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AMINO ACIDS QUANTITATION IN BIOLOGICAL MEDIA

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The development of simple and sensitive analytical methods for the determination of amino acids in different biological specimen samples involves some steps of purification by ion exchange extraction technique followed by derivatization and GC/MS analysis. Amino acids from different biological samples were derivatized in two steps to obtain trifluoroacetyl ester derivatives. Finally, the extracted analytes were detected by GC/MS, EI, scan or SIM modes. The addition of the appropriate internal standard before the preparation steps is very important for the quantitative work. The methods were validated by using amino acid standard samples. Some application studies are presented.

Introduction

The purpose of this study was to develop a simple, rapid and sensitive analytical method for determination of amino acids in biological specimen samples. The developed method involves the employment of derivatization and purification by ion exchange extraction technique together with gas chromatography/mass spectrometry (GC/MS). Amino acids from biological samples were derivatized in two steps to obtain trifluoroacetyl ester derivatives [1,2]. Finally, the extracted analytes were detected by GC/MS in electron impact (EI) mode [3]. GC-MS is a high sensitive and specific technique used in organic analysis. In the selective ion monitoring (SIM) mode, using a few selected ions, the sensitivity is increased by one or two orders of magnitude. SIM-GC/MS is very useful in quantitative work and is usually achieved by isotopic dilution (ID).

Applications for the total amino acids determination during some dairy processing and for diagnosis of metabolic diseases are presented. Phenylketonuria (PKU) is caused by a deficiency of phenylalanine hydroxylase. The normal catabolism of phenylalanine (Phe) in mammals requires its initial conversion to tyrosine (Tyr) in the liver. The enzyme defect leads to a specific pattern of plasma amino acids with increased Phe or decreased Tyr. PKU detection relies on amino acid (Phe) screening of newborn blood. At present, GC-MS is an indispensable method for diagnosing inborn errors in metabolism. By GC-MS quantitative determination 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,

it is possible to diagnose PKU, maple syrup urine disease (MSUD) and other aminoacidemias /2,3/.

Experimental

Materials and Methods

Standard amino acids and trifluoroacetic anhydride were obtained from Merck (Darmstadt, Germany). Acetyl chloride and the ion exchange resin Dowex 50W-X8 were from Fluka (Buchs, Switzerland). [^{15}N]-glycine (Gly: 98.98%) was produced by chemical synthesis. All other chemicals were from Comchim (Bucharest).

Amino acid purification and derivatization

The amino acids were purified on a Dowex 50W-W8 exchange resin, on a 2x40mm column and eluted with 4M NH_4OH (Table 1). A two step derivatization procedure was applied: esterification with butanol-acetyl chloride (4:1 v/v) for 1 h at 100°C and trifluoroacetylation with 200 µl trifluoroacetic anhydride at 60°C for 20 min [1].

Table 1

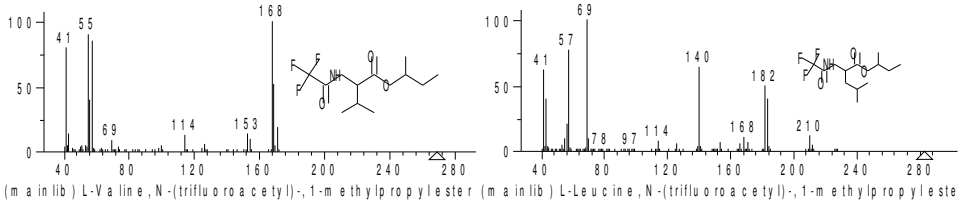
Extraction procedure for amino acids (AA) and drugs from plasma

Amino acids extraction	**AA dirivatization**	
cation exchange resin Dowex 50W-X8, 100mesh, 40x2mm column; Elution: 2ml 3M NH_4OH; Evaporate	1.esterification: 500µl butanol: acetyl chloride, 5:1,v/v, 1h, 100°C	2.acetylation 200µl TFAA[1], 60°C, 20 min, cool, dry, 1ml ethyl acetate

[1]TFAA=trifluotoacetic anhydride; Resin is kept into refrigerator with distilled water and 1N NaOH; H+ form made with 1N HCl. Free AA are analyzed by adding 1ml 1N acetic acid to 0.5ml plasma. Total AA are analyzed after protein hydrolysis: 6NHCl at 110°C.

Apparatus

A Thermo Finnigan GC-MS equipped with a Rtx-5MS (15 or 30 m x 0.25 mm, 0,25 µm) column was used. The GC/MS interface line and the ion source were maintained to 200°C and 250 °C. Electron energy was 70eV and electron emission 100µA.



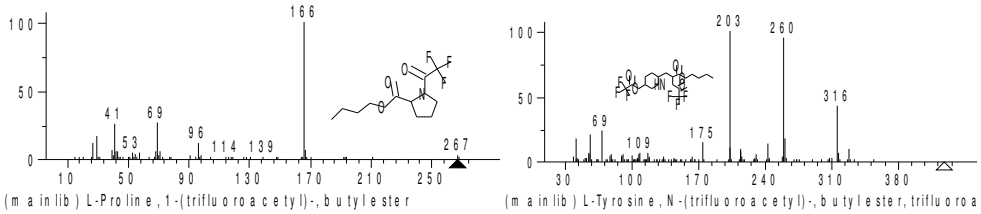


Fig. 1 EI mass spectra of Val, Leu, Pro and Tyr

In the SIM mode the following important ions from the mass spectra of the amino acids of interest were used: m/z 168 for Val, m/z 182 Leu, m/z 166 for Pro, m/z 91 for Phe, m/z 203 for Tyr and m/z 155 for the internal standard. The method was validated in the range 0 – 150 µg and linearity, precision, accuracy and limit of detection parameters were studied.

The EI mass spectra of some amino acid standards of interest are presented in Fig. 1.The total ion chromatogram TIC for all amino acid found in a plasma sample is presented in Fig.2.

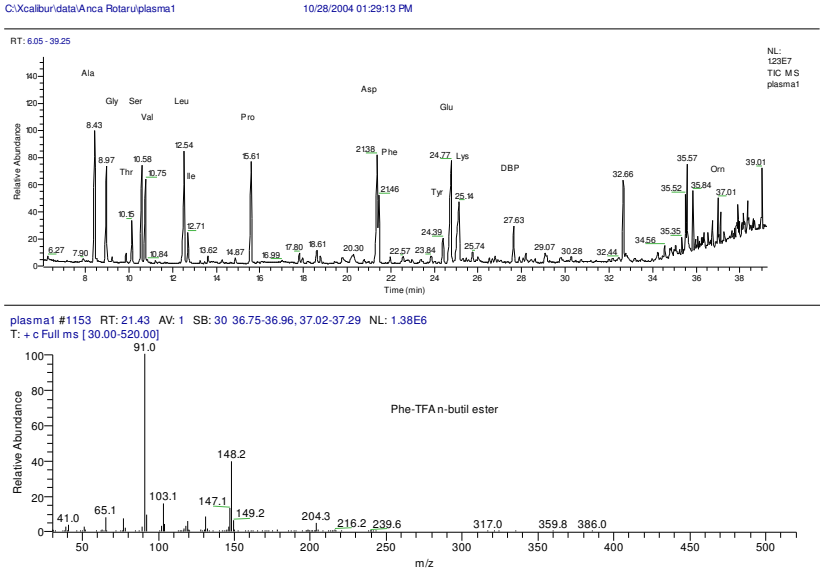


Fig. 2 TIC of a plasma amino acids and the mass spectrum of Phe

Quantitation is performed by addition of known amounts of internal standards to the sample before extraction. The method was validated by using amino acid standard samples.

3. Results and Discussions

The results obtained for the five amino acids of interest were calculated in GC analyses by using ^{15}N-Gly as internal standard. Table 1 presents the levels for the amino acids in control and diseases blood samples. Significant differences were found to all amino acids studied compared with control. PKU shows higher levels in Phe/Tyr than control, but in MSUD the diagnosis can be done on bases of the ratio of aliphatic amino acids to aromatic amino acids.

Significant differences among the three steps tested in the production processes of dairy products were observed in the concentration of the amino acids found in proteins. The proteins were hydrolyzed before derivatization. In the fermentation processes amino acids from proteins are changed, especially Phe, Tyr, Pro, Leu. The measured changes are presented in Table 2 in (yoghurt (I), "sana"(S), butter milk (LB)).

Table 1

Control and diseases amino acids results

	Control	**Diabet**	**Cirrhosis**	**Kidney disfunction**
Val	3.005	3.05	3.14	1.71
Leu	2.507	3.1	2.63	1.55
Pro	1.752	2.7	3.05	2.04
Phe	2.192	2.55	2.5	2.51
Tyr	0.606	2.18	1.67	0.53
Phe/Tyr	3.6	1.2	1.5	4.7

Table 2

Quantitative distribution of amino acids in the steps of dairy products preparation

.**Total amino acids**	**1**	**2**	**3**
I(mg/g)	128.18	80.30	52.50
S(mg/g)	254.61	159.45	104.12
LB(mg/g)	509.03	318.77	208.13

Conclusions

GC-MS is an indispensable method for diagnosing inborn errors in metabolism and is very useful for food industrial processes study. When the disease is discovered before the third month, the child could develop normal with a proper diet in the case of PKU disease or his life is saved in the case of MSUD disease.

In our method there is not distinguish between aspartic acid and asparagine and glutamic acid and glutamine, because in the derivatization steps asparagine and glutamine are transformed into the corresponding acid.

References

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