Analysis of amino acids and amines in biological tissues and food matrices by HPLC

Theses of doctoral (PhD) dissertation

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Introduction

Amino acids are the building blocks of proteins, taking part in the energetics of organism and play a key role in the synthesis of N-containing molecules (i.e. nucleotides, hormones, porphyrines). Of the 20 standard proteinogenic amino acids, 9, called essential amino acids, are to be draw from food. The shortage of the essential amino acids might result in serious deficiency (kwashiorkor). In addition to amino acids, also amines are of primary importance participating in physiological processes.

Biogenic amines, like putrescine, cadaverine, spermidine and spermine are the products both of normal and pathogenic metabolic processes in cases of animals, plants and microorganisms, equally. They are common constituents of many foods and beverages (cheese, wine, beer, fish, chocolate). However their elevated level in food is associated with food deterioration. Biogenic amines in low concentrations are essential for many physiological functions, whereas at high concentrations they could have deleterious effects. Subsequently to excessive oral intake of biogenic amines, might result in headaches, nausea, cardiac palpitations, hypo- or hypertension, renal intoxication and/or haemorrhages. Regarding toxicity, histamine, tyramine and phenylethylamine are the most hazardous biogenic amines for human health, whereas putrescine, cadaverine, spermidine and spermine, do act indirectly, by increasing the toxic effects of histamine and tyramine.

Polyamines (putrescine, spermidine and spermine) are growth factors in eucaryotic cells, and play role in cell growth and differentiation. Increased polyamine levels in blood and urine might be associated with various types of cancers.

Identification and quantification of amino acids and amines in biological matrices (blood, urea, tissues) and in food is a challenging analytical task.

Aim&objectives

This work aimed to optimise the extraction/deproteinization processes for the quantitation of putrescine, cadaverine, spermidine and spermine content of biological tissues. The method was developed in the presence of the rest of proteinogen amino acids, and extended to the determination of ornithine and lysine (being precursors of biogenic amines).

On the basis of our recent experiences, HPLC analysis of amines was studied with the *orto*-phthalaldehyde/ethanethiol (OPA/ET) reagent, applying perchloric acid deproteinization. In order to quantitate spermidine and spermine

−in the first step OPA/ET reagent,

−in the second step (to derivatize secondary amino groups), 9-fluorenylmethyl chloroformate (FMOC) reagent was performed.

This two step derivatization protocol was extended to quantification of amino acids, aliphatic and biogenic amines, simultaneously in a single run. The second aim was to identify and to quantify 32 amino acids and amines as their OPA/ET/FMOC-derivatives, and to demonstrate the practical utility of the method in biological tissues and food matrices.

Methods

HPLC system

The system was a Waters HPLC instrument (Waters Pharmaceutical Division, Milford, MA, USA), equipped with a Waters 996 diode array detection (DAD) and a Waters 474 fluorescence (Fl) detection system, a Waters 600 Controller quaternary pump with a thermostattable column area and a Waters 717 Autosampler, operating with the Millennium Software (version 2010, 1992-95, validated by ISO 9002).

On-line HPLC-MS system

On-line HPLC–ESI-MS studies was carried with a Thermo Finnigan TSQ Quantum AH apparatus (Thermo Finnigan, LC–MSDivision, San Jose, CA, USA), consisted of a Surveyor DAD detector, a TSQ Quantum AH detector, a Surveyor Autosampler, operating with the Xcalibur software 1.4 SRI.

The coloumns

1. BST Hypersil ODS bonded phase (5 µm), 200 mm x 4 mm+ 30 mm x 4 mm guard column (column1),
2. Thermo Hypersil ODS (5 µm), 200 mm x 4.6 mm+ 30 mm x 4 mm guard column (column2),
3. BST Hypersil ODS bonded phase (5 µm), 150 mm x 4 mm + 30 mm x 4 mm guard column (column3),
4. BST Hypersil ODS bonded phase (3 µm), 150 mm x 4 mm + 30 mm x 4 mm guard column, (column4),
5. Thermo Hypersil ODS (5 µm), 250 mm x 4.6 mm+ 20 mm x 4 mm guard column (column5).

Chromatography

Gradient elution, 50 °C column temperature and 1,8 ml/min flow rate were performed.

Determination of biogenic amines, ornithine and lysine – three eluents were applied: Eluent A (prepared from 0.1 M sodium acetate-acetonitrile-methanol=46/44/10, mixed in volume ratios and titrated with acetic acid or sodium hydroxide to pH=7.2), as well as eluent B (methanol) and C (acetonitrile), on column1–4, at 50 °C, during 18 min. Final analytical studies were performed on column2.

Determinetion of amino acids and amines: elution was performed with four eluents {(A)=acetonitrile/0,36 M sodium acetate /distilled water (10/5/85), pH=7; (B)=methanol/0,36 M sodium acetate /distilled water (55/8/37),

pH=7, (C)=acetonitrile/0,36 M sodium acetate /distilled water (55/5/40), pH=8; (D)=acetonitrile}, during 62 min, on column5.

Standard solutions

Standard solutions of free amino acids and amines have been prepared with distilled water in the concentrations of ~5×10^{-2} M (with analytical precision).

Stock solution of OPA contained 0.55 g OPA (weighed with analytical precision) in 25 mL methanol.

Reagent solutions

OPA/ET=1/10 reagent were obtained by mixing, in order of listing, 500 µL OPA stock solution, 2.0 mL borate buffer and 60 µL ethanethiol–completed with methanol up to 10.0 mL.

FMOC reagent was prepared from 0.11 g FMOC (weighed with analytical precision) and made up to 10.0 mL with acetonitrile.

It means that the molar ratios of the reagent's constituents were [OPA]/[ET]/[FMOC]=1/10/0.5.

Derivatization procedure

Two step reactions have been carried out as follows:

1. first, the OPA/ET=1/10 reagent with the molar ratios of OPA/amino acids+amines ≥ 20/1, followed with the

2. FMOC reagent, with the mole ratios of OPA/amino acids+amines/FMOC ≥ 20/1/0.5, was added to 20 µl sample. After 60+60 sec reaction time 40 µl solution was injected.

Sample preparation of biological tissues

Sample preparation were performed at State University of New York, in Syracuse (USA). Three types of genetic modified mouse tissues (testis, epi-head, and liver) were examined. Frozen tissues (0.12-0.15 g testis, 0.03-0.08 g epi-head or 0.06-0.12 g liver samples, weighed with analytical accuracy)

have been homogenized with 500 µL 1M perchloric acid, for 1 min, by means of the Ultra-Turrax T8 apparatus (IKA-Werke GmbH Co.KG, Staufen, Germany) in a 2 mL screw capped Eppendorf tube. The homogenized sample was transferred into a 3 mL screw-capped vial. Homogenizator and the 2 mL vial have been washed - in 4 additional, consecutive steps - with 500+400+300+300 µL distilled water and the sample and its washing solutions were unified in the 3 mL vial. In order for quantitative cell tissue homogenization samples were frozen/thawed three times.

The homogenized, unified samples were centrifuged, the supernatant was filtered by means of a syringe of acetate brands, (0.45 µM, Fisher, Ireland), and the filtrate was transferred into a 3mL screw-capped vial (previously weighed with analytical accuracy). In case of *samples without neutralization* the residue was washed two times with 200-200 µL distilled water subsequently to its softening with a spatula and vortexing. The washing solutions were also filtered and unified with the supernatant.

To the *neutralized samples* saturated potassium hydrogen carbonate solution; or 10 M potassium hydroxide solution was added, then the unified filtrate was dried by lyophilization.

Sample preparation of cheese samples

0.2 g cheese (porte salut-, or blue- or smoked cheese) samples, homogenized by a household turmix apparatus, weighed with analytical accuracy into a centrifuge tube, were mixed with 2.0 mL of the selected acid solution (1 M hydrochloric acid, perchloric acid, trichloroacetic and 0,5 M sulfosalicylic) and sonicated at room temperature, for 10 min or 30 min, respectively. The mixture was centrifuged and the supernatant filtrated into a 5 mL volumetric flask through a funnel covered by GF/F glass microfibre paper. The protein residue was repeatedly mixed, homogenized

with 2 x 0.5 mL acid, thereafter centrifuged and the supernatants filtrated into the flask through the funnel, and the volume was then made up to 5.0 mL with distilled water.

Results and conclusion

HPLC analysis of the derivatives of amino acids, aliphatic mono- and diamines, formed with the OPA/ET/FMOC reagents were examined. Products were detected by fluorescence and photodiode array detection, simultaneously.

1. Detailed investigation of the OPA-derivatives of biogenic amines (putrescine, cadaverine, spermidine and spermine) was carried out. It was proven that in case of the secondary amino groups containing spermidine and particularly in the case of spermine, their measurement, with response values similar to those obtained with other amino acids/amines, is only made possible by derivatizing their secondary amino groups as well.

–Based on this recognition, the two-step (1st: OPA/ET; 2nd: FMOC) derivatization of spermidine and spermine was described here the first time.

–Simultaneously, the OPA/ET/FMOC products were identified by HPLC-MS as well.

2. The procedure, elaborated for the analysis of biogenic amines, was utilized to determine them in biological tissues. In the frame of these studies the deproteinization of biological tissues was optimized in order

–to get quantitative recovery of biogenic amines, as well as

–to obtain optimum medium for the two step derivatization process.

3. Extending the analysis described above, the quantitation of ornithine and lysine, simultaneously with the biogenic amines, was elaborated. Beyond the method's novelty, unexpected reaction of ornithine and lysine with the OPA/ET/FMOC reagent was experienced. It has been proven, that the

reaction of ornithine and lysine with the OPA/ET/FMOC reagent yields derivatives of mixed ligands. This experience, verified by MS data, demonstrates the difference in the reactivity of the δ-/ε- and α-amino group of ornithine/lysine.

4. The possibility of the simultaneous analysis of 32 compounds, from a single solution by one injection has been examined.

–For this purpose a quaternary elution system was elaborated containing pH and ionic strength gradient, simultaneously.

–The practical utility of the method was demonstrated by the analyis of three different mouse tissues.

5. The method elaborated for the simultaneous analysis of amino acids and amines was extended to the identification and quantification of the constituents of various cheeses: as a result of changes regarding deproteinization, the constituents and the elution conditions the utility of the method was confirmed by the analysis of the composition of three different Hungarian cheeses.

6. As a result of my thesis, I could prove

–the optimal conditions for the 'OPA-derivatization', being the most popular preparation for HPLC separations, as well as

–its usefulness in the analysis of biological and food matrices.

Publications

1. Á. Kőrös, R. Hanczkó, A. Jámbor, Y. Quian, A. Perl, I. Molnár-Perl: Analysis of amino acids and biogenic amines in biological tissues as their o-phthalaldehyde/ethanethiol/fluorenylmethyl chloroformate derivatives by high-performance liquid chromatography: A deproteinization study
Journal of Chromatography A, 1149 (2007) 46-55.

2. Á. Kőrös, Zs. Varga, I. Molnár-Perl: Simultaneous analysis of amino acids and amines as their o-phthalaldehyde-ethanethiol-9-fluorenylmethyl chloroformate derivatives in cheese by high-performance liquid chromatography
Journal of Chromatography A, 1203 (2008) 146-152.

3. R. Hanczkó, Á. Kőrös, F. Tóth, I. Molnár-Perl: Behavior and characteristics of biogenic amines, ornithine and lysine derivatized with the o-phthalaldehyde-ethanethiol-fluorenylmethyl chloroformate reagent
Journal of Chromatography A, 1087 (2005) 210-222

4. Á. Kőrös, Zs. Varga, I. Molnár-Perl: Simultaneous analysis of amino acids and amines as their *o*-phthalaldehyde /ethanethiol/fluorenylmethyl chloroformate derivatives in biological tissues,
Journal of Chromatography B, összeállítás alatt.

Oral presentation

„Analysis of biogenic amines, ornithine and lysine as their *o*-phthalaldehyde derivatives" − presentation at „Magyar Kémikusok Egyesülete", Budapest, Hungary, 2005.

Poster presentations

1 „HPLC of Amino Acids and Biogenic Amines in Biological tissues as Their OPA/ ET/ FMOC Derivatives" – poster at „Sixth Balaton Symposium" Siófok, Hungary, 2005.

2 „The Stability And Characteristics of the o-Phthalaldehyde Derivatives of Amino Acids and Amines" − poster at „Pharmacy: Smart Molecules for Therapy" Budapest, Hungary, 2005.

3 „Analysis of amino acids and biogenic amines in biological tissues as their o-phthalaldehyde /ethanethiol/fluorenylmethyl chloroformate derivatives by HPLC" − poster at „International Symposium on Chromatography" Copenhagen, Denmark, 2006.

4 „Analysis of amino acids and amines in biological tissues and cheese by HPLC as their o-phthalaldehyde/ethanethiol/fluorenylmethyl chloroformate derivatives" − poster at „HPLC 2007" Ghent, Belgium, 2007.