**Elisabet Fuguet[1, 2]**
**Mario Reta[3]**
**Carme Gibert[1]**
**Martí Rosés[1, 2]**
**Elisabeth Bosch[1, 2]**
**Clara Ràfols[1, 2]**

[1]Departament de Química Analítica, Universitat de Barcelona, Barcelona, Spain
[2]Institut de Biomedicina Universitat de Barcelona, Barcelona, Spain
[3]División Química Analítica, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Buenos Aires, Argentina

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Research Article

# Critical evaluation of buffering solutions for p$K_a$ determination by capillary electrophoresis

The performance of the most common and also some other less common CE buffers has been tested for the p$K_a$ determination of several types of compounds (pyridine, amines, and phenols). The selected buffers cover a pH ranging from 3.7 to 11.8. Whereas some buffers, like acetic acid/acetate, BisTrisH$^+$/BisTris, TrisH$^+$/Tris, CHES/CHES$^-$, and CAPS/CAPS$^-$ can be used with all type of analytes, others like ammonium/ammonia, butylammonium/butylammonia, ethylammonium/ethylammonia, diethylammonium/diethylammonia, and hydrogenphosphate/phosphate are not recommended because they interact with a wide range of compounds. The rest of the tested buffers (dihydrogenphosphate/hydrogenphosphate, MES/MES$^-$, HEPES/HEPES$^-$, and boric acid/borate) can show specific interactions depending on the nature of the analytes, and their use in some applications should be restricted.

**Keywords:**
Buffer-analyte interactions / Capillary electrophoresis buffers / Capillary electrophoresis p$K_a$ determination
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## 1 Introduction

In the last years the growing in the development of new compounds, especially in the pharmaceutical industry, has caused an increase in the demand of fast and highly automated methods for the determination of physicochemical properties of different types of compounds. Furthermore, physicochemical properties have to be fast determined in a very early stage of the design, many times with very low amounts of material available and low purity levels, in order to discard the compound when it does not accomplish the requirements for a certain application. A very relevant property for an acid–base compound is its dissociation constant since it is a key parameter in processes such as chemical reactivity, adsorption, distribution, and biological activity among others [1, 2].

Potentiometric and spectrophotometric titrations are reference methods for p$K_a$ determination [3]. They are well-established methods with strong commercial support [2, 4–9]. CE has been gaining importance in the last years as a method for p$K_a$ determination because it requires very low amounts of samples and solvents and, as it is a separation technique, problems with impurities are overcome [10–15].

**Correspondence:** Dr. Clara Ràfols, Departament de Química Analítica, Universitat de Barcelona, Martí i Franquès 1-11, E-08028 Barcelona, Spain
**E-mail:** crafols@ub.edu
**Fax:** +34-934021233

The CE method is based on the measure of the electrophoretic mobility of the analyte as a function of the pH of the BGE. Then p$K_a$ is easily calculated fitting the experimental points to a suitable model, which depends on the nature of the compound and the number of ionizable groups. Anyway, to obtain reliable results several general considerations have to be taken into account before performing a p$K_a$ determination by CE. The main parameters affecting the mobility are pH, temperature, and ionic strength [12–16]. Some other items that also have some influence on mobility are the viscosity of the BGE, the electrolysis of the buffers due to the applied voltage, and the dissolution of atmospheric $CO_2$ in the BGE solutions [14, 15, 17].

The most common CE BGEs are phosphate, acetate, formate, borate, and several zwitterionic compounds [12–14, 17–19]. Nevertheless, sometimes there are interactions concerning these buffers, which can change the migration behavior of the analyte. Then deviations in the mobility *versus* pH plot are observed, and the p$K_a$ can not be accurately determined. This is the case, for example, of borate buffer which can complex vicinal diol-groups modifying in this way the electrophoretic mobility of diol compounds [17, 20]. In previous work we also observed a distortion in the mobility values of protonated amines when phosphate or borate was used as buffer [21]. In a number of cases literature report different p$K_a$ values for the same compound, all of them determined by CE but with different buffers, which presumably can be attributed to interactions between the compound and some of the buffers. For instance, we can found

$pK_a$ values for codeine of 7.97 (determined with acetate and phosphate buffers) [22], 8.20 (with Tris, ethanolamine, and acetate) [23], or 8.25 (with Tris, ethanolamine, and diethylamine) [24].

The aim of this work is to test the suitability of the most common and some other less common CE buffers for $pK_a$ determination by CE, discuss their possible interactions with some typical types of compounds and select the best buffers for a fast and accurate $pK_a$ determination. The buffers selected involve a wide range of pH, from 3.7 to 11.8 and have been tested with compounds of different nature (pyridine, amines, and phenols) that cover $pK_a$ values between 5 and 10.

## 2 Theory

The effective electrophoretic mobility, $\mu_{eff}$, of a compound can be expressed as a function of the $pK_a$ values of each species and the pH of the BGE by the following general equation [17]:

$$\mu_{eff} = \frac{\mu_{H_nA^z} + \sum_{i=1}^{n} 10^{ipH-\sum_{j=1}^{i} pK'_{aj}} \mu_{H_{n-i}A^{z-i}}}{1 + \sum_{i=1}^{n} 10^{ipH-\sum_{j=1}^{i} pK'_{aj}}} \quad (1)$$

where $\mu_{H_nA^z}$ is the mobility of the fully protonated species, $\mu_{H_{n-i}A^{z-i}}$ the mobility of the successive deprotonated species and $K'_a$ is a conditional acidity constant which is related to the thermodynamic acidity constant by the activity coefficient ($\gamma$) of the involved solutes. This coefficient corrects the effects of the ionic strength on solute ionization.

The following equation can be derived from Eq. (1) for a neutral monoprotic acid (HA):

$$\mu_{eff} = \frac{\mu_{A^-}}{1 + 10^{pK'_a - pH}} \quad (2)$$

where $\mu_A$ is the mobility of the deprotonated species and $pK_a'$ is related to the thermodynamic $pK_a$ through Eq. (3):

$$pK_a = pK'_a - \log \gamma_{A^-} \quad (3)$$

For neutral monoprotic bases (B) Eq. (1) can be expressed as Eq. (4):

$$\mu_{eff} = \frac{\mu_{BH^+}}{1 + 10^{pH - pK'_a}} \quad (4)$$

where $\mu^+_{BH}$ is the mobility of the protonated species and $pK'_a$ and $pK_a$ are related through Eq. (5):

$$pK_a = pK'_a + \log \gamma_{BH^+} \quad (5)$$

The $\mu_{eff}$ of analytes are determined at different buffer pH using Eq. (6), where $V$ is the applied voltage, $L_D$ the effective capillary length to the detector, $L_T$ the total capillary length, $t_m$ the migration time of the analyte, and $t_{EOF}$ is the migration time of the neutral marker due to the EOF.

$$\mu_{eff} = \frac{L_D L_T}{V}\left(\frac{1}{t_m} - \frac{1}{t_{EOF}}\right) \quad (6)$$

The experimental $\mu_{eff}$ values were fitted to Eq. (2) (acids) or Eq. (4) (bases) by the commercial software origin, version 7.0 from OriginLabs.

## 3 Materials and methods

### 3.1 Reagents and chemicals

Sodium dihydrogenphosphate monohydrate (>99%), sodium hydrogenphosphate (>99%), ammonium chloride (>99.8%), benzyl alcohol (p.a.), sodium hydroxide 0.5 M, hydrochloric acid (25%), pyridine (>99.7%), and 1-aminoethylbenzene (>99%) were from Merck (Darmstadt, Germany). Sodium acetate anhydrous (>99.6%) was purchased from J. T. Baker (Deventer, Holland). 2-Morpholinoethanesulfonic acid hydrate (MES, >99%), 2-(cyclohexylamino)ethanesulfonic acid (CHES, >99%), 3-(cyclohexylamino)1-propanesulfonic acid (CAPS, >98%), ethanolamine hydrochloride (>98%), ampicillin (>96%), codeine, diphenhydramine hydrochloride (>98%), nortriptyline hydrochloride (>98%), procainamide hydrochloride (>98%), propranolol hydrochloride, quinine, trimipramine maleate salt (>99%), trazodone hydrochloride, 3-nitrophenol (>99%), and resorcinol (>99%) were from Sigma (St. Louis, MO, USA). *N*-2-(Hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES, >99%), BisTris (2,2-Bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol), diethanolamine, 4-nitrophenol (99%), and catechol (>99%) were from Fluka (Buchs, Switzerland). Tris (>99.9%), butylamine (>99.5), 3-nitrophenol (99%), 3-bromophenol (98%), 4-bromophenol (99%), and 3,5-dichlorophenol (97%) were purchased from Aldrich (Milwaukee, WI, USA). Sodium tetraborate decahydrate (>99%) was from Probus (Barcelona, Spain). All solutions were prepared with ultrapure water (Milli-Q deionizer, Millipore, Bedford, MA, USA).

### 3.2 Instrumentation and operational conditions

Experiments were performed using two different Beckman (Palo Alto, CA, USA) instruments: A P/ACE 5500 equipped with a diode-array spectrophotometric detector, and a P/ACE 2210 equipped with an UV detector. A fused-silica capillary of 50 μm id, 375 μm od, and 47 cm of total length (40 cm to the detector) obtained from Composite Metal Services (Ilkley, UK) was used to carry out the experimental mobility determinations. The temperature of the capillary was kept at 25.0°C (±0.1°C). Samples were injected hydrodynamically at 0.5 psi for 2 s (1 psi = 6894.76 Pa) and the applied voltage was 20 kV. UV detection was carried out at 214 nm.

 

Several methodologies were followed for conditioning the capillary. Before the first use, the capillary was conditioned at 25°C as follows: 1 M NaOH (20 min), water (10 min), and finally the running buffer (35 min). Between runs the capillary was rinsed 6 min with the running buffer, and after five or six runs the running electrolyte was renewed. When the pH of the running buffer was changed but the electrolyte was the same, the capillary was conditioned with water (1 min), 0.1 M NaOH (1 min), water (1 min), and the new running buffer (6 min). When a buffer of different nature was used, then the capillary was conditioned with 0.1 M NaOH (6 min), water (10 min), and the new running buffer (35 min). At the end of each working session, the capillary was rinsed with 0.1 M NaOH (10 min), water (15 min), and $N_2$ (1 min).

pH measurements were taken with a Ross combination electrode Orion 8102 in a Crison micropH 2002 potentiometer with a precision of ±0.1 mV (±0.002 pH units).

### 3.3 Buffer and sample preparation

Several running solutions covering the pH range between 3.70 and 11.80 were prepared. Table 1 shows the employed buffers, its p$K_a$ value, the covered pH range, and the stock solutions used in their preparation. In order to obtain a desired pH value, the stock solutions were mixed in an adequate proportion, and diluted to have an ionic strength of 0.05 M. At least five running solutions at different pH values were prepared with the same electrolyte, covering the pH range indicated in Table 1. The pH of each running buffer was measured immediately after its preparation, and just before the mobility measurements.

In a previous paper [17], we studied the influence of the electrolysis of running buffers caused by the CE separation voltage and the absorption of the $CO_2$ from the air on the final buffer's pH. These factors are relevant for running buffers with a pH higher than 10 [14, 22]. In order to minimize these effects in our determinations, the running buffer has been changed every five or six runs, and the alkaline running buffers have been used immediately after preparation.

Stock solutions of analytes were prepared at a concentration of 1000 μg/mL in water. After that, they were diluted with water to a concentration of 100 μg/mL. Benzyl alcohol was added (100 μg/mL) and used as EOF marker for the calculation of the effective mobility ($\mu_{eff}$). All the samples were injected at least three times in order to assure reproducibility. Usually the RSD of mobility values was less than 4%. Sometimes, when the $\mu_{eff}$ was close to zero or when a running buffer with high pH was used, the RSD was higher than 4% but less than 8%.

All running buffers and samples were filtered through a 0.45 μm pore size nylon filter (Whatman, Maidstone, Kent, UK) and stored at 4°C until used.

## 4 Results and discussion

The precise determination of the p$K_a$ of an ionizable compound by CE requires the measurement of the effective mobility in several electrolyte solutions of different pH with a low and constant ionic strength. The first step is the choice of the different buffers needed to cover the desired pH range. These buffers must be adequate as running buffers in CE [14, 15, 17, 25] and the ionic strength has to be kept constant

**Table 1.** Stock solutions used for the preparation running buffers at 0.05 M ionic strength

| Buffer constituents | p$K_a$[a)] | Covered pH range | Stock solutions |
|---|---|---|---|
| $CH_3COOH/CH_3COO^-$ | 4.76 | 3.70– 5.80 | 0.1 M $CH_3COONa$ + 0.5 M HCl |
| $MES/MES^-$ | 6.15 | 5.00– 7.20 | 0.1 M NaMES + 0.5 M HCl |
| $BisTrisH^+$/BisTris | 6.48 | 5.50– 7.50 | BisTrisHCl + 0.5 M NaOH |
| $H_2PO_4^-/HPO_4^{2-}$ | 7.21 | 5.80– 8.20 | 0.1 M $NaH_2PO_4 \cdot H_2O$ + 0.1 M $Na_2HPO_4$ |
| $HEPES/HEPES^-$ | 7.51 | 6.50– 8.70 | 0.2 M NaHEPES + 0.5 M HCl |
| $TrisH^+$/Tris | 8.08 | 7.00– 9.00 | 0.1 M TrisHCl + 0.5 M NaOH |
| $(EtO)_2NH_2^+/(EtO)_2NH$[b)] | 8.88 | 8.00–10.10 | 0.1 M $(EtO)_2NH_2Cl$ + 0.5 M NaOH |
| $H_3BO_3/H_3BO_2^-$ | 9.50 | 8.00–10.60 | 0.1 M $H_3BO_2Na$ + 0.5 M HCl |
| $NH_4^+/NH_3$ | 9.25 | 8.20–10.20 | 0.1 M $NH_4Cl$ + 0.5 M NaOH |
| $CHES/CHES^-$ | 9.50 | 8.40–10.10 | 0.2 M NaCHES + 0.5 M HCl |
| $EtONH_3^+/EtONH_2$[c)] | 9.50 | 8.50–10.80 | 0.2 M $EtONH_3Cl$ + 0.5 M NaOH |
| $BuNH_3^+/BuNH_2$[d)] | 10.66 | 9.20–11.10 | 0.2 M $BuNH_3Cl$ + 0.5 M HCl |
| $CAPS/CAPS^-$ | 10.40 | 9.40–11.60 | 0.2 M NaCAPS + 0.5 M HCl |
| $HPO_4^{2-}/PO_4^{3-}$ | 12.32 | 10.80–11.80 | 0.1 M $Na_3PO_4 \cdot 12H_2O$ + 0.1 M $Na_2HPO_4$ |

a) From ref. [15, 45].
b) Diethanolammonium/diethanolamine.
c) Ethanolammonium/ethanolamine.
d) Butylammonium/butylamine.

 

throughout the series, in order to eliminate the influence of this parameter on raw effective mobilities [17, 26]. Moreover, the concentration of the running buffers must be low enough to minimize activity corrections, temperature gradients, and viscosity differences, but they must have enough buffer capacity to maintain a fixed pH [14]. An ionic strength of 0.05 M has been selected as a reasonable compromise between the need to minimize the Joule heat production inside the capillary and the requirement of enough buffering capacity [12–15, 27]. The chosen buffers allow an acceptable current intensity between 20 and 60 μA without any appreciable Joule effect.

The buffers for this study have been selected in such a way that any specific pH range can be covered by more than one buffering agent, as shown in Table 1. These buffers have been tested with different types of compounds: pyridine, amino compounds, and phenol derivatives, which are listed in Tables 2 and 3. These compounds have been chosen because of their well known and convenient p$K_a$ values (between 5 and 10). At acidic pH, the phenols are neutral compounds but pyridine and amino derivatives are cationic species. Ampicillin is a diprotic compound which is in its zwitterionic form in the pH range of 4–5.5 and the anionic species predominates at higher pH. The effective mobilities ($\mu_{eff}$) of the different compounds have been plotted in front of pH for each running buffer. As a typical example Fig. 1 shows the plots obtained for propranolol (base) and 3-bromophenol (acid).

In order to test the performance of the different buffers, the experimental mobility data for each solute was fitted to either Eq. (2) (acids) or Eq. (4) (bases) by using the average literature p$K_a$ at the working ionic strength (p$K'_a$) (see Tables 2 and 3). That is, only the $\mu_A^-$ (acids) or $\mu_{BH}^+$ (bases) value was calculated to obtain the best fit for each solute. The $\mu_{eff}$ *versus* pH curve obtained in this way is also plotted in Fig. 1. The variation of the experimental mobility data with pH fits these lines with small deviations that for most buffers can be attributed to random variability of electroosmotic mobility. In some instances (at high pH for bases and low pH for acids) this random variability may produce $\mu_{eff}$ values very close to zero or even slightly negative (*e.g.*, see quinine at pH 10.7 or trazodone at pH 10.7 or 11.3 in Fig. 2). However, it is evident that deviations from the expected plot much larger than those attributable to random variations of $t_m$ or $t_{EOF}$ can be clearly observed for some buffers: $NH_4^+/NH_3$, $BuNH_3^+/BuNH_2$, and $HPO_4^{2-}/PO_4^{3-}$. This is a general behavior observed for all studied solutes (acids and bases) which can be attributed to undesired buffer properties or interactions with solutes or the capillary.

The use of ammonium buffer often produces anomalous $\mu_{eff}$ values because of the ammonia volatility and to obtain reproducible $\mu_{eff}$ values is a difficult task. When ammonia is volatilized in the buffer reservoirs, the buffer properties, such as pH and ionic strength, change and then the $\mu_{eff}$ of the analyte is different than the expected one. Thus, we do not recommend this buffer for secure p$K_a$ determination.

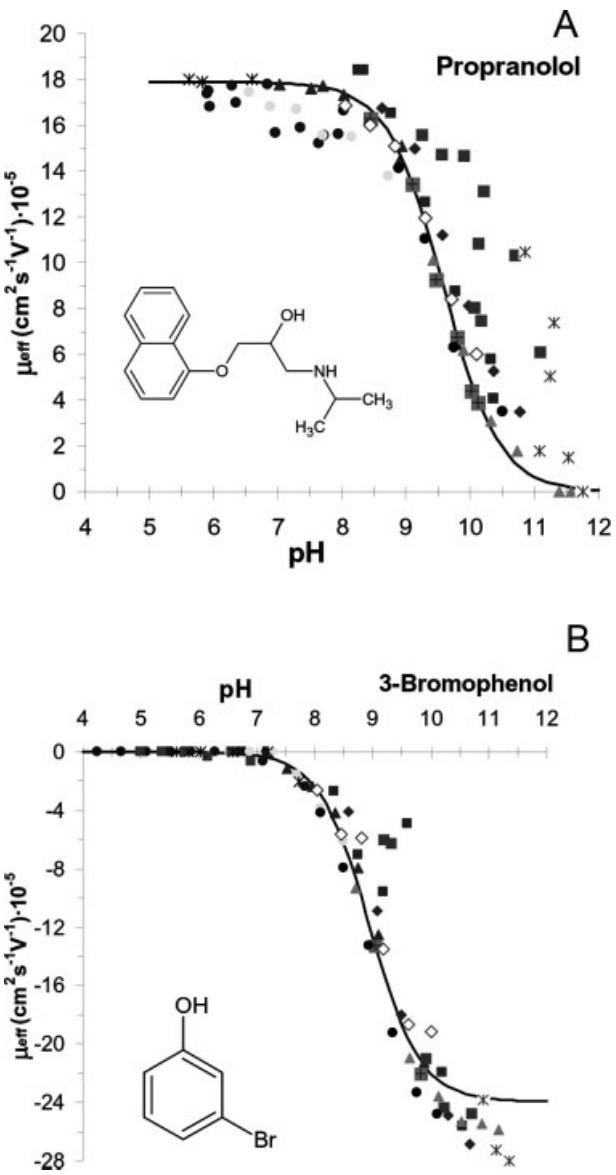


**Figure 1.** Electrophoretic mobilities of propranolol (A) and 3-nitrophenol (B) *versus* pH of the BGE, with curves fitted according to Eq. (4) and Eq. (2) respectively and with fixed literature p$K'_a$ value. The used buffers are represented by the following symbols: $CH_3COOH/CH_3COO^-$ (•), MES/$MES^-$ (■), $BisTrisH^+$/BisTris (*), $H_2PO_4^-/HPO_4^{2-}$ (•), HEPES/$HEPES^-$ (•), $TrisH^+$/Tris (▲), $(EtO)_2NH_2^+/(EtO)_2NH$ (◇), $H_3BO_3/H_3BO_2^-$ (•), $NH_4^+/NH_3$ (■), CHES/$CHES^-$ (■), $EtONH_3^+/EtONH_2$ (◆),$BuNH_3^+/BuNH_2$ (■), CAPS/$CAPS^-$ (▲), $HPO_4^{2-}/PO_4^{3-}$ (*).

The use of butylammonium buffer produces $\mu_{eff}$ values higher (more positive for amines and less negative for phenols) than those obtained with the other buffers. It is well known that alkylamines attach to the capillary wall through ionic interactions with the silanolate groups reducing the activity of the silanols, and then reducing the electroosmotic flow [15, 16, 28–30]. Nevertheless, this statement does not

 

**Table 2.** $pK'_a$ ($I$ = 50 mM), $pK_a$ and $pK_{a(bibl)}$ for the studied amines and pyridine

| | $pK'_a$ [a] | sd | $pK_a$ [b] | $pK_{a(bibl)}$ [c] | $pK_a - pK_{a(bibl)}$ |
|---|---|---|---|---|---|
| Pyridine | 5.33 ± 0.02 | 0.706 | 5.24 | 5.22[d] | 0.02 |
| | | | | 5.25[e] | −0.01 |
| Trazodone | 6.96 ± 0.02 | 0.331 | 6.87 | 6.69[f] | 0.18 |
| | | | | 6.93[f] | −0.06 |
| | | | | 6.78[f] | 0.09 |
| Ampicillin | 7.13 ± 0.03 | 0.495 | 7.22 | 7.24[d] | −0.02 |
| Codeine | 8.32 ± 0.03 | 0.527 | 8.24 | 8.20[d] | 0.04 |
| | | | | 8.21[e] | 0.03 |
| | | | | 8.25[g] | −0.01 |
| | | | | 8.22[g] | 0.02 |
| Quinine | 8.56 ± 0.02 | 0.405 | 8.48 | 8.52[g] | −0.04 |
| Diphenhydramine | 9.25 ± 0.02 | 0.426 | 9.17 | 9.12[g] | 0.05 |
| | | | | 9.10[h] | 0.07 |
| Procainamide | 9.43 ± 0.01 | 0.323 | 9.35 | 9.24[h] | 0.11 |
| Propranolol | 9.56 ± 0.01 | 0.333 | 9.48 | 9.53[i] | −0.05 |
| | | | | 9.45[j] | 0.03 |
| | | | | 9.48[k] | 0.00 |
| | | | | 9.50[k] | −0.02 |
| 1-Aminoethylbenzene | 9.58 ± 0.03 | 0.754 | 9.49 | 9.43[e] | 0.06 |
| Nortriptyline | 10.23 ± 0.04 | 0.787 | 10.14 | 10.02[f] | 0.12 |
| | | | | 10.14[f] | 0.00 |
| | | | | 10.10[f] | 0.04 |

a) Buffers selected: Acetate, BisTris, Tris, CHES and CAPS.
b) $pK_a$ value after ionic strength correction ($\log\gamma = -0.09$).
c) Literature values.
d) From ref. [46].
e) From ref. [47].
f) From ref. [44].
g) From ref. [48].
h) From ref. [49].
i) From ref. [50].
j) From ref. [51].
k) From ref. [4].

**Table 3.** $pK'_a$ ($I$ = 50 mM), $pK_a$ and $pK_{a(bibl)}$ for the studied phenols

| | $pK'_a$ [a] | sd | $pK_a$ [c] | $pK_{a(bibl)}$ | $pK_a - pK_{a(bibl)}$ |
|---|---|---|---|---|---|
| 4-Nitrophenol | 7.10 ± 0.02 | 0.826 | 7.19 | 7.18[d] | 0.01 |
| | | | | 7.16[e] | 0.03 |
| 3,5-Dichloro-phenol | 8.16 ± 0.02 | 0.695 | 8.21 | 8.18[e] | 0.07 |
| 3-Nitrophenol | 8.37 ± 0.02 | 0.645 | 8.42 | 8.36[d] | 0.09 |
| | | | | 8.38[e] | 0.07 |
| 3-Bromophenol | 8.98 ± 0.02 | 0.638 | 9.01 | 9.01[d] | 0.06 |
| | | | | 9.06[e] | 0.01 |
| | | | | 9.03[e] | 0.04 |
| 4-Bromophenol | 9.36 ± 0.02 | 0.454 | 9.40 | 9.34[e] | 0.10 |
| | | | | 9.36[e] | 0.08 |
| Catechol | 9.45 ± 0.01[b] | 0.363 | 9.54 | 9.46[f] | 0.08 |
| Resorcinol | 9.43 ± 0.02 | 0.647 | 9.50 | 9.49[f] | 0.02 |

a) Buffers selected: acetate, Bis-Tris, Tris, MES, hydrogenphosphate, HEPES, borate, CHES, and CAPS.
b) Borate is excluded.
c) $pK_a$ value after ionic strength correction ($\log\gamma = -0.09$).
d) From ref. [46].
e) From ref. [52].
f) Obtained in our laboratory.

justify the observed behavior for amines, whose protonated form migrates to the detector before than expected. In the case of phenols, we attribute the decrease in mobility to partial formation of a neutral ion pair between the phenolate anion and the butylammonium cation. This behavior is more significant when the running buffer has a pH value lower than the $pK_a$ of butylammonium, *i.e.*, most of the buffering agent is in its protonated form. In fact, ion pair formation between phenolate and quaternary ammonium salts has been already described in the literature [31].

Phosphate buffer presents also some troubles at high pH values. With this buffer it is difficult to obtain reproducible values of $\mu_{eff}$ and often the value obtained is not consistent with the expected one, *i.e.*, deviations from the general plot are observed. This behavior has been attributed to the electrochemical reactions at the electrodes and the absorption of carbon dioxide from the air, which can make pH drop up to 0.24 pH units [14, 32]. In order to minimize possible causes for the deviations, the phosphate running buffers used in this work were freshly prepared and the pH was measured just before their use. Moreover, the buffer reservoirs were changed very frequently (every four or five runs). Despite these cautions, we obtained poor reproducibility in agreement with the statement of Boček and co-workers [33–35], who pointed out that phosphate buffer, in particular at high pH values, can be not adequate as a buffer because it may affect crucially the migration behavior of analytes.

Because of the troubles commented above, we rejected $NH_4^+/NH_3$, $BuNH_3^+/BuNH_2$, and $HPO_4^{2-}/PO_4^{3-}$ buffers in the $pK_a$ determination. Then, in Figs. 2–4 we plotted the experimental data for all the compounds studied except the data corresponding to the buffers mentioned above. Like in Fig. 1 we fitted the data to Eq. (2) or Eq. (4) using the known $pK_a$ values of the compounds (Tables 2 and 3) after ionic strength correction. For some compounds it is observed that the $\mu_{eff}$ values obtained by different buffers in the same pH region differ. For instance, for quinine in the pH range of 7–8, the $\mu_{eff}$ obtained from $H_2PO^{4-}/HPO_4^{2-}$ buffers are lower than the ones obtained with HEPES/$HEPES^-$ buffers, and

 

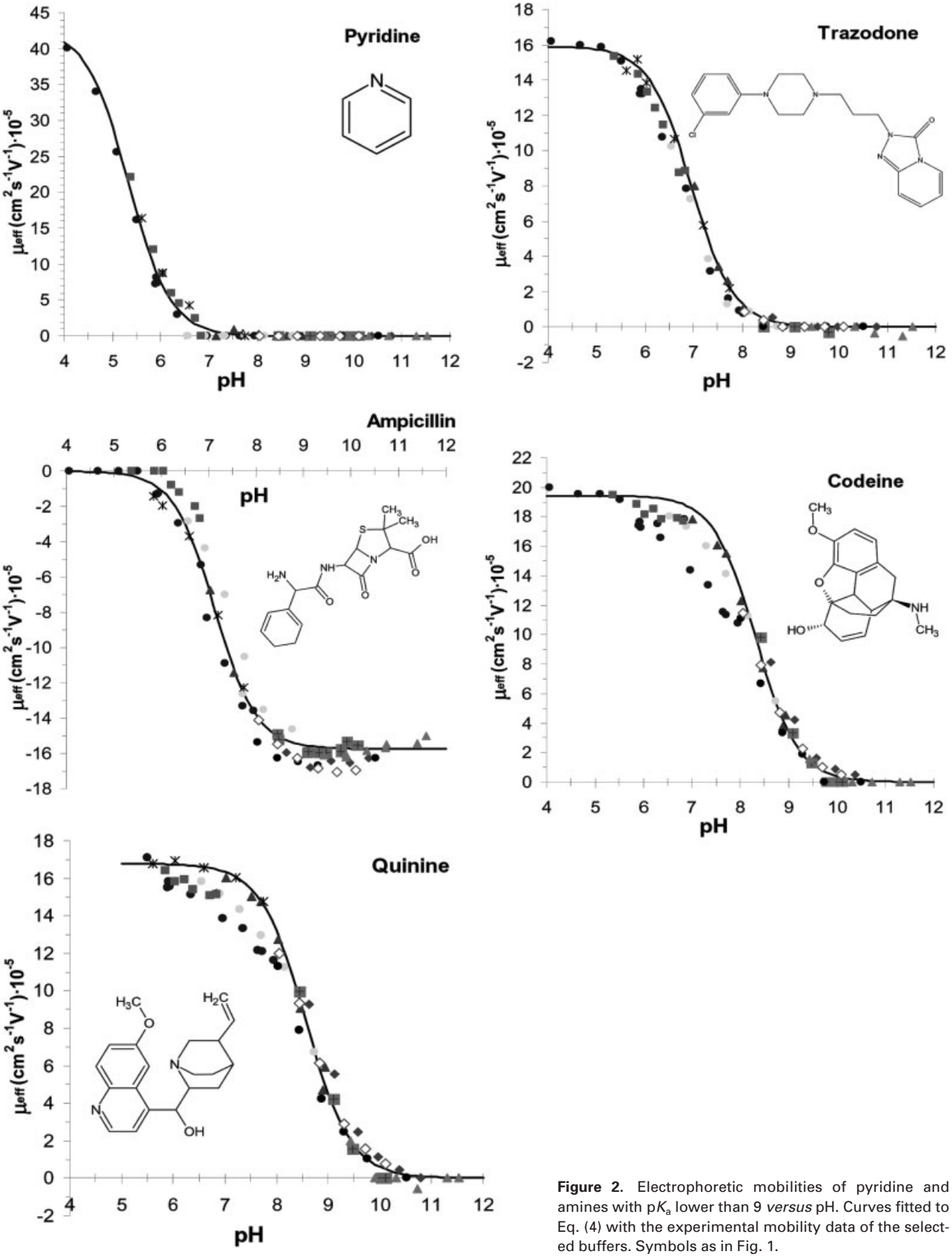


**Figure 2.** Electrophoretic mobilities of pyridine and amines with p$K_a$ lower than 9 *versus* pH. Curves fitted to Eq. (4) with the experimental mobility data of the selected buffers. Symbols as in Fig. 1.

these are lower than those obtained with $TrisH^+$/Tris and $BisTrisH^+$/BisTris buffers. In order to select the best buffers for accurate p$K_a$ determination, a systematic study for each type of buffer and solute was done.

### 4.1 Inorganic buffers

The $\mu_{eff}$ obtained using $H_2PO_4^-/HPO_4^{2-}$ buffers for basic compounds with p$K_a$ higher than 8 clearly deviate from the fitting line and show $\mu_{eff}$ values lower than those expected (Figs. 2 and 3). In fact, these deviations can be observed in the pH range where the phosphate ions and the protonated species of the base coexist, *i.e.*, at pH higher than 6.5, and they are more pronounced in the pH range of predominance of $HPO_4^{2-}$ ions, that is at pH higher than 7. Thus, strong deviations are observed for codeine, quinine, diphenhydramine, procainamide, propranolol, 1-aminoethylbenzene, and nortriptyline (p$K_a$>8), but only small ones for trazodone and ampicillin (p$K_a$<8) and no deviations are shown for pyridine (p$K_a$ = 5.22<<8) (Figs. 2–4). This behavior was already observed in the determination of p$K_a$ values of some drugs with amino groups by CE in 50% methanol/water [21] and was attributed to the ionic interactions between $HPO_4^{2-}$ and the protonated amine. In fact, literature already reports the ion pair formation between protonated amines and $HPO_4^{2-}$ [36, 37]. It was observed that the binding constants of the organic amines to phosphate become significant when the charge of phosphate is at least of two [37].

As already reported in 50% methanol/water medium [21], measurements of $\mu_{eff}$ using running buffers prepared with $H_3BO_3/H_2BO_3^-$ give also slightly lower mobility values than those expected for amines. These deviations occur when the deprotonated form of the base and the boric acid coexist, that is, in the region of the mobility curve at a pH equal or lower than the p$K_a$ of boric acid (9.24).

As in the case of $H_2PO^{4-}/H_2PO_4^{2-}$ buffers, no deviation is observed for phenols when $H_3BO_3/H_2BO_3^-$ buffers are used except, as expected, for catechol (Fig. 4). It is well known that boric acid may interact strongly with compounds with consecutive diols, like catechol, and thus modify the effective mobility of the compound [17, 20].

### 4.2 Zwitterionic buffers

Four zwitterionic buffers (MES/$MES^-$, HEPES/$HEPES^-$, CHES/$CHES^-$, and CAPS/$CAPS^-$) have been also tested. All of them have a sulfonic group which is completely deprotonated at any working pH, as well as an amino group which may be protonated or deprotonated depending on the working pH. Systematic deviations are observed for basic compounds when MES/$MES^-$ or HEPES/$HEPES^-$ are used, that is, the $\mu_{eff}$ obtained with these buffers show values lower than those expected. These deviations are larger when the negatively charged species of the buffer predominate (*i.e.*, at pH higher than the p$K_a$ of the buffer) and the solute amine is mostly in its protonated form. On the contrary, a slight or no deviation in $\mu_{eff}$ is observed when the buffer is mostly in its zwitterionic form (*i.e.*, at pH lower than its p$K_a$ value) and neither when the solute amine is in its neutral form. Then, some interactions between the protonated solute amine and the anionic species of the buffer could explain this experimental behavior. Ion pair formation between sulfonic groups and aliphatic amines in the protonated form or quaternary ammonium ions is widely reported in the literature [38–41], and it is pointed out that the formation constants are higher for tertiary amines than for primary amines and increase with the amine molecular weight [40]. Specific interaction between HEPES and protonated amines with high molecular weight was also observed by Lukkari *et al.* [42] in MEKC measurements. Thus, ion pair formation between the sulfonic group of the buffer and the protonated amine is likely the cause of deviation for MES and HEPES buffers.

Similar behavior could be expected with CHES/$CHES^-$ and CAPS/$CAPS^-$, but with these buffers practically no deviations in $\mu_{eff}$ values are observed. This is because these two buffers have p$K_a$ values similar or higher than the p$K_a$ of the studied amines, and in the buffered pH range the amines are in their neutral form. Therefore, the ionic interaction cannot be observed.

As expected, no deviations have been observed in the phenol derivatives study with any of the zwitterionic buffers tested.

Although ampicillin is an amino derivative, its plot is similar to that of phenols, because it has also a carboxylic group completely dissociated from pH 3.5 (p$K_{a1}$ = 2.52). Thus, in the working pH range it goes from the zwitterionic form ($\mu_{eff}$ = 0) to an anionic species ($\mu_{eff}$ <0). Nevertheless, the interactions between the protonated amino group of the zwitterion and the sulfonic group of the buffer produce an anion of higher mass and volume, and thus lower negative mobility (*i.e.*, closer to zero). In this case, the obtained $\mu_{eff}$ is higher than the expected one.

### 4.3 Cationic buffers

Figures 2–4 show some electrophoretic deviations when $EtONH_3^+/EtONH_2$ or $(EtO)_2NH_2^+/(EtO)_2NH$ are used as buffers. These deviations are observed for all the compounds studied (basic compounds and phenols) and are similar, but in minor degree, than the ones observed with $BuNH_3^+/BuNH_2$ buffers. It is known that the interaction of the amine with the capillary wall increases with the alkyl chain length and the hydrophobic character [28, 30]. Ethanolamine and diethanolamine have shorter alkyl chain length and are less hydrophobic than butylamine, and consequently we only observe small deviations in the $\mu_{eff}$ of solute with these buffers.

### 4.4 Recommended buffers for p$K_a$ determination

As we have shown before, specific interactions between the buffer and the analyte produce variations on the expected $\mu_{eff}$

 

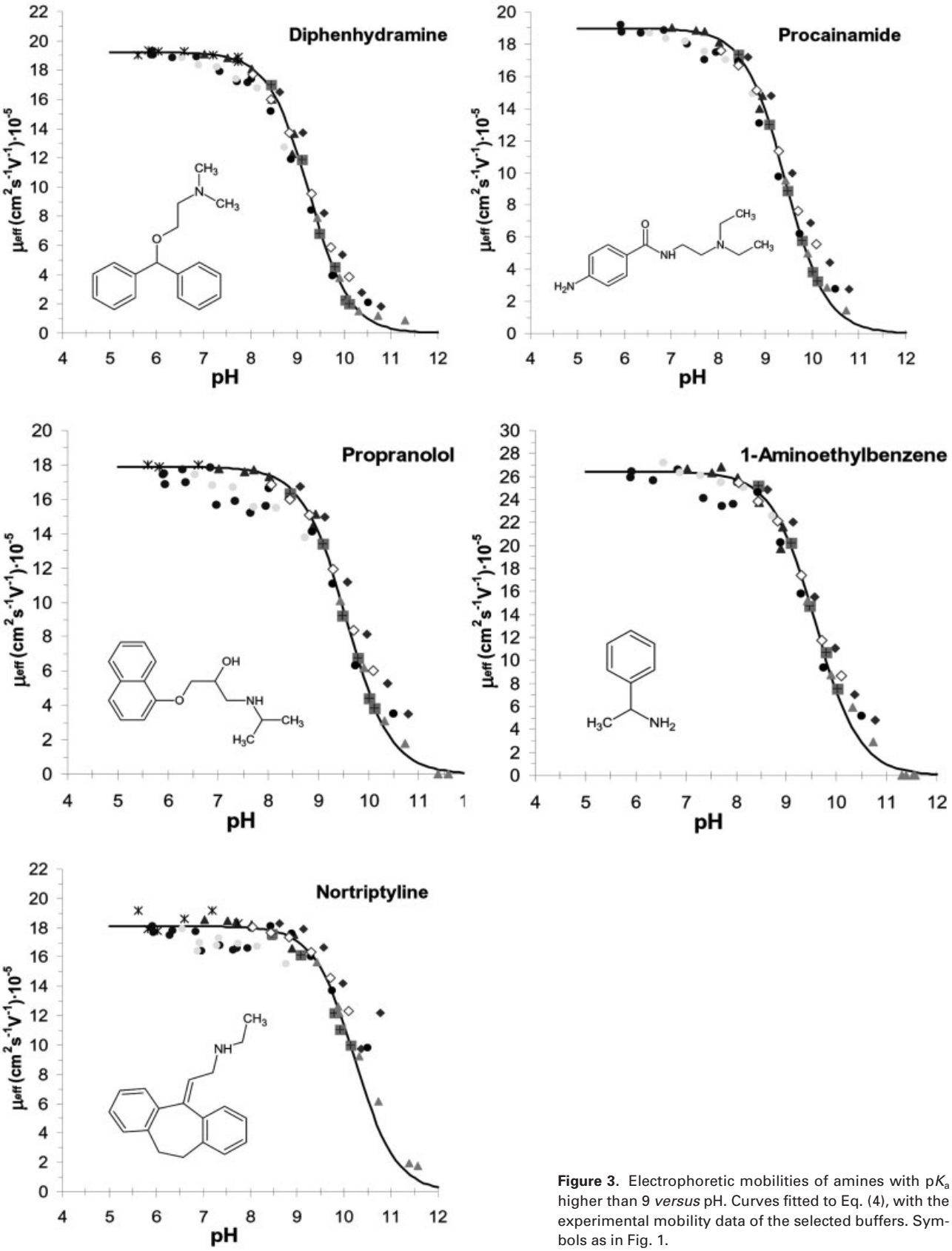


**Figure 3.** Electrophoretic mobilities of amines with p$K_a$ higher than 9 *versus* pH. Curves fitted to Eq. (4), with the experimental mobility data of the selected buffers. Symbols as in Fig. 1.

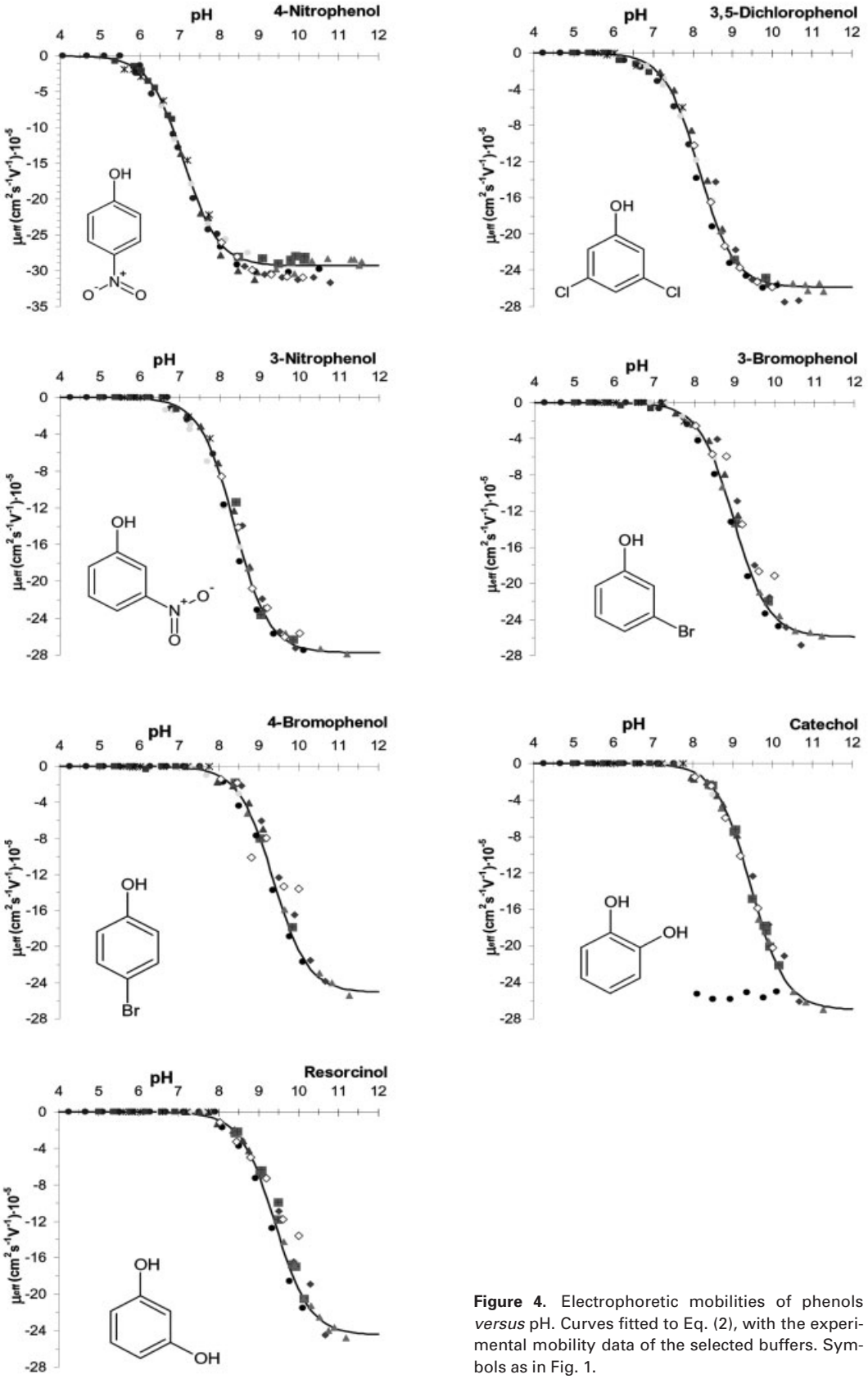


**Figure 4.** Electrophoretic mobilities of phenols *versus* pH. Curves fitted to Eq. (2), with the experimental mobility data of the selected buffers. Symbols as in Fig. 1.

 

value. Then, for accurate p$K_a$ determination all the buffers that can interact with the compound must be rejected. This means that in p$K_a$ determination of amines and pyridine we will consider only the following buffers among the ones studied: $CH_3COOH/CH_3COO^-$, $BisTrisH^+$/BisTris, $TrisH^+$/Tris, CHES/$CHES^-$, and CAPS/$CAPS^-$, which do not show any interaction with amines, nor with pyridine. Phenols present fewer interactions with buffers than basic compounds, therefore the buffers to use in p$K_a$ determination of phenols can be: $CH_3COOH/CH_3COO^-$, $BisTrisH^+$/BisTris, MES/$MES^-$, $H_2/PO_4^-/HPO_4^{2-}$, HEPES/$HEPES^-$, $TrisH^+$/Tris, $H_3BO_3/H_3BO_2^-$, CHES/$CHES^-$, and CAPS/$CAPS^-$. $H_3BO_3/H_3BO_2^-$ buffer is excluded for catechol because its ability to complex vicinal diol-compounds [20, 43].

The continuous line in Figs. 2–4 has been calculated fitting the data obtained with these recommended buffers when both p$K_a'$ and $\mu_A^-$ (Eq. 2) or $\mu_{BH}^+$ (Eq. 4) are calculated. The line fits very well the data obtained with the recommended buffers, as clearly shown in Fig. 5 for propranolol and 3-bromophenol, where only the data of these buffers have been plotted. Tables 2 and 3 present the p$K_a'$ obtained from the fits for the complete set of studied compounds, as well as the thermodynamic p$K_a$ values after ionic strength correction, and the potentiometric p$K_a$ reported in literature. Differences lower than 0.1 units are obtained between the determined and literature p$K_a$ values that can be attributed to the random error involved in the experimental procedure. The highest differences correspond to trazodone and nortriptyline whose potentiometric values can embody a slight error since they were not directly obtained in aqueous solution but were estimated by extrapolation from different methanol/water solutions [44].

## 5 Concluding remarks

Capillary electrophoresis is a suitable technique for the determination of dissociation constants of acids and bases. Several recommendations as temperature control and adequate and constant ionic strength have been already given in the literature, and have to be followed in order to obtain reliable results. However, all CE buffers do not behave in the same way in the CE system and its use for p$K_a$ determinations has to be individually tested for each analyte.

Some typical CE buffers such as $NH_4^+/NH_3$ and $HPO_4^{2-}/PO_4^{3-}$ are not adequate for p$K_a$ determination, since volatility, electrolysis and $CO_2$ dissolution in the buffer reservoirs leads to low reproducibility of mobility values. Amino-based buffers, like $BuNH_3^+/BuNH_2$, $EtOHNH_3^+/EtOHNH_2$, and $(EtO)_2NH_2^+/(EtO)_2NH$ are also not recommended since they interact with the capillary wall and distortions in the mobility *versus* pH curve are always observed, although in different degrees according to the alkyl chain length of the buffer.

Some other buffers present interactions depending on the type of analyte being analyzed. This is the case of

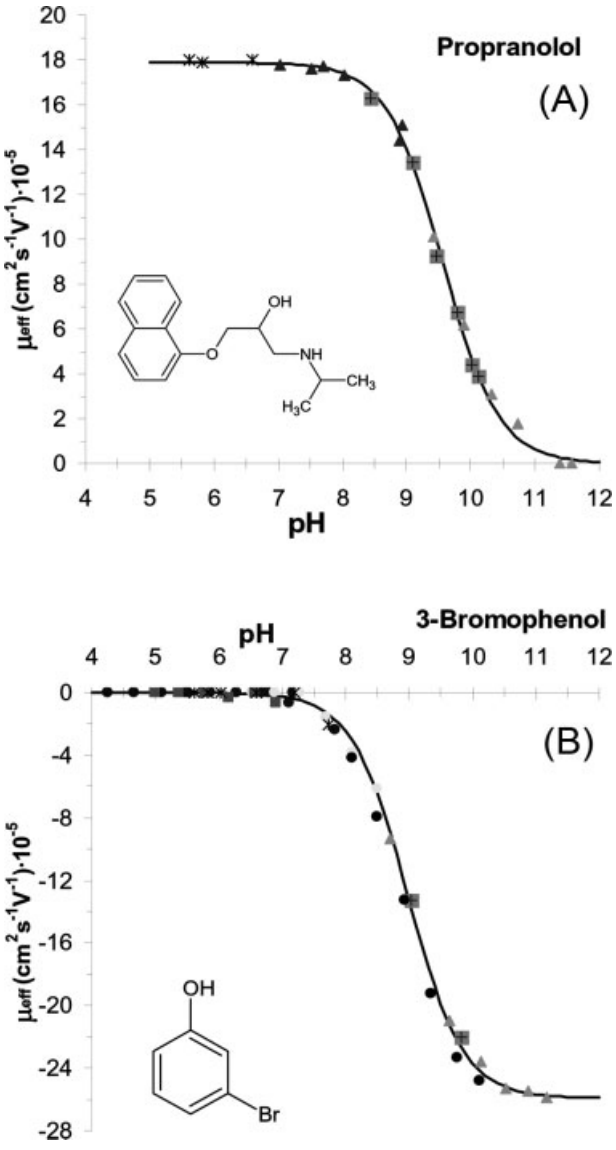


**Figure 5.** Electrophoretic mobilities of the selected buffers *versus* pH, and curve fit of the experimental data of the selected buffers to Eq. (4) or Eq. (2) for propranolol (A) and 3-nitrophenol (B). Symbols as in Fig. 1.

$H_2PO_4^-/HPO_4^{2-}$, HEPES/$HEPES^-$, and MES/$MES^-$, which interact with protonated amines when pH is higher than the buffer p$K_a$, or $H_3BO_3/H_3BO_2^-$ which interact with the deprotonated form when pH is lower than the buffer p$K_a$. Although CHES/$CHES^-$ and CAPS/$CAPS^-$ are also expected to interact with protonated amines, like HEPES/$HEPES^-$, and MES/$MES^-$, its higher p$K_a$ value reduces the range where protonated amines and the deprotonated species of the buffer coexist, being therefore suitable buffers to measure the p$K_a$ value of many different kinds of compounds. In the same way $CH_3COOH/CH_3COO^-$, $BisTrisH^+$/BisTris, and $TrisH^+$/Tris buffers can be also used for a wide range of determinations.

 

If all these factors are considered, p$K_a$ values obtained through CE measurements for both acids and bases, agree with the values given in the literature and obtained by other techniques (error lower than 0.1 p$K_a$ units), and thus CE can be recommended as a versatile, fast and accurate technique for p$K_a$ determination.

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