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Evaluation of Enantioresolution of (±)-Catechin using Electrokinetic Chromatography and Molecular Docking

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ABSTRACT

This study involves the enantioresolution of (±) catechin with the highly sulphated beta cyclodextrin (HS-β-CD) as a chiral selector using capillary electrophoresis (CE). The purpose of this study was to better understand enantioresolution amongst host-guest interactions. Furthermore, molecular docking was carried out to elucidate the mechanism of the enantioselective separations of (±) catechin enantiomers obtained in Electrokinetic chromatography (EKC). A large difference in the interaction energies observed between the two enantiomers represents significant enantiodifferentiation. Our results also suggest that the host-guest interactions between the phenyl ring of the ligand and the open cavity of the HS-β-CD are due mainly to hydrophobic interactions. Interestingly, the stronger interactions observed with (+)-catechin is consistent with the elution order observed in the CE experiments.

Keywords: Enantioresolution, Catechin, Electrokinetic chromatography (EKC), Molecular docking.

INTRODUCTION

Enantioselective chromatography using capillary electrophoresis (CE) are extensively employed for the analysis of the enantiomeric composition (enantiomeric excess, optical purity) of chiral compounds. A chiral selector can be an appropriate chiral molecule or a chiral surface due to the enantioselectivity of the interaction with the two enantiomers [1-3]. The chiral selector either transforms the enantiomers at a different rate into new chemical entities (kinetic enantioselectivity) or forms labile molecular adducts of differing stabilities with the enantiomers (thermodynamic enantioselectivity). Catechin has two chiral centers (carbons 2 and 3 shown in **Fig. 1**) resulting in four diastereoisomers; with (+)-catechin (2R-3S) and (-)-catechin (2S-3R) being the *trans* configurations, while the (+)-epicatechin (2S-3S) and (-)-epicatechin (2R-3R) referred to as the *cis* configurations [4, 5].

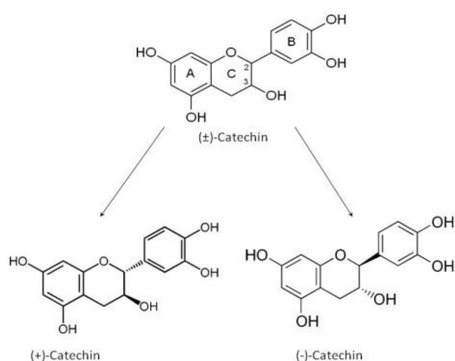

Fig. 1: **Structure of (±)-catechin and it *trans*diasteriomers**

Some differential biological activity between the enantiomers of (±)-catechin has extensively been reported. Specifically, the (+)-catechin and (-)-catechin enantiomers seem to have stereospecific opposite effects on the glycogen metabolism in isolated rat hepatocytes [6]. Bais and co-workers reported that (-)-catechin, had allelochemical activities [7] while several inhibitory effects of (+)-catechin have been observed, for instance, on intestinal tumor formation in mice [8], oxidation of low density lipoproteins [9], histidine decarboxylase (i.e. inhibiting the conversion of histidine to histamine) [10] or monoamine oxidase of the type MAO-B, which could be used as part of the treatment of Parkinson's and Alzheimer's patients [11]. Incubation experiments with (+)-catechin shows the prevention of human plasma oxidation [12].On the other hand, it has been reported that (-)-catechin suppresses the expression of Kruppel-like factor 7 and increases the expression and secretion of adiponectin protein in 3T3-L1 cells [13].These observations prompted our interest in expanding the studies of the biodifferentation of these enantiomers. Since two enantiomers possess similar physicochemical properties for a successful enantioresolution, it was therefore necessary to selectively modify the effective mobilities of these two analytes.

Cyclodextrins (CDs) are cyclic oligosaccharides consisting of six (α-CDs), seven (β-CDs) and eight (γ-CDs) glucopyranose units with a truncated cone providing a hydrophobic cavity [14]. The outer cavity is hydrophilic, making the CDs soluble in aqueous solution, while the inside cavity is less hydrophilic than the surrounding water molecules depicted in **Fig. 2** below [15].

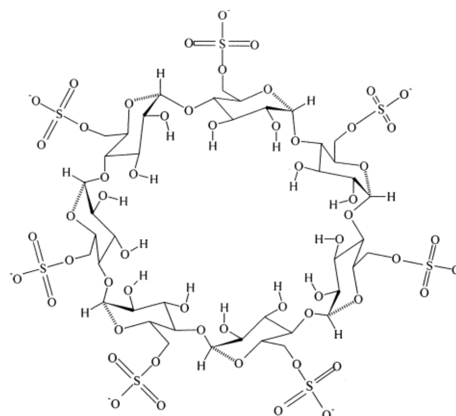

Fig. 2: Structure of highly sulphated-beta-cyclodextrin (HS-β-CD)

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As can be seen in **Fig. 2**, the hydroxyl groups present on the outer rim of the CDs can easily be modified by chemical reactions in order to obtain CD derivatives with a different degree of substitution; the composition of the modified CD depends on several experimental parameters. Modified CDs can exhibit different properties in relation to the native ones, which can easily be used for improving the selectivity the enantiomeric separation [16]. In this study we used a highly sulphated beta cyclodextrin (HS-β-CDs) as chiral selectors for CE [15]. The general merits of CE such as impressive peak efficiency, rapidity of analysis, low consumption of sample and reagents and the ease of changing chiral selectors are the most important factors contributing to the successful development of this technique. One of the most common modes of chiral CE is Electrokinetic Chromatography (EKC) in the presence of a chiral selector in the electrolyte solutions (BGE). The EKC counter-current modality, where the entire capillary is filled with the chiral selector, can be employed if the chiral selector possesses sufficient self-electrophoretic mobility in the opposite direction to the chiral analyte [17]. This technique allows for the use of chiral selectors to which the given detectors have a significant response and highly reduces the consumption of a chiral selector [18]. Currently, cyclodextrins (CDs) and their derivatives are the most popular resolving agents (chiral selector) used in CE analysis [17, 19, 20]. On the other hand, the applications of computational chemistry in the area of cyclodextrins have, until recently, been somewhat limited due to the fact that cyclodextrins are relatively large flexible molecules that are often studied experimentally in aqueous environments [2, 3, 21, 22]. However most computational studies of cyclodextrins involve host-guest complexes based on the energetics of their binding and structural analyses of inclusion complexes [23]. Accordingly, a molecular docking study was undertaken to better understand the mechanism of the enantiomeric separation between (±) catechin and HS-β-CD.

EXPERIMENTAL

Instrumentation:

A^{3D}CE capillary electrophoresis system (Hewlett-Packard, Waldbronn, Germany) equipped with a diode array detector (DAD) and ^{3D}CE Chemstation software was used throughout this. A 50 µm inner diameter (id) and 363 µm outer diameter (od) fused-silica capillary with total and effective lengths of 56 and 47.5 cm respectively was employed (Agilent Technologies, Germany). Electrophoretic solutions and samples were filtered through 0.45 µm pore size nylon membranes (Micron Separation, Weestboro, MA, USA) and degassed in an ultrasonic bath (JP Selecta, Barcelona, Spain) prior to use. A Crison Micro-pH 2000 pH meter (Crison Instruments, Barcelona, Spain) was employed to adjust the pH of buffer solutions.

Chemicals and solutions:

All reagents were of analytical grade. Sodium dihydrogen phosphate dihydrate, sodium chloride and sodium hydroxide were purchased from Scharlab (Barcelona, Spain); (+)-catechin hydrate was purchased from Cayman Chemical Company (Ann Arbor, MI, USA); (±)-catechin hydrate was purchased from Sigma and 20%w/v highly sulfated-β-cyclodextrin (HS-β-CD) aqueous solution was purchased from Beckman Coulter (Fullterton, CA, USA). Ultra Clear TWF UV ultra pure water (Siemens Water Technologies, Barsbüttel, Germany) was used to prepare solutions. For the separation in CD-EKC, a 30 mM pH 7 phosphate buffer solutions was used as a background electrolyte. 2000 µM of (±)-catechin stock solution was prepared by weighing an adequate amount of (±)-catechin powder and dissolving it with a phosphate buffer saline (PBS).

Capillary conditioning:

New capillaries were conditioned by flushing for 15 min with 1 M NaOH at 60 °C. Thereafter, they were rinsed for 5 min with deionized water and 15 min with phosphate buffer at 37 °C. The capillary was cleaned and conditioned prior to each injection as follows: (i) 2 min rinse with deionized water, (ii) 2 min rinse with 0.1M NaOH, (iii) 2 min rinse with deionized water and (iv) and 2 min with phosphate buffer at 1000 mbar.

Enantioseparation of (±)-catechin by CD-EKC:

A solution of 0.25 % w/v HS-β-CD was prepared by diluting 20 % w/v HS-β-CD with phosphate buffer used as a chiral selector. This solution was flushed through the capillary by applying approximately 1000 mbar for 120s, thus the capillary was completely filled ensuring that the inlet and outlet vials of the separation system are free of chiral selector assuring an extremely low consumption of the CD. Subsequently standard solutions were injected hydrodynamically at 50 mbar for 5 s. Separation was performed in normal polarity by applying 15 kV. The capillary was thermostated at 37 ºC and the UV-detection wavelength was set at 220 nm. Since the resolution at the maximum calibration concentration (250 µM) was adequate (*Rs* ~ 2.5), the effect of experimental variables such as pH was evaluated. The pure enantiomer (+)-catechin was used for the identification of each enantiomer by spiking the racemic mixture. Resolution of the enantiomers was calculated according to the following equation:

Where t_1 and t_2 are migration times of first and second eluted peaks respectively, w_1 and w_2 are the corresponding widths at the base peak.

$$R_s = \frac{1.18(t_2 - t_1)}{w_1 + w_2} \quad (1)$$

Computational Methodology

The molecular structure of β-cyclodextrin (CD) was obtained from the crystal structure and the highly sulfated-β-cyclodextrin (HS-β-CD) was built by adding seven sulphite (SO_3^-) functionalities to each of the primary hydroxyl groups of β-CD. The initial structures of (+)-catechin and (-)-catechin were energetically minimized using the Builder module in Discovery Studio (DS) 3.1 [24, 25]. Docking studies were performed using the CDOCKER module of DS. CDOCKER is a grid-based molecular docking method where the receptor is held rigid while the ligands are allowed to flex during the refinement. Due to a lack of information about the binding site, the whole CD molecule was selected as a target and a binding sphere with dimensions of 0.587 (X), 0.421 (Y) and 0.485 (Z) was generated. The CHARMm Force Field (FF) was used as an energy grid for docking calculations. Random ligand conformations were generated from the initial structure through high temperature molecular dynamics, followed by random rotations which were further refined by a grid-based (GRID 1) simulated annealing and a final grid-based minimization [25]. Of the 10 best poses (conformation), those having the highest docking score were used for the binding energy calculations and further analysis.

RESULTS AND DISCUSSION

Chiral resolution with Electrokinetic Chromatography (EKC):

The resolution of the enantiomers is based on the interaction between the host and guest molecules. It is apparent that the size and geometry of a guest molecule compared with that of the cyclodextrin cavity is also an important factor in the complex formation.The HS-β-CD was investigated for the chiral recognition of the (±)-catechin enantiomers, and no separation was achieved in the absence of HS-β-CD as shown in **Fig. 3**.

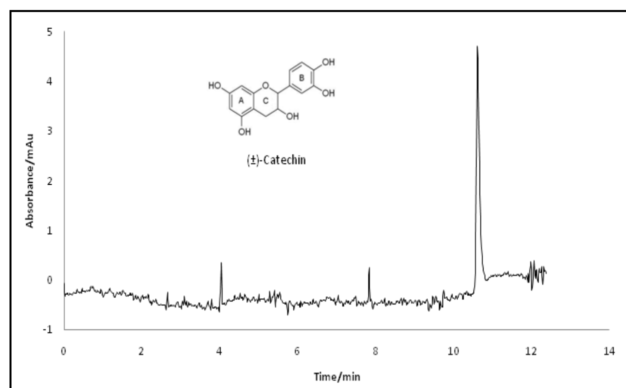

Fig. 3: Electropherogram showing unresolved enantiomers in the absence of the chiral selector.

The enantiomers of catechin were separated with EKC using HS-β-CD as a chiral selector. However the migration time was increased in the presence of the chiral selector as can be seen in **Fig. 4**. This separation was achieved using a phosphate buffer of 30 mM at pH 7. The influence of the pH was also studied by varying the pH from 6 to 7, but no significant differences were observed. The maximum resolution of 1.68 was observed at pH 7.0. Subsequently the enantiomers were identified by spiking the enantiomeric mixture with equal concentrations of (+)-catechin. It was observed that the peak area of the first eluted enantiomer increased (as shown in **Fig. 4**) hence from an experimental point of view, it was evident that (+)-catechin interacted to a greater extent with HS-β-CD in contrast to (-)-catechin.

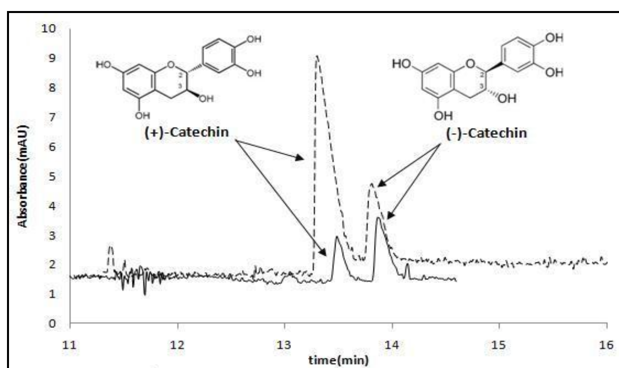

Fig. 4: EKC electropherogramm of (—) 100 µM (±)-catechin baseline resolved in the presence of 0.1 w/v HS-β-CD and (---) superimposed electropharogramm for a 100 µM (+)-catechin spiked solution.

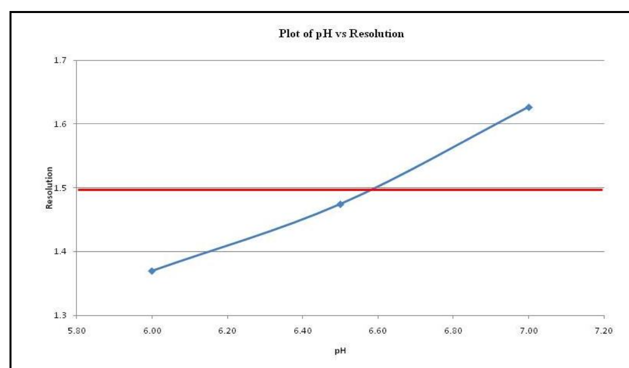

Fig. 5: Effect of pH on the resolution of (±)-catechin in the presence of the 0.1 w/v HS-β-CD. 15 kV, 30 mM Phosphate buffer

Thereafter, the enantiomeric excess was quantified by preparing calibrations with three standards of the mixture with concentrations 50, 120 and 250 M. Calibration solutions were prepared by diluting the adequate amount of (±)-catechin with the phosphate buffer at pH 7. These solutions were analysed according to the procedure described above for the sample preparation. Calibration curves were constructed by linear regression analysis of the corrected peak-area vs. concentration of the free drug for each enantiomer. The respective linear regression equations were y = 0.0117x-0016 (r = 0.9957) and y = 0.0138x-0.1164 (r = 0.9914) for (+)-catechin and (-)-catechin respectively. Using the mean ratio (-)-catechin/(+)-catechin of the three standards, it was found that the mixture constituted of 62.87 and 37.13 % of (-)-catechin and (+)-catechin respectively.

Effect of pH on chiral resolution:

Poor resolutions were observed at low pH due to the high mobility of the positively charged ions which are attracted towards the negatively charged electrode. As the pH increased, it was observed that the system shifted in an opposite direction hence improving the resolution of the two enantiomers. It was observed that on increasing the pH of the buffer, an optimum resolution (R_S =1.5) was reached at pH 6.6 as shown in **Fig. 5**, and a further increase in pH improved the resolution.

Apart from the high-resolution capability of HS-β- CDs in CE, it is worth mentioning that peak tailing were rarely observed within the evaluated pH range. Optimum conditions for the separation of the two studied enantiomers resulted in the baseline resolution of the enantiomers.

Molecular Docking:

Molecular docking was further used to assess the interactions between HS-β-CD and each enantiomer of catechin and to explain their differential separation in CE. In principle, two possible binding modes can be obtained from the interaction of (±)-catechin with HS-β-CD as shown in **Fig. 6**.

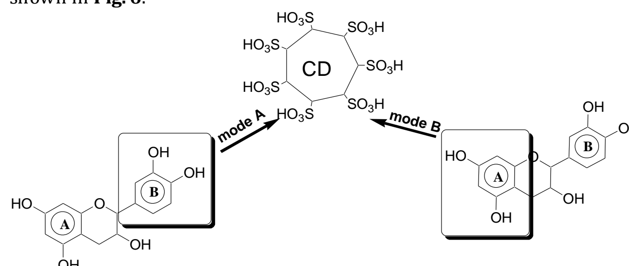

Fig. 6: Two different binding modes of Catechin towards the HS-β-CD.

In mode (i), the B ring of catechin approaches the rim of HS-β-CD while in mode (ii), it is the A ring of catechin that interacts with chiral center. Interestingly, the docked complexes of (+)-catechin and (-)-catechin with the HS-β-CD showed some important interactions and are diagrammatically shown in **Fig. 7**.

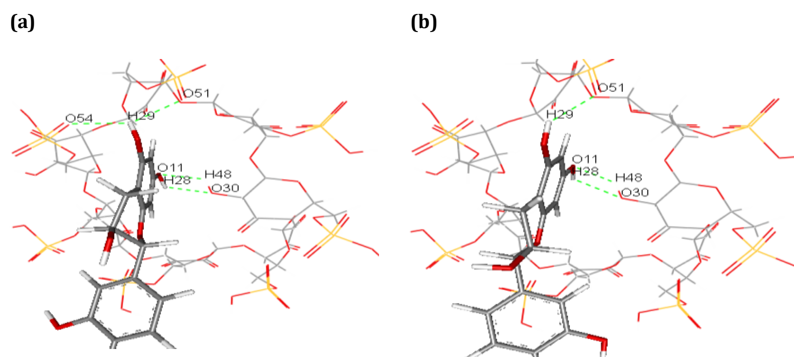

Fig. 7: Docked complexes of (+)-catechin (a) and (-)-catechin (b) with HS-β-CD. Ligand is presented in stick form, while CD is shown as Lines. All interacting atoms are labeled. Hydrogen bonds are shown as dotted green lines.

A closer inspection of **Fig. 7** revealed that the geometric orientations of both enantiomers were similar in both the complexes, stabilized by electrostatic (O…H) and hydrophobic interactions between the phenyl ring of the ligands and cavity of the HS-β-CD. Of the two possible binding modes (shown in **Fig. 6**), only one mode (ii) was observed for both enantiomers in their complexes. Both (+)- and (-)-catechin penetrate partially into the cavity of HS-β-CD, where two hydroxyl (-OH) functionalities in the A-ring of both enantiomers form hydrogen bonds with the hydroxyl and sulfate groups of the HS-β-CD. However, the presence of hydrogen bonding (H…O) was found to be more predominant in case of (+)-enantiomer (4 bonds) relative to the (-)-enantiomer (3 bonds). The corresponding docking results are summarized in **Table 1**.

Table No. 1: Docking results obtained for complexes of plus catechin and minus catechin with HS-β-CD.

Ligand (Lig)	No. of H-bonds	Donor-acceptor	Bond distance	CDocker Energy	Binding energy* (kcalmol^{-1})
(+)-catechin	4	(Lig)29H...O51(CD)	1.91	-14.9	-31.4
		(Lig)29H...O54(CD)	1.88		
		(Lig)28H...O30(CD)	1.92		
		(Lig)11O...H48(CD)	1.89		
(-)-catechin	3	(Lig)29H...O51(CD)	1.95	-13.3	-25.3
		(Lig)28H...O30(CD)	2.01		
		(Lig)11O...H48(CD)	1.94		

*Binding energy = Energy of Complex - Energy of Ligand - Energy of Receptor

The measured hydrogen bond distances were comparatively shorter in the complex of (+)-catechin with the HS-β-CD. The lower CDocker energy (-14.9) of (+)-catechin further suggests that the energy required for proper interaction between the (+)-catechin and the HS-β-CD is lowered compared to the (-)-catechin. It is reported that the electrostatic interactions (H…O) between host and guest plays a significant role in the binding energy for a number of cyclodextrin inclusion complexes (see **Fig. 7**). The computed binding free energies (**Table 1**) for the present docking results suggests that the (+)-enantiomer has a stronger propensity to interact with HS-β-CD relative to the (-)-enantiomer. The binding energies of -31.4 kcal/mol and -25.3 kcal/mol for the complexes of HS-β-CD with the (+)-enantiomer and (-)-enantiomer respectively, were found to be consistent with the elution order observed in the CE. It is reported that the enantiomer with a stronger affinity for the chiral selector migrates relatively faster during the chiral separation in CE. The first elution order of (+)-catechin in CE could be explained on the basis of its stronger interaction with the HS-β-CD. The difference in the interaction energies between the two enantiomers (E = -6.1 kcal/mol), represents the energetic contribution to the enantioselectivity and thus plays a significant role in the enantioseparation.

CONCLUSION

A methodology for the chiral separation of catechin isomers with the highly sulphated β cyclodextrin (CD) in capillary electrophoresis is described.The experimental elution order of both the enantiomers has further been explained on the basis of docking calculations. The result of the present study revealed that the location of the A-ring of the guest molecule near the sulphate groups of the β-CD is probably the key factor in stabilizing the binding energies of inclusion complexes. We have also established the importance of using derivatized CDs for additional interactions as can be seen by the H-bonds formed with the enantiomers.

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