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Dedicated to Professor Mircea Diudea on the Occasion of His 65th Anniversary

GC-MS METHODS FOR AMINO ACIDS DETERMINATION IN DIFFERENT BIOLOGICAL EXTRACTS

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ABSTRACT. Sensitive, precise and accurate analytical methods for free amino acids determination in biological samples were developed. Purification by ion exchange technique was followed by two steps derivatization method to obtain trifluoroacetyl ester derivatives. GC/MS analysis was performed by using scan or SIM mode. Known amounts of internal standard, the isotopic labelled analogue of glycine, methionine or isoleucine were added to the sample, before extraction, for the quantitative analysis, followed by matrix and regression curves calculation. The methods were validated using amino acid standard samples. Analyses of dairy, corn grain, fish plasma and meat are presented. Also a trace level (picogram) analysis method of blood spots, for diagnosis of inborn errors of metabolism, is described.

Keywords: *amino acids, GC-MS, isotopic dilution, blood*

INTRODUCTION

The paper presents the development of sensitive, simple and precise analytical methods for determination of amino acids in biological samples. The method involves different extraction methods, purification of extracts by

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ion exchange technique, derivatization in two steps of the amino acids and gas chromatography-mass spectrometry (GC-MS) analysis. The derivatization was applied to obtain trifluoroacetyl ester derivatives [1-14]. Analytical methods by GC/MS was performed in the electron impact (EI) mode [2- 14]. The high sensitivity and specificity of GC-MS technique for volatile and semivolatile compounds was increased by one or two orders of magnitude when selected ion monitoring (SIM) mode was used. SIM-GC/MS is very useful for low level, nanogram and picogram, quantitative work and is usually achieved by isotopic dilution (ID).

Applications for the amino acids determination during some dairy processing, for free amino acid in corn grain, in fish plasma and for diagnosis of metabolic diseases are presented. GC-MS is an indispensable method for diagnosing inborn errors of metabolism. GC-MS quantitative determination method of five amino acids L-phenylalanine (Phe), L-tyrosine (Tyr), L-proline (Pro), L-leucine (Leu) and L-valine (Val) as n-butyl trifluoroacetyl esters was developed, to diagnose PKU, maple syrup urine disease (MSUD) and other aminoacidemias. Phenylketonuria (PKU) is caused by phenylalanine hydroxylase enzyme deficiency.

RESULTS AND DISCUSSION

Method validation

Methionine quantitative method gave a good linearity regression curve, y=0.0355x +0.1319, r=0.9995, obtained with standards with known concentration of methionine, in the range 0-100μg.mL^{-1} and 20μg.mL^{-1} addition of internal standard. The internal standard ^{15}N-methionine, (99 atom % ^{15}N) and methionine required correction by deconvolution and matrix calculation. Fractional isotopic abundances for natural methionine and isotopomer were obtained experimentally [5,17-19] (Table 1).

Table 1. The matrix design (left) and the pseudoinverse matrix (right) used for methionine calculation [5]

methionine	[M]	[M+1	methionine	[M]	[M+1]
n.a.	0.95	0.05	n.a.	1.05	-0.05
^{15}N	0.01	0.99	^{15}N	-0.01	1.01

Methionine was calculated by matrix and regression curve calculation. Very good correlation between the two methods was obtained, the correlation coefficient of 0.998. Precision and accuracy for methionine, measured for standards of 20 and 30 μg mL^{-1} (n=7), showed very good results, lower than 6% and respectively 11%.

Method validation, using amino acid standards following the extraction and derivatization procedure (n=3), gave precision lower than 20% (R.S.D.), except Tyr and L.O.D. value 1ng of amino acid injected. Good linearity results for amino acids were found (Table 2).

Figure 1 presents the total ion chromatogram (TIC) of a standard solution of amino acids. The components were identified by using NIST library. The amino acids elution order was: alanine (Ala), glycine (Gly), threonine (Thr), serine (Ser), valine (Val), leucine (Leu), isoleucine (Ile), cysteine (Cys), gamma-aminobutiric acid (GABA), proline (Pro), hydroxyproline (Hy-Pro), methionine (Met), aspartic acid (Asp), ornitine (Orn), phenylalanine (Phe), lysine (Lys), glutamic acid (Glu), tyrosine (Tyr), histidine (His), tryptophan (Trp), cysteine (Cis).

Table 2. The regression curve and the coefficient of correlation, r, obtained for the studied amino acids [5]

Amino acid	**Regression curve**	**r**
Alanine (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
Gama-aminobutiric acid(GABA)	y = 2.207x - 8.8747	0.980
Proline (Pro)	y = 1.0331x + 20.49	0.991
Hydroxyproline (Hy-Pro)	y = 1.3504x - 4.257	0.993
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
Histidine (His)	y = 0.6916x - 10.196	0.957

Amino acids in meat

The method was applied for determination of free amino acid in beef, pork and a salami mixture meat, as presented in Table 3.

Significant differences were observed between the amino acid levels measured in different sorts of meat. Essential amino acids were higher in beef and pork meat while flavor amino acids were higher in mixture meat [5].

Table 3. Comparative values of amino acids levels in a mixture, beef and pork meat [5]

	mg/g	mixture	pork	beef
1	Ala[3]	1.18	1.29	2.43
2	Gly[3]	0.33	0.42	0.67
3	Thr[1,3]	0.24	0.29	0.55
4	Ser[3]	0.13	0.10	0.18
5	Val[1]	4.89	7.60	6.39
6	Leu[1]	0.5	0.56	1.38
7	Ile[1]	0.44	0.43	1.08
8	Pro	0.57	0.59	1.06
9	Met[1]	0.06	0.01	0.01
10	Asp[1]	0.51	0.48	1.18
11	Phe[1,4]	0.32	0.36	0.85
12	Orn[1]	0.11	0.00	0.15
13	Glu[2]	15.83	4.95	13.77
14	Lys[1]	1.23	1.09	2.21
15	Tyr[4]	0.1	0.33	0.20
16	His[1]	4.86	10.01	12.29
	total(mg/g)	31.29	28.51	44.40
	eAA	12.84	20.46	25.23
	fAA	15.83	4.95	13.77
	sAA	1.64	1.81	3.29
	frAA	0.42	0.70	1.05

Note: [1]-essential amino acids (eAA); [2]-flavor-related amino acids (fAA); [3]-sacharinity-related AA(sAA); [4]-fragrant-related amino acids (frAA

Cheese amino acids

Application for study the free amino acids (FAA) in cheese at various stages of ripening, by using two different starter enzymes, a lipolitic (L) one and a proteolitic (P) one, in comparison with a control (M) cheese was performed. The main amino acids identified in all samples were: Ala, Gly, Thr, Ser, Val, Leu, Ile, Pro, Asp, Phe, Orn, Glu, Lys, Tyr. Proteolitic cheese showed higher quantity of Ala, Thr, Ser, Pro, (sweet amino acids) and much higher Tyr (bitter

taste) than in control sample. These amino acids had no cheese taste, but they can contribute to the complex taste of maturated cheese. The use of different starter bacteria caused differences in the quality of cheese and the starter culture had contributed to proteolysis at different degrees [7].

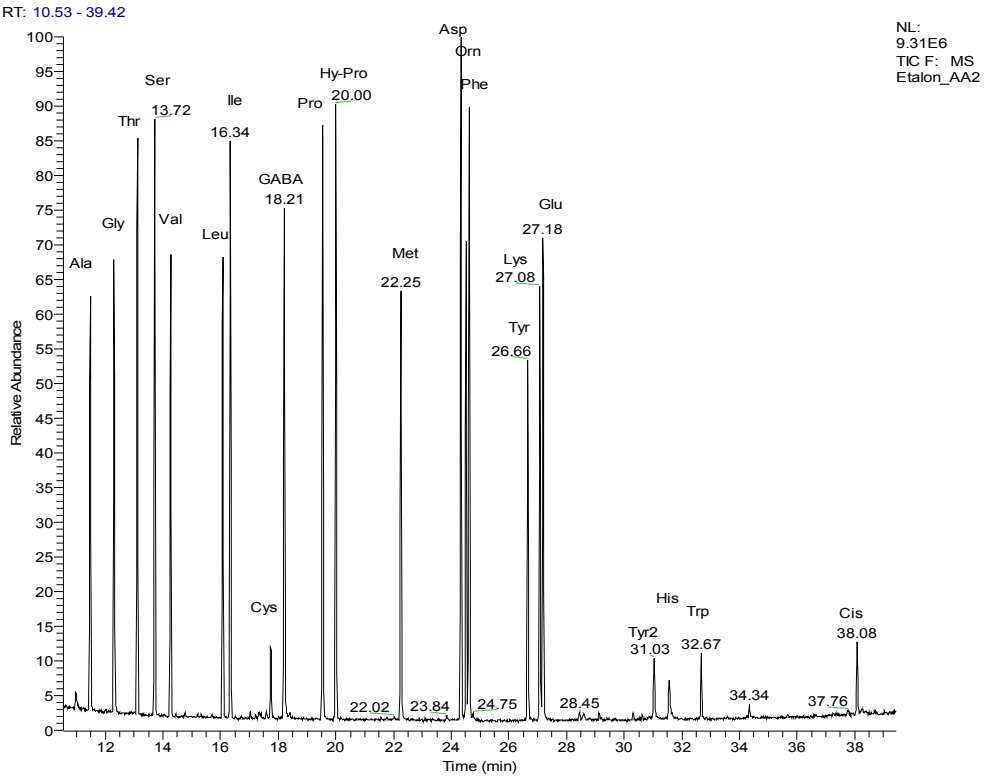

Figure 1. GC-MS amino acids standard separation and identification [5]

Yoghurt amino acids

The method was applied for the study of different steps in the technical processes of preparation of some dairy products, after protein hydrolysation. (yoghurt (I), “sana” (S), butter milk (LB)). Significant differences among the three steps tested in the concentration of the amino acids were found. In the fermentation processes amino acids from proteins were changed, especially Phe, Tyr, Pro, Leu [8].

Salami amino acids

The salami samples of different days of storage (dry fermentation) were purchased from a local producer as meat mixture after reddening (r, day 0), salami (day 0), salami (day 14), salami (day 30), salami (day 45) and preserved at - 20°C, until analysis. Two salami samples, extracted twice, were used for a day of study. Batches containing 0.3% glucono delta-lactone (GDL) and 0.05% sodium ascorbate (ASC), and batches containing only 0.1 % sodium ascorbate were manufactured. The content of FAA (Figure 2) was measured at different time intervals over 45 days of storage.

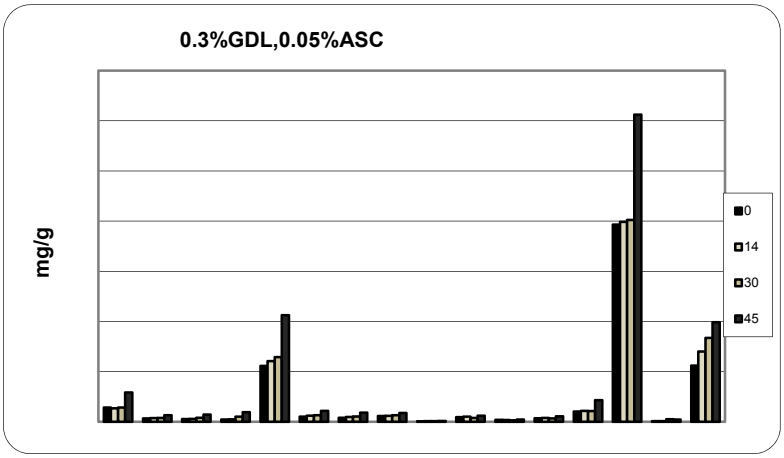

Figure 2. Comparison of FAA in a salami variety

The amino acids increased significantly with storage especially in GDL variety. A higher increase with fermentation time was observed especially for glutamic acid (flavor-related amino acid), serine, tyrosine (fragrant-related amino acid), isoleucine, threonine, lysine, alanine and glycine. The total amount of free amino acids may be influenced by the pH decrease of salami containing GDL and by fermentation time.

The method is suitable (e.g. RSD was lower than 20% for precision and lower than 23% for accuracy, LOD lower than 1ng and linearity (r>0.98),) for different purposes as: food quality control, food processing control, animals' diet control and metabolic studies [12,16].

Corn grain amino acids

The method was used for the determination of free amino acids from corn inbred lines flour (25 samples have been compared).

Table 4. FAA values, in $\mu g \cdot g^{-1}$, in corn seed nucleus and inbred lines*

1.1	2.1	3.1	4.1	5.1
1293.39±15.22	713.44±7.13	2404.68±31.49	5798.44±44.89	933.87±9.79
1.2	2.2	3.2	4.2	5.2
1315.09.±7.76	2075.06±6.14	4785.51±20.39	1556.46±20.39	977.90±6.28
1.3	2.3	3.3	4.3	5.3
831.67±4.36	1898.42±6.82	4443.38±80.20	1551.73±14.72	772.43±7.86
1.4	2.4	3.4	4.4	5.4
1858.99±19.14	3911.90±47.82	4290.14±39.53	2123.28±26.84	1685.26±12.66
1.5	2.5	3.5	4.5	5.5
1285.69±7.71	1226.75±8.00	4284.74±39.98	1317.92±8.63	924.76±3.28

* FAA: total free amino acids; the corn inbred lines were noted: 1.2, 1.3, 1.4, 1.5; and similar 2.2- 2.5; 3.2- 3.5; 4.2- 4.5; 5.2- 5.5. Seed nucleas: 1.1, 2.1, 3.1, 4.1, 5.1.

Among the free amino acids determined, the highest were aspartic acid, proline, gamma-aminobutyric acid, lysine, alanine, glutamic acid and histidine. The study established the variation of the free amino acids within the different inbred lines. The results suggest that FAA determination in inbred lines studied (Table 4) could help the selection of genotype and may improve its functional and nutritional qualities [13].

Fish amino acids

We have determined seasonal differences of blood plasma amino acids concentration of rainbow trout (*Oncorhynchus mykiss*). The essential amino acids determined were: threonine, valine, leucine, isoleucine, methionine, phenylalanine, tyrosine and lysine. Nonessential amino acids determined was: alanine, glycine, serine, proline, hydroxiproline, ornithine, aspartic acid and glutamic acid. For most amino acids, their concentration decreased in summer, compared with those recorded in the spring season[14].

Also the free amino acids in two carp varieties (Galitian and Lausitz) occurred at a dietary Se-methionine level of 0.05 mg/kg was compared with control carp plasma. A significant increase of about 3.8 times of methionine was observed in Galitian carp experimental group in comparison with control. Galitian carp variety showed almost two times higher values for total free amino acids in experimental fish than control, and also higher values were obtained in experimental Lausitz variety in comparison with control. The methods are very useful for nutrient and diet control [15].

Diagnosis of inborn errors of metabolism

Isotopic dilution GC-MS rapid method developed for the early diagnosing of inborn error of metabolism of some neonatal diseases was used for the screening of phenylketonuria or maple syrup urine disease in newborns. ID-GC-MS is a fast and reliable method which has the advantages of using small volumes of neonatal blood spots. The blood samples were derivatized as trifluoroacetylbutyl esters and analyzed by gas chromatography coupled with mass spectrometry in the selected ion monitoring (SIM) mode. Regression curves for standard amino acids were used for quantitative determination of valine, leucine, proline, phenylalanine and tyrosine using ^{15}N-isoleucine as internal standard.

Good regression curves were obtained by injecting standard solutions containing amino acids in concentration of 1, 5, 10, 20, 30 and 40 μg/ml with 25 μg of 15N-Ile, added to each standard solution and per ml of blood sample. The regression curves obtained were very good, with coefficient of correlation over 0.998 for Val, Leu, Pro, Phe except Tyr (r=0.984). Precision studied for standard of 30 and 40 μg/ml gave R.S.D. values between 9-12.9% for 30μg/ml and 6.7-18.6 % for 40 μg /ml. Accuracy values were lower than 5.5 for 40μg/ml (n=4). The limit of detection (L.O.D.) was lower than 0.1 μg/ml.

Significant differences between PKU subjects and control was observed. In control subjects (*n*=53) the ratio of Phe/Tyr was less than 1 while the PKU positive blood (n=20) gave more than 2. MSUD cases was not found in our study [3,4,9-11].

CONCLUSIONS

The methods developed are useful for the analysis of nutrients from different biological media. Good validation parameters, linearity, correlation coefficients, precision, accuracy, were obtained in the range of interest.

The use of isotopic labeled internal standard increased precision and avoids the overlapping of analytes with different contaminants.

Important differences in the free amino acids among varieties of meat and dairy products were observed. The free amino acids determined during the ripening period could characterise the quality of cheese. FAA determination in corn inbred lines studied could help the selection of genotype and may improve its nutritional qualities. The methods are useful for nutrient and diet control.

The minim invasive method was used for amino acids quantitation from dried blood spots. Diagnosis in the first 3 months of newborn saves lives (MSUD) or normal intellectual development (PKU) and also the treatment and diet could be controlled for this patients.

EXPERIMENTAL SECTION

A gas chromatograph coupled with a quadrupole mass spectrometer Trace DSQ (Thermo Finnigan, Proanalysis, Bucharest, Romania) was equipped with a capillary column using an adequate temperature program (Table 5).

Table 5. Amino acid analytical conditions

Capillary column	Temperature program	MS conditions:
Rtx-5MS had 30 m x 0.25 mm I.D., film thickness of 0.25 μm; split mode (10:1)	50°C for 1 min, 6°C/min to 100°C, 4°C/min to 200°C, 20°C/min to 300°C, 300°C for 3 min. Carrier gas: He, 6.0; 1ml/min.	Mode: EI; electron energy: 70 eV; emission current: 100μA. Mass range: 50-500 a.m.u. Transfer line: 250°C, injector: 200°C; ion source: 250°C.

The amino acids extraction and derivatization steps were followed as presented in Table 6.

Table 6. Extraction procedure of amino acids from sample

Amino acids extraction [1]	Amino acids derivatization	
cation exchange resin Dowex 50W-X8 100mesh, 40x2mm column; Activation of resin; sample+IS; Elution: 2ml 3M NH_4OH; Evaporate	1. esterification:100μl butanol/HCl 3M for 1h, 110°C;	2. acetylation:100μl TFAA, 80°C, 20 min; dry at 4°C; 1ml ethyl acetate

One microliter of each sample was injected into the GC/MS by using a TriPlus autosampler (Proanalysis, Bucharest, Romania).

The blood spot samples were separated on the same capillary column in a temperature gradient of 14 min. and in the selected ion monitoring (SIM) mode. The following important ions from the mass spectra of Phe, Pro, Val, Leu and Tyr were used: m/z 91, 148, 204 for Phe, m/z 166 for Pro, m/z 168 for Val, m/z 182 Leu, m/z 203, 260, 316 for Tyr and m/z 183 for the internal standard. This minimum invasive method was based on profiling and quantitative determination of some amino acids in blood samples of 20 μl by using filter paperblood specimens and the GC-MS technique [4].

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