Advanced Techniques of TLC, HPLC, GC, and Applications for Study and Analysis Amino Acids & Peptide Compounds

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Abstract – Amino acids are very important organic compounds in nature. The biological activity of amino acids depends mainly on their stereoisomeric configuration (D- or L-). Thus, the stereochemical analysis of amino acids and peptides is an important aspect of their characterization. Owing to the increasing role of amino acid configuration in biomedical and pharmaceutical studies, numerous analytical methods have been described in the literature. Among a wide range of analytical techniques available for the stereoselective separation of different amino acids, which were obtained from plants or biological samples, chromatographic methods such as thin-layer chromatography, high-performance chromatography and also gas chromatography are very useful. This review presents such systems developed for direct stereoisomeric separation and quantitative determination of amino acid and peptide enantiomers with emphasis on selected literature published during two last decades. Almost all aspects, including sample preparation prior chromatographic analysis, stationary phase, solvent system and detection system, are discussed. New possibilities in chiral amino acid analysis have been opened up by the application of mass spectrometry and infrared detection in thin-layer chromatography and mass spectrometry selected ion monitoring detection system in gas chromatography. A modern trend in high performance chromatography is two-dimensional chromatography. These innovations have led to decreased development time and increased amino acid resolution and detectability.

Keywords – TLC, HPLC, GC Amino Acids, Peptide Enantiomers, Detection, Separation.

I. INTRODUCTION

Amino acids are organic compounds belonging to the carboxylic acids, in which a hydrogen atom in the side chain (usually at α -carbon) has been replaced by the amino group. On the basis of the number of carboxylic groups as acidic (-COOH) and amino groups (-NH 2) as basic in the molecule, the amino acids are divided into three groups: neutral (e.g. serine), acidic (e.g. glutamic acid) and basic (e.g. arginine).

An asymmetric carbon atom in amino acids plays a role of a chiral center. For this reason, the amino acid molecules are opti-cally active and exist in form of respective enantiomers, which are designated by the symbols D and L (nomenclature developed by Fischer and determined on the base of D-glyceraldehyde structure; Lepri and Cincinelli, 2010b; Czerwenka and Lindner, 2005). The naturally found protein amino acids are generally composed of L-enantiomers. D -Enantiomers can be found in plants, bacterial cells or in several antibiotics (Hasegawa et al., 2011). The amino acid enantiomers have identical chemical and physical properties but possess different biological activities. Therefore, the separation of enantiomers is important for pharmaceutical (e.g. antibiotics), industrial (e.g. chiral catalysts) and toxicological (e.g. xenobiotics) applications. Nowadays, the examination of enantiomeric purity of drugs is an important step in pharmacokinetic and pharmacodynamic studies of chiral drugs and their xenobiotics.

Generally, the analysis of peptides is based on the determination of amino acid composition in peptide hydrolysates and consists of two main steps: hydrolysis of peptides to free amino acids and analysis of released amino acids (Zahradnicková et al., 2008). Numerous analytical methods have been developed for enantiomeric separation and determination of peptides and amino acids. As reported by B aćzek and Radkowska (2007), the choice of the analytical method for direct enantiomeric resolution of amino acids depends mainly on the chemical structure of amino acids (e.g. peptide chain length, peptide sequence) and also on available apparatus and sensitivity needed for the analysis.

At present, the chiral analysis of amino acids is carried out by chromatographic methods. For the enantiomeric separation of single L-amino acids and their corresponding D-enantiomers, the following chromatographic techniques have been applied: thin-layer chromatography (TLC), high-performance liquid chro-matography (HPLC) and gas chromatography (GC).

The review discusses the advantages of various chromato-graphic techniques: TLC and HPLC, published mainly from 2000 on, and GC, from 1990 to April 2013, for the direct enantiomeric separation of a single amino acids and peptide hydrolysates. This review summarizes important aspects including stationary phases, mobile phases and methods of detection of amino acid enantiomers, which were separated by means of TLC, HPLC and and GC techniques.

II. PREPARATION OF AMINO ACID SAMPLES FROM PEPTIDES

The chromatographic analysis of peptides involves the following steps: hydrolysis, separation, derivatization and detection. From the available literature, it is obvious that the isolation of amino acids from biological material (e.g. urine, plasma), food and from environmental samples such as water is carried out using different extraction methods (Lepri and Cincinelli, 2010a). The amino acids can be isolated from biological samples after prior peptide hydrolysis using 6 M HCl (liquid phase hydrolysis) under
vacuum for 20–24 h. To improve the recovery, a protective substance such as phenol (0.1%) is used. The temperature of this process is about 110°C. Alternatively, peptide samples can be hydrolyzed by HCl vapor (gas phase hydrolysis). It was reported that a better recovery is given by alkaline hydrolysis and hydrolysis by the use of methane sulfonic acid, which is applied in tryptophan and proteolipids analysis.

**Thin-layer Chromatography**

Thin-layer chromatography in normal (NP) and reversed (RP) phase systems is one of the most promising separation methods for amino acid analysis from peptides and proteins because it is simple, sensitive, economic and fast. Different mixtures of peptides and proteins were satisfac-torily separated under various chromatographic conditions which are presented in many papers (Marutoiu and Soran, 2010; Lepri and Cincinelli, 2005). To improve the separation of free amino acids, two-dimensional TLC (2D-TLC) can be used. In addition to two-dimensional systems, a new strategy in amino acid separation is to use of multi development thin-layer chromatography and instrumental mul tiple developments. Both methods have been successfully applied to the analysis of amino acids from blood pla sma on cellulose plates by mean so facetonitrile–water (8:2, v/v) (Flieger et a1., 2006).

**III. Separation of Amino Acid Enantiomers by TLC and HPTLC**

Of all chromatographic techniques, TLC is widely applied for direct resolution of enantiomeric pairs of amino acids (racemi mixture) into L- and D-form, because it is suitable, simple and inexpensive. TLC is used in one of the promising methods of amino acid separation for these types of amino acids for which GC is not suitable. Most of the reports on TLC chromatographic separation of amino acids involve the following procedure, which improves the resolution of amino acid enantiomers (Lepri and Cincinelli, 2010a, 2010b):

-**chiral derivatization reactions;**
-**separation of enantiomers by the use of chiral stationary phases or chiral coated phases;**
-**separation of enantiomers using chiral mobile phases;**
-**incorporation of some suitable reagent such as base, acid or metal complex with the adsorbent before developing the chromatogram or plate making.**

**IV. Separation of the Amino Acid Enantiomers on Impregnated and on Ion Exchange TLC and RP-TLC Plates**

Impregnation of a thin layer of commer-cially available adsor-bent–silica gel, c ellulose, po lyamide and a lu mi na – with various r eagents to achieve direct enantiomeric separation of amino acids is discussed in many papers. Modi-fication of respective adsorbent by means of impregnation is a simpler and inexpensive technique. The impregnation of TLC plates on one method: complex formation, ligand exchange and ion pairing formation lead to resolution of amino acid enantiomers. There are a few methods of TLC plate impregnation, such as:

- mixing of the impregnating reagent with the inert support;
- immersion of plates into an appropriate solution of the impregnated reagent or exposing the layers of adsorbent to the vapors of the impregnating reagent has been used;

Of all the above-mentioned methods, the simplest one is to dip the plates in solution of the respective impregnating agent. Depending on the chemical nature and structure of the compound being separated, the role of the impregnating agent can vary. Immersion of chromatographic plates precoated with silica gel in the solution of the respective chiral selector is successfully used for the resolution of various amino acid enantiomers via ligand exchange TLC. The commercially available ligand exchange TLC plates namely by producers Chiralplate (produced by Macherey and Nagel) and also HPTLC Chir (for high-performance thin-layer chromatography, produced by E. Merck) have been widely ap-plied in the resolution of D- and L-amino acids. Chiral plates have been applied for the separation of different enantio-mers of DL-amino acids such as thyr oxyenantiomers with the use of acetonitrile–methanol–water (Jork and Ganz, 1994).

An example of the ligand exchange resolution via Chiralplate of DL-amino acids has been performed in enantiomeric purity control of optical isomers of methyl-dopa (Martens et al., 1986). In this work, resolution of DL-methyl-dopa and DL-dopa was achieved on Chiralplate with the use of the mobile phase methanol–water–acetonitrile (5:5:3, v/v/v). Solution of ninhydrin was used as the visualizing agent.

The use of mobile ph as e a cetonitrile methanol water phosphate buffer in volume composition 70:10:10:10 for enantiomeric resolution of various amino acids on commercial plates (RP-TLC system).

The ligand exchange method with the use of chromatographic plates prepared with Cu2+ complex of L-proline and L-arginine was capable of resolving a large number of racemates of amino acids. The enantiomers of nine amino acids (Ala, Phe, Val, Leu, Ile, Trp, Tyr, Asp and Glu) were successfully separated on RP 18 plates impregnated with a hydroxyproline derivatives and Cu2+ ions using methanol water acetonitrile (1:1:4, v/v/v) as a mobile phase (Kowalski and Nowak, 2 0 0 1). Significant selectivity to wards RP HPTLC plates treated with copper acetate was observed for α-amino acid enantiomers investigated with different binary aqueous solvents as mobile phases (Remelli et al., 1991).

A literature (Thiong’O, 2000) survey reveals separation of many racemates on the chromatographic plates, which were mod-ified by impregnation with the following agents as chiral selectors: brucine, macrocyclic antibiotics, (+)-tartaric acid, L-ascorbic acid, c omplex of Cu (II) with L-
proline and L-arginine. In the case of the RP-TLC C system used in enantiomeric separation of amino acids, good resolution was achieved using α-cyclodextrin as chiral mobile phase additive. Mixing of the above-mentioned chiral selectors with the respective adsorbent (e.g., silica gel) during chro-matographic plate development resulted in formation of diastereoisomeric pairs. As reported in many papers, the resolution of f-t-butamino acids into D and L forms is possible during development of the chromatogram because they indicate the differences in retention. Moreover, the application of a chiral selector with stationary phase is important in amino acid separation. Increased sensitivity of detection and location of amino acids, which were separated on those stationary phases, was stated.

In the past few years, the application of macrocyclic antibiotics as the chiral selectors added to the stationary phase has been observed (Armstrong and Zhou, 1994). The use of macrocyclic antibiotics such as vancomycin, erythromycin or rifamycin-Brithostrepton as bonded chiral selectors has resulted in successful separation of enantiomeric compounds. It was reported that impregnation of stationary phase with both antibiotics erythro-mycin and vancomycin was used to resolve dansyl- DL-amino acids on RP-TLC plates (Parshad, 1996; Thiong ‘O, 2000). Silica gel impregnated with vancomycin and the mobile phase acetonitrile –0.5 M NaCl (aqueous) in the ratios 5:2 (v/v) and 14:3 (v/v) was used to separate a mixture of enantiomers of dansyl DL-amino acids (Thiong ‘O, 2000). Detection in both cases was under UV at λ = 254 nm.

The next chiral selector developed by Ali (1987b) was named ( β )-brucine, belonging to the alkaloid group, and slurred with silica gel for making TLC plates. The prepared plates were applied for separation of amino acid enantiomers with the use of butanol–acetic acid – chloroform (3:1:4, v/v/v). Ninhydrin was used as a visualizing agent.

Among numerous stationary phases, a new type of chiral stationary phase used in TLC separation of amino acids is silica gel impregnated with cetrimide. Surface modifi-
cation of silica gel with aqueous solution of surfactants, for example, cetrimide and cetylpyridinium chloride improves separation of biological important amino acids such as lysine from arginine. This method is free from the use of toxic volatile organic solvents, so it belongs to the green chemical methods (Mohammad and Haq, 2010).

Of many stationary phases presented above, the cellulose plates are most widely used for TLC enantiomeric resolution of amino acids. Cellulose plates are also useful in detection of blood spots containing homocysteine among patients with homocystinuria (Clift et al., 1994; Ohtake et al., 1995). Another report performs the application of microcrystalline cellulose and native cellulose as chiral stationary phase for TLC separation of D- and L-tyrphophans and their derivatives. Solution of NaCl (1 M) was used as a mobile phase. The spots were visualized by ninhydrin. Better separations were achieved on microcrystalline cellulose (Lederer, 1992).

Lepri (1997) reported the use of mixture of microcrystalline cellulose triacetate as a stationary phase for the resolution of 21 racemates developed with aqueous/organic mixture (ethanol, metha nol or 2-propanol). As a stationary phase consisting of a mixture of microcrystalline cellulose triacetate and silicagel (3:1) were also successfully applied for separation of enantiomers of PTH-amino acids and N- and C-substituted amino acids (Lepri et a., 1999). For PTH–Phe amino acids, a mixture of ethanol–water (4:1, v/v) was effective; in the case of PTH– Tyr, 2-propanol–water (4:1, v/v) was used (Lepri et al., 1999).

Direct enantiomeric resolution of other amino acids such as DL-arginine, DL-histidine, DL-valine and DL-leucine (from pharmaceutical industry) – into their enantiomers was achieved on silica gel TLC plates impregnated with butanol–acetic acid – chloroform (3:1:4, v/v/v). Ninhydrin was used as a visualizing agent. Good results of the enantiomeric separation by TLC plates precoated with silica gel and impregnated with the above-mentioned reagent were obtained in the case of D-amino acids, which were resolved using various mobile phases containing aqueous NaCl-acetonitrile–methanol. Dansyl-DL-amino acids were visualized under UV at 254 nm. Other amino acids were detected using ninhydrin.

V. SEPARATION OF THE AMINO ACID ENANTIOMERS WITH THE USE OF CHIRAL MOBILE PHASES AND CHIRAL SELECTORS AS MOBILE PHASE ADDITIVES

The resolution of amino acid enantiomers can be affected by the use of respective chiral selectors as mobile phase additives. The chiral selectors, which are most useful in enantiomeric separation of amino acids, are natural polymeric compounds such as β-cyclodextrin, macrocyclic antibiotic (e.g., vancomycin) and bovine serum albumin.

Cyclodextrins (CD) have been used extensively as mobile phase additives for chiral resolutions of variety amino acids and their derivatives (Flieger et al., 2006). Of different types of cyclodextrins, only β-cyclodextrin has been successfully applied for amino acid resolution from small peptides (short length, containing not more than 10 amino acids). γ-Cyclodextrin is not popular for this purpose because it is expensive and for this reason is rarely recommended in comparison with β-CD. Excellent resolution of D- and L-racemates of substituted amino acids and alkaloids was achieved on silica gel plates (Sil C 18 -50E) with the use of mobile phase 0.1 M β-cyclodextrin in urea solution (Lepri et al., 1990). Another work shows that the enantiomers of six amino acids – arginine, histidine, lysine, citrulline, glutamine and valine – could be successfully performed on silica gel TLC plates.
developed with the use of acetonitrile–water containing 6.5 mM 2-hydroxypropyl-β-cyclodextrin (Hao et al., 1995).

Le Fevre et al., (2000a, 2000b) reported a series studies on dansyl amino acid enantiomeric separation with the use of RP-TLC and β-cyclodextrin as a chiral additive of mobile phase. Selected α-amino acids from small peptides were effectively resolved on RP-TLC plates with the use of aqueous solution of cyclodextrin as a chiral additive containing various proportions of acetonitrile or methanol in mobile phase.

Among different chiral selectors used as the components of mobile phases in enantiomeric separation of D- and L-amino acids, the most important is macrocyclic antibiotic (vancomycin). It has been found very useful as a mobile phase additive in the separation of dansyl- DL-amino acids on RP-TLC plates (Parshad, 1996; Mohammad et al., 2012).

In 1992 Lepri and coworkers reported that the successful enantiomeric separation of free and derivatized amino acids was achieved by TLC on RP-18 W (Si C18-50) using UV detection and mobile phases containing from 6 to 9% of bovine serum albumin as chiral agent, from 2 to 20% propan-2-ol as organic modifier and acetic acid for pH control.

VI. RESOLUTION OF AMINO ACID DERIVATIVES

In practice, different methods of amino acid derivatization are used, which improves the sensitivity and the range of their detection. A mong various compounds reacting with amino acids, the most popular, which have been investigated as derivatizing agents, are 2,4-dinitrophenyl (DNP), 5-dimethylaminonaphthalen-1- sulfonil (dansyl, DNS), phenyl-isothiocyanate (PITC), 4- (dimethylamino)azobenzene-4′-phenyl-isothiocyanate (DABITC) and 4-(diethylamino)azobenzene-4′-phenyl-isothiocyanate (DAEBITC) (Lepri and Cincinelli, 2010a). Processes of derivatization by the use of the above-mentioned reagents enable direct separation and quantification of amino acids and peptides.

DNP-amino acids are the compounds obtained from amino acids and peptides by dinitrophenylation. Water-soluble DNP-amino acids are usually investigated by TLC on silica gel plates using an n-propanol–ammonia system (7:3, v/v). Ether-soluble DNP-amino acids have been studied by 1D and by 2D chroma-tography. Ammonia vapor is used as a suitable visualizing agent for DNP-amino acids. The limit of detection under UV light was 0.02 μg and for 2D about 0.5 μg (Lepri and Cincinelli, 2010a). Derivatization to PTH-amino acids is one of the main steps in amino acid analysis of amino acid sequences in proteins and peptides. Identification of these compounds is conducted using a TLC method using 1D technique. Various TLC plates such as alumina, polyamide and silica gel (Lepri and Cincinelli, 2010a) are used. Various solvent systems are applied: n-heptane –n-butanol – acetic acid (40:30:9, v/v/v), toluene –n-pentane-acetic acid (60:30:35, v/v/v), and ethylene chloride–acetic acid (90: 16, v/v) in the case of polyamide plates. For TLC analysis of PTH-amino acids on silica gel, mixtures of n-heptane –methylen chloride –propionic acid (45:25:30, v/v/v), xylene –methanol (80:10, v/v), chloroform –ethanol (98:2, v/v), disopropyl ether–ethanol (95:5, v/v) and pyridine–benzene (2.5:20, v/v) are used. Detection of the PTH spots is successful by the use of ninhydrin as a visualizing agent and UV light (Lepri and Cincinelli, 2010a).

A very sensitive method for quantification of N-terminal amino acids in protein is also derivatization in acidic solution of amino acids by the use of DABTC (4- (dimethylamino)azobenzene-4′-phenyl-isothiocyanate). The DNS-amino acid study has been focused on silica gel and polyamide plates using 1D and 2D methods. Among various TLC systems, a very useful DNS-amino acid analysis is the one based on polyamide plates developed in the first direction by water–formic acid, in the second direction by methanol–benzene–acetic acid, and in the third, ammonia-ethanol (1: 1) or et hyl acetate –acetic acid –methanol (20:1:1, v/v/v) (Lepri and Cincinelli, 2010a).

According to Lepri and Cincinelli (2010a), all DABTH-amino acids except the Leu/Ile pair can be separated by 2D technique on polyamide plates, developed in the first direction with the use of water–acetic acid (2:1, v/v). In the second direction, a mixture of toluene–hexane–acetic acid (2:1:1, v/v/v) was used. For the above-mentioned Leu/Ile pair of DABTH derivatives, a mixture of formic acid-ethanol (10:9, v/v) and polyamide plates is effective. On silica gel, chloroform–ethanol (100:3, v/v) was used. Wawrzycki and Pyra (2000) reported that derivatization in the range of their detection. Among various compounds reacting with amino acids, the most popular, which have been investigated as derivatizing agents, are 2,4-dinitrophenyl (DNP), 5-dimethylaminonaphthalen-1-sulfonil (dansyl, DNS), phenyl-isothiocyanate (PITC), 4-(dimethylamino)azobenzene-4′-phenyl-isothiocyanate (DABITC) and 4-(diethylamino)azobenzene-4′-phenyl-isothiocyanate (DAEBITC) (Lepri and Cincinelli, 2010a). Processes of derivatization by the use of the above-mentioned reagents enable direct separation and quantification of amino acids and peptides.

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VII. DETECTION OF AMINO ACID ENANTIOMERS SEPARATED BY THE USE OF TLC AND HPTLC

Because most amino acids have generally weak chromophores, for detection purpose of the amino acids after their separation by TLC or HPTLC, derivatization is needed to improve their detectability. The following methods are used in amino acid detection:

Chemical methods. Based on detection with the use of the respective visualizing agent ninhydrin, salicylaldehyde, 4-dime thylaminobenzaldehyde and hexane thyl disilazane, the absence of UV or fluorescence activity of amino acids is observed (Pachuski et al., 2002; Sherma and Fried, 2005). Of the above mentioned reagents, the most popular is ninhydrin for all amino acids except proline and its derivates. In the case of proline, reaction with iodineazide is proposed. This reagent is effective for detection of different amino acids including proline and hydroxyproline (Ka zmierzczak et al., 2005; Zakrzewski et al., 2002). Another reagent which is very useful because it produces a stable color with many amino acids is 2, 3 - dichloro - 1, 4 - n a phthoquin one, developed by Samanta and Laskar (2006). For detection of tryptophan a solution
of 1% p-dimethylaminobenzaldehyde in ethanol–hydrochloric acid (1:1, v/v) is used (Pachuski et al., 2002; Sherma and Fried, 2005).

Similarly, as in the case of single amino acids, the detection of peptide hydrolysates is achieved using different visualizing agents. The kind of visualizing agent depends on the chemical structure of peptides. According to Marutoiu and Soran report (2010), the peptides which are composed of hexa- and penta-amino acids are visualized under UV-light after spraying with 4-benzenediazonium hydrazine in 1 M Na2 CO3 (named Paul’s reagent) or ninhydrin (Marutoiu and Soran, 2010). The cyclic peptides are visualized by the use of 20% trichloroacetic acid. The tri- and tetra-peptides are identified under UV-light or by spraying with ninhydrin or iodine vapors or by the use of Sakaguchi reagent (Marutoiu and Soran, 2010). Among the numerous visualizing reagents, which are very popular in peptide detection, a new alternative is the Morin’s reagent (3,5,7,2’,4’-pentahydroxy fl avone) in methanol solution, which forms with peptides dark absorption spots (Marutoiu and Soran, 2010).

VIII. ENANTIOMERIC SEPARATION OF AMINO ACIDS BY HPLC METHOD

Developments in column technologies, modification of stationary phase and modern instrumentation of RP-HPLC method during the last decade show that at present it is a main technique used in resolution of amino acid derivatives and racemates. HPLC offers a short time analysis and high resolution (Papadoyannis and Theodoridis, 2010; Silverman and Christenson, 1996; Matthew and van Holde, 1995). Separation of amino acid enantiomers by the use of HPLC was summarized by Yoshi (1993). Our review paper included the selected literature mainly from 2000 to April 2013, surveying HPLC resolution of amino acids and their derivatives from various samples.

Two main strategies have been developed for the HPLC separation of amino acids and their analogs: an indirect method and a direct method. The indirect approach includes co-nversion of amino acid e enantiomers into their diastereomeric isomers using a pure derivatization reagent before HPLC analysis. This method is used when the enantiomer has an easy derivatization functional group such as carboxylic, c arbo nyl, amino or alcoholic. M any t ypes of de-rivatization r eagents suitable f or the r esolution o f enantiomers are described in the literature (Ilisz et al., 2008; Papadoyannis and Theodoridis, 2010). They form with the amino acids various compounds: amides, c arbo nates, ureas and thioureas. The most frequently used in enantiomeric analysis of amino acids are fl uoro mino acids such as phenylthiohydantoin (PTH), methylisothiocyanate, methylthiohydantoin, o-phenthaldehydes (OPA), (S)-N-(4-nitrophenoxycarbonyl) phenylalanine methox yethyl ester [(S)-NIFE], 1-fluoro-2, 4-dinitrophenyl-5-L-alanineamide (FDAA or FDNP-Ala-NH2) named Marfe y’s reagent (Papadoyannis and Theodoridis, 2010). The chio ice of chro matographic s ystem from both (direct and indirect) d ep ends on the type of the samp le to be analyzed (b io logical or r e pharmaceutical). Indirect chromatography is not suitable for pharmaceutical analysis of amino acids, but is very efficient in enantiomeric resolution of amino acids in complex matrices such as biological samples.

IX. INDIRECT ENANTIOMERIC SEPARATION OF AMINO ACIDS BY HPLC METHOD

Derivatization of amino acids for HPLC separation, many reports and books suggest that HPLC enantiomeric analysis of free amino acids and amino acids from peptides can be improved via the derivatization process. The method of derivatization depends on various factors such as sample size, time of the analysis, specific equipment and application requirements. Derivatization of amino acids can be provided via two systems: precolumn and postcolumn.

The main reactions used in the derivatization process are based on formation of the following compounds: amides, carbamates, ureas and thioureas (Ilisz et al., 2008; Brückner, 2004, 2011). Péteret al. (2000a) developed a new chiral derivatizing agent designated by (S)-NIFE for proteinogenic amino acid analysis by HPLC. Excellent resolution of 19 unnatural secondary α-amino acids and their analogs was achieved using a reversed-phase mobile system on a LiChrospher RP18 column, 150 x 4 mm. The chromatogram was equipped with a UV – vis detector. It was stated that (S)-NIFE is suitable for indirect separation of stereoisomers of amino acids by means of RP-HPLC method I water–acetoni trite or water – methanol mobile phase containing 0.1M TFA (trifl uoroacetic acid). The elution order of these compounds was found to be L < D (Péter et al., 2002).

Among the derivatization agents, Marfe y’s reagent is a very interesting, because it allows successful resolution of DL-amino acids from a mixture. Application of Marfe y’s reagent for chiral amino acid analysis was described by Brückner (2011) in review papers. Derivatization with different variants of Marfe y’s reagent (FDNP-L-Ala-NH2 or in combination with Val, Phe, Leu and Pro) was applied for assay of baclofen enantiomers in pharmaceutical preparations (tablets) using HPLC and RPTLC methods. HPLC quantitative analysis of baclofen in tablets was performed on a C18 column (250 x 4.6 mm) using a linear gradient of acetonitrile and 0.01% TFA with UV detection (Kumar, 2008a).

Another derivatizing agents from isocyanate and isothiocyanate groups such as 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate, 2,3,4,6-tetra-O-benzoyl-β-D-glucopyranosyl iso-thiocyanate and also
promising antibiotics as chiral selectors in the form of chiral bonded phases in HPLC. Different mechanisms of the separation of amino acid enantiomers (e.g., hydrogen bonding, electrostatic, van der Waals etc.) on macrocyclic glycopeptide stationary phases and the physicochemical properties of macrocyclic antibiotics used as the chiral selectors were reported by Ilisz et al. (2006a).

A macrocyclic antibio tic ristocetin A was used as chiral stationary phase f or HPLC separa ti on of amino acids (analogs of phenylalanine, tyrosine and tryptophan). Two mobile phase systems were used: reversed phase mode and new polar-organic mode. The reversed phase mobile phase was water purified by Mili-Q and methanol. The polar-organic mode mobile phase was prepared by mixing methanol, HOAc (acetic acid) and TEA (triethylamine). The column Chirobiotic R bonded with ristocetin a (250 x 4.6 mm) was used. The HPLC system was equipped with a photodiode-array detector (Pété r et al., 2000b).

Another glycopeptides-bonded chiral stationary phase, which was successfully applied for direct chromatographic resolution of carnitine and O-acetyl carnitines, is a chromatographic column (250 x 4.6 mm) packed with LiChrosorb Si 100, 5 μm, modifi ed with teicoplanin. The recomates of carnitine and its derivatives were eluted with mobile phases containing one of the organic solvents, methanol, ethanol, and acetoniitrile and ammonium acetate. Evaporative light scattering and optical rotation of detections were used in this analysis (D’Acquarica et al., 1999). Direct separation on stationary phase containing teicoplanin (Astec Chirobiotic T and T2), teicoplanin aglycone (Chirobiotic TAG) and vancomycin aglycone (Chirobiotic V) as chiral selectors was obtained for enantiomers of selected β-amino acids containing isoxazoline moiety with known antibacterial activity: cispentacin, icofungipen and orzyoxymycin. HPLC was carried out on a chromatograph equipped with a photodiode-array detector. The separation was achieved in reversed-phase mode using 0.1% TEAA (triethylammonium acetate, pH 4.1) – methanol as mobile phase in different compositions (Sipos et al., 2012).

Berkeczet et al. (2009) applied the above-mentioned chromatographic conditions for direct separation of β-amino acids from derivatives of 2-amino mono- and dihydroxycyclohexanecarboxylic acid. HPLC was equipped with a photodiode-array detector. It was stated that Chirobiotic TAG (containing teicoplanin aglycone) and Chirobiotic T (containing ristocetin) are most useful for these investigations.

Another work showed that chiral stationary phase containing teicoplanin and teicoplanin aglycone was used by Pataj et al. (2009) for HPLC enantioseparation of β 2-homo amino acids by using 0.1% TEAA (pH 4.1) – methanol at different temperatures and various compositions. HPLC measurements were carried out on HPLC system equipped with a photodiode-array detector. The results indicated that the elution sequence of enantiomers was R < S.

In another paper Péter and coworkers (Péter et al., 2004a) performed a similar enantioseparation of 18 unnatural β –
amino acids by HPLC. In this case, both direct and indirect reversed-phase HPLC methods were developed. Of all applied macrocyclic chiral selectors, the most suitable for the separation of β-homoamino acids was native teicoplanin. The indirect HPLC was performed with a pre-column and derivatization using (S)-NIFE. It was suggested that the indirect method gave better results for the separation than the direct method.

Ilisz et al., (2006b) described direct HPLC enantioseparation of unusual secondary amino acids using HPLC method on a D-penicillamine-based column (150 × 4.6 mm). The HPLC was equipped with a photodiode-array detector. The results indicated that in most cases the S isomer was eluted before the R.

A new approach in enantiomeric separation of amino acids and di- and tri-peptides on teicoplanin stationary phase (Chirobiotic T) is to use HPLC/APCI/MS (HPLC combined with atmospheric pres-sure ionization mass spectrometry). A mobile phase containing 1% ammonium tri fluoroacetate in ethanol −0.1% formic acid in water improved the sensitivity of this method. It was suggested that the developed method can be an alternative to the traditional techniques, especially for the peptides of molar mass ≥300 Da (Desai and Armstrong, 2004).

In recent years, a new type of HPLC column (coated dynamically by the use of nonionic RP 18 and vancomycin-derivatives as chiral selector) has been widely applied in enantioseparation of dansyl amino acids by HPLC (Pittler and Schmid, 2010). HPLC analysis was performed on the commercially available monolithic column Chromolith (RP 18, 100 × 4.6 mm). A mixture of 0.1% TEAA (pH 6) – methanol in various volume compositions, e.g. 50:50 (v/v), was used. Detection was made under UV (Pittler and Schmid, 2010). Another approach is the use of D-penicillamine-Cu (II) complex as a chiral selector to chromatographic separation of cycliβ substituted α-amino acids (Schlauch et al., 2000).

Moreover, it was stated that the chromatographic resolution of β3-homoamino acids depended on the nature of amino acids, and the concentration of alcoholic and acidic modifiers (Berkecz et al., 2001).

Next a chiral stationary phase based on optically active (3,3′-diphenyl-1,1′-binaphthyl)-20-crown-6-covalently bonded to silica gel was successfully utilized for the resolution of different β-amino acids using HPLC. Chromatography was performed with an HPLC system equipped with an absorbance detector.

Farkas et al., (2006) reported a new and very interesting variant of enantiomeric separation of different aromatic amino acids on chiral stationary phase containing crown ether. In this case a new stationary phase, (S, S)-dimethylpyrirdino-18-crown-6-ether bonded, was applied for resolution of aromatic amino acid mixture in form of hydrochloride salts.

The next types of stationary phases used in direct amino acid separation are polysaccharide-bonded stationary phases Po lysacc haride-type stationary phase was effective for chiral separa-tion of selected pharma ceutical impo rtant amino a cids belonging to proline derivatives (boc-proline, boc-2-methylproline, boc-2-methylproline benzyl ester and boc-2-methylproline-4-hydroxyproline benzyl ester). HPLC analysis was performed using a Chiralpak AD-H column and diode-array detector. For proline containing carboxyl or hydroxyl group, the resolution was changed corresponding to changes as small as 1% of ethanol in the mobile phase used. A small amount of ethanol in hexane may have caused significant cant con firmation changes of the chiral stationary phase applied (Zhao and Pritts, 2007).

Wenget et al., (2006) indicated that the amino acid enantiomers can be successfully separated on polysaccharide-based stationary phase (commercial Chiralcel OD-H column) by means of HPLC analysis in a normal-phase system. A mixture of n-hexane – polar alcohol was applied as mobile phase. The in fl uence of different parameters including amount of alcohol for the analysis was discussed in this paper (Wenget al., 2006).

Another polysaccharide used as a chiral selector in direct separation of the amino acid mixture is amylose. It was stated that a direct purification of four amino acid isomers in the form of derivatives was achieved on an amylose-based stationary phase (Chiralpak AD-H column; Nogle et al., 2006).

Among the related chiral selectors tested for enantiomeric resolution of the amino acids, good results were observed on a quinine-derived chiral anion exchange stationary phase. A quinine-derived weak anion exchange was successfully applied or the enantiomeric separation of apolar β-amino acids (e.g. β-substituted-β-alanines). Before HPLC analysis the amino acids were converted to N-2,4-dinitrophenyl (N-2,4-DNP) or N-3,5-dinitrobenzoyl (N-3,5-DNB)-derivatized form (Péter et al., 2002). The derivatized β-amino acids were detected at 360 or 250 nm.

In another paper presented by Török et al., (2006), the thermo-dynamic study of enantiomeric separation of α-substitutedglycine analogs on a quinine-based anion exchange chiral stationary phase was studied. The HPLC measurements were carried out on an HPLC system containing photodiode array. The amino acids were analyzed in the form of the respective derivatives: N-benzylcarboxyl, N -3,5-dinitrobenzoylcarboxyl, N -benzoyl and N-3,5-dinitrobenzoyl. A mixture of ammonium acetate buffer–organic solvent was used as a mobile phase. The thermodynamic parameters depended on the structure of the examined amino acids. Separation of Gly and analogs can be optimized quickly by adjusting the column temperature.

Nowadays, a new developed cinchona alkaloid derivative-based stationary phase is widely applied for the peptide enantiomeric separation. Under these conditions, an excellent separation of tripeptides is possible. Quantification limit and detection of separated amino acids were determined by means of MS detector (Czerwenka et al., 2005).

It was reported that cyclodextrin-bonded phase was efficient for enantiomeric separation of di- and tri-peptides after their prior derivatization using 9-fluorenylmethyl chloroformate. Chromatographic investigations of peptides were carried out on an HPLC column packed with α-, β- and γ-cyclodextrin bonded to silica gel and using
The use of chiral selectors added to mobile phases enabled successful separation of different amino acid enantiomers. Enantiomeric separation of racemates is based on various mechanisms: formation of labile diastereomeric complexes with different elution properties, complex reaction between chiral selector and stereoisomers in mobile phase and adsorption of chiral selector on stationary phase (Joshi, 1993).

Cycloexetrins are one of the most important chiral selectors used as additives to mobile phases. They can be either added to the mobile phase or bonded to stationary phase. Modification of the CD surface influences the enantioselective separation of various amino acids (Waldhier et al., 2009).

Other chiral selectors important for amino acid separation are metal chelate additives to the chiral ligand exchange system. Davy and Fr a n c i s (1987) described the use of the complex L-prolyl-n-octyl amide - N (II) in the mobile phase for direct resolution of the enantiomers of fluazifop and other phenoxypropionic acids with HPLC. It was suggested that, of all parameters, only temperature had a major effect on the retention of the fl uazifop and other phenoxypropionic acid enantiomers. Organic phase modifier or concentration had less effect on the chiral resolution.

Another work used bis-(L-amino acid amidato) copper (II) complexes as chiral eluents in amino acid enantiomeric separation in the form of DL-dansyl derivatives by HPLC. HPLC analysis was performed on a chromatograph equipped with fl uorescence detector. A Novapack (C18) 15 x 0.4 cm column was used (Armani et al., 1988).

HPLC with the use of copper (II) acetate and N,N-di-n-propyl-L-alanine (L-DPA) as a chiral mobile phase additive was effectively used for the separation of α-amino acids (α-AA) and α-amino acid amides (α-AA-NH2). The chromatographic analysis was performed on different C18 columns (Nucleosil, Polygosil) and on Hypersil ODS. The HPLC system was equipped with a fluorescence detector. The mobile phase consisted of copper (II) acetate, L-DPA and TEA in different volume compositions. The results indicate that the copper complex of L-DPA and addition of Cu (II) ions to the mobile phase will cause a decrease in the retention of the examined α-amino acids and their amides (Duchateau et al., 1989). The detection limit of the investigated compounds in the form of α-AA and α-AA-NH2 was expressed in picomoles.

Similar investigations of the enantioselective separation using L-proline and copper as chiral eluent additives were presented in the case of several α-ornithine analogs. HPLC procedure was performed on a C18 column using a fluorescence detector. The results presented the usefulness of the HPLC method for the separation of different substituted ornithine and lysine analogs using L-Pro/Cu(II) and a reversed-phase column. The chiral eluent HPLC procedure is suitable for semipreparative resolution of various ornithine analogs as ornithine decarboxylase inhibitors in vitro and in vivo (Wagner et al., 1987).

The effect of mobile phase composition on the chromatographic resolution of tryptophan enantiomers on a Leu-Leu-Leu-C18 column using mobile phase containing Leu-Leu-Leu peptide was described by Kaufman et al., (2000). A Chiral HPLC method was performed on an ODS CAP column (100 x 4.6 mm) packed with Leu-Leu-Leu peptide. A mobile phase containing 1mM phosphate buffer (pH 6.3)–0.1 mM DPA and other phenoxypropionic acids was used as mobile phases (Laod and Gani, 2009).

**XI. Separation Using Chiral Mobile Phases**

The use of chiral selectors added to mobile phases enabled successful separation of different amino acid enantiomers. Enantiomeric separation of racemates is based on various mechanisms: formation of labile diastereomeric complexes with different elution properties, complex reaction between chiral selector and stereoisomers in mobile phase and adsorption of chiral selector on stationary phase (Joshi, 1993).

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synchronous determination of proline and hydroxyprolinol ecounters in mammalian samples. By using a 2D-HPLC system equipped with a microbore-ODS column (500 x 0.53 mm) and two mobile phases: fi rst MeCN–TFA–water (2.0:0.2:98, v/v/v) and second Me CN–TFA–water (22.0:0.2:78, v/v/v) and by means of photoreactivity detection, successful determination of D-enantiomer of proline and hydroxyproline in the serum and collagen-rich skin was performed. D-Amino acid enantiomers were analyzed after prior derivatization with NBD-F (Tojo et al., 2008).

XII. GAS CHROMATOGRAPHY IN AMINO ACID AND PEPTIDE SEPARATION

Gas chromatography is one of the most important chromatographic methods in enantiomeric separation of a variety of classes of compounds. The main application of the separation of enantiomers by GC is connected with precise determination of enantiomeric composition of important chiral compound groups in nature including amino acids and peptides (Schurig, 2000; Czerwenka and Lindner, 2005). The GC method has been widely applied to biological and pharmaceutical samples because, in comparison to HPLC technique, it needs less expensive equipment and short analysis time. Gas chromatography is especially useful in the case of compounds containing polar groups, which require high column temperature.

Similarly, as in the case of HPLC technique, there are two strategies used in GC enantioseparation of amino acids and peptides: an indirect method and a direct method.

Direct GC enantioseparation of amino acids and peptides in both enantioselective GC methods, the main role in analysis of physiological fi ltrate of mammals involves a direct method. Prior to direct GC analysis the amino acids have to be chemically derivatized into volatile and stable compounds, suitable for GC resolution. A variety of derivatizing reagents are used for this purpose. The methods used in derivatization process are divided into two main groups (Zenkevich, 2010):

- derivatization of the functional groups – carboxylic (-COOH) and also amino (-NH2) separately;
- Protection of both groups by the respective reagent.

Zenkevich (2010) reported that the main amino acid derivatives obtained using the fi rst method belong various esters such as methyl, ethyl, iso-propyl, sec-butyl, amyl and iso-amyl of various N-acyl (acetyl, tri fi l uroacetyl, penta fi l uropropionyl, heptafl uorobutyryl) amino acids.

The bove-described process of derivatization of amino acids into the r and s stereoisomers can be performed in one or two stages. A very important in the derivatezation method is the basic reaction with methyl or phenyl isothiocyanate nates is not stable in the presence of thiophenyl hydantoins. Another important method of single-s tage amino acid derivative formation is the introduction of N(O,S)-tert butyldimethylsilyl derivatives using tert-butyldimethylsilyl trif l uoracetamide. This method was presented by Zenkevich (2010).

The direct method of peptide enantiomeric separation studies using GC is performed on various chiral stationary phases. Application of respective stationary phase in GC is limited by temperature stability. The chiral stationary phases used in GC are classified on the basis of the interaction between the chiral selector and the investigated compound into the following groups (Schurig, 2000; Scott, 2010):

- chiral metal coor dination com plexes (based on hydrogen bonding), e.g. commercially available Chiralsil-L-Val column or OV-225. In this case derivatization of separated substances (amino acids) to increase their volatility is required;
- chiral metal coor dination complexes (based on formation of inclusion complex), e.g. Chiralsil-β-Dex and Chiralsil-γ-Dex columns.

Helium and hydrogen are usually used as the carrier gases in GC enantiomeric separation. Quantitative GC measurements are performed with the use of flame ionization detection (GC-FID) or selected ion monitoring mass spectrometry (GC-MS-SIM) as the high selectivity module detection. GC is very useful in determination of various amino acid enantiomers on a small scale with good efficiency after their prior derivatization. Among various derivatizing reagents, the most commonly used are alkyl chloroformates or anhydrides, which react very rapidly with primary and secondary amino groups (Waldhier et al., 2009). Interesting variants of the derivatizing agents are those which produce fl uorinated amino acid derivatives. They offer, as reported Waldhier et al. (2009), the highest sensitivity with detection limit in the lower range (picomoles).

Several works have focused on direct GC enantioseparation of various amino acids and peptides with the use of the above-mentioned derivatizing reagents. The one-step derivatization reaction using alkyl chloroformate–alcohol–pyridine as a reagent to obtain N(O,S)-alkyl alkoxy carbonyl esters of amino acids was suitable for resolution of 20 proteinogenic amino acids in their D- and L-enantiomeric form and glycine. Enantiomeric pairs of 19 amino acids and glycine were separated after their derivatiza-tion with chloroformate and hepta fi l uro-1-butanol to obtain N-metoxycarbonyl-hepta fl uorobutyryl esters of amino acids on different combination of Chiral-L-Val column (25 x 0.25 mm). The column temperature was from 90 to 180 °C at the rate of 4 °C/min. On the studied phase, the D-form was eluted first. Methyl laurate was selected as the internal standard. Helium GC-MS analysis was carried out on a QMD 1000 GC-MS system. The carrier was helium. The procedure was fast and enabled the enantiomeric separation of mixture containing glycine and 16 enantiomeric pairs of amino acids: Ala, Val, Pro, Ile, Leu, Asp, Thr, Asn, Met, Cys, Glu, Phe, His, Lys, Tyr and Trp (Zampolli et al., 2007).

Zahradnicková et al. (2007) developed a simple and rapid GC-MS and GC-FID method for determination of D- and L-amino acids produced by cyanobacteria, which may alter biological activity and the safety of human food. Identification of these amino acids in natural waters was
conducted after preliminary derivatization of these compounds with propyl chloroformate as a derivatizing reagent. For chiral measurements, to achieve more volatile compounds, the amino acids were converted to corresponding N,O-pentafluoropropionyl pentafluoropropyl esters. Sep-aration of the above-mentioned esters was performed on a Chirasil-L-Val column (25 × 0.25 mm). Hydrogen was used as a carrier gas. The oven temperature was in the range from 80 to 200 °C. A GC-MS DSQ quadrupole instrument was equipped with electron ionization.

Derivatization of amino acid enantiomers with pentafluoropropyl chlorofluorone on chiral column Chirasil-L-Val was successfully applied for GC-FID analysis of DL-amino acids (pharmaceutically important, e.g. Carbococin), which stimulates lactation. This method can be suitable for rapid determination of optically purity pharmaceutical importance peptides (Zahradnicková et al., 2008).

Another paper shows that conversion of amino acids to N(O)-pentfluoropropanoyl 2-propyl esters is useful in separation and quantification of D- and L-amino acids in food chemistry, for example in wines and sparkling wines. GC-MS-SIM of amino acids in bottled wines in form of N(O)-pentafluoropropionyl 2-propyl esters was performed on a capillary column Chirasil-L-Val. This method is used in classifi cation and proof of au-thenticity of wines and juices or in determining of the correla-tion between storage time of bottled wine and quantities of D-amino acids in wine (Mohammed Ali et al., 2010). Hasegawa et al., (2005) performed stereoselective determination of the endogenous and labeled D- and L-enantiomers of methionine and [2H3] methionine in rat plasma with the use of GC-MS-SIM. The amino acids were derivatized to methyl esters and then N-acetylated with the use of optically active (+)-α-methoxy-α-trifluoromethylphenylacetyl chloride to form diastereoisomeric amides (Hasegawa et al., 2005). Separation of those amino acid enantiomers was performed on a methylsilicone-bonded phase fused-silica capillary column SPB-1 (15 m × 0.25 mm). Helium was used as a carrier gas. It was stated that the described method can be used in metabolic studies of endogenous amino acids and conversion of D-methionine to L-enantiomer in vivo. GC-MS-SIM method and isotopically labelled analog of serine DL-[2H3] as an internal standard was successfully applied to determine the serine enantiomers in biological fluids, for example, in plasma using Mosher’s reagent. D-Serine is present in mammalian brain and it is proposed as a potential therapeutic agent for schizophrenia. After purification by cation-exchange chromatography using a BondElut S CX cartridge, serine was derivatized with Mo sher’s reagent–optically active (+)-α-methoxy-α-trifluoromethylphenylacetic acid to form epimeric amide. GC-MS analysis was performed using quadrupole with selected ion monitoring (GC-MS-SIM) on an SPB-1 column (15 m × 0.25 mm). Helium was used as a carrier gas (Hassegawa et al., 2011).

The enantioselective analysis was also applied for detection of selected amino acid enantiomers from water samples. Eighteen biogenic amino acids converted to alkyl esters (methyl and propyl) of N-TFA amino acids were studied on three different chiral stationary phases: Chirasil-L-Val (l-valine-tertbutylamide coupled to 2-carboxypropyl-methylsiloxane), G-PROP [octakis (2,6-dipentyl-3-propionyl)-γ-cyclodextrin] stationary phase and also on G-BUT (octakis-2,6-dipentyl-3-butyryl-γ-cyclodextrin) to achieve optimal resolution of amino acids and their enantiomers. GC was performed using a gas chromatograph equipped with FID. Hydrogen was used as a carrier gas. The best results of the enantiomeric separation were obtained on a Chirasil-L-Val column (Spanik et al., 2007).

An automated GC-system was used for the enantiomeric separation of natural and non-proteinogenic amino acids and hydrolysates of proteins and peptides from cow and human milk. Food samples were hydrolyzed using 6M HCl. Then, amino propyl and methyl esters of amino acids were acylated into tri fl uoroacetyl or isopropylchloroformate derivatives. The separation was performed on Chirasil-L-Val and Chirasil-γ-Dex columns. Hydrogen was used as a carrier gas. Detection was by flame ionization. The temperature of analysis was 67 – 200 °C (Nokihara and Gerhardt, 2001). The presented method can be used, for example, in brain tissue analysis of phospholipids.

In another paper, to provide rapid enantiomeric separation by non-chiral GC of a mixture of 20 proteinogenic amino acids, which were converted by means of ethyl chloroforulinate and 2-chloropropanol into N(O,S)-ethoxy carbonyl-2-chloropropyl esters, GC analysis was performed on a chiral column (Chirasil-L-Val, 25 m × 0.25 mm) and a non-chiral column (CP-Sil 19CB from Varian, 30 m × 0.25 mm). Helium was used as a carrier gas. The oven temperature in the case of the fi rst column was from 70 to 180 °C. For the second one it was set at 100 °C and programmed to reach 275 °C. Selected ion monitoring was used as a detection mode. The best resolution was obtained after derivatization of amino acid mixture with ethanol and ethyl chloroforulinate on a Chirasil-L-Val column. This method allows separation of 14 enantiomeric pairs of 19 pairs and it can be used in many fi els, for example, in food analysis (Bertrand et al., 2008).

Reiner et al., (2007) showed accuracy and precision in the determination of enantiomeric fraction of DL-α-amino acids by using GC of their N(O)-tri fl uoroacetyl/ethyl ester derivatives on various chiral stationary phases: Chirasil-D-Val, Chirasil-L-Val and prepared in-house Lipodex E – octakis-3-O-butanol-2,6-di-O-n-pentyl-γ-cyclodextrin in PS255 (dimethylpolysiloxane, 20 cm × 0.25 mm). Gas chromatographic measurements were carried out using a gas chromatograph equipped with FID and MS detection systems. Hydrogen was used as a carrier gas in the case of FID detection. For GC-MS analysis helium was used. The choice of the derivatives is related to the choice of stationary phase that is used. Of all applied chiral
stationary phases, the best resolution of all α-amino acid enantiomers was obtained in the form of their ethyl esters on 30% Lipodex E–70% PS 255.

Thermodynamic investigations of the enantioseparation of N-ethoxy carbonylpropylamide of the selected amino acids vs N-trifluoroacetyl ethyl esters on the diamide-type chiral stationary phase, Chirasil-L-Val-C 11 with N-ethoxycarbonyl propylamide were performed. Hydrogen was used as carrier gas. GC was equipped with FID. It was found that the decrease of temperature to ~120°C resulted in complete loss of enantioselectivity of Chiral-L-Val column for all the analyzed amino acids except proline. It was observed that the further decrease of temperature restored the enantioselectivity of Chirasil- L-Val-C 11 for all amino acids and inversion of elution order was observed. Below 120 °C, L was after D. Based on the obtained results, the conclusion was that, in order to optimize the enantimetric separation of racemate mixtures using GC, it is recommended to conduct temperature-dependent studies (Levkin et al., 2007).

To enable simultaneous determination of various D- and L-amino acids in biological fluids (cerebrospinal fluid) two chromatographic techniques were performed: non-chiral and chiral GC (Chirasil- L-Val column, 25 m x 0.25 mm). Derivatization with the use of Marfey’s reagent and LC-MS analysis was applied. Both methods were validated for the determination of D-serine, L-serine and glycine in cerebrospinal fluid. After extraction from biological fluids, the amino acids were converted into N-(O)-trifluoroacetylpropyl ester derivatives. The GC analysis was performed using helium as a carrier gas. The temperature oven was in the range from 80 to 190 °C. The MS system was run in SIM mode. Comparison of the results obtained from GC-MS-SIM analysis with those obtained by LC-MS shows that both techniques correlated well, and separation of small quantities in clinical laboratories of amino acids in biological samples by use of both chromatographic methods was similar but, the time of preparing biological samples in the case of GC-MS was longer (6–8 h). It was concluded that, in medical laboratories without LC-MS, the GC method is a very suitable alternative (Fuchset al., 2008).

Effective GC separation of 20 proteinogenic amino acid enantiomers was achieved via derivatization with heptafluorobutyryl chlorofromate (HFBCF) on a Chirasil- L-Val column including ornithine, cystine and 4-fluorophenylalanine (as an internal standard). Nineteen amino acid enantiomers were ef ficiently separated in 43 min except proline, arginine and cysteine. Resolution of the HFBCF derivatives of the studied amino acids shows the improvement of the DL-amino acid separation (Zahradnicková et al., 2009).

XIII. INDIRECT GC ENANTIOSEPARATION OF AMINO ACIDS AND PEPTIDES

Indirect enantioselective amino acid analysis by GC is the method that requires prior conversion of the amino acids into their volatile diastereoisomeric derivatives. This is possible by introducing a second asymmetric center into analytes via estri fi cation of the COOH group in amino acid with the use of (S)-2-butanol in the presence of TFAA (Waldhier et al., 2009). Similarly to Waldhir, Zenkevich (2010) reported that, in the case of enantiomeric separation of amino acids into D- and L-form using nonchiral phases, their conversion into diastereoisomers with the use of optically active alcohol such as (R)- or (S)-2-BuOH, 2-AmOH, pinacolol or ( )-menthol or using acylation of amino group by chiral reagents, for example, α-methoxy-α-trifl uoromethylphenylacetyl chloride, N-trifluoroacetyl- L-prolyl chloride or N-trifluoroacetylthiazolidine-4-carbonyl chloride, enables enantiomeric separation of amino acids by the use of indirect method on a nonchiral column.

Pätzold et al., (2006) presented an example of the application of direct and indirect methods for enantioseparation of selected amino acids by GC. In this work, several synthetic dipeptides containing a mixture of enantiomers and diastereoisomers were analyzed by GC-MS-SIM on the chiral stationary phase: Chirasil-L-Val and Lipodex®-E (25 x 0.25 mm) at 200 °C using helium as a carrier gas after conversion into theirN-perfluoroacetyl dipeptide esters: TFA, pentafluorocetyl, heptafluorobutyryl, m et h yl, 1- pr opyl, 2-propyl, 2,2,2-trifluoroethyl esters. Next derivatization of investi-gated peptides into their N-trifluoroacetyl dipeptide S(+)-2-buty1 esters enabled further separation of compounds on an achiral phenylmethylpolysiloxane capillary column (HP-5MS). Compari-son of the resolution of TFA and pentafluoroacetyl methyl esters of the examined dipeptides showed the best separation on Chirasil®- L-Val (Pätzold et al., 2006). Comparison of both indirect and direct methods shows that of the two enantioselective GC methods only the direct method is suitable for enantioseparation of amino acids in biological samples.

XIV. CONCLUSIONS

The selected literature review relating to study of enantiomeric separation of various amino acids indicates that chromatographic techniques such as TLC, HPLC and GC play a crucial role in resolution of various D- and L-amino acids and peptide enantiomers from their racemates, because they are very sensitive to a detail. The choice of the chromatographic method used for separation and quantitative determination of various amino acids extracted from human samples (brain, serum) or those present in plant and food depends on the kind of amino acid, available instrumentation and needed sensitivity. Moreover, the literature study on enantiomeric separation of various amino acids conducted in this paper indicates that the increasing development of the chiral systems used in stereoisomeric analysis of amino acids enables direct separation of amino acid racemates using all chromatographic techniques, but especially HPLC and GC. Newpossibilities in chiral amino acid analysis using chromatographic methods were opened up by the application of MS and IR detection in TLC and MS-SIM detection systems in GC. A modern trend in high-
performance chromatography is two-dimensional chromatography. This innovation leads to decreased development time, increased amino acid resolution and detectability of amino acids in picomoles and femtomoles.

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