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Research Article

Chiral recognition of imperanene enantiomers by various cyclodextrins: A capillary electrophoresis and NMR spectroscopy study

The enantiomers of imperanene, a novel polyphenolic compound of *Imperata cylindrica* (L.), were separated via cyclodextrin-modified capillary electrophoresis. The anionic form of the analyte at pH 9.0 was subject to complexation and enantioseparation CE studies with neutral and charged cyclodextrins. As chiral selectors 27 CDs were applied differing in cavity size, sidechain, degree of substitution (*DS*) and charge. Three hydroxypropylated and three sulfoalkylated CD preparations provided enantioseparation and the migration order was successfully interpreted in each case in terms of complex mobilities and stability constants. The best enantioresolution ($R_S = 1.26$) was achieved using sulfobutyl-ether-γ-CD ($DS \sim 4$), but it could be enhanced by extensive investigations on dual selector systems. After optimization (CD concentrations and pH) $R_S = 4.47$ was achieved using a 12.5 mM sulfobutyl-ether-γ-CD and 10 mM 6-monodeoxy-6-mono-(3-hydroxy)-propylamino-β-cyclodextrin dual system. The average stoichiometry of the complex was determined with Job's method using NMR-titration and resulted in a 1:1 complex for both (2-hydroxy)propyl-β- and sulfobutyl-ether-γ-CD. Further NMR experiments suggest that the coniferyl moiety of imperanene is involved in the host-guest interaction.

Keywords:
Dual cyclodextrin / Enantioseparation / Lignan enantiomers / NMR / Reversal of migration order
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1 Introduction

Different enantiomers of a bioactive molecule frequently show substantially different biological effects, thus their enantioselective synthesis, pharmacological study, and chiral analysis are of fundamental interest. To control the enantiomeric purity, the development of sensitive, selective, fast, and robust methods is important [1, 2]. As capillary electrophoresis eminently meets all these requirements, it has rapidly been established as one of the major techniques for analytical-scale enantioseparations [3]. This technique enables the detection of rather weak intermolecular host-guest association or very low stereoselectivity. Chiral additives are commonly used as complex-forming agents in the background electrolyte (BGE) as pseudostationary phase for enantioseparation. Among the chiral selectors, cyclodextrins represent an outstanding class to achieve and optimize the separation of enantiomers via complexation [4, 5].

Cyclodextrins (CDs), the drug carrier family of cyclic oligosaccharides are composed of α-(1→4) linked D-glucopyranoside units with a shape of a truncated cone. The hydrophobic interior surface of the cavity provides the possibility to form diastereomeric host-guest inclusion complexes with a wide range of pharmaceuticals in aqueous solution [6]. As CDs of different cavity size, sidechain, degree of substitution (*DS*), and charge are available, the interaction between the guest and the host can be fine tuned and thoroughly characterized. Both stability constants and enantioseparating properties of CDs with various guest molecules can be investigated and characterized using capillary electrophoresis.

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Abbreviations: ***DS***, degree of substitution (number of substituted hydroxyl groups per CD); **EMO**, enantiomer migration order; **Ipn**, imperanene; **HP-α-CD**, (2-hydroxy)propyl-α-CD; **HP-β-CD**, (2-hydroxy)propyl-β-CD; **L-DOPA**, L-3,4-dihydroxyphenylalanine; **PA-β-CD**, 6-monodeoxy-6-mono (3-hydroxy)propylamino-β-cyclodextrin hydrochloride; **SB-α-CD**, sulfobutyl-ether-α-CD sodium salt; **SB-β-CD**, sulfobutyl-ether-β-CD sodium salt; **SB-γ-CD**, sulfobutyl-ether-γ-CD sodium salt; **SP-β-CD**, sulfopropylated-β-CD sodium salt

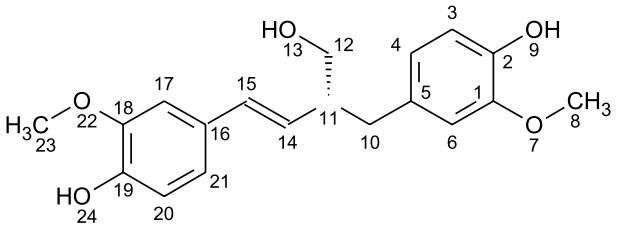

Figure 1. Chemical structure and numbering of (*S*)-imperanene.

Lignans constitute a group of important plant polyphenols that are structurally characterized by the coupling of two phenylpropanoid monomers by a bond between the β-positions in the propane sidechains. The single (*S*) isomer of imperanene (Ipn) (Fig. 1) is a member of the rare class of lignans with C_6-C_4-C_6 skeleton. This compound possesses platelet aggregation inhibitory activity and is also a competitive inhibitor of the human tyrosinase enzyme (IC_{50} = 1.85 mM) [7, 8]. The biological activity of the (*R*)-enantiomer has not been confirmed yet. Inhibition of platelet aggregation makes (*S*)-imperanene a suitable candidate for the treatment of diseases like stroke or heart attack [9]. The human tyrosinase enzyme converts tyrosine to L-DOPA and oxidizes L-DOPA to form DOPAquinone [10], which process is involved in the determination of skin color and local hyperpigmentation such as melasma and lentigo. Therefore, the well-known tyrosinase inhibitors (such as arbutin, catechins, kojic acid, resveratrol, and hydroquinone) are used for the treatment of hyperpigmentation and as cosmetics. In this area (*S*)-imperanene is a potent drug candidate as well.

Racemic and enantiopure Ipn has been synthesized and characterized by various methods so far [11–16]. In these studies, the enantiomeric excess was solely determined by HPLC using either a Chiralcel OD-H, Chiralpak AD-RH, or (*R*,*R*)-Whelk-O 1 chiral analytical column coupled to UV detection at 254 nm [12–14, 16].

To the best of our knowledge, capillary electrophoresis has not been used to separate the enantiomers of Ipn. However, cyclodextrin complexes of structurally similar compounds such as resveratrol, rosmarinic acid, or pterostilbene have been characterized. In these studies, the average complex stoichiometry was consistently found to be 1:1. Moreover, molecular modeling and ^{1}H NMR experiments were also applied to elucidate the structure of resveratrol-β-CD and resveratrol-hydroxypropyl-β-CD complexes [17–20].

Here, we report an electrophoretic separation method for imperanene enantiomers using cyclodextrin-modified capillary zone electrophoresis at alkaline pH. The enantioseparation of chiral organic acids in CE is usually performed at neutral or basic pH, where they are negatively charged and possess an electrophoretic mobility toward the anode, while the EOF is directed toward the cathode with usually higher mobility than the analyte [21]. In our study, 27 CDs were used to investigate the effect of cavity size, sidechain, degree of substitution, and the cyclodextrins' charge on the stability and mobility of the inclusion complex and the enantioseparation. Among the experimental parameters affecting chiral separation performance, pH of the background electrolyte, and the nature and concentration of the CD additive were examined. Following the study of single CD chiral CE systems, dual CD systems were extensively investigated. Among the charged chiral selectors, sulfoalkylated cyclodextrins are frequently used in dual systems to improve the enantioseparation [22, 23], therefore a wide selection of these hosts was tested in our study.

NMR spectroscopy is one of the most powerful techniques to characterize inclusion complexation at the molecular level [24–26]. To determine the complex stoichiometry, NMR titration experiments were carried out according to Job's method of continuous variation [27]. To reveal the structure of the complexes several 1D and 2D NMR experiments were also performed.

2 Materials and methods

2.1 Chemicals

Racemic and (*S*)-imperanene were synthesized and kindly provided by Yuichi Kobayashi and coworkers at the Department of Biomolecular Engineering, Tokyo Institute of Technology, Yokohama, Japan. The compounds were dissolved in MeOH (5 mg/1.5 mL) and stored at −20 °C prior to analysis. All native CDs and their derivatives (listed with their abbreviations in Supporting Information Table S1) were products of Cyclolab, Budapest, Hungary. While most of the applied CDs were multicomponent mixtures with a declared average *DS*, native CDs as well as the di- and trimethylated and the amino derivatives were single isomers. H_3BO_3, Na_2HPO_4, HCl, and NaOH used for the preparation of buffer solutions for CE and NMR titrations were of analytical grade and purchased from commercial suppliers. DMSO (Reanal, Budapest, Hungary) was used as EOF marker in CE experiments. As NMR solvent D_2O (>99.8 atom% D) from Sigma was used. All reagents were applied without further purification. Bidistilled Millipore water (Billerica, MA, USA) was used throughout this study.

2.2 Capillary electrophoresis

All CE experiments were performed on a ^{3D}CE instrument (Agilent Technologies, Waldbronn, Germany), equipped with a photodiode array detector and the Chemstation software for data handling. An untreated fused-silica capillary (50 μm id, 64.5 cm total, 56 cm effective length) was purchased from Agilent. Conditioning of new capillaries was conducted by flushing with 1 M NaOH for 30 min followed by 0.1 M NaOH and water for 60 min each. Prior to all runs the capillary was preconditioned by flushing with water (0.5 min), 0.1 M NaOH (1 min), water (1 min), and BGE (2 min). The running buffer was 75 mM borate of pH 9.0, chosen after a set of preliminary experiments with the following buffers: 75 mM borate at

pH 9.5, 9.0, 8.5, and 8.0, 50 mM phosphate at pH 7.0, 6.0, and 5.0, and 50 mM acetate at pH 5.0. The BGE contained CDs at concentrations ranging from 2 mM to 75 mM.

The Ipn sample solution (0.2 mM) contained 1.5% v/v MeOH and 0.001% v/v DMSO in H_2O and was injected hydrodynamically (50 mbar, 4 s). The temperature of the capillary was set to 25°C and +30 kV voltage was applied. UV detection was performed at 210 nm and samples were run in triplicate.

Effective mobility values were calculated with the usual formula:

$$\mu_{\text{eff}} = \frac{l_c l_d}{U} \cdot \left(\frac{1}{t} - \frac{1}{t_{\text{EOF}}} \right), \quad (1)$$

where l_c is the total length of the capillary, l_d is the length of the capillary to the detector, U is the applied voltage, while t and t_{EOF} are the peak appearance times of the analyte and the EOF marker, respectively [28].

In the absence of enantioseparation, μ_{eff} refers to racemic imperanene. The experimental μ_{eff} versus c_{CD} dataset was fitted by the following function in Microcal Origin (OriginLabs),

$$\mu_{\text{eff}} = \frac{\mu_{\text{Ipn}} + \mu_{\text{Ipn·CD}} K[\text{CD}]}{1 + K[\text{CD}]} \quad (2)$$

where $\mu_{\text{Ipn}} = -5.9 \cdot 10^{-5}$ cm^2 V^{-1} s^{-1} is the mobility of imperanene in the absence of CD and the two fitting parameters are $\mu_{\text{Ipn·CD}}$ and K, the electrophoretic mobility and stability (binding) constant of the transient Ipn·CD complex, respectively. Considering the low concentration of Ipn, the cyclodextrin equilibrium concentrations were approximated by the analytical (total) concentrations: [CD] $\cong c_{\text{CD}}$. Weighted nonlinear regression was proven to yield less biased, more reliable parameters than linearizations [29, 30], even if the most informative part (covering 20–80% complexation) of the binding isotherm cannot be recorded due to CD solubility or availability limitations, which was the case with some cyclodextrins in our study. Changes in ionic strength as a result of charged selectors were neglected in the calculations. Some of the applied CDs are multicomponent mixtures of CD derivatives differing in number and location of the substituents, in these cases only apparent complex stabilities and mobilities could be determined, which are weighted averages, composed of fractions of each additive component. *DS* and location of the substituent may strongly modulate chiral selectivity of the system and peak shape as well [5, 23]. Previous CE and NMR analysis of SB-γ-CD – being of highest importance in our study – showed that isomers of *DS* ranging from 1 to 8 exist in the mixture and the substituents are preferably located on the 6-OH group of the host.

In the "single-CD" systems with chiral resolution, Eqs. (1) and (2) were applied separately for the (*S*)- and (*R*)-Ipn peaks (the enantiomer migration order was determined by spiking with the (*S*)-Ipn sample), leading to $\mu_{\text{R·CD}}$, $\mu_{\text{S·CD}}$, K_{R}, and K_{S} parameter estimates of the diastereomeric complexes. According to the simple mathematical model by Wren and Rowe [31] (which was originally developed for the electrophoretic separation of cations [32]), the optimum CD concentration for the enantioseparation was estimated as $c_{\text{W}} = (K_{\text{R}} K_{\text{S}})^{-1/2}$, then an additional chiral CE run was performed at this CD concentration and enantioresolution (R_{S}) values were calculated as:

$$R_S = \frac{2(t_R - t_S)}{w_R + w_S} \quad (3)$$

where w_{R} and w_{S} stand for the extrapolated peak width at the baseline.

2.3 NMR experiments

All NMR experiments were carried out on a 600 MHz Varian DDR NMR spectrometer equipped with a 5 mm inverse-detection gradient (IDPFG) probehead. Standard pulse sequences and processing routines available in VnmrJ 2.2 C/Chempack 4.0 were used for structure identifications. The probe temperature was maintained at 298 K and standard 5 mm NMR tubes were used. ROESY (t-roesy) spectra were recorded on samples in D_2O containing 1 mM (*S*)-Ipn and either 3 mM HP-β-CD (*DS*∼4.6) or 3 mM β-CD or 3 mM SB-γ-CD in separate experiments. As mixing times 300 ms and 500 ms were applied with 3.0 and 5.1 kHz spin-lock. A total of 512 increments were collected with 32 repetitions and the measured data matrix was processed as a matrix of 2 K (F2) by 1 K (F1) data points. Job's analysis was carried out using 1 mM (*S*)-imperanene and 1 mM HP-β-CD or 1 mM SB-γ-CD as stock solutions at pH 9.0 in 50 mM phosphate buffer (in H_2O/D_2O 9/1). As both CDs applied for the determination of complex stoichiometry were multicomponent mixtures, the obtained stoichiometry can only be defined as an average-binding ratio. In Job's titrations chemical shift values were referenced to internal MeOH (0.05% v/v). A range of 128–512 scans (depending on the experiment) with a spectral window of 5100 Hz were collected into 32 000 data points, giving a digital resolution of 0.32 Hz/point. For solvent suppression the presaturation sequence was used. NMR spectra were processed with MestreNova 5.3.1-4825 software.

3 Results and discussion

3.1 Capillary electrophoresis

Host-guest based enantioseparation in CE requires that at least one of the interacting species has to carry a charge. Imperanene is neutral at pH 7.0, therefore it comigrates with the EOF. The predicted dissociation constants for Ipn phenolic groups are $pK_{a1} = 9.81$ and $pK_{a2} = 10.45$ (Marvin). Since also neutral CDs were subject to screening for chiral recognition in our study, the analyte was preferable to carry charge. Preliminary experiments yielded an optimum pH of 9.0, at which Ipn bears a slightly negative charge.

Twenty-one of the 27 tested cyclodextrins failed to separate the Ipn enantiomers up to 33 mM CD concentration

(the successful enantioseparations will be discussed in Section 3.2). Supporting Information Table S1 summarizes the estimated stability constants and mobilities of the Ipn-CD complexes. The native CDs showed weak interaction to Ipn and only upper limits of the respective stability constants could be deduced. Random *O*-acetylation or permethylation of β-CD lead to a ca. threefold increase in the affinity of Ipn.

Considering all substituted CDs in Supporting Information Table S1, β-CD derivatives with an average *DS* of 4 (HP-β-CD, SP-β-CD, SB-β-CD) exhibit remarkably high (averaged) complex stabilities, but only SP-β-CD and the HP-β-CD substances with lower and higher substitution degrees enabled enantioseparation. This corroborates the observation that stronger interaction may overturn the fine enantioselective forces and spoil chiral resolution [33].

Charged chiral selectors have been shown to be superior to neutrally substituted CDs in several studies [24, 33–35]. The potential of applying oppositely charged selectors and selectands was early emphasized [31, 36] and later demonstrated experimentally [33, 37]. In our study, 6-monoamino-6-monodeoxy-β-CD accelerated Ipn toward the cathode, without enantioseparation.

Applying an identically charged chiral selector may seem at first glance unfavorable, but the effective enantioseparations of this kind demonstrate that analyte-selector repulsion is not always the major contributor to the enhanced selectivity [33]. In our study, the carboxyalkylated or succinylated CDs interacted very weakly with Ipn, without chiral separation.

3.1.1 Enantioseparations in single-CD systems

Theoretical models of chiral capillary electrophoresis are now well developed [3, 31, 33, 38–42]. Briefly, in order to separate enantiomers, difference should be generated between their (vectorial) effective mobilities. If complex formation takes place instantaneously on the electrophoretic time scale, only a 1:1 complex is formed by both enantiomers and the [CD] ≅ c_{CD} approximation also holds (see Section 2.2), the difference of observed mobilities can be modeled as follows:

$$\Delta\mu_{RS} = \mu_R - \mu_S = \frac{\mu_{Ipn} + \mu_{R\cdot CD} K_R c_{CD}}{1 + K_R c_{CD}} - \frac{\mu_{Ipn} + \mu_{S\cdot CD} K_S c_{CD}}{1 + K_S c_{CD}}. \quad (4)$$

It is now generally accepted that enantioseparation in CE can be based not only on the affinity difference of the enantiomers ($K_R \neq K_S$) but also solely on difference in complex mobilities ($\mu_{R\cdot CD} \neq \mu_{S\cdot CD}$ while $K_R = K_S$ [3, 37, 38, 40]), or both [41].

Each separation of Ipn enantiomers are now analyzed using these concepts in order to identify the underlying mechanism. Using the calculated stability constants and mobilities of diastereomeric complexes from Supporting Information Table S1, the mobility difference $\Delta\mu_{RS}$ (and thus EMO) is modeled by Eq. (4) as function of CD concentration and plotted for four systems in Fig. 2.

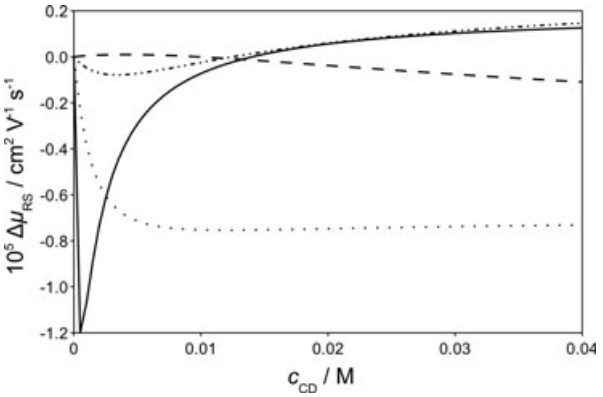

Figure 2. Mobility difference of (*R*)- and (*S*)-Ipn/CD complexes as function of the CD concentration, calculated by Eq. (4). Legend to curves: dash: HP-α-CD *DS*∼3; solid: SP-β-CD *DS*∼4; dot: SB-γ-CD *DS*∼4; dash-dot-dot: SB-α-CD *DS*∼4.

Although Wren's formula for optimum CD concentration c_W (see Section 2.2) is based on approximations, it worked well in our systems (see Supporting Information Table S1): the CD additives gave chiral resolution at $c_{CD} > c_W$, when the bound fraction exceeded 50% for both enantiomers.

For HP-α-CD and the HP-β-CDs with *DS* 3 and 6.3, the EMO was consistently S before R. The opposite order is expected on the basis of stability constants ($K_R > K_S$), so difference in the complex mobilities should be the determining factor of EMO. Indeed, the complexes with (*R*)-Ipn have a larger negative self-mobility ($-3.5 < -3.1$ in 10^{-5} cm^2·V^{-1}·s^{-1} units), so this enantiomer is more decelerated when migrating toward the cathode. This effect is probably even more pronounced for HP-β-CD (*DS* 3), since there is a greater difference in the stability constants. No reliable estimate could be deduced in this case for $\mu_{S\cdot CD}$ of the less stable complex, but its average over the isomeric CD mixture should be much lower in magnitude than $\mu_{R\cdot CD} = -3.3$. For HP-β-CD (*DS* 6.3), the observed mobilities of Ipn enantiomers did not vary in a monotonic fashion with c_{CD} (maybe due to the greater affinity dispersion of the constituting chiral selectors), thus no firm conclusion can be drawn in this case.

Comparing the Ipn complex stabilities of the hydroxypropylated and sulfoalkylated CDs of the same cavity size and DS, the negatively charged substituents enhance affinity.

SP-β-CD (*DS* 4) formed outstandingly stable complexes with both Ipn enantiomers. Since the complex mobilities are practically equal (−30), thermodynamic selectivity ($\alpha = K_R/K_S = 1987/1600 = 1.24$) seems to govern the separation. The preferentially bound *R* enantiomer gains more negative effective mobility thus decelerated when migrating toward the cathode, explaining the observed EMO *S* before *R*. Figure 2 predicts that chiral separation is restricted to low (<10 mM) CD concentrations only, which is confirmed by experiment.

SB-α-CD was exceptional among the studied systems since the EMO was here (*R*)- before (*S*)-Ipn. The reason lies in the opposite mobility pattern: the (*S*)-Ipn·CD complex has a more negative mobility (−19.5 versus −19.2), although it is

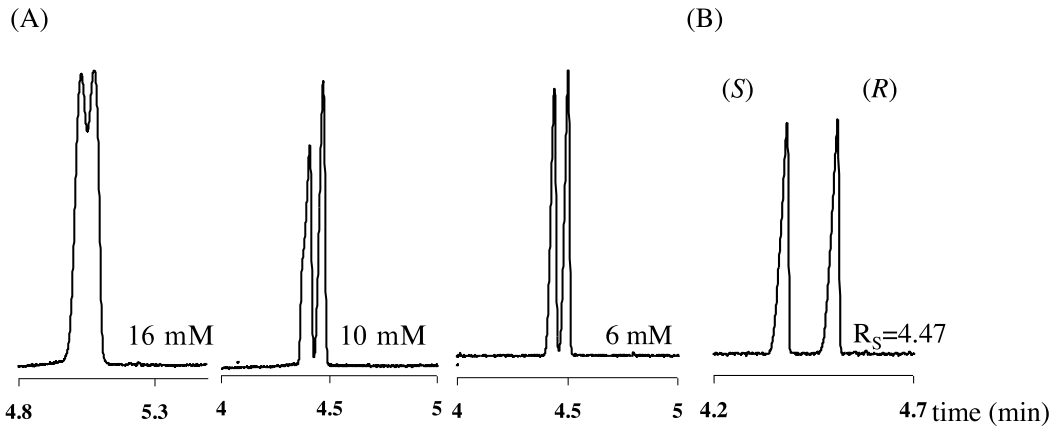

Figure 3. Enantioseparation of imperanene enantiomers at various SB-γ-CD concentrations (A) and with a dual CD system of 12.5 mM SB-γ-CD and 10 mM PA-β-CD (B).

the (slightly) less stable complex (130 versus 138 M^{-1}). From counteraction of mobility and affinity terms a reversal of EMO near 13 mM CD concentration is predicted by Eq. (4) (see Fig. 2). Experimentally, no chiral resolution was observed at $c_{CD} < 15$ mM.

Baseline separation ($R_s = 1.26$) of Ipn enantiomers was achieved only with SB-γ-CD as chiral selector (Fig. 3A). Both mobility and affinity terms act in the same direction here: the more stable (*R*)-Ipn·CD complex (336 versus 288 M^{-1}) has the more negative electrophoretic mobility (−14.1 versus −13.4), leading to strong deceleration of *R*. The μ_R–μ_S mobility difference remains practically constant at $c_{CD} > 5$ mM, as predicted by theory [40].

In summary, mobility difference of the transient diastereomeric complexes offers a plausible explanation for the separation and migration order of Ipn enantiomers at pH 9.0, which pH is close to the pK_{a1} value of this biphenol. A similar importance of the complex mobilities at pH values around the analyte pK_a has been demonstrated for CD complexes of dansylated amino acids [43] and dipeptides [44]. Since all the effective CD preparations in our study are in fact mixtures of a large number of chiral selectors, the estimated averaged complex mobility data should be interpreted with caution. Although their similarly "simplistic" presentation and interpretation is currently common in the literature [44], the recently introduced "multi-chiral selector enantioseparation model" [45, 46] shows that in mixtures of chiral selectors thermodynamic and electrophoretic enantioselective mechanisms are mixed together.

3.1.2 Enantioseparations in dual-CD systems

As the single CD systems provided rather weak enantioseparations, investigations were continued on dual CD systems. In the dual systems, a charged and a neutral CD or two ionized CDs were combined. On addition of a neutral CD to a buffer containing an anionic CD, the apparent electrophoretic mobilities of the analyte enantiomers decrease if complexed with the neutral CD [22]. Among the single CD systems SB-γ-CD (*DS*∼4) resulted in the best enantioresolution, so this CD was chosen as primary selector. As the second component of the dual host systems, several neutral (HP-α-CD, HP-β-CD DS∼3, HP-β-CD∼4.6, HP-β-CD∼6.3) and charged (SP-β-CD∼3, SB-β-CD∼4, SB-α-CD∼4, PA-β-CD) cyclodextrins were tested at various concentrations. HP-α-CD, HP-β-CD∼3, HP-β-CD∼6.3, and SB-α-CD∼4 had opposite enantiomer affinity to that of SB-γ-CD, thus these CDs were tested first. Although PA-β-CD alone did not enable enantioseparation, it may enhance enantioseparation via electrostatic interaction with imperanene or by influencing the mobilities of the enantiomers. The potential of eight dual CD systems (listed in Table 1) were tested. The selectors were mixed at different concentrations to determine the optimum ratio for the separation. Optimization of the best dual system yielded the combination of 12.5 mM SB-γ-CD and 10 mM PA-β-CD that provided a spectacular enantioresolution with an R_s value of 4.47 (Fig. 3B). In this system, SB-γ-CD provides the enantioselectivity and determines EMO. The effect of BGE pH changes on the separation was (re)investigated in this optimized system (pH ranging from 8.6 to 9.4 by 0.2 steps) and pH 9.0 was found to be optimal again.

Table 1. The applied dual CD systems along with the highest achieved resolution values at pH 9.0

CD–1	CD–2	Resolution value
SB-γ-CD (5 mM)	HP-α-CD (30 mM)	1.45
SB-γ-CD (5 mM)	HP-β-CD *DS*∼3 (50 mM)	0.69
SB-γ-CD (7.5 mM)	HP-β-CD *DS*∼4.5 (30 mM)	1.73
SB-γ-CD (5 mM)	HP-β-CD *DS*∼6.3 (50 mM)	2.22
SB-γ-CD (12.5 mM)	PA-β-CD (10 mM)	4.47
SB-γ-CD (10 mM)	SB-α-CD (5 mM)	1.72
SB-γ-CD (7.5 mM)	SB-β-CD (5 mM)	0
SB-γ-CD (12.5 mM)	SHP-β-CD (10 mM)	1.04

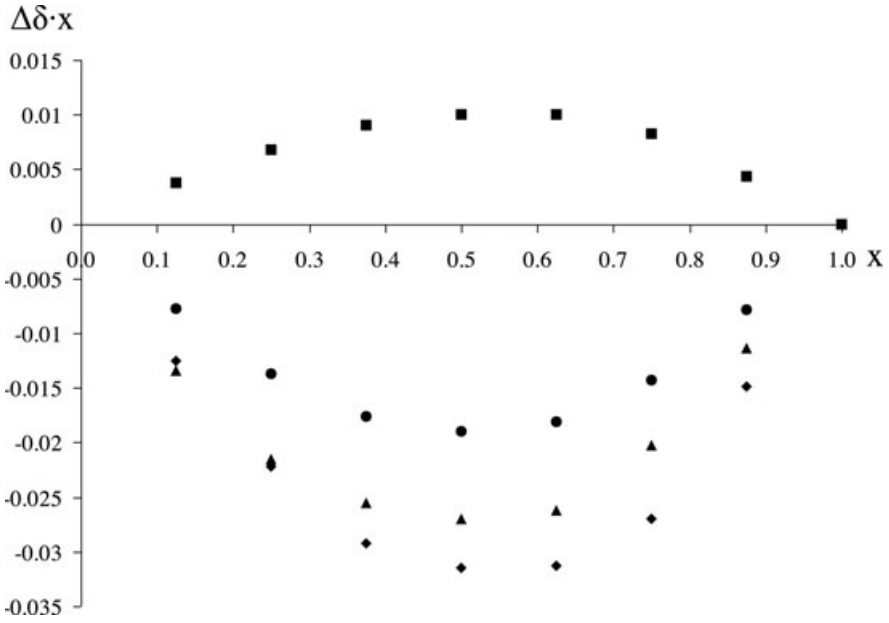

Figure 4. Job's plots derived from the downfield changes in chemical shift of ^{8}H (square), and upfield changes of ^{10}H (triangle), ^{14}H (circle), and ^{17}H (rhombus) imperanene resonances recorded on imperanene/SB-γ-CD samples.

3.2 ^{1}H NMR experiments

3.2.1 Titrations according to Job's method

The average stoichiometry for the binary Ipn-CD inclusion complexes was determined by Job's method, using 1 mM (*S*)-Ipn (due to its low solubility) and 1 mM HP-β-CD DS∼4.6 or 1 mM SB-γ-CD (in separate experiments) in a 50 mM phosphate buffer (pH 9.0) in H_2O/D_2O 9/1. The stock solutions were mixed at different ratios and chemical shift changes were recorded for both the guest and host protons. The chemical shift displacements weighted with the actual molar ratio (Δδ·x) were then plotted as a function of the molar ratio (see Fig. 4, for the Ipn-SB-γ-CD system). Job's plots showed maxima at 0.5 for both CDs proving the 1:1 average-binding stoichiometry of the complexes.

3.2.2 Structural NMR studies

To explore the structure of the inclusion complexes 2D ROESY NMR experiments were run on Ipn-β-CD, Ipn-HP-β-CD, and Ipn-SB-γ-CD samples. Although several spectra with different mixing times (300 and 500 ms) were recorded no intermolecular cross-peaks could be observed. Thus these experiments did not provide useful information on the direction of penetration and the interacting moieties. The complexation-induced chemical shift changes may also be used to identify the protons mostly involved in the host-guest interaction [47]. The most pronounced changes were observed at ^{17}H and ^{20}H Ipn protons in the case of 0.90 mM SB-γ-CD that indicate their active participation in the complexation process (Table 2). These results suggest that the coniferyl moiety of imperanene is more likely to interact with the cyclodextrin hosts. This finding is consistent with those of previous studies on the plant polyphenol-cyclodextrin structures discussed in Section 1.

Table 2. ^{1}H NMR chemical shifts of free and complexed (SB-γ-CD) imperanene protons (in ppm)

Proton	δ_{free}	δ_{cplx}	Δδ
^{3}H	6.775	6.784	−0.009
^{4}H	6.712	6.692	0.020
^{6}H	6.655	6.615	0.040
^{8}H	3.701	3.731	−0.030
^{10}H	2.533	2.516	0.017
^{11}H	3.584	n.d.	
^{12}H	2.73	2.725	0.005
^{14}H	5.893	5.838	0.055
^{15}H	6.252	6.244	0.008
^{17}H	6.942	6.841	0.101
^{20}H	6.834	6.735	0.099
^{21}H	6.791	6.798	−0.007
^{23}H	3.756	3.752	0.004

Due to spectral overlap the δ_{cplx} of ^{11}H resonance could not be detected.

4 Concluding remarks

Cyclodextrin-hosted diastereomeric complexation with 27 CDs and the concomitant enantioseparation of imperanene optical isomers by CE are reported at alkaline pH. Three hydroxypropylated and three sulfoalkylated CD preparations provided enantioseparation and the importance of inequality of complex mobilities in determining the EMO was demonstrated. The best enantioresolution ($R_S = 1.26$) was achieved using sulfobutyl-ether-γ-CD (DS ∼4), but it could be enhanced by extensive investigations on dual selector systems.

The combination of 12.5 mM SB-γ-CD and 10 mM PA-β-CD resulted in the highest resolution value, $R_S = 4.47$. The average stoichiometry and structure of the inclusion complex were investigated by ^{1}H NMR experiments. Titrations according to Job's method proved that 1:1 complex is formed between the negatively charged guest and various hosts. Complexation-induced chemical shift values of Ipn suggest that the coniferyl moiety of imperanene is favorable to interact with the selector.

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