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# Study on complex formation of biologically active pyridine derivatives with cyclodextrins by capillary electrophoresis

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**Abstract**

The capillary electrophoretic separation of the pyridine derivatives pyridoxine, pyridoxal, nicotinamide, nicotinic acid and isonicotinic acid in phosphate buffer using cyclodextrins as buffer additives was studied at pH 2.0 and 3.5. Superior separation was achieved at pH 2.0. Addition of α- and β-cyclodextrin and the respective 2-hydroxypropyl derivatives as well as carboxymethyl-α-cyclodextrin to the running buffer did not significantly improve the resolution of the compounds. The interactions of α- and β-cyclodextrin as well as their hydroxypropyl derivatives with the pyridine derivatives were investigated by capillary electrophoresis at pH 2.0. No complex formation was observed between the cyclodextrins and pyridoxine, pyridoxal and nicotinamide. α-Cyclodextrin and 2-hydroxypropyl-α-cyclodextrin form weak 1:1 complexes with nicotinic and isonicotinic acids in aqueous media at 298.15 K, while β-cyclodextrin and its hydroxypropyl derivative did not form complexes. The apparent stability constants ($K$) of the complexes calculated from the electrophoretic mobility data ranged between 3 and 33 kg/mol. The negative values of enthalpy and entropy of complex formation obtained from the graphical plot of the van't Hoff equation indicate an important role of van der Waals and electrostatic interactions in the binding of nicotinic acid with α-cyclodextrin.


*Keywords:* B-vitamins; Capillary electrophoresis; Complex formation; Cyclodextrins; Thermodynamics

## 1. Introduction

Inclusion complexes of cyclodextrins (CDs) with various biologically active molecules have received much attention in recent years due to their significance for the understanding of the phenomenon of biochemical specificity (e.g. molecular recognition, separation) and some biochemical processes (e.g. enzyme catalysis, membrane transport), as well as for their industrial use in pharmaceutical, cosmetic and food technologies [1–3]. All these applications are based on the ability of CDs to selectively interact with the guest molecules and to form inclusion (or host–guest) complexes. This property of CDs is determined by their particular structure. The CD molecule possesses a hydrophilic exterior and hydrophobic interior cavity, which is capable to accommodate a wide range of guest molecules both in the solid state and in solutions [4,5]. Selectivity of interaction and stability of the complexes are mainly governed by the principles of geometric and energetic complementarity. The forces involved in the complexation process are of non-covalent nature (mainly hydrogen bonding, hydrophobic, van der Waals and electrostatic interactions) [6]. In some cases, substitution of OH groups surrounding the CD cavity by functional groups (2-hydroxypropyl, methyl, carboxymethyl etc.) can change the cavity size, the CD's conformational flexibility, polarity, hydrophobicity and capability to bind the guest molecules. All these factors can influence the complex formation process and promote more selective interaction and more strong binding.

In recent years capillary electrophoresis (CE) has been applied to study binding equilibria between various guest and host molecules including CDs [7,8]. CE offers several advantages for the determination of complexation constants such as the consumption of only small amounts of chemicals. Moreover, it is possible to study closely related analytes simultaneously provided that they can be separated by CE. For the same reason, the analytes do not have to be pure as it is required for other techniques (e. g. calorimetry).

B-vitamins such as nicotinic acid, nicotinamide, pyridoxine and pyridoxal are important compounds in pharmaceutical,

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cosmetic and food products. Because the stability of compounds may be increased by complexation with CDs [1,2] the interaction between CDs and B-vitamins is an important topic for industry. Only few studies on complex formation of CDs with pyridine [9] and pyridine derivatives [10–13] have been published. Pyridine forms a 1:1 complex only with α-CD, whereas β-CD was found to be a more suitable complexing agent for substituted pyridines. To the best of our knowledge no studies on the interactions of CDs with B-vitamins such as nicotinic acid, nicotinamide, pyridoxal, or pyridoxine have been published except an investigation of the interaction of nicotinic acid with native α- and β-CD by calorimetry [13]. Therefore, the aims of the present study were the following: (1) to determine conditions for CE separations of B-vitamins; (2) to study the ability of native and 2-hydroxypropylated α- and β-CDs to form complexes with B-vitamins; (3) to analyze the influence of guest structure, the CD cavity dimensions, and the availability of the 2-hydroxypropyl substituents and the pH on the selectivity of complex formation.

## 2. Experimental

### 2.1. Chemicals

Pyridoxine, pyridoxal, nicotinamide, nicotinic and isonicotinic acids were from MP Biomedicals (Solon, OH, USA), and were used without further purification. Riboflavin-5′-phosphate, α-CD, β-CD, hydroxypropyl-α-cyclodextrin (HP-α-CD) and hydroxypropyl-β-cyclodextrin (HP-β-CD) with average degree of substitution 0.6 per glucose unit were obtained from Sigma–Aldrich (Taufkirchen, Germany). Carboxymethyl-α-cyclodextrin (CM-α-CD) with the substitution degree 3.5 was from Cyclolab (Budapest, Hungary). All other chemicals were of analytical grade. Buffers containing β-CD were prepared with 2 M urea.

Sodium phosphate buffers and sample solutions were prepared in double-distilled, deionized water, filtered (0.47 μm) and degassed by sonication. All solutions were prepared by weight.

### 2.2. Apparatus

All experiments were performed on a Beckman P/ACE 5510 instrument (Beckman Coulter GmbH, Unterschleißheim, Germany) equipped with a diode array detector at 298.15 K using 50 μm I.D. fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA). The effective length of the capillary was 40 cm, the total length was 47 cm. UV detection at 215 nm was performed at the cathodic end for all experiments with the exception of CM-α-CD when the polarity was reversed. Sample solutions at a concentration of $1.5 \times 10^{-3}$ mol/kg were introduced at a pressure of 3.45 kPa. The separations were carried out at 25 kV. The EOF was monitored with mesityl oxide. Before all separations, the capillary was washed for 5 min with water, 2 min with 100 mM phosphoric acid and 15 min with the running buffer. For the determination of the binding constants each sample was analyzed three times and the mean of the migration times was used for the calculation.

### 2.3. Viscosity correction

For the accurate calculation of the binding constants and other thermodynamic parameters the correction of buffer viscosity is necessary [14]. The viscosity measurements were carried out using riboflavin-5′-phosphate and a capillary electrophoresis instrument as a viscosimeter according to reference [15]. Triplicate measurements were made for each CD concentration and temperature.

Viscosity-corrected effective mobility, $\mu_{\text{eff}}$, was calculated by the following equation:

$$\mu_{\text{eff}} = \nu_\eta \frac{l_{\text{eff}} l_{\text{t}}}{V} \left( \frac{1}{t} - \frac{1}{t_{\text{EOF}}} \right) \quad (1)$$

where $\nu_\eta$ is viscosity correction factor, $l_{\text{eff}}$ and $l_{\text{t}}$ are effective and total lengths of capillary, respectively, $V$ the applied voltage, $t$ and $t_{\text{EOF}}$ are the migration times of analyte and EOF marker, respectively.

## 3. Results and discussion

### 3.1. Separation of some B-vitamins by CE

The separation of B-vitamins nicotinic acid, nicotinamide, pyridoxal, and pyridoxine (Fig. 1) was studied at pH 2.0 and 3.5. Isonicotinic acid, as a structural isomer of nicotinic acid, with the carboxylic group in *para*-position was also examined. Nicotinic acid and isonicotinic acid exist as zwitterions at pH 3.5 and as a mixture of cations and zwitterions at pH 2.0 [16]. Pyridoxine, pyridoxal and nicotinamide are predominantly cations at the considered pH values [17,18].

The separation of B-vitamins in fused-silica capillaries at pH 3.5 and 2.0 is shown in Fig. 2A and B, respectively. Nicotinamide, pyridoxal and pyridoxine are resolved at pH 3.5 while nicotinic acid and isonicotinic acid comigrate as electrically neutral zwitterions with the EOF at pH 3.5 and can therefore not be separated (Fig. 2A). Addition of the charged CD derivative CM-α-CD at concentrations between $4 \times 10^{-4}$ and $6 \times 10^{-2}$ mol/kg did not result in a significant improvement of the separation. CM-α-CD was chosen as it is known from calorimetry that only α-CD

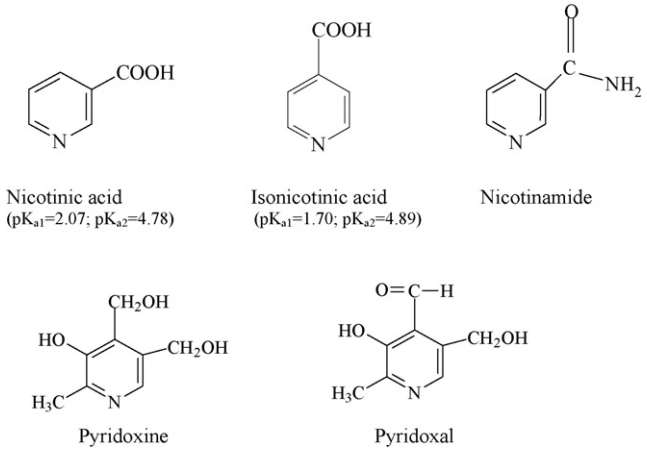


Fig. 1. Structures of the pyridine derivatives molecules under study.

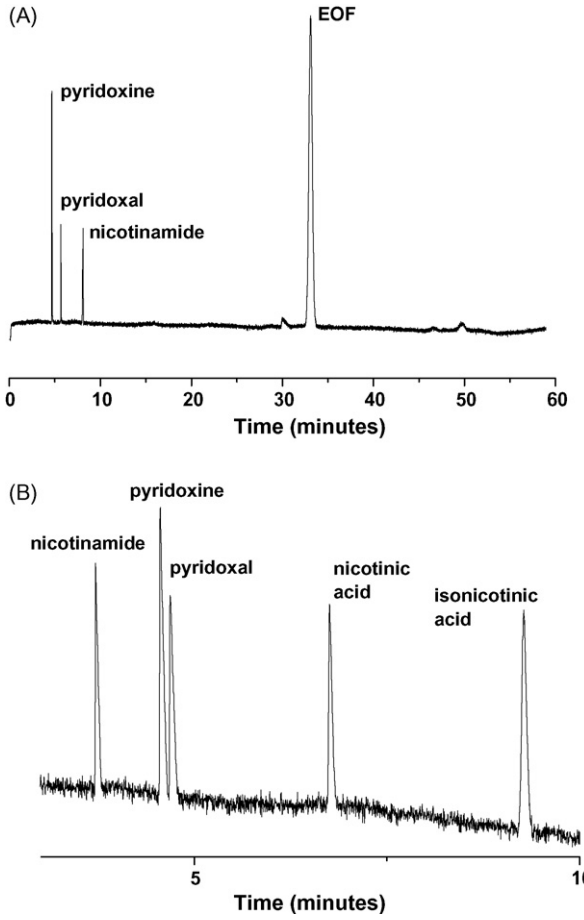


Fig. 2. Electropherograms of the separation of the pyridine derivatives at pH 3.5 (A) and 2.0 (B). Conditions: 47 cm (40 cm effective length) fused-silica capillary, 50 mM sodium phosphate buffer, 25 kV, UV at 215 nm, 298.15 K.

forms complexes with nicotinic acid [13]. CM-α-CD may not be able to include nicotinic acid and isonicotinic acid. Thus, further methods such as calorimetry and UV-spectroscopy were additionally employed to study the interaction between CM-α-CD and the pyridinecarboxylic acids in more detail. Using calorimetry we found that interactions of CM-α-CD with nicotinic acid are accompanied by very small endothermal effects caused by the partial dehydration of solutes. Subsequently, UV-spectra of nicotinic acid and isonicotinic acid in pure buffer and buffer containing different amounts of CM-α-CD were recorded. No changes in absorption spectra of nicotinic and isonicotinic acids in the presence of excess amounts of CM-α-CD were observed. Thus, it can be concluded that no significant complex formation between CM-α-CD and the investigated pyridinecarboxylic acids occurs.

Compared to pH 3.5, the separation of all compounds is achieved at pH 2.0 although pyridoxal and pyridoxine are not completely separated (Fig. 2B). Addition of α-CD, β-CD and HP-α-CD to the running buffer did not result in an improvement of the separation. This may be due to the fact that pyridoxine and pyridoxal do not bind to the investigated CDs. Complex formation of the pyridine derivatives with CDs is discussed below.

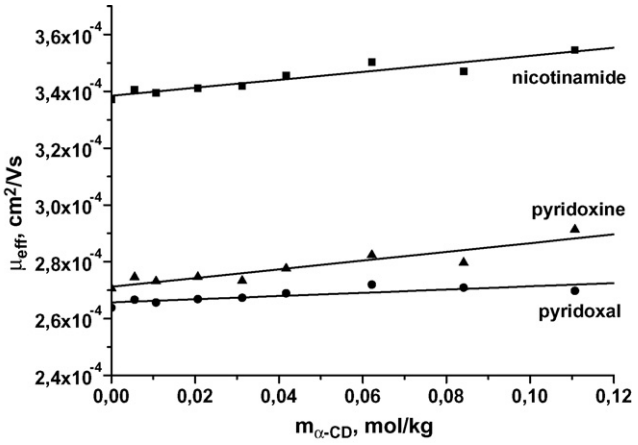


Fig. 3. Dependence of viscosity-corrected effective mobility ($\mu_{eff}$) of nicotinamide, pyridoxine and pyridoxal on the α-CD concentration. Conditions: sodium phosphate buffer, pH 2.0, $T = 298.15$ K, other conditions as in Fig. 2.

### 3.2. Investigation of complex formation by CE

The interactions of the CDs with the examined pyridine derivatives were studied by CE at 298.15 K and pH 2.0. Figs. 3 and 4, as examples, show the influence of increasing α-CD concentrations on the effective mobility of the analytes. The linear dependences shown in Fig. 3 reveal the absence of complex formation of α-CD with nicotinamide, pyridoxine and pyridoxal. The minor increase of the effective mobility with increasing CD concentrations can be attributed to a decrease in the bulk dielectric constant of the solution as reported by Britz-McKibbin and Chen [7]. Similar linear dependences were found for all other systems with the exception of α-CD and nicotinic acid, HP-α-CD and nicotinic acid as well as α-CD and isonicotinic acid. The binding isotherms of these systems displayed a nonlinear dependence of the mobility on the concentration of α-CD and HP-α-CD as shown in Fig. 4 indicating complex formation between the CDs and the analytes.

Pyridoxal, pyridoxine and nicotinamide, which have polar substituents in the pyridine ring and are positively charged at pH 2.0, apparently do not form complexes with the investigated

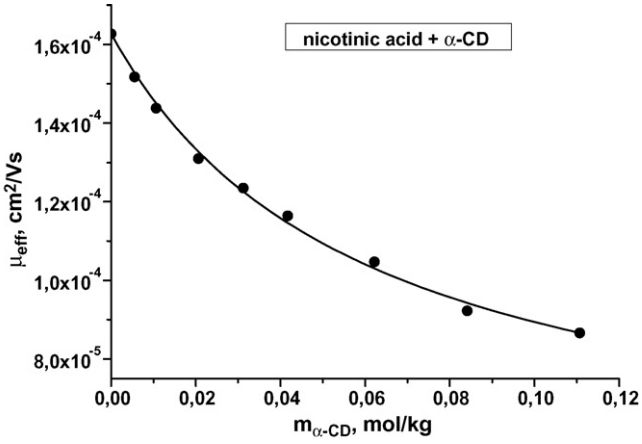


Fig. 4. Dependence of viscosity-corrected effective mobility ($\mu_{eff}$) of nicotinic acid on the α-CD concentration. Experimental conditions as in Fig. 3.

CDs. The substituents may be too bulky hindering inclusion of the compounds into the CD cavity. Interestingly, only the pyridinecarboxylic acids with a free carboxyl group form complexes with α-CD, while nicotinamide with an amide group is not complexed. Binding of the acids by β-CDs did not occur due to geometric discrepancy of the guest size with regard to the β-CD cavity dimensions.

For a correct calculation of the binding constants of the complexes the following equilibria have to be considered:

1. Equilibrium between the protonated and neutral (zwitterionic) species of nicotinic (or isonicotinic) acid:

$$AH^{\pm} + H^{+} = AH_2{}^{+}, \qquad K_a = \frac{[AH_2{}^{+}]}{[AH^{\pm}] \cdot [H^{+}]} \tag{2}$$

2. Complex formation of the zwitterions with the CD:

$$AH^{\pm} + CD = AH^{\pm} \cdot CD, \qquad K_1 = \frac{[AH^{\pm} \cdot CD]}{[AH^{\pm} \cdot CD]} \tag{3}$$

3. Complex formation of the cationic species with the CD:

$$AH_2{}^{+} + CD = AH_2{}^{+} \cdot CD, \qquad K_2 = \frac{[AH_2{}^{+} \cdot CD]}{[CD] \cdot [AH_2{}^{+}]} \tag{4}$$

Taking into account all equilibria, the effective mobility can be expressed as

$$\mu_{eff} = \mu_{AH_2{}^{+}} \alpha_{AH_2{}^{+}} + \mu_{AH^{\pm}} \alpha_{AH^{\pm}} + \mu_{AH_2{}^{+}\cdot CD} \alpha_{AH_2{}^{+}\cdot CD} + \mu_{AH^{\pm}\cdot CD} \alpha_{AH^{\pm}\cdot CD} \tag{5}$$

Since the mobility of the uncharged zwitterions as free or complexed species is equal to zero, the Eq. (5) is simplified to

$$\mu_{eff} = \mu_{AH_2{}^{+}} \alpha_{AH_2{}^{+}} + \mu_{AH_2{}^{+}\cdot CD} \alpha_{AH_2{}^{+}\cdot CD} \tag{6}$$

In Eqs. (5) and (6) the mol fraction of the species, $\alpha$, can be written:

$$\alpha_{AH_2{}^{+}} = \frac{[AH_2{}^{+}]}{c} \tag{7}$$

$$\alpha_{AH_2{}^{+}\cdot CD} = \frac{[AH_2{}^{+} \cdot CD]}{c} = K_2 \frac{[CD] \cdot [AH_2{}^{+}]}{c} \tag{8}$$

Combining Eqs. (7) and (8) with Eq. (6) yields:

$$\mu_{eff} = \frac{[AH_2{}^{+}]}{c} (\mu_{AH_2{}^{+}} + \mu_{AH_2{}^{+}\cdot CD} K_2[CD]) \tag{9}$$

where $c$ is the total analyte concentration which can be expressed by

$$c = [AH_2{}^{+}] + [AH^{\pm}] + [AH_2{}^{+} \cdot CD] + [AH^{\pm} \cdot CD] = [AH_2{}^{+}] \left(1 + \frac{1}{K_a[H^{+}]} + K_2[CD] + \frac{K_1[CD]}{K_a[H^{+}]}\right) \tag{10}$$

Thus, Eq. (9) becomes:

$$\mu_{eff} = \frac{\mu_{AH_2{}^{+}} + \mu_{AH_2{}^{+}\cdot CD} K_2[CD]}{1 + 1/K_a[H^{+}] + K_2[CD] + K_1[CD]/K_a[H^{+}]} \tag{11}$$

Table 1
Apparent binding constants for complex formation of nicotinic acid and isonicotinic acid with CDs in aqueous solution at 298.15 K

| Complex | $K_1^{CE}$ (kg/mol) | $K_1^{Cal}$ (kg/mol) | $K_2^{CE}$ (kg/mol) |
|---|---|---|---|
| α-CD/nicotinic acid | 30 ± 2 | 33 ± 5 | 3.2 ± 0.6 |
| HP-α-CD/nicotinic acid | 17 ± 1 | 23 ± 4 | nc |
| α-CD/isonicotinic acid | 12 ± 6 | 7 ± 3 | 3.5 ± 2.1 |

nc: no complex formation; $K^{CE}$: obtained by CE; $K_1^{Cal}$: early obtained from calorimetric measurements [13].

where $K_a$ is the dissociation constant known from the literature [16], $K_1$ and $K_2$ are the apparent binding constants of the zwitterionic and the cationic species with the CDs, respectively, [CD] the concentration of the CD, and $[H^+]$ is the concentration of protons. Two assumptions were made in order to simplify the calculations. First, binding of the analytes by the CD does not alter the dissociation constant $K_a$. It is well known that the $K_a$ shifts upon complexation but as the exact extend is unknown such a shift was not considered. Second, concentrations were used for the calculations instead of activities as the activity coefficients are not known either. Moreover, as the concentrations of ionic species were rather low, one can assume that the coefficients are close to unity.

The values of $K_1$ and $K_2$ were calculated according to Eq. (11) by non-linear least-squares regression analysis assuming the formation of 1:1 complexes. To test the validity of the proposed model the calculated $K_1$ values were compared with values earlier obtained by calorimetry [13]. The constants obtained by CE are in good agreement with the reported data (Table 1). The constant reported for HP-α-CD is an apparent constant averaged for all CD isomers because HP-a-CD is a randomly substituted derivative. The Benesi–Hildebrand procedure [19] was used to confirm the 1:1 stoichiometry of the complexes. As an example, the linear *x*-reciprocal plot which is typical for a 1:1 complex is shown in Fig. 5.

As can be seen from the data in Table 1, the complexes of α-CD and HP-α-CD with nicotinic and isonicotinic acids are weak, the values of the binding constants fall in the range 3–33 kg/mol. Relatively more stable complexes were formed with the zwitte-

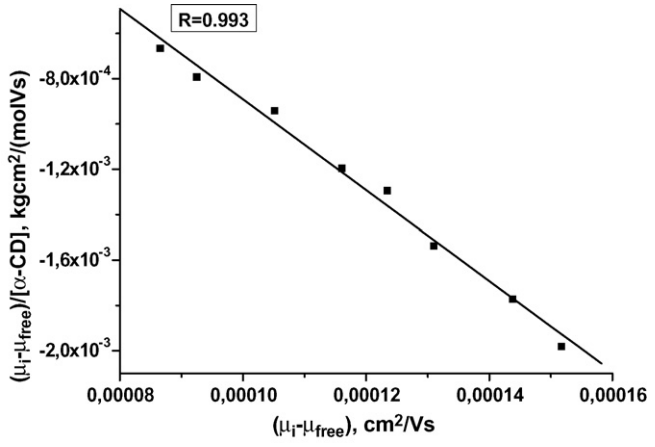


Fig. 5. X-reciprocal plot of the complex formation between nicotinic acid and α-CD.

rionic species. The remarkable difference between the values of $K_1$ and $K_2$ can be explained by the different ionization state of the carboxyl group. The hydrophility of the carboxylate group is higher compared to the protonated carboxyl group. Thus, van der Waals interactions and electrostatic interactions of the ionized carboxylate group with the CD cavity, which is polarized, can stabilize the complexes formed by zwitterions [6,20]. The fact that carboxyl group of carboxylic acids can in fact be located inside the CD cavity has been described for nicotinic acid [21] and substituted benzoic acids [22,23]. Alternatively, it may be speculated that the carboxylate anion is able to form hydrogen bonds with the hydroxyl groups located on the rim of the CDs.

The position of the carboxyl group versus the nitrogen in the pyridine ring significantly influenced the $K_1$ values, while the influence on $K_2$ values is not appreciable (see Table 1). More stable complexes were formed when the carboxylate group occupies *meta*-position in the aromatic ring of the zwitterions. "Moving" the carboxylate group into the *para*-position resulted in an approximately 50% reduction of the capability of isonicotinic acid to form a complex with α-CD and HP-α-CD. Thus, the location of the carboxylate group closer to the nitrogen atom appears to favor the binding of nicotinic acid with α-CD and HP-α-CD. Maybe, placing the negatively charged group closer to the protonated positively charged nitrogen atom induces a change of the hydration state of the functional groups as well as their acid-base properties. Moreover, the electronic charge distribution of the molecule is changed depending on the position of the carboxylate group versus the nitrogen [24]. It should be also mentioned that nicotinic acid can exist in two conformations which differ in the orientation of the carboxyl group, whereas only one stable conformer has been described for isonicotinic acid [24]. All these factors determine the different ability of nicotinic and isonicotinic acids to form complexes with α-CD and HP-α-CD.

The introduction of hydroxypropyl substituents to the rims of the α-CD molecule results in a decrease of the stability constants of the complexes. The bulky substituents obstructed the binding of nicotinic acid with HP-α-CD, and in the case with isonicotinic acid prevented the complex formation process. Steric effects may be responsible for this effect.

The temperature effect on the stability of the α-CD/nicotinic acid complex was examined in the 293.15–300.15 K temperature range. The stability constants determined at different temperatures are summarized in Table 2. The stability of the α-CD/nicotinic acid complex decreased with increasing temperatures. A linear relationship between ln $K$ and $1/T$ was observed (Fig. 6). The thermodynamic parameters such as enthalpy and entropy of complex formation were calculated from the relationship according to the van't Hoff equation:

$$\ln K = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} \quad (12)$$

where $\Delta H^\circ$ and $\Delta S^\circ$ are the enthalpy and entropy of complex formation, respectively, $T$ is the temperature, $R$ is the gas constant. Linear least-squares analysis of the ln K versus $1/T$ gave the following results: $\Delta H^\circ = -24.5 \pm 3.5$ kJ/mol and $\Delta S^\circ = -54 \pm 11$ J/mol K. The data are in accordance with the values determined by calorimetric measurements: $\Delta H^\circ = -26.4 \pm 0.3$ kJ/mol and $\Delta S^\circ = -59 \pm 11$ J/mol K [13] clearly demonstrating the suitability of CE for the determination of such data. As currents ranged between 40 and 50 μA in all experiments a significant effect of Joule heating on the data can be excluded. The relatively large negative values of the enthalpy and entropy of complex formation can be attributed to binding occurring due to electrostatic and van der Waals interactions or hydrogen bonding that are more important than hydrophobic interactions and solvent effects.

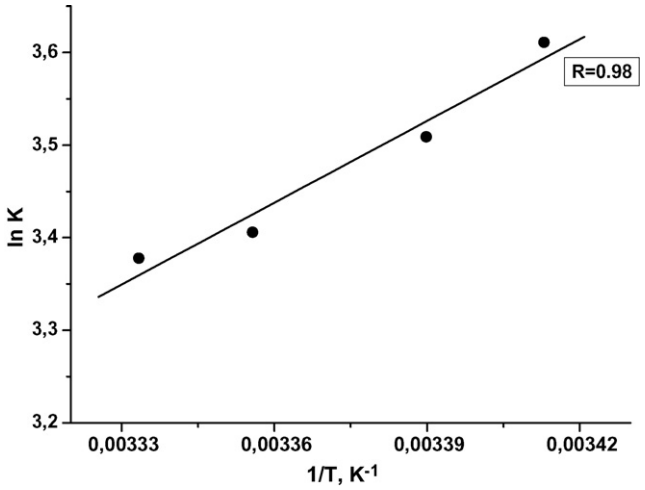


Fig. 6. Dependence of ln $K$ vs. $1/T$ of the complex formation between the zwitterionic species of nicotinic acid and α-CD.

Table 2
Apparent stability constants of α-CD complexes with nicotinic acid at different temperatures

| $T$ (K) | $K_1$ (kg/mol) |
|---|---|
| 293.15 | 37 ± 3 |
| 295.15 | 33 ± 5 |
| 298.15 | 30 ± 2 |
| 300.15 | 29 ± 4 |

## 4. Conclusions

The present study on the CE separation of pyridoxine, pyridoxal, nicotinamide, nicotinic acid and isonicotinic acid in phosphate buffer demonstrated that a better separation is achieved at pH 2.0 as compared to pH 3.5. The presence of different CDs in the run buffer did not result in improvement of the compounds resolution due to weak interactions that occur in the majority of the studied systems and are not accompanied by complex formation. Only nicotinic acid and isonicotinic acid formed 1:1 complexes with α-CD and HP-α-CD in aqueous buffers at 298.15 K. The apparent stability constants of the complexes of the CDs with the protonated and zwitterionic species of the pyridinecarboxylic acids were simultaneously calculated using mobility data from CE experiments. Apparently, the ionization state of the carboxyl group and its position in the pyridine ring influence the stability of the complexes. The partial substitution

of hydroxyl groups surrounding the CD cavity by hydroxypropyl groups weakened the binding. Negative values of enthalpy and entropy of complex formation indicate an important role of electrostatic and van der Waals interactions in the binding of α-CD with nicotinic acid.

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