# Chiral Separation of Deprenyl-*N*-Oxide Isomers by Capillary Electrophoresis Using Various Cyclodextrin Derivatives

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## Abstract

**Chiral separation of deprenyl-*N*-oxide isomers is presented using capillary electrophoresis in the presence of various cyclodextrin (CD) derivatives. This recently identified metabolite of *R*-(–)-deprenyl may possess desirable pharmacological activities. The effect of the cavity size and the substituents of the CD are examined on the enantiomer resolution of the compound having an asymmetric center on a heteroatom. The importance of hydrophilic or hydrogen bonding interaction, as well as the position of the interacting groups is demonstrated. Outstanding selectivity and resolution values are achieved using the chargeable carboxymethyl-β-CD. 2-Hydroxypropyl-β-CD is also suitable for the enantiomer separation of the analyte. Native β-CD and carboxyethyl-β-CD provide only poor enantioselectivity, whereas heptakis-(2,6-di-*O*-methyl)-β-CD is capable of separating only the diastereomers. No chiral resolution can be observed in the presence of γ-CD.**

## Introduction

Chiral analysis of drugs, as well as their metabolites, is important to characterize the pharmacodynamic and pharmacokinetic properties of the compounds. The study of the action and toxicity of the single enantiomers and the clarification of the stereochemical aspects of the metabolism are also required (1).

Capillary electrophoresis (CE) is especially suitable for chiral separations because of its high efficiency, low reagent and sample requirement, and quick method development (1–5). The chiral selector dissolved in the background electrolyte acts as a pseudo-stationary phase that can interact with one of the enantiomers preferably. The most widely used chiral selectors are cyclodextrin (CD) derivatives. These are cyclic oligosaccharides built up from glucopyranose units forming a hydrophobic cavity and having a hydrophilic exterior (6). The chiral recognition is based on the inclusion complex formation stabilized by the stereoselective interactions between the substituents on the rim of the CD ring and the groups situated around the asymmetric center of the analyte. The hydroxyl groups bound to the chiral carbon atoms of the glucose units (three per units) can be chemically modified, resulting in a great versatility that provides the opportunity to separate the enantiomers of the majority of compounds (7).

Although inclusion of the hydrophobic part of the analyte into the CD cavity is essential, it is not sufficient for the enantiorecognition. The chiral discrimination depends rather on the interaction between the select and the asymmetric groups on the rim, which provide different stability to the complexes of the enantiomers, thus the quality of the substituents is determinant on the enantioseparation.

The interaction between a compound and a chiral selector can be examined by NMR spectroscopy, mass spectrometry, and X-ray crystallography, however the applicability of a selector for the enantiomer separation can be determined only on the basis of CE experiments (8).

Deprenyl is established as a selective and irreversible inhibitor of monoamine oxidase B enzyme (MAO-B) (9). *R*-(–)-deprenyl, also called selegiline, proved to be much more potent and selective inhibitor than its antipode (10). Based on its dopamine-sparing effects, selegiline is widely used in the treatment of Parkinson's disease. Neuroprotective and neuronal rescue activities of *R*-(–)-deprenyl are unrelated to MAO-B inhibition, but are also highly stereoselective (11–13). These pharmacological activities of selegiline were abolished by SKF 525A, an inhibitor of the drug-metabolizing enzymes (14,15), indicating that a metabolite (or some metabolites) rather than the parent compound is responsible for these effects. However, the main metabolites methamphetamine and amphetamine, formed by N-dealkylation, were found lacking of the neuroprotective and neuronal rescue activities characteristics of *R*-(–)-deprenyl (11,15).

Recently, a new metabolic pathway, N-oxidation of the tertiary amine of deprenyl by flavin-containing monoxygenase enzymes, was proposed by Wu and Ichikawa (16). This compound was later

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identified in vitro in microsomal preparation (from Lévai et al., submitted for publication) and in vivo in rat (17) and human urine (18,19). The *N*-oxide generation also has stereochemical significance, as it is accompanied by the creation of a new chiral center on the quaternary nitrogen atom; thus, four diastereomers (i.e., two pairs of enantiomers) of deprenyl-*N*-oxide exist (Figure 1). The chiral behavior of compounds with an asymmetric heteroatom is hardly examined. Although Hadley et al. separated the enantiomers of pargyline-*N*-oxide, as well as a series of *N*-alkyl-*N*-methylaniline-*N*-oxides, the effect of CD type and concentration were not examined in detail (20).

In our recent paper, we have developed a method for the simultaneous separation of the enantiomers of deprenyl and eight of its metabolites, among them deprenyl-*N*-oxides (17). This method utilizes a dual CD system to overcome some achiral co-migrations and is validated for the quantitation of deprenyl and deprenyl metabolites in rat urine to examine in vivo metabolism of the drug. We have observed that the deprenyl-*N*-oxides differed from that of the amine metabolites, as heptakis-(2,6-di-*O*-methyl)-β-CD (DMBCD) was capable of separating only its diastereomers, although this chiral selector could be previously used successfully for the enantioseparation of deprenyl and its dealkylated metabolites (21,22).

## Experimental

### Chemicals

The standard compounds, hydrochloride salts of *1R,NR*-(+)- and *1S,NS*-(–)-, and the diastereomeric mixture (1:1) of *1R,NR*-(+)- and *1R,NS*-(–)- and *1S,NS*-(–)- and *1S,NR*-(+)-deprenyl-*N*-oxide were kindly provided by Chinoin Pharmaceutical and Chemical Works Co. Ltd., member of Sanofi-Synthelabo Group (Budapest, Hungary). Standard stock solutions ($10^{-2}$ and $10^{-3}$M) of all compounds were prepared in distilled water, stored frozen (–20ºC), and diluted just prior to use. The chiral selectors, CE-grade β-CD, γ-CD, DMBCD, and (2-hydroxy-propyl)-β-CD (HPBCD) (degree of substitution 3) were supplied by Beckman Coulter (Fullerton, CA), and carboxymethyl-β-CD (CMBCD) (degree of substitution 3.5) and carboxyethyl-β-CD (CEBCD) (degree of substitution 3.5) were purchased from Cyclolab Ltd. (Budapest, Hungary). Tris, sodium hydroxide, and phosphoric acid were supplied by Reanal (Budapest, Hungary) and were analytical grade. Hydroxypropylmethyl-cellulose (HPMC) (viscosity of 2% solution is 2000 cP at 20ºC) was obtained from Sigma (St. Louis, MO). The ultrapure water used in the CE experiments was obtained from a MilliQ water system (Millipore, Bedford, MA).

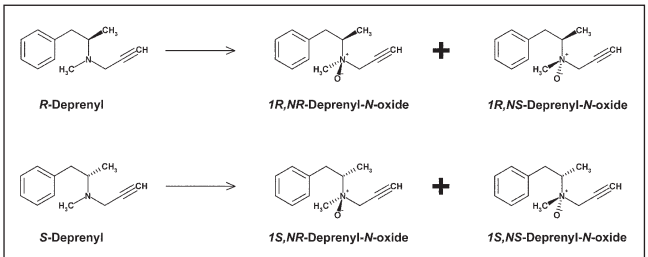


**Figure 1.** Chemical structure of the deprenyl-*N*-oxide isomers and their parent compounds.

### Apparatus

All experiments were performed with a PRINCE (Prince Technology, Emmen, the Netherlands) CE system equipped with a UV detector set at 200 nm. Separations were carried out in uncoated fused-silica capillaries (75-µm i.d., 365-µm o.d., 75-cm total length, 55 cm to the detector) (Polymicro Technology, Phoenix, AZ). The capillary was washed successively with 0.1M sodium hydroxide, water, and the separation buffer (2000 mbar, 5 min each). Axxiom 727 (Axxiom Chromatography Inc., Moorpark, CA) software was used for data collection.

### Electrophoretic conditions

The composition of the separation buffer was 20mM Tris-phosphate, pH 2.7, containing 0.5% HPMC and various concentrations of the chiral selectors. Samples were introduced into the capillary by pressure (300 mbar, 24 s); the concentration of the standard compounds was in the range of 7.5–20µM. The separations were carried out at ambient temperature by applying constant voltage of 22.5kV (300V/cm).

### Calculations

The following equations were used for the calculation of selectivity, resolution, and migration retardation factor, respectively:

$$\alpha = t_2/t_1 \qquad \text{Eq. 1}$$

where α is separation selectivity and $t_1$ and $t_2$ are the migration times of the enantiomers.

$$R_s = 2(t_2 - t_1)/(w_1 + w_2) \qquad \text{Eq. 2}$$

where $R_s$ is the peak resolution, $t_1$ and $t_2$ are the migration times of the enantiomers, and $w_1$ and $w_2$ are the width of the peaks at the baseline.

$$R_m = t_2/t_0 \qquad \text{Eq. 3}$$

where $R_m$ is the migration retardation factor, $t_2$ is the migration time of the later-eluted enantiomer, and $t_0$ is the migration time of the analyte in the absence of chiral selector.

## Results and Discussion

### Separation conditions

The composition of the background electrolyte was the same used in our previous experiments (17,21,22). The low pH (i.e., pH 2.7) is suitable for the separation of basic compounds in their cationic form. Although amines, such as deprenyl and its dealkylated metabolites, also can be easily separated at higher pH, the *N*-oxide formation results in a significant decrease in the basicity of the compound, leading to decreased electrophoretic mobility at higher pH (data not shown).

The rather high concentration of HPMC used in the background electrolyte not only decreased the electroosmotic flow and the interaction between the capillary wall and the analytes, but also resulted in a wider migration window and improved peak

shapes and resolution (21). The relatively high viscosity also permitted neglect of the viscosity differences caused by the application of various concentrations of CDs.

**The effect of CD cavity size**

The size of the CD cavity is dependent on the number of the glucopyranose unites in the ring. The α-, β-, and γ-CDs are built up from 6, 7, and 8 glucose units, respectively (6). Guttman (23,24) has proposed that the enantioseparation of aromatic compounds requires β- or γ-CDs, as the cavity size of the α-CDs is not wide enough for the inclusion complex formation. Koppenhoefer et al. showed that β-CDs are the most suitable selectors for the complexation of aromatic compounds, because the benzene ring is fitted exactly with the diameter of its cavity (25). However, γ-CDs also can be useful when aromatic compounds with bulky substituents or condensed rings are to be separated.

First we examined the effect of the cavity size on the enantiomer separation. Native β- and γ-CD were used as chiral selectors in their low, medium, and high concentrations (5, 10, and 15 and 10, 30, and 50mM β- and γ-CD, respectively). We have found that the interaction between the deprenyl-*N*-oxide isomers and β-CD was relatively strong, whereas that of with γ-CD was somewhat weaker, characterized by the migration retardation factor as was suggested by Koppenhoefer et al. (25). However, this interaction was stereoselective only in the case of β-CD, as partial resolution of the enantiomers could be achieved, whereas γ-CD was not capable of resolving even the diastereomers of the compounds (Table I).

These results show that the enantioselective interaction requires the exact fit of the compound into the CD cavity. In this case, β-CD containing seven glucopyranose units provides the proper cavity size. Although the wider cavity of γ-CD can complex the analyte, the strength of the interaction is not different for the stereoisomers.

The effect of the CD concentration was also examined. Only partial resolution of the enantiomers could be achieved using 15mM β-CD. (Because of the low solubility of the chiral selector, its concentration could not be further increased.) However, the separation selectivity and enantiomer resolution reached the maximum values in the presence of 10mM β-CD and slightly decreased when the concentration of the chiral selector was increased to 15mM (Table I). This type of concentration dependence is characteristic of chiral separations, as was examined by Wren and Rowe in detail (26,27). These data indicate that β-CD possesses only poor enantioselectivity for this compound.

**Table I. Migration Retardation Factor ($R_m$), Separation Selectivity (α), and Enantiomeric Resolution ($R_s$) in the Presence of Various Concentrations of Native β- and η-CD**

| CD | | 1R,NR- and 1S,NS-DNO* | | | 1R,NS- and 1S,NR-DNO | | |
|---|---|---|---|---|---|---|---|
| Type | Concentration | $R_m$ | α | $R_s$ | $R_m$ | α | $R_s$ |
| β-CD | 5mM | 1.40 | 1.011 | 0.78 | 1.52 | 1.008 | 0.55 |
| | 10mM | 1.77 | 1.012 | 0.90 | 1.88 | 1.012 | 0.81 |
| | 15mM | 1.74 | 1.010 | 0.76 | 1.86 | 1.011 | 0.69 |
| γ-CD | 10mM | 1.16 | NR† | NR | 1.16 | NR | NR |
| | 30mM | 1.23 | NR | NR | 1.23 | NR | NR |
| | 50mM | 1.54 | NR | NR | 1.54 | NR | NR |

* DNO = deprenyl-*N*-oxide.
† NR = not resolved.

**The effect of the substitution of β-CD**

We have also examined the applicability of the most widely used β-CD derivatives; the neutral DMBCD and HPBCD, as well as the chargeable CMBCD and CEBCD were analyzed. DMBCD, as we have previously reported, was only capable of the separation of the diastereomers, whereas the enantiomer pairs comigrated (17). Although no enantioselectivity was observed, the migration retardation factors indicated a rather strong interaction between DMBCD and the deprenyl-*N*-oxide isomers (Table II). These data reveal that inclusion complex formation occurs, but there is no difference in the stability of the complexes of the enantiomers.

The other neutral CD, the HPBCD, was found highly enantioselective (Figure 2). In as low as 3mM concentration, baseline resolution of the enantiomer pairs were observed. The concentration dependence of the selectivity and resolution followed the pattern described by Wren and Rowe (26,27), with a maximum value in the concentration range of 10–20mM. The interaction strength characterized by the migration retardation factors was found to be relatively high, however somewhat lower compared with that of DMBCD (Figure 3). The difference between the two neutral CD could reveal that hydrophilic or even hydrogen-bonding interac-

**Table II. Migration Retardation Factor ($R_m$) in the Presence of Various Concentrations of DMBCD**

| CD concentration | 1N,NR- and 1S,NS-DNO | 1R,NS- and 1S,NR-DNO |
|---|---|---|
| 10mM | 1.85 | 2.02 |
| 20mM | 2.22 | 2.34 |
| 30mM | 2.58 | 2.67 |

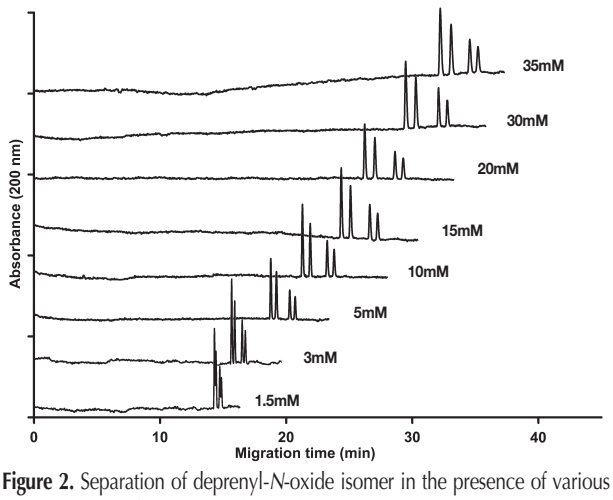


**Figure 2.** Separation of deprenyl-*N*-oxide isomer in the presence of various concentrations of HPBCD as chiral selector. Sparation conditions are given in the Experimental section. Peaks in the order of elution: 1R,NR-; 1S,NS-; and 1S,NR-deprenyl-*N*-oxide.

tions are essential for the enantiodiscrimination. In the case of DMBCD, the 2- and 6-hydroxyl groups are methylated, indicating that one or both of these positions must play an important role in the enantioselective interaction through hydrophilic or hydrogen bonds. As the 6-hydroxyl group is situated on the primary and the 2-hydroxyl group on the secondary side of the CD ring, the latter one seems to be a more critical site of interaction for the chiral recognition, but further experiments are required to clarify these speculations.

Among the chargeable CDs, the CMBCD was extensively examined in our previous paper (17). In this present work, similar results were obtained. The interactions were very strong, and excellent selectivities and resolutions were found in the concentration range of 1–3mM of the chiral selector (Figure 4). The concentration dependence of these parameters was different from those found in the case of native β-CD and HPBCD in this concentration range, as no maximum values could be achieved (Figure 5). Higher concentrations of the selector could not be examined because of the highly increased migration times (in the concentration of 4mM, no peaks were obtained within 90 min). This outstanding interaction strength might derive from the residual charge of the CD, even in this low pH, that might lead to strong ionic interaction between the cationic analyte and the anionic chiral selector (28). This prominently strong interaction was accompanied by distinguished selectivity and resolution, too.

Interestingly, the separation parameters in the presence of the other chargeable CD resembled those of β-CD rather than CMBCD. The migration retardation factors revealed a medium strength of interaction, but slight selectivity and only partial resolution could be achieved even when higher concentrations were used (Table III). This probably indicates that the chargeable groups that also possess hydrophilic and hydrogen-bonding properties in their neutral forms are too far from the rim of the CD, in the case of CEBCD. The distance between the rim and the hydrophilic groups of the substituents are two methylene groups, in the case of HPBCD and CMBCD, and three in CEBCD. This may explain the difference between the chiral resolutions provided by the two chargeable CDs, as well as may also explain the similar behavior of CMBCD and HPBCD, the CDs that provide good enantioselectivity.

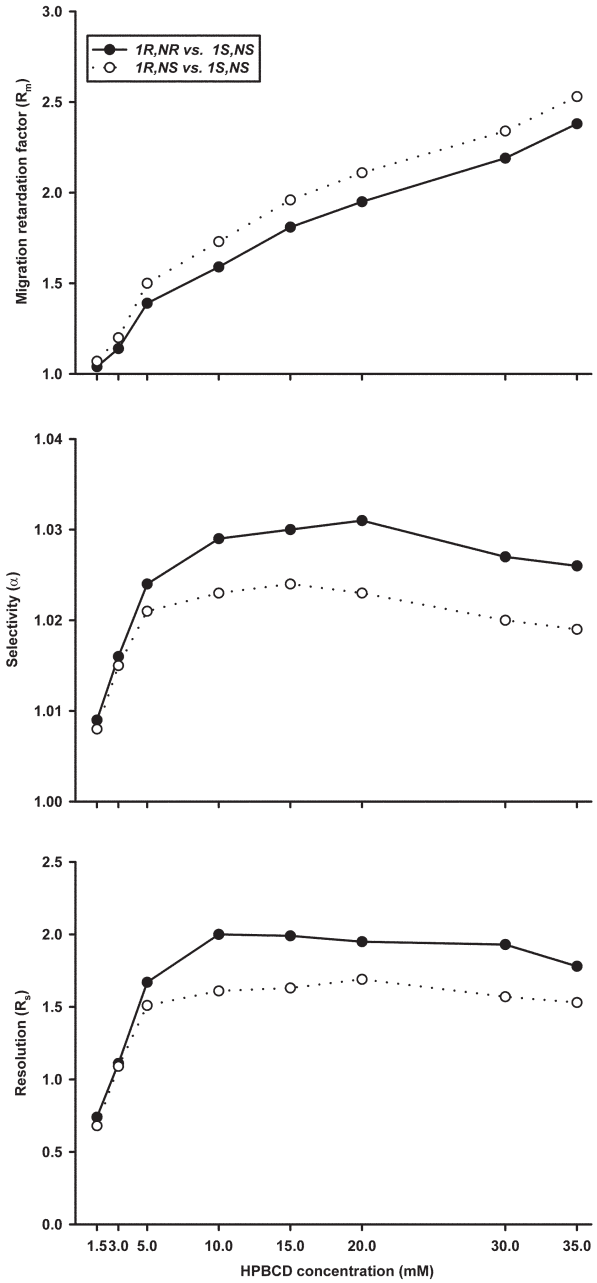


**Figure 3.** Migration retardation factor ($R_m$), selectivity (α), and resolution ($R_s$) values of the deprenyl-*N*-oxide isomers in the presence of various concentrations of HPBCD as chiral selector.

## Conclusion

In this work, we have examined the effect of CD type on the chiral resolution of deprenyl-*N*-oxide isomers. The β-CD derivatives containing hydrophilic substituent at two methylene-group distances from the CD rim (CMBCD, HPBCD) were found suitable for enantiomer separation. Outstanding resolution and selectivity values were obtained in the case of CMBCD, likely related to its

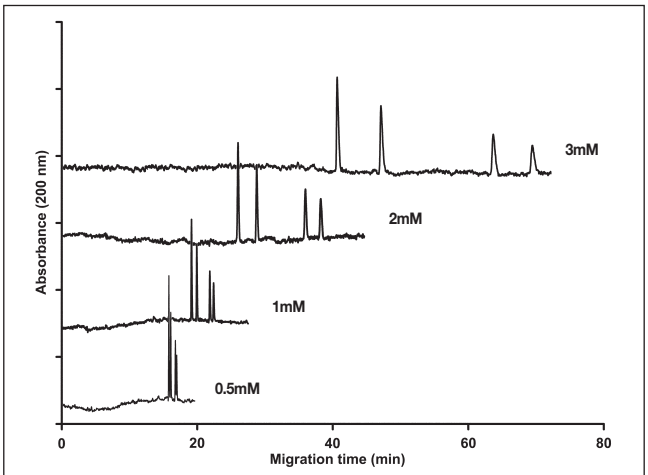


**Figure 4.** Separation of deprenyl-*N*-oxide isomers in the presence of various concentration of CMBCD as chiral selector. Separation conditions are given in the Experimental section. Peaks in the order of election: 1R,NR-; 1S,NS; 1R,NS-; and 1S,NR-deprenyl-*N*-oxide.

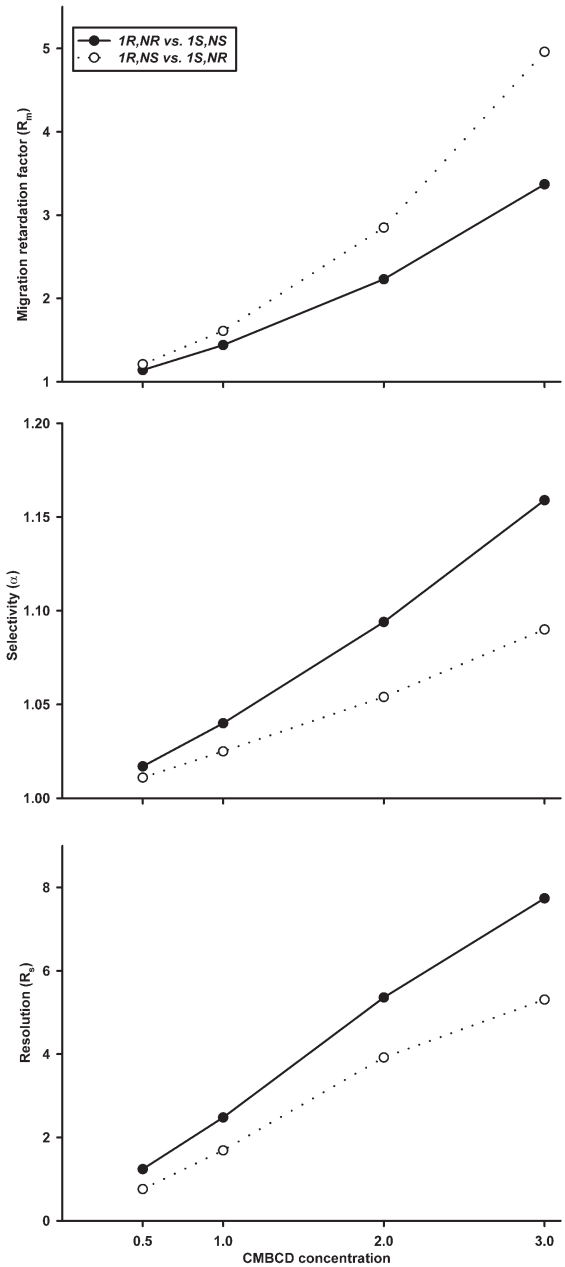


**Figure 5.** Migration retardation factor ($R_m$), selectivity (α), and resolution ($R_s$) values of the deprenyl-*N*-oxide isomers in the presence of various concentrations of CMBCD as chiral selector.

**Table III. Migration Retardation Factor ($R_m$), Separation Selectivity (α), and Enantiomeric Resolution ($R_s$) in the Presence of Various Concentrations of CEBCD**

| | 1R,NR- and 1S,NS-DNO* | | | 1R,NS- and 1S,NR-DNO | | |
|---|---|---|---|---|---|---|
| CD concentration | $R_m$ | α | $R_s$ | $R_m$ | α | $R_s$ |
| 2.5mM | 1.25 | 1.011 | 0.88 | 1.38 | 1.007 | 0.52 |
| 5mM | 1.48 | 1.014 | 1.10 | 1.66 | 1.008 | 0.65 |
| 10mM | 1.78 | 1.015 | 0.90 | 1.97 | 1.007 | 0.54 |

* DNO = deprenyl-*N*-oxide.

slight ionization. The chiral selectors possessing the hydrophilic substituent nearer (β-CD) or farther from the rim (CEBCD) provided only poor selectivity and partial resolution. No enantiomer resolution was found when mainly hydrophobic interactions were preferred, like in the case of DMBCD.

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