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Determination of Amino Acid Composition of Two Carp Species by GC-MS

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An isotopic dilution gas chromatography-mass spectrometric (ID-GC/MS) technique was used to evaluate the effects in quantitative amino acids of fish plasma after Se addition in food in comparison with control. The stable isotope internal standard used was ^{15}N-methionine. A trace DSQ ThermoFinnigan quadrupole mass spectrometer coupled with a trace gas chromatography was used. Amino acids were separated on a Rtx-5MS capillary column, 30 m × 0.25 mm, 0.25 μm film thickness, using a temperature program from 50 ºC, 1 min, 6 ºC/min at 100 ºC, 4 ºC/min at 200 ºC, 20 ºC/min at 300 ºC, (3 min). The transfer line temperature was 250 ºC, the injector temperature 200 ºC and ion source temperature 250 ºC; splitter: 10:1. The amino acids were purified on a Dowex 50W-W8 exchange resin and derivatized in a procedure following two steps to obtain trifluoroacetyl butyl esters. Concentrations of majority of free amino acids as methionine, glutamic acid, aspartic acid, lysine, phenylalanine, increased with Se supplementation.

Key Words: Amino acids, GC-MS, Derivatization, Selenium.

INTRODUCTION

Isotopic dilution gas chromatography-mass spectrometry (ID-GC/MS) is a technique used for quantitative analysis of compounds from different biological specimens. Stable isotopes provide the ideal internal standards in quantitative information[1-7].

Selenium is a very potent antioxidant protecting the body from damage due to oxidation by free radicals. Dietary supplementation with selenium in animals increased selenium content in several tissues. The antioxidant effect of selenium on lipid peroxidation, enzyme activities and biochemical parameters might be beneficial in antagonizing aluminium toxicity[8]. Oral nutritional supplements infusion improves duodenal protein balance in healthy humans[9]. Changes in the plasma amino acids pool reflect the nutritional state of fish and help us to understand the complex amino acids metabolism. Plasma amino acid levels can be used as an index of nutritional status and to evaluate the quality of a diet[10,11].

The aim of the paper was the development of a simple and sensitive analytical method for determination of free amino acids to evaluate effects in quantitative amino acids in fish plasma after Se addition in food in comparison with control.

EXPERIMENTAL

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

Application of the method was to study the influence of food on amino acids level in plasma. Two different varieties of carps (*Cyprinus carpio*) were studied: Galitian and Lausitz. The fodder consisted of: 38 % proteins, 5 % fat, 3.5 % pulp and 9 % humidity. For the experimental batch the fodder included also 0.03 mg organic Se (Sel-plex, All-Tech, USA) per kg.

Plasma samples collected for this study were ten controls, five of each variety and ten experimental carps, five of the two varieties.

General procedure: The determination of methionine was obtained by isotopic dilution (ID). 20 μg of ^{15}N-Met was added before extraction to 1 mL of each sample. By selecting the specific ions m/z 171 and 172 from the mass spectrum of methionine (Met), respectively of labelled methionine, Met could be determined by using regression curve calculation or

by matrix calculation. The other amino acids were calculated according with the internal standard quantity and by using the response factors (for detector response correction) or the regression curves obtained by repetitive injections into GC/MS of a standard mixture containing known quantity of each amino acid. The response factors were calculated from standard samples with the relation:

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

The amino acid calculations in biological samples were performed following the formula:

$$C_i(\%\ \text{weight}) = \frac{\frac{m_j \times A_i}{F_i \times A_j}}{\sum_{i=1}^{n}\left(\frac{A_i}{F_i}\right)} \times 100 \quad (2)$$

where C_i (or m_i) is the quantity corresponding to the compound i; m_j is the internal standard quantity added before sample preparation; A_i and A_j are the peak areas of the compounds i and j, respectively; F_i is the response factor for compound i. Also, the amino acid levels in plasma were calculated by using the regression curve of each amino acid using as internal standard ^{15}N-Met.

Isotopic deconvolutions: The use of the isotopic labellled analogue of the analyte (the amino acid of interest) as internal standard and the presence of the analyte (tracer) with its natural isotopic abundance in plasma necessitate careful correction of the mass spectrum, to deconvolute the information of interest. Fractional isotopic abundances for natural methionine and isotopomer were calculated from experimentally measured isotopic ratios. The set of simultaneous linear equations each describing the isotopic contributors had to be solved having the general form:

$$I_x = \sum_{x=i,j} A_i X_j \quad (3)$$

where I_x represents the relative ion abundance for the xth ion; X_j represents the unknown fractional abundance. The relative abundance of the contributors (A_i) was calculated for the two ions expressing the simultaneous equations in matrix notations:

$$I = AX \quad (4)$$

The least squares solution of X can be obtained by using the inverse of A transpose:

$$X = (A^T A)^{-1} A^T I \quad (5)$$

The amino acids were purified on a Dowex 50W-W8 exchange resin, on a 2 mm × 40 mm column and eluted with 4M NH_4OH. A two step derivatization procedure was applied: esterification with butanol-acetyl chloride (4:1 v/v) for 1 h at 100 ºC and trifluoroacetylation with 100 µL trifluoroacetic anhydride at 60 ºC for 20 min. The method was validated and some validation parameters, precision and sensitivity were tested. GC/MS analyses were performed for the determination of amino acids in plasma samples.

Detection method: A trace DSQ ThermoFinnigan quadrupol mass spectrometer coupled with a trace GC was used. The derivatized amino acids were separated on a Rtx-5MS capillary column, 30 m × 0.25 mm, 0.25 µm film thickness, using a temperature program from 50 ºC, 1 min, 6 ºC/min la 100 ºC, 4 ºC/min la 200 ºC, 20 ºC/min la 310 ºC, (5 min). The following conditions were followed: transfer line temperature 250 ºC, injector temperature 200 ºC; ion source temperature 250 ºC; splitter: 10:1. Electron energy was 70 eV and emission current, 100 µA.

RESULTS AND DISCUSSION

The method was validated by using amino acid standards. The elution order of the amino acids is presented in Fig. 1: alanine (Ala), glycine (Gly), threonine (Thr), serine (Ser), valine (Val), leucine (Leu), isoleucine (Ile), proline (Pro), methionine (Met), aspartic acid (Asp), phenylalanine (Phe), glutamic acid (Glu), lysine (Lys), tyrosine(Tyr), histidine (His). The standards have followed the described extraction and derivatization procedure (n = 3). Precision gave lower value than 20 % for RSD, except Tyr and sensitivity value lower than 10 ng of amino acid injected. All the samples followed the same extraction and derivatization steps.

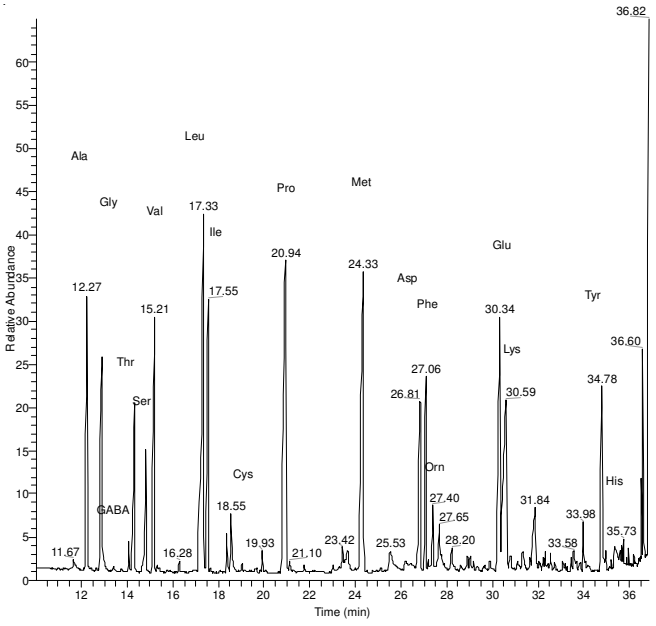

Fig. 1. Amino acid chromatogram of a plasma sample: Ala, Gly, Thr, Ser, Val, Leu, Ile, Cys, Pro, Met, Asp, Phe, Glu, Lys, Tyr, His

Fig. 2 presents the mass spectrum of methionine. The ion m/z 171 was selected for quantitative measurements of methionine together with the corresponding ion of the labelled methionine, m/z 172.

The amino acids measured for different control carp fishes, of the two varieties, gave similar results. A significant increase of methionine was observed in carp experiment in comparison with control, higher than 3 times in Lausitz variety. The majority of the free essential amino acids have increased in the both variety of carps. The essential amino acids[12] are marked in Table-1: Arg, His, Leu, Ile, Lys, Met, Phe, Thr, Trp, Val. The non-essential free amino acids have also increased especially in plasma of Galitian carp (Figs. 4 and 5). The important differences observed in the total free amino acids in Galitian carps and in Lausitz varieties are presented in Fig. 6. The

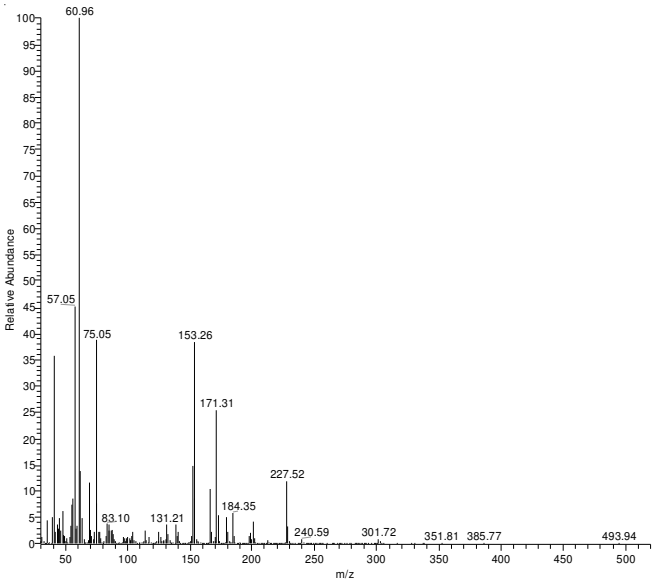

Fig. 2. Mass spectrum of ^{15}N-methionine trifluoroacetyl butyl ester, M = 228

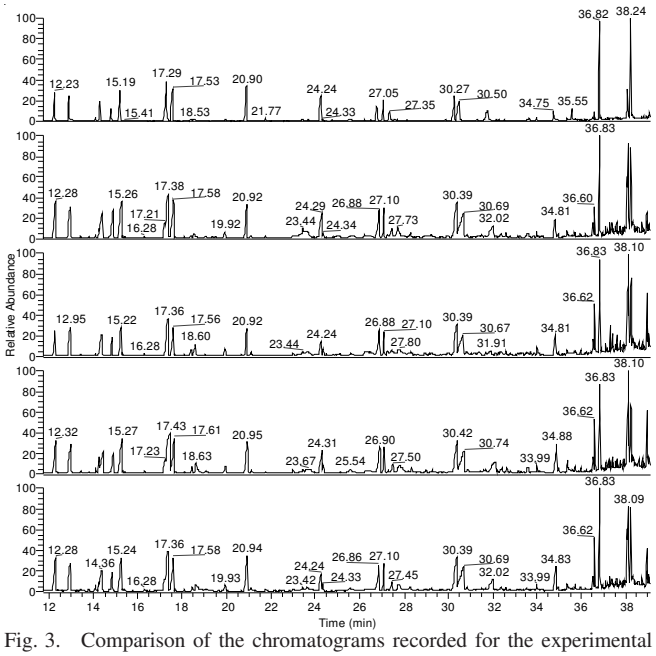

Fig. 3. Comparison of the chromatograms recorded for the experimental fish (Galitian carp)

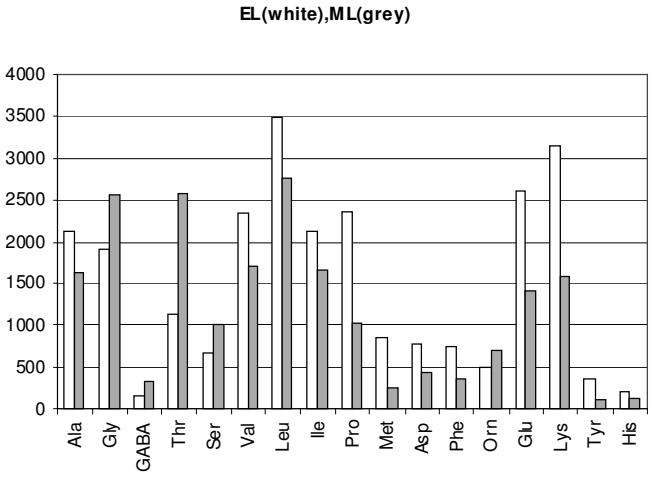

Fig. 4. Comparison of free amino acid of Lausitz carp plasma, experiment (EL) and control (ML)

TABLE-1
CHANGES OF FREE AMINO ACIDS CONCENTRATIONS IN CONTROL AND EXPERIMENTS (μM)

AA	M	EL	EO	ML	MO
Ala	89	2123	2383	1632	1225
Gly	75	1917	2651	2541	1217
GABA	103	151	214	330	101
Thr[e]	119	1138	1305	2566	898
Ser	105	673	1026	1014	305
Val[e]	117	2330	2544	1698	1319
Leu[e]	131	3433	3654	2769	2660
Ile[e]	131	2121	2531	1658	1456
Pro	115	2350	1778	1018	1170
Met[e]	149	856	648	251	438
Asp	133	779	993	440	403
Phe[e]	165	749	877	376	472
Orn	132	308	387	424	441
Glu	147	2599	2774	1401	1101
Lys[e]	146	3150	3480	1592	1930
Tyr	181	360	510	116	153
His[e]	155	212	225	142	140
–	–	25499	28230	20243	15713

[e]Essential amino acids.

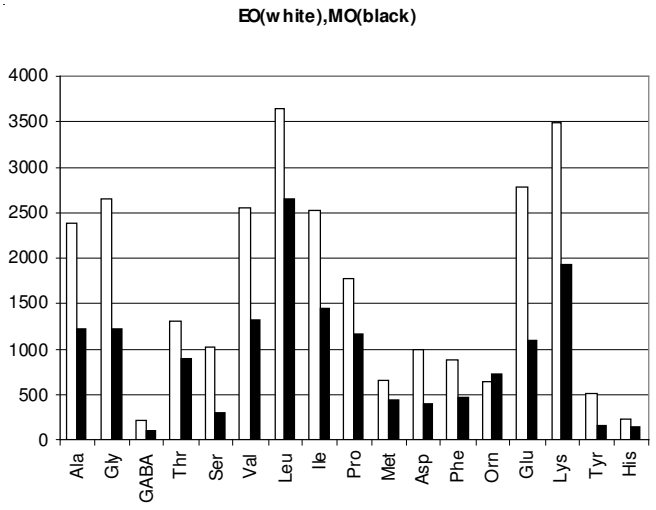

Fig. 5. Comparison of free amino acids of Galitian carp plasma, experiment (EO) and control (MO)

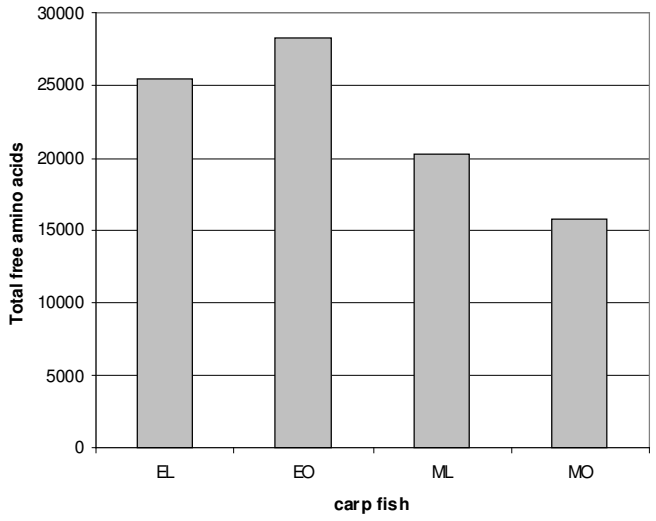

Fig. 6. Total free amino acids levels in experiments (E) and controls (M), in Lausitz (L) and Galitian (O) carp

changes in the plasma amino acids pool clearly reflect the nutritional state of fish and help us to understand the complex amino acids metabolism.

Conclusion

The method is precise and useful in the analysis of amino acids from different biological media. Good validation parameters were obtained in the range of interest. The use of isotopic labellled internal standard permits precise determination of the amino acids and avoids the overlapping with different contaminants. Important differences were observed in plasma total free amino acids between varieties and also between experiments and control fish. The method is very useful for nutrient and diet control.

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