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REVIEW OF THE MOST APPROPRIATE METHODS FOR DETECTION OF AMINO ACIDS IN PHARMACEUTICAL PRODUCTS

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ABSTRACT

One of the most used food additives are products containing amino acids. Amino acids play an important role in human nutrition and health. The supplementation with amino acids gives many benefits in health aspect. Because of the increased consumption of amino acids containing products the chromatographic study of these molecules is very important. This review describes the most appropriate chromatographic systems used for the analysis of amino acids. The analytical control is the most important element of the safety of these products. One of the most common method for the analysis of amino acids is the high performance liquid chromatography (HPLC). The Gas Chromatography is also a rapid and very effective method for detection of amino acids. The polar nature of amino acids requires derivatization prior to Gas Chromatography. HPTLC method is also very appropriate for detection of amino acids.

Keywords: amino acids, HPLC, TLC, food additives

INTRODUCTION

The market of food additives is growing.[9] The fact that food additives have plant or animal origin, does not make them safe. The food additives are tested for efficiency, which is supported by scientific evidence, but in some supplements the qualitative and quantitative composition of their components is not declared. [8,9] One of the most used food additives are products containing amino acids. Amino acids play an important role in human nutrition and health maintenance.[7] Amino acids are biologically important organic compounds that contain amine and carboxylic acid functional groups, along with a side-chain specific to each amino acid. Amino acids can be divided to two types, essential and non-essential. Eight amino acids have been

found necessary and essential. The amino acids have a biological activity and are components in food and food additives. The food and food additives contain a different variety of essential and non-essential amino acids that play a critical role in metabolizing nutrients, building muscle tissue, and protecting the body against disease.[8] Food supplements, containing amino acids are very important for human health: L – Tyrosine, as a precursor of catecholamines; L – Tryptophan, as a precursor of serotonin, melatonin and niacin; L – Glutamic acid, as a fuel for the brain.[3,4,5]

L – Arginine is an amino acid, involved in numerous areas of human biochemistry, including: changing into powerful neurotransmitter nitric oxide, which mediates it's biological effects by activating the soluble isoform of guanylyl cyclase and increasing the synthesis from GTP of secondary messenger cyclic GMP synthesis, reversing of endothelial dysfunction in hypertensive cardiac transplant recipients, hypercholesterolemic patients and in cigarette smokers, ammonia detoxification, regulation of growth hormone production, enhancement of the spermatogenesis, stimulation of: a) insuline secretion from pancreas, b) synthesis of the pituitary hormone vasopressin; c) immune system, by increasing the output of T – lymphocytes from the thymus gland. [2] Both the fermentation and enzyme-based production methods play a central role in the production of l-amino acids used as ingredients in the pharmaceutical industry and provide the constant growth of the amino acid market. [7] The choice of industrial method depends on the available technology, costs of raw material, market characteristics, cost of running fermentation versus synthesis reactions, and environmental impact of the process itself. [8]

The enlarged application of these pharmaceutical products leads to the increasing of the problem with their quality control.[6] The analytical control is key for the quality of these products. Methods for determination of aminoacids include postcolumn derivatization with ninhydrin or o-phthalaldehyde (OPA) by commercially available aminoacid analyzers or HPLC with precolumn derivatization with different reagents, such as dansyl chloride, phenylisothiocyanate, fluorenylmethyl, chloroformate. [6] In the scientific literature are describes various analytical techniques for their qualitative and quantitative analysis. Most preferred methods is HPLC because of its range, accuracy and speed. Despite the wide variety of methods, there isn't coherent analytical system associated with the standartization of food additives containing amino acids. [10] Recent studies show that non-hormonal supplements such as vitamins, minerals, amino acids or other food

additives can contain not declared substances [11], that demonstrates the great need of better analytical control of these products.

Amino acids supplementation:

L-arginine was first isolated in 1886, reportedly from the extract of a lupine (Lupinus spp.) seedling. Lupinus is a genus in the legume (Fabaceae) plant family. Arginine serves as a precursor for creatine, which plays an essential role in the energy metabolism of muscle, nerve and testis. Via its ability to increase growth hormone secretion it influences immune function. Depending on nutritional status and developmental stage, normal plasma Arginine concentrations in humans and animals range from 95 to 250 micromol/l. [30] Arginine supplementation improves cardiovascular function and reduces myocardial ischemia in coronary artery disease patients. It reduces blood pressure and renal vascular resistance in essential hypertensive patients with normal or insufficient renal function. The main importance of Arginine is attributed to its role as a precursor for the synthesis of nitric oxide (NO) [30] The supplements with L – Arginine offers many benefits like: improving of endothelial, cardiovascular and renal function in patients with chronic heart failure. Supplementation with L-arginine increases of the exercise tolerance in stable coronary artery disease patients, increases the human growth and hormone levels. L- arginine is considered also as testosterone stimulator and has a hight performance-enchancing potential. Food additives containing L-arginine are also used for treatment of erectile dysfunction : Liderin Walmark (It contains L-arginine , Pycnogenol, Ginseng extr.); Permen Walmark (It contains L-arginine , Tribulus Terrestris extr, Ginseng extr.); Eromaks 2 (combination from L-arginine , Maca extr., Tribuli terrestris, extr., Epimedium extr.,Se, Panax Ginseng extr., Fl. Onopordonis acanthii, extr., Vit. B3, Zn);Kamagra 30 capsules 800 mg (combination of L- arginine , Ginseng , Tribulus Terrestris extr.,yohimbe extr.) and many others.[30] Tryptophan (TRYP), is a precursor for serotonin, a brain neurotransmitter theorized to suppress pain. Free tryptophan (fTRYP) enters the brain cells to form serotonin. Thus, tryptophan supplementation has been used to increase serotonin production in attempts to increase tolerance to pain during intense exercise.[31]Glutamine may be theorized to be ergogenic in various ways.[31,32] Taurine is a non-essential sulfur-containing amino acid, but it lacks a genetic codon to be incorporated into proteins or enzymes. Nevertheless, it plays a role in several metabolic processes, such as heart contraction and antioxidant activity. Tyrosine is a precursor

for the catecholamine hormones and neurotransmitters, specifically epinephrine, norepinephrine, and dopamine. Some have suggested that inadequate production of these hormones or transmitters could compromise optimal physical performance. Thus, as a precursor for the formation of these hormones and neurotransmitters, tyrosine has been suggested to be ergogenic [31].

Methods for analyses of amino acids

Analysis of amino acids is a current scientific issue due to the important role they play in many biological processes. Up to now, a variety of methods to separate and detect specific amino acids in samples of interest have been developed. However, since most amino acids lack large hydrophobic sides and natural strong chromophore, fluorophore or electroactive groups for photometric, fluorometric or amperometric detection, respectively, the majority of the present methods employ some form of pre- or post-column derivatization procedures in order to enhance detection or chromatographic separation. In general, it is now accepted that the most difficult part of amino acid analysis is not their separation but their detection. Direct detection of amino acids without per- forming derivatization is preferred, when it is available, not only for convenience, flexibility, simplicity, and accuracy but also for avoiding problems introduced by derivatizing procedures such as repeatability, derivative instability, side reactions, and reagent interferences.[14] Several HPLC methods for the detection of underivatized amino acids have been described. These methods include direct detection by UV absorbance at low wave- lengths when high sensitivity is not necessary [14,15,16,17,18,19], conductivity detection [14,16,20,21] electrospray MS methods, in simple and tandem MS mode [14,16], detection via native fluorescence [14], and some other detection techniques, e.g., evaporative light scattering (ELS) [14], refractive index [14], chemiluminescent nitrogen or NMR detection as well as a combination of two different methods of detection such as NMR-ELS [14, 22] or UV-ELS detection.[14] Thin layer chromatography is also a very important method for the identification of amino acids. Many specific and non-specific reagents have been used to detect amino acids on thin-layer chromatographic plates, among them ninhydrin is most widely used for its remarkable high sensitivity. It produces same purple/violet colour with all amino acids, except proline and hydroxyproline which produce yellow colour. During the last three decades, a good number of new reagents have been introduced to overcome the colour problem of ninhydrin with different amino acids as well as to en-

hance the degree of sensitivity for such detection.[27]

The reaction of ninhydrin could be also used for determination of amino acids. The reaction of ninhydrin with primary amino groups to form the purple dye now called Ruhemann's purple was discovered by Siegfried Ruhemann in 1910. In addition, imines such as pipecolic acid and proline, the guanidino group of arginine, the amide groups of asparagine, the indole ring of tryptophan, the sulfhydryl group of cysteine, amino groups of cytosine and guanine, and cyanide ions also react with ninhydrin to form various chromophores of analytical interest. Since its discovery, extensive efforts have been made to apply manual and automated ninhydrin reactions as well as ninhydrin spray reagents to the detection, isolation, and analysis of numerous compounds of interest across a broad spectrum of disciplines. These include agricultural, biochemical, clinical, environmental, food, forensic, histochemical, microbiological, medical, nutritional, plant, and protein sciences. This reaction is unique among chromogenic reactions in that at pH 5.5 it results in the formation of the same soluble chromophore by all primary amines which react, be they amines, amino acids, peptides, proteins, and even ammonia. Because the chromophore is not chemically bound to the protein or other insoluble material, it is not lost when the insoluble substrate is removed by centrifugation or filtration after the reaction is completed. The visible colour of the chromophore is distinctive and is generally not affected by the yellow colours present in many food, plant, and tissue extracts. Adaptations of the classical ninhydrin reaction to specialised needs in analytical chemistry and biochemistry include the use of acid, alkaline, and fluorogenic ninhydrin reagents. A better understanding of these multifaceted ninhydrin reactions provide a scientific basis for further improvements of this important analytical technique [24].

Table 1 demonstrates the most appropriate methods for analyses of amino acids in food supplements.

Table1: Methods for determination of amino acids in food supplements

Method for analys	Samples	Analysed substance	Ref.
Chromatographic method with flame ionization detector (GC – FID)	**Food additives**	**17 amino acids : L – Alanine, L – Glycine, L – Valine, L – Leucine, L – Isoleucine, L – Threonine, L – Serine, L – Proline, L – Aspartate, L – Methionine, L – Glutamate, L – Phenylalanine, L – Cystine, L – Lysine, L – Histidine, L – Tyrosine,L – Triptophan.**	**[1]**
HPLC Mobile phase (MPh): 20 mM ammonia acetate; flow rate: 1.0 ml/min; column temperature: 40 0C; UV – detection at λ = 254 nm; volume for injection – 20 µl. Before using MPh was filtered throught membrane filter with pore size 0.45 µm.	**Food additive**	**L – ARGININE IN TONOTYL SOLUTION**	**[2]**
HPLC method with UV – detection. Liquid chromatograph Shimadzu (Japan) (LC – 10 Advp), equipped with: column Spherisorb ODS RP – C18 (250 mm/4.6 mm i.d./5 µm), column oven (CTO – 10 Asvp); isocratic pump (LC – 10 A); 20 µl injector loop; UV – VIS – detector at fixed wavelengths (SPD – 10 Avvp).	**Food additives**	**L – Glutamic acid and L – Arginine**	**[3]**
Gas chromatographic method with flame-ionization detector (GC-FID)	**Food additive: Aminogame 1500 solution**	**L-Alanine, L-Glycine, L-Valine, L-Leucine, L-Isoleucine, L-Threonine, L- Serine, L-Proline, L-Aspartate, L-Methionine, L-Glutamate, L-Phenylalanine, L-Cystine, L-Lysine, L-Histidine, L-Tyrosine, L-Triptophan**	**[6]**
HPLC with electrochemical detection method for the isocratic separation	**Amino acid neurotransmitters from brain tissue and microdialysis perfusates. Tissue measurements from caudate, globus pallidus and substantia nigra.**	**aspartate, glutamate, taurine, tyrosine and GABA**	**[12]**
HPLC by precolumn fluorescence derivatization with o-phthaldialdehyde	**Urine samples**	**25 amino acids**	**[13]**
Direct RP-HPLC determination of underivatized amino acids with online dual UV absorbance, fluorescence, and multiple electrochemical detection. The LC system consisted of a Shimadzu LC-20AD pump, a model 7125 syringe loading sample injector fitted with a 20 lL loop, a 250 mm64.6 mm MZ-Analytical column (MZ-Aqua Perfect C18 5lm), a Shimadzu dual UV–Vis spectrophotometric detector (Model SPD-10A), a Shi- madzu spectrofluorometric detector RF-10AXL and a lab- oratory-made multiple EC detector equipped with a labo- ratory-modified dual electrode.	**Mixture of amino acids**	**18 underivatized amino acids and related compounds**	**[14]**
Two-dimensional thin-layer chromatography	**amino acids in normal and pathological body fluids**	**amino acids**	**[23]**

HPTLC detection with ninhydrin chromogenic reagent solution, and automated visible mode densitometry	**Dietary supplements**	**L-arginine**	**[25]**
Thin-layer chromatography and pressurized planar electrochromatography	**amino acids**	**amino acids**	**[26]**
TLC: Chromatography plates (20×20 cm; thickness 0.1 mm), prepared with silica gel G (Merck, India) using Unoplan Coating apparatus (Shandon, London, U.K.).Detection limits for the amino acids after use of ninhydrin reagent .		**Glycine,Alanine, Valine,Leucine, Isoleucine, Serine, Threonine,Aspartic acid,Asparagine, Glutamic acid,Glutamine, Lysine Histidine, Arginine, Phenyl alanine, Tyrosine,Tryptophan, Cysteine, Cystine, Methionine, Proline, Hydroxy proline**	**[28]**

Even the HPLC is the most commonly used method for the analysis of amino acids the GC can also be used, and in some cases availability of instrumentation or operation costs can make it a better choice. The polar nature of amino acids requires derivatization prior to GC analysis. The goal of derivatization is to make an analyte more volatile, less reactive, and thus improve its chromatographic behaviour. In the case of amino acids, derivatization replaces active hydrogens on OH, NH2, and SH polar functional groups with a nonpolar moiety. Silylation is a very common derivatization technique, and is useful for a wide variety of compounds. The main disadvantage of this method is its sensitivity to moisture. The presence of moisture results in poor reaction yield and instability of the derivatized analytes. For this study, we evaluated the use of the silylation reagent N-tert-butyldimethylsilyl- N-methyltrifluoroacetamide (MTBSTFA) for the derivatization of amino acids. MTBSTFA, forms tert-butyl dimethylsilyl (TBDMS) derivatives when reacted with polar functional groups containing an active hydrogen.[29]

CONCLUSION

There are many analytical approaches for determination of amino acids. Every method has its own benefits and disadvantages. One of the most used method for analyse of these products is the HPLC. HPLC method could separate fast and effective mixture of amino acids in pharmaceutical products of biological samples. The Gas Chromatography is also a rapid and very effective method for detection of amino acids but the polar nature of amino acids requires derivatization. Thin layer chromatography finds also its place because it requires relatively costly equipment. The development of new analytical methods for determination would be a current issue for the modern scientists because the global market of products containing amino acids is growing.

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