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Enantioresolution, chiral recognition mechanisms and binding of xanthone derivatives on immobilized human serum albumin by liquid chromatography

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“All artificial bodies and all minerals have superposable images. Opposed to these are nearly all organic substances which play an important role in plant and animal life. These are asymmetric, and indeed have the kind of asymmetry in which the image is not superposable with the object.”

Louis Pasteur, 1860

Publications

Under the terms of the Decree-Law no 216/92, of October 13th, is hereby declared that the author afforded a major contribution to the conceptual design and technical execution of the work and interpretation of the results included in this dissertation. Under the terms of the referred Decree-Law, is hereby declared that the following communications were prepared in the scope of this dissertation.

The results presented in this dissertation are part of the following scientific communications:

Poster Communications:

João Carmo; Carla Fernandes; Maria Elizabeth Tiritan; Carlos Afonso; Madalena M.M. Pinto. *Liquid chromatography enantioseparation of xanthone derivatives on a human serum albumin stationary phase.* 10º Encontro Nacional de Cromatografia (10 ENC), Bragança, Portugal, December 04-06, 2017, PC-49 (**Appendix A.**).

João Carmo; Carla Fernandes; Maria Elizabeth Tiritan; Carlos Afonso; Madalena M.M. Pinto. *Enantioresolution of bioactive compounds on human serum albumin stationary phase.* Escola de Inverno de Farmácia, 3rd ed, Porto, Portugal, March 07-15, 2018 (**Appendix B.**).

Carla Fernandes; **João Carmo**; Andreia Palmeira; Maria Elizabeth Tiritan; Carlos Afonso; Madalena M.M. Pinto. *Chiral carboxyxanthone derivatives: enantioselectivity studies in binding interaction with human serum albumin.* Italian-Spanish-Portuguese Joint Meeting in Medicinal Chemistry, MedChemSicily2018, Palermo, Italy, July 17-20, 2018 (**Appendix C.**).

Ye Zaw Phyo; **João Carmo**; Andreia Palmeira; Maria Elizabeth Tiritan; Carla Fernandes; Carlos Afonso; Anake Kijjoa; Madalena M.M. Pinto. *Enantioresolution and docking studies of xanthone derivatives on a human serum albumin stationary phase.* 11th International Symposium on Drug Analysis and 29th International Symposium on Pharmaceutical and Biomedical Analysis, DA-PBA 2018, Leuven, Belgium, September 9-12, 2018, ID 254 (**Appendix D.**).

Oral Communications:

João Carmo; Carla Fernandes; Maria Elizabeth Tiritan; Carlos Afonso; Madalena M.M. Pinto. *Chiral recognition of xanthone derivatives on human serum albumin stationary phase: effect of chromatographic conditions.* 11th Meeting of Young Researchers of University of Porto (IJUP18); February 07-09, 2018 (**Appendix E.**).

João Carmo; Ye Zaw Phyo; Carla Fernandes; Maria Elizabeth Tiritan; Carlos Afonso; Madalena M.M. Pinto. *Enantioresolution, chiral recognition mechanisms and binding of xanthone derivatives on immobilized human serum albumin by liquid chromatography.* XXIV Encontro Luso-Galego de Química (XXIV ELGQ); November 21-23, 2018 (**Appendix F.**).

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Abstract

High-performance affinity chromatography (HPAC) is a type of liquid chromatography in which solutes are separated based on their affinity to the mobile phase and a stationary phase that is a biologically-related agent.

Human serum albumin (HSA), the most abundant protein in the blood, plays an important role in the transport of drugs, metabolites, and endogenous ligands. Binding to HSA controls the free and active concentration of a drug, provides a reservoir for a long duration of action, and affects drug absorption, metabolism, distribution, excretion and toxicology profile (ADMET), determining the overall pharmacological activity of a drug.

The interactions of drugs with HSA are often stereoselective, and information on these interactions can be valuable in the design and optimization of chiral separations. Another important aspect of these interactions is predicting how drugs may behave in the human body, understanding drug-drug interactions, and determining the optimum dosages that should be used with such agents for treating patients.

Chiral stationary phases based on human serum albumin (HSA-CSP) exhibit a broad chiral recognition and enantioselectivity for a variety of classes of analytes due to multiple binding sites in the HSA selector.

During the last few decades, there has been a widespread interest in oxygenated heterocyclic compounds, such as molecules with a xanthone scaffold, mainly taking in consideration their variety of biological activities. Among them, chiral derivatives of xanthones (CDXs) have interesting biological activities being, in some cases, dependent on the stereochemistry. Consequently, the development of improved and accurate methodologies to enantioresolve, and evaluate the enantiomeric purity of these compounds are necessary.

In this dissertation, a systematic study of enantioresolution of a library of CDXs, prepared “in house”, was successfully carried out using a commercially available HSA-CSP, namely CHIRALPAK® HSA.

The enantioseparations were explored using different mobile phases, under reversed-phase elution mode. Chromatographic conditions such as mobile phase pH, buffer type and ionic strength, type and content of organic modifiers and temperature were optimized. Herein, thirteen among thirty-one enantiomeric mixtures of CDXs were enantioseparated by liquid chromatography on HSA-CSP. The obtained results showed very high enantioselectivity and resolution, with α and R_S ranging from 1.27 to 12.53 and from 0.90 to 6.41, respectively.

The binding to HSA of sixty-two enantiomers of CDXs has been determined by bioaffinity chromatography by measuring the retention times on an HSA-CSP using mobile phases

with different proportions of organic modifier, to allow the extrapolation to 100% aqueous buffer, and further calculation of compound bound percentage. In general, high affinity for HSA-CSP was observed, with bound percentage ranging from 79.02% to 99.99%.
Considering the importance of understanding the chiral recognition mechanisms associated with the chromatographic enantioresolution, computational studies by molecular docking were also carried out. Data regarding the CSP-CDX molecular conformations and interactions were retrieved. The docking calculations were in accordance with the experimental chromatographic parameters regarding enantioselectivity and enantiomer elution order, with a success rate of 77%.

Keywords: Chiral Derivatives of Xanthones; Liquid Chromatography; Chiral Recognition; Chirality; Enantioresolution; Human Serum Albumin; Bioaffinity.

Resumo

A cromatografia de afinidade de alta eficiência (CAAE) é um tipo de cromatografia líquida na qual os solutos são separados tendo como base a sua afinidade para a fase estacionária e para uma fase estacionária cujo seletor é constituído por uma molécula de origem biológica.

A albumina sérica humana (ASH) é a proteína mais abundante no sangue e desempenha um papel importante no transporte de fármacos, metabolitos e ligandos endógenos. A ligação dos fármacos à ASH controla a sua concentração livre e ativa, providencia uma reserva para uma longa duração de ação e afeta a absorção, o metabolismo, a distribuição, a excreção e o perfil toxicológico (ADMET), determinando a atividade farmacológica global de um fármaco.

As interações dos fármacos com a ASH são frequentemente estereosseletivas, sendo seu estudo valioso para a otimização de resoluções enantioméricas, prever o comportamento dos fármacos no organismo, assim como possíveis interações entre fármacos e determinar a dose ideal que deve ser administrada.

As fases estacionárias quirais baseadas em albumina sérica humana (FEQ-ASH), apresentam um amplo reconhecimento e capacidade enantiosseletiva para uma variedade de classes de analitos, devido aos múltiplos locais de ligação.

Nas últimas décadas, tem havido um crescente interesse em compostos heterocíclicos oxigenados, tais como moléculas contendo um núcleo xantónico, tendo em consideração a grande diversidade de atividades biológicas. Os derivados xantónicos quirais (DXQs) demonstraram atividades biológicas interessantes sendo, em alguns casos, dependentes da sua estereoquímica. Consequentemente, é necessário o desenvolvimento de metodologias para resolver e avaliar a pureza enantiomérica destes compostos quirais.

Nesta dissertação, foi realizado um estudo sistemático de enantiorresolução de uma biblioteca de DXQs utilizando uma coluna comercial de FEQ-ASH, nomeadamente CHIRALPAK® HSA.

A separação enantiomérica destes compostos quirais foi otimizada usando diferentes fases móveis, em modo de eluição inversa. Condições cromatográficas como o pH da fase móvel, tipo de tampão e força iônica, natureza e proporção de modificador orgânico, e temperatura foram testadas. Treze das trinta e uma misturas enantioméricas de DXQs foram resolvidas na FEQ-ASH. Os resultados obtidos mostraram uma boa enantiosseletividade e resolução, com valores de α e R_S variando de 1,27 a 12,53 e de 0,90 a 6,41, respetivamente.

A afinidade de sessenta e dois enantiómeros de DXQs para a ASH foi determinada por cromatografia de bioafinidade através dos tempos de retenção obtidos usando fases móveis com diferentes proporções de modificador orgânico. Estas medições permitiram o

cálculo da percentagem de ligação dos compostos, por regressão linear a 100% de solução tampão aquosa, com percentagens de ligação que variaram de 79,02% a 99,99%
Tendo em consideração a importância dos mecanismos de reconhecimento quiral associados à enantiorresolução cromatográfica, foram realizados estudos de química computacional por docking molecular. Os dados referentes às conformações e interações moleculares entre a FEQ e os enantiómeros dos DXQ foram analisados. Os valores obtidos foram concordantes com os dados cromatográficos experimentais, em relação à enantiosseletividade e à ordem de eluição dos enantiómeros, com uma taxa de sucesso de 77%.

Palavras-chave: Derivados Xantónicos Quirais; Cromatografia Líquida; Reconhecimento Quiral; Quiralidade; Enantiorresolução; Albumina Sérica Humana; Bioafinidade.

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Abbreviations and Symbols

µg	Micrograms
µL	Microliters
2-PrOH	2-Propanol
ACN	Acetonitrile
AGP	α1-acid glycoprotein
BSA	Bovine Serum Albumin
CBB	Chiral Building Block
CBH	Cellobiohydrolase
CDX	Chiral Derivative of Xanthone
CIIMAR	Centro Interdisciplinar de Investigação Marinha e Ambiental
cm	Centimeter
COX	Cyclooxygenase
CSP	Chiral Stationary Phase
CX	Carboxyxanthone
EtOH	Ethanol
HPAC	High Performance Affinity Chromatography
HPLC	High Performance Liquid Chromatography
HPLC-DAD	High Performance Liquid Chromatography Diode Array Detector
HSA	Human Serum Albumin
I.D.	Inner Diameter
k	Retention Factor
kcal.mol^{-1}	Kilocalorie per mole
LC-MS	Liquid Chromatography Mass Spectrometry
LQOF	Laboratory of Organic and Pharmaceutical Chemistry
MeOH	Methanol
mg	Milligram
min	Minute
mM	Millimolar
MS	Mass Spectrometry
ng	Nanogram
OM	Organic Modifier
OVM	Ovomucoid
R_S	Resolution Factor
t_0	Dead Time

TBTU	*O*-(Benzotriazol-1-yl)-*N*,*N*,*N'*,*N'*-tetramethyluronium tetrafluoroborate
t_R	Retention Time
UPW	Ultra-Pure Water
UV-Vis	Ultraviolet - Visible
v*/*v	Volume per Volume
XD	Xanthone Derivative
α	Separation Factor

Outline of the Dissertation

This dissertation is divided into seven chapters:

I. Introduction

In this chapter, general concepts and importance of chirality are presented, as well as the methods for enantiomeric resolution. In addition, the different types of chiral stationary phases (CSPs) are described, giving more importance to protein-based CSPs, specifically human serum albumin (HSA). Affinity and chiral recognition studies are also discussed. The importance of xanthone derivatives, namely chiral derivatives of xanthones (CDXs) are also highlighted.

II. Aims

This chapter summarizes the main and secondary objectives of the present dissertation.

III. Experimental

The third chapter describes the experimental methodologies used in this work, including information about the chemicals, chromatographic instrumentation and conditions, mobile phases, sample preparation and chromatographic parameters used for liquid chromatographic analysis, as well as computational study.

IV. Results and Discussion

In this chapter, the results from the original work concerning the systematic study of enantioresolution of a library of CDXs and the affinity studies on a HSA-based column are described. The results of computational study by docking approach are also discussed.

V. Conclusions

This chapter sum up the general conclusions of the developed work, based on the proposal aims.

VI. References

All the references used as support for this dissertation are listed in this chapter. The databases used are also mentioned.

VII. Appendixes

Appendix A. – Abstract and Poster of 10º Encontro Nacional de Cromatografia (10 ENC), December 04-06, 2017.

Appendix B. – Abstract and Poster of Escola de Inverno de Farmácia, 3rd ed, Porto, Portugal, March 07-15, 2018.

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Appendix G. – Technical data sheet of CHIRALPAK® HSA provided by the column manufacturer (Chiral Technologies, Daicel Group).

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Chapter I

INTRODUCTION

1. Chirality: general concepts and importance

One of the main characteristics of nature and features of the living world is its chirality [1]. Chirality is a fundamental property in analysis of three-dimensional shapes of molecules applied in the development of agrochemical, food, flavours, and to pursue new drug candidates to the pharmaceutical industry [2].

Molecules are termed chiral when they do not have a plane of symmetry, and exist in two enantiomeric forms, which are mirror images of each other and are non-superimposable in three dimensions [3]. Since the term chirality comes from the Greek *cheir* which means hand, the spatial relationship between two enantiomers is classically represented by the right and left hands [4] (**Figure 1**).

Generally, chiral molecules are characterized by the presence of one asymmetric center known as stereogenic center or stereocenter [3]. In fact, most chiral molecules present central chirality, with one or more stereogenic centers, usually sp^3 (tetrahedral) carbon atoms bonded to four different substituents (atoms or groups of atoms). In addition to carbon, nitrogen, phosphorus, sulphur, selenium and boron can also produce stereogenic centers [2]. Nevertheless, other types of chirality are considered, including planar, axial and helical chirality [2].

Chemically, enantiomers (Greek *enantios* = opposite, *meros* = part) have the same chemical formula and physicochemical properties when they are in an achiral environment, such as density, melting point, boiling point, among others. However, it is possible to distinguish between them when they interact with chiral systems, and by their optical activity [5]. In fact, the enantiomers rotate plane-polarized light in equal amounts but in opposite directions: one enantiomer rotates the light to clockwise (dextrorotatory) and the other to counterclockwise (levorotatory), referred by the symbols *d* or (+) and *l* or (-), respectively. This is consistent with absence of optical activity in racemic mixtures or racemates, i.e., equimolar mixtures of enantiomers [3].

Configuration is the term used to refer the spatial arrangement of the substituents around the stereogenic center, and the nomenclature Cahn-Ingold-Prelog is the most used system to distinguish enantiomers based on their configuration [6]. This system is based on the application of a set of priority rules, and subsequent assignment of (*R*) or (*S*) configurations. Accordingly, the configuration (*R*) is attributed when the order of priority of the groups follows the clockwise direction (from the Latin word *rectus* which means right), and the configuration (*S*) when the sequence follows the counterclockwise direction (from the Latin word *sinister* which means left) [6] (**Figure 1**).

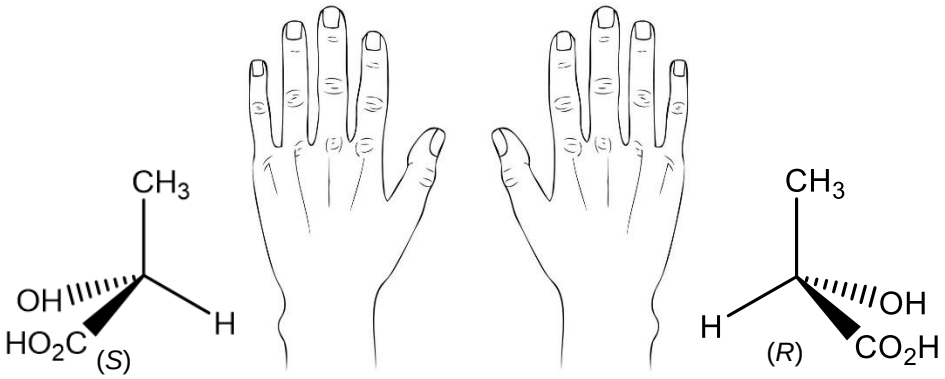


Figure 1 - (S) and (R) enantiomers of lactic acid.

Biosystems comprise many chiral components such as proteins, nucleic acids, and sugars, tending to be highly stereoselective environments [7]. The individual enantiomers of drugs can interact differently with biomolecules and, consequently, may exhibit different pharmacodynamic, pharmacokinetic and toxicological properties [8-10].

When one enantiomer is responsible for the biological activity of interest, the other could be inactive, possess lower activity, be an antagonist of the active enantiomer or have a different activity that could be desirable or undesirable [11-13]. Some examples illustrating stereoselective responses of chiral drugs are presented in **Table 1**.

For a specific biological activity, the enantiomer that most effectively establishes the interactions with the biological target is designated as eutomer. The less active enantiomer is the distomer, being the ratio between both called eudismic ratio [14].

Table 1 - Examples of different stereoselective responses of chiral drugs in their biological activity.

Drug	Biological activity
Thalidomide	(*S*)-enantiomer is teratogenic, (*R*)-enantiomer is sedative [15]
Timolol	(*S*)-enantiomer used for treatment of cardiovascular disease, (*R*)-enantiomer for glaucoma [16]
Ibuprofen	(*S*)-enantiomer is the active isomer as anti-inflammatory, (*R*)-enantiomer has no effect [3]
Citalopram	(*S*)-enantiomer is 100 times more potent than (*R*)-enantiomer [11]

Many chiral drugs used in clinical practice were administered as racemates. However, this trend was changed mainly because of the regulatory requirements [17, 18]. In fact, the investigation and production of chiral drugs began to have real significance from new guidelines adopted since 1992, by the Food and Drug Administration (FDA), in U.S.A., present in a document entitled *Policy Statement for the Development of New*

Stereoisomeric Drugs [19]. In 1994, the European Committee for Proprietary Medical Products (CPMP) also issued formal guidelines called *Investigation of Chiral Active Substances* [20]. In addition to the regulatory requirements, also the advances in enantioselective synthesis [21-24], enantiomeric resolution and analytic methodologies [25-27], as well as the "chiral switch" phenomena [28-30] contributed to the increasing development of enantiomerically pure drugs rather than racemates [31].

2. Enantiomeric resolution

The enantiomerically pure drugs can be obtained either by enantioselective synthesis of a desired enantiomer or by preparative resolution of a racemate or mixture of enantiomers [32].

Enantioselective synthesis is not so suitable in the early phases of drug discovery process, since large quantities of reagents or chiral raw material are required, and a large time is needed to perform the synthesis, and if both enantiomers are needed, it is necessary to develop two independent syntheses [33]. Enantiomeric resolution has the advantage to obtain both enantiomers with high enantiomeric purity essential for biological testing [34].

Several methods can be used for enantiomeric resolution purpose, including enzymatic resolution, electrophoresis, membranes, and chromatography [35, 36]. The separation of enantiomers can be carried out by indirect or direct methods [37]. The indirect method is based on the synthesis of a pair of diastereomers from the reaction of the racemate with an enantiomerically pure reagent, and further separation of the obtained diastereomers by conventional procedures. Then, the enantiomers can be recovered by overturn the derivatization procedure [38]. The direct method requires the formation of energetically distinct transient diastereomers, by interaction of enantiomers with a chiral selector. In liquid chromatography (LC) the chiral selector can be attached to a stationary phase or be a component of the mobile phase [39].

LC is often the first choice for enantioresolution showing several advantages, such as: (i) vast applicability; (ii) reproducibility, high speed and sensitivity; (iii) remarkable assay precision, with the possibility of coupling different complementary equipment's. In fact, most industrial and academic laboratories are equipped with several LC apparatus. Nowadays, LC enantioresolution using chiral stationary phases (CSPs) is widely used for pharmacokinetic [40] and toxicity studies [41], preparative resolution of enantiomers [42], evaluation of enantiomeric purity [43], as well as environmental studies [44].

3. Chiral stationary phases

In the last 40 years, scientific and commercial interest in enantiomeric resolution using CSPs has been increasing [45]. There is a wide variety of commercially available CSPs for both analytical and preparative scales [46, 47]. CSPs are usually prepared from chiral molecules obtained from natural chiral pool or synthesized in a stereoselective form, being covalent bond or coated onto a chromatographic support [48]. **Table 2** shows examples of different CSPs organized according to their source [46].

Table 2 – Different types of CSPs.

Chiral Stationary Phase	Source
Pirkle-type Ion-exchange-based Crown ether-based Polymer-based	Synthetic
Protein-based Polysaccharide-based Cyclodextrin-based Macrocyclic antibiotic-based Cinchona-based	Natural

Pirkle's [49, 50], Okamoto's [51, 52], Armstrong's [53-56], Lindner's [7, 57] and Hage's [58] research groups were responsible for several studies describing the development and applicability of new CSPs for LC enantioselectivity studies.

4. Protein-based chiral stationary phases

The commercial and scientific interest on plasma proteins, enzymes, and other biomolecules as chiral selectors increased due to their ability of chiral recognition [59]. On their large surface, proteins present different binding sites which allow to multiple possibilities of intermolecular interactions with small molecules [60]. Therefore, it is not an easy task to predict retention and enantioselectivity using such CSPs [61]. When used as chiral selectors, proteins demonstrate enantioselectivity for a wide range of chiral compounds, including pharmaceutical compounds [62].

The first report demonstrating that a protein has different binding properties in a LC analysis was in 1973, for tryptophan resolution using bovine serum albumin (BSA) coupled to an agarose support [63]. After that, Allenmark [64], Haginaka [60], Hage [58, 65], and other groups have contributed to the development of several and exhaustive studies to assess the ability of various proteins to be used as chiral selectors, and also in biological assays.

Nevertheless, only a limited number of proteins are commercially available as CSPs (**Table 3**).

Table 3 - Protein-based CSPs, column tradenames and type of resolved racemates **[38, 59]**.

Proteins	Mr (kDa)	Type of resolved racemates	Column tradename
Serum albumin			
Human (HSA)	67	Acidic and neutral	CHIRAL-HSA®[a]
Bovine (BSA)	68	Acidic and neutral	CHIRAL-BSA®[a]
α1-Acid glycoprotein (AGP)	44	Basic, neutral and acidic	CHIRAL-AGP®[a]
Ovomucoid (OVM)	28	Basic, neutral and acidic	Ultron ES-OVM®[b]
Cellobiohydrolases (CBH)	60-70	Basic and neutral	CHIRAL-CBH®[a]
Avidin	68	Basic, neutral and acidic	Bioptic AV-1®[c]
Pepsin	34.6	Basic and neutral	Ultron ES-Pepsin®[b]

[a]ChromTech Lda., Congelton, Cheshire, UK
[b]Shinwa Chemical Industries Lda., Fushimi-ku, Kyoto, JP
[c]GL Sciences Inc., Torrance, California, USA

α1-Acid glycoprotein (AGP) and ovomucoid (OVM) from chicken egg are mostly applied for resolution of a wide range of basic, acidic and neutral drugs [66-68]. Nevertheless, bovine serum albumin (BSA), human serum albumin (HSA) and cellobiohydrolase (CBH), as immobilized chiral selectors, are well documented for chromatographic enantioseparations and for binding studies [69-71]. Moreover, the retention and enantioselectivity obtained on immobilized proteins also reflect the binding behavior of native proteins [72].

In the immobilization process of proteins onto a chromatographic support, to obtain the LC columns, the main aim is to preserve their natural function, conformation and chiral discrimination. Accordingly, for preparation of this type of CSPs, several techniques were developed for physical adsorption as well as covalent binding of the proteins to the chromatographic support [73]. Silica-based materials, zirconia particles or polymeric materials can be used as chromatographic supports [74].

Variations on chromatographic conditions such as pH, buffer type and ionic strength of the mobile phase, type and proportions of organic modifiers (commonly isopropanol (2-PrOH) and acetonitrile (ACN)), charged additives and temperature are decisive to adjust retention times, enantioselectivity and resolution [70]. It was found that pH, temperature and organic modifier variations may quite differently affect reversible spatial conformation of the protein leading to changes of the number of available selective and non-selective binding sites [72]. For example, the presence and variation of organic modifiers and high temperatures respectively, may lead to a significant shortening of CSPs lifetime [75], although immobilized proteins are probably more stable than native proteins.

5. Human serum albumin as stationary phase

HSA is one of the most abundant plasma proteins being one of its main functions to act as drug transporter [76]. HSA assists in the transport, distribution, metabolism and facilitates transfer athwart organ-circulatory interfaces of several ligands, including fatty acids, amino acids, steroids, metals, and many pharmaceutical compounds [77]. HSA concentration in blood plasma is about 0.7 mM, its molecular weight is 67 kDa, isoelectric point around pH 5.9 and comprises a peptide chain consisting of 535 amino acids, mostly ionic such as glutamate and lysine [78].

Once systemic absorption is completed, most drugs experience some degree of reversible binding to HSA, thereby restricting their free and active concentration [79]. High affinity for plasma proteins (over 90%) may consequently be a benefit or a drawback for efficacy, depending on the drug and target [80]. Moreover, when two drugs are absorbed into the bloodstream the concentration of the free fraction can change because they may interact with each other and compete for the same albumin binding site, which brings the possibilities of contraindications or toxicity [79]. Additionally, the presence of a chiral drug as racemate could induce enantioselective binding, if both enantiomers have different binding affinity to the HSA [81]. Consequently, as an impact of stereoselective protein binding to albumin, not only the transport mechanism of both enantiomers will be affected but also, they may exhibit different pharmacodynamic, pharmacokinetic and toxicological properties [79].

Crystallographic data about the primary structure of HSA reveals that it has three homologous helical domains, namely I, II and III [82]. Individually, each domain is divided in two subdomains linked by a random coil, respectively A and B. Interdomain helices linked the terminal regions of sequential domains, namely IB to IIA and IIB to IIIA [82]. The nature of this structural organization provides a wide variety of ligand binding sites [78]. Most drugs and endogenous hormones interact with two major binding sites on HSA known, according to Sudlow's nomenclature [83], as Sudlow's site I (located in subdomain IIA), and Sudlow's site II (located in subdomain IIIA), and also as warfarin–azapropazone (Site I) and indole–benzodiazepine sites (Site II). Site I is the highest-affinity binding site for, among others, anticoagulants (like warfarin) and NSAIDs (like phenylbutazone, indomethacin and salicylate) [84]. Site II is also a specific site for "profens" (like ibuprofen, fenoprofen, or ketoprofen), psychoactive drugs (like diazepam), and anesthetics (like halothane and propofol) [85] (**Figure 2**). Other minor bindings have also been proposed, including those that bind to bilirubin, digitoxin and tamoxifen [86].

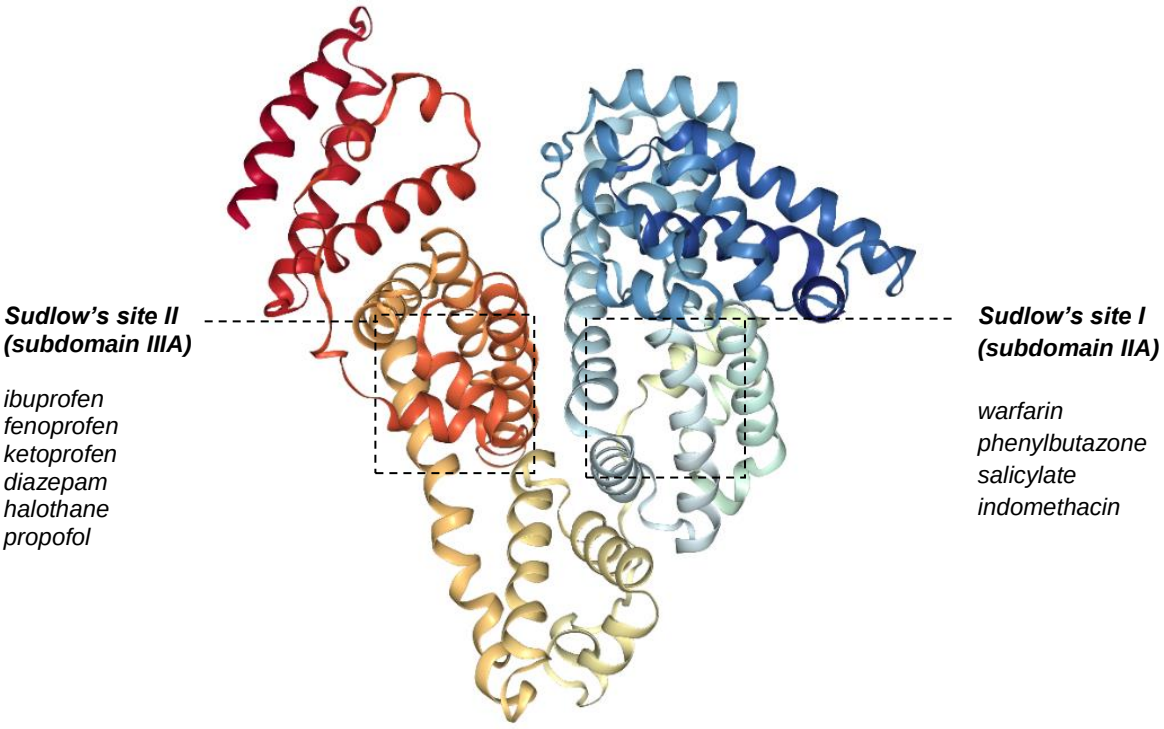


Figure 2 - Illustration summarizing the various ligand-binding sites on the structure of HSA. Structure obtained from PDB ID 1AO6, in protein data bank of the Research Collaboratory for Structure Bioinformatics (http://www.rcbs.org/pdb/).

However, a thorough understanding is needed to explain the variabilities that occurred in drug binding properties as consequence, for example, the presence of covalently or reversibly bound compounds that can alter the conformation of the binding sites [79], as well as expand the knowledge on stereospecific binding of chiral compounds.

6. Ligand-Protein binding studies

In the early stages of drug discovery, beyond the biological activity evaluation, each compound should also be evaluated and characterized by its lipophilicity, ionization, solubility, metabolic properties, and protein-binding properties. Although there are other proteins in human blood plasma capable of binding drugs, HSA is the most abundant protein [82] and, consequently, the most studied in high-performance affinity chromatography (HPAC) [87].

HPAC is a separation technique that has become increasingly important when working with biological samples and pharmaceutical agents. This chromatographic technique is based on the use of a biologically related agent as a stationary phase that selectively retain analytes or to study biological interactions. A great advantage of this technique is that only

a small amount of protein is required to carried out several studies because the same amount of immobilized protein (CSP) can be used for the experiments [88].

This technique is very useful and presents other advantages since it is coupled to an LC system, making the process automatic and reproducible, and with the possibility of working under near-physiological conditions (buffer pH, ionic strength and temperature). Additionally, being a CSP, it can be used to analyze the behavior of both enantiomers of an enantiomeric mixture in the same run [58, 65].

One disadvantage of this technique is related to the basis of its concept: the immobilization of the protein to a chromatographic support. The immobilization procedure can bring some changes comparatively to the protein in its soluble form, namely conformational changes, denaturation, steric hindrance at the binding sites, nonspecific binding by the support and anchor matrix, leading to the change of the retention time [89].

There are various approaches used for the immobilization process of HSA to a silica or monolithic chromatographic support which can counterbalance this disadvantage, namely covalent immobilization, biospecific adsorption, and entrapment [90].

Ligand-protein binding studies by HPAC can be achieved through two main outlines: zonal elution and frontal analysis [91].

In frontal analysis, a solution with a known concentration of an analyte is continuously injected into a column containing the immobilized protein and, as soon as the analyte binds to the protein, becomes saturated. At the detection, an increase of the absorbance (mAU) is observed as saturation occurs until the constants of association and dissociation change to an equilibrium, and the mAU stabilizes [92]. This equilibrium curve is known as a breakthrough curve and combining its start and end times, its average position and its format, it is possible to obtain relevant information about the number of binding sites or the strength and affinity of the binding. The major disadvantage of this technique is that a large amount of analyte is required, making it totally unfeasible in drug discovery [93].

On the contrary, zonal elution analysis uses a small amount of analyte injected into a column while the time elution of the analyte is monitored, like almost all analytical methods of chromatography, making it the most used due to its simplicity [94]. Several factors must be taking into account such as the type and proportion of organic modifier, temperature and the presence of charged additives or a displacement agent since they may affect the results. In this type of studies, the mobile phase is characterized by having a buffer that mimics the physiological conditions [95].

In zonal elution method, protein binding studies are performed by injecting a small amount of a compound of interest into the chromatographic system, and through analysis of its retention time the relative binding or percent binding can be achieved [96]. Several studies

have demonstrated a high concordance of this method compared to other techniques, such as ultrafiltration [97]. When an equilibrium is stablished, the retention factor (*k*) is equal to the number of moles or fraction of the ligand bound to the protein (*b*) divided by the number of moles or fraction of the free ligand (*f*) in the mobile phase ($k = b/f$) [98]. Considering that the free or protein bound ligand fraction is equal to 1, the relative binding is calculated through the retention factor (*k*) by the following **equation (1)** [99]:

$$b = k/(1 + k) \quad \textbf{(1)}$$

In addition, it should be kept in mind that the obtained results are dependent on the ratio between the amount of compound injected and the amount of protein immobilized on the column [100]. Consequently, two distinct situations can occur [65]: (i) small amount of compound injected into a large amount of protein; (ii) an excess of compound injected compared with the amount of protein entrapped in the chromatographic column; the second situation should be avoided. In the immobilization process of the selector, some molecules can lost their binding activity by the change of the conformation of its different binding sites [100]. For these reasons, the results from this type of study are only indicative, comparing with the binding results to the protein in solution form.

Displacement studies are most commonly used in zone elution by HPAC [91]. These studies are very useful knowing that an analyte will experience some binding competition to the protein by another agent. This technique consists in analyzing the retention of one or more injected analytes while a determined concentration of a presumed competitive agent present in the mobile phase passes through the column [91]. The competitor may interfere in the retention time of the analyte by different reasons: (i) competition between the injected analyte and the competitor for the same binding site (when the analyte and the competitor do not bind to any other site) [101]; (ii) competition between the injected analyte and the competitor for two types of binding sites [101]; (iii) competition between the injected analyte and the competitor agent for different binding site (allosteric competition) [102]; (iv) competition between the injected analyte and the competitor for the same binding site (when the competitor does not bind to any other site and the analyte binds to two) [79]; (v) competition between the injected analyte and the competitor for the same binding site (when the analyte does not bind to any other site and the competitor binds to two). The results of competition between single or multiple binding sites as well as allosteric competition are relatively simple to analyze using reciprocal plots, concentration of the competitive agent *versus* 1/*k* (or other models based on equation 1) [102].

From displacement studies, relevant information can be withdrawn regarding the site or multiple protein binding sites of a given analyte and, consequently, the binding force between each ligand-protein interaction. In competition studies, it is possible to determine whether other compounds bind to this same protein site as well as to obtain information about the type of interactions that may occur. In addition, with the constants of association of the competitive agent it is possible to establish quantitative structure-retention relationships [103].

In summary, additionally to the use of biologically-based CSPs on zone elution technique to study the relative binding of bioactive compounds to the protein [104], other kind of studies can be performed, including: displacement studies with the presence of a competitive agent [105], thermodynamic and variability studies of chromatographic conditions [106], quantitative structure-retention relationships studies [107], and enantioresolution of racemic mixtures [108].

7. Enantioresolution and chiral recognition studies on HSA-CSPs

In the last years, several studies have shown that HSA-CSPs can be efficiently used for enantioseparation of chiral analytes, and that the chromatographic data may reflect their *in vivo* binding to HSA [72, 75, 91, 101, 109]. Examples of chiral drugs that have been enantioseparated and studied include benzodiazepines [110], coumarins (e.g., warfarin) [58, 65], NSAIDs (e.g., ibuprofen) [101], thyronine's (e.g., thyroid hormones) [88, 111], tryptophan [106], among others [91, 112]. The referred examples include measurement of binding of analytes to HSA, equilibrium constants studies, number of binding sites which the analyte interact, rates of the interactions, as well as the effect of the chromatographic conditions on binding to HSA, for example, mobile phase composition or temperature.

In the case of the influence of mobile phase, the variations of pH, ionic strength and type or proportion of organic modifier can be examined [113]. Chiral recognition properties of protein are dependent on the balance between electrostatic and hydrophobic interactions [114]. Both pH and ionic strength can bring conformational modifications to the protein structure itself, modify the interactions between the protein and analyte, and also influence the enantioselectivity [115]. Changes in the conformation of the molecule can also occur by the addition of organic modifiers to the mobile phase (never more than 40%) such as alcohols or ACN, thus leading to a decrease in the binding of the analytes to the protein [116]. This decrease in binding, caused by disturbance of interactions of non-polar nature, leads to a decrease in the width and tailing of the chromatogram peaks. Temperature

variation can also be a factor of study, to discover the thermodynamic constant of a particular analyte, to promote a decrease in the binding force and, consequently, a decrease in retention factor, or finally to set the temperature equal to the physiological [89].

Stereospecific recognition of chiral compounds is a significant issue in various aspects of chemistry and life sciences. Diverse intermolecular interactions namely ionic, ion-dipole or dipole–dipole, π-π, van der Waals, and hydrogen bond interactions can be established [117, 118]. Initially, strong or long-range interactions such as ionic are alleged to be involved on non-stereoselective binding. On the contrary, short-range directional interactions such as hydrogen bonds and π-π interactions would be responsible, in most cases, for stereoselective binding of HSA-CSP to analytes [119]. Furthermore, chiral recognition can be a result of steric hindrance factors from the spatial arrangement of the binding cavity of the selector. Thus, conformational change of the selector may also be considered. Moreover, the interactions can be attractive or repulsive [117].

In order to rationalize the observed stereoselective behavior of a chiral selector with an enantiomeric compound, such as a drug, Pirkle's research group adapted a model to explain the chiral recognition mechanism called "3-point interaction" model (**Figure 3**) [120]. This model was previously proposed by Easson and Stedman [121] to justify the differences in pharmacodynamic activity observed between enantiomers, considering their different interactions with a crystallographic structure, such a protein.

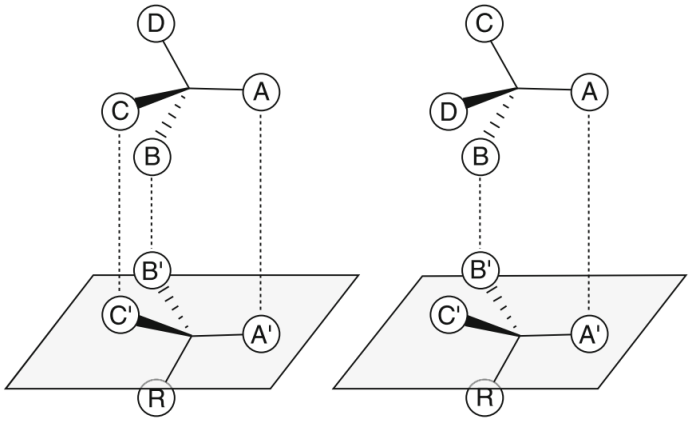


Figure 3 - "3-Point interaction" model [117]. The enantiomer on the left presents three groups that match exactly three sites of the selector, while its mirror image, on the right, can interact with a maximum of two sites of the selector.

This model stipulates that at least one of the enantiomers must undergo a minimum of three simultaneous interactions with the CSP, and that the overall interactions of the two enantiomers with the CSP must be energetically distinct. The predominant type of

interactions that occur are dependent upon the functional groups present on both the analyte and CSP, and also the mobile phase used [122]. Moreover, interactions may rather be mediated via multiple points instead of single points.

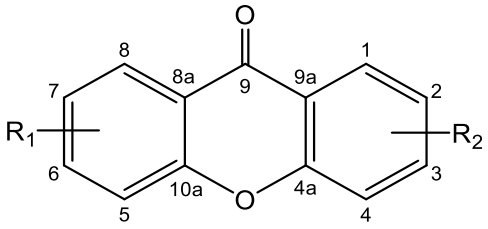
This model is very useful but it is a relatively simplistic representation of drug-CSP interactions since it assumes that the drug must adopt a particular orientation in relation to the binding site [123]. In addition, the interaction between the drug and the chiral selector may result in conformational changes in both the drug and selector macromolecule. The final interaction model may be complex and, therefore, both the stereochemistry and conformational flexibility of the ligand should be considered.

The difficulty in understanding the chiral recognition process of analytes increase when large biologically related molecules, such as HSA, are used as chiral selectors. Computational studies, including molecular docking approach of ligand-protein complexation can provide insights of the ability of the chiral selectors to discriminate enantiomers, and to understand the basis of multiple intermolecular interactions that can be established [124-129].

8. Chiral derivatives of xanthones (CDXs)

One of the most important class of oxygenated heterocycles are xanthones or 9*H*-xanthen-9-ones comprising a dibenzo-*γ*-pyrone scaffold (**Figure 4**) [130]. Xanthone derivatives (XDs) have an important role in Medicinal Chemistry, mainly considering their biological and pharmacological activities [131, 132].

XDs can be found in plants, fungi, lichens and bacteria, whether in terrestrial or marine origin [133, 134], or by synthesis [135, 136]. They are considered privileged structures [137] been associated with several biological and pharmaceutical activities of interest, such as antifungal [138], anticoagulant [139], anticonvulsant [140], antitumor [141], among others. The biological activities of this class of compounds are associated with their tricyclic scaffold but vary depending on the nature and/or position of the different substituents [135].



R_1, R_2: diverse substituents

Figure 4 - Xanthone scaffold and numbering.

In the last several years, one of the main aims of the research Group of Medicinal Chemistry

of Laboratory of Organic and Pharmaceutical Chemistry (LQOF) of the Faculty of Pharmacy of the University of Porto/CIIMAR is the synthesis of new XDs for biological activity evaluation, as example in [142-144].

Regardless of the large structural diversity of bioactive XDs, only a limited number of synthetic chiral derivatives of xanthones (CDXs) were reported [145, 146]. To expand the library of chiral compounds, new CDXs were synthetized by LQOF/CIIMAR research group [147, 148].

The CDXs were synthesized in enantiomerically pure form, by coupling carboxyxanthones with both enantiomers of commercially available chiral reagents, including for example amines and amino alcohols, using *O*-(benzotriazol-1-yl)-*N*,*N*,*N'*,*N'*-tetramethyluronium tetrafluoroborate (TBTU) as coupling reagent [148].

Some of the synthetized CDXs proved to have interesting biological activities, acting as blockers of sciatic nerve transmission [148], or as inhibitors of human tumor cell lines growth [147], and as inhibitors of enzymes involved in inflammatory processes, cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2) [149]. Furthermore, CDXs proved to have analytical applications as chiral selectors in LC [142].

Additionally, enantioseparation studies and enantiomeric purity evaluation of the new CDXs have been conducted in LQOF/CIIMAR research group using different types of CSPs, namely Pirkle-type [50, 150], macrocyclic glycopeptide-based [79, 151, 152], and polysaccharide-based [153].

The same group reported *in vitro* assays, by spectrofluorimetry, as well as *in silico* studies, by docking technique, to evaluate the binding interaction of three enantiomeric pairs of CDXs, synthetized “in house”, with HSA [149]. For both assays, all tested CDXs demonstrated to bind with high affinity to HSA, and enantioselectivity was observed for one enantiomeric pair. Good agreement between *in silico* and *in vitro* data (bind affinity and enantioselectivity) was achieved.

Chapter II

AIMS

One of the main objectives of this dissertation was the expansion of enantioresolution by liquid chromatography (LC) and chiral recognition studies of a library of chiral derivatives of xanthones (CDXs), synthetized in our research group, on a commercially available human serum albumin chiral stationary phase (HSA-CSP), specifically CHIRALPAK® HSA.

For this main aim to be fulfilled, the following specific objectives had to be contemplated:

- Systematic study of enantioresolution by optimization of the chromatographic conditions, under reversed-phase elution mode, using different mobile phases, exploring:
    - buffer type and ionic strength
    - type and content of organic modifiers
    - mobile phase pH
    - as well as temperature of analysis;
- Carrying out computational studies, via molecular docking approach, to confirm the results obtained in LC studies and to better understand the chromatographic behavior at a molecular level, as well as the structural features associated with the chiral recognition mechanism.

Another main objective was the measurement of the protein binding affinity of each enantiomer of CDXs no HSA-CSP under chromatographic conditions that mimetic the physiological environmental, in order to optimize a fast and reliable method of screening for the affinity for HSA of this class of compounds.

Additionally, to contribute to expand the applications of HSA-CSP on the enantioseparation of a new class of chiral compounds.

Chapter III

EXPERIMENTAL

1. Chemical and reagents

CDXs were previously synthesized in the research Group of Medicinal Chemistry of Laboratory of Organic and Pharmaceutical Chemistry (LQOF) of the Faculty of Pharmacy of the University of Porto/CIIMAR in enantiomerically pure form, by coupling different carboxyxanthones with both enantiomers of commercially available chiral reagents, according to described procedure [148]. Chlorpromazine, indomethacin and metronidazole (all in p.a. grade) were purchased from Merck (Darmstadt, Germany). Methanol (MeOH), ethanol (EtOH), 2-propanol (2-PrOH), acetonitrile (ACN) for HPLC were purchased from Merck (Darmstadt, Germany). Ammonium acetate (CH_3COONH_4), sodium phosphate dibasic (Na_2HPO_4), sodium phosphate monobasic (NaH_2PO_4), potassium phosphate monobasic (KH_2PO_4), di-potassium hydrogen phosphate 3-hydrate ($K_2HPO_4.3H_2O$) (all in p.a. grade) were purchased from Carlo Erba (Barcelona, Spain) or from Merck (Darmstadt, Germany). Ultrapure water was generated by a Milli-Q system (Millipore, Bedford, MA). All other reagents and solvents were of analytical grade or ultrapure.

2. Instrumentation and chromatographic conditions

Analytical LC analyses were performed on a Thermo® Scientific HPLC equipped with an Thermo® Scientific Spectra System P4000 pump, detection was carried out using an Thermo® Scientific Spectra System UV8000 diode array detector (DAD) and the samples were injected by an autosampler Thermo® Scientific Spectra System AS3000. Data acquisition was performed using ChromQuest 5.0™. The chromatography analyses were achieved in a reversed-phase mode on a CHIRALPAK® HSA column (150 mm × 40 mm, I.D., 5 µm) purchased at ChromTech, Lda (Congelton, Cheshire, UK). The flow rate used was 0.9 mL/min and the chromatograms were monitored by a DAD detector with the wavelength of 254 nm. The sample injections (20 µL) were carried out in duplicate and the mean of both values were considered. The HPLC temperature and mobile phases were controlled by AS3000 autosampler oven and during the systematic study ranged from 22 ± 2°C to 37 ± 2°C), and in binding studies were 37 ± 2°C.

3. Mobile phases

Aqueous buffers of sodium phosphate, potassium phosphate, ammonium acetate and sodium acetate were prepared at 10 mM; potassium phosphate buffer was also prepared at 67 mM. Buffers at 10 mM were prepared at pH 5.0 and 7.0, and the 67 mM buffers were prepared at pH 7.0 and 7.4. All aqueous buffers pH was controlled by a Gondo® PL-700PV

pH monitor. The mobile phases were prepared in a volume/volume relation (*v*/*v*), degassed in an ultrasonic bath (Sonorex Digitec, Bandelin) for at least 15 min before use and were filtered through polyamide membrane filters of 0.2 µm pore size from Whatman® GmbH (Dassel, Germany).

In the systematic study and optimization of the method, several mobile phases were tested. The organic modifiers used were ACN, MeOH, EtOH and 2-PrOH.

For binding studies, the mobile phase composition used was a mixture of 67 mM potassium phosphate buffer at pH 7.4 with ACN as organic modifier in nine different proportions between 98:2 and 75:25 (*v*/*v*).

4. Sample solutions

Stock solutions of enantiomerically pure CDXs were prepared in EtOH at the concentration of 1 mg/mL and further diluted to working solutions of 100 µg/mL. Working solutions of enantiomeric mixtures were prepared mixing equal aliquots of each enantiomer. The ligand-protein binding determinations were performed with the working solutions of each enantiomer diluted at the concentration of 50 µg/mL. The same principle was used to prepare the working solutions of the drugs, prepared in EtOH at the concentration of 1 mg/mL and further diluted at the concentration of 50 µg/mL.

5. Chromatographic parameters

The chromatographic parameters determined by LC analyses on the systematic study of enantioresolution and for binding studies of CDXs were: the retention factor (***k***), the separation factor (***α***) and resolution ($\mathbf{R_S}$). The dead time (t_0) was considered to be equal to the peak of the solvent front. An example of a general chromatogram of an enantioseparation is represented in **Figure 5**.

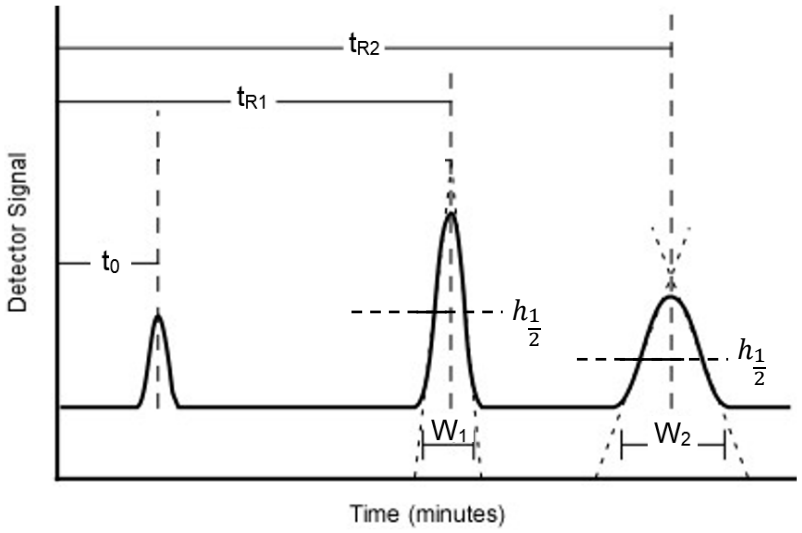


Figure 5 - Illustration of an enantiomeric separation chromatogram with the measurements related to chromatographic parameters [154].

The retention factor (***k***) is the ratio between the retention time (t_R) of the analyte in the chromatographic column and the dead time (t_0), and is calculated by the following equation **(2)**:

$$k = \frac{(t_R - t_0)}{t_0} \quad \textbf{(2)}$$

The separation factor (***α***) measures the capability of the chromatographic system to separate the enantiomers in a sample and it is calculated by the following equation **(3)**:

$$\alpha = \frac{k_2}{k_1} \quad \textbf{(3)}$$

The resolution (***R*$_S$**) measures the quality of a separation and it is calculated by the following equation **(4)**:

$$R_S = 1.18 \times \frac{t_{R2} - t_{R1}}{W_{1\,h_{\frac{1}{2}}} - W_{2\,h_{\frac{1}{2}}}} \quad \textbf{(4)}$$

were, $W_{1\,h_{\frac{1}{2}}}$ and $W_{2\,h_{\frac{1}{2}}}$ are the width of the band mid-height.

6. Computational

The X-ray crystal structure of HSA (PDB code: 2bxg) was downloaded from the Protein Data Bank of Brookhaven [155], and both enantiomers of all CDXs were drawn and minimized using an Austin Model 1 (AM1) semi-empirical quantum mechanics force field [156]. The calculation was finished when the gradient between any two successive steps in the geometry search was less than 10^{-1} kcal/mol/Å or the maximum steps were reached, whichever comes first. The line search used was the Broyden-Fletcher-Golfarb-Shanno search which uses an approximate Hessian matrix to guide the search [157]. Docking simulations between the chiral selector and the CDXs were performed in AutoDock Vina (Molecular Graphics Lab, La Jolla, CA, USA) [158]. AutoDock Vina considered the target conformation as a rigid unit while the ligands were allowed to be flexible and adaptable to the target. Vina searched for the lowest binding affinity conformations and returned nine different conformations for each small molecule. The lowest binding energy docking poses of each compound were chosen. AutoDock Vina was run using an exhaustiveness of 8 and a grid boxes with the dimension of X: 2.8, Y: 0.669, Z: 0.128 for HSA. PyMol v1.3 (Schrödinger, New York, NY, USA) [159] was used for visual inspection of results and graphical representations.

Chapter IV

RESULTS AND DISCUSSION

In this work, a CHIRALPAK® HSA chromatographic column was employed for a systematic study of enantioseparation of a library of thirty-one enantiomeric mixtures of CDXs (**Figure 6**), where several chromatographic conditions were explored. Affinity studies, under chromatographic conditions that mimetic *in vivo* environment, and computational studies *via* docking technique were also conducted.

Both enantiomers of all enantiomeric mixtures of CDXs **1-31** were previously synthesized, in enantiomerically pure form, by coupling carboxyxanthones (CXs) with both enantiomers of commercially available chiral reagents using *O*-(benzotriazol-1-yl)-*N*,*N*,*N'*,*N'*-tetramethyluronium tetrafluoroborate (TBTU) as coupling reagent (**Scheme 1**), according to reported methods [147, 148].

As mentioned previously, systematic studies of enantioseparation and determination of enantiomeric purity were carried out with some of these chiral compounds through polysaccharide-based [153], macrocyclic antibiotic-based [79, 151, 152], and Pirkle-type [50, 150] CSPs. Thus, this systematic study using a column with an immobilized HSA is essential to increase the chiral recognition studies of these compounds on different types of CSPs. Previously three CDXs have demonstrated a high affinity for HSA in *in vitro* studies [149]. Thus, to prove and extend the binding study for the sixty-two enantiomers to HSA, herein, affinity studies were performed by liquid chromatography.

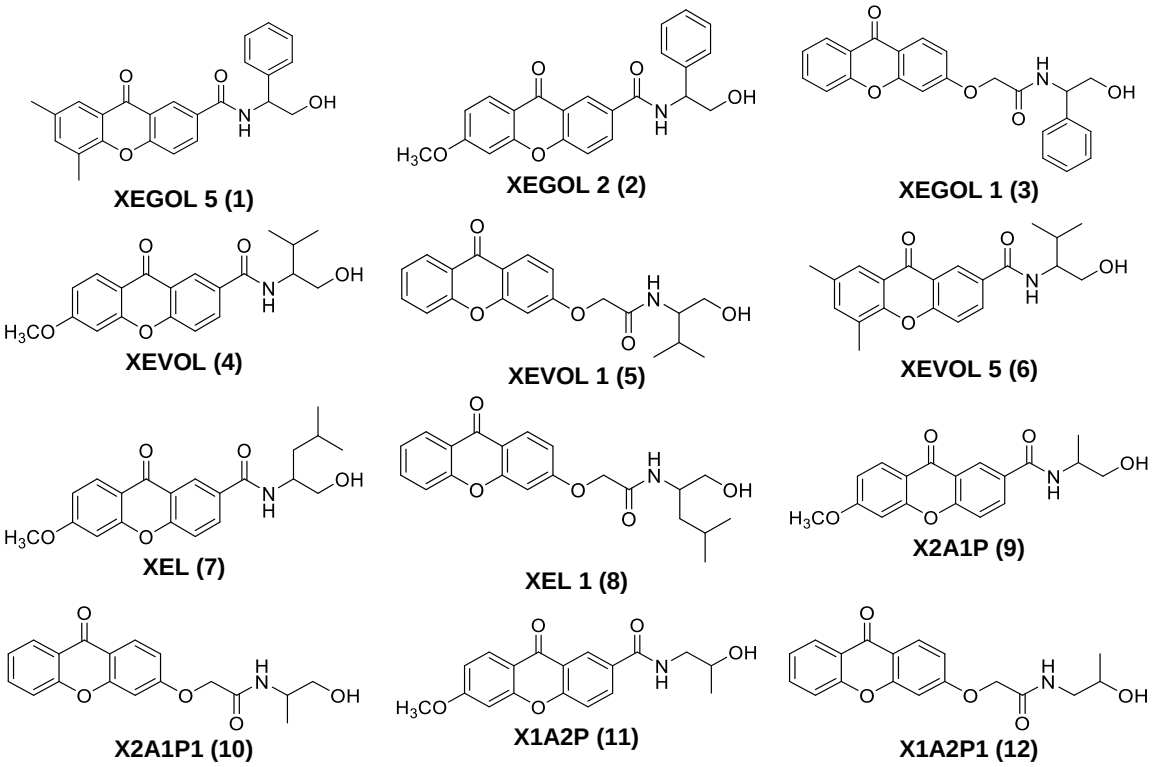
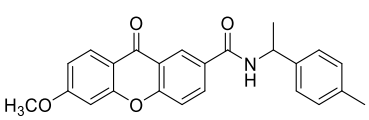
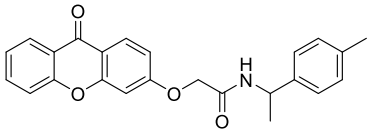


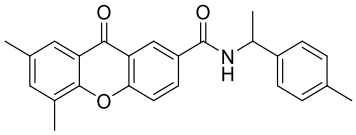
Figure 6 - Chemical structures of enantiomeric mixtures of CDXs **1-31**.



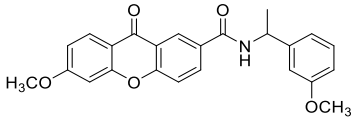
XEA (13)



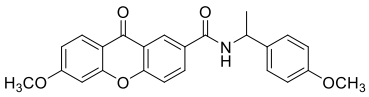
XEA 1 (14)



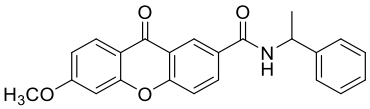
XEA 5 (15)



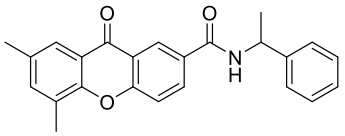
XEA 3 MET (16)



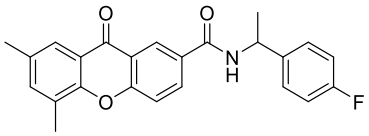
XEA 4 MET (17)



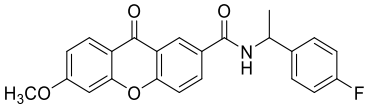
XEA DES (18)



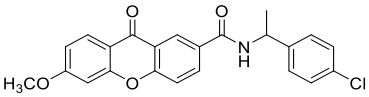
XEA 5 DES (19)



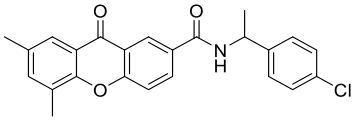
XEA 5 4 FLU (20)



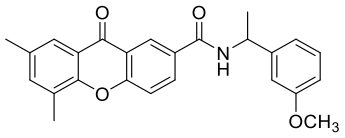
XEA 4 FLU (21)



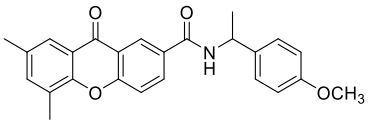
XEA 4 CLO (22)



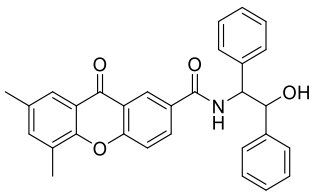
XEA 5 4 CLO (23)



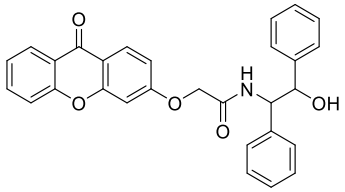
XEA 5 3 MET (24)



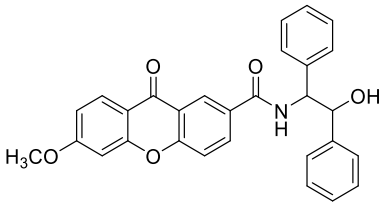
XEA 5 4 MET (25)



X2ADF 5
(RR, SS) **(26)**
(SR, RS) **(27)**

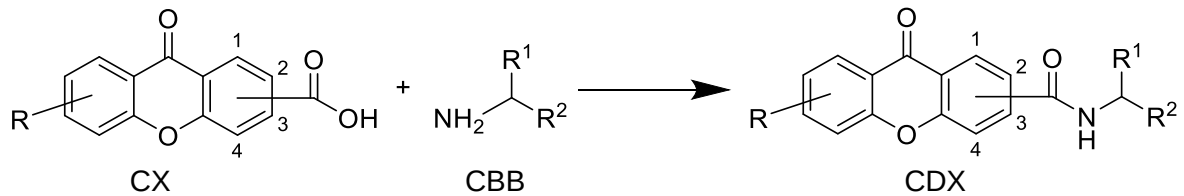


X2ADF 1
(RR, SS) **(28)**
(SR, RS) **(29)**



X2ADF
(SR, RS) **(30)**
(RR, SS) **(31)**

Figure 6 - Continuation.



R, R^1 and R^2: diverse substituents
CX: carboxyxanthone
CBB: chiral building block
CDX: chiral derivatives of xanthone

Scheme 2 – General representation of the synthesis of CDXs.

1. Systematic enantiorresolution of CDXs

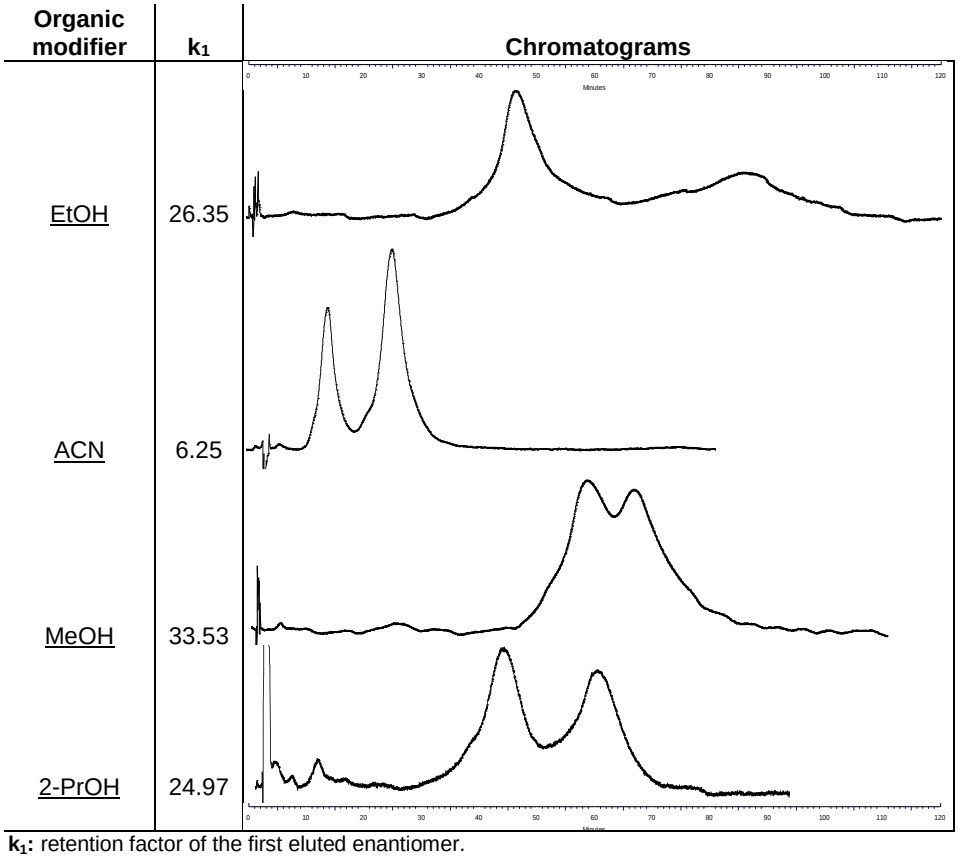
In this systematic study, optimization of the chromatographic conditions were performed to achieve the best conditions to obtain a higher number of separations of enantiomeric mixtures of CDXs **1-31** (**Figure 6**), with good resolution and short analysis time, using a CHIRALPAK® HSA column. Firstly, the organic modifier type and content, the buffer type and mobile phase pH were studied and optimized. Then, modifications of the chromatographic conditions were carried out to approach to the physiological environment, by changing the ionic strength of the buffer to 67 mM, the mobile phase pH to 7.4 and the temperature of analysis to 37ºC.

1.1. Organic modifier selection

The first step was to choose the most suitable organic modifier for the resolution of enantiomeric mixtures of CDXs **1-31**. Columns with immobilized HSA, such as a CHIRALPAK® HSA, are very susceptible to the presence and variation of organic modifiers [160]. Organic modifiers may also differentially affect the reversible spatial conformation of the protein leading to changes in the number of available selective and non-selective binding sites, and may further accelerate protein denaturation leading to a significant shortening of CSP lifetime. This type of columns works in reversed phase mode, knowing that the presence of organic modifier may be harmful [161]. The purpose of choosing the best organic modifier must take into consideration three factors: (i) minor proportion as possible of organic modifier; (ii) shorten CDXs retention and, consequently, analysis time; (iii) maintain the enantioselective capacity of the CSP.

Two possible mechanisms can explain the change of retention of analytes in the column with an increase of organic modifier in mobile phase [70, 113]. On the one hand, the molecules of organic modifier can act as hydrogen bonding acceptors or donors and easily form strong hydrogen interactions with immobilized HSA and analytes. Consequently, solvent molecules occupy binding sites of HSA through formation of hydrogen bond interactions, and the interactions between these sites and analytes will be reduced, resulting in a trend for retention decrease. On the other hand, hydrophobic interactions between analytes and CSP will become weaker with increasing hydrophobicity of the mobile phase, which also causes a reduction of retention of analytes in the column. In addition, an increase of resolution (Rs) might be due to a change of spatial conformation and steric environment of HSA induced by organic solvent, which contributes to improvement of a protein's chiral recognition ability [161].

From the technical data sheet provided by the column manufacturer (**Appendix G.**) and considering that the tested CDXs are mostly neutral, a mobile phase comprising 10 mM ammonium acetate buffer at pH 7.0 was chosen for starting the systematic study of enantioresolution. The ratio of solvent chosen (15%) was due to the high retention demonstrated by these compounds in previous works [150-153, 162]. Four organic modifiers were described in the technical data sheet, namely ACN, EtOH, 2-PrOH and MeOH. Initially, all enantiomeric mixtures were tested using four different mobile phases comprising a 10 mM ammonium acetate buffer at pH 7.0 and each of the organic modifiers described previously, in a proportion of 85:15 *v/v*. Under these conditions, the majority of the enantiomeric mixtures of CDXs were not enantioseparated. Nevertheless, for some enantiomeric mixtures enantioselectivity was observed; however, very high retention and poor resolution were observed. As example, **Figure 7** shows the chromatograms obtained for enantiomeric mixture of X2ADF *SR RS* (**30**) (**Figure 7**) using different mobile phases.



k_1: retention factor of the first eluted enantiomer.

Figure 7 - Chromatograms for enantiomeric mixture X2ADF *SR RS* (**30**) on a CHIRALPAK® HSA using different organic modifiers: EtOH, ACN, MeOH and 2-PrOH. Mobile phase, 10 mM ammonium acetate buffer/organic modifier (85:15 *v/v*); Flow rate, 0.9 mL/min.; Detection, 254 nm.

According to **Figure 7**, the mobiles phases comprising MeOH, EtOH and 2-PrOH as organic modifiers afforded the highest retention factors, with k_1 = 33.53, k_1 = 26.35 and k_1 = 24.97,

respectively. The organic modifier ACN showed lower retention factors, with k_1 = 6.25 as well as better resolution, when compared to the other mobile phases. Considering retention time, similar behavior was observed for the other enantiomeric mixtures of CDXs. The main objectives for the selection of the organic modifier were the reduction of the analysis time as well as its proportion used in the mobile phase, in addition to enantioselectivity. Accordingly, ACN was selected for the development and method optimization because it allowed short retention times for the tested compounds with enantioselectivity, for some mixtures.

1.2. Buffer and pH selection

After choosing ACN as the most suitable organic modifier for the systematic study of enantioseparation, different buffers were tested as aqueous phase. Based on the technical sheet of the manufacturer (**Appendix G.**), four types of buffers were chosen, namely ammonium acetate, sodium acetate, potassium phosphate and sodium phosphate.

The choice of phosphate buffers is intrinsically related to the stability of the HSA as chiral selector as already reported in other studies [100]. In addition, several studies described the use of this type of buffers mimicking the physiological conditions existing in human plasma consequently used in protein binding studies [71, 104, 160]. Acetate buffers were also widely used in several enantioseparation works, demonstrating good applicability [163, 164]. The advantage of acetate buffers coincides with one of the disadvantages of the phosphate buffers, i.e., their applicability in LC-MS systems. In fact, phosphate buffers cannot be used in the mobile phase for LC analysis using MS detection because they are non-volatile buffers being responsible for ion suppression [165].

The effect of pH variation is also a determining factor for this type of study. The pH variation interferes in Coulomb interactions, leading to conformational alterations of the albumin molecule which possesses negative or positive charges when the buffer pH is higher or lower than its isoelectric point [166]. HSA will be more negatively charged with increasing pH, for example. In addition, the control of pH is essential for analysis ofionized samples [167]. In this work, since all tested CDXs are mostly neutral and weak oxygenated heterocycles acids, no significant changes in their ionization occurred varying the pH of the mobile phase. According to the manufacturer of the column (**Appendix G.**), a variation of mobile phase pH from 5.0 to 7.0 is possible to be used without shortening the life of the CSP. Thus, to verify the effect of pH, and using ACN as the organic modifier (ranging from 25 to 0%), two different pH were selected for the various buffers studied: (i) 10 mM ammonium acetate at pH 5.0; (ii) 10 mM ammonium acetate at pH 7.0; (iii) 10 mM sodium acetate at pH 5.0; (iv) 10 mM sodium acetate at pH 7.0; (v) 10 mM potassium phosphate at

pH 5.0; (vi) 10 mM potassium phosphate at pH 7.0; (vii) 10 mM sodium phosphate at pH 5.0; (viii) 10 mM sodium phosphate at pH 7.0.

All thirty-one enantiomeric mixtures of CDXs (**1-31**) were injected into the HSA-CSP using the buffers described above (i – viii) and with different proportions of ACN as organic modifier ranging from 25 to 0%.

1.2.1. Ammonium acetate buffer

Among all aqueous buffers, ammonium acetate (10 mM) at pH 5.0 was the first to be tested to perform the resolution of the enantiomeric mixtures of CDXs **1-31**. Using ACN as organic modifier, eight among of thirty-one enantiomeric mixtures of CDXs injected into the HSA-CSP were baseline enantioseparated, with α ranging from 1.51 to 4.91 and Rs ranging from 1.61 to 3.87. The overall best results are shown on **Table 4**.

Table 4 - The best chromatographic data obtained for separation of enantiomeric mixtures of CDXs **1-31**, on CHIRALPAK® HSA, using 10 mM ammonium acetate at pH 5.0 as buffer.

Enantiomeric mixture	t_1	t_2	k_1	k_2	α	R_s	% of ACN
XEGOL 5 (**1**)	8.29	20.27	3.36	9.67	2.87	3.27	18
XEA 5 (**15**)	7.26	18.77	2.82	8.88	3.15	2.79	22
XEA 3 MET (**16**)	26.62	39.11	13.01	19.58	1.51	1.61	10
XEA 5 DES (**19**)	5.76	20.86	2.03	9.98	4.91	3.87	22
XEA 5 4 FLU (**20**)	5.76	16.25	2.03	7.55	3.72	3.08	22
XEA 5 3 MET (**24**)	13.11	33.04	5.90	16.39	2.78	3.07	18
XEA 5 4 MET (**25**)	14.69	30.82	6.73	15.22	2.26	2.40	18
X2ADF 5 *SR RS* (**27**)	6.45	15.63	2.39	7.23	3.02	2.64	22

CHIRALPAK® HSA column; Mobile phase acetate buffer (pH 5.0):ACN (variable proportion); Flow rate 0.9 mL/min; Temperature 22 ± 2°C; Detection UV 254nm

As showed in **Table 4**, the enantiomeric mixture X2ADF 5 SR RS (**27**) was separated in the shortest analysis time, k_1 = 2.39 and k_2 = 7.23, with good enantioselectivity and resolution, α = 3.02 and R_S = 2.64 respectively, using a ratio of 22% of organic modifier. Using the same mobile phase, the enantiomeric mixture XEA 5 DES (**19**) demonstrated the best enantioselectivity and resolution, with α = 4.91 and R_S = 3.87 respectively (**Figure 8**).

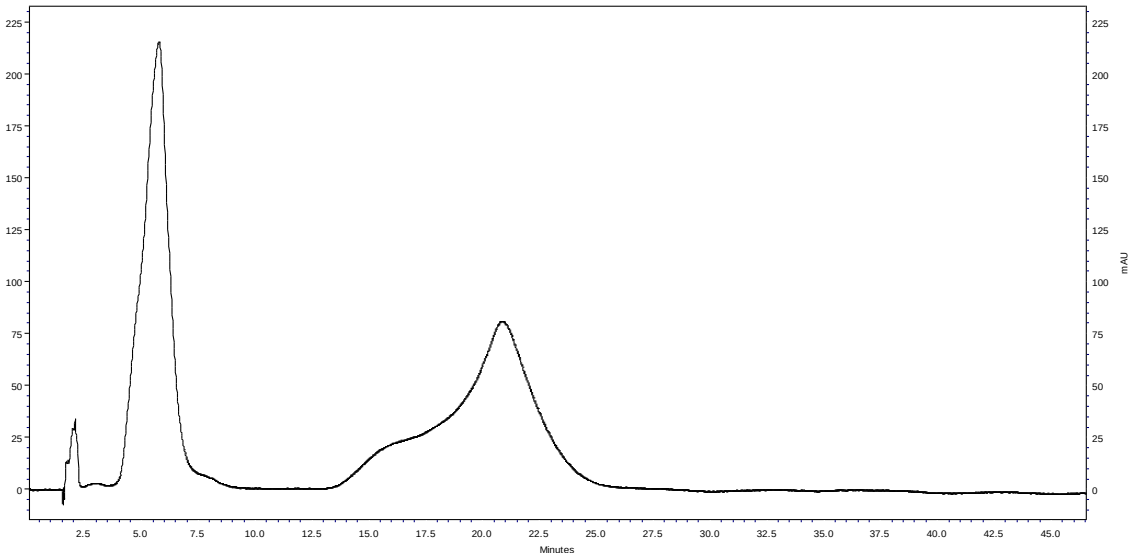


Figure 8 - Chromatogram of enantioresolution of enantiomeric mixture of CDX XEA 5 DES **(19)** on CHIRALPAK® HSA column. Conditions: Flow rate: 0.9 mL/min; Mobile Phase, 10 mM ammonium acetate at pH 5.0:ACN (78:22 *v/v*); Detection, 254 nm; temperature, 22 ± 2°C.

Similar results with good separation and resolution were also achieved for enantiomeric mixture XEGOL 5 (**1**) (α = 2.87; R_S = 3.27) (**Figure 9**).

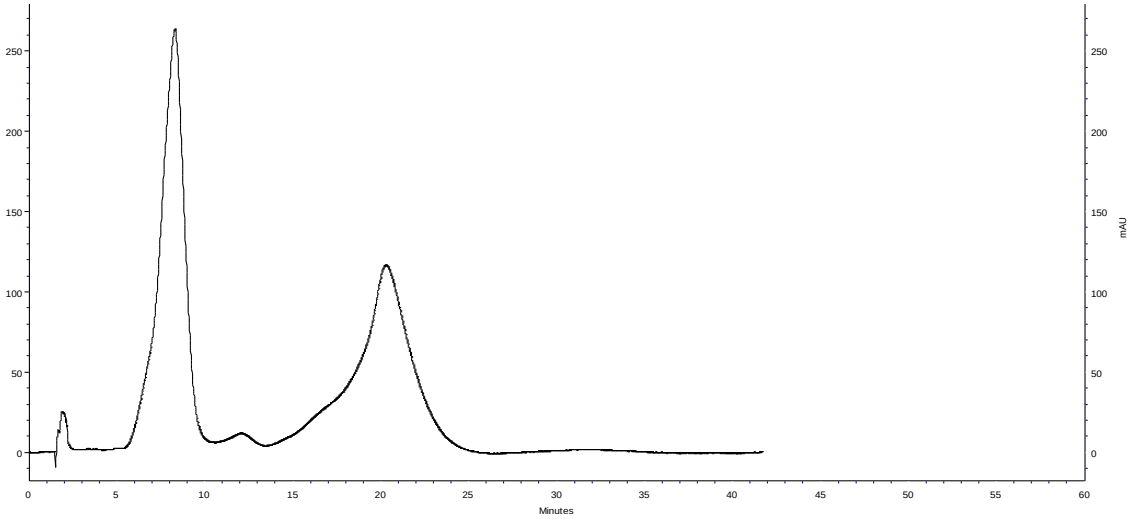


Figure 9 –Chromatogram of the enantioresolution of enantiomeric mixture of CDX XEGOL 5 (**1**) on the CHIRALPAK® HSA column; Conditions: mobile phase, 10 mM ammonium acetate (pH 5.0):ACN (82:18 *v/v*); flow rate: 0.9 mL/min; detection, 254 nm; temperature, 22 ± 2°C.

Regarding the use of pH 7.0 on mobile phase, the overall best results are shown on **Table 5**. Among the thirty-one enantiomeric mixtures tested on HSA column, eight of them were baseline enantioseparated, with α ranging from 1.86 to 6.63 and R_S ranging from 2.20 to 5.24, respectively. The same aqueous buffer (10 mM ammonium acetate buffer) and

organic modifier (ACN) were used as mobile phase, only changing the pH and proportions of solvents to achieve the best chromatographic results.

Table 5 - The best chromatographic data obtained for the separation of enantiomeric mixtures of CDXs on CHIRALPAK® HSA, using 10 mM ammonium acetate at pH 7.0 as buffer.

Enantiomeric mixture	t_1	t_2	k_1	k_2	α	R_s	% of ACN
XEGOL 5 (**1**)	7.01	17.15	2.69	8.03	2.98	3.55	20
XEA 5 (**15**)	11.09	37.44	4.84	18.71	3.87	4.73	22
XEA 5 DES (**19**)	7.72	40.47	3.06	20.30	6.63	5.24	22
XEA 5 4 FLU (**20**)	7.83	30.67	3.12	15.14	4.85	4.38	22
XEA 5 3 MET (**24**)	7.15	15.29	2.76	7.05	2.55	2.87	22
XEA 5 4 MET (**25**)	13.76	30.04	6.24	14.81	2.37	2.30	20
X2ADF 5 *SR RS* (**27**)	9.20	32.03	3.84	15.86	4.13	3.97	22
X2ADF *SR RS* (**30**)	26.51	47.61	12.95	24.06	1.86	2.20	13

CHIRALPAK® HSA column; Mobile phase: ammonium acetate buffer (pH 7.0):ACN (variable proportion); Flow rate 0.9 mL/min; Temperature 22 ± 2°C; Detection UV 254nm

The enantiomeric mixture XEA 5 3 MET (**24**) was enantioseparated in the shortest analysis time, with k_1 = 2.76 and k_2 = 7.05, and with good enantioselectivity and resolution, with α = 2.55 and R_S = 2.87 respectively, using a ratio of 22% of organic modifier. Using the same solvent ratio, the enantiomeric mixture XEA 5 DES (**19**) (**Figure 10**) showed the best enantioselectivity and resolution, α = 6.63 and R_S = 5.24 respectively.

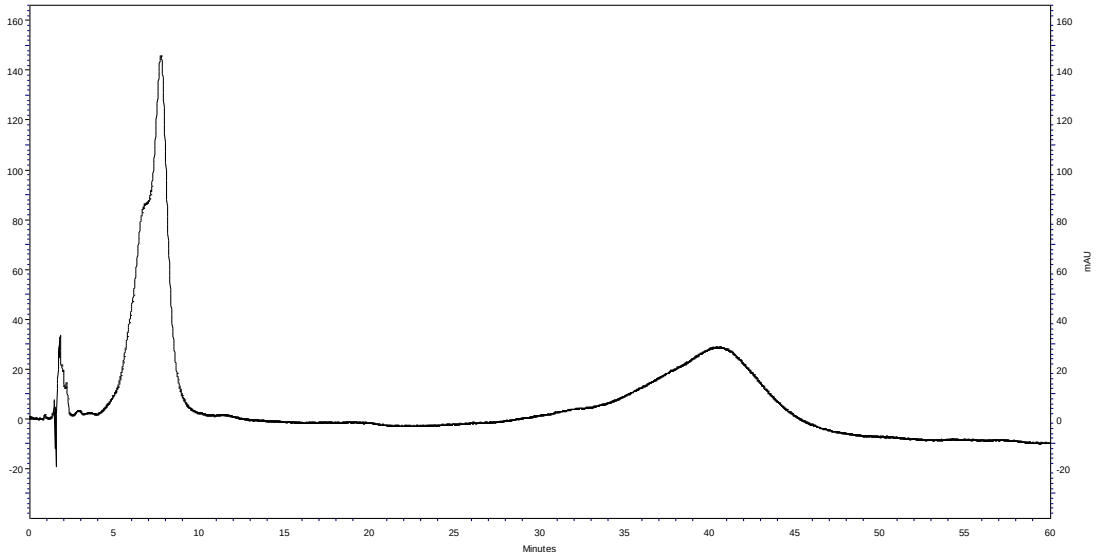


Figure 10 - Chromatogram of enantioresolution of enantiomeric mixture of CDX XEA 5 DES (**19**) on the CHIRALPAK® HSA column; Conditions: flow rate: 0.9 mL/min; mobile phase, 10 mM ammonium acetate (pH 7.0):ACN (78:22 *v/v*); detection, 254 nm; temperature, 22 ± 2°C.

Regarding the effect that the change of mobile phase pH caused, **Figure 11** shows, as example, the variations of the chromatographic parameters k_1, k_2 and Rs, for three

enantiomeric mixtures (**1**, **20** and **25**), according to the mobile phase pH. For the three enantiomeric mixtures, at pH 7.0 both enantiomers were more retained, as well as the quality of the separation increased. The same behavior was obtained for the other baseline separated enantiomeric mixtures.

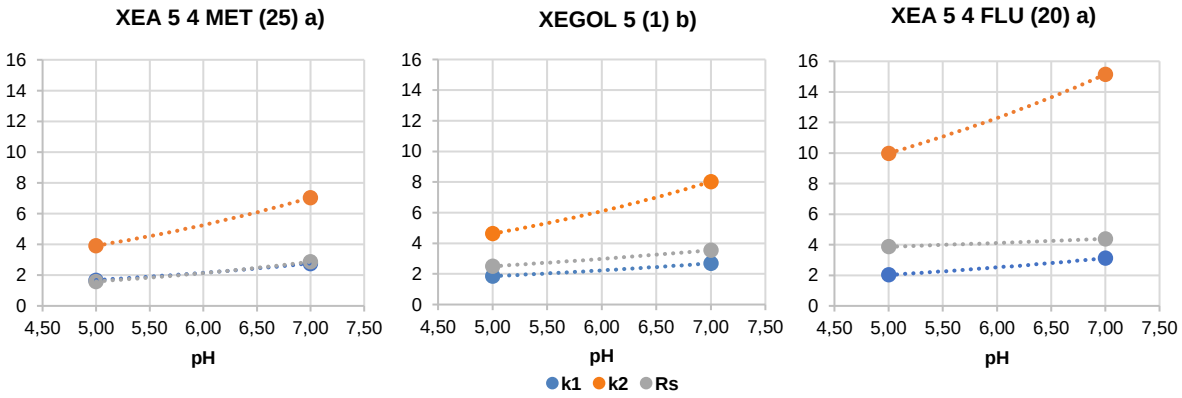


Figure 11 – Effect of mobile phase pH on retention factors and resolution of enantiomeric mixtures XEA 5 4 MET (**25**), XEGOL 5 (**1**) and XEA 5 4 FLU (**20**); Conditions: flow rate: 0.9 mL/min; mobile phase a), 10 mM ammonium acetate (pH 5.0 and 7.0): ACN 78:22 (*v/v*); mobile phase b), 10 mM ammonium acetate (pH 5.0 and 7.0): ACN 80:20 (*v/v*).

The only exceptions were observed for enantiomeric mixture XEA 3 MET (**16**), being enantioseparated at pH 5.0 but at pH 7.0 no baseline separation was observed, and enantiomeric mixture X2ADF *SR RS* (**30**), being only enantioseparated when using a mobile phase at pH 7.0.

1.2.2. Sodium acetate buffer

According to the results, a total of ten among thirty-one tested enantiomeric mixtures of CDXs were baseline enantioseparated using 10 mM sodium acetate at pH 5.0 and ACN, in variable proportion, as mobile phase. Good enantioselectivity and resolution were obtained, with α values ranging from 1.53 to 4.88 and Rs ranging from 1.38 to 3.10. The overall best results are shown on **Table 6**.

Table 6 - The best chromatographic data obtained for separation of enantiomeric mixtures of CDXs **1-31**, on CHIRALPAK® HSA, using 10 mM sodium acetate (pH 5.0) as buffer.

Enantiomeric mixture	t_1	t_2	k_1	k_2	α	R_s	% of ACN
XEGOL 5 (**1**)	10.83	28.59	4.70	14.05	2.99	2.70	16
XEA 5 (**15**)	9.59	26.03	4.05	12.84	3.17	2.49	21
XEA 3 MET (**16**)	30.94	46.23	15.28	23.33	1.53	2.08	11
XEA 5 DES (**19**)	7.46	22.15	2.93	10.66	3.64	2.57	21
XEA 5 4 FLU (**20**)	6.19	22.84	2.26	11.02	4.88	3.10	22
XEA 5 4 CLO (**23**)	8.72	16.71	3.59	7.79	2.17	1.38	20
XEA 5 3 MET (**24**)	11.48	26.99	5.04	13.21	2.62	2.70	18
XEA 5 4 MET (**25**)	19.71	42.39	9.37	21.31	2.27	2.45	16
X2ADF 5 *SR RS* (**27**)	8.49	21.51	3.47	10.32	2.98	2.38	21
X2ADF *SR RS* (**30**)	39.31	70.75	19.69	36.24	1.84	3.04	11

CHIRALPAK® HSA column; Mobile phase sodium acetate buffer (pH 5.0):ACN (variable proportion); Flow rate 0.9 mL/min; Temperature 22 ± 2°C; Detection UV 254nm

Regarding baseline separation, the enantiomeric mixture XEA 5 4 CLO (**23**) showed the shortest retention time, with k_1 = 3.59 and k_2 = 7.79, as well as good enantioselectivity and resolution, with α = 2.17 and R_S = 1.38 respectively, when using a ratio of 20% of organic modifier in the mobile phase. Using a different ACN ratio (22%), it should be noted that the enantiomeric mixture XEA 5 4 FLU (**20**) showed the best enantioselectivity and resolution, with α = 4.88 and R_S = 3.10, respectively (**Figure 12**).

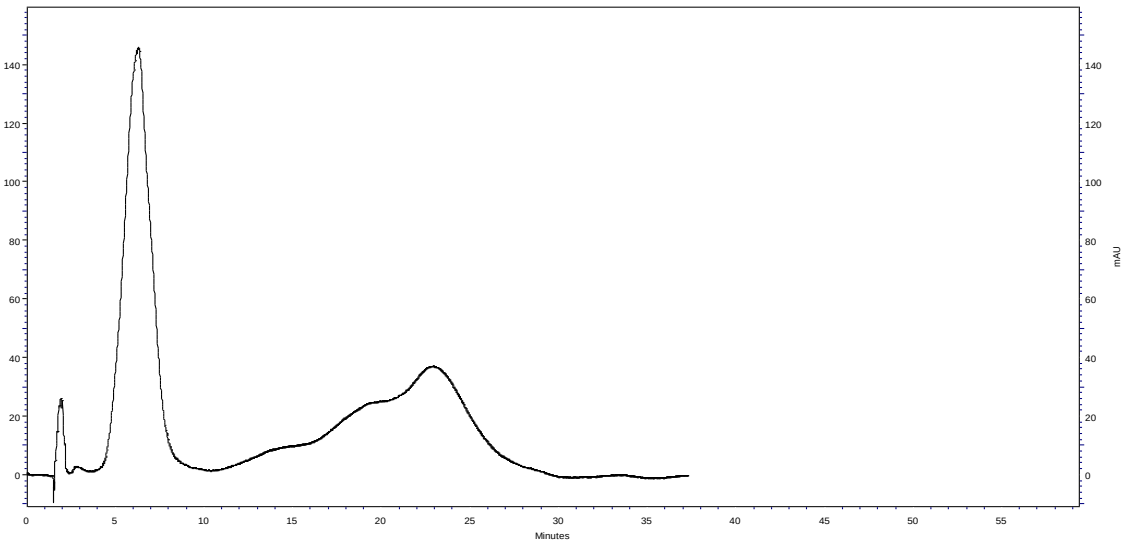


Figure 12 - Chromatogram for enantioresolution of enantiomeric mixture XEA 5 4 FLU (**20**) on CHIRALPAK® HSA column; Conditions: flow rate: 0.9 mL/min; mobile phase, 10 mM sodium acetate (pH 5.0):ACN (78:22 *v/v*); detection, 254 nm; temperature, 22 ± 2°C.

When the pH of the mobile phase was changed to 7.0, from the thirty-one tested enantiomeric mixtures of CDXs nine of them were baseline enantioseparated, with α values ranging from 1.88 to 4.11 and resolution from 1.78 to 3.30 (**Table 7**).

Table 7 - The best chromatographic data obtained for separation of enantiomeric mixtures of CDXs **1-31**, on CHIRALPAK® HSA, using 10 mM sodium acetate (pH 7.0) as buffer.

Enantiomeric mixture	t_1	t_2	k_1	k_2	α	R_s	% of ACN
XEGOL 5 (**1**)	13.05	38.83	5.87	19.44	3.31	3.30	20
XEA 5 (**15**)	9.22	25.81	3.85	12.58	3.27	1.78	21
XEA 5 DES (**19**)	6.77	21.91	2.56	10.53	4.11	2.03	21
XEA 5 4 FLU (**20**)	7.03	21.58	2.70	10.36	3.84	2.37	21
XEA 4 CLO (**22**)	11.29	23.32	4.94	11.27	2.28	2.52	20
XEA 5 3 MET (**24**)	13.38	33.73	6.04	16.75	2.77	2.52	18
XEA 5 4 MET (**25**)	10.43	20.36	4.49	9.72	2.16	2.27	20
X2ADF 5 *SR RS* (**27**)	9.02	22.38	3.22	10.78	3.35	2.35	21
X2ADF *SR RS* (**30**)	46.48	85.90	23.46	44.21	1.88	2.12	10

CHIRALPAK® HSA column; Mobile phase sodium acetate buffer (pH 7.0):ACN (variable proportion); Flow rate 0.9 mL/min; Temperature 22 ± 2°C; Detection UV 254nm

Under these chromatographic conditions, the enantiomeric mixture separated in the shortest analysis time, with k_1 = 4.49 and k_2 = 9.72, was XEA 5 4 MET (**25**)**,** using a ratio of 20% of organic modifier in the mobile phase. Moreover, a good enantioselectivity and resolution were also achieved, with α = 2.16 and R_S = 2.27 respectively. Using the same ACN ratio, the enantiomeric mixture XEGOL 5 (**1**) showed the best enantioselectivity and resolution, with α = 3.31 and R_S = 3.30 respectively (**Figure 13**).

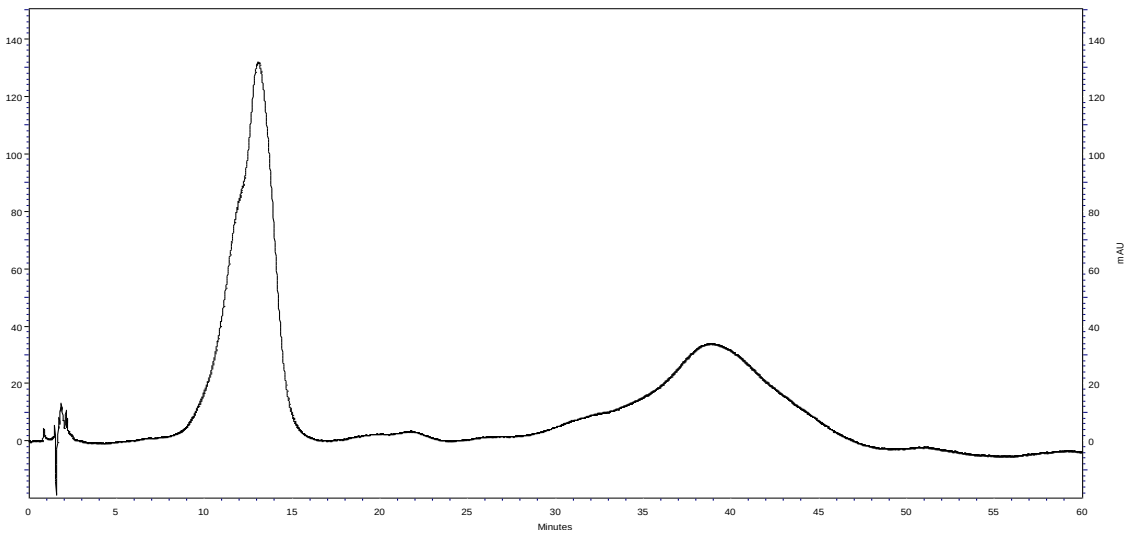


Figure 13 - Chromatogram for enantioresolution of enantiomeric mixture of CDX XEGOL 5 (**1**) on CHIRALPAK® HSA column; Conditions: flow rate: 0.9 mL/min; mobile phase, 10 mM sodium acetate (pH 7.0):ACN (80:20 *v/v*); detection, 254 nm; temperature, 22 ± 2°C.

Similar results were also achieved for enantiomeric mixtures XEA 5 3 MET (**24**), with α = 2.77 and R_S = 2.52 (**Figure 14**), and XEA 5 4 FLU (**20**), with α = 3.84 and R_S = 2.37 (**Figure 15**).

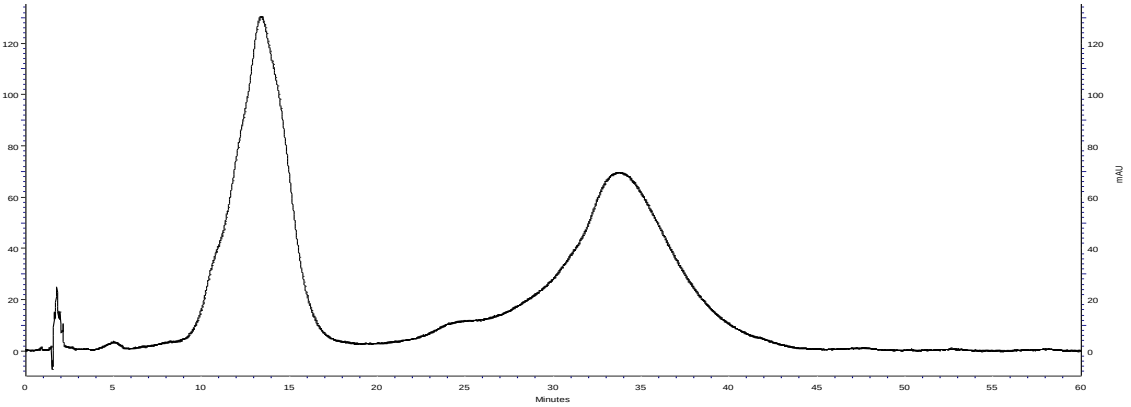


Figure 14 –Chromatogram for enantioresolution of enantiomeric mixture of CDX XEA 5 3 MET (**24**), on CHIRALPAK® HSA column; Conditions: flow rate: 0.9 mL/min; mobile phase, 10 mM sodium acetate (pH 7.0):ACN (82:18 *v/v*); detection, 254 nm; temperature, 22 ± 2°C.

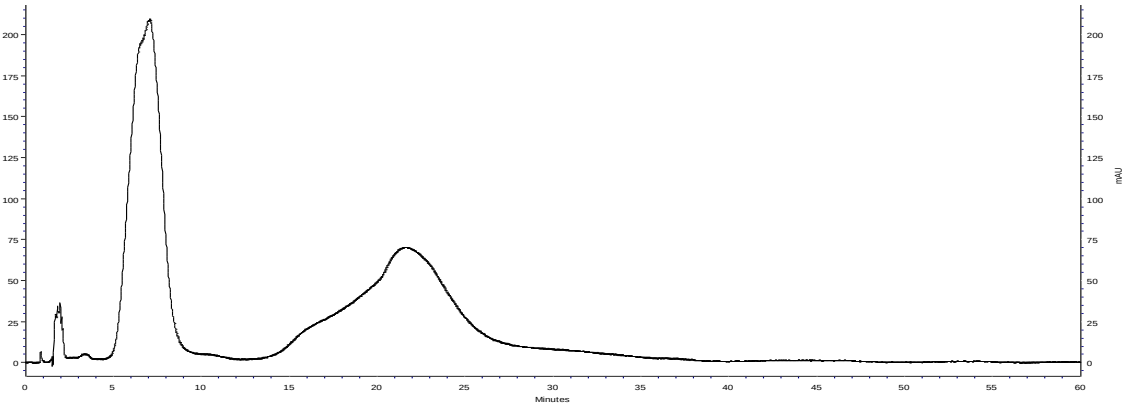


Figure 15 – Chromatogram for enantioresolution of enantiomeric mixture CDX XEA 5 4 FLU (**20**), on CHIRALPAK® HSA column; Conditions: flow rate: 0.9 mL/min; mobile phase, 10 mM sodium acetate (pH 7.0):ACN (79:21 *v/v*); detection, 254 nm; temperature, 22 ± 2°C.

As seen in **Figure 16**, the pH variation of sodium acetate buffer from 5.0 to 7.0 does not exhibit significant differences on either retention factors or even resolution of the enantiomeric mixtures.

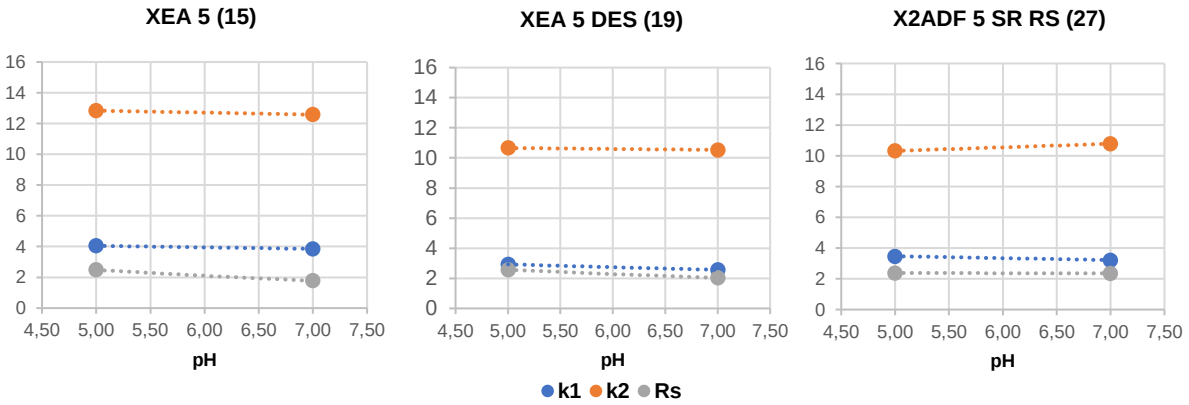


Figure 16 - Effect of mobile phase pH on retention factors and resolution of enantiomeric mixtures XEA 5 (**15**), XEA 5 DES (**19**) and X2ADF 5 SR RS (**27**); Conditions: flow rate: 0.9 mL/min; mobile phase, 10 mM sodium acetate (pH 5.0 and 7.0): ACN= 79:21 (*v/v*).

The other baseline resolved enantiomeric mixtures of CDXs had a similar behavior. The only exceptions were for enantiomeric mixtures XEA 3 MET **(16)**, XEA 5 4 FLU **(20)** and XEA 5 4 CLO **(23)**, which were baseline enantioseparated at pH 5.0 but at pH 7.0 no baseline separations were observed. In contrast, the enantiomeric mixture XEA 4 CLO **(22)** was only possible to enantioseparate using a mobile phase at pH 7.0.

1.2.3. Potassium phosphate buffer

Ten of thirty-one enantiomeric mixtures of CDXs were enantioseparated with excellent enantioselectivity on CHIRALPAK® HSA using a mobile phase comprising potassium phosphate buffer (10 mM, pH 5.0) and ACN in variable proportion, with α ranging from 1.47 to 8.19 and resolutions from 1.02 to 5.29. The overall best results are shown on **Table 8**.

Table 8 - The best chromatographic data obtained for separation of enantiomeric mixtures of CDXs **1-31**, on CHIRALPAK® HSA, using 10 mM potassium phosphate at pH 5.0 as buffer.

Enantiomeric mixture	t_1	t_2	k_1	k_2	α	R_s	% of ACN
XEGOL 5 (**1**)	6.91	20.67	2.64	9.88	3.75	4.04	20
XEA 5 (**15**)	6.52	21.94	2.43	10.55	4.34	4.36	24
XEA 3 MET (**16**)	30.29	43.67	14.94	21.98	1.47	1.02	12
XEA DES (**18**)	4,12	20,09	1,17	9,57	8,19	5,29	24
XEA 5 DES (**19**)	5.17	18.79	1.72	8.89	5.17	4.88	24
XEA 5 4 FLU (**20**)	8.59	40.60	3.52	20.37	5.78	3.94	22
XEA 5 4 CLO (**23**)	17.36	67.53	8.14	34.53	4.25	1.97	22
XEA 5 3 MET (**24**)	9.73	27.61	4.12	13.53	3.28	3.28	20
XEA 5 4 MET (**25**)	13.01	35.46	5.85	17.66	3.02	4.27	18
X2ADF 5 *SR RS* (**27**)	9.59	39.51	4.05	19.79	4.89	4.53	22

CHIRALPAK® HSA column; Mobile phase potassium phosphate buffer (pH 5.0):ACN (variable proportion); Flow rate 0.9 mL/min; Temperature 22 ± 2°C; Detection UV 254nm

Using 24% of organic modifier in mobile phase, XEA 5 DES (**19**) was the enantiomeric mixture that showed the shortest retention factors, with k_1 = 1.72 and k_2 = 8.89, as well as good enantioselectivity and resolution, with **α =** 5.17 and R_S = 4.88 respectively. Using the same mobile phase, the enantiomeric mixture XEA DES (**18**) (**Figure 17**) demonstrated the best enantioselectivity and resolution values, with **α =** 8.19 and R_S = 5.29, respectively.

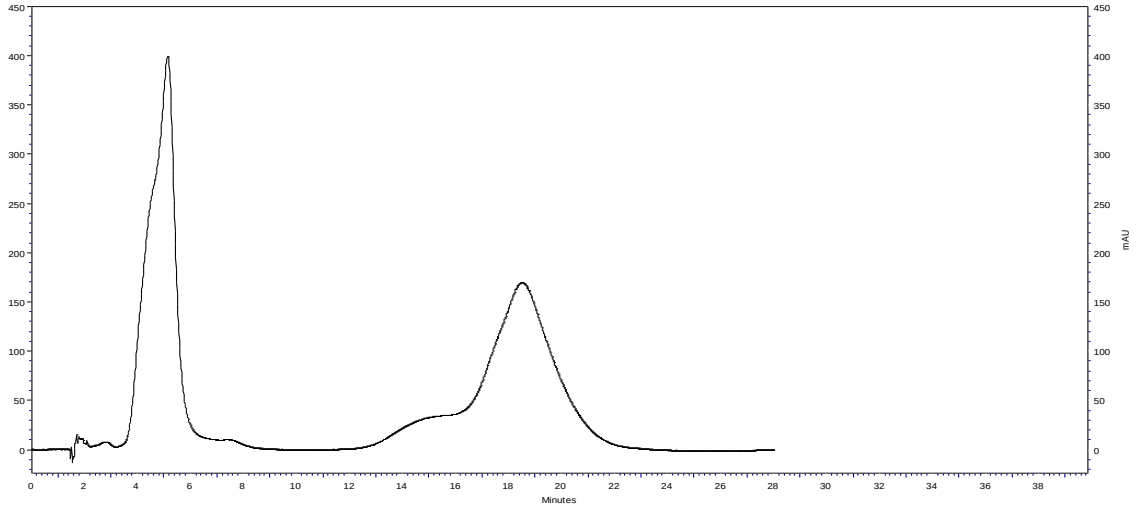


Figure 17 - Chromatogram for enantioresolution of enantiomeric mixture CDX XEA DES (**18**), on CHIRALPAK® HSA column; Conditions: flow rate: 0.9 mL/min; mobile phase, 10 mM potassium phosphate (pH 5.0):ACN (76:24 *v/v*); detection, 254 nm; temperature, 22 ± 2°C.

Similar chromatographic results were also achieved for enantiomeric mixture XEA 5 (**15**) (α = 4.34; R_S = 4.36) (**Figure 18**).

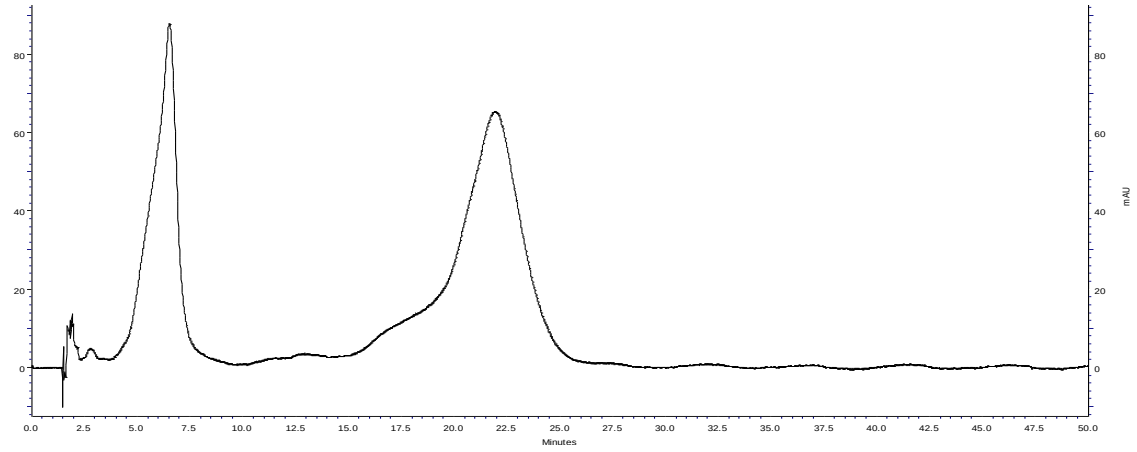


Figure 18 –Chromatogram for enantioresolution of enantiomeric mixture of CDX XEA 5 (**15**), on CHIRALPAK® HSA column; Conditions: flow rate: 0.9 mL/min; mobile phase, 10 mM potassium phosphate (pH 5.0):ACN (76:24 *v/v*); detection, 254 nm; temperature, 22 ± 2°C.

Then, the resolving ability of CHIRALPAK® HSA was evaluated using an aqueous buffer of potassium phosphate (10 mM) at pH 7.0, and ACN as organic modifier, in variable

proportions. From thirty-one enantiomeric mixtures injected, thirteen of them were baseline enantioseparated, with α and Rs values ranging from 1.27 to 12.53 and from 0.90 to 6.11, respectively. The overall best results are shown on **Table 9**.

Table 9 - The best chromatographic data obtained for separation of enantiomeric mixtures of CDXs **1-31**, on CHIRALPAK® HSA, using 10 mM potassium phosphate at pH 7.0 as buffer.

Enantiomeric mixture	t_1	t_2	k_1	k_2	α	R_s	% of ACN
XEGOL 5 (**1**)	10.96	39.21	4.77	19.64	4.12	2.04	18
XEVOL 5 (**6**)	3.46	21.44	0.82	10.28	12.53	4.81	20
XEA 1 (**14**)	14.28	19.06	6.52	9.03	1.39	1.02	16
XEA 5 (**15**)	9.61	39.20	4.06	19.63	4.84	6.11	21
XEA DES (**18**)	5.31	34.98	1.79	17.41	9.70	3.93	21
XEA 5 DES (**19**)	6.95	31.72	2.66	15.69	5.90	5.72	21
XEA 5 4 FLU (**20**)	9.48	40.74	3.99	20.44	5.12	3.41	20
XEA 4 CLO (**22**)	31.82	38.81	15.75	19.95	1.27	0.90	18
XEA 5 4 CLO (**23**)	14.73	52.81	6.75	26.79	3.97	2.31	22
XEA 5 3 MET (**24**)	9.67	24.82	4.09	12.06	2.95	4.20	19
XEA 5 4 MET (**25**)	15.76	43.07	7.29	21.67	2.97	4.90	19
X2ADF 5 *SR RS* (**27**)	7.89	34.34	3.15	17.07	5.42	4.45	21
X2ADF *SR RS* (**30**)	19.08	46.53	9.04	23.49	2.60	3.73	15

CHIRALPAK® HSA column; Mobile phase potassium phosphate buffer (pH 7.0):ACN (variable proportion); Flow rate 0.9 mL/min; Temperature 22 ± 2°C; Detection UV 254nm

The enantiomeric mixture XEA 5 3 MET (**24**) was the one that was enantioseparated in the shortest analysis time, with k_1 = 4.09 and k_2 = 12.06. Moreover, a good enantioselectivity and resolution were observed, with α = 2.95 and R_S = 4.20 respectively, using a ratio of 19% of organic modifier in mobile phase. Using a different organic solvent ratio (21%), the enantiomeric mixture XEA 5 (**15**) (**Figure 19**) showed the best enantioselectivity and resolution, with α = 4.84 and R_S = 6.11, respectively.

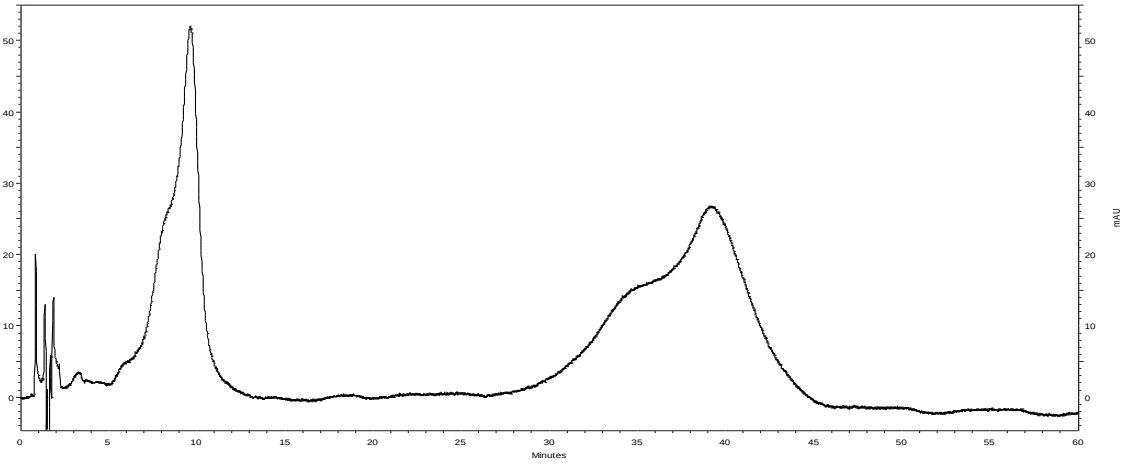


Figure 19 - Chromatogram for enantioresolution of enantiomeric mixture XEA 5 (**15**), on CHIRALPAK® HSA column; Conditions: flow rate: 0.9 mL/min; mobile phase, 10 mM potassium phosphate (pH 7.0):ACN (79:21 *v/v*); detection, 254 nm; temperature, 22 ± 2°C.

Similar chromatographic results were achieved for enantiomeric mixtures XEA 5 4 CLO (**23**), with α = 3.97 and R_S = 2.31 (**Figure 20**), and X2ADF 5 *SR RS* (**27**), with α = 5.42 and R_S = 4.45 (**Figure 21**).

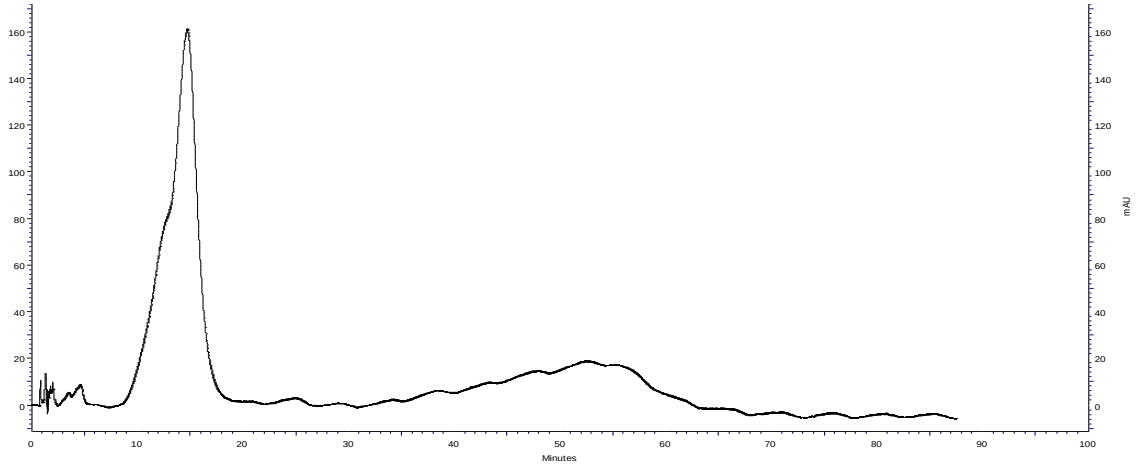


Figure 20 – Chromatogram for enantioresolution of enantiomeric mixture of CDX XEA 5 4 CLO (**23**), on CHIRALPAK® HSA column; Conditions: flow rate: 0.9 mL/min; mobile phase, 10 mM potassium phosphate (pH 7.0):ACN (78:22 *v/v*); detection, 254 nm; temperature, 22 ± 2°C.

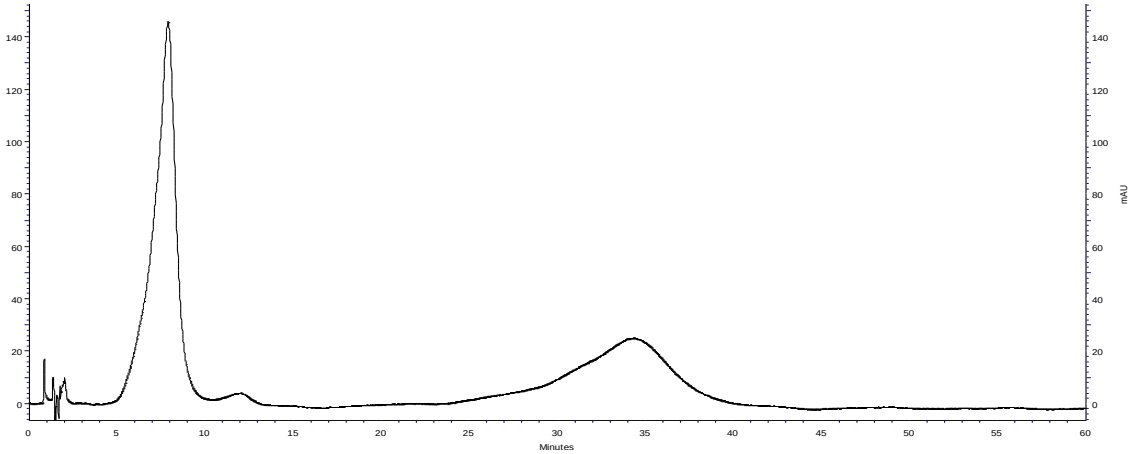


Figure 21 – Chromatogram for enantioresolution of enantiomeric resolution of CDX X2ADF 5 SR RS (**27**), on CHIRALPAK® HSA column; Conditions: flow rate: 0.9 mL/min; mobile phase, 10 mM potassium phosphate (pH 7.0):ACN (79:21 *v/v*); detection, 254 nm; temperature, 22 ± 2°C.

In **Figure 22** the chromatographic parameters k_1, k_2 and Rs of three enantiomeric mixtures obtained at two different mobile phase pH are compared. In general, at pH 5.0 both enantiomers are comparably more retained in the column, thus increasing the retention factors. For the baseline separated enantiomeric mixtures, the quality of the enantioseparation was similar in both conditions; however, some enantiomeric mixtures exhibited an increase in their resolution.

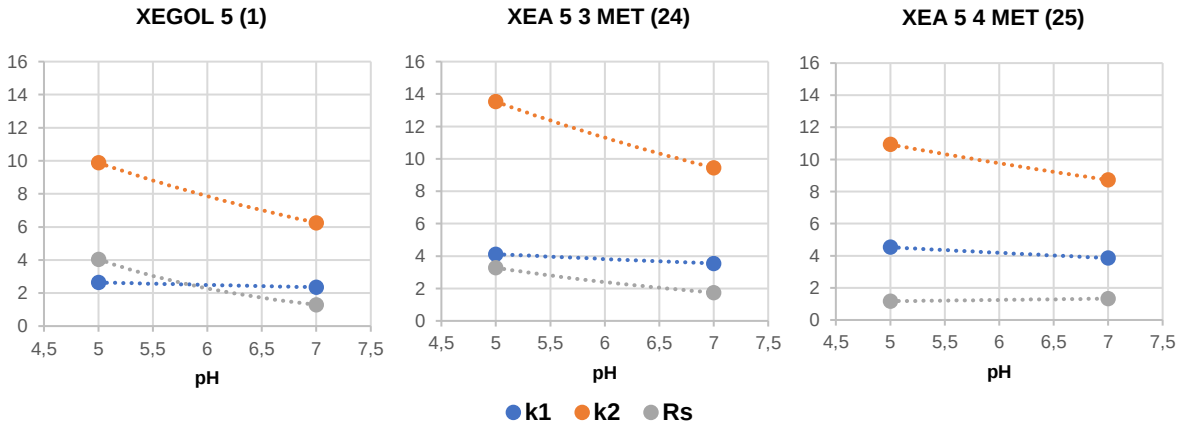


Figure 22 - Effect of mobile phase pH on retention factors and resolution of enantiomeric mixtures XEGOL 5 (**1**), XEA 5 3 MET (**24**) and XEA 5 4 MET (**25**); Conditions: flow rate: 0.9 mL/min; mobile phase, 10 mM potassium phosphate (pH 5.0 and 7.0): ACN= 79:21 (*v/v*).

The only exceptions were for enantiomeric mixtures XEGOL 2 (**2**), XEA 4 CLO (**22**) and X2ADF SR RS (**30**) being enantioseparated at pH 7.0, while no baseline enantioseparation was achieved with a mobile phase at pH 5.0.

1.2.4. Sodium phosphate buffer

The thirty-one enantiomeric mixtures of CDXs were also evaluated using mobile phases comprising sodium phosphate (10 mM, pH 5.0) as buffer and ACN in variable proportion, into a CHIRALPAK® HSA column. Only eight of them were enantioseparated (**Table 10**).

Table 10 - The best chromatographic data obtained for separation of enantiomeric mixtures of CDXs **1-31**, on CHIRALPAK® HSA, using 10 mM sodium phosphate at pH 5.0 as buffer.

Enantiomeric mixture	t_1	t_2	k_1	k_2	α	R_s	% of ACN
XEGOL 5 (**1**)	6.70	20.30	2.53	9.68	3.83	3.12	20
XEA 5 (**15**)	8.65	32.85	3.55	16.29	4.59	4.91	22
XEA 5 DES (**19**)	6.60	40.26	2.47	20.19	8.16	4.32	22
XEA 5 4 FLU (**20**)	6.63	28.98	2.49	14.25	5.73	4.17	22
XEA 5 3 MET (**24**)	9.68	25.83	4.09	12.59	3.08	2.86	20
XEA 5 4 MET (**25**)	17.33	45.89	8.12	23.15	2.85	3.49	18
X2ADF 5 *SR RS* (**27**)	7.46	27.19	2.93	13.31	4.55	3.46	22
X2ADF *SR RS* (**30**)	24.71	52.86	12.00	26.82	2.23	2.36	13

CHIRALPAK® HSA column; Mobile phase sodium phosphate buffer (pH 5.0):ACN (variable proportion); Flow rate 0.9 mL/min; Temperature 22 ± 2°C; Detection UV 254nm

For the enantiomeric mixtures efficiently enantioseparated on HSA-CSP the α and R_S values ranged from 2.23 to 8.16 and from 2.36 to 4.91, respectively. The enantiomeric mixture X2ADF 5 *SR RS* (**27**) (**Figure 23**) was separated in the shortest time, with k_1 = 2.93 and k_2 = 13.31, with a good enantioselectivity and resolution, (α = 4.55 and R_S = 3.46 respectively), using a ratio of 22% of organic modifier. Using the same mobile phase, the enantiomeric mixture XEA 5 (**15**) demonstrated good enantioselectivity and the best resolution, with α = 4.59 and R_S = 4.91, respectively.

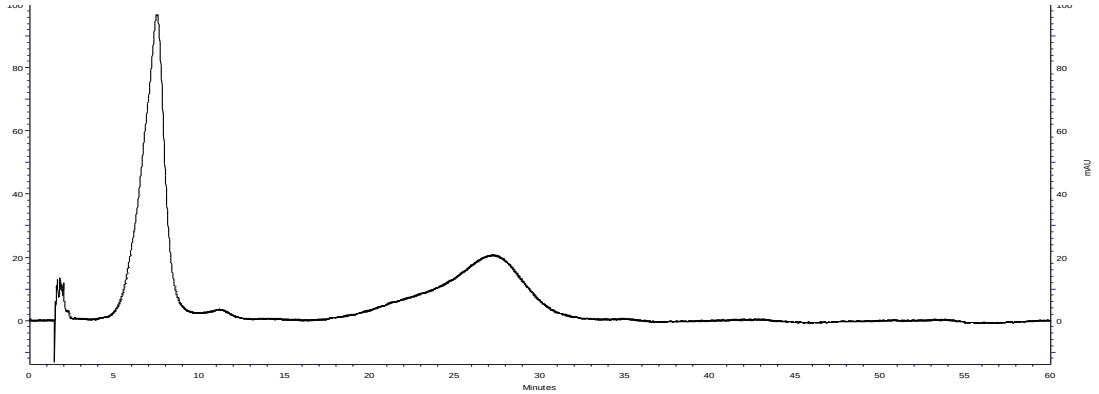


Figure 23 - Chromatogram for enantioresolution of enantiomeric mixture X2ADF 5 SR RS (**27**) on the CHIRALPAK® HSA column; Conditions: flow rate: 0.9 mL/min; mobile phase, 10 mM sodium phosphate (pH 5.0):ACN (78:22 *v/v*); detection, 254 nm; temperature, 22 ± 2°C.

When the pH of the mobile phase was changed to 7.0, from the thirty-one enantiomeric mixtures of CDXs injected ten of them were baseline enantioseparated, with α values ranging from 1.36 to 7.62 and resolutions ranging from 1.07 to 6.41. The overall best results are shown on **Table 11**.

Table 11 - The best chromatographic data obtained for separation of enantiomeric mixtures of CDXs **1-31**, on CHIRALPAK® HSA, using 10 mM sodium phosphate at pH 7.0 as buffer.

Enantiomeric mixture	t_1	t_2	k_1	k_2	α	R_s	% of ACN
XEGOL 5 (**1**)	7.19	25.56	2.78	12.45	4.47	6.41	20
XEA 1 (**14**)	21.86	30.16	10.51	14.87	1.42	1.27	15
XEA 5 (**15**)	8.36	29.48	3.40	14.52	4.27	4.77	23
XEA 5 DES (**19**)	8.41	41.60	3.43	20.89	6.10	5.17	22
XEA 5 4 FLU (**20**)	6.08	33.77	2.20	16.77	7.62	4.40	23
XEA 4 CLO (**22**)	14.64	19.25	6.71	19.13	1.36	1.07	20
XEA 5 3 MET (**24**)	12.35	37.90	5.50	18.95	3.44	4.93	20
XEA 5 4 MET (**25**)	12.78	33.94	5.73	16.86	2.94	5.23	20
X2ADF 5 *SR RS* (**27**)	9.61	44.28	4.06	22.31	5.50	3.97	22
X2ADF *SR RS* (**30**)	18.97	40.97	8.98	20.56	2.29	2.90	15

CHIRALPAK® HSA column; Mobile phase sodium phosphate buffer (pH 7.0):ACN (variable proportion); Flow rate 0.9 mL/min; Temperature 22 ± 2°C; Detection UV 254nm

Regarding baseline separation, the enantiomeric mixture XEGOL 5 (**1**) (**Figure 24**) showed a good enantioselectivity and resolution, with α = 4.47 and R_S = 6.41 respectively, when using a ratio of 20% of organic modifier in mobile phase. In addition, the enantiomeric mixture of CDXs separated in the shortest time was XEA 4 CLO (**22**), with k_1 = 6.71 and k_2 = 19.13, with good enantioselectivity and resolution, with a α = 1.36 and R_S = 1.07 respectively.

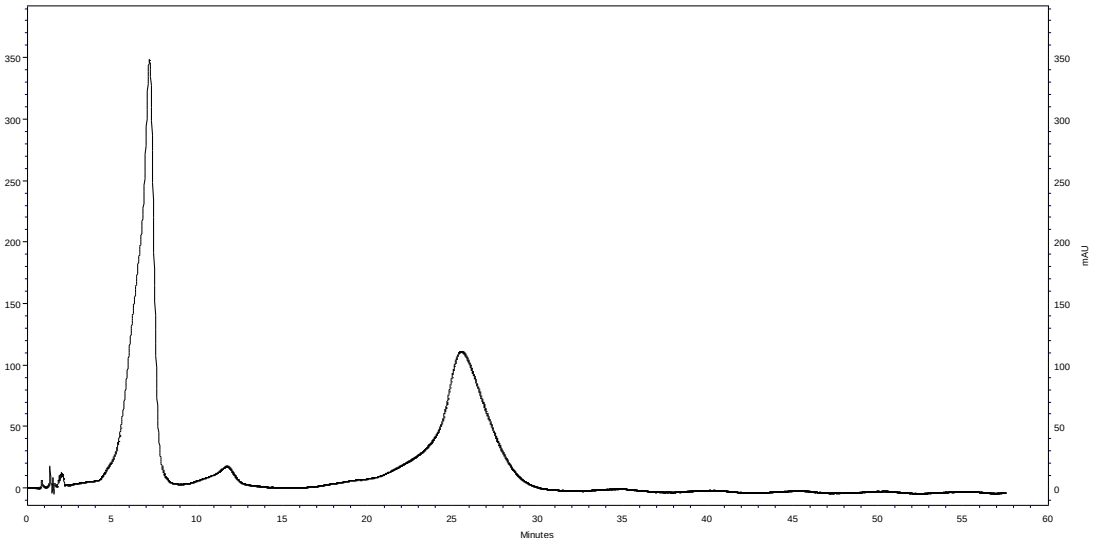


Figure 24 - Chromatogram for enantioresolution of enantiomeric mixture of CDX XEGOL 5 (**1**), on CHIRALPAK® HSA column; Conditions: flow rate: 0.9 mL/min; mobile phase, 10 mM sodium phosphate (pH 7.0):ACN (80:20 *v/v*); detection, 254 nm; temperature, 22 ± 2°C.

Regarding the effect that the change of mobile phase pH caused on chromatographic parameters k_1, k_2 and R_S, **Figure 25** shows, as example, the obtained results for three enantiomeric mixtures of CDXs (**15**, **19** and **20**) according to the pH of the mobile phase. At pH 7.0, both enantiomers of the three enantiomeric mixtures of CDXs were strongly retained, , however, the resolution increased. The same behavior was obtained for the other baseline separated enantiomeric mixtures.

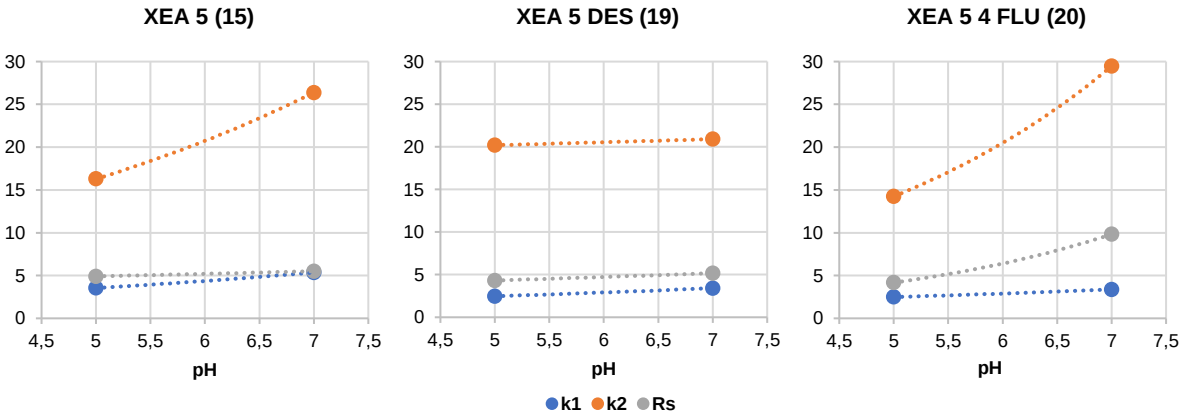


Figure 25 - Effect of mobile phase pH on retention factors and resolution of enantiomeric mixtures XEA 5 (**15**), XEA 5 DES (**19**) and XEA 5 4 FLU (**20**); Conditions: flow rate: 0.9 mL/min; mobile phase, 10 mM sodium phosphate (pH 5.0 and 7.0): ACN= 78:22 (*v/v*).

Exceptions were for enantiomeric mixtures XEA 1 (**14**) and XEA 4 CLO (**22**) since at pH 5.0 no baseline enantioseparations were observed, while at pH 7.0 have been separated.

Figure 26 shows an example of the effect that pH modification can cause on chiral recognition of an enantiomeric mixture. When the pH falls below the isoelectric point of the albumin selector, no separation can be obtained; however, when the pH of the mobile phase is above the isoelectric point, there is already an indication of enantiomeric separation and resolution.

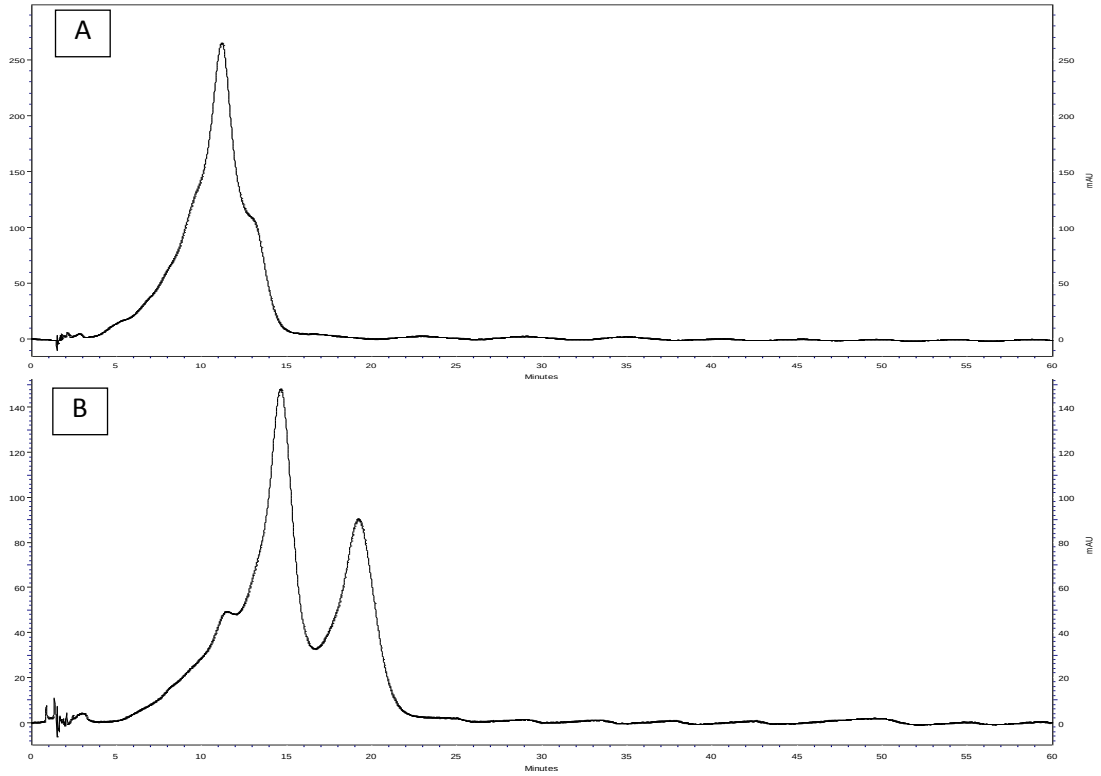


Figure 26 - Chromatograms on the enantioresolution of enantiomeric mixture of CDX XEA 4 CLO (**22**) on the CHIRALPAK® HSA column; Conditions: flow rate: 0.9 mL/min; mobile phase, (**A**) 10 mM sodium phosphate (pH 5.0):ACN (80:20 *v/v*), (**B**) 10 mM sodium phosphate (pH 7.0):ACN (80:20 *v/v*); detection, 254 nm; temperature, 22 ± 2°C.

1.2.5. Summary

From all thirty-one enantiomeric mixtures of CDXs injected on CHIRALPAK® HSA column under all the chromatographic conditions described before, only fourteen were baseline enantioseparated as summarized in **Table 12**.

Table 12 – Summary of baseline separations of enantiomeric mixtures of CDXs using mobile phases with different buffer types and pH, and ACN as organic modifier, in variable proportions.

Enantiomeric mixture	Ammonium acetate		Sodium acetate		Potassium phosphate		Sodium phosphate	
	pH 5.0	pH 7.0	pH 5.0	pH 7.0	pH 5.0	pH 7.0	pH 5.0	pH 7.0
XEGOL 5 (**1**)	✓	✓	✓	✓	✓	✓	✓	✓
XEVOL 5 (**6**)	-	-	-	-	-	✓	-	-
XEA 1 (**14**)	-	-	-	-	-	✓	-	✓
XEA 5 (**15**)	✓	✓	✓	✓	✓	✓	✓	✓
XEA 3 MET (**16**)	✓	-	✓	-	✓	-	-	-
XEA DES (**18**)	-	-	-	-	✓	✓	-	-
XEA 5 DES (**19**)	✓	✓	✓	✓	✓	✓	✓	✓
XEA 5 4 FLU (**20**)	✓	✓	✓	✓	✓	✓	✓	✓
XEA 4 CLO (**22**)	-	-	-	✓	-	✓	-	✓
XEA 5 4 CLO (**23**)	-	-	✓	-	✓	✓	-	-
XEA 5 3 MET (**24**)	✓	✓	✓	✓	✓	✓	✓	✓
XEA 5 4 MET (**25**)	✓	✓	✓	✓	✓	✓	✓	✓
X2ADF 5 *SR RS* (**27**)	✓	-	✓	✓	✓	✓	✓	✓
X2ADF *SR RS* (**30**)	-	✓	✓	✓	-	✓	✓	✓

CHIRALPAK® HSA column; Conditions: flow rate: 0.9 mL/min; temperature, 22 ± 2°C; detection, 254nm.

As demonstrated in **Table 12**, the mobile phase comprising 10 mM potassium phosphate buffer solution afforded a higher number of baseline enantioseparations, compared to the others. Our analysis had been performed at the pH range of 5.0 – 7.0, which means that the protein has a net negative charge. Regarding the influence of pH, in general, a decrease in the retention time has been noted at pH 7.0. Moreover, at pH 7.0 buffer the highest number of baseline enantioseparations were observed, specifically thirteen enantiomeric mixtures. Additionally, a wide number of protein-binding studies use phosphate buffer solutions to approximate to the physiological conditions. Consequently, considering the tested chromatographic conditions, the best mobile phase for this class of compounds was a mixture of potassium phosphate buffer (10 mM, pH 7.0) using ACN as organic modifier.
Nevertheless, for each enantiomeric mixture of CDX different proportions of organic modifier in the mobile phase needed to be used. Moreover, it is important to highlight that for the same enantiomeric mixture slight variations of organic modifier content in mobile phase caused a significant change on chromatographic parameters, as demonstrated in **Figure 27** for enantiomeric mixture XEA 5 4 MET (**25**). By reducing the proportion of organic

modifier, at both pH 5.0 and pH 7.0, a substantial increase of retention factors was observed. Regarding resolution, the behavior was different depending on the mobile phase pH; at pH 5.0, the resolution increased as the proportion of organic modifier reduces, while at pH 7.0 the opposite was observed.

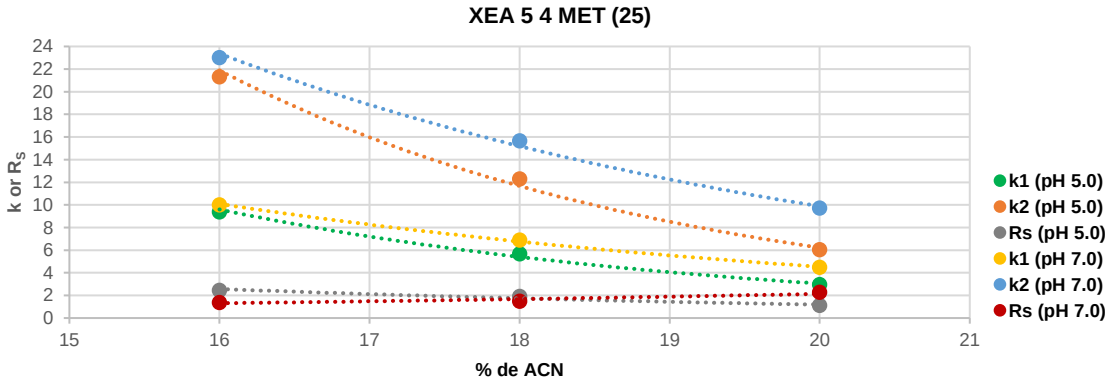


Figure 27 - Effect of mobile phase proportion of ACN as organic modifier on retention factors and resolution of enantiomeric mixture of XEA 5 4 MET **(25)**, both on buffer at pH 5.0 and 7.0; Conditions: flow rate: 0.9 mL/min; mobile phase, 10 mM sodium acetate (pH 5.0 and 7.0): ACN;

In some cases, the chromatographic parameter that was most influenced by changing the proportion of organic modifier in the mobile phase was K_2, as shown in **Figure 28**, for enantiomeric mixture XEGOL 5 (**1**).

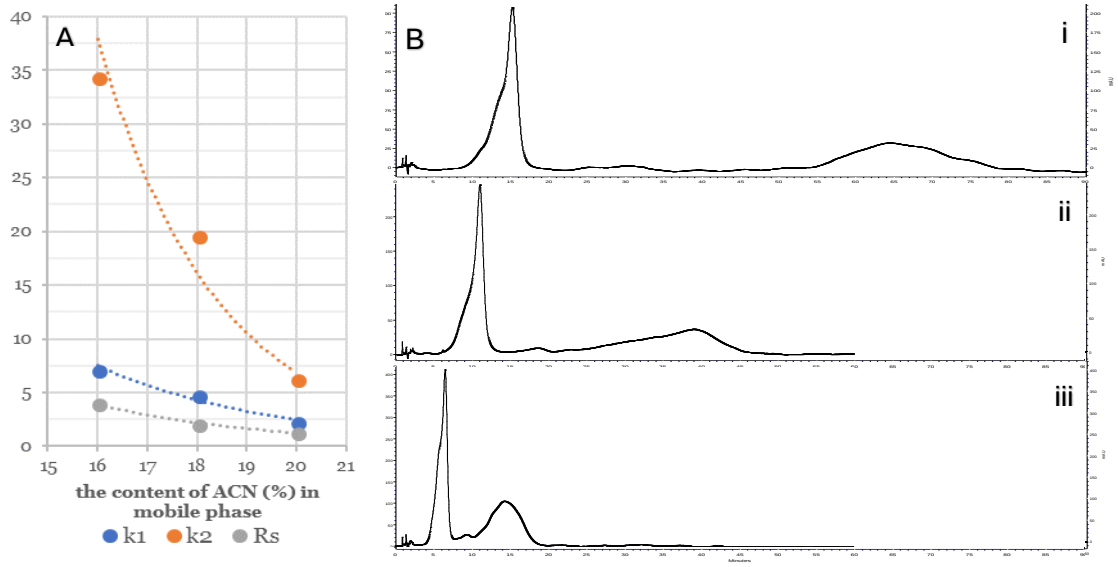


Figure 28 – (**A**) Effect of mobile phase proportion of ACN as organic modifier on retention factors and resolution of enantiomeric mixture XEGOL 5 (**1**); Conditions: flow rate: 0.9 mL/min; mobile phase, 10 mM potassium phosphate at pH 7.0:ACN; detection, 254 nm; (**B**) Chromatograms for resolution of enantiomeric mixture XEGOL 5 (**1**) on CHIRALPAK® HSA column using 16 (**i**), 18 (**ii**) and 20% (**iii**) ACN as organic modifier; Conditions: flow rate: 0.9 mL/min; mobile phase, 10 mM potassium phosphate at pH 7.0:ACN; detection, 254 nm; temperature, 22 ± 2°C.

1.3. Optimizations to approach the physiological conditions

Modifications of the chromatographic conditions were carried out to approach to the physiological environment, by changing the ionic strength of the buffer to 67 mM, the mobile phase pH to 7.4 and the temperature of analysis to 37 ^{o}C. From this step forward, only the thirteen enantiomeric mixtures of CDXs successfully baseline enantioseparated were tested.

1.3.1. Increase the mobile phase ionic strength

An important chromatographic condition for optimization is also the buffer ionic strength [79, 168]. Previous systematic studies have proved its influence on chiral recognition of a CSP [169]. Nevertheless, its influence on retention time is not linear being always dependent on the characteristics of the compounds to be analyzed [170]. Moreover, an increase in buffer concentration has been shown to be an important ionization suppressor, increasing the quality of the separations [171].

Many drug binding studies to a protein-based CSP utilize the 67 mM concentration in buffer in order to mimic the physiological conditions [62]. In addition, that concentration is also used for immobilization procedures of HSA to a chromatographic support, for packing of CSP into columns as well as in their storage [100].

In this work, a total of ten among thirteen tested enantiomeric mixtures of CDXs were baseline enantioseparated using 67 mM potassium phosphate at pH 7.0 and ACN in variable proportion, as mobile phase. Good enantioselectivity and resolution were obtained, with α values ranging from 2.37 to 10.35 and R_S from 2.01 to 4.12. The overall best results are shown on **Table 13**.

Table 13 - The best chromatographic data obtained for separation of enantiomeric mixture of CDXs, on CHIRALPAK® HSA, using 67 mM potassium phosphate at pH 7.0 as buffer.

Enantiomeric mixture	t_1	t_2	k_1	k_2	α	R_s	% of ACN
XEGOL 5 (**1**)	6.38	13.86	2.36	6.30	2.67	2.62	20
XEVOL 5 (**6**)	3.97	17.32	1.09	8.12	7.49	3.92	20
XEA 5 (**15**)	7.53	18.39	2.96	8.68	2.93	2.59	24
XEA DES (**18**)	3.77	21.24	0.98	10.18	10.35	3.81	24
XEA 5 DES (**19**)	5.71	20.87	2.00	9.98	4.98	3.43	24
XEA 5 4 FLU (**20**)	5.74	15.92	2.02	7.38	3.65	2.26	24
XEA 5 4 CLO (**23**)	20.81	60.23	9.95	30.70	3.08	2.30	21
XEA 5 3 MET (**24**)	9.86	20.75	4.19	9.92	2.37	2.01	20
XEA 5 4 MET (**25**)	20.74	58.83	9.92	29.96	3.02	4.12	21
X2ADF 5 *SR RS* (**27**)	13.21	52.26	5.95	26.51	4.45	3.48	20

CHIRALPAK® HSA column; Mobile phase potassium phosphate buffer (pH 7.0):ACN (variable proportion); Flow rate 0.9 mL/min; Temperature 22 ± 2°C; Detection UV 254nm

As example, the enantiomeric mixture XEGOL 5 (**1**) using a ratio of 20% of organic modifier, was enantioseparated in the shortest retention time, with k_1 = 2.36 and k_2 = 6.30. In addition, good enantioselectivity and resolution were obtained, with **α =** 2.67 and R_S = 2.62, respectively. Moreover, the enantiomeric mixture XEA 5 DES (**19**) was also an example of the best enantioselectivity and resolution, with **α =** 4.98 and R_S = 3.43 respectively, using 24% of ACN in the mobile phase (**Figure 29**).

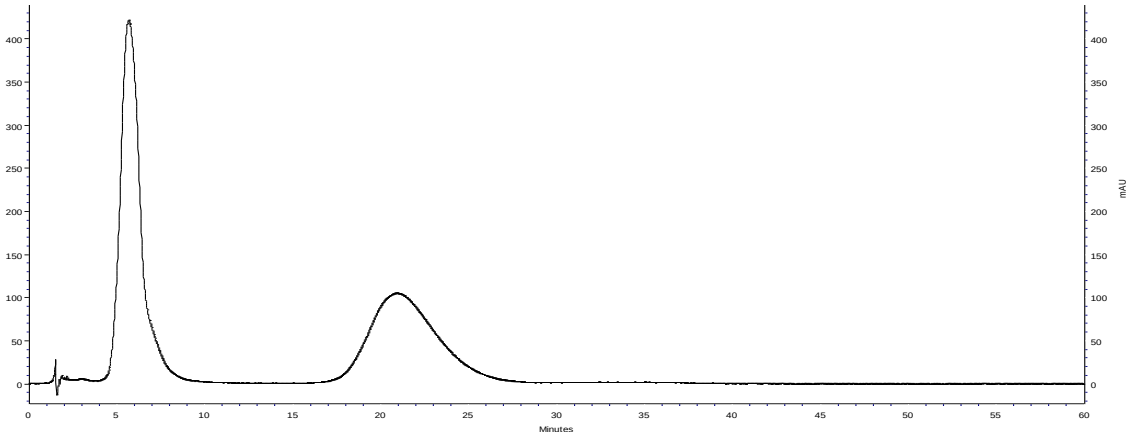


Figure 29 - Chromatogram on the enantioresolution of enantiomeric mixture of CDX XEA 5 DES (**19**) on the CHIRALPAK® HSA column; Conditions: flow rate: 0.9 mL/min; mobile phase, 67 mM potassium phosphate (pH 7.0):ACN (76:24 *v/v*); detection, 254 nm; temperature, 22 ± 2°C.

It is also important to emphasize the good resolution obtained in a relatively short analysis time (25 min) for the enantiomeric mixture XEA 5 (**15**) (**α =** 2.93; R_S = 2.59) (**Figure 30**).

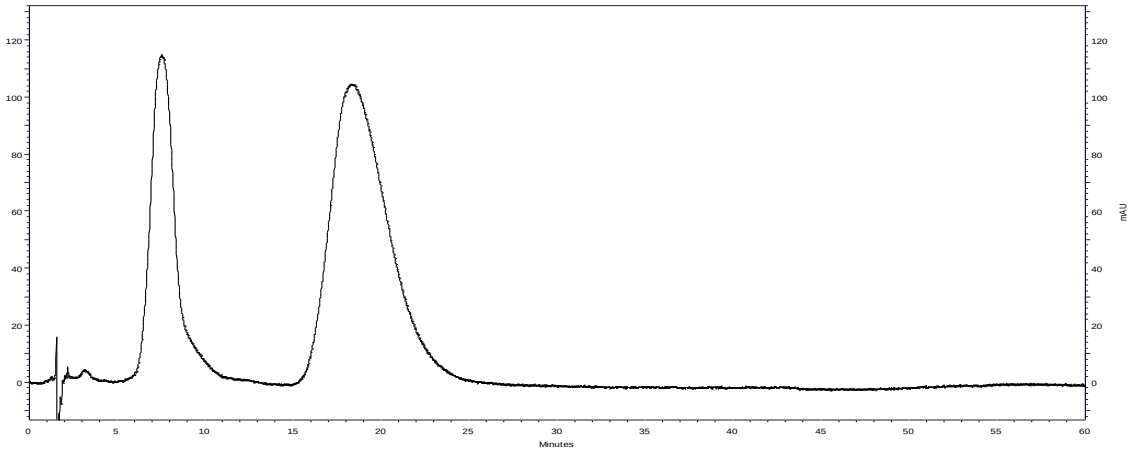


Figure 30 - Chromatogram on the enantioresolution of enantiomeric mixture of CDX XEA 5 (**15**) on the CHIRALPAK® HSA column; Conditions: flow rate: 0.9 mL/min; mobile phase, 67 mM potassium phosphate (pH 7.0):ACN (76:24 *v/v*); detection, 254 nm; temperature, 22 ± 2°C.

As expected, the result obtained by increasing in ionic concentration of the mobile phase to 67 mM afforded a slight increase in the retention times of the enantiomeric mixtures of CDXs but for the same mixtures a large difference on resolution values as showed in **Figure 31**.

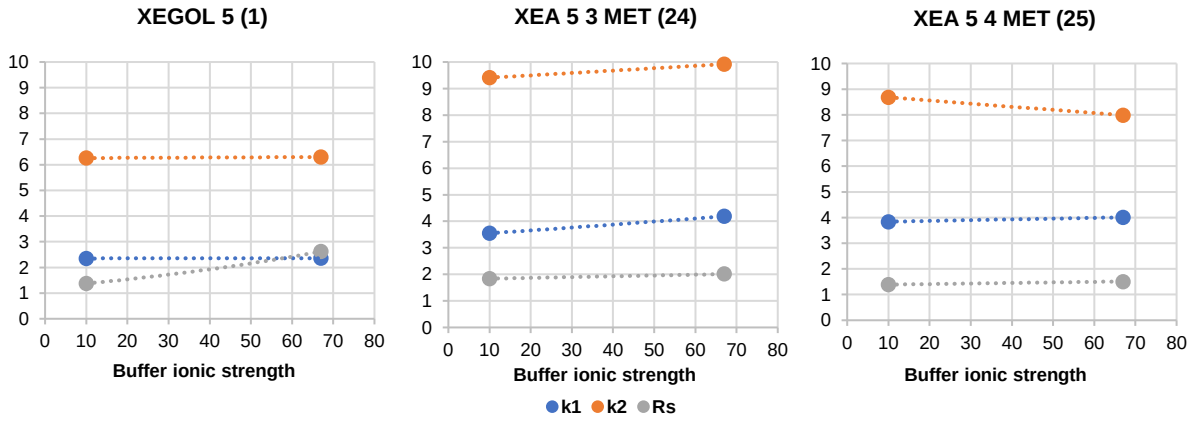


Figure 31 - Effect of mobile phase ionic strength on retention factors and resolution of enantiomeric mixtures XEGOL 5 (**1**), XEA 5 3 MET (**24**) and XEA 5 4 MET (**25**); Conditions: flow rate: 0.9 mL/min; mobile phase, (10 or 67 mM) potassium phosphate (pH 7.0): ACN= 80:20 (*v/v*).

1.3.2. Increase the mobile phase pH

As stated before, HSA is the most abundant protein in human plasma [172]. The physiological pH of human plasma is pH 7.4 and it is assumed that HSA has a higher stability in this condition. To mimic the physiological conditions and aiming to optimize the enantioresolution method, the mobile phase pH was increased to pH 7.4 [59, 92, 173].

Among the thirteen enantiomeric mixtures of CDXs injected into the column using the mobile phase 67 mM potassium phosphate at pH 7.4 and ACN, in variable proportion, as mobile phase, twelve of them were baseline enantioseparated, with α values ranging from 1.40 to 11.76 and R_S from 1.21 to 5.41 (**Table 16**).

Table 16 - The best chromatographic data obtained for separation of enantiomeric mixture of CDXs, on CHIRALPAK® HSA, using 67 mM potassium phosphate at pH 7.4 as buffer.

Enantiomeric mixture	t_1	t_2	k_1	k_2	α	R_S	% of ACN
XEGOL 5 (**1**)	5.36	10.69	1.82	4.62	2.54	2.35	22
XEVOL 5 (**6**)	3.22	16.40	0.69	7.63	11.01	5.29	22
XEA 1 (**14**)	29.47	40.60	14.51	20.37	1.40	1.51	14
XEA 5 (**15**)	7.79	17.94	3.10	8.44	2.72	2.27	23
XEA DES (**18**)	5.08	39.26	1.67	19.66	11.76	5.41	22
XEA 5 DES (**19**)	8.59	37.21	3.52	18.58	5.28	3.71	22
XEA 5 4 FLU (**20**)	8.95	29.33	3.71	14.44	3.89	2.89	22
XEA 5 4 CLO (**23**)	17.44	46.29	8.18	23.36	2.86	2.27	22
XEA 5 3 MET (**24**)	7.61	14.65	3.01	6.71	2.23	1.83	22
XEA 5 4 MET (**25**)	13.31	24.84	6.00	12.07	2.01	1.81	20
X2ADF 5 *SR RS* (**27**)	7.55	20.22	2.97	9.64	3.24	2.83	23
X2ADF *SR RS* (**30**)	18.10	27.97	8.53	13.72	1.61	1.21	16

CHIRALPAK® HSA column; Mobile phase potassium phosphate buffer (pH 7.4):ACN (variable proportion); Flow rate 0.9 mL/min; Temperature 22 ± 2°C; Detection UV 254nm

The enantiomeric mixture XEGOL 5 (**1**) continued to be the one that was enantioseparated in the shortest analysis time, with k_1 = 1.82 and k_2 = 4.62, as well as with good enantioselectivity and resolution, with **α =** 2.54 and R_S = 2.35 respectively, using 22% of organic modifier in mobile phase. At the same solvent percentage, the enantiomeric mixture XEA DES (**18**) showed the best enantioselectivity and resolution, with **α =** 11.76 and R_S = 5.41 respectively (**Figure 32**).

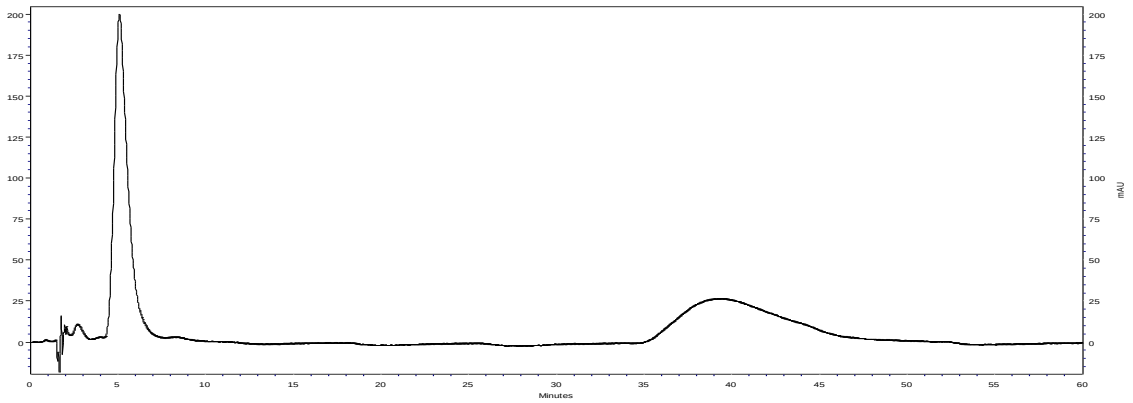


Figure 32 - Chromatogram on the enantioresolution of enantiomeric mixture of CDX XEA DES (**18**) on the CHIRALPAK® HSA column; Conditions: flow rate: 0.9 mL/min; mobile phase, 67 mM potassium phosphate (pH 7.4):ACN (78:22 *v/v*); detection, 254 nm; temperature, 22 ± 2°C.

Similar results were also achieved for enantiomeric mixture XEA 5 4 MET (**25**) (**α =** 2.01; R_S = 1.81) (**Figure 33**).

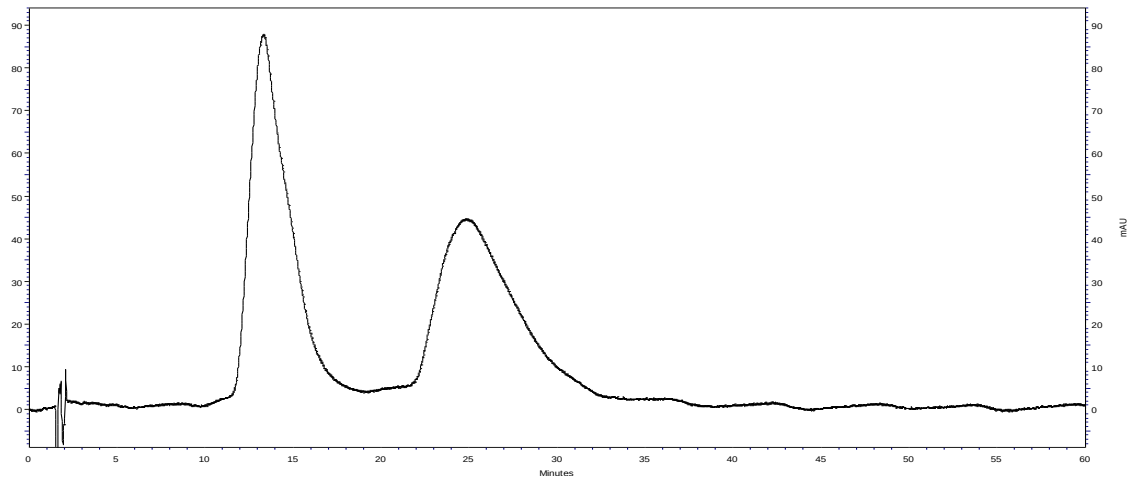


Figure 33 - Chromatogram on the enantioresolution of enantiomeric mixture of CDX XEA 5 4 MET (**25**) on the CHIRALPAK® HSA column; Conditions: flow rate: 0.9 mL/min; mobile phase, 67 mM potassium phosphate (pH 7.4):ACN (80:20 *v/v*); detection, 254 nm; temperature, 22 ± 2°C.

Figure 34 shows examples of how the chromatographic parameters k_1, k_2 and Rs of some enantiomeric mixtures of CDXs changed according to the increment of mobile phase pH to 7.4. In general, the referred pH increase led to a decrease in the retention factors, being

more slightly for k_1 and more significant for k_2, as exemplified in **Figure 35**. Regarding resolution, it was found to be similar in both mobile phase pH. The only exception was observed for enantiomeric mixture XEA 5 4 MET (**24**), which although the enantioseparation and resolution improved, the retention time was longer.

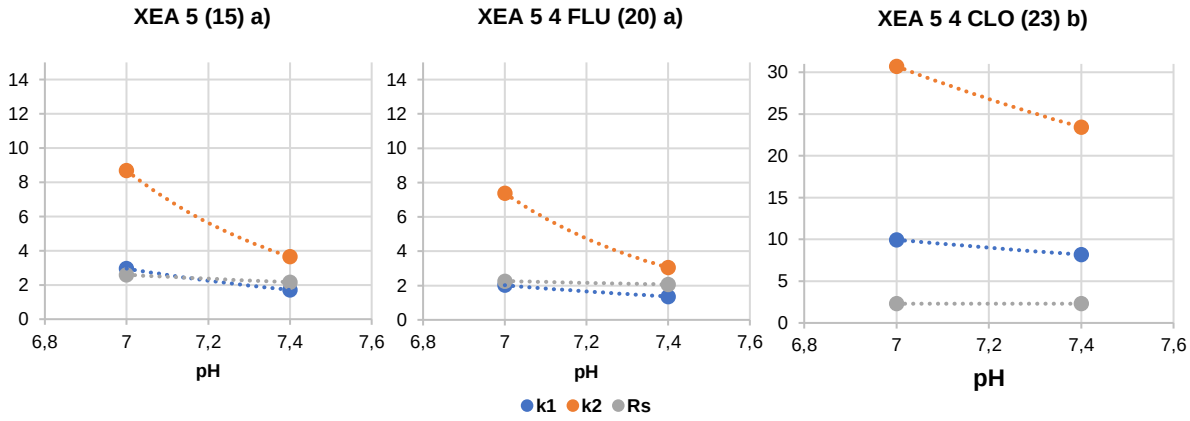


Figure 34 – Effect of mobile phase pH on retention factors and resolution of enantiomeric mixtures XEA 5 (**15**), XEA 5 4 FLU (**20**) and XEA 5 4 CLO (**23**). Conditions: flow rate: 0.9 mL/min; mobile phase a), 67 mM potassium phosphate (pH 7.0 and 7.4): ACN (76:24 *v/v*); mobile phase b), 67 mM potassium phosphate (pH 7.0 and 7.4): ACN (79:21 *v/v*).

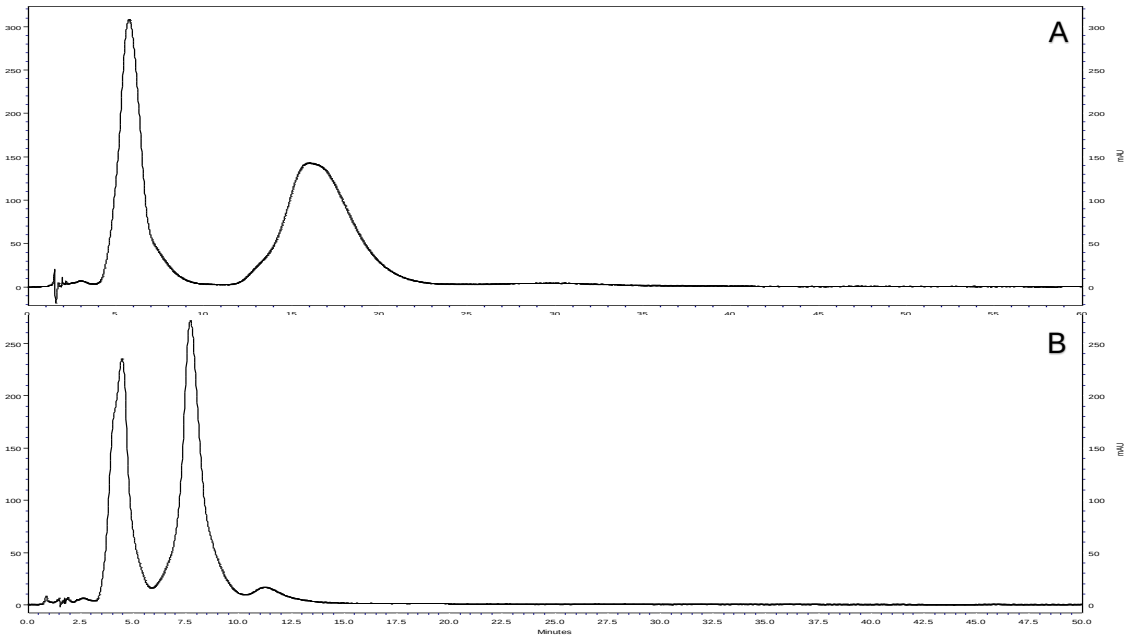


Figure 35 - Chromatograms on the enantioresolution of enantiomeric mixture of CDX XEA 5 4 FLU on the CHIRALPAK® HSA column; Conditions: flow rate: 0.9 mL/min; mobile phase, (**A**) 67 mM potassium phosphate (pH 7.0):ACN (76:24 *v/v*) (**B**) 67 mM potassium phosphate (pH 7.4):ACN (76:24 v/v); detection, 254 nm; temperature, 22 ± 2°C.

1.3.3. Change the organic modifier

Taking into account that: a) several protein binding studies describes 2-PrOH as a solvent used to modify the interactions between strong retained compounds and the CSP [163, 174, 175], and b) in the beginning of this systematic work, enantioselectivity was observed for some enantiomeric mixtures of CDXs, we decided, in this optimization step, to use mixtures of 2-PrOH and 67 mM potassium phosphate buffer at pH 7.4 as mobile phase, and evaluate its influence on chromatographic results. Ten among thirteen enantiomeric mixtures of CDXs were baseline enantioseparated with excellent enantioselectivity on CHIRALPAK® HSA, using potassium phosphate (67 mM, pH 7.4) with 2-PrOH in variable proportion, as mobile phase, with α values ranging from 1.52 to 8.68 and R_S from 1.20 to 4.12. The overall best results are shown on **Table 17**.

Table 17 - The best chromatographic data obtained on separation of enantiomeric mixtures of CDXs on CHIRALPAK® HSA, using mixtures of 67 mM potassium phosphatebuffer at pH 7.4 and 2-PrOH as mobile phase.

Enantiomeric mixture	t_1	t_2	k_1	k_2	α	R_s	% of 2-PrOH
XEGOL 5 (**1**)	9.24	20.08	3.86	9.57	2.48	1.80	25
XEVOL 5 (**6**)	4.48	24.31	1.36	11.79	8.68	4.12	25
XEA 1 (**14**)	22.06	32.51	10.61	16.11	1.52	1.20	20
XEA 5 (**15**)	7.78	17.98	3.09	8.46	2.73	1.80	27
XEA DES (**18**)	6.27	18.24	2.31	8.60	3.72	2.14	27
XEA 5 DES (**19**)	6.27	23.97	2.30	11.61	5.06	3.13	27
XEA 5 4 FLU (**20**)	6.29	18.24	2.31	8.60	3.72	2.14	27
XEA 5 4 CLO (**23**)	21.55	56.18	10.34	28.57	2.76	2.84	25
XEA 5 3 MET (**24**)	9.50	24.02	4.00	11.64	2.91	3.05	25
XEA 5 4 MET (**25**)	9.87	18.42	4.19	8.69	2.07	2.12	25

CHIRALPAK® HSA column; Mobile phase phosphate buffer (pH 7.4):2-PrOH (variable proportion); Flow rate 0.9 mL/min; Temperature 22 ± 2°C; Detection UV 254nm

The enantiomeric mixture XEA 5 (**15**) was enantioseparated in the shortest retention time, k_1 = 3.09 and k_2 = 8.46, with good enantioselectivity and resolution, α = 2.73 and R_S = 1.80 respectively, using a ratio of 27% of organic modifier. Using a different solvent proportion (25%), the enantiomeric mixture XEVOL 5 (**6**) (**Figure 36**) demonstrated the best enantioselectivity and resolution, with α = 8.68 and R_S = 4.12 respectively.

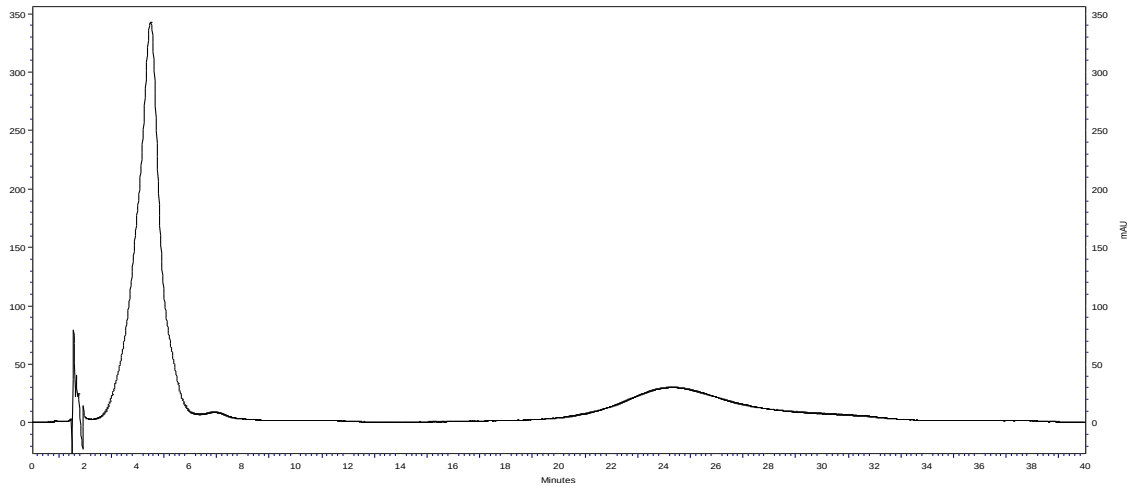


Figure 36 - Chromatogram forresolution of enantiomeric mixture of CDX XEVOL 5 (**6**) on the CHIRALPAK® HSA column; Conditions: flow rate: 0.9 mL/min; mobile phase, 67 mM potassium phosphate (pH 7.4):2-PrOH (75:25 *v/v*); detection, 254 nm; temperature, 22 ± 2°C.

In general, mobile phases comprising 2-PrOH as an organic modifier, demonstrated to afford good enantioselectivity values for these highly hydrophobic compounds, such as for separation of enantiomeric mixture XEA 5 3 MET (**24**) (**Figure 37**). However, it was less efficient when compared to ACN. Actually, a higher proportion of solvent was required to perform similar work, and the higher viscosity of 2-PrOH leaded to an undesirable increase in system pressure. Moreover, as already mentioned, the presence of large proportions of organic modifier in mobile phase reduces the chiral selector life time.

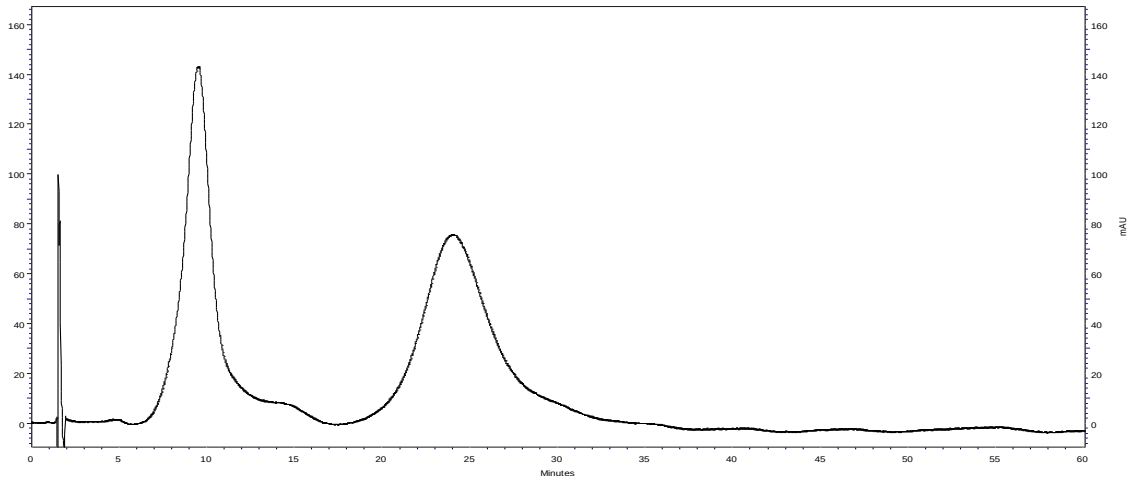


Figure 37 - Chromatogram for resolution of enantiomeric mixture XEA 5 3 MET (**24**) on CHIRALPAK® HSA column; Conditions: flow rate: 0.9 mL/min; mobile phase, 67 mM potassium phosphate (pH 7.4):2-PrOH (75:25 *v/v*); detection, 254 nm; temperature, 22 ± 2°C.

1.3.4. Increase the system temperature

As a major protein in human blood, the normal function of HSA is carried out at the physiological temperature of 37°C. Thus, this temperature was evaluated on this systematic study aiming to improve the chromatographic results. Both ACN and 2-PrOH were used as organic modifiers in mobile phase.

Firstly, retention factors, enantioselectivity and resolution values of thirteen enantiomeric mixtures of CDXs injected on HSA-CSP column were determined at physiological temperature of 37°C ± 2°C, using mobile phases comprising potassium phosphate buffer (67 mM, pH 7.4) and ACN in variable proportion. From this analysis, only eleven of them were baseline enantioseparated, with α values ranging from 1.34 to 9.16 and R_S from 1.00 to 4.97. The overall best results are shown on **Table 18**.

Table 18 - The best chromatographic data obtained for separation of enantiomeric mixtures of CDXs using CHIRALPAK® HSA, using 67 mM potassium phosphate (pH 7.4) as buffer and ACN as organic modifier, in mobile phase, at 37°C ± 2°C.

Enantiomeric mixture	t_1	t_2	k_1	k_2	α	R_s	% of ACN
XEGOL 5 (**1**)	9.72	20.95	4.11	10.03	2.44	3.14	16
XEVOL 5 (**6**)	2.81	10.22	0.48	4.38	9.16	4.31	22
XEA 1 (**14**)	13.83	17.92	6.28	8.43	1.34	1.00	14
XEA 5 (**15**)	9.97	20.29	4.25	9.68	2.28	2.51	20
XEA DES (**18**)	3.84	15.72	1.02	7.28	7.14	4.97	22
XEA 5 DES (**19**)	6.03	15.13	2.17	6.96	3.20	3.39	22
XEA 5 4 FLU (**20**)	6.31	12.23	2.32	5.44	2.34	2.20	22
XEA 5 4 CLO (**23**)	10.93	20.28	4.75	9.67	2.04	2.45	22
XEA 5 3 MET (**24**)	11.84	23.10	5.23	11.16	2.13	2.66	18
XEA 5 4 MET (**25**)	17.45	29.73	8.18	14.65	1.79	2.32	16
X2ADF 5 *SR RS* (**27**)	2.81	10.22	0.48	4.38	9.16	4.31	23

CHIRALPAK® HSA column; Mobile phase phosphate buffer (pH 7.4):ACN (variable proportion); Flow rate 0.9 mL/min; Temperature 37 ± 2°C; Detection UV 254nm

The enantiomeric mixtures XEVOL 5 (**6**) and X2ADF 5 SR RS (**27**) can be distinguished because both were enantioseparated in the shortest retention time, k_1 = 0.48 and k_2 = 4.38. In addition, good enantioselectivity and resolution were obtained, with **α =** 9.16 and R_S = 4.31 respectively for both mixtures, using an organic modifier percentage of 22% and 23%, respectively, for XEVOL 5 (**1**) and X2ADF 5 SR RS (**27**) enantiomeric mixtures. By using a solvent proportion of 22%, the enantiomeric mixture XEA DES (**18**) demonstrated the best enantioselectivity and resolution, with **α =** 7.14 and R_S = 4.97 respectively (**Figure 38**).

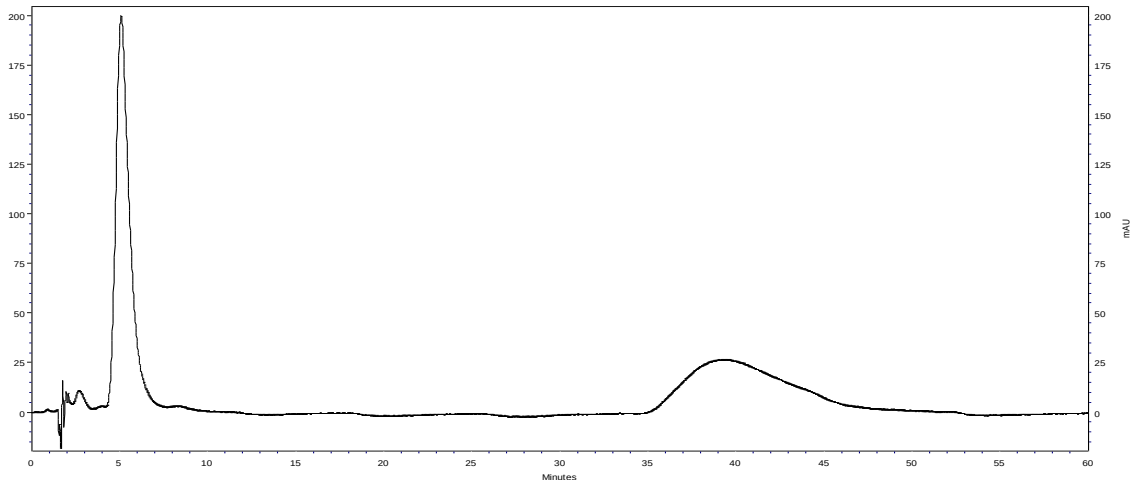


Figure 38 - Chromatogram for resolution of enantiomeric mixture of CDX XEA DES (**18**) on CHIRALPAK® HSA column; Conditions: flow rate: 0.9 mL/min; mobile phase, 67 mM potassium phosphate (pH 7.4):ACN (78:22 *v/v*); detection, 254 nm; temperature, 37 ± 2°C.

Regarding the effect that the change of temperature caused, **Figure 39** shows, as example, the variations of chromatographic parameters of six enantiomeric mixtures of CDXs by changing the temperature of analysis. As expected, a strong decrease in the retention times of all the enantiomeric mixtures of CDXs was observed at the physiological temperature. The proportion of organic modifier had to be decreased to maintain similar enantioselectivity. The temperature increase may decrease the hydrophobicity of the CDXs, as well as the viscosity of the mobile phase and the pressure of the HPLC system.

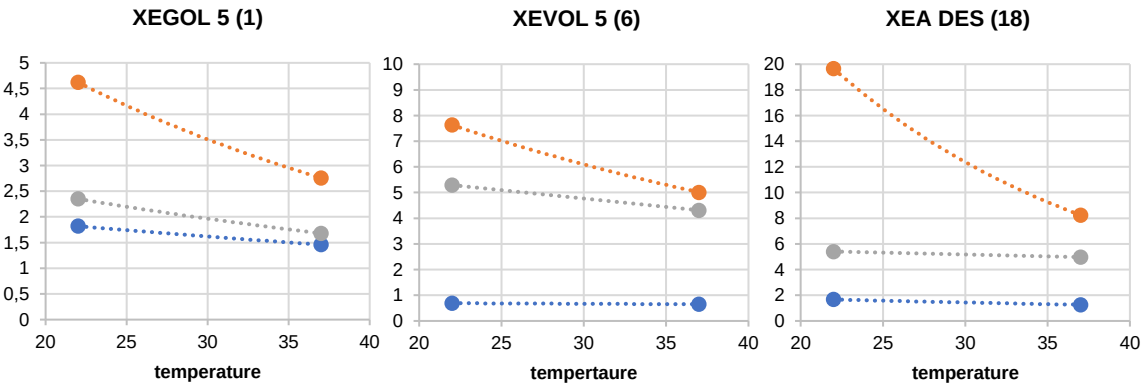


Figure 39 - Effect of the temperature on retention factors and resolution of enantiomeric mixtures XEGOL 5 (**1**), XEVOL 5 (**6**), XEA DES (**18**), XEA 5 DES (**19**), XEA 5 4 FLU (**20**) and XEA 5 4 CLO (**23**); Conditions: flow rate: 0.9 mL/min; mobile phase, 67 mM potassium phosphate (pH 7.4): ACN= 78:22 (*v/v*); temperature: 22°C ± 2°C and 37 ± 2°C.

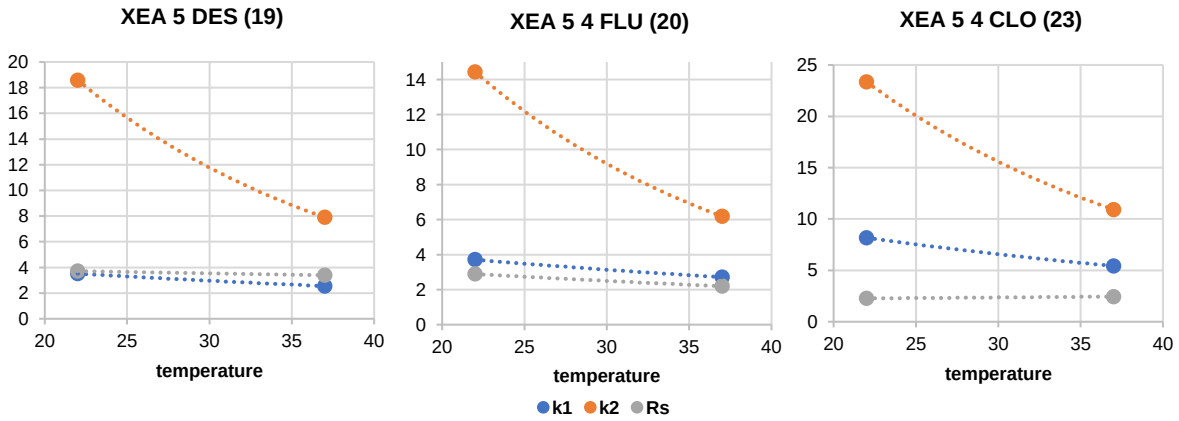


Figure 39 – Continue.

Similar chromatographic results were also achieved for enantiomeric mixture X2ADF 5 *SR RS* (**27**) (α = 9.16; R_S = 4.31) (**Figure 40**).

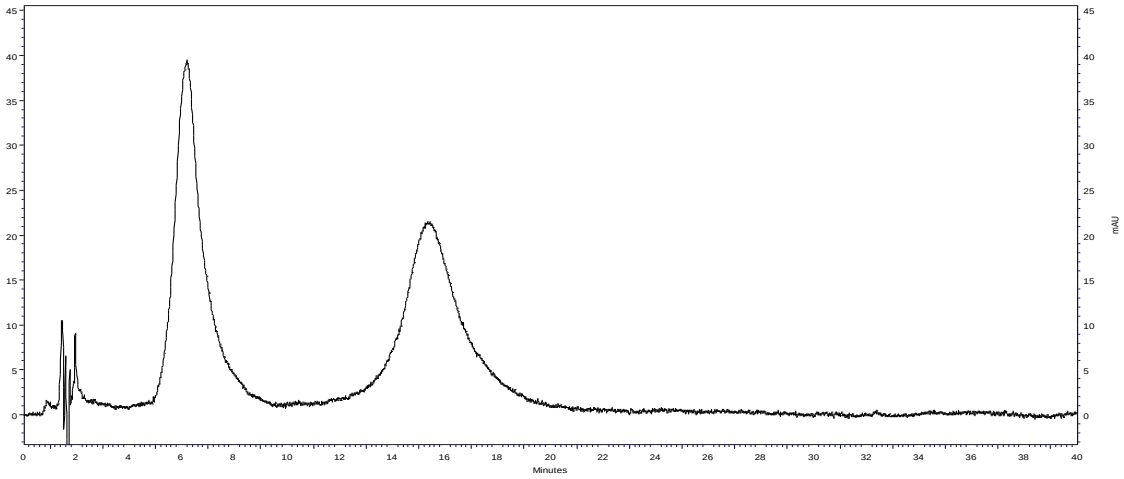


Figure 40 - Chromatogram for resolution of enantiomeric mixture of CDX X2ADF 5 SR RS (**27**) on the CHIRALPAK® HSA column; Conditions: flow rate: 0.9 mL/min; mobile phase, 67 mM potassium phosphate (pH 7.4):ACN (77:23 *v/v*); detection, 254 nm; temperature, 37 ± 2°C.

Then, the same type of study was performed by using the same chromatographic conditions but changing the organic modifier to 2-PrOH. Similarly, the retention factors, enantioselectivity and resolution values of the same thirteen enantiomeric mixtures of CDXs were analyzed. in this case, only nine of them were baseline enantioseparated, with α values ranging from 2.01 to 9.43 and resolutions from 1.54 to 4.04 (**Table 19**).

Table 19 - The best chromatographic data obtained on separation of enantiomeric mixtures of CDXs using CHIRALPAK® HSA, using 67 mM potassium phosphate (pH 7.4) as buffer and 2-PrOH as organic modifier, in mobile phase, at 37 ± 2°C.

Enantiomeric mixture	t_1	t_2	k_1	k_2	α	R_s	% of 2-PrOH
XEGOL 5 (**1**)	9.03	19.34	3.75	9.18	2.44	1.85	20
XEVOL 5 (**6**)	4.56	26.95	1.40	13.18	9.43	4.04	20
XEA 5 (**15**)	20.57	53.66	9.83	27.24	2.77	3.52	20
XEA DES (**18**)	4.16	19.96	1.19	9.51	7.98	3.35	23
XEA 5 DES (**19**)	4.56	11.35	1.40	4.97	3.55	1.94	25
XEA 5 4 FLU (**20**)	15.65	51.68	7.69	15.46	2.01	1.54	20
XEA 5 4 CLO (**23**)	15.14	30.79	6.97	15.21	2.18	1.55	21
XEA 5 3 MET (**24**)	15.72	40.36	7.27	20.24	2.78	2.52	18
XEA 5 4 MET (**25**)	16.52	31.28	7.69	15.46	2.01	1.54	18

CHIRALPAK® HSA column; Mobile phase phosphate buffer (pH 7.4):2-PrOH (variable proportion); Flow rate 0.9 mL/min; Temperature 37 ± 2°C; Detection UV 254nm

It was founf that the enantiomeric mixture XEA 5 DES (**19**) was enantioseparated in the shortest analysis time, k_1 = 1.40 and k_2 = 4.97, and with good enantioselectivity and resolution, α = 3.55 and R_S = 1.94 respectively, using 25% of organic modifier. Using the same solvent percentage, the enantiomeric mixture XEVOL 5 (**6**) (**Figure 41**) demonstrated the best enantioselectivity and resolution, with α = 9.43 and R_S = 4.04 respectively.

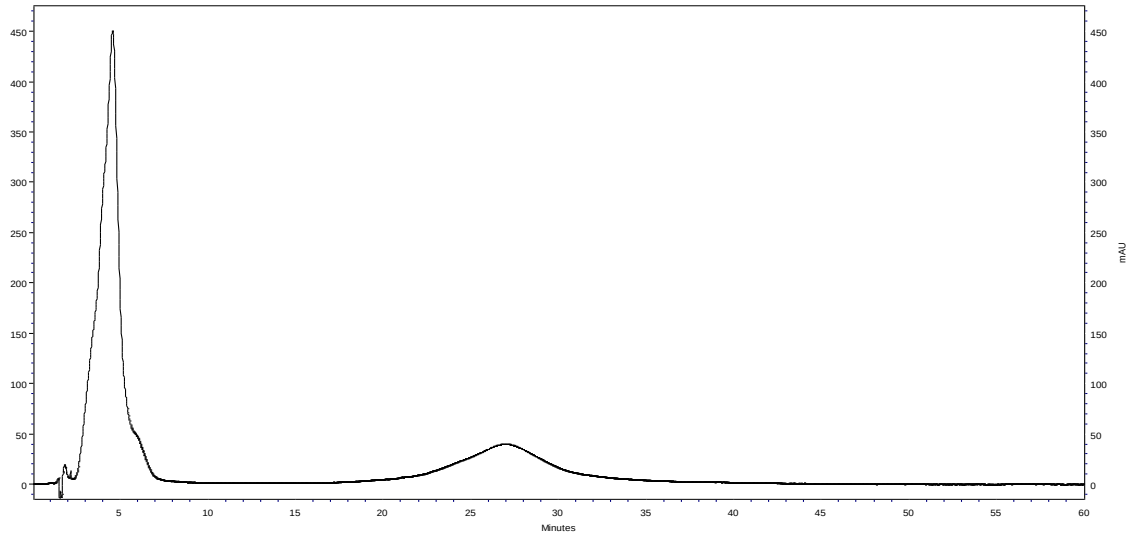


Figure 41 - Chromatogram on the enantioresolution of enantiomeric mixture of CDX XEVOL 5 (**6**) on the CHIRALPAK® HSA column; Conditions: flow rate: 0.9 mL/min; mobile phase, 67 mM potassium phosphate (pH 7.4):2-PrOH (80:20 *v/v*); detection, 254 nm; temperature, 37 ± 2°C.

Similar results and good separations were also achieved for the enantiomeric mixture XEA 5 4 CLO (**23**) (α = 2.18; R_S = 1.55) (**Figure 42**).

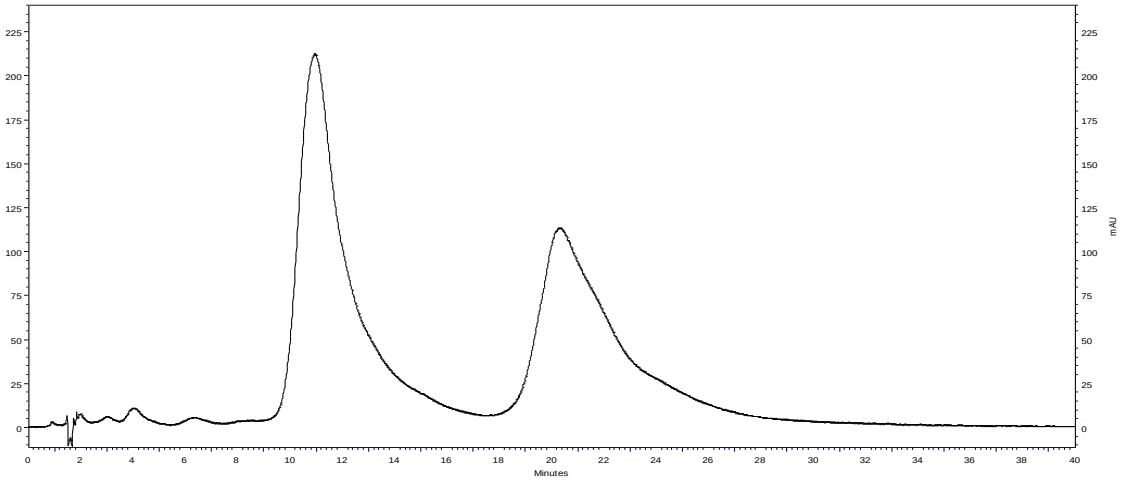


Figure 42 - Chromatogram on the enantioresolution of enantiomeric mixture of CDX XEA 5 4 CLO (**23**) on the CHIRALPAK® HSA column; Conditions: flow rate: 0.9 mL/min; mobile phase, 67 mM potassium phosphate (pH 7.4):2-PrOH (78:22 *v/v*); detection, 254 nm; temperature, 37 ± 2°C.

Based on the results obtained by using the two types of mobile phase organic modifiers, namely ACN and 2-PrOH, it was found that ACN afforded a greater chiral recognition differentiation for a higher number of enantiomeric mixtures of CDXs compared to 2-PrOH. In addition, the separations with ACN as organic modifier occurred using a lower percentage of solvent and afforded a better peak profile and higher resolutions. Moreover, the injections were performed with a lower back pressure in the HPLC system.

1.4. Best performance of CHIRALPAK® HSA column for enantioresolution of CDXs

During the development of this systematic study of enantioresolution of CDXs, several experimental conditions were investigated, including the nature and proportion of the organic modifier, the pH of the mobile phase, the buffer type and concentration, and analysis temperature. **Table 20** summarizes the best chromatographic results obtained for thirteen enantiomeric mixtures of CDXs on CHIRALPAK® HSA column, under reversed-phase mode, in less than 60 minutes and with a resolution factor ≥1.00.

Table 20 - The best chromatographic data obtained on CHIRALPAK® HSA column, under reversed-phase elutions conditions, for the enantiomeric mixtures of CDXs.

Enantiomeric mixture	Chromatographic conditions	t_1	t_2	k_1	k_2	α	R_s
XEGOL 5 (**1**)	67 mM PP (pH 7.4):ACN (84:16 v/v); Temp.: 37 ± 2°C	9.72	20.95	4.11	10.03	2.44	3.14
XEVOL 5 (**6**)	67 mM PP (pH 7.4):ACN (78:22 v/v); Temp.: 37 ± 2°C	2.81	10.22	0.48	4.38	9.16	4.31
XEA 1 (**14**)	67 mM PP (pH 7.4):ACN (86:14 v/v); Temp.: 37 ± 2°C	13.83	17.92	6.28	8.43	1.34	1.00
XEA 5 (**15**)	67 mM PP (pH 7.4):ACN (80:20 v/v); Temp.: 37 ± 2°C	9.97	20.29	4.25	9.68	2.28	2.51
XEA DES (**18**)	67 mM PP (pH 7.4):ACN (78:22 v/v); Temp.: 37 ± 2°C	3.84	15.72	1.02	7.28	7.14	4.97
XEA 5 DES (**19**)	67 mM PP (pH 7.4):ACN (78:22 v/v); Temp.: 37 ± 2°C	6.03	15.13	2.17	6.96	3.20	3.39
XEA 5 4 FLU (**20**)	67 mM PP (pH 7.4):ACN (78:22 v/v); Temp.: 37 ± 2°C	6.31	12.23	2.32	5.44	2.34	2.20
XEA 4 CLO (**22**)	10 mM SP (pH 7.0):ACN (80:20 v/v); Temp.: 22 ± 2°C	14.64	19.25	6.71	19.13	1.36	1.07
XEA 5 4 CLO (**23**)	67 mM PP (pH 7.4):ACN (78:22 v/v); Temp.: 37 ± 2°C	10.93	20.28	4.75	9.67	2.04	2.45
XEA 5 3 MET (**24**)	67 mM PP (pH 7.4):ACN (82:18 v/v); Temp.: 37 ± 2°C	11.84	23.10	5.23	11.16	2.13	2.66
XEA 5 4 MET (**25**)	67 mM PP (pH 7.4):ACN (84:16 v/v); Temp.: 37 ± 2°C	17.45	29.73	8.18	14.65	1.79	2.32
X2ADF 5 *SR RS* (**27**)	67 mM PP (pH 7.4):ACN (77:23 v/v); Temp.: 37 ± 2°C	2.81	10.22	0.48	4.38	9.16	4.31
X2ADF *SR RS* (**30**)	67 mM PP (pH 7.4):ACN (82:18 v/v); Temp.: 22 ± 2°C	18.10	27.97	8.53	13.72	1.61	1.21

Chromatographic conditions: flow rate: 0.9 mL/min, UV detection at 254 nm.
PP: potassium phosphate buffer

According to the results, the best enantioselectivities and resolutions for the enantiomeric mixtures of CDXs, obtained in a shorter time as possible, were achieved using mixtures of potassium phosphate buffer (67 mM, pH 7.4) and ACN as mobile phase, at 37 ± 2°C. Exceptions were enantiomeric mixtures X2ADF *SR RS* (**30**), which obtained better results at 22 ± 2°C, and XEA 4 CLO (**22**), which the best results were obtained by using sodium phosphate buffer (10mM, pH 7.0) and ACN as organic modifier in mobile phase, at a temperature of 37 °C.

2. Elution order of CDXs

The determination of the elution order of the separated enantiomers in a chromatographic experiment is important to identify the enantiomers in the respective chiral separations, to confirm the elution order as well to predict events such as inversion of the elution order by modification of the chromatographic conditions.

It also becomes important in the planning of future chromatographic work, saving time, solvent and facilitates the automation of the process. For further studies by computational chemistry, via molecular docking, for example, it is essential to know the enantiomer that is more retained on CSP to compare to computational data.

Thus, to know the elution order of the thirteen enantioseparated mixtures, the twenty-six CDXs as single enantiomers were injected on CHIRALPAK® HSA column using the same chromatographic conditions as presented in **Table 21**.

Table 21 - Elution order and retention factor of CDXs enantiomers on CHIRALPAK® HSA, using as mobile phase 67 mM potassium phosphate (pH 7.4):ACN (76:24 *v/v*), temperature of 22 ± 2°C, Flow rate 0.9 mL/min, detection UV 254nm.

Analyte	First eluted enantiomer	*k*	Second eluted enantiomer	*k*
XEGOL 5 (**1**)	(*S*)	0.81	(*R*)	1.71
XEVOL 5 (**6**)	(*S*)	0.12	(*R*)	3.51
XEA 1 (**14**)	(*R*)	13.88	(*S*)	20.86
XEA 5 (**15**)	(*R*)	1.84	(*S*)	4.64
XEA DES (**18**)	(*R*)	1.01	(*S*)	5.42
XEA 5 DES (**19**)	(*R*)	1.39	(*S*)	5.16
XEA 5 4 FLU (**20**)	(*R*)	1.42	(*S*)	3.85
XEA 4 CLO (**22**)	(*R*)	1.33	(*S*)	2.67
XEA 5 4 CLO (**23**)	(*R*)	2.84	(*S*)	6.69
XEA 5 3 MET (**24**)	(*R*)	0.99	(*S*)	2.21
XEA 5 4 MET (**25**)	(*R*)	0.97	(*S*)	1.66
X2ADF 5 *SR RS* (**27**)	(*R,S*)	1.64	(*S,R*)	5.30
X2ADF *SR RS* (**30**)	(*R,S*)	0.63	(*S,R*)	0.84

According to results, among the twenty-six CDX injected as pure enantiomer, the (*R*)-enantiomer eluted first for nine enantiomeric mixtures. Exceptions were obtained for enantiomeric mixtures XEGOL 5 (**1**) and XEVOL 5 (**6**), where the (*S*)-enantiomer eluted first. For the enantiomeric mixtures X2ADF 5 *SR RS* (**27**) and X2ADF *SR RS* (**30**), the (*R,S*)-enantiomer eluted first comparatively to (*S,R*).

3. Ligand-Protein binding studies

Reversible binding to serum proteins modulates the distribution of drugs, and then affects their pharmacokinetics and pharmacodynamic properties [79]. Once administered, the free concentration of a drug can change due to its interaction with other drugs and endogenous factors, to its binding to plasma proteins, or to significant changes of the serum carrier concentration. Therefore, plasma protein drug binding studies with determination of binding parameters are relevant when studying a drug profile.

Previously, the *in vitro* binding of three enantiomeric mixtures of CDXs to HSA has been studied through a spectrofluorimetric method as well as *in silico* [149] allowed to obtain information on the binding sites on HSA, and to determine binding parameters. Moreover, the reported data showed a high affinity of CDXs to HSA.

In this work, ligand-protein binding studies were performed by bioaffinity liquid chromatography. The experimental conditions to carrying out this bioaffinity experiments were selected to overcome the very low solubility of CDXs in aqueous solution and to approach to the physiological conditions.

Both enantiomers of the thirty-one enantiomeric mixtures of CDXs were tested, and their affinity to the protein was measured based on their retention times on a CHIRALPAK® HSA column using a zonal chromatography approach. The binding of the sixty-two single enantiomers of CDXs were monitored at different percentages of organic solvent which proved to be a fast and convenient method for analysis of relatively large number of compounds with a retention time of less than 60 minutes. The chosen mobile phases were mixtures of 67 mM potassium phosphate (pH 7.4) buffer and ACN ranging from 75:25 to 98:2, at the physiological temperature of 37 ± 2°C. As positive controls, the affinity of three reported drugs was also measured. Chlorpromazine, described as binding to HSA in subdomain IIIA [176], indomethacin, described as having affinity mainly for subdomain IIA [176] and metronidazole, described as having affinity for both subdomain IIA and IIIA [177] were chosen.

The bound drug percentage (*%b*) was calculated from the *k*-values obtained according to the equation (**1**), and the *%b* calculation in only aqueous phase was extrapolated by linearly plotting the log *k* values (averages of duplicate measurements) against the percentage (*v/v*) of ACN in the mobile phase. The results (all values were rounded up to 2 decimals) are presented in **Table 22** (full results are detailed in the **Appendix H.**).

Table 22 – Binding percentage (*%b*) values of CDXs, injected as single enantiomers on a CHIRALPAK® HSA column, using different ACN proportions in mobile phase, calculated based on *k/(k+1)* equation **(1)** and by extrapolation to 100% aqueous buffer.

Compound	Bound percentage, *%b*										R^2
	25[a]	**22[a]**	**20[a]**	**17[a]**	**15[a]**	**12[a]**	**10[a]**	**7[a]**	**2[a]**	**HSA[b]**	
Chlorpromazine	-	-	87.79	89.26	92.75	94.31	94.75	-	-	98.12	0.948
Indomethacin	-	-	86.30	90.78	92.02	91.93	-	-	-	96.89	0.961
Metronidazole	-	-	-	-	5.16	7.27	8.22	-	16.78	19.78	0.997
XEGOL 5 (*S*) (**1**)	-	-	66.54	85.22	90.96	95.77	96.48	-	-	99.79	0.975
XEGOL 5 (*R*) (**1**)	64.74	77.34	88.01	92.42	95.99	-	-	-	-	99.90	0.988
XEGOL 2 (*S*) (**2**)	-	-	48.02	74.77	83.11	90.96	92.39	-	-	99.50	0.962
XEGOL 2 (*R*) (**2**)	-	-	48.07	74.60	83.08	90.87	92.26	-	-	99.48	0.961
XEGOL 1 (*S*) (**3**)	-	-	36.48	67.70	76.86	86.48	88.36	-	-	99.19	0.976
XEGOL 1 (*R*) (**3**)	-	-	38.42	68.90	78.29	88.06	89.87	-	-	99.36	0.953
XEVOL (*S*) (**4**)	-	-	23.97	58.51	67.74	78.28	80.28	-	-	98.46	0.963
XEVOL (*R*) (**4**)	-	-	-	57.56	66.82	77.17	79.02	85.85	-	94.48	0.982
XEVOL 1 (*S*) (**5**)	-	-	-	56.12	65.63	76.84	79.57	82.81	-	93.33	0.948
XEVOL 1 (*R*) (**5**)	-	-	21.33	59.86	69.14	80.78	83.07	-	-	99.09	0.987
XEVOL 5 (*S*) (**6**)	-	-	44.59	72.80	81.06	89.29	90.79	-	-	99.33	0.951
XEVOL 5 (*R*) (**6**)	74.88	86.21	92.76	95.67	97.70	-	-	-	-	99.95	0.992
XEL (*S*) (**7**)	-	-	-	67.19	76.89	86.63	88.49	93.01	-	98.05	0.982
XEL (*R*) (**7**)	-	-	-	66.49	76.36	85.97	87.67	91.92	-	97.59	0.976
XEL 1 (*S*) (**8**)	-	-	-	62.69	72.88	84.16	86.21	91.50	-	97.60	0.983
XEL 1 (*R*) (**8**)	-	-	-	65.97	75.59	86.38	88.34	92.55	-	97.99	0.980
X2A1P (*S*) (**9**)	-	-	-	50.53	58.99	69.35	71.00	73.63	-	86.52	0.973
X2A1P (*R*) (**9**)	-	-	-	50.79	60.10	69.99	71.94	74.45	-	87.25	0.941
X2A1P1 (*S*) (**10**)	-	-	-	47.41	55.20	65.11	67.28	67.91	-	81.94	0.951
X2A1P1 (*R*) (**10**)	-	-	-	51.19	56.31	62.90	67.55	67.10	-	79.02	0.956
X1A2P (*S*) (**11**)	-	-	-	52.20	59.76	70.41	73.22	74.70	-	87.52	0.942
X1A2P (*R*) (**11**)	-	-	-	52.54	59.48	69.98	72.47	73.83	-	86.51	0.957
X1A2P1 (*S*) (**12**)	-	-	-	47.63	54.20	64.60	67.11	66.72	-	80.91	0.951
X1A2P1 (*R*) (**12**)	-	-	-	48.07	54.45	64.78	67.22	66.82	-	80.82	0.941
XEA (*S*) (**13**)	-	74.37	84.05	90.06	94.04	97.51	-	-	-	99.86	0.991
XEA (*R*) (**13**)	-	75.11	84.42	90.44	94.39	97.77	-	-	-	99.89	0.990
XEA 1 (*S*) (**14**)	-	66.52	78.10	85.76	91.27	96.38	-	-	-	99.79	0.989
XEA 1 (*R*) (**14**)	-	64.31	75.19	83.14	89.19	95.15	-	-	-	99.65	0.989
XEA 5 (*S*) (**15**)	78.69	89.73	95.74	98.08	-	-	-	-	-	99.99	0.990
XEA 5 (*R*) (**15**)	71.26	83.55	90.43	94.57	96.85	-	-	-	-	99.92	0.997
XEA 3 MET (*S*) (**16**)	-	-	61.30	84.94	90.44	95.79	96.68	-	-	99.85	0.972

This table continues on the next page.

Table 22 – Continuation.

Compound	Bound percentage, *%b*										R^2
	25[a]	**22[a]**	**20[a]**	**17[a]**	**15[a]**	**12[a]**	**10[a]**	**7[a]**	**2[a]**	**HSA[b]**	
Chlorpromazine	-	-	87.79	89.26	92.75	94.31	94.75	-	-	98.12	0.948
XEA 3 MET (*R*) (**16**)	-	-	58.43	82.51	88.72	94.86	95.98	-	-	99.80	0.975
XEA 4 MET (*S*) (**17**)	-	-	60.73	84.74	90.33	95.74	96.63	-	-	99.85	0.971
XEA 4 MET (*R*) (**17**)	-	-	63.47	87.15	92.16	96.77	97.64	-	-	99.92	0.976
XEA DES (*S*) (**18**)	77.95	89.04	95.69	98.16	-	-	-	-	-	99.99	0.988
XEA DES (*R*) (**18**)	-	-	61.90	84.02	89.47	94.98	95.96	-	-	99.76	0.971
XEA 5 DES (*S*) (**19**)	77.46	88.70	95.63	97.89	-	-	-	-	-	99.99	0.983
XEA 5 DES (*R*) (**19**)	-	-	76.78	91.44	94.88	97.77	-	-	-	99.95	0.992
XEA 5 4 FLU (*S*) (**20**)	-	-	56.88	79.56	92.24	97.06	-	-	-	99.98	0.993
XEA 5 4 FLU (*R*) (**20**)	-	-	78.13	90.28	95.25	97.97	-	-	-	99.96	0.998
XEA 4 FLU (*S*) (**21**)	-	-	66.07	87.07	91.75	95.90	97.89	-	-	99.90	0.988
XEA 4 FLU (*R*) (**21**)	-	-	63.81	85.13	90.47	95.22	97.67	-	-	99.88	0.991
XEA 4 CLO (*S*) (**22**)	-	80.05	87.96	92.65	95.62	96.47		-	-	99.70	0.964
XEA 4 CLO (*R*) (**22**)	-	78.96	87.46	92.29	95.31	-	-	-	-	99.85	0.990
XEA 5 4 CLO (*S*) (**23**)	83.11	92.15	95.02	97.07	-	-	-	-	-	99.95	0.987
XEA 5 4 CLO (*R*) (**23**)	77.30	87.64	93.02	96.07	98.13	-	-	-	-	99.96	0.993
XEA 5 3 MET (*S*) (**24**)	69.11	82.71	91.40	95.52	97.96	-	-	-	-	99.98	0.993
XEA 5 3 MET (*R*) (**24**)	-	76.08	85.10	90.90	95.21	-	-	-	-	99.87	0.987
XEA 5 4 MET (*S*) (**25**)	67.50	81.42	90.55	94.99	97.61	-	-	-	-	99.97	0.993
XEA 5 4 MET (*R*) (**25**)	-	77.14	86.15	91.80	95.77	-	-	-	-	99.91	0.988
X2ADF 5 (*S,S*) (**26**)	74.45	86.00	92.13	95.58	98.00	-	-	-	-	99.96	0.991
X2ADF 5 (*R,R*) (**26**)	74.75	86.30	92.78	96.28	-	-	-	-	-	99.97	0.995
X2ADF 5 (*S,R*) (**27**)	82.18	91.99	95.00	97.63	-	-	-	-	-	99.98	0.998
X2ADF 5 (*R,S*) (**27**)	69.66	82.19	90.93	93.70	95.51	-	-	-	-	99.85	0.971
X2ADF 1 (*S,S*) (**28**)	-	70.70	81.38	87.83	93.58	-	-	-	-	99.81	0.981
X2ADF 1 (*R,R*) (**28**)	-	67.92	77.69	84.59	91.12	-	-	-	-	99.68	0.994
X2ADF 1 (*S,R*) (**29**)	-	64.26	-	82.43	89.27	94.02	96.07	-	-	99.54	0.997
X2ADF 1 (*R,S*) (**29**)	-	65.07	78.86	84.54	90.63	94.90	96.54	-	-	99.61	0.991
X2ADF (*S,R*) (**30**)	-	72.13	82.66	90.19	94.10	97.12		-	-	99.86	0.998
X2ADF (*R,S*) (**30**)	-	-	78.86	86.43	91.39	95.22	96.70	-	-	99.59	0.998
X2ADF (*S,S*) (**31**)	-	75.02	83.95	90.56	94.29	97.02	-	-	-	99.82	0.998
X2ADF (*R,R*) (**31**)	-	74.33	83.34	90.23	94.12	97.04	-	-	-	99.83	0.998

[a] Acetonitrile %

[b] Extrapolated to 100% buffer

According to the results, all sixty-two enantiomers of CDXs injected into the HSA column showed a high affinity for the protein. All values presented a good correlation with the

decrease of organic modifier, increasing its affinity with the protein, praising the importance of the hydrophobic forces between this type of ligands and the protein. Extrapolation by linearly plotting the log *k* values against the percentage of ACN in the mobile phase indicates that, in 100% aqueous buffer, the *%b* of the CDXs enantiomers to the HSA protein ranges from 79.02 to 99.99%, with a R^2 normally higher than 0.941. The **Figure 43** represents a stratification of the number of enantiomers per each range of *%b* and shows that 87% of the compounds have a high affinity (> 90%) for the HSA.

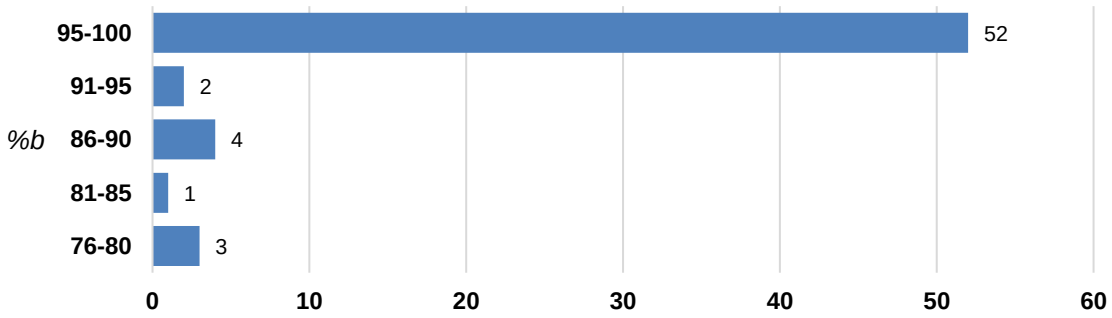


Figure 43 – Screening of CDXs enantiomers: number of enantiomers for each range of bound percent (*%b*), values extrapolated for 100% aqueous buffer solution.

The drugs used as positive controls, namely chlorpromazine, indomethacin and metronidazole, after extrapolation have shown a good agreement between the experimental and the described in the literature, respectively 98.12% (Lit. value: > 90% [178]), 96.89% (Lit. value: 97.8% [179]) and 19.78% (Lit. value: 20% [180]).

It is known that the presence of an organic solvent may affect the drug–protein binding process, either by generating a change in the secondary or tertiary structure of the protein, and/or modifying the microenvironment at the binding site so that low energy interactions (hydrophobic and dipole–dipole interactions, coulombic forces, hydrogen bonds) cannot take place [61]. A study of the variation of log *k* values of each enantiomer of CDXs when changing the ACN concentration in the mobile phase showed a nearly linear relationship between log *k* and the organic solvent concentration as in the two examples below (**Figure 44**), specifically for XEA 5 (*R*) (**15**) and XEA 4 MET (*R*) (**17**). Due to the different nature of the enantiomers, they had a different behavior for the same proportions of organic modifier, but when extrapolated by linear regression, both had a similar *%b* of 99.92%.

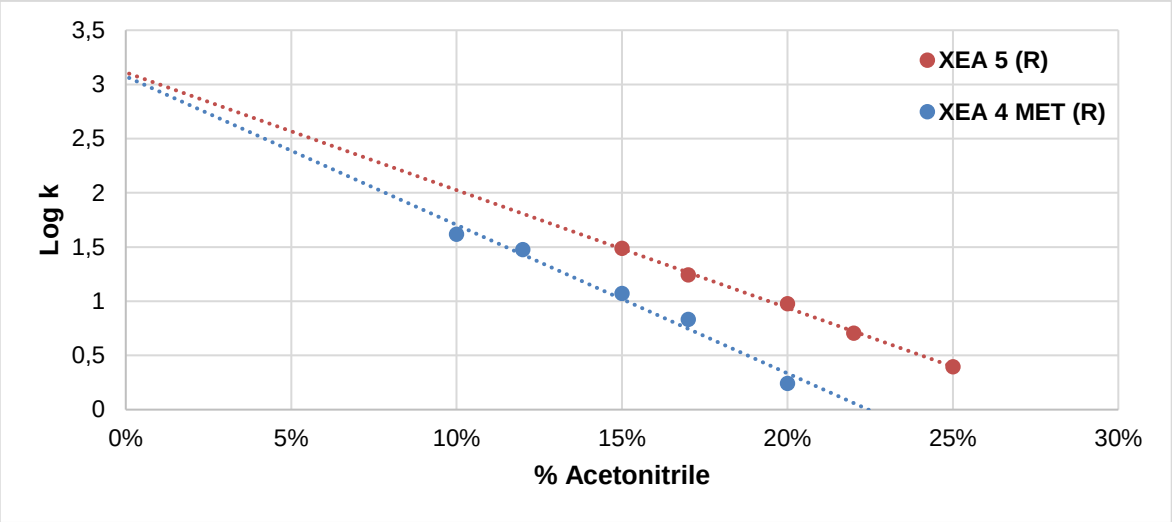


Figure 44 – Linear plotting of log k values of XEA 5 (*R*) (**15**) and XEA 4 MET (*R*) (**17**), obtained with the CHIRALPAK® HSA column, against various concentrations of ACN in the mobile phase; Conditions: flow rate: 0.9 mL/min; mobile phase, 67 mM potassium phosphate (pH 7.4):ACN; detection, 254 nm; temperature, 37 ± 2°C.

4. Computational docking studies

In order to understand the chiral recognition mechanism behind the observed results of enantioresolution, docking studies were performed, using the thirteen enantiomeric mixtures of CDXs that were previously enantioseparated on a CHIRALPAK® HSA column by LC with resolution factors ≥0.50. Values of docking scores are presented on **Table 23**. The lower the docking score, the more stable is the analyte-selector complex. The differences in energy of the docking score of (*S*)-enantiomer-CSP complex versus (*R*)-enantiomer-CSP complex were used to calculate the energy difference values. In the case of CDXs comprising two stereogenic centers, the energy differences were calculated by the difference between (*SR*)-enantiomer-CSP complex and (*RS*)-enantiomer-CSP complex. A previous study indicates that CDXs fit within the hydrophobic pocket of subdomain IIIA, presenting low negative docking scores [149]. In this study this pocket was selected for the docking studies as it was described as being the binding pocket for (*S*)-ibuprofen and most ligands [179]. Actually, it was found that CDXs bind in a similar position in the binding groove, with the central xanthone ring aligned with ibuprofen ring [179].

Table 23 – Chromatographic data, docking scores of both enantiomers of CDXs on a CHIRALPAK® HSA column and the energy difference between (*S*) and (*R*) or (*S,R*) and (*R,S*) enantiomers.

Enantiomeric mixture	Mobile Phase	k_1	α	Rs	First Eluted Enantiomer	Docking Score (kcal.mol^{-1})	Energy difference (kcal.mol^{-1})
XEGOL 5 (**1**)	PB:ACN (83:17 *v/v*)	3.78	2.38	3.57	(*S*)	(*S*): -8.9 (*R*): -9.5	0.6
XEVOL 5 (**6**)	PB:ACN (83:17 *v/v*)	1.58	10.82	4.69	(*S*)	(*S*): -8.4 (*R*): -8.8	0.4
XEA 1 (**14**)	PB:ACN (83:17 *v/v*)	3.32	1.25	0.86	(*R*)	(*S*): -9.8 (*R*): -8.6	-1.2
XEA 5 (**15**)	PB:ACN (83:17 *v/v*)	11.56	2.49	4.04	(*R*)	(*S*): -10.6 (*R*): -9.8	-0.8
XEA DES (**18**)	PB:ACN (83:17 *v/v*)	3.34	8.31	4.17	(*R*)	(*S*): -9.5 (*R*): -9.1	-0.4
XEA 5 DES (**19**)*	PB:ACN (76:24 *v/v*)	7.25	3.78	3.21	(*R*)	(*S*): -10.3 (*R*): -9.8	-0.5
XEA 5 4 FLU (**20**)	PB:ACN (83:17 *v/v*)	7.56	2.59	2.22	(*R*)	(*S*): -9.8 (*R*): -10.1	0.3
XEA 4 CLO (**22**)	PB:ACN (83:17 *v/v*)	6.97	2,18	1,55	(*R*)	(*S*): -9.0 (*R*): -8.9	-0.1
XEA 5 4 CLO (**23**)	PB:2-PrOH (78:22 *v/v*)	16.38	2.08	2.60	(*R*)	(*S*): -11.2 (*R*): -9.9	-1.3
XEA 5 3 MET (**24**)	PB:ACN (83:17 *v/v*)	7.08	2.11	3.67	(*R*)	(*S*): -10.1 (*R*): -9.4	-0.7
XEA 5 4 MET (**25**)	PB:ACN (83:17 *v/v*)	7.63	1.68	2.50	(*R*)	(*S*): -10.1 (*R*): -9.4	-0.7

This table continues on the next page.

Table 23 – Continue.

Enantiomeric mixture	Mobile Phase	k_1	α	Rs	First Eluted Enantiomer	Docking Score (kcal.mol^{-1})	Energy difference (kcal.mol^{-1})
X2ADF 5 SR RS (**27**)	PB:ACN (83:17 *v/v*)	10.15	3.78	7.00	(*R,S*)	(*S,R*): -9.9 (*R,S*): -10.0	0.1
X2ADF SR RS (**30**)	PB:ACN (83:17 *v/v*)	3.95	1.24	0.59	(*R,S*)	(*S,R*): -9.0 (*R,S*): -9.6	0.6

Buffer: PB (potassium phosphate buffer) 67 mM, pH 7.4; Chromatographic conditions: flow rate: 0.9 mL/min, UV detection at 254 nm, temperature at 37 ± 2°C or *22 ± 2°C.
Energy difference = (*S*)-enantiomer-CSP complex docking score - (*R*)-enantiomer-CSP complex docking score or (*SR*)-enantiomer-CSP complex docking score - (*RS*)-enantiomer-CSP complex docking score

It was found that, an 77% agreement between docking scores and experimental chromatographic results were achieved concerning enantiomers elution order.

The differences observed may be due to the complex structure of the HSA selector allowing different binding patterns with the molecules of CDXs. Moreover, it is not sure that all CDXs molecules bind only to one pocket of the protein structure, or if there may be a competition between both enantiomers for the same binding site when they are injected into the column as enantiomeric mixture.

Moreover, computationally, Autodock Vina has a hybrid scoring function (empirical and knowledge-based function) inspired in the X-Score function that consists of a weighted sum of steric interactions, hydrophobic interaction, and number of active rotatable bonds with different weights [158]. Therefore, similar ligands can originate very different docking scores, depending not only on the type of interactions established, but also the number of interactions, maximizing favorable and minimizing unfavorable interactions, shape and property complementarities [181].

In order to understand the binding energy scores, a visual inspection of the binding conformations was performed for the CDXs molecules on HAS-CSP. **Figure 45** and **46** illustrate representative examples of the most stable docked conformations for enantiomers complexes with the HSA-CSP. As shown in both **Figures 45**, the docking poses of the enantiomers were diverse, leading to different binding interactions. The superimposition of (*R*) and (*S*) enantiomers of the same enantiomeric mixture shows that their positions are not in fact similar.

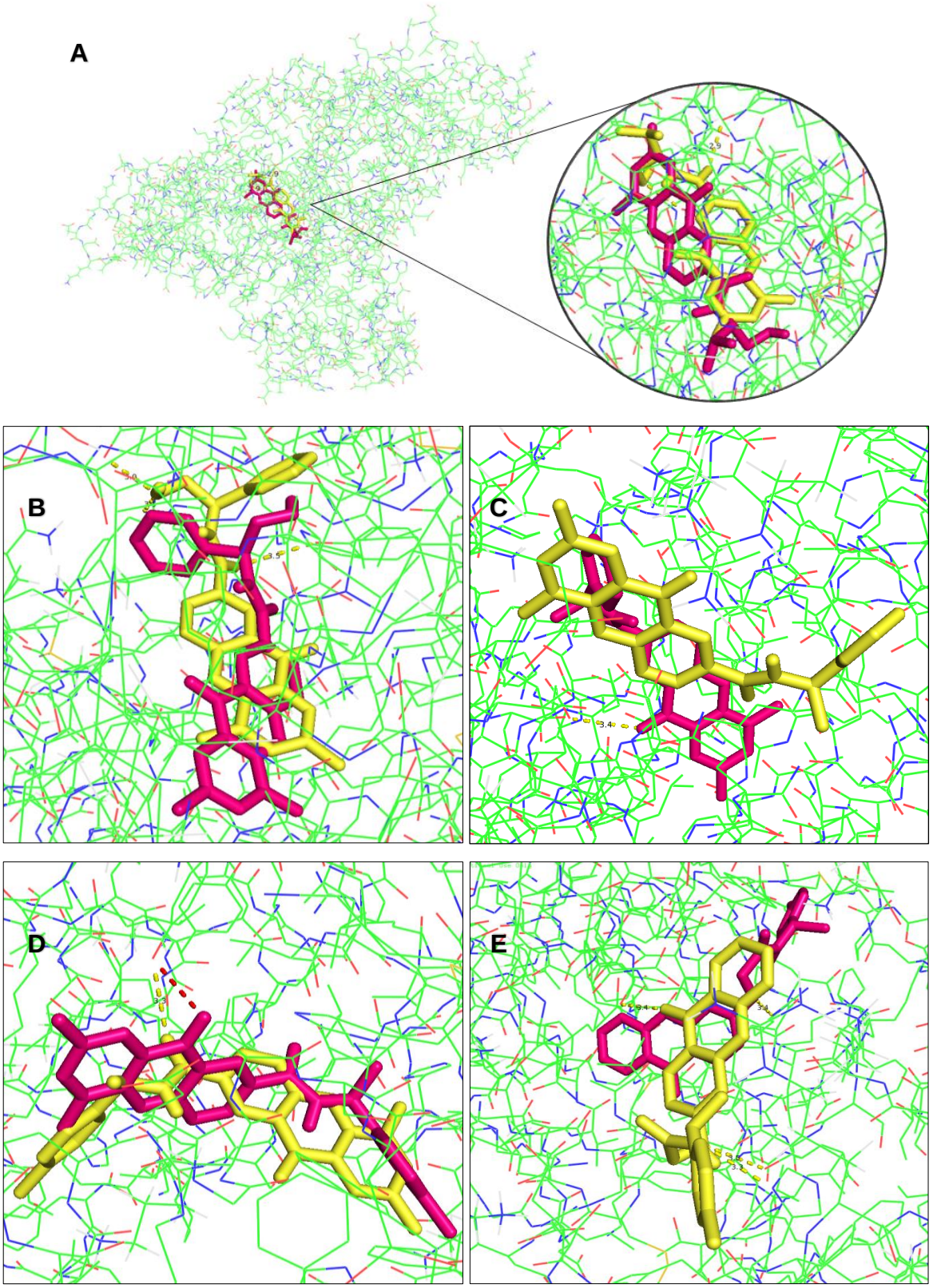


Figure 45 – XEVOL 5 (**6**) (**A**), XEGOL 5 (**1**) (**B**), XEA 5 DES (**19**) (**C**), XEA 5 4 CLO (**23**) (**D**), and XEA 1 (**14**) (**E**) enantiomers, docked on a HSA selector. Chiral selector is represented in green, blue and red sticks. (*S*) and (*R*) enantiomers are represented in with magenta and yellow sticks, respectively.

Chapter V

CONCLUSIONS

A systematic study of enantioseparation of thirty-one enantiomeric mixtures of chiral derivatives of xanthones (CDXs) on a human serum albumin (HSA)-based column, namely CHIRALPAK® HSA CSP, was performed by using different mobile phases, under reversed-phase elution mode.

Several chromatographic conditions, such as mobile phase pH, buffer type and ionic strength, type and content of organic modifiers and temperature, were explored.

Under the optimized chromatographic conditions, thirteen out of thirty-one enantiomeric mixtures of CDXs were successfully enantioseparated with good enantioselectivity and resolution, with α ranging from 1.27 to 12.53 and R_S from 0.90 to 6.41.

The best performances were achieved using mixtures of potassium phosphate buffer and ACN as mobile phases. For the majority of the enantiomeric mixtures, the mobile phase pH of 7.4 and temperature of 37 ± 2°C afforded the best chromatographic data.

Additionally, binding affinity studies of sixty-two enantiomers of CDXs on HSA-based CSP were performed. The bound percent of compound was determined based on retention data obtained using different proportions of organic modifier in the mobile phase. Calculation by extrapolation of each enantiomer bound percentage by linear plotting of the Log *k* was made, showing in general high affinity for HSA-CSP, with bound percentage ranging from 79.02% to 99.99% in 100% aqueous buffer.

Docking studies yielded scores that were in accordance with the chromatographic data regarding enantioselectivity and enantiomer elution order, with a success rate of 77%.

The results of the present study fulfilled the initial objectives of this work and confirmed the applicability of HSA-based column to this class of chiral compounds.

Chapter VI

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Chapter VII

APPENDIXES

Appendix A.

Abstract and Poster of 10º Encontro Nacional de Cromatografia (10 ENC), December 04-06, 2017.

10º Encontro de Cromatografia

PC-49

Liquid chromatography enantioseparation of xanthone derivatives on a human serum albumin stationary phase

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Chiral derivatives of xanthones (CDXs) have important biological activities being, in some cases, dependent on the stereochemistry [1,2]. Therefore, accurate methodology to enantioseparate and evaluate their enantiomeric purity plays a very important role.

In our group, liquid chromatography (LC) methods using different type of chiral stationary phases (CSPs), specifically macrocyclic glycopeptide antibiotic-based [3], Pirkle-type [4,5], and polysaccharide-based [6], have demonstrated to be efficient for enantioresolution of xanthone derivatives.

In order to expand the systematic investigation on enantioseparation of this important class of compounds using different types of CSPs, herein we report the development of enantioselective LC method for the resolution of enantiomeric mixtures of a series of CDXs by using a human serum albumin CSP. The enantioseparation was explored using different mobile phases, under reversed-phase elution conditions.

For some CDXs high enantioselectivity and resolution were obtained.

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This work was partially supported through national funds from Foundation for Science and Technology (FCT) and European Regional Development Fund (ERDF) and COMPETE under the projects UID/Multi/04423/2013, PTDC/MAR-BIO/4694/2014 (POCI-01-0145-FEDER-016790), QOPNA (FCT UID/QUI/00062/2013), and INNOVMAR (Innovation and Sustainability in the Management and Exploitation of Marine Resources) - NORTE-01-0145-FEDER-000035, Research Line NOVELMAR, Chiral–Drugs-CESPU-2017.

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10º Encontro Nacional de Cromatografia
Bragança 2017

Liquid chromatography enantioseparation of xanthones derivatives on a human serum albumin stationary phase

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INTRODUCTION

Figure 1. Structure of human serum albumin used as CSP.

Chiral derivatives of xanthones (CDXs) have important biological activities being, in some cases, dependent on the stereochemistry [1,2]. Therefore, accurate methodology to enantioseparate and evaluate their enantiomeric purity plays a very important role.
In our group, liquid chromatography (LC) methods using different type of chiral stationary phases (CSPs), specifically macrocyclic glycopeptide antibiotic-based [3], Pirkle-type [4,5], and polysaccharide-based [6], have demonstrated to be efficient for enantioresolution of xanthone derivatives.
In order to expand the systematic investigation on enantioseparation of this important class of compounds using different types of CSPs, herein we report the development of enantioselective LC method using a human serum albumin CSP (Figure 1) for the resolution of enantiomeric mixtures of a series of CDXs (Figure 2).

RESULTS

Chromatographic conditions:

HPLC system: Thermo® Scientific HPLC;
Detector: Thermo® Scientific Spectra System UV8000 diode array detector;
Column: CHIRALPAK® HSA (150 mm × **4.0** mm, I.D., 5 µm particles size);
Mobile phase: different percentages (10-24%) of acetonitrile, as organic modifier, in 10 mM aqueous buffers of potassium phosphate, sodium phosphate, sodium acetate and ammonium acetate at pH of 5.0 or 7.0.
Flow rate: 0.9 mL/min;
Sample concentration: 50 µg/mL;
Sample injections: 20 µL;
Temperature: 22 ± 2° C, in isocratic mode.

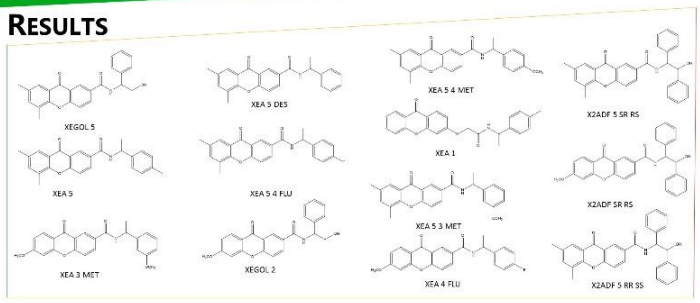


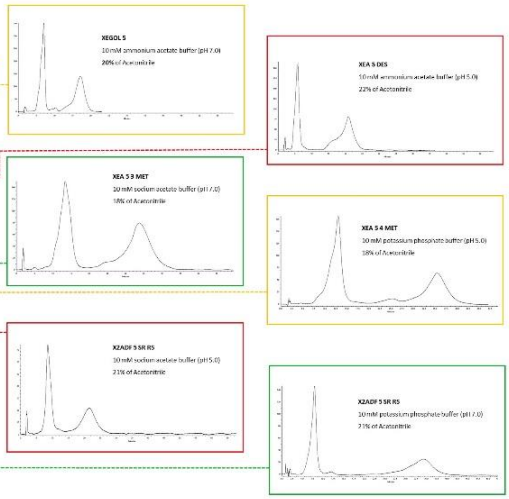
Figure 2. Structures of enantioseparated CDXs.

Chromatographic data:

Table 1. Best enantioseparation achieved on a human serum albumin CSP.

	10 mM Potassium phosphate				10 mM Sodium phosphate				10 mM Sodium acetate				10 mM Ammonium acetate			
	pH 5		pH 7		pH 5		pH 7		pH 5		pH 7		pH 5		pH 7	
	α	R_s	α	R_s	α	R_s	α	R_s	α	R_s	α	R_s	α	R_s	α	R_s
XEGOL 5	3.75	4.04	4.12	3.29	3.83	3.12	4.47	6.41	2.99	2.70	3.31	3.30	**2.87**	**3.27**	2.99	3.55
% organic modifier	20		18		20		20		16		20		18		20	
XEA 5	4.34	4.36	4.84	6.11	4.59	4.91	4.27	4.77	3.17	2.49	3.27	1.78	3.15	2.79	3.87	4.73
% organic modifier	24		21		22		23		21		21		22		22	
XEA 3 MET	-	-	1.64	2.63	-	-	-	-	1.53	2.08	-	-	1.51	1.61	-	-
% organic modifier	-		12		-		-		11		-		10		-	
XEA 5 DES	5.17	4.88	5.90	5.72	8.16	4.32	6.10	5.17	3.84	2.57	4.11	2.03	**4.91**	**3.87**	6.83	5.24
% organic modifier	24		21		22		22		21		21		22		22	
XEA 5 4 FLU	5.78	3.94	5.12	3.41	5.73	4.17	7.62	4.40	4.88	3.10	3.84	2.37	5.72	3.08	4.85	4.38
% organic modifier	22		20		22		23		22		21		22		22	
XEA 4 FLU	-	-	1.89	3.71	1.71	1.78	1.79	2.60	1.55	2.21	-	-	-	-	-	-
% organic modifier	-		12		13		13		11		-		-		-	
XEA 5 3 MET	3.28	3.28	2.95	4.20	3.08	2.86	3.44	4.93	2.62	2.70	**2.77**	**2.52**	2.78	3.07	2.55	2.87
% organic modifier	20		19		20		20		18		18		18		22	
XEA 5 4 MET	**3.02**	**4.27**	2.97	4.90	2.85	3.49	2.94	5.23	2.27	2.45	2.16	2.27	2.26	2.40	2.37	2.80
% organic modifier	18		19		18		20		16		20		18		20	
X2ADF 5 SR RS	4.89	4.53	**5.42**	**4.45**	4.55	3.46	5.50	3.97	**2.98**	**2.38**	3.35	2.35	3.02	2.64	4.13	3.97
% organic modifier	22		21		22		22		21		21		22		22	
X2ADF SR RS	-	-	2.60	3.73	2.23	2.36	2.29	2.90	1.84	3.04	1.88	2.12	-	-	1.86	2.20
% organic modifier	-		15		13		15		11		10		-		13	
XEGOL 2	-	-	1.55	1.81	-	-	-	-	-	-	-	-	-	-	-	-
% organic modifier	-		8		-		-		-		-		-		-	
XEA 1	-	-	-	-	-	-	1.42	1.27	-	-	-	-	-	-	-	-
% organic modifier	-		-		-		15		-		-		-		-	
X2ADF 5 RR SS	-	-	-	-	3.03	2.77	-	-	-	-	-	-	-	-	-	-
% organic modifier	-		-		20		-		-		-		-		-	

Chromatograms:



CONCLUSION & FUTURE WORK

In this study, the enantioseparation of a series of CDXs was explored using an immobilized human serum albumin CSP and several mobile phases, under reversed-phase elution conditions. For some CDXs high enantioselectivity and resolution were obtained. In the future, the optimized chromatographic conditions will be used for further high performance affinity chromatography (HPAC) studies to measure the global affinity constants and site-specific binding constants of drug-protein interactions. Additionally, *in silico* studies by docking approach will be conducted aiming to establish the active site and the type interactions between the CDXs and the human serum albumin CSP.

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This research was partially supported by the Strategic Funding UID/Multi/04423/2013 through national funds provided by FCT – Foundation for Science and Technology and European Regional Development Fund (ERDF), in the framework of the programme PT2020, the project PTDC/MAR-BIO/4694/2014 (reference POCI-01-0145-FEDER-016790; Project 3599 – Promover a Produção Científica e Desenvolvimento Tecnológico e a Constituição de Redes Temáticas (3599-PPCDT)) as well as by the project INNOVMAR - Innovation and Sustainability in the Management and Exploitation of Marine Resources (reference NORTE-01-0145-FEDER-000035, within Research Line NOVELMAR), supported by North Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF), and Chiral_Drugs_CESPU_2017.

Appendix B.

Abstract and Poster of Escola de Inverno de Farmácia, 3rd ed, Porto, Portugal, March 07-15, 2018.

ESCOLA DE INVERNO DE FARMÁCIA - 3ª EDIÇÃO
FARMÁCIA CLÍNICA CENTRADA NA SEGURANÇA DO PACIENTE
7 a 15 de Março, 2018

Chiral recognition through liquid chromatography: optimization process leading to displacement studies

Protein-based Chiral separations of xanthones derivatives: optimization process leading to displacement studies

João P. do Carmo[1], Carla Fernandes[1,2*], Maria Elizabeth Tiritan[1,2,3], Carlos Afonso[1,2], Madalena M. M. Pinto[1,2]

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Human serum albumin chiral stationary phase (HSA-CSP) demonstrated to exhibit good results for a variety of classes of analytes due to multiple binding sites [1].

Chiral recognition and enantioseparation process are fundamental to understand pharmacokinetic, pharmacodynamic and toxicological profiles leading to a great impact in chemical fields who are dealing with bioactive compounds that are dependent on their stereochemistry, particularly chiral derivatives of xanthones (CDXs)[1,2]. Displacement studies are important to understand how the presence of competitive agents may or may not compromise the interactions with this multiple binding sites, therefore, accurate methodology to enantioseparate and evaluate the chromatographic conditions play a very important role to advance to this.

Very high enantioselectivity and resolution were obtained and the enantioseparation was explored using different mobile phases, under reversed-phase elution mode. Chromatographic conditions such as mobile phase pH, buffer type and ionic strength, type and content of organic modifiers and temperature were optimized for separation of enantiomers.

This research was partially supported by the Strategic Funding UID/Multi/04423/2013 through national funds provided by FCT – Foundation for Science and Technology and European Regional Development Fund (ERDF), in the framework of the programme PT2020, the project PTDC/MAR-BIO/4694/2014 (reference POCI-01-0145-FEDER-016790; Project 3599 – Promover a Produção Científica e Desenvolvimento Tecnológico e a Constituição de Redes Temáticas (3599-PPCDT)) as well as by the project INNOVMAR - Innovation and Sustainability in the Management and Exploitation of Marine Resources (reference NORTE-01-0145-FEDER-000035, within Research Line NOVELMAR), supported by North Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF), and Chiral_Drugs_CESPU_2017.

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3ª Edição da ESCOLA DE INVERNO DE FARMÁCIA
7 a 15 de Março de 2018
Instituto ideHia
Porto, Portugal

Enantioresolution of bioactive compounds on human serum albumin stationary phase

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INTRODUCTION

Human serum albumin (HSA) plays a vital role in the transport of drugs, metabolites, and endogenous ligands. Binding to HSA controls the free, active concentration of a drug, provides a reservoir for a long duration of action, and affects drug absorption, metabolism, distribution and excretion, determining the overall pharmacological activity of a drug[1].

The interactions of drugs with HSA are often stereoselective, and information on these interactions can be valuable in the design and optimization of chiral separations, as well as in predicting how drugs may behave in the human body, in understanding drug-drug interactions, and in determining the optimum dosages that should be used with such agents for treating patients[2].

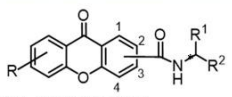
There are many techniques that have been utilized to study drug interactions with proteins[3], including liquid chromatography using HSA-CSP[4].

METHODS AND MATERIALS

Human serum albumin (HSA)

- HSA is one of the most **important plasma proteins**, which act as **drug carrier**.
- **Chiral recognition** of drugs on HSA has become essential to understand the **pharmacokinetic, pharmacodynamics and toxicological** profile of drugs.
- Contain different **binding sites**.
- Establish various types of **interactions.**

Chiral derivatives of xanthones (CDXs)



R, R^1, R^2: vary substituents

Interesting biological activities (EX. antitumor and anti-inflammatory.)

in some cases

dependent on the stereochemistry

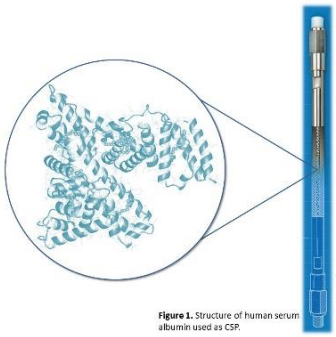


Figure 1. Structure of human serum albumin used as CSP.

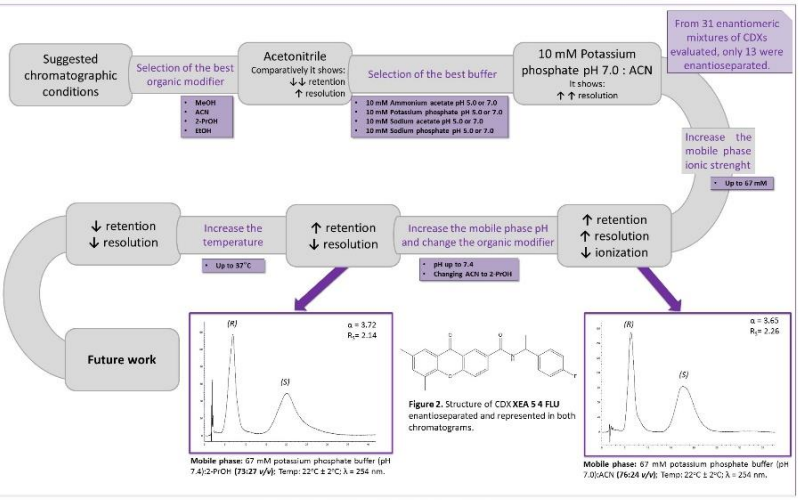
Chromatographic conditions

Column: CHIRALPAK® HSA (150 mm × 4.0 mm, I.D., 5 µm particle size)
Analytes: 31 enantiomeric mixtures of CDXs
Sample concentration: 50 µg/mL
Sample injections: 20 µL
Flow-rate: 0.9 mL/min

Buffer types: Ammonium acetate, sodium acetate, sodium phosphate, potassium phosphate (10 mM)
pH range: 5.0 – 7.0
Organic modifier type: MeOH, ACN, 2-PrOH, EtOH
Maximum % of organic modifier recommended: 20%
Maximum pressure recommended : 140 bar
Temperature: 20 – 25°C (max. 40°C)

RESULTS AND DISCUSSION

- Systematic study of enantioresolution of a library of **CDXs synthesized "in house"** by liquid chromatography (LC) using a HSA-CSP.
- Optimization of the chromatographic conditions by using different mobile phases, under **reversed-phase elution mode**, exploring:
  - **Buffer type and ionic strength.**
  - **Type and content of organic modifiers.**
  - **Mobile phase pH.**
  - **Temperature.**



CONCLUSIONS

- **13 among 31 enantiomeric mixtures of CDXs were separated** by LC on a HSA-CSP reversed-phase conditions.
- **High enantioselectivity and resolution were obtained** with α and R_S values ranging from 1.52 to 10.12 and from 1.20 to 4.12, respectively.
- The best mobile phases were mixtures of **67 mM potassium phosphate buffer (pH 7.0-7.4) with ACN or 2-PrOH**, as organic modifiers, with different proportions depending of the analyte.

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Appendix C.

Abstract of Italian-Spanish-Portuguese Joint Meeting in Medicinal Chemistry, MedChemSicily2018, July 17-20, 2018.

Chiral carboxyxanthone derivatives: synthesis and enantioselectivity studies

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Enantioselectivity may be considered an essential issue to take into consideration when studying chiral compounds, being the enantiomers able to interact differently with proteins, and other chiral biotargets. These events can be translated into implications in pharmacokinetics, pharmacodynamics as well as in toxicity[1].
The binding of drugs to plasma proteins, such as human serum albumin (HSA) is an important parameter since it has implications on drug action by affecting free concentration in serum. According to current point of views in the drug discovery pipeline, the binding of new compounds to HSA at an early stage is of crucial importance, since affects not only distribution and elimination but also duration and intensity of the pharmacological action of drugs, especially chiral compounds[2].
Marine carboxylated xanthones, obtained from the inner tissue of marine red algae, are very interesting bioactive compounds[3]. Carboxyxanthones are also very important as basis to molecular modifications in order to obtain new derivatives, such as chiral derivatives of xanthones (CDXs)[4,5]. In our group, a library of CDXs were synthetized, using analogues of marine carboxyxanthone derivatives as chemical substrates, and some of them revealed growth inhibitory activity[4], in three human tumor cell lines, as well as cyclooxygenase inhibition[5]. Moreover, enantioselectivity was observed for both biotargets. Additionally, HSA binding affinity was evaluated by spectrofluorimetry, as well as in *in silico*[5]; also by liquid chromatography using a HSA stationary phase. The results obtained by the different used methods are compared and discussed.

This research was partially supported by the Strategic Funding UID/Multi/04423/2013 through national funds provided by FCT and ERDF, in the framework of PT2020, the project PTDC/MAR-BIO/4694/2014 (reference POCI-01-0145-FEDER-016790; Project 3599-PPCDT) as well as by the Structural Program of R&D&I INNOVMAR (reference NORTE-01-0145-FEDER-000035, Research Line NOVELMAR) funded by the NORTE2020 through the ERDF, and Chiral_Drugs_CESPU_2017.

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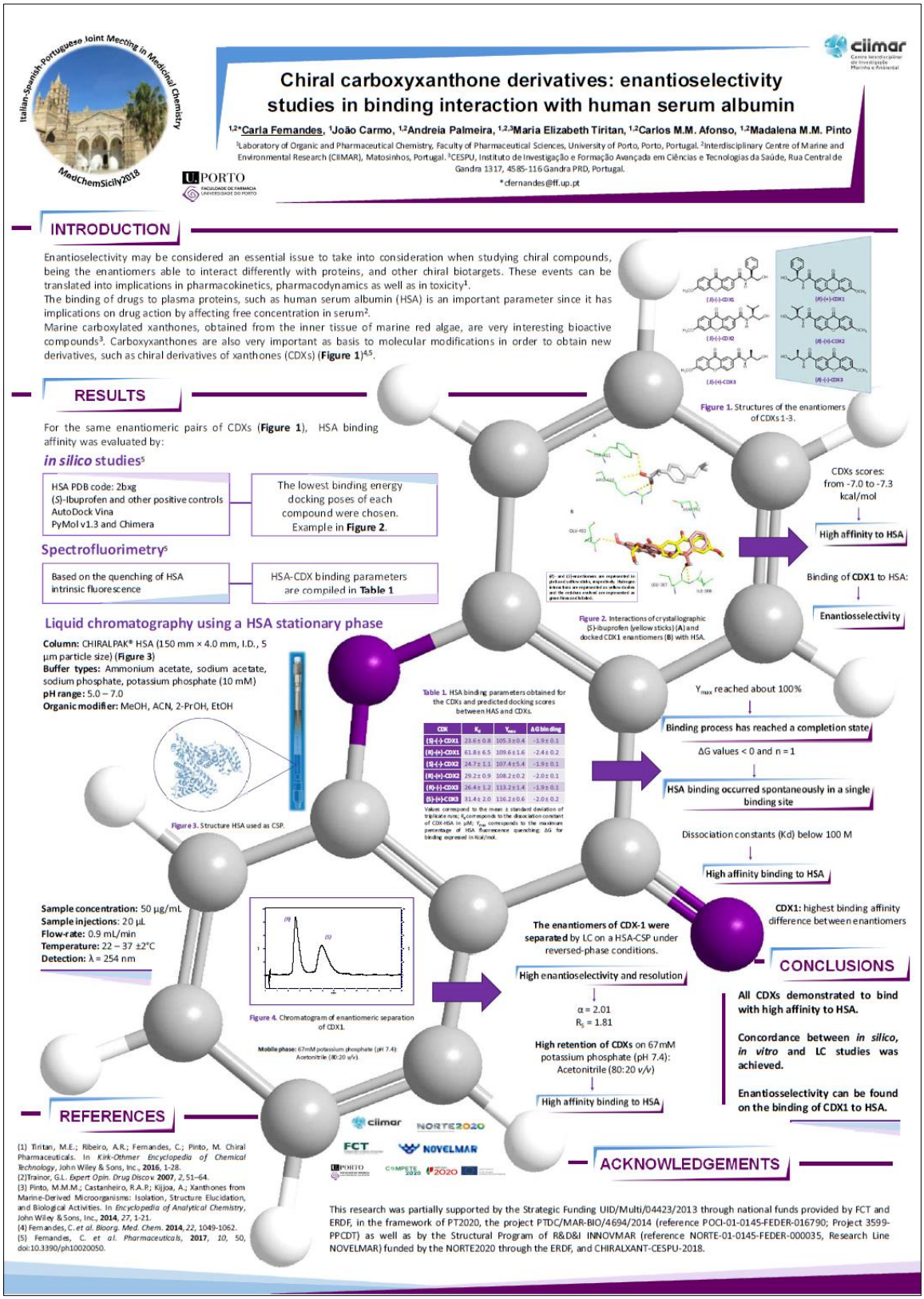
Italian-Spanish-Portuguese Joint Meeting in Medicinal Chemistry
MedChemSicily2018
Chiral carboxyxanthone derivatives: enantioselectivity studies in binding interaction with human serum albumin
[1,2*]Carla Fernandes, [1]João Carmo, [1,2]Andreia Palmeira, [1,2,3]Maria Elizabeth Tiritan, [1,2]Carlos M.M. Afonso, [1,2]Madalena M.M. Pinto
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INTRODUCTION
Enantioselectivity may be considered an essential issue to take into consideration when studying chiral compounds, being the enantiomers able to interact differently with proteins, and other chiral biotargets. These events can be translated into implications in pharmacokinetics, pharmacodynamics as well as in toxicity[1].
The binding of drugs to plasma proteins, such as human serum albumin (HSA) is an important parameter since it has implications on drug action by affecting free concentration in serum[2].
Marine carboxylated xanthones, obtained from the inner tissue of marine red algae, are very interesting bioactive compounds[3]. Carboxyxanthones are also very important as basis to molecular modifications in order to obtain new derivatives, such as chiral derivatives of xanthones (CDXs) (Figure 1)[4,5].
Figure 1. Structures of the enantiomers of CDXs 1-3.
RESULTS
For the same enantiomeric pairs of CDXs (Figure 1), HSA binding affinity was evaluated by:
in silico studies[5]
HSA PDB code: 2bxg
(S)-Ibuprofen and other positive controls
AutoDock Vina
PyMol v1.3 and Chimera
The lowest binding energy docking poses of each compound were chosen. Example in Figure 2.
Figure 2. Interactions of crystallographic (S)-ibuprofen (yellow sticks) (A) and docked CDX1 enantiomers (B) with HSA.
CDXs scores: from -7.0 to -7.3 kcal/mol
High affinity to HSA
Binding of CDX1 to HSA:
Enantioselectivity
Spectrofluorimetry[5]
Based on the quenching of HSA intrinsic fluorescence
HSA-CDX binding parameters are compiled in Table 1
Table 1. HSA binding parameters obtained for the CDXs and predicted docking scores between HAS and CDXs.
CDX | K_d | Y_{max} | ΔG binding
(S)-(-)-CDX1 | 23.6 ± 0.8 | 105.3 ± 0.4 | -1.9 ± 0.1
(R)-(+)-CDX1 | 61.8 ± 6.5 | 109.6 ± 1.6 | -2.4 ± 0.2
(S)-(-)-CDX2 | 24.7 ± 1.1 | 107.4 ± 5.4 | -1.9 ± 0.1
(R)-(+)-CDX2 | 29.2 ± 0.9 | 108.2 ± 0.2 | -2.0 ± 0.1
(R)-(-)-CDX3 | 26.4 ± 1.2 | 113.2 ± 1.4 | -1.9 ± 0.1
(S)-(+)-CDX3 | 31.4 ± 2.0 | 116.2 ± 0.6 | -2.0 ± 0.2
Values correspond to the mean ± standard deviation of triplicate runs; K_d corresponds to the dissociation constant of CDX-HSA in μM; Y_{max} corresponds to the maximum percentage of HSA fluorescence quenching; ΔG for binding expressed in kcal/mol.
Y_{max} reached about 100%
Binding process has reached a completion state
ΔG values < 0 and n = 1
HSA binding occurred spontaneously in a single binding site
Dissociation constants (Kd) below 100 M
High affinity binding to HSA
Liquid chromatography using a HSA stationary phase
Column: CHIRALPAK® HSA (150 mm × 4.0 mm, I.D., 5 μm particle size) (Figure 3)
Buffer types: Ammonium acetate, sodium acetate, sodium phosphate, potassium phosphate (10 mM)
pH range: 5.0 – 7.0
Organic modifier: MeOH, ACN, 2-PrOH, EtOH
Figure 3. Structure HSA used as CSP.
Sample concentration: 50 μg/mL
Sample injections: 20 μL
Flow-rate: 0.9 mL/min
Temperature: 22 – 37 ±2°C
Detection: λ = 254 nm
Figure 4. Chromatogram of enantiomeric separation of CDX1.
Mobile phase: 67mM potassium phosphate (pH 7.4): Acetonitrile (80:20 v/v).
The enantiomers of CDX-1 were separated by LC on a HSA-CSP under reversed-phase conditions.
High enantioselectivity and resolution
α = 2.01
R_s = 1.81
High retention of CDXs on 67mM potassium phosphate (pH 7.4): Acetonitrile (80:20 v/v)
High affinity binding to HSA
CDX1: highest binding affinity difference between enantiomers
CONCLUSIONS
All CDXs demonstrated to bind with high affinity to HSA.
Concordance between in silico, in vitro and LC studies was achieved.
Enantiosselectivity can be found on the binding of CDX1 to HSA.
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Appendix D.

Abstract and Poster of 29th International Symposium on Pharmaceutical and Biomedical Analysis, DA-PBA 2018, September 9-12, 2018.

Enantioresolution and docking studies of xanthone derivatives on a human serum albumin stationary phase

Ye Zaw Phyo[1,2], João Carmo[3], Andreia Palmeira[2,3], Maria Elizabeth Tiritan[2,3,4], Carla Fernandes[2,3*], Carlos Afonso[2,3], Anake Kijjoa[1,2], Madalena M.M. Pinto[2,3]

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During the last few decades, there has been a widespread interest in oxygenated heterocyclic compounds, such as molecules with a xanthone scaffold, mainly taking in consideration their variety of biological activities [1]. Among them, chiral derivatives of xanthones (CDXs) have interesting biological activities being, in some cases, dependent on the stereochemistry [2,3]. Consequently, the development of improved and accurate methodologies to enantioresolve and evaluate enantiomeric purity of these chiral compounds are necessary.

In our group, liquid chromatography (LC) methods using macrocyclic glycopeptide antibiotic-based [4], Pirkle-type [5], and polysaccharide-based [6] chiral stationary phases (CSPs), have demonstrated to be efficient for enantioresolution of CDXs.

Herein, we report the LC resolution of enantiomeric mixtures of a series of CDXs by using a human serum albumin (HSA) CSP, under reversed-phase elution mode. In general, all CDXs showed high affinity for HSA-CSP; for some enantiomeric mixtures high enantioselectivity and resolution were obtained.

Considering the importance of understanding the chiral recognition mechanisms associated with the chromatographic enantioresolution, computational studies by molecular docking were also carried out. Data regarding the CSP-CDX molecular conformations and interactions were retrieved. The docking calculations were in accordance with the experimental chromatographic parameters regarding enantioselectivity and enantiomer elution order, with a success rate of 80%.

This work was supported through national funds from Foundation for Science and Technology (FCT) and European Regional Development Fund (ERDF) and COMPETE under the projects PEst-C/MAR/LA0015/2013, PTDC/MAR-BIO/4694/2014 (POCI-01-0145-FEDER-016790), QOPNA (FCT UID/QUI/00062/2013), and INNOVMAR (Innovation and Sustainability in the Management and Exploitation of Marine Resources) - NORTE-01-0145-FEDER-000035, Research Line NOVELMAR, and CHIRALXANT-CESPU-2018. Y.Z. Phyo thanks the Erasmus Mundus Action 2 (Lotus Plus project) for a PhD's scholarship.

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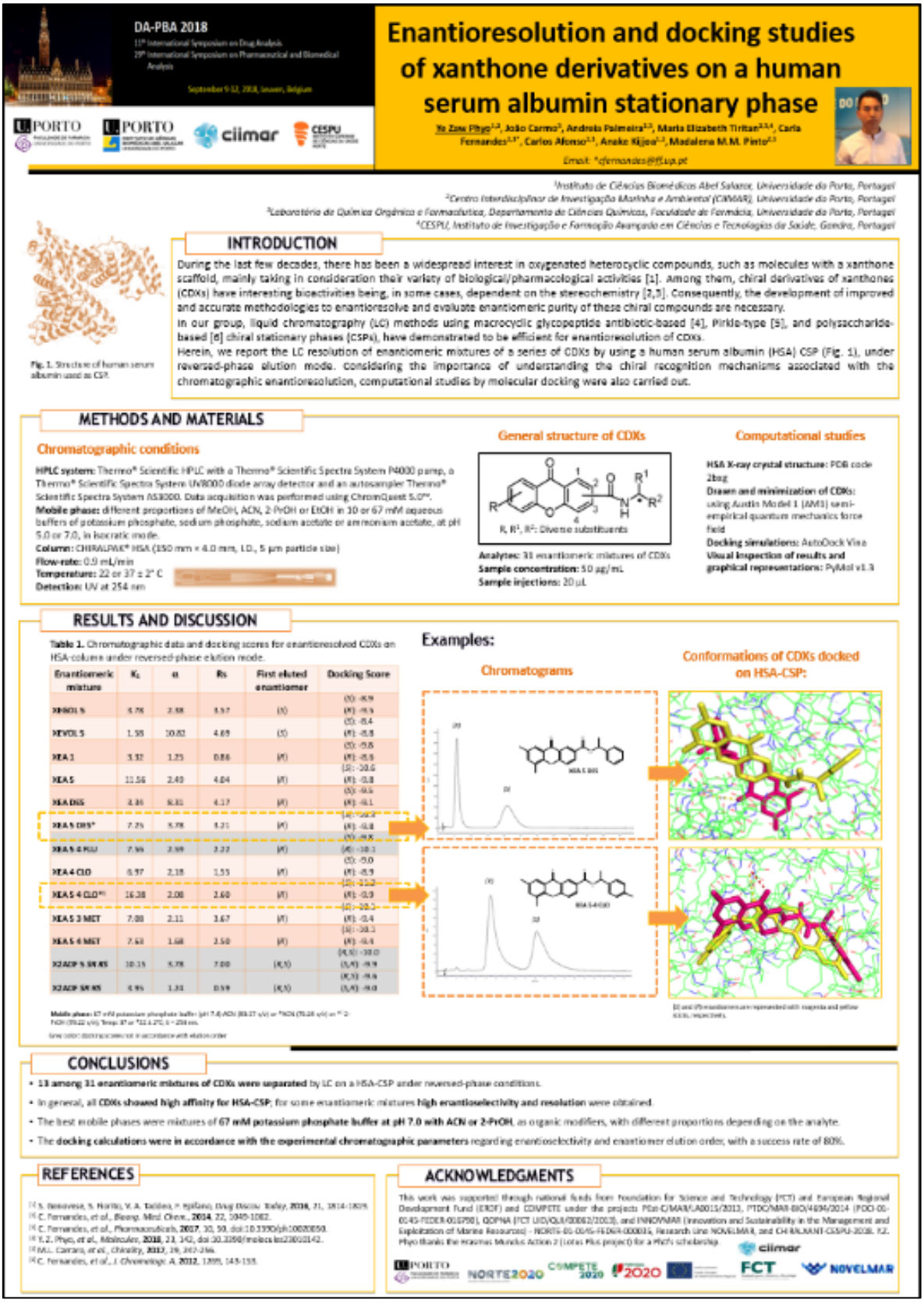
DA-PBA 2018
Enantioresolution and docking studies of xanthone derivatives on a human serum albumin stationary phase
INTRODUCTION
METHODS AND MATERIALS
Chromatographic conditions
General structure of CDXs
Computational studies
RESULTS AND DISCUSSION
Examples:
Chromatograms
Conformations of CDXs docked on HSA-CSP:
CONCLUSIONS
REFERENCES
ACKNOWLEDGMENTS

Appendix E.

Abstract of 11th Meeting of Young Researchers of University of Porto (IJUP18), February 07-09, 2018.

Chiral recognition of xanthone derivatives on human serum albumin stationary phase: effect of chromatographic conditions

João P. do Carmo[1], Carla Fernandes[1,2*], Maria Elizabeth Tiritan[1,2,3], Carlos Afonso[1,2], Madalena M. M. Pinto[1,2]

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Human serum albumin chiral stationary phase (HSA-CSP) demonstrated to exhibit broad chiral recognition and enantioselectivity for a variety of classes of analytes due to multiple binding sites [1].

Herein, enantiomers of thirteen among thirty-one xanthone derivatives synthesized "in house" were separated by liquid chromatography on HSA-CSP. The enantioseparation was explored using different mobile phases, under reversed-phase elution mode. Chromatographic conditions such as mobile phase pH, buffer type and ionic strength, type and content of organic modifiers and temperature were optimized for separation of enantiomers. Very high enantioselectivity and resolution were obtained with α and R_S ranging from 1.55 to 8.16 and from 1.81 to 6.41, respectively.

This study will contribute to expand the applications of HSA-CSP on the separation of new class of chiral compounds, as well as for future studies concerning a better understanding of chiral recognition involved in enantioselectivity.

This research was partially supported by the Strategic Funding UID/Multi/04423/2013 through national funds provided by FCT – Foundation for Science and Technology and European Regional Development Fund (ERDF), in the framework of the programme PT2020, the project PTDC/MAR-BIO/4694/2014 (reference POCI-01-0145-FEDER-016790; Project 3599 – Promover a Produção Científica e Desenvolvimento Tecnológico e a Constituição de Redes Temáticas (3599-PPCDT)) as well as by the project INNOVMAR - Innovation and Sustainability in the Management and Exploitation of Marine Resources (reference NORTE-01-0145-FEDER-000035, within Research Line NOVELMAR), supported by North Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF), and Chiral_Drugs_CESPU_2017.

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Appendix F.

Abstract of XXIV Encontro Luso-Galego de Química (XXIV ELGQ), November 21-23, 2018.

Enantioresolution, chiral recognition mechanisms and binding of xanthone derivatives on immobilized human serum albumin by liquid chromatography

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High-performance affinity chromatography (HPAC) is a type of liquid chromatography in which solutes are separated based on their affinity to the mobile phase and a stationary phase that is a biologically-related agent [1].
The interactions of drugs with HSA are often stereoselective, and information on these interactions can be valuable in the design and optimization of chiral separations. Another important aspect of these interactions is predicting how drugs may behave in the human body, understanding drug-drug interactions, and determining the optimum dosages that should be used with such agents for treating patients [2].
Chiral stationary phases based on human serum albumin (HSA-CSP) exhibit a broad chiral recognition and enantioselectivity for a variety of classes of analytes due to multiple binding sites [3].
Herein, enantiomers of thirteen among thirty-one xanthone derivatives synthesized "in house" were separated by liquid chromatography on HSA-CSP. The enantioseparation was explored using different mobile phases, under reversed-phase elution mode. Chromatographic conditions such as mobile phase pH, buffer type and ionic strength, type and content of organic modifiers and temperature were optimized for separation of enantiomers. Very high enantioselectivity and resolution were obtained with α and R_S ranging from 1.55 to 8.16 and from 1.81 to 6.41, respectively.
The binding to HSA of sixty-two enantiomers of CDXs has been also determined by bioaffinity chromatography. In general, high affinity for HSA-CSP was observed, with bound percentage ranging from 79.02% to 99.99%.
Considering the importance of understanding the chiral recognition mechanisms associated with the chromatographic enantioresolution, computational studies by molecular docking were also carried out. The docking calculations were in accordance with the experimental chromatographic parameters regarding enantioselectivity and enantiomer elution order, with a success rate of 77%.

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Appendix G.

Technical data sheet of CHIRALPAK® HSA provided by the column manufacturer (Chiral Technologies, Daicel Group).

DAICEL

CHIRAL TECHNOLOGIES
DAICEL GROUP

INSTRUCTION MANUAL FOR CHIRALPAK® HSA

Please read this instruction sheet completely before using this column

Column Description

CHIRALPAK® HSA :Human serum albumin immobilized on 5µm silica-gel.

Shipping solvent : **Water / 2-Propanol (2-PrOH) solvent mixture (90/10 v/v)**

All columns have been pre-tested before packaging. The test parameters and results, as well as the Column Lot Number, are included on a separate (enclosed) page.

Application Scope

CHIRALPAK® HSA can offer high enantioselectivity for compounds bearing carboxylic groups, including:

- strong and weak acids
- zwitter-ionic molecules
- non-ionisable compounds (amides, esters, alcohols, sulfoxides, etc)

For compounds of basic category, however, it is preferred to use CHIRALPAK® AGP and CHIRALPAK® CBH columns.

Operating Conditions

	50 x 2 mm i.d.*1 100 x 2 mm i.d.*1 150 x 2 mm i.d.*1 Analytical column	**50 x 3 mm i.d.*1 100 x 3 mm i.d. 150 x 3 mm i.d.** Analytical column	**50 x 4 mm i.d.*1 100 x 4 mm i.d. 150 x 4 mm i.d.** Analytical column	**100 x 10 mm i.d. 150 x 10 mm i.d.** Semi-prep. column
Flow direction	As indicated on the column label			
Typical Flow rate	0.2 mL/min	0.5 mL/min	0.9 mL/min	4.0 mL/min
pH range	5.0 - 7.0			
Recommended temperature*2	20 - 30°C			
Buffer concentration	up to 100mM, typically 10-20mM			
Organic modifier ratio	0-15% by volume			
Charged additive concentration	up to 10mM			

*1 It is very important that the HPLC system is optimized in terms of void volume for work with columns of small dimensions.

*2 The column lifetime might be reduced if used at higher temperature.

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Operating Procedure

A - Mobile Phase Starting Conditions

	ACIDIC Compounds	NEUTRAL Compounds
Typical starting conditions	**10mM Ammonium acetate buffer (pH 7.0)❶ / 2-PrOH = 85 / 15 (v/v)**	

❶ Refer to section B for preparation of the buffer.

B – Buffer Preparation - Example

➢ *Preparation of 10mM Ammonium acetate buffer (1Liter):*

1. Weigh 770.8 mg of ammonium acetate (CH_3COONH_4, purity > 99%) into a beaker.
2. Dissolve the salt with about 800mL water (HPLC grade), equilibrated at room temperature (20-25℃).
3. Adjust pH to the target value by using either diluted acetic acid or a diluted ammonium hydroxide solution.
4. Filter the solution through a membrane of 0.22µm into a measuring flask.
5. Add water until the limit line of the measuring flask. Place the stopper in the neck and homogenize the solution by agitation.

When buffer should be mixed with an organic modifier, the measurements are normally by volumes, using preferably volumetric flasks or measuring pipettes.
After mixing, degas the mobile phase in an ultrasonic bath.

Note that in the case where a charged additive is needed in the mobile phase, the charged additive should be added into the aqueous solution before the pH adjustment.

C – Mobile Phases

Bacteria grow fast in eluents containing no or low alcoholic organic modifier. Such mobiles phases must be freshly prepared.

❖ ***Buffer***
The salt concentration of ammonium acetate buffer is typically 10-20mM but can be varied up to 100mM. The other kinds of buffers, such as sodium or potassium phosphate buffers, sodium acetate buffers, formate or citrate buffers, can also be used. However, the LC-MS compatibility of the method may be sometimes compromised.

❖ ***Organic Modifiers***
2-PrOH is the most frequently used. However, methanol, ethanol and acetonitrile can also be investigated. The relative eluting strength can be ranked as follows: 2-PrOH > EtOH ≥ ACN > MeOH

❖ ***Charged additives***
Cationic and anionic additives, such as *N,N*-dimethyloctyl amine (DMOA), trifluoroacetic acid (TFA), octanoic acid (OA), heptafluorobutyric acid (HFBA), can also be used to regulate retention and enantioselectivity. However, some of these additives may be difficult to remove totally, due to very high affinity to the matrix. Thus, the properties of the column may be affected.

CAUTION: The miscibility of OA and DMOA to water is very limited. Only 2mM OA or 5mM DMOA can be homogeneously incorporated into the aqueous solution at ambient temperature. A phase separation may occur beyond these concentrations.

Once a charged additive is used in the mobile phase, the column should be dedicated for the purpose.

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D – Samples

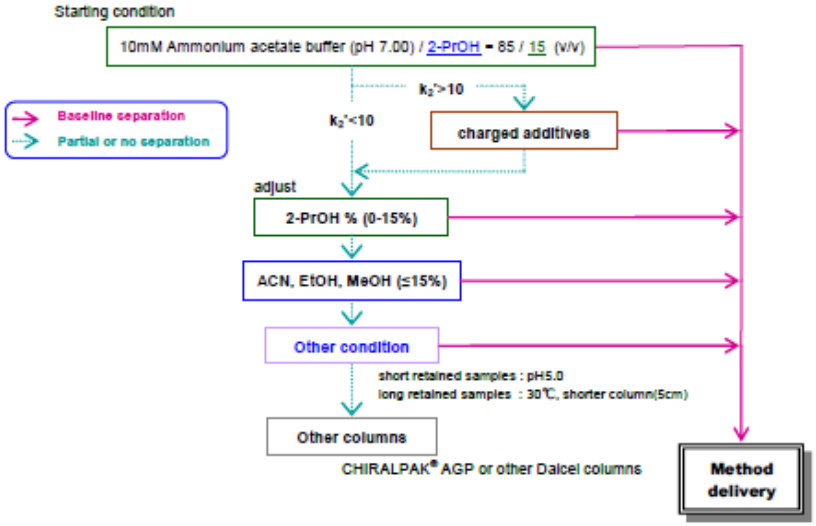
The sample amount injected onto the column should be kept low enough. The recommended sample concentration is 0.20 mg/mL or lower with an injection volume of 5-10 µL, preferably.

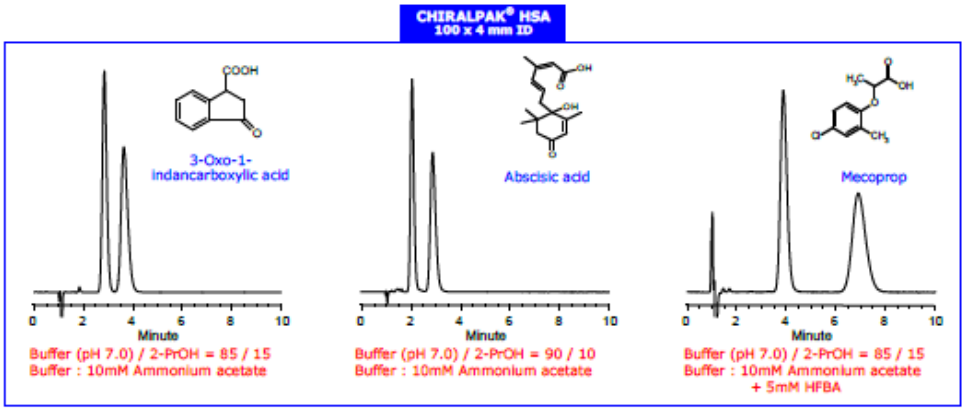
Dissolve the sample in the mobile phase when it is possible. If the sample is insoluble in the mobile phase, add a higher concentration of the organic modifier. The sample solution should be filtered through a membrane filter of approximately 0.5µm porosity to ensure that there is no precipitate before using.

CAUTION: Dissolution of the sample in pure or high percentage of organic solvents may cause on-line sample precipitation.
Do not inject unclear sample solutions or solutions containing undissolved compounds.

Method Development

The following scheme offers a guide for method development and method optimization





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Column Care / Maintenance

- The use of a guard cartridge is highly recommended for maximum column life.
- If the column has been contaminated with very hydrophobic material, wash the column backwards (no detector connected) over night with Water/2-PrOH 75/25(v/v) at a reduced flow-rate (e.g. 0.3 mL/min for 4mm ID columns).
- Before disconnecting the column from the HPLC system, flush the column with a mobile phase that does not contain any salts / buffers, e.g. Water/2-PrOH 90/10(v/v).
- For the storage of the column, it is recommended to fill it with Water/2-PrOH 90/10(v/v).
  For short storage period, the column can be placed at ambient temperature (<30°C).
  For longer storage periods, however, it is recommended to place it in a refrigerator.

Important Notice

We recommend the use of a CHIRALPAK® HSA guard column in order to protect the analytical column from any particulates and impurities with high affinity to the stationary phase. Change the guard column regularly, especially in bioanalysis.

Operating these columns in accordance with the guidelines outlined here will result in a long column life.

⇨ If you have any questions about the use of these columns, or encounter a problem, contact:

In the USA: questions@chiraltech.com or call 800-6-CHIRAL
In the EU: cte@chiral.fr or call +33 (0)3 88 79 52 00
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Appendix H.

Table 24 – Retention time (t_R), retention factor (*k*) and binding percentage (*%b*) (calculated based on *k/(k+1)* equation **(1)**) values of CDXs injected as single enantiomers for different ACN proportions, on a CHIRALPAK® HSA column, and extrapolation to 100% aqueous buffer.

Compounds	25% [a]			22% [a]			20% [a]			17% [a]			15% [a]			12% [a]			10% [a]			7% [a]			2% [a]			[0%] [b]		R^2
	t_R	*k*	*b%*	t_R	*k*	*b%*	t_R	*k*	*b%*	t_R	*k*	*b%*	t_R	*k*	*b%*	t_R	*K*	*b%*	t_R	*k*	*b%*	t_R	*k*	*b%*	t_R	*k*	*b%*	*k*	*b%*	
Chlorpromazine	-	-	-	-	-	-	10.24	7.19	87.79	11.64	8.31	89.26	17.25	12.80	92.75	21.97	16.57	94.31	23.79	18.03	94.75	-	-	-	-	-	-	52.31	98.12	0.948
Indomethacin	-	-	-	-	-	-	9.13	6.30	86.30	13.55	9.84	90.78	15.66	11.53	92.02	20.44	11.39	91.93	-	-	-	-	-	-	-	-	-	31.20	96.89	0.961
Metronidazole	-	-	-	-	-	-	-	-	-	-	-	-	1.32	0.05	5.16	1.35	0.08	7.27	1.36	0.09	8.22	-	-	-	1.50	0.20	16.78	0.25	19.78	0.997
XEGOL 5 (*S*)	-	-	-	-	-	-	3.74	1.99	66.54	8.46	5.76	85.22	13.83	10.06	90.96	29.57	22.66	95.77	35.55	27.44	96.48	-	-	-	-	-	-	483.95	99.79	0.975
XEGOL 5 (*R*)	3.55	1.84	64.74	5.52	3.41	77.34	10.42	7.34	88.01	16.48	12.19	92.42	31.20	23.96	95.99	-	-	-	-	-	-	-	-	-	-	-	-	1035.14	99.90	0.988
XEGOL 2 (*S*)	-	-	-	-	-	-	2.41	0.92	48.02	4.96	2.96	74.77	7.40	4.92	83.11	13.84	10.07	90.96	16.43	12.15	92.39	-	-	-	-	-	-	198.06	99.50	0.962
XEGOL 2 (*R*)	-	-	-	-	-	-	2.41	0.93	48.07	4.92	2.94	74.60	7.39	4.91	83.08	13.69	9.95	90.87	16.16	11.93	92.26	-	-	-	-	-	-	191.51	99.48	0.961
XEGOL 1 (*S*)	-	-	-	-	-	-	1.97	0.57	36.48	3.87	2.10	67.70	5.40	3.32	76.86	9.25	6.40	86.48	10.74	7.59	88.36	-	-	-	-	-	-	122.80	99.19	0.976
XEGOL 1 (*R*)	-	-	-	-	-	-	2.03	0.62	38.42	4.02	2.22	68.90	5.76	3.61	78.29	10.47	7.37	88.06	12.34	8.87	89.87	-	-	-	-	-	-	156.03	99.36	0.953
XEVOL (*S*)	-	-	-	-	-	-	1.64	0.32	23.97	3.01	1.41	58.51	3.88	2.10	67.74	5.76	3.60	78.28	6.34	4.07	80.28	-	-	-	-	-	-	64.00	98.46	0.963
XEVOL (*R*)	-	-	-	-	-	-	-	-	-	2.95	1.36	57.56	3.77	2.01	66.82	5.48	3.38	77.17	5.96	3.77	79.02	8.83	6.07	85.85	-	-	-	17.13	94.48	0.982
XEVOL 1 (*S*)	-	-	-	-	-	-	-	-	-	2.85	1.28	56.12	3.64	1.91	65.63	5.40	3.32	76.84	6.12	3.89	79.57	7.27	4.82	82.81	-	-	-	14.00	93.33	0.948
XEVOL 1 (*R*)	-	-	-	-	-	-	1.59	0.27	21.33	3.11	1.49	59.86	4.05	2.24	69.14	6.50	4.20	80.78	7.39	4.91	83.07	-	-	-	-	-	-	108.47	99.09	0.987
XEVOL 5 (*S*)	-	-	-	-	-	-	2.26	0.80	44.59	4.60	2.68	72.80	6.60	4.28	81.06	11.67	8.33	89.29	13.57	9.85	90.79	-	-	-	-	-	-	148.42	99.33	0.951
XEVOL 5 (*R*)	4.98	2.98	74.88	9.06	6.25	86.21	17.25	12.80	92.76	28.86	22.08	95.67	54.24	42.39	97.70	-	-	-	-	-	-	-	-	-	-	-	-	2086.89	99.95	0.992
XEL (*S*)	-	-	-	-	-	-	-	-	-	3.81	2.05	67.19	5.41	3.33	76.89	9.35	6.48	86.63	10.86	7.69	88.49	17.89	13.31	93.01	-	-	-	50.41	98.05	0.982
XEL (*R*)	-	-	-	-	-	-	-	-	-	3.73	1.98	66.49	5.29	3.23	76.36	8.91	6.13	85.97	10.14	7.11	87.67	15.48	11.38	91.92	-	-	-	40.51	97.59	0.976
XEL 1 (*S*)	-	-	-	-	-	-	-	-	-	3.35	1.68	62.69	4.61	2.69	72.88	7.89	5.31	84.16	9.07	6.25	86.21	14.70	10.76	91.50	-	-	-	40.63	97.60	0.983
XEL 1 (*R*)	-	-	-	-	-	-	-	-	-	3.67	1.94	65.97	5.12	3.10	75.59	9.18	6.34	86.38	10.72	7.57	88.34	16.77	12.42	92.55	-	-	-	48.73	97.99	0.980
X2A1P (*S*)	-	-	-	-	-	-	-	-	-	2.53	1.02	50.53	3.05	1.44	58.99	4.08	2.26	69.35	4.31	2.45	71.00	4.74	2.79	73.63	-	-	-	6.42	86.52	0.973
X2A1P (*R*)	-	-	-	-	-	-	-	-	-	2.54	1.03	50.79	3.13	1.51	60.10	4.17	2.33	69.99	4.45	2.56	71.94	4.89	2.91	74.45	-	-	-	6.84	87.25	0.941
X2A1P1 (*S*)	-	-	-	-	-	-	-	-	-	2.38	0.90	47.41	2.79	1.23	55.20	3.58	1.87	65.11	3.82	2.06	67.28	3.90	2.12	67.91	-	-	-	4.54	81.94	0.951
X2A1P1 (*R*)	-	-	-	-	-	-	-	-	-	2.56	1.05	51.19	2.86	1.29	56.31	3.37	1.70	62.90	3.85	2.08	67.55	3.80	2.04	67.10	-	-	-	3.77	79.02	0.956
X1A2P (*S*)	-	-	-	-	-	-	-	-	-	2.62	1.09	52.20	3.11	1.48	59.76	4.23	2.38	70.41	4.67	2.73	73.22	4.94	2.95	74.70	-	-	-	7.01	87.52	0.942
X1A2P (*R*)	-	-	-	-	-	-	-	-	-	2.63	1.11	52.54	3.09	1.47	59.48	4.16	2.33	69.98	4.54	2.63	72.47	4.78	2.82	73.83	-	-	-	6.41	86.51	0.957
X1A2P1 (*S*)	-	-	-	-	-	-	-	-	-	2.39	0.91	47.63	2.73	1.18	54.20	3.53	1.82	64.60	3.80	2.04	67.11	3.76	2.00	66.72	-	-	-	4.24	80.91	0.951
X1A2P1 (*R*)	-	-	-	-	-	-	-	-	-	2.41	0.93	48.07	2.74	1.20	54.45	3.55	1.84	64.78	3.81	2.05	67.22	3.77	2.01	66.82	-	-	-	4.21	80.82	0.941
XEA (*S*)	-	-	-	4.88	2.90	74.37	7.84	5.27	84.05	12.57	9.06	90.06	20.97	15.77	94.04	50.14	39.11	97.51	-	-	-	-	-	-	-	-	-	735.70	99.86	0.991
XEA (*R*)	-	-	-	5.02	3.02	75.11	8.02	5.42	84.42	13.08	9.46	90.44	22.28	16.82	94.39	56.10	43.88	97.77	-	-	-	-	-	-	-	-	-	889.41	99.89	0.990

This table continues on the next page.

Table 24 – Continuation.

Compounds	25% [a]			22% [a]			20% [a]			17% [a]			15% [a]			12% [a]			10% [a]			7% [a]			2% [a]			[0%] [b]		R^2
	t_R	*k*	*b%*	t_R	*k*	*b%*	t_R	*k*	*b%*	t_R	*k*	*b%*	t_R	*k*	*b%*	t_R	*K*	*b%*	t_R	*k*	*b%*	t_R	*k*	*b%*	t_R	*k*	*b%*	*k*	*b%*	
XEA 1 (*S*)	-	-	-	3.73	1.99	66.52	5.71	3.57	78.10	8.78	6.02	85.76	14.32	10.45	91.27	34.54	26.63	96.38	-	-	-	-	-	-	-	-	-	485.74	99.79	0.989
XEA 1 (*R*)	-	-	-	3.50	1.80	64.31	5.04	3.03	75.19	7.41	4.93	83.14	11.57	8.25	89.19	25.79	19.63	95.15	-	-	-	-	-	-	-	-	-	284.71	99.65	0.989
XEA 5 (*S*)	5.87	3.69	78.69	12.17	8.74	89.73	29.36	22.49	95.74	65.25	51.20	98.08	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	16481.62	99.99	0.990
XEA 5 (*R*)	4.35	2.48	71.26	7.60	5.08	83.55	13.07	9.45	90.43	23.01	17.41	94.57	39.69	30.75	96.85	-	-	-	-	-	-	-	-	-	-	-	-	1289.44	99.92	0.997
XEA 3 MET (*S*)	-	-	-	-	-	-	3.23	1.58	61.30	8.30	5.64	84.94	13.08	9.46	90.44	29.72	22.78	95.79	37.65	29.12	96.68	-	-	-	-	-	-	662.98	99.85	0.972
XEA 3 MET (*R*)	-	-	-	-	-	-	3.01	1.41	58.43	7.15	4.72	82.51	11.08	7.86	88.72	24.30	18.44	94.86	31.09	23.87	95.98	-	-	-	-	-	-	493.51	99.80	0.975
XEA 4 MET (*S*)	-	-	-	-	-	-	3.18	1.55	60.73	8.19	5.55	84.74	12.92	9.34	90.33	29.35	22.48	95.74	37.12	28.69	96.63	-	-	-	-	-	-	660.54	99.85	0.971
XEA 4 MET (*R*)	-	-	-	-	-	-	3.42	1.74	63.47	9.73	6.78	87.15	15.95	11.76	92.16	38.71	29.96	96.77	52.91	41.32	97.64	-	-	-	-	-	-	1185.77	99.92	0.976
XEA DES (*S*)	5.67	3.54	77.95	11.40	8.12	89.04	28.98	22.18	95.69	67.80	53.24	98.16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	20502.18	99.99	0.988
XEA DES (*R*)	-	-	-	-	-	-	3.28	1.62	61.90	7.82	5.26	84.02	11.87	8.50	89.47	24.88	18.90	94.98	30.97	23.77	95.96	-	-	-	-	-	-	422.57	99.76	0.971
XEA 5 DES (*S*)	5.55	3.44	77.46	11.06	7.85	88.70	28.59	21.87	95.63	59.11	46.28	97.89	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	14934.82	99.99	0.983
XEA 5 DES (*R*)	-	-	-	-	-	-	5.38	3.31	76.78	14.60	10.68	91.44	24.40	18.52	94.88	56.11	43.89	97.77	-	-	-	-	-	-	-	-	-	2192.30	99.95	0.992
XEA 5 4 FLU (*S*)	-	-	-	-	-	-	2.90	1.32	56.88	6.11	3.89	79.56	16.10	11.88	92.24	42.56	33.05	97.06	-	-	-	-	-	-	-	-	-	4867.43	99.98	0.993
XEA 5 4 FLU (*R*)	-	-	-	-	-	-	5.72	3.57	78.13	12.86	9.29	90.28	26.31	20.05	95.25	61.62	48.29	97.97	-	-	-	-	-	-	-	-	-	2587.02	99.96	0.998
XEA 4 FLU (*S*)	-	-	-	-	-	-	3.68	1.95	66.07	9.67	6.73	87.07	15.16	11.12	91.75	30.50	23.40	95.90	59.24	46.40	97.89	-	-	-	-	-	-	989.01	99.90	0.988
XEA 4 FLU (*R*)	-	-	-	-	-	-	3.45	1.76	63.81	8.41	5.72	85.13	13.12	9.49	90.47	26.14	19.91	95.22	53.71	41.97	97.67	-	-	-	-	-	-	857.24	99.88	0.991
XEA 4 CLO (*S*)	-	-	-	6.27	4.01	80.05	10.38	7.31	87.96	17.01	12.61	92.65	28.53	21.82	95.62	35.41	27.33	96.47	-	-	-	-	-	-	-	-	-	335.81	99.70	0.964
XEA 4 CLO (*R*)	-	-	-	5.94	3.75	78.96	9.97	6.97	87.46	16.21	11.96	92.29	26.67	20.34	95.31	-	-	-	-	-	-	-	-	-	-	-	-	651.33	99.85	0.990
XEA 5 4 CLO (*S*)	7.40	4.92	83.11	15.92	11.74	92.15	25.12	19.10	95.02	42.69	33.15	97.07	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2080.18	99.95	0.987
XEA 5 4 CLO (*R*)	5.51	3.41	77.30	10.11	7.09	87.64	17.90	13.32	93.02	31.82	24.46	96.07	66.83	52.46	98.13	-	-	-	-	-	-	-	-	-	-	-	-	2618.79	99.96	0.993
XEA 5 3 MET (*S*)	4.05	2.24	69.11	7.23	4.78	82.71	14.53	10.62	91.40	27.92	21.33	95.52	61.34	48.07	97.96	-	-	-	-	-	-	-	-	-	-	-	-	4192.76	99.98	0.993
XEA 5 3 MET (*R*)	-	-	-	5.23	3.18	76.08	8.39	5.71	85.10	13.73	9.98	90.90	26.11	19.89	95.21	-	-	-	-	-	-	-	-	-	-	-	-	792.14	99.87	0.987
XEA 5 4 MET (*S*)	3.85	2.08	67.50	6.73	4.38	81.42	13.23	9.58	90.55	24.94	18.95	94.99	52.23	40.78	97.61	-	-	-	-	-	-	-	-	-	-	-	-	3188.60	99.97	0.993
XEA 5 4 MET (*R*)	-	-	-	5.47	3.37	77.14	9.03	6.22	86.15	15.25	11.20	91.80	29.56	22.65	95.77	-	-	-	-	-	-	-	-	-	-	-	-	1052.45	99.91	0.988
X2ADF 5 (*S*,*S*)	4.89	2.91	74.45	8.93	6.14	86.00	15.88	11.71	92.13	28.27	21.62	95.58	62.49	48.99	98.00	-	-	-	-	-	-	-	-	-	-	-	-	2690.30	99.96	0.991
X2ADF 5 (*R*,*R*)	4.95	2.96	74.75	9.12	6.30	86.30	17.31	12.85	92.78	33.63	25.90	96.28	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2927.52	99.97	0.995
X2ADF 5 (*S*,*R*)	7.02	4.61	82.18	15.61	11.48	91.99	24.99	18.99	95.00	52.67	41.14	97.63	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4335.11	99.98	0.998
X2ADF 5 (*R*,*S*)	4.12	2.30	69.66	7.02	4.62	82.19	13.79	10.03	90.93	19.85	14.88	93.70	27.81	21.25	95.51	-	-	-	-	-	-	-	-	-	-	-	-	681.87	99.85	0.971
X2ADF 1 (*S*,*S*)	-	-	-	4.27	2.41	70.70	6.71	4.37	81.38	10.27	7.22	87.83	19.47	14.58	93.58	-	-	-	-	-	-	-	-	-	-	-	-	517.61	99.81	0.981
X2ADF 1 (*R*,*R*)	-	-	-	3.90	2.12	67.92	5.60	3.48	77.69	8.11	5.49	84.59	14.08	10.27	91.12	26.54	20.23	95.29	42.55	33.04	97.06	-	-	-	-	-	-	309.31	99.68	0.994
X2ADF 1 (*S*,*R*)	-	-	-	3.50	1.80	64.26	-	-	-	7.12	4.69	82.43	11.65	8.32	89.27	20.90	15.72	94.02	31.80	24.44	96.07	-	-	-	-	-	-	217.72	99.54	0.997
X2ADF 1 (*R*,*S*)	-	-	-	3.58	1.86	65.07	5.91	3.73	78.86	8.09	5.47	84.54	13.34	9.67	90.63	24.49	18.59	94.90	36.09	27.87	96.54	-	-	-	-	-	-	252.64	99.61	0.991

This table continues on the next page.

Table 24 – Continuation.

Compounds	25% [a]			22% [a]			20% [a]			17% [a]			15% [a]			12% [a]			10% [a]			7% [a]			2% [a]			[0%] [b]		R^2
	t_R	*k*	*b%*	t_R	*k*	*b%*	t_R	*k*	*b%*	t_R	*k*	*b%*	t_R	*k*	*b%*	t_R	*K*	*b%*	t_R	*k*	*b%*	t_R	*k*	*b%*	t_R	*k*	*b%*	*k*	*b%*	
X2ADF (*S*,*R*)	-	-	-	4.49	2.59	72.13	7.21	4.77	82.66	12.74	9.19	90.19	21.20	15.96	94.10	43.34	33.67	97.12	-	-	-	-	-	-	-	-	-	702.59	99.86	0.998
X2ADF (*R*,*S*)	-	-	-	-	-	-	5.91	3.73	78.86	9.21	6.37	86.43	14.52	10.61	91.39	26.17	19.94	95.22	37.89	29.31	96.70	-	-	-	-	-	-	242.55	99.59	0.998
X2ADF (*S*,*S*)	-	-	-	5.01	3.00	75.02	7.79	5.23	83.95	13.25	9.60	90.56	21.88	16.50	94.29	41.99	32.59	97.02	-	-	-	-	-	-	-	-	-	556.16	99.82	0.998
X2ADF (*R*,*R*)	-	-	-	4.87	2.90	74.33	7.50	5.00	83.34	12.79	9.23	90.23	21.25	16.00	94.12	42.16	32.73	97.04	-	-	-	-	-	-	-	-	-	577.96	99.83	0.998

[a] Acetonitrile %

[b] Extrapolated to 100% buffer