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Review

Determination of the pH of binary mobile phases for reversed-phase liquid chromatography

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Abstract

The measurement of pH in chromatographic mobile phases has been a constant subject of discussion during many years. The pH of the mobile phase is an important parameter that determines the chromatographic retention of many analytes with acid–base properties. In many instances a proper pH measurement is needed to assure the accuracy of retention-pH relationships or the reproducibility of chromatographic procedures. Three different methods are common in pH measurement of mobile phases: measurement of pH in the aqueous buffer before addition of the organic modifier, measurement of pH in the mobile phase prepared by mixing aqueous buffer and organic modifier after pH calibration with standard solutions prepared in the same mobile phase solvent, and measurement of pH in the mobile phase prepared by mixing aqueous buffer and organic modifier after pH calibration with aqueous standard solutions. This review discusses the different pH measurement and calibration procedures in terms of the theoretical and operational definitions of the different pH scales that can be applied to water–organic solvent mixtures. The advantages and disadvantages of each procedure are also presented through chromatographic examples. Finally, practical recommendations to select the most appropriate pH measurement procedure for particular chromatographic problems are given.

Keywords: Reviews; Mobile phase composition; pH determination

Contents

1. Introduction

In 1993, the *Liquid Chromatography Problem Solving and Troubleshooting* section of a popular liquid chromatography journal brought up the following question: “It is not always clear in the literature how the pH of a mobile phase is measured. Do they measure only the pH of the buffer solution, or do they measure the pH of the organic–buffer mixture?” [1]. The answer of the expert pointed out the importance of the proper measurement of pH to control the chromatogaphic separation of ionizable analytes and to prevent column damage. He also differentiated between the pH measured in water (pH) and the “apparent pH” or “pH*” used to report pH measurements in aqueous–organic solutions (such as many liquid chromatography mobile phases are). The general opinion

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was that the pH should be measured in the aqueous buffer before mixing it with the organic modifier because the meaning of pH* was not clear and its practical measurement might be troublesome. In any case, the expert summoned authors to report clearly for any procedure how the pH was measured, because "chromatography is difficult enough to set up and operate successfully without having the extra burden of guessing or interpreting what the proper procedure should be" [1].

The above example points out several facts related to the pH of chromatographic mobile phases. On one hand, the meaning and measurement of pH in water–organic solvents mixtures was not well understood by many practical chromatographers, despite the extensive studies that had been carried out by physical and analytical chemists, reported in many books and reviews [2–8]. These studies led to IUPAC recommendations in the field and compilation of appropriate standards for pH measurements [9–12]. On the other hand, chromatographers often did not clarify how they measure the pH of the mobile phase, probably because they assumed that was too obvious or were not aware that there were operational procedures different from the one they used. These different procedures include not only the pH measurement in the sample, but also selection and calibration of the proper pH scale and practical electrode system used.

During the last 10 years, the IUPAC has continued the compilation of reference standards and procedures for pH measurement in both aqueous–organic solvent mixtures [13,14] and water [14,15], but pH meaning, calibration and measurement in liquid chromatography mobile phases, is still nowadays a matter of discussion between chromatographers and the practical pH measurement used for a particular procedure is not yet mentioned in some works. As an example, the main effect of the pH of the mobile phase on the chromatographic retention of ionic analytes has been discussed in a publication from a chromatography supplies and reagents maker [16]. The effect of the mobile phase pH on the ionization and retention of acid–base analytes and the selection of appropriate buffers is clearly explained but the proper pH measurement (either in the aqueous buffer or in the mixed mobile phase) is not mentioned at all.

The example shows that the problems in mobile phase pH measurement are not limited to discussion in research journals since they have already reached current publications of popular chromatography. The *LC Troubleshooting* section of the *LC GC* journal, in both the North America and Europe editions, has recently published a series of three interesting articles by Tindall about interpretation of pH in partially aqueous mobile phases [17], buffer selection and capacity [18], and preparation of buffers [19]. A publication of Sýkora et al. in the same journal, just before the ones of Tindall, had already discussed the importance of the proper pH measurement in chromatographic mobile phases and the different pH scales that can be used [20]. The article of Sýkora et al. also presented several practical considerations about the influence of the addition of organic modifiers to the pH of aqueous chromatographic buffers and to the ionization of acid–base analytes in these mobile phases.

In the first part of the series [17], Tindall explains the meaning of pH in a simple but rigorous manner, understandable to many chromatographers regardless of their academic preparation. He also explains the changes that the addition of an organic modifier may produce to the pH of an aqueous buffer and to the pK_a of the analyte, which will lead to changes in the chromatographic retention of an ionizable analyte. The second part [18] is devoted to the appropriate selection of chromatographic buffers, which must have an adequate buffer capacity in the working pH range (i.e. the pH of the buffered mobile phase must be close to the pK_a of the buffer component). It is explained that by considering the changes in the pK_a of the buffer components produced by addition of the organic modifier (explained in Part I of the series), analysts can estimate relative changes for sample pK_a and buffer pH values, and cautiously apply this information to buffer selection [18]. The third part of the series [19] comments the preparation of buffers in the two main cases a chromatographer may need: when the exact target pH is known or when an approximate range is known and the optimum pH must be found. Tindall concludes that no matter what technique is used, the author should include in his report or publication an unambiguous description, so that others can reproduce the results.

Tindall illustrates the importance of the pH changes upon addition of organic modifier with an example based on a publication from Claessens et al. [21]. These authors prepared chromatographic mobile phases by mixing equal amounts of aqueous buffers of pH 10 with methanol. The pH 10 aqueous buffers were prepared from phosphate and from glycine, and the authors were surprised to find that silica dissolved 10 times faster in the phosphate-buffered mobile phase than in the glycine-buffered mobile phase, regardless that the aqueous pH was the same. Claessens et al. concluded that some unknown property of the phosphate ion resulted in the aggressive attack on the silica, but Tindall suggests another explanation based on the pH shifts caused by the addition of methanol [18]. He measured the pH of the mobile phases after the addition of methanol and found that the aqueous pH 10 phosphate buffer became more basic by 0.7 pH units (from pH 10 to 10.7), whereas the aqueous pH 10 glycine buffer became more acidic by 0.1 pH units (from pH 10 to 9.9). The 0.8 pH unit difference between the two mobile phases was consistent with the differences in the observed dissolution rate [18]. A further work of Tyndall and Perry [22] corrobarated these pH shifhts and extended the study to borate and bicarbonate buffers, which increased their pH values by 0.24 and 1.40 pH units, respectively, when 0.10 M aqueous buffers of pH 10 prepared from these compounds were diluted to 50% methanol.

Although pH meaning, pH scales and pH measurement in water–organic solvent mixtures used as chromatographic mobile phases have been discussed in a recent general review about the influence of mobile phase acid–base equilib-

ria on the retention of ionizable compounds [23], it seems appropriate to devote a specific review to these subjects. It may help chromatographers to decide when the pH can/must be measured in the aqueous buffer before mixing it with the organic modifier and when it should be measured in the mobile phase obtained after mixing. The decision must take in mind the purpose of the chromatographic procedure to be developed and be based on a complete understanding of the meaning of pH and on the advantages and limitations of the different experimental procedures that can be used to measure pH. The pH meaning and procedures used to measure it in chromatography are not different than those that are currently used in solution chemistry (e.g. in determination of acid–base constants in non-aqueous and mixed solvents) and even in other practical analytical techniques. For instance, Porras and Kenndler have prepared a very recent review about pH measurement of the background electrolyte in capillary zone electrophoresis in non-aqueous solutions [24], that could be as well applied to the water–organic solvent mixtures used as chromatographic mobile phases.

2. Theoretical definition of pH

The first definition of pH was proposed by Sørensen in 1909 [25]. Sørensen looked for a simple way to write the small hydrogen ion concentrations $[H^+]$ usual in water solutions, so he decided to take the negative decimal logarithm of the hydrogen ion concentration, and pH was defined as:

$$\mathrm{pH} = -\log[H^+] \tag{1}$$

Soon he discovered the system electrodes used to measure "pH" responded to hydrogen ion activity (a_H), not to concentration, and the pH definition was changed to the negative logarithm of the hydrogen ion activity [26]:

$$\mathrm{pH} = -\log a_H \tag{2}$$

Activity and pH are dimensionless quantities, but activity must be referred to a concentration scale and so must be pH. In fact, the activity can be related to the concentration through an activity coefficient (γ). This means that the same solution may have different pH values, which depend on the scale that hydrogen ion concentration is measured. The two most used concentration scales, accepted by the IUPAC for pH definition, are molality (m, mol kg^{-1}) and molarity (c, mol dm^{-3}). For pH definition, molality would be the number of moles of hydrogen ion per kilogram of solvent (mol kg^{-1}) and molarity the number of moles of hydrogen ion per litre of solution (mol dm^{-3}). This leads to two definitions of pH, either in the molality scale (pH_m) or in the molarity scale (pH_c). Since it is not correct to write, in isolation, the logarithm of a quantity other than a dimensionless number, the full forms of the equations for pH definition are:

$$\mathrm{pH}_m = -\log\left(\frac{m_H \gamma_{m,H}}{m^0}\right) \tag{3}$$

$$\mathrm{pH}_c = -\log\left(\frac{c_H \gamma_{c,H}}{c^0}\right) \tag{4}$$

where c^0 and m^0 are arbitrary constants, representing the standard state condition, numerically equivalent to either 1 mol dm^{-3} or 1 mol kg^{-1}, respectively, and $\gamma_{c,H}$ and $\gamma_{m,H}$ are the single-ion activity coefficients of the hydrogen ion in the two scales, respectively. In general, physical chemists prefer to work in the molality scale because the weight of the solvent does not change with temperature, whereas the volume of the solution, used in the molarity scale, does change. Because of this reason, some IUPAC documents [11,15] only report pH definition in the molality scale (Eq. (3)). However, in analytical chemistry practice, including chromatography, molarity is almost always used because of its simplicity for preparation of solutions and IUPAC documents related to this field usually include the pH definitions in the two scales [12,14].

In solutions diluted enough, one may neglect the weights of the solutes in the overall solution mass and thus assume that the volume of the solution is the weight of solvent divided by the density of the solvent (ρ, kg dm^{-3}). Therefore, pH in one scale can be easily converted to pH in the other scale through Eq. (5) with $\rho^0 = 1\,\mathrm{kg\,dm^{-3}}$.

$$\mathrm{pH}_c = \mathrm{pH}_m + \log\left(\frac{\rho}{\rho^0}\right) \tag{5}$$

The density of water is close to 1 kg dm^{-3}, and therefore the two pH scales in water are practically identical (the pH difference is about 0.001 at 0 °C rising to 0.02 at 100 °C). However, the density of some non-aqueous solvents and aqueous organic solvent mixtures can be quite different from 1 kg dm^{-3}, and the transfer term $\log \rho/\rho^0$ may achieve several tenths of pH units. Densities at 25 °C of the most used chromatographic mobile phases (methanol–water, acetonitrile–water, and tetrahydrofuran–water) are well known and the ρ values and the term $\log \rho/\rho^0$ have been reported elsewhere [23]. For instance in a mobile phase 50% methanol–50% water (v/v), the $\log \rho/\rho^0$ term is −0.045 at 25 °C, and this would be the difference between the pH of this mobile phase measured in the molarity or in the molality pH scales. The particular pH scale employed in a procedure, which will depend on the buffers used to calibrate the electrode system, should be clearly indicated in the final report.

Since pH is defined in terms of activity it also depends on the standard state of the activity, i.e. the conditions for which the activity coefficient of hydrogen ion is considered to be equal to unity, and thus in this standard state activity becomes numerically equal to concentration. In water, the standard state for a_H is infinite dilution of hydrogen ion in water (i.e. pure water), for which $\gamma_H \to 1$. In a solvent s different from water (e.g. a water–organic solvent mixture used as a chromatographic mobile phase), two different standard states can be chosen. One is infinite dilution of the hydrogen ion in the same solvent *s*, and the other is infinite dilution

of the ion in water. This leads to two different pH scales, one relative to each particular solvent (${}^{s}_{s}$pH), and the other relative to water (${}^{s}_{w}$pH), which is also called "absolute pH scale".

In order to distinguish between the two pH scales, the IUPAC [9,14] recommends the notation used by Robinson and Stokes [27] for their discussion of the effect of the medium on transferring a binary electrolyte from water (w) to a non-aqueous or mixed solvent (s). Thus, lower-case left-hand superscripts indicate the solvent (w or s) in which measurements are being made; lower-case left-hand subscripts indicate the solvent in which the ionic activity coefficient γ is referred to unity at infinite dilution (w or s) [9,14].

The transfer of one mol of hydrogen ions from infinite dilution in water to infinite dilution in a different solvent s requires some work that can be measured by a free energy change ($\Delta^{s}_{w}G^{\circ}_{H}$). This free energy change is negative when the hydrogen ion is more stable in solvent s and positive when it is more stable in water. The free energy change can be considered proportional to the logarithm of an activity coefficient [3,6], indicated as ${}^{s}_{w}\gamma^{\circ}_{H}$ according to IUPAC notation [14], that relates the activity coefficient of hydrogen ion in the standard state in water to the activity coefficient in the standard state in solvent s:

$$\Delta^{s}_{w}G^{\circ}_{H} = 2.303RT \log {}^{s}_{w}\gamma^{\circ}_{H} \quad (6)$$

The "transfer activity coefficient" is called the "primary medium effect" and its logarithm determines the shift of the ${}^{s}_{w}$pH scale in reference to the ${}^{s}_{s}$pH, since the two pH scales are related by means of the following equation:

$${}^{s}_{w}\mathrm{pH} = {}^{s}_{s}\mathrm{pH} - \log {}^{s}_{w}\gamma^{\circ}_{H} \quad (7)$$

where ${}^{s}_{w}\gamma^{\circ}_{H} \rightarrow 1$ as s $\rightarrow$ w.

The ${}^{s}_{s}$pH scale is different for each solvent and solvent composition since it is a scale relative to each solvent, i.e. with a different standard state for each solvent. It allows comparison of acidities only between the same solvent. However, the ${}^{s}_{w}$pH scale is an "absolute" or "universal" pH scale that allows comparison of acidities between solutions in different solvents because the standard state is the same in all solvents. This universal pH scale illustrates clearly the fact that the concepts of the "strength" of an acid and the "acidity" of a solution should be distinguished [4]. For example, a 1 mol dm^{-3} solution of acetic acid in water has a pH value (${}^{s}_{s}$pH or ${}^{w}_{w}$pH or simply pH) of about 2.5 ([H_3O^+] = $10^{-2.5}$ mol dm^{-3}). In liquid ammonia, a strong basic solvent that has been studied at low temperatures [4,8], the acetic acid behaves as a strong acid and it is fully dissociated. Thus, in liquid ammonia the concentration of hydrogen ion (NH_4^+ in this solvent) is 1 mol dm^{-3} and ${}^{s}_{s}\mathrm{pH} = 0$. From the relative pH scales, a solution of acetic acid in liquid ammonia appears to be much more acidic (2.5-orders of magnitude) than a solution of the same concentration of acetic acid in water. This contradicts chemical reasoning because a solution in liquid ammonia must be much less active as acid than the same solution in water. Nevertheless, the $\log {}^{s}_{w}\gamma^{\circ}_{H}$ value for liquid ammonia is about −16.0 because the activity of NH_4^+ in liquid ammonia is about 16-orders of magnitude lower than that of H_3O^+ in water [4]. Thus, the absolute pH value of the acetic acid solution in liquid ammonia is ${}^{s}_{w}\mathrm{pH} = 16.0$, which indicates a solution much less acidic than that in water with ${}^{s}_{w}\mathrm{pH} = 2.5$.

The IUPAC remarks that the above definitions of pH are only notional because they involve a single ion activity (a_H) which is immeasurable [9–15]. Therefore, operational definitions of pH have been established.

3. Operational definition of pH and pH measurement

It is universally agreed that the definition of pH is a practical one, based in a series of operations that have been extensively studied for pH measurement in water [2–15]. The pH of a solution is obtained by comparison of the electromotive force of the test solution in an appropriate potentiometric cell to the electromotive force of one or several standard reference solutions of known pH. Thus, the procedure includes pH calibration and pH measurement and both operations must be adequately done to assure the quality of the quantity obtained (pH).

There are standard reference solutions S of different quality for pH calibration. The IUPAC divides these standards into primary (PS) and secondary (SS). The primary pH standard values were determined with a Harned type cell, which does not show liquid junction. The Harned cell consists of a hydrogen gas and a silver–silver chloride electrodes and it contains the standard buffer, S, and chloride ions, in the form of potassium or sodium chloride, which are added to use the silver–silver chloride electrode. The cell is defined by:

$$\mathrm{Pt|H_2|buffer\ S,\ Cl^-|AgCl|Ag} \quad (8)$$

The standard potential of the Harned cell (E^0) is calculated by measuring the potential difference of the cell for buffers S prepared from known concentrations of HCl (no KCl or NaCl are added in this instance). The potential of the cell is related to the calculated activity of hydrogen and chloride ions (assumed to be equal) and the E^0 parameter is obtained. The potential values obtained must be corrected to 1 atm partial pressure of hydrogen gas and the activity is calculated from concentration through known activity coefficients or extrapolated to zero ionic strength. The Harned cell is later filled with the standard buffer, which pH wants to be determined, and the potential of the cell is measured for at least three different concentrations of chloride ion. The pH of the buffer is calculated from the potential readings and chloride ion concentrations by linear extrapolation to zero ionic strength. For more details see reference [15]. Primary buffer solutions must fulfil a series of requisites to assure the highest metrological quality in pH measurement. Buffers that do not fulfil strictly these conditions or which pH has not been determined by the primary method based

in the Harned cell can be considered secondary standards if their pH value can be traced to the one of a primary standard (i.e. obtained by comparison to the pH and potential of the primary standard in the same cell).

Everyday pH measurement of an unknown solution X is not usually done with a Harned cell, since this would be extremely complex and expensive. In chromatography and in many other analytical practices, the glass electrode combined with a reference electrode (silver–silver chloride or calomel) is almost always used:

$$\text{glass electrode}|\text{solution S or X}||\text{KCl}(c \geq 3.5\,\text{mol dm}^{-3})|\ \text{reference electrode} \quad (9)$$

where S and X are the standard reference solution (PS or SS) and the unknown test solution, respectively. The reference electrode contains a filling solution, which usually is a highly concentrated solution with equitransferent cation and anion, e.g. KCl, that minimizes the liquid junction potential. The potential difference of the cell (E) is the addition of the potentials of the glass electrode (E_{glass}), the reference electrode (E_{ref}) and the liquid junction potential (E_{j}). The liquid junction potential is the potential difference arising between two electrolyte solutions of different composition, i.e. between the solution S or X and the reference electrode filling solution. The overall electromotive force of this type of electrode systems may be given by the equation:

$$E = E^0 + E_j - g\text{pH} \quad (10)$$

where ideally $g = (RT/F)\ln 10$ and R is the gas constant, T the thermodynamic temperature, and F the Faraday constant. E^0 is a constant, combination of the standard potential of the glass electrode and the potential of the reference electrode. The recommended symbol for the term $(RT/F)\ln 10$ is k, but we shall use g to avoid any confusion with chromatographic retention factors.

Various random and systematic effects must be considered when using glass electrodes for pH measurement [15]. Glass electrodes may exhibit a slope (g) of the E versus pH function smaller than the theoretical $(RT/F)\ln 10$ value, often called a sub-Nernstian response, which is experimentally determinable. The potential of the glass electrode is strongly temperature-dependent and calibration and measurement should be carried out under temperature-controlled conditions. The liquid junction potential varies with the composition of the solutions forming the junction, i.e. it changes when S or X changes, and it also depends on the geometry and type (sleeve, ceramic, diaphragm, fibre, etc.) of the junction. Liquid junction potentials may suffer from clogging, memory, and hydrodynamic (stirring) effects. Since these effects introduce errors of unknown magnitude, the measurement of an unknown sample requires a suitable calibration procedure. Three procedures are common: one-point calibration, two-point calibration, and multipoint calibration.

In one point calibration, the pH of a test solution (pH_X) is determined by comparison of the electromotive forces E_X and E_S of two appropriate potentiometric cells. The two cells must be equal except for that one contains the test solution X (potential reading E_X) and the other a standard reference solution S (potential reading E_S) of known pH (pH_S). The pH_X is determined from:

$$\text{pH}_\text{X} = \text{pH}_\text{S} - \frac{E_\text{X} - E_\text{S}}{g} \quad (11)$$

ignoring the term $\Delta E_\text{J} = E_\text{JX} - E_\text{JS}$, which is called the residual liquid junction potential. g is taken as equal to the theoretical value. The standard chosen should have a pH_S value as close as possible to the pH_X value of the sample in order to minimize the error in the variation of g.

In the majority of practical applications, glass electrode cells are calibrated by two-point calibration, or bracketing, procedure using two standard buffer solutions, with pH values pH_{S1} and pH_{S2}. Many commercial instruments use buffers of aqueous pH 4 and 7 (or sometimes 9). This procedure determines the practical slope g and assumes the liquid junction potentials of the two standards and the test solution are equal. If the respective potentials of the two buffers are E_{S1} and E_{S2}, the pH value of the unknown is:

$$\text{pH}_\text{X} = \text{pH}_\text{S} - \frac{E_\text{X} - E_\text{S}}{g} \quad (12)$$

with

$$g = \frac{E_{\text{S1}} - E_{\text{S2}}}{\text{pH}_{\text{S2}} - \text{pH}_{\text{S1}}} \quad (13)$$

Calibration using more than two points (or multipoint calibration) should be carried out using up to five standard buffers, because using more buffers does not improve the quality of the information obtained [15]. Since again the liquid junction potentials are assumed to be constant, they can be included in the constant ($E^{0'} = E^{0'} + E_\text{j}$), and the calibration function is:

$$E_\text{S} = E^{0'} - g\text{pH}_\text{S} \quad (14)$$

The constant $E^{0'}$ and g are calculated by linear regression from the measured potentials (E_S) and known pH_S of the multiple standards. The pH of the unknown is later calculated from its measured potential (E_X) through the equation:

$$\text{pH}_\text{X} = \frac{E^{0'} - E_\text{X}}{g} \quad (15)$$

None of the procedures can correct the variation of the liquid junction potential between the different standards and between them and the test solution. Therefore, the variation of the junction potential introduces an error in pH measurement intrinsic to the method. This error cannot be avoided in cells with liquid junction, only minimized using reference filling solutions highly concentrated with a cation and an anion of similar mobility (equitransferent ions). Use of the same solvent composition in standard, sample and reference electrode filling solutions also minimizes liquid junction potential error. Of course, the same electrode pair and temperature should be used for both calibration and measurements.

The same three procedures for pH calibration in water, can be used for pH calibration and measurement in non-aqueous and mixed solvents. The procedure for one-point calibration implies the measurement of the electromotive forces (${}^{s}E_X$ and ${}^{s}E_S$) of two potentiometric cells, one containing the test solution X in solvent s and the other a standard reference solution S prepared in the same solvent s and of known ${}^{s}_{s}$pH (${}^{s}_{s}pH_S$). The ${}^{s}_{s}pH_X$ is determined from:

$$ {}^{s}_{s}pH_X = {}^{s}_{s}pH_S - \frac{{}^{s}E_X - {}^{s}E_S}{g} \quad (16) $$

The two-point calibration uses the equations:

$$ {}^{s}_{s}pH_X = {}^{s}_{s}pH_{S1} - \frac{{}^{s}E_X - {}^{s}E_{S1}}{g} \quad (17) $$

and

$$ g = \frac{{}^{s}E_{S1} - {}^{s}E_{S2}}{{}^{s}_{s}pH_{S2} - {}^{s}_{s}pH_{S1}} \quad (18) $$

And the multipoint calibration fits the potentiometric data (${}^{s}E_S$) to the equation:

$$ {}^{S}E_S = {}^{S}E^{0'} - g\,{}^{s}_{s}pH_S \quad (19) $$

and calculates ${}^{s}_{s}$pH from:

$$ {}^{s}_{s}pH_X = \frac{{}^{S}E^{0'} - {}^{S}E_X}{g} \quad (20) $$

In the three methods, the liquid junction potential (${}^{s}E_J$) of samples and reference solutions is assumed to be the same, and it is included in the ${}^{S}E^{0'}$ constant. The three calibration procedures considered require the assignment of reference ${}^{s}_{s}$pH values to standard solutions (primary or secondary) prepared in a solvent of exactly the same composition as the solvent where the pH will be measured. There are only a few reference ${}^{s}_{s}$pH values reported for aqueous–organic solvent mixtures. For common chromatographic mobile phases, the IUPAC [10,13,14] reports only data of 0.05 m potassium hydrogenphthalate buffer for some methanol–water and acetonitrile–water compositions, a few other buffers in 50% (w/w) methanol, and oxalate and succinate buffers for several compositions of methanol–water mixtures [28–32]. Notice that all these data are given in the molality pH scale, and a correction according to Eq. (5) is needed if they are used as standards to measure the pH in the molarity scale. Some more ${}^{s}_{s}$pH reference data in the molarity scale have been determined by Barbosa et al. for acetonitrile–water [33–36] and tetrahydrofuran–water [37] that can be directly used for ${}^{s}_{s}$pH standardization in these mobile phases.

The ${}^{s}_{w}$pH scale may also be used for pH measurement in non-aqueous or mixed solvents. In this instance, the ${}^{s}_{w}$pH of the test sample (${}^{s}_{w}pH_X$) is determined by measuring the electromotive forces of the test sample in the solvent s (${}^{s}E_X$) and one or several standard reference solution in water (${}^{w}E_S$) of known pH (${}^{w}_{w}$pH), i.e. primary or secondary standard reference buffer solutions in water.

For one point calibration, the ${}^{s}_{w}$pH is given by:

$$ {}^{s}_{w}pH_X = {}^{w}_{w}pH_S - \frac{{}^{s}E_X - {}^{w}E_S}{g} \quad (21) $$

For two-point calibration the equations used are:

$$ {}^{s}_{w}pH_X = {}^{w}_{w}pH_{S1} - \frac{{}^{s}E_X - {}^{w}E_{S1}}{g} \quad (22) $$

and

$$ g = \frac{{}^{w}E_{S1} - {}^{w}E_{S2}}{{}^{w}_{w}pH_{S2} - {}^{w}_{w}pH_{S1}} s \quad (23) $$

And the multipoint calibration fits the potentiometric data of the aqueous standards (${}^{w}E_S$) to their aqueous pH (${}^{w}_{w}pH_S$) through the equation:

$$ {}^{w}E_S = {}^{w}E^{0'} - g\,{}^{w}_{w}pH_S \quad (24) $$

and calculates the ${}^{s}_{w}$pH of the unknown (${}^{s}_{w}pH_X$) by measuring its potential (${}^{S}E_X$) and applying Eq. (25):

$$ {}^{s}_{w}pH_X = \frac{{}^{w}E^{0'} - {}^{S}E_X}{g} \quad (25) $$

Again the constancy of the liquid junction potential between standards and samples is assumed in the practical measurement of pH in the ${}^{s}_{w}$pH scale. It is important to notice that this assumption is intrinsic to pH measurement in any solvent, aqueous, non-aqueous or mixed, because there is no way to measure the contribution of the liquid junction potential to pH. However, the error in pH determination introduced by this assumption is likely to be larger in the ${}^{s}_{w}$pH scale (i.e. when pH calibration and measurement are done in different solvents) than in the ${}^{s}_{s}$pH scale (i.e. when pH calibration and measurement are done in the same solvent), because the mobilities of ions that cause the liquid junction potential are solvent dependent. This error may arrive to 1–2 mV for some pure non-aqueous solvents.

The difference in the liquid junction potential between samples in solvent s and standards in water introduces another term of variation in the relationship between ${}^{s}_{w}$pH and ${}^{s}_{s}$pH scales, in addition to the medium effect $\log({}^{s}_{w}\gamma^{0}_{H})$ (Eq. (7)). The determination of this primary medium effect for a particular solvent s (i.e. the difference between ${}^{s}_{w}$pH and ${}^{s}_{s}$pH scales) would imply the measurement of the ${}^{s}_{w}$pH of one or several standards with ${}^{s}_{s}$pH known in solvent s, after calibration of the electrode system with aqueous standard reference solutions. The difference would include not only the medium effect but also the difference between the liquid junction potentials of the measured solution and aqueous standards ($\Delta E_J = {}^{s}E_{JX} - {}^{w}E_{JS}$). The addition of the two terms has been called the δ term [3,6,11,12], i.e.:

$$ \delta = \overline{E_j} - \log({}^{s}_{w}\gamma^{0}_{H}) = {}^{s}_{w}pH - {}^{s}_{s}pH \quad (26) $$

with

$$ \overline{E_j} = \frac{{}^{s}E_{JX} - {}^{w}E_{JS}}{g} \quad (27) $$

The knowledge of δ values for the water–organic solvents mixtures of interest (e.g. chromatographic mobile phases) is very practical because one may calibrate the pH electrode system with the usual aqueous standards, measure the ${}^{s}_{w}pH$ of the particular mobile phase of interest, and through the δ value (Eq. (26)) convert it easily to ${}^{s}_{s}pH$, which is the pH magnitude that can be directly related to the thermodynamic acid–base constants of the ionizable solute. ${}^{s}_{s}pH$ can be also related to solute concentrations through ionic activity coefficients estimated by means of Debye–Hückel type equations.

Unfortunately, there are not many δ values available for chromatographic mobile phases. Bates [3] reported δ values for methanol–water mixtures, which had been obtained from two different sets of authors by using the hydrogen gas electrode [38,39]. Some other δ values have been obtained recently for a glass electrode system [40], which agree quite well with the two set values reported by Bates. This agreement indicates that the residual liquid junction contribution to δ values (i.e. the difference between the liquid junction potentials of the methanol–water test solution and the aqueous standard solution) may be quite low for many well designed electrode systems (electrodes properly designed to minimize the liquid junction potential), and thus to be of general application. In fact, the contribution of the residual liquid junction potential can be insignificant for many practical pH measurements. For example, a 3 M KCl salt bridge in water can experience junction potentials on the order of 1 mV (about 0.02 pH units), which may be partially balanced by a similar junction potential in the mobile phase. Therefore, the error introduced by the residual liquid-junction potentials can be estimated to be about 0.01 pH units or less, which is indeed a low error for practical liquid chromatography measurements [41]. Therefore, conversion between ${}^{s}_{w}pH$ and ${}^{s}_{s}pH$ values can be easily done with the reported δ values. The available δ values for methanol–water mixtures have been fitted to the volume fraction of methanol (ϕ_{MeOH}) in the solvent mixture [40] through the equation:

$$\delta = \frac{0.09\phi_{MeOH} - 0.11\phi^2_{MeOH}}{1 - 3.15\phi_{MeOH} + 3.51\phi^2_{MeOH} - 1.35\phi^2_{MeOH}} \quad (28)$$

δ values for acetonitrile–water up to 60% of acetonitrile in volume have been reported too, but only from one glass–reference electrode system [41]. These δ values for acetonitrile–water can be estimated from the solvent composition (ϕ_{MeCN} or volume fraction of acetonitrile) through the equation [23,41]:

$$\delta = \frac{-0.446\phi^2_{MeCN}}{1 - 1.316\phi_{MeCN} + 0.433\phi^2_{MeCN}} \quad (29)$$

The difference between ${}^{s}_{w}pH$ and ${}^{s}_{s}pH$ is a constant value for each mobile phase composition. However, the difference between ${}^{s}_{w}pH$ and ${}^{s}_{s}pH$ (or ${}^{s}_{w}pH$) depends not only of the mobile phase composition, but also of the particular buffering solution measured [23,42]. Several examples have been presented to illustrate this fact. A 0.010 M solution of HCl in water has a ${}^{w}_{w}pH$ of 2.046 and if the same solution is prepared in 50% acetonitrile the ${}^{s}_{s}pH$ is 2.068. The pH variation between the two solutions is 0.022, due only to the variation of the activity coefficient. However, if the solution is 0.010 M in KOH, the pH value is 11.954 in water (${}^{w}_{w}pH$), but 13.412 in 50% acetonitrile (${}^{s}_{s}pH$). For this basic solution the pH variation is 1.458, quite more considerable than for the solution of HCl because of the variation of the autoprotolysis constant between the two solvents ($pK_{ap} = 14.00$ in water, but 15.48 in 50% acetonitrile) [23]. See also the example of Tindall [18,22] presented in the introduction: an aqueous buffer solution of pH 10.0 prepared from phosphate has a ${}^{s}_{s}pH$ value of 10.7 when diluted to 50% methanol, but if the aqueous buffer of pH 10.0 is prepared from glycine, the ${}^{s}_{s}pH$ value decreases to 9.9 when diluted to 50% methanol.

Table 1 presents some more examples of the pH variation of some aqueous buffers of chromatographic interest with the addition of organic modifiers [41–43]. It can be observed that in general, the ${}^{s}_{s}pH$ value of buffers prepared from neutral (phosphoric, acetic, citric and boric) or anionic (phosphates and citrates) acids and its conjugated base increases with the addition of organic solvent because the pK_a values of neutral and anionic acids in many water–organic solvent mixtures increase with the contents of organic solvent. The pH variation increases with the charge of the acid, i.e. the pH of hydrogen citrate/citrate buffer varies more with the addition of acetonitrile or methanol than the pH of dihydrogencitrate/hydrogencitrate, and the pH of this one changes more than the one of citric acid/dihydrogencitrate buffers. The ${}^{s}_{s}pH$ of buffer solutions prepared from neutral bases (ammonia and butylamine) shows a different behaviour. It slightly decreases with the addition of acetonitrile up to 60%, but shows a steeper decrease with the addition of methanol up to 80%. For larger concentrations of organic solvent, the ${}^{s}_{s}pH$ value is expected to increase, since the pK_a value of neutral bases in acetonitrile–water and methanol–water mixtures shows a minimum for acetonitrile or methanol concentrations between 50 and 90% of organic solvent. This differential behaviour of neutral or anionic acids, on one hand, and cationic acids, on the other hand, is caused by the decrease of the dielectric constant of the medium when the organic modifier is added to an aqueous buffer [22,23,41]. The dissociation of a neutral or anionic acid produces an increase in the concentration of charged species ($HA \Leftrightarrow H^+ + A^-$ or $HA^- \Leftrightarrow H^+ + A^{2-}$), whereas the dissociation of a cationic acid does not change the number of charged species ($HA^+ \Leftrightarrow H^+ + A$). The decrease of the dielectric constant of the medium favours aggregation of ionic species and thus unfavours dissociation of neutral and anionic acids (increasing the pK_a value), but it does not affect protonated bases. The smaller variation of the pK_a of bases with addition of organic modifier is caused by changes in the basicity of the solvent and in the solvation of the different species. These two effects contribute to the changes in the pK_a of neutral and anionic acids too, but in a minor degree than the change in the dielectric constant [23,41].

Table 1
Variation of the pH of aqueous buffers at overall concentration 0.01 mol dm^{-3} with the addition of organic modifiers [41–43]

Buffer	${}^{w}_{w}$pH	% Acetonitrile			% Methanol		
		ΔpH					
		20	40	60	40	60	80
H_3PO_4	2.00	0.10	0.34	0.70	0.49	0.85	0.95
H_3Cit/H_2Cit^-	3.00	0.27	0.67	1.23	0.70	1.18	1.34
$H_2Cit^-/HCit^{2-}$	4.00	0.35	0.85	1.60	–	–	–
HAc/Ac$^-$	4.00	–	–	–	0.69	1.31	1.80
HAc/Ac$^-$	5.00	0.41	1.13	1.81	0.77	1.33	1.85
$Hcit^{2-}/Cit^{3-}$	6.00	0.51	1.02	1.56	1.13	1.66	2.41
$H_2PO^{4-}/HPO_4{}^{2-}$	7.00	0.45	0.93	1.47	0.99	1.74	2.45
$H_2PO^{4-}/HPO_4{}^{2-}$	8.00	0.42	0.74	1.43	–	–	–
$NH_4{}^+/NH_3$	8.00	–	–	–	−0.40	−0.49	−0.70
$NH_4{}^+/NH_3$	9.00	–	–	–	−0.41	−0.64	−0.71
$H_3BO_3/H_2BO_3{}^-$	9.00	0.67	1.38	2.19	0.21	0.61	0.53
$BuNH_3{}^+/BuNH_2$	10.00	−0.22	−0.37	−0.21	−0.52	−0.70	−1.19
$BuNH_3{}^+/BuNH_2$	11.00	−0.14	−0.14	−0.13	−0.31	−0.46	−1.15
$PO_4{}^{3-}$	12.00	0.37	0.80	1.61	0.14	0.21	0.00

$\Delta pH = {}^{s}_{s}pH - {}^{w}_{w}pH$.

Table 2
Variation of the pK_a of acids and bases in acetonitrile–water mixtures with the percentage of acetonitrile [44]

	${}^{w}_{w}pK_a$	ΔpK_a (%)									
		10	20	30	40	50	60	70	80	90	100
Acetic	4.76	0.3	0.6	0.8	1.2	1.6	2.2	–	–	–	17.5
Benzoic	4.21	0.4	0.6	0.8	1.0	1.5	2.1	–	–	–	16.3
Phenol	9.98	0.7	0.9	1.1	1.4	2.0	2.8	–	–	–	13.5
Methylamine	10.62	−0.1	−0.1	−0.2	−0.3	−0.4	−0.3	–	–	–	7.8
Aniline	4.61	−0.1	−0.3	−0.4	−0.6	−0.7	−0.7	–	–	–	6.0
Pyridine	5.17	−0.2	−0.3	−0.5	−0.6	−0.8	−0.9	−1.0	−1.2	−0.4	7.2

Δp$K_a = {}^{s}_{s}pH - {}^{w}_{w}pK_a$.

The variation of the pK_a values of several representative neutral acids and bases with the addition of acetonitrile or methanol has been estimated from the equations and data given in literature [44,45] and it is presented in Tables 2 and 3.

4. Evolution of mobile phase pH measurement in reversed-phase liquid chromatography

Reversed-phase liquid chromatography is a relative new analytical technique. Although its roots can be traced to just one hundred years ago, when Tswett separated several vegetal pigments of leaf extracts in a chalk column, the technique could not be extensively applied until the end of the 1960s and early 1970s. At that time, reversed-phase liquid chromatography emerged as a common separation technique because of the extensive developments in high-pressure pumping systems and efficient column packings with chemically bonded phases [46,47].

In the 1970s, the development of reversed-phase liquid chromatography as an analytical technique of practical interest propitiated theoretical research in the fundamentals of the technique. Pioneer studies were carried out by Horváth and Melander who investigated and reviewed the rules that govern chromatographic retention in reversed-phase

Table 3
Variation of the pK_a of acids and bases in methanol–water mixtures with the percentage of methanol [45]

	${}^{w}_{w}pK_a$	ΔpK_a (%)									
		10	20	30	40	50	60	70	80	90	100
Acetic	4.76	0.2	0.4	0.6	0.8	1.1	1.3	1.5	1.8	2.2	5.0
Benzoic	4.21	0.2	0.4	0.6	0.8	1.1	1.4	1.7	2.1	2.6	3.0
Phenol	9.98	0.2	0.3	0.5	0.7	0.9	1.1	1.3	1.6	2.1	4.4
Methylamine	10.62	−0.1	−0.3	−0.4	−0.5	−0.7	−0.8	−0.9	−1.0	−0.9	0.8
Aniline	4.61	−0.1	−0.2	−0.3	−0.4	−0.5	−0.6	−0.7	−0.8	−0.7	1.0
Pyridine	5.17	−0.3	−0.5	−0.7	−0.9	−1.1	−1.3	−1.5	−1.5	−1.3	0.3

Δp$K_a = {}^{s}_{s}pH - {}^{w}_{w}pK_a$.

and reversed-phase ion-pair chromatography [48–50]. They studied the effect of solute ionization on the retention of weak acids, bases, and ampholytes and established equations that related retention (k, retention factor) to the pH of the mobile phase [48]. Horváth and Melander were aware that the pH must be measured in the eluent used for separation, but since the acid–base equilibria in the mixed solvents were more difficult to treat than in water, they limited their experiments to neat aqueous eluents, which contained no organic solvent [48].

Van de Venne et al. [51] extended the work of Horváth and Melander to mobile phases prepared from aqueous–organic solvent mixtures. They demonstrated that the retention of carboxylic acids was directly related to the pH of methanol–water mixtures used as mobile phases (with pH measured in these mobile phases) by means of the pK_a of the acid in the same methanol–water mobile phases. They recommended the measurement of pH in the mobile phase after calibration with standard buffer solutions of the same solvent composition as the mobile phase if they were available. However, as the preparation of standard pH-calibration buffer solutions for different aqueous–organic mixtures is time consuming, they suggested to calibrate the electrode system with aqueous standard buffer solutions and convert the pH readings to methanol–water pH values by using the δ values determined by de Ligny et al. for methanol–water solutions [29–31,52].

The lack of pH values for standard buffer solutions and δ values for pH calibration in mobile phases other than methanol–water (such as acetonitrile–water) hampered the measurement of pH in the same mobile phase used for separation. Thus, the common practice of measuring the pH of the mobile phase in the aqueous buffer before mixing it with the organic modifier was extended among many workers. Some misunderstandings about pH scales and pH measurement in non-aqueous and mixed solvents contributed to the extension of this practice. It was also argued, incorrectly, that the glass electrode could be damaged or give poor reproducibility when used in aqueous–organic mobile phases [1].

Schoenmakers and co-workers developed several models in the early 1990s to model retention as a function of pH and solvent compositions [53–55]. The equations derived to relate retention to pH at a fixed mobile phase composition were similar to those developed by Horváth and Melander. For a solute with an acid–base equilibria of the type:

$$HA^z \Leftrightarrow H^+ + A^{z-1}, \qquad K'_a = a_H \frac{[A^{z-1}]}{[HA^z]} \tag{30}$$

the overall retention factor of the analyte (k) is given by the expression:

$$k = \frac{k_{HA} + k_A 10^{pH-pK_a}}{1 + 10^{pH-pK_a}} \tag{31}$$

where k_{HA} and k_A are the retention factors of the acid and basic forms, respectively, of the analyte, i.e. the retention factor that is obtained when the analyte is completely in its acidic or basic form, respectively. The same type of expression (replacing k by the appropriate magnitude: t_R, V_R, t_R' or V_R') is obtained if the retention is measured in retention time or volume or adjusted retention time or volume instead of retention factor [23,55]. More complex expressions are obtained if the analyte has more than one acid–base equilibria [23,56].

Eq. (31) defines a sigmoidal plot for the retention of an acid–base analyte as a function of the pH of the mobile phase. The inflection point of the plot should agree with the acid–base pK_a value of the analyte. Schoenmakers and co-workers [53–55] discussed the different approaches to pH measurement (before and after mixing aqueous buffer and organic modifier) and concluded that measuring before mixing was more practical because pH has to be measured only once for each different buffer. The pH is always the same for all mobile phases prepared from the same aqueous buffer, regardless of the amount and type of organic modifier added. This is a practical advantage, especially for automated systems where it is technically difficult to measure the pH of the eluent after mixing [54]. The major shortcoming is that the pK_a values obtained in the fits of retention to pH (inflection point) do not have a physical meaning, i.e. do not agree with the expected thermodynamic pK_a values of the analyte [54,55].

McCalley [57–59] studied the protonation of bases in methanol–water, acetonitrile–water and tetrahydrofuran–water with phosphate buffers and concluded that half-protonation of the bases (inflection points of the sigmoidal plots) was produced at aqueous pH much lower than the aqueous pK_a value of the base. Kele and Guiochon [60] and Neue et al. [61] found that amines in a 65% methanol mobile phase buffered with phosphate at pH 7 measured before the addition of methanol were not as protonated as expected from its aqueous pK_a. This was attributed to the increase of the pH of the phosphate buffer and the decrease of the pK_a of the amine caused by the addition of methanol.

Sýkora et al. [62] studied the effect in the retention of neutral bases of mobile phase pH measured in the aqueous buffer. They observed apparent shifts of the retention versus pH plots towards pH values more acidic than the true pK_a value of the base. They demonstrated that the shifts were a combination of the two individual shifts caused by the change in the dissociation of the buffer (which produces a mobile phase pH change) and by the change in the pK_a of the basic analyte caused by the addition of organic modifier. The individual shifts are different for each buffer and analyte, respectively. The combined overall shift of the examples shown (acidic buffer and basic analyte) increases with the percentage of organic modifier because of the increase of both individual shifts. Sýkora et al. used normalized retention (r), which allows an easy comparison between the data in different mobile phases for which retention can be quite different. The use of r is equivalent to assume that $k_{HA} = 0$ and $k_A = 1$ in Eq. (31), and thus normalized retention

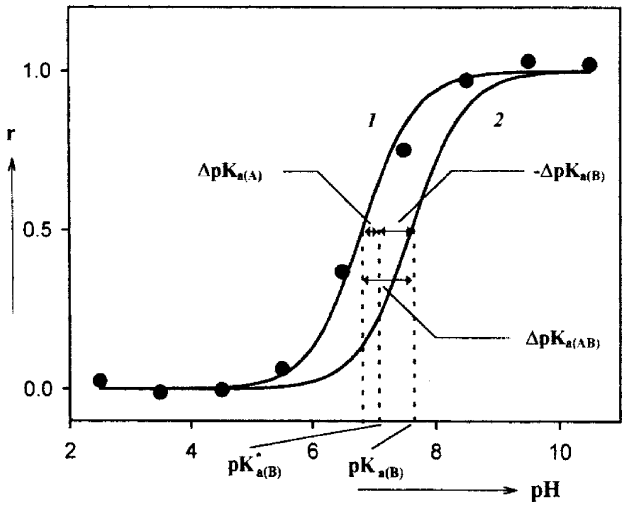

Fig. 1. Influence of methanol on the shift of normalized retention (r) vs. pH dependence. Stationary phase: HEMA-BIO 1000 C_{18}. Mobile phase: methanol–25 mM sodium phosphate buffer (20:80). Analyte: (●) 2,4,6-collidine. Curve 1: fitted to the experimental data. Curve 2: theoretical dependence according to the ${}^{w}_{w}$pH value of the aqueous buffer and the aqueous ${}^{w}_{w}pK_a$ value of the analyte. $\Delta pK_{a(A)}$ change in the pK_a value of the phosphate buffer (${}^{s}_{s}pK_{a(A)} - {}^{w}_{w}pK_{a(A)}$); $-\Delta pK_{a(B)}$ change in the pK_a value of the basic analyte (${}^{s}_{s}pK_{a(B)} - {}^{w}_{w}pK_{a(B)}$); $\Delta pK_{a(AB)}$ overall apparent pK_a change (−pH shift); $pK^{*}_{a(B)} \equiv {}^{s}_{s}pK_{a(B)}$; $pK_{a(B)} \equiv {}^{w}_{w}pK_{a(B)}$. From ref. [62], with permission.

depends only on the pH of the buffer and the pK_a of the analyte:

$$r = \frac{10^{\mathrm{pH}-\mathrm{p}K_a}}{1 + 10^{\mathrm{pH}-\mathrm{p}K_a}} \tag{32}$$

Two examples are depicted in Figs. 1 and 2. Comparison of the two figures shows that the shift in both, pH of the phosphate buffer ($\Delta pK_{a(A)}$) and pK_a of the base

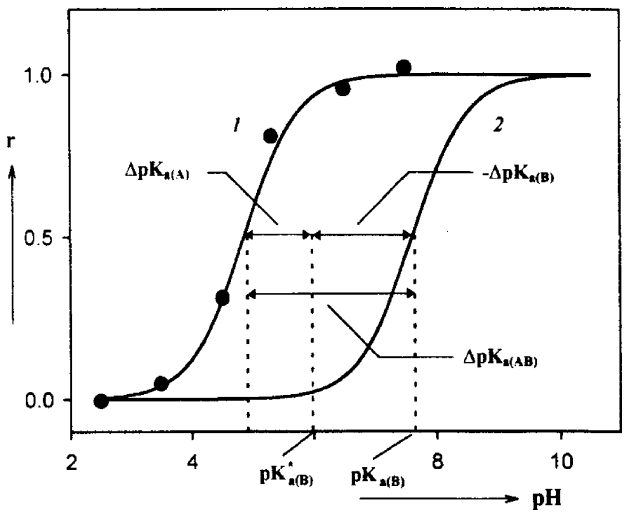

Fig. 2. Influence of methanol on the shift of normalized retention (r) vs. pH dependence. Stationary phase: symmetry C_{18}. Mobile phase: methanol–25 mM sodium phosphate buffer (60:40). Analyte: (●) 2,4,6-collidine. Curves 1 and 2 and other symbols as in Fig. 1. From ref. [62], with permission.

($\Delta pK_{a(B)}$) increase with the percentage of methanol in the mobile phase, as can be easily deduced from the data in Tables 1 and 3.

The shifts for different analytes were also studied by Canals et al. for ammonium acetate buffer in methanol–water mobile phases [63]. Four representative solutes are depicted in Fig. 3. Benzoic acid has a pK_a value close to that of acetic, and thus the pH range for variation of retention of this analyte is buffered by the acetic/acetate buffer. The variation of the pK_a of benzoic acid with the addition of methanol is slightly larger than that of acetic acid (Table 3) and therefore slightly larger than the pH variation of acetic/acetate buffer (Table 2). In consequence the normalized retention versus aqueous pH plot shifts to slightly higher pH values with the increase of methanol contents in the mobile phase (Fig. 3). 4-*tert*-Butylpyridine has also a pK_a value close to that of acetic acid, but since its pK_a decreases with the addition of methanol and the pH of the acetic/acetate buffer increases, the increase in the contents of methanol in the mobile phase produces large shifts to lower pH values in the normalized retention versus aqueous pH plots (similar to those of Figs. 1 and 2). Ephedrine is a stronger base than 4-*tert*-Butylpyridine, and thus the ammonia/ammonium buffer covers the pH of variation of retention. The decrease of the pK_a of ephedrine with the addition of methanol matches almost exactly the pK_a decrease of ammonia, and therefore the change in methanol contents in the mobile phase practically does not affect the position of the normalized retention versus aqueous pH plot. The behaviour of lidocaine is more complex. Its pK_a value is between that of acetic and ammonium acids and it may be buffered by one (acetic/acetate) or other (ammonium/ammonia) acid–base pair. Since lidocaine is a neutral base, an increase in methanol content decreases its pK_a and the overall trend is that the normalized retention versus aqueous pH plot moves towards lower pH values, although the sigmoidal shape is somewhat distorted.

The above examples show that good fits of retention to aqueous pH through Eqs. (31) and (32) can be obtained when the same acid–base buffer type is used for all measured points, because then the buffer shift is the same for all points. However, the use of the same buffer type is not possible when retention must be measured along a wide pH range. In this instance, different buffers must be used and the shift of each buffer can be different resulting in bad fits of retention to pH. This is illustrated in Fig. 4 for the normalized retention of a neutral acid. When the pH is measured in the mobile phase, after mixing aqueous buffer and organic modifier, normalized retention fits well Eq. (32) regardless that if the electrode system was calibrated with standard solutions in the same mobile phase solvent (${}^{s}_{s}$pH scale, full line) or calibrated with the aqueous buffers (${}^{s}_{w}$pH scale, dashed line). The difference between the two sets of pH data for all chromatographic buffers is the constant δ term. The inflection points of the two plots are indicated by ${}^{s}_{s}pK'_a$ and ${}^{s}_{w}pK'_a$, respectively, and their pH difference is δ.

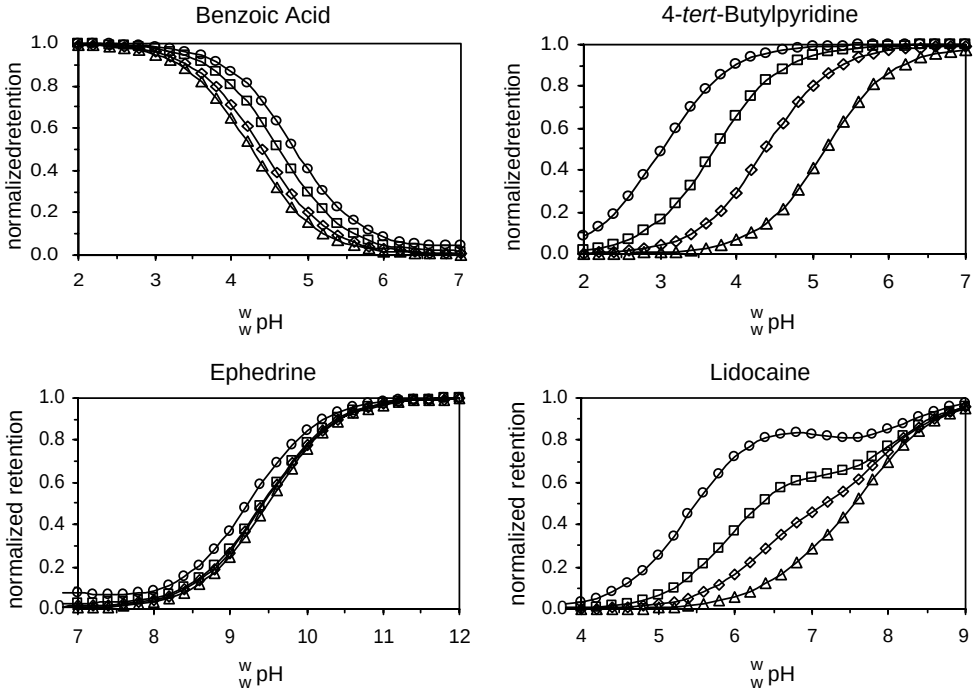

Fig. 3. Calculated retention plots for selected compounds in several isocratic methanol/water mobile phases. Methanol concentrations: (△) 20%, (◇) 40%, (□) 60%, and (○) 80%. From ref. [63], with permission, ©2001 American Chemical Society.

However, if the pH is measured in the aqueous buffer ($^{w}_{w}$pH scale) the ΔpH difference between the value measured in the mobile phase and that of the buffer is different for each buffer. For instance in a 60% methanol mobile phase, Table 1 shows that for an acetic/acetate buffer (HB/B^{-}) the pH variation would be about 1.3, for a dihydrogen phosphate/hydrogen phosphate buffer (HB^{-}/B^{2-}) about 1.7 and for an ammonium/ammonia buffer about −0.6 (HB^{+}/B).

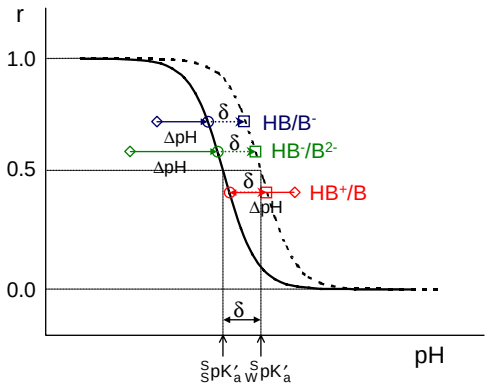

Fig. 4. Position of the pH points of chromatographic buffers of different type when measured in different pH scales: (◇) $^{w}_{w}$pH measured in the aqueous buffer before addition of organic modifier, (○) $^{s}_{s}$pH measured in the mobile phase after mixing aqueous buffer and organic modifier with calibration of the pH electrode system in the same mobile phase solvent, (□) $^{s}_{w}$pH measured in the mobile phase after mixing aqueous buffer and organic modifier with calibration of the pH electrode system in water. Continous line predicted by Eq. (32) for $^{s}_{s}$pH scale, dashed line predicted by Eq. (32) for $^{s}_{w}$pH scale.

The convenience of measuring the pH in the mobile phase and not in the aqueous buffer when buffers of different type are used has been discussed in several publications [23,40–43,64,65]. Graphical examples are presented in Figs. 5 and 6 for an acidic analyte (3-nitrophenol) and a basic analyte (triethylamine) in a 40% acetonitrile mobile

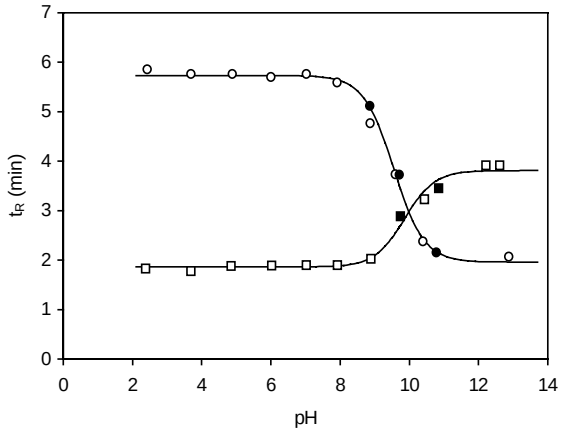

Fig. 5. Variation of the retention time of 3-nitrophenol and triethylamine in the polymeric column with the 40% acetonitrile mobile phase pH measured after mixing the aqueous buffer with the organic modifier ($^{s}_{s}$pH scale): (○) 3-nitrophenol in neutral and anionic acid buffers, (●) 3-nitrophenol in ammonia and butylamine buffers, (□) triethylamine in neutral and anionic acid buffers, (■) triethylamine in butylamine buffers. Neutral acid buffers: phosphoric acid, citric acid + dihydrogencitrate, acetic acid + acetate, and boric acid + borate. Anionic acid buffers: dihydrogencitrate + hydrogencitrate, hydrogencitrate + citrate, dihydrogenphosphate + hydrogenphosphate, hydrogenphosphate + phosphate, and phosphate. From ref. [41], with permission, ©2000 American Chemical Society.

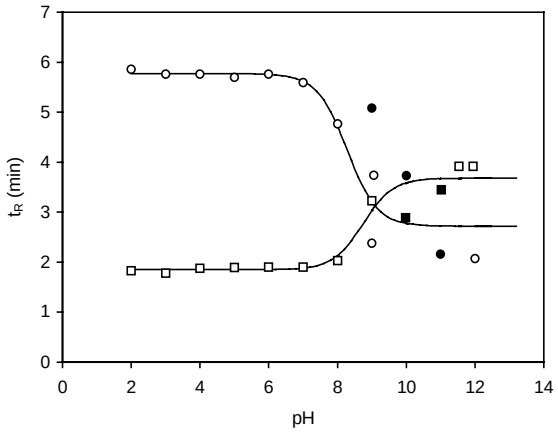

Fig. 6. Variation of the retention time of 3-nitrophenol and triethylamine in the polymeric column with the 40% acetonitrile mobile phase pH measured before mixing the aqueous buffer with the organic modifier ($^{w}_{w}$pH scale): symbols as in Fig. 5. From ref. [41], with permission, ©2000 American Chemical Society.

phase buffered by acid–base buffers of different type [40]. When the pH is measured in the mobile phase ($^{s}_{s}$pH scale), all data points fulfil Eq. (31), regardless of the buffer used (Fig. 5). However, when the pH is measured in the aqueous buffer ($^{w}_{w}$pH scale), the different shifts of the buffers placed in the region of variation of retention with pH distort the plot (Fig. 6). The practice of measuring the pH of the mobile phase is thus advisable when retention has to be related to the pH of buffers of different type. The calibration of the pH electrode system can be done with buffers prepared in the same mobile phase solvent where pH is measured ($^{s}_{s}$pH scale) or with the usual aqueous buffers ($^{w}_{w}$pH scale). Both procedures have been followed in the literature.

The preparation and validation of several standard pH buffers for pH electrode calibration in acetonitrile–water mixtures of different composition [10,13,14,33–36] allowed Barbosa and co-workers to measure the pH of acetonitrile–water mobile phases and relate retention to mobile phase pH [66–70]. A few standard pH buffers have been also proposed for tetrahydrofuran–water mobile phases [37], but they have not been used in liquid chromatography applications. The available pH reference data for methanol–water mixtures [10,13,14,28–32] has been used for calibration and measurement of pH in these mobile phases [71,72]. Measurement of pH in the mixed mobile phase after calibration with pH standards prepared in the same mobile phase is also common in capillary electrophoresis with non-aqueous or mixed organic solvents. This subject has been the object of a recent review [24]. The main shortcoming of direct measurement of the pH value of the mobile phase is that it requires calibration of the pH electrode system with standard buffers prepared in exactly the same solvent composition that the mobile phase has and with a reference pH value known for this solvent composition. Then, different calibration buffers are required for each mobile phase composition.

The problem of using different pH calibration buffers for each mobile phase composition can be obviated with calibration of all mobile phases with the same pH standards used for pH calibration in water. The pH is then measured in the mobile phase and it is shifted from the pH obtained by calibration with buffers in the same mobile phase in a constant δ value for all points in the retention versus pH plot, which results in the same fit quality (Fig. 4). This was the procedure used by Van den Venne et al. [50] in their pioneering work and that has been widely used by other authors [40–43,64,65,73–79]. δ Values are available for methanol–water [3,38–40] and acetonitrile–water mobile phases [41].

5. Practical recommendations for pH measurement in liquid chromatography

There are three main procedures to measure the pH of a chromatographic mobile phase. In the most common procedure, the pH is measured in the aqueous buffer before mixing it with the organic modifier ($^{w}_{w}$pH). Alternatively, the pH can be measured in the mobile phase after mixing the aqueous buffer and organic modifier with pH electrodes calibrated with standard solutions in the same mixed mobile phase ($^{s}_{s}$pH) or with aqueous standard solutions ($^{s}_{w}$pH). The three measurement methods have their advantages and disadvantages. These have to be evaluated for each procedure in order to select the most convenient pH measurement method.

5.1. pH measurement in the aqueous buffer before adding organic modifier

This method is the most currently used and may give acceptable results for repetitive routine procedures. For a routine analysis procedure, the pH can be measured in the aqueous buffer if the method reports exactly what buffer must be used (buffer components and concentration) and in what conditions the electrode system is calibrated and pH is measured (temperature and ionic strength), as well as all subsequent manipulation of the aqueous buffer in the preparation of the mobile phase. These conditions must be adequately described in the procedure in order to assure that when reproducing it, one always obtains the same mobile phase pH. Indiscriminate changes of buffer components should be avoided, even if the pH of the aqueous buffer is adjusted to the same value, because the pH variation with the addition of the organic modifier will be different. This fact is illustrated in Figs. 7 and 8. Two aqueous buffers of the same pH 8.0 value have been prepared from phosphate (buffer A) and from ammonia (buffer B), respectively [65]. Fig. 7 presents the ionization degree of four phenols and two bases (one amine and one pyridine) in these buffers and how this ionization changes with the addition of acetonitrile. Addition of acetonitrile increases the pK_a of the

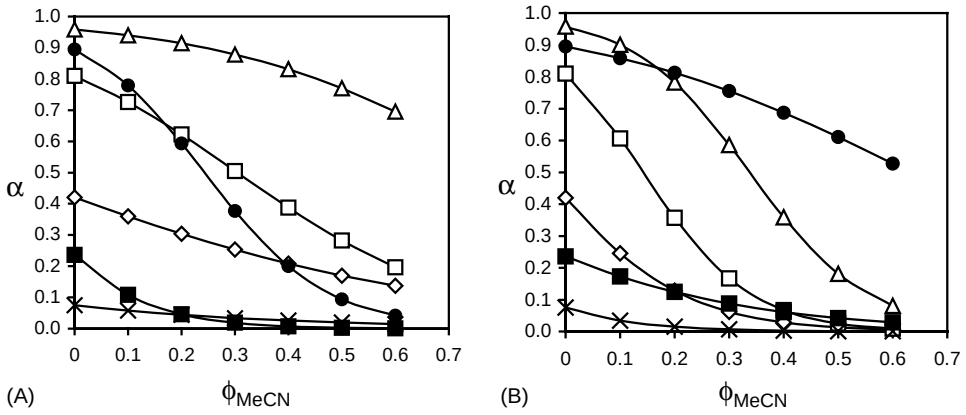

Fig. 7. Variation of the ionization of acid–base compounds with the addition of acetonitrile to aqueous buffers of ${}^{w}_{w}$pH = 8.0. (A) $H_2PO_4^-/HPO_4^{2-}$ buffer; (B) NH_4^+/NH_3 buffer. Compounds: (◇) 3,5-dichlorophenol, (□) 2,4-dichlorophenol, (△) 2-nitrophenol, (×) 3-bromophenol, (■) 2,4,6-trimethylpyridine, (●) *N*,*N*-dimethylbenzylamine. From ref. [65], with permission, ©2002 American Chemical Society.

phenols and decreases the pK_a of the bases. It also increases the pH of the phosphate buffer, but decreases the pH of the ammonia buffer. In consequence, the ionization of the compounds, equal in the two original aqueous pH 8.0 buffers, changes in a different way in the two buffers with acetonitrile addition. In particular, *N*,*N*-Dimethylbenzylamine becomes much less ionized in the phosphate buffer than in the ammonia buffer, whereas 2-nitrophenol becomes much less ionized in the ammonia buffer than in the phosphate buffer. The reverse behaviour of these two compounds produces an inversion in the elution order of them when they are chromatographed with a 60% acetonitrile mobile phase and aqueous pH 8.0 phosphate or ammonia buffer (Fig. 8) [65].

Measurement of the pH in the aqueous buffer may be also adequate when the analyst can work in an approximate pH range, although it should taken into account the pH changes that the addition of the organic modifier may produce in the buffer. This is the case when the analyst wants to get the acid–base analyte in an unique form, such as in the determination of physicochemical parameters of pharmaceutical drugs by chromatography. The determination of lipophilicity parameters such as the chromatographic hydrophobicity index (CHI) or even the octanol–water partition coefficient is often done by liquid chromatography [63,80–85]. The parameters are usually determined for the neutral form of the drug and an adequate pH of the mobile phase is required to assure that the drug is quantitatively in neutral form. The pH needed can be estimated by the Henderson–Hasselbach equation:

$$\mathrm{pH} = \mathrm{p}K_a + \log\frac{[A^{z-1}]}{[HA^{z}]} \quad (33)$$

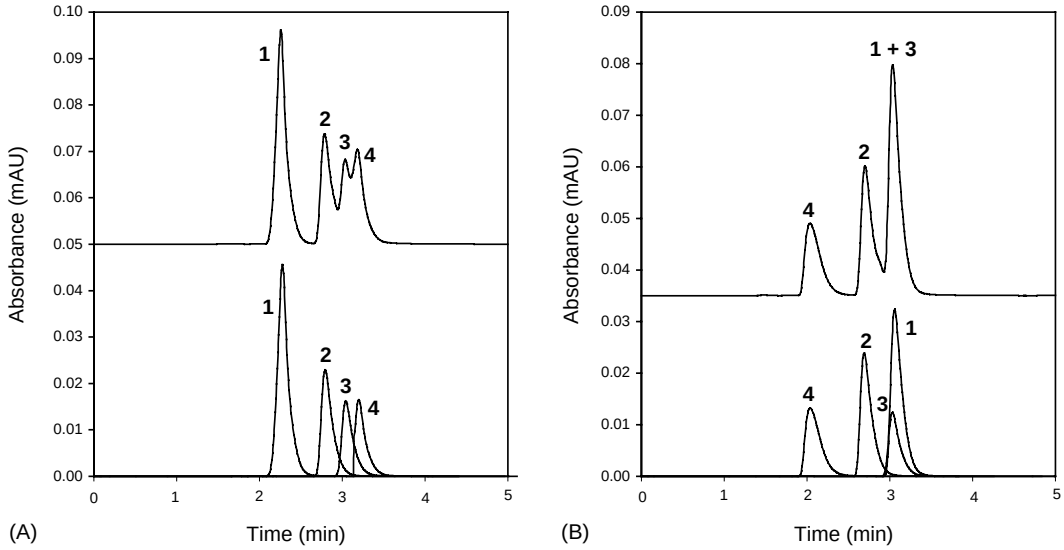

Fig. 8. Elution of a mixture of ionizable compounds in an X-Terra MS C_{18} column (Waters) with a 60% acetonitrile mobile phase prepared from aqueous buffers of ${}^{w}_{w}$pH = 8.0. (A) $H_2PO_4^-/HPO_4^{2-}$ buffer; (B) NH_4^+/NH_3 buffer. Compounds are 2-nitrophenol (1), 2,4,6-trimethylpyridine (2), 3-bromophenol (3), and *N*,*N*-dimethylbenzylamine (4). Chromatograms for the individual compounds in each mobile phase are also given. From ref. [65], with permission, ©2000 American Chemical Society.

To get a neutral acid ($z = 0$) 99% in the unionized form, the pH must be at least 2 units below the pK_a, and to get a neutral base ($z = +1$) 99% in the unionized form, the pH must be at least 2 units above the pK_a. If the pH is measured in the aqueous buffer, the addition of organic modifier will increase the pK_a of a neutral acid and the pH of buffers prepared from neutral or anionic acids, and it will decrease the pK_a of neutral bases and the pH of buffers prepared from neutral bases and cationic acids. If a neutral acid is chromatographed in a buffer prepared from another neutral or cationic acid, the degree of ionization of the acid calculated from the aqueous data may increase or decrease, depending on if the increase of the pK_a of the acid is lower or higher, respectively, than the increase of the pH of the buffer. The degree of ionization of a neutral base chromatographed with a buffer prepared from another base will also increase or decrease in reference to the one calculated in water, depending on if the decrease of the pK_a of the base with the addition of organic modifier is lower or larger, respectively, than the decrease in the pH of the buffer. For a neutral acid the best would be to use a buffer prepared from a neutral base and a cationic acid (e.g. NH_4^+/NH_3) because the increase of the pK_a of the acid and the decrease of the pH of the buffer with the addition of the organic modifier will favour formation of the uncharged form of the acid. For a neutral base, the use of a buffer prepared from neutral or anionic acids (e.g. HAc/Ac^-, $H_2PO_4^-/HPO_4^{2-}$, HPO_4^{2-}/PO_4^{3-}, etc.) will favour the unionization of the base because the addition of the organic modifier will decrease the pK_a of the base and increase the pH of the buffer.

Another instance where the pH of the mobile phase has to be measured in the aqueous buffer is when working with gradient elution. Since the mobile phase composition changes during elution, one particular mobile phase composition has to be selected for pH measurement and the aqueous buffer seems the most convenient. The pH change produced during the gradient elution with a particular aqueous buffer can be determined and related to gradient change or elution time by measuring the pH of the buffer for different mobile phase compositions [63,85]. It is advisable to choose a buffer as simple as possible, i.e. with a low number of components, in order to get simple relationships between pH change and mobile phase composition. It has been demonstrated that for fast gradient elution methods with an ammonium acetate aqueous buffer in methanol–water and acetonitrile–water mobile phases [63,85], the gradient retention time of acid–base analytes can be related to the initial pH of the aqueous buffer through equations similar to (31) with the simple inclusion of an additional parameter (s), i.e.:

$$t_R = \frac{t_{R(HA)} + t_{AR(A)} 10^{s(pH - pK'_a)}}{1 + 10^{s(pH - pK'_a)}} \quad (34)$$

The s parameter measures the change of the slope of the sigmoidal plot in the inflection point caused by the pH variation between the elution of the less retained species (the ionized one) and the most retained species (the uncharged form) of the acid–base analyte [63,85]. The uncharged form of the analyte is eluted later than the charged form and thus, it experiences a larger pH change of the mobile phase.

5.2. pH measurement in the mobile phase after mixing aqueous buffer and organic modifier

The measurement of the pH of the mobile phase after mixing aqueous buffer and organic modifier is advisable when one wants to get accurate relationships between retention and mobile phase pH in isocratic conditions, such as in optimization studies or pK_a determination by liquid chromatography [23]. This type of pH measurement is particularly recommended if buffers of different type are going to be used in the optimization procedure or pK_a determination. Models to fit retention to mobile phase pH are based on Eq. (31) or more complex equations of the same type if the analyte has more than one acid–base equilibrium [23,56]. Measurement of analyte retentions at several mobile phase pH allows determination of the pK_a value (or values) and of retention of the different acid–base forms of the analytes by fitting the data to the model. The fitting parameters can be later used to estimate retention of the different analytes at different mobile phase pH values and to optimize separation. In addition, some models have been proposed to fit the parameters obtained for particular mobile phases to mobile phase composition, which may allow simultaneous optimization of mobile phase composition and pH [42,43,76,78].

The electrode assembly used can be calibrated with standards of known pH prepared in the same mixed solvent used as mobile phase or with the common aqueous standards. In any case the pH in the appropriate pH scale (^s_spH for standards in the mixed solvent and ^w_wpH for standards in water) of the standards used must be precisely and accurately known. Accurate calibration will also require the pH of the standards to be traced to that of a primary standard. The chromatographer must know in which concentration scale (molality or molality) the pH of the standard solutions is given, since this will determine the concentration scale that the pH readings are obtained. Temperature of calibration and measurement must be controlled.

If standards prepared in the same mobile phase solvent are used, the pH readings will be in the ^s_spH scale and the fit of retention to pH through Eq. (31) provides the pK_a value of the analyte in the mobile phase solvent ($^s_s pK'_a$). This is the magnitude that can be directly related to analyte concentrations and pH through mass and charge balances and Debye–Hückel type equations for ionic activity coefficients. It is also the pK_a magnitude that it is usually found in tables and compilations of acidity constants for non-aqueous and mixed solvents.

If the electrode system is calibrated with the usual aqueous buffers, the pH values obtained will be in the ^s_wpH scale. If the δ value for the particular solvent composition is known, the ^s_wpH readings can be easily converted to ^s_spH through Eq. (26) and then one may work as reported in the

previous paragraph. Alternatively, retention can be fitted to ${}^{s}_{w}$pH through Eq. (31) and the analyte pK_a obtained will be in the ${}^{s}_{w}$pH scale (which we may indicate by ${}^{s}_{w}$pK'_a). This value can be converted to ${}^{s}_{s}$pK'_a through the δ value. Even, if the δ value is not known, the chromatographer may still use Eq. (31) for optimization by using the measured ${}^{s}_{w}$pH values because all pH readings and calculations will be displaced the same unknown, but constant, δ value in reference to the ${}^{s}_{s}$pH values. The optimization algorithm will suggest optimal mobile phase pH values that will be in the ${}^{s}_{w}$pH scale, and thus they must be measured in the mobile phase with pH calibration in water. This procedure provides an easy way of optimization of separations since pH calibration is done in water, but retention and pH measurements are in the working mobile phase.

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