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Enantioseparation of underivatized amino acids by capillary liquid chromatography. 1. Background on chiral separations

Enantioseparação de aminoácidos não derivatizados por cromatografia líquida capilar. 1. Antecedentes das separações quirais

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Abstract

The need to separate chiral compounds, that is, those with an asymmetric carbon linked to four different atoms or chemical groups, to later be used in the pharmaceutical, biological, biochemical areas, among others, led to the development of chiral chromatographic techniques. Chiral chromatography usually employs a solid chiral stationary phase with a non-chiral mobile phase. This configuration allows us to separate a racemic mixture in its pure enantiomers. The goal of this separation is that in many cases, a chiral compound's biological activity depends on a certain enantiomer. A historical case of this fact was thalidomide, where one enantiomer has a sedative effect, while the other isomer was responsible for one of the biggest medical mistakes in the world when it takes about 10,000 children to have congenital disabilities. This review aims to present and discuss the main chiral solid stationary phases employed in this type of chromatography. This includes the "brush type" phases, which are based on the bonding of chiral groups to the silica surface, the polymeric phases based on cellulose and amylose, the helical polymeric phases, the cavity-type phases based on cyclodextrins, crown ethers and macrocyclic antibiotics, and, the ligand exchange phases.

Keywords: Enantioseparation, chiral compounds, capillary liquid chromatography, amino acids.

Resumo

A necessidade em separar compostos quirais, ou seja, aqueles que possuem um carbono assimétrico ligado a quatro átomos ou grupos químicos diferentes, para posteriormente serem utilizados nas áreas farmacêutica, biológica, bioquímica, dentre outras, levou ao desenvolvimento de técnicas cromatográficas quirais, que empregam comumente uma fase estacionária sólida quiral com uma fase móvel não-quiral. Essa configuração permite separar uma mistura racêmica em seus enantiômeros puros. O objetivo dessa separação é o fato de que, em muitos casos, a atividade biológica de um composto quiral depende de um determinado enantiômero. Um exemplo é o uso da talidomida, onde um enantiômero possui efeito sedativo, enquanto o outro foi responsável por um dos maiores erros da medicina no mundo, ao levar cerca de 10.000 crianças a terem desabilidades congênitas. Nesse contexto, o objeto desse trabalho consiste em apresentar as principais fases estacionárias sólidas quirais empregadas nesse tipo de cromatografia, como as do tipo "brush", que são baseadas na ligação de grupos quirais a superfície da sílica; as fases poliméricas a base de celulose e amilose; as fases de polímeros helicoidais, as fases baseadas no tamanho e seletividade de cavidades como em ciclodextrinas, éteres de coroa e antibióticos macrocíclicos, e as fases baseadas em troca de ligantes.

Palavras chaves: Enantioseparação, compostos quirais, cromatografia líquida capilar, aminoácidos.

1. Introduction

This manuscript is the first of two reports on the separation of enantiomeric amino acids employing a home-packed capillary liquid chromatography column. The first part will discuss some background involving the chirality concept, moving towards the main approaches to separate chiral amino acids. The second part will present our experimental results referring to the actual separation of selected chiral amino acids using an approach termed chiral ligand-exchange chromatography in a capillary liquid chromatography column.

1.1. The concept of chirality

Chirality (from the Greek word χειρ (*kheir*), "hand") [1] is a property of asymmetry valuable in several branches of science. An object or a system is *chiral* if it is distinguishable from its mirror image, meaning it cannot be superimposed onto it. On the other hand, a mirror image of an *achiral* object, such as a sphere, cannot be distinguished from the object. A chiral object and its mirror image are called *enantiomorphs* (from the Greek, "opposite forms"); in the case of chemical substances, they are termed enantiomers. A non-chiral object is termed *achiral* or *amphichiral*, being superposed onto its mirror image.

It has been considered that the term was first used in 1894 as [2]: ... *"I call any geometrical figure, or group of points, 'chiral,' and say that it has chirality if its image in a plane mirror, ideally realized, cannot be brought to coincide with itself."*

However, it has to be pointed out that before Kelvin, at the beginning of his career, Luis Pasteur studying tartaric acid (Figure 1) and its salts, developed a good understanding of the subject as accepted now. In 1848 (almost 50 years before Kelvin's presentation), Pasteur separated by hands enantiomorphous crystals of racemic sodium ammonium tartrate [3-6].

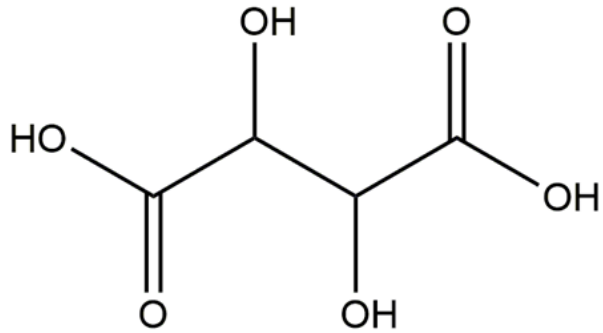

Figure 1. Tartaric acid chemical representation.

Pasteur's resolution immortalized his name in the area of chemistry. His discovery that one of the racemic acid salt forms consists of two optically active isomeric constituents (Figure 2) formed the basis for the born of stereochemistry, the study of the spatial arrangement of atoms in molecules. Pasteur assigned their activity to the molecular asymmetry [7]. This resolution belongs to a small group of classic experiments that radically changed our view of the world and opened up new research paths, yet are simple enough to be duplicated by a skilled undergraduate student [7-9].

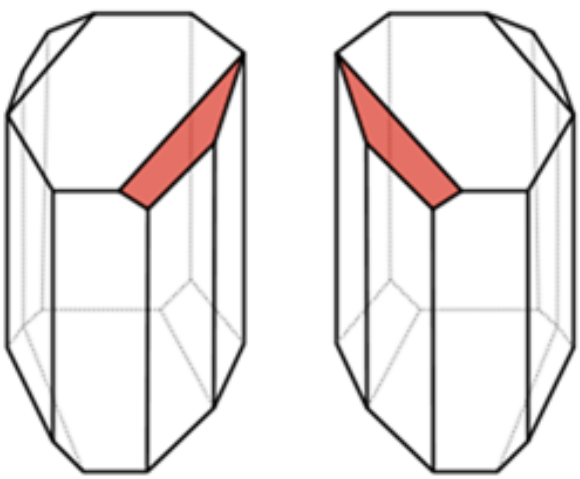

Figure 2. Enantiomorphous crystals of racemic sodium ammonium tartrate separated by Louis Pasteur in 1848 [4].

Human hands are perhaps the most universally recognized example of chirality. The left hand is a non-superimposable mirror image of the right hand; no matter how the two hands are oriented, all the significant features of both hands cannot coincide across all axes [10]. This difference in symmetry becomes obvious if someone attempts to shake the right hand of a person using their left hand, or if a left-handed glove is placed on a right hand. In mathematics, *chirality* is the property of a figure that is not identical to its mirror image. A molecule is said to be chiral if its all valence is occupied by different atom or group of atoms.

A *chiral molecule* has a non-superposable mirror image. The feature that is most often the cause of chirality in molecules is the presence of an asymmetric carbon atom – a carbon atom that is attached to four different types of atoms or groups of atoms. The term "*chiral*," in general, is used to describe the object that is non-superposable on its mirror image [11]. The term enantiomers or optical isomers (old-fashionedly also termed optical isomers, antipodes, or optical antipodes) refers to two images of a chiral compound (Figure 3).

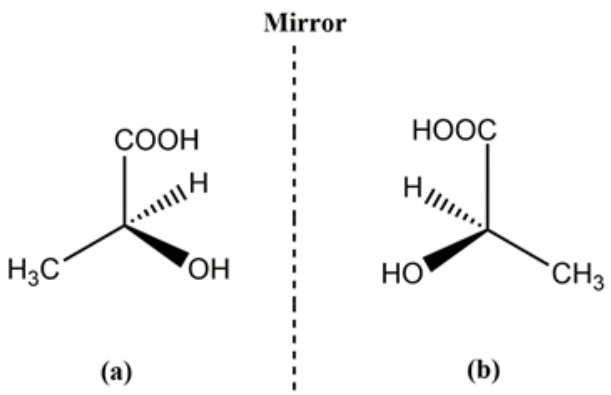

Figure 3. Lactic acid enantiomers. (a) (*S*)-(+)-lactic acid and (b) (*R*) -(–)-lactic acid. They are non-superposable mirror images of each other.

Enantiomers have identical chemical and physical properties, such as the same boiling and melting point, density, solubility, reactivity, vibrational and electronic frequencies, and refractivity [12]. However, they can rotate the plane-polarized light by equal amounts but in opposite directions (although the polarized light can be considered an asymmetric medium) [13]. Such compounds are therefore described as *optically active*, with specific terms for each enantiomer based on the direction: a dextrorotatory (d) compound rotates light a clockwise (+) direction whereas a levorotatory (l) compound rotates light in a counter-clockwise (–) direction.

The d- and l-isomers are enantiomers, isomers of the same compound. In an equimolar mixture of the two optical isomers – a racemic *mixture* or a *racemate* - the amount of positive rotation is precisely counteracted by the equal amount of negative rotation. The net rotation is zero (the mixture is not optically active). The presence of multiple chiral features in a given compound increases the number of geometric forms possible, though there may still be some perfect-mirror-image pairs.

Enantiomer members often have different chemical reactions with other enantiomer substances. Since enantiomers form many biological molecules, there is sometimes a marked difference in the effects of two enantiomers on biological organisms. In the pharmaceutical area, frequently, one of the enantiomers is responsible for the desired physiological effects, while the other enantiomer is less active, inactive, or sometimes even produces adverse effects [14]. Owing to this effect, nowadays drugs composed of only one enantiomer ("enantiopure") can be developed to make the drug more active and usually eliminating some of the common side effects.

2. Why separate enantiomers?

There are several practical reasons to separate enantiomers once in most cases, their biological activity depends on each enantiomer [15]. Some, but not all, reasons for enantioseparation includes:

- Most of the natural processes occur with remarkable stereospecificity;
- Interaction between biologically active compounds and receptor proteins often shows antipodal specificity;
- Antipodes shows different physiological behavior (such as taste and smell);
- Many drugs are synthetic racemic compounds and used as such;
- Chiral discrimination in physiological reactions is found in the hormone field.

3. Selected examples of relevant enantiomers

There are many examples of the importance of enantiseparations to be done, in particular in pharmaceutical, biochemical, and biological arenas. A few of them are exemplified in the following text.

3.1. Thalidomide – a sedative

An example of such an enantiomeric effect is the thalidomide (Figure 4), a sedative sold in several countries around the world from 1957 until 1961. It was withdrawn from the market when it was found to cause congenital disabilities. One enantiomer caused the desirable sedative effects, while the other, unavoidably [16] present in equal quantities, caused congenital disabilities [17,18]. In 1979 it was demonstrated that the (*S*)-(-)enantiomer has teratogenic activity.

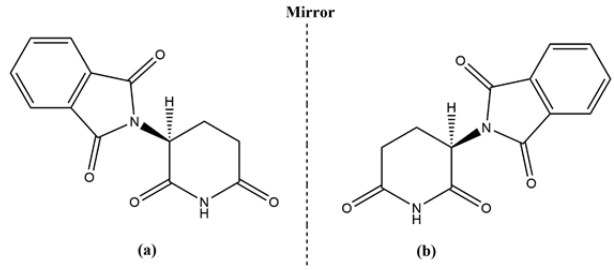

Figure 4. Chemical structure of the thalidomide enantiomers. (a) (*S*)-(-)-thalidomide and (b) (R)-(+)-thalidomide.

Thalidomide was first marketed in 1957 in Germany [19]. When first released, thalidomide was promoted for anxiety, tension, morning sickness, and trouble sleeping. While initially seemed to be safe in pregnancy, concerns regarding congenital disabilities were noted in 1961, and the medication was removed from the market in Europe that year [20]. The total number of people affected by use during pregnancy is estimated at 10,000; 40% died around the time of birth [21]. Those who survived had limb, eye, urinary tract, and heart problems. The congenital disabilities of thalidomide led to the development of better drug regulation and monitoring in many countries. It was approved for medical use in the United States in 1998 and has been used to treat leprosy, also known as Hansen's disease (HD), a long-term infection by the bacteria *Mycobacterium leprae* or *Mycobacterium lepromatosis*.

3.2. Citalopram – an antidepressant

The antidepressant drugs escitalopram and citalopram illustrate another example. Citalopram is a racemate [1:1 mixture of (*S*)-citalopram and (*R*)-citalopram] (Figure 5); escitalopram [(*S*)-citalopram] is a pure enantiomer. The dosages for escitalopram are typically 1/2 of those for citalopram, once the (*S*)-citalopram is almost entirely responsible for inhibiting serotonin reuptake. [22]. Citalopram, sold under the brand name Celexa and others, is an antidepressant of the SSRI (selective serotonin

reuptake inhibitor) class. It is used to treat a depressive disorder, social phobia, and panic disorder.

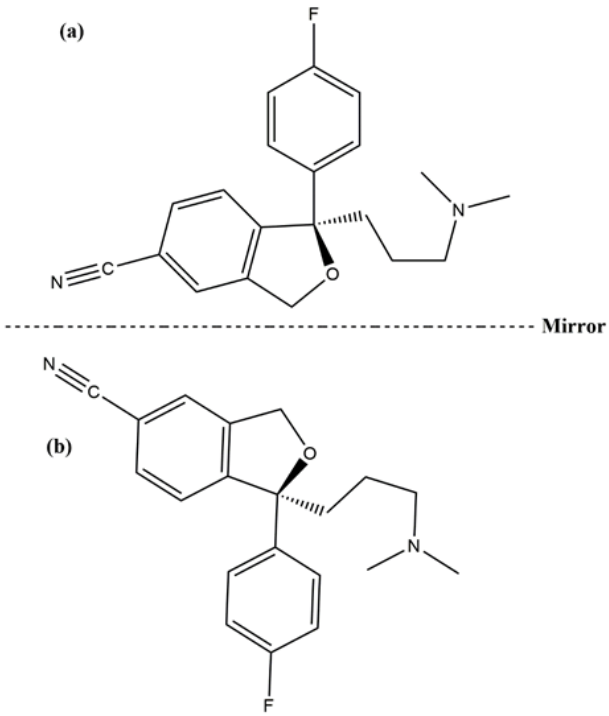

Figure 5. Chemical structure of the citalopram enantiomers. (a) (R)-citalopram e (b) (*S*)-citalopram.

3.3. Dopamine – a neurotransmitter

Dopamine is a type of neurotransmitter. The human body makes it, and the nervous system uses it to send messages between nerve cells [23]. That is why it is sometimes called a chemical messenger. Dopamine plays a role in how we feel pleasure. It is a large part of our uniquely human ability to think and plan. It helps us strive, focus, and find things interesting. The body spreads it along four main pathways in the brain. Like most other systems in the body, this is not noticed (or maybe even know about it) until there is a problem.

Too much or too little of it can lead to a vast range of health issues. Some are serious, like Parkinson's disease [24]. Others are much less dire. Too much of this chemical in certain parts of the brain can lead to schizophrenia, including hallucinations and delusion. A lack of it in other parts can cause different signs, such as lack of motivation and desire.

Dopamine enables neurons in the brain to communicate and control movement. In Parkinson's, one type of neuron steadily degenerates [24]. In the absence of a signal to send anymore, the body makes less dopamine. The chemical imbalance causes physical symptoms. These include tremor, stiffness, slowness of spontaneous movement, poor balance, and poor coordination.

Dopamine, norepinephrine, and epinephrine are synthesized through the multistep processing of tyrosine [25] (Figure 6). Tyrosine is a highly concentrated amino acid in catecholaminergic neurons. The main steps involved in the dopamine synthesis are:

- Phenylalanine hydroxylase converts phenylalanine to tyrosine;
- Tyrosine hydroxylase converts tyrosine to L-DOPA;
- L-DOPA is converted to dopamine by aromatic amino acid decarboxylase;
- Dopamine can subsequently be converted to epinephrine or norepinephrine.

4. Chromatographic separation of enantiomers

To achieve adequate separation of enantiomers, the chromatographic system has to present some type of asymmetry, i.e., it has to be chiral. This condition can be achieved in different ways:

1. A solid stationary phase is chiral, and the mobile phase is non-chiral (achiral);

2. A liquid stationary phase is chiral, and the mobile phase is non-chiral (similar to liquid-liquid partition chromatography);

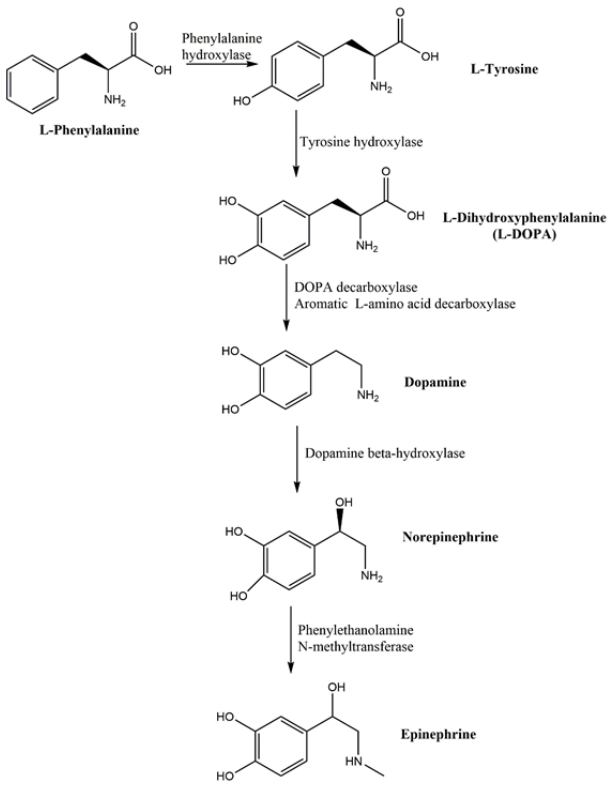

Figure 6. Biosynthesis of dopamine, norepinephrine and epinephrine from the tyrosine amino acid.

3. The stationary phase is non-chiral, and the mobile phase is chiral. The chiral mobile phase is obtaining by aiding a small amount of a chiral compound to it.

Another way to achieve a chromatographic separation of enantiomers consists of performing a previous derivatization step with a chiral compound, producing diastereomers, before the chromatographic separation [26]. The formed diastereomers can be separated using a regular (non-chiral) chromatographic setup, such as by normal phase HPLC. This mode is usually termed "indirect separation of enantiomers."

4.1. Chiral Stationary phases in liquid chromatography

The most common form to separate enantiomers directly (without the need for previous modification on their chemical structure) is the use of a solid chiral stationary (CSP) phase. As a function of the vast number of chiral compounds being separated and having an entirely different chemical identity, a large number of different CSPs are commercially available. Despite the considerable number of distinct CSPs, they can be grouped into five main categories:

- "Brush-type" CSPs, obtained by modification of the silica;
- Polymeric CSPs, based on cellulose and other polymers;
- CSPs based on cavities as crown ethers and cyclodextrins and derivatives;
- CSPs based on proteins;
- Ligand-exchange CSPs.

4.1.1. "Brush-type" Chiral Stationary Phases (CSPs)

These phases are mainly based on a chemical reaction on the silanol groups (Si-OH) from the silica surface with small molecules, usually containing aromatic units, such as modified amino acid, to generate monomers termed "brushes" [27,28]. These phases are also known as "Pirkle phases," honoring its inventor (W.H. Pirkle). Although the philosophy of making these phases is similar to the ones used for the normal and reversed-phase HPLC, also obtained by modifying the silanol group on the silica surface, the group to be introduced has to present a chiral center. This will provide a chiral character to the synthesized stationary phase.

Figure 7 depicts some examples of the "Brush-type" CSPs. Once several of these phases have similar structures (in many cases by just a small modification of a group in the main chain), some characteristics of the main Pirkle phase will be presented and commented.

The first Brush-type CSP to achieve commercial success was DNBPG (dinitrobenzoylphenylglycine), which

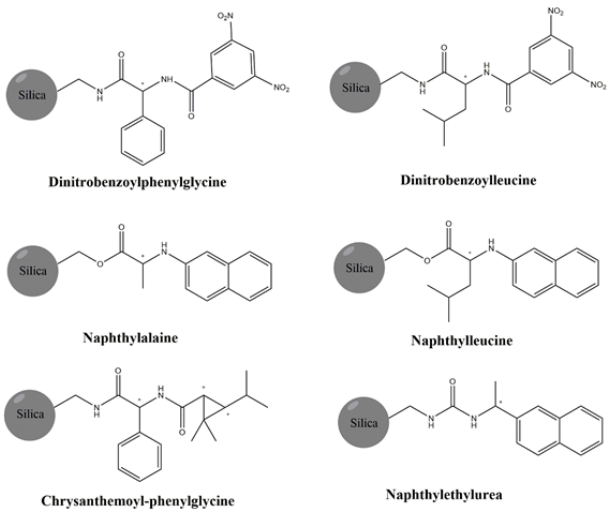

Figure 7. Examples of the main "brush-type chiral stationary phases.

served as a model to the synthesis of the vast majority of a phase of its kind. This phase is still widely utilized for chiral HPLC separations, showing good results for several enantiomeric separations [29]. Among the different groups in this phase, the dinitrobenzoyl group – being a π-acceptor - is considered to be the most relevant for the interactions with phenols, anilines, naphthalenes, and other molecules [30]. Even so, the other groups also participate in different degrees in the separation process, making it difficult to predict the best phase for a given separation. These phases are not expensive compared to other chiral phases and are robust in most cases (also compared to other CSPs).

In addition to periodicals (for instance, there is one dedicated only to chiral compounds, named Chirality) and books, an excellent source to help select a proper stationary phase is the literature supplied by the phase's manufacturers.

4.1.2. Helical Polymers Stationary Phases (CSPs)

Chemical modifications of crystalline cellulose arose to a large class of helical polymers CSPs (Figure 8) [31,32]. The most common ones are obtained through a derivatization process aiming to include specific radical groups (such as triacetate, tribenzoate, tristoluylate among several others) into the cellulose moieties. The produced phases are usually expensive and less robust than the Brush-type CSPs, but more selective for a broader range of enantiomers separation. The complete separation mechanism of this type of CSP is not yet fully understood.

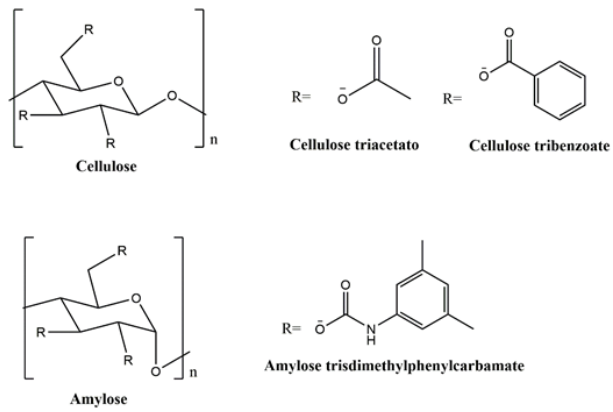

Figure 8. Most common carbohydrate units used in the helical polymers chiral stationary phases.

4.1.3. Cavity Phases

Although several classes of substances presenting closed rings were investigated to act as CSPs, in practice, only two showed the expected results: cyclodextrins and crown ethers [33].

4.1.3.a. Cyclodextrins

Cyclodextrins (CDs) are oligosaccharides with six (α), seven (β), or eight (γ) glucose units (Figure 9) that can undergo host-guest complexes with molecules that fit their conical cavity in a stereochemically controlled way [34]. Although many different CDs are available for chiral separations, in both gas and liquid chromatography, β-CD is by far the most used one.

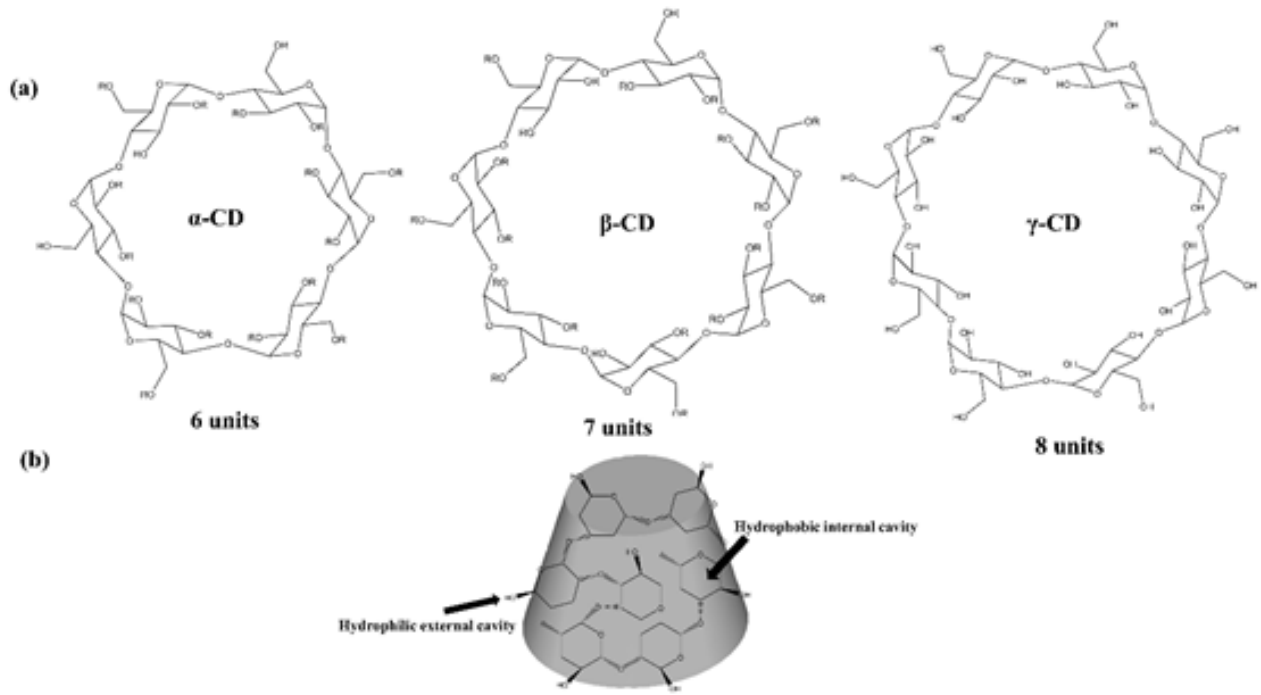

Figure 9. Chemical structure of cyclodextrins. (a) units of α, β, γ-CDs (b) Tridimensional structure of β-CD the most used CSP-CDs. Adapted from reference [1].

The CD's chemical structure reminders a truncated cone with a large number of hydroxyl groups available for acting as chiral binding points that can be derivatized to yield a large number of new phases such as acetylated, methylated and several others [35].

4.1.3.b. Crown ethers

Although attempts were made to use crown ethers as CSPs [36], the best results were obtained using 18-crown-6 [37,38]. In this type of phase, the interaction occurs between an amino proton from the guest analyte with ether oxygen from the host crown ether phase. To achieve an intended separation, the 18-crown-6 (Figure 10) can be modified with other chemical groups suitable for the target analytes.

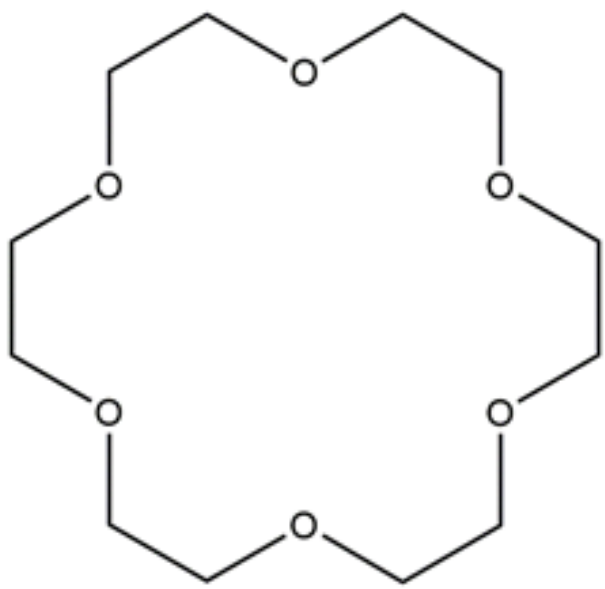

Figure 10. General structure of 18-crown-6 that can be derivatized with other groups to form the crown ether stationary phases.

4.1.3.c. Macrocyclic antibiotics

Some large glycosylated macrocyclic antibiotics [39] having several phenyl rings and peptide groups have been successfully employed as CSPs in liquid chromatography. Among them, vancomycin (Figure 11) is by far the most investigated and reported [40,41].

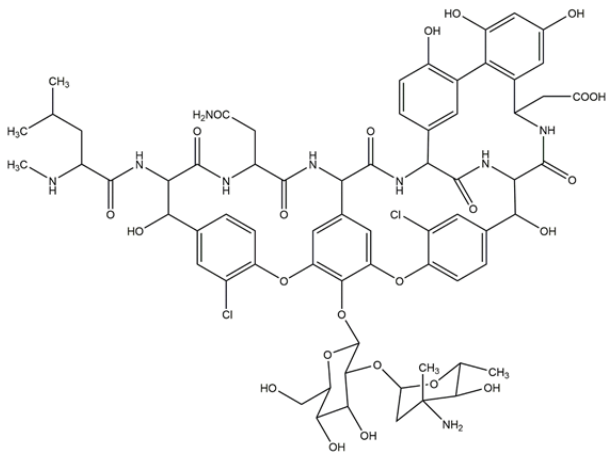

Figure 11. Chemical structure of Vancomycin.

4.1.4. Protein CSPs

Although presenting excellent enantioselectivity, protein-based CSPs, in general, are not physically robust and are very expensive, making their use restricted to specialized applications [42,43]. These phases present excellent enantioselective interactions with chiral molecules, leading to good enantioresolutions. Several classes of proteins presenting different chemical and physical characteristics have been successfully utilized as CSPs, including – to list a few - albumins, α-acid glycoprotein, avidin, and pepsin.

4.1.5. Ligand Exchange CSPs

This type of CSP takes advantage of the fact that silica-bonded amino acids containing Cu(II) ions present stereoselectivity to amino acids through the Cu(II) complexation [44]. Consequently, the main application of this kind of phase is in the enantioseparation of underivatized amino acids in opposite to traditional enantioseparations that require a derivatization step before analysis. Unfortunately, these columns require the use of Cu(II) in the mobile phase [45], and the column efficiency (plate number) is reduced.

5. Concluding Remarks

In this tutorial-review, after a background presentation of relevant chirality aspects presented by chemical compounds, the main types of chiral stationary phases (CSPs) were presented and discussed. Many different types of phases are now available for enantiomers separation, each presenting advantages, and limitations. Some are more specific (as in the case of ligand exchange); others show better selectivities (as the helical polymers); some are more robust (as the brush-type) while others are applied to a large class of analytes (as in the case of cyclodextrins). As a result, analysts have now a broad spectrum of CSPs to select from, according to his particular analytical approach. In the next report in this series, we will present our laboratory results of the enantioseparation of intact (nonderivatized) amino acids by using a capillary LC ligand exchange chiral stationary phase.

Acknowledgments

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