# Determination of the dissociation constants (p$K_a$) of secondary and tertiary amines in organic media by capillary electrophoresis and their role in the electrophoretic mobility order inversion

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## Abstract

Non-aqueous capillary electrophoresis (NACE) may provide a selectivity enhancement in separations since the analyte dissociation constants (p$K_a$) in organic media are different from those in aqueous solutions. In this work, we have studied the inversion in mobility order observed in the separation of tertiary (imipramine (IMI) and amitryptiline (AMI)) and secondary amines (desipramine (DES) and nortryptiline (NOR)) in water, methanol, and acetonitrile. We have determined the p$K_a$ values in those solvents and the variation of dissociation constants with the temperature. From these data, and applying the Van't Hoff equation, we have calculated the thermodynamic parameters $\Delta H$ and $\Delta S$. The p$K_a$ values found in methanol for DES, NOR, IMI, and AMI were 10.80, 10.79, 10.38, and 10.33, respectively. On the other hand, in acetonitrile an opposite relation was found since the values were 20.60, 20.67, 20.74, and 20.81 for DES, NOR, IMI, and AMI. This is the reason why a migration order inversion is observed in NACE for these solvents. The thermodynamic parameters were evaluated and presented a tendency that can be correlated with that observed for p$K_a$ values.
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## 1. Introduction

Non-aqueous capillary electrophoresis (NACE) [1–3] has emerged as an alternative to obtain good separations of hydrophobic analytes without using special additives such as surfactants, cyclodextrins or complexing agents [4–6]. Several studies have been carried out in order to explain the physical chemistry involved in electrophoretic separations where background electrolytes (BGEs) are in presence of organic solvents [7–12]. The first studies have focused on the bulk solvent properties, e.g., viscosity and dielectric constants of organic solvents and how these properties could affect electrophoretic behavior.

In a recent review by Riekkola [2] the properties that affect electrophoretic behavior in NACE were summarized.

Briefly, the solvent relative permittivity, $\varepsilon$, is the major factor that governs ion interactions. Solvents with $\varepsilon < 10$ have no practical use since very little or no ionic dissociation takes place. For $10 < \varepsilon < 30$, in which ionic dissociation occurs, ion-pair formation is the dominant effect. The solvent viscosity directly affects the mobility of ions and thus the electroosmosis. However, the viscosity itself cannot be studied separately since changes in solvent composition also affect other medium properties such as the electrical permittivity and the zeta potential [4,13,14].

Other important issue in NACE study is the observation that the analyte ionization constants in organic media are different from those in aqueous solutions. The mobility of the analyte is a function of the mobility of the fully charged species (actual mobility) and its degree of ionization, which, in turn, is a consequence of the dissociation equilibrium. The dissociation equilibrium depends on the thermodynamic stabilization of reagents and products. When shifting from

aqueous to organic medium the equilibrium is mostly related to the solvent ability in stabilizing charged species. In a series of papers [12,15–18], Kenndler and co-workers reported that the analyte $pK_a$ values are larger in almost all organic solvents commonly used in NACE when compared to aqueous media. The theoretical interpretation for this observation is based on the concept of transfer activity coefficient that consider all species involved in the acid-base equilibrium. A detailed description of such model in the context of CE can be found in [14] and the references cited within.

Since there are several organic solvents with different physical and chemical properties, the $pK_a$ of a single analyte can present different values within quite different scale ranges depending on the medium. Therefore, the selectivity can be enhanced using different pure organic solvents or their mixtures to achieve separations that are impossible to obtain in aqueous media. The pH scale is particular for each solvent and solutions with well-defined pH can be prepared according to the Henderson–Hasselback equation (Eq. (1)) if the $pK_a$ of the BGE in that solvent is known [3].

$$pH = pK_a + \log \frac{[R_3N]}{[R_3N^+H]} \qquad (1)$$

When studying the electrophoretic separation of aliphatic amines using different aqueous and organic media, an inversion in migration order is observed. An example of this behavior was reported by Bjornsdottir and Hansen [4,7] who separated primary, secondary and tertiary amines with closely related structures. In methanol, dimethylformamide, dimethylacetamide and dimethylsulfoxide media, primary amines eluted first, followed by secondary and tertiary amines. On the other hand, in acetonitrile and *N*-methylformamide the migration order was inversed, i.e., tertiary amines were followed by secondary and primary amines.

A detailed understanding of the dissociation equilibria involved in the inversion phenomena may help to construct a model that allows the prediction of separation behavior in NACE. In this work, we have studied the effect of the dissociation equilibrium on the separation of tertiary (imipramine and amitryptiline) and secondary amines (desipramine and nortryptiline) in water, methanol, and acetonitrile. We have determined the $pK_a$ values in those solvents and the variation of dissociation constants with the temperature. From these data and applying the Van't Hoff equation (Eq. (2)) we have calculated the thermodynamic parameters $\Delta H$ and $\Delta S$, which may help to elucidate the inversion in mobility order observed in NACE.

$$\ln K = \frac{\Delta S}{R} - \frac{\Delta H}{R}\frac{1}{T} \qquad (2)$$

## 2. Experimental

### 2.1. Chemicals

All chemicals and solvents were analytical grade. Methanol (MeOH), acetonitrile (ACN), perchloric acid (70%), and acetic acid were purchased from Merck (Darmstadt, Germany). Ammonium acetate, sodium tetraborate ($Na_2B_4O_7 \cdot 10H_2O$), and sodium hydroxide were purchased from Mallinckrodt Baker (Xalostoc, Mexico). The amines standards were purchased as hydrochloride salts from different suppliers as follows: imipramine (IMI) and desipramine (DES) from Ciba–Geigy (São Paulo, Brazil), amitryptiline (AMI) from Sigma (St. Louis, USA), and nortryptiline (NOR) from Sandoz (São Paulo, Brazil). Mesityl oxide was obtained from Rhodia (São Paulo, Brazil).

### 2.2. Sample and BGE solutions

Both analyte stock solutions ($2\,mg\,mL^{-1}$) and analyte mixtures ($100\,\mu g\,mL^{-1}$ for AMI and NOR, $50\,\mu g\,mL^{-1}$ for IMI and DES) were prepared in methanol and used throughout the work.

For $pK_a$ determination in aqueous medium $20\,mM$ sodium borate buffer solutions were prepared with pH 9.0, 9.5, 10.0, and 10.5. BGEs used were prepared with acetic acid (and ammonium acetate), whose $pK_a^*$ in ACN, and methanol are, respectively, 9.7 and 22.4 [19]. In ACN, the acetic acid concentration was fixed in $1\,M$ and the ammonium acetate concentration was varied from 10 to $70\,mM$ (10, 25, 50 and $70\,mM$), giving the pH 20.4, 20.8, 21.1, and 22.25, respectively. For methanol the pH of BGE solutions were 10.0 ($20\,mM$ acetic acid and $40\,mM$ ammonium acetate), and 10.5 ($20\,mM$ acetic acid and $126\,mM$ ammonium acetate).

### 2.3. Instrumentation and CE procedures

CE experiments were carried out in a HP³ᴰCE instrument (Agilent Technologies, Waldbronn, Germany) equipped with a diode-array detector. Analytes were monitored at 214 nm with a bandwidth of 16 nm. A fused silica capillary of 46.5 cm (38 cm to the detection window) × 50 µm i.d. was used and samples were introduced hydrodynamically with 50 mbar by 10 s and separated under 25 kV.

The determination of $pK_a$ by CE is based on the dependence of the analyte electrophoretic mobility as a function of the medium pH. A detailed description of the CE-based method for $pK_a$ determination can be found elsewhere [12,17]. First, the analyte mobilities were measured according to the method by Williams and Vigh [20]. In the first step, a sample plug containing the analyte and the electroosmotic flow (EOF) marker (mesityl oxide) was injected and transferred into the thermostated region of the capillary by applying pressure. It is important to ensure that the electrophoresis will take place within the thermostated region of the capillary since in most of commercial instru-

ments the capillary ends are not under temperature control. Furthermore, it is known that the temperature inside the capillary is not exactly the same as that measured by the instrument cooling system due to Joule effect and the radial heat diffusion. However, since we have worked with organic media, the current during the electrophoretic step was quite low, i.e., less than 30 µA for ACN-based BGE (under 25 kV), therefore such a temperature difference could be neglected. If needed, approaches to calculate the actual temperature inside the capillary can be found elsewhere [21]. With the analytes inside the thermostated region, a run voltage was applied allowing the analyte and the EOF marker to be separated. Next, a second sample plug containing only the EOF marker was injected, and finally all three bands were mobilized through the detection window by pressure. The electrophoretic mobility of the analyte was calculated using the distance between the analyte peak and the EOF marker from the first injection. Pressure delay time (0.1 min), pressure ramp-up time (0.01 min), and voltage ramp-up and rump-down times (both 0.05 min) were considered in the calculations.

The p$K_a$ value was determined by fitting a sigmoidal curve, represented by the (Eq. (3)), in a plot of electrophoretic mobility versus pH. The non-linear regression tool from Microcal Origin 6.0 (Northampton, USA) was used.

$$\mu_{eff} = \frac{\mu_{act}}{10^{(pH^* - pK_a^*)} + 1} \tag{3}$$

where $\mu_{eff}$ is the effective mobility and $\mu_{act}$ the actual mobility (fully charged analytes).

In principle, the determination of only two electrophoretic mobility values is sufficient for p$K_a$ determination of basic analytes [12,17]. The first at low pH, where the amine is fully protonated (actual mobility), and the second at a pH value where it is partially ionized. The actual mobilities were measured in 7.9 mM perchloric acid solutions in water, methanol, and ACN. In all cases the pH was close to 2 since perchloric acid is a strong acid in these media [12]. A third mobility value equal to 0 is obtained in pH about four units above the p$K_a$, where the analyte is completely deprotoned (neutral).

We have used two and four data points (measured in triplicate) for methanol and ACN, respectively for the non-linear curve fitting.

The use of Eq. (3) for p$K_a$ determination implies that the ionic strength should be low and constant. However, the electrolyte solution may have a sufficient buffer capacity in order to hold the pH in the presence of the sample. Therefore, a compromise between low and constant ionic strength and an adequate buffer capacity of the electrolyte is required [22]. In the present work, we did not perform all determinations using a constant ionic strength because, in order to adjust to a desired pH, a varied amount of salt content was needed. To evaluate the variation in mobility values due the change on the ionic strength we have determined the mobilities in ACN and methanol, at the same pH values with different ionic strengths. In ACN, we determined the mobilities in the pH 21.10 at ionic strengths 5 and 50 mM. In methanolic medium, we determined the mobilities in the pH 10 at ionic strengths 4 and 40 mM (data not shown). The observed variation in mobility values was slightly larger than the experimental error inherent to the measurement (triplicate). However, such variation when applied to the mobility curve (mobility versus pH) for p$K_a$ determination, is not statistically different from the error in the fitting process (determination of p$K_a$ value). Furthermore, even if the determined value does not present an extremely high accuracy its precision is reliable enough to allow us to conclude that the relative order of the values is correct.
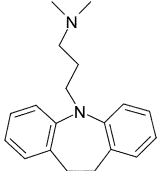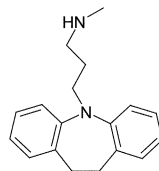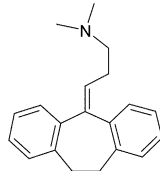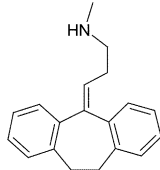
In order to assess thermodynamic parameters ($\Delta H$ and $\Delta S$), the variation of dissociation constants with the temperature was evaluated by determining the p$K_a$ at 16, 20, 25, 30, and 35 °C.

## 3. Results and discussion

### 3.1. Selectivity and p$K_a$ determination

The compounds presented in Table 1 are secondary (DES and NOR) and tertiary (IMI and AMI) amines with similar

Table 1
Structure and properties of model amines used in this work

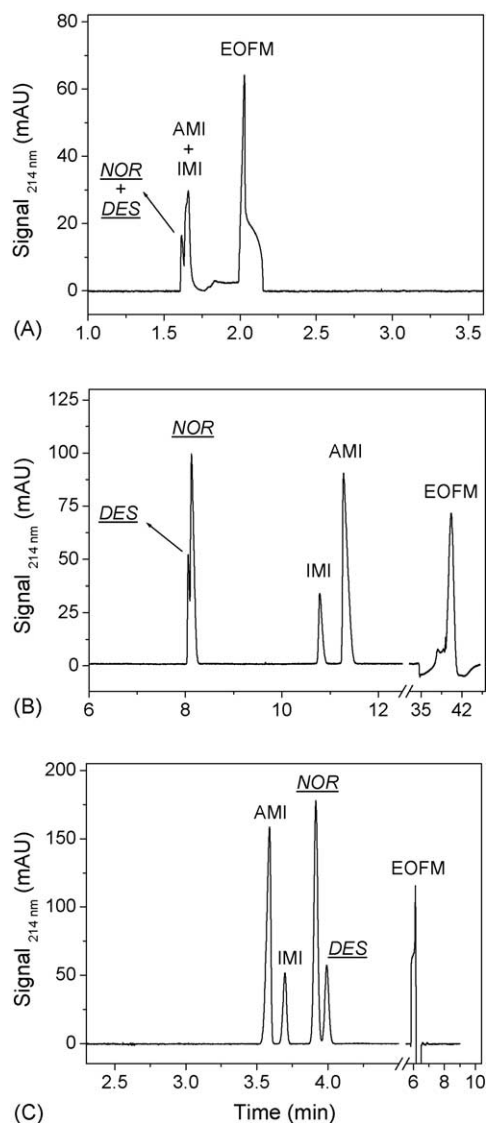| Properties | Compound | | | |
|---|---|---|---|---|
| | Imipramine | Desipramine | Amitryptiline | Nortryptiline |
| Structure |  |  |  |  |
| $M_r$ (g mol$^{-1}$) | 280.4 | 266.4 | 277.4 | 263.4 |
| p$K_a$ in water (Merck Index) | 9.50 | 10.20 | 9.40 | 10.08 |
| p$K_a$ (determined in) | | | | |
| Water | 9.45 | 10.22 | 9.41 | 10.10 |
| MeOH | 10.38 | 10.80 | 10.33 | 10.79 |
| ACN | 20.74 | 20.60 | 20.81 | 20.67 |

Fig. 1. Separation of amines in (A) aqueous buffer (20 mmol L$^{-1}$ borate, pH 10); (B) methanol electrolyte (160 mmol L$^{-1}$ ammonium acetate and 5 mmol L$^{-1}$ acetic acid, pH 11.2); and (C) acetonitrile electrolyte (50 mmol L$^{-1}$ ammonium acetate and 1 mol L$^{-1}$ acetic acid, pH 21.1). The underline/italic labels refer to secondary amines. Analyses were carried out in a 46.5 cm (38 cm to the detection window) × 50 μm i.d. fused silica capillary. The samples were injected by pressure (10 s, 50 mbar) and separated under 25 kV. Amine concentrations: AMI and NOR 100 μg mL$^{-1}$; IMI and DES 50 μg mL$^{-1}$. Mesityl oxide (0.5%, v/v) was used as EOFM.

structures. Using aqueous electrolytes, the separation of these four amines could not be achieved in a broad pH range and using different BGEs (data not shown), nor even in a pH close to the analyte p$K_a$ values (Fig. 1A) where the selectivity in CZE is supposed to be optimal [2]. Using non-aqueous background electrolytes the separations could be obtained partially [4,7] or even completely [23,24].

A curious fact concerning these analytes in NACE is the fact that the migration order is different depending on the organic solvent. An illustration of this fact can be seen in Fig. 1. In MeOH (Fig. 1B) secondary amines eluted first, followed

by tertiary amines. On the other hand, in ACN (Fig. 1C) migration order was completely inversed, tertiary amines were followed by secondary amines.

In order to understand the migration order observed for secondary and tertiary amines in the different solvents we have determined the p$K_a$ of the AMI, IMI, NOR, and DES in aqueous buffer, MeOH and ACN electrolytes using the CE method [20]. The results are presented in Table 1. The p$K_a$ values found for the amines in aqueous buffer are in good agreement with the literature thus confirming the reliability of the CE method used for p$K_a$ determination. Fig. 2 shows the obtained fitting curves for amitriptyline in both ACN and methanol media. The p$K_a$ values of the amines found in organic media were larger than those in water, as expected according to the theoretical model based on the transfer activity coefficients [14]. Furthermore, the p$K_a$ values in ACN are larger than in methanol. This behavior is related to the change of the ionization constants in non-aqueous media relatively to water and reflects the stabilization of the particular species by the solvents. Secondary and tertiary amines are basic compounds, which accept a proton to form an acidic cation (R$_3$N$^+$H). The acid–base equilibrium, which takes place for such an acidic cation under solution is described in Eq. (4).

$$R_3N^+H = R_3N + H^+ \tag{4}$$

As assumed in the theoretical model extensively described by Kenndler and co-workers [14,15] to support CE results, the p$K_a$ variation depends on the transfer activity coefficients, $_m\gamma$, of the species involved in the equilibrium. The Eq. (5) describes the p$K_a$ variation ($\Delta$p$K_{HB}^+$) observed when shifting from water to any organic solvent.

$$\Delta pK_{HB^+} = pK_{a\,HB^+}^{solvent} - pK_{a\,HB^+}^{water} = \log\left[\frac{(_m\gamma_{H^+})(_m\gamma_B)}{_m\gamma_{HB^+}}\right] \tag{5}$$

In the case of acidic cations (HB$^+$), the contribution of neutral molecules to the p$K_a$ variation may be neglected since it is small when compared with those of ionic species [14]. Therefore, the solvent ability to solvate only cations (H$^+$ and HB$^+$) determines the extension of p$K_a$ variation. ACN has almost no ability to solvate cations [14], what means that this solvent cannot accept the H$^+$ from the acid R$_3$NH$^+$. According to this model, the p$K_a$ is expected to be larger as the basicity of the solvent decreases. In conclusion, our results are in agreement with this theoretical model since ACN is far less basic than MeOH.

A closer look to the p$K_a$ values revealed that the secondary amines presented p$K_a$ values larger than the tertiary amines in MeOH. On the other hand, in ACN an opposite relation is found. This behavior is likely the answer to the migration order observed.

The theory of transfer activity coefficient concerns on a change in p$K_a$ when going from water to an organic solvent but do not anticipate quantitatively the extension of such change unless experimental measures are used. It means that
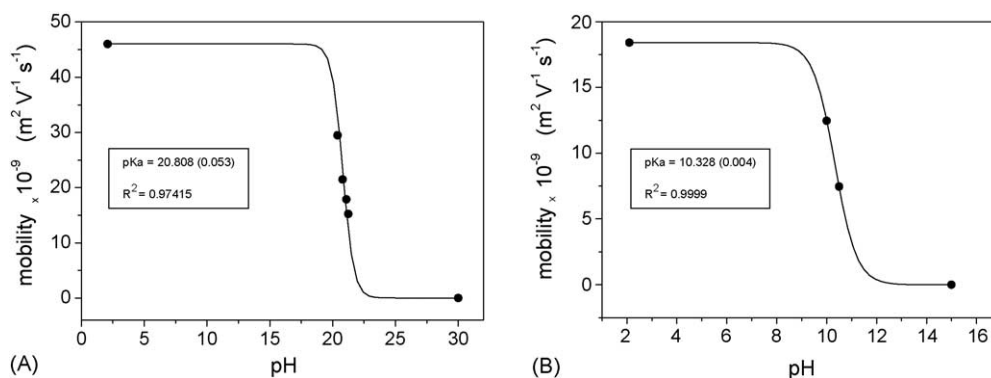
Fig. 2. Fitted curves of amitriptyline for $pK_a$ determination according to Eq. (3). (A) ACN medium and (B) methanolic medium. Experimental conditions for each data point are described in Section 2.

an inversion of $pK_a$ order cannot be intuitively drawn from such a model before experimental data, such as $pK_a$ measurements, are available. On the other hand, it is well known that solvent interactions with the analytes play an important role in mobility behavior since the basic nature of amines depends on such interactions. Basically, the basicity (or $pK_a$) order relies on the solvation power of the charged and neutral amine species, which in turn result from steric and electronic inductive effects of the substituents.

In aprotic protophobic solvents such as ACN, the interaction with the solvent plays a less important role and the electronic inductive effect prevails. Therefore, tertiary amines are more basic (higher $pK_a$) than secondary amines due to higher number of alkyl groups attached to the nitrogen. On the other hand, in methanol, an amphiprotic solvent, the possibility of forming hydrogen bonds, donating or accepting a proton makes the interaction between solvent and amines the most important factor. In this case, secondary amines are more basic than the tertiary since the former interact more closely due to lower steric effect from the substituents. Nevertheless, if one takes the example from the work by Hansen and co workers [4], the inversion order was also observed for solvent pairs as similar as dimethylformamide and *N*-methylformamide. In such a case, it is not straightforward to use the explanation described above to predict the basicity without experimental measures. Therefore, the approach presented in this work might be followed by other groups interested in getting experimental $pK_a$ or basicity series.

In conclusion, it is clear that the analyte $pK_a$ in each solvent is responsible for the migration order inversion. In order to understand better the thermodynamics of such $pK_a$ variations, we have tried to measure the enthalpic and entropic contributions associated to the dissociation process.

### 3.2. Determination of thermodynamic parameters

The determination of the thermodynamic parameters, $\Delta H$ and $\Delta S$, was performed by determining the $pK_a$ of each compound at different temperatures. From these values the respective equilibrium constants, $K_a$, were calculated and ap-

plied to the Van't Hoff equation (Eq. (2)) [25]. This equation relates the equilibrium constant with the temperature. Assuming that $\Delta H$ and $\Delta S$ are constant in the temperature range, a plot of $\ln K$ versus $1/T$ yields a straight line. The parameters $\Delta H$ and $\Delta S$ can than be obtained, respectively, from the slope and intercept coefficients.

The measurements were carried out for all four compounds in acetonitrile and methanol in temperatures ranging from 16 to 35 °C. The results are presented in Fig. 3 and Table 2.

It is of basic knowledge that the thermodynamic parameters $\Delta H$ and $\Delta S$ indicate the spontaneity of an equilibrium process [26]. In Fig. 3 each analyte pair (the tertiary and the respective secondary amine) the $\Delta H$ and $\Delta S$ have values in inverted order regarding the solvent. For instance, IMI
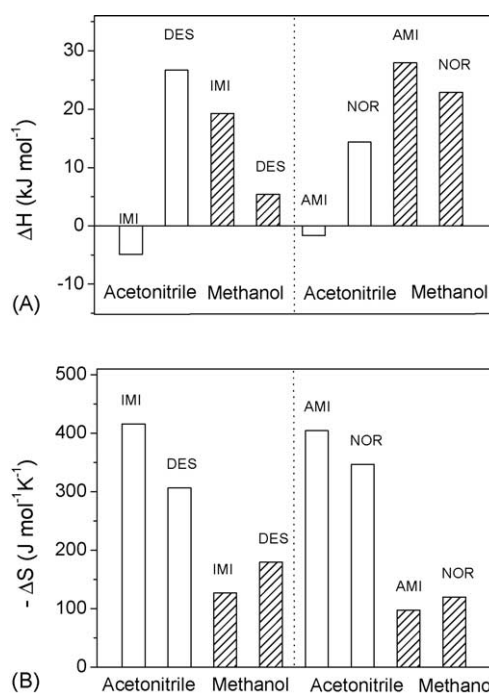


Fig. 3. Variation of enthalpy, $\Delta H$ (A) and entropy, $\Delta S$ (B) calculated for the compounds in methanol and acetonitrile.

Table 2
Thermodynamic parameters for all studied analytes determined in acetonitrile and methanol ($T = 298$ K)

| Solvent | Compound | $\Delta H$ (kJ mol$^{-1}$) | $T\Delta S$ (kJ mol$^{-1}$) |
|---------|----------|---------|---------|
| ACN | AMI | −1.7 | −120.5 |
| | IMI | −4.9 | −124.0 |
| | NOR | 14.4 | −103.3 |
| | DES | 26.7 | −91.4 |
| Methanol | AMI | 28.0 | −29.1 |
| | IMI | 19.3 | −37.7 |
| | NOR | 22.9 | −35.7 |
| | DES | 5.4 | −53.4 |

(tertiary amine) has a $\Delta H$ value lower than DES (secondary amine) in acetonitrile, while in methanol, the $\Delta H$ of IMI is higher than DES (Fig. 3A). This trend is also valid for the pair AMI (tertiary amine) and NOR (secondary amine). Furthermore, the same is observed for the parameter $\Delta S$ (Fig. 3B).

Since the thermodynamic parameters represent the spontaneity of the equilibrium reaction, the inversion in their values is likely to be the explanation for the inversion in p$K_a$ values and the consequent inversion in the electrophoretic mobility.

Another interesting feature regarding the equilibrium thermodynamics is the evaluation of the effect that governs the process, the enthalpy or the entropy. According to Eq. (6), an equilibrium process might be described by the Gibbs free energy, which sums up the contribution of the enthalpic term, $\Delta H$, and the entropic term, $T\Delta S$. The values of these parameters are presented in Table 2.

$$\Delta G = \Delta H - T\Delta S \tag{6}$$

In acetonitrile, the entropic contribution is much greater than the enthalpic one. It is well established that ACN presents poor cation solvation ability and almost none capability to act as a proton donor or acceptor to form hydrogen bonds [3,14]. These characteristics indicate that the possibility of interaction between the analyte and the solvent is quite small. This means that the enthalpic contribution is supposed to be also small, as experimentally observed. Regarding the entropic term, the signal is negative for all analytes and the magnitudes are greater than the enthalpic term. It implies that the dissociation process is not spontaneous and that somehow it increases the order of the system. Although the $\Delta H$ for IMI and DES are negative, their values are small in magnitude and the entropic contribution seams to be the driving force in the dissociation process for the four analytes.

When methanol is the solvent, the enthalpic and entropic effects are quite similar in magnitude indicating that there is not a predominant factor. Methanol is able to make several kinds of intermolecular interactions, including hydrogen bonds with the analyte and within itself. The enthalpic contribution could be assigned to the break and formation of

hydrogen bonds between analytes and the solvent. The entropic variation might be related to variations in the system order since this solvent is supposed to be fairly organized, for instance, in a hydrogen-bond network.

## 4. Conclusions

The secondary amines presented p$K_a$ values larger than the tertiary amines when in MeOH. On the other hand, in ACN an opposite relation is found. This is the reason why a migration order inversion is observed in NACE for these solvents. The thermodynamic parameters were evaluated and presented a tendency that can be correlated with that observed for p$K_a$ values. The entropic effect seams to play the major role in the dissociation process in acetonitrile. In methanol, both, the entropic and the enthalpic effects are similarly important.

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