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# Model of CE enantioseparation systems with a mixture of chiral selectors Part II. Determination of thermodynamic parameters of the interconversion in chiral and achiral environments separately☆

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ABSTRACT

The theoretical assumption that a multi-CS enantioseparation system, the model of which was described in Part I of this work, can be treated as a separation system with only one CS is confirmed by a set of experiments. The model assumes that each individual analyte–CS interaction is fast, fully independent on other interactions and the analyte: CS ratio is 1:1 and that the analyte is present in its concentration small enough not to considerably change the concentration of free CSs. An enantioselective environment in affinity capillary electrophoresis is created using a commercially available mixture of highly sulfated β-cyclodextrines as chiral selectors (CSs) and lorazepam as an analyte that undergoes interconversion during the separation process. Dependencies of the electrophoretic mobilities of the two enantiomers on concentration of the CSs mixture are proved to follow the proposed multi-CS model. Global rate constants of interconversion are determined at various temperatures and concentrations of the CSs mixture. In accord with the proposed theory, linear dependencies of the global rate constants on CSs concentration are achieved. Intercepts and slopes of these plots correspond to local rate constants of interconversion in achiral (without the mixture of CSs in background electrolyte (BGE)) and chiral (with the CSs mixture in BGE) environments, respectively. The experimentally obtained electropherograms show an excellent fit with those resulting from the computer simulation based on our model.



## 1. Introduction

Separation environment for successful resolution of enantiomers cannot be always formed using a single chiral selector (CS). In many cases mixtures of isomers or derivatives with various degrees of substitution and various positions of substituents are employed [1–6]. Often, CSs are commercially available only as mixtures. Many papers have shown strong improvement of enantioseparation if more than one CS has been added to the separation system [1,3,7–15]. Some quantitative approaches have been published describing dual-CS (namely dual-cyclodextrins) systems under the conditions that: (i) an analyte's enantiomer interact with any of CSs in 1:1 ratio, (ii) independent complexation occurs (i.e. no mixed complexes are formed) and (iii) the two cyclodextrin derivatives are well defined compounds [8,10,16–19]. Additional presumptions that (iv) complexation reaction between the enantiomer and any CS in the mixture is much faster than the separation – and possibly interconversion – and that (v) the enantiomers are present in a concentration small enough not to considerably change the concentration of free CSs are not usually mentioned but should also be considered. Part I [20] of this work has been aimed at a description of the behaviour of enantiomers on interactions with a mixture (i.e. two or more) of CSs present in the enatioseparation system ("multi-CS" enantioseparation system). Mathematical equations of the distribution equilibria, migration and dynamics of interconversion have been derived. For the first time it is shown there that under the preconditions mentioned above a multi-CS system actually behaves as there was only one CS with its particular properties as summarized bellow. Moreover, as a number of CSs in their mixture is not restricted, the preset model remains valid even if the precondition (iii) is not fulfilled, i.e. not pure selectors are used

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or even composition of the mixture is not exactly known. Similarly to single-CS systems, interaction of an analyte with a mixture of CSs in a multi-CS system is thus described by

$$K_i^{\text{over}} = \sum_q K_i^q \chi_q \tag{1}$$

$c_i^0$ and $c_i^q$ are concentrations of the $i$th enantiomer in the free and complexed form, respectively, $c_q^0$ is the concentration of the free form of the $q$th chiral selector. The subscript $i$ attains 1 or 2, for the 1st or the 2nd enantiomer, respectively.

$K_i^{\text{over}}$ is the overall equilibrium constant (subscript $i$ attains 1 or 2, for the 1st or the 2nd enantiomer), $\chi_i$ is a molar fraction of the $q$th CS in the mixture of CSs. $K_i^q$ is an (intrinsic) equilibrium constant of the complexation (association) between $i$th enantiomer and $q$th CS defined as $K_i^q = c_i^q / c_i^0 c_q^0$, where $c_i^0$ and $c_i^q$ are concentrations of the $i$th enantiomer in the free and complexed form, respectively, $c_q^0$ is the concentration of the free form of the $q$th chiral selector. If each CS is in sufficient excess when compared to the analyte, the quantity $c_q^0$ can be approximated by $c_q^{\text{tot}}$, the total (analytical) concentration of the $q$th CS, and $c_{\text{CS}}^{\text{tot}} = \sum_q c_q^{\text{tot}}$ is the total (analytical) concentration of the entire mixture of CSs. Further:

$$\mu_i^{\text{over}} = \frac{\sum_q \mu_i^q K_i^q \chi_q^{tot}}{K_i^{\text{over}}}, \tag{2}$$

$\mu_i^{\text{over}}$ stands for the overall mobility of the $i$th enantiomer when associated with the mixture of CSs as the whole, $\mu_i^q$ are individual mobilities of the associates of $i$th enantiomer with $q$th CS. The meaning of this quantity is described in detail in Part I. Effective mobility of an $i$th enantiomer, $\mu_i^{\text{eff}}$, in a multi-CS enantioseparation system is then expressed as

$$\mu_i^{\text{eff}} = \frac{\mu_i^0 + c_{\text{CS}}^{\text{tot}} K_i^{\text{over}} \mu_i^{\text{over}}}{1 + c_{\text{CS}}^{\text{tot}} K_i^{\text{over}}} \tag{3}$$

where $\mu_i^0$ stands for an effective mobility of a free form of an $i$th enantiomer.

Similarly:

$$k_i^{\text{over}} = \frac{\sum_q k_i^q K_i^q \chi_q^{\text{tot}}}{K_i^{\text{over}}} \tag{4}$$

$k_i^{\text{over}}$ is the overall rate constant of interconversion of the $i$th enantiomer in the mixture of CSs. In principle, the quantity $k_i^{\text{over}}$ does not describe the rate of interconversion of the analyte in a particular chiral environment of mixture of CSs, but should be considered as a limiting value of the rate of interconversion in the system when CS concentration tends to reach infinity, as discussed in the Part I. Meaning of symbols is analogous to that in Eqs. (1) and (2). Finally, the principle of microscopic reversibility [21] leads to the relations as follows:

$$k_1^0 = k_2^0 \quad \text{and} \quad K_1^{\text{over}} k_1^{\text{over}} = K_2^{\text{over}} k_2^{\text{over}} \tag{5}$$

$$k_{\text{glob}} = k_1^0 + c_{\text{CS}}^{\text{tot}} K_1^{\text{over}} k_1^{\text{over}} = k_2^0 + c_{\text{CS}}^{\text{tot}} K_2^{\text{over}} k_2^{\text{over}} \tag{6}$$

$$k_i^{\text{app}} = \frac{k_{\text{glob}}}{1 + c_{\text{CS}}^{\text{tot}} K_i^{\text{over}}} \tag{7}$$

Here $k_1^0$ and $k_2^0$ are "achiral" rate constants of interconversion of the enantiomers, $k_{\text{glob}}$ stands for the "global" and $k_i^{\text{app}}$ for the apparent rate constants of interconversion. Apparent rate constants control the interconversion in the whole system including both the chiral and achiral part. It is inherently impossible to discern between the local rate constants, $k_1^0$, $k_2^0$, $k_1^{\text{over}}$, $k_2^{\text{over}}$, of interconversion in chiral and achiral environments in a single dynamic enantioseparation experiment.

In our previous paper [22] we defined the global rate constant $k_{\text{glob}}$ for single-CS enantioseparation systems. We showed that a decomposition of the apparent rate constants ($k_1^{\text{app}}$, $k_2^{\text{app}}$) back to the local ones ($k_1^0$, $k_2^0$, $k_1^{\text{CS}}$, $k_2^{\text{CS}}$) is possible because of the linear dependence of $k_{\text{glob}}$ on concentration of the CS (here $k_1^{\text{CS}}, k_2^{\text{CS}}$ are rate constants of interconversion of the two enantiomers when associated with the CS present in a single-CS system). Eqs. (5)–(7) and the equations defined for a single-CS enantioseparation system are formally identical. This implies that the approach originally proposed for single-CS systems is also applicable for multi-CS systems.

Temperature dependences of the rate constants of interconversion enable to determine the corresponding thermodynamic activation parameters of interconversion from the respective Eyring plots:

$$\ln\left(\frac{k}{T}\right) = -\frac{\Delta H^{\#}}{R}\frac{1}{T} + \frac{\Delta S^{\#}}{R} - \ln\left(\frac{h}{k_B \kappa}\right) \tag{8}$$

Here, $k$ is a rate constant of interconversion, $T$ is the absolute temperature, $k_B$ and $h$ are the Boltzmann and the Planck constants, respectively, and $\kappa$ is the transmission factor (a value of $\kappa = 0.5$ is considered for the reversible first order reaction of interconversion) [23,24]. $k$ is the rate constant of interconversion and $\Delta H^{\#}$ and $\Delta S^{\#}$ are relating thermodynamic activation parameters (enthalpy and entropy, respectively). The Eyring analysis in multi-CS systems has been discussed in detail in Part I of this work.

Affinity capillary electrophoresis (ACE) [25,26] is a well-established method that can be used to acquire all the necessary data for evaluation of the parameters described above. In the ACE experiment, an analyte interacts with an additive present in the background electrolyte (BGE) and interaction-specific parameters such as equilibrium constant and limiting mobilities of analyte–additive complexes (associates) can be determined. In chiral separations, a chiral selector is used as the additive, its concentration can be simply varied; the effect of temperature can be also examined.

Cyclodextrins (CDs) and their derivatives are very popular chiral selectors widely used in chiral separation techniques [1–3,7,8,11,14,27]. A drawback of these commercially available compounds is that they are often supplied as poorly defined mixtures of various isomers or derivatives with various degrees of substitution [6,28–30]. An example of these commercial mixtures of CDs derivatives is highly sulfated β-CDs (HS-β-CDs) [28,31,32] that is used in this study as a multi-CS agent. Lorazepam, the analyte in our experiments, belongs to the family of diazepam drugs [33], some of them are known for their tendency to undergo interconversion in the time scale of the separation process [23,34–41]. As the rate of interconversion is dependent on temperature, evaluation of thermodynamic activation functions (enthalpy and entropy) is also possible from the ACE measurements [39].

The aim of the present work is verification of the model of migration and interconversion proposed and described in detail in Part I of this study. The model is further utilized for computer simulation and consequently for determination of all local rate constants in a mixture of chiral selectors, namely HS-β-CDs. Separations performed at different temperatures allow to evaluate thermodynamic parameters, such as activation enthalpies and entropies for both enantiomers in both, chiral and achiral, environment separately.

## 2. Experimental

### 2.1. Chemicals

All chemicals used were of analytical reagent grade purity. Sodium tetraborate decahydrate was a product of Lachema (Brno,

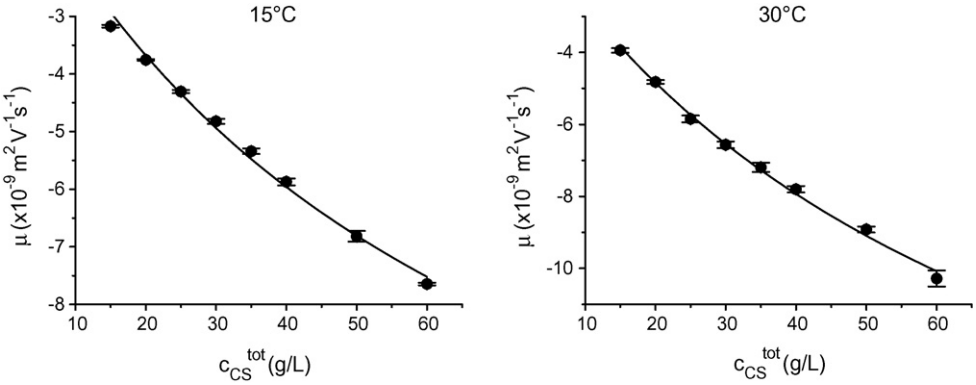


**Fig. 1.** Dependence of the effective mobilities $\mu_i$ of the 1st enantiomer on the total concentration $c_{CS}^{tot}$ of CSs mixture in a BGE at the lowest and the highest experimental temperatures measured. Error bars: standard deviations. Similar curves were obtained for the 2nd enantiomer.

Czech Republic). The chiral selector β-cyclodextrin, sulfated sodium salt (HS-β-CD) was purchased from Sigma–Aldrich (Prague, Czech Republic). Lorazepam racemate was obtained from the State Institute of Drug Control (SUKL, Prague, Czech Republic). Water used for preparation of all solutions was purified with a Milli-Q water purification system (Millipore, Bedford, USA).

### 2.2. Instruments

All experiments were performed using the $^{3D}$CE capillary electrophoresis system with the ChemStation software (Agilent Technologies, Waldbronn, Germany). Fused silica capillary of 50 μm I.D. and 375 μm O.D. was provided by CaCo (Bratislava, Slovakia). The total capillary length was 80.5 cm, the length to a detector was 72 cm. Detection was performed with the photometric diode-array detector (DAD) at the detection wavelength of 210 nm. The capillary was thermostated at various temperatures as described below. Samples were introduced hydrodynamically, 300 mbar s. Applied voltage was 15 kV (cathode at the detector side). The capillary was flushed with $H_2O$ for 8 min and with BGE for 4.5 min before each run. pH was measured with a PHM 220 instrument (Radiometer, Copenhagen, Denmark).

### 2.3. Experimental conditions

Running buffer was composed of 20 mM sodium tetraborate, pH 9.20. Eight background electrolytes with various concentrations of HS-β-CD (15, 20, 25, 30, 35, 40, 50 and 60 mg/L) were prepared by dissolving the appropriate amounts of HS-β-CD in the running buffer. The sample solution was prepared as follows: 1 mg of lorazepam was dissolved in 1 mL of methanol, 150 μL of the methanolic solution was mixed with 450 μL of the running buffer (without HS-β-CD). All solutions were filtered and then degassed in the ultrasonic bath before measurements. Electroosmotic flow was determined by means of the stationary injection system peak (we verified by our simulation program Simul [42] that it has zero mobility so it can be used for this purpose). To determine thermodynamic activation parameters the experimental runs were performed at the temperatures of 15, 17, 20, 23, 27 and 30 °C. The measurements at each combination of temperature and CSs concentration were repeated at least three times.

### 2.4. Software

Owing to the formal similarity of the single-CS enantioseparation system [22] with the multi-CS enantioseparation system (viz. Part I of this article [20]) the software SimulChir, previously used for numerical simulation of the former system, can also be applied for simulations of the latter. In contrary to our previous work [22], we use a simplified model of continuity equations for computer simulation of elution profiles in this work. Electrophoretic velocity of each enantiomer is regarded constant and its value is obtained experimentally, concentration of mixture of CSs is also regarded constant, axially uniform and equal to its analytical concentration in the BGE. The other constituents of the electromigration separation system (except the two enantiomers) are not explicitly involved in the simplified model. The simplified model has a better stability and its computation is less time consuming. The numerical simulation is employed for determination of the global rate constant $k_{glob}$: it runs iteratively several times until the simulated electropherogram and the experimental one have the same "relative plateau height", $H_{pm}$. This parameter was introduced by Bürkle et al. [21] and is defined as

$$H_{pm}(\%) = \frac{2V_p}{V_1 + V_2} \times 100 \quad (9)$$

where $V_p$ is the height of the plateau in the middle between the peaks, $V_1$ and $V_2$ are heights of the first and second peak, respectively. In the iteration process the initial dispersion of the analyte profile at time $t = 0$ s is varied as well to achieve the same experimental and simulated peak widths.

**Table 1**
Experimentally determined overall equilibrium constants, $K_i^{over}$, and the related overall mobilities, $\mu_i^{over}$

| $T$ (°C) | $K_1^{over}$ (L g$^{-1}$) | $\mu_1^{over}$ ($x$ 10$^{-9}$ m$^2$ s$^{-1}$ V$^{-1}$) | $K_2^{over}$ (L g$^{-1}$) | $\mu_2^{over}$ ($x$ 10$^{-9}$ m$^2$ s$^{-1}$ V$^{-1}$) |
|---|---|---|---|---|
| 15 | 0.0153 ± 0.0042 | −0.240 ± 0.026 | 0.0413 ± 0.0081 | −0.883 ± 0.105 |
| 17 | 0.0160 ± 0.0038 | −0.255 ± 0.024 | 0.0424 ± 0.0080 | −0.929 ± 0.107 |
| 20 | 0.0155 ± 0.0048 | −0.267 ± 0.032 | 0.0413 ± 0.0090 | −0.960 ± 0.126 |
| 23 | 0.0147 ± 0.0025 | −0.278 ± 0.018 | 0.0381 ± 0.0050 | −0.972 ± 0.075 |
| 27 | 0.0141 ± 0.0025 | −0.293 ± 0.019 | 0.0363 ± 0.0024 | −0.999 ± 0.038 |
| 30 | 0.0142 ± 0.0030 | −0.311 ± 0.024 | 0.0368 ± 0.0039 | −1.055 ± 0.065 |

Errors are expressed as 95% confidence intervals. Values are given for the 1st and the 2nd enantiomer at the given temperature.

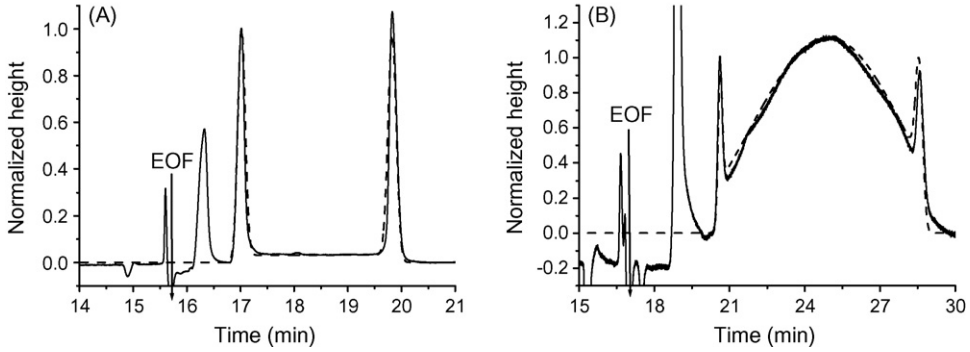


**Fig. 2.** Examples of the experimental and simulated electropherograms at different temperatures and different CSs concentrations: (A) 15 °C, 15 g L$^{-1}$ HS-β-CDs; (B) 30 °C, 30 g L$^{-1}$ HS-β-CDs. Solid curve: experiment. Dashed curve: simulation. EOF: system peak with zero electrophoretic mobility—the marker of the electroosmotic flow.

The SimulChir was programmed in Delphi 7.0 environment (Borland Software Corporation, USA). The Graphical User Interface allows a user-friendly input of parameters and the results of computation to be presented as time-dependent concentration profiles of constituents at the site of the detector. The algorithms searching for peak widths and the relative plateau height, Eq. (9), are included. More information on the software and simulation algorithm used can be found in Refs. [22,42,43].

## 3. Results and discussion

A set of measurements should be carried out at various concentrations of CSs and various temperatures in order to evaluate all thermodynamic parameters of the interconversion of enantiomers individually in the achiral and chiral part of the enantioseparation system. More than 170 measurements have been performed with plateau heights $H_{pm}$ resulting in the range from 1% up to 150% (or even to 300%). At least three data points have been measured at each combination of temperature and CSs concentration, and average values, standard deviations and variances (squared standard deviations) have been calculated. Standard deviations have been considered as *y*-errors and reciprocal variances have been used as weights for least-square fitting. The error propagation law has been applied to evaluate a variance of a variable after its transformation if needed (Eyring analysis, evaluation of $k_i^{over}$).

The evaluation of the experimental results has been performed in five consecutive steps as follows:

(i) ACE experiments. The experiments have been carried out at various CSs concentrations and temperatures. Observed effective mobilities have been plotted against the concentration of the CSs at each individual temperature and non-linear curve fits have been obtained according to Eq. (3). As the result, overall equilibrium constants, $K_1^{over}$ and $K_2^{over}$, and overall mobilities, $\mu_1^{over}$ and $\mu_2^{over}$, have come out. (In our particular case, the enantiomers non-complexed (non-associated) with CSs are uncharged and have zero mobilities: $\mu_1^0 = \mu_2^0 = 0$.)

(ii) Simulation of elution profiles. This step has resulted in determination of the global rate constant, $k_{glob}$, and consequently, the apparent rate constants, $k_i^{app}$, by means of Eqs. (6) and (7).

(iii) Plotting the apparent rate constants $k_i^{app}$ against $1/T$ (Eq. (8)) for obtaining the apparent thermodynamic activation parameters, i.e., the apparent activation enthalpies and entropies at various concentrations of CSs mixture.

(iv) Plotting the global rate constant $k_{glab}$ against concentration of CSs according to Eq. (6) in order to evaluate the local rate constants of interconversion of the enantiomers in the chiral environment, $k_1^{over}$, $k_2^{over}$, and achiral environment $k_1^0$, $k_2^0$, at various temperatures.

(v) Plotting the local rate constants against $1/T$ (Eq. (8)) and evaluation of the local activation entropies and enthalpies of the interconversion of enantiomers.

If the regression in (v) shows considerable linearity, this may be considered as a proof of the plausibility of the model proposed in this work.

### 3.1. Determination of equilibrium constants

Overall equilibrium constants $K_1^{over}$ and $K_2^{over}$ quantify the affinity of both enantiomers to interact with the mixture of HS-β-CDs. These constants, defined by Eq. (1), can be evaluated through the ACE experiments as described above. HS-β-CDs concentration was given in g/L because of the unknown molar mass of the CDs mixture; this, however, does not affect the final results, as the product $c_{CS}^{tot} K_i^{over}$, which appears in all the related equations, is dimensionless. Examples of the obtained regression curves for the first enantiomer at two experimental temperatures (the lowest and the highest temperature values studied) are shown in Fig. 1. The resulting values of $K_i^{over}$ and $\mu_i^{over}$ are given in Table 1.

**Table 2**
Apparent activation parameters calculated from Eyring analysis at various concentrations of the CSs mixture

| $c_{CS}^{tot}$ (g L$^{-1}$) | $\Delta H_1^{\#,app}$ (kJ mol$^{-1}$) | $\Delta S_1^{\#,app}$ (J mol$^{-1}$ K$^{-1}$) | $\Delta H_2^{\#,app}$ (kJ mol$^{-1}$) | $\Delta S_2^{\#,app}$ (J mol$^{-1}$ K$^{-1}$) |
|---|---|---|---|---|
| 15 | 107 ± 22 | 62 ± 75 | 109 ± 23 | 66 ± 78 |
| 20 | 89 ± 14 | 2 ± 48 | 90 ± 14 | 3 ± 48 |
| 25 | 104 ± 9 | 54 ± 29 | 107 ± 9 | 61 ± 29 |
| 30 | 97 ± 8 | 32 ± 25 | 99 ± 8 | 33 ± 25 |
| 35 | 96 ± 9 | 26 ± 31 | 98 ± 9 | 29 ± 31 |
| 40 | 92 ± 13 | 15 ± 43 | 95 ± 13 | 20 ± 43 |
| 50 | 96 ± 14 | 28 ± 47 | 99 ± 14 | 35 ± 47 |
| 60 | 100 ± 7 | 43 ± 23 | 104 ± 7 | 52 ± 23 |

Errors are expressed as 95% confidence intervals.

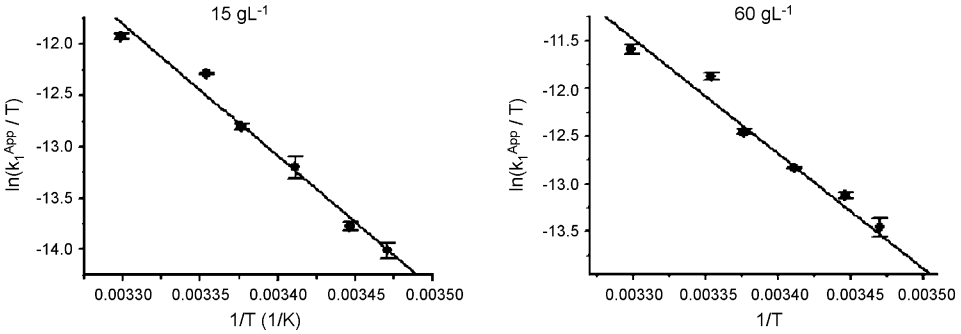


**Fig. 3.** Determination of the apparent activation parameters from the apparent Eyring plots at the lowest and the highest experimental concentrations of the CSs mixture. Error bars: standard deviations. The data are plotted for the 1st enantiomer. Similar curves were obtained for the 2nd enantiomer.

Experimental errors expressed as 95% confidence intervals are comparable with those usually obtained in single-CS systems. The good fit of experimental results proves that the mixture of HS-β-CDs behaves as there was only one CS in the separation system and the interaction with each enantiomer can be integrally described by means of the overall equilibrium constant $K_i^{over}$ and overall mobility $\mu_i^{over}$.

### 3.2. Global and apparent thermodynamic parameters

The global rate constants are evaluated through numerical simulation of the continuity equations (see Part I of this article [20]) as briefly described in Section 2.4. Two examples of experimentally obtained and simulated electropherograms are shown in Fig. 2. Generally, a very good agreement between experimental and simulated electropherograms could be attained within the whole range of CSs concentrations and temperatures.

Apparent rate constants were evaluated from the global rate constants, which result from the computer simulation, according to Eq. (7). The Approximation Function (AF) introduced by Trapp and Schurig [44] has been utilized to assess the firs measure of the apparent (and subsequently the global) rate constant of interconversion for the purpose of simulation. As expected, a good agreement was achieved when relative plateau height values was small while the situation was becoming worse with increasing the relative plateau height. At very high plateau heights the AF could not be used at all and simulation is the only possible tool to evaluate the apparent rate constants of interconversion from the elution profile.

The apparent rate constants serve for "apparent" Eyring plots and provide apparent activation functions. The results obtained for both enantiomers are summarized in Table 2. The apparent thermodynamic parameters can be found for some analytes and CSs in the literature [23,37,45–50]. Examples of the apparent Eyring plots for the 1st enantiomer at the lowest and the highest experimental concentrations of the mixture of CSs are shown in Fig. 3. As the Joule heating may slightly shift the results, additionally the current in the capillary was measured at various voltages applied. This dependency was found linear up to the experimental voltage, even at the highest concentration of CSs. This indicates that the effect

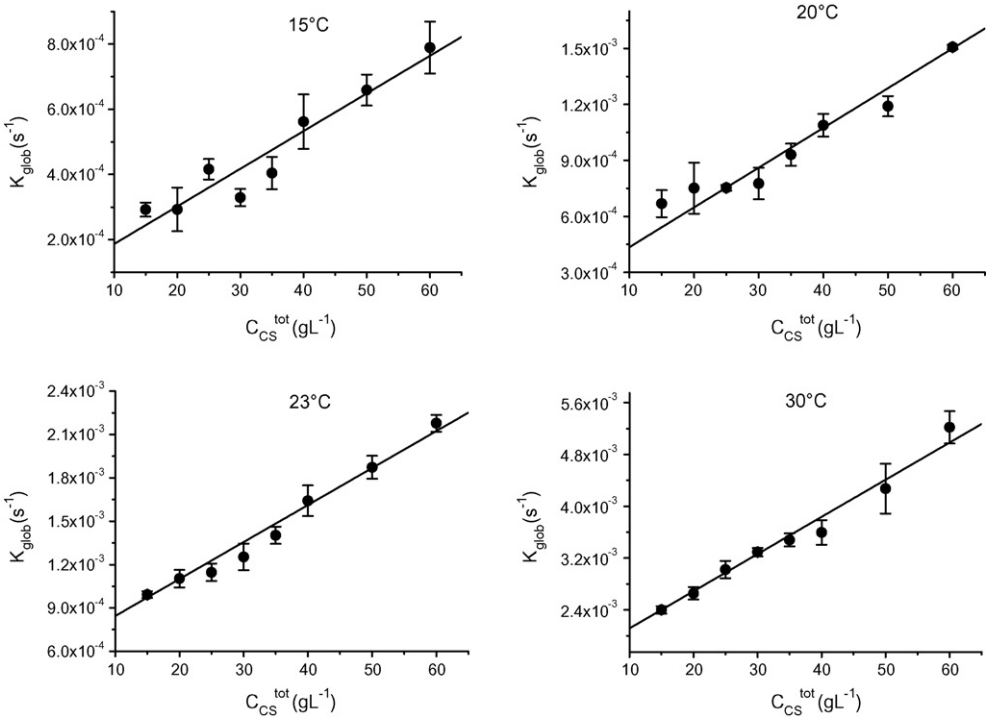


**Fig. 4.** Determination of the local rate constants from the dependences of the global rate constants $k_{glob}$ on the total concentration of CSs, $c_{CS}^{tot}$, at various temperatures. Error bars: standard deviations. The values are plotted for the 1st enantiomer. Similar curves were obtained for the 2nd enantiomer.

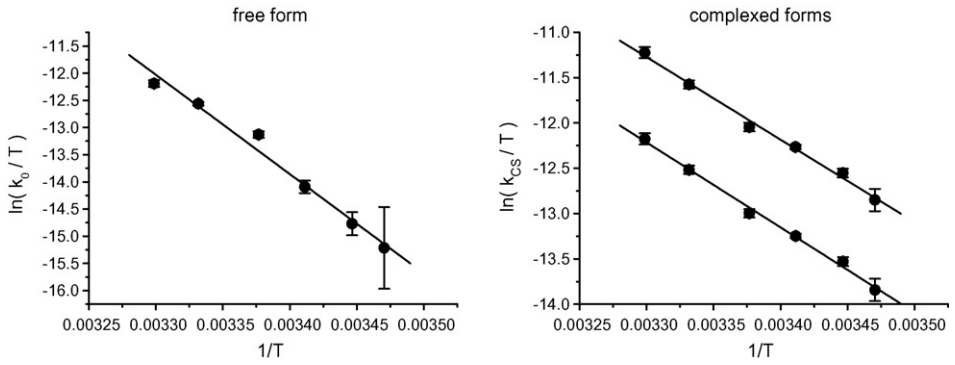


**Fig. 5.** Determination of the local thermodynamic parameters from the local Eyring plots of $k_1^0 = k_2^0$ (denoted $k_0$ in the plot; left dependence) and $k_1^{over}$ and $k_2^{over}$ (denoted $k_{CS}$ in the plot; upper line for the 1st enantiomer, lower line for the 2nd enantiomer; right graph). Error-bars: standard deviations.

of the Joule heating should not be too significant. In addition, all dependences measured in this study meet very well the expected linear trends that would have hardly arisen if the Joule heating had significantly affected the results.

Although the Eyring plots show a good linearity, the activation entropy is determined with a relatively large error of estimation. This is due to the fact that the measurements are performed in a narrow temperature range (from 15 to 30 °C) while activation entropy is determined by the extrapolation to the infinite temperature. This is a general problem of the Eyring analysis [51]. Examples of evaluation of apparent activation entropies with unrealistically low errors of estimates can be found in some papers. We have recalculated some of the literature data and found that the authors probably did not realise that not only the resulting slopes and intercepts but also their errors must be multiplied by $R$ (universal gas constant) when transforming to the corresponding activation parameters. Moreover, to give the 95% confidence interval, the resulting error should be further multiplied by the factor of, typically, 2.0–2.5. This increases a final error of the estimate by a multiplication factor of about 20. In addition, every dynamic-separation measurement in CE/HPLC is performed in a typical temperature range, say, 15–40 °C, that predestinates the error of measurement to be approximately the same for each measurements regardless of the actual value of apparent activation parameters. In summary, the determined errors of the activation parameters are comparable with those accompanying single-CS experiments. This fact, along with the good linearity of the apparent Eyring plots at any CSs concentration, supports the reliability of the determination of the rate constants by the computer simulation approach.

### 3.3. Local thermodynamic parameters

As proposed theoretically in our previous work [22] for single-CS enantioseparation systems and further generalized in Part I for multi-CS systems, the global rate constant $k_{glob}$ should exhibit a linear dependence on the total concentration $c_{CS}^{tot}$ of the mixture of CSs, i.e., the mixture of HS-β-CDs in this study. This prediction is fully in agreement with experimental results. Fig. 4 illustrates these dependences at four different temperatures. According to the theory, $k_{glob} = k_1^0 + c_{CS}^{tot} K_1^{over} k_1^{over} = k_2^0 + c_{CS}^{tot} K_2^{over} k_2^{over}$, so intercept and slope of the regressions relates to the rates of interconversion in achiral and chiral environments, respectively, as described in Part I. The apparent constants (discussed in the previous section) can be decomposed into the local ones in this way, so it can be distinguished which portion of the interconversion takes place in the chiral and achiral part of the enantioseparation system. The interconversion in the chiral and achiral part of the enantioseparation system is governed by the achiral, $k_i^0$, and overall, $k_i^{over}$, rate constants of interconversion, respectively. It has been already discussed in the Part I, that although the overall rate constant $k_i^{over}$ is not the true (intrinsic) rate constant, it still provides information on the rate of interconversion in the chiral environment of the mixture of CSs. Unfortunately, the individual rate constants of interconversion, $k_i^q$, of an $i$th enantiomer when associated with a particular $q$th CS in the mixture, are unrecognisable.

Local rate constants of interconversion at various temperatures are summarized in Table 3. It is worth noting that the overall distribution constants are larger when compared to the achiral rate constant $k_i^0$, which implies that lorazepam undergoes the interconversion faster if associated with HS-β-CDs than in the free BGE, within the studied temperature range. The mixture of HS-β-CDs seems to speed up the interconversion of lorazepam. However, this is just a primary observation; the theory implies that this effect should depend on temperature. However, a wider examination of the topic is beyond the scope of this article.

Rate constant of interconversion in the achiral environment, $k_i^0$, should provide a linear Eyring plot that allows the corresponding activation parameters to be determined. The dependence of the overall rate constants of interconversion $k_i^{over}$ on temperature is generally more complicated (viz. discussion in the Part I). The Eyring plot based on these overall rate constants may still show linearity in a certain rather narrow temperature range and

**Table 3**
Local rate constants of interconversion, $k_i^0$, in the achiral part (CSs free BGE) and overall rate constants of interconversion, $k_i^{over}$, in the chiral part of the separation system (BGE with the mixture of HS-β-CDs)

| $T$ (°C) | $k_1^0 = k_2^0$ ($x$ 10$^{-3}$ s$^{-1}$) | $k_1^{over}$ ($x$ 10$^{-3}$ s$^{-1}$) | $k_2^{over}$ ($x$ 10$^{-3}$ s$^{-1}$) |
|---|---|---|---|
| 15 | 0.0711 ± 0.0134 | 0.7570 ± 0.0237 | 0.2798 ± 0.0088 |
| 17 | 0.1115 ± 0.0167 | 1.0249 ± 0.0353 | 0.3855 ± 0.0133 |
| 20 | 0.2222 ± 0.0181 | 1.3813 ± 0.0243 | 0.5170 ± 0.0092 |
| 23 | 0.5881 ± 0.0247 | 1.7381 ± 0.0578 | 0.6704 ± 0.0223 |
| 27 | 1.0500 ± 0.0248 | 2.8194 ± 0.0906 | 1.0989 ± 0.0353 |
| 30 | 1.5400 ± 0.0673 | 4.0474 ± 0.1785 | 1.5604 ± 0.0688 |

Errors are expressed as 95% confidence intervals.

**Table 4**
Local activation parameters calculated from Eyring analysis. $\Delta H^{\#} \equiv \left\{ \Delta H_1^{\#,0} = \Delta H_2^{\#,0}; \Delta H_1^{\#,over}; \Delta H_2^{\#,over} \right\}$ for the achiral and overall part of the system and the 1st and the 2nd enantiomer; the same for $\Delta S^{\#}$

| Parameter | Free form | 1st enantiomer | 2nd enantiomer |
|---|---|---|---|
| $\Delta H^{\#}$ (kJ mol$^{-1}$) | 151 ± 29 | 75 ± 8 | 78 ± 7 |
| $\Delta S^{\#}$ (J mol$^{-1}$ K$^{-1}$) | 209 ± 98 | −36 ± 28 | −36 ± 25 |

Errors are expressed as 95% confidence intervals.

the overall activation functions may be evaluated. However, these thermodynamic parameters are valid only in the given temperature range. The local Eyring plots are depicted in Fig. 5 for both enantiomers in their free form and in the chiral environment of the HS-β-CD mixture. Table 4 gives both the achiral and overall activation parameters evaluated directly through the Eyring analysis.

## 4. Conclusion and remarks

This study (Part I and Part II) aimed to: (i) propose a new mathematical model for enantioseparation (including on-column interconversion) when mixture of CSs is employed, (ii) verify this model experimentally and (iii) verify our formerly proposed method to distinguish between true and apparent thermodynamics and to show that the method is utilizable even for mixtures of CSs. In Part I [20], which has been focused on the first stage (i), we have made a theoretical assumption that an enantioseparation system involving more than one CS (multi-CS systems) may formally be treated, if some approximations are considered, as there has been only one CS. This has been fully confirmed experimentally in Part II as described herein. All experimentally obtained dependences of either mobilities or global rate constants on concentration of the CSs mixture have shown the expected trends. Simulated electropherograms have matched very well the experimental ones and the resulting apparent rate constants of interconversion have provided linear Eyring dependences. Also Eyring plots of local rate constants of interconversion, as resulting from the linear dependences of the global rate constants on the CSs concentration, have proved a significant linearity. The equations derived could fit well the experimental data in both the temperature and CSs concentration coordinates. This result supports our approach introduced earlier [22] by which we succeeded to resolve the apparent rate constants of interconversion back to the local ones in the chiral and achiral environments individually.

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