PRODUCTION AND REGULATORY ANALYTICAL CONTROL OF AMINO ACIDS INCLUDE IN FOOD ADDITIVES

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Abstract. Called "the building blocks of life", amino acids have long played and important role in human nutrition and health maintenance. The amino acids have a biological activity and are components in foods and food additives. The food additives contains 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. The fact that one food additive has a plant or animal origin, does not make it safe. Mostly there is no regulatory analytical control, qualitative and quantitative analysis of the input amino acids in food additives, leading to compromise of quality and in some cases to adverse risk. This paper reviews the most used manufacturing methods for the production of some amino acids and variety of analytical methods for their characterization.

Key Words: amino acids, analytical, control, food additives, production

Introduction

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 foods and food additives. The foods 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. Amino acids are produced by protein hydrolysis, chemical synthesis or microbiological (biotechnological) methods. 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. The fact that one food additive has a plant or animal origin, does not make it safe. Mostly there is no regulatory analytical control, qualitative and quantitative analysis of the input amino acids in food additives, leading to compromise of quality and in some cases to adverse risk. There are various analytical methods for their characterization, but no evidence of coherent analytical system, associated with the standardization of products containing amino acids.

This review focuses on the industrial methods of amino acids production and regulatory analytical control of amino acids included in food additives.

Discussion

According to the European Community Directive of 2002 (Directive 2002/46/EC) and according to Bulgarian legislation, issued by the Ministry of Health 01.08.2005, food additives are defined as substances that contain concentrated nutrients or other elements with a nutritional or physiological effect, alone or in combination, distributed in certain dosage forms designed to enrich your diet. Dietary supplements contains a number of biologically active substances, including amino acids. The fact that one food additive has a plant or animal origin, does not make it safe. Low consumer`s awareness of food additives in many cases makes this type of product risk. The food additives are tested for efficiency, which is supported by scientific evidence, but some of them don`t

declared the qualitative and quantitative composition of their components. The food additives market is growing. Control of the food additives often absent. Registration mode on food additives is relatively liberal. Market monitoring of food additives should be strengthened because of the high rates of consumption. The users are not always sufficiently informed.

The amino acids have a biological activity and are components in nutritional supplements. Amino acids are produced by protein hydrolysis, chemical synthesis or microbiological (biotechnological) methods. 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 [1]. The manufacture of amino acids has its roots in the food preparation practices in Japan where Ajinomoto Co began extracting monosodium glutamate from acidhydrolyzed wheat gluten or defatted soybean [2]. The original method for amino acid manufacture is protein hydrolysis (extraction method). Before 1950, most amino acids were produced by denaturing and hydrolysis of various protein sources [3]. Glycine and L-alanine are produced by chemical synthesis. In the series of chemical reactions known as Strecker amino acid synthesis, an amino acid is synthesized from an aldehyde (or ketone). The process includes condensation of the aldehyde with ammonium chloride in the presence of potassium cyanide, forming an α-aminonitrile, which is then hydrolyzed to obtain the desired amino acid. The original Strecker reaction combines acetaldehyde, ammonia, and hydrogen cyanide to give alanine after hydrolysis [4]. In the traditional synthesis of Adolph Strecker from 1850, racemic α-amino nitriles are formed, but several protocols employing asymmetric catalysts or auxiliaries have been developed since [5, 6, 7]. An example present-day use of the Strecker synthesis is the industrial chemical synthesis of an L-valine derivative starting from 3-methyl-2-butanone [8]. According to Ault [9], chemical synthesis is currently used only for the production of alanine, glycine, methionine, phenylalanine, threonine, tryptophan and valine. Microbiological (biotechnology) methods for the industrial production of amino acids are of three types: use of microbial enzymes or immobilized cells (enzymatic method), semi-fermentation, and direct fermentation. Enzyme catalysis is well established in the chemical industry [10]. With the enzymatic method, an amino acid precursor is converted to the target amino acid using one or two enzymes. The fermentation method is being applied to industrial production of most L-amino acids. This method utilizes the phenomenon that microorganisms convert nutrients to various vital components they need. 10 to 30 types of enzymes are involved in the process of fermentation, and various amino acids are produced as a result of these reactions [11]. The first report on the introduction of fermentation in the industrial production of amino acids was that of Kinoshita et al. in 1957 [12]. They highlighted the discovery of Corynebacterium glutamicum: a soil bacteria with the unique ability to produce considerable amounts of L-glutamine from sugar and ammonia. A few years later, a homoserineauxotrophic mutant of C. glutamicum produced a large amount of L-lysine by fermentation [13, 14, 15, 16]. Other amino acids produced by Corynebacteria include L-valine, L-isoleucine, L-threonine, L-aspartic acid and L-alanine [17, 18, 19, 20, 21]. The amino acids L-phenylalanine, L-threoine and L-cysteine can be obtained by fermentation with E. Coli [22, 23, 24].

Mostly there is no regulatory analytical control, qualitative and quantitative analysis of the input amino acids in food additives, leading to compromise of quality and in some cases to adverse risk. There are various analytical methods for their characterization, but no evidence of coherent analytical system, associated with the standardization of products containing amino acids.

Analytical methods for determination of amino acids:

The components in biological fluids - amino acids, peptides, and proteins must be isolated and purified to allow their evaluation, and standardizing. The separation is achieved by various methods based on differences in aggregate charge, molecular size, solubility, affinity for specific substances and others properties. In clinical practice, these methods are used for diagnosis of diseases and for the preparative purification and preparation of other organic products.

Electrophoresis is a method for separating charged molecules, or larger particles, based mainly on differences in the cumulative charge. The direction of movement of one of the poles of an external electric field is determined by the sign of the combined charge of the molecules. The speed is determined by the ratio of charge/molecular weight. Furthermore, the separation depends on the intensity of the field and the shape of the molecules in the mixture and the structure of the carrier gel (agar, agarose, cellulose acetate, polyacrylamide gel, paper, or other). Upon selection of the various experimental conditions electrophoresis methods are successfully applied for separation of mixtures of amino acids.

Capillary electrophoresis is a method for analysis and diagnosis due to the high speed of the separation (a few minutes) and therefore, it takes a very small amount of the test substances. When this method is carried out in open capillary electrophoresis. Around the negatively charged walls of the capillary flow of cations move to the cathode, dragging with her spliting molecules, regardless of their charge. Positively charged molecule, move with this flow quickly followed by neutral and negative -charged molecules.

Chromatographic methods are widely applied to identify and quantify amino acids, taking account of their chemical properties and select the appropriate chromatographic conditions. In chromatographic techniques, substances are allocated between static and mobile phase. Separation depends on the relative ability of compounds to bind more strongly with one or the other phase. Thin layer chromatography (TLC) - except for purposes of a qualitative analysis may be used in semi-quantitative analysis of the amino acids. This analytical approach used to determine the amino acid components with relevant conditions. Quantitative TLC methods can be classified into the following groups:

- Semi-quantitative TLC spot of the test substance is compared with a series of standards, it is necessary intensity of the spot of the test substance into a space between or coincide therewith.
- Quantitative determination (usually spectrophotometrically) of a component after elution of the stain from the resulting chromatogram.
- Direct measurement of the spots directly on the resulting chromatographic plate.

Densitometry - a method for quantitative thin-layer chromatographic determination based on irradiation of the stain from the TLC-plate wave with a certain intensity and the Law of the Bouguer-Lambert-Beer is measured decrease in light intensity, which is proportional to the concentration of a substance or measured: intensity of reflected light (remission) or the intensity of fluorescence. The HPLC method is, wherein the separation of the substances is carried out in a system consisting of a stationary phase (sorbent) and a liquid mobile phase (eluent). HPLC is a modern method of analysis, characterized by a high rate of separation, using a small volume analytical sample reproducibility and accuracy of analytical results, high sensitivity. It is possible the separation of complex mixtures of substances (10 or more components) in low concentrations and chemical properties close within the 15 -20min. It is widely used for separation and analysis of volatile and difficult thermo labile substances. By means of HPLC assay is carried out on amino acids in the formulation of nutritional supplements.

In ion exchange chromatography the amino acids, peptides and proteins are separated on the basis of the difference-coupled. This is one of the most used modern methods of separation, proof and determination of amino acids in protein hydrolysates. In it the hydrolyzate is passed through a column containing ion exchange resin with charged groups that bind oppositely charged amino acids. By creating a gradient of pH and salt concentration of the eluent, the individual amino acids are eluted gradually from the column and separated. Nowadays the ion exchange chromatography is applied in special high pressure apparatus providing an extremely high-resolution, and reproducibility of the results. Upon reverse phase HPLC technique the resin contains hydrophobic groups to bind hydrophobic molecules and slow. In this case, the elution of the column becomes solvents. As a higher percentage of the organic solvent, the faster the non-polar components are extracted. The whole procedure in the amino acid analyzer (elution, collecting separate fractions, analysis of each fraction, recording and printing of results) is fully automated.

A high performance liquid chromatography method for the determination of 20 amino acids (AAs), using 1,2,5,6-dibenzocarbazole-9-ethyl chloroformate as a novel fluorescent labeling reagent, has been developed and applied for the analysis of amino acids. The simultaneous separation of 20 AA derivatives was achieved on a Hypersil BDS C(18) column with gradient elution. And the identification of amino acids derivatives was carried out by on-line electrospray ionization mass spectrometry in positive ion mode. The amino acids derivatives were detected with excitation and emission at 300 nm and 395 nm, respectively [25].

There is a report of use of LC–MS/MS method for analysing the free amino acid composition. Reliable and reproducible techniques for identification and quantification of amino acids usually require derivatization. However, techniques such as LC–MS/MS may perhaps sideline the derivatization with significant accuracy. The analysis revealed a higher concentration of essential amino acids especially Threonine and Tryptophan than non essential amino acids [26].

Simple, rapid and economical method for routine determination of 24 amino acids is the following one. No sample clean-up is required and total run time including column re-equilibration is less than 40min. Following automated in-loop automated

pre-column derivatisation with an o-phthaldialdehyde, N-acetyl-l-cysteine reagent, compounds were separated on a 3mm×25cm C(18) column using a binary mobile phase. The method was validated in the range 0.25-10mg/l; repeatability was less than 3% RSD and the intermediate precision ranged from 2 to 7% RSD. The method was shown to be linear by the 'lack of fit' test and the accuracy was between 97 and 101%. The LLOQ varied between 10µg/l for aspartic and glutamic acids, ethanolamine and GABA, and 100µg/l for tyrosine, phenylalanine, putrescine and cadaverine [27].

A rapid and comprehensive analytical method for D- and L-enantiomers of proteinogenic amino acids was developed using ultra-high performance liquid chromatography (UHPLC) equipped with a circular dichroism (CD) detector. Pre-column derivatization reagents were examined for enhanced sensitivity and selectivity for UV and CD detection: 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) was selected. The method, using a CD detector, does not require separation of optical isomers on a column to calculate the enantio ratio (%D) using the g-factor value and produces a simple chromatogram in comparison to other reported methods. Using this advantage, combined with UHPLC technology, analysis time for the derivatized proteinogenic amino acids was within 5.5 min. The UV detection limit was 4.9-23 pmol/injection and the CD detection limit was 11-64 pmol/injection. The method was applied to the analysis of D- and L-amino acids [28].

A simple, rapid microwave digestion procedure for protein hydrolysis preceding the determination of amino acids in yeast using gas chromatography–mass spectrometry (GC–MS) is described. Protein hydrolysis was performed in a focused microwave using 4 M methanesulfonic acid (MAS). Amino acids were derivatized with methyl chlorofomate (MCF) and extracted into chloroform prior to GC–MS analysis. The microwave parameters, including power, temperature and heating time, were optimized. It was found that temperature and heating time were the most influential factors. A total of 17 amino acids were determined with use of standard addition calibration. Limits of detection and quantitation (LODs/LOQs) of the amino acids measured were in the sub-nmol range, suitable for monitoring of amino acids [29].

Another method to derivatize and analyze amino acids have developed and optimized, using hexafluoroacetone as the derivatizing agent. The temperature, duration of the derivative transfer to the analyser, and chromatographic separation parameters have been optimized. The work presented in this rationale has established that hexafluoroacetone, in addition to its intrinsic qualities, such as the production of lightweight derivatives (no racemization) and great resistance to the drastic operating conditions, has indeed facilitated simple and fast derivatization that appears to be suitable for analysis. By using hexafluoroacetone as the derivatizing agent, we successfully identified, 21 amino acids including 12 of the 20 proteinic amino acids without stirring or extraction steps. Ten of these derivatized amino acids were enantioselectively separated. The precision and accuracy measurements for the D/L ratio showed that the proposed method was also suitable for the determination of both enantioselective forms of most of the tested amino acids [30].

A sensitive and rapid analytical method has been proposed for the first time for the simultaneous determination of twenty amino acids using solid-phase microextraction (SPME). The protein samples were hydrolyzed by 6M HCl under microwave radiation for 120 min. Then the amino acids were derivatized by ethyl chloroformate (ECF) and the ethoxy carbonyl ethyl esters of amino acids formed were extracted using SPME by direct immersion. Finally the extracted analytes on the SPME fiber were desorbed at 260°C and analyzed by gas chromatography-mass spectrometer (GC-MS) in electron ionization mode. Factors which affect the SPME efficiency were screened by Plackett-Burmann design; most significant factors were optimized with response surface methodology. The optimum conditions for SPME are as follows: pH of 1.7, ionic strength of 733 mg, extraction time of 30 min and fiber of divinyl benzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS). The method can find wide applications in the routine analysis of amino acids in food additives.(31)

Concentrations of amino acids were determined using four independent quantification methods. Orthogonal separation schemes (LC, GC, or GC x GC) and detection systems (triple quadrupole or time-of-flight mass spectrometry) are shown to demonstrate excellent consistency among platforms for quantifying 18 amino acids in NIST Standard Reference Material (SRM) 1950 using a well-characterized isotope dilution (ID) quantification method. Certified mass fraction values for amino acids in NIST SRM 1950 will be established from statistically weighted means of all experimental results [32].

A single experiment can be performed in order to determine simultaneously the free amino acid profiles. The analytes of interest are rapidly extracted from the

matrix and derivatized by using methyl chloroformate. This reagent allows the transformation of the analytes into the corresponding N-methyloxycarbonyl amino acid methyl esters that can easily be extracted and sampled for their further identification and quantitation. The measurement of the obtained amino acid derivatives is performed by GC/MS analysis and their concentrations are calculated by using appropriate internal standards. The main advantage of the proposed protocol is the determination at the same time of important classes of analytes that are of great importance in food analysis and characterization. The optimized protocol successfully allowed the determination of thirteen free amino acids [33].

A simple and rapid method for quantitative analysis of amino acids, including valine (Val), leucine (Leu), isoleucine (Ile), methionine (Met) and phenylalanine (Phe), has been developed using GC/MS. Amino acids were extracted from the sample, and the extracts were purified using cation-exchange resins. The isotope dilution method using $^2H_8$-Val, $^2H_3$-Leu, $^2H_3$-Met and $^2H_5$-Phe as internal standards was applied. Following propyl chloroformate derivatization, the derivatives were analyzed using fast-GC/MS. The extraction recoveries using these techniques ranged from 69.8% to 87.9%, and analysis time for each sample was approximately 26 min. Calibration curves at concentrations from 0.0 to 1666.7 μmol/l for Val, Leu, Ile and Phe and from 0.0 to 333.3 μmol/l for Met showed good linearity with regression coefficients=1. The method detection limits for Val, Leu, Ile, Met and Phe were 24.2, 16.7, 8.7, 1.5 and 12.9 μmol/l, respectively. This method was applied to blood spot samples obtained from patients with phenylketonuria (PKU), maple syrup urine disease (MSUD), hypermethionine and neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD), and the analysis results showed that the concentrations of amino acids that characterize these diseases were increased. These results indicate that this method provides a simple and rapid procedure for precise determination of amino acids [34].

There is variety of analytical methods for determination of amino acids. These methods include HPLC, GC FID, TLC densitometry (35, 36, 37, 38, 39), but, there isn't analytical system associated with the standardization of food additives containing amino acids.

Conclusion

The amino acids are very important for the vital functions of the human body. The amino acids are components a number of food additives. The food additives market is growing very fast. This results in an increase of the production of amino acids. There is variety of methods for amino acids production. Biotechnology methods are widely established in the production of proteinogenic amino acids. Enzyme catalysis will remain the preferred production method for nonproteinogenic amino acids and amino acid derivatives. 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. The need for analytical control of food supplements containing amino acids is huge. In the literature there are describes various analytical techniques for their qualitative and quantitative analysis. Most preferred methods are HPLC and GCMS, because of their range, accuracy and speed. Despite the wide variety of methods, there isn't coherent analytical system associated with the standardization of food additives containing amino acids.

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