion source temperature, 250ºC and the split ratio 10:1. An Rtx-5MS capillary column, 30 m x 0.25 mm, 0.25 µm film thickness was used. Helium 5.5 (1 mL/min flow) was the gas carrier.

The GC temperature program started at 50°C (1 min) then increased to 260°C, at 20°C/min and then to 300°C, at 100°C/min (0.3 min). The mass spectrometer operated in SIM (selected ion monitoring) mode.

Table 8. The amino acids used as biomarkers and the selected ions.

Amino acids	Selected ions (m/z)
Val	168
Leu	182
Pro	166
Phe	91, 148
Tyr	203, 260, 316

For unlabeled methionine and ^{15}N-Met, the ions 171 and 172 were selected.

Results and discussion

Figure 12 presents the separation chromatogram of the amino acids, extracted from 20 µL of blood and then derivatized, as it was previously described.

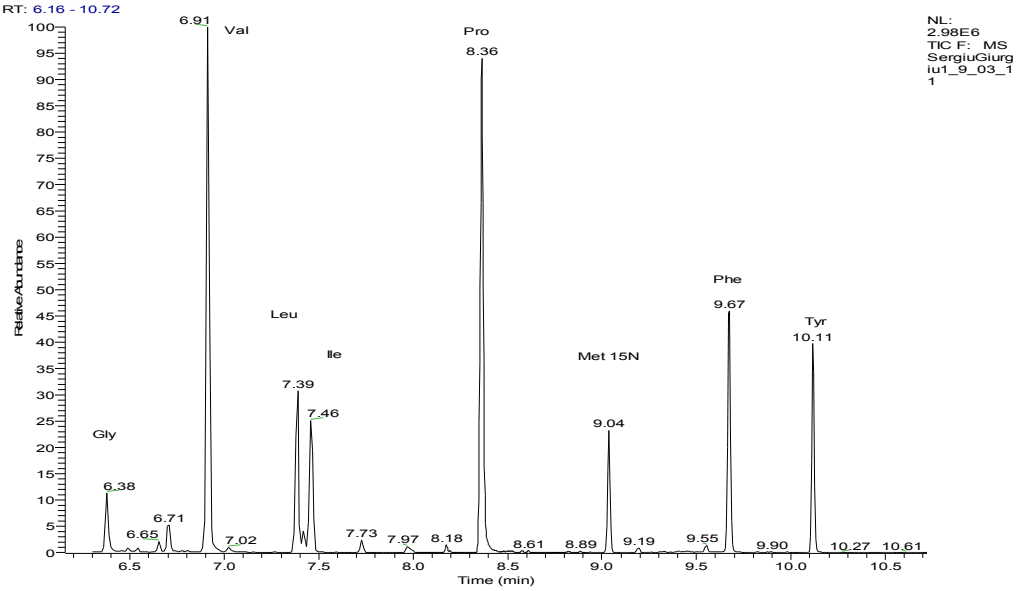


Fig. 12. The SIM GC-MS chromatogram of a blood spot sample: Val (t_R = 6.91 min), Leu (t_R = 7.39 min), Pro (t_R = 8.36 min), ^{15}N-Met *IS* (t_R = 9.04min), Phe (t_R = 9.67 min), Tyr (t_R = 10.11 min).

For patient AS, who was 9 months old, when the analysis was performed for the first time, significant differences were observed. Over the two months of treatment monitoring, the Phe concentration decresead by 37 times (Fig. 13) and the Phe/Tyr ratio decreased from 11.9 to 2.01 (Fig. 19 a).

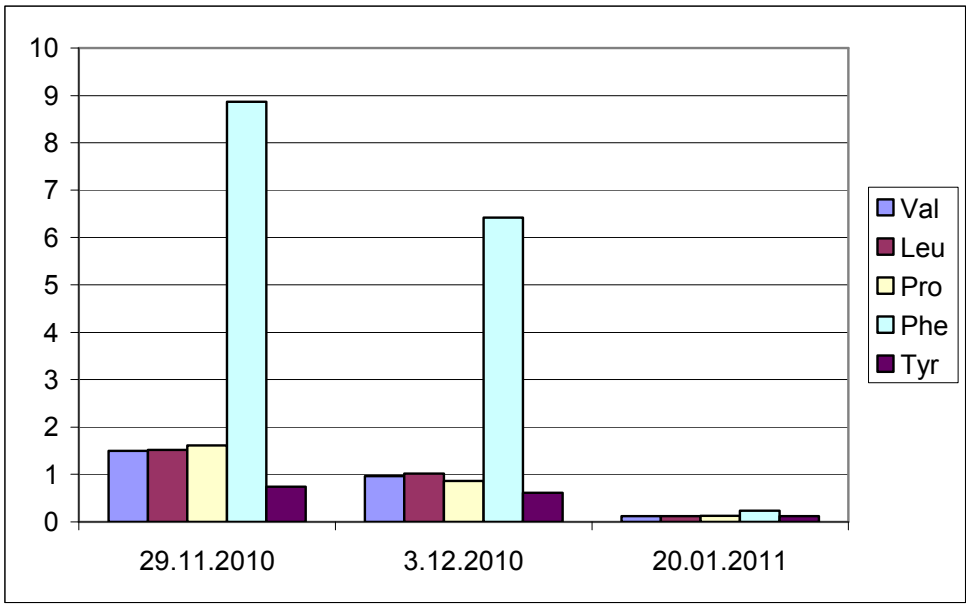


Fig. 13. The variation of amino acid concentrations during the monitoring period for patient AS (9 months old).

For patient SG (Fig. 14), the special diet and the appropriate medication determined the maintainance of Phe and Tyr concentrations in normal limits. The normal values are: for Phe 0.61-1.45 mg/100 mL and for Tyr, 0.39-1.58 mg/100 mL (for adults).

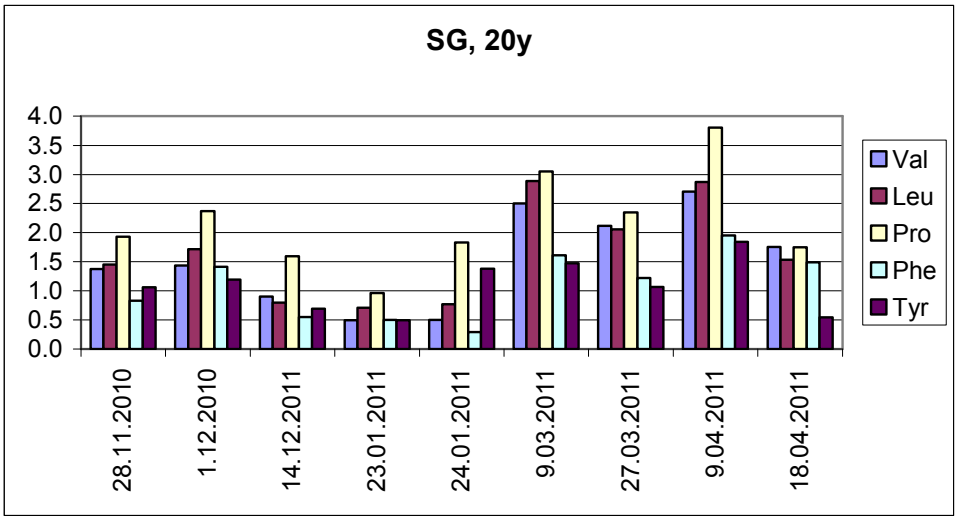


Fig. 14. The variation of amino acid concentrations during the monitoring period for patient SG (20 years old).

For patient DA, the concentration of Phe is particularly high in the second month of monitoring (Fig. 15), about 5.5 times higher than the average value of 0.88 mg/100 mL, for an adult. During the following months, the appropriate treatment decreased this value and the Phe/Tyr ratio, which sometimes became lower than 1 (Fig. 17 c).

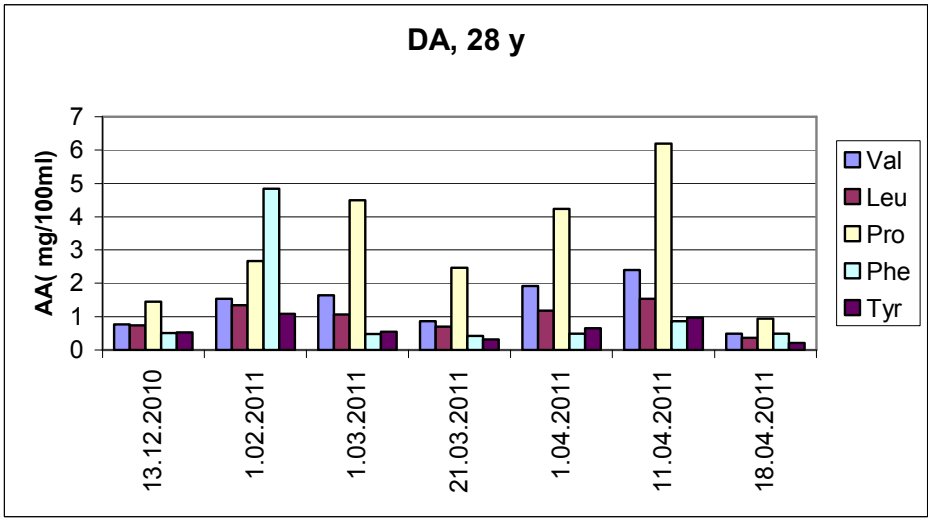


Fig. 15. The variation of amino acids concentration during the monitoring period for patient DA (28 years old).

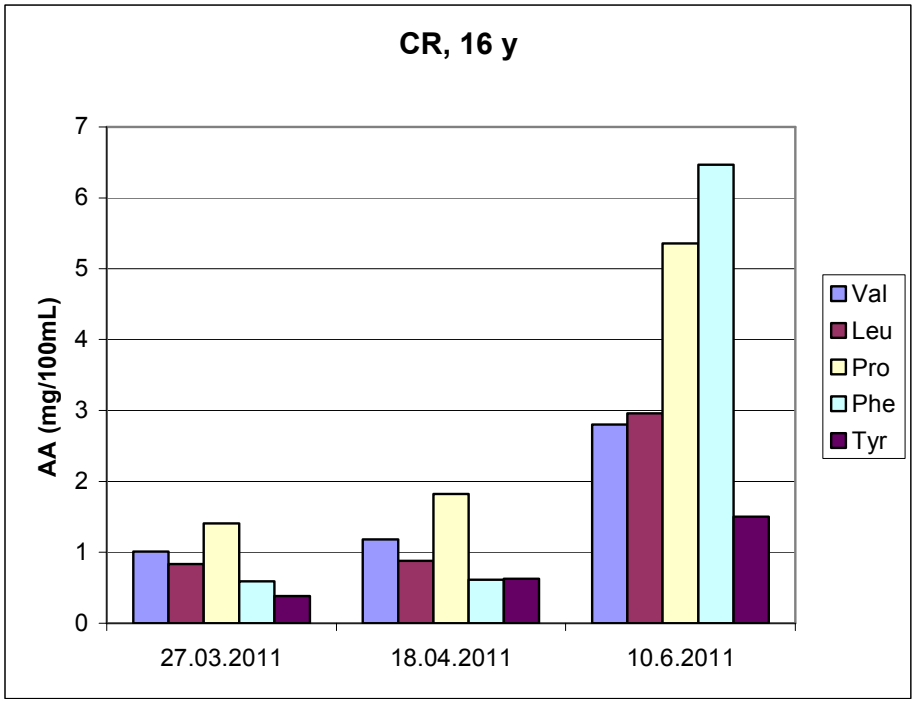
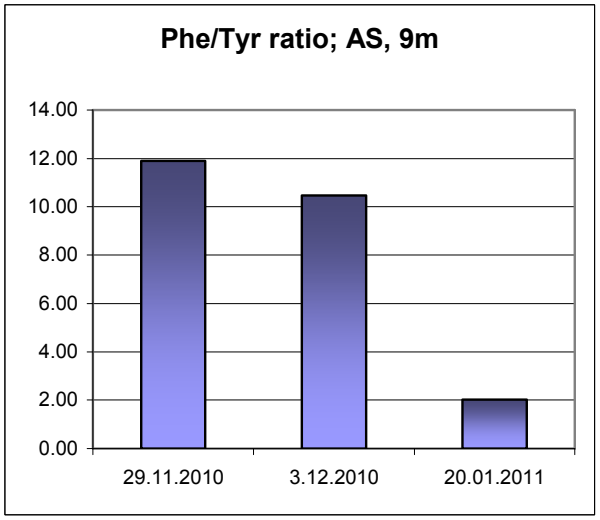
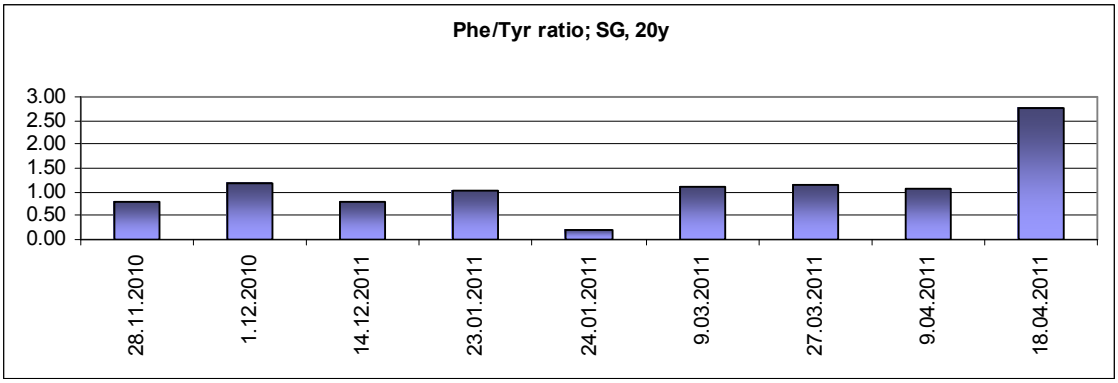


Fig. 16. The variation of amino acid concentrations during the monitoring period for patient CR (16 years old).

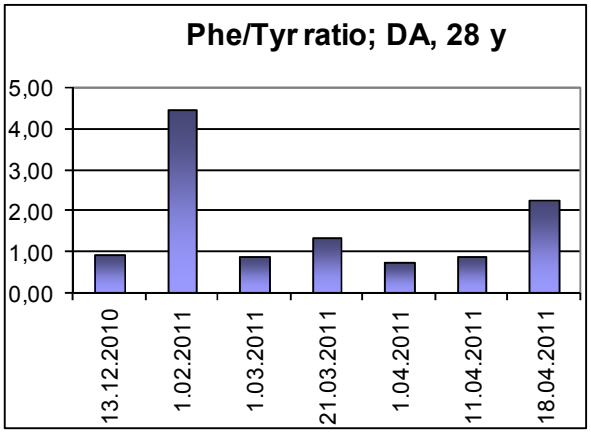
The treatment's efficiency can be controlled by determining the value of Phe/Tyr ratio. The most pronounced decrease of this value was observed for patient AS, in response to the appropriate treatment for PKU. For patients SG and DA, the Phe/Tyr ratio was kept below 3, with one exception for DA: 4.5 on 01.02.2011. In the last month of analysis, patient CR presented a Phe/Tyr ratio greater than 4.



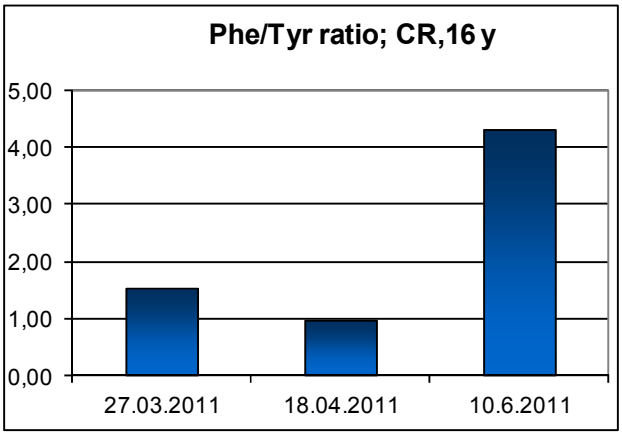
a)



b)



c)



d)

Fig. 17. The values of the Phe/Tyr ratio for each patient involved in this study.

Research studies show that the early exposure at a high level of Phe, over a long period of time, is extremely harmful for the executive functions. A higher Phe/Tyr ratio affects dopamine synthesis as tyrosine is a precursor of the neurotransmitters from catecholamines' group (dopamine, norepinephrine and epinephrine).

Conclusions

- GC-MS is a sensitive, accurate and rapid method, and a cheap alternative for diagnosis and monitoring errors of metabolism.
- The proposed method was validated in the range: 1-20 µg/mL and it shows a good linearity for each amino acid of interest.
- The method is simple, rapid and reproducible and the use of isotopic dilution increased the precision and accuracy of the method.
- The method allows monitoring treatment and determining the Phe/Tyr ratio as a quick indicator of PKU.
- This method can also be used for the diagnosis of other errors of amino acid metabolism as: maple syrup urine disease (MSUD), prolinemia, methioninemia, and tyrosinemia.

Selective bibliography

1. M. Culea, *Mass Spectrometry. Principles and applications*, Risoprint, Cluj-Napoca, (2008), p.15-21, 41-45, 48-51, 27-28, 256-260.
2. J. H. Gross, *Mass spectrometry – A textbook*, Springer-Verlag Berlin Heidelberg, (2004), p. 154-157, 146-148, 475-480.
3. R. P. W. Scott, *Principles and practice of chromatography*, Chrom-Ed Book Series, Vol. 2, (2003), p.1, 46-54, http://www.library4science.com/
4. S. Gocan, *Cromatografia de înaltă performanță. Partea I: Cromatografia de gaze*, Ed. Dacia, Cluj-Napoca, (1998), p. 9, 10, 11, 53-55, 12-17, 29, 34, 75, 115-117, 120-128.
5. R. J. Mishur, S. L. Rea, *Applications of mass spectrometry to metabolomics and metabonomics: detection of biomarkers of aging and of age-related diseases*, Mass Spectrometry Reviews, 31, (2012), p. 70-95.
6. A. R. Ani, *Influence of organic selenium on carp (Cyprinus Carpio) and grass carp (Ctenopharyngodon Idella) production rates and health*, PhD Thesis, (2011), Cluj-Napoca, p. 70-74.
7. D. I. Cocan, *Morpho-physiological peculiarities of rainbow trout of different growth and operating systems,* PhD Thesis, (2011), Cluj-Napoca, p. 232-281.
8. S. L. Andrew, *The omega-3 connection: the groundbreaking omega-3antidepression diet and brain program,* Elena Francisc Publishing, (2005), p. 35-60.
9. M. P. Rayman, *The importance of selenium to human health*, Lancet, 356, (2000), p. 233-241.
10. S. K. Jin, I. S. Kim, D. H. Kim, Y. J. Choi, S. J. Hur, *The Development of Sausage Including Meat from Spent Laying Hen Surimi*, Poultry Science, 86(12), (2007), p. 2676–2684.
11. F. Toldrá, *Biochemistry of Fermented Meat*, Food Biochemistry & Food Processing, editori: Y. H. Hui, W.-K. Nip, L. M. L. Nollet, G. Paliyath, B. K. Simpson, Oxford, Ed. Blackwell Publishing Ltd, (2006), p. 641–658.
12. M. Yudkoff, Cap.13. *Disorders of Amino Acid Metabolism,* Handbook of Neurochemistry and Molecular Neurobiology: Amino Acids and Peptides in the Nervous System, Editori: S. S. Oja, P. Saransaari, & A. Schousboe, p. 279–287, Berlin: Springer-Verlag, 2007.
13. C. Deng, C. Shang, Y. Hu, X. J. Zhang, , *Rapid diagnosis of phenylketonuria and other aminoacidemias by quantitative analysis of amino acids in neonatal blood spots by gas chromatography–mass spectrometry*, Chromatogr. B, 775, p. 115-120, 2002.
14. C. Mesaroş, A. Iordache, O. Cozar, C. Cosma, M. Culea, *Diagnosis by Mass Spectrometry*, Romanian J. Biophys., 20, No. 1, p. 71–82, 2010.
15. C. Mesaroş, M. Culea, A. M. Iordache, Chap. 3, *Diagnostics by GC/MS,* Applications of gas chromatography and mass spectrometry in studies of biology and medicine, Ed. Risoprint, Cluj-Napoca, 2012, pp. 67-82.
16. A. M. Iordache, M. Culea, C. Mesaroş, I. Ştefănescu, Chap.2, *Validation of quantitative analysis*, Biomedical and environmental applications of mass spectrometry, Ed. Risoprint, pp. 40-43, 46-47, Cluj-Napoca, 2012.

17. M. Culea, M. Chiriac, E. Culea, C. Lehene, A. Iordache, *ID-GC-MS diagnosis of metabolic diseases*, Romanian Journal of Biophysics, 17(3), (2007), pp. 169–175.
18. U. Ceglarek, P. Müller, B. Stach, P. Bührdel, J. Thiery, W. Kiess, *Validation of the Phenylalanine/Tyrosine Ratio Determined by Tandem Mass Spectrometry: Sensitive Newborn Screening for Phenylketonuria*, Clinical Chemistry and Laboratory Medicine, 40(7), (2002), pp. 693-697, D.O.I.10.1515/CCLM.2002.119.
19. C. Deng, B. Wang, L. Liu, *Fast diagnosis of neonatal phenylketonuria by gas chromatography–mass spectrometry following microwave-assisted silylation*, Journal of Chromatography B, 62, (2005), pp. 617-621.
20. S. Kawana, K. Nakagawa, Y. Hasegawa, S. Yamaguchi, *Simple and rapid analytical method for detection of amino acids in blood using blood spot on filter paper, fast-GC/MS and isotope dilution technique*, J. Chromatogr. B (2010), doi:10.1016/j.jchromb.2010.09.015.

List of publications

ISI Publications

1. Andreea Iordache, **Elena Horj**, Alina Rodica Ani, Cornelia Mesaros, Simina Morar, O. Cozar, Monica Culea, *Amino Acids Profiles In Biological Media*, AIP Conference Proceedings, vol. 1262, p.192-197, 2010, ISSN 0094-243X, ISBN 978-0-7354-0810-4.
2. Andreea Iordache, Monica Culea, **Elena Horj**, Onuc Cozar, *Determination of amino acids and selenium in fish plasma*, Romanian Journal of Physics, vol. 56(7-8), p. 963-970, 2011.
3. Andreea Iordache, **Elena Horj**, Alina Toma, Onuc Cozar, Monica Culea, *Amino acid determination of two carp species by GC-MS*, Asian Journal of Chemistry, 23(11), p. 4757-4760, 2011.
4. **Elena Horj**, Andreea Iordache, Alina Toma, Onuc Cozar, Monica Culea, *Fatty acid composition of two carp species by GC-MS*, Asian Journal of Chemistry, 23(11), p. 4768-4770, 2011.
5. Monica Culea, **Elena Horj**, Andreea Iordache, Onuc Cozar, *Determination of glycine in biological fluids by isotopic dilution mass spectrometry*, Asian Journal of Chemistry, 23(11), p. 4279-4281, 2011.
6. **Elena Horj**, Andreea Iordache, Monica Culea, *Isotopic Dilution GC/MS Method for Methionine Determination in Biological Media*, AIP-Conference Proceedings 1387, p. 241-246, doi:10.1063/1.3647081, 2011, ISSN 0094-243X, ISBN 978-0-7354-0951-4.
7. **Elena Horj**, Diana Florescu, Andreea Iordache, Silvia Neamțu, Monica Culea, *Leucine transmembranar transport study in the human red cell*, Chem. Listy 105, s1048 - s1049, 2011.

8. **Elena Horj**, Diana Florescu, Andreea Iordache, Cornelia Mesaros, Romana Vulturar, Monica Culea, *Diagnosis and monitoring by GC-MS*, Chem. Listy 105, s1047 - s1048, 2011.
9. Andreea Iordache, Diana Florescu, **Elena Horj**, Monica Culea, *Fatty acids determination in biological samples by GC-MS*, Chem. Listy 105, s1015, 2011.
10. Monica Culea, **Elena Horj**, Diana Florescu, Andreea Iordache, Levente Bara, Codrean Pop, *Amino acids determination in dry fermented salami*, Chem. Listy 105, s996, 2011.
11. Diana Florescu, Roxana Elena Ionete, Andreea Iordache, **Elena Horj**, Ioan Ştefănescu, Monica Culea, *Optimal Measurements of Surface Water Pollution-Case Study on Southern Part of Romania***,** Asian Journal of Chemistry, 23(12), p. 5209-5212, 2011.
12. Diana Florescu, Andreea Iordache, **Elena Horj**, Roxana Ionete, Ioan Ştefănescu, Monica Culea, *SIM-GC/MS determination of BTEX in ambiental air***,** Chem. Listy 105, s984, 2011.
13. Diana Florescu, Andreea Iordache, **Elena Horj**, Roxana Ionete, Claudia Şandru, Monica Culea, *Trace of heavy metals in fish tissues, sediment and water from Danube river in Romania***,** Chem. Listy 105, s984, 2011.
14. Diana Florescu, Andreea Iordache, Diana Costinel, **Elena Horj**, Roxana Ionete, Monica Culea, *Validation procedure for assessing the total organic carbon in water samples***,** articol acceptat spre publicare în *Romanian Journal of Physics.*

BDI Publications

1. Daniel Cocan, **Elena Horj**, Monica Culea, Vioara Mireşan, Adela Pintea, *Amino Acids Levels of Rainbow Trout (Oncorhynchus mykiss) Plasma During Spring – Summer Seasons*, Bulletin of UASVM, Agriculture, vol.67(1-2)132-136, 2010, ISSN 1843-5262, Electronic ISSN 1843-536X.
2. Daniel Cocan, **Elena Horj**, Monica Culea, Vioara Mireşan, Adela Pintea, *Variation of Free Fatty Acid in Rainbow Trout (Oncorhynchus mykiss) Plasma During Spring – Summer Seasons*, Bulletin of UASVM, Agriculture, vol.67(1-2)137-141, 2010, ISSN 1843-5262, Electronic ISSN 1843-536X.
3. Codrean Pop, Levente Bara, **Elena Horj**, Andreea Iordache, Cornel Laslo, Monica Culea, *Determination of free fatty acids in sausage meat*, Bulletin of UASVM, Agriculture, vol.67(2)373-377, 2010, ISSN 1843-5246, Electronic ISSN 1843-5386.
4. Levente Bara, Codrean Pop, **Elena Horj**, Andreea Iordache, Cornel Laslo, Monica Culea, *Amino acids determination in meat by GC-MS*, Bulletin of UASVM, Cluj-Napoca, Agriculture, vol.67(2)162-167, 2010, ISSN 1843-5246, Electronic ISSN 1843-5386.
5. Andreea Iordache, **Elena Horj**, Alina Rodica Ani, Cornelia Mesaros, Simina Morar, Monica Culea, *Fatty Acids Profile in Fish Plasma,* Analele Universităţii de Vest, Timişoara, seria FIZICA, LIV, p. 55-61, 2010.

6. **Elena Horj**, Andreea-Maria Iordache, Monica Culea, *Method validation for free amino acids determination in biological samples*, STUDIA UNIVERSITATIS BABES BOLYAI. PHYSICA, LV, 2, p. 61 – 68, 2010.
7. Diana Florescu, Andreea Iordache, Claudia Şandru, **Elena Horj**, Roxana Ionete, Monica Culea, *Heavy Metals Concentration in Contaminated Soils from Southern Part of Romania***,** Bulletin UASVM Agriculture, 68(2) 160, 2011, ISSN 1843-5246; Electronic ISSN 1843-5386.