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Chiral HPLC separation and simulation studies of two chiral centered bis-imino flavans (Schiff base) --Manuscript Draft--

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Corresponding Author:	Imran Ali Jamia Millia Islamia Central University New Delhi, India
First Author:	Imran Ali
Order of Authors:	Imran Ali Mohammed El Amin Zaid Nasser Belboukhari Khaled Sekkoum Wahidah H. Al-Qahtani Abdulnasser Mahmoud Karami Marcello Locatelli
Abstract:	<p>The biological activities of flavanone and hesperetin were enhanced by synthesizing Schiff base types molecules (bis-imino-flavans; BHF4, BHF8 and BHF10) by combining flavanone and hesperetin. These molecules were characterized by spectroscopic studies. The four enantiomers of these molecules were separated by HPLC due to the presence of two chiral centers in these molecules. The best separation was achieved with Chiralcel[®] OD-H column under normal mobile phase mode. BHF4 and BHF8 racemates separated completely with k_1, k_2, k_3 & k_4; α_1, α_2 & α_3 and $R_s 1$, $R_s 2$ & $R_s 3$ values of 3.00, 3.55, 3.80 & 4.25; 1.18, 1.07 & 1.12 and 1.26, 1.10 & 1.00 for BHF4 while these values were 5.70, 6.30, 9.08 & 9.83; 1.11, 1.44 & 1.08 and 1.08, 1.37, 6.35 and 1.71. On the other hand, BHF10 could not separate completely. The free energy (ΔG) was calculated for the best separation conditions, and the correlation accurately shows the favorable range of the intercalated length. The chiral mechanism was proposed based on the carbon lengths between flavanone and hesperetin molecules in bis-imino-flavans. The modeling results confirmed the binding order of the enantiomers in BHF4 > BHF8 > BHF10; with maximum binding of SR-enantiomers. The synthesized and separated Schiff base types bis-imino-flavans were evaluated in urine samples with satisfactory results.</p>

JAMIA MILLIA ISLAMIA (A Central University by an Act of parliament)	Prof. Imran Ali, PhD, FRSC, C Chem (UK) Highly Cited Researcher, Clarivate, USA Rank: 01 Indian & 24 Global (Anal. Chem.)	
Department of Chemistry Faculty of Natural Science Maulana Mohammad Ali Jauhar Marg, New Delhi-110025 Tel.: 011-269981717 Extn. 3253 E-mails: drimran_ali@yahoo.com , drimran.chiral@gmail.com Phone: +91-9211458226 Editor-in-Chief: 02 Journals; Editor: 02 Journals http://jmi.ac.in/iali2 Associate & Section Editor: 5 Journals; On Editorial Board: 40 Journals		

March 21, 2022

Prof. Justyna Płotka-Wasyłka
Editor-in-Chief
Microchemical Journal

Dear Prof. Justyna Płotka-Wasyłka,

Good day and Greetings...

Thank you very much for giving us a chance to revise our manuscript. The manuscript is revised as per the suggestions made by the learned reviewers. The changes made are highlighted in red color.

Kindly proceed for publication.

Title: Chiral HPLC separation and simulation studies of two chiral centered bis-imino flavans (Schiff base)

Novelty:

- Synthesis and characterization of most active Schiff bases (bis-imino-flavans).
- Chiral resolution of two centered bis-imino-flavans.
- Fast (20 minutes) and reproducible chiral separation with mechanism determination.
- Simulation study to determine the mechanism of chiral resolution
- Application in biological samples (urine).

Looking forward to hear from you a positive response.

Thanking you with regards,

Yours sincerely,



Thanking you with regards and stay blessed,

Yours sincerely,

Prof. & Dr. Imran Ali

Ph.D., C. Chem., FRSC, London (UK)

Web of Science Highly Cited Researcher

Rank: 01 Indian & 24 Global (Anal. Chem.); Stanford Univ. Survey

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Address:

Department of Chemistry

Jamia Millia Islamia (Central University), Jamia Nagar, New Delhi - 110025, India.

Phone and WhatsApp: 0091-9211458226

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Second Point wise replies

Journal: Microchemical Journal

Manuscript Number: MICROC-D-22-00048R2

Title: Chiral HPLC separation and simulation studies of two chiral centered bis-imino flavans (Schiff base)

Again, I would like to thank Professor Justyna Płotka-Wasyłka, the learned Editor to give us a chance for revising this manuscript. Besides, thanks are also the scholarly reviewers to give fruitful suggestions. Really, the incorporation of all the suggestions made this manuscript more useful and attractive to the readers. The point-wise replies to the comments of reviewers are given below.

Editor and Reviewer comments:

Reviewer 2: I don't think the authors understand the problem that the peak areas of the two peaks of the enantiomer are equal. According to the literature (Journal of Chromatographic Science, 2014; 52:1051-1058) provided by the author, the drug Nebivolol has four enantiomeric pairs that include Isomers 1 and 2 (RSSR,SRRS), Isomers 4 and 5 (SRRR, RSSS), Isomers 6 and 8 (RSRR,SRSS) and Isomers 7 and 10 (RRRR, SSSS). In Figure 2 (Journal of Chromatographic Science, 2014; 52:1051-1058), we can see that the peak areas of two enantiomers for each enantiomeric pair are basically equal, namely, RSSR and SRRS, SRRR and RSSS, RSRR and SRSS, RRRR and SSSS, respectively. In this manuscript, the BHF4, BHF8 and BHF10 possess two enantiomeric pairs including RR and SS, RS and SR. That is to say, the peak areas of RR and SS, RS and SR should be equal, respectively. Based on the elution order (SS- > RS- > RR- > SR-) for BHF4 according to the simulation study, the areas of the first peak and the third peak should be equal, while the areas of the second peak and the fourth peak should be equal. However, as can be seen from Figure 2 in this manuscript, their peak areas are very different. Why? Besides, the authors did not provide the chromatograms for the purity analysis of these compounds.

Reply:

Once again a great thanks to this reviewer for his/her appreciation of our work in the sense that he/she did not reject.

Also, thanks for sparing his/her valuable time reviewing this manuscript and giving fruitful suggestions.

We have already submitted the following.

The statement of “the peak areas of the two enantiomers corresponding to each chiral center should be equal” IS NOT TRUE BECAUSE ENANTIOMER MAY BE IN DIFFERENT PROPORTIONS. The enantiomers may or may not be in equal amounts.

There are many examples in the literature where enantiomers have different amounts. For example. We already gave one example of nebivolol where 1 and 2 enantiomers are not of equal concentrations (Journal of Chromatographic Science, 2014; 52:1051-1058).

ONCE AGAIN, WE ARE CITING BELOW SOME REFERENCES WHERE ENANTIOMERS ARE NOT IN EQUAL AMOUNTS. Therefore, the presence of enantiomers in **EQUAL AMOUNT IS NOT UNIVERSAL PHENOMENON.**

- Journal of Chromatography A, 799 (1998) 301–307; Please see Fig 1.
- Advanced Materials Research Vols. 706-708 (2013) pp 36-39, Please see Fig 2.
- Egyptian Pharmaceutical Journal 2016, 15:88–97 Please see Fig 2-9.
- Scientific Reports volume 5, Article number: 11523 (2015); please see Fig 4 (7-Hydroxyflavanone) and Fig. 7.

Besides, there are other papers describing unequal amounts of the enantiomers.

We have also included the purity peak in the Supplementary information.

Highlights

- Synthesis and characterization of most active Schiff bases (bis-imino-flavans).
- Chiral resolution of two centered bis-imino-flavans.
- Fast (20 minutes) and reproducible chiral separation with mechanism determination.
- Simulation study to determine the mechanism of chiral resolution
- Application in biological samples (urine).

Chiral HPLC separation and simulation studies of two chiral centered bis-imino flavans (Schiff base)

*Imran Ali¹, Mohammed El Amin Zaid², Nasser Belboukhari², Khaled Sekkoum²,
Wahidah H. Al-Qahtani³, Abdunnasser Mahmoud Karami⁴, Marcello Locatelli⁵

¹Department of Chemistry, Jamia Millia Islamia (Central University),
New Delhi-110025, India

²Bioactive Molecules and Chiral Separation Laboratory, Faculty of Exacte Science,
University Tahri Mohamed of Bechar, Bechar, 08000, Algeria

³Department of Food Sciences & Nutrition, College of Food & Agriculture Sciences, King
Saud University, Riyadh 11451, Saudi Arabia

⁴Department of Chemistry, College of Science, King Saud University, Riyadh 11451, Saudi
Arabia

⁵Analytical and Bioanalytical Chemistry, University "G. d'Annunzio" of Chieti-Pescara;
Department of Pharmacy, Build B, level 2; Via dei Vestini, 31; 66100 Chieti, Italy

Abstract:

The biological activities of flavanone and hesperetin were enhanced by synthesizing Schiff base types molecules (bis-imino-flavans; **BHF4**, **BHF8** and **BHF10**) by combining flavanone and hesperetin. These molecules were characterized by spectroscopic studies. The four enantiomers of these molecules were separated by HPLC due to the presence of two chiral centers in these molecules. The best separation was achieved with Chiralcel[®]OD-H column under normal mobile phase mode. BFH4 and BFH8 racemates separated completely with k_1 , k_2 , k_3 & k_4 ; α_1 , α_2 & α_3 and Rs_1 , Rs_2 & Rs_3 values of 3.00, 3.55, 3.80 & 4.25; 1.18, 1.07 & 1.12 and 1.26, 1.10 & 1.00 for BFH4 while these values were 5.70, 6.30, 9.08 & 9.83; 1.11, 1.44 & 1.08 and 1.08, 1.37, 6.35 and 1.71. On the other hand, BHF10 could not separate completely. The free energy (ΔG) was calculated for the best separation conditions, and the correlation accurately shows the favorable range of the intercalated length. The chiral mechanism was proposed based on the carbon lengths between flavanone and hesperetin molecules in bis-imino-flavans. The modeling results confirmed the binding order of the enantiomers in BHF4 > BHF8 > BHF10; with maximum binding of SR-enantiomers. The synthesized and separated Schiff base types bis-imino-flavans were evaluated in urine samples with satisfactory results.

Keywords: Schiff base (bis-imino-flavans), Flavone and hesperetin, Chiral-HPLC separation, Chiral recognition mechanism.

*Correspondence: drimran.chiral@gmail.com; drimran_ali@yahoo.com

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4 **45 1. Introduction:**

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6 **46** The flavonoids are a very significant group of molecules and appeal considerable
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9 **47** devotion because of their pharmacological and physiological impact [1,2]. Hesperetin is
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11 **48** identified to have strong chemo-preventive and antitumor possessions against abdominal
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14 **49** carcinoma in the treatment of a diversity of vascular and cancers diseases [3-5]. As a vital
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16 **50** bioactive Chinese traditional medication, hesperetin has manifold pharmacological and
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19 **51** biological activities. It is an antibacterial, anticancer, antioxidant, antiallergenic and anti-
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21 **52** inflammatory agent since it stimulates or inhibits a wide diversity of enzyme systems as a
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24 **53** pharmacological agent including inhibition of cancer development, effects on the blood-brain
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26 **54** barrier, signal transduction pathways, etc. [6-9]. These properties strongly depend on the
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29 **55** chemical structure; especially the presence and location of hydroxyl groups [3]. The reactivity of
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31 **56** the flavonoids with reagents at C4 carbonyl group has been getting growing interest and led to
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33 **57** interesting new synthetic compounds [10-11]. The flavanones having 2-aryl chroman-4-one
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36 **58** skeleton embedded chemical structures are extensively distributed in plants [12] and synthesized
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38 **59** as well [13-18]. Thus, the chemical modification through synthetic routes is a new direction in
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41 **60** flavanone research [19]. Belboukhari et al. [20,21] synthesized flavanone derivatives such as 4-
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43 **61** iminoflavan [22] and imino-4-hesperidin [23-25] derivatives. The modification of such types of
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46 **62** molecules is always encouraged to enhance biological activities. Therefore, it was considered
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49 **63** worthwhile to synthesize two chiral centers bis-imino flavans by using flavanone and hesperetin
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51 **64** molecules; with varying lengths of the carbon chain of intercalations. It is important to mention
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53 **65** here that the resulting bis-imino flavans were Schiff base. Therefore, it is assumed that the
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4 68 As mentioned above, the synthesized bis-imino flavans are having two chiral centers and
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6 69 exist with four enantiomers in each molecule. This made these molecules more important than
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9 70 the other flavonoids [4,26]. The chiral separation has been of great significance, particularly in
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11 71 the pharmacological industry. This attention is because of the dissimilar pharmacological and
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14 72 pharmacokinetic activities of the enantiomers [27]. The compounds with more than one
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16 73 asymmetric center are now a challenge in chiral separation to have all possible enantiomers
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19 74 because of the complex structure of these analytes and that the chiral selectors must have the
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21 75 ability to differentiate the chiral centers simultaneously [28-31], especially under isocratic
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24 76 conditions [32]. Polysaccharide-based CSPs are the most widespread, among various chiral
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26 77 stationary phases [33-38]. The benzoate ester, acetate ester, or phenyl carbamate derivatives of
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29 78 cellulose and amylose have revealed extensive enantio-selectivity and resolution abilities [39].
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31 79 They are effective under normal-phase and reversed-phase conditions. Most commonly used
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33 80 chiral separation techniques are High-Performance Liquid Chromatography (HPLC) and
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36 81 Capillary Electrophoresis (CE). It is important to mention that HPLC is better than CE because
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38 82 of the high reproducibility of HPLC in comparison to CE. Moreover, chiral separation is
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41 83 achieved on Chiral Stationary Phases (CSPs) in HPLC while CE needs addition of chiral selector
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43 84 in background electrolytes. This made the method costly in CE because everytime costly chiral
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46 85 selectors are added, which is wastage. Besides, the separated enantiomers in HPLC are pure
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48 86 while in the case of CE the separated enantiomers are the diastereomers formed with chiral
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51 87 selector [40,41]. In this way, HPLC is much better than CE in chiral separation. Therefore,
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53 88 HPLC was used as the separation technique in this article. Therefore, efforts are made to resolve
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56 89 four enantiomers of the reported bis-imino flavans by using a variety of chiral columns and
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59 90 mobile phases. Finally, the developed chiral HPLC methods were applied in urine samples for
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61 91 enantiomeric resolution of the reported molecules

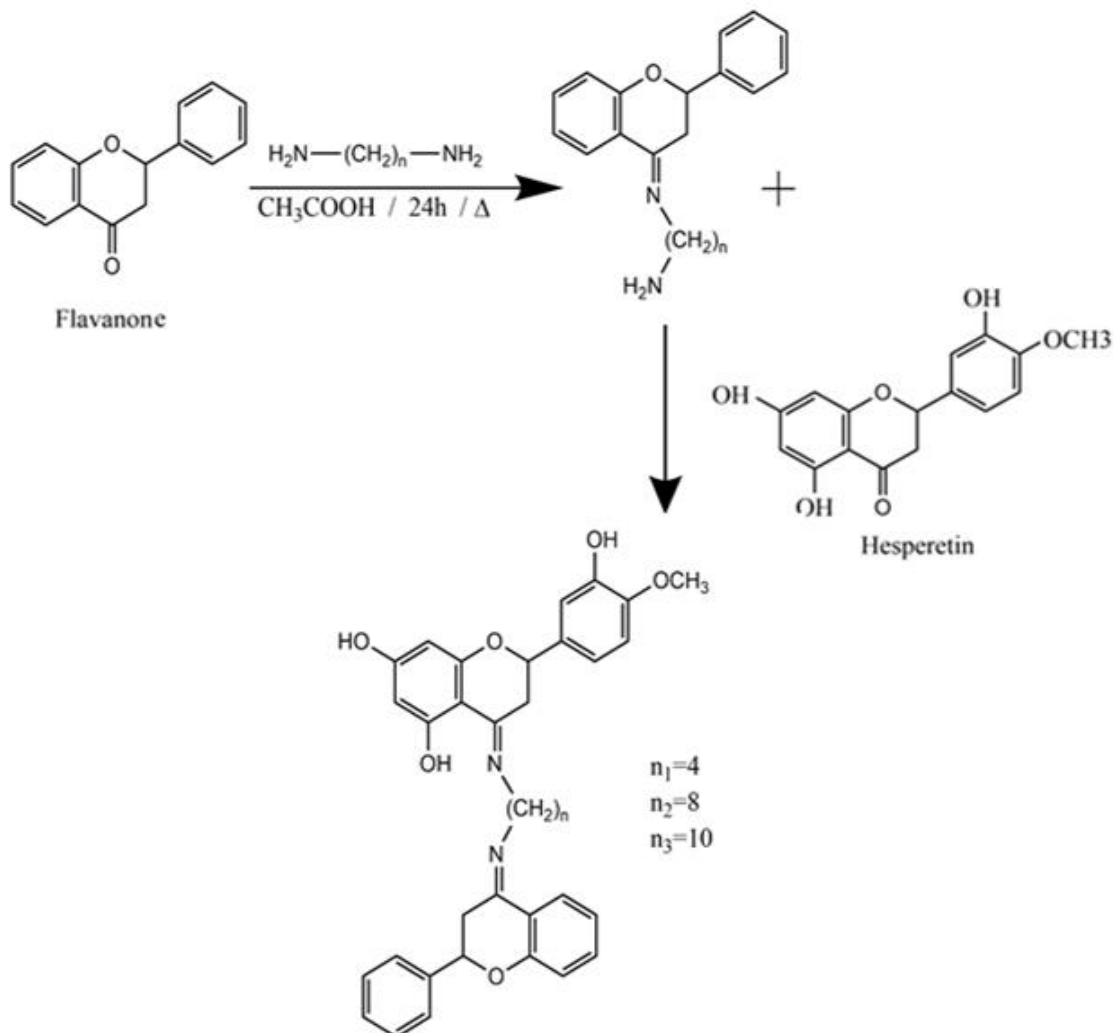
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92 **2. Experimental:**

93 The chemicals, reagents, and instruments are given in supplementary information.

94 **2.1 Synthesis of bis-imino flavans**

95 To synthesize the asymmetric compounds, 0.5 mmol of flavanone was dissolved in methanol
96 and added to an acetic acid/ethanol (1.5 mL/25mL) hot stirring solution. The solutions of 5 mmol
97 of each appropriate primary diamine dissolved in ethanol were added to the mixture. Then, 0.5
98 mmol of hesperetin dissolved in methanol was added dropwise to the reactional medium. After
99 24 hours, the mixture was concentrated, chilled and the solid was separated out. The precipitate was
100 filtered, washed with water, and recrystallized from methanol to give the desired products
101 (Figure 1). The synthesized molecules were characterized by UV-Visb., FT-IR, ¹H NMR and ¹³C
102 NMR methods.



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BHF4, BHF8 and BHF10 represents bis-hesperitin-flavanone at $-(\text{CH}_2)-$ equal to 4, 6 and 10

Figure 1: The synthesis of bis-imino flavans (Schiff base).

2.3 Sample preparation

A very small amount of each compound was accurately weighed and dissolved with 5.0 mL methanol with 10^{-5} M concentration.

2.4 Analysis in biological samples

To check the applicability of the established chiral HPLC methods, the racemates of BHF4 and BHF8 were examined in urine samples. 50 mL urine was sampled and the

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4 112 BHF4 and BHF8 molecules were added discretely and correspondingly to get 10^{-5} M
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6 113 concentration. The pointed urine trials were conceded through the multi-walled carbon
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9 114 nanotubes (MWCNTs) solid-phase extraction unit as developed in our lab. [42].

11 115 **3. Results and discussion**

13 116 **3.1 Synthesis of bis-imino flavans**

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16 117 As clear from Figure 1 that total 3 compounds were synthesized. The synthesized
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18 118 compounds were 2-(3-hydroxy-4-methoxyphenyl)-4-((4-(2-phenylchroman-4-
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21 119 ylidene)amino)butyl)imino)chromane-5,7-diol (BHF4), 2-(3-hydroxy-4-methoxyphenyl)-4-((8-
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23 120 (2-phenylchroman-4-ylidene)amino)octyl)imino)chroman-5,7-diol (BHF8) and 2-(3-hydroxy-4-
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25
26 121 methoxyphenyl)-4-((10-(2-phenylchroman-4-ylidene)amino)decyl)imino)chroman-5,7-diol
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28 122 (BHF10). By the structural point of view, BHF4, BHF8 and BHF10 represents bis-hesperetin-
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31 123 flavanone at $-(\text{CH}_2)-$ equal to 4, 6 and 10.

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33 124 The reported bis-imino flavans (Schiff base) were synthesized by a typical procedure
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35 125 described by Bouanini et al. [43]. The synthesis of imino-flavans was performed by refluxing
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38 126 several flavanone with the suitable different primary diamines in methanol in attendance of a few
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40 127 drops of acetic acid. The results showed that the yields depend on the nature of primary diamine,
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43 128 the carbon bridge length and the nature of flavanes (the presence or not of hydroxyl groups). The
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45 129 reactions yields ranged between 69 to 89%. The formation of Schiff bases took place under acid
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48 130 or base catalysis and preference with heat. A Schiff base acts as a flexi-dentate ligand and
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50 131 generally coordinates via the O atom of the deprotonated phenolic group and the N atom of the
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53 132 azomethine group. The Schiff bases formation is actually an arrangement of two types of
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55 133 reactions *i.e.* addition followed by elimination. Ther Schiff bases syntheses are best performed at
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57 134 mildly acidic pH. The Schiff base formation mechanism is another difference on the theme of
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60 135 nucleophile addition to the carbonyl group. In this case, the nucleophile is the diamine. Firstly,

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4 136 the diamine reacted with ketone or aldehyde to give an unsteady addition compound termed
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6 137 carbinol diamine. The carbinol diamine loosed water either by base or acid catalyzed pathways.
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9 138 Since the carbinol amine is an alcohol, it went acid-catalyzed dehydration [44]. The reactions
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11 139 could be achieved only with carbon bridge length superior to four (CH₂) groups of the diamine,
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14 140 but are not successful with carbon bridge length less than that because of the steric gene which
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16 141 prohibits the diamine's end to reach the carbonyl site.

142 3.2 Characterization of the compounds

143 The above-reported compounds were characterized by different techniques. The structures of
144 the products have been established by spectral studies as UV-Visb., FT-IR, ¹H NMR and ¹³C
145 NMR methods. Their characterization is discussed below.

146 3.2.1 2-(3-hydroxy-4-methoxyphenyl)-4-((4-(2-phenylchroman-4-ylidene) amino) butyl) 147 imino) chromane-5,7-diol (BHF4)

148
149 $C_{35}H_{34}N_2O_6$, Dark Brown powder; yield: 77%; M-P: 246-247°C; UVmax (MeOH, nm):
150 283 (band I); 333 (band II); IR (neat, cm⁻¹): 3378 (-OH), 3065 (-CH arom.), 2823 and 2956
151 (CH₂, CH₃), 1593 (C=C arom.), 1377(OH), 1279 and 1120 (C-O) , 721 (CH₂), 675(OH) , 650
152 (CH arom).

153 ¹H NMR (400 MHz, DMSO-d₆, ppm) :7.55(t, 2H, F: H-3', H-5', 5.6Hz), 7.45(d, 1H,
154 Hes : H-2', 3.1Hz) , 7.41(m, 3H, H-2', H-4',H-6'), 7.36 (td, 1H, F: H-7, 5.7, 2.9 Hz), 7.20(s,
155 OH-5), 7.24 (dd, 1H, F: H-5, 5.7, 2.9 Hz), 7.06 (td, 1H, H-6, 5.7, 2.9 Hz), 6.97 (dd, 1H, F: H-8,
156 5.7, 2.9 Hz), 6.81 (dd, 1H, Hes: H-2', 5.7, 2.9 Hz), 6.74(d, 1H, Hes: H-6', 5.6 Hz), 6.04 (d,
157 1H, Hes: H-5', 5.6Hz), 6.01(d, 1H, Hes: H-8, 2.9Hz), 5.85(d, 1H, H-6, 2.9Hz), 5.33(dd, 1H, H-
158 2,6.01Hz, 5.7Hz), 4.99(dd, 1H, F: H-2, 5.9Hz, 5.4Hz), 3.79(s, 3H, OCH₃), 3.75(t, 2H, CH₂-N),
159 3.69(t, 2H, CH₂-N), 3.64(s, 1H, OH-3'), 3.23(dd, 1H, H-3a, 12.3Hz, 6.01Hz), 2.87(dd, 1H, H-
160 3a, 11.8Hz,5.9Hz), 2.75(dd, 1H, H-3b, 12.3Hz, 5.7Hz), 2.61(dd, 1H, H-3b, 11.8Hz,5.4Hz),

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4 161 1.78(m, 4H, CH₂-CH₂), 0.61 (s, 1H, OH-7). ¹³C NMR (75 MHz, DMSO-d₆, ppm) δ 166.86,
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6 162 163.81, 163.23, 159.13, 147.70, 147.04, 140.62, 132.00, 128.87, 128.45 – 128.01 (m), 127.43 –
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9 163 127.02 (m), 123.09, 119.99, 118.49, 117.55, 113.72, 112.79, 101.22, 97.80, 95.93, 78.64, 56.98 –
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11 164 56.47 (m), 35.65, 27.06 – 26.65 (m).
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14 165 **3.2.2 2-(3-hydroxy-4-methoxyphenyl)-4-((8-(2-phenylchroman-4-ylidene) amino)**
15 166 **octyl)imino) chroman-5,7-diol (BHF8)**

16 167
17 168 C₃₉H₄₂N₂O₆ ; Brown powder; yield: 69%; M-P: 259-260°C; UVmax (MeOH, nm): 285
18
19
20 169 (band I); 331 (band II); IR (neat, cm⁻¹): 3380 (-OH), 3061 (-CH arom.), 2852 and 2957 (CH₂ ,
21
22 170 CH₃), 1590 (C=C arom.),1377(OH), 1273 and 1120 (C-O) , 719 (CH₂), 670(OH) , 655 (CH
23
24 171 arom).
25

26
27 172 ¹H NMR (400 MHz, DMSO-d₆, ppm) : 7.53(t, 2H, F: H-3', H-5', 5.6Hz), 7.41(d, 1H,
28
29 173 Hes : H-2', 3.1Hz) , 7.39(m, 3H, H-2', H-4',H-6'), 7.33 (td, 1H, F: H-7, 5.7, 2.9 Hz), 7.20(s,
30
31
32 174 OH-5), 7.21 (dd, 1H, F: H-5, 5.7, 2.9 Hz), 7.03 (td, 1H, H-6, 5.7, 2.9 Hz), 6.94 (dd, 1H, F: H-8,
33
34 175 5.7, 2.9 Hz), 6.76 (dd, 1H, Hes: H-2', 5.7, 2.9 Hz), 6.70(d, 1H, Hes: H-6', 5.6 Hz), 6.0 (d, 1H,
35
36 176 Hes: H-5', 5.6Hz), 5.95(d, 1H, Hes: H-8, 2.9Hz), 5.84(d, 1H, H-6, 2.9Hz), 5.29(dd, 1H, H-
37
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39 177 2,6.01Hz, 5.7Hz), 4.99(dd, 1H, F: H-2, 5.9Hz, 5.4Hz), 3.78(s, 3H, OCH₃), 3.70(m, 8H, CH₂-
40
41
42 178 CH₂-N), 3.64(s, 1H, OH-3'), 3.25(dd, 1H, H-3a, 12.3Hz, 6.01Hz), 2.84(dd, 1H, H-3a,
43
44 179 11.8Hz,5.9Hz), 2.75(dd, 1H, H-3b, 12.3Hz, 5.7Hz), 2.61(dd, 1H, H-3b, 11.8Hz,5.4Hz), 1.77-
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46
47 180 1.29 (m, 8H, CH₂-CH₂), 0.91 (s, 1H, OH-7). ¹³C NMR (75 MHz, DMSO-d₆, ppm) δ 163.21,
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49 181 161.35, 156.34, 144.83, 143.17, 138.41, 130.13, 128.17, 127.00, 125.22 – 124.93 (m), 122.13 –
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51 182 121.21 (m), 120.54, 117.76, 115.12, 113.76, 110.16, 108.14, 107.08, 93.12, 91.15, 73.31, 53.92 –
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54 183 52.40 (m), 32.12, 29.20 – 28.19 (m), 27.65 – 26.11 (m), 25.83 – 24.21 (m).
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56 184
57 185 **3.2.3 2-(3-hydroxy-4-methoxyphenyl)-4-((10-(2-phenylchroman-4-ylidene) amino) decyl)**
58 186 **imino) chroman-5,7-diol (BHF10)**
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4 188 $C_{41}H_{46}N_2O_6$; Dark Orange powder: yield: 73%; M-P: 265-266 °C; UVmax (MeOH, nm):
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6 189 286 (band I); 338 (band II); IR (neat, cm^{-1}): 3258 (-OH), 3061 (-CH arom.), 2824 and 2957
7
8
9 190 (CH_2 , CH_3), 1578 (C=C arom.), 1377(OH), 1278 and 1100 (C-O) , 723 (CH_2), 673(OH) , 654
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11 191 (CH arom).

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14 192 1H NMR (400 MHz, DMSO- d_6 , ppm) : 7.61(t, 2H, F: H-3', H-5', 5.6Hz), 7.45(d, 1H,
15
16 193 Hes : H-2', 3.1Hz) , 7.39(m, 3H, H-2', H-4',H-6'), 7.29 (td, 1H, F: H-7, 5.7, 2.9 Hz), 7.11 (dd,
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18 194 1H, F: H-5, 5.7, 2.9 Hz), 7.01 (td, 1H, H-6, 5.7, 2.9 Hz), 6.85 (dd, 1H, F: H-8, 5.7, 2.9 Hz),
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21 195 6.78 (dd, 1H, Hes: H-2', 5.7, 2.9 Hz), 6.70(d, 1H, Hes: H-6', 5.6 Hz), 6.0 (d, 1H, Hes: H-5',
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23 196 5.6Hz), 5.95(d, 1H, Hes: H-8, 2.9Hz), 5.84(d, 1H, H-6, 2.9Hz), 5.38(dd, 1H, H-2,6.01Hz,
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26 197 5.7Hz), 5.03(dd, 1H, F: H-2, 5.9Hz, 5.4Hz), 3.86(s, 3H, OCH3), 3.73(m, 8H, CH_2-CH_2-N),
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28 198 3.62(s, 1H, OH-3'), 3.27(dd, 1H, H-3a, 12.3Hz, 6.01Hz), 2.89(dd, 1H, H-3a, 11.8Hz,5.9Hz),
29
30 199 2.75(dd, 1H, H-3b, 12.3Hz, 5.7Hz), 2.60(dd, 1H, H-3b, 11.8Hz,5.4Hz), 1.84-1.32 (m, 12H, CH_2-
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32
33 200 CH_2), 0.87 (s, 1H, OH-7). ^{13}C NMR (75 MHz, DMSO- d_6 , ppm) δ 168.87, 167.12, 166.01,
34
35
36 201 160.42, 148.21, 146.10, 141.04, 135.16, 131.11, 126.23 – 125.10 (m), 123.33 – 122.32 (m),
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38 202 122.12, 120.14, 119.42, 115.76, 113.23, 107.35, 97.15, 92.11, 81.98, 59.87 – 58.45 (m), 45.39,
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40 203 43.39, 32.12 – 29.93 (m), 27.13 – 25.19 (m), 24.79 – 23.19 (m).

42 204 **3.3 Chiral Separation:**

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45 205 In this work, we used two chiral separation approaches *i.e.* normal and polar organic
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48 206 mobile phase modes under isocratic or gradient elution system. The chiral columns used were
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50 207 Chiralpak®AD, Chiralpak®IA, Chiralpak®IB, Chiralcel®OJ, Chiralcel®OZ and Chiralcel®OD,
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52
53 208 Chiralcel®OD-H. The chiral separation of BHF4, BHF8 and BHF10 is given in Table 1 and the
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55 209 chromatograms are shown in Figure 2. It is clear from Table 1 and Figure 2 that BHF4 got
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58 210 resolved completely with four sharp peaks using Chiralcel®OD-H and Chiralpak®IB with
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60 211 70%HEX-30%ISP mobile phase. The retention times were in the range of 6.01 to 19.66 minutes.

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4 212 The values of retention factors, separation factors and resolution factors of BHF4 with
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6 213 Chiralcel®OD-H column were 2.01, 2.56, 2.98 & 4.27; 1.27, 1.16 & 1.43 and 4.20, 2.73 & 1.78
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9 214 while these values with Chiralpak®IB were 2.30, 2.87, 3.87 & 4.19; 1.24, 1.35 & 1.27 and 0.59,
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11 215 1.09 & 2.38. These values clearly showed better resolution with Chiralcel®OD-H in comparison
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14 216 to Chiralpak®IB column. BHF8 could only be got resolved with Chiralcel®OD-H column by
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16 217 using 65%HEX-35%ISP mobile phase. The values of retention factors, separation factors and
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18 218 resolution factors were 1.79, 2.16, 3.54 & 3.92; 1.21, 1.64 & 1.11 and 2.68, 9.99 & 2.68 while
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21 219 these values with Chiralpak®IB were 2.30, 2.87, 3.87 & 4.19; 1.24, 1.35 & 1.27 and 0.59, 1.09
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23 220 & 2.38. On the other hand, BHF10 could not get resolved with any chiral column and mobile
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26 221 phases used. The maximum three peaks could be obtained with both Chiralcel®OD-H and
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28 222 Chiralpak®IB columns by using 70%HEX-30%ISP and 75%HEX-25%ISP mobile phases,
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30
31 223 respectively. All the separated compounds were almost always baseline separated ($R_s > 1.0$) on
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33 224 cellulose-based CSPs i.e. Chiralcel®OD-H and Chiralpak®IB; showing good chiral recognition.
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35 225 Chiralpak®IB and Chiralcel®OD-H have a similar chiral selector with the former having
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38 226 polysaccharides immobilized onto silica. The retention times and separation factors of
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40 227 enantiomers were different on both columns under the same conditions. The immobilization of
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42
43 228 the cellulose tris-(3, 5- dimethyl phenyl carbamate) on silica affected the chiral recognition
44
45 229 capability may be because of the change in configuration of polysaccharide during the
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48 230 immobilization procedure; showing lower resolving capability than coated column [7]. It is
49
50 231 important to mention here that the best chiral separation was on Chiralcel OD-H and Chiralcel IB
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52 232 columns. The reason is this the side chains in both cases have phenyl group with two methyl
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55 233 constituents. The methyl group have increased the electronic density on phenyl ring; facilitating
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57 234 π - π interactions. And π - π interactions are the most important ones in the chiral separations [40-
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60 235 42].

236 **Table 1: HPLC parameters of BHF4, BHF8 and BHF10 with different CSPs and mobile**
 237 **phases.**
 238

Racemates	CSPs	Mobile phases	FR	K ₁	K ₂	K ₃	K ₄	α ₁	α ₂	α ₃	Rs ₁	Rs ₂	Rs ₃
BHF4	Chiralpak®AD	-	-	-	-	-	-	-	-	-	-	-	-
	Chiralpak®IA	-	-	-	-	-	-	-	-	-	-	-	-
	Chiralcel®OJ	100% MeOH	0.5	4.45	5.23	-	-	1.18	-	-	2.77	-	-
	Chiralcel®OZ	-	-	-	-	-	-	-	-	-	-	-	-
	Chiralcel®OD	70% HEX-30% ISP	0.5	2.57	5.24	-	-	2.04	-	-	3.30	-	-
	Chiralcel®OD-H	70% HEX-30% ISP	0.3	3.00	3.55	3.80	4.25	1.18	1.07	1.12	1.26	1.10	1.00
	Chiralpak®IB	70% HEX-30% ISP	0.5	2.30	2.87	3.87	4.19	1.25	1.35	1.08	1.50	1.00	1.00
BHF8	Chiralpak®AD	-	-	-	-	-	-	-	-	-	-	-	-
	Chiralpak®IA	-	-	-	-	-	-	-	-	-	-	-	-
	Chiralcel®OJ	100% MeOH	0.5	4.50	5.26	-	-	1.17	-	-	2.68	-	-
	Chiralcel®OZ	30% HEX-70% ISP	0.5	6.52	9.13	-	-	1.40	-	-	3.15	-	-
	Chiralcel®OD	100% MeOH	0.5	2.60	5.17	-	-	2.76	-	-	4.45	-	-
	Chiralcel®OD-H	70% HEX-30% ISP	0.3	5.70	6.30	9.08	9.83	1.11	1.44	1.08	1.37	6.35	1.71
	Chiralpak®IB	70% HEX-30% ISP	0.4	3.97	4.64	5.76	-	1.17	1.24	-	1.69	1.67	-
BHF10	Chiralpak®AD	-	-	-	-	-	-	-	-	-	-	-	-
	Chiralpak®IA	-	-	-	-	-	-	-	-	-	-	-	-
	Chiralcel®OJ	30% HEX-70% ISP	0.5	2.56	3.00	-	-	1.17	-	-	3.06	-	-
	Chiralcel®OZ	-	-	-	-	-	-	-	-	-	-	-	-
	Chiralcel®OD	100% MeOH	0.5	3.62	4.56	-	-	1.26	-	-	2.55	-	-
	Chiralcel®OD-H	70% HEX-30% ISP	0.5	5.25	5.65	6.05	-	1.08	1.07	-	0.85	0.71	-
	Chiralpak®IB	75% HEX-25% ISP	0.5	4.31	4.70	5.09	-	1.09	1.08	-	0.80	0.65	-

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 240 FR: Flow rate (mL/min)
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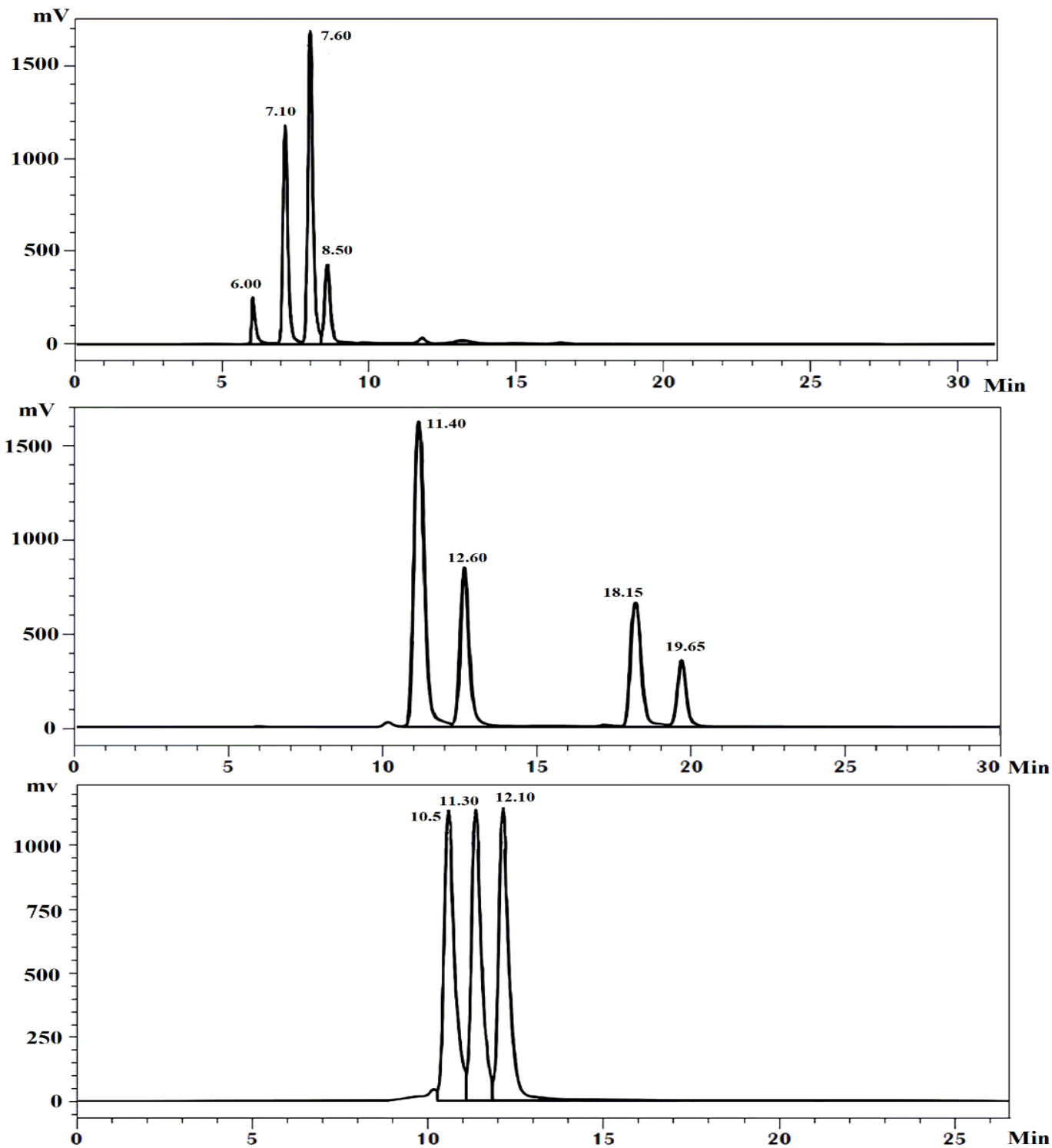


Figure 2: Chiral separation of BHF4, BHF8 and BHF10 with Chiralcel®OD-H column by using 70%HEX-30%EtOH mobile at 0.3 mL/min flow.

3.3.1 Optimization of chiral separation

For optimization purposes, many mobile phases with different combinations were examined by varying the nature and percentage of alcohol (from 5% to 95%). The various CSPs such as Chiralcel[®]OJ, Chiralcel[®]OZ, Chiralcel[®]OD, Chiralcel[®]OD-H, Chiralpak[®]IB, Chiralpak[®]AD and Chiralpak[®]IA were tried to optimize the chiral separations. To streamline the procedure, merely the chromatographic results attained by the optimum mobile phase composition and/or the situations that gave the best resolution on dissimilar columns are offered in this article.

3.4. Thermodynamic study

Thermodynamics is a very crucial study to get data around the recognition mechanism. It depends on the types of both stationary and mobile phases and the type of the enantiomers. The diastereomers formation or transitory complexes is controlled by several parameters including free energy. The change in free energy (ΔG) accompanying the separation of two enantiomers is directly related to the selectivity factor (α) and is given by $(\Delta G) = -RT \ln \alpha$; where R is the gas constant, T is the absolute temperature and α is the selectivity factor [45,46]. Free energy values of the best separations on CSPs (Chiralcel[®]OD-H, Chiralcel[®]IB) were calculated. The values of free energy for BHF4 with Chiralcel[®]OD-H were -0.141, -0.088 and -0.0212 Kcal/mol corresponding to α_1 , α_2 and α_3 separation factors while these values were -0.113, -0.134 and -0.062 Kcal/mol for BHF8 racemate (Table 2). The values of free energy for BHF10 with Chiralcel[®]OD-H were -0.45 and -0.51 Kcal/mol corresponding to α_1 and α_2 separation factors. The values of free energy for BHF4 with Chiralcel[®]IB were -0.127, -0.177 and -0.141 Kcal/mol corresponding to α_1 , α_2 and α_3 separation factors. The values of free energies of BHF8 were -0.093 and -0.127 Kcal/mol while these values were -0.051 and -0.045 for BHF10. The values of

free energy were negative and these signs are a suggestion of good interactions of the enantiomers with CSPs.

Table 2. A comparison of free energies values on Chiralcel®OD-H column.

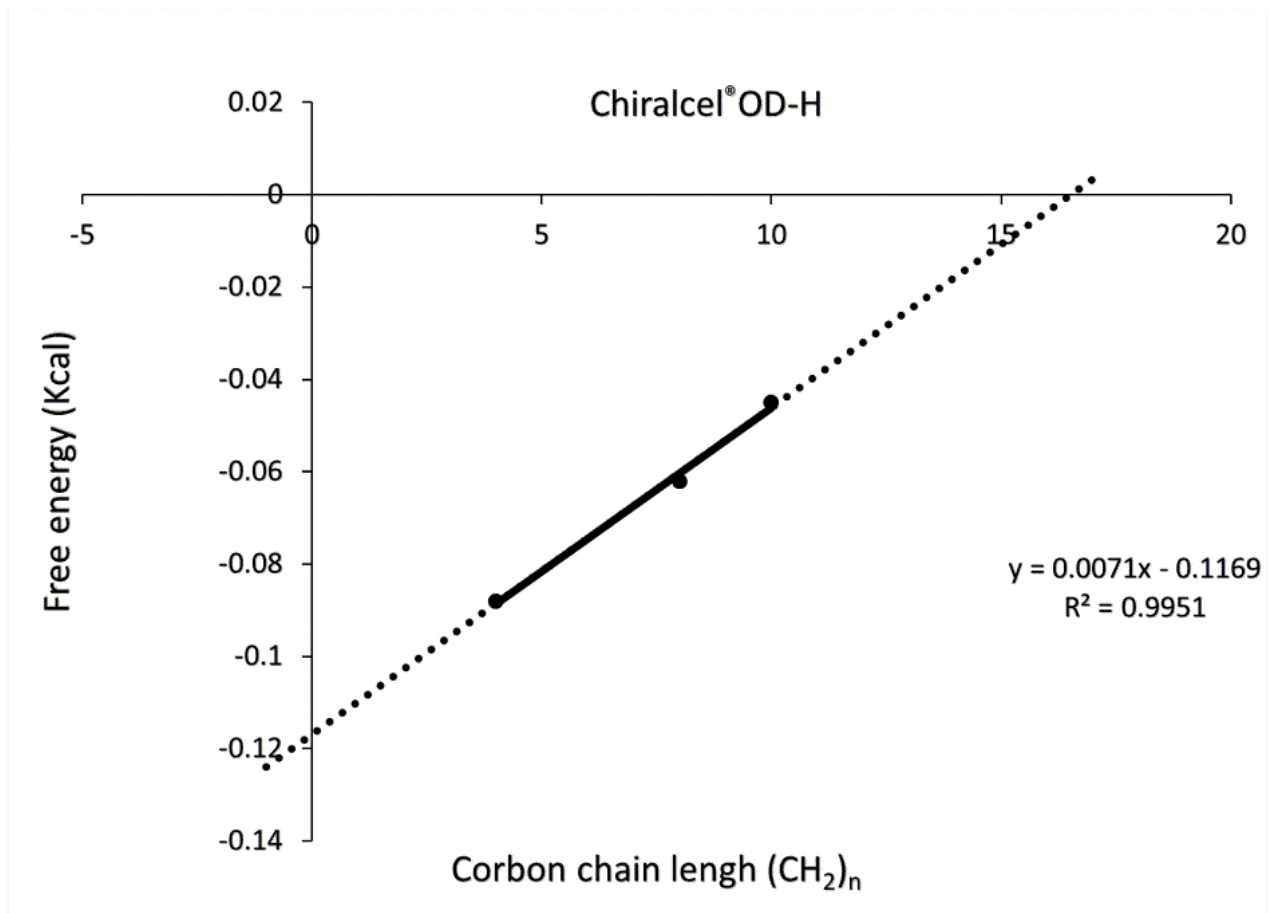
Compounds	CSP	α	ΔG
BHF4	Chiralcel®OD-H	$\alpha_1 = 1.27$	$\Delta G_1 = -0.141$ Kcal/mol
		$\alpha_2 = 1.16$	$\Delta G_2 = -0.088$ Kcal/mol
		$\alpha_3 = 1.43$	$\Delta G_3 = -0.212$ Kcal/mol
BHF8	Chiralcel®OD-H	$\alpha_1 = 1.21$	$\Delta G_1 = -0.113$ Kcal/mol
		$\alpha_2 = 1.64$	$\Delta G_2 = -0.134$ Kcal/mol
		$\alpha_3 = 1.11$	$\Delta G_3 = -0.062$ Kcal/mol
BHF10	Chiralcel®OD-H	$\alpha_1 = 1.08$	$\Delta G_1 = -0.045$ Kcal/mol
		$\alpha_2 = 1.09$	$\Delta G_2 = -0.051$ Kcal/mol

3.5 Thermodynamics, carbon-chain and molar masses of the enantiomers

The free energy (ΔG) permits determining if a chemical reaction can occur due to its enthalpy (ΔH) and entropy (ΔS) changes under specific conditions of pressure and temperature [47]. It has almost a linear correlation with the carbon chain length and the molar mass of the three bis asymmetric compounds (BHF4, BHF8 and BHF10) on both Chiralcel®OD-H and Chiralcel®IB columns, with a linear correlation coefficient $R^2 \approx 1$ (0.92 to 0.99). With this study, we can observe the minimum free energy needed to have a separation and the maximum free energy so the last separation could be done with the CSP. On Chiralcel®OD-H, when the free energy tends towards zero $\Delta G = 0$ kcal/mol (when $\alpha = 1$), which means there is no separation and with an extrapolation, we can notice that this value coincided with a carbon chain length equal to $n=16.56 \approx 17$ (Figure 3) so $(CH_2)_{17}$, which is confirmed by the correlation between the free energy and the molar mass (Figure 4) with $\Delta G = 0$ kcal (when $\alpha = 1$) which was equal to $M=760$ g/mol, and if we omit the molar mass of the compound from the total mass of the carbon chain length we can remark that the molar mass of the carbon chain length was about

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292 238.26g/mol, after divided it on 14 which is equivalent to CH₂ we can found n (number of carbon
293 chain length) = 17.01 so (CH₂)₁₇. We remark that the number of carbon chain length or the molar
294 mass is inversely proportional to the free energy (ΔG), the augmentation of carbon chain length
295 or the molar mass means the diminution of free energy (ΔG), the chiral separation is still can be
296 done when free energy (ΔG) begins from -7648.10^{-3} Kcal and stopped when it reached -0.1169
297 Kcal so between $n=1$ to $n=16$; The separations were impossible out this range (under $n=1$ so
298 $M < 536.34$ g/mol and above $n=16$ so $M > 746.34$ g/mol), which was confirmed also by the
299 correlation between the free energy(ΔG) the molar mass.



300
301 **Figure 3: The correlation between the free energy (ΔG) and the carbon chain length with**
302 **Chiralcel®OD-H column.**

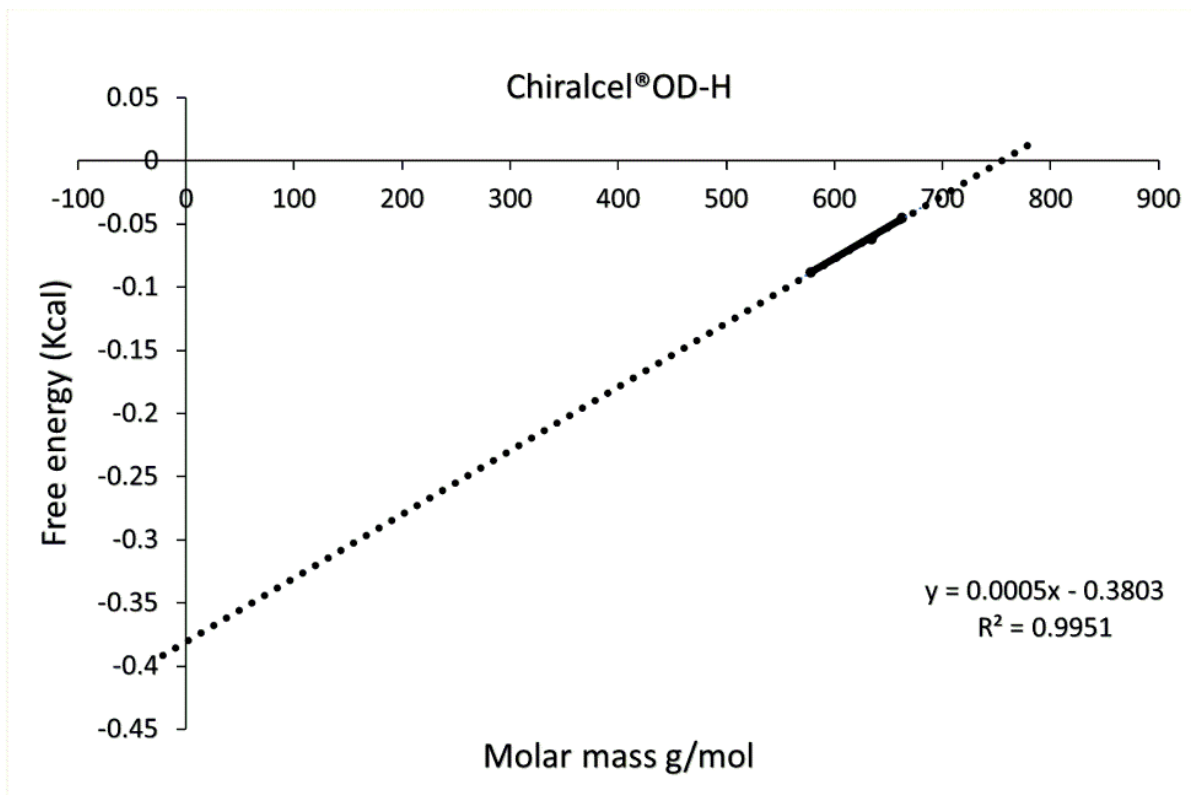


Figure 4: The correlation between the free energy (ΔG) and the molar mass with Chiralcel®OD-H column.

On Chiralcel®IB when $\Delta G = 0$ kcal (when $\alpha=1$), the carbon chain length $n=14.20 \approx 15$ (Figure 5) so $(CH_2)_{15}$; which was confirmed by the correlation between the free energy and the molar mass (Figure 6) so when $\Delta G = 0$, $M=738.56$ g/mol, and if we neglect the molar mass of the compound from the total mass of the carbon chain length, we can remark that the molar mass of the carbon chain length was about 216.22g/mol, after divided it on 14 which is equivalent to CH_2 we can found n (number of carbon chain length) = 15.44 so $(CH_2)_{15}$. The chiral separation still could be done when free energy (ΔG) began from -0.007648 Kcal and stopped when it reached -0.1831 Kcal; so between $n=1$ to $n=14$; The separations were impossible out this range (under $n=1$ so $M < 536.34$ g/mol and above $n=14$ so $M > 746.34$ g/mol), which coincided also with the correlation between the free energy (ΔG) the molar mass.

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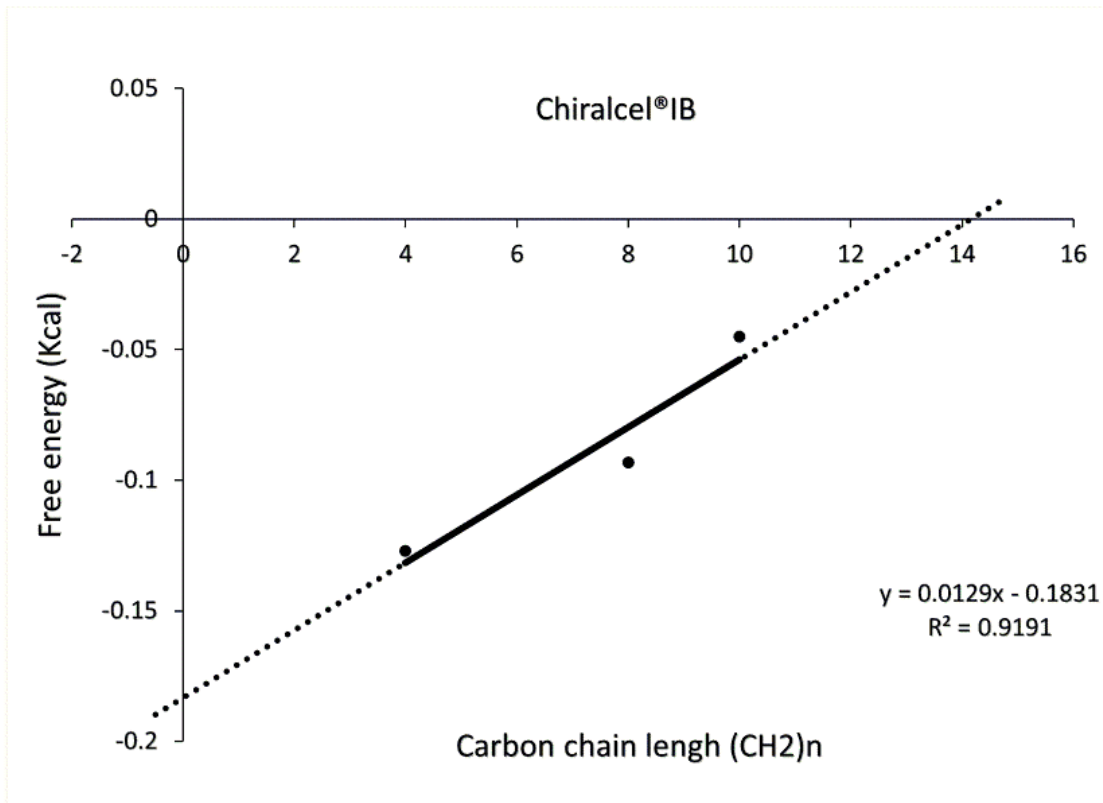


Figure 5: The correlation between free energy (ΔG) and the carbon chain length with Chiralcel®IB column.

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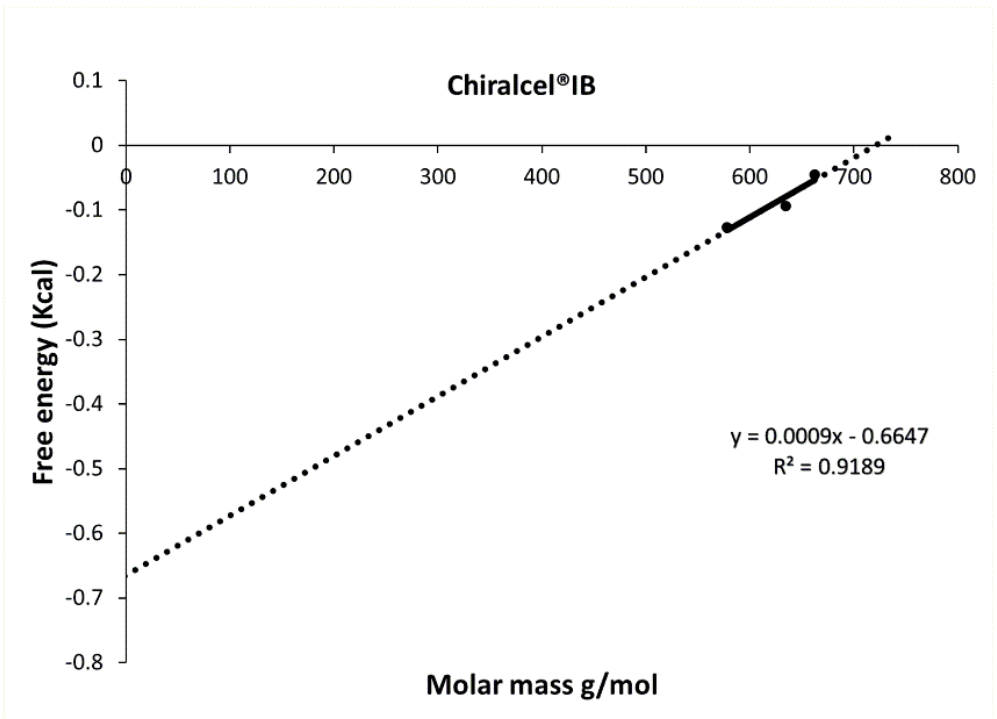


Figure 6: The correlation between the free energy (ΔG) and the molar mass with Chiralcel®IB column.

3.6 Mechanism of chiral separation

The discernment control of these polysaccharide-based phases stemmed from complex relations with the solutes. The chiral selector has chiral grooves offering a stereoselective situation to the enantiomers. The enantiomers fit in these chiral grooves to dissimilar degrees as per the lock and key arrangement. These polysaccharides comprise a large number of optically active sites and, thus, have a relatively high chance of interaction with the solute, giving the separation of the stereoisomers. The hydrophobic interactions combination, attractive forces (e.g., hydrogen bonding), charge transfer (π - π) formation and dipole-dipole interactions were supposed to clarify the molecule recognition process [28,48]. From the point of view of the mobile phase, we notice that the normal organic phase is more efficient than the polar organic phase for the separation of the majority of our compounds. It appears that the hydrogen bonding and π - π interactions are playing a separation role among aromatic moieties of the CSPs and the enantiomers [4,33-36]. The carbon chain length of the compounds also affected the separation of polysaccharide CSPs. A comparison of the separation was carried out on Chiralcel®OD-H column. It was observed that the order of the separation was the racemates BHF4 and BHF8 were resolved while racemate BHF10 could not be separated. Further, it was observed that BHF4 had good separation than BHF8. It may be concluded that the carbon chain length a playing crucial role in chiral recognition mechanism. The carbon chain $n = 4$ provides the ideal size to the racemates to fit sufficiently on the chiral grooves on the CSPs. Contrarily, the molecular size of BHF8 provided fair chances of fitting the enantiomers on the chiral groove. Moreover, the molecular size of BHF10 provided poor chances of fitting the enantiomers on the chiral groove. This is the reason that BHF4 could be resolved better than BHF8 while BHF10 could not be separated.

3.7 Simulation study

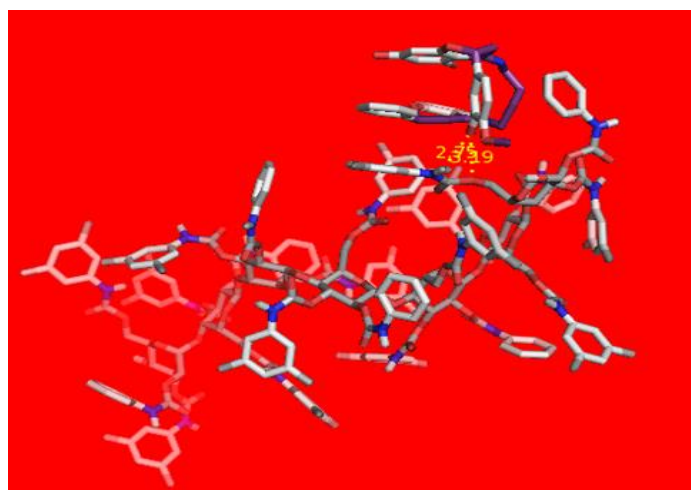
The modeling of all 12 enantiomers (4 enantiomers of each molecule) was carried out on the polysaccharide-based chiral stationary phases as per the procedure described in the experimental part. It was observed that each enantiomers of BHF4 interacted with CSP in different fashions with different binding energies. For example, the binding energies of the four enantiomers of BHF4 were -5.9, -5.4, -6.0 and -5.3 kcal/mol for RR-, RS-, SR- and SS-enantiomers (Table 3). It was also observed that the hydrogen bondings were 1 in RR-, RS-, and SS-enantiomers while it was 2 in SR-enantiomers (Figure 7). It is clear from these results that the binding order was SR- > RR- > RS- > SS. The same the trend was observed with enantiomers of BHF8 and BHF10 molecules. For example, the binding energies were -5.0, -4.8, -5.1 and -5.8 kcal/mol for RR-, RS-, SR- and SS-enantiomers with 1 hydrogen bonding in RR-, RS- and SS-enantiomers while 2 hydrogen bonds were observed in SR-enantiomers. In the case of BHF10, the binding energies were -3.6, -4.6, -4.1 and -4.8 kcal/mol for RR-, RS-, SR- and SS-enantiomers with 1 hydrogen bonding in RR-, RS- and SS-enantiomers while 2 hydrogen bonds were observed in SR-enantiomers. A comparison of the overall bondings was carried out among all 12 enantiomers and it was observed that the binding order was BHF4 enantiomers > BHF8 enantiomers > BHF10 enantiomers. It may be due to the fact that by increasing the carbon chain in these enantiomers the bonding is become weak due to the large size effect. Therefore, the enantiomers of BHF4 were bonded to the CSP strongly. These results are in agreement with the findings of the above mention chiral recognition mechanism. Only the models of the maximum bonded SR-enantiomers of all the three molecules (BHF4, BHF8 and BHF10) are given in Figure. 7.

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373 **Table 3.** Modeling data of enantiomers with CSP.

Enantiomers		Binding affinity (kcal/mol)	Number of hydrogen bonds
n	Enantiomers		
BHF4	RR	-5.9	1
	RS	-5.4	1
	SR	-6.0	2
	SS	-5.3	1
BHF 8	RR	-5.0	1
	RS	-4.8	1
	SR	-5.1	2
	SS	-5.8	1
BHF	RR	-3.6	1
	RS	-4.6	1
	SR	-4.1	2
	SS	-4.8	1

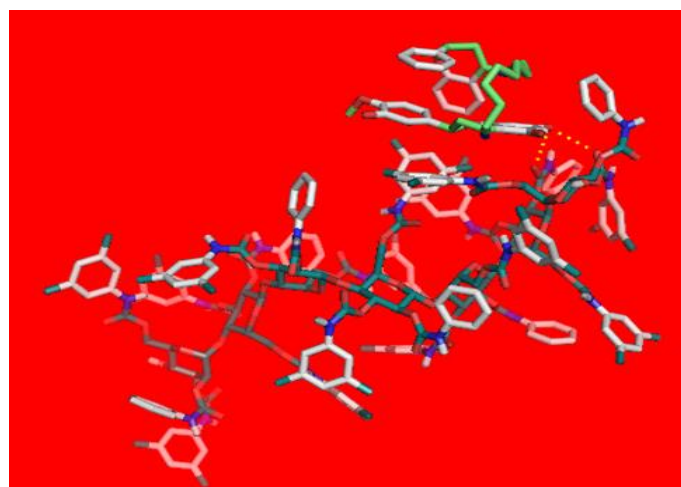
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SR-Enantiomer of BHF4 molecule.

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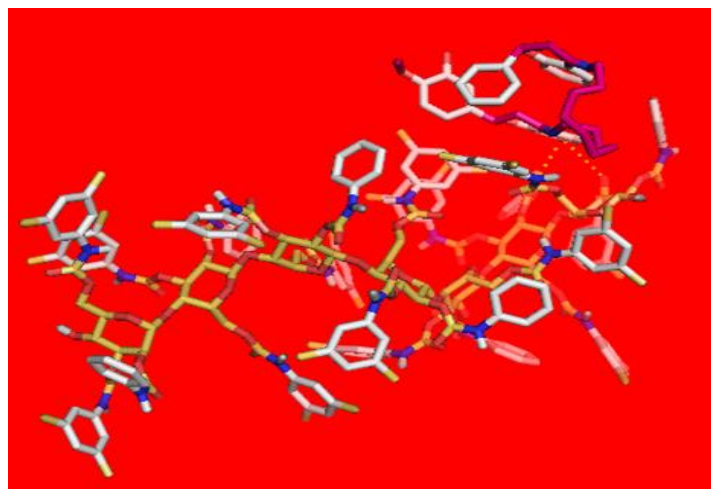
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SR-Enantiomer of BHF8 molecule.

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SR-Enantiomer of BHF10 molecule.

Figure 7. The models of the maximum bonded SR-enantiomers of all the three molecules (BHF4, BHF8 and BHF10).

3.8 Application biological samples

The utility of the defined chiral HPLC methods was verified in urine bio-samples. 50 mL urine samples were obtained and the BHF4 and BHF8 molecules were added independently and correspondingly to get 10^{-5} M concentrations. The so obtained urine samples were allowed to pass through MWCNTs solid-phase extraction, which was developed in our lab. These urine samples were examined by the reported chiral HPLC methods. It was seen that the peaks were alike as in the standard solutions. The regainings of the BHF4 and BHF8 molecules were in the range of 89.5 to 91.7%. No extra peak was gotten in the chromatograms; approving the appropriateness of the described chiral HPLC methods. The analytical data was authenticated and the degrees of standard deviation were ranging from 0.66 to ± 0.81 while the correlation values coefficients values and confidence levels values were 0.9994 to 0.9995 and 93.4 to 94.5. These values established the utility of the stated method.

4. Conclusion

The novelty of this work lies in the fact that almost all the papers in chiral separations reported the simple HPLC method development of one chiral centered racemates, which were

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4 399 obtained from different suppliers. In the present paper, we described first the synthesis,
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6 400 characterization and chiral separations. The resolved enantiomers will have different potencies
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9 401 and will be highly useful in pharmacological and physiological applications. Besides, most of the
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11 402 papers are describing the separation of one chiral centered racemates i.e. a separation of only two
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14 403 enantiomers while this article describes the chiral separation of four enantiomers of a single
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16 404 racemate. Definitely, it is an innovative work and will be useful in future research.

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18 405 The expected most pharmaceutical active Schiff base type three new imino-flavans were
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21 406 well synthesized starting from commercially available materials in acceptable yields. Chiral
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23 407 HPLC investigation was then used to separate the diastereomer by using seven CSPs in normal
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26 408 and polar organic mobile phases. Out of 3 two racemates *i.e.* BHF4 and BHF8 were resolved
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28 409 successfully. The thermodynamics and the length of the carbon chain were studied for chiral
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31 410 resolution. The study of the relationship between the free energy (ΔG) and the carbon chain
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33 411 length enabled us to know the possible domain of separation. The chiral recognition mechanism
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36 412 was also developed and it was found that BHF4 fitted the best in chiral groves of CSP following
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38 413 BHF8 and BHF10. The modeling results confirmed the binding order of the enantiomers in
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40 414 BHF4 > BHF8 > BHF10; with maximum binding of SR-enantiomers. The synthesized and
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43 415 separated Schiff's base types bis-imino-flavans were evaluated in urine samples with satisfactory
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45 416 results. Therefore, the developed HPLC methods may be applied for the enantiomeric resolution
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47
48 417 of BHF4 and BHF8 racemates.

50 418 **5. Acknowledgment:**

51
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4 423 **6. Conflict of interest:**

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6 424 The authors declare no conflict of interest.
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9 425 **References:**

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Declaration of interests

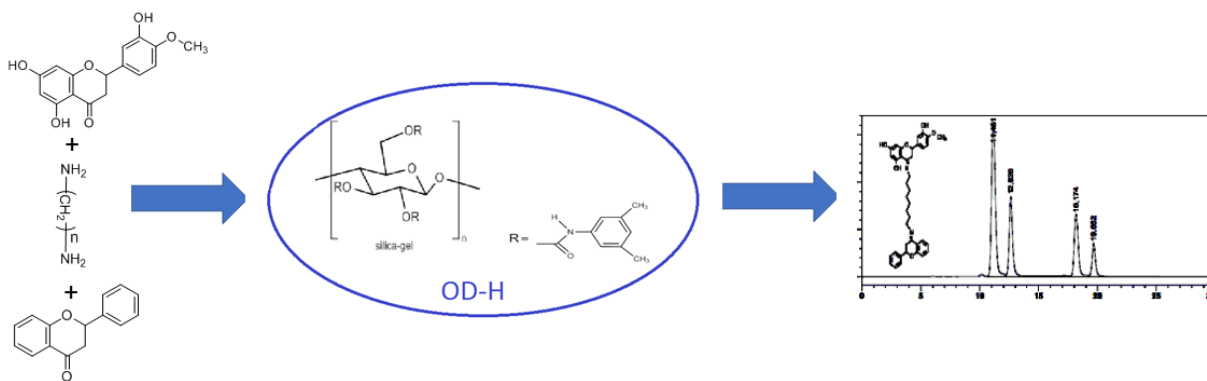
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Chiral HPLC separation and simulation studies of two chiral centered bis-imino flavans (Schiff base)

Credit author statement

Imran Ali: Conceptualization, writing, reviewing; **Mohammed El Amin Zaid:** Methodology, data curation, writing; **Nasser Belboukhari:** Conceptualization, writing, reviewing; **Khaled Sekkoum:** Conceptualization, writing, reviewing; **Wahidah H. Al-Qahtani:** Software, formal analysis; writing; **Abdulnasser Mahmoud Karami:** Writing, editing, Figures preparation; **Marcello Locatelli:** Revision, editing, reviewing, software.



Graphical Abstract

Chiral HPLC separation and simulation studies of two chiral centered bis-imino flavans (Schiff base)

*Imran Ali¹, Mohammed El Amin Zaid², Nasser Belboukhari², Khaled Sekkoum², Wahidah H. Al-Qahtani³, Abdunasser Mahmoud Karami⁴, Marcello Locatelli⁵

¹Department of Chemistry, Jamia Millia Islamia (Central University),
New Delhi-110025, India

²Bioactive Molecules and Chiral Separation Laboratory, Faculty of Exacte Science,
University Tahri Mohamed of Bechar, Bechar, 08000, Algeria

³Department of Food Sciences & Nutrition, College of Food & Agriculture Sciences, King
Saud University, Riyadh 11451, Saudi Arabia

⁴Department of Chemistry, College of Science, King Saud University, Riyadh 11451, Saudi
Arabia

⁵Analytical and Bioanalytical Chemistry, University "G. d'Annunzio" of Chieti-Pescara;
Department of Pharmacy, Build B, level 2; Via dei Vestini, 31; 66100 Chieti, Italy

Abstract:

The biological activities of flavanone and hesperetin were enhanced by synthesizing Schiff base types molecules (bis-imino-flavans; BHF4, BHF8 and BHF10) by combining flavanone and hesperetin. These molecules were characterized by spectroscopic studies. The four enantiomers of these molecules were separated by HPLC due to the presence of two chiral centers in these molecules. The best separation was achieved with Chiralcel[®]OD-H column under normal mobile phase mode. BHF4 and BHF8 racemates separated completely with k_1 , k_2 , k_3 & k_4 ; α_1 , α_2 & α_3 and Rs_1 , Rs_2 & Rs_3 values of 3.00, 3.55, 3.80 & 4.25; 1.18, 1.07 & 1.12 and 1.26, 1.10 & 1.00 for BHF4 while these values were 5.70, 6.30, 9.08 & 9.83; 1.11, 1.44 & 1.08 and 1.08, 1.37, 6.35 and 1.71. On the other hand, BHF10 could not separate completely. The free energy (ΔG) was calculated for the best separation conditions, and the correlation accurately shows the favorable range of the intercalated length. The chiral mechanism was proposed based on the carbon lengths between flavanone and hesperetin molecules in bis-imino-flavans. The modeling results confirmed the binding order of the enantiomers in BHF4 > BHF8 > BHF10; with maximum binding of SR-enantiomers. The synthesized and separated Schiff base types bis-imino-flavans were evaluated in urine samples with satisfactory results.

Keywords: Schiff base (bis-imino-flavans), Flavone and hesperetin, Chiral-HPLC separation, Chiral recognition mechanism.

*Correspondence: drimran.chiral@gmail.com; drimran_ali@yahoo.com

45 **1. Introduction:**

46 The flavonoids are a very significant group of molecules and appeal considerable
47 devotion because of their pharmacological and physiological impact [1,2]. Hesperetin is
48 identified to have strong chemo-preventive and antitumor possessions against abdominal
49 carcinoma in the treatment of a diversity of vascular and cancers diseases [3-5]. As a vital
50 bioactive Chinese traditional medication, hesperetin has manifold pharmacological and
51 biological activities. It is an antibacterial, anticancer, antioxidant, antiallergenic and anti-
52 inflammatory agent since it stimulates or inhibits a wide diversity of enzyme systems as a
53 pharmacological agent including inhibition of cancer development, effects on the blood-brain
54 barrier, signal transduction pathways, etc. [6-9]. These properties strongly depend on the
55 chemical structure; especially the presence and location of hydroxyl groups [3]. The reactivity of
56 the flavonoids with reagents at C4 carbonyl group has been getting growing interest and led to
57 interesting new synthetic compounds [10-11]. The flavanones having 2-aryl chroman-4-one
58 skeleton embedded chemical structures are extensively distributed in plants [12] and synthesized
59 as well [13-18]. Thus, the chemical modification through synthetic routes is a new direction in
60 flavanone research [19]. Belboukhari et al. [20,21] synthesized flavanone derivatives such as 4-
61 iminoflavan [22] and imino-4-hesperidin [23-25] derivatives. The modification of such types of
62 molecules is always encouraged to enhance biological activities. Therefore, it was considered
63 worthwhile to synthesize two chiral centers bis-imino flavans by using flavanone and hesperetin
64 molecules; with varying lengths of the carbon chain of intercalations. It is important to mention
65 here that the resulting bis-imino flavans were Schiff base. Therefore, it is assumed that the
66 reported molecules will be of high biological values having properties of flavanone, hesperetin
67 and Schiff bases.

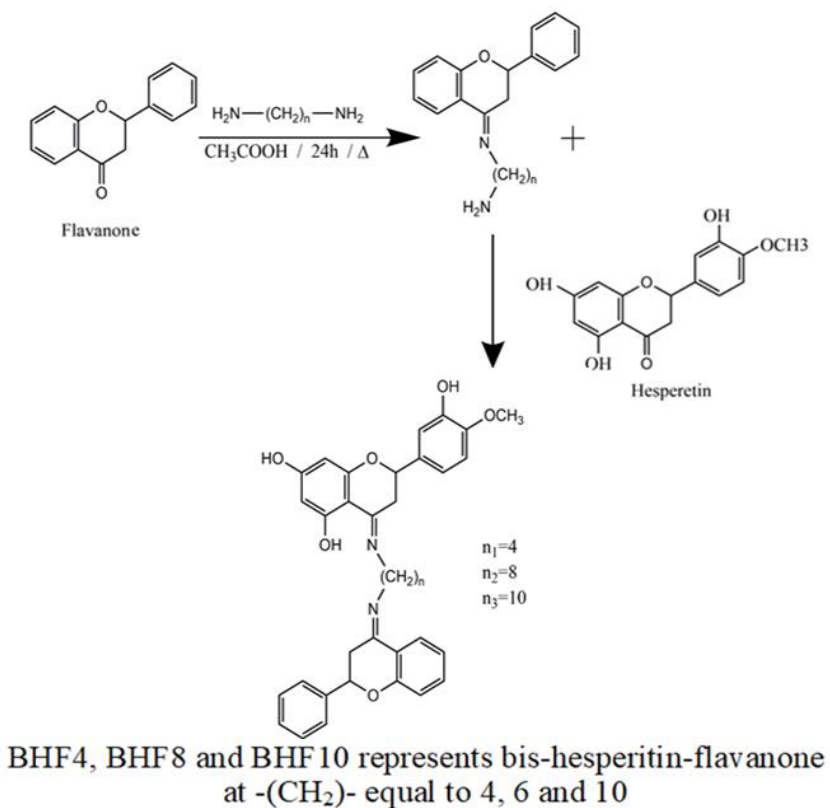
68 As mentioned above, the synthesized bis-imino flavans are having two chiral centers and
69 exist with four enantiomers in each molecule. This made these molecules more important than
70 the other flavonoids [4,26]. The chiral separation has been of great significance, particularly in
71 the pharmacological industry. This attention is because of the dissimilar pharmacological and
72 pharmacokinetic activities of the enantiomers [27]. The compounds with more than one
73 asymmetric center are now a challenge in chiral separation to have all possible enantiomers
74 because of the complex structure of these analytes and that the chiral selectors must have the
75 ability to differentiate the chiral centers simultaneously [28-31], especially under isocratic
76 conditions [32]. Polysaccharide-based CSPs are the most widespread, among various chiral
77 stationary phases [33-38]. The benzoate ester, acetate ester, or phenyl carbamate derivatives of
78 cellulose and amylose have revealed extensive enantio-selectivity and resolution abilities [39].
79 They are effective under normal-phase and reversed-phase conditions. The most commonly used
80 chiral separation techniques are High-Performance Liquid Chromatography (HPLC) and
81 Capillary Electrophoresis (CE). It is important to mention that HPLC is better than CE because
82 of the high reproducibility of HPLC in comparison to CE. Moreover, chiral separation is
83 achieved on Chiral Stationary Phases (CSPs) in HPLC while CE needs the addition of chiral
84 selector in background electrolytes. This made the method costly in CE because every time-
85 costly chiral selectors are added, which is wastage. Besides, the separated enantiomers in HPLC
86 are pure while in the case of CE the separated enantiomers are the diastereomers formed with
87 chiral selectors [40,41]. In this way, HPLC is much better than CE in chiral separation.
88 Therefore, HPLC was used as the separation technique in this article. Therefore, efforts are made
89 to resolve four enantiomers of the reported bis-imino flavans by using a variety of chiral columns
90 and mobile phases. Finally, the developed chiral HPLC methods were applied in urine samples
91 for enantiomeric resolution of the reported molecules

92 **2. Experimental:**

93 The chemicals, reagents, and instruments are given in supplementary information.

94 **2.1 Synthesis of bis-imino flavans**

95 To synthesize the asymmetric compounds, 0.5 mmol of flavanone was dissolved in methanol
96 and added to an acetic acid/ethanol (1.5 mL/25mL) hot stirring solution. The solutions of 5 mmol
97 of each appropriate primary diamine dissolved in ethanol were added to the mixture. Then, 0.5
98 mmol of hesperetin dissolved in methanol was added dropwise to the reactional medium. After
99 24 hours, the mixture was concentrated, chilled and the solid was separated. The precipitate was
100 filtered, washed with water, and recrystallized from methanol to give the desired products
101 (Figure 1). The synthesized molecules were characterized by UV-Visb., FT-IR, ¹H NMR and ¹³C
102 NMR methods.



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Figure 1: The synthesis of bis-imino flavans (Schiff base).

106 **2.3 Sample preparation**

107 A very small amount of each compound was accurately weighed and dissolved with 5.0
108 mL methanol with 10^{-5} M concentration.

109 **2.4 Analysis in biological samples**

110 To check the applicability of the established chiral HPLC methods, the racemates
111 of BHF4 and BHF8 were examined in urine samples. 50 mL urine was sampled and the
112 BHF4 and BHF8 molecules were added discretely and correspondingly to get 10^{-5} M
113 concentration. The pointed urine trials were conceded through the multi-walled carbon
114 nanotubes (MWCNTs) solid-phase extraction unit as developed in our lab. [42].

115 **3. Results and discussion**

116 **3.1 Synthesis of bis-imino flavans**

117 As clear from Figure 1 that total 3 compounds were synthesized. The synthesized
118 compounds were 2-(3-hydroxy-4-methoxyphenyl)-4-((4-(2-phenylchroman-4-
119 ylidene)amino)butyl)imino)chromane-5,7-diol (BHF4), 2-(3-hydroxy-4-methoxyphenyl)-4-((8-
120 (2-phenylchroman-4-ylidene)amino)octyl)imino)chroman-5,7-diol (BHF8) and 2-(3-hydroxy-4-
121 methoxyphenyl)-4-((10-(2-phenylchroman-4-ylidene)amino)decyl)imino)chroman-5,7-diol
122 (BHF10). By the structural point of view, BHF4, BHF8 and BHF10 represents bis-hesperetin-
123 flavanone at -(CH₂)- equal to 4, 6 and 10.

124 The reported bis-imino flavans (Schiff base) were synthesized by a typical procedure
125 described by Bouanini et al. [43]. The synthesis of imino-flavans was performed by refluxing
126 several flavanone with the suitable different primary diamines in methanol in attendance of a few
127 drops of acetic acid. The results showed that the yields depend on the nature of primary diamine,
128 the carbon bridge length and the nature of flavanes (the presence or not of hydroxyl groups). The
129 reactions yields ranged between 69 to 89%. The formation of Schiff bases took place under acid

130 or base catalysis and preference with heat. A Schiff base acts as a flexi-dentate ligand and
131 generally coordinates via the O atom of the deprotonated phenolic group and the N atom of the
132 azomethine group. The Schiff bases formation is actually an arrangement of two types of
133 reactions *i.e.* addition followed by elimination. The Schiff bases syntheses are best performed at
134 mildly acidic pH. The Schiff base formation mechanism is another difference on the theme of
135 nucleophile addition to the carbonyl group. In this case, the nucleophile is the diamine. Firstly,
136 the diamine reacted with ketone or aldehyde to give an unsteady addition compound termed
137 carbinol diamine. The carbinol diamine loosed water either by base or acid-catalyzed pathways.
138 Since the carbinol amine is an alcohol, it went acid-catalyzed dehydration [44]. The reactions
139 could be achieved only with carbon bridge length superior to four (CH₂) groups of the diamine,
140 but are not successful with carbon bridge length less than that because of the steric gene which
141 prohibits the diamine's end to reach the carbonyl site.

142 **3.2 Characterization of the compounds**

143 The above-reported compounds were characterized by different techniques. The structures of
144 the products have been established by spectral studies as UV-Visb., FT-IR, ¹H NMR and ¹³C
145 NMR methods. Their characterization is discussed below.

146 **3.2.1 2-(3-hydroxy-4-methoxyphenyl)-4-((4-(2-phenylchroman-4-ylidene) amino) butyl)** 147 **imino) chromane-5,7-diol (BHF4)**

148
149 C₃₅H₃₄N₂O₆ , Dark Brown powder; yield: 77%; M-P: 246-247°C; UVmax (MeOH, nm):
150 283 (band I); 333 (band II); IR (neat, cm⁻¹): 3378 (-OH), 3065 (-CH arom.), 2823 and 2956
151 (CH₂, CH₃), 1593 (C=C arom.), 1377(OH), 1279 and 1120 (C-O) , 721 (CH₂), 675(OH) , 650
152 (CH arom).

153 ¹H NMR (400 MHz, DMSO-d₆, ppm) :7.55(t, 2H, F: H-3', H-5', 5.6Hz), 7.45(d, 1H,
154 Hes : H-2', 3.1Hz) , 7.41(m, 3H, H-2', H-4',H-6'), 7.36 (td, 1H, F: H-7, 5.7, 2.9 Hz), 7.20(s,

155 OH-5), 7.24 (dd, 1H, F: H-5, 5.7, 2.9 Hz), 7.06 (td, 1H, H-6, 5.7, 2.9 Hz), 6.97 (dd, 1H, F: H-8,
156 5.7, 2.9 Hz), 6.81 (dd, 1H, Hes: H-2', 5.7, 2.9 Hz), 6.74(d, 1H, Hes: H-6', 5.6 Hz), 6.04 (d,
157 1H, Hes: H-5', 5.6Hz), 6.01(d, 1H, Hes: H-8, 2.9Hz), 5.85(d, 1H, H-6, 2.9Hz), 5.33(dd, 1H, H-
158 2,6.01Hz, 5.7Hz), 4.99(dd, 1H, F: H-2, 5.9Hz, 5.4Hz), 3.79(s, 3H, OCH3), 3.75(t, 2H, CH₂-N),
159 3.69(t, 2H, CH₂-N), 3.64(s, 1H, OH-3'), 3.23(dd, 1H, H-3a, 12.3Hz, 6.01Hz), 2.87(dd, 1H, H-
160 3a, 11.8Hz,5.9Hz), 2.75(dd, 1H, H-3b, 12.3Hz, 5.7Hz), 2.61(dd, 1H, H-3b, 11.8Hz,5.4Hz),
161 1.78(m, 4H, CH₂-CH₂), 0.61 (s, 1H, OH-7). ¹³C NMR (75 MHz, DMSO-d₆, ppm) δ 166.86,
162 163.81, 163.23, 159.13, 147.70, 147.04, 140.62, 132.00, 128.87, 128.45 – 128.01 (m), 127.43 –
163 127.02 (m), 123.09, 119.99, 118.49, 117.55, 113.72, 112.79, 101.22, 97.80, 95.93, 78.64, 56.98 –
164 56.47 (m), 35.65, 27.06 – 26.65 (m).

165 **3.2.2 2-(3-hydroxy-4-methoxyphenyl)-4-((8-(2-phenylchroman-4-ylidene) amino)**
166 **octyl)imino) chroman-5,7-diol (BHF8)**

167
168 C₃₉H₄₂N₂O₆ ; Brown powder; yield: 69%; M-P: 259-260°C; UVmax (MeOH, nm): 285
169 (band I); 331 (band II); IR (neat, cm⁻¹): 3380 (-OH), 3061 (-CH arom.), 2852 and 2957 (CH₂ ,
170 CH₃), 1590 (C=C arom.),1377(OH), 1273 and 1120 (C-O) , 719 (CH₂), 670(OH) , 655 (CH
171 arom).

172 ¹H NMR (400 MHz, DMSO-d₆, ppm) : 7.53(t, 2H, F: H-3', H-5', 5.6Hz), 7.41(d, 1H,
173 Hes : H-2', 3.1Hz) , 7.39(m, 3H, H-2', H-4',H-6'), 7.33 (td, 1H, F: H-7, 5.7, 2.9 Hz), 7.20(s,
174 OH-5), 7.21 (dd, 1H, F: H-5, 5.7, 2.9 Hz), 7.03 (td, 1H, H-6, 5.7, 2.9 Hz), 6.94 (dd, 1H, F: H-8,
175 5.7, 2.9 Hz), 6.76 (dd, 1H, Hes: H-2', 5.7, 2.9 Hz), 6.70(d, 1H, Hes: H-6', 5.6 Hz), 6.0 (d, 1H,
176 Hes: H-5', 5.6Hz), 5.95(d, 1H, Hes: H-8, 2.9Hz), 5.84(d, 1H, H-6, 2.9Hz), 5.29(dd, 1H, H-
177 2,6.01Hz, 5.7Hz), 4.99(dd, 1H, F: H-2, 5.9Hz, 5.4Hz), 3.78(s, 3H, OCH3), 3.70(m, 8H, CH₂-
178 CH₂-N), 3.64(s, 1H, OH-3'), 3.25(dd, 1H, H-3a, 12.3Hz, 6.01Hz), 2.84(dd, 1H, H-3a,
179 11.8Hz,5.9Hz), 2.75(dd, 1H, H-3b, 12.3Hz, 5.7Hz), 2.61(dd, 1H, H-3b, 11.8Hz,5.4Hz), 1.77-

180 1.29 (m, 8H, CH₂-CH₂), 0.91 (s, 1H, OH-7). ¹³C NMR (75 MHz, DMSO-d₆, ppm) δ 163.21,
181 161.35, 156.34, 144.83, 143.17, 138.41, 130.13, 128.17, 127.00, 125.22 – 124.93 (m), 122.13 –
182 121.21 (m), 120.54, 117.76, 115.12, 113.76, 110.16, 108.14, 107.08, 93.12, 91.15, 73.31, 53.92 –
183 52.40 (m), 32.12, 29.20 – 28.19 (m), 27.65 – 26.11 (m), 25.83 – 24.21 (m).

184
185 **3.2.3 2-(3-hydroxy-4-methoxyphenyl)-4-((10-(2-phenylchroman-4-ylidene) amino) decyl)**
186 **imino) chroman-5,7-diol (BHF10)**

187
188 C₄₁H₄₆N₂O₆; Dark Orange powder: yield: 73%; M-P: 265-266 °C; UVmax (MeOH, nm):
189 286 (band I); 338 (band II); IR (neat, cm⁻¹): 3258 (-OH), 3061 (-CH arom.), 2824 and 2957
190 (CH₂, CH₃), 1578 (C=C arom.), 1377(OH), 1278 and 1100 (C-O) , 723 (CH₂), 673(OH) , 654
191 (CH arom).

192 ¹H NMR (400 MHz, DMSO-d₆, ppm) : 7.61(t, 2H, F: H-3', H-5', 5.6Hz), 7.45(d, 1H,
193 Hes : H-2', 3.1Hz) , 7.39(m, 3H, H-2', H-4',H-6'), 7.29 (td, 1H, F: H-7, 5.7, 2.9 Hz), 7.11 (dd,
194 1H, F: H-5, 5.7, 2.9 Hz), 7.01 (td, 1H, H-6, 5.7, 2.9 Hz), 6.85 (dd, 1H, F: H-8, 5.7, 2.9 Hz),
195 6.78 (dd, 1H, Hes: H-2', 5.7, 2.9 Hz), 6.70(d, 1H, Hes: H-6', 5.6 Hz), 6.0 (d, 1H, Hes: H-5',
196 5.6Hz), 5.95(d, 1H, Hes: H-8, 2.9Hz), 5.84(d, 1H, H-6, 2.9Hz), 5.38(dd, 1H, H-2,6.01Hz,
197 5.7Hz), 5.03(dd, 1H, F: H-2, 5.9Hz, 5.4Hz), 3.86(s, 3H, OCH₃), 3.73(m, 8H, CH₂-CH₂-N),
198 3.62(s, 1H, OH-3'), 3.27(dd, 1H, H-3a, 12.3Hz, 6.01Hz), 2.89(dd, 1H, H-3a, 11.8Hz,5.9Hz),
199 2.75(dd, 1H, H-3b, 12.3Hz, 5.7Hz), 2.60(dd, 1H, H-3b, 11.8Hz,5.4Hz), 1.84-1.32 (m, 12H, CH₂-
200 CH₂), 0.87 (s, 1H, OH-7). ¹³C NMR (75 MHz, DMSO-d₆, ppm) δ 168.87, 167.12, 166.01,
201 160.42, 148.21, 146.10, 141.04, 135.16, 131.11, 126.23 – 125.10 (m), 123.33 – 122.32 (m),
202 122.12, 120.14, 119.42, 115.76, 113.23, 107.35, 97.15, 92.11, 81.98, 59.87 – 58.45 (m), 45.39,
203 43.39, 32.12 – 29.93 (m), 27.13 – 25.19 (m), 24.79 – 23.19 (m).

204

205

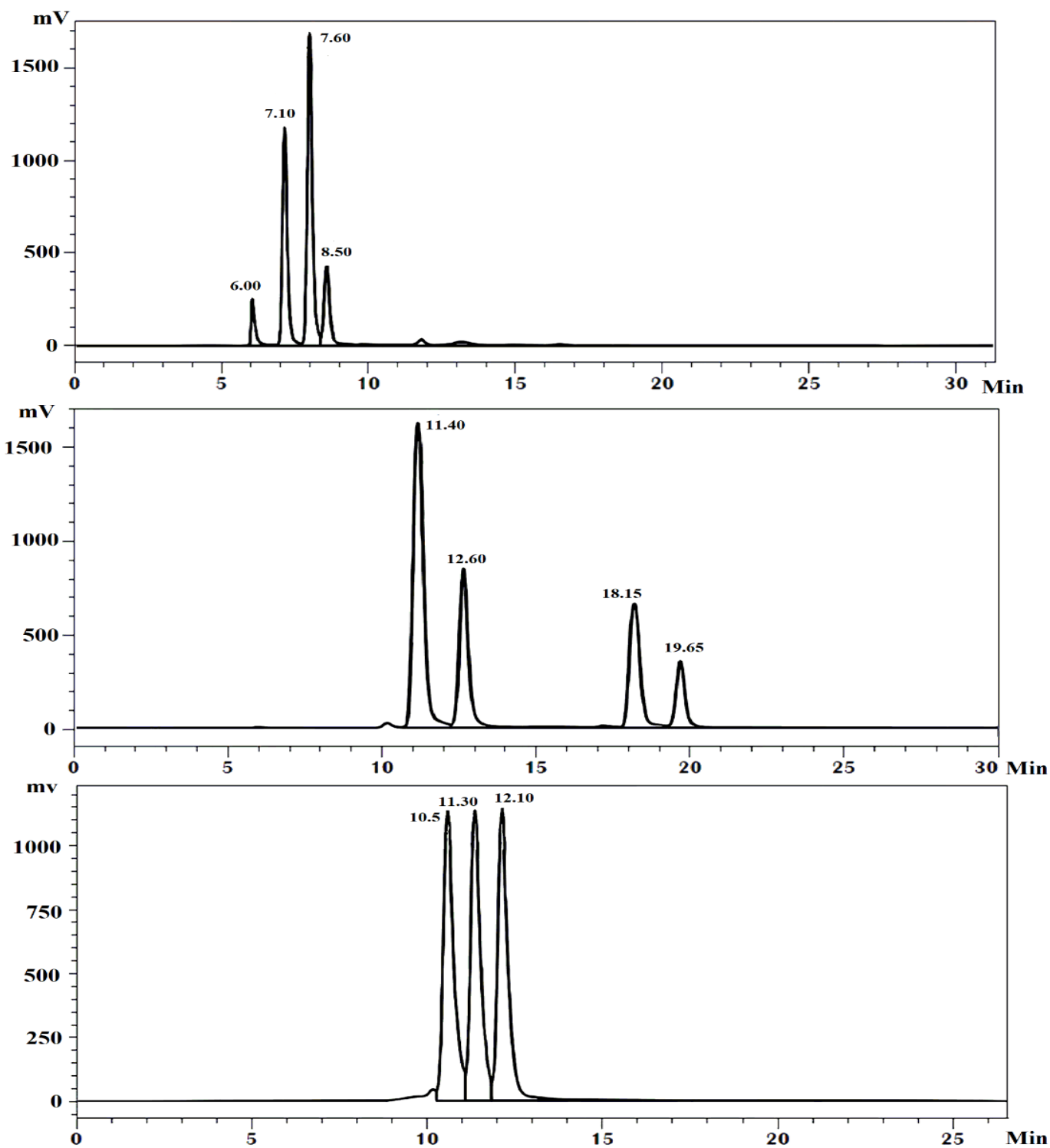
206 3.3 Chiral Separation:

207 In this work, we used two chiral separation approaches *i.e.* normal and polar organic
208 mobile phase modes under isocratic or gradient elution system. The chiral columns used were
209 Chiralpak®AD, Chiralpak®IA, Chiralpak®IB, Chiralcel®OJ, Chiralcel®OZ and Chiralcel®OD,
210 Chiralcel®OD-H. The chiral separation of BHF4, BHF8 and BHF10 is given in Table 1 and the
211 chromatograms are shown in Figure 2. It is clear from Table 1 and Figures 2 that BHF4 got
212 resolved completely with four sharp peaks using Chiralcel®OD-H and Chiralpak®IB with
213 70%HEX-30%ISP mobile phase. The retention times were in the range of 6.01 to 19.66 minutes.
214 The values of retention factors, separation factors and resolution factors of BHF4 with
215 Chiralcel®OD-H column were 2.01, 2.56, 2.98 & 4.27; 1.27, 1.16 & 1.43 and 4.20, 2.73 & 1.78
216 while these values with Chiralpak®IB were 2.30, 2.87, 3.87 & 4.19; 1.24, 1.35 & 1.27 and 0.59,
217 1.09 & 2.38. These values clearly showed better resolution with Chiralcel®OD-H in comparison
218 to Chiralpak®IB column. BHF8 could only be got resolved with Chiralcel®OD-H column by
219 using 65%HEX-35%ISP mobile phase. The values of retention factors, separation factors and
220 resolution factors were 1.79, 2.16, 3.54 & 3.92; 1.21, 1.64 & 1.11 and 2.68, 9.99 & 2.68 while
221 these values with Chiralpak®IB were 2.30, 2.87, 3.87 & 4.19; 1.24, 1.35 & 1.27 and 0.59, 1.09
222 & 2.38. On the other hand, BHF10 could not get resolved with any chiral column and mobile
223 phases used. The maximum three peaks could be obtained with both Chiralcel®OD-H and
224 Chiralpak®IB columns by using 70%HEX-30%ISP and 75%HEX-25%ISP mobile phases,
225 respectively. All the separated compounds were almost always baseline separated ($R_s > 1.0$) on
226 cellulose-based CSPs *i.e.* Chiralcel®OD-H and Chiralpak®IB; showing good chiral recognition.
227 Chiralpak®IB and Chiralcel®OD-H have a similar chiral selector with the former having
228 polysaccharides immobilized onto silica. The retention times and separation factors of
229 enantiomers were different on both columns under the same conditions. The immobilization of

230 the cellulose tris-(3, 5- dimethyl phenyl carbamate) on silica affected the chiral recognition
 231 capability may be because of the change in configuration of polysaccharide during the
 232 immobilization procedure; showing lower resolving capability than coated column [7]. It is
 233 important to mention here that the best chiral separation was on Chiralcel OD-H and Chiralcel IB
 234 columns. The reason is this the side chains in both cases have a phenyl group with two methyl
 235 constituents. The methyl group has increased the electronic density on the phenyl ring;
 236 facilitating π - π interactions. And π - π interactions are the most important ones in the chiral
 237 separations [40-42].

238 **Table 1: HPLC parameters of BHF4, BHF8 and BHF10 with different CSPs and mobile**
 239 **phases.**
 240

Racemates	CSPs	Mobile phases	FR	K ₁	K ₂	K ₃	K ₄	α_1	α_2	α_3	Rs ₁	Rs ₂	Rs ₃
BHF4	Chiralpak®AD	-	-	-	-	-	-	-	-	-	-	-	-
	Chiralpak®IA	-	-	-	-	-	-	-	-	-	-	-	-
	Chiralcel®OJ	100%MeOH	0.5	4.45	5.23	-	-	1.18	-	-	2.77	-	-
	Chiralcel®OZ	-	-	-	-	-	-	-	-	-	-	-	-
	Chiralcel®OD	70%HEX-30%ISP	0.5	2.57	5.24	-	-	2.04	-	-	3.30	-	-
	Chiralcel®OD-H	70%HEX-30%ISP	0.3	3.00	3.55	3.80	4.25	1.18	1.07	1.12	1.26	1.10	1.00
	Chiralpak®IB	70%HEX-30%ISP	0.5	2.30	2.87	3.87	4.19	1.25	1.35	1.08	1.50	1.00	1.00
BHF8	Chiralpak®AD	-	-	-	-	-	-	-	-	-	-	-	-
	Chiralpak®IA	-	-	-	-	-	-	-	-	-	-	-	-
	Chiralcel®OJ	100%MeOH	0.5	4.50	5.26	-	-	1.17	-	-	2.68	-	-
	Chiralcel®OZ	30%HEX-70%ISP	0.5	6.52	9.13	-	-	1.40	-	-	3.15	-	-
	Chiralcel®OD	100%MeOH	0.5	2.60	5.17	-	-	2.76	-	-	4.45	-	-
	Chiralcel®OD-H	70%HEX-30%ISP	0.3	5.70	6.30	9.08	9.83	1.11	1.44	1.08	1.37	6.35	1.71
	Chiralpak®IB	70%HEX-30%ISP	0.4	3.97	4.64	5.76	-	1.17	1.24	-	1.69	1.67	-
BHF10	Chiralpak®AD	-	-	-	-	-	-	-	-	-	-	-	-
	Chiralpak®IA	-	-	-	-	-	-	-	-	-	-	-	-
	Chiralcel®OJ	30%HEX-70%ISP	0.5	2.56	3.00	-	-	1.17	-	-	3.06	-	-
	Chiralcel®OZ	-	-	-	-	-	-	-	-	-	-	-	-
	Chiralcel®OD	100%MeOH	0.5	3.62	4.56	-	-	1.26	-	-	2.55	-	-
	Chiralcel®OD-H	70%HEX-30%ISP	0.5	5.25	5.65	6.05	-	1.08	1.07	-	0.85	0.71	-
	Chiralpak®IB	75%HEX-25%ISP	0.5	4.31	4.70	5.09	-	1.09	1.08	-	0.80	0.65	-



241
 242 **Figure 2: Chiral separation of BHF4, BHF8 and BHF10 with Chiralcel®OD-H column by**
 243 **using 70%HEX-30%EtOH mobile at 0.3 mL/min flow.**

244
 245
 246
 247

248 **3.3.1 Optimization of chiral separation**

249 For optimization purposes, many mobile phases with different combinations were
250 examined by varying the nature and percentage of alcohol (from 5% to 95%). The various CSPs
251 such as Chiralcel®OJ, Chiralcel®OZ, Chiralcel®OD, Chiralcel®OD-H, Chiralpak®IB,
252 Chiralpak®AD and Chiralpak®IA were tried to optimize the chiral separations. To streamline the
253 procedure, merely the chromatographic results attained by the optimum mobile phase
254 composition and/or the situations that gave the best resolution on dissimilar columns are offered
255 in this article.

256 **3.4. Thermodynamic study**

257 Thermodynamics is a very crucial study to get data around the recognition mechanism. It
258 depends on the types of both stationary and mobile phases and the type of the enantiomers. The
259 diastereomers formation or transitory complexes is controlled by several parameters including
260 free energy. The change in free energy (ΔG) accompanying the separation of two enantiomers is
261 directly related to the selectivity factor (α) and is given by $(\Delta G) = -RT \ln \alpha$; where R is the gas
262 constant, T is the absolute temperature and α is the selectivity factor [45,46]. Free energy values
263 of the best separations on CSPs (Chiralcel®OD-H, Chiralcel®IB) were calculated. The values of
264 free energy for BHF4 with Chiralcel®OD-H were -0.141, -0.088 and -0.0212 Kcal/mol
265 corresponding to α_1 , α_2 and α_3 separation factors while these values were -0.113, -0.134 and -
266 0.062 Kcal/mol for BHF8 racemate (Table 2). The values of free energy for BHF10 with
267 Chiralcel®OD-H were -0.45 and -0.51 Kcal/mol corresponding to α_1 and α_2 separation factors.
268 The values of free energy for BHF4 with Chiralcel®IB were -0.127, -0.177 and -0.141 Kcal/mol
269 corresponding to α_1 , α_2 and α_3 separation factors. The values of free energies of BHF8 were -
270 0.093 and -0.127 Kcal/mol while these values were -0.051 and -0.045 for BHF10. The values of

271 free energy were negative and these signs are a suggestion of good interactions of the
 272 enantiomers with CSPs.

273 **Table 2. A comparison of free energies values on Chiralcel®OD-H column.**
 274

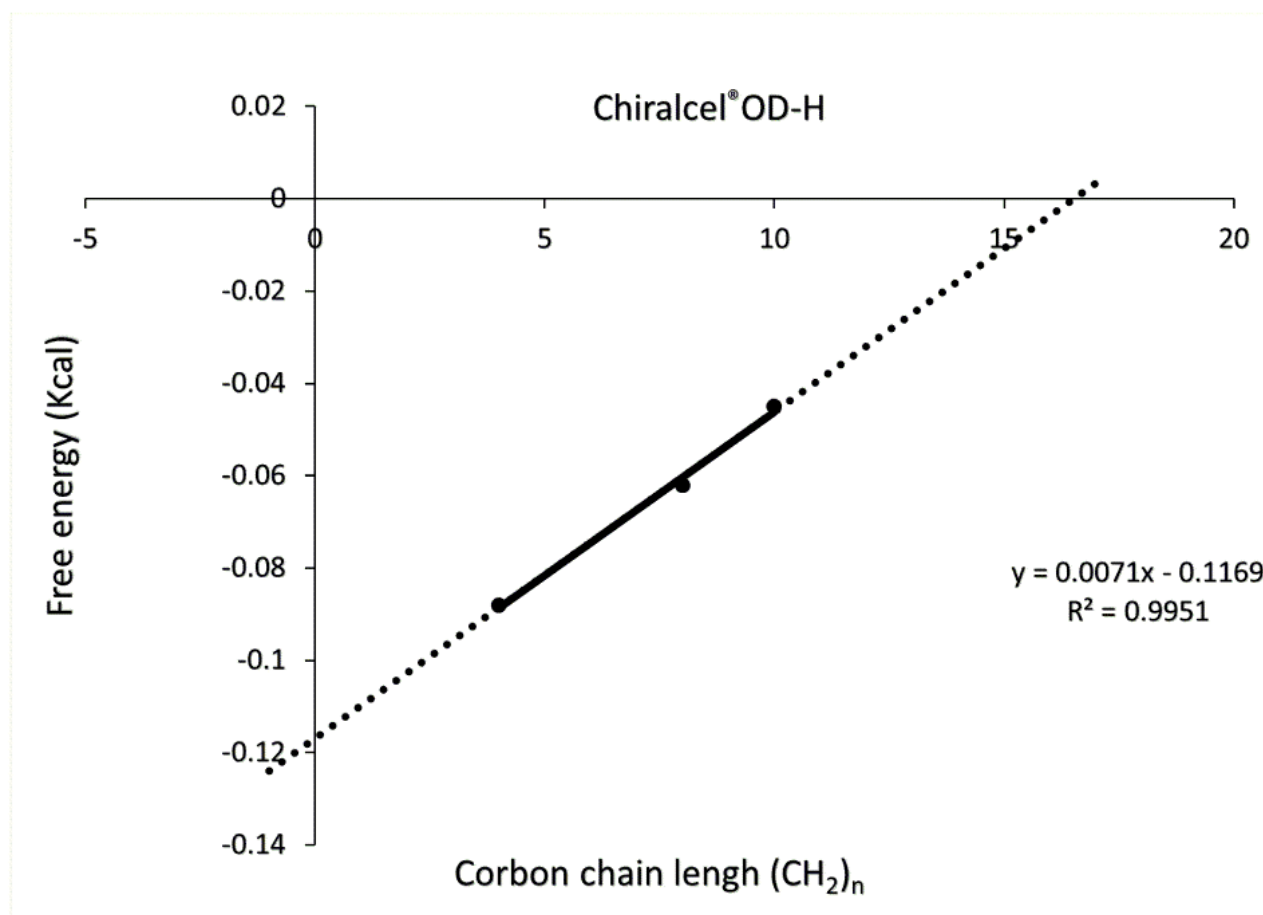
Compounds	CSP	α	ΔG
BHF4	Chiralcel®OD-H	$\alpha_1 = 1.27$	$\Delta G_1 = -0.141$ Kcal/mol
		$\alpha_2 = 1.16$	$\Delta G_2 = -0.088$ Kcal/mol
		$\alpha_3 = 1.43$	$\Delta G_3 = -0.212$ Kcal/mol
BHF8	Chiralcel®OD-H	$\alpha_1 = 1.21$	$\Delta G_1 = -0.113$ Kcal/mol
		$\alpha_2 = 1.64$	$\Delta G_2 = -0.134$ Kcal/mol
		$\alpha_3 = 1.11$	$\Delta G_3 = -0.062$ Kcal/mol
BHF10	Chiralcel®OD-H	$\alpha_1 = 1.08$	$\Delta G_1 = -0.045$ Kcal/mol
		$\alpha_2 = 1.09$	$\Delta G_2 = -0.051$ Kcal/mol

275
 276

277 3.5 Thermodynamics, carbon-chain and molar masses of the enantiomers

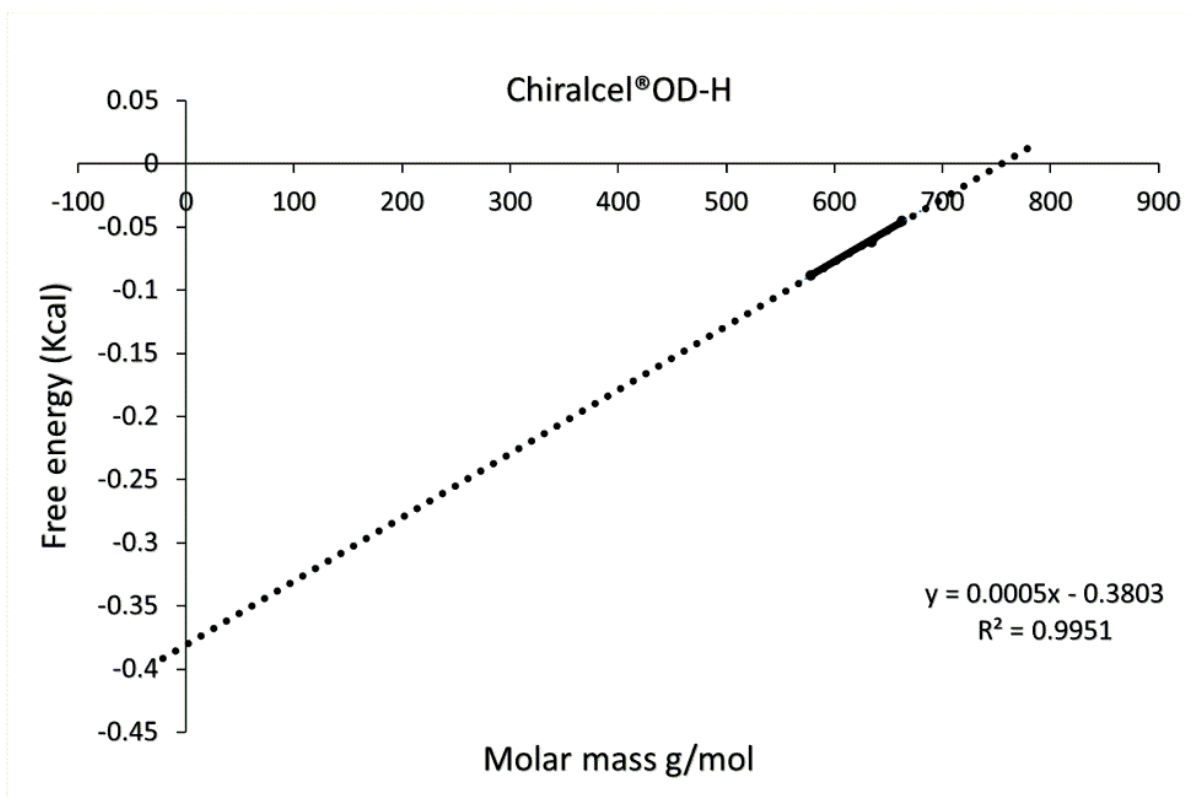
278 The free energy (ΔG) permits determining if a chemical reaction can occur due to its
 279 enthalpy (ΔH) and entropy (ΔS) changes under specific conditions of pressure and temperature
 280 [47]. It has almost a linear correlation with the carbon chain length and the molar mass of the
 281 three bis asymmetric compounds (BHF4, BHF8 and BHF10) on both Chiralcel®OD-H and
 282 Chiralcel®IB columns, with a linear correlation coefficient $R^2 \approx 1$ (0.92 to 0.99). With this study,
 283 we can observe the minimum free energy needed to have a separation and the maximum free
 284 energy so the last separation could be done with the CSP. On Chiralcel®OD-H, when the free
 285 energy tends towards zero $\Delta G = 0$ kcal/mol (when $\alpha = 1$), which means there is no separation
 286 and with an extrapolation, we can notice that this value coincided with a carbon chain length
 287 equal to $n = 16.56 \approx 17$ (Figure 3) so $(CH_2)_{17}$, which is confirmed by the correlation between the
 288 free energy and the molar mass (Figure 4) with $\Delta G = 0$ kcal (when $\alpha = 1$) which was equal to
 289 $M = 760$ g/mol, and if we omit the molar mass of the compound from the total mass of the carbon
 290 chain length we can remark that the molar mass of the carbon chain length was about

291 238.26g/mol, after divided it on 14 which is equivalent to CH₂ we can found n (number of carbon
292 chain length) = 17.01 so (CH₂)₁₇. We remark that the number of carbon chain length or the molar
293 mass is inversely proportional to the free energy (ΔG), the augmentation of carbon chain length
294 or the molar mass means the diminution of free energy (ΔG), the chiral separation is still can be
295 done when free energy (ΔG) begins from -7648.10^{-3} Kcal and stoped when it reached -0.1169
296 Kcal so between n=1 to n=16; The separations were impossible out this range (under n=1 so
297 $M < 536.34$ g/mol and above n=16 so $M > 746.34$ g/mol), which was confirmed also by the
298 correlation between the free energy(ΔG) the molar mass.



299
300 **Figure 3: The correlation between the free energy (ΔG) and the carbon chain length with**
301 **Chiralcel®OD-H column.**

302



303

304 **Figure 4: The correlation between the free energy (ΔG) and the molar mass with**
 305 **Chiralcel®OD-H column.**

306

307 On Chiralcel®IB when $\Delta G = 0$ kcal (when $\alpha=1$), the carbon chain length $n=14.20 \approx 15$

308 (Figure 5) so $(CH_2)_{15}$; which was confirmed by the correlation between the free energy and the

309 molar mass (Figure 6) so when $\Delta G = 0$, $M=738.56$ g/mol, and if we neglect the molar mass of

310 the compound from the total mass of the carbon chain length, we can remark that the molar mass

311 of the carbon chain length was about 216.22g/mol, after divided it on 14 which is equivalent to

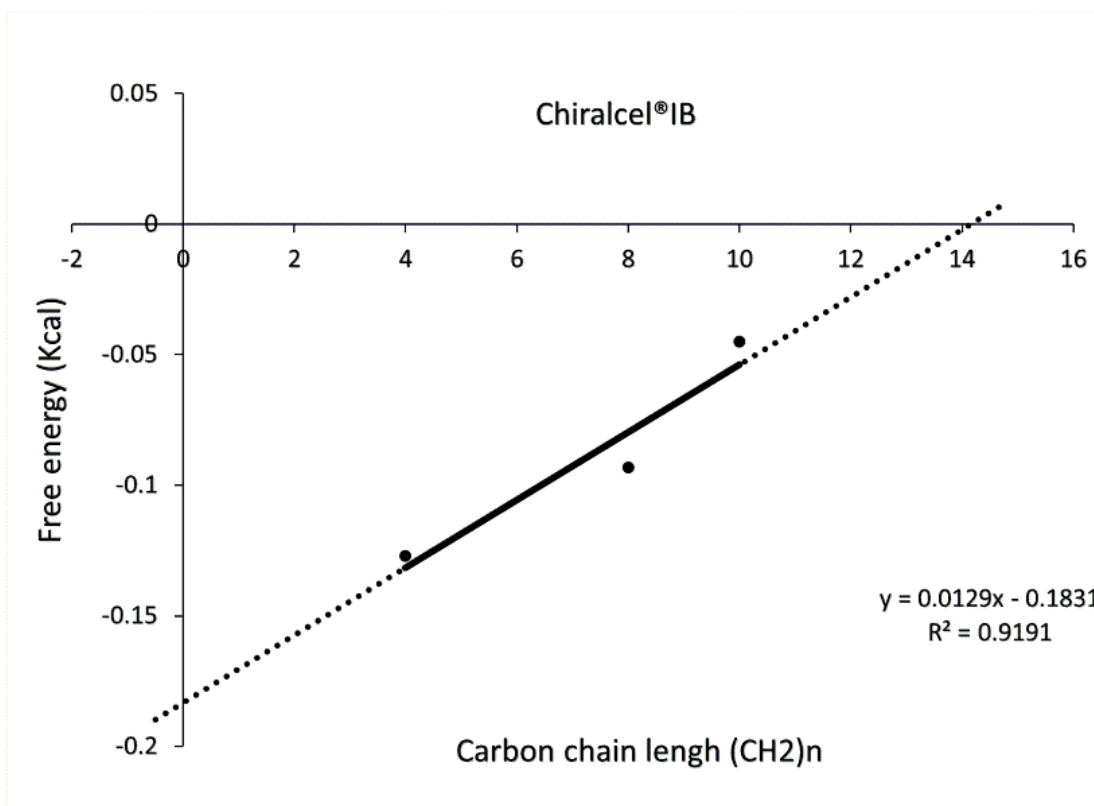
312 CH_2 we can found n (number of carbon chain length) = 15.44 so $(CH_2)_{15}$. The chiral separation

313 still could be done when free energy (ΔG) began from -0.007648 Kcal and stopped when it

314 reached -0.1831 Kcal; so between $n=1$ to $n=14$; The separations were impossible out this range

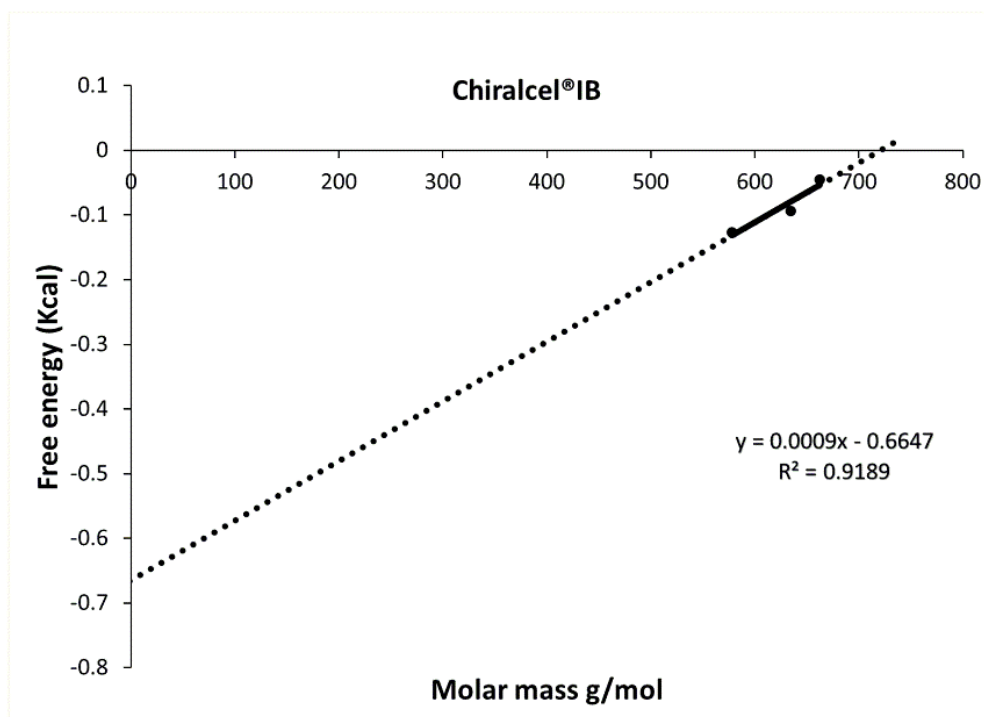
315 (under $n=1$ so $M<536.34$ g/mol and above $n=14$ so $M>746.34$ g/mol), which coincided also with

316 the correlation between the free energy (ΔG) the molar mass.



317
318
319
320

Figure 5: The correlation between free energy (ΔG) and the carbon chain length with Chiralcel®IB column.



321
322
323

Figure 6: The correlation between the free energy (ΔG) and the molar with Chiralcel®IB column.

324 **3.6 Mechanism of chiral separation**

325 The discernment control of these polysaccharide-based phases stemmed from complex
326 relations with the solutes. The chiral selector has chiral grooves offering a stereoselective
327 situation to the enantiomers. The enantiomers fit in these chiral grooves to dissimilar degrees as
328 per the lock and key arrangement. These polysaccharides comprise a large number of optically
329 active sites and, thus, have a relatively high chance of interaction with the solute, giving the
330 separation of the stereoisomers. The hydrophobic interactions combination, attractive forces
331 (e.g., hydrogen bonding), charge transfer (π - π) formation and dipole-dipole interactions were
332 supposed to clarify the molecule recognition process [28,48]. From the point of view of the
333 mobile phase, we notice that the normal organic phase is more efficient than the polar organic
334 phase for the separation of the majority of our compounds. It appears that the hydrogen bonding
335 and π - π interactions are playing a separation role among aromatic moieties of the CSPs and the
336 enantiomers [4,33-36]. The carbon chain length of the compounds also affected the separation of
337 polysaccharide CSPs. A comparison of the separation was carried out on Chiralcel®OD-H
338 column. It was observed that the order of the separation was the racemates BHF4 and BHF8
339 were resolved while racemate BHF10 could not be separated. Further, it was observed that BHF4
340 had good separation than BHF8. It may be concluded that the carbon chain length a playing
341 crucial role in chiral recognition mechanism. The carbon chain $n = 4$ provides the ideal size to
342 the racemates to fit sufficiently on the chiral grooves on the CSPs. Contrarily, the molecular size
343 of BHF8 provided fair chances of fitting the enantiomers on the chiral groove. Moreover, the
344 molecular size of BHF10 provided poor chances of fitting the enantiomers on the chiral groove.
345 This is the reason that BHF4 could be resolved better than BHF8 while BHF10 could not be
346 separated.

347

348 **3.7 Simulation study**

349 The modeling of all 12 enantiomers (4 enantiomers of each molecule) was carried out on
350 the polysaccharide-based chiral stationary phases as per the procedure described in the
351 experimental part. It was observed that each enantiomer of BHF4 interacted with CSP in
352 different fashions with different binding energies. For example, the binding energies of the four
353 enantiomers of BHF4 were -5.5, -5.4, -5.0 and -4.8 kcal/mol for SR-, SS-, RS- and RR-
354 enantiomers (Table 3). Consequently, the elution order may be considered as RR- > RS- > SS- >
355 SR- enantiomers. It was also observed that the hydrogen bondings were 1 in RR-, RS- and SS-
356 enantiomers while 2 hydrogen bonds were observed in SR-enantiomers (Figure 7). The same
357 trend was observed with enantiomers of BHF8 and BHF10 molecules. For example, the binding
358 energies in the enantiomers of BHF8 were -6.0, -5.9, -5.4 and -5.3 kcal/mol for SR-, SS-, RS-
359 and RR- enantiomers with 1 hydrogen bonding in RR-, RS- and SS-enantiomers while 2
360 hydrogen bonds were observed in SR-enantiomers. In the case of BHF10, the binding energies
361 were -5.6, -5.5, -5.2 and -5.1 kcal/mol for SR-, SS-, RS- and RR- enantiomers with 1 hydrogen
362 bonding in RR-, RS- and SS-enantiomers while 2 hydrogen bonds were observed in SR-
363 enantiomers. A comparison of the overall bondings was carried out among all 12 enantiomers
364 and it was observed that the binding order was BHF4 enantiomers > BHF10 enantiomers >
365 BHF8 enantiomers. These results are in agreement with the findings of the above mention chiral
366 recognition mechanism. Only the models of the maximum bonded SR-enantiomers of all the
367 three molecules (BHF4, BHF8 and BHF10) are given in Figure. 7.

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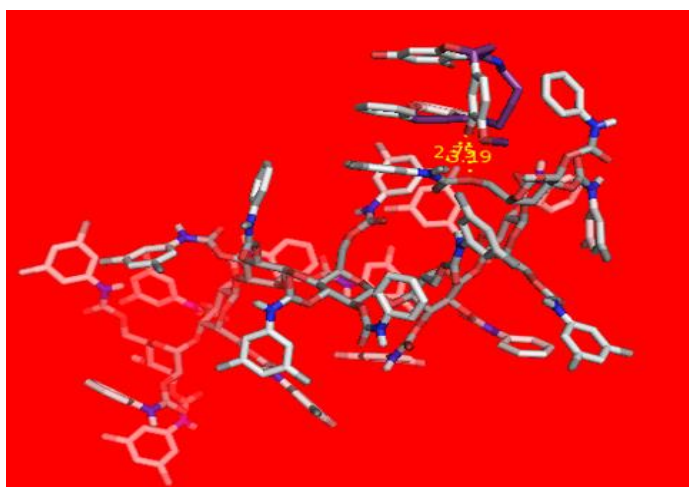
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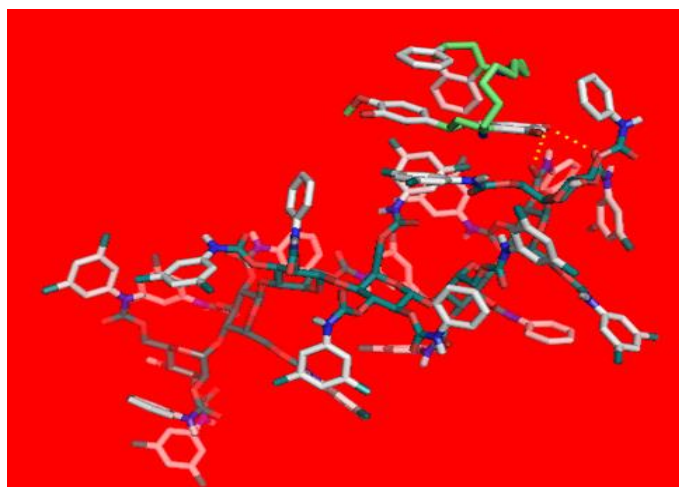
Table 3. Modeling data of enantiomers with CSP.

Compounds/Enantiomers		Binding affinity (kcal/mol)	Number of hydrogen bonds
Compounds	Enantiomers		
BHF4	SR	-5.5	1
	SS	-5.4	1
	RS	-5.0	1
	SR	-4.8	2
BHF 8	SR	-6.0	1
	SS	-5.9	1
	RS	-5.4	1
	SR	-5.3	2
BHF	SR	-5.6	1
	SS	-5.5	1
	RS	-5.2	1
	SR	-5.1	2

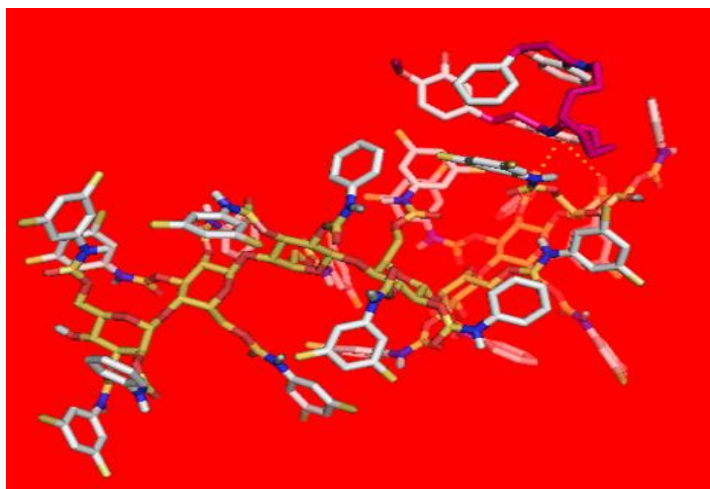
373

374
375

SR-Enantiomer of BHF4 molecule.

376
377

SR-Enantiomer of BHF8 molecule.



SR-Enantiomer of BHF10 molecule.

378
379

380 **Figure 7.** The models of the maximum bonded SR-enantiomers of all the three molecules
381 (BHF4, BHF8 and BHF10).

382

383 **3.8 Application biological samples**

384 The utility of the defined chiral HPLC methods was verified in urine bio-samples. 50 mL
385 urine samples were obtained and the BHF4 and BHF8 molecules were added independently and
386 correspondingly to get 10^{-5} M concentrations. The so obtained urine samples were allowed to
387 pass through MWCNTs solid-phase extraction, which was developed in our lab. These urine
388 samples were examined by the reported chiral HPLC methods. It was seen that the peaks were
389 alike as in the standard solutions. The regainings of the BHF4 and BHF8 molecules were in the
390 range of 89.5 to 91.7%. No extra peak was gotten in the chromatograms; approving the
391 appropriateness of the described chiral HPLC methods. The analytical data was authenticated
392 and the degrees of standard deviation were ranging from 0.66 to ± 0.81 while the correlation
393 values coefficients values and confidence levels values were 0.9994 to 0.9995 and 93.4 to 94.5.
394 These values established the utility of the stated method.

395 **4. Conclusion**

396 The novelty of this work lies in the fact that almost all the papers in chiral separations
397 reported the simple HPLC method development of one chiral-centered racemates, which was

398 obtained from different suppliers. In the present paper, we described first the synthesis,
399 characterization and chiral separations. The resolved enantiomers will have different potencies
400 and will be highly useful in pharmacological and physiological applications. Besides, most of the
401 papers are describing the separation of one chiral-centered racemates *i.e.* separation of only two
402 enantiomers while this article describes the chiral separation of four enantiomers of a single
403 racemate. Definitely, it is an innovative work and will be useful in future research.

404 The expected most pharmaceutical active Schiff base type three new imino-flavans were
405 well synthesized starting from commercially available materials in acceptable yields. Chiral
406 HPLC investigation was then used to separate the diastereomer by using seven CSPs in normal
407 and polar organic mobile phases. Out of 3 two racemates *i.e.* BHF4 and BHF8 were resolved
408 successfully. The thermodynamics and the length of the carbon chain were studied for chiral
409 resolution. The study of the relationship between the free energy (ΔG) and the carbon chain
410 length enabled us to know the possible domain of separation. The chiral recognition mechanism
411 was also developed and it was found that BHF4 fitted the best in chiral groves of CSP following
412 BHF8 and BHF10. The modeling results confirmed the binding order of the enantiomers in
413 BHF4 > BHF8 > BHF10; with maximum binding of SR-enantiomers. The synthesized and
414 separated Schiff's base types bis-imino-flavans were evaluated in urine samples with satisfactory
415 results. Therefore, the developed HPLC methods may be applied for the enantiomeric resolution
416 of BHF4 and BHF8 racemates.

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422 **6. Conflict of interest:**

423 The authors declare no conflict of interest.

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Chiral HPLC separation and simulation studies of two chiral centered bis-imino flavans (Schiff base)

*Imran Ali¹, Mohammed El Amin Zaid², Nasser Belboukhari², Khaled Sekkoum², Wahidah H. Al-Qahtani³, Abdunasser Mahmoud Karami⁴, Marcello Locatelli⁵

¹Department of Chemistry, Jamia Millia Islamia (Central University), New Delhi-110025, India

²Bioactive Molecules and Chiral Separation Laboratory, Faculty of Exacte Science, University Tahri Mohamed of Bechar, Bechar, 08000, Algeria

³Department of Food Sciences & Nutrition, College of Food & Agriculture Sciences, King Saud University, Riyadh 11451, Saudi Arabia

⁴Department of Chemistry, College of Science, King Saud University, Riyadh 11451, Saudi Arabia

⁵Analytical and Bioanalytical Chemistry, University "G. d'Annunzio" of Chieti-Pescara; Department of Pharmacy, Build B, level 2; Via dei Vestini, 31; 66100 Chieti, Italy

Abstract:

The biological activities of flavanone and hesperetin were enhanced by synthesizing Schiff base types molecules (bis-imino-flavans; BHF4, BHF8 and BHF10) by combining flavanone and hesperetin. These molecules were characterized by spectroscopic studies. The four enantiomers of these molecules were separated by HPLC due to the presence of two chiral centers in these molecules. The best separation was achieved with Chiralcel[®]OD-H column under normal mobile phase mode. **BHF4** and **BHF8** racemates separated completely with k_1 , k_2 , k_3 & k_4 ; α_1 , α_2 & α_3 and Rs_1 , Rs_2 & Rs_3 values of 3.00, 3.55, 3.80 & 4.25; 1.18, 1.07 & 1.12 and 1.26, 1.10 & 1.00 for **BHF4** while these values were 5.70, 6.30, 9.08 & 9.83; 1.11, 1.44 & 1.08 and 1.08, 1.37, 6.35 and 1.71. On the other hand, BHF10 could not separate completely. The free energy (ΔG) was calculated for the best separation conditions, and the correlation accurately shows the favorable range of the intercalated length. The chiral mechanism was proposed based on the carbon lengths between flavanone and hesperetin molecules in bis-imino-flavans. The modeling results confirmed the binding order of the enantiomers in BHF4 > BHF8 > BHF10; with maximum binding of SR-enantiomers. The synthesized and separated Schiff base types bis-imino-flavans were evaluated in urine samples with satisfactory results.

Keywords: Schiff base (bis-imino-flavans), Flavone and hesperetin, Chiral-HPLC separation, Chiral recognition mechanism.

*Correspondence: drimran.chiral@gmail.com; drimran_ali@yahoo.com

45 **1. Introduction:**

46 The flavonoids are a very significant group of molecules and appeal considerable
47 devotion because of their pharmacological and physiological impact [1,2]. Hesperetin is
48 identified to have strong chemo-preventive and antitumor possessions against abdominal
49 carcinoma in the treatment of a diversity of vascular and cancers diseases [3-5]. As a vital
50 bioactive Chinese traditional medication, hesperetin has manifold pharmacological and
51 biological activities. It is an antibacterial, anticancer, antioxidant, antiallergenic and anti-
52 inflammatory agent since it stimulates or inhibits a wide diversity of enzyme systems as a
53 pharmacological agent including inhibition of cancer development, effects on the blood-brain
54 barrier, signal transduction pathways, etc. [6-9]. These properties strongly depend on the
55 chemical structure; especially the presence and location of hydroxyl groups [3]. The reactivity of
56 the flavonoids with reagents at C4 carbonyl group has been getting growing interest and led to
57 interesting new synthetic compounds [10-11]. The flavanones having 2-aryl chroman-4-one
58 skeleton embedded chemical structures are extensively distributed in plants [12] and synthesized
59 as well [13-18]. Thus, the chemical modification through synthetic routes is a new direction in
60 flavanone research [19]. Belboukhari et al. [20,21] synthesized flavanone derivatives such as 4-
61 iminoflavan [22] and imino-4-hesperidin [23-25] derivatives. The modification of such types of
62 molecules is always encouraged to enhance biological activities. Therefore, it was considered
63 worthwhile to synthesize two chiral centers bis-imino flavans by using flavanone and hesperetin
64 molecules; with varying lengths of the carbon chain of intercalations. It is important to mention
65 here that the resulting bis-imino flavans were Schiff base. Therefore, it is assumed that the
66 reported molecules will be of high biological values having properties of flavanone, hesperetin
67 and Schiff bases.

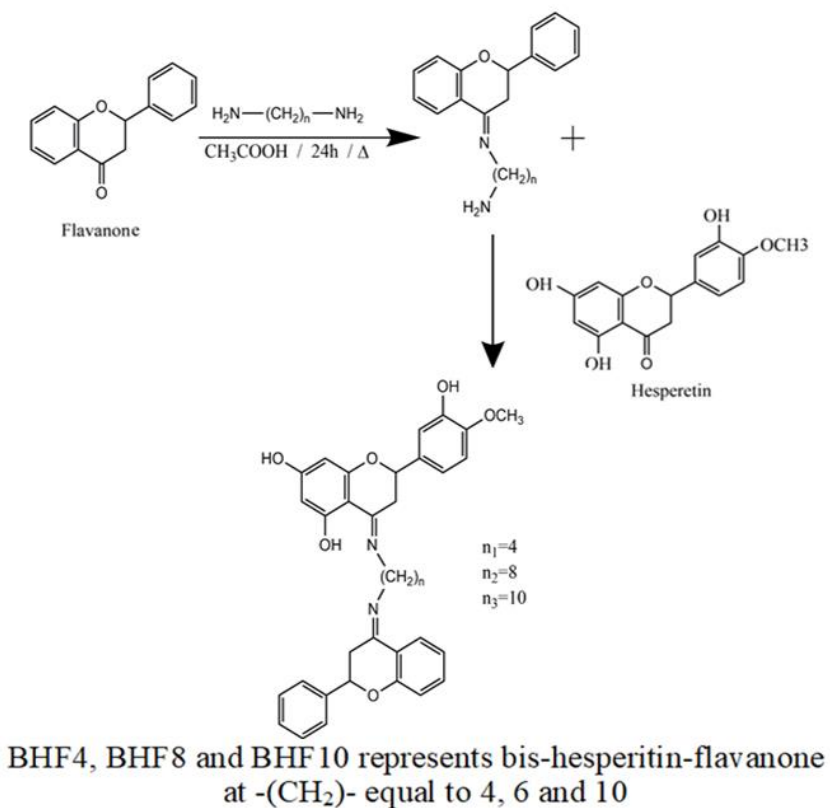
68 As mentioned above, the synthesized bis-imino flavans are having two chiral centers and
69 exist with four enantiomers in each molecule. This made these molecules more important than
70 the other flavonoids [4,26]. The chiral separation has been of great significance, particularly in
71 the pharmacological industry. This attention is because of the dissimilar pharmacological and
72 pharmacokinetic activities of the enantiomers [27]. The compounds with more than one
73 asymmetric center are now a challenge in chiral separation to have all possible enantiomers
74 because of the complex structure of these analytes and that the chiral selectors must have the
75 ability to differentiate the chiral centers simultaneously [28-31], especially under isocratic
76 conditions [32]. Polysaccharide-based CSPs are the most widespread, among various chiral
77 stationary phases [33-38]. The benzoate ester, acetate ester, or phenyl carbamate derivatives of
78 cellulose and amylose have revealed extensive enantio-selectivity and resolution abilities [39].
79 They are effective under normal-phase and reversed-phase conditions. **The most** commonly used
80 chiral separation techniques are High-Performance Liquid Chromatography (HPLC) and
81 Capillary Electrophoresis (CE). It is important to mention that HPLC is better than CE because
82 of the high reproducibility of HPLC in comparison to CE. Moreover, chiral separation is
83 achieved on Chiral Stationary Phases (CSPs) in HPLC while CE needs **the** addition of chiral
84 selector in background electrolytes. This made the method costly in CE because **every time-**
85 **costly** chiral selectors are added, which is wastage. Besides, the separated enantiomers in HPLC
86 are pure while in the case of CE the separated enantiomers are the diastereomers formed with
87 chiral **selectors** [40,41]. In this way, HPLC is much better than CE in chiral separation.
88 Therefore, HPLC was used as the separation technique in this article. Therefore, efforts are made
89 to resolve four enantiomers of the reported bis-imino flavans by using a variety of chiral columns
90 and mobile phases. Finally, the developed chiral HPLC methods were applied in urine samples
91 for enantiomeric resolution of the reported molecules

92 **2. Experimental:**

93 The chemicals, reagents, and instruments are given in supplementary information.

94 **2.1 Synthesis of bis-imino flavans**

95 To synthesize the asymmetric compounds, 0.5 mmol of flavanone was dissolved in methanol
96 and added to an acetic acid/ethanol (1.5 mL/25mL) hot stirring solution. The solutions of 5 mmol
97 of each appropriate primary diamine dissolved in ethanol were added to the mixture. Then, 0.5
98 mmol of hesperetin dissolved in methanol was added dropwise to the reactional medium. After
99 24 hours, the mixture was concentrated, chilled and the solid was separated. The precipitate was
100 filtered, washed with water, and recrystallized from methanol to give the desired products
101 (Figure 1). The synthesized molecules were characterized by UV-Visb., FT-IR, ¹H NMR and ¹³C
102 NMR methods.



103

104

105

Figure 1: The synthesis of bis-imino flavans (Schiff base).

106 **2.3 Sample preparation**

107 A very small amount of each compound was accurately weighed and dissolved with 5.0
108 mL methanol with 10^{-5} M concentration.

109 **2.4 Analysis in biological samples**

110 To check the applicability of the established chiral HPLC methods, the racemates
111 of BHF4 and BHF8 were examined in urine samples. 50 mL urine was sampled and the
112 BHF4 and BHF8 molecules were added discretely and correspondingly to get 10^{-5} M
113 concentration. The pointed urine trials were conceded through the multi-walled carbon
114 nanotubes (MWCNTs) solid-phase extraction unit as developed in our lab. [42].

115 **3. Results and discussion**

116 **3.1 Synthesis of bis-imino flavans**

117 As clear from Figure 1 that total 3 compounds were synthesized. The synthesized
118 compounds were 2-(3-hydroxy-4-methoxyphenyl)-4-((4-(2-phenylchroman-4-
119 ylidene)amino)butyl)imino)chromane-5,7-diol (BHF4), 2-(3-hydroxy-4-methoxyphenyl)-4-((8-
120 (2-phenylchroman-4-ylidene)amino)octyl)imino)chroman-5,7-diol (BHF8) and 2-(3-hydroxy-4-
121 methoxyphenyl)-4-((10-(2-phenylchroman-4-ylidene)amino)decyl)imino)chroman-5,7-diol
122 (BHF10). By the structural point of view, BHF4, BHF8 and BHF10 represents bis-hesperetin-
123 flavanone at -(CH₂)- equal to 4, 6 and 10.

124 The reported bis-imino flavans (Schiff base) were synthesized by a typical procedure
125 described by Bouanini et al. [43]. The synthesis of imino-flavans was performed by refluxing
126 several flavanone with the suitable different primary diamines in methanol in attendance of a few
127 drops of acetic acid. The results showed that the yields depend on the nature of primary diamine,
128 the carbon bridge length and the nature of flavanes (the presence or not of hydroxyl groups). The
129 reactions yields ranged between 69 to 89%. The formation of Schiff bases took place under acid

130 or base catalysis and preference with heat. A Schiff base acts as a flexi-dentate ligand and
131 generally coordinates via the O atom of the deprotonated phenolic group and the N atom of the
132 azomethine group. The Schiff bases formation is actually an arrangement of two types of
133 reactions *i.e.* addition followed by elimination. The Schiff bases syntheses are best performed at
134 mildly acidic pH. The Schiff base formation mechanism is another difference on the theme of
135 nucleophile addition to the carbonyl group. In this case, the nucleophile is the diamine. Firstly,
136 the diamine reacted with ketone or aldehyde to give an unsteady addition compound termed
137 carbinol diamine. The carbinol diamine loosed water either by base or **acid-catalyzed** pathways.
138 Since the carbinol amine is an alcohol, it went acid-catalyzed dehydration [44]. The reactions
139 could be achieved only with carbon bridge length superior to four (CH₂) groups of the diamine,
140 but are not successful with carbon bridge length less than that because of the steric gene which
141 prohibits the diamine's end to reach the carbonyl site.

142 **3.2 Characterization of the compounds**

143 The above-reported compounds were characterized by different techniques. The structures of
144 the products have been established by spectral studies as UV-Visb., FT-IR, ¹H NMR and ¹³C
145 NMR methods. Their characterization is discussed below.

146 **3.2.1 2-(3-hydroxy-4-methoxyphenyl)-4-((4-(2-phenylchroman-4-ylidene) amino) butyl)** 147 **imino) chromane-5,7-diol (BHF4)**

148
149 C₃₅H₃₄N₂O₆ , Dark Brown powder; yield: 77%; M-P: 246-247°C; UVmax (MeOH, nm):
150 283 (band I); 333 (band II); IR (neat, cm⁻¹): 3378 (-OH), 3065 (-CH arom.), 2823 and 2956
151 (CH₂, CH₃), 1593 (C=C arom.), 1377(OH), 1279 and 1120 (C-O) , 721 (CH₂), 675(OH) , 650
152 (CH arom).

153 ¹H NMR (400 MHz, DMSO-d₆, ppm) :7.55(t, 2H, F: H-3', H-5', 5.6Hz), 7.45(d, 1H,
154 Hes : H-2', 3.1Hz) , 7.41(m, 3H, H-2', H-4',H-6'), 7.36 (td, 1H, F: H-7, 5.7, 2.9 Hz), 7.20(s,

155 OH-5), 7.24 (dd, 1H, F: H-5, 5.7, 2.9 Hz), 7.06 (td, 1H, H-6, 5.7, 2.9 Hz), 6.97 (dd, 1H, F: H-8,
156 5.7, 2.9 Hz), 6.81 (dd, 1H, Hes: H-2', 5.7, 2.9 Hz), 6.74(d, 1H, Hes: H-6', 5.6 Hz), 6.04 (d,
157 1H, Hes: H-5', 5.6Hz), 6.01(d, 1H, Hes: H-8, 2.9Hz), 5.85(d, 1H, H-6, 2.9Hz), 5.33(dd, 1H, H-
158 2,6.01Hz, 5.7Hz), 4.99(dd, 1H, F: H-2, 5.9Hz, 5.4Hz), 3.79(s, 3H, OCH₃), 3.75(t, 2H, CH₂-N),
159 3.69(t, 2H, CH₂-N), 3.64(s, 1H, OH-3'), 3.23(dd, 1H, H-3a, 12.3Hz, 6.01Hz), 2.87(dd, 1H, H-
160 3a, 11.8Hz,5.9Hz), 2.75(dd, 1H, H-3b, 12.3Hz, 5.7Hz), 2.61(dd, 1H, H-3b, 11.8Hz,5.4Hz),
161 1.78(m, 4H, CH₂-CH₂), 0.61 (s, 1H, OH-7). ¹³C NMR (75 MHz, DMSO-d₆, ppm) δ 166.86,
162 163.81, 163.23, 159.13, 147.70, 147.04, 140.62, 132.00, 128.87, 128.45 – 128.01 (m), 127.43 –
163 127.02 (m), 123.09, 119.99, 118.49, 117.55, 113.72, 112.79, 101.22, 97.80, 95.93, 78.64, 56.98 –
164 56.47 (m), 35.65, 27.06 – 26.65 (m).

165 **3.2.2 2-(3-hydroxy-4-methoxyphenyl)-4-((8-(2-phenylchroman-4-ylidene) amino)**
166 **octyl)imino) chroman-5,7-diol (BHF8)**

167
168 C₃₉H₄₂N₂O₆ ; Brown powder; yield: 69%; M-P: 259-260°C; UVmax (MeOH, nm): 285
169 (band I); 331 (band II); IR (neat, cm⁻¹): 3380 (-OH), 3061 (-CH arom.), 2852 and 2957 (CH₂ ,
170 CH₃), 1590 (C=C arom.),1377(OH), 1273 and 1120 (C-O) , 719 (CH₂), 670(OH) , 655 (CH
171 arom).

172 ¹H NMR (400 MHz, DMSO-d₆, ppm) : 7.53(t, 2H, F: H-3', H-5', 5.6Hz), 7.41(d, 1H,
173 Hes : H-2', 3.1Hz) , 7.39(m, 3H, H-2', H-4',H-6'), 7.33 (td, 1H, F: H-7, 5.7, 2.9 Hz), 7.20(s,
174 OH-5), 7.21 (dd, 1H, F: H-5, 5.7, 2.9 Hz), 7.03 (td, 1H, H-6, 5.7, 2.9 Hz), 6.94 (dd, 1H, F: H-8,
175 5.7, 2.9 Hz), 6.76 (dd, 1H, Hes: H-2', 5.7, 2.9 Hz), 6.70(d, 1H, Hes: H-6', 5.6 Hz), 6.0 (d, 1H,
176 Hes: H-5', 5.6Hz), 5.95(d, 1H, Hes: H-8, 2.9Hz), 5.84(d, 1H, H-6, 2.9Hz), 5.29(dd, 1H, H-
177 2,6.01Hz, 5.7Hz), 4.99(dd, 1H, F: H-2, 5.9Hz, 5.4Hz), 3.78(s, 3H, OCH₃), 3.70(m, 8H, CH₂-
178 CH₂-N), 3.64(s, 1H, OH-3'), 3.25(dd, 1H, H-3a, 12.3Hz, 6.01Hz), 2.84(dd, 1H, H-3a,
179 11.8Hz,5.9Hz), 2.75(dd, 1H, H-3b, 12.3Hz, 5.7Hz), 2.61(dd, 1H, H-3b, 11.8Hz,5.4Hz), 1.77-

180 1.29 (m, 8H, CH₂-CH₂), 0.91 (s, 1H, OH-7). ¹³C NMR (75 MHz, DMSO-d₆, ppm) δ 163.21,
181 161.35, 156.34, 144.83, 143.17, 138.41, 130.13, 128.17, 127.00, 125.22 – 124.93 (m), 122.13 –
182 121.21 (m), 120.54, 117.76, 115.12, 113.76, 110.16, 108.14, 107.08, 93.12, 91.15, 73.31, 53.92 –
183 52.40 (m), 32.12, 29.20 – 28.19 (m), 27.65 – 26.11 (m), 25.83 – 24.21 (m).

184
185 **3.2.3 2-(3-hydroxy-4-methoxyphenyl)-4-((10-(2-phenylchroman-4-ylidene) amino) decyl)**
186 **imino) chroman-5,7-diol (BHF10)**

187
188 C₄₁H₄₆N₂O₆; Dark Orange powder: yield: 73%; M-P: 265-266 °C; UVmax (MeOH, nm):
189 286 (band I); 338 (band II); IR (neat, cm⁻¹): 3258 (-OH), 3061 (-CH arom.), 2824 and 2957
190 (CH₂, CH₃), 1578 (C=C arom.), 1377(OH), 1278 and 1100 (C-O) , 723 (CH₂), 673(OH) , 654
191 (CH arom).

192 ¹H NMR (400 MHz, DMSO-d₆, ppm) : 7.61(t, 2H, F: H-3', H-5', 5.6Hz), 7.45(d, 1H,
193 Hes : H-2', 3.1Hz) , 7.39(m, 3H, H-2', H-4',H-6'), 7.29 (td, 1H, F: H-7, 5.7, 2.9 Hz), 7.11 (dd,
194 1H, F: H-5, 5.7, 2.9 Hz), 7.01 (td, 1H, H-6, 5.7, 2.9 Hz), 6.85 (dd, 1H, F: H-8, 5.7, 2.9 Hz),
195 6.78 (dd, 1H, Hes: H-2', 5.7, 2.9 Hz), 6.70(d, 1H, Hes: H-6', 5.6 Hz), 6.0 (d, 1H, Hes: H-5',
196 5.6Hz), 5.95(d, 1H, Hes: H-8, 2.9Hz), 5.84(d, 1H, H-6, 2.9Hz), 5.38(dd, 1H, H-2,6.01Hz,
197 5.7Hz), 5.03(dd, 1H, F: H-2, 5.9Hz, 5.4Hz), 3.86(s, 3H, OCH₃), 3.73(m, 8H, CH₂-CH₂-N),
198 3.62(s, 1H, OH-3'), 3.27(dd, 1H, H-3a, 12.3Hz, 6.01Hz), 2.89(dd, 1H, H-3a, 11.8Hz,5.9Hz),
199 2.75(dd, 1H, H-3b, 12.3Hz, 5.7Hz), 2.60(dd, 1H, H-3b, 11.8Hz,5.4Hz), 1.84-1.32 (m, 12H, CH₂-
200 CH₂), 0.87 (s, 1H, OH-7). ¹³C NMR (75 MHz, DMSO-d₆, ppm) δ 168.87, 167.12, 166.01,
201 160.42, 148.21, 146.10, 141.04, 135.16, 131.11, 126.23 – 125.10 (m), 123.33 – 122.32 (m),
202 122.12, 120.14, 119.42, 115.76, 113.23, 107.35, 97.15, 92.11, 81.98, 59.87 – 58.45 (m), 45.39,
203 43.39, 32.12 – 29.93 (m), 27.13 – 25.19 (m), 24.79 – 23.19 (m).

204

205

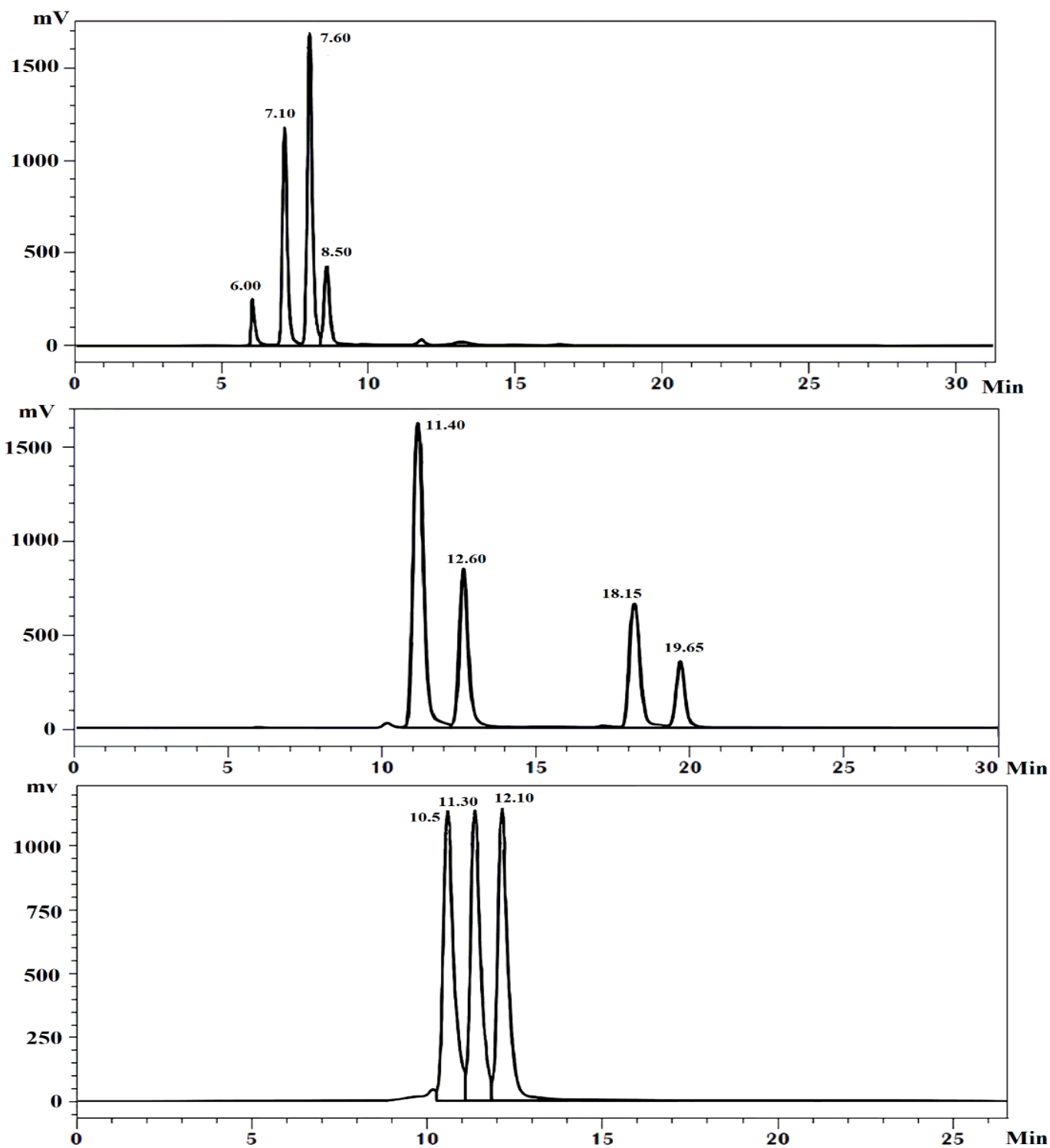
206 3.3 Chiral Separation:

207 In this work, we used two chiral separation approaches *i.e.* normal and polar organic
208 mobile phase modes under isocratic or gradient elution system. The chiral columns used were
209 Chiralpak®AD, Chiralpak®IA, Chiralpak®IB, Chiralcel®OJ, Chiralcel®OZ and Chiralcel®OD,
210 Chiralcel®OD-H. The chiral separation of BHF4, BHF8 and BHF10 is given in Table 1 and the
211 chromatograms are shown in Figure 2. It is clear from Table 1 and Figures 2 that BHF4 got
212 resolved completely with four sharp peaks using Chiralcel®OD-H and Chiralpak®IB with
213 70%HEX-30%ISP mobile phase. The retention times were in the range of 6.01 to 19.66 minutes.
214 The values of retention factors, separation factors and resolution factors of BHF4 with
215 Chiralcel®OD-H column were 2.01, 2.56, 2.98 & 4.27; 1.27, 1.16 & 1.43 and 4.20, 2.73 & 1.78
216 while these values with Chiralpak®IB were 2.30, 2.87, 3.87 & 4.19; 1.24, 1.35 & 1.27 and 0.59,
217 1.09 & 2.38. These values clearly showed better resolution with Chiralcel®OD-H in comparison
218 to Chiralpak®IB column. BHF8 could only be got resolved with Chiralcel®OD-H column by
219 using 65%HEX-35%ISP mobile phase. The values of retention factors, separation factors and
220 resolution factors were 1.79, 2.16, 3.54 & 3.92; 1.21, 1.64 & 1.11 and 2.68, 9.99 & 2.68 while
221 these values with Chiralpak®IB were 2.30, 2.87, 3.87 & 4.19; 1.24, 1.35 & 1.27 and 0.59, 1.09
222 & 2.38. On the other hand, BHF10 could not get resolved with any chiral column and mobile
223 phases used. The maximum three peaks could be obtained with both Chiralcel®OD-H and
224 Chiralpak®IB columns by using 70%HEX-30%ISP and 75%HEX-25%ISP mobile phases,
225 respectively. All the separated compounds were almost always baseline separated ($R_s > 1.0$) on
226 cellulose-based CSPs *i.e.* Chiralcel®OD-H and Chiralpak®IB; showing good chiral recognition.
227 Chiralpak®IB and Chiralcel®OD-H have a similar chiral selector with the former having
228 polysaccharides immobilized onto silica. The retention times and separation factors of
229 enantiomers were different on both columns under the same conditions. The immobilization of

230 the cellulose tris-(3, 5- dimethyl phenyl carbamate) on silica affected the chiral recognition
 231 capability may be because of the change in configuration of polysaccharide during the
 232 immobilization procedure; showing lower resolving capability than coated column [7]. It is
 233 important to mention here that the best chiral separation was on Chiralcel OD-H and Chiralcel IB
 234 columns. The reason is this the side chains in both cases have a phenyl group with two methyl
 235 constituents. The methyl group has increased the electronic density on the phenyl ring;
 236 facilitating π - π interactions. And π - π interactions are the most important ones in the chiral
 237 separations [40-42].

238 **Table 1: HPLC parameters of BHF4, BHF8 and BHF10 with different CSPs and mobile**
 239 **phases.**
 240

Racemates	CSPs	Mobile phases	FR	K ₁	K ₂	K ₃	K ₄	α_1	α_2	α_3	Rs ₁	Rs ₂	Rs ₃
BHF4	Chiralpak®AD	-	-	-	-	-	-	-	-	-	-	-	-
	Chiralpak®IA	-	-	-	-	-	-	-	-	-	-	-	-
	Chiralcel®OJ	100%MeOH	0.5	4.45	5.23	-	-	1.18	-	-	2.77	-	-
	Chiralcel®OZ	-	-	-	-	-	-	-	-	-	-	-	-
	Chiralcel®OD	70%HEX-30%ISP	0.5	2.57	5.24	-	-	2.04	-	-	3.30	-	-
	Chiralcel®OD-H	70%HEX-30%ISP	0.3	3.00	3.55	3.80	4.25	1.18	1.07	1.12	1.26	1.10	1.00
	Chiralpak®IB	70%HEX-30%ISP	0.5	2.30	2.87	3.87	4.19	1.25	1.35	1.08	1.50	1.00	1.00
BHF8	Chiralpak®AD	-	-	-	-	-	-	-	-	-	-	-	-
	Chiralpak®IA	-	-	-	-	-	-	-	-	-	-	-	-
	Chiralcel®OJ	100%MeOH	0.5	4.50	5.26	-	-	1.17	-	-	2.68	-	-
	Chiralcel®OZ	30%HEX-70%ISP	0.5	6.52	9.13	-	-	1.40	-	-	3.15	-	-
	Chiralcel®OD	100%MeOH	0.5	2.60	5.17	-	-	2.76	-	-	4.45	-	-
	Chiralcel®OD-H	70%HEX-30%ISP	0.3	5.70	6.30	9.08	9.83	1.11	1.44	1.08	1.37	6.35	1.71
	Chiralpak®IB	70%HEX-30%ISP	0.4	3.97	4.64	5.76	-	1.17	1.24	-	1.69	1.67	-
BHF10	Chiralpak®AD	-	-	-	-	-	-	-	-	-	-	-	-
	Chiralpak®IA	-	-	-	-	-	-	-	-	-	-	-	-
	Chiralcel®OJ	30%HEX-70%ISP	0.5	2.56	3.00	-	-	1.17	-	-	3.06	-	-
	Chiralcel®OZ	-	-	-	-	-	-	-	-	-	-	-	-
	Chiralcel®OD	100%MeOH	0.5	3.62	4.56	-	-	1.26	-	-	2.55	-	-
	Chiralcel®OD-H	70%HEX-30%ISP	0.5	5.25	5.65	6.05	-	1.08	1.07	-	0.85	0.71	-
	Chiralpak®IB	75%HEX-25%ISP	0.5	4.31	4.70	5.09	-	1.09	1.08	-	0.80	0.65	-



241
 242 **Figure 2: Chiral separation of BHF4, BHF8 and BHF10 with Chiralcel®OD-H column by**
 243 **using 70%HEX-30%EtOH mobile at 0.3 mL/min flow.**

244
 245
 246
 247

248 **3.3.1 Optimization of chiral separation**

249 For optimization purposes, many mobile phases with different combinations were
250 examined by varying the nature and percentage of alcohol (from 5% to 95%). The various CSPs
251 such as Chiralcel®OJ, Chiralcel®OZ, Chiralcel®OD, Chiralcel®OD-H, Chiralpak®IB,
252 Chiralpak®AD and Chiralpak®IA were tried to optimize the chiral separations. To streamline the
253 procedure, merely the chromatographic results attained by the optimum mobile phase
254 composition and/or the situations that gave the best resolution on dissimilar columns are offered
255 in this article.

256 **3.4. Thermodynamic study**

257 Thermodynamics is a very crucial study to get data around the recognition mechanism. It
258 depends on the types of both stationary and mobile phases and the type of the enantiomers. The
259 diastereomers formation or transitory complexes is controlled by several parameters including
260 free energy. The change in free energy (ΔG) accompanying the separation of two enantiomers is
261 directly related to the selectivity factor (α) and is given by $(\Delta G) = -RT \ln \alpha$; where R is the gas
262 constant, T is the absolute temperature and α is the selectivity factor [45,46]. Free energy values
263 of the best separations on CSPs (Chiralcel®OD-H, Chiralcel®IB) were calculated. The values of
264 free energy for BHF4 with Chiralcel®OD-H were -0.141, -0.088 and -0.0212 Kcal/mol
265 corresponding to α_1 α_2 and α_3 separation factors while these values were -0.113, -0.134 and -
266 0.062 Kcal/mol for BHF8 racemate (Table 2). The values of free energy for BHF10 with
267 Chiralcel®OD-H were -0.45 and -0.51 Kcal/mol corresponding to α_1 and α_2 separation factors.
268 The values of free energy for BHF4 with Chiralcel®IB were -0.127, -0.177 and -0.141 Kcal/mol
269 corresponding to α_1 α_2 and α_3 separation factors. The values of free energies of BHF8 were -
270 0.093 and -0.127 Kcal/mol while these values were -0.051 and -0.045 for BHF10. The values of

271 free energy were negative and these signs are a suggestion of good interactions of the
 272 enantiomers with CSPs.

273 **Table 2. A comparison of free energies values on Chiralcel®OD-H column.**
 274

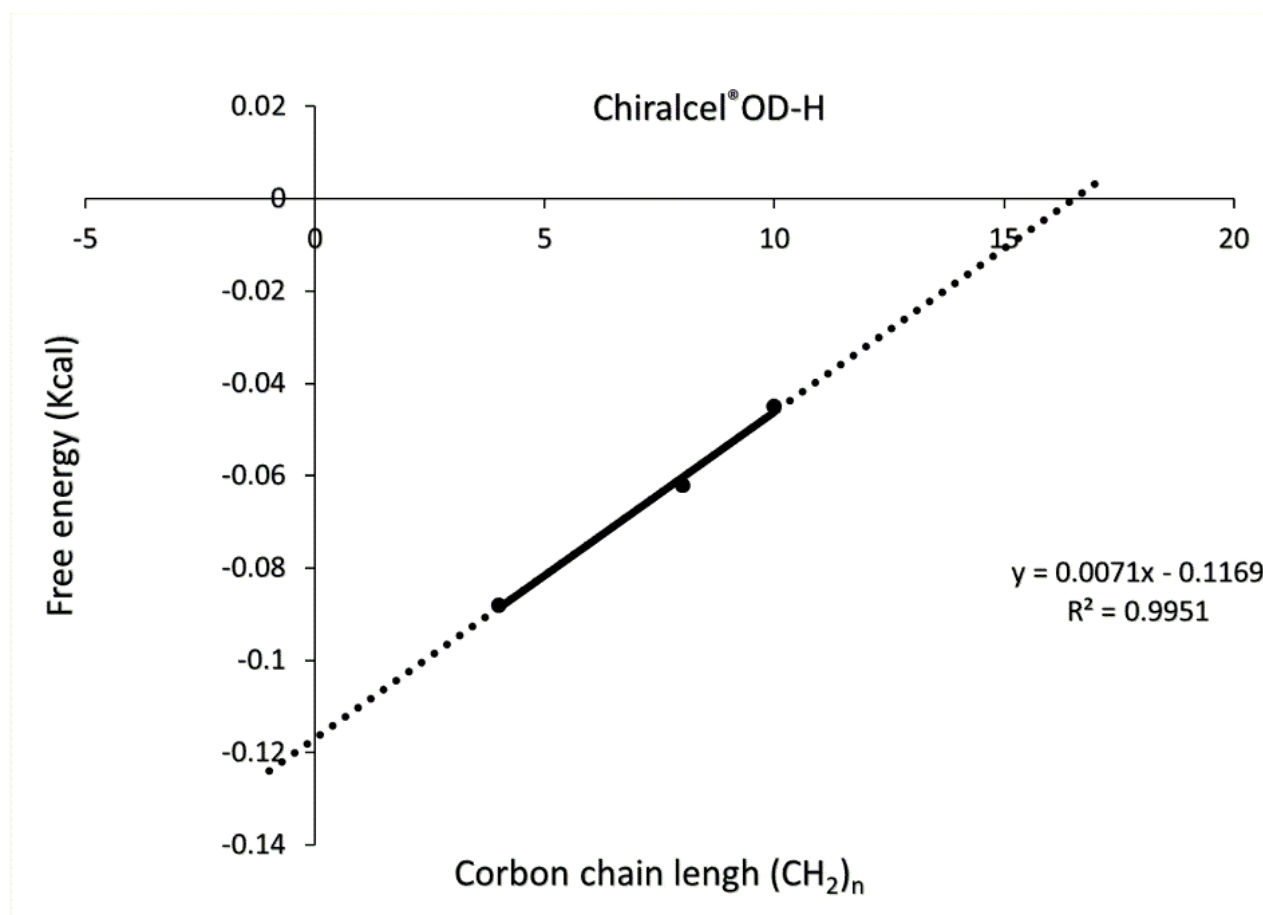
Compounds	CSP	α	ΔG
BHF4	Chiralcel®OD-H	$\alpha_1 = 1.27$	$\Delta G_1 = -0.141$ Kcal/mol
		$\alpha_2 = 1.16$	$\Delta G_2 = -0.088$ Kcal/mol
		$\alpha_3 = 1.43$	$\Delta G_3 = -0.212$ Kcal/mol
BHF8	Chiralcel®OD-H	$\alpha_1 = 1.21$	$\Delta G_1 = -0.113$ Kcal/mol
		$\alpha_2 = 1.64$	$\Delta G_2 = -0.134$ Kcal/mol
		$\alpha_3 = 1.11$	$\Delta G_3 = -0.062$ Kcal/mol
BHF10	Chiralcel®OD-H	$\alpha_1 = 1.08$	$\Delta G_1 = -0.045$ Kcal/mol
		$\alpha_2 = 1.09$	$\Delta G_2 = -0.051$ Kcal/mol

275
 276

277 3.5 Thermodynamics, carbon-chain and molar masses of the enantiomers

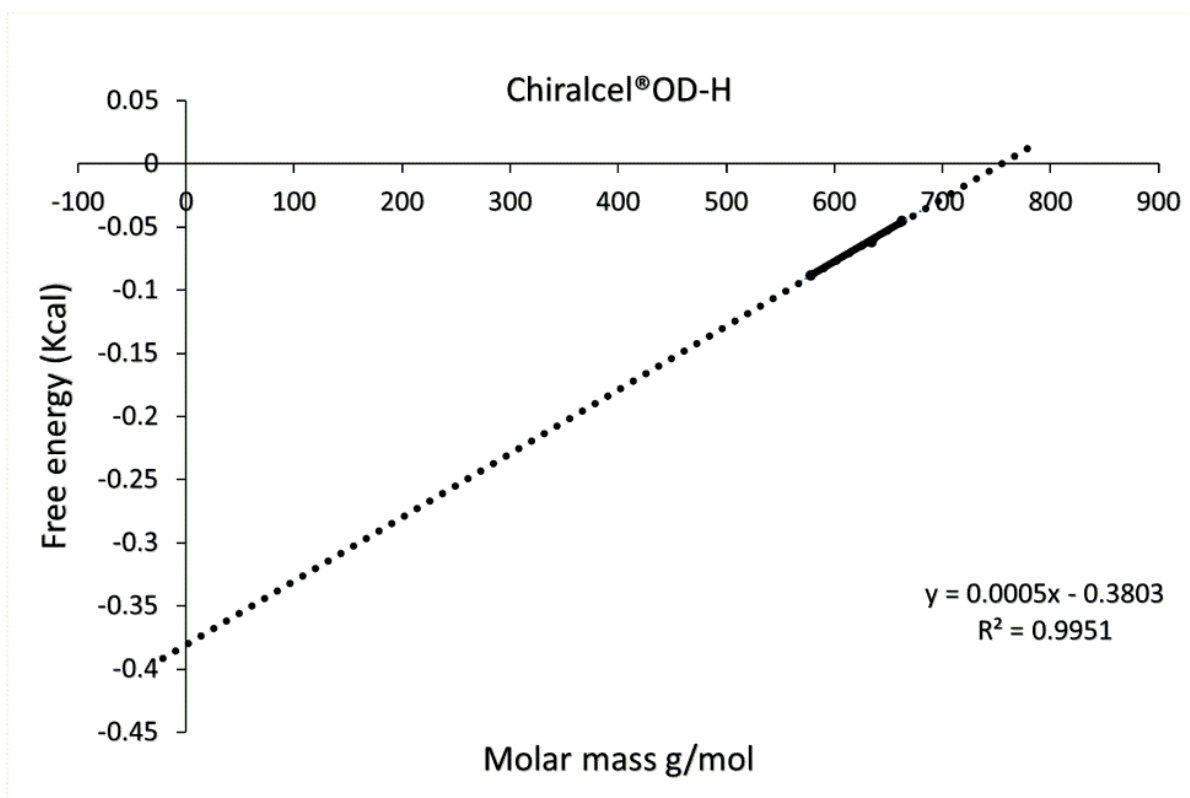
278 The free energy (ΔG) permits determining if a chemical reaction can occur due to its
 279 enthalpy (ΔH) and entropy (ΔS) changes under specific conditions of pressure and temperature
 280 [47]. It has almost a linear correlation with the carbon chain length and the molar mass of the
 281 three bis asymmetric compounds (BHF4, BHF8 and BHF10) on both Chiralcel®OD-H and
 282 Chiralcel®IB columns, with a linear correlation coefficient $R^2 \approx 1$ (0.92 to 0.99). With this study,
 283 we can observe the minimum free energy needed to have a separation and the maximum free
 284 energy so the last separation could be done with the CSP. On Chiralcel®OD-H, when the free
 285 energy tends towards zero $\Delta G = 0$ kcal/mol (when $\alpha = 1$), which means there is no separation
 286 and with an extrapolation, we can notice that this value coincided with a carbon chain length
 287 equal to $n = 16.56 \approx 17$ (Figure 3) so $(CH_2)_{17}$, which is confirmed by the correlation between the
 288 free energy and the molar mass (Figure 4) with $\Delta G = 0$ kcal (when $\alpha = 1$) which was equal to
 289 $M = 760$ g/mol, and if we omit the molar mass of the compound from the total mass of the carbon
 290 chain length we can remark that the molar mass of the carbon chain length was about

291 238.26g/mol, after divided it on 14 which is equivalent to CH₂ we can found n (number of carbon
292 chain length) = 17.01 so (CH₂)₁₇. We remark that the number of carbon chain length or the molar
293 mass is inversely proportional to the free energy (ΔG), the augmentation of carbon chain length
294 or the molar mass means the diminution of free energy (ΔG), the chiral separation is still can be
295 done when free energy (ΔG) begins from -7648.10^{-3} Kcal and stopped when it reached -0.1169
296 Kcal so between n=1 to n=16; The separations were impossible out this range (under n=1 so
297 $M < 536.34$ g/mol and above n=16 so $M > 746.34$ g/mol), which was confirmed also by the
298 correlation between the free energy(ΔG) the molar mass.



299
300 **Figure 3: The correlation between the free energy (ΔG) and the carbon chain length with**
301 **Chiralcel®OD-H column.**

302



303

304 **Figure 4: The correlation between the free energy (ΔG) and the molar mass with**
 305 **Chiralcel®OD-H column.**

306

307 On Chiralcel®IB when $\Delta G = 0$ kcal (when $\alpha=1$), the carbon chain length $n=14.20 \approx 15$

308 (Figure 5) so $(CH_2)_{15}$; which was confirmed by the correlation between the free energy and the

309 molar mass (Figure 6) so when $\Delta G = 0$, $M=738.56$ g/mol, and if we neglect the molar mass of

310 the compound from the total mass of the carbon chain length, we can remark that the molar mass

311 of the carbon chain length was about 216.22g/mol, after divided it on 14 which is equivalent to

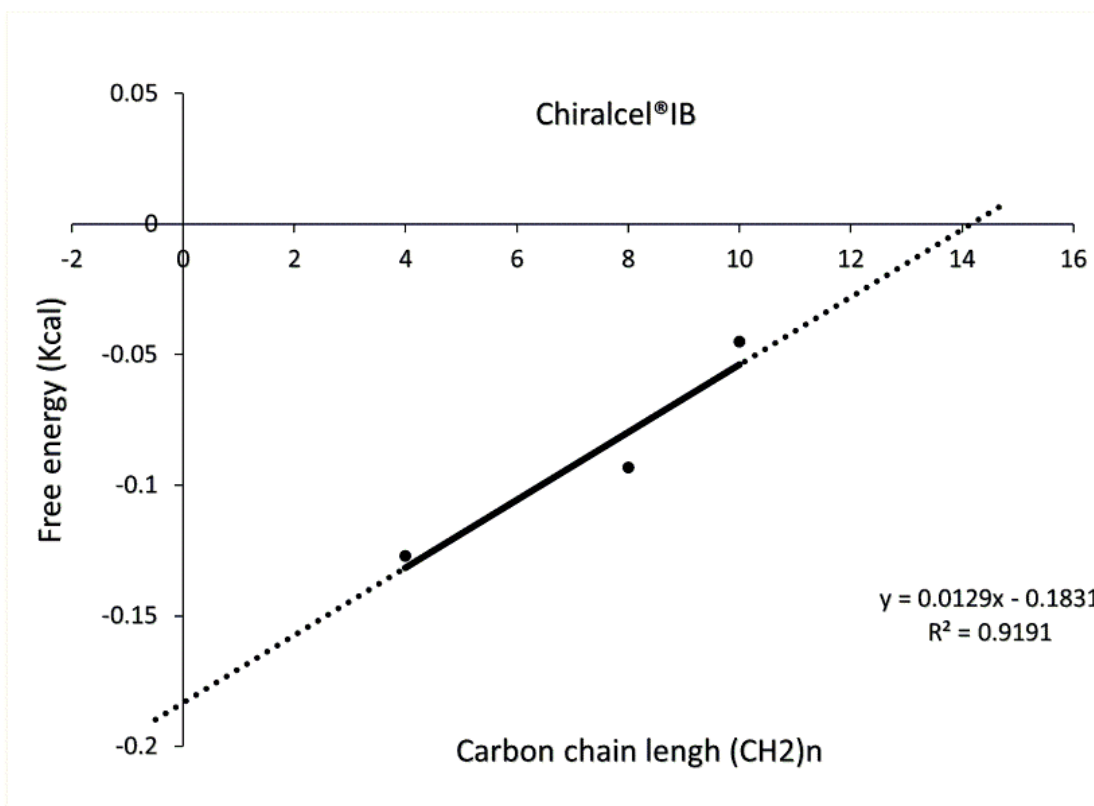
312 CH_2 we can found n (number of carbon chain length) = 15.44 so $(CH_2)_{15}$. The chiral separation

313 still could be done when free energy (ΔG) began from -0.007648 Kcal and **stopped** when it

314 reached -0.1831 Kcal; so between $n=1$ to $n=14$; The separations were impossible out this range

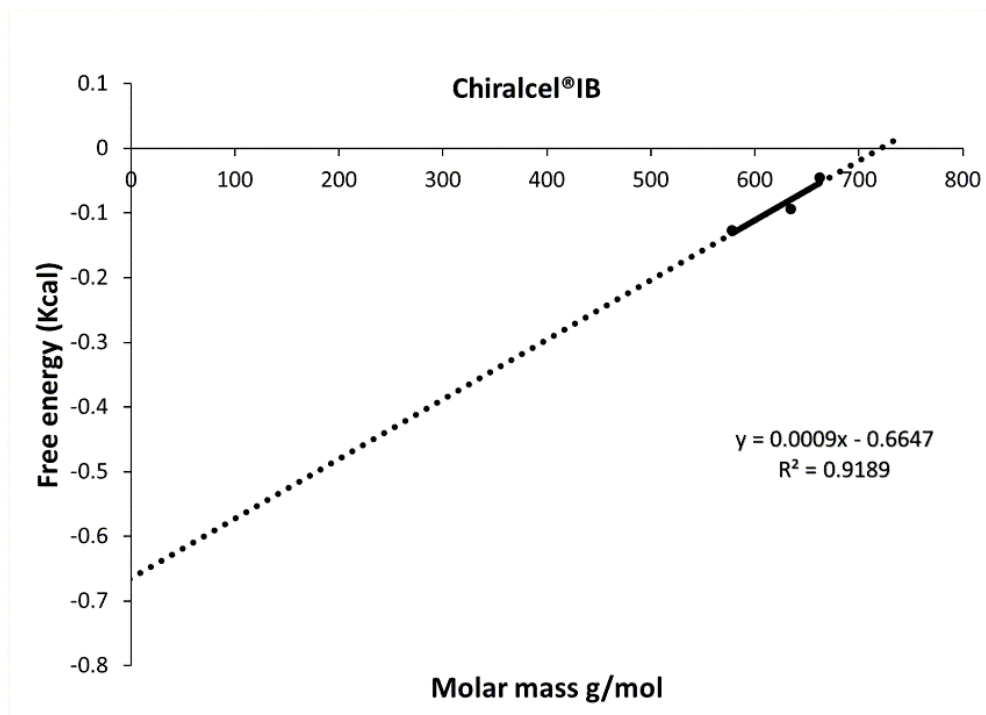
315 (under $n=1$ so $M<536.34$ g/mol and above $n=14$ so $M>746.34$ g/mol), which coincided also with

316 the correlation between the free energy (ΔG) the molar mass.



317
 318
 319
 320

Figure 5: The correlation between free energy (ΔG) and the carbon chain length with Chiralcel®IB column.



321
 322
 323

Figure 6: The correlation between the free energy (ΔG) and the molar with Chiralcel®IB column.

324 **3.6 Mechanism of chiral separation**

325 The discernment control of these polysaccharide-based phases stemmed from complex
326 relations with the solutes. The chiral selector has chiral grooves offering a stereoselective
327 situation to the enantiomers. The enantiomers fit in these chiral grooves to dissimilar degrees as
328 per the lock and key arrangement. These polysaccharides comprise a large number of optically
329 active sites and, thus, have a relatively high chance of interaction with the solute, giving the
330 separation of the stereoisomers. The hydrophobic interactions combination, attractive forces
331 (e.g., hydrogen bonding), charge transfer (π - π) formation and dipole-dipole interactions were
332 supposed to clarify the molecule recognition process [28,48]. From the point of view of the
333 mobile phase, we notice that the normal organic phase is more efficient than the polar organic
334 phase for the separation of the majority of our compounds. It appears that the hydrogen bonding
335 and π - π interactions are playing a separation role among aromatic moieties of the CSPs and the
336 enantiomers [4,33-36]. The carbon chain length of the compounds also affected the separation of
337 polysaccharide CSPs. A comparison of the separation was carried out on Chiralcel®OD-H
338 column. It was observed that the order of the separation was the racemates BHF4 and BHF8
339 were resolved while racemate BHF10 could not be separated. Further, it was observed that BHF4
340 had good separation than BHF8. It may be concluded that the carbon chain length a playing
341 crucial role in chiral recognition mechanism. The carbon chain $n = 4$ provides the ideal size to
342 the racemates to fit sufficiently on the chiral grooves on the CSPs. Contrarily, the molecular size
343 of BHF8 provided fair chances of fitting the enantiomers on the chiral groove. Moreover, the
344 molecular size of BHF10 provided poor chances of fitting the enantiomers on the chiral groove.
345 This is the reason that BHF4 could be resolved better than BHF8 while BHF10 could not be
346 separated.

347

348 **3.7 Simulation study**

349 The modeling of all 12 enantiomers (4 enantiomers of each molecule) was carried out on
350 the polysaccharide-based chiral stationary phases as per the procedure described in the
351 experimental part. It was observed that each enantiomer of BHF4 interacted with CSP in
352 different fashions with different binding energies. For example, the binding energies of the four
353 enantiomers of BHF4 were -5.5, -5.4, -5.0 and -4.8 kcal/mol for SR-, SS-, RS- and RR-
354 enantiomers (Table 3). Consequently, the elution order may be considered as RR- > RS- > SS- >
355 SR- enantiomers. It was also observed that the hydrogen bondings were 1 in RR-, RS- and SS-
356 enantiomers while 2 hydrogen bonds were observed in SR-enantiomers (Figure 7). The same
357 trend was observed with enantiomers of BHF8 and BHF10 molecules. For example, the binding
358 energies in the enantiomers of BHF8 were -6.0, -5.9, -5.4 and -5.3 kcal/mol for SR-, SS-, RS-
359 and RR- enantiomers with 1 hydrogen bonding in RR-, RS- and SS-enantiomers while 2
360 hydrogen bonds were observed in SR-enantiomers. In the case of BHF10, the binding energies
361 were -5.6, -5.5, -5.2 and -5.1 kcal/mol for SR-, SS-, RS- and RR- enantiomers with 1 hydrogen
362 bonding in RR-, RS- and SS-enantiomers while 2 hydrogen bonds were observed in SR-
363 enantiomers. A comparison of the overall bondings was carried out among all 12 enantiomers
364 and it was observed that the binding order was BHF4 enantiomers > BHF10 enantiomers >
365 BHF8 enantiomers. These results are in agreement with the findings of the above mention chiral
366 recognition mechanism. Only the models of the maximum bonded SR-enantiomers of all the
367 three molecules (BHF4, BHF8 and BHF10) are given in Figure. 7.

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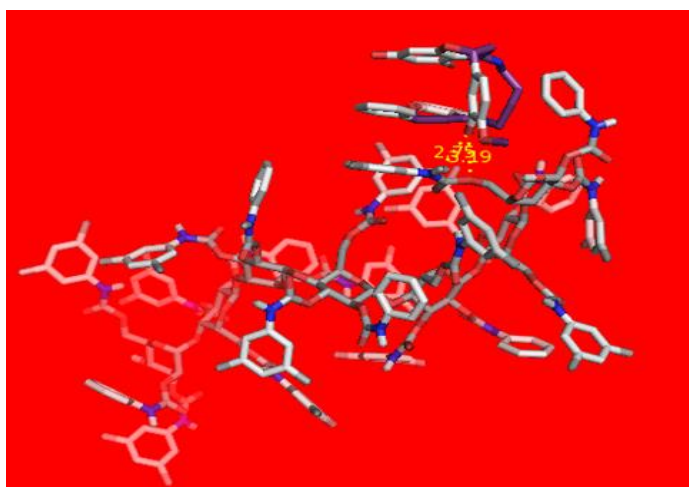
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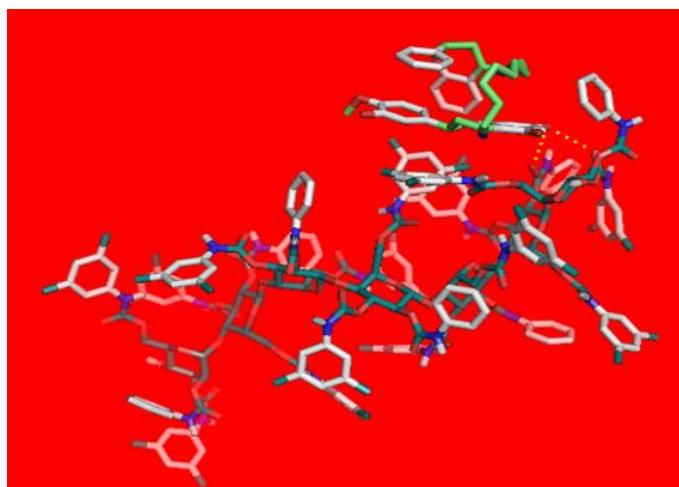
Table 3. Modeling data of enantiomers with CSP.

Compounds/Enantiomers		Binding affinity (kcal/mol)	Number of hydrogen bonds
Compounds	Enantiomers		
BHF4	SR	-5.5	1
	SS	-5.4	1
	RS	-5.0	1
	SR	-4.8	2
BHF 8	SR	-6.0	1
	SS	-5.9	1
	RS	-5.4	1
	SR	-5.3	2
BHF	SR	-5.6	1
	SS	-5.5	1
	RS	-5.2	1
	SR	-5.1	2

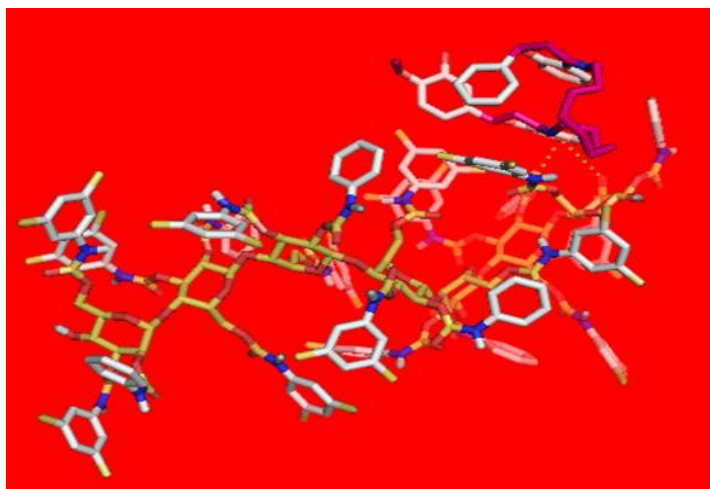
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374
375

SR-Enantiomer of BHF4 molecule.

376
377

SR-Enantiomer of BHF8 molecule.



SR-Enantiomer of BHF10 molecule.

378
379

380 **Figure 7.** The models of the maximum bonded SR-enantiomers of all the three molecules
381 (BHF4, BHF8 and BHF10).

382

383 **3.8 Application biological samples**

384 The utility of the defined chiral HPLC methods was verified in urine bio-samples. 50 mL
385 urine samples were obtained and the BHF4 and BHF8 molecules were added independently and
386 correspondingly to get 10^{-5} M concentrations. The so obtained urine samples were allowed to
387 pass through MWCNTs solid-phase extraction, which was developed in our lab. These urine
388 samples were examined by the reported chiral HPLC methods. It was seen that the peaks were
389 alike as in the standard solutions. The regainings of the BHF4 and BHF8 molecules were in the
390 range of 89.5 to 91.7%. No extra peak was gotten in the chromatograms; approving the
391 appropriateness of the described chiral HPLC methods. The analytical data was authenticated
392 and the degrees of standard deviation were ranging from 0.66 to ± 0.81 while the correlation
393 values coefficients values and confidence levels values were 0.9994 to 0.9995 and 93.4 to 94.5.
394 These values established the utility of the stated method.

395 **4. Conclusion**

396 The novelty of this work lies in the fact that almost all the papers in chiral separations
397 reported the simple HPLC method development of one **chiral-centered** racemates, which was

398 obtained from different suppliers. In the present paper, we described first the synthesis,
399 characterization and chiral separations. The resolved enantiomers will have different potencies
400 and will be highly useful in pharmacological and physiological applications. Besides, most of the
401 papers are describing the separation of one **chiral-centered** racemates *i.e.* separation of only two
402 enantiomers while this article describes the chiral separation of four enantiomers of a single
403 racemate. Definitely, it is an innovative work and will be useful in future research.

404 The expected most pharmaceutical active Schiff base type three new imino-flavans were
405 well synthesized starting from commercially available materials in acceptable yields. Chiral
406 HPLC investigation was then used to separate the diastereomer by using seven CSPs in normal
407 and polar organic mobile phases. Out of 3 two racemates *i.e.* BHF4 and BHF8 were resolved
408 successfully. The thermodynamics and the length of the carbon chain were studied for chiral
409 resolution. The study of the relationship between the free energy (ΔG) and the carbon chain
410 length enabled us to know the possible domain of separation. The chiral recognition mechanism
411 was also developed and it was found that BHF4 fitted the best in chiral grooves of CSP following
412 BHF8 and BHF10. The modeling results confirmed the binding order of the enantiomers in
413 BHF4 > BHF8 > BHF10; with maximum **binding** of SR-enantiomers. The synthesized and
414 separated Schiff's base types bis-imino-flavans were evaluated in urine samples with satisfactory
415 results. Therefore, the developed HPLC methods may be applied for the enantiomeric resolution
416 of BHF4 and BHF8 racemates.

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422 **6. Conflict of interest:**

423 The authors declare no conflict of interest.

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