

Dissertation of the 2nd cycle of studies leading to the Master's degree in
Pharmaceutical Chemistry

**Development of new chiral selectors based on xanthone
derivatives for liquid chromatography**

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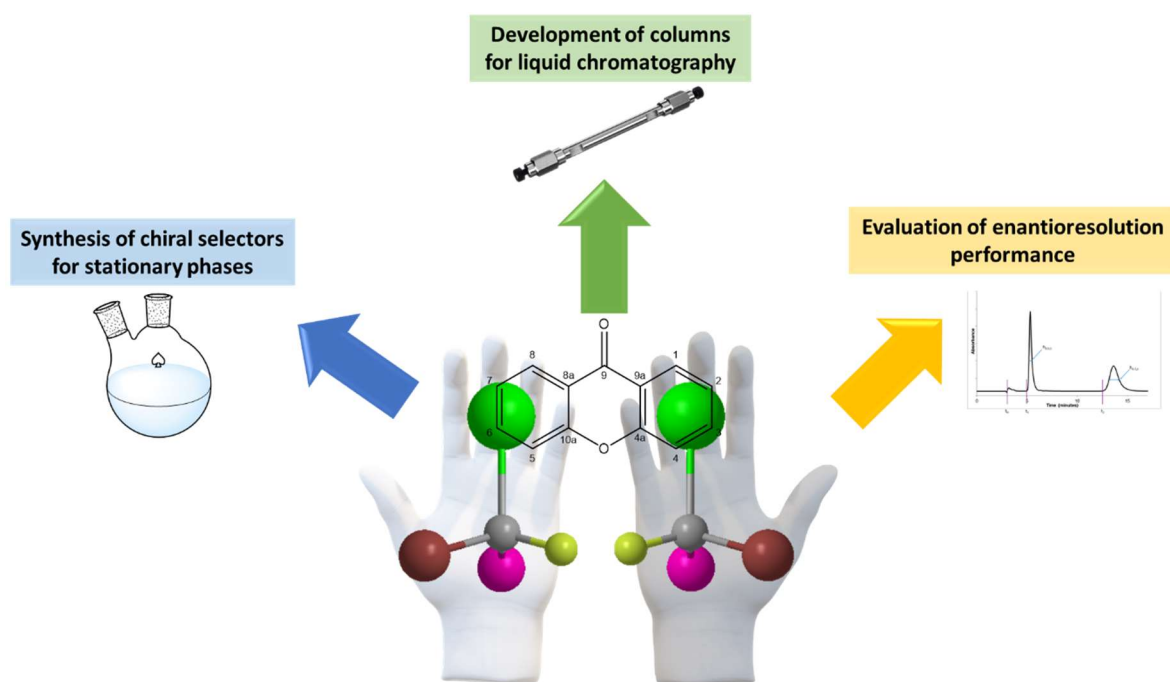
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GRAPHICAL ABSTRACT

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.

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Review

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RESUMO

A quiralidade assume um papel crucial em diversas áreas, como por exemplo na química analítica e medicinal, estando presente no nosso cotidiano. O desenvolvimento de metodologias associadas a este conceito é fundamental de modo a permitir a análise, preparação, monitorização e identificação de compostos quirais. A resolução de enantiómeros por cromatografia líquida (CL) com o uso de fases estacionárias quirais (FEQs) é um dos métodos mais versáteis e amplamente aplicado para analisar e isolar enantiómeros. As FEQs revolucionaram a resolução enantiomérica estando em desenvolvimento desde 1939. As estratégias adotadas para o seu desenvolvimento incluem, essencialmente, a introdução de novos seletos quiais (SQs) e suportes cromatográficos, assim como diferentes técnicas de ligação/adsorção dos SQs ao suporte cromatográfico.

O objetivo deste trabalho é o desenvolvimento de novos SQs baseados em derivados xantônicos quirais (DXQs) e a sua aplicação para o desenvolvimento de novas FEQs do “tipo Pirkle”. Além das atividades biológicas e farmacológicas promissoras, os DXQs também já demonstraram potencial de aplicação em cromatografia.

Tendo por base quatro derivados xantônicos (DXs) diferentes, foi planeada o desenvolvimento de cinco novas FEQs. A estratégia geral para obter os SQs consistiu, na ligação direta da porção quiral ao DX ou, alternativamente, na ligação de uma ponte química entre o DX e a porção quiral. Duas abordagens diferentes foram exploradas: introdução da mesma porção quiral ou a introdução de diferentes porções quirais em diferentes posições do DX. A ligação covalente dos SQs ao suporte cromatográfico, envolveu, primeiro, a síntese de derivados siliados. O desenvolvimento de três novas FEQs foi alcançada com sucesso. Além disso, são aqui descritos pela primeira vez catorze compostos novos. A estrutura dos SQs e seus intermediários foi confirmada por RMN de ^1H e de ^{13}C e por análise de IV. Para as FEQs preparadas foi efetuada a análise elementar com o objetivo de calcular o *loading* dos SQs. Os valores de *loading* dos seletos obtidos para **CSP1**, **CSP2** e **CSP3** foram 1.50, 1.00 e 0.32 μmolm^{-2} , respetivamente.

A capacidade de enantio-resolução em CL das novas FEQs foi avaliada no modo de eluição de fase normal com misturas de *n*-hexano, iso-propanol ou etanol em diferentes proporções como fases móveis. Duas FEQs apresentaram enantiosselectividade e resolução à linha de base para dois analitos enquanto que a outra FEQ apresentou enantiosselectividade para três analitos. Os fatores de separação obtidos variaram entre 1.41 e 16.4 e os valores de resolução entre 1.08 e 6.10. O melhor resultado cromatográfico foi obtido para o analito XEGOL2 com um fator de separação e resolução de 16.4 e 5.45, respetivamente.

Palavras-chave: quiralidade, síntese, derivados xantônico, fases estacionárias quirais, cromatografia líquida, enantiosseletividade.

ABSTRACT

Chirality assumes a crucial role on diverse areas, such as on analytical and medicinal chemistry, being present on our quotidian. The development of methodologies associated to this concept becomes fundamental to allow the analysis, preparation, monitorization and identification of chiral compounds. The resolution of enantiomers by liquid chromatography (LC) using chiral stationary phases (CSPs) is one of the most versatile and widely applied method to analyse and to isolate enantiomers. CSPs revolutionised the enantiomeric resolution being on development since 1939. The strategies adopted for their development include, essentially, the introduction of new chiral selectors (CSs) and chromatographic supports as well as different techniques of binding/coating of the CSs onto a chromatographic support.

The focus of this work was the development of new CSs based on chiral derivatives of xanthenes (CDXs) to be applied on the development of new Pirkle-type CSPs.

Starting from four different suitable functionalized xanthone derivatives (XD), the development of five CSPs was planned. The general strategy to obtain the CSs consisted, on the direct bond of a chiral moiety to the XD or, alternatively, a chemical bridge between the XD and the chiral moiety was firstly introduced. Two different approaches were explored: the introduction of the same chiral moiety or the introduction of different chiral moieties in different positions of the xanthone scaffold. The covalent linkage of the CSs to a chromatographic support involved, firstly, the synthesis of silylated derivatives. The development of three new CSPs was successfully achieved. Moreover, fourteen new compounds were described herein for the first time. The structure of the CSs and intermediates was established by ^1H and ^{13}C NMR and IR analysis. For the prepared CSPs, elemental analysis was performed to allow the calculation of the CS loading. The values of selector loading obtained for **CSP1**, **CSP2** and **CSP3** were 1.50, 1.00 and 0.32 μmolm^{-2} , respectively.

The LC enantioresolution performance of the new CSPs was evaluated under normal-phase elution mode using mixtures of *n*-hexane with isopropanol or ethanol, at different proportions, as mobile phases. Two CSPs presented enantioselectivity and baseline resolution for two analytes, while the other CSP demonstrated enantioselectivity for three analytes. Separation factors ranging from 1.41 to 16.4 and resolution values ranging from 1.08 to 6.10 were obtained. The best chromatographic result was obtained for the analyte XEGOL2 with a separation factor and resolution of 16.4 and 5.45, respectively.

Keywords: chirality, synthesis, xanthone derivatives, chiral stationary phases, liquid chromatography, enantioselectivity

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ABBREVIATIONS AND SYMBOLS

$[\alpha]_D$	Specific rotation
^{13}C NMR	Carbon Nuclear Magnetic Resonance
^1H NMR	Proton Nuclear Magnetic Resonance
2-PrOH	Isopropanol
CDX	Chiral derivative of xanthone
COMU	(1-cyano-2-ethoxy-2-oxoethylidenaminoxy) dimethylamino-morpholino-carbenium hexafluorophosphate
CS	Chiral selector
CSP	Chiral stationary phase
d	Doublet
dd	Double doublet
ddd	Double double doublet
DMSO	Dimethyl sulfoxide
DMSO- d_6	Hexadeuterodimethyl sulfoxide
EtOH	Ethanol
FDA	Food and Drug Administration
Hz	Hertz
IR	Infrared Spectroscopy
J	Coupling constant (Hz)
k	Retention factor
LC	Liquid chromatography
m	Multiplet
N	Number of theoretical plates
<i>n</i> -Hex	<i>n</i> -hexane
q	Quartet
R_s	Resolution
s	Singlet
SAR	Structure-activity relationship
t	Triplet
TBTU	2-(1 <i>H</i> -benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate
TEA	Triethylamine
THF	Tetrahydrofuran
TLC	Thin-layer chromatography
t_0	Dead-time
t_r	Retention time
UV	Ultra-violet
$W_{0.5}$	Width of chromatographic peaks at half height
XD	Xanthone derivative
α	Separation factor
δ	Chemical shift (ppm)

OUTLINE OF THE DISSERTATION

This dissertation is divided into seven chapters:

I. Introduction

In the beginning of the first chapter, general concepts related with chirality and chiral stationary phases (CSPs) are described. The Pirkle-type CSPs, based on small molecules as chiral selectors (CSs), are emphasized. A brief description of xanthone derivatives (XDs), their structure and biological/pharmacological activities are highlighted. The importance of XDs as chemical substrates for the development of new CSPs. Finally, the different pathways for the synthesis of xanthenes are summarized.

II. Aims and planning

In this chapter, the aims and planning of the work developed are presented.

III. Results and discussion

The third chapter is divided in two sections: A – Planning and development of Pirkle-type CSPs with chiral derivatives of xanthenes (CDXs) as CSs, and B – Evaluation of enantioresolution by liquid chromatography (LC). In the first section, the results related with the synthesis and structure elucidation of CSs and their intermediates are described. The strategy for the covalent bonding of CSs to the chromatographic support and the packing of CSPs in LC stainless-steel columns were described. In the second section, the experimental data obtained from the preliminary evaluation of the LC performance of the CSPs are presented and discussed.

IV. Experimental

This chapter includes the experimental methodologies, reagents, experimental conditions and procedures adopted for development of CSs and CSPs as well as the methods for LC evaluation.

V. Conclusions

The main conclusions of the developed work are presented.

VI. References

In this chapter, the references that support the work developed as well as the databases are presented.

VII. Appendixes

Appendix 1 includes the abstract and poster of the work presented on the 48th International Symposium on High-Performance Liquid Phase Separations and Related Techniques.

Appendix 2 contains the abstract and poster presented in Escola de Inverno de Farmácia-2019.

Appendix 3 includes the abstract and poster of the work presented in 12nd Encontro de Investigação Jovem da Universidade do Porto.

Appendix 4 includes a review, published in the International Peer-Reviewed Journal Molecules, entitled “Chiral Stationary Phases for Liquid Chromatography: Recent Developments”.

I. INTRODUCTION

The synthesis on organic chemistry had a great impulse in the beginning of the nineteenth century with the first report of a synthesis of a molecule that only occurred in Nature, the urea, by Friedrich Wöhler¹. This landmark set that natural compounds could be synthesized from inorganic compounds being not necessary the traditional organic sources such as live organisms². The synthetic chemistry started a revolution leading to the introduction of new concepts related with the chemical structure and reactivity, enabling the creation and modification of a wide diversity of organic molecules³. Since the majority of natural compounds as well as live organisms are chiral, the knowledge related with chirality should also be explored alongside with synthetic chemistry.

In order to mimic natural compounds, it could be necessary to synthesize compounds in enantiomerically pure form. The main strategies to obtain single enantiomers include the enantioselective synthesis and chiral separation⁴. The enantioselective synthesis involves the synthesis of the desired enantiomer being mainly useful when a large amount of only one enantiomer is required. The enantioselective synthesis can be perform with enantiomerically pure starting material and several achiral reagents, the chiral pool strategy, or start from achiral precursors and using chiral synthons and auxiliaries, stereoselective catalysts or enzymes to obtain the desired enantiomer⁴. The first report of an asymmetric stereoselective synthesis was attributed to Marckwald who also presented the first definition of this type of reaction⁵. Over the last few years, several progresses in the catalytic asymmetric synthesis have been reported⁶⁻⁸.

The chiral separation involves the separation of a racemate or enantiomeric mixture into the single enantiomers, using several methodologies including liquid chromatography (LC)⁹⁻¹². The isolation of enantiomerically pure compounds based on preparative chromatography has a recognized importance in many fields¹³⁻¹⁴. The separation of enantiomers can be achieved by direct method due to the presence of a chiral selector (CS) in the chromatographic system. The CS can be incorporated on the stationary phase by binding or coating onto packing material, affording a chiral stationary phase (CSP), or it can be an additive in the mobile phase¹⁵.

Nowadays, direct LC method using CSPs proved to be as one of the most significant separation approach for preparative applications⁹⁻¹⁰ as well as for analytical purposes¹⁶⁻¹⁸. Although many CSPs have been described in the last years, the development of new CSPs continues to be a field of research with crucial importance in order to follow the evolution of many research areas.

The main focus of this dissertation was the synthesis of new CSs for the development of CSPs to be applied for LC.

1. Chirality: general concepts and importance

Chirality concept was firstly referred in 1815 by Jean-Baptiste Biot, a French physicist who observed that some natural organic compounds presented an intrinsic optical activity when they were in solution or liquid¹⁹. He was succeeded by Louis Pasteur, a French chemist and biologist, who reported the first chiral separation in 1848 through crystallization²⁰. The chirality is a geometric property associated with three-dimensionality and asymmetry of a molecule.

Chiral compounds do not present a symmetry plan and the presence of one or more stereogenic centres can confer chirality¹⁹. Typically, the stereogenic centre is an atom of carbon surrounded by four different substituents. Nevertheless, other atoms such as sulphur, phosphorus or nitrogen could also be stereogenic centres²¹. Despite that the majority of chiral compounds present stereogenic centres on their structure, some compounds are chiral, and they have not a stereogenic centre. Other elements that can confer chirality are an axis of chirality, a plan of chirality and a helix of chirality²⁰. As examples of these types of chiral compounds are the atropisomers and the akamptisomers. Atropisomers are nonplanar chiral compounds which the asymmetry is not associated with a specific atom so they do not possess a stereogenic centre²². The chirality of these compounds is associated to their axis since the optical activity is promoted by the linkage²³. Akamptisomers were recently described (2018) being similar to the atropisomers. For this type of compounds occurred a bond-angle inversion being the resulted isomers correspondent to the opposite orientations of the bond angle²⁴.

Chiral compounds are represented by two non-overlapping structures and related to each other as an object and its mirror image, designated as enantiomers²⁰ (**Figure 1**).

Enantiomers have identical physicochemical properties being the differences based on their optically properties, their behaviour on chiral environments²⁰ and their spatial arrangement²¹. They present distinctive aspects related with their nomenclature, synthesis and separation²⁵. The identification and classification of enantiomers can be based on their ability to rotate plane-polarized light. If the light is rotated to clockwise the enantiomer is called as dextrorotatory (+), while if it is rotated to counter clockwise is called as levorotatory (-)²⁰. Consequently, the enantiomers are frequently called optical isomers. Configuration of enantiomers can be assigned by the system Cahn-Ingold-Prelog, which expresses the configurations as (*R*) and (*S*) (from the Latin words *rectus* and *sinister* which means right

and left, respectively) by a set of priority rules for the substituents of the stereogenic centre²⁶. Diastereomers (**Figure 1**) are molecules with two or more stereogenic centres but they are not chiral. In contrast to enantiomers, they have different physicochemical properties, which facilitates its chemical characterization. Diastereomers and enantiomers are called estereoisomers²¹.

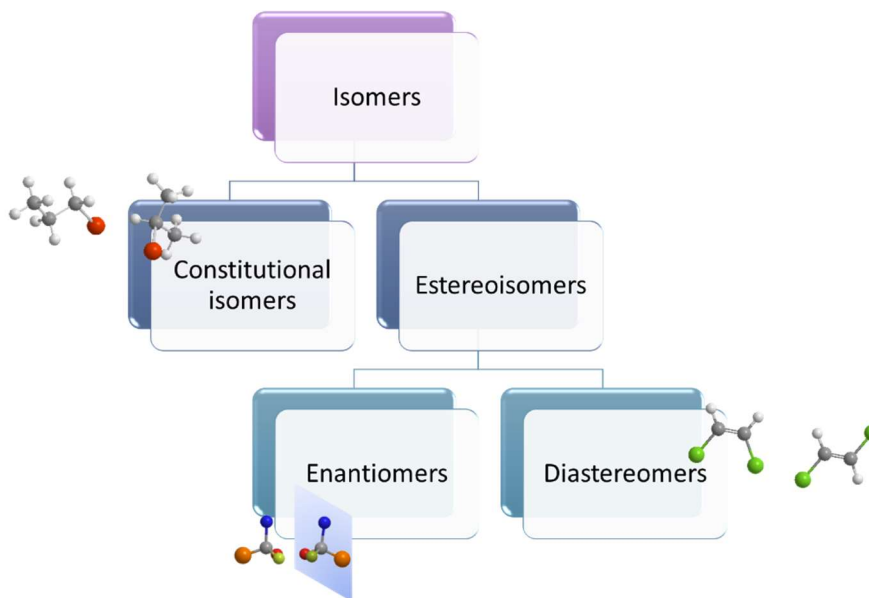


Figure 1 – Classification of isomers.

Biological systems comprise molecules with intrinsic chirality (proteins, sugars and nucleic acids) which can recognize and discriminate between enantiomers (chiral recognition) resulting on different biological responses²⁰. Chiral recognition is dependent on the complementary configuration which conditionate the interactions with binding sites of the target²⁰. This phenomenon is called enantioselectivity whereby an enantiomer is expressed exclusively or predominantly over the other²¹. The chiral recognition plays an essential role in pharmacodynamic, pharmacokinetic and toxicological events²⁷, being a factor that should be taking into account during drug development in order to potentiate the therapeutic benefits and to develop more effective and safer drugs²⁸. In 1992, the Food and Drug Administration (FDA) approved a stricter policy related with the development of stereoisomeric drugs recommending the preferential production of single-isomer drugs²⁹.

Despite the pharmacology³⁰, toxicology³¹ and chemistry³², the chirality also plays an important role in other areas such as environment^{17, 33}, food³⁴⁻³⁵ as well as forensic sciences³⁶ being a topic with relevant economic repercussions (**Figure 2**).

As referred previously, enantiomers can be obtained by synthesis or by a process of separation. The implementation of technologies which improve the efficiency of processes related with chiral compounds has been the focus of many companies. As an example, the European Commission 7th Framework implemented a project, the KYROBIO, which the

main objective is to improve the range of high value single enantiomer chiral chemicals manufactured in Europe using biotechnological routes³⁷.

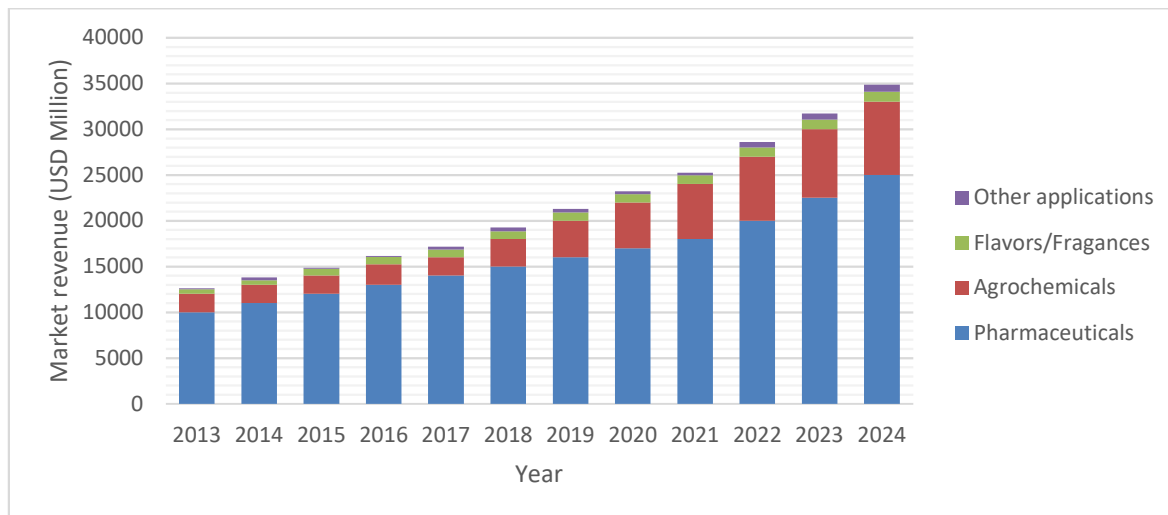


Figure 2 - U.S. chiral chemicals market revenue, by application, between 2013 and 2024 (USD Million)³⁸.

The separation of chiral compounds allowed the obtainment and analysis of each enantiomer separately which could be useful for pharmacologic and toxicological studies, for example. Separation of racemic mixtures by chromatography was firstly described in 1938, by Henderson and Rule³⁹ who applied a column based on lactose. They were followed by Karagunis and Coumoulos⁴⁰ who resorted to enantioselectivity of quartz. In 1952, Dalgliesh, an English scientist studied the enantiomeric separation of amino acids by paper chromatography attributing the enantioselectivity to the cellulose of the paper⁴¹.

The development of CSPs improved the chromatographic performance for a wide range of compounds and the extension of application areas, with a considerable economic impact in the industry^{16, 33-34, 36, 42}. The value of chiral columns was 73.4\$ million in 2016 with a prevision of 124.0\$ million in 2024 being the regulatory field and the development of proteomic area some of the promoters of this growth⁴³.

These progresses established the advancement in this area being the chiral chromatography applied not only in the first steps of the drug discovery but also in a full-scale process.

2. Chiral stationary phases

Since the introduction of the first CSP³⁹, the planning and development of new CSPs for LC can be considered as continuous and evolutionary issues. The main objectives of the developed strategies were to attempt the improvement of the chromatographic enantioresolution performance of the CSPs as well as to enlarge their versatility and range of applications. Additionally, the transition to ultra-high-performance LC had also been underscored⁴⁴.

Over the last few years, CSPs have been applied in LC with different purposes such as preparative resolution of racemates⁹⁻¹⁰, determination of the enantiomeric composition¹¹⁻¹², monitorization of asymmetric reactions⁴⁵⁻⁴⁶, analysis of the stereochemistry of natural compounds⁴⁷⁻⁴⁸, pharmacokinetic⁴⁹⁻⁵⁰ and enantioselective studies⁵¹⁻⁵², among others. Several types of CSPs are available (**Figure 3**) and, among them, more than a hundred were currently commercially available⁵³. Other types have been introduced more recently such as the molecularly-imprinted CSPs⁵⁴⁻⁶³ and those comprising synthetic polymers as CSS^{54, 64-70}.

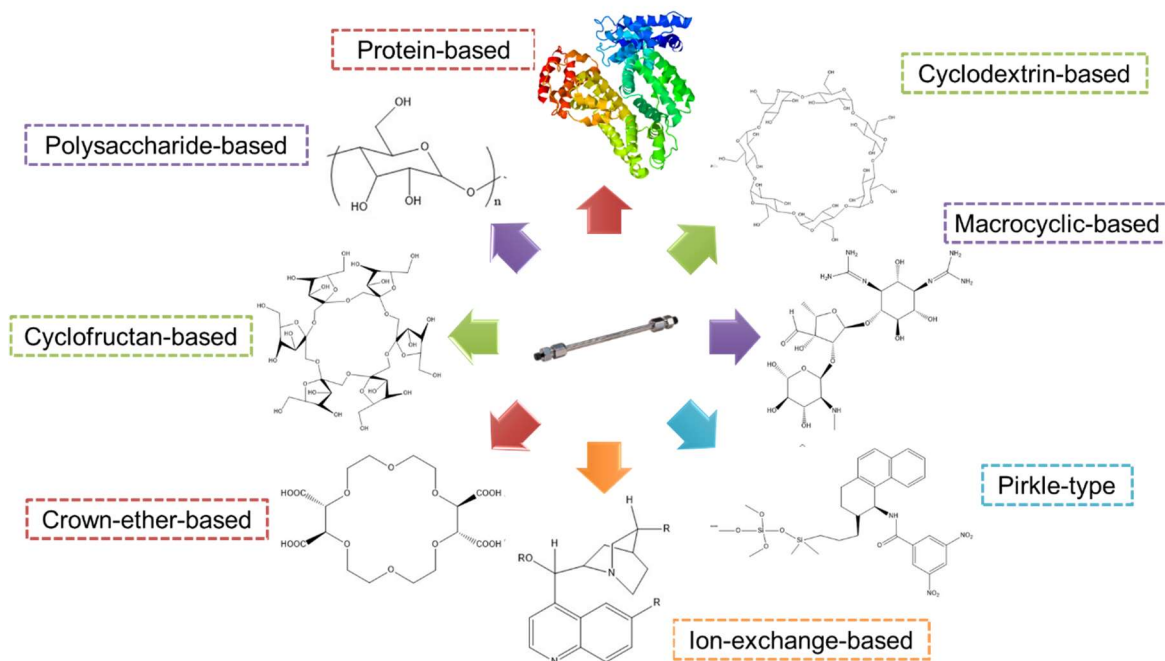


Figure 3 – Examples of the different types of CSPs.

Despite the variety of CSPs already introduced, the development of new CSPs continues to be a field of research with great importance. Nevertheless, the various strategies adopted for their development (**Figure 4**) constitute an important trigger aiming to achieve new CSPs, as commercially viable products with high versatility and broad range of analytical and preparative applications or, on the contrary, be exceptionally efficient for

the enantioresolution of specific target analytes. The most recent strategies for the development of new CSPs (**Figure 4**) comprised the introduction of new CSs, the use of new materials as chromatographic supports or the reduction of its particle size, and the application of different synthetic approaches for preparation of CSPs.

Recently, we performed a literature survey covering the report on recent developments of diverse CSPs⁴⁴ (**Appendix 4**).

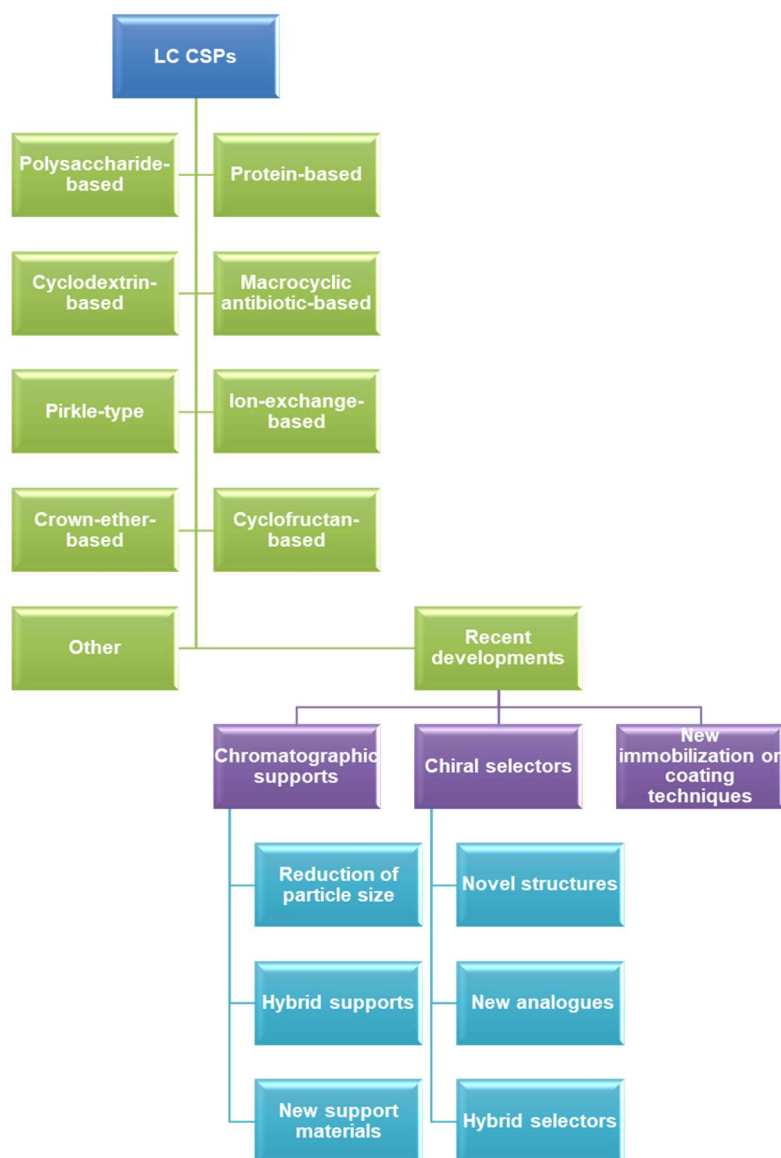


Figure 4 - Recent strategies for development of new CSPs for LC.

The focus of this work was the development of new CSPs based on small molecules (Pirkle-type) which present a wide variety of advantages⁷¹ being highlighted as one of the most promising type of CSPs. Actually, Pirkle-type CSPs are characterized by their diversity and versatility since a variety of different small molecules as CSs can be used as well as different substituents can be introduced to improve their enantioselectivity⁷².

2.1. Pirkle-type CSPs

Brush-type or Pirkle-type CSPs were introduced, in 1979, by Pirkle and House⁷³. They evolved over the years, being the type of CSPs with more reported progresses⁷¹, mainly due to the possibility of use of a wide variety of small molecules as CSs. The base of this type of CSPs are neutral synthetic chiral low-molecular mass molecules as CSs⁷⁴. These molecules should promote donor-acceptor interactions as hydrogen-bond, π - π or dipole-dipole, in addition to attractive and/or repulsive Van der Waals interactions⁵⁴. Considering that the CSs are based on small molecules, the mechanism of chiral recognition is, frequently, based on the “three-points” model, which refers that the establishment of at least three interactions between one of the enantiomers to be resolved and the CSP are crucial for chiral recognition⁷⁵.

The CSs are usually covalently linked to a silica support, which can have monosubstituted or trisubstituted silane groups, through a spacer⁶⁴. Over several years, Pirkle's group has developed successive generations of CSPs⁷⁶. Among them, Whelk-O1 CSP, created by a rational approach, is the most applied and versatile CSP in both academic and industrial fields⁷⁷.

Initially, the preferred elution mode was the normal phase since it provides a favourable environment for the interactions needed for the enantioseparation of the analytes⁵⁵. The most commonly used solvents are ethanol (EtOH), isopropanol (2-PrOH), *n*-hexane (*n*-Hex) and CH₂Cl₂. Nevertheless, this type of CSPs can also be used in polar organic and reversed-phase elution modes⁷⁸⁻⁸⁰.

The advantages inherent to this type of CSPs are the compatibility with a wide range of solvents used as mobile phase, the stability to a wide range of temperature and pressure, the considerable loading capacity and the possibility to be easily scale-up to preparative chromatography⁷¹. Another important advantage is the possibility of switching the configuration of the CS and use the inverted configuration column approach⁷⁴. Its kinetic performance is reasonable and the fact that the structure of the CS is relatively “simple” allows an easier knowledge of the chiral recognition mechanisms as well as a consecutive optimization of the selector⁸¹. In addition, they can be highly specific for certain types of chiral compounds⁷².

3. One-step and multi-step synthesis

An organic synthesis aiming to achieve a specific final product could be performed through a one-step synthesis or a multi-step approach.

A one-step synthesis (**Figure 5a**) comprises the junction of many synthetic reactions in one-step, in other words, from different raw materials, a complex product could be achieved in a single step⁸². The advantages inherent to this type of synthesis are the possibility to perform a reaction with many reagents without a sequence of many steps and additionally, the purification and isolation of the intermediates⁸² is not necessary.

Despite the advantages presented, some disadvantages could be enumerated. It could not be possible the junction of some synthetic pathways due to incompatibility of reaction conditions or even the purification of the final product could be compromised. The moderation of reactivity and minimization of by-products and side-reactions could also be challenging⁸³.

Multi-step synthesis (**Figure 5b**), which could also be designated by cascade reactions, consists on a sequence of chemical reactions where a product becomes the substrate for the next reaction and successively, until the desired product is obtained⁸⁴. This approach present many advantages which include the possibility to increase molecular complexity and stereoselectivity⁸⁵, atom and time economy, management of resources and waste, some of the characteristics aspects of green chemistry⁸⁶. Multi-step synthesis raised as an important field on organic chemistry allowing the production of complex molecules including the synthesis of biomimetic molecules, which is an important feature for medicinal chemistry research area.

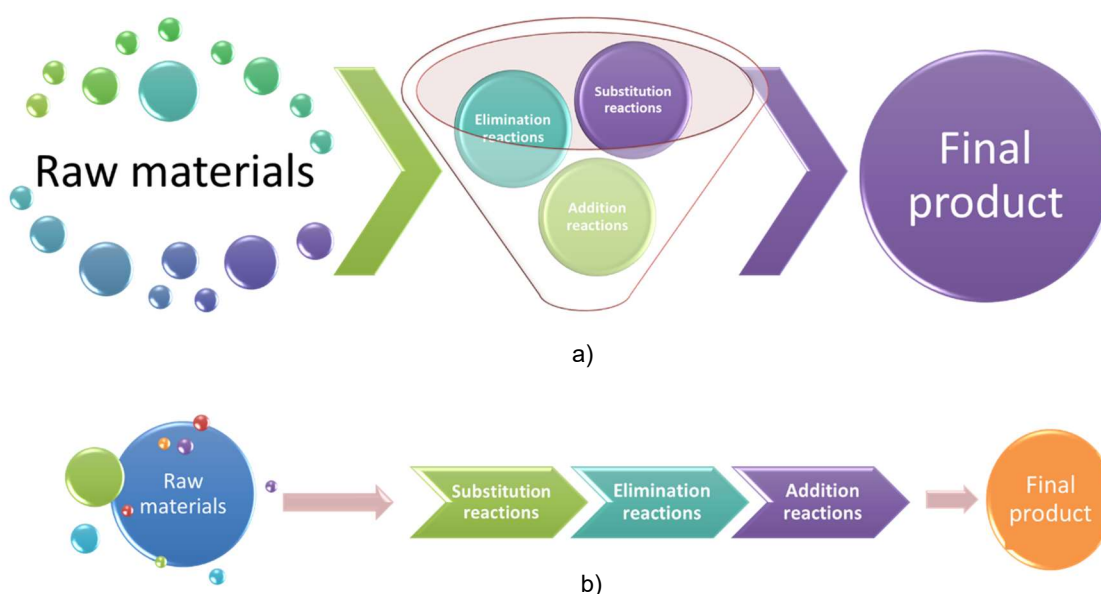


Figure 5 - Schematic representation of one-step (a) and multi-step (b) synthesis.

These concepts could be applied for the synthesis of xanthone derivatives (XDs) and each strategy presents different aspects that should be taken into account.

4. Xanthone derivatives

Xanthones are secondary metabolites⁸⁷ being characterized as heterocyclic compounds with a scaffold based on dibenzo- γ -pyrone (**Figure 6**). Its crystal structure was reported, firstly, in 1982 by Biswas and Sen⁸⁸. Xanthones could be obtained from terrestrial^{89,90} or marine⁹¹ sources as well as by synthesis⁹². It has been considered as a privileged structure on Medicinal Chemistry⁹³ since their derivatives present a broad spectrum of pharmacological and biological properties, such as anticoagulant⁹⁴, antipyretic⁹⁵, analgesic⁹⁶, anti-HIV⁹⁷, among others^{93, 98-99} (**Figure 6**).

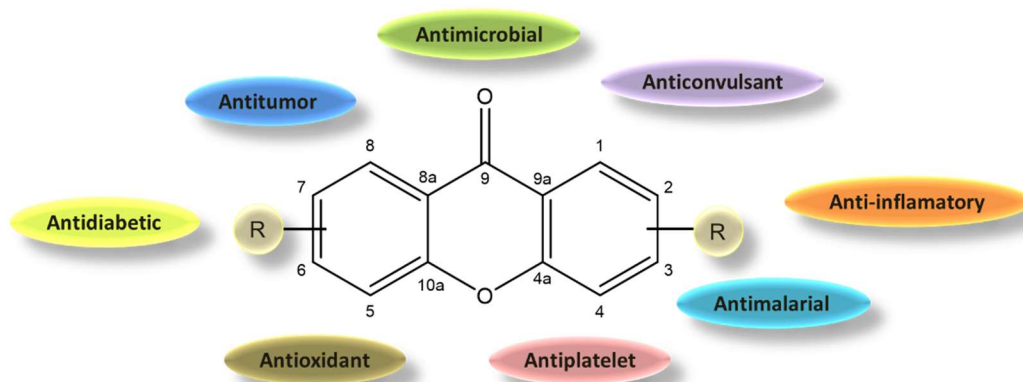


Figure 6 - The xanthone scaffold and some pharmacological activities of its derivatives.

Although, XDs could occur naturally, their chemical synthesis allow to obtain a wide diversity of structures with different patterns of substitution⁹³. Chiral derivatives of xanthones (CDXs) are also present on Nature being, usually, more complex structures and they comprising a superior number of stereogenic centres when comparing with synthetic chiral derivatives¹⁰⁰. The chirality of the XDs may play an important role on their pharmacological and biological properties since their activities could be conditionate by this feature^{93, 101}.

The synthesis of CDXs presented some advantages such as the possibility to obtain different analogues that allow structure-activity relationship (SAR) studies as well as the preparation of both enantiomers in order to perform enantioselectivity studies. Actually, at a clinical level, a single enantiomer is usually more beneficial than a racemate⁹³ considering toxicological and pharmacokinetic aspects, for example²⁸. Despite the applications already mentioned, CDXs could also be applied as CSPs¹⁰².

4.1. Synthesis of xanthenes

The majority of synthetic xanthenes are obtained by three classical synthetic methods: the synthesis by one-step condensation of two aryl blocks, or a multi-step pathway *via* diaryl ether or benzophenone intermediates. In addition, other methods have been described such as those involving chromone derivatives¹⁰³.

4.1.1. Synthesis of xanthenes by one-step condensation of two aryl blocks

The synthesis of xanthenes could be performed by condensation of two aryl blocks, namely salicylic derivatives and another aromatic building block, using a strong catalyst acid, such as zinc chloride/phosphoryl chloride⁹². One advantage of this approach is the accessibility of the reaction conditions¹⁰⁴, while the main disadvantage is a considerable limitation of the scaffold of the building blocks^{92, 104}. The condition to obtain a xanthone is that the benzophenone intermediate should present at least three hydroxyl groups at 2, 2', 6 or 6' positions⁹². The most recognized method is denominated as Grover, Shah and Shah¹⁰⁵. The multi-step synthesis appeared as a feasible alternative to this approach.

4.1.2. Multi-step synthesis of xanthenes

The multi-step synthesis of xanthenes includes the routes through a diaryl ether intermediate, a benzophenone intermediate or a chromone derivative¹⁰⁴ (**Figure 7**). The first step of the diaryl ether approach consists on the ether linkage of two aromatic rings followed by intramolecular cyclization⁹². The synthesis *via* benzophenone route is similar although the first step is the synthesis of a benzophenone intermediate⁹². Comparing these two methods, the synthesis through benzophenone intermediate usually presents superior yields^{92, 104}, being the most applied one. The synthesis using chromone derivatives is not so commonly applied, however, it presents as advantage the potential to obtain XDs with a more complex pattern of substitution⁹².

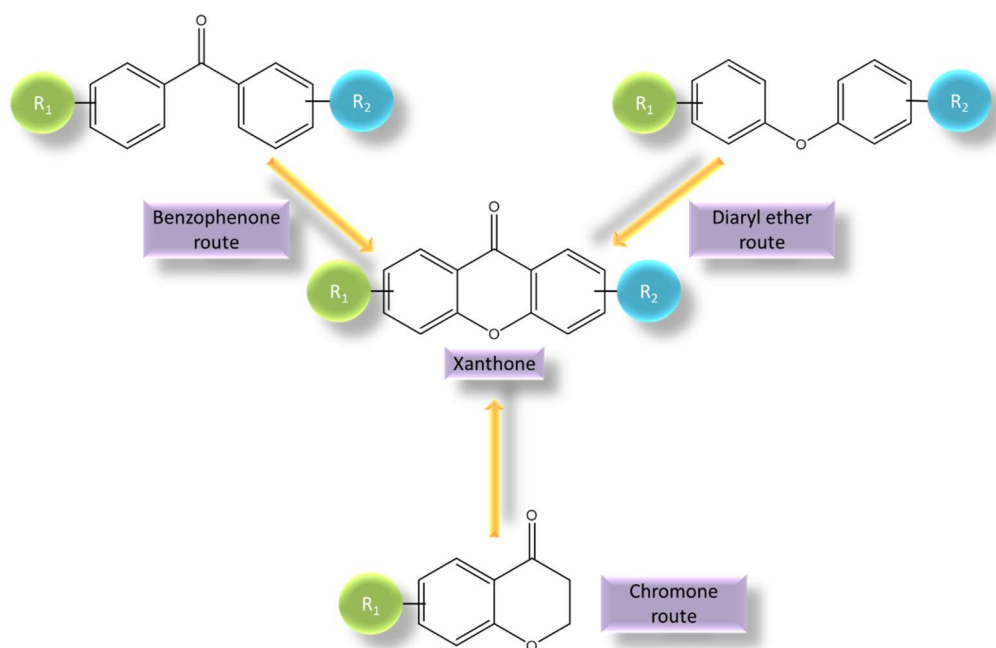


Figure 7 - Strategies for multi-step synthesis of xanthenes.

II. AIMS AND PLANNING

The main objective of this dissertation was the development and evaluation of new CSPs for LC based on XDs as CSs.

The specific aims were the following:

- Total synthesis of XDs with different patterns of substitution, by multi-step pathways *via* benzophenone, to be used as chemical substrates;
- Synthesis of new CDXs by coupling reactions of XDs with different enantiomerically pure building blocks;
- Structure elucidation of the CDXs, the XDs and all the intermediates by spectroscopic techniques (IR, ^1H and ^{13}C NMR);
- Synthesis of silylated derivatives of CDXs followed by covalent bonding to a chromatographic support (silica gel) to obtain CSPs;
- To determine the CS loadings of the CSPs by elemental analysis;
- Packing of the CSPs in LC stainless-steel columns and further evaluation of their performance using different chiral analytes.

The general strategy for the planning of the new CSPs (**CSP1-CSP5**) was based on a previous work of our group¹⁰². To obtain some of the desired CSs, a chemical bridge between the XD and the chiral moiety was firstly introduced. In other cases, the direct coupling of the chiral moiety to a suitable functionalized XD was performed. Two different approaches were explored to obtain the CDXs to be used as CSs: introduction of the same chiral moiety (**Figure 8a and 8b**) or the introduction of different chiral moieties in different positions on the xanthone scaffold (**Figure 8c and 8d**). The aims were to explore the effect of the number, chemical nature and position of these substituents in the xanthone scaffold and, consequently, their effect on the enantioselective performance of the CSPs. After the preparation of the CDXs as selectors, the synthesis of silica-reactive selectors was performed to allow their covalent linkage to the chromatographic support (silica) *via* a spacer. In some cases, the CSs were covalently bonded through two sites (**Figure 8a and 8b**), while in other cases by one side (**Figure 8c and 8d**) in order to promote a more flexibility of the CS.

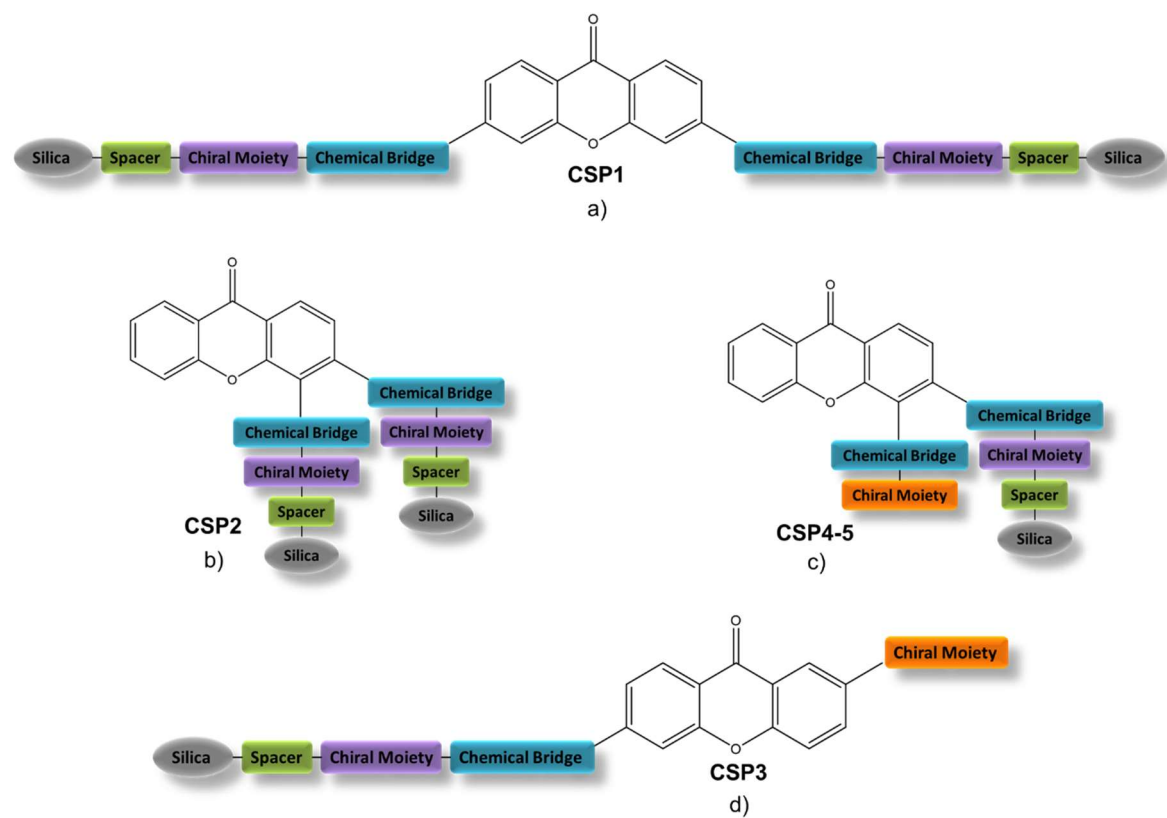


Figure 8 - Schematic representation of the planned CSPs.

III. RESULTS AND DISCUSSION

In this chapter, the discussion of the results is presented being the first part related to the planning and development of the CSPs (**CSP1-CSP5**), followed by the results of structure elucidation of the CSs and their intermediates. The determination of the loading of the CSs is also described.

In the second part, the preliminary results of the evaluation of the LC performance of the developed CSPs (**CSP1-CSP3**) are presented and discussed.

Five CSPs were planned, however only three CSPs were achieved during this master dissertation. The enantioselectivity of the new CSPs was evaluated using diverse chiral analytes.

A. Planning and development of new CSPs with CDXs as selectors

Inspired by the enantioselective properties of proteins¹⁰⁶, in the last years, many amino acids-based CSPs have been developed^{102, 107-109}. Since they present a more simplistic structure, one of the advantages of these CSPs is their mechanism of chiral recognition, which is more easily to be described¹⁰⁶. Other advantages for the amino acids-based CSPs are the enhancement of the hydrogen-bond and π - π interactions with the analytes to be enantioseparated, their conformational rigidity and steric hindrance, which reflects on a promising enantioresolution performance⁷¹. The interesting results of CSPs comprising chiral moieties on their structures derived from amino-acids units were described by some authors^{102, 107-109}.

The presence of a derived-amino alcohol moiety on CSPs was also been reported¹¹⁰⁻¹¹¹. Besides the facility on the description of their mechanism of chiral recognition, the simplicity of their synthetic procedures and the results of their enantioresolution performance were pointed out as key factors for their development¹⁰⁷.

Pirkle *et al.*¹¹² developed the first amino alcohol-based CSPs, including a phenylglycine-derived CSP. Their work, inspired many other research groups to developed amino alcohol-derived CSPs and, among the chiral units chosen, the CSPs derived from (*R*)-phenylglycinol demonstrated good results for the LC enantioseparation of many chiral analytes¹¹³⁻¹²⁰. In our group, the development of CSPs comprising amino-alcohols was also reported¹⁰². Based on the main conclusions of that work¹⁰², which also included the

development of (*R*)-phenylglycinol-derived CSPs as the most promising ones, this chiral unit was selected for the development of new CSPs in this work. The chemical substrates for the CSPs were suitable functionalized XDs. These small molecules were chosen because our group has a great experience in synthesis of XDs^{94, 121-131}, and besides the variety of the interesting bioactivities of CDXs^{93, 98, 101, 103, 127, 129, 132}, they already demonstrated potential as CSPs¹⁰².

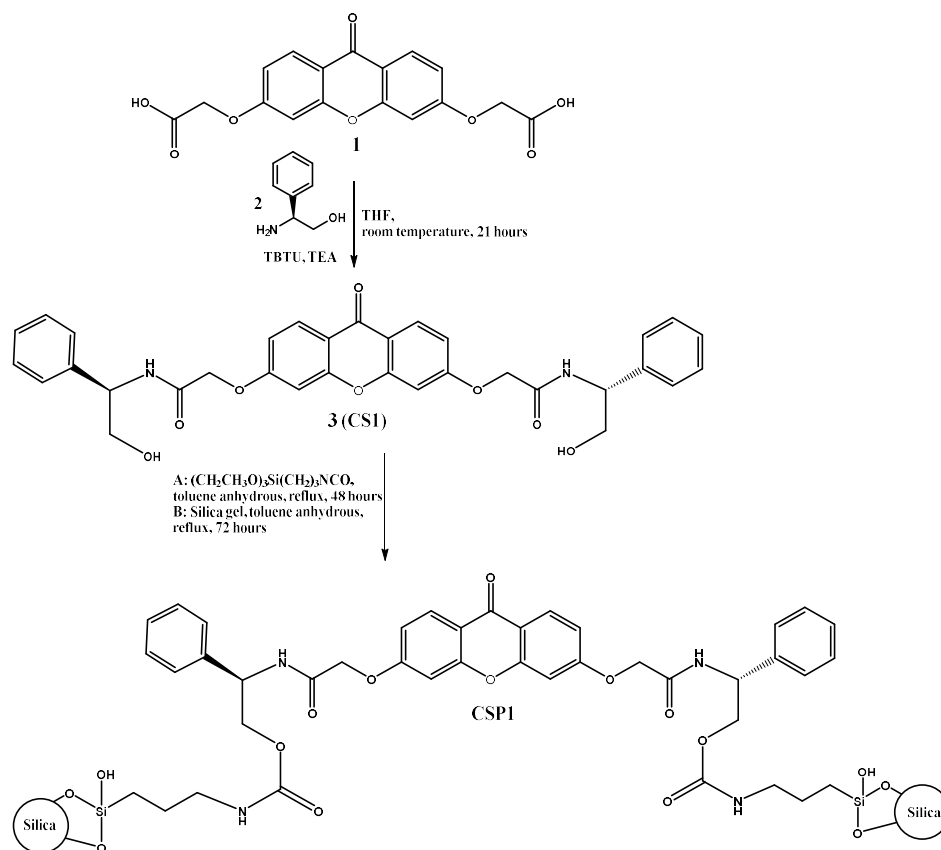
In this study, the new CSPs were planned by using multi-step pathways starting from suitable functionalized small molecules based on the most promising selectors (CDXs) previously reported¹⁰². Herein, the synthesized CSs were xanthone-(*R*)-phenylglycinol derivatives comprising one or two chiral moieties, being covalently bonded through one or two sites to a chromatographic support. The influence of the chiral moiety, (*R*)-(-)-2-phenylglycinol, in different positions of the xanthone scaffold was studied, specifically, the presence of this chiral moiety in the different aromatic rings of the xanthone scaffold and in the same aromatic ring. The introduction of other chiral units with different structural characteristics, in addition with this amino alcohol moiety, was also investigated.

1. Planning and development of CSP1

The synthetic pathway for the development of **CSP1** is presented on **Scheme 1**. **CSP1** was prepared by the linkage of the chiral amino alcohol (*R*)-(-)-2-phenylglycinol (**2**) to the carboxyxanthone derivative **1**, and further covalent bonding to a chromatographic support by two sites.

Compound **1** was synthesized previously *via* benzophenone intermediate, which was kept at the furnace at 200°C for 8 hours¹²² obtaining the 3,6-dihydroxy-9*H*-xanthen-9-one. A Williamson ether synthesis allowed the introduction of a chemical bridge using an alkyl halide with an ester group (BrCH₂COOCH₃), and a base (K₂CO₃)¹²¹. After this step, an alkaline hydrolysis of the esters, with NaOH¹²¹ was performed allowing to obtain the compound **1**.

To prepare the **CSP1**, the (*R*)-(-)-2-phenylglycinol (**2**) was binding in the different aromatic rings of the xanthone scaffold of compound **1**, specifically in symmetric positions (3 and 6), affording the **CS1** (**3**). The reagent 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate (TBTU) was chosen to perform the coupling reaction, using as catalyst the triethylamine (TEA)¹⁰². The next step was the silylation of **CS1** (**3**) using 3-(triethoxysilyl)propylisocyanate to introduce the spacer and allow the covalent linkage of the selector to the chromatographic support (silica)¹⁰², affording **CSP1**.

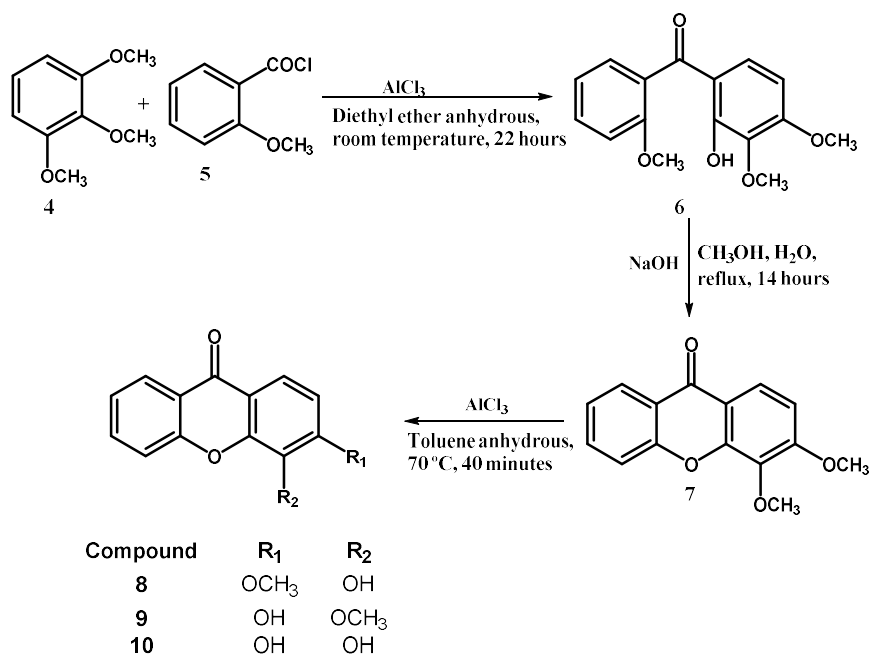


Scheme 1 - Synthetic route of the development of CSP1.

2. Planning and development of CSP2

CSP2 was developed in order to study the effect of the presence of the same chiral moiety derived from (*R*)-(-)-2-phenylglycinol (**2**) in the same aromatic ring of the xanthone scaffold, specifically at adjacent positions (3 and 4). The synthetic pathway for the development of **CSP2** is presented on **Scheme 2**.

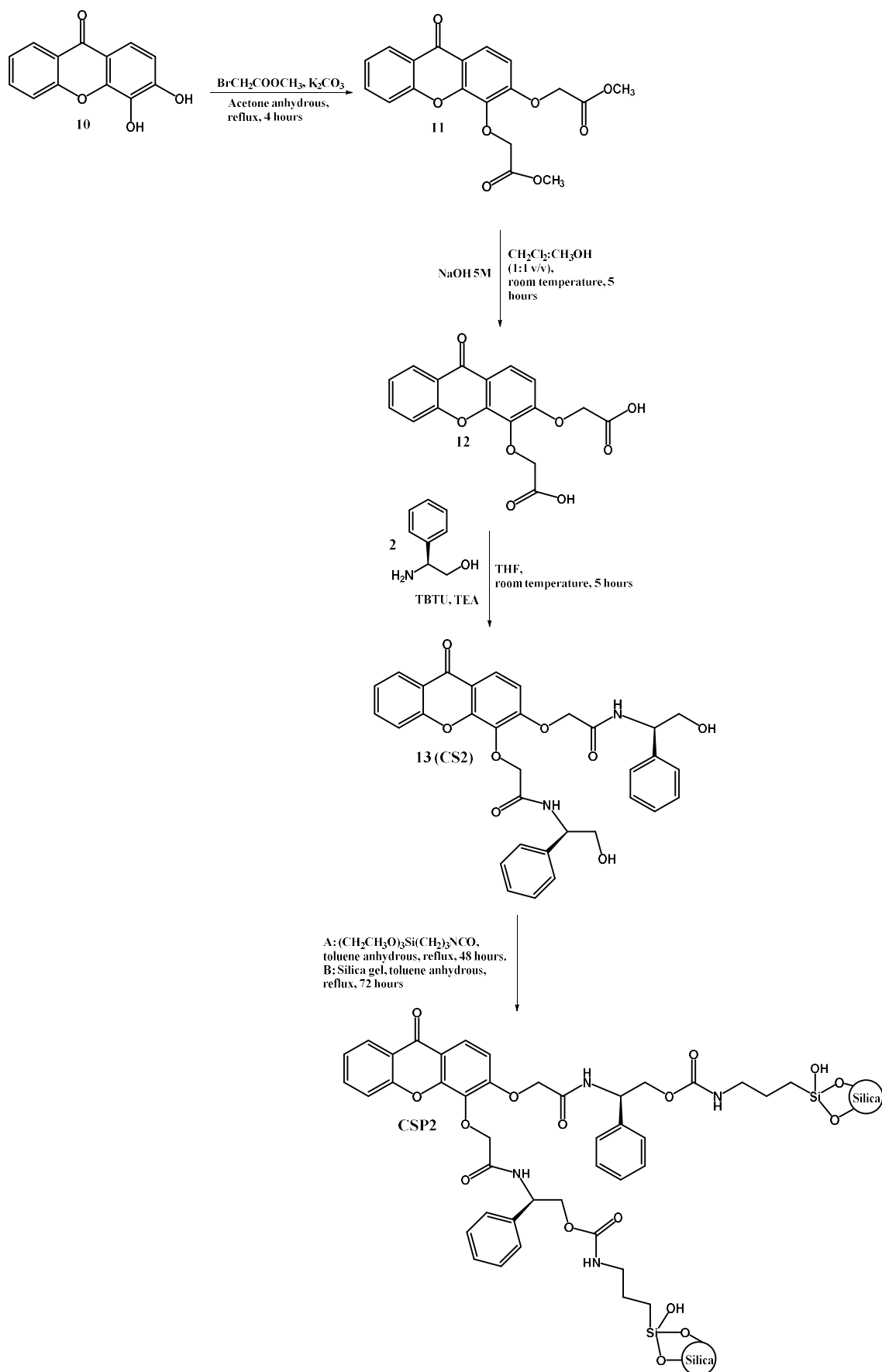
For the development of **CSP2**, the total synthesis of the chemical substrate the 3,4-dihydroxy-9*H*-xanthen-9-one (**10**) was carried out by a multi-step pathway based on a previous work¹²³ (**Scheme 2**). The strategy for its synthesis was the benzophenone route, being a secondary product of the demethylation of the 3,4-dimethoxy-9*H*-xanthen-9-one (**7**). So, the first step to obtain compound **10** consisted on the synthesis of the benzophenone [(2-hydroxy-3,4-dimethoxyphenyl) (2-methoxyphenyl)-methanone] (**6**) through a Friedel-Crafts acylation, promoted by AlCl₃. Then a cyclization of the benzophenone **6** occurred by an intramolecular nucleophilic aromatic substitution with a basic catalyst, the NaOH to afford the 3,4-dimethoxy-9*H*-xanthen-9-one (**7**). The final step consisted on the demethylation of the 3,4-dimethoxy-9*H*-xanthen-9-one (**7**) through AlCl₃. The main product was the 3-methoxy-4-hydroxy-9*H*-xanthen-9-one (**8**), although the formation of secondary products, 3-hydroxy-4-methoxy-9*H*-xanthen-9-one (**9**) and 3,4-dihydroxy-9*H*-xanthen-9-one (**10**) occurred¹²³.



*Scheme 2 – Synthetic route of the 3-methoxy-4-hydroxy-9*H*-xanthen-9-one (**8**), 3-hydroxy-4-methoxy-9*H*-xanthen-9-one (**9**) and 3,4-dihydroxy-9*H*-xanthen-9-one (**10**).*

After the synthesis of the XD **10**, a chemical bridge was added, through a Williamson ether reaction¹²¹. The alkyl halide chosen was the BrCH₂COOCH₃ and the base was K₂CO₃.

This reaction was followed by an alkaline hydrolysis of the esters of compound **11**, in the two positions, in an alkaline environment¹²¹. The next step, enabled the introduction of the chiral moiety derived from (*R*)-(-)-2-phenylglycinol (**2**), at positions 3 and 4 of the xanthone scaffold, using the coupling reagent TBTU and the catalyst TEA¹⁰² to afford **CS2 (13)**. The covalent linkage of **CS2 (13)** to the silica gel to obtain **CSP2** was also through two sites based on the same methodology referred for the development of **CSP1**¹⁰² (**Scheme 3**).



Scheme 3 - Synthetic route of the development of CSP2.

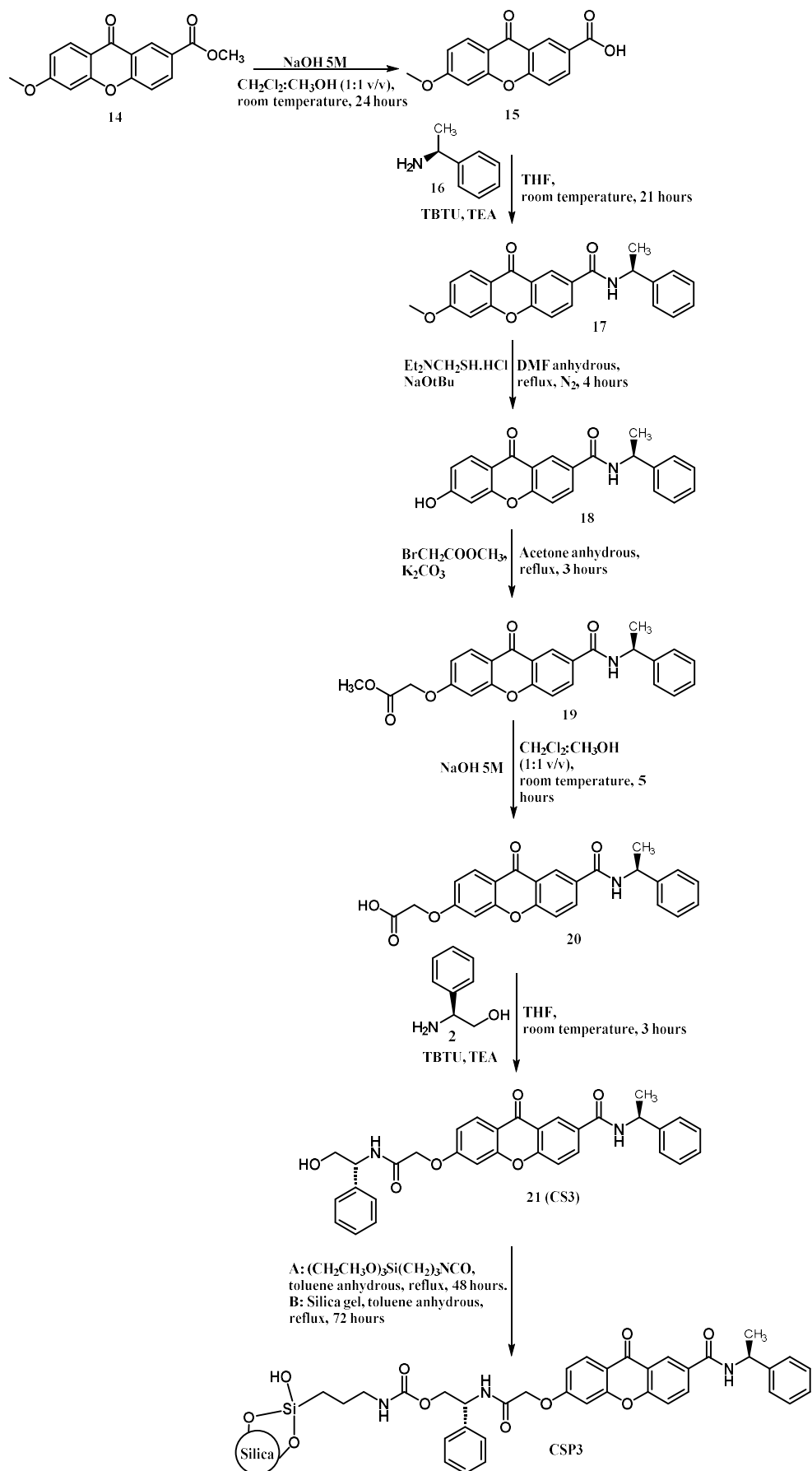
3. Planning and development of CSP3

In order to achieve a remarkable performance, a Pirkle-type CSP should present moieties that promote interactions with different moieties and functional groups in order to expand the diversity of analytes that it could separate. Taking this into account, **CSP3** was planned and developed comprising two different chiral units, derived from (*R*)-(-)-2-phenylglycinol (**2**) and (*S*)-(α)-methylbenzylamine (**16**), to enlarge the variety of interactions.

CSP3 comprises two different chiral moieties in different aromatic rings of xanthone scaffold, specifically in the positions 2 and 6. It can be considered as a hybrid CSP since it resulted from the combination of two previously developed CSPs¹⁰². For **CSP3**, the linkage of the CS to the chromatographic support was performed just through one side in order to allow a superior flexibility of the CS. The synthetic pathway that allowed the development of **CSP3** is presented on **Scheme 4**.

The compound **14** was synthesized previously through a multi-step pathway with the diaryl ether as intermediate¹³². Firstly, a Fischer esterification with H₂SO₄ as catalyst was performed, followed by an Ullmann reaction with CuI, Cs₂CO₃ and *N,N*-dimethyl glycine¹³². The third reaction consisted on an alkaline hydrolysis with NaOH which allowed the Friedel-Crafts intramolecular acylation obtaining the compound **14**¹³². The ester of the chemical substrate of **CSP3** (compound **14**) was purified by *flash* chromatography (*n*-Hex/ethyl acetate in gradient) and hydrolysed in order to obtain the chemical substrate as a carboxylic acid (compound **15**).

After the obtainment of compound **15**, the following step on the synthesis of **CSP3** consisted on a coupling reaction with TBTU and TEA¹⁰² allowing the bonding of the chiral moiety derived from (*S*)-(α)-methylbenzylamine (**16**), obtaining the compound **17**. The next step consisted on the demethylation of the methoxy group of the compound **17** on the position 6, promoted by the NaOt-Bu and 2-(diethylamino)ethanethiol hydrochloride¹³³. The demethylation was followed by the introduction of the chemical bridge, for compound **18**, resorting to Williamson ether synthesis, with the same reagents described above¹²¹. Then, an alkaline hydrolysis of compound **19** with NaOH was performed, yielding the compound **20**¹²¹. After this step, a coupling reaction with TBTU and TEA¹⁰² was performed to introduce a second chiral moiety, the (*R*)-(-)-2-phenylglycinol (**2**), obtaining the **CS3** (**21**). The introduction of the spacer and the covalent linkage to the silica gel was performed with the methodology referred previously¹⁰² affording **CSP3**.



Scheme 4 - Synthetic route of the development of **CSP3**.

4. Planning of CSP4

A new CSP was planned (**CSP4**) comprising the same chiral units of **CSP3** although, in this case, they were linked to the same aromatic ring of the xanthone scaffold and in adjacent positions (3 and 4) to achieve a more rigid cleft. Like **CSP3**, the strategy to bond the CS to a chromatographic support is through only one side. The synthetic pathway for the development of **CSP4** is presented on **Scheme 5**. The synthesis of this CSP was not concluded, although in this chapter will be present the reaction steps performed successfully.

The first part for the development of **CSP4** consisted on the total synthesis of the XD, the 3-methoxy-4-hydro-9*H*-xanthen-9-one (**8**) by a multi-step pathway (**Scheme 2**), based on previous work¹²³. Then, a chemical bridge was linked on position 4 of the compound **8**, through a Williamson ether synthesis, using the alkyl halide BrCH₂COOCH₃ and the base K₂CO₃¹²¹. This reaction was followed by an alkaline hydrolysis using NaOH¹²¹. For the introduction of the chiral moiety (*S*)-(α)-methylbenzylamine (**16**), at position 4 of the xanthone scaffold of **8**, the coupling reagent TBTU and the catalyst TEA were applied¹⁰² yielding the compound **24**. To pursue the introduction of the second chiral moiety at position 3, a demethylation of the methoxy group present on this carbon was performed, using NaOt-Bu and 2-(diethylamino)ethanethiol hydrochloride¹³³, affording compound **25**.

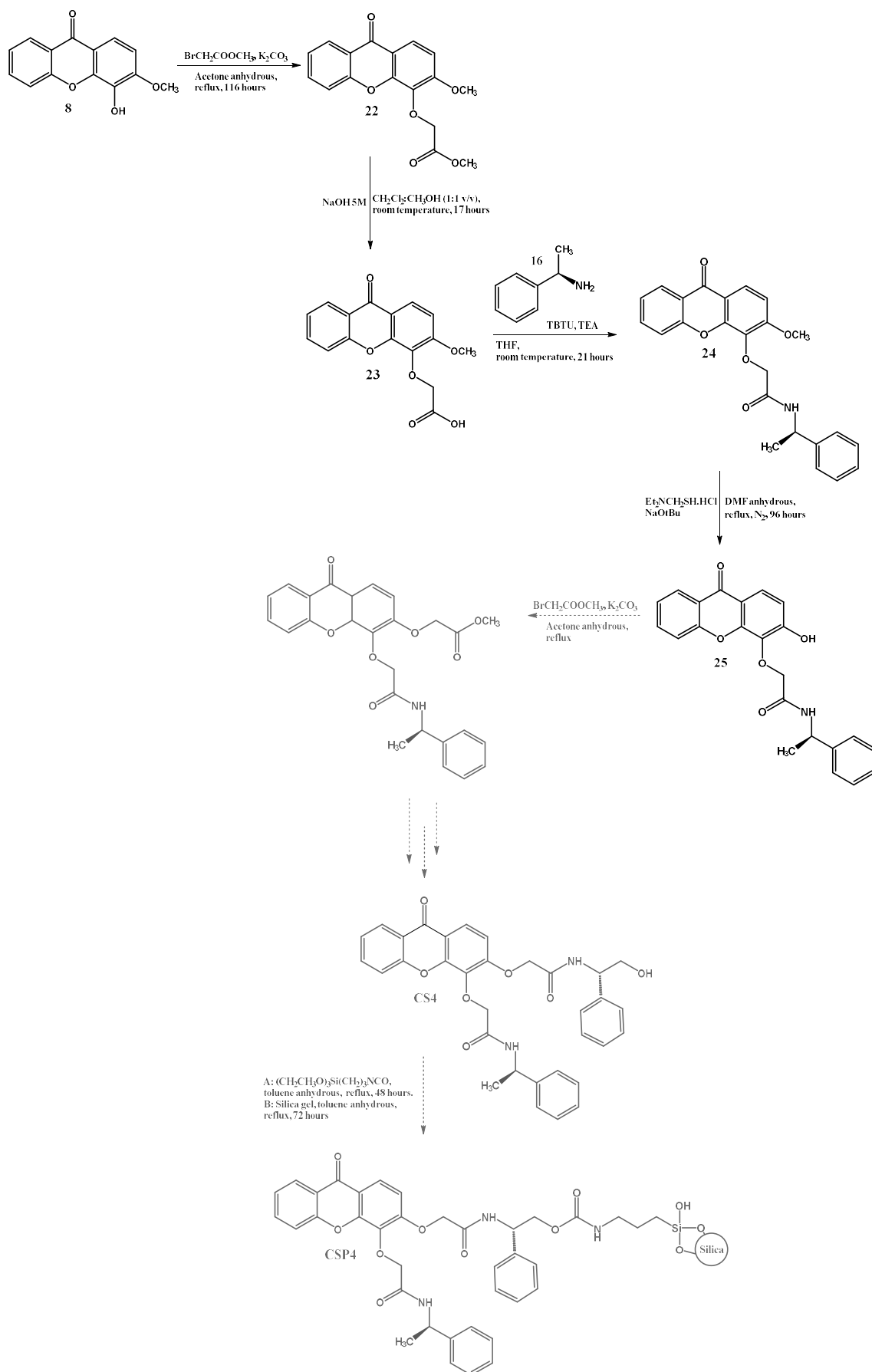
Aiming to introduce a chemical bridge on position 3 of compound **25**, for further coupling to a second chiral moiety, some reactions were attempted. The first attempt consisted on the same procedure applied for the previous CSPs, with acetone as solvent, K₂CO₃ as base and the alkyl halide BrCH₂COOCH₃¹²¹. Unfortunately, the reaction did not occur, and the raw material (compound **25**) was preserved.

In a second attempt, the general procedure was adjusted using tetrahydrofuran (THF) as solvent and NaH as base¹³⁴. Since the NaH is a stronger base than K₂CO₃, the deprotonation of the phenol was expected to occur easier, however, the reaction did not occur.

A third attempt was carried out, consisting on a reaction performed on microwave system with NaOH as base¹³⁵. This reaction was performed with different reaction times, although after the purification process, the product was isolate, and its structure elucidated through ¹H and ¹³C NMR and IR analysis.

Probably, the aromatic moiety present on the chain on position 4 of the xanthone scaffold caused steric hindrance preventing the occurrence of the reaction on position 3. This hindrance could also be explained by the possibility of an intramolecular hydrogen-bond between the phenol on position 3 and the amide group of the side chain at position 4.

Other strategies will have to be explored in order to synthesize the proposed compound and to pursue the development to achieve the planned CSP (**CSP4**).



Scheme 5 - Synthetic route of the development of CSP4.

5. Planning of CSP5

The planning of **CSP5** was inspired in Whelk-01 CSP, which presents chiral units with π -basic and π -acidic character as well as a semi-rigid cleft¹³⁶. The objective was to obtain a new CSP by combining chiral moieties with these characteristics in order to expand the variety of analytes that could be enantioseparated. Analytes comprising π -electron-accepting (π -acidic) units may interact with the π -electron-donating (π -basic) units of the CSP and vice versa¹³⁷. Moreover, the semi-rigid cleft may also be crucial for enantioselectivity⁷¹.

The chosen chiral unit with π -acidic character was the (*R*)-(-)-*N*-(3,5-dinitrobenzoyl)- α -phenylglycine (**27**) to be linked at position 4 of xanthone scaffold, and the π -basic chiral unit was the (*R*)-(-)-2-phenylglycinol (**2**) linked at position 3. The linkage of the CS to the chromatographic support is through one site. The synthetic pathway for the development of **CSP5** is presented on **Scheme 6**. Despite the synthesis of this CSP was not concluded, in this chapter will be present the reaction steps performed successfully.

The first part of the synthesis of **CSP5** consisted on the total synthesis of the chemical substrate 3-methoxy-4-hydro-9*H*-xanthen-9-one (**8**) (**Scheme 2**), through a synthetic procedure described previously¹²³.

Firstly, for the synthesis of **CS5**, a direct coupling between compound **8** with the chiral unit (*R*)-(-)-*N*-(3,5-dinitrobenzoyl)- α -phenylglycine (**27**) was attempted, using as coupling reagent the (1-cyano-2-ethoxy-2-oxoethylidenaminoxy) dimethylamino-morpholino-carbenium hexafluorophosphate (COMU). After two days of reaction, many secondary products were obtained being the purification process too complex. This procedure demonstrated to be not efficient¹³⁸.

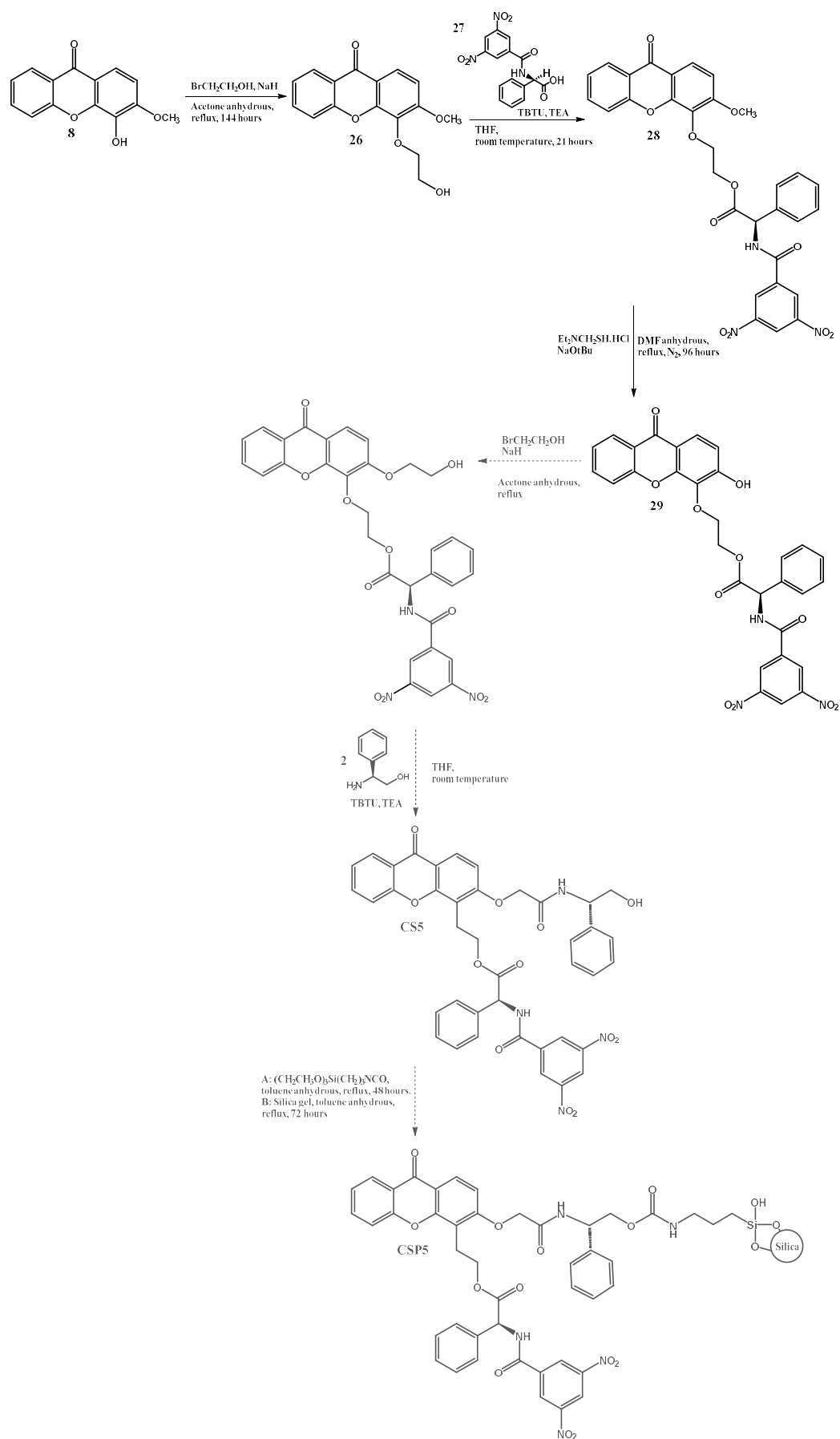
Following, the TBTU was applied as coupling reagent. Despite the reduction of the number of secondary products, the reaction time was extensive as well as the presence of the impurities in the final product is significant. The isolation of the main product and consequently its characterization, were not possible to be performed so another strategy was adopted.

The new strategy consisted on the introduction of a chemical bridge with a shorter size containing an alcohol at the extremity, specifically the alkyl halide BrCH₂CH₂OH.

In a first attempt, the reaction was carried out with K₂CO₃ as base¹²¹ being an incomplete reaction with a long reaction time and a low yield (59%). Since the phenol is a weak acid, its reactivity for this type of reaction is reduced. Thus, a second attempt was tried selecting a stronger base in order to promote a more efficient deprotonation of the acid. The chosen base was the NaH¹³⁴ affording a higher yield (80%) in the same reaction time.

Then, the coupling of the chiral moiety derived from (*R*)-(-)-*N*-(3,5-dinitrobenzoyl)- α -phenylglycine (**27**) to compound **26** was performed with the coupling reagent TBTU and the catalyst TEA¹⁰², affording the compound **28**. The followed reaction was the demethylation of the methoxy on position 3 with the reagents mentioned before. After these steps it was obtained the compound **29**.

Since the compound **29** presented a bulky chain on position 4, it is expected a similar behaviour to the compound **25** (the intermediate for the synthesis of **CSP4**). As for the compound **25**, the following reactions will be conditionate by the steric hindrance of the bulky groups on chain at position 4. Other strategies will have to be explored in order to achieve the total synthesis of the proposed compounds in order to develop the planned CSP (**CSP5**).



Scheme 6 - Synthetic route of the development of CSP5.

6. Structure elucidation of the chiral selectors and intermediates

The structure elucidation of the CSs and intermediates of the reactions performed during their development is presented.

6.1.CSP1

6.1.1. Structure elucidation of 2,2'-[9-oxo-9H-xanthene-3,6-diyl]bis(oxy)]bis{N-[(R)-2-hydroxy-1-phenylethyl]acetamide}, CS1, (3)

The **CS1 (3)** was obtained after a coupling reaction with TBTU, to introduce the chiral moiety derived from (*R*)-(-)-2-phenylglycinol (**2**) in two opposite positions of the 3,6-dihydroxy-9H-xanthen-9-one, the XD used as chemical substrate. The ^1H and ^{13}C NMR (**Figure 9**) and IR analysis (**Table 1**) allowed to confirm its structure.

The symmetry of the compound **3** was reflected on the values obtained from ^1H and ^{13}C NMR analysis. The highest values correspond to the protons from amides ($\delta = 8.24$ ppm) since the electronegativity of the nitrogen has influence. Between the carbons C1' and C1'' ($\delta = 4.72$ ppm) and the carbons C11' and C11'' ($\delta = 3.75$ ppm), the last ones presented an inferior chemical shift value since the alcohol group presents a superior electron-donating capacity leading to a higher protection of the carbons C11' and C11''.

The values obtained from the ^{13}C NMR spectrum also reflected the symmetry of the compound **3**. In this spectrum was possible to observe the presence of two signals at 166.7 and 55.0 ppm corresponding to the carbons of the amide group and the alcohol group, respectively.

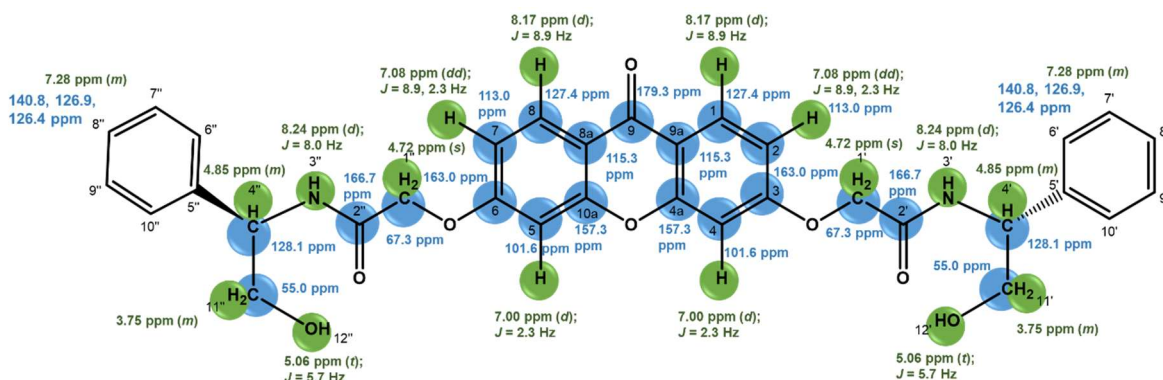


Figure 9 - ^1H and ^{13}C NMR values of compound 3.

The values obtained from the IR analysis of compound **3** are presented in **Table 1**. It is important to highlight the bands at 3444.4, 3286.8 and 1651.1 cm^{-1} representative of the

amide groups and the bands at 3062.2 and 295.8 cm^{-1} from the alcohol indicative of the introduction of the chiral moiety.

Table 1 – IR data of compound 3.

Bond	ν (cm^{-1})
C=O (xanthone)	1624.0
Xanthonic ether	1251.5
C=C (aromatic)	1547.7, 1455.3, 842.6, 769.5
C-O (ether)	1063.3
CONH (amide)	3444.4, 3286.8, 1651.1
OH (primary alcohol)	3062.2, 2954.8

6.2. CSP2

6.2.1. Structure elucidation of 3,4-dihydroxy-9H-xanthen-9-one (10)

The structure elucidation of the 3,4-dihydroxy-9H-xanthen-9-one (**10**) was performed by ^1H and ^{13}C NMR (**Figure 10**) and IR analysis (**Table 2**).

The most significant ^1H NMR data of compound **10** was the presence of one singlet which integrate for two protons with a chemical shift value at 9.72 ppm, corresponding to the protons of the phenol groups.

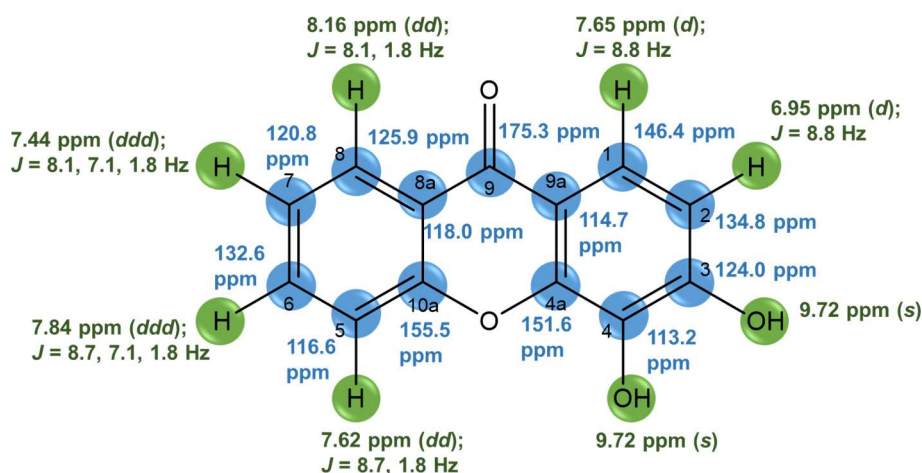


Figure 10 - ^1H and ^{13}C NMR values of compound 10.

The values of the IR analysis of compound **10** are presented on **Table 2**. It is important to highlight the presence of a band with a shift at 3138.4 cm^{-1} representative of a phenol

group and of a band with a shift at 1604.8 cm^{-1} representative of a carbonyl group of xanthone.

Table 2 - IR data of compound 10.

Bond	$\nu\text{ (cm}^{-1}\text{)}$
C=O (xanthone)	1604.8
C-O-C (xanthone)	1224.0
C=C (aromatic)	1526.2, 1458.7, 904.6
OH (phenol)	3138.4

6.2.2. Structure elucidation of dimethyl 2,2'-[(9-oxo-9H-xanthene-3,4-diyl)bis(oxy)]diacetate (11)

The diacetate derivative (compound 11) was the product of the Williamson ether synthesis of the compound 10 being its structure elucidation performed by ^1H and ^{13}C NMR (Figure 11) and IR (Table 3).

The ^1H NMR spectrum of compound 11, compared with the spectrum of its precursor (compound 10), presented new signals such as a duplet with a chemical shift at 3.71 ppm corresponding to the three protons of the ester group which integrated for six protons. It is also important to refer the difference on the chemical shift of H1' and H1'' which was due to the greater proximity of H1'' to the xanthonic ether group, which has electron donating properties contributing for a higher protection of the H1''.

Considering the data from ^{13}C NMR spectrum it is important to consider the signals at 69.1 and 65.4 ppm from the ether group of the chemical bridge. The signals at 52.1 and 51.8 ppm indicated the presence of the ester group.

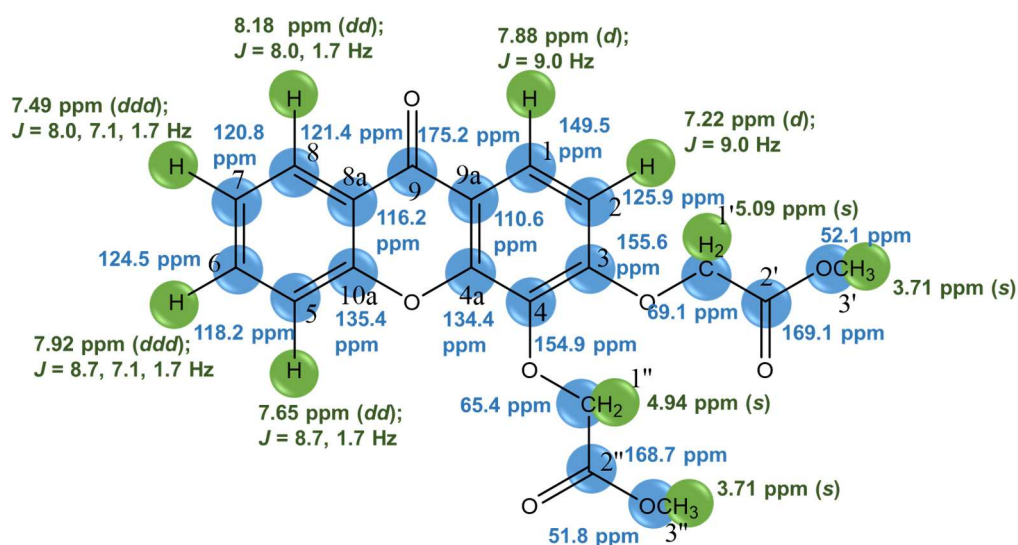


Figure 11 - ^1H and ^{13}C NMR values of compound 11.

The values obtained from the IR analysis of compound **11** are presented on **Table 3**. Comparing with the IR spectrum of the compound **10**, it is important to refer the presence of the bands with a shift at 1767.2 and 1337.8 cm^{-1} representative of the ester group which were not presented on the spectrum of the compound **10**. The presence of a band at 1086.9 cm^{-1} indicated the presence of the ether group, which was also considered to confirm the introduction of the chemical bridge.

Table 3 – IR data of compound 11.

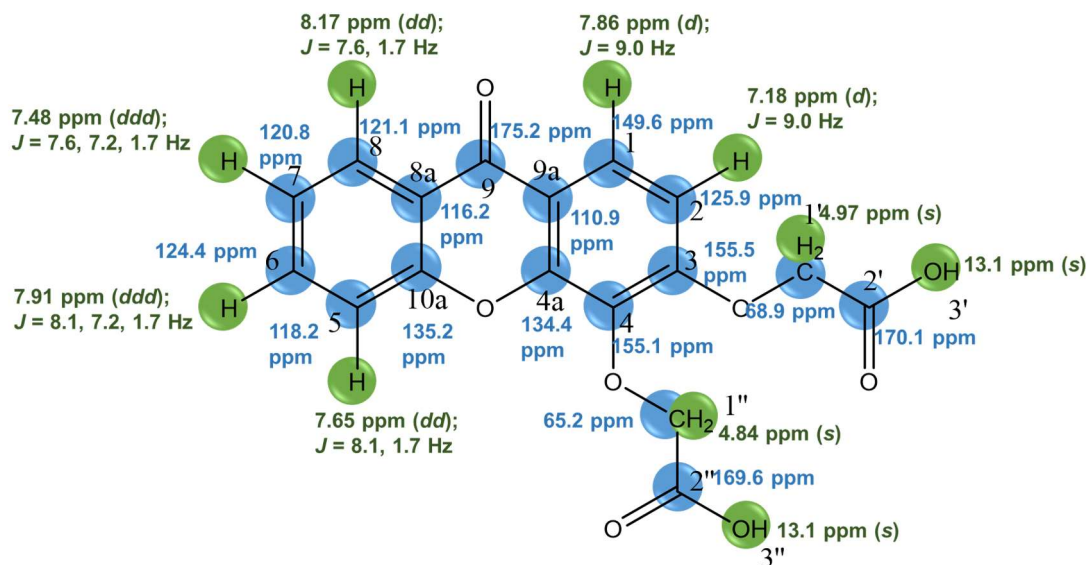
Bond	ν (cm^{-1})
C=O (xanthone)	1605.4
C-O-C (xanthone)	1266.8
C=C (aromatic)	1470.2, 1430.7, 832.5, 763.3
C-O (ether)	1086.9
COOCH ₃ (ester)	1767.2, 1337.8

6.2.3. Structure elucidation of 2,2'-[(9-oxo-9H-xanthene-3,4-diyl)bis(oxy)]diacetic acid (**12**)

The diacetic acid derivative (compound **12**) was obtained after an alkaline hydrolysis of compound **11** being its structure elucidate through ^1H and ^{13}C NMR (**Figure 12**) and IR analysis (**Table 4**).

From the analysis of the ^1H NMR, it is important to refer the presence of a singlet with a chemical shift at 13.1 ppm which integrate for two protons that corresponded to the protons of hydroxyl from the carboxylic acid groups. The difference on the chemical shift between H1' and H1'' was also denoted. Comparing with the data from the previous compound, the singlet correspondent to the ester group ($\delta = 3.71$ ppm) was not detected.

Considering the ^{13}C NMR spectrum and comparing with the spectrum from compound **11**, the signals correspondent to the ester group (52.1 and 51.8 ppm) were not detected.

Figure 12 - ^1H and ^{13}C NMR values of compound **12**.

The values of the IR analysis of compound **12** are presented on **Table 4**. In this spectrum, it is important to highlight the bands with a shift at 3413.2, 2955.0 and 1745.7 cm^{-1} representative of a carboxylic acid group, which indicated a successful hydrolysis of the ester groups.

Table 4 – IR data of compound **12**.

Bond	ν (cm^{-1})
C=O (xanthone)	1605.1
C-O-C (xanthone)	1217.3
C=C (aromatic)	1514.9, 1467.0, 819.2, 760.9
C-O (ether)	1067.3
COOH (carboxylic acid)	3413.2, 2955.0, 1745.7

6.2.4. Structure elucidation of 2,2'-[(9-oxo-9H-xanthene-3,4-diyl)bis(oxy)]bis{N-[(R)-2-hydroxy-1-phenylethyl]acetamide}, **CS2 (13)**

The chiral selector **CS2 (13)** was obtained after the coupling of the (R)-(-)-2-phenylglycinol (**2**) moiety with the compound **12** using the coupling reagent TBTU. The structure elucidation was performed by ^1H and ^{13}C NMR (**Figure 13**) and IR analysis (**Table 5**).

Considering the ^1H NMR data, the most characteristics chemical shifts belong to the protons from the amide group with chemical shifts at 8.63 and 8.51 ppm, and the protons from the hydroxyl groups ($\delta = 5.07$ and 5.00 ppm). These chemical shifts were not present

in the spectrum of the compound **12**. The difference between the values of chemical shifts of the side chains at the positions 3 and 4 was also exhibited.

From the analysis of the ^{13}C NMR spectrum and comparing with the data from the previous compound, the presence of the signals at 166.6 and 167.8 ppm from the amide group were indicative of the introduction of the chiral moiety.

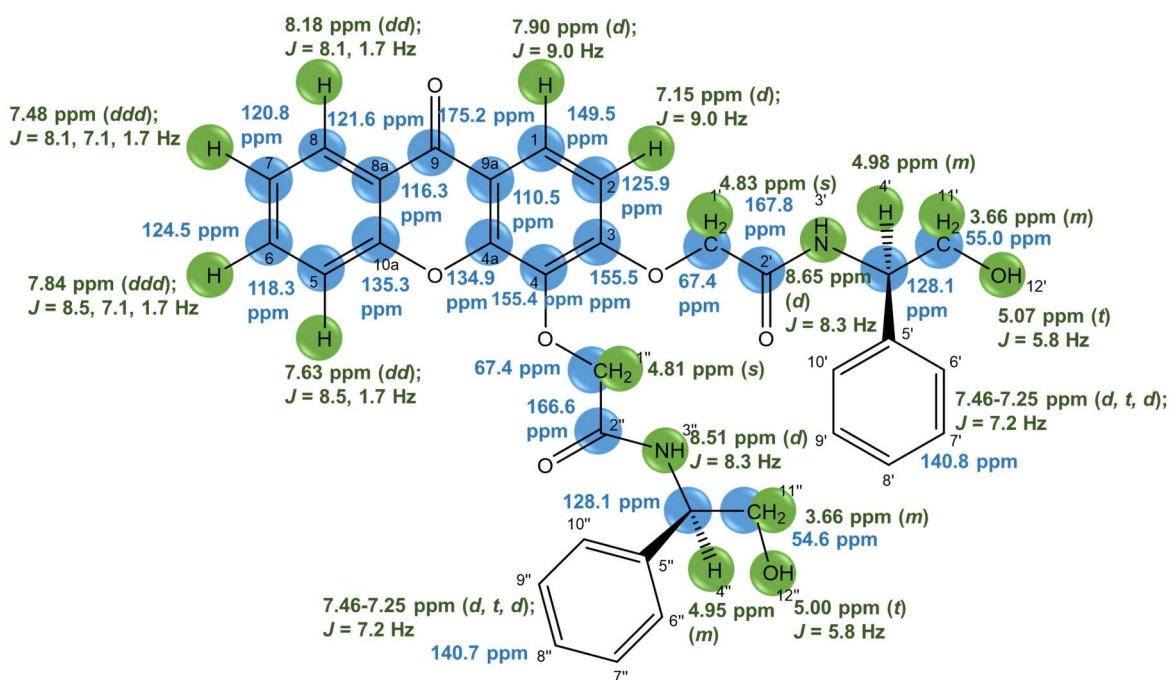


Figure 13 - ^1H and ^{13}C NMR values of compound **13**.

The values obtained from the IR analysis of compound **13** are presented on **Table 5**. Comparing the IR data with the spectrum of compound **12**, it is important to refer the presence of the bands with a shift at 3093.3 and 2940.9 cm⁻¹ representative of the alcohol and the bands with a shift at 3388.6, 3271.5 and 1668.2 cm⁻¹ representative of the amide group.

Table 5 – IR data of compound 13.

Bond	ν (cm ⁻¹)
C=O (xanthone)	1603.8
C-O-C (xanthone)	1218.6
C=C (aromatic)	1529.6, 1463.3, 831.8, 764.2
C-O (ether)	1048.5
CONH (amide)	3388.6, 3271.5, 1668.2
OH (primary alcohol)	3093.3, 2940.9

6.3.CSP3

6.3.1. Structure elucidation of methyl (S)-2-[[9-oxo-7-((1-phenylethyl) carbamoyl)-9H-xanthen-3-yl]oxy} acetate (**19**)

The acetate derivative (compound **19**) was obtained after a Williamson ether synthesis being its structure confirmed by ^1H and ^{13}C NMR (**Figure 14**) and IR analysis (**Table 6**).

Considering the data from ^1H NMR, it is highlighted the duplet with a chemical shift at 9.20 ppm, which integrate for one proton characteristic of the amide group. This proton was the most unshielded since it is linked directly to an electronegative atom and it is influence by the carbonyl and benzyl groups. A singlet with a chemical shift value at 3.74 ppm, which integrate for three protons, was representative of the ester group. These protons have the influence of the ether group, which has electron donating properties, and the carbonyl group with electron withdrawing properties.

From the ^{13}C NMR spectrum, it is important to refer the signal at 168.6 and 52.1 ppm that indicate the presence of the amide and ester groups.

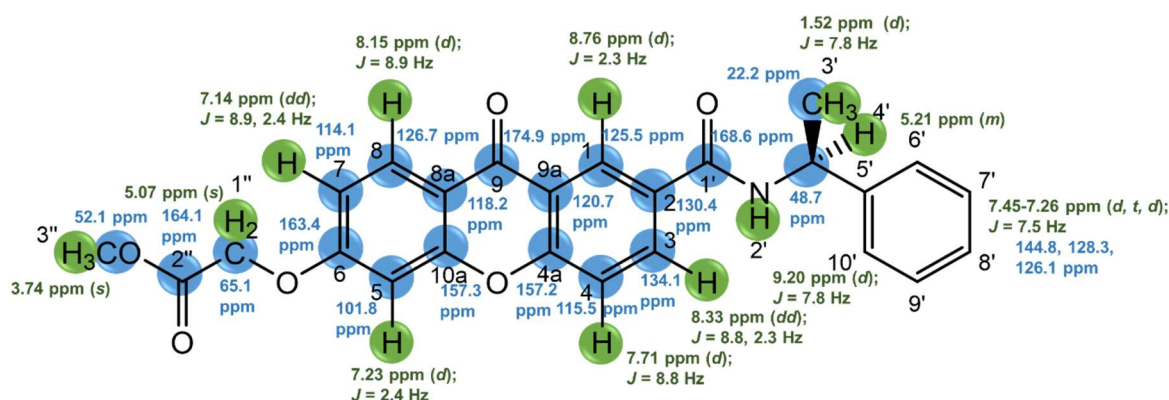


Figure 14 - ^1H and ^{13}C NMR values of compound **19**.

The values correspondent to IR analysis of compound **19** are presented on **Table 6**. From the analysis of the IR data, it is important to emphasize a band with a shift at 1759.3 cm^{-1} which represents the carbonyl of the ester group and the band with a shift at 1348.7 cm^{-1} from the methoxy of the ester. The bands with a shift at 3431.7, 3295.6 and 1648.4 cm^{-1} are indicative of the amide group.

Table 6 - IR data of compound **19**.

Bond	ν (cm ⁻¹)
C=O (xanthone)	1617.9
C-O-C (xanthone)	1213.5
C=C (aromatic)	1540.7, 1479.7, 828.6, 781.6
C-O (ether)	1067.7
CONH (amide)	3431.7, 3295.6, 1648.4
COOCH ₃ (ester)	1759.3, 1348.7

6.3.2. Structure elucidation of (S)-2-[[9-oxo-7-((1-phenylethyl)carbamoyl)-9H-xanthen-3-yl]oxy}acetic acid (**20**)

The compound **19** was hydrolysed through an alkaline hydrolysis allowing to obtain the compound **20**. Its structure was confirmed by ¹H and ¹³C NMR (**Figure 15**) and IR analysis (**Table 7**).

Comparing the ¹H and ¹³C NMR spectra of the compounds **19** and **20**, the absence of signals correspondent to the ester group in the spectrum of compound **20** was observed. In the ¹H NMR, a singlet with a chemical shift value at 1.20 ppm was observed which corresponding to a hydroxyl group of the carboxylic acid. These values were indicative of the success of the hydrolysis.

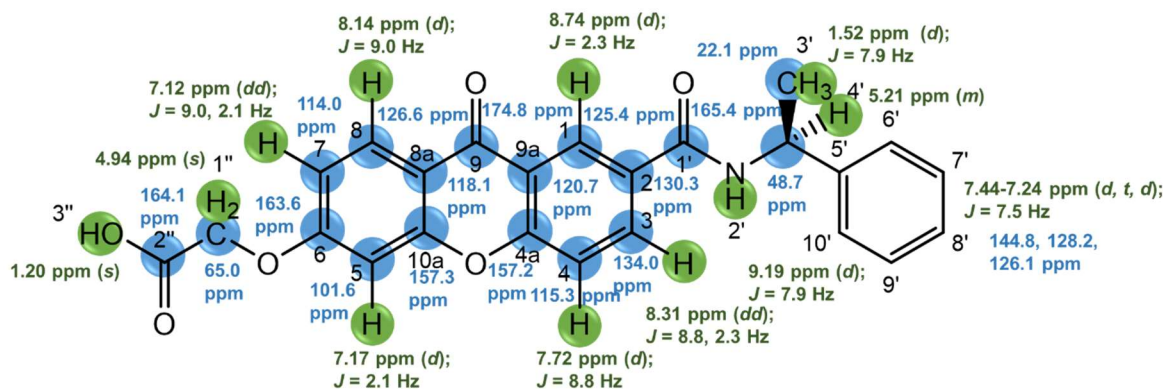


Table 7 - IR data of compound 20.

Bond	ν (cm ⁻¹)
C=O (xanthone)	1619.8
C-O-C (xanthone)	1236.6
C=C (aromatic)	1552.4, 1480.6, 830.9, 779.2
C-O (ether)	1065.8
CONH (amide)	3306.9, 1664.0
COOH (carboxylic acid)	3082.7, 2977.7, 1718.6

6.3.3. Structure elucidation of 6-{2-[[*(R)*-2-hydroxy-1-phenylethyl]amino]-2-oxoethoxy}-9-oxo-*N*-[(*S*)-1-phenylethyl]-9*H*-xanthene-2-carboxamide, CS3, (21)

The chiral selector **CS3** (**21**) was obtained after the coupling between the chiral moiety, *R*-(−)-2-phenylglycinol (**2**) with the compound **20**. The ¹H and ¹³C NMR (**Figure 16**) and IR analysis (**Table 8**) allowed to confirm its structure.

From the analysis of the ^1H NMR, it is important to highlight the presence of a duplet corresponding to the amide group ($\delta = 8.78$ ppm), which integrate for one proton and a singlet which integrated for one proton corresponding to the alcohol group ($\delta = 5.05$ ppm). It was found that, the chemical shift value of the proton of this second amide introduced was inferior than the previous one since it is shielder due to the presence of the alcohol and ether groups which have an electron donating capacity. These shifts were not present in the spectrum of the compound **20**.

In the ^{13}C NMR spectrum, a signal at 164.1 ppm was identified as originating by the amide from the chiral moiety introduced.

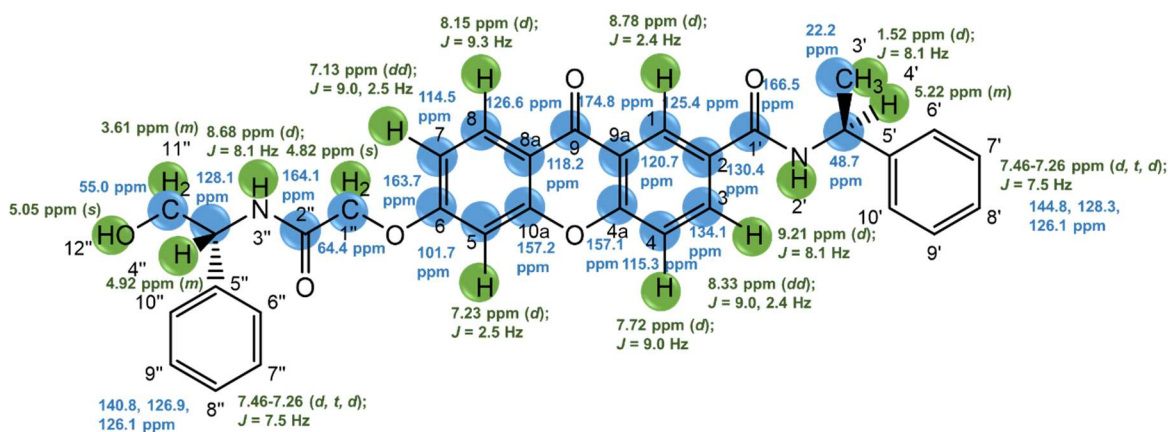


Figure 16 – ^1H and ^{13}C NMR values of compound **21**.

The values obtained from the IR analysis of compound **21** are presented on **Table 8**. From the IR data collected and comparing with the compound **20**, it is important to highlight the band with a shift at 1654.3 cm^{-1} representative of the carbonyl from the amide group, and the band with a shift at 3261.5 cm^{-1} from the alcohol.

Table 8 - IR data of compound 21.

Bond	ν (cm ⁻¹)
C=O (xanthone)	1616.5
C-O-C (xanthone)	1222.4
C=C (aromatic)	1471.4, 1140.4, 911.8, 763.0
C-O (ether)	1060.4
CONH (amide)	1654.3
OH (primary alcohol)	3261.5

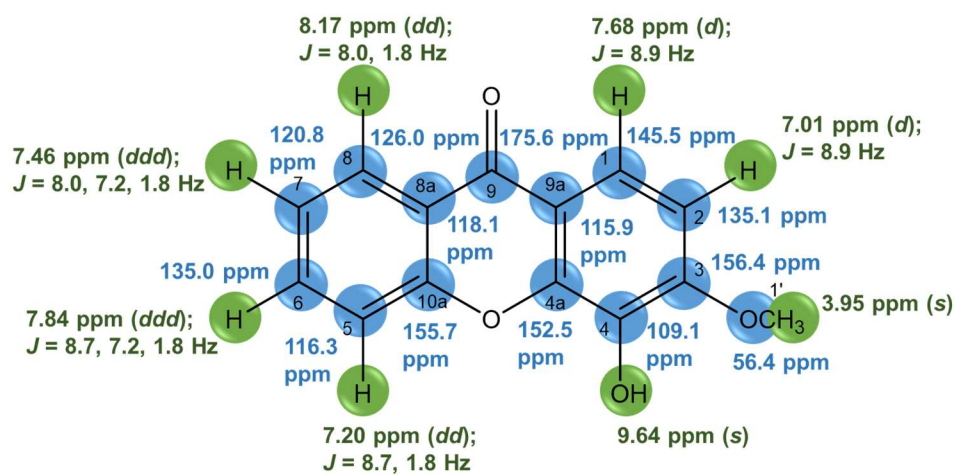
6.4. CSP4

6.4.1. Structure elucidation of 3-methoxy-4-hydro-9H-xanthen-9-one (8)

The 3-methoxy-4-hydro-9H-xanthen-9-one (**8**) was obtained through a multi-step pathway *via* benzophenone. The structure elucidation was performed by ¹H and ¹³C NMR (Figure 17) and IR analysis (Table 9).

Considering the data from the ¹H NMR, it is important to highlight the presence of one singlet which integrate for one proton with a chemical shift value at 9.64 ppm, corresponding to the proton of the phenol group, and another singlet which integrate for three protons (δ = 3.95 ppm) corresponding to the protons of the methoxy group. Comparing with the ¹H NMR data obtained from compound **10**, an extra chemical shift corresponding to the methoxy group was observed.

The analysis of the ¹³C NMR spectrum also revealed an extra signal at 56.4 ppm, relatively to compound **10**, representative of the methoxy group.

Figure 17 - ¹H and ¹³C NMR values of compound 8.

The values obtained after the IR analysis of compound **8** are presented on **Table 9**. From the analysis to the IR data, it is important to highlight a band with a shift at 3243.0 cm^{-1} representative of the phenol group and a band with a shift at 1287.0 cm^{-1} from the aromatic ether. This last band was not present in the IR spectrum of compound **10**.

Table 9 – IR data of compound 8.

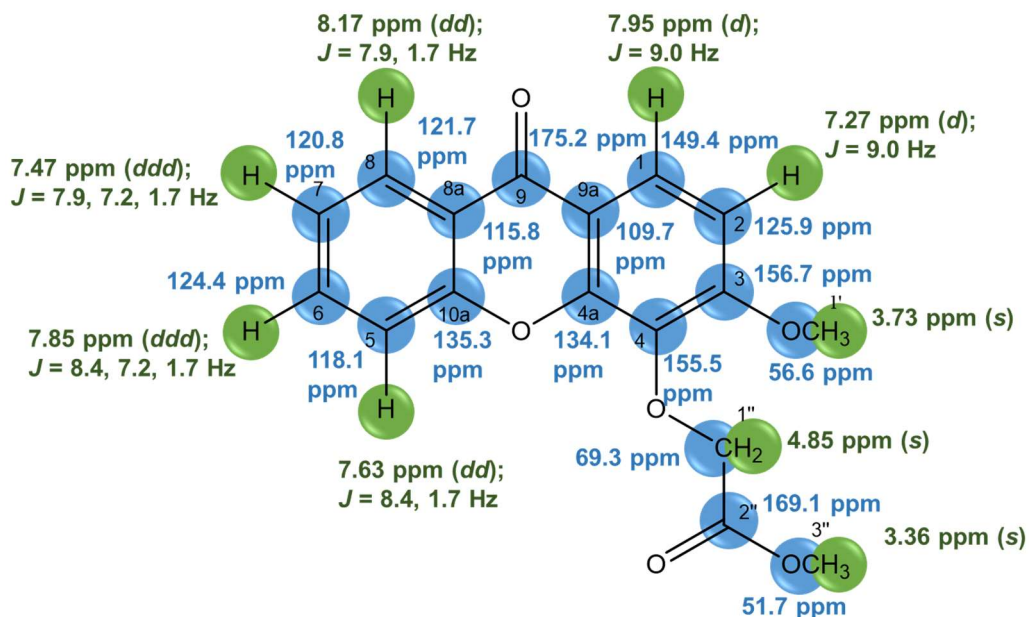
Bond	$\nu\text{ (cm}^{-1}\text{)}$
C=O (xanthone)	1605.6
C-O-C (xanthone)	1226.1
C=C (aromatic)	1515.4, 1465.9, 821.1, 759.4
OH (phenol)	3243.0
OCH ₃ (aromatic ether)	1287.0

6.4.2. Structure elucidation of methyl 2-[(3-methoxy-9-oxo-9*H*-xanthen-4-yl)oxy]acetate (**22**)

The acetate derivative (compound **22**) was obtained after a Williamson ether synthesis and its structure was confirmed by ^1H and ^{13}C NMR (**Figure 18**) and IR analysis (**Table 10**).

From the analysis of the ^1H NMR data, it is important to highlight the presence of a singlet with a chemical shift value at 3.36 ppm, which integrate for three protons, corresponding to the methoxy from the ester group. Comparing with the compound **8**, this additional chemical shift indicated the introduction of the desired chemical bridge. It could be referred that the value of the chemical shift of the proton at position 1 of the xanthone scaffold ($\delta = 7.95\text{ ppm}$) is higher than the chemical shift from the proton at position 5 ($\delta = 7.63\text{ ppm}$) since the presence of carbonyl group at *ortho* and *para* positions contributes for a superior unshielded due to its electron withdrawing properties.

Concerning the ^{13}C NMR data, the signal at 51.7 ppm, which was not present in the spectrum of the compound **8**, was indicative of the introduction of the desired chemical bridge.

Figure 18 - ^1H and ^{13}C NMR values of compound **22**.

The values correspondent to the IR analysis of compound **22** are presented on **Table 10**. It is important to refer at the bands with a shift at 1750.8 and 1388.0 cm^{-1} representative of the ester group. A band with a shift at 1033.5 cm^{-1} was also indicative of the introduction of the desired chemical bridge.

Table 10 – IR data of compound **22**.

Bond	ν (cm^{-1})
C=O (xanthone)	1603.4
C-O-C (xanthone)	1216.3
C=C (aromatic)	1509.1, 1464.6, 819.5, 767.6
C-O (ether)	1033.5
COOCH ₃ (ester)	1750.8, 1388.0
OCH ₃ (aromatic ether)	1288.2

6.4.3. Structure elucidation of 2-[(3-methoxy-9-oxo-9H-xanthene-4-yl)oxy]acetic acid (**23**)

An alkaline hydrolysis allowed to obtain an acidic derivative (compound **23**) from compound **22** being its structure elucidate resorting to ^1H and ^{13}C NMR (**Figure 19**) and IR (**Table 11**) analysis.

By the analysis of the spectrum of ^1H NMR and comparing with the spectrum of compound **22**, the absence of a singlet correspondent to the ester was confirmed. The

absence of a singlet correspondent to the hydroxyl group of the carboxylic acid was also revealed. This phenomenon could be explained by the possibility of intermolecular exchange that could occurred between the protons of hydroxyl and the solvent or even the possibility to establish intermolecular hydrogen-bonds¹³⁹.

Considering the ^{13}C NMR spectrum and, also, taken into account the spectrum of the compound **22**, the signal correspondent to the ester group was not present.

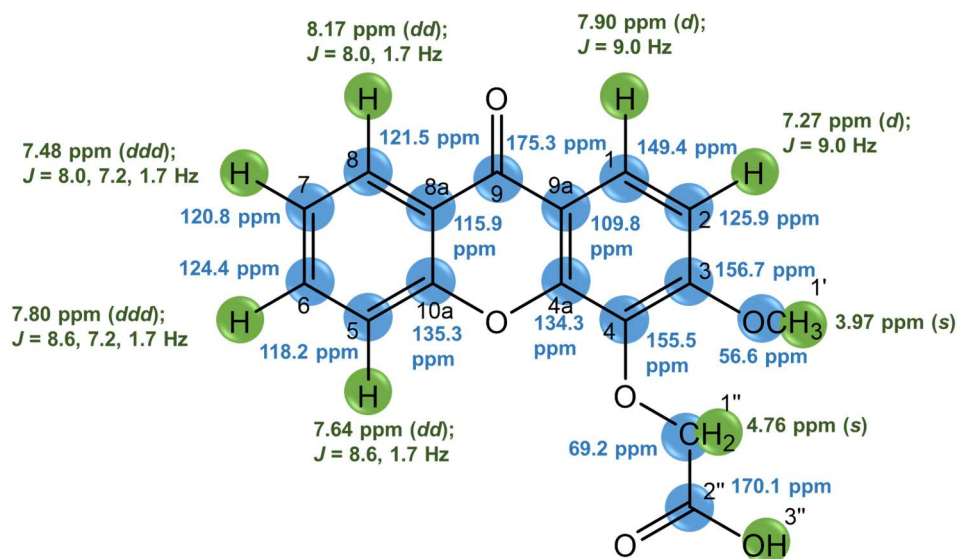


Figure 19 - ^1H and ^{13}C NMR values of compound **23**.

The values obtained from the IR analysis of compound **23** are present on **Table 11**. From the IR spectrum analysis, it is important to refer the bands with a shift at 3204.0, 2925.0 and 1758.6 cm^{-1} being representative of the carboxylic acid group which confirmed the efficiency of the alkaline hydrolysis.

Table 11 – IR data of compound **23**.

Bond	ν (cm^{-1})
C=O (xanthone)	1604.1
C-O-C (xanthone)	1229.6
C=C (aromatic)	1585.2, 1467.8, 819.1, 766.6
C-O (ether)	1033.1
COOH (carboxylic acid)	3204.0, 2925.0, 1758.6
OCH ₃ (aromatic ether)	1290.4

6.4.4. Structure elucidation of (S)-2-[(3-methoxy-9-oxo-9H-xanthen-4-yl)oxy]-N-(1-phenylethyl)acetamide (**24**)

The coupling reaction resorting to TBTU allowed the linkage of the chiral unit, (S)-(α)-methylbenzylamine (**16**) to the compound **23** obtaining the compound **24**. Its structure was elucidated by ^1H and ^{13}C NMR (**Figure 20**) and IR analysis (**Table 12**).

From the analysis of the ^1H NMR spectra of compound **24**, the chemical shift with a value at 8.38 ppm confirmed the presence of the amide group, which was not present on the spectrum of its precursor (compound **23**). It was found that the proton of the amide group is the most unshielded due to the influence of the carbonyl group and the presence of the nitrogen, an electronegative atom. The chemical shift with a value at 1.48 ppm, from the methyl group, was also indicative of the presence of the chiral unit.

Concerning the ^{13}C NMR spectrum, the signal at 167.1 and 22.1 ppm indicated the presence of the amide and the methyl group being these signals not presented on the spectrum of the compound **23**.

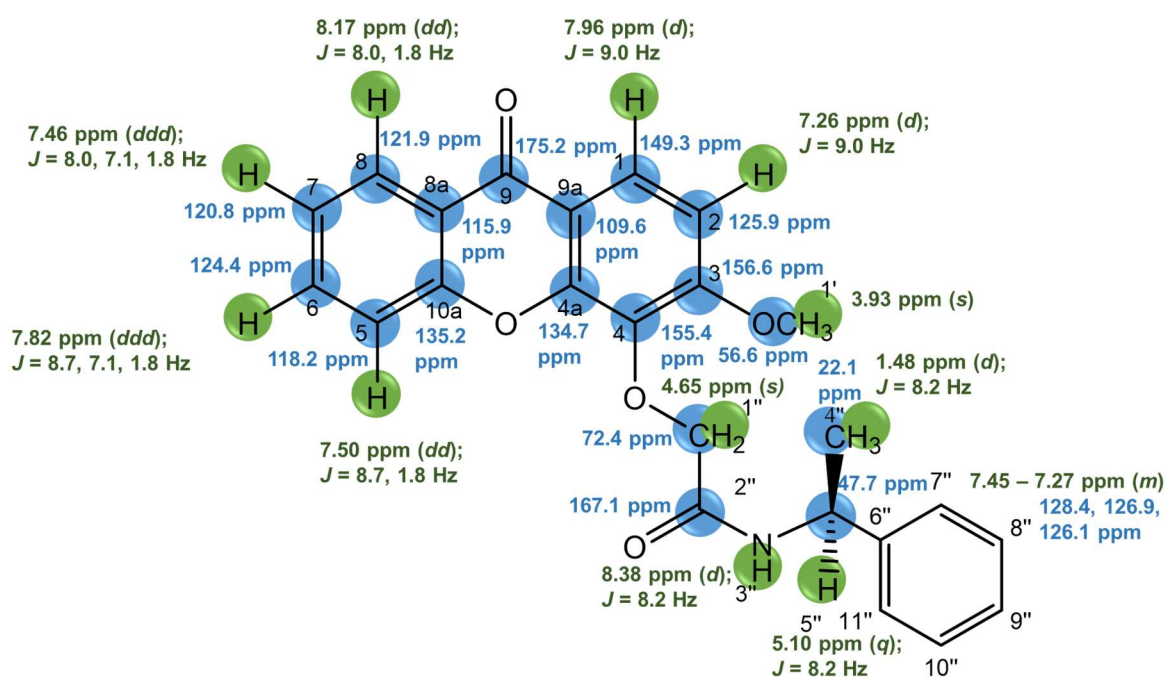


Figure 20 - ^1H and ^{13}C NMR values of compound **24**.

The values of the IR analysis of compound **24** are presented on **Table 12**. From the analysis of the IR spectrum, it is important to highlight bands with a shift at 3348.1 and 1665.1 cm^{-1} representative of the amide group which were not present on the spectrum of the compound **22**.

Table 12 – IR data of compound 23.

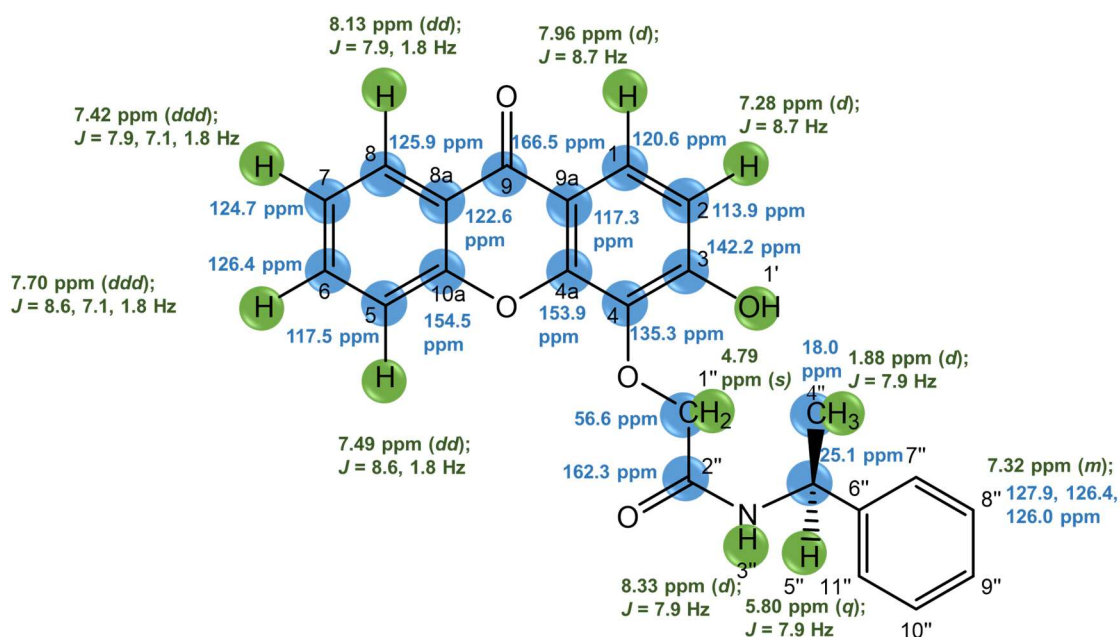
Bond	ν (cm ⁻¹)
C=O (xanthone)	1604.8
C-O-C (xanthone)	1230.2
C=C (aromatic)	1587.5, 1466.9, 817.7, 769.1
C-O (ether)	1023.4
CONH (amide)	3348.1, 1665.1
OCH ₃ (aromatic ether)	1289.1

6.4.5. Structure elucidation of (S)-2-[(3-hydroxy-9-oxo-9H-xanthen-4-yl)oxy]-N-(1-phenylethyl)acetamide (25)

The compound **25** was obtained after the demethylation of the methoxy group at position 3 of the xanthone scaffold of compound **24**. Its structure was confirmed by ¹H and ¹³C NMR (Figure 21) and IR analysis (Table 13).

The analysis of the spectrum of ¹H NMR of compound **25** and by comparison with the spectrum of its precursor (compound **24**) it was found the absence of a singlet characteristic of the methoxy group. Once again, a singlet corresponding to the phenol group was not observed.

From the ¹³C NMR spectrum and considering the spectrum from the compound **24**, the absence of the methoxy was also confirmed.

Figure 21 - ¹H and ¹³C NMR values of compound 25.

The values obtained from the IR analysis of compound **25** are presented on **Table 13**. By the analysis to the IR spectrum, it was observed the presence of a band with a shift at 1702.0 cm^{-1} representative of the amide group and a band with a shift at 3442.9 cm^{-1} indicative of the phenol group. This last band was not present on the IR spectrum of compound **24**.

Table 13 – IR data of compound 25.

Bond	$\nu\text{ (cm}^{-1}\text{)}$
C=O (xanthone)	1615.2
C-O-C (xanthone)	1220.9
C=C (aromatic)	1598.7, 1465.9, 834.4, 759.5
C-O (ether)	1032.5
CONH (amide)	1702.0
OH (phenol)	3442.9

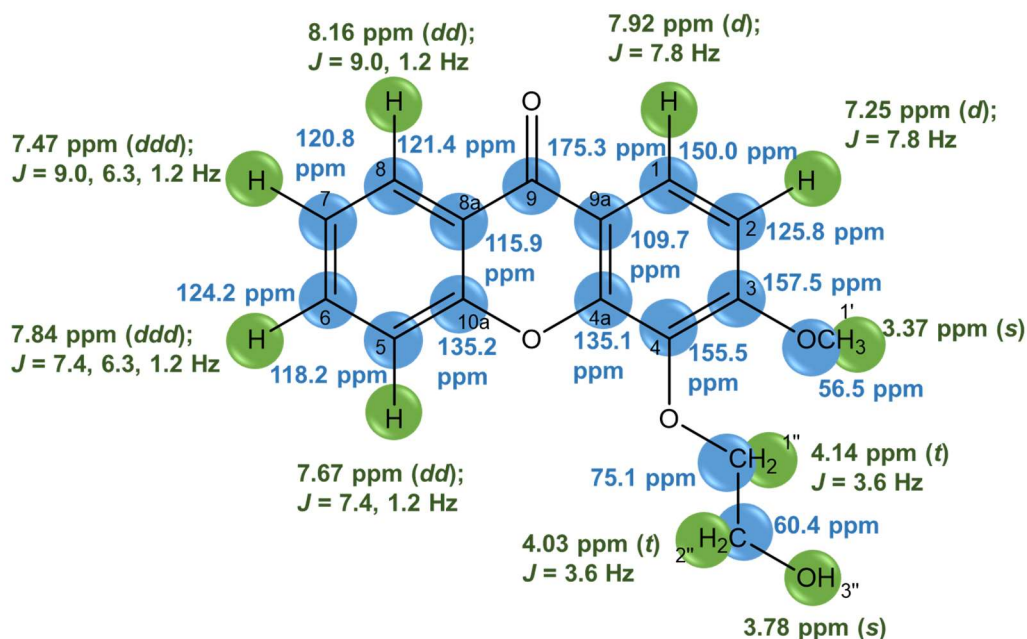
6.5. CSP5

6.5.1. Structure elucidation of 4-(hydroxymethoxy)-3-methoxy-9H-xanthen-9-one (26)

The alcohol derivative (compound **26**) was obtained by a Williamson ether synthesis and its structure was confirmed by ^1H and ^{13}C NMR (**Figure 22**) and IR analysis (**Table 14**).

The analysis of the ^1H NMR spectrum revealed the presence of two duplets with chemical shift values at 4.14 and 4.03 ppm which integrate for two protons each corresponding to the ethyl groups of the chemical bridge. A singlet with a chemical shift at 3.78 ppm indicated the presence of the alcohol group. These three shifts were not present on the spectrum of the compound **8** which confirmed the introduction of the desired chemical bridge. The difference in the chemical shift values of the H1'' and H2'' it is explained by the electron donating property of the alcohol. The proximity of the H2'' to the alcohol was reflected on the lower chemical shift value ($\delta = 4.03\text{ ppm}$) relatively to H2''.

Concerning the ^{13}C NMR spectrum, the signals at 75.1 and 60.4 ppm were also indicative of the introduction of the desired chemical bridge since these signals were not present on the spectrum of the compound **8**.

Figure 22 - ^1H and ^{13}C NMR values of compound **26**.

The values correspondent to the IR analysis of compound **26** are presented on **Table 14**. From the analysis to the IR data, it is important to emphasize the presence of a band with a shift at 3423.2 cm^{-1} representative of the alcohol group. A band with a shift at 1068.1 cm^{-1} was also indicative of the introduction of the desired chemical bridge since it belongs to the aliphatic ether. These bands were not observed on the IR spectrum of its precursor (compound **8**).

Table 14 – IR data of compound **26**.

Bond	$\nu\text{ (cm}^{-1}\text{)}$
C=O (xanthone)	1602.0
C-O-C (xanthone)	1224.5
C=C (aromatic)	1467.0, 905.5, 823.5, 760.4
OCH ₃ (aromatic ether)	1290.5
OCH ₂ (aliphatic ether)	1068.1
OH (primary alcohol)	3423.2

6.5.2. Structure elucidation of 2-[(3-methoxy-9-oxo-9H-xanthen-4-yl)oxy]ethyl (*R*)-2-(3,4-dinitrobenzamido)-2-phenylacetate (**28**)

After the introduction of the chemical bridge, a coupling reaction was performed to introduce the first chiral moiety, the (*R*)-(-)-*N*-(3,5-dinitrobenzoyl)- α -phenylglycine (**27**). The structure of the compound **28** was elucidated by ^1H and ^{13}C NMR (**Figure 23**) and IR analysis (**Table 15**).

By comparing the ^1H NMR spectrum of compounds **26** and **28**, it was observed that the chemical shifts with the values at 9.02, 8.90 and 7.61 ppm were not present at the spectrum of compound **26**. These chemical shifts confirmed the introduction of the chiral moiety since they correspond to the protons next to the nitro, amide and benzyl groups, respectively. Since the ester and the amide groups on side chain of the position 4 have electron-donating properties the values of the chemical shifts of H1'' and H2'' are lower in comparison with compound **26**. The highest value of chemical shift belongs to the protons next to the dinitrobenzyl group since the nitro groups presents electron-withdrawing properties.

From the ^{13}C NMR spectrum, the introduction of the chiral moiety was also confirmed by the presence of new signs when comparing with its precursor (compound **26**).

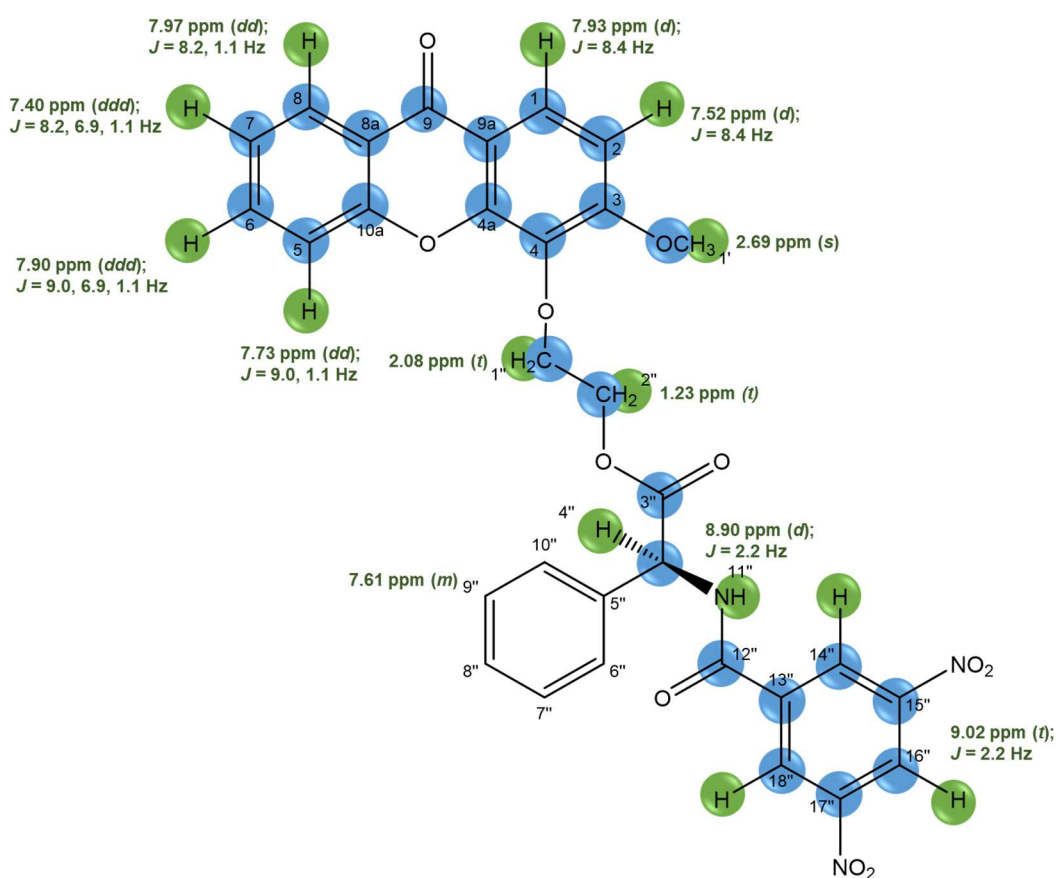


Figure 23 - ^1H and ^{13}C NMR values of compound **28**.

The values correspondent to the IR analysis of compound **28** are presented on **Table 15**. Concerning the IR data, it is important to refer the bands with a shift at 423.7 and 408.9 cm^{-1} representative of the nitro groups. The presence of bands with a shift at 1741.6 and 3454.8 cm^{-1} were also indicative of the introduction of the chiral unit since they correspond to the ester and amide groups, respectively. These bands were not observed on the IR spectrum of the compound **26**.

Table 15 - IR data of compound 28.

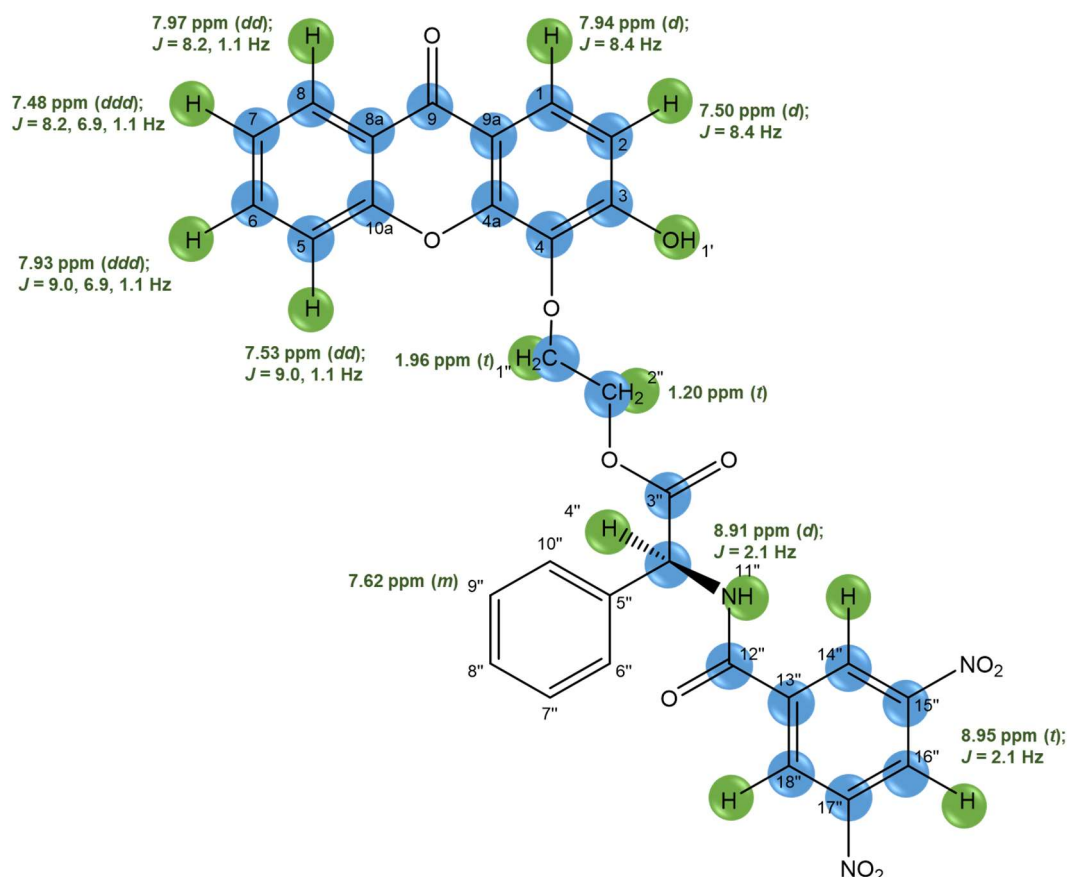
Bond	ν (cm ⁻¹)
C=O (xanthone)	1630.1
C-O-C (xanthone)	1219.4
C=C (aromatic)	1471.6, 923.5, 808.5, 777.9
OCH ₃ (aromatic ether)	1288.0
OCH ₂ (aliphatic ether)	1077.2
COOCH ₃ (ester)	1741.6, 1382.6
CONH (amide)	3454.8, 1685.7
NO ₂ (nitro group)	423.7, 408.9

6.5.3. Structure elucidation of 2-[(3-hydroxy-9-oxo-9H-xanthen-4-yl)oxy]ethyl (R)-2-(3,4-dinitrobenzamido)-2-phenylacetate (29)

Compound **28** was subjected to a demethylation of methoxy group on position 3 of xanthone scaffold resulting on compound **29**. Its structure was elucidated by ¹H and ¹³C NMR (**Figure 24**) and IR analysis (**Table 16**).

By analysis of the spectrum of ¹H NMR of compound **29** and comparing with the spectrum of the compound **28**, the absence of the singlet correspondent to the methoxy group was confirmed.

From the ¹³C NMR spectrum and considering the spectrum from the compound **28**, the absence of the methoxy was also confirmed.

Figure 24 - ¹H and ¹³C NMR values of compound **29**.

The values correspondent to the IR analysis of compound **29** are presented on **Table 16**. Considering the IR data, the presence of a band with a shift at 1291.9 cm⁻¹ is representative of the phenol group being indicative of the demethylation of the aromatic ether. The presence of this band was not observed on the IR spectrum of the compound **28**.

Table 16 - IR data of compound **29**.

Bond	ν (cm ⁻¹)
C=O (xanthone)	1634.2
C-O-C (xanthone)	1179.7
C=C (aromatic)	1453.8, 931.7, 718.8, 707.4
OH (phenol)	1291.9
OCH ₂ (aliphatic ether)	1072.6
COOCH ₃ (ester)	1702.3, 1398.2
CONH (amide)	3444.7, 1683.0
NO ₂ (nitro group)	552.3, 480.3

8. Loading of the chiral selectors

For each CSP prepared (**CSP1-CSP3**), the loading of chiral selector was calculated according to the equation presented below¹⁴⁰. The values obtained from the elemental analysis, specifically the percentage of carbon were used for loading determination, being expressed in μmolm^{-2} (**Table 17**). This parameter indicates the quantity of selector bonded *per* area of the surface of silica particles. The loading selector can influence the enantiorecognition, the selectivity, the resolution and the time of analysis¹⁴¹.

$$\text{Loading} = \frac{10^6 \times \%C}{S[(100 \times n \times 12) - (\%C \times M_w)]} \quad (1)$$

The %C refers the percentage of carbon atoms of the selector while n is the number of carbon atoms; S is the surface area of silica particles and M_w is the relative molecular mass of the selector¹⁴⁰.

The values of selector loading obtained for **CSP1**, **CSP2** and **CSP3** were 1.50, 1.00 and 0.32 μmolm^{-2} , respectively

Table 17 - Elemental analysis data from **CSP1**, **CSP2** and **CSP3**.

	CSP1			CSP2			CSP3		
	%C	%H	%N	%C	%H	%N	%C	%H	%N
Real	17.5%	2.67%	3.81%	13.1%	2.02%	2.55%	4.73%	1.02%	0.88%
Theory	56.0%	5.85%	5.81%	56.0%	5.85%	5.81%	62.7%	5.68%	5.77%
Loading (μmolm^{-2})	1.50			1.00			0.32		

The majority of the CSPs prepared was covalently bonded to silica gel within appropriate levels (1.00-1.50 μmolm^{-2}) when compared to previous CSPs¹⁰². The silica gel in **CSP3** contain about 0.32 μmolm^{-2} . The lower value of the loaded chiral selector in **CSP3** could be explained by the fact that the covalent linkage to the silica was performed through one side.

B. Evaluation of enantioresolution performance by liquid chromatography

A preliminary evaluation of the LC enantioresolution for all prepared CSPs (**CSP1-CSP3**) was performed using five commercial standard racemates (**Figure 25**) and thirty-one enantiomeric mixtures of CDXs synthesized “in house” (**Figure 26**).

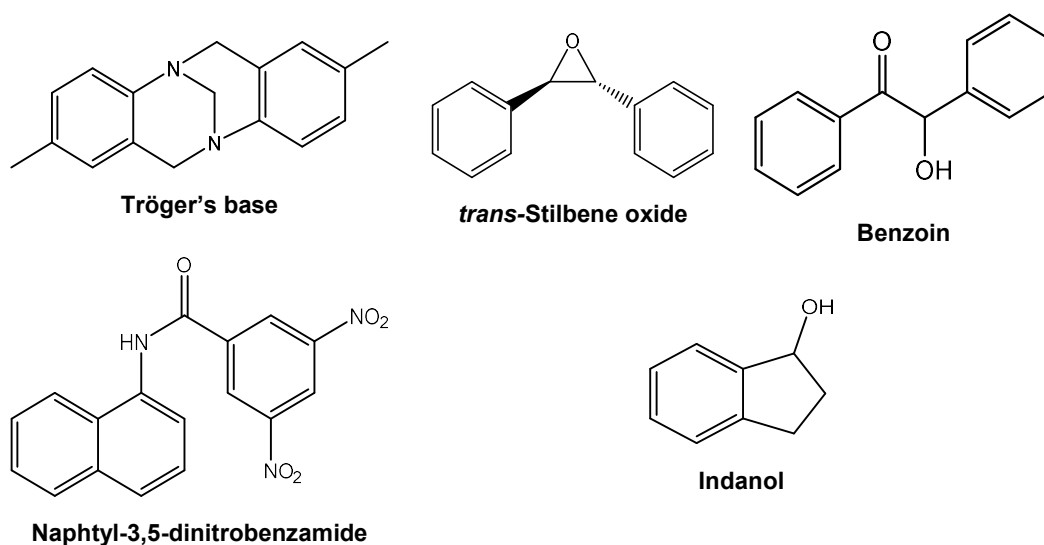


Figure 25 - Chemical structures of the commercial analytes tested.

For all the analytes tested, the chromatographic parameters including the retention factor (k), enantioseparation factor (α) and resolution (R_s) were calculated, according to the equations described in Experimental section. The theoretical number plates (N) of **CSP1-CSP3** were also calculated for the analytes which presented the best chromatographic data. The performance of all CSPs was tested under normal phase elution conditions using different proportions of *n*-Hex with 2-PrOH or EtOH as mobile phases. The flow rate chosen was 0.2 mLmin^{-1} , the temperature of analysis was the room temperature and UV detection at 254 nm was chosen.

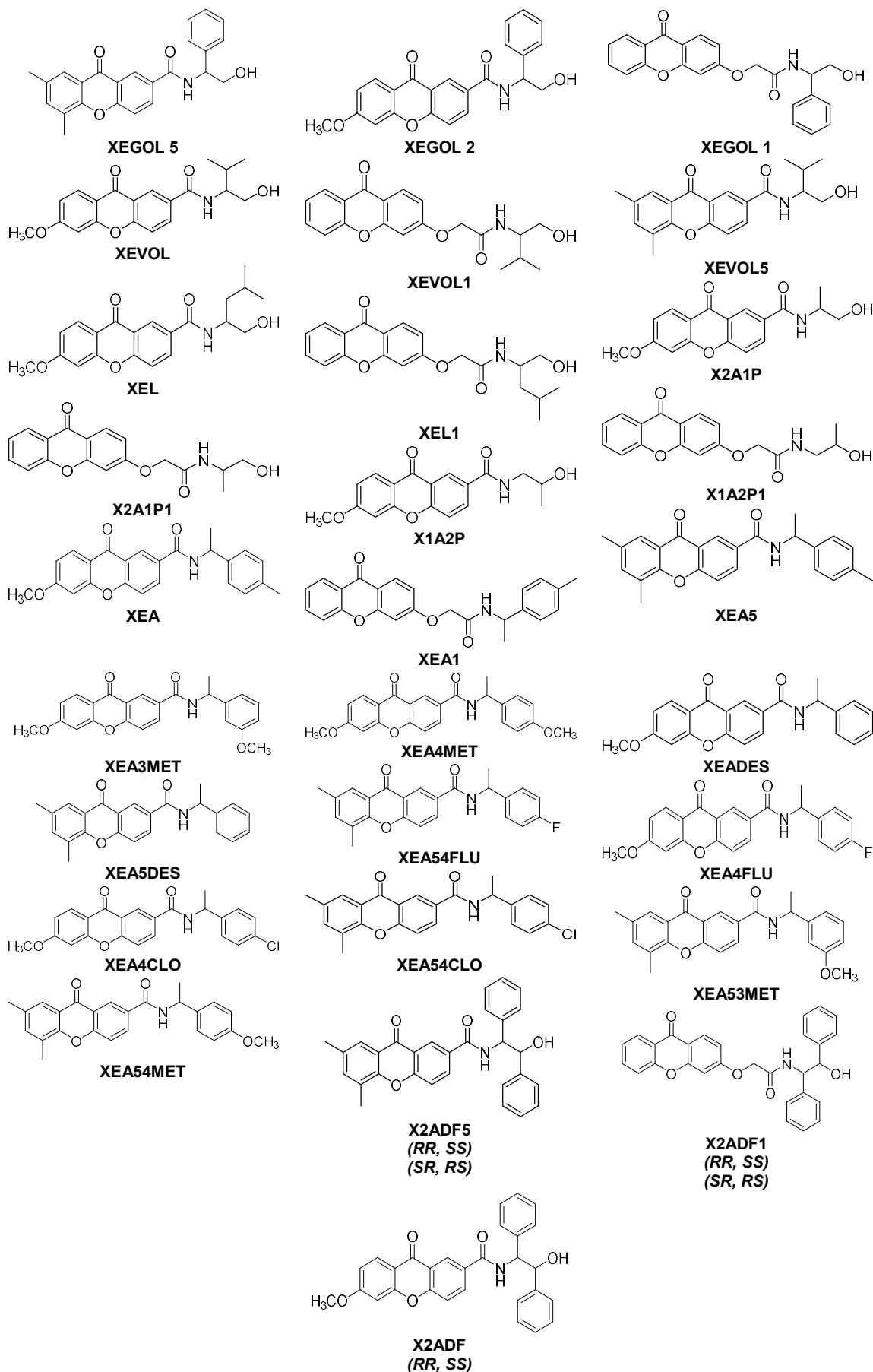


Figure 26 - Chemical structures of the enantiomeric mixtures of CDXs prepared "in house".

1. Enantioresolution performance of CSP1

The LC performance of **CSP1** was evaluated using as mobile phase *n*-Hex:EtOH (80:20 v/v), for a preliminary evaluation (**Table 19**).

Among all the chiral analytes (**Figure 25** and **26**), the **CSP1** presented enantiorecognition and baseline separation for two enantiomeric pairs of CDXs, XEADES ($\alpha = 2.29$ and $R_s = 1.33$) and XEVOL5 ($\alpha = 3.33$ and $R_s = 1.76$) (**Table 19**). For these two enantiomeric pairs, in order to improve the chromatographic data, three additional mobile phases were tested, specifically *n*-Hex:EtOH 70:30 v/v, *n*-Hex:2-PrOH 80:20 v/v and 70:30 v/v) being these results presented on **Table 18**. It was found that the mobile phase *n*-Hex:EtOH (70:30 v/v) allowed to obtain better results, namely, an improved enantioseparation factor with a reduced time of analysis. The analyte XEVOL5 presented the best resolution ($R_s = 1.76$) for the mobile phase *n*-Hex:2-PrOH (80:20 v/v) and the best enantioselectivity ($\alpha = 3.48$) was obtained for the same analyte with the mobile phase *n*-Hex:EtOH (70:30 v/v). For this analyte is presented a chromatogram in **Figure 27**. The enantiomeric pair XEADES also presented enantiorecognition being the best results obtained with *n*-Hex:2-PrOH (80:20 v/v) as mobile phase since for the same enantioseparation factor ($\alpha = 2.29$), the time analysis was inferior.

Table 18 - Chromatographic parameters for enantiomeric pairs XEVOL5 and XEADES on **CSP1** using different mobile phases.

	<i>n</i> -Hex:EtOH 70:30 v/v			<i>n</i> -Hex:2-PrOH 80:20 v/v			<i>n</i> -Hex:2-PrOH 70:30 v/v		
	k_1	α	R_s	k_1	α	R_s	k_1	α	R_s
XEVOL5	1.06	3.48	1.64	3.28	2.24	1.76	1.10	3.01	1.59
XEADES	1.44	2.16	1.11	1.82	2.29	1.09	1.73	2.08	1.08

The number of theoretical plates was 175 for the analyte XEVOL5 based on the results obtained for the mobile phase *n*-Hex:EtOH (80:20 v/v).

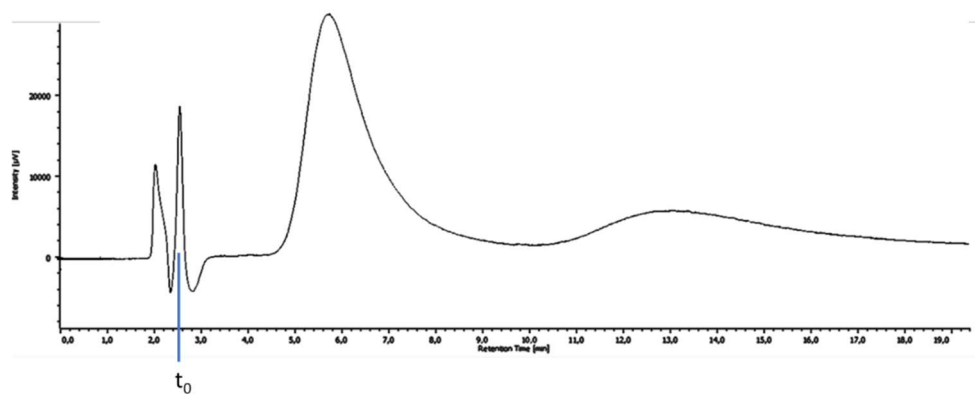


Figure 27 - Chromatogram for XEVL5 on **CSP1** using *n*-Hex:EtOH 80:20 v/v.

2. Enantioresolution performance of CSP2

A preliminary evaluation of the LC enantiorecognition performance of **CSP2** was performed using *n*-Hex:EtOH (80:20 v/v) as mobile phase. The results are presented on **Table 19**.

Table 19 - Enantioresolution performance of CSP1 and CSP2.

	CSP1			CSP2		
	<i>k</i> ₁	α	<i>R</i> _s	<i>k</i> ₁	α	<i>R</i> _s
XEGOL5	8.49	1.00	-	4.76	1.00	-
XEGOL2	10.4	1.00	-	0.59	1.00	-
XEGOL1	18.2	1.00	-	7.11	1.00	-
XEVOL	-	-	-	3.56	1.00	-
XEVOL1	18.1	1.00	-	3.32	1.00	-
XEVOL5	1.75	3.33	1.74	2.08	2.37	2.91
XEL	5.34	1.00	-	2.96	1.00	-
XEL1	5.32	1.00	-	2.93	1.00	-
X2A1P	12.7	1.00	-	5.66	1.00	-
X2A1P1	3.31	1.00	-	5.02	1.00	-
X1A2P	12.0	1.00	-	5.25	1.00	-
X1A2P1	11.1	1.00	-	4.82	1.00	-
XEA	5.90	1.00	-	2.77	1.00	-
XEA5	2.83	1.00	-	1.76	1.00	-
XEA3MET	10.5	1.00	-	4.64	1.00	-
XEA4MET	10.8	1.00	-	3.41	1.00	-
XEADES	2.09	2.29	1.33	2.13	1.61	1.61
XEA54FLU	3.31	1.00	-	1.93	1.00	-
XEA4FLU	6.96	1.00	-	2.71	1.00	-
XEA5DES	3.54	1.00	-	1.90	1.00	-
XEA4CLO	6.28	1.00	-	0.85	1.00	-
XEA53MET	4.98	1.00	-	2.64	1.00	-
XEA54MET	4.67	1.00	-	2.71	1.00	-
XEA54CLO	-	-	-	1.81	1.00	-
X2ADF5 SRRS	8.09	1.00	-	4.73	1.00	-
X2ADF5 RRSS	18.9	1.00	-	8.54	1.00	-
X2ADF1 SRRS	6.45	1.00	-	6.54	1.00	-
X2ADF1 RRSS	-	-	-	6.56	1.00	-
X2ADF RRSS	-	-	-	8.59	1.00	-
Bis XEVOL1	6.45	1.00	-	0.11	1.00	-
Transtilbene oxide	0.46	1.00	-	0.38	1.00	-
Benzoin	1.84	1.00	-	1.19	1.00	-
Naphthyl benzamide	5.87	1.00	-	-	-	-
Tröger's base	0.45	1.00	-	0.41	1.00	-
Indanol	0.94	1.00	-	-	-	-

Similar results were obtained for **CSP2**, compared with **CSP1**, since the same enantiomeric pairs of CDXs were enantioseparated, i.e. XEADES and XEVOL5, using *n*-Hex:EtOH (80:20 v/v) as mobile phase. For some analytes, additional mobile phases were tested (*n*-Hex:2-PrOH 90:10 and 80:20 v/v) to improve their chromatographic parameters being the results presented on **Table 20**. The mobile phase *n*-Hex:2-PrOH 80:20 v/v afforded an improved enantioselectivity with lower retention time. Nevertheless, the best resolution ($R_s = 2.91$) was achieved for enantiomeric pair XEVOL5 using the mobile phase *n*-Hex:EtOH (80:20 v/v) being its chromatogram presented in **Figure 28**. The enantiomeric pair XEADES also presented enantio recognition with a good resolution ($R_s = 1.48$) and enantioselectivity ($\alpha = 1.61$). For the mobile phase *n*-Hex:2-PrOH 80:20 v/v, the enantioselectivity was superior ($\alpha = 1.81$) combined with an improved retention factor ($k_1=2.01$).

Table 20 - Chromatographic parameters for enantiomeric pair XEVOL5 and XEADES on **CSP2** using different mobile phases.

	<i>n</i> -Hex:2-PrOH 90:10 v/v			<i>n</i> -Hex:2-PrOH 80:20 v/v		
	k_1	α	R_s	k_1	α	R_s
XEGOL2	9.18	1.00	-	-	-	-
XEVOL5	-	-	-	1.65	2.98	2.54
XEADES	-	-	-	2.01	1.81	1.48
Bis XEVOL1	0.49	1.00	-	-	-	-
Transtilbene oxide	0.44	1.00	-	-	-	-
Benzoin	1.91	1.00	-	-	-	-
Tröger's base	0.41	1.00	-	-	-	-

The number of theoretical plates was 271 and calculated taking into account the chromatographic parameters of XEVOL5 in the mobile phase *n*-Hex:EtOH (80:20 v/v).

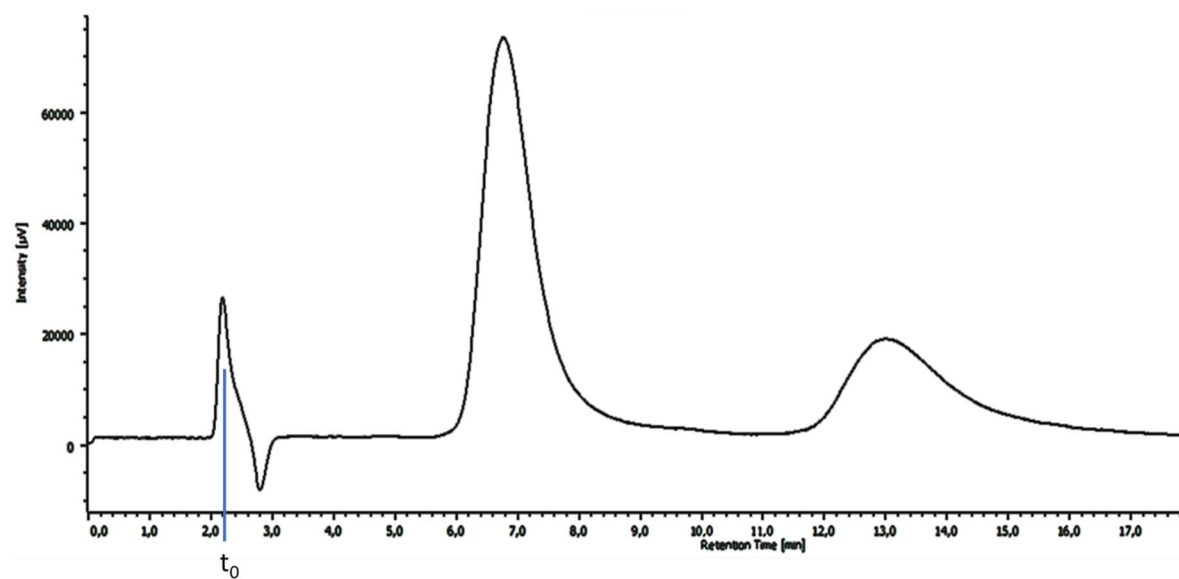


Figure 28 – Chromatogram for analyte XEVOL5 on **CSP2** using *n*-Hex:EtOH 80:20 v/v.

3. Enantioresolution performance of CSP3

A preliminary LC evaluation of the performance of **CSP3** was performed using the mobile phase *n*-Hex:EtOH 80:20 v/v, being the results presented on **Table 21**.

Table 21 - Enantioresolution performance of CSP3.

	<i>n</i> -Hex:EtOH 80:20 v/v			<i>n</i> -Hex:EtOH 90:10 v/v			<i>n</i> -Hex:2-PrOH 90:10 v/v		
	<i>k</i> ₁	α	<i>R</i> _s	<i>k</i> ₁	α	<i>R</i> _s	<i>k</i> ₁	α	<i>R</i> _s
XEGOL5	1.21	1.00	-	2.01	1.00	-	-	-	-
XEGOL2	0.68	3.62	2.29	0.69	6.84	5.04	0.54	16.4	5.45
XEGOL1	2.62	1.00	-	4.98	1.00	-	-	-	-
XEVOL	1.76	1.00	-	3.10	1.00	-	-	-	-
XEVOL1	1.91	1.00	-	3.38	1.00	-	-	-	-
XEVOL5	0.89	1.00	-	1.32	1.42	<1.00	1.88	1.41	<1.00
XEL	1.42	1.00	-	2.45	1.00	-	-	-	-
XEL1	1.58	1.00	-	2.79	1.00	-	-	-	-
X2A1P	2.77	1.00	-	4.88	1.00	-	-	-	-
X2A1P1	3.20	1.00	-	5.53	1.00	-	-	-	-
X1A2P	2.68	1.00	-	4.67	1.00	-	-	-	-
X1A2P1	3.04	1.00	-	5.21	1.00	-	-	-	-
XEA	1.13	1.00	-	1.75	1.00	-	-	-	-
XEA1	1.21	1.00	-	1.74	1.00	-	-	-	-
XEA5	0.57	1.00	-	0.76	1.00	-	0.55	1.00	-
XEA3MET	1.6	1.00	-	2.66	1.00	-	-	-	-
XEA4MET	1.62	1.00	-	2.74	1.00	-	-	-	-
XEADES	0.64	1.91	1.36	0.88	2.31	1.73	0.77	4.15	6.10
XEA54FLU	0.62	1.00	-	0.85	1.00	-	0.72	1.00	-
XEA4FLU	1.23	1.00	-	1.97	1.00	-	-	-	-
XEA5DES	0.67	1.00	-	0.84	1.00	-	0.83	1.00	-
XEA4CLO	1.17	1.00	-	1.87	1.00	-	-	-	-
XEA53MET	0.80	1.00	-	1.18	1.00	-	-	-	-
XEA54MET	0.81	1.00	-	1.26	1.00	-	-	-	-
XEA54CLO	0.62	1.00	-	0.82	1.00	-	0.62	1.00	-
X2ADF5 SRRS	1.05	1.00	-	1.75	1.00	-	-	-	-
X2ADF5 RRSS	2.19	1.00	-	4.20	1.00	-	-	-	-
X2ADF1 SRRS	1.81	1.00	-	3.39	1.00	-	-	-	-
X2ADF1 RRSS	1.83	1.00	-	-	1.00	-	-	-	-
X2ADF RRSS	2.21	1.00	-	4.19	1.00	-	-	-	-
Bis XEVOL1	0.64	1.00	-	-	1.00	-	-	-	-
Transtilbene oxide	3.83	1.00	-	0.20	1.00	-	-	-	-
Benzoin	0.66	1.00	-	0.74	1.00	-	-	-	-
Naphthyl benzamide	0.95	1.00	-	1.64	1.00	-	-	-	-
Tröger's base	0.65	1.00	-	0.55	1.00	-	-	-	-
Indanol	0.47	1.00	-	0.57	1.00	-	-	-	-

CSP3 presented enantiorecognition for three analytes (XEADES, XEVOL5 and XEGOL2) although a baseline separation was just achieved for two of them (XEADES and XEGOL2). For some enantiomeric pairs of CDXs, two additional mobile phases were tested, specifically *n*-Hex:EtOH and *n*-Hex:2-PrOH 90:10 v/v to improve their chromatographic parameters.

In order to attempt a baseline separation of analyte XEVOL5, an additional mobile phase was tested (*n*-Hex:EtOH 95:5 v/v) at different flow rates (**Table 22**). The reduction on the flow rate resulted on the loss of enantioselectivity.

Table 22 – Chromatographic parameters for XEVOL5 on **CSP3** with different flow rates.

Flow rate (mLmin ⁻¹)	k ₁	α	R _s
0.15	1.53	1.47	<1.00
0.10	2.81	1.39	<1.00
0.05	6.67	1.33	<1.00

Concerning the compounds with baseline separation (XEADES and XEGOL2), with the mobile phase *n*-Hex:2-PrOH 90:10 v/v, the enantioselectivity and resolution obtained were superior. The best resolution (R_s = 6.10) was obtained for analyte XEADES, and the best enantioselectivity (α = 16.4) was achieved for analyte XEGOL2.

The analyte XEADES presented values similar to those obtained with **CSP1** and **CSP2**, for resolution (R_s = 1.36) and enantioselectivity (α = 1.91) with the mobile phase *n*-Hex:EtOH (80:20 v/v). A chromatogram obtained for this analyte in **CSP3** is presented in **Figure 29**. The mobile phase *n*-Hex:2-PrOH 90:10 v/v also afforded the conditions to obtain the best results for this analyte (R_s = 6.10 and α = 4.15).

The number of theoretical plates was calculated taken into consideration the chromatographic parameters obtained for analyte XEGOL2 with the mobile phase *n*-Hex:2-PrOH 90:10 v/v being the number obtained 173.

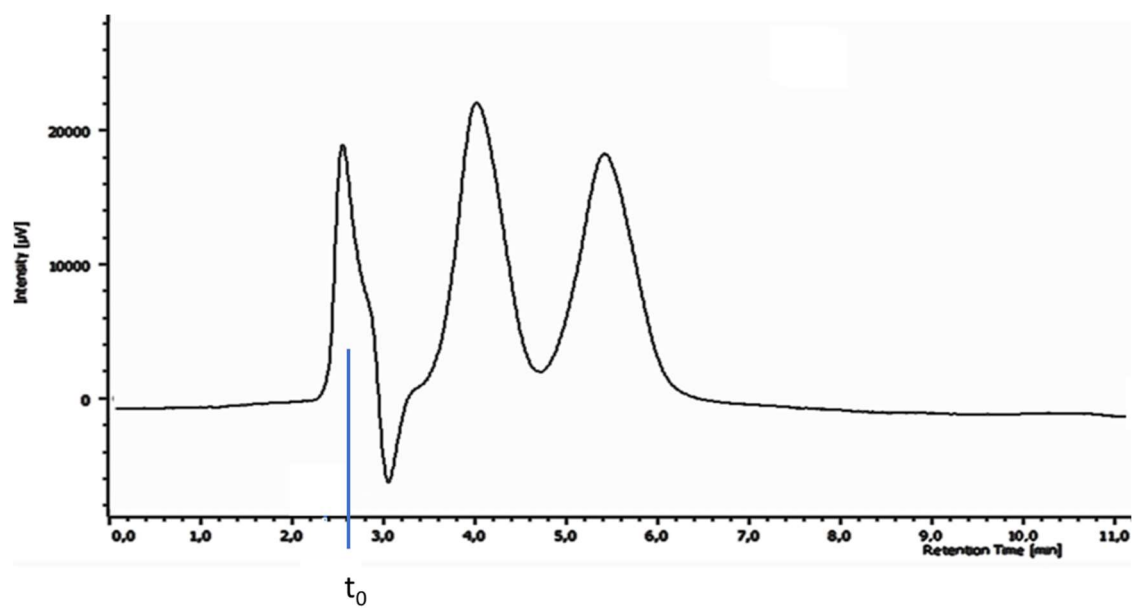


Figure 29 - Chromatogram for XEADES on **CSP3** using *n*-Hex:EtOH 80:20 v/v.

4. Comparison of the enantioresolution performance of the evaluated CSPs

Taken into consideration the selector loading values and the chromatographic data obtained, the enantioresolution performance of **CSP1-CSP3** can be compared.

The **CSP2** presented a superior selector loading ($1.00 \mu\text{molm}^{-2}$) relatively to the **CSP3** ($0.32 \mu\text{molm}^{-2}$) being a factor that may influence the chromatographic parameters. The selector loading of **CSP1** was the highest ($1.50 \mu\text{molm}^{-2}$). A higher selector loading may be reflected on a superior retention factor, an improve selectivity¹⁴¹, resolution and efficiency¹⁴².

The structure of the CSs has major importance on the results obtained in the evaluation of the enantioresolution performance of the CSPs. The **CSP3** presented the most promising results with superior values for resolution and enantioselectivity and reduced retention factors. This CSP also demonstrated enantiorecognition for three compounds while the other CSPs had enantiorecognition for two compounds. From the comparison between the results for **CSP1** and **CSP2**, the main conclusions were that **CSP1** exhibited higher enantioselectivity, for XEVOL5 and XEADES, while for **CSP2** the resolution and retention factors were greater.

Analysing this behaviour and considering the structure of each CSP, the main conclusions were that the presence of chiral units at opposite positions (**CS3**) contribute for a superior enantioresolution performance. This scaffold contains chiral units with different properties which contributes to establish interactions with a wide variety of analytes. The position of these units also potentiated the interactions. The presence of the chiral moieties at symmetric positions (**CS1**), in comparison with the chiral units at adjacent positions (**CS2**), are preferable for enantioselectivity. Since the chiral moieties are at adjacent positions, this could conditionate the enantioselectivity whereas the analytes could not have the possibility to establish interactions with the CS due to steric hindrance. This factor could also explain the higher retention factors of the **CSP2**. The chiral units of **CSP1** and **CSP2** are the same so it just could be analysed the influence of their positions.

IV. EXPERIMENTAL

1. General Methods

The reactions were controlled by thin-layer chromatography (TLC) using Merck Silica Gel 60 GF₂₅₄ precoated plates (Merck, Darmstadt, Germany), with appropriate mobile phases and UV detection at 254 and 365 nm.

Microwave reactions were performed on a MicroSYNTH 1600 from Milestone (ThermoUnicam, Portugal) synthesizer in sealed reaction vessels.

The evaporation of the organic solvents was performed on a rotary evaporator under reduced pressure (rotative evaporator Büchi).

The compounds were purified by *flash* chromatography using MerckTM silica gel 60 (0.04-0.063 mm), liquid-liquid extraction and crystallization.

The column packing was performed using the system SSI Lab Alliance® – Pack in a Box, portable HPLC column packing system model CP constant pressure pump, non-flush, 0.1-24 mLmin⁻¹, 10.000 psig, with transducer and RS-232 control, SS, 110/220V column packer assembly (50 mL reservoir, column adapter 5/16" 4.6 mm) Quick-Set pump control software. A Shandon column packing pump was also used. The empty HPLC stainless-steel columns (150 × 2.0 mm ID) were purchased from Phenomenex.

Melting points were obtained in a Köfler microscope. The optical rotations were recorded on a Polartronic Universal polarimeter (ADP 410 polarimeter).

The IR spectra were obtained in a KBr microplate in a FTIR spectrometer Nicolet iS10 from Thermo Scientific (Waltham, MA, USA) with Smart OMNI- transmission accessory (Software 188 OMNIC 8.3).

¹H and ¹³C NMR spectra were performed in University of Aveiro, Department of Chemistry, and they were taken in DMSO-d₆ at room temperature on Bruker Avance 300 instrument (300.13 MHz for ¹H NMR and 75.47 MHz for ¹³C NMR). Chemical shifts are expressed in ppm values relative to tetramethylsilane (TMS) as an internal reference. Coupling constants are reported in hertz (Hz).

The element analysis was performed in Centro de Apoio Científico e Tecnológico á Investigación (CACTI, University of Vigo, Spain) on an Elemental Carlo Erba 1108 apparatus.

1.1. Chemicals and reagents

The organic solvents used were *pro analysis* or HPLC grade from Sigma-Aldrich, ACROS and Fluka.

For the synthesis of 2-hydroxy-2',3,4-trimethoxybenzophenone, the 2-methoxybenzoyl chloride (CAS 21615-34-9) was purchased from TCI and the 1,2,3-trimethoxybenzene (CAS 634-36-6) from Sigma-Aldrich. The aluminium chloride (CAS 7446-70-0) was obtained from Fluka.

For the Williamson ether synthesis, the potassium carbonate was purchased from Proralab and the sodium hydride (CAS 7646-69-7) was obtained from TCI. The anhydrous acetone (CAS 67-64-1) was from Biosolve. The methyl bromoacetate (CAS 96-32-2) was purchased from Fluka and the 2-bromoethanol (CAS 540-51-2) was obtained from Sigma-Aldrich.

For the demethylation, the 2-(diethylamino) ethanethiol hydrochloride (CAS 1942-52-5) and *tert*-butoxide sodium (CAS 865-48-5) were also purchased from Sigma-Aldrich.

For the performance of coupling reactions, the coupling reagents TBTU (CAS 125700-67-6) and COMU (CAS 1075198-30-9) were purchased from ACROS Organics. The triethylamine (CAS 121-4-8) was obtained from TCI. The (*S*)- α -methylbenzylamine (CAS 2627-86-3) was purchased from TCI and (*R*)-2-phenylglycinol (CAS 56613-80-0) as well as (*R*)-(-)-*N*-(3,5-dinitrobenzoyl)- α -phenylglycine (CAS 74927-72-3) from Sigma-Aldrich.

For the performance of silylation, the 3-(triethoxysilyl)propylisocyanate (CAS 24801-88-5) was obtained from TCI and the spherical silica gel 100Å for covalent linkage was purchased from Silicycle.

The anhydrous sodium sulphate, the sodium hydroxide was obtained from, the formic acid was from and the hydrochloric acid 37.0 % was purchased from PanReac Applichem, JMS, CarloErba and VWR, respectively.

A. Development of new CSPs with CDXs as selectors

2. Development of CSP1

2.1. Synthesis of 2,2'-[(9-oxo-9H-xanthene-3,6-diyl)bis(oxy)]bis{N-[(R)-2-hydroxy-1-phenylethyl]acetamide}, **CS1**, (**3**)

The 2,2'-[(9-oxo-9H-xanthene-3,6-diyl)bis(oxy)]diacetic acid (compound **1**) (0.200 g, 0.584 mmol) was dissolved in THF and TBTU (0.375 g, 1.169 mmol) was added. Following, TEA (300 μ L, 2.340 mmol) and the chiral reagent, the (R)-(-)-2-phenylglycinol (0.160 g, 1.169 mmol) were added. The mixture was stirred at room temperature for 13 hours. After the reaction was finished, the solvent was evaporated under reduced pressure and the crude product was dissolved in CH₂Cl₂. This solution was washed with 1 M HCl solution (2 $\times$ 25 mL), NaHCO₃ saturated solution (2 $\times$ 30 mL), and H₂O (3 $\times$ 50 mL). The organic layer was dried with anhydrous Na₂SO₄, filtered and the organic solvent was evaporated under reduced pressure affording the **CS1** (**3**), as yellow solid.

Yield: 85%. m.p.: 177-179°C. $[\alpha]_D^{25^\circ\text{C}}$ -455.0 ($c = 0.088 \times 10^{-3}$ g mL⁻¹ in CHCl₃). IR ν_{max} (cm⁻¹) (KBr): 3444.4, 3286.8, 3062.2, 2954.8, 1651.1, 1624.0, 1547.7, 1455.3, 1251.5, 1063.3, 842.6, 769.5. ¹H NMR (300.13 MHz, DMSO-d₆) δ : 8.24 (d, $J = 8.0$ Hz, 2H, H-3' and H-3''), 8.17 (d, $J = 8.9$ Hz, 2H, H-1 and H-8), 7.28 (m, 10H, H-5' to H-10' and H-5'' to H-10''), 7.08 (dd, $J = 8.9, 2.3$ Hz, 2H, H-2 and H-7), 7.00 (d, $J = 2.3$ Hz, 2H, H-4 and H-5), 5.06 (t, $J = 5.7$ Hz, 2H, H-12' and H-12''), 4.85 (m, 2H, H-4' and H-4''), 4.72 (s, 4H, H-1' and H-1''), 3.75 ppm (m, 4H, H-11' and H-11''). ¹³C NMR (75.47 MHz, DMSO-d₆) δ : 174.3 (C-9), 166.7 (C-2' and C-2''), 163.0 (C-3 and C-6), 157.3 (C-4a and C-10a), 140.8 (C-5' and C-5''), 127.4 (C-1 and C-8), 126.9 (C-7' to C-9' and C-7'' to C-9''), 126.4 (C-6', C-10', C-6'' and C-10''), 115.3 (C-8a and C-9a), 113.0 (C-2 and C-7), 101.6 (C-4 and C-5), 67.3 (C-1' and C-1''), 55.0 ppm (C-11' and C-11'').

2.2. Synthesis of a silylated derivative and covalent bonding onto the silica gel to obtain CSP1

The **CS1** (**3**) (0.200 g, 0.343 mmol) was dissolved in anhydrous toluene and some drops of dimethyl sulfoxide (DMSO) were added, to improve its solubility. After dissolution, TEA (200 μ L, 1.413 mmol) and 3-(triethoxysilyl)propylisocyanate (400 μ L, 1.441 mmol) were added. The mixture was refluxed for 72 hours. Then, the solvent was evaporated under

reduced pressure affording a crude product. To silica gel Nucleosil (800 mg, 100 Å-5µm, Macherey-Nagel), previously dried in a desiccator under vacuum and with phosphorus pentoxide for 24 hours, anhydrous toluene (30 mL) was added. The mixture was kept under magnetic stirring for 15 minutes. The crude product was dissolved in the same solvent and the solution was added to the silica. This mixture was gently stirred at reflux for 72 hours. The solid was then filtered and washed successively with toluene, CH₃OH, acetone, ethyl acetate, CH₂Cl₂, and *n*-Hex, affording **CSP1**.

3. Development of CSP2

3.1. Synthesis of 2-hydroxy-2',3,4-trimethoxybenzophenone (6)

2-Methoxybenzoyl chloride (**5**) (8.1 mL, 54.415 mmol) and 1,2,3-trimethoxybenzene (**4**) (10.067 g, 59.855 mmol) were dissolved in anhydrous diethyl ether and anhydrous AlCl₃ (29.10 g, 218.239 mmol) was added. The mixture was kept with magnetic stirring at room temperature, for 24 hours. After the evaporation of the organic solvent, a viscous crude product was obtained, which was poured in crushed ice. The obtained suspension was acidified with 1 M HCl solution and extracted with CH₂Cl₂. The organic phase was washed with H₂O, dried with anhydrous Na₂SO₄ and filtered under reduced pressure. Then, the organic solvent was evaporated under reduced pressure, affording 2-hydroxy-2',3,4-trimethoxybenzophenone (**6**) as an orange oil.

This compound was synthesized previously by our group being the final product obtained compared by TLC with a known pattern.

Yield: 80%

3.2. Synthesis of 3,4-dimethoxyxanthone (7)

The 2-hydroxy-2',3,4-trimethoxybenzophenone (**6**) (23.98 g, 83.178 mmol) was dissolved on a mixture of CH₃OH (265 mL), H₂O (177 mL) and NaOH (53.0 g, 1.325 mol). The mixture was kept under reflux with magnetic stirring for 40 hours. Then, the mixture was stored at 4°C, overnight. The obtained solid was filtered under reduced pressure and washed with CH₃OH, to afford the 3,4-dimethoxyxanthone (**7**), as light-yellow solid.

This compound was synthesized previously by our group being the final product obtained compared by TLC with a known pattern.

Yield: 50%

3.3. Synthesis of 3-methoxy-4-hydro-9H-xanthen-9-one (8)

The 3,4-dimethoxyxanthone (**7**) (10.670 g, 41.637 mmol) was dissolved in anhydrous toluene and AlCl_3 (16.005 g, 120.031 mmol) was added. The mixture was kept under 70°C for 40 minutes. To the mixture, 5 M HCl solution was added and extracted with CH_2Cl_2 . The organic phase was extracted with 0.5 M NaOH solution followed by acidification with 5 M HCl solution. The solid obtained was recrystallized from CHCl_3 :*n*-Hex yielding the main by product, the 3,4-hydro-9H-xanthen-9-one (**10**) as green solid. The recrystallization of the mother waters allowed to obtain the 3-methoxy-4-hydro-9H-xanthen-9-one (**8**) as white solid and the 3-hydro-4-methoxy-9H-xanthen-9-one (**9**) as white solid.

These compounds were synthesized previously by our group being the final products obtained compared by TLC with a known pattern.

3-methoxy-4-hydro-9H-xanthen-9-one (8)

Yield: 28%. m.p.: $> 330^\circ\text{C}$. IR ν_{max} (cm^{-1}) (KBr): 3243.0, 1605.6, 1515.4, 1465.9, 1287.0, 1226.1, 821.1, 759.4. ^1H NMR (300.13 MHz, $\text{DMSO}-d_6$) δ : 9.64 (s, 1H, H-4), 8.17 (dd, $J = 8.0, 1.8$ Hz, 1H, H-8), 7.84 (ddd, $J = 8.7, 7.2, 1.8$ Hz, 1H, H-6), 7.68 (d, $J = 8.9$ Hz, 1H, H-1), 7.46 (ddd, $J = 8.0, 7.2, 1.8$ Hz, 1H, H-7), 7.20 (dd, $J = 8.7, 1.8$ Hz, 1H, H-5), 7.01 (d, $J = 8.9$ Hz, 1H, H-2), 3.95 (s, 3H, H-1'). ^{13}C NMR (75.47 MHz, $\text{DMSO}-d_6$) δ : 175.6 (C-9), 156.4 (C-3), 155.7 (C-10a), 152.5 (C-4a), 145.5 (C-1), 135.1 (C-2), 135.0 (C-6), 126.0 (C-8), 120.8 (C-7), 118.1 (C-8a), 116.3 (C-5), 115.9 (C-9a), 109.1 (C-4), 56.4 (C-1').

3-hydro-4-methoxy-9H-xanthen-9-one (9)

Yield: $< 1\%$. m.p.: $> 330^\circ\text{C}$. IR ν_{max} (cm^{-1}) (KBr): 3245.1, 1606.8, 1522.0, 1466.2, 1284.7, 1227.2, 820.6, 752.8. ^1H NMR (300.13 MHz, $\text{DMSO}-d_6$) δ : 8.17 (dd, $J = 8.0, 1.8$ Hz, 1H, H-8), 7.84 (ddd, $J = 9.0, 7.0, 1.8$ Hz, 1H, H-6), 7.70 (d, $J = 8.8$ Hz, 1H, H-1), 7.40 (ddd, $J = 8.0, 7.0, 1.8$ Hz, 1H, H-7), 7.25 (dd, $J = 9.0, 1.8$ Hz, 1H, H-5), 7.01 (d, $J = 8.8$ Hz, 1H, H-2), 3.94 (s, 3H, H-1'). ^{13}C NMR (75.47 MHz, $\text{DMSO}-d_6$) δ : 175.0 (C-9), 156.4 (C-3), 155.5 (C-10a), 150.7 (C-4a), 134.9 (C-6), 134.6 (C-4), 125.9 (C-8), 124.3 (C-7), 121.6 (C-1), 120.9 (C-8a), 118.2 (C-5), 114.8 (C-9a), 114.1 (C-2), 60.8 (C-1').

3,4-dihydroxy-9H-xanthen-9-one (10)

Yield: 3%. m.p.: $> 330^\circ\text{C}$. IR ν_{max} (cm^{-1}) (KBr): 3138.4, 1604.8, 1526.2, 1458.7, 1224.0, 904.6. ^1H NMR (300.13 MHz, $\text{DMSO}-d_6$) δ : 9.72 (s, 2H, H-3 and H-4), 8.16 (dd, $J = 8.1, 1.8$ Hz, 1H, H-8), 7.84 (ddd, $J = 8.7, 7.1, 1.8$ Hz, 1H, H-6), 7.65 (d, $J = 8.8$ Hz, 1H, H-1), 7.62 (dd, $J = 8.7, 1.8$ Hz, 1H, H-5), 7.44 (ddd, $J = 8.1, 7.1, 1.8$ Hz, 1H, H-7), 6.95 (d, $J = 8.8$ Hz, 1H, H-2). ^{13}C NMR (75.47 MHz, $\text{DMSO}-d_6$) δ : 175.3 (C-9), 155.5 (C-10a), 151.6 (C-4a),

146.4 (C-1), 134.8 (C-2), 132.6 (C-6), 125.9 (C-8), 124.0 (C-3), 120.8 (C-7), 118.0 (C-8a), 116.6 (C-5), 114.7 (C-9a), 113.2 (C-4).

3.4. Synthesis of dimethyl 2,2'-[(9-oxo-9*H*-xanthene-3,4-diyl)bis(oxy)]diacetate (**11**)

The 3,4-dihydroxy-9*H*-xanthen-9-one (**10**) (0.249 g, 1.091 mmol) was dissolved in anhydrous acetone and K₂CO₃ (0.336 g, 2.431 mmol) and BrCH₂COOCH₃ (400 μ L, 4.184 mmol) were added. The mixture was kept under reflux and magnetic stirring for 4 hours. Then, the organic solvent was evaporated under reduced pressure and the solid obtained was washed with *n*-Hex, affording the 2,2'-[(9-oxo-9*H*-xanthene-3,4-diyl)bis(oxy)]diacetate (compound **11**) as white solid.

Yield: 95%. m.p.: > 330°C. IR $\nu_{\max}$ (cm⁻¹) (KBr): 1767.2, 1605.4, 1470.2, 1430.7, 1337.8, 1266.8, 1086.9, 832.5, 763.3. ¹H NMR (300.13 MHz, DMSO-*d*₆) δ : 8.18 (dd, *J* = 8.0, 1.7 Hz, 1H, H-8), 7.92 (ddd, *J* = 8.7, 7.1, 1.7 Hz, 1H, H-6), 7.88 (d, *J* = 9.0 Hz, 1H, H-1), 7.65 (dd, *J* = 8.7, 1.7 Hz, 1H, H-5), 7.49 (ddd, *J* = 8.0, 7.1, 1.7 Hz, 1H, H-7), 7.22 (d, *J* = 9.0 Hz, 1H, H-2), 5.09 (s, 2H, H-1'), 4.95 (s, 2H, H-1''), 3.71 (s, 6H, H-3' and H-3''). ¹³C NMR (75.47 MHz, DMSO-*d*₆) δ : 175.2 (C-9), 169.1 (C-2'), 168.7 (C-2''), 155.6 (C-3), 154.9 (C-4), 149.5 (C-1), 135.40 (C-10a), 134.4 (C-4a), 125.9 (C-2), 124.5 (C-6), 121.4 (C-8), 120.8 (C-7), 118.2 (C-5), 116.2 (C-8a), 110.6 (C-9a), 69.1 (C-1'), 65.4 (C-1''), 52.1 (C-3'), 51.8 (C-3'').

3.5. Synthesis of 2,2'-[(9-oxo-9*H*-xanthene-3,4-diyl)bis(oxy)]diacetic acid (**12**)

The 2,2'-[(9-oxo-9*H*-xanthene-3,4-diyl)bis(oxy)]diacetate (compound **11**) (0.388 g, 1.040 mmol) was dissolved in CH₂Cl₂:CH₃OH (250 mL, 50:50 v/v) and 5 M NaOH solution (3 mL, 15.000 mmol) was added. The mixture was kept at room temperature and magnetic stirring for 4 hours. After this time, the organic solvents were evaporated under reduced pressure. To the suspension was added H₂O and it was washed with CH₂Cl₂ (2 $\times$ 100 mL). The aqueous phase was acidified with 5 M HCl solution and the precipitate was collected by filtration under reduced pressure affording the 2,2'-[(9-oxo-9*H*-xanthene-3,4-diyl)bis(oxy)]diacetic acid (compound **12**) as white solid.

Yield: 81%. m.p.: 257-260°C. IR $\nu_{\max}$ (cm⁻¹) (KBr): 3413.2, 2955.0, 1745.7, 1605.1, 1514.9, 1467.0, 1217.3, 1067.3, 819.2, 760.9. ¹H NMR (300.13 MHz, DMSO-*d*₆) δ : 13.09 (s, 2H, H-3' and H-3''), 8.17 (dd, *J* = 7.6, 1.7 Hz, 1H, H-8), 7.91 (ddd, *J* = 8.1, 7.2, 1.7 Hz,

¹H, H-6), 7.86 (d, $J = 9.0$ Hz, 1H, H-1), 7.65 (dd, $J = 8.1, 1.7$ Hz, 1H, H-5), 7.48 (ddd, $J = 7.6, 7.2, 1.7$ Hz, 1H, H-7), 7.18 (d, $J = 9.0$ Hz, 1H, H-2), 4.97 (s, 2H, H-1'), 4.84 (s, 2H, H-1''). ¹³C NMR (75.47 MHz, DMSO-*d*₆) δ : 175.2 (C-9), 170.1 (C-2'), 169.6 (C-2''), 155.5 (C-3), 155.1 (C-4), 149.6 (C-1), 135.2 (C-10a), 134.4 (C-4a), 125.9 (C-2), 124.4 (C-6), 121.1 (C-8), 120.8 (C-7), 118.2 (C-5), 116.2 (C-8a), 110.5 (C-9a), 68.9 (C-1'), 65.2 (C-1'').

3.6. Synthesis of 2,2'-[(9-oxo-9H-xanthene-3,4-diyl)bis(oxy)]bis{N-[(*R*)-2-hydroxy-1-phenylethyl]acetamide}, **CS2**, (**13**)

CS2 (**13**) was prepared from a suitable carboxylated compound (2,2'-[(9-oxo-9H-xanthene-3,4-diyl)bis(oxy)]diacetic acid, **12**) using the same procedure as described for compound **3**, being the amount of TBTU and TEA calculated according to the number of carboxyl groups of the chemical substrate (**12**) and the stoichiometry of the reaction.

The chiral reagent used for development of compound **13** was the (*R*)-(-)-2-phenylglycinol being the final product obtained as white solid.

Yield: 57%. m.p.: 215-218°C. $[\alpha]_D^{25^\circ\text{C}} -300.0$ ($c = 0.1 \times 10^{-3}$ g mL⁻¹ in CHCl₃). IR ν_{max} (cm⁻¹) (KBr): 3388.6, 3271.5, 3093.3, 2940.9, 1668.2, 1603.8, 1529.6, 1463.3, 1218.6, 1048.5, 831.8, 764.2. ¹H NMR (300.13 MHz, DMSO-*d*₆) δ : 8.65 (d, $J = 8.3$ Hz, 1H, H-3'), 8.51 (d, $J = 8.3$ Hz, 1H, H-3''), 8.18 (dd, $J = 8.1, 1.7$ Hz, 1H, H-8), 7.90 (d, $J = 9.0$ Hz, 1H, H-1), 7.84 (ddd, $J = 8.5, 7.1, 1.7$ Hz, 1H, H-6), 7.63 (dd, $J = 8.5, 1.7$ Hz, 1H, H-5), 7.48 (ddd, $J = 8.1, 7.1, 1.7$ Hz, 1H, H-7), 7.46-7.25 (d,t,d, $J = 7.2$ Hz, 10H, H-5' to H-10' and H-5'' to H-10''), 7.15 (d, $J = 9.0$ Hz, 1H, H-2), 5.07 (t, $J = 5.8$ Hz, 1H, H-12'), 5.00 (t, $J = 5.8$ Hz, 1H, H-12''), 4.98 (m, 1H, H-4'), 4.95 (m, 1H, H-4''), 4.83 (s, 2H, H-1'), 4.81 (s, 2H, H-1''), 3.66 ppm (m, 4H, H-11' and H-11''). ¹³C NMR (75.47 MHz, DMSO-*d*₆) δ : 175.2 (C-9), 167.8 (C-2'), 166.6 (C-2''), 155.5 (C-3), 155.4 (C-4), 140.8 (C-5' to C-10'), 140.7 (C-5'' to C-10''), 135.3 (C-10a), 134.9 (C-4a), 128.1 (C-4' and C-4''), 125.9 (C-2), 124.5 (C-6), 121.6 (C-8), 120.8 (C-7), 118.3 (C-5), 116.3 (C-8a), 110.5 (C-9a), 67.4 (C-1' and C-1''), 55.0 (C-11'), 54.6 (C-11'').

3.7. Synthesis of a silylated derivative and covalent bonding onto the silica gel to obtain **CSP2**

The silylation reaction of the **CS2** (**12**) was performed using the same conditions as described for **CS1** (**3**). The solvent was anhydrous toluene with a few drops of DMSO due to the low solubility of **CS2** (**12**). The covalent linkage of silylated selector onto the silica gel to afford **CSP2** was performed using the same method as described for **CS1** (**3**).

4. Development of CSP3

4.1. Synthesis of 6-methoxy-9-oxo-9*H*-xanthene-2-carboxylic acid (**15**)

First, an impure sample of methyl 6-methoxy-9-oxo-9*H*-xanthene-2-carboxylate (compound **14**) previously synthesized in our group¹³² was purified by column chromatography (silica gel, *n*-Hex/ethyl acetate in gradient) in order to obtain pure compound **14**.

The methyl 6-methoxy-9-oxo-9*H*-xanthene-2-carboxylate (compound **14**) (1.00 g, 3.520 mmol) was dissolved in CH₂Cl₂:CH₃OH (50:50 *v/v*) and a solution of 5 M NaOH solution was added (7 mL, 35.000 mmol). This mixture was kept at room temperature and magnetic stirring for 24 hours. After this period, the organic solvents were evaporated under reduced pressure. The solid obtained was dissolved in H₂O being the aqueous solution acidified with 5 M HCl solution resulting in the formation of a precipitate that was collected by filtration under reduced pressure affording the 6-methoxy-9-oxo-9*H*-xanthene-2-carboxylic acid (compound **15**) as a white solid.

Yield: 50%.

4.2. Synthesis of (S)-6-methoxy-9-oxo-*N*-(1-phenylethyl)-9*H*-xanthene-2-carboxamide (**17**)

(S)-6-methoxy-9-oxo-*N*-(1-phenylethyl)-9*H*-xanthene-2-carboxamide (compound **17**) was prepared from a suitable carboxylated compound (6-methoxy-9-oxo-9*H*-xanthene-2-carboxylic acid, **15**) using the same procedure as described for compound **3**, being the amount of TBTU and TEA calculated according to the number of carboxyl groups of the chemical substrate (**15**) and the stoichiometry of the reaction.

The chiral reagent used for preparation of compound **17** was the (S)-(α)-methylbenzylamine.

Yield: 95%.

4.3. Synthesis of (S)-6-hydroxy-9-oxo-*N*-(1-phenylethyl)-9*H*-xanthene-2-carboxamide (**18**)

The reaction was carried out under anhydrous conditions and nitrogen atmosphere. The solid NaOt-Bu (0.297 g, 3.091 mmol) was added and the flask was cooled in an ice water bath. A solution of the 2-(diethylamino) ethanethiol HCl (0.300 g, 2.118 mmol)

dissolved in *N,N*-dimethylformamide (10 mL) was added via syringe. After 5 minutes, the cooling bath was removed, and the white suspension warmed up until the room temperature. After 15 minutes, the (*S*)-6-methoxy-9-oxo-*N*-(1-phenylethyl)-9*H*-xanthene-2-carboxamide (compound **17**) (0.620 g, 1.660 mmol) dissolved in the same solvent was added and the reaction mixture was heated to reflux for 96 hours. The mixture was cooled to room temperature, and the flask was placed in an ice water bath. A solution of 1 M HCl was added dropwise until the pH = 1 followed by H₂O (25 mL). The aqueous phase was extracted with diethyl ether (3 × 50 mL), and the combined organic layers were washed with 0.5 M NaOH solution (3 × 50 mL). The aqueous phase was acidified with HCl and extracted with diethyl ether (3 × 75 mL). Then, the organic phase was washed with H₂O (2 × 50 mL), saturated brine (1 × 50 mL) and dried with anhydrous NaSO₄. The solvent was evaporated under reduced pressure affording the (*S*)-6-hydroxy-9-oxo-*N*-(1-phenylethyl)-9*H*-xanthene-2-carboxamide (compound **18**) as an orange oil.

This compound was synthesized previously by our group being the final product obtained compared by TLC with a known pattern.

Yield: 50%.

4.4. Synthesis of methyl (*S*)-2-[[9-oxo-7-((1-phenylethyl) carbamoyl)-9*H*-xanthen-3-yl]oxy}acetate (**19**)

(*S*)-2-[[9-oxo-7-((1-phenylethyl) carbamoyl)-9*H*-xanthen-3-yl]oxy}acetate (compound **19**) was prepared using the hydroxylated compound (*S*)-6-hydroxy-9-oxo-*N*-(1-phenylethyl)-9*H*-xanthene-2-carboxamide (**18**) according to the same method as described for compound **11**. The amount of K₂CO₃ and BrCH₂COOCH₃ was calculated according with the number of hydroxyl groups of the chemical substrate and the stoichiometry of the reaction.

Yield: 18%. m.p.: > 330°C. $[\alpha]_D^{25^\circ\text{C}} +686.0$ ($c = 0.86 \times 10^{-3}$ g mL⁻¹ in CH₃OH). IR ν_{max} (cm⁻¹) (KBr): 3431.7, 3295.6, 1759.3, 1648.4, 1617.9, 1540.7, 1479.7, 1348.7, 1213.5, 1067.7, 828.6, 781.6. ¹H NMR (300.13 MHz, DMSO-*d*₆) δ : 9.20 (d, $J = 7.8$ Hz, 1H, H-2'), 8.76 (d, $J = 2.3$ Hz, 1H, H-1), 8.33 (dd, $J = 8.8, 2.3$ Hz, 1H, H-3), 8.15 (d, $J = 8.9$ Hz, 1H, H-8), 7.71 (d, $J = 8.8$ Hz, 1H, H-4), 7.45 – 7.26 (d,t,d, $J = 7.5$ Hz, 5H, H-5' to H-10'), 7.23 (d, $J = 2.4$ Hz, 1H, H-5), 5.21 (m, 1H, H-4'), 5.07 (s, 2H, H-1''), 3.74 (s, 3H, H-3''), 1.52 (d, $J = 7.8$ Hz, 3H, H-3'). ¹³C NMR (75.47 MHz, DMSO-*d*₆) δ : 174.9 (C-9), 168.6 (C-1'), 164.1 (C-2''), 163.4 (C-6), 157.3 (C-10a), 157.2 (C-4a), 144.8 (C-5'), 134.1 (C-3), 130.4 (C-2), 128.3 (C-7' to C-

9'), 126.7 (C-8), 126.1 (C-6' and C-10'), 125.5 (C-1), 118.2 (C-8a), 114.1 (C-7), 101.8 (C-5), 65.1 (C-1''), 52.1 (C-3''), 48.7 (C-4'), 22.2 (C-3').

4.5. Synthesis of (S)-2-[[9-oxo-7-((1-phenylethyl)carbamoyl)-9H-xanthen-3-yl]oxy}acetic acid (20)

(S)-2-[[9-oxo-7-((1-phenylethyl)carbamoyl)-9H-xanthen-3-yl]oxy}acetic acid (**20**) was prepared from a suitable acetate compound methyl (S)-2-[[9-oxo-7-((1-phenylethyl)carbamoyl)-9H-xanthen-3-yl]oxy}acetate (compound **19**) using the same method as described for compound **12**, adapting the volume of 5 M NaOH solution, according to the number of acetate groups of the chemical substrate and the stoichiometry of the reaction.

Yield: 51%. m.p.: > 330°C. $[\alpha]_D^{25^\circ\text{C}} +450.0$ ($c = 0.75 \times 10^{-3} \text{ g mL}^{-1}$ in CH_3OH). IR ν_{max} (cm^{-1}) (KBr): 3306.9, 3082.7, 2977.7, 1718.6, 1664.0, 1619.8, 1552.4, 1480.6, 1236.6, 1065.8, 830.9, 779.2. ^1H NMR (300.13 MHz, $\text{DMSO}-d_6$) δ : 9.19 (d, $J = 7.9 \text{ Hz}$, 1H, H-2'), 8.74 (d, $J = 2.3 \text{ Hz}$, 1H, H-1), 8.31 (dd, $J = 8.8, 2.3 \text{ Hz}$, 1H, H-3), 8.14 (d, $J = 9.0 \text{ Hz}$, 1H, H-8), 7.72 (d, $J = 8.8 \text{ Hz}$, 1H, H-4), 7.44 – 7.24 (d,t,d, $J = 7.5 \text{ Hz}$, 5H, H-5' to H-10'), 7.17 (d, $J = 2.1 \text{ Hz}$, 1H, H-5), 7.12 (dd, $J = 9.0, 2.1 \text{ Hz}$, 1H, H-7), 5.21 (m, 1H, H-4'), 4.94 (s, 2H, H-1''), 1.52 (d, $J = 7.9 \text{ Hz}$, 3H, H-3'), 1.20 (s, 1H, H-3''). ^{13}C NMR (75.47 MHz, $\text{DMSO}-d_6$) δ : 174.8 (C-9), 165.4 (C-1'), 164.1 (C-2''), 163.6 (C-6), 157.3 (C-10a), 157.2 (C-4a), 144.8 (C-5'), 134.0 (C-3), 130.3 (C-2), 128.2 (C-7' to C-9'), 126.6 (C-8), 126.1 (C-6' and C-10'), 125.4 (C-1), 118.1 (C-8a), 114.0 (C-7), 101.6 (C-5), 65.0 (C-1''), 48.7 (C-4'), 22.1 (C-3').

4.6. Synthesis of 6-{2-[[*(R)*-2-hydroxy-1-phenylethyl]amino]-2-oxoethoxy}-9-oxo-N-((*S*)-1-phenylethyl)-9H-xanthene-2-carboxamide, CS3, (21)

CS3 (21) was prepared resorting to the same procedure as described for **CS1 (3)**, being the amount of TBTU and TEA calculated according to the number of carboxyl groups of the chemical substrate (**20**) and the stoichiometry of the reaction. The chiral reagent was the (*R*)-(-)-2-phenylglycinol.

Yield: 65%. m.p.: > 330°C. $[\alpha]_D^{25^\circ\text{C}} -211.3$ ($c = 0.71 \times 10^{-3} \text{ g mL}^{-1}$ in CH_3OH). IR ν_{max} (cm^{-1}) (KBr): 3261.5, 1654.3, 1616.5, 1471.4, 1222.4, 1140.4, 1060.4, 911.8, 763.0. ^1H NMR (300.13 MHz, $\text{DMSO}-d_6$) δ : 9.21 (d, $J = 8.1 \text{ Hz}$, 1H, H-2'), 8.78 (d, $J = 2.4 \text{ Hz}$, 1H, H-1), 8.68 (d, $J = 8.1 \text{ Hz}$, 1H, H-3''), 8.33 (dd, $J = 9.0, 2.4 \text{ Hz}$, 1H, H-3), 8.15 (d, $J = 9.0 \text{ Hz}$, 1H, H-8), 7.72 (d, $J = 9.0 \text{ Hz}$, 1H, H-4), 7.46 – 7.26 (d,t,d, $J = 7.5 \text{ Hz}$, 10H, H-5' to H-10' and H-5'' to H-10''), 7.23 (d, $J = 2.5 \text{ Hz}$, 1H, H-5), 7.13 (dd, $J = 9.0, 2.5 \text{ Hz}$, 1H, H-7), 5.22 (m, 1H, H-4'),

5.05 (s, 1H, H-12''), 4.92 (m, 1H, H-4''), 4.82 (s, 2H, H-1''), 3.61 (m, 2H, H-11''), 1.52 (d, J = 8.1 Hz, 3H, H-3'). ^{13}C NMR (75.47 MHz, DMSO- d_6) δ : 174.8 (C-9), 166.5 (C-1'), 164.1 (C-2''), 163.7 (C-6), 157.2 (C-10a), 157.1 (C-4a), 144.8 (C-5'), 140.8 (C-5''), 134.1 (C-3), 130.4 (C-2), 128.3 (C-7' to C-9'), 128.1 (C-4''), 126.9 (C-7'' to C-9''), 126.6 (C-8), 126.1 (C-6', C-9', C-6'' and C-9''), 125.4 (C-1), 118.2 (C-8a), 115.3 (C-4), 114.5 (C-7), 101.7 (C-6), 64.4 (C-1''), 55.0 (C-11''), 48.7 (C-4'), 22.2 (C-3').

4.7. Synthesis of a silylated derivative and covalent bonding onto the silica gel to obtain CSP3

The silylation reaction of **CS3** (**21**) was performed using the same conditions as described for **CS1** (**3**), being the amount of TEA and 3-(triethoxysilyl)propylisocyanate adjusted according to the number of chiral units of the selector and the stoichiometry of the reaction. The solvent was anhydrous toluene with a few drops of DMSO due to the low solubility of **CS3** (**21**). The covalent linkage of silylated selector onto the silica gel to afford **CSP3** was performed using the same method as described for **CS1** (**3**).

5. Development of CSP4

The planned CSP4 was not achieved although from the nine planned reaction steps they were performed four reaction steps successfully.

5.1. Synthesis of methyl 2-[(3-methoxy-9-oxo-9H-xanthen-4-yl)oxy]acetate (**22**)

Methyl 2-[(3-methoxy-9-oxo-9H-xanthen-4-yl)oxy]acetate (**22**) was prepared from a suitable hydroxylated compound, the 3-methoxy-4-hydro-9H-xanthen-9-one (**8**), using the same method as described for 2'-[(9-oxo-9H-xanthene-3,4-diyl)bis(oxy)]diacetate (compound **11**), adjusting the amount of K_2CO_3 and $\text{BrCH}_2\text{COOCH}_3$ according to the number of hydroxyl groups of the chemical substrate and the stoichiometry of the reaction. The product was purified by *flash* chromatography (*n*-Hex/ethyl acetate in gradient) affording the 2-[(3-methoxy-9-oxo-9H-xanthen-4-yl)oxy]acetate (**22**) as white solid.

Yield: 40%. m.p.: 135-138°C. IR ν_{max} (cm^{-1}) (KBr): 1750.8, 1603.4, 1509.1, 1464.6, 1388.0, 1288.2, 1216.3, 1033.5, 819.5, 767.6. ^1H NMR (300.13 MHz, DMSO- d_6) δ : 8.17 (dd, J = 7.9, 1.7 Hz, 1H, H-8), 7.95 (d, J = 9.0 Hz, 1H, H-1), 7.85 (ddd, J = 8.4, 7.2, 1.7 Hz,

1H, H-6), 7.63 (dd, $J = 8.4, 1.7$ Hz, 1H, H-5), 7.47 (ddd, $J = 7.9, 7.2, 1.7$ Hz, 1H, H-7), 7.27 (d, $J = 9.0$ Hz, 1H, H-2), 4.85 (s, 2H, H-1''), 3.73 (s, 3H, H-1'), 3.36 (s, 3H, H-3''). ^{13}C NMR (75.47 MHz, DMSO- d_6) δ : 175.2 (C-9), 169.1 (C-2''), 156.7 (C-3), 155.5 (C-4), 149.4 (C-1), 135.3 (C-10a), 134.1 (C-4a), 125.9 (C-2), 124.4 (C-6), 121.7 (C-8), 120.8 (C-7), 118.1 (C-5), 115.8 (C-8a), 109.7 (C-9a), 69.3 (C-1''), 56.6 (C-1'), 51.7 (C-3'').

5.2. Synthesis of 2-[(3-methoxy-9-oxo-9H-xanthen-4-yl)oxy]acetic acid (**23**)

2-[(3-methoxy-9-oxo-9H-xanthen-4-yl)oxy]acetic acid (**23**) was prepared from a suitable acetate compound, 2-[(3-methoxy-9-oxo-9H-xanthen-4-yl)oxy]acetate (**22**), using the same method as described for 2,2'-[(9-oxo-9H-xanthene-3,4-diyl)bis(oxy)]diacetic acid (**12**), adjusting the volume of the solution of 5 M NaOH according to the number of acetate groups of the chemical substrate and the stoichiometry of the reaction. The 2-[(3-methoxy-9-oxo-9H-xanthen-4-yl)oxy]acetic acid (**23**) was obtained as white solid.

Yield: 97%. m.p.: 205-208°C. IR ν_{max} (cm^{-1}) (KBr): 3204.0, 2925.0, 1758.6, 1604.1, 1585.2, 1467.8, 1290.4, 1229.6, 1033.1, 819.1, 766.6. ^1H NMR (300.13 MHz, DMSO- d_6) δ : 8.17 (dd, $J = 8.0, 1.7$ Hz, 1H, H-8), 7.90 (d, $J = 9.0$ Hz, 1H, H-1), 7.80 (ddd, $J = 8.6, 7.2, 1.7$ Hz, 1H, H-6), 7.64 (dd, $J = 8.6, 1.7$ Hz, 1H, H-5), 7.48 (ddd, $J = 8.0, 7.2, 1.7$ Hz, 1H, H-7), 7.27 (d, $J = 9.0$ Hz, 1H, H-1), 4.76 (s, 2H, H-1''), 3.97 (s, 3H, H-1'). ^{13}C NMR (75.47 MHz, DMSO- d_6) δ : 175.3 (C-9), 170.1 (C-2''), 156.7 (C-3), 155.5 (C-4), 149.4 (C-1), 135.3 (C-10a), 134.3 (C-4a), 125.9 (C-2), 124.4 (C-6), 121.5 (C-8), 120.8 (C-7), 118.2 (C-5), 115.9 (C-8a), 109.8 (C-9a), 69.2 (C-1''), 56.6 (C-1').

5.3. Synthesis of (S)-2-[(3-methoxy-9-oxo-9H-xanthen-4-yl)oxy]-N-(1-phenylethyl)acetamide (**24**)

(S)-2-[(3-methoxy-9-oxo-9H-xanthen-4-yl)oxy]-N-(1-phenylethyl)acetamide (**24**) was prepared from a suitable carboxylated compound, 2-[(3-methoxy-9-oxo-9H-xanthen-4-yl)oxy]acetic acid (**23**), using the same method as described for compound **3**, being the amount of TBTU and TEA adapted according to the number of carboxyl groups of the chemical substrate and the stoichiometry of the reaction. The chiral reagent was the (S)-(α)-methylbenzylamine. The final product was obtained as white solid.

Yield: 45%. m.p.: 164-167°C. $[\alpha]_{\text{D}}^{25^\circ\text{C}} +48.2$ ($c = 0.83 \times 10^{-3} \text{ g mL}^{-1}$ in CH_3OH). IR ν_{max} (cm^{-1}) (KBr): 3348.1, 1665.1, 1604.8, 1587.5, 1466.9, 1289.1, 1230.2, 1023.4, 817.7, 769.1.

^1H NMR (300.13 MHz, $\text{DMSO}-d_6$) δ : 8.38 (d, $J = 8.2$ Hz, 1H, H-3''), 8.17 (dd $J = 8.0$, 1.8 Hz, 1H, H-8), 7.96 (d, $J = 9.0$ Hz, 1H, H-1), 7.82 (ddd, $J = 8.7$, 7.1, 1.8 Hz, 1H, H-6), 7.50 (dd, $J = 8.7$, 1.8 Hz, 1H, H-5), 7.46 (ddd, $J = 8.0$, 7.1, 1.8 Hz, 1H, H-7), 7.45 – 7.27 (m, 5H, H-6'' to H-11''), 7.26 (d, $J = 9.0$ Hz, 1H, H-2), 5.10 (q, $J = 8.2$ Hz, 1H, H-5''), 4.65 (s, 2H, H-1''), 3.93 (s, 3H, H-1'), 1.48 (d, $J = 8.2$ Hz, 3H, H-4''). ^{13}C NMR (75.47 MHz, $\text{DMSO}-d_6$) δ : 175.2 (C-9), 167.1 (C-2''), 156.6 (C-3), 155.4 (C-4), 149.3 (C-1), 135.2 (C-10a), 134.7 (C-4a), 128.4 (C-6''), 126.9 (C-8'' to C-10''), 126.1 (C-7'' and C-11''), 125.9 (C-2), 124.4 (C-6), 121.9 (C-8), 120.8 (C-7), 118.2 (C-5), 109.6 (C-9a), 72.4 (C-1''), 56.6 (C-1'), 47.7 (C-5''), 22.1 (C-4'').

5.4. Synthesis of the (S)-2-[(3-hydroxy-9-oxo-9H-xanthen-4-yl)oxy]-N-(1-phenylethyl)acetamide (25)

Compound **25** was prepared from the suitable methoxylated compound (S)-2-[(3-methoxy-9-oxo-9H-xanthen-4-yl)oxy]-N-(1-phenylethyl)acetamide (**24**) using the same method as described for (S)-6-hydroxy-9-oxo-N-(1-phenylethyl)-9H-xanthene-2-carboxamide (**18**). (S)-2-[(3-hydroxy-9-oxo-9H-xanthen-4-yl)oxy]-N-(1-phenylethyl)acetamide (**25**) was obtained as an orange oil.

Yield: 75%. m.p.: 126-129°C. $[\alpha]_D^{25^\circ\text{C}} +57.1$ ($c = 1.40 \times 10^{-3}$ gmL^{-1} in CH_3OH). IR ν_{max} (cm^{-1}) (KBr): 3442.9, 1702.0, 1615.2, 1598.7, 1465.9, 1220.9, 1032.5, 834.4, 759.5. ^1H NMR (300.13 MHz, $\text{DMSO}-d_6$) δ : 8.33 (d, $J = 7.9$ Hz, 1H, H-3''), 8.13 (dd, $J = 7.9$, 1.8 Hz, 1H, H-8), 7.96 (d, $J = 8.7$ Hz, 1H, H-1), 7.70 (ddd, $J = 8.6$, 7.1, 1.8 Hz, 1H, H-6), 7.49 (dd, $J = 8.6$, 1.8 Hz, 1H, H-5), 7.42 (ddd, $J = 7.9$, 7.1, 1.8 Hz, 1H, H-7), 7.32 (m, 5H, H-6'' to H-11''), 7.28 (d, $J = 8.7$ Hz, 1H, H-2), 5.80 (q, $J = 7.9$ Hz, 1H, H-5''), 4.79 (s, 2H, H-1''), 1.88 (d, $J = 7.9$ Hz, 3H, H-4''). ^{13}C NMR (75.47 MHz, $\text{DMSO}-d_6$) δ : 166.5 (C-9), 162.3 (C-2''), 154.5 (C-10a), 153.9 (C-4a), 142.2 (C-3), 135.3 (C-4), 127.9 (C-6''), 126.4 (C-6 and C-8'' to C-10''), 126.0 (C-7'' and C-11''), 125.9 (C-8), 124.7 (C-7), 122.6 (C-8a), 120.6 (C-1), 117.5 (C-5), 117.3 (C-9a), 113.9 (C-2), 56.6 (C-1''), 25.1 (C-5''), 18.0 (C-4'').

5.5. Williamson ether synthesis to attempt methyl 2-((9-oxo-4-(2-oxo-2-(((R)-1-phenylethyl)amino)ethoxy)-4a,9a-dihydro-9H-xanthen-3-yl)oxy)acetate

First attempt:

The (S)-2-[(3-hydroxy-9-oxo-9H-xanthen-4-yl)oxy]-N-(1-phenylethyl)acetamide (**25**) (0.920 g, 1.97 mmol) was dissolved in anhydrous acetone and K_2CO_3 (0.661 g, 4.78 mmol)

and $\text{BrCH}_2\text{COOCH}_3$ (0.5 mL, 5.29 mmol) were added. The mixture was kept under reflux and magnetic stirring for 3 days. Then, the organic solvent was evaporated under reduced pressure and the solid obtained was washed with *n*-Hex. After the spectral analysis to the solid obtained it was concluded that it not corresponded to the desired product.

Second attempt:

The (S)-2-[(3-hydroxy-9-oxo-9*H*-xanthen-4-yl)oxy]-*N*-(1-phenylethyl)acetamide (**25**) (0.920 g, 1.97 mmol) was dissolved in anhydrous THF and NaH (0.400 g, 16.7 mmol) and $\text{BrCH}_2\text{COOCH}_3$ (0.4 mL, 4.23 mmol) were added. The mixture was kept under reflux and magnetic stirring for 3 days. To stop the reaction, some drops of CH_3OH were added. Then, the organic solvent was evaporated under reduced pressure and the crude product was dissolved in CHCl_3 . This solution was washed with H_2O . After the spectral analysis to the solid obtained it was concluded that it not corresponded to the desired product.

Third attempt:

The (S)-2-[(3-hydroxy-9-oxo-9*H*-xanthen-4-yl)oxy]-*N*-(1-phenylethyl)acetamide (**25**) (0.920 g, 1.97 mmol) was dissolved in H_2O . A 5 M NaOH solution (2.4 mL, 12.000 mmol) and $\text{BrCH}_2\text{COOCH}_3$ (0.4 mL, 4.23 mmol) were added. The mixture was irradiated by microwaves (200 W) during a specific period (cycles of 10 minutes each). After the spectral analysis to the solid obtained it was concluded that it not corresponded to the desired product. the mixture was cooled to room temperature and the raw material was separated. This solution was washed with an organic solvent (CH_2Cl_2 and ethyl acetate). After the spectral analysis to the solid obtained it was concluded that it not corresponded to the desired product.

6. Development of CSP5

The planned CSP5 was not achieved although from the seven planned reaction steps they were performed four reaction steps successfully.

6.1. Attempt the direct coupling reaction of 3-methoxy-4-hydro-9H-xanthen-9-one (8) to (R)-(-)-N-(3,5-dinitrobenzoyl)- α -phenylglycine

First attempt:

The chiral reagent, (R)-(-)-N-(3,5-dinitrobenzoyl)- α -phenylglycine (**27**) (0.073 g, 0.211 mmol) was dissolved in THF and COMU (0.090 g, 0.210 mmol) was added. Following, TEA (60 μ L, 0.430 mmol) and the 3-methoxy-4-hydro-9H-xanthen-9-one (compound **8**) (0.050 g, 0.211 mmol) were added. The mixture was stirred at room temperature for 72 hours. After this time, the solvent was evaporated under reduced pressure and the crude product was dissolved in CH₂Cl₂. The organic solution was washed with 1 M HCl solution (2 $\times$ 25 mL), 0.5 M NaOH solution (2 $\times$ 30 mL), and H₂O (3 $\times$ 50 mL). The organic layer was dried with anhydrous NaSO₄, filtered and the organic solvent was evaporated under reduced pressure. After the spectral analysis to the solid obtained it was concluded that it not corresponded to the desired product.

Second attempt:

The chiral reagent, (R)-(-)-N-(3,5-dinitrobenzoyl)- α -phenylglycine (**27**), (0.030 g, 0.087 mmol) was dissolved in THF and TBTU (0.030, 0.104 mmol) was added. Then, TEA (20 μ L, 0.174 mmol) was added and the mixture was kept with magnetic stirring for 15 minutes, followed the 3-methoxy-4-hydro-9H-xanthen-9-one (compound **8**) (0.020 g, 0.087 mmol). The mixture was stirred at room temperature for 72 hours. After this time the solvent was evaporated under reduced pressure and the crude product was dissolved in CH₂Cl₂. This solution was washed with 1 M HCl solution (2 $\times$ 25 mL), 0.5 M NaOH solution (2 $\times$ 30 mL), and H₂O (3 $\times$ 50 mL). The organic layer was dried with anhydrous NaSO₄, filtered and the organic solvent was evaporated under reduced pressure. After the spectral analysis to the solid obtained it was concluded that it not corresponded to the desired product.

6.2. Synthesis of 4-(hydroxymethoxy)-3-methoxy-9H-xanthen-9-one (26)

The 3-methoxy-4-hydro-9H-xanthen-9-one (compound **8**) (0.23 g, 0.95 mmol) was dissolved in anhydrous acetone and NaH (0.1 g, 4.17 mmol) and BrCH₂CH₂OH (0.3 mL, 4.23 mmol) were added. The mixture was kept under reflux and magnetic stirring for 4 days. Then, the organic solvent was evaporated under reduced pressure and the crude product was dissolved in ethyl acetate. This solution was washed with 0.5 M NaOH solution (3 $\times$

100 mL) and H₂O (2 × 50 mL). The organic layer was dried with anhydrous NaSO₄, filtered and the organic solvent was evaporated under reduced pressure affording a yellow solid.

Yield: 80%. m.p.: 137-140°C. IR $\nu_{\max}$ (cm⁻¹) (KBr): 3423.2, 1602.0, 1467.0, 1290.5, 1224.5, 1068.1, 905.5, 823.5, 760.4. ¹H NMR (300.13 MHz, DMSO-*d*₆) δ : 8.16 (dd, *J* = 9.0, 1.2 Hz, 1H, H-8), 7.92 (d, *J* = 7.8 Hz, 1H, H-1), 7.84 (ddd, *J* = 7.4, 6.3, 1.2 Hz, 1H, H-6), 7.67 (dd, *J* = 7.4, 1.2 Hz, 1H, H-5), 7.47 (ddd, *J* = 9.0, 6.3, 1.2 Hz, 1H, H-7), 7.25 (d, *J* = 7.8 Hz, 1H, H-2), 4.14 (s, 2H, H-1''), 4.03 (s, 2H, H-2''), 3.78 (s, 1H, H-3''), 3.37 (s, 3H, H-1'). ¹³C NMR (75.47 MHz, DMSO-*d*₆) δ : 175.3 (C-9), 157.5 (C-3), 155.5 (C-4), 150.0 (C-1), 135.2 (C-10a), 135.1 (C-4a), 125.8 (C-2), 124.2 (C-6), 121.4 (C-8), 120.8 (C-7), 118.2 (C-5), 115.9 (C-8a), 109.7 (C-9a), 75.1 (C-1''), 60.4 (C-2''), 56.5 (C-1').

6.3. Synthesis of 2-[(3-methoxy-9-oxo-9*H*-xanthen-4-yl)oxy]ethyl (*R*)-2-(3,4-dinitrobenzamido)-2-phenylacetate (**28**)

2-((3-methoxy-9-oxo-9*H*-xanthen-4-yl)oxy)ethyl (*R*)-2-(3,4-dinitrobenzamido)-2-phenylacetate (**28**) was prepared from a suitable hydroxylated, the 4-(hydroxymethoxy)-3-methoxy-9*H*-xanthen-9-one (**26**), using the same method as described for compound **3**, being the amount of TBTU and TEA adapted according to the number of carboxyl groups of the chemical substrate and the stoichiometry of the reaction. The chiral reagent was the (*R*)-(-)-*N*-(3,5-dinitrobenzoyl)- α -phenylglycine. The final product was obtained as an orange solid.

Yield: 75%. m.p.: > 330°C. [α]_D^{25°C} -70.7 (*c* = 1.84 × 10⁻³ g mL⁻¹ in CH₃OH). IR $\nu_{\max}$ (cm⁻¹) (KBr): 3454.8, 1741.6, 1685.7, 1630.1, 1471.6, 1382.6, 1288.0, 1219.4, 1077.2, 923.5, 808.5, 777.9, 423.7, 408.9. ¹H NMR (300.13 MHz, DMSO-*d*₆) δ : 9.02 (t, *J* = 2.2 Hz, 3H, H-14'', H-16'', H-18''), 8.90 (d, *J* = 2.2 Hz, 1H, H-11''), 7.97 (dd, *J* = 8.2, 1.1 Hz, 1H, H-8), 7.93 (d, *J* = 8.4 Hz, 1H, H-1), 7.90 (ddd, *J* = 9.0, 6.9, 1.1 Hz, 1H, H-6), 7.73 (dd, *J* = 9.0, 1.1 Hz, 1H, H-5), 7.52 (d, *J* = 8.4 Hz, 1H, H-2), 7.40 (ddd, *J* = 8.2, 6.9, 1.1 Hz, 1H, H-7), 5.64 (m, 1H, H-4''), 2.69 (s, 3H, H-1'), 2.08 (s, 2H, H-1''), 1.23 (s, 2H, H-2'').

6.4. Synthesis of 2-[(3-hydroxy-9-oxo-9*H*-xanthen-4-yl)oxy]ethyl (*R*)-2-(3,4-dinitrobenzamido)-2-phenylacetate (**29**)

2-((3-hydroxy-9-oxo-9*H*-xanthen-4-yl)oxy)ethyl (*R*)-2-(3,4-dinitrobenzamido)-2-phenylacetate (**29**) was prepared from a suitable methoxylated compound, the 2-((3-

methoxy-9-oxo-9*H*-xanthen-4-yl)oxy)ethyl (*R*)-2-(3,4-dinitrobenzamido)-2-phenylacetate (**28**), using the same method as described for (*S*)-6-hydroxy-9-oxo-*N*-(1-phenylethyl)-9*H*-xanthene-2-carboxamide (compound **18**). The final product was obtained as an orange oil.

Yield: 50%. m.p.: > 330°C. $[\alpha]_D^{25^\circ\text{C}}$ -228.9 ($c = 0.83 \times 10^{-3} \text{ g mL}^{-1}$ in CH_3OH). IR ν_{max} (cm^{-1}) (KBr): 3454.8, 1741.6, 1685.7, 1630.1, 1471.6, 1382.6, 1288.0, 1219.4, 1077.2, 923.5, 808.5, 777.9, 423.7, 408.9. ^1H NMR (300.13 MHz, $\text{DMSO}-d_6$) δ : 8.95 (t, $J = 2.1 \text{ Hz}$, 3H, H-14'', H-16'', H-18''), 8.91 (d, $J = 2.1 \text{ Hz}$, 1H, H-11''), 7.97 (dd, $J = 8.2, 1.1 \text{ Hz}$, 1H, H-8), 7.94 (d, $J = 8.4 \text{ Hz}$, 1H, H-1), 7.93 (ddd, $J = 9.0, 6.9, 1.1 \text{ Hz}$, 1H, H-6), 7.53 (dd, $J = 9.0, 1.1 \text{ Hz}$, 1H, H-5), 7.50 (d, $J = 8.4 \text{ Hz}$, 1H, H-2), 7.48 (ddd, $J = 8.2, 6.9, 1.1 \text{ Hz}$, 1H, H-7), 5.62 (m, 1H, H-4''), 2.96 (s, 2H, H-4'), 1.96 (s, 2H, H-1''), 1.20 (s, 2H, H-2'').

7. General procedure for LC column packing

The prepared CSPs (**CSP1-CSP3**) (~ 800 mg) were dried in a desiccator under vacuum for 24 hours. Each new CSP was mixed with *n*-Hex:2-PrOH (50:50 v/v) (30 mL), and then sonicated for 3 minutes. The suspension was poured into the chamber of a column packer and it was packed into an empty LC column (150 x 2.1 mm i.d.) with *n*-Hex:2-PrOH (90:10 v/v) as packing solvent, under a pressure no more than 7500 psi.

B. Evaluation of enantioresolution by liquid chromatography

1. Instrumentation and chromatographic conditions

The analytical LC analyses were performed on a JASCO LC-NetII/ADC equipped with an 880-PU pump, an 880-30 solvent mixing module, an injector and an UV detector 875-UV.

The solvents used were HPLC grade and purchased from Sigma-Aldrich Co (St. Louis, Missouri, USA).

The LC analysis were performed at room temperature in normal phase elution mode using different mobile phases: *n*-Hex:2-PrOH (90:10, 80:20 or 70:30 v/v) and *n*-Hex:EtOH (95:5, 90:10, 80:20 or 70:30 v/v). The mobile phases were degassed in a sonicator bath for 15 minutes before use.

The dead-time (t_0) was assumed to be equal to the peak of the solvent front and was taken from each run. The working flow rate was, mainly, 0.2 mLmin⁻¹ although, in some analysis, the flow rates 0.15, 0.10 and 0.05 mLmin⁻¹ were also chosen. The chromatograms were monitored by UV detection at a wavelength of 254 nm.

The racemic working solutions of tested analytes were prepared from stock solutions (1 mgmL⁻¹ in EtOH) and were diluted, in the same solvent, to a final concentration of 50 µgmL⁻¹. The working solutions of enantiomeric mixtures of tested analytes were prepared by mixing an equal quantity of each enantiomer in order to obtain a final concentration of 50 µgmL⁻¹.

The analyte injections (10 µL) were performed in duplicate.

2. Chromatographic parameters

The chromatographic parameters determined during the LC analysis (the retention factor (k), the separation factor (α), the resolution (R_s) and the number of theoretical plates (N)) were calculated using the equations presented below and after chromatogram interpretation (**Figure 29**). The number of theoretical plates (N) for **CSP1-CSP3** was determined for the analyte which presented the best chromatographic data.

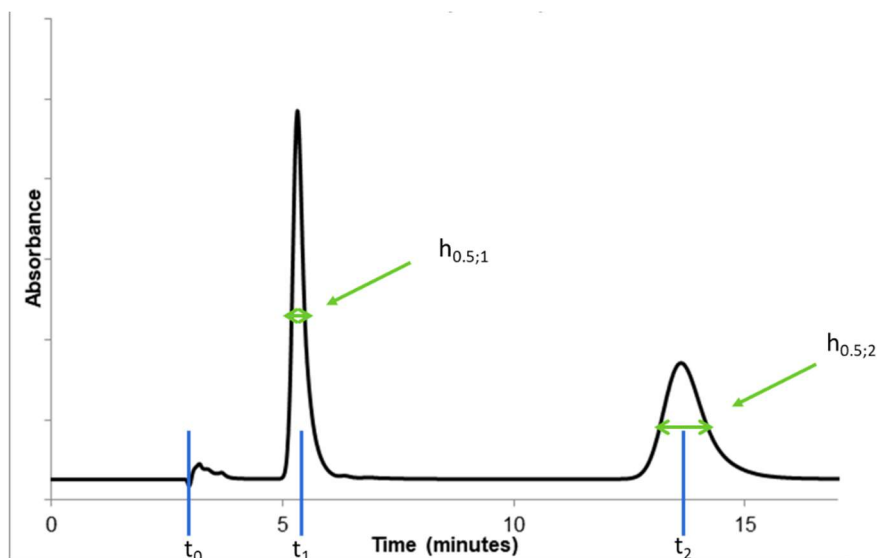


Figure 30 - Chromatogram of an enantiomeric separation and indication of parameters that can be obtained by its analysis.

Retention factor (k) is calculated according the following equation⁵⁶:

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

where t_R is the retention time for each enantiomer and t_0 is the dead time.

Separation factor (α) is calculated considering the retention factors following the equation 3⁵⁶.

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

where k_1 is the retention factor of the enantiomer which is eluted first and k_2 is the retention factor of the most retained enantiomer.

Resolution factor (R_S) is obtained from the ratio between the difference of retention time of each enantiomers and the sum of peak lengths at half height. The equation is presented below:

$$R_S = 1.18 \times \frac{t_2 - t_1}{w_{0.5,2} + w_{0.5,1}} \quad (4)$$

t_2 and $h_{0.5,2}$ are the retention time and the peak length for enantiomer which eluted at last and t_1 and $h_{0.5,1}$ are the retention time and peak length for enantiomer which is the first to elute¹⁶⁷.

The number of theoretical plates (N) evaluates the performance of the CSP being that a higher value indicates a superior efficiency of the column as the number of equilibrium steps established is superior.

$$N = 5.54 \left(\frac{t_r}{w_{0.5}} \right)^2$$

V. CONCLUSIONS AND FUTURE PERSPECTIVES

The development of new CSPs for LC is a continuous and challenging issue covering various types of CSPs.

The CSPs developed on this work were prepared by multi-step pathways starting from four suitable functionalized XDs based on the most promising selectors (CDXs) previously reported. Herein, the synthesized CSs were (*R*)-phenylglycinol derivatives comprising one or two different chiral moieties, and they were covalently bonded through one or more sites to a chromatographic support. The influence of the chiral moiety, (*R*)-(-)-2-phenylglycinol, in different positions of the xanthone scaffold was studied. The development of five CSPs was planned being possible to conclude the synthesis of three new CSPs. The description of fourteen new compounds (CSs and intermediates) was also presented.

For the three CSPs prepared, a preliminary evaluation of their LC enantioresolution performance was performed. The LC enantioresolution performance was evaluated under normal-phase elution mode using mixtures of *n*-Hex with 2-PrOH or EtOH, at different proportions, as mobile phases. Two CSPs (**CSP1-CSP2**) presented enantioselectivity and baseline separation for two analytes, while the other CSP (**CSP3**) demonstrated enantioselectivity for three analytes. Separation factors ranging from 1.41 to 16.4 and resolution values ranging from 1.08 to 6.10 were obtained. The best chromatographic result was obtained for the analyte XEGOL2 with a separation factor and resolution of 16.4 and 5.45, respectively.

The selector loading was calculated for each CSP according to the data obtained from the elementary analysis. The values obtained for **CSP1**, **CSP2** and **CSP3** were 1.50, 1.00 and 0.32 $\mu\text{mol m}^{-2}$, respectively.

The results of the enantioresolution performance were evaluated considering the scaffold of the CS. The number and position of the chiral units linked to XD can interfere with the chiral recognition mechanism by steric hindrance. The presence of different chiral moieties at opposite positions demonstrated to be beneficial (**CSP3**) leading to a superior enantioselectivity and reduction of the retention factors. The possibility of interactions that this CSP can establish is superior, due to the different chiral moieties, which was reflected on a higher enantiorecognition potential. Since the **CSP1** and **CSP2** presented the same chiral unit it was expected a similar behaviour. This behaviour was confirmed by the results obtained since their enantioresolution performance was identical.

As future work, the attempt to conclude the synthesis of **CS4** and **CS5** should be performed. Taking into consideration the conclusions of the work developed, the scaffold of these selectors could be modified in order to introduce the planned chiral moieties in different positions more specifically, at opposite positions.

The evaluation of the LC enantioresolution performance of the developed CSPs (**CSP1-CSP3**) should be proceeded being evaluated their behaviour at different elution modes (as polar organic or reverse-phase mode) and extend the range of the analytes evaluated.

The development of efficient chromatographic tools for LC enantioresolution is a subject that should continue to receive special attention since it has constructive repercussions in several other research areas, such as biomedical, toxicology and forensic sciences, environment, food and fragrances, industry, among others. Besides, the goal of developing a universal CSP remains a dream to be reached by those working in this research area.

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APPENDIX 1

Chiral stationary phases based on small molecules: development, enantioresolution evaluation and chiral recognition mechanisms

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High-performance liquid chromatography (HPLC) enantioresolution using chiral stationary phases (CSPs) is one of the most significant separation approaches in academic research and industry, for both analytical and preparative purposes [1]. A large number of CSPs for HPLC have been commercially available; however, there is no universal CSP and some chiral compounds remain to be resolved [2]. Therefore, the development of new chromatographic CSPs possessing effective enantioselective separation ability continues to be an important issue to overcome these gaps in chiral HPLC. Recently, we reported the development of new CSPs based on chiral derivatives of xanthenes (CDXs) [3], a different type of small molecules from the commercially available CSPs [4]. Herein, twelve CSPs were successfully prepared by multi-step pathways starting from diverse suitable functionalized small molecules. The chiral selectors were covalently bonded through one or more sites to a chromatographic support, further by packing into HPLC stainless-steel columns (150 x 2.1 mm i.d.). The enantioresolution performance of the CSPs was evaluated using different standard racemates as well as a library of enantiomeric mixtures of CDXs prepared “in-house”. The chromatographic results showed that these CSPs have enantiomeric specificity for CDXs. Besides, exploration of chiral recognition mechanisms was performed by computational studies using molecular docking approach, being in accordance with the chromatographic parameters.

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CHIRAL STATIONARY PHASES BASED ON SMALL MOLECULES: DEVELOPMENT, ENANTIORESOLUTION EVALUATION AND CHIRAL RECOGNITION MECHANISMS

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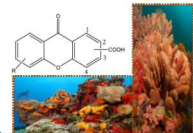
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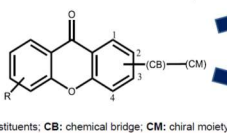
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INTRODUCTION

Xanthone derivatives (Marine like)



Chiral derivatives of xanthones (CDXs)^{1,2}



R: diverse substituents; CB: chemical bridge; CM: chiral moiety

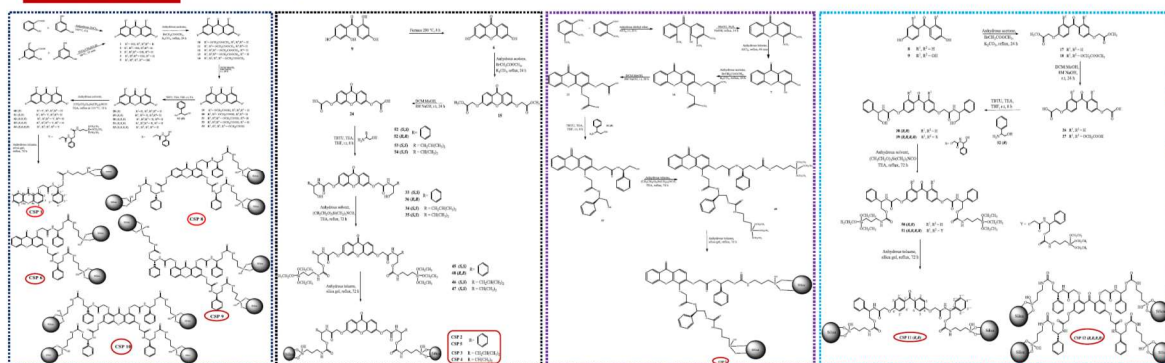
I. Development of new chiral stationary phases (CSPs) for liquid chromatography (LC)³

II. LC enantioresolution and chiral recognition mechanisms⁴⁻⁷

Aims of the research

RESULTS

1. Development of CSPs based on Xanthone and Benzophenone scaffolds (CSPs 1-12)



2. LC enantioresolution

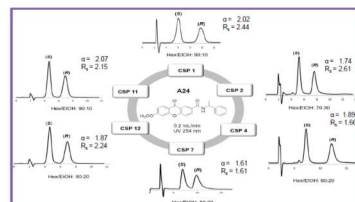


FIGURE 1. Chromatograms for the enantioseparation of analyte 24 on different CSPs, at optimized chromatographic conditions.

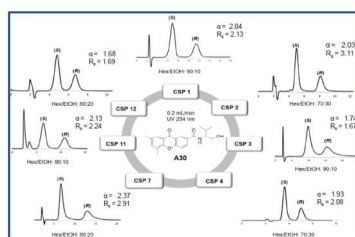


FIGURE 2. Chromatograms for the enantioseparation of analyte 30 on different CSPs, at optimized chromatographic conditions.

3. Computational studies

CSP	A24		A30	
	Docking Score (kcal/mol ⁻¹)	Energy difference (kcal/mol ⁻¹)	Docking Score (kcal/mol ⁻¹)	Energy difference (kcal/mol ⁻¹)
CSP 1 (X-ray structure)	(S): -4.5 (R): -4.8	-0.3	(S): -4.5 (R): -4.7	-0.2
CSP 2	(S): -5.4 (R): -5.5	-0.1	(S): -5.2 (R): -5.4	-0.2
CSP 3	(S): -5.0 (R): -5.1	-0.1	(S): -4.6 (R): -4.9	-0.3
CSP 4	(S): -5.5 (R): -5.7	-0.2	(S): -5.1 (R): -5.4	-0.3
CSP 5	(S): -5.1 (R): -5.6	-0.5	(S): -5.0 (R): -5.3	-0.2
CSP 6	(S): -5.5 (R): -5.7	-0.2	(S): -5.2 (R): -5.2	0.0
CSP 7	(S): -3.4 (R): -3.5	-0.1	(S): -4.0 (R): -4.4	-0.4
CSP 8	(S): -5.0 (R): -5.9	-0.9	(S): -4.1 (R): -4.2	-0.1
CSP 9	(S): -6.3 (R): -6.5	-0.2	(S): -6.1 (R): -6.1	0.0
CSP 10	(S): -6.0 (R): -6.1	-0.1	(S): -5.7 (R): -5.7	-0.1
CSP 11	(S): -5.3 (R): -5.4	-0.1	(S): -5.0 (R): -5.2	-0.2
CSP 12	(S): -6.0 (R): -6.1	-0.1	(S): -5.6 (R): -5.7	-0.1

FIGURE 3. Ortep view of the crystal structure of chiral selector of CSP 1. Cambridge Crystallographic Data Centre (CCDC 1908277).

100% agreement between docking scores and chromatographic data for enantioselectivity and elution order

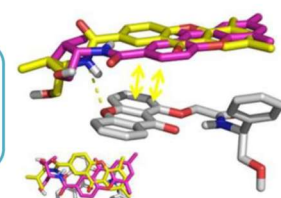


FIGURE 4. (A) Crystallographic CSP 1 and docked A30 enantiomers (top-view of the docking poses is represented on the bottom-left corner of the image). Selector is represented as grey sticks; (R) and (S) enantiomers are represented as yellow and pink sticks, respectively. Hydrogen interactions and π -stacking interactions are represented in yellow broken lines and yellow double arrow, respectively.

CONCLUSIONS

- Twelve new CSPs based on xanthone and benzophenone derivatives were successfully developed to explore the role of some structural characteristics that could be crucial for enantioselectivity.
- The number and position of the chiral units positioned in the xanthone scaffold of the chiral selectors can interfere with the chiral recognition mechanism by steric hindrance.
- The central aromatic core of the selector, either xanthone or benzophenone scaffolds, aligned with the xanthone tricyclic ring of the analytes to maximize π -stacking interactions, as well as the hydrogen bonding interactions between the donor and acceptor polar groups of analytes and selectors, demonstrated to be essential for enantioselective recognition.

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Figure A 1 - Poster from 48th International Symposium on High-Performance Liquid Phase Separations and Related Techniques.

APPENDIX 2

Development of liquid chromatography chiral stationary phases for separation of enantiomeric drugs

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Liquid chromatography (LC) using chiral stationary phases (CSPs) is an essential tool with applications in preparative separation of enantiomers of chiral drugs, determination of enantiomeric composition, monitorization of asymmetric reactions, analysis of the stereochemistry of natural compounds, pharmacokinetic, and enantioselective studies¹⁴³¹. This work, describes the successful development of several CSPs, by multi-step pathways, based on the most promising selectors recently obtained in our group [5].

The versatility and flexibility are two of the advantages of the strategy adopted since it is possible to design and modify a variety of different small molecules for use as CSPs. In addition, they are compatible with a wide range of solvents used as mobile phases.

Acknowledgments: This research was supported by the Strategic Funding UID/Multi/04423/2013 through national funds provided by FCT and ERDF, in the framework of the programme PT2020, the project PTDC/MAR-BIO/4694/2014 (reference POCI-01-0145-FEDER-016790; Project 3599-PPCDT), by the Project No. POCI-01-0145-FEDER-028736, co-financed by COMPETE 2020, under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF) as well as CHIRALXANT-CESPU-2018 and the Portuguese NMR Network. Y.Z. Phyo thanks the Erasmus Mundus Action 2 (Lotus Plus project) for a PhD's scholarship.

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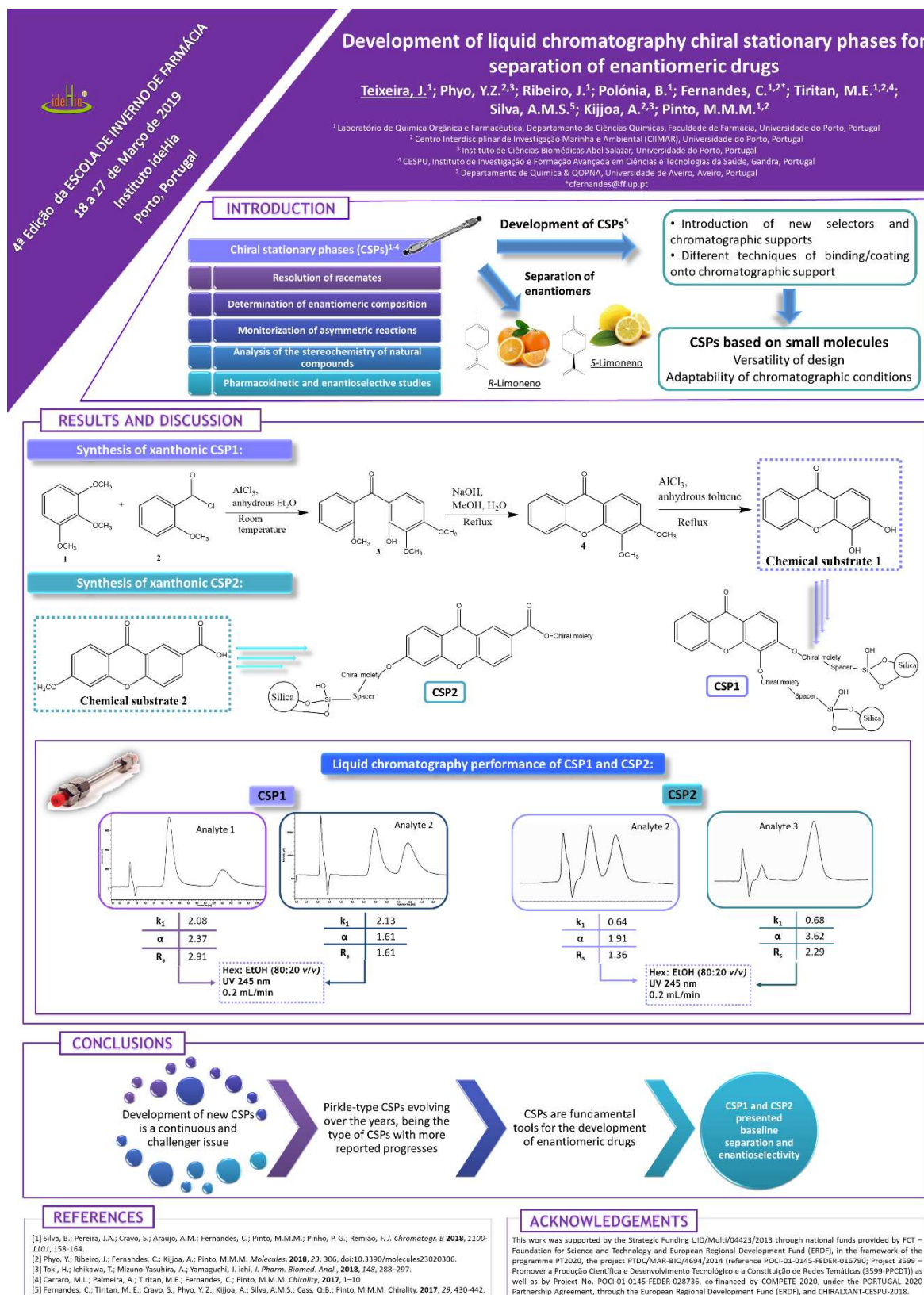


Figure A 2 - Poster from Escola de Inverno de Farmácia - 2019.

APPENDIX 3

Synthesis and structure elucidation of new chiral selectors for liquid chromatography

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The enantioseparation resorting to liquid chromatography and to chiral stationary phases (CSPs) is one of the most versatile and widely applied method to analyse and isolate enantiomers [1]. Over the last few years, CSPs have been applied for resolution of racemates, determination of enantiomeric composition, monitorization of asymmetric reactions, analysis of the stereochemistry of natural compounds, pharmacokinetic, and enantioselective studies [1-4].

The strategies adopted for development of CSPs include, essentially, the introduction of new selectors and chromatographic supports, and different techniques of binding/coating onto chromatographic support [5].

Herein, the synthesis and structure elucidation of new selectors inspired on Pirkle-type CSPs were performed. This type of CSPs are known by their versatility and flexibility since it is possible to create and adapt a variety of small molecules to chiral selectors. In addition, they are compatible with a wide range of solvents used as mobile phase and stable to temperature and pressure [5].

Acknowledgements

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APPENDIX 4

Review

Chiral Stationary Phases for Liquid Chromatography: Recent Developments

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Abstract: The planning and development of new chiral stationary phases (CSPs) for liquid chromatography (LC) are considered as continuous and evolutionary issues since the introduction of the first CSP in 1938. The main objectives of the development strategies were to attempt the improvement of the chromatographic enantioresolution performance of the CSPs as well as enlarge their versatility and range of applications. Additionally, the transition to ultra-high-performance LC were underscored. The most recent strategies have comprised the introduction of new chiral selectors, the use of new materials as chromatographic supports or the reduction of its particle size, and the application of different synthetic approaches for preparation of CSPs. This review gathered the most recent developments associated to the different types of CSPs providing an overview of the relevant advances that are arising on LC.

Keywords: liquid chromatography; enantioseparation; chiral stationary phase; chiral selector; chromatographic support

1. Introduction

Now more than ever, analytical and preparative enantiomeric separations play a crucial role in industry and academic research [1]. There are a wide variety of methods to achieve and analyze enantiomerically pure compounds, including liquid chromatography (LC) [2,3], supercritical fluid chromatography [4–6], diastereomeric crystallization [7,8], membranes [9,10], asymmetric catalysis [11], simulated moving bed [12,13], dynamic and enzyme-mediated kinetic resolution [14,15], among others.

LC using chiral stationary phases (CSPs) proved to be an essential tool with a wide range of applications, including preparative separation of enantiomers of diverse analytes [16,17], determination of enantiomeric composition [18,19], monitorization of asymmetric reactions [20,21], analysis of the stereochemistry of natural compounds [22,23], pharmacokinetic [24,25], forensic [26–28], environmental [29–31], and enantioselective studies [32,33], among others.

The development of CSPs for LC combined with the improvement of chromatography instrumentation revolutionized the enantioseparation approaches. LC using CSPs has demonstrated to be extremely useful, accurate, versatile, and it has been a widely used technique in diverse fields and applications, emphasizing, for example, the enantioseparation of underivatized amino acids

[34,35], diverse classes of pharmaceuticals [36–40], atropisomers [41], as well as the study of intermolecular interactions between biomolecules and drugs [42], among others.

Over the last decades, several types of CSPs have been developed [43–46] and, among them, more than a hundred are currently commercially available [39]. These comprise Pirkle-type, ligand-exchange-type, molecularly-imprinted, and based on macrocyclic antibiotics, proteins, polysaccharides, cyclodextrins, crown ethers, cyclofructans, synthetic polymers, among others [43–46]. Nevertheless, although many different types of CSPs are described, the development of new CSPs continues to be a field of research with great importance.

2. Chiral Stationary Phases: Recent Developments

Since the first description of a CSP, in 1938, by Henderson and Rule [47], and to follow the constant challenges on different areas as well as the advances in chromatographic instrumentation, the development of new CSPs for LC has been a continuous and evolutionary subject.

In this review, for each type of CSPs, the most recent CSPs were presented describing the strategies used for their development (Figure 1).

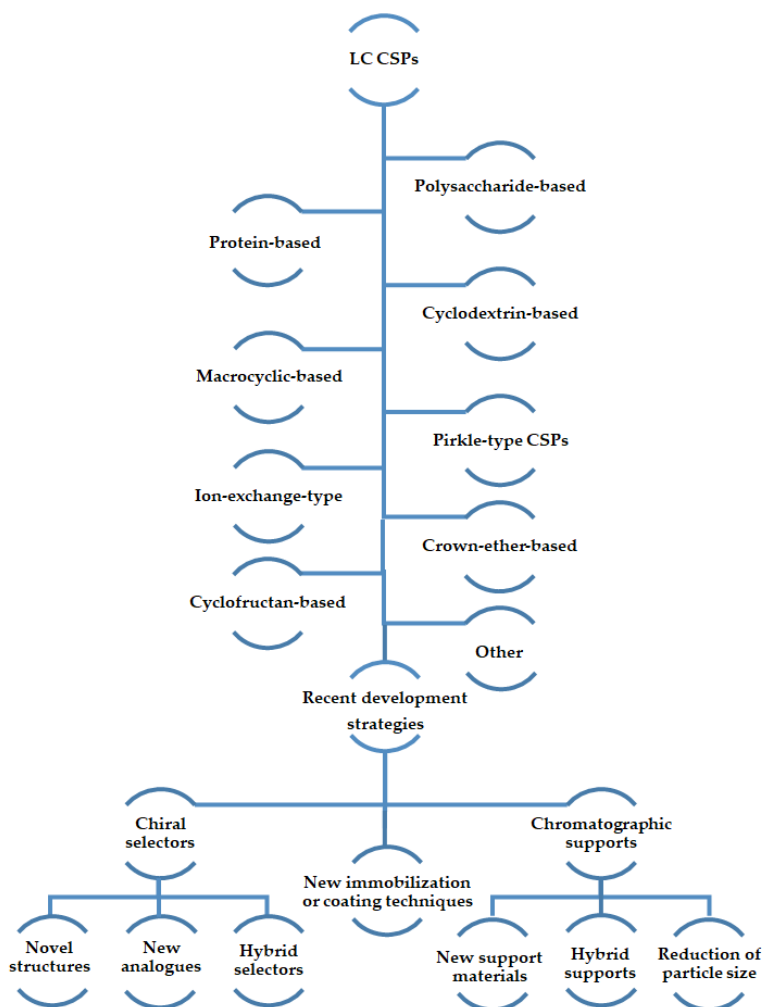


Figure 1. Summary of recent strategies for development of new chiral stationary phases (CSPs) for liquid chromatography (LC).

Herein, only the CSPs that were not reported in previous fundamental reviews will be presented, highlighting the main advantages of the strategy used for their preparation, the best chromatographic results and the objectives to be achieved.

2.1. Polysaccharide-Based CSPs

The first application of a polysaccharide as a chromatographic chiral selector was described by Hessen and Hagel, in 1976 [48]. Since then, different polysaccharides were extensively used as CSPs due to their high enantioselectivity properties after derivatization [49]. Nevertheless, amylose and cellulose are the main polysaccharides used to obtain CSPs [50], followed by chitosan and chitin [51]. The chiral recognition ability of polysaccharide derivatives is dependent on diverse structural features, including sugar units, stereogenic centres of the glucopyranose units, type of the linkage and its position, as well as the adjacent polymer chains [49]. The helical twist of the polymer backbone is also essential for enantioselectivity [43].

Polysaccharide derivatives as efficient chiral selectors can include phenyl, alkyl or benzylcarbamates, esters, benzoate, or aryl or cycloalkyl groups [52]. Benzoate or phenylcarbamate moieties may comprise methyl, methoxy, among other groups, and/or chlorine substituents in the aromatic ring [52], affording different solubility and chiral recognition ability [52]. Moreover, the position of the substituents in the aromatic ring influences the enantioseparation performance of the chiral selector [49].

Polysaccharide derivatives can be coated onto a chromatographic support, as silica or derivatives, by an adsorption process [53,54] allowing a larger surface area [55] and high efficiency [43]. CSPs comprising coated polysaccharide derivatives can operate in normal phase, polar organic and reversed-phase elution mode; however, they have restrictions due to the non-compatibility with “non-standard” solvents, such as dichloromethane, chloroform, toluene, or acetone [43]. The use of those solvents in the mobile phase may cause the dissolution of the adsorbed polymer and, consequently, removal of the selector from the chromatographic column [43]. Immobilized polysaccharides emerged as a reliable alternative allowing the use of a broader selection of solvents as mobile phases [56–60]. Different procedures can be used for covalently bonded the polysaccharide derivatives to the chromatographic support, such as a polymerization reaction and photoinduced and enzymatic polymerization [56]. Nevertheless, despite the solvent versatility, in general, the potential of chiral recognition of immobilized polysaccharide-based CSPs is lower than the coated due to modification of stereospecific conformation that can occur during the immobilization process [56,57].

This type of CSPs is recognized as being the most successful and widely applied for both analytical [61–69] and preparative enantioseparations [17,70–76], being responsible for about 99% of reported chiral separations [50]. Among the developed polysaccharide-based CSPs, the 3,5-dimethylphenyl *tris*-phenylcarbamates of amylose and cellulose proved to have the best enantiorecognition performance [77–80]. In our group, this type of CSPs has proved to be effective for analytical as well as preparative applications [26,81–84].

The chiral recognition mechanisms concerning these CSPs are not yet completely understood. In an attempt to improve the knowledge related with structural features associated with the chiral recognition mechanisms and their chromatographic behavior at a molecular level, several studies concerning to docking, spectroscopy, molecular modelling, and quantum chemical calculations were recently performed and compiled by Scriba et al. [45].

Several reviews have assembled the advances on preparation and evaluation of this type of CSPs over the years [43,45,49–52,57–59,80,85–93]. Nevertheless, this research field is always evolving being the most recent polysaccharide-based CSPs presented on Table S1 (supplementary material).

Recent developments on polysaccharide-based CSPs comprise different approaches, with the general objective being the improvement of the enantioseparation performance. The strategies include the introduction of new polysaccharide derivatives (mainly new chitin and chitosan derivatives but also cellulose derivatives), hybrid selectors, and different chromatographic supports (monoliths, core-shell, microspheres), as well as the application of different methodologies for coating or immobilization procedures. Generally, for the new derivatives, the effect of different substituents on chiral recognition has been discussed [94–97]. Additionally, the effect of mobile phase composition on enantioseparation was also explored [94–96,98,99].

Recently, Han et al. [94] developed two CSPs, using a derivative of cellulose *tris* (3,5-dimethylphenylcarbamate) (CSP1) and the same derivative functionalized with carboxylic acid (CSP2) (Figure 2). They concluded that a large variety of substituents could avoid the chiral recognition properties of the cellulose derivatives, reducing the performance of the CSP. The best chromatographic results were obtained for *trans*-stilbene oxide, with α and R_s values of 1.84 and 9.59, respectively.

Shen et al. [100] synthesized cellulose derivatives with different combination of carbamate substituents and prepared 25 new CSPs (CSP3–27) (Figure 2). The effect of the carbamate substituents at 2,3-positions and 6-position of the glucose moiety were the main focus of the study. It was found that the chiral recognition properties of the CSPs comprising derivatives with two different phenylcarbamates were higher than if CSPs only had one substituent. The resolution was improved by the presence of different carbamate substituents, suggesting that the chiral recognition was dependent on the electronic properties, position and number of substituents of the glucose unit [100]. The highest separation factor obtained by using these recent CSPs was 2.87, for Pirkle alcohol.

Chitin and chitosan-based CSPs have received particular attention in the last few years [51]. Through continuous efforts to develop effective CSPs, other recent reports describing the use of chitin [101,102] and chitosan [95–97] derivatives are rising, with the carbamates as one of the most studied [49]. The growing interest in these polysaccharides comes from the fact that they have low solubility, which allows the use of a wide variety of mobile phases [52]. The influence of substituents on chitin and chitosan derivatives was also investigated. For some analytes, these CSPs possessed an enhanced chiral recognition when compared to cellulose and amylose derivatives, which may be attributed to the variety of solvents that can be used [103].

Tang et al. [97] developed eight CSPs (CSP28–35) comprising chitosan 3,6-*bis*(arylcarbamate)-2-(*p*-methylbenzylurea) with diverse substituents in the aromatic rings of the carbamates as well as in the amide group (Figure 2). Selectors with electron-donating substituents demonstrated a higher ability of enantioseparation. Previous reports emphasized that an electron-donating substituent at the 4-position of the aromatic ring was beneficial for the chiral separation [44]. Despite the selectors with 4-methyl substituent and 3-chloro-4-methyl portion presented a superior enantioseparation, the highest resolution ($R_s = 18.1$) and separation factor ($\alpha = 6.72$) were obtained by the CSP with 3,5-dimethyl substituent [97].

In another study, Zhang et al. [95] prepared seven CSPs (CSP36–42) comprising derivatives of chitosan *bis*(phenylcarbamate)-(N-cyclobutylformamide) (Figure 2). The same substituent on different positions resulted on modifications on the suprastructure of the selector leading to different size of cavities, for example, due to different electronic effects. The obtained CSPs proved to have considerable stability on different solvents and a good enantioselectivity, allowing the researchers to obtain a separation factor of 8.64 for voriconazole [95].

Other new chitosan-based CSPs were developed, in this case, comprised of derivatives of chitosan (*bis*(methylphenylcarbamate)-(isobutyrylamide)) (CSP43–48) (Figure 2) [96]. The introduction of some substituents on specific positions of the aromatic ring linked to the carbamate were favorable for enantioseparation, such as methyl substituents. Additionally, the low solubility of chitosan was proved to be an advantage for the solvent tolerance and good enantioselectivity performance achieved. As an example of its performance, high enantioselectivity and resolution were obtained for voriconazole, with α and R_s values 4.32 and 11.9, respectively [96].

Zhang et al. [102] synthesized derivatives of chitin using three different phenyl isocyanates (4-trifluoromethoxy, 3-chloro-4-methyl, 4-chloro-3-trifluoromethylphenylcarbamate) to develop three CSPs (CSP49–51) (Figure 2). All CSPs were applied for enantioseparation of tadalafil and its intermediate, demonstrating great enantioselectivity potential, with resolution and separation factor values of 4.72 and of 2.15, respectively [102].

Mei et al. [101] derivatized natural and regenerated chitins with 3,5-dimethylphenyl isocyanate, to prepare CSP52 and CSP53–55, respectively (Figure 2), with the difference between them only related to the raw material. The regenerated chitins were obtained from natural chitins, after a

treatment with acetic anhydride, showing a more promising performance. They pointed out that CSP prepared from selectors with lower molecular weight provided an improved resolution [101]. The best chromatographic results were obtained for voricomazole, with $R_s = 11.7$ and $\alpha = 3.06$.

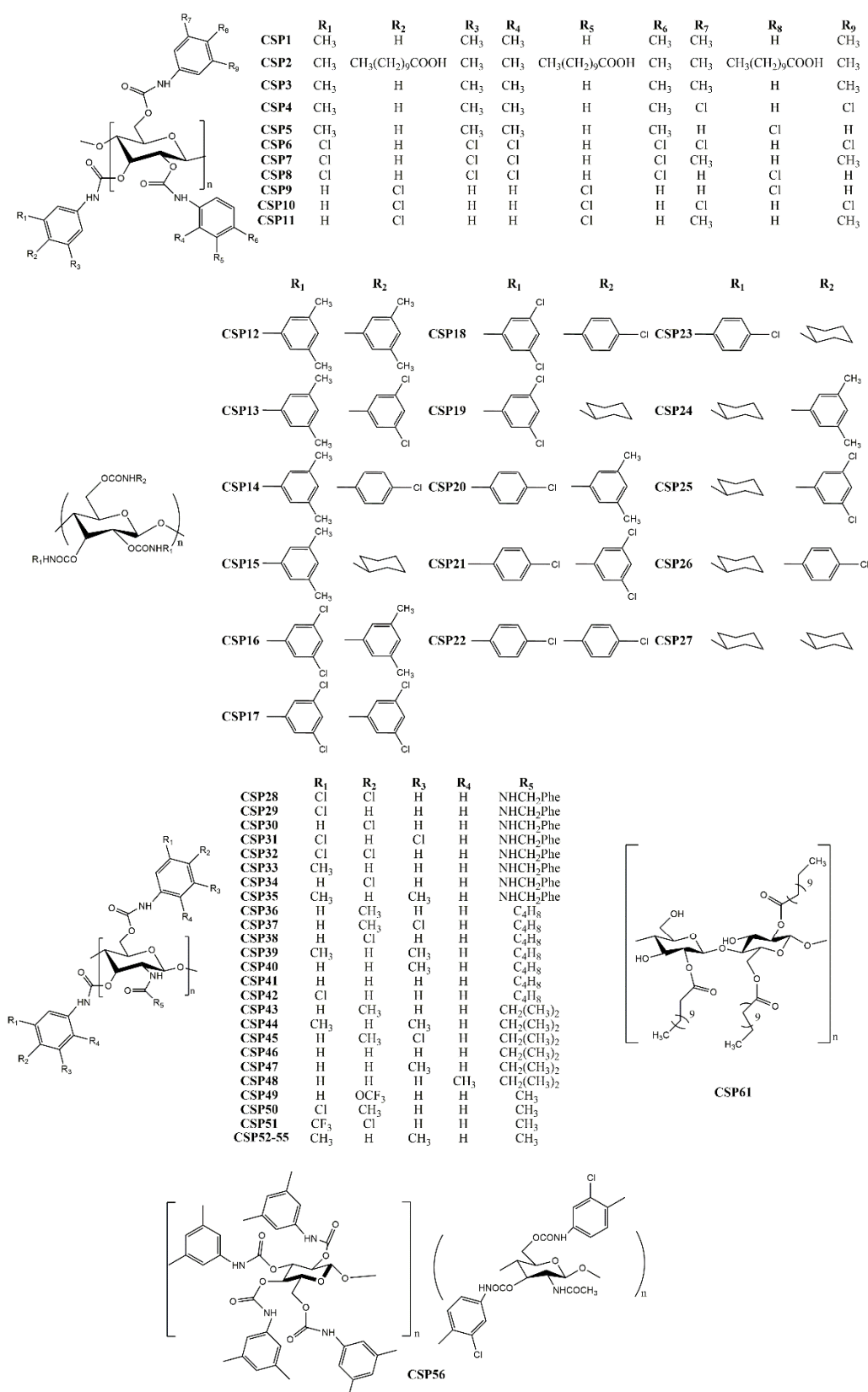


Figure 2. Chemical structures of polysaccharide-based CSP1–56 and CSP61.

Another strategy was the development of hybrid selectors [98]. Hybrid selectors or biselectors comprise two different polysaccharide derivatives coated on a chromatographic support [104]. Zhang et al. prepared the CSP56, which comprised a biselector based on derivatives of amylose and chitin (Figure 2), combining the low solubility of chitin derivatives with the excellent chiral recognition properties of amylose derivatives [98]. The obtained CSP presented an improved resistance against organic solvents with high enantioselectivity, with a R_s value of 8.49 for mephobarbital, and an α value of 4.32 for 2-(5-chloro-2-((4-methoxybenzyl)-amido)phenyl)-4-cyclopropyl-1,1,1-trifluorobut-3-yn-2-ol.

Regarding the use and preparation of chromatographic supports, a new technique of encapsulation in organic polymer monolith was reported, by Fouad et al. [103], as an alternative preparation of the chromatographic support. They functionalized an organic polymer monolith with carbamylated amylose as selector to obtain the CSP57. The synthesis of the amylose derivative was described previously [105]. The encapsulation of amylose was described as an economic methodology and it allowed the conjugation of amylose with reversed-phase elution mode for several analytes [103]. This promising technique allowed good results with a maximum resolution of 2.80 and a separation factor of 3.90 for the testes analytes.

Another different approach for preparation of chromatographic support was reported by Li et al. [99], who functionalized core-shell silver particles with cellulose derivatives through coating and intermolecular polycondensation and developed CSP58. A synergetic effect between silver and cellulose was observed considering the high values of resolution ($R_s = 2.61$) and enantioseparation ($\alpha = 8.42$). This CSP demonstrated a particular selectivity toward analytes having the functional group ketone [99].

In another study, Bezhitashvili et al. [106] reported the covalent immobilization of a cellulose derivative (cellulose-(3,5-dichlorophenylcarbamate)) onto the surface of core-shell particles to obtain CSP59. The synthesis of the cellulose derivative was described previously [77]. The click chemistry was the synthetic methodology applied for the immobilization of the cellulose derivative to the chromatographic support [107]. The authors emphasized the short time of analysis achieved with baseline separations [106]. The highest separation factor obtained was 15.3 with a resolution of 11.0 for 2-(4-methylbenzylsulfinyl)-benzamide.

Huang et al. [108] developed a new methodology for coating cellulose *tris*(3,5-dimethylphenyl carbamate) derivative on silica microspheres, without any surface pre-treatment since no aggregation occurred, and prepared CSP60 (range of pore size 10–150 nm). The synthesis of the cellulose derivative was previously described [109]. The silica microspheres with reduced size have functionalized polymeric beads being highly crosslinked [108]. This technique allowed a high-loading of the chiral selector and the obtained CSP provided a good performance, being the best separation factor of 2.41 for 2,2,2-trifluoro-1-(9-anthryl) ethanol.

The CSP61 (Figure 2) was reported by Vieira et al. [110] using a technique of thermal immobilization of cellulose dodecanoate on silica particles without the use of chemical reagents. Despite of the absence of a chemical reagent during the procedure of immobilization, the selector was strongly linked to the chromatographic support allowing an exceptional selectivity. Some advantages of the immobilization technique were highlighted, including its low cost and eco-friendly feature [110]. The separation factor obtained was 3.10.

Besides the recent developments on polysaccharide-based CSPs, it is important to emphasize that this type of CSPs also cover a wide range of recent applications [111–113]. For example, Padró et al. [89] recently reviewed applications of polysaccharide-based CSPs in different fields. Studies comparing the enantioresolution performance of coated and covalently immobilized CSPs based on polysaccharides were also found [114].

Additionally, the influence of mobile phase is a common focus in several studies [81,115].

2.2. Protein-Based CSPs

The intrinsic ability of chiral recognition by enzymes, plasma proteins and receptors inspired the application of proteins in enantioseparation techniques [43]. Proteins are complex structures with a large surface area comprising a variety of stereogenic centers and different binding sites, which allow multiple possibilities of intermolecular interactions with small molecules [55]. The first application of a protein as CSP was reported in 1973, describing the separation of tryptophan enantiomers using a bovin serum albumin (BSA)-sepharose CSP [116]. After this first report, many CSPs based on proteins have been developed, with the most used proteins the human serum albumin (HSA), α_1 -acid glycoprotein (AGP), crude ovomucoid (OVM), and cellobiohydrolase I (CBH I) [86]. All these proteins as chiral selectors have been well documented for chromatographic enantioseparation for a wide range of chiral compounds and for binding affinity studies [117].

Proteins as CSPs are applied on affinity and pharmacokinetic studies since they can mimic the *in vivo* systems [118], being this feature very important in drug discovery [43]. The possibility of using aqueous or aqueous-organic as mobile phases was pointed out as other advantage of protein-based CSPs considering its compatibility with mass spectrometric detection. The disadvantages of this type of CSPs are the low capacity and efficiency. Moreover, the possibility of denaturation of protein limit the ranges of pH, ionic strength, temperature, and organic modifier composition of mobile phase [88], which is a result of its reduced chemical and biochemical stabilities.

HSA is the most applied protein-based CSP, and it is used predominantly on studies of drug-protein binding [43]. In separation techniques, it is applied for weakly acidic and neutral compounds [119] as well as zwitterionic molecules [120]. For preparation of this type of CSPs, the protein can be physically adsorbed onto the packing material or it can be covalently bound [121].

A number of reviews on CSPs based on proteins have appeared over the years, focusing on their developments and applications [43,45,50,85,87,88,118,120–122]. Recently, Bocian et al. [117] briefly reported several studies related to protein-based CSPs, including the most common (HSA and AGP) as well as the more unusual, namely, avidin and fatty acid binding proteins. The developed strategies presented on that review were mainly related with the chromatographic support as the introduction of monoliths. The introduction of new selectors was also described [117]. Bertucci and Tedesco [42] recently reviewed the advances concerning the HSA as chiral selector highlighting the application of competitors for a particular binding site of HSA as the greatest advance. Scriba et al. [45] compiled some studies, focusing on the understanding of the binding sites and the main interactions between protein-based CSPs with diverse analytes, by molecular modeling.

The most recent protein-based CSPs was not reported in those reviews; their chromatographic performance are presented on Table S2 (supplementary material). It was found that different proteins as new selectors were not introduced. Nevertheless, diverse techniques of preparation of chromatographic support, namely new techniques of immobilization [42,117,123], entrapment [124], or the application of monoliths [125] have been reported attempting to overcome the stability problems of the proteins

Proteins are usually immobilized on silica and its pore size can be defined in order to optimize the separation. Matsunaga and Haginaka [126] immobilized AGP on silica particles with different sizes, 5, 3 and 2.1 μm (CSP62–64). The use of this protein as chiral selector was reported, in 1985, by Hellerstein et al. [127]. In another study, Bi et al. [124] entrapped the same protein on a silica support with 100 Å and 300 Å (CSP65–66). Relatively to the first study mentioned, the resolution and efficiency of CSPs with lower particle size were superior. As an example, an excellent resolution was obtained for benzoin ($R_s = 14.2$) [126]. In the latter study, the described methodology using CSP65–66 proved to be an alternative to high-throughput screening and analysis of biological interactions due to the good affinity results, a maximum of $2.10 \times 10^6 \text{ M}^{-1}$ [124].

Matsunaga and Haginaka [128] also studied the effect of particle size, on the efficiency of the column, with cellulase as chiral selector (CSP67). The first application of cellulase as chiral selector was reported by Vandebosch et al. in 1992 [129]. As in the previous studies, the column with the

lowest particle size provided the greatest efficiency and enantioselectivity [128], with a resolution value of 10.7 for propranolol, for example.

Zheng et al. combined a covalent immobilization process with a cross-linking/modification methodology, using HSA as chiral selector, to achieve CSP68. The aim of this new immobilization strategy was to enhance the protein retention [130]. The CSP obtained presented a high binding affinity for warfarin, with a maximum affinity constant of $2.60 \times 10^5 \text{ M}^{-1}$.

A polyclonal antibody CSP (CSP69) was developed by Bi et al. [131] through an alternative methodology, which consisted of the isolation and immobilization of the selector presented on a serum sample (on-line immunoextraction). This methodology was previously described by Matsuda et al. [132]. Once again, it was found that it could be an alternative to the traditional immobilization methodologies since it is not necessary extra steps of protein purification and immobilization [131]. The stability and the robustness of the CSP were also highlighted [131]. This methodology allowed the preparation of a CSP with considerable affinity. For example, a binding affinity value of $90.0 \times 10^6 \text{ M}^{-1}$ was obtained for disopyramide.

A new protein-based CSP was reported by Fedorova et al. [133] using a different adsorption methodology, which consisted of BSA adsorbed on eremomycin and grafted on silica (CSP70). An improved resolution, with a good resolution ($R_s = 2.14$) was provided, in comparison with a CSP comprising only eremomycin.

One of the most recent developments concerning this type of CSPs was the functionalization of monoliths with proteins. Monoliths are based on silica and present the advantage of optimizing the proportion of monomers and cross-linkers. This optimization enables the control of the average size of the throughput channels and the porous [134]. Monoliths can be prepared with different materials and techniques; the advantages comprise a superior flow as well as an enhanced mass transfer resulting on a more efficient separation [121]. Pfaunmiller et al. [125] immobilized HSA on monoliths to obtain CSP71–72. The main objective was to optimize the amount of protein that could be immobilized. As a consequence, the prepared monoliths allowed an improvement on all chromatographic parameters [125].

The recent applications described for this type of CSPs are more diversified than the developments and some of them are related with the optimization of the chromatographic conditions [133–135]. Nevertheless, the number of publications describing the use of protein-based CSPs has been decreasing over the years [117,136]. Binding affinity studies between drugs and proteins and drug-protein interactions were also found [137–139].

2.3. Cyclodextrin-Based CSPs

The first application of cyclodextrins as CSPs was described by Armstrong and DeMond in 1984 [140]. Since then, several cyclodextrin-based CSPs have been reported [137,140–142]. Cyclodextrins consist on cyclic oligosaccharides [88]; this type of macrocycles can be divided into three classes, α , β , and γ [43]. The structure of a cyclodextrin consists on a truncated cone [43] with an interior non-polar cavity and free hydroxyl groups located on larger and tiny edges [143]. The hydroxyl groups can be derivatized with diverse polar or apolar substituents [55], which can influence the conformational flexibility of a given cyclodextrin, modifying the size of its cavity and creating additional binding sites [43].

The chiral recognition mechanism is typically based on the formation of an inclusion complex between the analytes and the internal cavity of the cyclodextrin [43]. Additionally, the analytes can establish different types of interactions with the exterior side, including dipole-dipole, hydrogen-bond, ionic, π - π , or London interactions [120]. Cyclodextrins present a considerable number of stereogenic centres, which also contributes to enantiorecognition [55].

Cyclodextrin derivatives can be prepared through physical coating or covalently bonding to a chromatographic support [144]. Covalent bonding of cyclodextrin derivatives is the most applied methodology, since it provides a powerful and resistant linkage to the chromatographic support. The most common linkers are ether, amino, and urea. Recently, a triazole linker was also described [144].

The high stability of this type of CSPs allows the use of an extensive variety of solvents as components of mobile phases, with a wide range of polarities, affording an efficient enantioseparation for different analytes [55]. This type of CSPs can be applied in multimodal elution conditions [142].

Several reviews have been devoted to the developments and applications of cyclodextrin-based CSPs [43,45,85,87,88,140,142,144]. Additionally, Guo et al. [145] reviewed the most recent developments concerning on cyclodextrin functionalized monolithic columns.

Studies related to chiral recognition mechanisms of this type of CSPs using diverse methodologies, such as nuclear magnetic resonance, docking, or molecular modeling, were also addressed to understand the molecular interactions as well as the effect of some chromatographic conditions, such as pH, temperature, or organic modifier, in the enantioseparation [146,147].

The recent cyclodextrin-based CSPs and the evaluation of their chromatographic performance are described on Table S3 (supplementary material). The most recent developments are comprised mainly of the introduction of new derivatives and application of different methodologies of immobilization to the chromatographic support. The preparation of hybrid CSPs to enhance the interactions between the analyte and the stationary phase was also emphasized. It was found that the majority of the new cyclodextrin-based CSPs were prepared based on the most widely used cyclodextrin as CSP, i.e., the β -cyclodextrin [148–151]. Moreover, the immobilization strategy of the chiral selector on the chromatographic support was, mainly, by click chemistry [149–152]. The main advantages of this approach are the mild reaction conditions and the enhanced tolerance of the CSPs to solvents and the range of pH values [153]. The introduction of new methodologies to prepare the chromatographic support was also focused, including the preparation of hybrid supports [154,155], the introduction of monoliths [156] and new chromatographic supports [157], or using a different technique to prepare the support [158].

Zhou et al. [152] reported the linkage of a C6-disubstituted cationic β -cyclodextrin onto an alkynylated β -cyclodextrin bonded to a silica support to afford the CSP73 (Figure 3). The obtained bilayer cationic β -cyclodextrin presented a remarkable enantioselectivity for the tested analytes. As an example of its excellent enantioseparation and resolution, α and R_s values of 2.39 and 4.40, respectively, were obtained for 4-(chlorophenyl) propyl ester [152].

Tang et al. [151] resorted to thiol-ene click chemistry to prepare a sulfoether-bridged cationic per(3,5-dimethyl) phenylcarbamoylated- β -cyclodextrin-based CSP (CSP74) (Figure 3) being able to establish π - π interactions and hydrogen bonding interactions with the tested analytes. Its enantiorecognition ability was demonstrated by a separation factor of 1.70 and resolution of 6.03 for 3-(chlorophenyl) propyl ester.

Zhou et al. prepared a perphenylcarbamate β -cyclodextrin chloride linked by click chemistry to an alkynyl silica support to obtain the CSP75 (Figure 3) [159]. After evaluation of its enantioseparation performance using diverse analytes, they concluded that the introduction of the 3-methoxypropylammonium substituent promoted favorable intermolecular interactions with the analytes. In addition, it was suggested that the mobile phase could cause steric hindrance which prevented the establishment of interactions that were crucial for enantiorecognition [159]. The performance of the CSP was promising with a maximum resolution value of 9.84 and a separation factor of 2.76 for 7-methoxyflavanone and 6-methoxyflavanone, respectively.

A new *N*-benzyl-phenethylamino- β -cyclodextrin was synthesized and bonded to mesoporous nanoparticles of silica obtaining the CSP76 (Figure 3) [149]. The new CSP demonstrated to have a superior flexibility and stability, in comparison with the native β -cyclodextrin-based CSP, being obtained through a more economic process [149]. Relatively to its performance, the higher separation factor and resolution values were 1.30 and 1.97, respectively, for carvedilol.

Four new cyclodextrin-based CSPs (CSP77–80) (Figure 3) were developed by chemical bonding of carboxymethyl- β -cyclodextrin derivatives to silica gel by an amidation reaction on aqueous solution [160]. The carboxymethyl moiety provided additional interactions with the tested analytes, in comparison with the native β -cyclodextrin demonstrating a superior enantioselectivity and

resolution [160]. For example, an excellent separation factor value ($\alpha = 6.08$) was achieved for methyl 2-amino-3-(3-(methylsulfonyl)phenyl)propanoate hydrochloride. Moreover, for 1-((benzyloxy)carbonyl)-4-hydroxypyrrolidine-2-carboxylic acid, the resolution value was 9.56 [160].

The effect of different substituents on CSPs has also been investigated. Chen et al. [148] synthesized β -cyclodextrin derivatives with a phenylcarbamate moiety with different patterns of substituents, which were subsequently immobilized onto the silica gel through intermolecular polycondensation of the triethoxysilyl groups (CSP81–85) (Figure 3). They reported that the presence of an aromatic ring with electron-withdrawing groups on the β -cyclodextrin improved the chiral recognition for analytes with electron-donor groups since the number of possible π - π interactions was superior [148]. The hydrogen-bond interactions between the carbonyl group or nitrogen of the analytes and the amino group of phenylcarbamate of cyclodextrin-based CSP were also improved [148]. Relatively to the performance of the CSPs, the highest separation factor value achieved was 2.87 for Pirkle alcohol.

Li et al. [150] arrived at similar conclusions after preparing oxazolinyl-functionalized β -cyclodextrins covalently bonded to silica support (CSP86–88) (Figure 3). They described that analytes with electron-donating or hydrogen-bonding groups were easily enantioseparated due to a higher number of π - π and hydrogen-bonding interactions [150]. CSP88 was more suitable for enantioseparation of polar compounds since it promoted electrostatic interactions due to the presence of an ionic group [150]. Additional factors that could influence the enantioseparation performance of the CSPs, such as the spacer length, selector concentration, and rim functionalities, were also investigated [150]. A reduced surface concentration and a superior flexibility of the spacer decreased the enantioselectivity, since it weakened the interactions between the selector and the analyte [150]. Additionally, a superior selector concentration could be beneficial for enantioseparation of some racemates. The performance of the CSP was promising with an excellent resolution for ketoprofen ($R_s = 22.0$) and a separation factor value of 15.5 for loxoprofen.

The same group developed four thioether bridged cationic cyclodextrin-based CSPs (CSP89–92) (Figure 3) and the influence of the spacer length, selector concentration, and rim functionalities on the performance of the CSP were studied [161]. In this case, it was found that CSPs comprising a spacer with a superior length could compromise their ability of enantiorecognition; however, a higher concentration of the selector was positive [161]. The higher resolution value achieved was 12.7 for 4-nitrophenyl propyl oxide, and the separation factor value was 3.30 for styrene oxide.

Regarding the development of hybrid CSPs, a spherical β -cyclodextrin-silica hybrid CSP (CSP93) (Figure 3) was reported by Wang et al. [154] highlighted by the presence of multiple functional groups, which expanded the spectrum of possible interactions with analytes. The β -cyclodextrin derivative was introduced into the pore channels and pore wall framework, and the linker was attached just into the pore channels [154]. Both the interior and exterior of the CSP participate in the process of the enantiorecognition. For example, separation factor and resolution values of 1.63 and 4.65 were obtained for diclofenac and mandelonitrile, respectively.

Regarding the use of new materials as chromatographic supports, a modification of the most common chromatographic support was performed by Zhao et al. [155] to obtain new CSPs. The modified silica gel was named hydride silica, and its surface was covered by silica-hydrogen bond instead of silica-hydroxyl. The hydride silica presents a superior resistance to water, a reduced polarity, and an improved separation rate and stability and it can be used with a wide variety of solvents [155,162]. Zhao et al. [155] prepared polar group derivatives of β -cyclodextrin bonded to hydride silica to obtain CSP94–97 (Figure 3). The higher resolution value achieved was 9.31 for methyl (2*R*,3*R*,4*S*,5*R*)-5-(4-fluorophenyl)-4-nitro-3-phenyl-3-(trifluoromethyl)-pyrrolidine-2-carboxylate; the best separation factor value was 3.65 for methyl (2*R*,3*S*,4*S*,5*R*)-5-(4-fluorophenyl)-4-nitro-3-(*p*-tolyl)-pyrrolidine-2-carboxylate.

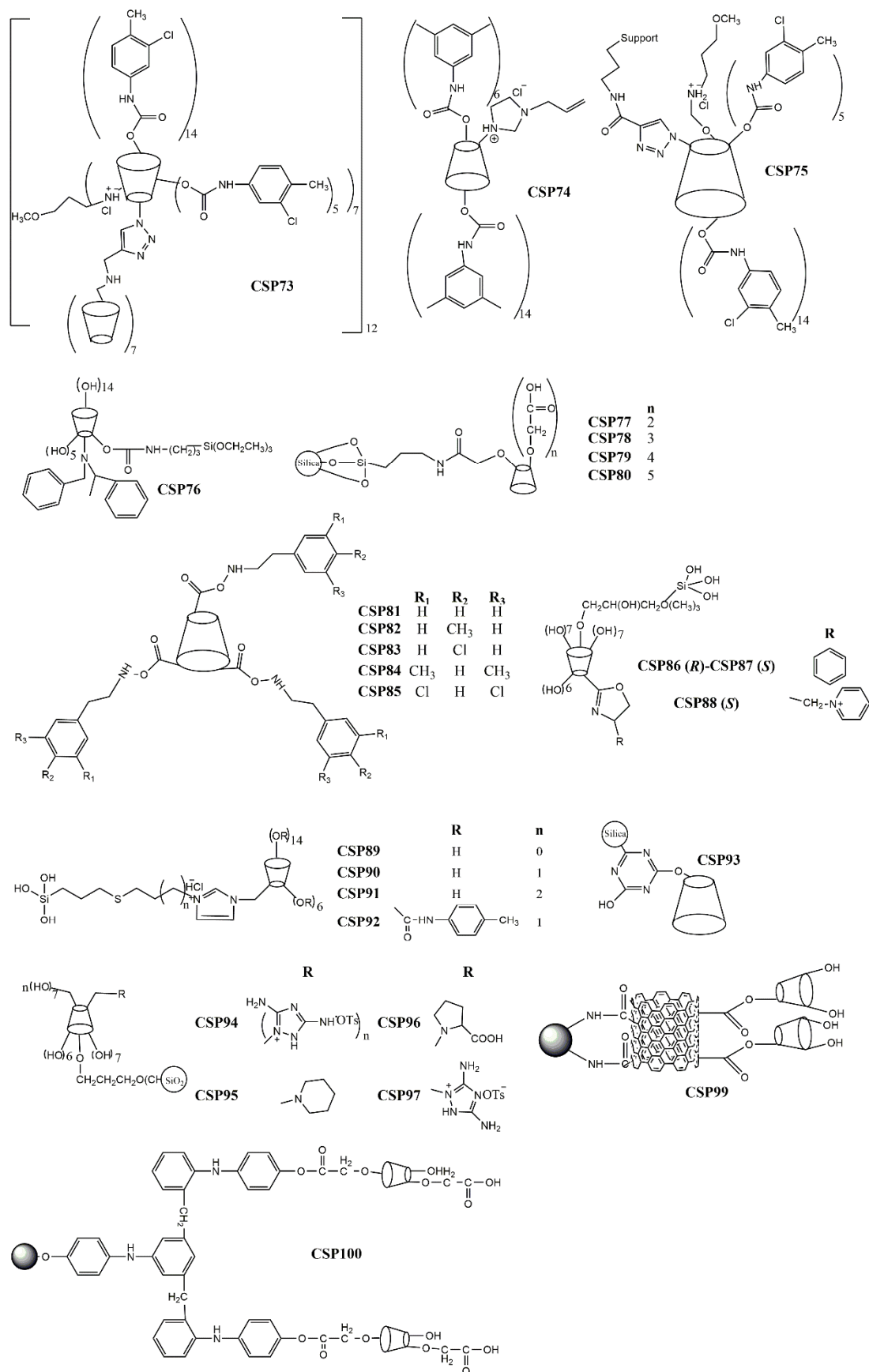


Figure 3. Chemical structure of cyclodextrin-based CSP73–100.

Ghanem et al. [156] used a different strategy to prepared new cyclodextrin-based CSPs. They encapsulated the trimethylated- β -cyclodextrin to a polymeric monolithic, to obtain a superior surface area, reduced pore size, and enhanced total pore volume, and developed the CSP98. They also studied the physical characteristics of the CSP to established relationships with the potential of enantio-recognition and concluded that a superior concentration of the selector improved the enantioseparation [156]. The CSP98 demonstrated a suitable mechanical and thermal stability as well as reproducibility [156] with a maximum resolution value of 2.51 for flavanone, and a separation factor of 1.42 for carprofen.

A different chromatographic support was also proposed by Qiang et al. [157], who described a β -cyclodextrin CSP based on graphene oxide (CSP99) (Figure 3), which was covalently linked to amino silica gel by an amide bond. The graphene oxide and cyclodextrin presented a synergetic effect for enantio-recognition being the hydrogen bonding and π - π interactions the main interactions between the CSP and analyte. The CSP99 was also applied for hydrophilic interaction chromatography. Regarding the chromatographic results, a separation factor of 38.8 was achieved for equol, and a resolution value of 2.17 for 1-phenylethanol [157].

A light-assisted preparation of carboxyl methyl β -cyclodextrin-based CSP (CSP100) (Figure 3) was described by Tang et al. [158] who used ultra-violet light to link the chiral selector to silica, which promoted the modification of ionic bonds into covalent bonds. This technique proved to be eco-friendly and efficient [158]. The morphology and chemical composition of CSP100 was characterized. Moreover, it was concluded that its enantio-recognition ability was dependent of hydrogen bonding and dipole-dipole interactions [158]. The maximum resolution value achieved was 8.04 for chlorthalidone.

2.4. Macrocyclic Antibiotic-Based CSPs

Macrocyclic antibiotics are the second most versatile group of CSPs, after polysaccharides; their planning was inspired by cyclodextrins. The first report of macrocyclic antibiotics as CSP was in 1994, describing the application of vancomycin, thiostrepton, and rifamycin B as CSPs [163].

Macrocyclic antibiotics are divided into four groups: ansamycins, polypeptides, glycopeptides, and aminoglycosides [164]. Ansamycins comprise an aromatic unit linked to an aliphatic chain, and their classification is based on the aromatic moiety. If the aromatic unit is a naphthalene or naphthoquinone, it is denominated naphthalenic ansamycin, while if it is a benzene or benzoquinone, it is a benzenic ansamycin [164]. The most common ansamycins used as CSPs are rifamycins B and SV; the first one is enantioselective for cationic compounds and the second for neutral and anionic [165]. Polypeptides have few aromatic ring units while aminoglycosides do not have this type of structural feature [165]. Only one polypeptide is used as CSP, thiostrepton, whereas aminoglycoside class comprises more CSPs, such as fradiomycin, kanamycin, and streptomycin [166].

Glycopeptides are the most promising class of macrocyclic antibiotic-based CSPs, including avoparcin, ristocetin A, teicoplanin, vancomycin, and derivatized analogues from vancomycin, among others [165]. The chemical structure of glycopeptides consists on a glycosylated cyclic or polycyclic peptide. The central framework is a heptapeptide, in which five of the seven amino acid residues are common to all glycopeptides [164]. Glycopeptides have some flexibility due to the possibility of rotation of sugar groups [165].

The structure of macrocyclic antibiotics allows a variety of interactions with the analytes, such as hydrophobic, π - π , dipole-dipole, hydrogen-bond, electrostatic, ionic, and Van der Waals interactions [166], being either attractive or repulsive. It is possible for the formation of inclusion complexes to occur [167]. The high number of stereogenic centers in their structures also contributes for its high capacity of chiral recognition [55]. Nevertheless, the chiral recognition mechanism of this type of CSPs it is not currently quite understood [43].

The chromatographic support of this type of CSPs is, predominantly, silica gel [164]. Macrocyclic antibiotic-based CSPs are able to operate in all chromatographic elution modes [88]. Besides that, the macrocyclic antibiotic-based CSPs provide a complementary enantioselective profile [167].

Over the years, the developments carried out to obtain diverse macrocyclic antibiotic-based CSPs as well as their applications have been compiled [43,45,85,87,88,164,165,167,168]. Additionally, some authors focused their studies on the mechanism of chiral recognition [169,170].

The most recent reports related to new macrocyclic antibiotic-based CSPs as well as their chromatographic parameters are presented in Table S4 (supplementary material). The developments did not comprise the introduction of new antibiotics as chiral selectors but rather the use of new chromatographic supports, specifically the use of silica particles with sub-2- μm size. In fact, they are mainly based on the preparation of new teicoplanin and vancomycin-based CSPs by reducing the size of the packaging material [171–173]. The main objective was the improvement on chromatographic performance by reduction on analysis time and enhance of resolution and enantioselectivity.

Min et al. [171] described the preparation of a teicoplanin-based CSP bonded to sub-2 μm superficially porous particles (CSP101). The main focus was to avoid aggregation and to uniformize the size distribution, enhancing the surface area. The high resolution and enantioselectivity obtained in a short time of analysis were highlighted. The maximum resolution value was 5.60 for methionine, and the separation factor was 9.40 for norvaline.

Ismail et al. [172] developed a teicoplanin-based CSP with a sub-2 μm chromatographic support; however, in this case, they used totally porous silica particles (CSP102). They pointed out the flexibility of the CSP to operate on different elution modes. The selectivity, efficiency, and the short analysis time on ultra-high-performance liquid chromatographic (UHPLC) were also emphasized [172]. Its performance was promising achieving a resolution value of 10.7 for alanine, and a separation factor of 3.45 for mandelic acid.

Vancomycin was bonded to sub 2- μm diol hydride-based silica particles by Rocchi et al. [173]. Four new CSPs were developed (CSP103–106) with the same main objective: reduction of analysis time. It was inferred that this technique could be applied to other chiral selectors due to the promising results [173]. The maximum resolution value was 3.36 and the separation factor was 2.69 for haloxyfop.

Despite the reduction of particle size of the support, new materials were introduced as chromatographic support. Recently, Xu et al. [174] described the preparation of a vancomycin-based CSP through the combination of monoliths and polymeric cross-linking (CSP107). The new CSP possessed a good mechanical stability, permeability, and enantioselectivity [174]. The influence of some chromatographic conditions was also investigated. The performance of the CSP was satisfactory, with a resolution value of 1.47 for salbutamol, and a separation factor of 1.23 for carteolol. Hellinghausen et al. [175] prepared the CSP108 through the prior synthesis of vancomycin, by Edman degradation, and further binding it to superficially porous particles through a primary amine group of vancomycin, which resulted from the removal of an *N*-terminus leucine residue. The CSP108 presented promising results with a good resolution and separation factor values for 2-amino-2-phenylbutyric acid ($R_s = 2.70$ and $\alpha = 1.57$).

A vancomycin-based CSP was recently prepared (CSP109) by a photochemistry-based method [176]. Additionally, the influence of flow rate, elution mode, buffer, and the mass of analyte were also investigated. The addition of 2-propanol, buffer and an increase on analyte mass improved its enantioresolution performance, since π - π interactions were superior. The chromatographic performance was good, with a maximum resolution of 3.08 and a separation factor of 4.23.

It is also important to highlight that the complementary behavior of the different macrocyclic antibiotic-based CSPs continues to be a subject of great relevance. In fact, several recent studies can be found in literature [166,167,177]. Most of them compared the enantioresolution performance of teicoplanin and teicoplanin aglycone CSPs [166,167] or of vancomycin and teicoplanin CSPs [177].

2.5. Brush-Type or Pirkle-Type CSPs

Brush-type or Pirkle-type CSPs were introduced in 1979, when Pirkle and House described the development and application of a chiral fluoro alcoholic CSP able to enantioseparate diverse classes of analytes [178].

Neutral synthetic chiral low-molecular mass molecules are the base of this type of CSPs [43]. These molecules should promote donor-acceptor interactions as a hydrogen-bond, π - π , or dipole-dipole, in addition to attractive and/or repulsive Van der Waals interactions [45]. As they comprise small molecules as chiral selectors, the mechanism of chiral recognition is, frequently, based on the “three-points” model, which refers that the establishment of at least three interactions between one of the enantiomers to be resolved and the CSP are essential for chiral recognition [179].

The chiral selectors are usually covalently linked to a silica support, which can have monosubstituted or trisubstituted silane groups, through a spacer [55]. Over several years, Pirkle's group has developed successive generations of CSPs [180], based on the principle of reciprocity [181] and on chromatographic [182,183] and spectroscopic [184,185] methods to understand the chiral recognition mechanisms. Among them, Whelk-O1 CSP, created by a rational approach, is the most applied and versatile CSP in both academic and industrial fields [186].

Initially, the preferred elution mode was the normal phase since it provides a favorable environment for the interactions needed to enantioseparate the analytes [88]. Nevertheless, this type of CSPs can also be used in polar organic and reversed-phase elution modes [187–189].

The advantages inherent to this type of CSPs are the compatibility with a wide range of solvents used as mobile phase, the stability to temperature and pressure, the considerable loading capacity and the possibility to be easily scaled up to preparative chromatography [190]. Another key advantage is the possibility of switching the configuration of the chiral selector and to use the inverted configuration column approach [43]. Its kinetic performance is reasonable and the fact that the structure of the chiral selector is relatively “simple” allows an easier knowledge of the chiral recognition mechanisms as well as a consecutive optimization of the selector [191]. Pirkle-type CSPs are characterized by their diversity and versatility since it is possible to use a variety of different small molecules as chiral selectors as well as introduce different substituents that can improve enantioselectivity. In addition, they can be highly specific for certain types of chiral compounds [120].

Pirkle-type CSPs have evolved over the years. Certain types of CSPs have more reported progresses, mainly due to the possibility of use of a wide variety of small molecules as chiral selectors. Several Pirkle-type CSPs can be found in literature comprising chiral selectors closely related to the original Pirkle's group CSPs and others structurally different [43,45,85,87,88,180,186,190,191]. Recently, a literature survey made by our group covering the report on Pirkle-type CSPs developed during the last 17 years was published [190]. We described 226 new CSPs, including a wide diversity of small molecules as chiral selectors, including amine, amino alcohol and amino acids derivatives, peptides, drugs, selectors based on natural products, and xanthone derivatives, among others [190].

The recent developments of this type of CSPs also include the use of new chromatographic supports, such as monolith supports, core-shell particles, or particles with a reduced size (sub 2- μ m). The reduction of particle size enables the adaptation of Pirkle-type CSPs to UHPLC, the first ones to be converted, which are associated with the inherent advantages such as the reduction of analysis time and quantity of the solvent, improved efficiency, and enantioresolution [192,193]. Regarding the use of core-shell particles, it was found that the packaging with this type of material or, alternatively, with fully porous particles led to differences on chromatographic performance. The diffusion on core-shell particles is lower than in fully porous particles, which is especially beneficial for large analytes, since it prevents a decrease on efficiency due to an inefficient mass transfer. The distribution of particle size of core-shell particles is nearly unimodal, which increases efficiency on enantioseparation of small analytes [194,195].

Different synthetic methodologies to obtain the chiral selectors as well as for its immobilization on the chromatographic support were also introduced. The synthesis of biselectors was another approach [190]. The most recent Pirkle-type CSPs and the chromatographic parameters obtained after

evaluation of their enantioresolution performance are presented on Table S5 (supplementary material).

Qiao et al. [196] developed a CSP based on *N*-ferrocenyl benzoyl-(1*S*,2*R*)-1,2-diphenyl ethanol as a chiral selector (CSP110) (Figure 4). The conjugation of a cyclopentadienyl carbon ring with an aromatic ring demonstrated to improve enantioselectivity. The chiral recognition mechanisms were also explored, revealing that hydrophobic, hydrogen-bond, π - π , and dipole-dipole interactions between the chiral ferrocene CSP and acidic and basic groups of the analytes were crucial. The performance of the CSP was promising achieving a maximum resolution value of 4.13 and a separation factor of 2.43 for 3-nitrophenol.

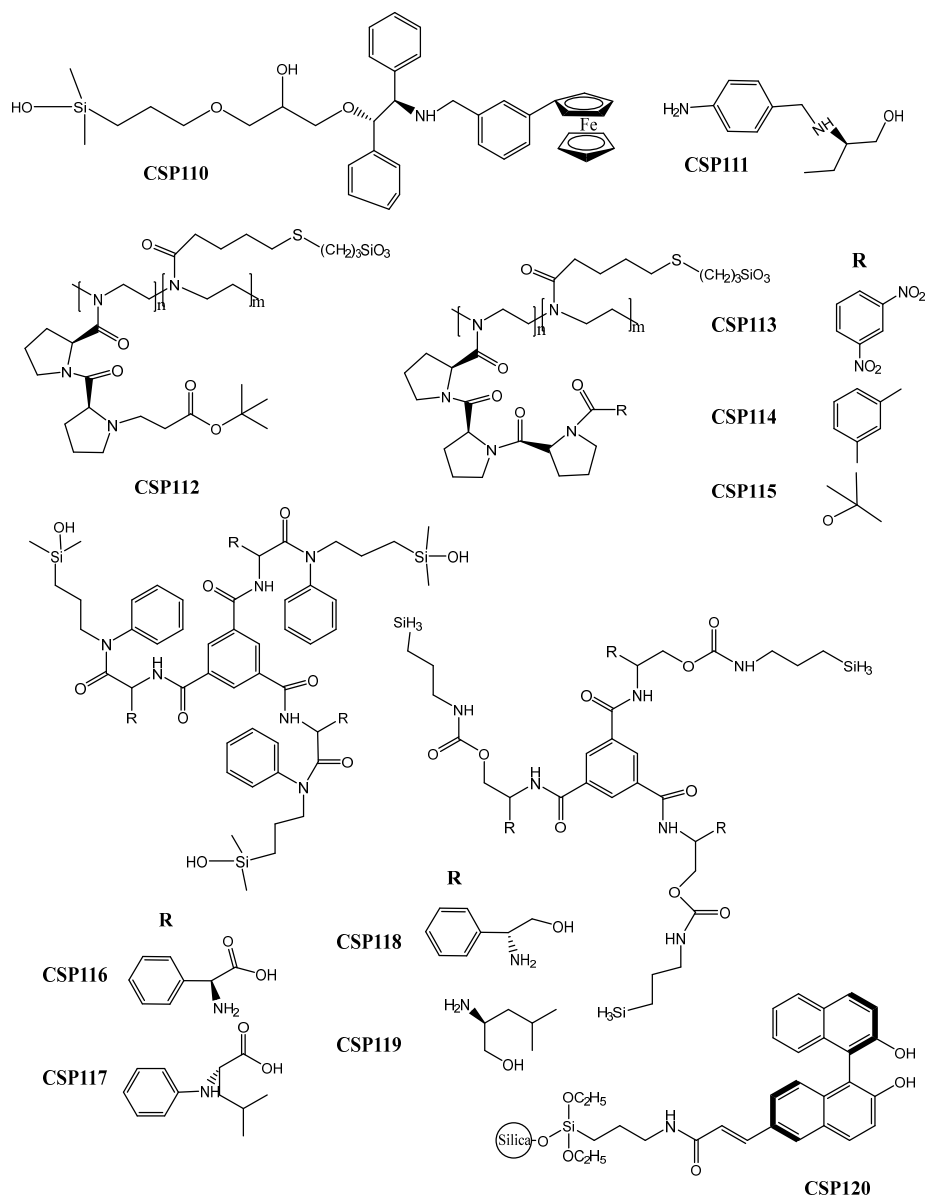


Figure 4. Chemical structures of Pirkle-type CSP110–120.

Çakmak et al. [197] synthesized an aromatic amine derivative of (*R*)-2-amino-1-butanol for the application as chiral selector of a new CSP (CSP111) (Figure 4). In the same study, they used docking, molecular dynamics simulation, and quantum mechanical computation methods to characterize the mechanisms of chiral recognition. The performance of the new CSP was good with a high resolution value of 3.85 for 2-phenylpropionic acid, and a separation factor of 2.75 for mandelic acid.

Four new pseudopeptide-based CSPs were developed (CSP112–115) (Figure 4) inspired by the possibility of enantioselective ability of an organocatalyst [198]. It was found that the enantioselectivity of the CSPs was dependent of the degree of derivatization of diproline portion and of the length of polymeric chain. The chromatographic results were promising, achieving, for example, separation factor and resolution values of 9.80 and 2.89, respectively, for 1-phenylethan-1-amine and *N*-(1-(naphthalen-2-yl) ethyl)-3,5-dinitrobenzamide.

Additionally, in another recent work, derivatives of amino acids and amino alcohols as CSPs were prepared by Yu et al. [199], based on C3-symmetric CSPs (CSP116–119) (Figure 4). It was found that a phenyl group linked to amide was crucial for chiral recognition and, despite the chiral selectors did not possess a π -acidic or π -basic group, their performance was promising. For example, a separation factor value of 2.58 was achieved for 2-phenyl-2-pentanol.

Wang et al. [200] synthesized the (*R*)-6-acrylic-binaphthol as chiral selector through addition of the acrylic group to the (*R*)-binaphthol and developed the CSP120 (Figure 4). The mechanisms of chiral recognition, the effect of the temperature and mobile phase composition were also discussed. It was found that the flexibility of the CSP and the π - π stacking event allowed the retention of the analytes without compromise the enantioseparation [200]. Regarding the chromatographic results, a separation factor value of 1.12 was achieved for 3,5-dinitro-*N*-(1-phenylethyl) benzamide.

Along with the continuous developments of this type of CSPs, it is important to emphasize that a broad range of recent applications have also been reported [201–205].

2.6. Ion-Exchange-Type CSPs

Ion-exchange-type CSPs were introduced by Salvadori et al. in 1985, who described the application of cinchona alkaloids as CSPs [206]. Nevertheless, Lindner group developed the majority of this type of CSPs [207]. Ion-exchanger selectors can be subdivided into three groups: anionic, cationic, or zwitterionic [208].

The most common anion-exchangers as chiral selectors are cinchona alkaloids [45] and terguride [43]. Anion-exchanger selectors are appropriate for enantioseparation of acidic compounds; their enantioselectivity are attributed to the five stereogenic centers of the basic nucleus common to quinine and quinidine [209]. Cation-exchanger selectors are useful for enantioseparation of basic analytes, which are structurally based on chiral sulfonic or carboxylic acid compounds as selectors [45]. Zwitterionic selectors were introduced, more recently, by Lindner et al. [210] by merging key cation- and anion-exchange moieties in one single chiral selector [45]. Those CSPs can be applied for the enantioresolution of acid, basic, and amphoteric compounds [209]. Zwitterionic CSPs have overcome the main disadvantage of anion and cation-exchanger CSPs, since these two groups only separate enantiomers with opposite charge [210].

The chiral mechanism of recognition is mainly based on ionic interactions between the charged analytes and the opposite charged groups of the CSPs [208]. Hydrogen bonds as well as π - π interactions are also important for complex formation [45]. The ion-pairing of solvent controls the adsorption and retention of the analytes [43]. Polar-organic and reversed-phase elution modes are the preferential elution modes for this type of CSPs [43]. The retention and enantioselectivity are affected by the pH and the nature and concentration of acid or base added to the mobile phase [43].

The progresses resorting to this type of CSPs have been reviewed over the years [43,45,85,88,207,209,211–213]. Recently, Ilisz et al. [213] compiled the most recent developments concerning to anionic and zwitterionic-exchange-based CSPs, which are related to the application of different techniques of preparation of chromatographic support to attempt the optimization of chromatographic parameters. The most recent ion-exchanger-type CSPs and their chromatographic parameters are presented on Table S6 (supplementary material). The majority of the recent developments focused on quinine and quinidine derivatives as chiral selectors [214–217], the key structural moiety representative of anion-exchanger selectors.

Todoroki et al. [214] developed a new technique to prepare new ion-exchange CSPs, specifically cinchona alkaloid-based quinine and quinidine-type fluorinated-tagged-CSPs (CSP121–125) (Figure 5).

The main objective was to improve the enantioseparation properties enabling a sensitive, selective, robust, and reproducible analysis methodology. The versatility of the new CSPs was another advantage, as it was capable to enantioseparate bulky, aromatic compounds, in addition to amino acids, such as threonine with a resolution value of 11.8, and asparagine, with a separation factor of 4.56.

The complementarity profile between anion-exchange-type CSPs was the focus of a recent study reported by Lämmerhofer et al. [218], who prepared several cinchona carbamate selectors with distinct carbamate residues to obtain CSP126–131 (Figure 5). Different structural moieties were introduced to enhance the possibility of complementary; for example, the introduction of bulky groups to create steric hindrance or aromatic rings to provide π - π interactions. The complementary accomplished with the new CSPs allowed the expansion of the enantioselectivity range. The performance of the CSPs was promising, achieving, for example, a separation factor of 17.0 for leucine.

De Martino et al. [216] reported the synthesis of an anion exchange hybrid selector, the 3,5-dinitrobenzoyl-9-amino-9-deoxy-9-epiquinine, to develop CSP132 (Figure 5). The strategy applied was the association of typical moieties of Pirkle-type selectors with key moieties of anionic-exchange-based selectors, enlarging the possibility of multiple interactions with the analytes. The performance of this hybrid CSP was promising, with separation factor and resolution values of 2.06 and 11.0, respectively, for diazepam *N*-oxide.

A new immobilization technique based on click chemistry, was described by Lämmerhofer et al. [219], who prepared a cross-linked *tert*-butylcarbamoyl quinine-based CSP (CSP133). The technique allowed achieving a CSP with reduced resistance to mass transfer and retention times, as well as an improved stability. During the optimization of the procedure, some features were discussed, such as the amount of polysiloxane, chiral selector, radical initiator, and reaction solvent, as well as reaction time and size of the chromatographic support particles [219]. The performance of the CSP was promising; for example, with separation factor and resolution values of 1.54 and 5.20, respectively, for *N*-[(9*H*-fluoren-9-ylmethoxy) carbonyl]-phenylalanine.

The same group also resorted to click chemistry to prepare other CSPs based on *tert*-butylcarbamoyl quinine (CSP134–137) (Figure 5) [215]. The optimization of the selector's structure allowed the avoidance of non-specific interactions that could reduce chiral recognition [215]. The introduction of a sulfonic group afforded a reduction on the retention times and an improvement, in some cases, of separation factors since its negative charge provided electrostatic interactions, promoting an effect similar to the counterion effect [215]. The performance of the CSP was satisfactory achieving a maximum resolution value of 6.20 for *N*-(9-fluorenylmethoxycarbonyl)-phenylalanine and a separation factor of 1.66 for *N*-acetyl-phenylalanine.

The application of core-shell particles was another strategy. A new CSP based on *tert*-butylcarbamoylquinine selector (CSP138) (Figure 5) was described by the same group, to promote the enantioseparation of several proteinogenic amino acids [217]. Core-shell particles were introduced in order to improve the analysis time, which was a promising methodology for the bioanalytical area, since it could be combined with sensitive fluorescence detection or highly sensitive and selective mass spectrometric detection. The column presented a reasonable performance with good enantioselectivity and resolution. For example, α and R_s values of 1.55 and 4.08, respectively, were achieved for threonine [217].

Armstrong et al. [220] also resorted to core-shell particles to develop two new quinine-based CSPs (CSP139–140) (Figure 5) for ultrafast liquid and supercritical fluid chromatography. The new CSPs allowed fast analysis with high enantioselectivity and efficiency for the tested analytes. The performance of the new CSPs was promising, affording, for example, a maximum resolution of 25.5 and a separation factor of 14.5 for *N*-(3,5-dinitrobenzoyl)-leucine.

It is important to highlight that recent applications of this type of CSPs were diversified, with the focus, mainly, on the application of anion and zwitterionic-type CSPs with amino acids [221–225].

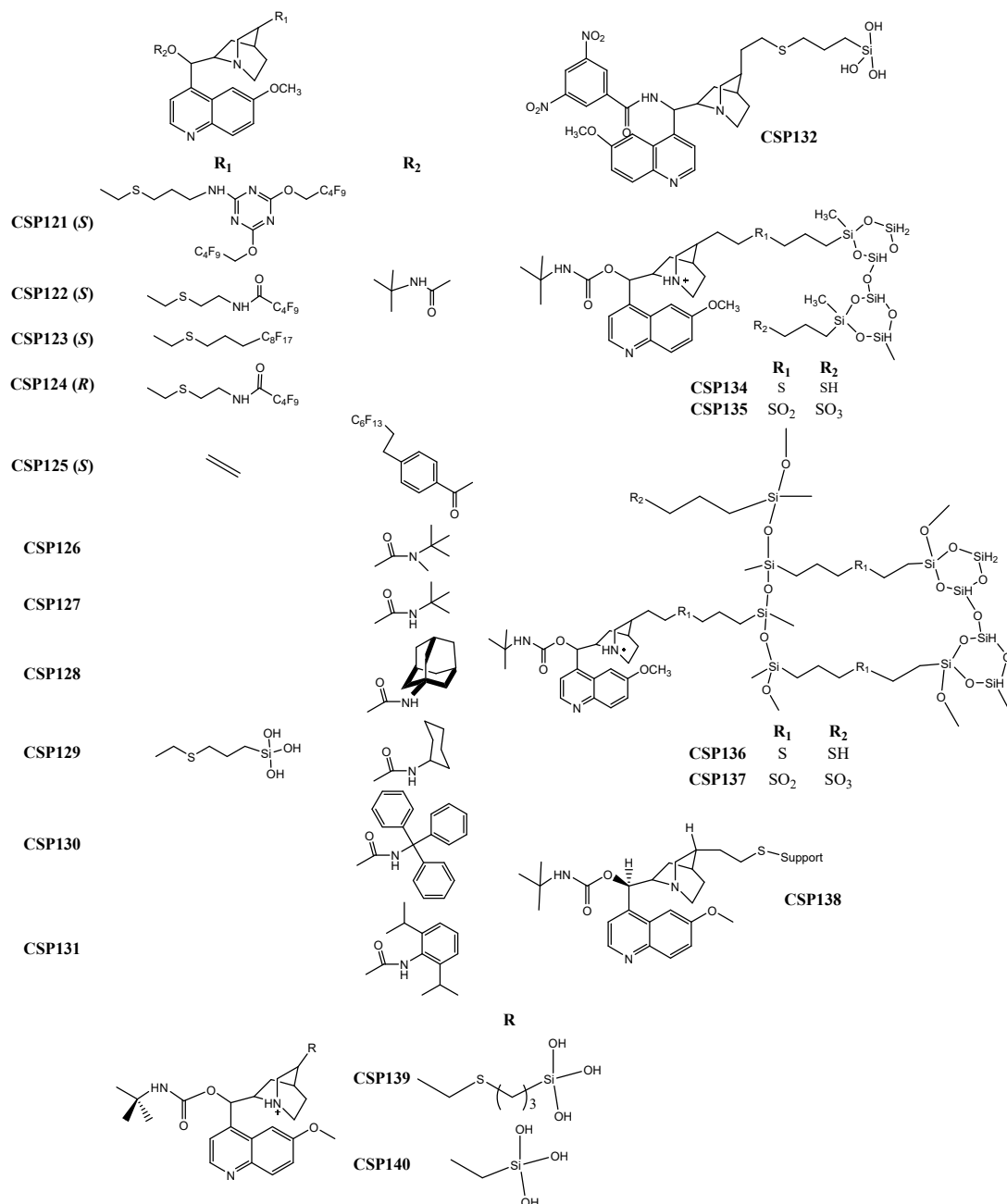


Figure 5. Chemical structures of Ion-exchange-type CSP132–140.

2.7. Crown-Ether-Based CSPs

Crown-ethers as CSPs were firstly described by Sousa et al. [226] for the enantioseparation of primary amine salts. Crown-ethers consist on macrocyclic polyethers, with a cavity of a specific size, being able to form complexes with analytes [227]. CSPs based on crown-ethers can be divided into two major groups: the crown-ethers comprising a 1,1'-binaphthyl group and those containing two tartaric acid groups [228]. The enantiomers of α -amino acids and primary amines may be separated by the first type of crown-ethers CSPs [229]; the second group can be applied for enantioseparation of primary and secondary amino compounds and non-amino compounds [229].

The mechanisms of chiral recognition are typically driven by triple hydrogen bonds established by an ionized ammonium group of the analytes and three oxygen of the CSP, leading to the formation of an inclusion complex [43]. The electron-donor oxygen particles are distributed on inside of the cavity of the crown-ether [88]. Steric hindrance from the substituents of the analyte ions and the

functional groups of the crown-ethers can influence the enantioseparation [43]. Additional interactions are essential to complement the formation of the complex, including π - π , hydrogen-bond, and dipole-dipole interactions [230]. Mobile phases should be strong acidic aqueous solutions to achieve the total protonation of the amino group of the analytes [43]. Crown-ether-based CSPs can be obtained by a coating process or by immobilization [231]. To avoid the leaching of the CSP from the column and to allow the analysis of hydrophobic compounds the use of covalently bonded CSPs it can be preferable than the coated [232].

The developments of crown-ether-based CSPs have been revised over the years [43,45,85,87,88,228,229]. Hyun et al. [228] reviewed the most recent developments, related to both classes of crown-ethers as CSPs, highlighting techniques for the preparation of the chromatographic support or the protection of unreacted residues.

Crown-ether-based CSPs have some restrictions related to the target analytes; however, their preparation and the chromatographic conditions can be modified to improve the chromatographic results. The majority of the recent developments encompassed the use of different strategies for immobilization of the chiral selectors to the chromatographic support (click chemistry) and the introduction of different functional groups on previously described selectors. The most recent crown-ether-based CSPs as well as their chromatographic performance presented on Table S7 (supplementary material). Some of the recent CSPs are based on calix[4]arene derivatives as chiral selectors [233–235]. The new derivatives are mainly prepared using click chemistry [234,235].

Accordingly, the CSP141 (Figure 6) comprising the aza-15-crown-5-capped methylcalix[4]resorcinarene derivative was developed by Ma et al. [233]. Structurally, the CSP possesses two key recognition sites, enhancing the possibility of interactions and, consequently, enantioseparation of analytes. The robustness of the CSP was highlighted since it could operate on different elution modes with a short analysis time (for example, $k_1 = 0.08$ for *m*-nitrophenol).

Yaghoobnejad et al. [234] prepared a calix[4]arene functionalized with two L-alanine units to develop CSP142 (Figure 6), through covalent binding between the allyl groups at the lower rim of the chiral selector and the chromatographic support, using click chemistry. The CSP142 was able to enantioseparate both π -acidic and π -basic analytes. It was suggested that the used technique could easily be adapted to other derivatives to obtain improved CSPs. The maximum resolution value achieved was 1.43 and the separation factor was 2.00 for mandelic acid.

Click chemistry has also been explored, by Li et al. [235], for the preparation of a click-dibenzo-18-crown-6-ether-based CSP (CSP143) (Figure 6). The effect of pH and concentration of salt in the mobile phase on chromatographic parameters was evaluated [235]. It was found that the retention of strong acids decreased with the increment on salt concentration. Regarding the pH values, the retention of both acidic and basic analytes decreased with its reduction. The retention factors were good with a minimum of 0.10 for uracil.

CSPs comprising carboxyl derivatives of crown ethers as chiral selectors were also prepared. Németh et al. [236] synthesized derivatives of acridino-crown ethers containing a carboxyl group to obtain eleven CSPs (CSP144–154) (Figure 6). The CSPs were developed taking into account some structural features that could favor the interactions crucial for chiral recognition mechanisms. Due to the rigidity of the tricyclic ring system, the enantioselectivity was improved [236]. The obtained performance was reasonable with a maximum resolution value of 1.20 for 1-(1-naphthyl)-ethylamine, and a separation factor of 2.05 for 1-(4-nitrophenyl)-ethylamine hydrogen chloride.

The development of hybrid crown-ether-based CSPs was also reported. Deoxycholic-calix[4]arene hybrid-type selectors were synthesized, by Yaghoobnejad et al. [237], aiming to enhance the interactions of the obtained CSPs (CSP155–156) (Figure 6). The calix[4]arene unit was fundamental for the mechanisms of chiral recognition being responsible for the establishment of hydrophobic and π - π interactions, important for inclusion complexes formation. The presence of an acidic or basic modifier in mobile phase was beneficial for enantioresolution of acidic or basic analytes [237]. Relatively to its performance, a maximum resolution value of 3.93 and a separation factor of 4.30 were obtained for mandelic acid.

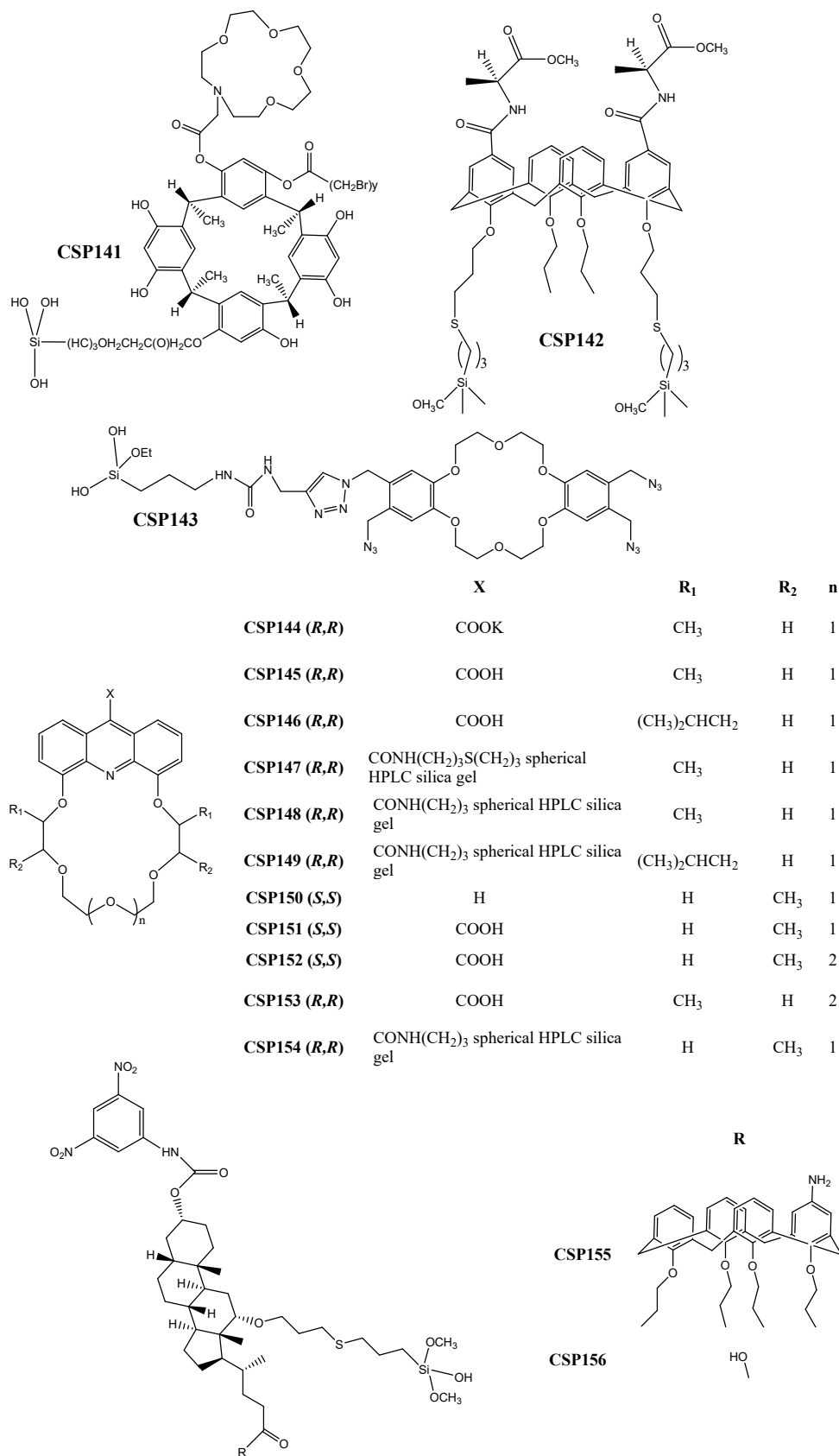


Figure 6. Chemical structures of crown-ether-based CSP141–156.

2.8. Cyclofructan-Based CSPs

Cyclofructans are the most recent type of CSPs being introduced, in 2009, by Armstrong et al. [238]. Moreover, they demonstrated that suitable derivatized cyclofructans presented a superior enantioselectivity in comparison with native cyclofructans [238].

Cyclofructans are cyclic oligosaccharides formed by units of D-fructofuranose $\beta(2\rightarrow1)$ linked together [45]. They are also described as a crown-ether nucleus rounded by fructofuranose units, with its number between 6 and 8 [239]. Each unit has four stereogenic centres [88]. In opposition to cyclodextrins, the interior of the nucleus is hydrophilic [45]. The mechanism of chiral recognition is based on the formation of a complex, which is driven by polar interactions, including dipole-dipole and hydrogen-bond interactions [45]. Therefore, the analytes to be enantioseparated should not be hydrophobic and may have hydrogen-acceptor and polarizable groups next to stereogenic center [240]. The acidic hydrogen-bond play an important role on chiral recognition, thus, the presence of a polarizable group that causes steric hindrance to the basic portion of the cyclofructan is favorable [240]. The main advantages of this type of CSPs are their high loadability and versatility, as it is able to enantioseparate basic, acidic, and neutral analytes [239]. Moreover, they can be used in different elution modes [239]. Although cyclofructan-based CSPs are recent, some reviews can be found related to its developments and applications [45,55,85,87,88,90]. The elucidation of their chiral recognition mechanisms has been the focus of some studies to clarify the interactions between the CSP and the analytes [45]. The most recent cyclofructan-based CSPs and its chromatographic behavior are presented in Table S8 (supplementary material). The latest developments include the synthesis of new derivatives of cyclofructan as chiral selectors, the preparation of new chromatographic supports, and the application of different immobilization strategies.

In order to evaluate the effect of electron-donating and electron-withdrawing groups on enantioselectivity, Khan et al. [239] synthesized chlorinated aromatic derivatives of cyclofructan 6 and developed CSP157–166 (Figure 7). The presence of a chlorine proved to be beneficial for enantioselectivity, in opposition to nitro group, especially in the ortho position of the aromatic ring, which negatively affected the chiral recognition [239]. A maximum resolution value of 6.90 for 2-2'-binaphthol, and a separation factor of 2.05 for Tröger's base were obtained.

The influence of the degree of substitution, as well as the size of the substituents, was researched by Padivitage et al. [241] by preparing CSP167–171 (Figure 7), with basic derivatives cyclofructan 6 as a selector. It was concluded that bulky groups caused steric hindrance and that a high degree of substitution (up to six substituents) negatively affected the enantioselectivity. Moreover, it was found that charged cyclofructans did not possess a superior ability of enantioselectivity [241]. Relatively to the performance of the CSPs, as an example, separation factor and resolution values of 1.43 and 3.10 were obtained for warfarin.

An alternative technique for preparation of a CSP was presented by Qiu et al. [242] (CSP172) (Figure 7), using click chemistry to immobilize the chiral selector, cyclofructan 6, to a resin. The resin was chosen as chromatographic support due the advantages inherent to this material, such as high adsorption capacity, high mechanical strength, lower cost, and reduced sensitivity to pH. Although the chromatographic results were only reasonable, with a resolution of 1.40 for *trans*-1-amino-2-indanol, and a separation factor of 1.41 for *N*-*p*-tosyl-1,2-diphenylethylenediamine, the stability and reproducibility of the CSP were emphasized [242].

Similarly to other types of CSPs, the applications of cyclofructan-based CSPs are becoming more prominent [243,244].

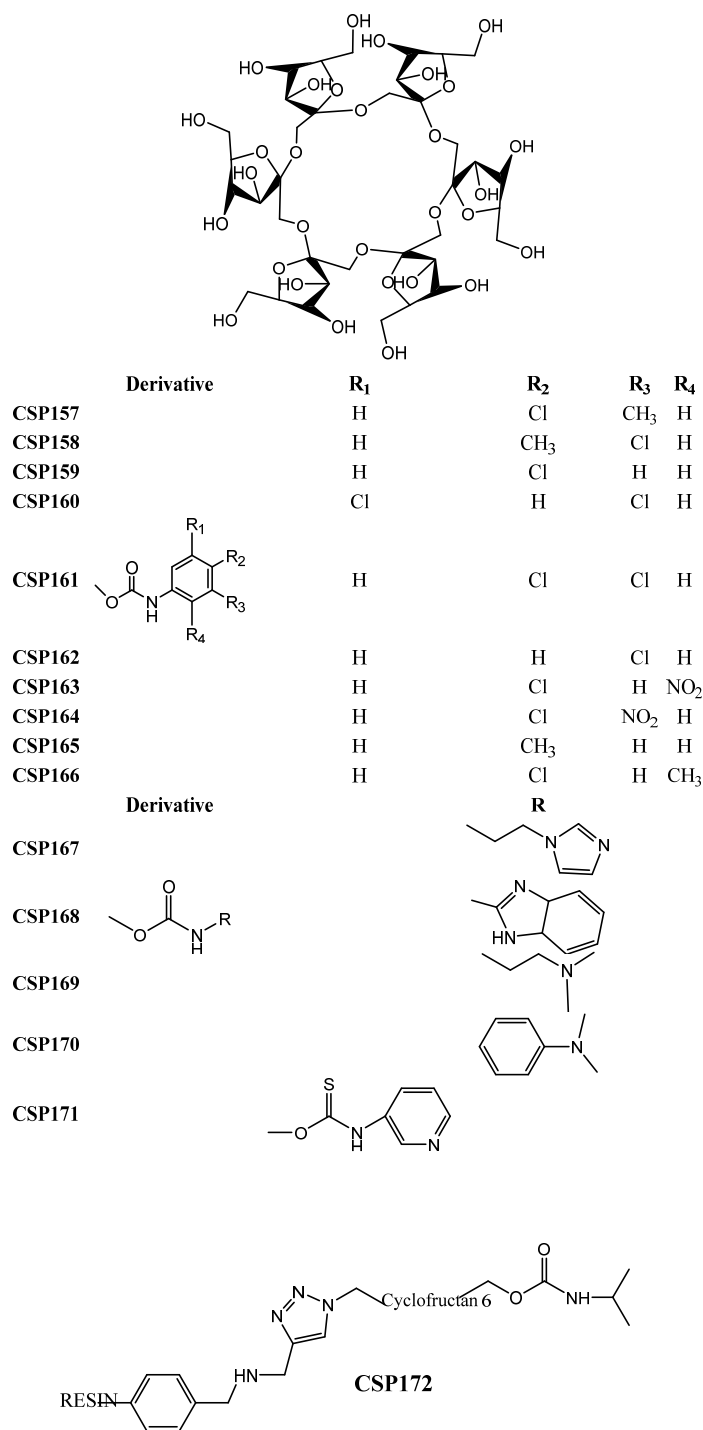


Figure 7. Chemical structures of cyclofructan-based CSP157–172.

2.9. Molecularly-Imprinted CSPs

A different approach to chiral separation has been applied by using molecularly-imprinted CSPs. The synthesis of artificial selectors that are specific for a selected target (template) [245] is the principle of this type of CSPs. Each molecular imprinted CSP can only be applied for a specific type of analytes, as they are frequently applied on preparative enantioseparation and extraction of the desired compounds [246].

Several reviews have been devoted to molecularly-imprinted CSPs, mainly focusing on their developments and the different fields of application [45,88,246–253]. Although the developments concerning this type of CSPs are becoming more usual, their enantioresolution performance is

currently not competitive in comparison to the existing CSPs. The most recent developments comprised the adaptation of this type of CSP to monoliths, nanoparticles, and predominantly to polymers. The introduction of different supports such as alginate microspheres [254] or polymer functionalized with quantum dots [255] were also reported as well as the description of different functional monomers and crosslinking agents [256–259].

Recently, Gutierrez-Climente et al. [245] prepared a new CSP by molecularly-imprinted nanoparticles on silica beads to reduce the tailing effect, commonly observed with this type of CSPs, through the reduction of particle size. The influence of some factors, such as the buffer percentage and concentration, pH, temperature, and column length, on chromatographic performance was evaluated. Regarding the chromatographic results, a maximum resolution value of 1.44 and a separation factor of 1.45 were obtained for citalopram.

In another study, Yang et al. [260] prepared a molecularly-imprinted polymer on porous silica gel microspheres to improve the chromatographic performance of a previous developed CSP and to reduce the analysis time. The new CSP demonstrated a higher affinity than the nonimprinted polymer with the silica gel, and selectivity for the target analyte, oseltamivir, with a retention factor of 13.5.

The optimization of the capacity of enantioseparation of a molecularly-imprinted monolith using a molecular crowding agent was recently reported by Wang et al. [261]. The main aim was to enhance the interactions between the CSP and the target analyte (*S*)-amlodipine. The composition of mobile phase, ionic strength, pH, and content of organic modifier were also taken into account when attempting to improve chromatographic performance [261].

2.10. Other CSPs

Despite the CSPs already mentioned, there are other types of CSPs, such as ligand-exchange, based on synthetic polymers, among others. The ligand-exchange CSPs do not present significant recent developments. Regarding polymers, several synthetic polymers can be used as selectors of CSPs [262]. Nevertheless, despite the interest in this type of material, synthetic polymer-based CSPs are not yet commercialized.

Regarding synthetic polymers, their classification can be based on the type of polymerization, as addition or condensation polymers and cross-linked gels, which are prepared resorting to molecular imprinted technique [263]. A synthetic and optically active polymer can be used for preparation of CSPs if it possesses a helical conformation, which contributes to the wide range of applications and effective separations [264]. The chiral recognition mechanism is based on hydrogen-bond, π - π interactions, and steric factors [45].

As for the other types of CSPs, the CSPs comprising synthetic polymers were the focus of several reviews [45,55,262–267]. The most recent developments are related with the introduction of monoliths [267], specifically of nanoparticles and hybrid monoliths. Ding et al. [264] also reported the use of smart polymers.

Recently, Maeda et al. [268] synthesized derivatives of optically active poly(diphenylacetylene) with chiral and achiral substituents. The helical structure of the polymer demonstrated to influence the enantioselectivity. The performance of the CSP was promising, affording excellent enantioselectivity and resolution, with $\alpha = 19.3$ and $R_s = 15.7$ for ruthenium (III) acetylacetonate.

In another recent study, optically active π -conjugated polymers formed by alternated units of thieno[3-*b*]thiophene and glucose-linked biphenyl were prepared, with its backbone conformation important for the enantioseparation of the obtained CSP [269]. Its performance was satisfactory with a maximum separation factor of 1.56 for cobalt (II) acetylacetonate.

A stable, porous, and crystalline organic polymer was introduced by Zhang et al. [270] highlighting its enhanced stability and resolution. The enantioresolution performance of the obtained CSP was reasonable with a separation factor value of 1.21 for *trans*-metoconazole, and a resolution of 2.56 value for *p*-nitrochlorobenzene.

Additionally, it is important to highlight the introduction of chip-based columns, motivated by the same factors than the transition for UHPLC, i.e., reduction of analysis time and improve the efficiency; the adaptation of LC remains challenging due to technical aspects [271–273]. An advantage focused for this type of columns was the simplicity of the process of its production as referred in the first report by Manz et al. [274]. More recently, they have been applied to extraction methodologies [275,276]. Despite the incorporation of micro and nanoparticles, this remains a challenging issue [273]; the introduction of monolith chip-based columns has already been reported [277].

3. Conclusions

The development of new CSPs for LC is a continuous and challenger issue covering various types of CSPs. This review gathered the most recent developments associated to different types of CSPs providing an overview of the advances that are occurring on this research area.

The most recent strategies, summarized in Figure 1, comprised the introduction of new chiral selectors or new chromatographic supports, and the application of different immobilization or coating methodologies for preparation of the CSPs. Regarding the chiral selectors, novel structures or analogues related to previously reported selectors were described as well as the use of hybrid selectors. The focus in chromatographic supports with lower particle size, the innovation related to the application of new materials such as monoliths and core-shell particles, as well as the use of hybrid supports, were also reported. In addition, several non-conventional approaches for immobilization or coating the chiral selectors to the chromatographic support were included, with particular emphasis to click chemistry as well as new encapsulation techniques or thermal immobilization without the use of chemical reagents, among others. Regardless of the type of CSPs, the main objectives of the development strategies were similar, concerning the improvement of the enantioresolution performance of the CSPs, as well as the increase of versatility and range of applications. Additionally, the transition to UHPLC and the possibility of the new CSPs to be used in all elution modes or using mobile phases compatible with mass spectrometric detection has also been underscored.

Even though several innovative strategies have been applied and several new CSPs were recently developed, they do not yet go beyond the exploratory stage. Nevertheless, the various strategies presented constitute an important trigger aiming to achieve new CSPs, as commercially viable products with high versatility and broad range of analytical and preparative applications or, on the contrary, as exceptionally efficient for the enantioresolution of specific target analytes.

In our opinion, the development of efficient chromatographic tools for LC enantioresolution is a subject that should continue to receive special attention, since it has constructive repercussions in several other research areas, such as biomedical, toxicology and forensic sciences, environment, food and fragrances, industry, among others. Moreover, the goal of developing a universal CSP remains a dream to be reached by those working in this research area.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: Recent developments of polysaccharide-based CSPs, Table S2: Recent developments of protein-based CSPs, Table S3: Recent developments of cyclodextrin-based CSPs, Table S4: Recent developments of macrocyclic-based CSPs, Table S5: Recent developments of donor-acceptor or Pirkle-type CSPs, Table S6: Recent developments of ion-exchange-based CSPs, Table S7: Recent developments of crown-ether-based CSPs, Table S8: Recent developments of cyclofructan-based CSPs.

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