

Review

# Capillary zone electrophoresis in non-aqueous solutions: pH of the background electrolyte

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## Abstract

Although the establishment of a pH scale and the determination of the pH in water is not problematic, it is not a straightforward task in non-aqueous solvents. As capillary zone electrophoresis (CZE) in organic solvents has gained increasing interest, it seems to be valuable to re-discuss the concept of the pH in such media, especially pointing to those aspects, which make pH measurement uncertain in non-aqueous solvents. In this review, the relevant aspects when dealing with primary standard (PS) and secondary standard (SS) as recommended by the International Union of Pure and Applied Chemistry (IUPAC), and the usage of the operational pH are discussed with special emphasis to non-aqueous solvents. Here, different liquid junction potentials, incomplete dissociation of the electrolytes (especially in solvents with low or moderate relative permittivity) and the occurrence of homo- and heteroconjugation must be taken into account. Problems arising in capillary zone electrophoresis practice are addressed, e.g. when the background electrolyte (BGE) consists of organic solvents, but the measuring electrode (normally the glass electrode) is calibrated with aqueous buffers, and the liquid junction potentials between the solvents do not cancel each other. The alternative concept of establishing a certain pH is described, using mixtures of reference acids or bases with known  $pK_a$  in the organic solvent, and their respective salts, at a certain concentration ratio, relying to the Henderson–Hasselbalch equation. Special discussion is directed to those organic solvents most common in capillary zone electrophoresis, methanol (MeOH) and acetonitrile (ACN), but other solvents are included as well. The potential significance of small amounts of water present in the organic solvent on changes in  $pK_a$  values, and thus on the pH of the buffering components is pointed out.

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*Keywords:* Capillary electrophoresis; Reviews; pH; Background electrolytes; Non-aqueous organic solvents

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## 1. Introduction

Capillary zone electrophoresis (CZE) using non-aqueous background electrolytes (BGEs) is an interesting alternative to traditional aqueous or aqueous–organic BGEs. The advan-

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tage of organic solvents is their influence on all the decisive parameters CZE separations are based on [1]. Like in aqueous media, the most significant parameter for modification of the separation selectivity is the pH of the BGE. However, pH adjustment in organic solvents is not as straightforward as in aqueous media. Accordingly, in many CZE applications in non-aqueous solutions the pH established is not unambiguous. Separation selectivity is often changed either by varying the composition of two or more solvents, or by applying different electrolyte/additive components. Most often this strategy has led to extremely complicated BGE systems, where the obtained results might be caused by several phenomena simultaneously operative. However, for an appropriate interpretation of the results, the experimental set-up should be kept as simple as possible. This can be done, e.g. by starting the experimental work with a single organic solvent system and by changing the pH of the solution in a defined manner.

It is therefore the goal of this work to discuss the determination of the pH in non-aqueous solvent systems, and to give examples about different approaches how pH can be tuned in CZE experiments in non-aqueous BGEs. A brief discussion on the pH in non-aqueous BGEs has already been given in a previous work [1], but the present contribution gives a more fundamental view on the topic. In order to take full advantage on the discussion, a short introduction is given into the concept of pH and into the standard buffer determination methods according to recent recommendations of the International Union of Pure and Applied Chemistry (IUPAC) [2]. It should be noted that we do not include a discussion on pH measurements or pH standardisation in mixed aqueous–organic solvent systems even though many of the conclusions in the present work are authoritative also in these systems. Detailed discussions on mixed aqueous–organic solvent systems can be found elsewhere (see, e.g. [3–8]).

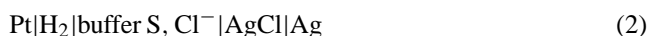
## 2. Concept of pH

The pH definition recommended by IUPAC [2] is based on the proposal given by Sørensen and Linderstrøm-Lang [9]:

$$\text{pH} = -\log a_{\text{H}^+} \quad (1)$$

where  $a_{\text{H}^+}$  is an activity of the hydrogen ion. As the activity of a single ion cannot be measured by thermodynamic methods, appropriate conventions are thus needed to determine the pH.

According to IUPAC recommendations, standard pH buffer solutions are divided into primary standard (PS) and secondary standard (SS) according to the reproducibility and uncertainty in their measurements [2]. The primary pH standard values are determined with the following cell without transference (i.e. without liquid junction):



where  $\text{Pt}|\text{H}_2$  is the hydrogen gas electrode, buffer S is the standard buffer and  $\text{AgCl}|\text{Ag}$  is the silver–silver chloride reference electrode.  $\text{Cl}^-$  ions (added either as potassium or sodium chloride) are required to function the  $\text{AgCl}|\text{Ag}$  electrode. Arrangement (2) is known as the Harned cell [10].

The acidity function,  $p(a_{\text{H}^+}\gamma_{\text{Cl}^-})$ , of the Harned cell is [2]:

$$\begin{aligned} p(a_{\text{H}^+}\gamma_{\text{Cl}^-}) &= -\log(a_{\text{H}^+}\gamma_{\text{Cl}^-}) \\ &= \frac{(E - E^0)F}{RT \ln 10} + \log \frac{m_{\text{Cl}^-}}{m^0} \end{aligned} \quad (3)$$

where  $\gamma_{\text{Cl}^-}$  is the activity coefficient of the chloride ion,  $E$  the potential difference of the cell,  $E^0$  the standard potential difference of the cell,  $F$  and  $R$  are the Faraday constant and the gas constant, respectively.  $T$  is the absolute temperature,  $m_{\text{Cl}^-}$  the molality (mol/kg) of chloride and  $m^0$  the standard molality (1 mol/kg). The standard potential difference  $E^0$  can be determined by filling the cell (Eq. (2)) with hydrochloric acid (HCl) solution of fixed molality (e.g. 0.01 mol/kg) and taking into account the respective mean activity coefficient of HCl ( $\gamma_{\pm\text{HCl}}$ ; values can be found from the literature). In order to obtain  $-\log a_{\text{H}^+}$  (i.e. the pH) from Eq. (3), the contribution of  $-\log \gamma_{\text{Cl}^-}$  has to be evaluated. This is done with the following two-step procedure: (i) the acidity function  $p(a_{\text{H}^+}\gamma_{\text{Cl}^-})$  is determined in at least three fixed molalities of sodium or potassium chloride. Then, linear extrapolating to zero chloride concentration will lead to  $p(a_{\text{H}^+}\gamma_{\text{Cl}^-})^0$ , superscript '0' denoting zero concentration. (ii) The acidity function at zero chloride concentration can be written:

$$\begin{aligned} p(a_{\text{H}^+}\gamma_{\text{Cl}^-})^0 &= -\log(a_{\text{H}^+}\gamma_{\text{Cl}^-})^0 \\ &= -\log a_{\text{H}^+} - \log \gamma_{\text{Cl}^-}^0 \end{aligned} \quad (4)$$

where the limiting activity coefficient of chloride  $\gamma_{\text{Cl}^-}^0$  is derived from the Debye–Hückel law [11,12] as:

$$\log \gamma_{\text{Cl}^-}^0 = -\frac{A\sqrt{I}}{1 + Ba\sqrt{I}/m^0} \quad (5)$$

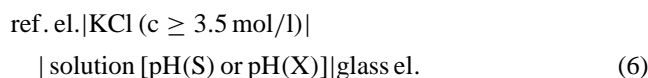
In Eq. (5),  $A$  and  $B$  are the Debye–Hückel constants (for numerical values see, e.g. [13,14]),  $a$  is the mean distance of closest approach of the ions (also known as the ion size parameter) and  $I$  is the ionic strength (mol/kg) of the buffer. By the Bates–Guggenheim convention [15], the dimensionless parameter  $Ba$  is set to 1.5 for all aqueous buffers in the temperature range from 5 to 50 °C. It should be noted that Eq. (5) is valid for ionic strengths below 0.1 mol/kg. Primary pH standard buffers at different temperatures can be found from the literature [2,13]. For a more detailed discussion on the determination of the primary standard pH values, see [2,16].

Secondary pH standards are buffers, which either (i) do not meet the requirements for primary standards or (ii) which are not subjected to determination methods of primary standards due to economic, time or quality reasons [2,16]. Uncertainty of pH(SS) values is higher than pH(PS) values. The

pH(SS) values can be determined by comparison to pH(PS) value(s) using either a cell without salt bridge, a cell with salt bridge, a cell with salt bridge and glass electrode, or the Harned cell (Eq. (2)) (for details, see [2,16]). In the latter case, the buffer does not meet the criteria for primary standard, e.g. due to inappropriateness in quality of the chemicals or of the Bates–Guggenheim convention (e.g. buffers with other charge-type) [2].

It should be noted that according to the IUPAC recommendation the pH is given in molal scale ( $\text{pH}_m$ ). Conversion to molar scale ( $\text{pH}_c$ ) in diluted solutions can be done with the following relation:  $\text{pH}_c = \text{pH}_m - \log(\rho/\rho^0)$  where  $\rho$  is the density of the solvent and  $\rho^0 = 1 \text{ kg/l}$ . Similar conversion can be applied to acid dissociation constants as well.

Practical pH measurements are most often done with the following cell, which consists of a reference electrode (typically the silver–silver chloride electrode) including a reference electrode filling solution (typically a highly concentrated aqueous solution of equitransferent cation and anion; these are ions with the same transference number, e.g. KCl), a liquid junction (marked as “||”) and a glass electrode:



where S and X are the standard solution and the unknown sample solution, respectively. The device forming a liquid junction (salt bridge) can be a ceramic plug, a frit or a ground glass sleeve [2]. If the mercury–mercury(I) chloride (calomel) electrode is used as a reference, the electrode filling solution and the solution to be measured (standard or sample) are separated by another solution, called salt bridge electrolyte solution (e.g. saturated KCl in water). In this case, a second liquid junction is present.

The pH values obtained with the aid of the cell depicted by Eq. (6) are called operational pHs. In case that a liquid junction is present, its potential has to be considered together with those of the glass electrode and the reference electrode [16]. The liquid junction potential is the potential difference arising between two electrolyte solutions of different composition (e.g. between standard buffer or unknown sample solution and the reference electrode filling solution). Also, construction of the junction, memory, clogging or hydrodynamic effects (e.g. stirring) might have an effect on the liquid junction potential.

Upon using the cell in Eq. (6) for pH measurements, several points must be taken into account [2,16,17]. The glass electrode, for example, may exhibit a slope of  $E$  versus pH smaller than the theoretical value,  $k = (RT/F) \ln 10$  (which is 59.16 mV at 25.0 °C). Such behavior is often called a sub-Nernstian response or a practical slope (denoted with  $k'$ ); it is experimentally determinable. Also, the response of the glass electrode may vary with time, memory effects and history of use. In addition, all measurements should be done under temperature-controlled conditions. Accordingly, a suitable calibration procedure should be selected in or-

der to use the cell described in Eq. (6) for pH measurements.

Either one-, two- or multi-point calibration can be used for the cell presented in Eq. (6) [2,16]. One-point calibration is done as:

$$\text{pH}(\text{X}) = \text{pH}(\text{S}) - \frac{E(\text{X}) - E(\text{S})}{(RT/F) \ln 10} \quad (7)$$

where  $\text{pH}(\text{X})$  and  $\text{pH}(\text{S})$  are the pH values of unknown sample (X) and the standard buffer (S),  $E(\text{X})$  and  $E(\text{S})$  are the potential differences of the respective solutions. The limitation of one-point calibration is that it relies on the theoretical Nernstian response,  $k$ .

The most commonly used method is the two-point calibration, which consists of two standard buffer solutions of pH values  $\text{pH}(\text{S1})$  and  $\text{pH}(\text{S2})$ , preferably immediately below and above of pH of the unknown sample solution. The practical slope factor,  $k'$ , can be given as:

$$k' = \frac{E(\text{S1}) - E(\text{S2})}{\text{pH}(\text{S2}) - \text{pH}(\text{S1})} \quad (8)$$

where  $E(\text{S1})$ ,  $E(\text{S2})$  are the respective potential differences;  $\text{pH}(\text{X})$  is then obtained from

$$\text{pH}(\text{X}) = \text{pH}(\text{S1}) - \frac{E(\text{X}) - E(\text{S1})}{k'} \quad (9)$$

Given that the practical slope ( $k'$ ) is used instead of the theoretical Nernstian response ( $k$ ), uncertainty in pH measurements with two-point (as well as with multi-point) calibration procedure is obviously smaller than that with the one-point calibration method.

The following conditions should be fulfilled for correct pH measurements [18]. (i) The same electrode pair should be used for both, calibration and measurements, (ii) temperature should be same, and (iii) the solvent composition should be the same for the calibration solution(s), the sample solution and for the equitransferent reference electrode filling electrolyte (and salt bridge electrolyte). As the concept of the operational pH relies on the assumption that the liquid junction potential is the same during calibration step(s) and sample measurement, i.e. the liquid potentials cancel each other. An error called residual liquid junction potential will arise [2] when this assumption is not fulfilled.

### 3. pH in non-aqueous media

In general, the above discussion related to the primary and secondary standard buffers as well as to the operational pH is not limited to aqueous solutions but is valid for organic solvents as well. However, there are several points, which have to be first taken into account [19].

When the Bates–Guggenheim convention [15] is applied for the determination of primary standards in non-aqueous systems, the following modifications for Eq. (5) have to be done [19]. Parameter  $A$  should be replaced by  $A^Z =$

$A^W(\varepsilon^W/\varepsilon^Z)^{3/2}$ , parameter  $Ba$  (which is set to 1.5; see above) by the expression  $Ba(\varepsilon^W\rho^Z/\varepsilon^Z\rho^W)^{1/2}$ .  $\log \gamma_{\text{Cl}^-}^0$  expressed by Eq. (5) is then:

$$\log \gamma_{\text{Cl}^-}^0 = -\frac{A^W(\varepsilon^W/\varepsilon^Z)^{3/2}\sqrt{I}}{1 + Ba(\varepsilon^W\rho^Z/\varepsilon^Z\rho^W)^{1/2}\sqrt{I/m^0}} \quad (10)$$

where  $\varepsilon$  and  $\rho$  are the relative permittivity and density, superscripts denoting water (W) and solvent/solvent mixture (Z). The lower limit for  $\varepsilon^Z$  is considered to be 35 at given working temperature because at lower relative permittivity values potassium or sodium chloride added (see cell in Eq. (2)) and hydrochloric acid (used in  $E^0$  determination) might not be completely dissociated [19]. It should be noted that the relative permittivity of methanol (MeOH), which is the most commonly used organic solvent in CZE, is 32.66 at 25.0 °C [20], and hydrochloric acid is a weak acid with  $\text{p}K_a$  1.2 in this solvent [21]. Obviously, this means that the above-mentioned IUPAC method for primary standardisation cannot be applied in MeOH, unless possible ion-pair formation of chloride salts and an incomplete dissociation of HCl is taken into account. The same is true for another popular solvent in CZE, acetonitrile (ACN). Even though ACN has a relative permittivity of 35.94 at 25.0 °C [20], which is slightly above the recommended lower limit, HCl is a weak acid also in this solvent ( $\text{p}K_a$  8.9 at 25.0 °C [21]). In addition, due to the low stabilisation of anions in ACN, possible homo- and heteroconjugation as well as triple-ion formation (see, e.g. [22,23]) can make the use of the Harned cell (Eq. (2)) inappropriate.

Accordingly, it is not a surprise that there are hardly any primary pH standards, which meet the IUPAC criteria, available for organic solvents. One exception has recently been introduced, namely potassium hydrogen phthalate buffer in formamide (FA) [18]. FA is a solvent with high relative permittivity (109.5 at 25.0 °C [20]), in which complete dissociation of the salts takes place; it is thus suitable for the IUPAC primary pH standard measurements using the Harned cell. The pH value of 6.43 at 25.0 °C for 0.05 mol/kg potassium hydrogen phthalate buffer in FA was reported [18].

de Ligny and coworkers [24–26] have introduced  $\text{pH}^*$  standards in pure MeOH, namely oxalate, salicylate and succinate buffers of  $\text{pH}^*$  values 5.79, 7.53 and 8.75 at 25.0 °C, respectively (Table 1). Note that here the asterisk (\*) means that the pH is referred to the infinitely diluted solution in MeOH as selected standard state. The Harned cell (Eq. (2)) was used for the pH determinations, incomplete dissociation of salts in MeOH was taken into account, and  $\log \gamma_{\text{Cl}^-}^0$  was estimated from the equation given by Gronwall et al. [27]. In a later contribution of the same group, salicylate and diethylbarbiturate buffer solutions with respective  $\text{pH}^*$  values of 8.3 and 13.2 in pure ethanol at 25.0 °C were reported (for details see, e.g. [13]).

Even though there are no standard pH buffers (IUPAC or others) available for many potentially interesting organic

Table 1  
Standard buffers in methanol introduced by de Ligny and coworkers [24–26]

Buffer	$\text{pH}^*$ at 25.0 °C	Composition
Oxalate	5.79	10 mmol/kg oxalic acid and 10 mmol/kg ammonium hydrogen oxalate
Salicylate	7.53	10 mmol/kg salicylic acid and 10 mmol/kg sodium salicylate
Succinate	8.75	10 mmol/kg succinic acid and 10 mmol/kg lithium hydrogen succinate

solvents, this does not mean that the pH-meter cannot be calibrated in these solvents. In fact, several buffers have been used for calibration of the glass electrode in organic solvents. Most common calibration solutions are prepared from picric acid (2,4,6-trinitrophenol) or 2,6-dinitrophenol, and from their respective tetraalkylammonium salts. The anions of these phenols have delocalised charges, which means that homoconjugation of the acid with their anionic forms is either weak or negligible [22]. Thus, buffer solutions prepared from an equimolar mixture of such a phenol and its tetraalkylammonium salt are especially suitable for calibration of the glass electrode in solvents where homoconjugation can be otherwise serious (e.g. ACN, propylene carbonate (PC)). In Table 2,  $\text{p}K_a$  values of picric acid and 2,6-dinitrophenol are given in some selected solvents determined either by conductometric, potentiometric or spectrophotometric methods (for details, see the original literature [21,28–33]). Dissociation constants of many other acids and bases in organic solvents have been determined by potentiometric titration using a glass electrode calibrated with buffers prepared from picric acid or 2,6-dinitrophenol (see, e.g. [21,22,31,32,34–44] and references cited therein). Typically, a one-point calibration has been applied and correction for the effect of the ionic strength has been done with the aid of the Debye–Hückel theory. Often a modified calomel reference electrode has been used (aqueous KCl filling solution has been replaced by tetraalkylammonium chloride in the given solvent), and the tetraalkylammonium perchlorate has been used for the salt bridge solution (see, e.g. [36,37]). The linearity of the response of the glass electrode versus the reference electrode, the Nernstian response, has been confirmed by measuring the potential difference of the cell by varying the concentra-

Table 2  
 $\text{p}K_a$  values of picric acid and 2,6-dinitrophenol at 25.0 °C

Solvent	$\text{p}K_a$	
	Picric acid	2,6-Dinitrophenol
MeOH	3.8 [21]	7.74 [28]
ACN	11.0 [29]	16.45 [30]
PC	9.29 [31]	13.45 [32]
DMSO	~–1.0 <sup>a</sup> [33]	4.9 [33]
Water	0.38 [21]	3.70 [21]

Data for water is included for comparison of the acid strengths.

<sup>a</sup> Too strong acid to be used for calibration of the electrode.

tion ratio of the buffer acid and its salt (e.g. picric acid and tetraethylammonium picrate).

#### 4. pH adjustments in CZE with non-aqueous BGEs

Concerning non-aqueous background electrolytes in CZE, several methods have been used to adjust the pH. For example, calibration of the glass electrode has been carried out with non-aqueous standard buffers. The easiest and thus the most commonly used method is, however, the measurement of the pH with glass electrode calibrated using aqueous standard buffers. Recently, BGEs based on known  $pK_a$  values of buffering acids in the given solvent have been applied. The advantages and disadvantages of these methods are discussed in the following sections.

##### 4.1. Calibration of pH-meter

###### 4.1.1. Non-aqueous calibration buffers

As discussed above, an operational pH can be measured in organic solvents using a glass electrode calibrated with suitable non-aqueous calibration buffers and non-aqueous reference electrode filling electrolyte solutions. The calibration buffer can be either a standard buffer solution with predetermined pH or a solution based on the known  $pK_a$  value of the buffer acid (or base). It is clear that this approach is the most accurate way of measuring the pH in non-aqueous media. To our knowledge, however, it has not been used in CZE, most probably because of the tedious maintenance of electrodes and chemical purity. In order to maintain the electrode potentials constant, carefully purified solvents and electrolyte components are needed and the system should be protected from atmospheric moisture and other impurities. In addition, the solvent composition of the calibration buffer(s), reference electrode filling, salt bridge electrolyte and sample should be the same. This means that every time the solvent composition of the sample is changed, the other solutions should be changed as well.

Accordingly, some authors have preferred to calibrate the pH-meter with non-aqueous calibration buffers using aqueous reference electrode filling solution. Belder et al. [45] have used the above-mentioned methanolic oxalate and succinate standard buffers of  $pH^*$  5.79 and 8.75 (Table 1), respectively, to calibrate the pH-meter. Setting the pH-meter with these reference pH values gave the possibility to read pH values directly for unknown methanolic BGEs. This approach is not limited to standard buffers of known pH values in a given solvent (which are rare, see discussion above) but reference acids of known  $pK_a$  values can also be used.

Even though the method of using non-aqueous calibration solutions and an aqueous electrode set-up seems to be rather useful for CZE applications in non-aqueous BGEs, it also includes some limitations. A small drift in potential can occur due to mixing of the non-aqueous measuring solution and the aqueous reference electrode filling solution (typi-

cally concentrated KCl solution) through the junction or due to the hygroscopic nature of the sample organic solvent (the latter effect can be minimised by avoiding the contact with air). Clogging of the device forming a liquid junction can occur due to the limited solubility of some components. The most important limitation is, however, the complexity of the liquid junction potential. As discussed above, the concept of operational pH relies on the fact that the liquid junction potentials in the calibration step and in the sample measurement step cancel each other. As pointed out by Izutsu [21], the liquid junction between two different solvent compositions consists of three components: (i) differences in electrolyte concentrations on the two sides of the junction and the differences between cationic and anionic mobilities, (ii) differences between ion solvation on the two sides of the junction, and (iii) solvent–solvent interactions at the junction (for details, see [21]). The first issue is relevant also for the junction formed with two solutions of the identical solvent composition. The latter two issues are relevant when the solvent composition is different at the each side of the junction. Accordingly, it is clear that deviations in liquid junction potentials between the calibration step(s) and the sample measurement step (i.e. the residual liquid junction potential error) can easily occur, which may cause deviations in measured pH values.

An alternative way to calibrate the pH-meter in non-aqueous media is to use a solution of a strong acid or base as calibration solution [21]. Carabias-Martínez et al. have used this method for calibration in MeOH–ACN (1/1 (v/v)) medium using perchloric acid as reference acid [46]. The limitation of this approach is that very few acids (or bases) are strong in organic solvents. For example, perchloric acid is one of the rare acids known to be strong in ACN, and even for this assumption some discrepancies exist in the literature (see, e.g. [34] and papers cited therein). What makes the use of perchloric acid tedious is that it is not available as pure substance but only as a solution containing about 30% water.

###### 4.1.2. Aqueous calibration buffers

A much easier and more practical alternative to measure the pH in non-aqueous media uses an all-aqueous pH-meter set-up consisting of aqueous calibration buffers and aqueous reference electrode filling solution. The pH values measured with this procedure are often termed as apparent pH values or  $pH_{app}$  (occasionally also marked with an asterisk, which can be misleading; c.f. above). The apparent pH values measured with the above-mentioned procedure do not have a fundamental meaning because they cannot be related to true  $-\log a_{H^+}$ . The same discussion given in Section 4.1.1 for liquid junctions is authoritative here as well. It should be noted that with aqueous calibration buffers the deviation in junction potential is even more pronounced compared to non-aqueous calibration solution (Section 4.1.1). This is because the two solvents forming the junction are different in the calibration step and in the sample measuring step.

However, this procedure can be useful to approximate relative acidities in non-aqueous media given that the solvent composition of the samples stays constant. While applying this approach, it is very important to give detailed information about the pH-meter set-up used (e.g. electrode types, filling/salt bridge solutions, calibration buffer composition and ionic strength). Unfortunately, such information is often not detailed, which can make the comparison of the results difficult.

de Ligny and Rehbach [47] have introduced a method for the approximation of the  $\text{pH}^*$  of oxalate and succinate buffers in MeOH using a glass electrode calibrated with aqueous standard buffer solutions and aqueous KCl salt bridge electrolyte. The method is based on the following relation [13]:

$$\text{pH}^* = \text{pH} - \delta \quad (11)$$

where  $\text{pH}$  denotes the apparent pH measured with the all-aqueous pH-meter set-up and the constant  $\delta = E_j - \log_m \gamma_{\text{H}^+}$  where  $E_j$  is the liquid junction potential (expressed in pH units after transformation). The term  $\log_m \gamma_{\text{H}^+}$  is known as the medium effect on the proton, which is proportional to the reversible work of transferring 1 mol of proton from infinite dilution in water to infinite dilution in solvent, Z (in this case MeOH) [13]. It has a negative value when the proton is more stable in solvent Z, a positive one when it is more stable in water. The value of  $-2.3$  for  $\delta$  in pure MeOH at  $25.0^\circ\text{C}$  has been reported [47]. This means that the  $\text{pH}^*$  of oxalate and succinate buffer in MeOH using an all-methanolic calibration set-up is 2.3 pH units higher than the apparent pH of the same methanolic buffers using an all-aqueous calibration system.

In principle, a very useful way to approximate pH values in non-aqueous media would be to calibrate the pH-meter with all-aqueous systems and to apply tabulated  $\delta$  values to correct the measured apparent pH values to  $\text{pH}^*$  (Eq. (11)). Indeed, this method has recently been applied to CZE in MeOH [48]. Unfortunately, data for  $\delta$  values in other solvents are hardly available. Similar to other methods in which a liquid junction between two solvents with different compositions exists, mixing of solvents through the junction and clogging of the KCl at the junction can cause instability in measurements. To avoid this, de Ligny and Rehbach [47] used the cell where the contact between methanolic standard buffer and aqueous KCl salt bridge electrolyte was allowed only for very short time period during the potential measurement. In between the measurements the solutions were isolated from each other.

It should be noted that when an all-aqueous pH-meter set-up is used for non-aqueous sample solutions, where the aqueous reference electrode filling electrolyte or the salt bridge electrolyte (in both cases typically KCl) has limited solubility or is even insoluble, clogging of the liquid junction device may cause a permanent damage to the electrode used. Also, replacement of the aqueous reference electrode filling solution (and salt bridge solution) may frequently be

Table 3

Conventional  $\text{pK}_a^*$  values (at  $25.0^\circ\text{C}$ ) of some weak carboxylic acids used as BGE constituents in MeOH

Acid	$\text{pK}_a^*$
Acetic	9.7
Chloroacetic	7.8
Dichloroacetic	6.3
Trichloroacetic	4.9

Data from [22,23] and references cited therein.

needed due to the diffusion of organic solvent(s) or other components from the sample into it.

#### 4.2. Concept of reference BGE acids

It is obvious from the discussion above that, independently of the method applied, the calibration of the pH-meter in non-aqueous media is not an easy task. An alternative approach is to avoid the use of the pH-meter in non-aqueous media and to use solutions of known pH values directly as BGEs. The oxalate and succinate standard buffers (see Table 1) introduced by de Ligny and coworkers have already been used as BGEs in CZE [45,48]. With an appropriate selection of detection methods, the methanolic salicylate standard buffer (Table 1) and the phthalate primary standard buffer in FA [18] can be used as well. Recently, an approach based on known  $\text{pK}_a$  values of reference BGE acids has been introduced to capillary electrophoresis [49,50]. The different aspects of the latter approach are discussed in the following sections.

##### 4.2.1. Methanol as solvent

Based on the fundamentals of thermodynamics, the pH of a solution consisting of an equimolar mixture of a weak acid and its salt is equal to the  $\text{pK}_a$  value of the acid in the given solvent. By selecting reference acids with different  $\text{pK}_a$  values as BGE constituents, the pH can be changed in an elegant way without the use of a pH-meter. In a recent work, monoprotic weak carboxylic acids and their sodium (or potassium) salts were selected as BGE components in MeOH [50]. The conventional  $\text{pK}_a^*$  values of these acids in MeOH are given in Table 3. These  $\text{pK}_a^*$  values are determined with a cell without transference (Eq. (2)) taking into account the conventional definition of the activity coefficient of the chloride ion (from the Debye–Hückel theory; Eq. (5)). The asterisk (\*) means that the value is related to infinite dilution in the given solvent as selected standard state [23].

$\text{pK}_a$  values of several weak cation acids ( $\text{BH}^+$ ) in MeOH were determined by CZE using BGEs with pH values from 4.9 to 9.7 [50]. The pH of the BGE was enlarged to 10.7 using a 1:10 molar ratio of acetic acid and sodium acetate. Low pH BGE was prepared from perchloric acid, which is a strong acid in MeOH.

A practical way to adjust the pH in organic solvents, when the  $\text{pK}_a$  value of the buffer acid is known, is to use the Henderson–Hasselbalch equation [51,52]:

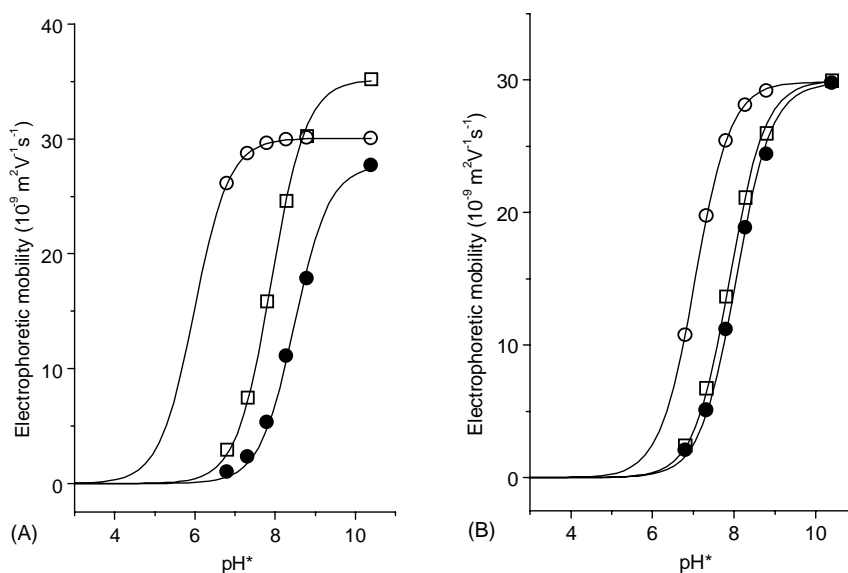


Fig. 1. Electrophoretic mobility of analytes vs.  $\text{pH}^*$  of the BGE in MeOH at  $25.0^\circ\text{C}$ . BGE: either mixture of chloroacetic acid and sodium chloroacetate, or mixture of acetic acid and sodium acetate (salt concentration  $0.01\text{ mol/l}$  at each  $\text{pH}^*$ ) in MeOH. Data for acetate BGE is from [60]. Analytes: (A) (○) 2,4,6-trihydroxybenzoic acid, (□) 2-hydroxybenzoic acid, (●) 2,4-dihydroxybenzoic acid; (B) (○) 2,6-dichlorobenzoic acid, (□) 2,4-dichlorobenzoic acid, (●) 2,5-dihydroxybenzoic acid (reproduced from [55], with permission).

$$\text{pH} = \text{p}K_a - \log \frac{[\text{acid}]}{[\text{base}]} \quad (12)$$

where the acid can be either a neutral acid (HA) or a cation acid ( $\text{HB}^+$ ), and the base is then either the conjugate form of the neutral acid ( $\text{A}^-$ ) or the conjugate form of the cation acid (B). Note that the brackets indicate the concentration of the given species, and no activity correction is applied for ionic species (for limitations of the Henderson–Hasselbalch equation, see [53]). The pH can be adjusted within the buffering range of the acid (typically  $\text{p}K_a \pm 1$ ) by varying the ratio of acid and base in Eq. (12). This approach has been used in methanolic BGEs to determine  $\text{p}K_a$  values of cation acids [54], neutral acids [55] and zwitterionic peptides [56] by using buffers prepared from acids given in Table 3. An example of pH adjustment in MeOH can be seen in Fig. 1 where the mobilities of some substituted benzoic acids are depicted versus the pH of the BGE. Recently, BGEs prepared from varying ratios of diprotic carboxylic acids and tetrabutylammonium hydroxide (TBAOH) have also been used in CZE [57]. It should be noted that the dissociation constants of the reference BGE acids do not need to be conventional ones (see definition given above) but other  $\text{p}K_a$  values (e.g. based on operational pH values) can be used as well. It is needed, however, to give appropriate information about the  $\text{p}K_a$  data used as reference.

Despite the method of reference BGE acids seems to be a very suitable choice for pH adjustment in methanolic BGEs, there is one drawback, which somewhat limits its use, namely the occurrence of ion-pair formation. Even though, to our knowledge, there are no data available for ion-pair formation constants for salts of high interest in CZE

(namely, e.g. potassium, sodium and tetraalkylammonium salts of the acids given in Table 3), some data for simple 1:1 electrolytes indicate that ion-pair formation takes place in this solvent (see, e.g. [58,59]). This phenomenon has following two consequences:

- (i) Possible incomplete dissociation of the acid salt means that the theoretical pH calculated from the Henderson–Hasselbalch equation (Eq. (12)) is lower than it is supposed to be. The following examples should illustrate the magnitude of this effect. According to Barthel et al. [59], the association constants ( $K_A$ ) of 1:1 electrolytes in MeOH at  $25.0^\circ\text{C}$  are in the range from 10 to  $60\text{ l/mol}$ . If we take an equimolar mixture of a monoprotic weak acid and its salt (concentration  $0.01\text{ mol/l}$ ), and consider that the association constant of the latter is  $10\text{ l/mol}$ , the true concentration of the dissociated salt (determining the ionic strength) is ca.  $0.0092\text{ mol/l}$  (activity correction not taken into account in this calculation). According to Eq. (12), such a small deviation in salt (base) concentration decreases the pH of the solution by only few hundredths of pH units (0.04). The same calculation using an association constant of  $60\text{ l/mol}$  leads to a salt concentration of ca.  $0.007\text{ mol/l}$ , and accordingly to a decrease in pH of 0.15 units (Eq. (12)). The respective calculations for  $0.05\text{ mol/l}$  as initial concentration of acid and salt would lead to a pH lower by 0.14 ( $K_A = 10$ ) and 0.36 ( $K_A = 60$ ) units. Ion-pair formation also causes the reduction of the ionic strength of the BGE, which affects the mobility of the analyte ions (it is noteworthy that the effect of ionic strength on mobility in many organic solvents can be more pronounced than in water [1]).

- (ii) Ion pairing between the analyte anion or cation and the oppositely charged BGE ion can take place. As the analyte ion has typically a much lower concentration than the BGE ions, pairing does not affect the pH or ionic strength of the BGE to a considerable extent, but it clearly affects the mobility of the analyte ion. In addition, if different BGE counter ions are applied the mobility of the analyte ion may vary depending on the strength of ion-pair formation with the counter ion [54,60]. It should be mentioned that in Fig. 1 the concentration of the BGE counter ion (sodium) is the same at each pH, which means that, if ion-pair formation took place between the anionic analytes and the sodium ions under the conditions applied, the strength of the formation should have been the same.

In order to minimise the possible effect of incomplete dissociation of the BGE salts on the pH of the BGE and on the mobility of analyte ions, it is preferable to use relatively low concentrations of the BGE salt (i.e. ionic strength). Clearly, the concentrations of the BGE components should be high enough to allow appropriate buffering of the BGE.

#### 4.2.2. Acetonitrile as solvent

The reference BGE acid concept has also been applied to adjust the pH in ACN using either monoprotic [49,61–63] or diprotic carboxylic acids [49,62] as buffer components. Similar to MeOH, the possible occurrence of ion pairing of the BGE salts can have an effect on the pH of the BGE and the mobilities of analytes (see, e.g. [64] for association constants of simple electrolytes in ACN). However, in ACN there is a more serious drawback, which hinders the application of the Henderson–Hasselbalch equation, namely homoconjugation due to hydrogen bonding of the neutral BGE acid with its own anionic form, and heteroconjugation from hydrogen bonding of the acid and/or anion with other neutral or charged species present in the solution. Additional limitations lay in the formation of triple ionic or higher ionic aggregates (see, e.g. [65,66]).

If the pH of the BGE is adjusted by changing the concentration ratio of a monoprotic weak acid and its salt, homoconjugation of the buffer acid with its anionic form (i.e. the base) [29] affects the logarithmic term of Eq. (12) and accordingly the pH. In case that the initial acid concentration is higher than the initial anion concentration homoconjugation leads to a decrease in both concentrations to an equal amount. The concentration ratio of acid and salt (see Eq. (12)) is then higher than in the case without conjugation (obviously depending on the extent of homoconjugation). The consequence is a decrease in pH of the BGE. In case that the initial anion concentration is higher than the acid concentration, the situation is opposite, and the pH of the BGE is higher. The pH of an equimolar solution of acid and its salt, on the other hand, is theoretically not affected by homoconjugation. However, the buffering capacity and ionic strength of the buffer are decreased (the latter can affect the

pH as well). For further details on the topic, see the seminal work of Kolthoff and Chantooni Jr. [29] and [21–23,67–69].

Homoconjugation of the BGE components in ACN (namely chloroacetic acid— $pK_a$  15.8 in ACN at 25.0 °C [70]—and chloroacetate) can explain slight deviations found from the theoretical behavior of the mobilities of basic drugs when the pH was adjusted according to the Henderson–Hasselbalch equation [61].

In case of BGEs prepared with weak diprotic acids and with pH adjusted to the buffering range of the second  $pK_a$  value of the acid, homoconjugation does not take place as the two anionic forms of the acid ( $HA^-$  and  $A_2^-$ ) repel each other [22]. Thus, pH adjustment with mono- and disalts of suitable weak dicarboxylic acids would be a good approach for CZE in ACN. Unfortunately, such salts (preferably tetraalkylammonium ones) are hardly commercially available. Moreover, synthesis of the salts does not seem to be an easy task (see, e.g. [71]). Accordingly, Miller et al. [49] have used ACN-based BGEs prepared from diprotic carboxylic acids using tetrabutylammonium hydroxide to adjust the pH to the second  $pK_a$  value of the acid. The same approach has recently been used for the separation of methyl-substituted phenols in ACN [62]. A disadvantage of the use of TBAOH or other hydroxides is that they are available only as solutions typically prepared in water, MeOH or MeOH–2-propanol. By using them one introduces additional solvent(s) to the BGE. If the molecules of the second solvent are capable for hydrogen bonding with the BGE anions (note that MeOH and water molecules are capable for donation of hydrogen bond to the anion), the pH of the BGE can be substantially affected (see [71,72]).

Strictly speaking, the Henderson–Hasselbalch equation (Eq. (12)) cannot be used to adjust the pH in ACN unless buffer components with very low or negligible tendency for homo- and heteroconjugation are applied. As discussed above, only picric acid and 2,6-dinitrophenol fulfil such a prerequisite. However, with the limitations given above, an equimolar mixture of some other acids and their respective salts can be an appropriate choice for an approximation of the pH in ACN within an acceptably small bias.

#### 4.2.3. Other solvents

The concept of the reference BGE acids and the application of the Henderson–Hasselbalch equation would be suitable in organic solvents like formamide or *N*-methylformamide with high relative permittivity, and where additional effects like homo- and heteroconjugation are negligible. So far such investigations have not been published. However, the concept has been applied to some other organic solvents, which do not fully meet these demands.

Propylene carbonate, a solvent with rather high relative permittivity (64.92 at 25.0 °C [20]), has been used as a medium to determine mobilities and ionisation constants of aliphatic amines by CZE [73]. It should be noted, however, that homo- and heteroconjugation are present also in PC [21,31,35] with the effects discussed above. Acetic acid



and tetrabutylammonium acetate in equimolar proportion has been used in PC for separation of phenols [63]. In this work, Kuldvee et al. used heteroconjugation of BGE acetates and uncharged analyte phenols as a separation mechanism. An equimolar mixture of 2,6-dihydroxybenzoic acid and 2,6-dihydroxybenzoate was the electrolyte in PC, *N,N*-dimethylformamide and *N,N*-dimethylacetamide [74]. The pH was adjusted to the  $pK_a$  value of the acid with an aqueous solution of tetraethylammonium hydroxide.

Some investigations have also been carried out with ethanol ( $\epsilon = 24.55$  at  $25.0^\circ\text{C}$  [20]), 1-propanol ( $\epsilon = 20.45$  at  $25.0^\circ\text{C}$  [20]) and 1-butanol ( $\epsilon = 17.51$  at  $25.0^\circ\text{C}$  [20]) as BGE solvents [75–77]. In these investigations, the BGEs were prepared from acetic acid and its salt in equimolar proportion. Due to strong ion pairing in solvents with such low relative permittivity (for ionic association in ethanol and 1-propanol, see [78,79], respectively), salts are only partly dissociated, which means that the pH of equimolar acetic acid and acetate BGEs is lower than the theoretical value. Accordingly, it is clear that the Henderson–Hasselbalch equation cannot appropriately be applied for pH adjustments in such solvents.

From the practical point of view mixed organic solvents are of high interest because of the wide variety of the physicochemical properties solvent mixtures can offer [80]. Most commonly used mixture in CZE is MeOH–ACN. Recently, Wróbel et al. [41] and Wróbel and Chmurzyński [42] have determined the  $pK_a$  value of 2,6-dinitrophenol in MeOH–ACN systems. With suitable detection, this data can be used as reference values to adjust the pH of the BGE in CZE as well.

#### 4.3. Effect of water content

It should be pointed out that for capillary electromigration experiments in BGEs consisting of electrolyte components dissolved in organic solvent(s), the term ‘non-aqueous’ is typically applied to make a clear distinction from aqueous BGEs. However, this does not necessarily mean that the BGEs are really water-free. In fact, the organic solvents are commonly used as commercially obtainable, and during handling they may take up traces of water, e.g. from the air. This holds especially for hygroscopic solvents; EtOH, e.g. is very, MeOH and ACN are slightly hygroscopic. Also, chemicals used for preparation of BGEs may contain a considerable amount of water (e.g. perchloric acid, see above). In addition, chemical reactions of BGE components, e.g. during neutralisation, may produce small amounts of water. Accordingly, in most CZE investigations the term ‘non-aqueous’ simply means that no water is intentionally added to the electrophoresis medium. This constitutes a difference from non-aqueous solution chemistry and electrochemistry, where even commercial solvents are further purified and carefully protected from atmospheric moisture and from any other source of impurities. Accordingly, this aspect should be taken into account when literature data for dissociation

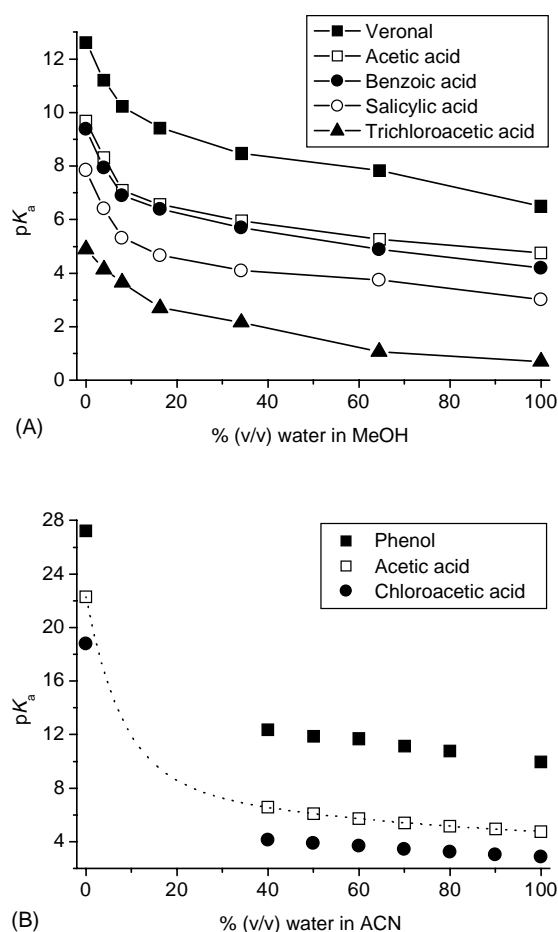


Fig. 2.  $pK_a$  of some acids in mixtures of (A) MeOH–water; (B) ACN–water at  $25.0^\circ\text{C}$ . Data for MeOH–water is from [81] (original solvent compositions given in weight percents are converted to volume percents using solvent densities from [20]). ACN–water data is from [85,86], data for pure ACN is from [29,33]. See text for details.

or other complexation constants are used in CZE related work.

In this context, it is very important, but often overseen, that the presence of a small amount of water in the non-aqueous solution can seriously affect the  $pK_a$  and pH values. In general, presence of water increases the acid strength of substances and thus reduces their  $pK_a$  values. Fig. 2A shows  $pK_a$  values of some acids in MeOH–water mixtures [81]. It can be seen from the following examples that a low concentration of water in the solvent can have a considerable effect on the  $pK_a$  of the acids. One percent (v/v) of water, e.g. reduces the  $pK_a$  of acetic acid by about 0.4 pH units from 9.7 to ca. 9.3. In contrast, traces of water, say in the range below 0.1%, decrease the  $pK_a$  only by few hundredths of units.

In case of ACN the difference between  $pK_a$  in pure ACN and pure water is rather large (e.g. for phenol the difference is about 17 units), and the decrease in  $pK_a$  with increasing water content is quite pronounced, as seen from Fig. 2B. Unfortunately, no data for water contents below 40% (v/v) in ACN is available for neutral acids (for cation acids see, e.g. [82]). An approximated curve for acetic acid shown in

Fig. 2B indicates, however, that the effect of small amounts of water has a more distinct effect on the  $pK_a$  than for the MeOH–water solvent system. According to this curve addition of, e.g. 1% (v/v) water to ACN reduces the  $pK_a$  by as much as about 1.6 units from 22.3 to ca. 20.7. It should be noted that the decay of the % (v/v) water versus  $pK_a$  curve in the low water region might be even steeper, and the change of  $pK_a$  thus larger.

Addition of water to the buffer solution consisting of a solvent where homo- and heteroconjugation can take place could affect the pH considerable. The pH of a buffer prepared, e.g. from 3.6 mmol/l benzoic acid and 30.5 mmol/l tetraethylammonium benzoate in ACN ( $pH = 23.4$ ) is decreased seriously upon addition of water [83], because the concentration of benzoate ( $A^-$ ) is reduced by hydrogen bonding with water molecules (i.e.  $A^- + H_2O \rightarrow A(H_2O)^-$ ). The pH of the solution consisting of 65 mmol/l benzoic acid and 2.18 mmol/l tetraethylammonium benzoate in ACN ( $pH = 18.6$ ), on the other hand, is not much affected upon addition of water [83], because the benzoate ion is present as a homoconjugate with the neutral benzoic acid molecule, and thus addition of water has no influence on the pH. It is obvious that the pH of picric acid–picrate buffers in ACN are not much affected by the addition of small amounts of water (or alcohols) because of the weak or negligible tendency of the buffer components for homo- and heteroconjugation [84].

## Acknowledgements

The Austrian Academic Exchange Service is acknowledged for financial support (an Ernst Mach fellowship for SPP).

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