DETERMINATION OF AMINO ACIDS AND SELENIUM IN FISH PLASMA*

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Two different mass spectrometric methods were developed for amino acids and selenium monitoring in plasma. The stable isotope internal standard used was ^{15}N-Methionine for amino acids quantitation. The aim of this study was to evaluate the differences in plasma free amino acids and selenium occurred at a dietary Se-methionine level of 0.05 mg/kg in comparison with control carp plasma.

Key words: GC-MS, ICP-MS, selenium.

1. INTRODUCTION

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 aluminum toxicity [1]. Oral nutritional supplements infusion improves duodenal protein balance in healthy humans [2]. Changes in the plasma amino acids (AA) pool reflect the nutritional state of fish, help us to understand the complex amino acids metabolism and to evaluate the quality of a diet.

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 [3-6].

Inductively coupled plasma mass spectrometry (ICP-MS) is a relatively new and powerful technique for the determination of metals in aqueous solution. The aqueous sample is turned in aerosols by continuous nebulization, then is ionized in

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an argon plasma at atmospheric pressure and analyzed offering fast multielement capabilities, a high dynamic range and excellent detection limits in a large number of matrices [7-12].

The aim of this paper was to evaluate the differences in plasma free amino acids and selenium occurred at a dietary Se-methionine level of 0.05 mg/kg in comparison with control carp plasma.

2. EXPERIMENTAL AND THEORETICAL DETAILS

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

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 the standard mixture containing known quantity of each amino acid. The response factors were calculated from standard samples with the relation:

$$F_i = \frac{\frac{A_i}{A_j}}{\frac{m_i}{m_j}} \tag{1}$$

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

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

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 respectively j; F_i and F_j are the response factors for compound i and respectively j (the internal standard) calculated by using standards.

Also, the amino acid levels in plasma were calculated using the regression curve of each amino acid using as internal standard ^{15}N-Met.

Isotopic deconvolutions. Methionine was determined using matrix calculation. The use of the isotopic labelled analogue of the analyte (the AA of

interest) as internal standard and the presence of the analyte (tracer) with their 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 and synthetic isotopic ratios in the case when the isotopomer needed was missing. The set of simultaneous linear equations each describing the isotopic contributors had to be solved having the general form:

$$I_x = \sum_{x=i,j} A_i X_j \tag{3}$$

where I_x represents the relative ion abundance for the x^{th} 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:

$$I=AX \tag{4}$$

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

$$X = \left(A^T A\right)^{-1} A^T I \tag{5}$$

Chemicals. 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). 18 MΩ cm^{-1} ultrapure water prepared in the laboratory using a Milli-Q system (Millipore, Watford, Hertfordshire, UK) and high purity grade reagents (AristaR or Specroscol): 25% NH3 solution, EDTA, Triton-X, butan-1-ol and an ultra pure 16 mol l-1 HNO3 solution (Merck, Darmstadt, Germany) for ICP-MS were used throughout this study. A diluent solution containing 5 g l-1NH3, 0.5 g l-1 Triton-X, 0.5 g l-1 EDTA and 6 ml l-1 butan-1-ol according to Varian procedure was prepared by dilution with Milli-Q DI water.

GC-MS method. The amino acids were purified on a Dowex 50W-W8 exchange resin, on a 2 x 40mm 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^{o}C and trifluoroacetylation with 100 µl trifluoroacetic anhydride at 60^{o}C 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. 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, 30mx0.25mm, 0.25 µm film thickness, using a temperature program from 50^{o}C, 1 min, 6^{o}C/min la 100^{o}C, 4^{o}C/min la

200°C, 20°C/min la 310°C, (5 min). The working conditions were: transfer line temperature 250°C, injector temperature 200°C; ion source temperature 250°C; Splitter: 10:1. Electron energy was 70eV and emission current, 100 μA. The application of the method was to study the influence of food on AA 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.

ICP-MS method: 18 MΩ cm^{-1} ultrapure water prepared in the laboratory using a Milli-Q system (Millipore, Watford, Hertfordshire, UK) and high purity grade reagents (AristaR or Specroscol): 25% NH_3 solution, EDTA, Triton-X, butan-1-ol and an ultra pure 16 mol l-1 HNO_3 solution (Merck, Darmstadt, Germany) for ICP-MS were used throughout this study. A diluent solution containing 5 g l-1NH_3, 0.5 g l-1 Triton-X, 0.5 g l-1 EDTA and 6 ml l-1 butan-1-ol according to Varian procedure was prepared by dilution with Milli-Q DI water.

Inductively coupled plasma mass spectrometric measurements for multi-element determination of trace elements were performed with a Varian 820 –MS (Varian, Australia). The extract solution was introduced by pneumatic nebulization into a radiofrequency- generated argon plasma where energy transfer processes caused analyte desolvation, atomization, and ionization. The ions transmitted through the quadrupole were registered by a continuous dynode electron multiplier and processed by a PC-based data handling system.

Prior to analysis of samples, a quality control sample prepared from ICP Multi Element Standard Solution XXI CertiPUR and 10 μg/L Selenium was analyzed to verify the calibration. A six point calibration curve for the analyte element was linear. A correlation coefficient of 0.9993 was assigned in the software for QC check. Each sample was analyzed in duplicate, each analysis consisting of five replicates. The quantitative data were obtained in segmented scan mode, dwell time 1 ms, integration time 395.08 s.

Instrumental operation parameters are given in Table 1.

Table 1

Instrumental parameters of ICP-MS

RF power	1400 W
Argon gas flow	
nebulizer	1.0 L/min
Auxillary	1.8 L/min
Plasma	18 L/min
Lens voltage	
Mirror Lens Left	37 V
Mirror Lens Right	17 V
Mirror Lens Bottom	30 V
Sample uptake rate	90 s

3. RESULTS AND DISCUSSIONS

The GC-MS method was validated using amino acid standards. The separation chromatogram of the amino acids is presented in Fig.1: alanine (Ala), glicine (Gly), thronine (Thr), serine (Ser), valine (Val), leucine (Leu), isoleucine (Ile), proline (Pro), metionine (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.

Precision gave lower value than 20% for R.S.D., except Arg, Cys and Tyr and a sensitivity value lower than 10 ng of amino acid injected. All the samples followed the same extraction and derivatization steps.

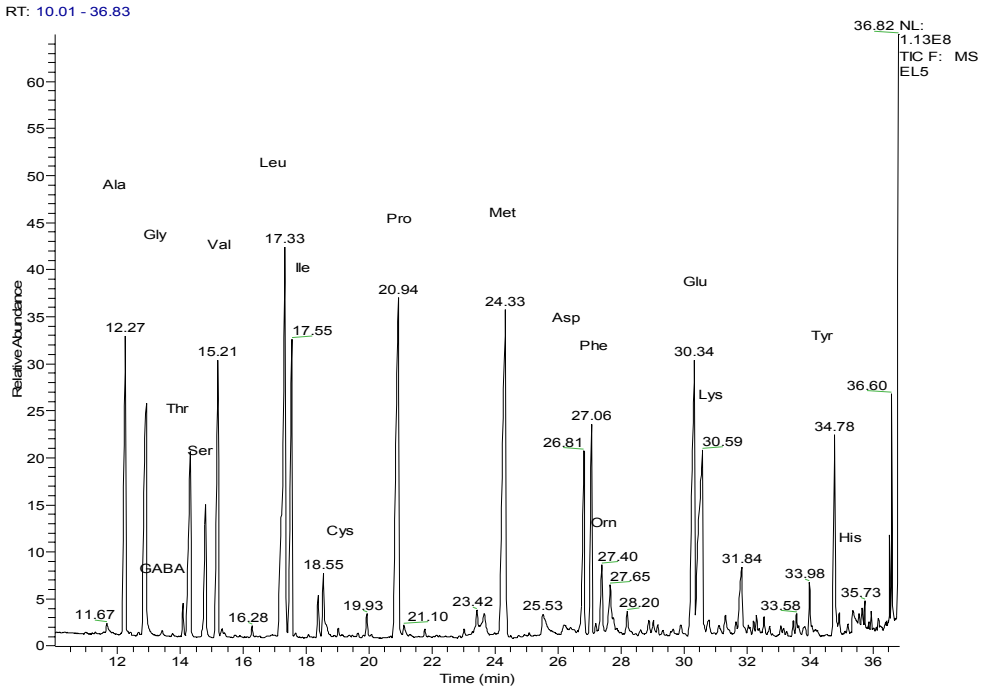

Fig. 1 – The separation chromatogram of amino acids in a plasma sample: Ala, Gly, Thr, Ser, Val, Leu, Ile, Cys, Pro, Met, Asp, Phe, Glu, Lys, Tyr, His.

The majority of the free essential amino acids have increased in the both variety of carps (Table 1). The essential amino acids are marked with * 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.

Table 2

Changes of free AA concentrations in control and experiments (μM)

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

*-essential amino acids, M: molecular mass

The mean values of selenium in fish plasma showed increased values for experiments in comparison with control in Lausitz (L) and Galitian (O) carp varieties respectively (Table 3). The coefficients of variation (RSD) were lower than 9% in control and lower than 22% in experiments.

Table 3

Selenium concentrations mean value in control (C) and experiments (E) (ppm)

EL	EO	CL	CO
43.512	34.605	29.664	28.941

The amino acids measured for the control carp fishes of the two varieties gave similar results. A significant increase of about 3.8 times of methionine was observed in Galitian carp experimental group in comparison with control.

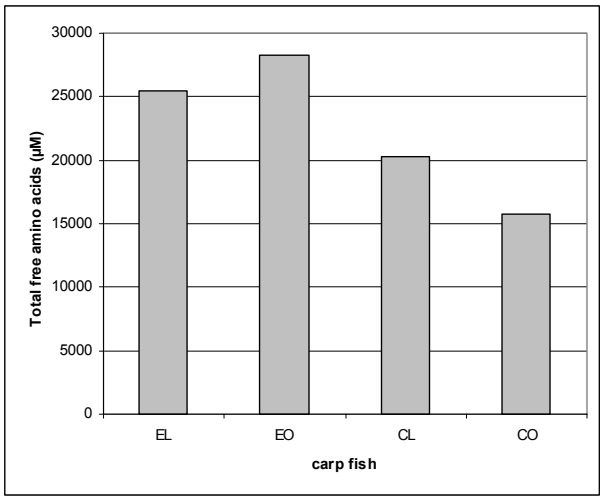

Fig. 2 – Total free amino acid levels in experiments (E) and controls (C), in Lausitz (L) and Galitian (O) carp.

Galitian carp variety showed almost two times higher values for total free amino acids in experimental fish than control, but also higher values were obtained in experimental Lausitz variety in comparison with control (Fig. 2).

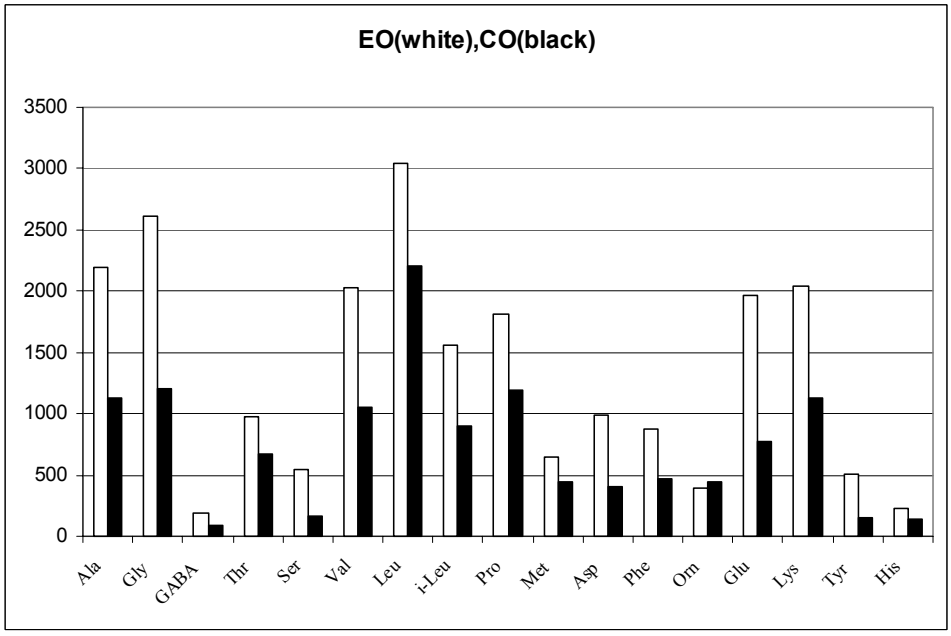

Fig. 3 – Comparison of free AA in Galitian carp plasma, experiment (EO) and control (CO).

Fig. 3 presents the comparison of different free amino acids measured in experimental and control fish, variety Galitian. All measured amino acids showed increased values in experimental plasma.

4. CONCLUSIONS

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

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