Chapter 6

Peptide and Amino Acids Separation and Identification from Natural Products

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1. Introduction

The natural extracts of plants are an important source for the identification of new biologically active compounds with possible applications in the pharmaceutical field. Phytotherapy embraces especially the isolation from herbs, of compounds with unique chemical structures, which are considered to be pharmacologically active. Recent statistics show that, on an annual basis, there are identified over 1500 new compounds from different species of plants, and about one quarter of prescription drugs contain substances of plant origin.

Although globally there are many studies on natural products, research in this area continues to be of great latent potential, in part due to the existing and still unexploited, potential and perspective development of new opportunities to recovery in major industrial areas and because of their socio-economic impact. A large number of plant extracts that have been used in the traditional medicine have also applications nowadays, both in the pharmaceutical and food industries (Busquet et al., 2005 [1]).

It is known that native Carpathian flora represents about 30% of plant species on the entire European continent. In Romanian, traditional medicine, there are used a number of plants with powerful therapeutic effects, and, as a result, an extensive investigation of their content is necessary. There are many classes of compounds that can be found in an alcoholic, natural extracts: amino acids, peptides, small proteins, phenols, polyphenols, saponins, flavonoids and sugars. Compounds of great interest are the free amino acids and the peptides from these extracts, which show important antitumor activities.

The process of structure elucidation of a natural product involves the determination of many physical-chemical properties: melting point, optical rotation, solubility, absorption, optical rotatory dispersion, circular dichroism, infrared spectroscopy, as well as mass and

nuclear magnetic resonance spectroscopies. On the basis of such information will be proposed a likely and reasonable structure(s) for the studied natural product.

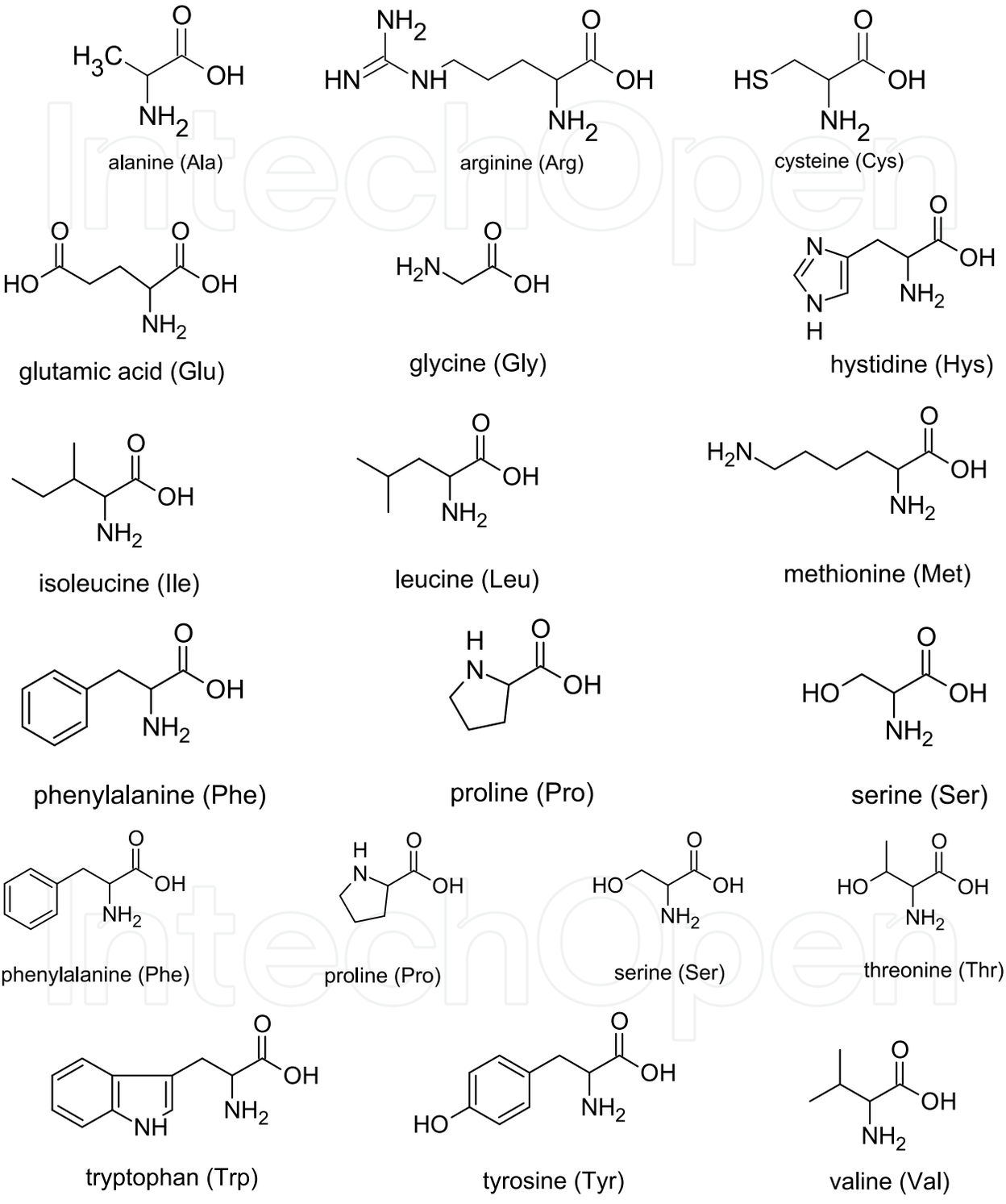

Figure 1. Amino acids chemical structures

If early methods for organic molecule characterisation used only a few physico-chemical parameters, such as: melting point, solubility, elemental analysis, molecular weight, and/or

specific rotation, yet now more modern techniques, especially various spectroscopies, of analysis and characterization are extremely useful tools for a complete chemical screening of the natural extracts.

The chemistry of natural products includes three main areas: isolation, structure elucidation, and synthetic methods. The isolation step is considered to be a part of structure elucidation, and therefore analysis and characterization methods, such as the UV-Vis and infrared spectroscopy, mass spectrometry and various chromatographic techniques, are all important tools for a proper identification of the components of an extract. Figure 1 illustrates the chemical structures for most, widely found, naturally occurring amino acids.

2. Sample preparation

A preliminary step, required for the proper separation of amino acids and peptides, consists in finding a suitable, partitioning scheme of the extract between various solvents, in order to remove the unwanted compounds, such as: polysaccharides, lipids, phenols and others.

Capillary electrophoresis (CE) allows the separation of amino acids without prior derivatization. A derivatization step is often necessary in order to improve the detectability using optical detection. A wide variety of labeling reagents have been reported, such as: FMOC, NDA, OPA or FITC (fluorescein isothiocyanate).

Typically, in amino acid analysis, peptide bonds must first be broken, into the individual amino acid constituents. It is known, that the sequence and nature of amino acids in a protein or peptide determines the properties of the molecule. There are different hydrolyzing methods commonly utilized before amino acid analysis, but the most common is acid hydrolysis. However, some of the amino acids can be destroyed using such an approach. Thus, methionine and cystine were either partially destroyed or oxidised to methionine sulphone and cysteic acid. Usually, it is often best to use a hot hydrochloric acid solution and 0.1% to 1.0% of phenol, which is added to prevent halogenation of tyrosine.

Alkaline hydrolysis method has limited applications due the destruction of arginine, serine, threonine, cysteine and cystine. Enzymatic hydrolysis represents perhaps the best method for the complete hydrolysis of peptide bonds, because it does not affect tryptophan, glutamine and asparagines. However, their applications are restricted, due to the difficulties often involved with the use of enzymes.

Separation and elucidation of the chemical composition of a natural product, from a medicinal plant, involves a very laborious procedure. For instance, in the case of *Chelidonium majus L,* a well –known herb, it was necessary to perform successive extractions with hexane, ethyl acetate, chloroform, and n-butyl alcohol. Every fraction obtained was analyzed in detail by various spectroscopic and chromatographic techniques.

Recent scientific research has reported a number of increased and improved techniques for the identification of free amino acids, such as, spectroscopic identification by means of colorimetric methods. These have often used reagents such as 2,4-dinitrofluorobenzene [2]

and genipin [3]. Also, it has also been reported on the use of IR spectroscopy for the study of various extracts of *Angelica* [4].

3. UV-Vis spectroscopy

The UV-Vis spectra of natural compounds contain information about different properties (such as: chemical composition and structure). Such methods are simple, fast, inexpensive, and safe to perform; which accounts for their popularity. However, these methods have disadvantages, because the result's accuracy depends on many factors: e.g., variations in the length of the polypeptide chain, amount and types of amino acid residues, accessibility of dye reagents, presence of final buffers, stabilizers, and other excipients, which can react with dyes or absorb at the detection wavelength.

4. IR spectroscopy

Infrared spectroscopy is based on molecular vibrations, characteristic to the specific chemical bonds or groups. The energy of most molecular vibrations (stretching, twisting and rotating) corresponds to that of the infrared region of the electromagnetic spectrum. There are many vibrational modes that do not represent a single type of bond oscillation but are strongly dependent on the neighbouring bonds and functional groups. One of the great advantages of this analytical technique for natural products, is due to the fact that spectra can be obtained form almost any environment (aqueous solution, organic solvents, etc.) and from relatively small quantities of sample.

There are a large number of IR spectroscopic studies regarding the structure of amino acids and peptides; some of the approached subjects are the following: infrared spectra of potassium ion tagged amino acids and peptides (Polfer et al., 2005[5]), IR spectra of deprotonated amino acids (Oomens et al., 2009[6]). Also, there have been reported studies regarding the IR spectra of some derivatives of the amino acids, namely the amides (Kasai et al., 1979[7]). The results show the appearance of the C=O group around 1675-1680 cm-1 for most of the studied compounds. An exception is represented by the L-tyrozine amide, which shows a vibration frequency of the C=O group at 1705 cm-1, fact that is probably due to the intermolecular hydrogen bonds between the N atom of the amidic group and the phenolic OH group (Kasai et al, 1979[7]). Linder et al. have presented the IR spectra of 5 natural amino acids, namely valine, proline, isoleucine, phenylalanine and leucine (Linder et al., 2005[8]). The five spectra are very similar as regards the position of C=O group, and the absorption frequencies of OH groups. Slight differences appear only in the case of the stretching vibrations of the C-H groups (Linder et al., 2005[8]).

The amino acid and peptide absorption bands in the 3400 cm^{-1} region is due to O–H and N–H, bond stretching. The broad absorption bands in the region 3030-3130 cm^{-1} are attributed to asymmetric valence vibrations of the ammonium (NH_3^{+}) group. The symmetric absorption vibrations in 2080 -2140 cm^{-1} or 2530-2760 cm^{-1}, depend on amino acid chemical structures. The ammonium group deformation vibrations are located at 1500-1600 cm^{-1}, together with

the absorptions characteristic of the carboxylate ion. The asymmetrical deformation bands from 1610-1660 cm^{-1} is associated with a carboxylate (COO^{-}) group, and it usually represents a weak absorption. The bands in the 1724-1754 cm^{-1} region correspond to the carbonyl (C=O) vibration.

In the next figure (Figure 2), is presented the FT-IR spectra of L-leucine.

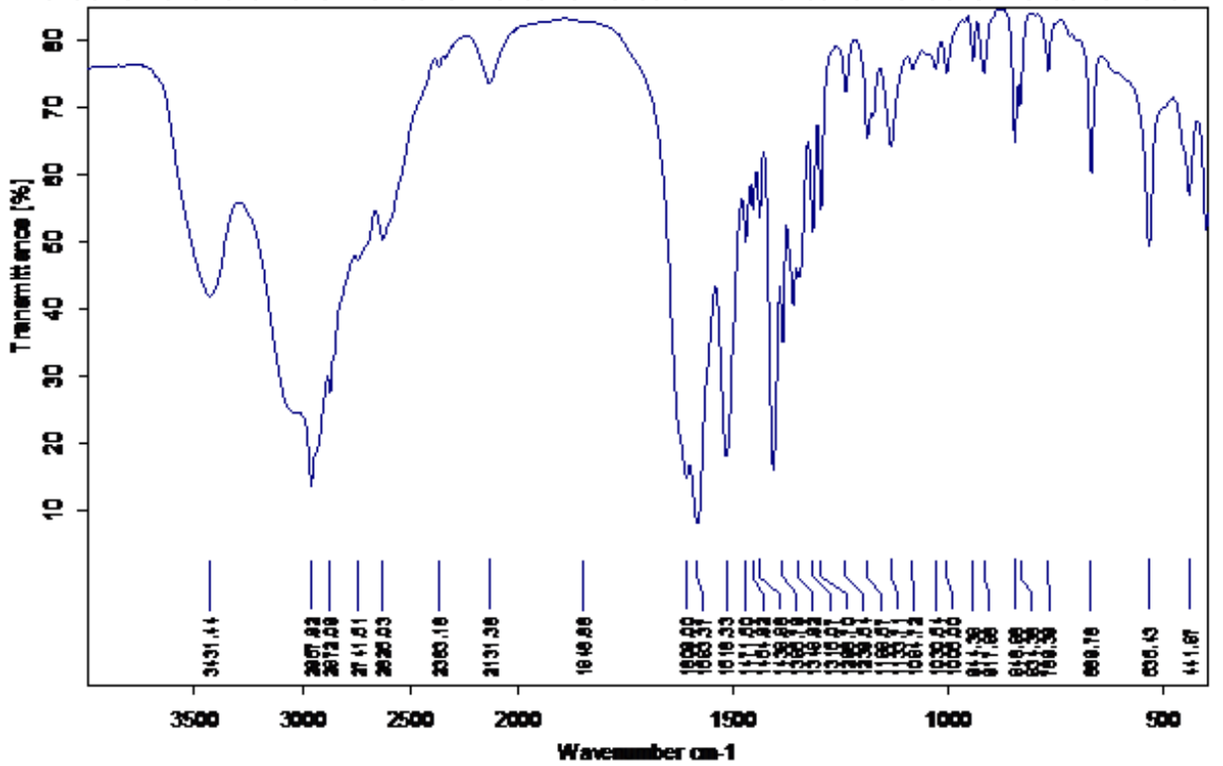

Figure 2. FT-IR spectra of leucine

In the following Figure 3, the IR spectrum of the *Chelidonium majus L.* extract is presented:

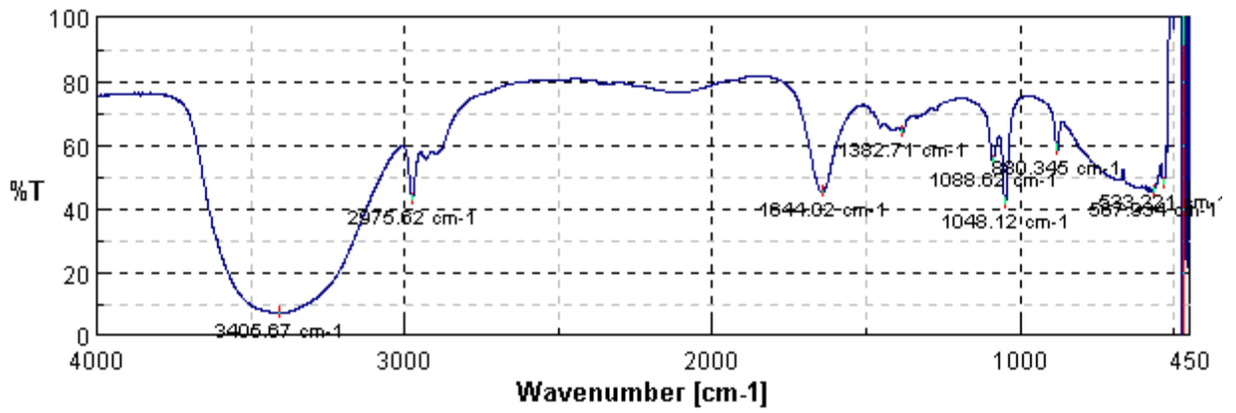

Figure 3. IR spectrum of the aqueous part of the *Chelidonium majus L.* extract (after successive extractions with hexane, ethyl acetate, chloroform and n-butyl alcohol)

The wavenumbers that appear in the IR spectra can be attributed to: OH (3405.67 cm^{-1}), CH_2 and CH_3 (2975.62 cm^{-1}), C=C (1644.02 cm^{-1}), and C-O (1382.71 cm^{-1}). Also, the UV-Vis spectra of the aqueous part of *Chelidonium majus L.*, showed the existence of three absorption bands: 734 nm, 268 nm and 198 nm, respectively. For a complete study, further analysis (including derivatization and HPLC) are usually performed.

5. Chromatographic methods

These techniques insure the separation of closely related compounds in a mixture, by differences in the equilibrium or partition distributions of the components between two immiscible phases, the stationary and the mobile phases. These differences in the equilibrium distribution are a result of chemical structures and the degree of interactions of the components between these two phases. Under the influence of a mobile phase (one or a mixture of solvents), the target compounds percolate through the stationary phase, which is a porous medium (usually, silica or alumina). For successful amino acid and peptide isolations and purifications from natural products, have been developed different chromatographic methods (e.g., paper, thin layer, gas chromatography, column and high performance liquid chromatography, etc.). From the enormous variety of methods of separation and isolation useful for natural products, adsorption or partition chromatography represents one of the most useful techniques of general application.

Thin layer chromatographic (TLC) is the simplest technique used to separate and identify natural products of interest. This method readily provides qualitative information and possibly quantitative data. The stationary phase is usually silica gel on the TLC or HPTLC (high performance TLC) plate, which is made up of silica adhered to glass or aluminium or a plastic, for support. The eluent (solvent mixture) acts as the mobile phase. Practically, the compounds of interest need to be soluble to varying degrees. Separation again results from the partition equilibrium of the components in the mixture. The separation depends on several factors: 1) solubility in the mobile phase, 2) attractions or adsorption between the compound and silica, the more the compound interacts with silica, the less it moves upwards, 3) size or MW of the compound, for the larger the compound, the slower it moves up the plate.

Since amino acids are colourless compounds, ninhydrin is routinely used to detect them, with the result of a coloured product, due to the formation of Ruhemann'purple complex. The familiar violet color which is associated with the reaction of amino acids with ninhydrin is attributed to the anion of the reagent (derivatizing agent). Another technique uses anisaldehyde-H2SO4 reagent for detection of amino acids, followed by heating (120° C, 5 minutes).

Different organic solvents (e.g., alcohol, dioxane, methyl cellosolve, pyridine, and phenol) are used to accelerate the development of color, to varying degrees. Ultimately a phenol-pyridine system was adopted as the most effective solvent. Exposure to 105°C for 3 to 5 minutes gives quantitative yields of color for all amino acids, except for tryptophan and lysine. The Rf (retardation factor) value for each compound can be calculated and compared

with their reference values, in order to identify specific amino acids. The Rf value for each known compound should remain the same, provided the development of the plate is done with the same solvents, type of TLC plates, method of spotting and under exactly the same conditions.

High performance liquid chromatography (HPLC) allows for the most efficient and appropiate separations of consitutents from natural product, complex mixtures. It has been shown that HPLC is the premier separation method that can be used for amino acid analysis (AAA), from natural products, allowing for the separation and detection by UV absorbance or fluorescence. However, most common amino acids do not contain a chromophoric group, and thus some form of derivatization is usually required before HPLC or post-column.

Amino acids are highly polar molecules, and therefore, conventional chromatographic methods of analysis, such as, reversed-phase high performance liquid chromatography (RP-HPLC) or gas-chromatography (GC) cannot be used without derivatization. The derivatization procedure has several goals, such as: to increase the volatily, to reduce the reactivity, or to improve the chromatographic behaviour and performance of compounds of interest. In the case of amino acids, derivatization replaces active hydrogens on hydroxyl, amino and SH polar functional groups, with a nonpolar moiety. The great majority of derivatization procedures involve reaction with amino groups: usually primary amines, but also secondary amines (proline and hydroxyproline), or the derivatization of a carboxyl function of the amino acids. Some of the most common derivatization reagents are presented in the Table 1.

As it was mentioned before, prior derivatization of the amino acids is necessary due to the lack of UV absorbance in the 220-254 range. The paper of Moore and Stein [9] is actual even nowadays. Their method, that used a modified nynhidrin reagent for the photometric determination of the amino acids, represents the basis for various derivatization methods. There is a continuous increasing number of amino acids derivation reagents. There will be mentioned, as follows, some of the them: Melucci et al. [10] presents a method for the quantization of free amino acids that implies a pre-column derivatization with 9-fluorenylmethylchloroformate, followed by separation by reversed-phase high-performance liquid chromatography. Kochhar et al. [11] use the reverse-phase high-performance liquid chromatography for quantitative amino acids analysis and, as derivatization agent, 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide, known as Marfey's reagent. The method was successfully applied for the quantization of 19 L-amino acids and it is based on the stoichiometric reaction between the reagent and the amino group of the amino acids [11]. Ngo Bum et al. [12] have been used the cation exchange chromatography and post-column derivatization with ninhydrin for the detection of the free amino acids from the plant extracts. Culea et al. [13] have used the derivatization of amino acids with trifluoroacetic anhydride, followed by the extraction with ion exchangers and GC/MS analysis. Warren proposed a version of CE, CE-LIF, for quantifying the amino acids from soil extracts. The advantage of the method is represented by the low detection limits that are similar to the ones corresponding to the chromatographic techniques. In 2010, Sun et al. [15] have presented another method for the detection of amino acids from Stellera chamaejasme L., a

widely-used plant in the Chinese traditional medicine; DBCEC (2-[2-(dibenzocarbazole)-ethoxy] ethyl chloro-formate) was used as derivatization reagent, and the modified amino acids were detected by means of liquid chromatography with fluorescence detection. Li et al. [16] have proposed a new method for the detection of amino acids from the asparagus tin. After performing the derivatization of the samples with 4-chloro-3,5-dinitro-benzotrifluoride (CNBF), solid phase extractions on C18 cartridges have been performed. The purified amino acid derivatives were then subjected to the HPLC analysis. Zhang et al.[17] have proposed an improved chromatographic method (by the optimization of mobile phases and gradients) for the simultaneous detection of 21 free amino acids in tea leaves.

DERIVATIZATION REAGENT	Reference
Nynhidrin	9, 12
9-fluorenylmethylchloroformate	10
trifluoroacetic anhydride	13
1-fluoro-2,4-dinitrophenyl-5-L-alanine amide	11
2-[2-(dibenzocarbazole)-ethoxy] ethyl chloroformate	15
4-chloro-3,5-dinitrobenzotrifluoride	16
ortho-phthaldehyde (OPA)	
phenylisothiocyanate	
dimethylamino-azobenzenesulfonyl chloride	
7-fluoro-4-nitro-2-aza-1,3-diazole	

Table 1. Reagents for Derivatization

There have been already developed, several liquid chromatography methods for amino acid quantification. General approaches are ion-exchange chromatography (IEC) and reversed-phase HPLC (RP-HPLC). Both approaches require either a post-column or pre-column derivatization step. Even this technique offers satisfactory resolutions and sensitivity, but the necessary derivatization step provides an increased complexity, cost, and analysis times.

Ion-exchange chromatography with postcolumn ninhydrin detection is one of the most commonly used methods employed for quantitative amino acid analysis. Separation of the amino acids on an ion-exchange column is accomplished through a combination of changes in pH and ionic (cation) strength. A temperature gradient is often employed to enhance separation.

But, perhaps the most effective method is cation exchange chromatography (CEC) in the presence of a buffer system (usually a lithium buffer system), and a post-column derivatization step with ninhydrin. Detection is performed with UV absorbance. In this way one achieves the desired amino acid separation, according to the colour (structure) of the derivatived compound formed. Amino acids which contain primary amines, except an imino acid, give a purple color, and show the maximum absorption at 570 nm. The imino acids such as proline give a yellow color, and show the maximum absorption at 440 nm. The postcolumn reaction between ninhydrin and an amino acid eluted from the column is monitored at 440 and 570 nm.

OPA is another reagent used both for post-column or pre-column derivatization. Ortho-phthaldehyde (OPA) reacts at an amino group, generally in the presence of a thiol (mercaptoethanol), resulting in a fluorescent derivative, UV active at 340 nm. Other reagents for the precolumn derivatization of free amino groups from amino acids, are: PITC (phenylisothiocyanate), DABS-Cl (dimethylamino-azobenzenesulfonyl chloride), Fmoc-Cl (9-fluorenylmethyl-chloroformate), NBD-F (7-fluoro-4-nitro-2-aza-1,3-diazole), and others. The reaction time depends on the type of derivatization reagent and the reacting, functional group involved. For instance, from practically nearly instantaneous derivative formation in the case of the reagent, fluorene chloroformate, OPA is 1 minute and PITC is about 20 minutes.

Ion pair, reverse phase liquid chromatography coupled with mass spectroscopy, IPRPLC-MS/MS, is a technique which allows for the analysis of amino acids without derivatization, thus reducing the possible errors introduced by reagent, interferences and derivative instability, side reactions, etc. Using volatile reagents, the IP separation is based on two different mechanisms: a) the IP-reagent is adsorbed at the interface between stationary and mobile phases; and b) the formation of a diffuse layer and the electrostatic surface potential depends on superficial (surface) concentration of IP reagent. There are other, possible mechanisms suggested in the literature for how IPRPLC operates.

Gas chromatography (GC) can be used for the separation and analysis of compounds that can be vaporized without decomposition. The derivatization procedure most commonly employed is silylation, a method through which acidic hydrogens are replaced by an alkylsilyl group. Typically, silylation reagents are: BSTFA (*N*,*O*-bis-(tri-methyl-silyl)-trifluoroacetamide and MSTFA (*N*-methyl-silyl-trifluoro-acetamide). A possible disadvantage of this approach, is due to the reagent and derivative being sensitive to moisture and possible, derivative instability. Some amino acids are unstable (e.g., arginine and glutamic acid). Arginine descomposes to ornithine, and glutamic acid undergo a rearrangement to pyro-glutamic acid. Another GC-derivatization method includes acylation or esterification, now using an aldehyde and alcohol (pentafluorpropyl or trifluoracetic aldehyde and isopropanol) or alkyl chloroformate and alcohol. Silylation takes place through the direct conversion of carboxylic groups to esters and amino groups to carbamates. Such reactions are catalyzed by a base (pyridine or picoline). Alkyl esters are extremely stable and can be stored for long periods of time.

GC-MS represents an analysis method with excellent reproducibility of retention times, and the method can be easily automated. The major disadvantage is due to the possible temperature instabiity of some compounds and/or their derivatives, which then cannot be easily analyzed under most GC conditions.

Mass spectrometry represents one of most efficient techniques for natural product, structure elucidation. It functions by a separation of the ions formed in the ionisation source of the mass spectrometer, according to their mass-to-charge (m/z) ratios. The technique allows for accurate MW measurements, sample confirmation, demonstration of the purity of a sample, verification of amino acid substitutions, and amino acid sequencing. This procedure is

useful for the structural elucidation of organic compounds and for peptide or oligo-nucleotide sequencing. The major advantadge in using MS is due to the need for very small amounts of sample (ng to pg). A disadvantage of conventional ionization methods (e.g., electron impact, API) is that they are limited to compounds with sufficient volatility, polarity and MW. Volatility can be increased by chemical modifications (derivatizations, such as: methylation, trimethylsilylation or trifluoro-acetylation). For peptides, there has been developed certain new, very efficient techniques, such as: electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI).

In the next Figure 4, is presented the mass spectrum of pure valine, recorded on a Bruker Daltonics High Capacity Ion Trap Ultra (HCT Ultra, PTM discovery) instrument.

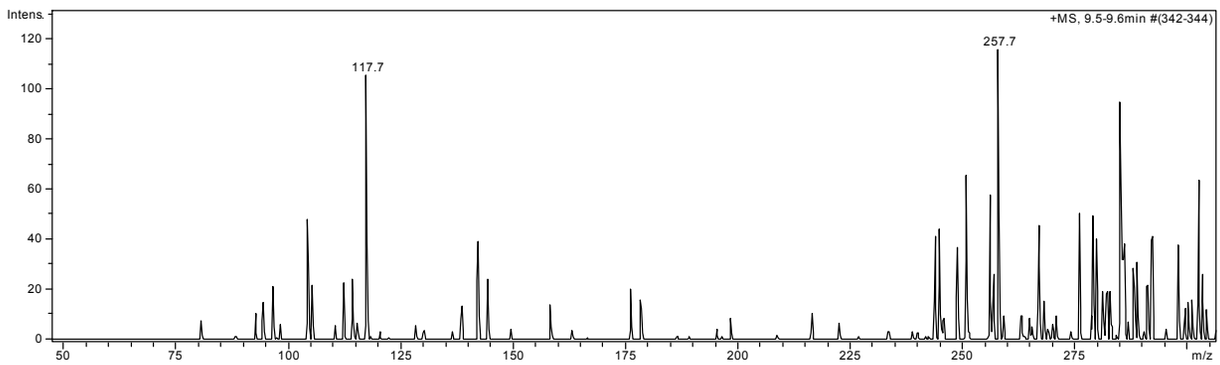

Figure 4. Valine MS-specta

NMR spectroscopy offers the most useful and valuable information about the structure of perhaps any natural product. The method has the advantage of excellent reproducibility. Even though it is considered to be one of the more expensive techniques, NMR is relatively cheap, fast sensitive and easily used as a routine application for amino acid analysis.

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