Retention of Ionizable Compounds on HPLC. 5. pH Scales and the Retention of Acids and Bases with Acetonitrile−Water Mobile Phases

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The pH calibration procedures that lead to the different pH scales in acetonitrile−water mixtures used as mobile phases in reversed-phase liquid chromatography are discussed. Appropriate buffers of known pH value in acetonitrile−water mixtures are selected and used to establish the relationship (δ values) between the two rigorous acetonitrile−water pH scales: ${}_{s}^{s}\text{pH}$ and ${}_{w}^{s}\text{pH}$ (pH measured in acetonitrile−water mixtures and referred to acetonitrile−water or water, respectively, as standard state). These δ values allow one to convert pH values measured in acetonitrile−water with electrode systems calibrated with aqueous buffers (${}_{w}^{s}\text{pH}$ scale) to ${}_{s}^{s}\text{pH}$ values, which are directly related to the thermodynamic acid−base constants. This offers an easy way to measure the pH of acetonitrile−water mobile phases and to relate this pH to the chromatographic retention of acids and bases through the thermodynamic acid−base constants. The relationships are tested for the variation of the retention of acids and bases with the pH of the mobile phase at several mobile-phase compositions and favorably compared with the relationships obtained with the common ${}_{w}^{w}\text{pH}$ scale (pH measured in the aqueous buffer before mixing it with the organic modifier). The use of the rigorous ${}_{s}^{s}\text{pH}$ and ${}_{w}^{s}\text{pH}$ scales allows one to explain the retention behavior of bases, which in many instances cannot be justified from the pH measurement in the ill-founded ${}_{w}^{w}\text{pH}$ scale.

Successful optimization of HPLC separations of ionizable compounds requires, in many instances, an accurate measurement and control of mobile-phase pH. Although the IUPAC has endorsed rules and procedures for the measurement of pH in aqueous organic solvent mixtures,[1−3] the lack of appropriate reference pH data in mixed solvents and some common misunderstandings on pH definition have limited the application of these procedures in practical HPLC.

In a previous work,[4] we revised the most common procedures used to measure the pH of liquid chromatography mobile phases and we evaluated them for methanol−water mobile phases in terms of the IUPAC definitions and rules. Methanol−water and acetonitrile−water mixed solvents are the most used mobile phases for reversed-phase liquid chromatography.[5] The chemical equilibria in methanol−water have been studied for a long time,[6] and there are numerous literature pH and pK data concerning these media[7] that we have analyzed in various publications.[8−13]

However, the literature pH and pK data in acetonitrile−water are very limited.[14−23] In a previous study,[24] we analyzed the variation of pK data of neutral and anionic acids with the acetonitrile contents and evaluated them in order to identify the most reliable data for preparation of buffer solutions in acetonitrile−water mixtures. These buffer solutions should allow a rigorous calibration of potentiometric sensors in acetonitrile−water. In this paper, we propose operational pH standards in acetonitrile−water and from them we establish the two rigorous pH scales (${}_{w}^{s}\text{pH}$ and ${}_{s}^{s}\text{pH}$) and pH measurement procedures that should be used in acetonitrile−water mobile phases to obtain accurate chromatographic retention−pH relationships. Some

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Table 1. Macroscopic Properties of Relevant Interest for pH Measurement in Acetonitrile−Water Mixtures at 25 °C[10,18,24]a

% MeCN	x_{MeCN}	ρ (kg dm^{-3})	log ρ	ϵ	A	a_0B	$^s_s pK_{ap}$
0	0.000	0.999	−0.001	76.6	0.528	1.52	14.00
10	0.040	0.983	−0.007	73.2	0.566	1.55	14.24
20	0.079	0.968	−0.014	70.1	0.604	1.59	14.47
30	0.130	0.950	−0.022	66.4	0.655	1.63	14.74
40	0.186	0.931	−0.031	62.8	0.712	1.68	15.08
50	0.260	0.908	−0.042	58.6	0.791	1.74	15.48
60	0.339	0.885	−0.053	54.7	0.877	1.80	15.90
70	0.440	0.859	−0.066	50.4	0.992	1.87	16.42
80	0.578	0.829	−0.081	45.5	1.156	1.97	17.14
90	0.755	0.802	−0.096	40.4	1.378	2.09	17.13
100	1.000	0.782	−0.107	35.1	1.707	2.24	34.40

^{a} Properties calculated through the equations: $\rho = 0.9985 - 0.397x_{MeCN} + 0.180(x_{MeCN})^2$; $\epsilon^{-1} = 0.01305 + 0.01547x_{MeCN}$; $A = 354.4\epsilon^{-3/2}$; $a_0B = 13.28\epsilon^{-1/2}$; $^s_s pK_{ap} = -\log(x_{H_2O}{}^2 10^{-14.00x_{H_2O}+19.96x_{MeCN}} + x_{H_2O}x_{MeCN}10^{-13.80x_{H_2O}+18.58x_{MeCN}} + x_{MeCN}{}^2 10^{-34.40})$.

chromatographical examples are presented that illustrate the good agreement between the parameters obtained from solute retention/mobile-phase pH relationships and the thermodynamic acid−base solute constants, when the pH is measured in the appropriate pH scales. The retention of bases is especially discussed because of the common deviations observed, between expected and measured retention, that come out from mobile-phase pH measurements in the ill-founded aqueous pH scale ($^w_w pH$).

pH SCALES IN ACETONITRILE−WATER

Two rigorous notional pH scales can be used in acetonitrile−water mixtures (solvent s) depending on the standard-state solvent for ionic activity coefficients, γ, (the solvent in which they tend to unity at infinite dilution). If the standard-state solvent is water (w), the scale obtained is $^s_w pH$ (absolute pH), but if the standard state is chosen to be the same acetonitrile−water mixture in which the pH is measured, the working pH scale is $^s_s pH$ (pH relative to the acetonitrile−water solvent). Both scales differ in the primary medium effect for the transfer of the H$^+$ ion from water (w) to the solvent s, $-\log {}^s_w \gamma^0_H$.[1] Earlier, the pH scale relative to the solvent ($^s_s pH$) was usually indicated by pH*,[25] whereas the absolute pH scale ($^s_w pH$) was sometimes indicated by pH^{app}.[26,27]

Operationally, the two pH scales correspond to measurement of the pH in the acetonitrile−water mixture with the system electrode calibrated with aqueous buffers ($^s_w pH$) or calibrated with buffers of known pH prepared in the same acetonitrile−water mixture ($^s_s pH$). Both operational scales differ in the δ term

$$\delta = \bar{E}_j - \log({}^s_w \gamma^0_H) = {}^s_w pH - {}^s_s pH \qquad (1)$$

which includes the primary medium effect $-\log({}^s_w\gamma^0_H)$ and the difference between the liquid-junction potentials ($\bar{E}_j$) of the electrode system in solvents s and w.

It must be remarked here that the primary medium effect ($-\log({}^s_w\gamma^0_H)$) depends only on the solvent s at which pH is measured, but that the liquid-junction potential depends also on

the particular electrode system, pH standards, and sample used. Therefore, general interlaboratory conversion between both pH scales is only possible if the different electrode systems are designed to have a negligible residual liquid-junction potential ($\bar{E}_j$), i.e., if the junction potential of the electrode system in the measurement solution in solvent s ($^s E_j$) is close to the junction potential in the calibration solution in water ($^w E_j$). Usually this can be achieved with a salt bridge containing a solution of an equitransferent binary salt at a much higher concentration than the sample and standard solutions.[1] For example, a 3 M KCl salt bridge in water can experience junction potentials on the order of 1 mV (∼0.02 pH unit), which would be usually partially balanced by a similar junction potential in the pH measurement acetonitrile−water mobile phase. Therefore, the error introduced by the residual liquid-junction potentials can be estimated to be ∼0.01 pH unit or less, which is indeed a low error for practical liquid chromatography measurements. In this instance, $\delta \equiv -\log({}^s_w \gamma^0_H)$.

However, many chromatographers prefer to work in the aqueous $^w_w pH$ scale, i.e., measuring the pH in the aqueous pH buffer before mixing it with acetonitrile to obtain the mobile phase. There is not a general relationship between the $^w_w pH$ scale and the rigorous $^s_w pH$ and $^s_s pH$ scales.[4]

Another parameter to clarify is the concentration scale at which pH is referred. The most used concentration scales are molality and molarity, and the two corresponding pH scales are related by the logarithm of the density of the solvent (ρ).[4] Although, the IUPAC prefers the molality scale, molarity is more used in analytical chemistry and we have used this scale throughout all this work.

Table 1 reports parameters and equations of interest for pH definition in acetonitrile−water mobile phases, including the Debye−Hückel A and a_0B parameters for estimation of ionic activity coefficients relative to the acetonitrile−water mixture. The autoprotolysis constants of the mixtures are also given because they define the limits of the pH scales.[10] $^s_s pH$ scale ranges from 0.00 to $^s_s pK_{ap}$, $^s_w pH$ from δ to $^s_w pK_{ap}$ (equivalent to $^s_s pK_{ap} + \delta$), and obviously $^w_w pH$ ranges from 0.00 to 14.00.

CHROMATOGRAPHIC RETENTION AND MOBILE-PHASE pH SCALES

The rigorous thermodynamic constant that rules distribution of the analyte between the mobile and stationary phases is the

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distribution constant that relates the activities of the analyte in these two phases. Since it would be extremely difficult to estimate the activity of ionic species in the stationary phase, in chromatography the distribution constant (K_c) is usually defined in terms of the concentration of the analyte in the stationary (c_S) and mobile (c_M) phases.[1]

$$K_c = c_S/c_M \quad (2)$$

For an analyte with an acid−base equilibrium ruled by an acidity constant K_a, the distribution constant can be given as an average of the distribution constants of the two solute species, HA and A ($K_{c(HA)}$ and $K_{c(A)}$) according to the mole fraction of each species at the mobile-phase pH

$$K_c = ([HA]K_{c(HA)} + [A]K_{c(A)})/([HA] + [A]) =$$
$$(K_{c(HA)} + K_{c(A)}10^{pH - pK_a'})/(1 + 10^{pH - pK_a'}) \quad (3)$$

where pK_a' is the pK_a in terms of the concentration of the two species, instead of activities.[4]

Determination of HPLC distribution constants requires measurement of the mobile- and stationary-phase volumes, which is not so simple. Therefore, retention parameters, such as retention factor (k), adjusted retention time (t'_R), and retention time (t_R) are used in practice. These retention parameters can be related to distribution constant through the stationary (V_S) and mobile (V_M) -phase volumes, the mobile-phase flow (F_c), and the column holdup time (t_M):

$$K_c = kV_M/V_S = kF_c t_M/V_S = t'_R F_c/V_S = (t_R - t_M)F_c/V_S \quad (4)$$

If V_S, F_c, and t_M remain constant, expressions analogous to (3) can be derived for the different retention parameters.

$$k = (k_{HA} + k_A 10^{pH - pK_a'})/(1 + 10^{pH - pK_a'}) \quad (5)$$

$$t'_R = (t'_{R(HA)} + t'_{R(A)}10^{pH - pK_a'})/(1 + 10^{pH - pK_a'}) \quad (6)$$

$$t_R = (t_{R(HA)} + t_{R(A)}10^{pH - pK_a'})/(1 + 10^{pH - pK_a'}) \quad (7)$$

From a practical point of view, eq 7 is the most useful because t_R is the parameter directly measured and fitted to the equation. Determination of t'_R and k requires measurement of the column holdup time (t_M):

$$k = t'_R/t_M = (t_R - t_M)/t_M \quad (8)$$

Several methods have been proposed to measure holdup time, but they usually lead to different t_M values. When working with ionizable analytes, the most appropriate seems to be to measure the elution time of an ionic solute, such as KBr, that is not retained by the stationary phase.[9] However, the t_M values obtained are different for each pH buffer and therefore the constancy of the t_M parameter is not observed. The variation of the holdup time may be especially important for k, since for a highly ionized solute, the numerator ($t_R - t_M$) of eq 8 is usually much lower than the

denominator (t_M). Therefore, the most appropriate parameters to fit to the pH of the mobile phase are t_R and t'_R. In this work, we have used t_R because it does not require measurement of the holdup time.

Equations 3 and 5−7 predict a sigmoidal relationship between the retention of the solute and the pH of the mobile phase. This mobile-phase pH can be measured in the different pH scales.[4,26,27] If the ^s_spH scale is used, the inflection point of the sigmoidal plot must agree with the pK_a' value of the solute in the particular acetonitrile−water mixture and referred also to this mixture as standard state ($^s_s pK_a'$). For the ^s_wpH scale, the pH of inflection corresponds to the pK_a' value of the solute in the acetonitrile−water mixture but referred to water as standard state ($^s_w pK_a'$). Both pK_a' values differ in the δ term. However, the most employed procedure for pH measurement in chromatography consists of calibrating the electrode system with aqueous standards and measuring the pH of the aqueous buffer before mixing it with the organic modifier (^w_wpH scale). It must be pointed again that there is not a general relationship between this ^w_wpH scale and the ^s_wpH and ^s_spH scales. It has been demonstrated[4] that the difference between ^w_wpH and ^s_wpH or ^s_spH is buffer dependent. Therefore, eqs 3 and 5−7 do not hold for the ^w_wpH scale. Only approximate relationships, without any thermodynamic meaning for the inflection point, can be obtained if buffers of the same type are used in the pH range of variation of retention.

EXPERIMENTAL SECTION

Apparatus. Potentiometric and pH measurements were taken with a Ross combination electrode Orion 8102 (glass electrode and a reference electrode with a 3.0 M KCl solution in water as salt bridge) in a Crison micropH 2002 potentiometer with a precision of ±0.1 mV (±0.002 pH unit). The retention data were measured on a 25 cm × 4.0 mm i.d. Merck LiChrospher 100 RP-18 column (5 μm) or on a 15 cm × 4.6 mm i.d. Polymer Labs PLRP-S 100-Å column (15−20 μm) with a flow of 1 mL min^{-1} in an Isco model 2350 dual-pump system with a 20-μL loop valve. A variable-wavelength V^4 absorbance detector (Isco) set at 254 nm and a refractive index detector (Shimadzu) were used. All data was taken by triplicate at 25 °C with the potentiometric cell and columns thermostated with water jackets.

Chemicals. Acetonitrile for chromatography from Merck and water purified by the Milli-Q plus system from Millipore were used. Other chemicals were reagent grade or better and obtained from Fluka, Aldrich, Merck, Carlo Erba, or Baker.

RESULTS AND DISCUSSION

Selection of Reference Buffers in Acetonitrile−Water. The establishment of the ^s_wpH and ^s_spH scales requires the selection of appropriate reference solutions of assigned pH value for electrode system calibration. The acetonitrile−water ^s_wpH scale requires calibration with reference solutions prepared in water. Therefore, the same primary or operational standard reference solutions proposed for the IUPAC in water for the ^w_wpH scale and the ^w_wpH values assigned to these standards should be used for the ^s_wpH scale, with independence of the particular solvent s in which the pH measurements are being done. However, the acetonitrile−water ^s_spH scale requires calibration of the electrode system with reference standards prepared in the same acetonitrile−water mixture where the pH will be measured and with ^s_spH values in

Table 2. Composition and $_s^s$pH Values of Equimolar Mixtures of Acids and Their Conjugate Base (Potassium or Sodium Salts) Studied as Buffered Solutions in Acetonitrile−Water Mixtures up to 60% (v/v) Acetonitrile

buffer	acid	c_a (M)	c_b (M)	$_s^s$pH in % MeCN						
				0%	10%	20%	30%	40%	50%	60%
B	2,3-dichloropropionic acid	0.05	0.05	2.31	2.50	2.77	3.02	3.35	3.79	4.29
C	2,3-dibromopropionic acid	0.05	0.05	2.29	2.51	2.78	3.08	3.44	3.85	4.27
D	2-nitrobenzoic acid	0.05	0.05		2.59	2.90	3.25	3.66	4.08	4.61
E	chloroacetic acid	0.05	0.05		3.00	3.21	3.46	3.78	4.15	4.60
F	2-chloropropionic acid	0.05	0.05	2.84	3.05	3.32	3.62	3.95	4.44	4.88
G	citric acid	0.05	0.05	3.00	3.16	3.32	3.51	3.72	3.95	4.25
H	3-nitrobenzoic acid	0.025	0.025		3.67					
		0.05	0.05			3.94	4.20	4.52	4.89	5.39
I	4-nitrobenzoic acid	0.0025	0.0025		3.72					
		0.005	0.005			4.04	4.26			
		0.01	0.01					4.61	4.89	5.40
J	3-bromopropionic acid	0.05	0.05	3.99	3.96	4.26	4.45	4.73	5.34	5.57
K	hydrogen tartrate	0.014	0.014	4.20	4.38					
		0.01	0.01			4.59	4.95			
		0.008	0.008					5.01		
		0.007	0.007						5.47	
		0.003	0.003							5.72
L	benzoic acid	0.05	0.05		4.41	4.71	5.06	5.42	5.82	6.26
M	dihydrogen citrate	0.05	0.05	4.38	4.57	4.76	4.94	5.14	5.35	
		0.025	0.025							5.74
N	cinnamic acid	0.005	0.005		4.67					
		0.01	0.01			4.87	5.27			
		0.02	0.02					5.72	6.12	6.54
O	acetic acid	0.05	0.05	4.67	4.86	5.08	5.34	5.68	6.04	6.46
P	propionic acid	0.05	0.05	4.79	5.07	5.32	5.59	5.95	6.37	6.82
Q	valeric acid	0.05	0.05	4.77	5.07	5.31	5.68	6.07	6.52	6.99
R	isobutyric acid	0.05	0.05	4.75	5.08	5.34	5.67	6.03	6.50	6.96
S	hydrogen phthalate	0.05	0.05	5.01	5.34	5.69	6.09	6.48	6.86	7.24
T	hydrogen citrate	0.05	0.05	5.61	5.85	6.03	6.18	6.35	6.41	
		0.0125	0.0125							7.09
U	dihydrogen phosphate	0.05	0.05	6.75	7.00	7.19	7.37	7.54	7.71	
		0.025	0.025							8.10
V	boric acid	0.05	0.05	9.15	9.54	9.85	10.23	10.57	10.94	
		0.025	0.025							11.35

the same solvent mixture assigned to these standards. We recommend to select equimolar mixtures of acids and their conjugate base, which correspond to the maximum buffer capacity, such as those we used in our previous publication to evaluate the reliability of literature pK data in acetonitrile−water mixtures.[24]

Assignment of Reference pH Values for Buffers in Acetonitrile−Water. The $_s^s$pH value of the buffer solutions selected must be determined in order to achieve an effective electrode calibration. The composition of some equimolar mixtures of a weak acid and its conjugate base (at 0.05 M concentration of each species, except for solutions of low solubility, which are more diluted) prepared in a previous work,[24] is given in Table 2. In the previous study,[24] the fem (^sE_X) of each buffer solution (solutions of Table 2 and some other solutions of strong acids−HCl− and bases−KOH) was measured and related with the $_s^s$pH$_X$ value, theoretically calculated from the literature $_s^s$pK value and buffer composition, through the Nernst equation

$$^sE_X = (^sE_0 + {}^sE_{jX}) - g({}_s^s\text{pH}_X)\qquad(9)$$

where $^sE_0$ is the standard potential (constant at each temperature) and $^sE_{jX}$ the liquid-junction potential (assumed to be constant for all buffers). The ($^sE_0 + {}^sE_{jX}$) and g constants were calculated for each acetonitrile−water composition. g values close to the Nernst constant ($g = 0.059157$ V at 25 °C) were found in all instances

after removal of some outliers attributed to unaccurate literature pK data.

The knowledgment of ($^sE_0 + {}^sE_{jX}$) and g constants allows back-calculation of reliable $_s^s$pH values for each buffer at each solvent composition, including the outliers. These pH values are presented in Table 2 for each buffer solution and can be taken as reference $_s^s$pH values for electrode calibration in acetonitrile−water mixtures.

Determination of the δ Parameter for Acetonitrile−Water Mixtures. The assignment of reliable $_s^s$pH values in acetonitrile−water to buffer solutions allows determination of the δ term, which relates the $_w^s$pH and $_s^s$pH scales. Some of the solutions presented in Table 2 with known $_s^s$pH value were selected (based on criteria of solubility, purity, and easy availability), and the pH of each solution was measured after calibration of the electrode system with aqueous buffers. This gave a direct measure of the $_w^s$pH value of each solution, and the δ term was estimated from the difference between $_w^s$pH and $_s^s$pH values of the same solution. The results obtained are presented in Table 3. The selected solutions gave a very good agreement in δ values up to 40% acetonitrile, with standard deviations less than 0.05 pH unit. The scattering of the results (standard deviations ~0.1) is slightly larger for higher acetonitrile contents where the pH and literature pK measurements are less reproducible because of a slower response of the electrode systems. The variation of the δ values

Table 3. $_{w}^{s}$pH Values and δ Terms for Some of the Buffered Solutions of Table 2 for Acetonitrile−Water Mixtures up to 60% (v/v) Acetonitrile

	% MeCN mixtures											
	10%		20%		30%		40%		50%		60%	
buffer	$_{w}^{s}$pH	δ	$_{w}^{s}$pH	δ	$_{w}^{s}$pH	δ	$_{w}^{s}$pH	δ	$_{w}^{s}$pH	δ	$_{w}^{s}$pH	δ
B	2.47	−0.03	2.70	−0.07	2.95	−0.07	3.25	−0.10	3.45	−0.34	3.65	−0.64
E	2.96	−0.04	3.19	−0.02	3.38	−0.08	3.60	−0.18	3.86	−0.29	4.08	−0.52
G	3.15	−0.01	3.32	0.00	3.48	−0.03	3.53	−0.19	3.66	−0.29	3.73	−0.52
M	4.53	−0.04	4.74	−0.02	4.87	−0.07	4.97	−0.17	5.09	−0.26	5.30	−0.44
O	4.87	0.01	5.05	−0.03	5.31	−0.03	5.53	−0.15	5.81	−0.23	6.04	−0.42
S	5.34	0.00	5.68	−0.01	6.09	0.00	6.37	−0.11	6.68	−0.18	6.81	−0.43
T	5.82	−0.03	5.96	−0.07	6.14	−0.04	6.20	−0.15	6.47	−0.12	6.59	−0.50
U	7.01	0.01	7.13	−0.06	7.32	−0.05	7.42	−0.12	7.53	−0.18	7.74	−0.36
V	9.54	0.00	9.84	−0.01	10.21	−0.02	10.47	−0.10	10.87	−0.07	11.08	−0.27
δ_{av}		−0.01		−0.03		−0.04		−0.14		−0.22		−0.46
SD		0.02		0.03		0.03		0.03		0.09		0.11
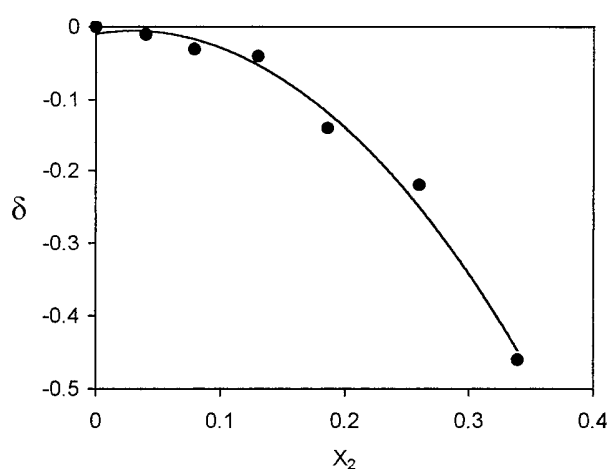
Figure 1. Variation of the δ quantity in molar scale with solvent composition in acetonitrile−water mixtures.

with solvent composition is also presented in Figure 1. The δ values have been fitted to the empirical equation

$$\delta = (-3.81 \pm 0.15)x^2_{\text{MeCN}} \qquad \text{SD} = 0.02 \qquad r^2 = 0.984$$
$$F = 376 \quad (10)$$

where x_{MeCN} is the mole fraction of acetonitrile in the mixture. This equation allows an accurate calculation of δ for any acetonitrile−water mixture up to 60% acetonitrile. It has to be pointed that we prepare the mixed acetonitrile−water solvent according to the usual procedure in liquid chromatography; i.e., 60% acetonitrile refers to 60 volumes of acetonitrile mixed with 40 volumes of water or aqueous buffer.

As far as we know, this is the first time that the δ term has been determined for acetonitrile−water mixtures and we believe that the values obtained are reasonably precise and accurate. Moreover, we think that residual liquid-junction potential is negligible for our electrode system (this was first checked for the same electrode system in methanol−water[4]), and therefore, the δ values are a good estimation of the primary medium effect $-\log(_{w}^{s}\gamma_{H}^{0})$. This should allow our δ values to be directly used with other electrode systems or by other laboratories, provided they ensure that the residual-liquid junction potential of their

system is also negligible. In case of doubt, the reference $_{s}^{s}$pH values of Table 2 provide an easy way to check this hypothesis or calculate the δ values for other electrode systems.

Chromatographic Retention of Acids and Bases. The relationships between the retention of acids and bases and the several pH scales have been tested at three different acetonitrile−water mixtures (20, 40, and 60% acetonitrile by volume) for a typical neutral acid (benzoic acid) and for a typical neutral base (pyridine), which is the conjugated base of a cationic acid (pyridinium cation).

Retention of benzoic acid was studied on a C_{18} column, and the same aqueous buffers of $_{w}^{w}$pH ranging between 2 and 7 used in a previous work[24] were selected for this study. The pH of the buffers was measured before ($_{w}^{w}$pH) and after ($_{s}^{s}$pH) the addition of acetonitrile. In the latter instance, the electrode system was calibrated with buffers prepared in the same acetonitrile−water mixture (buffers G, O, U, and V of Table 2), the ($^{s}E_{0} + {}^{s}E_{jX}$) and g parameters of eq 9 were obtained from the potential readings ($^{s}E_{X}$) and the assigned $_{s}^{s}$pH$_{X}$ values of the calibration buffers (Table 2). The $_{s}^{s}$pH$_{X}$ values of the HPLC buffers were later obtained from the potential readings and calibration parameters.

The retention times of benzoic acid for the different buffered mobile phases were fitted to eq 7 using the two pH scales, $_{w}^{w}$pH and $_{s}^{s}$pH, and the parameters obtained are presented in Table 4. As expected, the retention time of the neutral form of benzoic acid ($t_{R(HA)}$) is larger than the retention time of the ionized form of the acid ($t_{R(A)}$) and for both species the retention decreases when the percentage of acetonitrile in the mobile phase increases. Comparison of the results obtained with the two pH scales shows that the fits are slightly better for the $_{s}^{s}$pH scale, although for both pH scales the relative standard deviation is less than 2% of the retention time of the more retained species ($t_{R(HA)}$). There are no significant differences between the retention parameters $t_{R(HA)}$ and $t_{R(A)}$ obtained from both pH scales, but the values of the pK_{a}' parameter differ considerably.

When the pH scale in water is used ($_{w}^{w}$pH), one may expect a pK_{a}' value close to the pK_{a} value of the solute (benzoic acid) in water ($_{w}^{w}pK_{a}$), but this would be only true if the pK_{a} variation (ΔpK_{a}) for benzoic acid between the particular mobile phase used and water would match the ΔpK_{a} value of the HPLC buffers employed (from which pK_{a}' is computed). The largest variation

Table 4. Retention Parameters for Test Solutes Obtained Using Eq 7 with the ^w_wpH and ^s_spH Values of the Mobile Phase

% MeCN	^w_wpH scale				^s_spH scale					
	$t_{R(HA)}$	$t_{R(A)}$	pK_a'	SD	$t_{R(HA)}$	$t_{R(A)}$	pK_a'	SD	$^w_w pK_a$	$^s_s pK_a$
benzoic acid										
20	17.67	2.45	4.32 ± 0.04	0.35	17.43	2.48	4.70 ± 0.03	0.24	4.18	4.87,[a] 4.82[b]
40	5.11	2.05	4.54 ± 0.05	0.09	5.07	2.06	5.48 ± 0.04	0.07	4.18	5.67,[a] 5.54[b]
60	3.38	1.99	4.64 ± 0.11	0.10	3.38	1.99	6.43 ± 0.06	0.05	4.18	6.63,[a] 6.40[b]
pyridine										
20	2.12	4.53	4.52 ± 0.03	0.04	2.14	4.52	4.95 ± 0.03	0.03	5.23	4.94[c]
40	2.06	2.97	3.88 ± 0.05	0.02	2.08	2.96	4.75 ± 0.05	0.02	5.23	4.73[c]
60	1.94	2.61	3.00 ± 0.03	0.01	1.99	2.61	4.49 ± 0.04	0.01	5.23	4.50[c]
3-nitrophenol										
40	5.77	2.72	8.28 ± 0.40	0.80	5.73	1.96	9.55 ± 0.05	0.15	8.43	
triethylamine										
40	1.85	3.60	8.69 ± 0.29	0.28	1.86	3.81	9.86 ± 0.11	0.12	10.67	

[a] From ref 23. [b] Calculated from the potentiometric data of Table 2. [c] From ref 17.
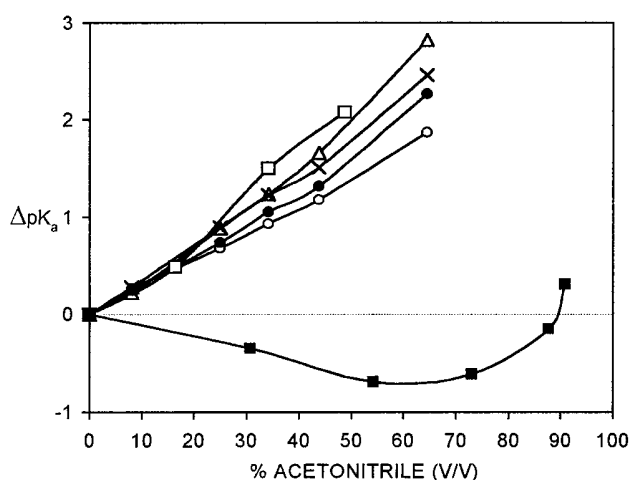
Figure 2. Variation of the pK_a of acids ($\Delta pK_a = {}^s_s pK_a - {}^w_w pK_a$) in acetonitrile−water mixtures with solvent composition: (○) pK_{a1} of citric acid, (●) pK_{a2} of citric acid, (×) pK_{a3} of citric acid, (△) acetic acid, (□) benzoic acid, and (■) pyridinium.

of the retention of the solute with the pH of the mobile phase is observed for pH values close to the pK_a'. For benzoic acid, the variation is observed in the ^w_wpH range between 3.5 and 5.5 approximately, and this pH range was covered with acetic/acetate and dihydrogen citrate/hydrogen citrate buffers. Figure 2 shows that the ΔpK_a values for benzoic acid are larger than the ΔpK_a values for acetic acid and dihydrogen citrate, and this explains that the pK_a' values obtained are higher than the $^w_w pK_a$ (Table 4 for ^w_wpH scale). In addition, the differences between the ΔpK_a values of benzoic acid and dihydrogen citrate and acetic acid increase with the acetonitrile contents, and so do the differences between pK_a' and $^w_w pK_a$. When the ^s_spH scale is used, the obtained pK_a' value is expected to be close to the pK_a value of the solute in the same mobile phase ($^s_s pK_a$), and the results obtained confirm this.

For pyridine, the C$_{18}$ column gave very poor results which were attributed to the interaction of pyridine with the residual silanol groups,[28] and a polymeric column was selected. For this test solute, the alternative procedure of measuring the pH after mixing

the aqueous buffer and the acetonitrile, but calibrating the electrode system with the common aqueous buffers of pH 4 and 7 (^s_wpH scale), was used. Several aqueous buffers at round ^w_wpH values from 2.0 to 12.0 scaled at 1.0 pH unit were prepared using different acid−base pairs based on phosphate, acetate, citrate, borate, and butylamine (see Tables of Supporting Information). These were later mixed with acetonitrile, and the ^s_wpH values were measured and converted to ^s_spH values by means of eq 1 and the δ values of Table 3. The retention times obtained for the HPLC different buffers and mobile phases were related to the ^w_wpH and ^s_spH values of the buffers through eq 7 and the fits obtained are given in Table 4. The results are similar to those obtained for benzoic acid.

The retention time of the neutral form of the solute ($t_{R(A)}$ in this example) is larger than the retention time of the ionized species (piridinium cation), and the retention of both species decreases when the percentage of acetonitrile in the mobile phase increases. The standard deviations and the $t_{R(A)}$ and $t_{R(HA)}$ values obtained from both pH scales are very similar, but the pK_a' parameters differ considerably. In this case, the pK_a' parameters obtained from the ^w_wpH scale are markedly lower than the pK_a value of pyridinium in water ($^w_w pK_a$), because the ΔpK_a values for this acid are slightly negative (see Figure 2), whereas the ΔpK_a values for the HPLC buffers on the range of variation of the solute retention (similar to that of benzoic acid) are positive.

The factors that contribute to the variation of pK_a when a solute is transferred from water (w) to another solvent (s) can be summarized in the equation[13,29]

$$\Delta pK_a = {}^s_s pK_a - {}^w_w pK_a = {}^{vac}_{vac} pK_{H_3O^+} - {}^{vac}_{vac} pK_{HS^+} - \frac{e^2(z-1)}{2.303rkT}\left(\frac{1}{{}^s\epsilon} - \frac{1}{{}^w\epsilon}\right) - \frac{\left(\sum {}^s_s G_{SOLV} - \sum {}^w_w G_{SOLV}\right)}{2.303RT} \quad (11)$$

where $^{vac}_{vac} pK_{H_3O^+}$ and $^{vac}_{vac} pK_{HS^+}$ indicate the intrinsic acidities of the protonated water and solvent s (the acetonitrile−water mixture) in the vacuum. The term $[e^2(z-1)/2.303rkT(1/^s\epsilon - 1/^w\epsilon)$, where e is the electron charge and kT the energy of thermal

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agitation, stands out for the differences on the electrostatic interactions between the ions present in the solutions in solvent s and in water, which depends on the charge z of the acid, the static dielectric constants of the two solvents $^s\epsilon$ and $^w\epsilon$, and on the radius of the ions r. The last term of the equation stands out for the differences on the energies of specific solvation (such as hydrogen bonding) of the acid in the two solvents.

The different behavior of cationic acids (e.g., pyridinium) and neutral (e.g., acetic) or anionic (e.g., dihydrogen citrate) acids comes from the different contributions of the electrostatic term of eq 11. For a cationic acid, $z = 1$ and the electrostatic term cancels out. Therefore, ΔpK_a depends only on the differences between the acidities of the protonated water and acetonitrile–water mixture and on the differences between the specific solvation terms, which in general are small and negative as compared with the electrostatic contribution, which is positive for $z \leq 0$. As expected from a close observation of Figure 2, the differences between $^w_wpK_a$ and pK_a' increase with the acetonitrile contents of the mobile phase.

The opposite variation of the $^s_spK_a$ values of neutral and anionic acids, on one hand, and neutral bases (or cationic acids), on the other, with the addition of organic modifiers is a general behavior[4,17] that has important consequences in HPLC retention–pH studies.[27] Most chromatographic buffers are prepared from neutral and cationic acids, which may approximately match the pK_a variation of acids but not the one of bases. The literature reports good relationships between the aqueous pK_a values of acids[30,31] estimated by HPLC using the ^w_wpH scale and the accepted thermodynamic values, but the relations worsen for bases.[30]

McCalley[28,32,33] has studied the protonation of bases in methanol–water, acetonitrile–water, and tetrahydrofuran–water and concluded that half-protonation is produced at an aqueous pH much lower than the aqueous $^w_wpK_a$ value of the base. Since protonation of the base causes tailing and peak asymmetry in many C_{18} columns, it is possible to obtain good efficiencies for bases at mobile phase ^w_wpH values much lower than expected from the aqueous $^w_wpK_a$ values of the bases.

Recently, Kele and Guiochon[34] reported good batch-to-batch reproducibilities for the basic compounds amitriptyline and propanolol in a C_{18} column and a methanol–water (65:35) mobile phase with a ^w_wpH 7.0 buffer. At this pH value, the silanol groups of the column are dissociated, whereas the aqueous $^w_wpK_a$ values of the amines (9.4 and 9.5) suggest that they should be still completely protonated. Thus, strong ion-exchange interactions are expected between the silica surface of the column and the amines that should produce pronounced differences between the column batches. However, the increase of the aqueous pH of the buffer (to a ^s_spH of ~8.3) and the decrease of the pK_a value of the amines (in ~0.5 pH units), caused by the addition of the organic modifier, combine to get only half-protonation of the amines and, therefore, a decrease on the amine–silica interactions.

The same argument was used by Neue et al.[35] for the same amines and mobile phase on a C_8 column to explain the large shifts obtained (relative to acenaphthene) for small variations in the mobile-phase conditions (buffer concentration, pH, methanol percentage, and temperature). At mobile phase pH 7.0 and for pK_a values of the bases close to 9, the bases should be completely protonated. In this instance, a small variation in the mobile-phase conditions should not influence ionization of the bases and therefore the relative retention should not change. However, the "apparent" pK_a values of the bases (caused by combination of the buffer pH and base pK_a variations) were found to be around 6.5–7, i.e., around the ^w_wpH value of the buffer. Therefore, in fact, the bases are more or less half-protonated and small variations of the conditions cause appreciable variation of ionization and retention.

The ^s_spH scale allows one to obtain good direct relationships between the mobile-phase pH and base $^s_spK_a$ value. Table 4 shows that the obtained pK_a' parameters agree very well with the thermodynamic $^s_spK_a$ values of pyridinium at the different acetonitrile–water percentages. The literature pK_a values were obtained by interpolation from the values reported by Pawlak.[17] Since the literature values were given in the molal scale for a ionic strength of 0.01 m, they were corrected using the A and a_0B constants of Table 1. The correction was −0.11, −0.12, and −0.15 for 20, 40, and 60% acetonitrile. In a rigorous approach, the pK_a' values obtained should be also corrected for the activity coefficients before comparison with thermodynamic pK values, but the ionic strength of the HPLC buffers is very low and the correction is similar to the standard deviations obtained for pK_a'.

In addition to the discrepancies between the thermodynamic and "HPLC apparent" aqueous pK_a values of bases, the buffer-dependent differences between the ^w_wpH and ^s_spH scales may also produce bad retention–pH relationships. Many chromatographers prefer to work with buffers prepared from a polyprotic acid (e.g., phosphoric or citric) to minimize the differences, but even in this instance, eq 11 predicts different variation for the different charged species of the buffer (e.g., different for a $H_2PO_4^-/HPO_4^{2-}$ than for a $H_3PO_4/H_2PO_4^-$ or a HPO_4^{2-}/PO_4^{3-} buffer).

The variability of the differences may be notable if buffers prepared from neutral acids, for which ΔpK_a is positive, and bases, for which ΔpK_a is negative, are combined. We have included several buffers prepared from ammonia and butylamine in this study. The effect of the pH variation of these buffers, opposite to the pH variation of the other buffers, is not appreciable for benzoic acid ($^w_wpK_a = 4.18$) and pyridine ($^w_wpK_a = 5.23$), since they are completely in their basic form in these buffers (^w_wpH values of 10.0 and 11.0).

To show the effect of buffers prepared from neutral bases, we have included in this study two other test solutes (3-nitrophenol and triethylamine) with more basic pK_a values. Table 4 and Figures 3 and 4 present the results obtained in 40% acetonitrile as mobile phase. The fits obtained for the plots of retention time vs the ^s_spH values of the buffers are very good (Figure 3), with small standard deviations, but they are much worse for the ^w_wpH values (Figure 4). Ammonia and butylamine buffers show ^w_wpH values higher than the ^s_spH values of the same buffers, whereas the other buffers (prepared from phosphates, citrates, acetates,

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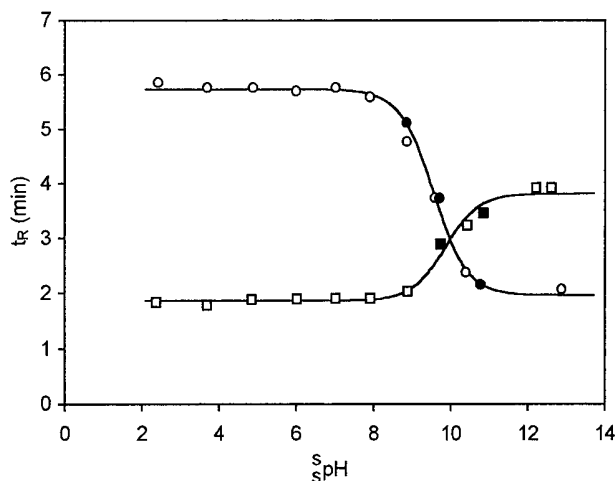
Figure 3. Variation of the retention time of 3-nitrophenol and triethylamine in the polymeric column with the 40% acetonitrile mobile-phase pH measured after mixing the aqueous buffer with the organic modifier ($_s^s$pH scale): (○) 3-nitrophenol in neutral and anionic acid buffers, (●) 3-nitrophenol in ammonia and butylamine buffers, (□) triethylamine in neutral and anionic acid buffers, and (■) triethylamine in butylamine buffers.
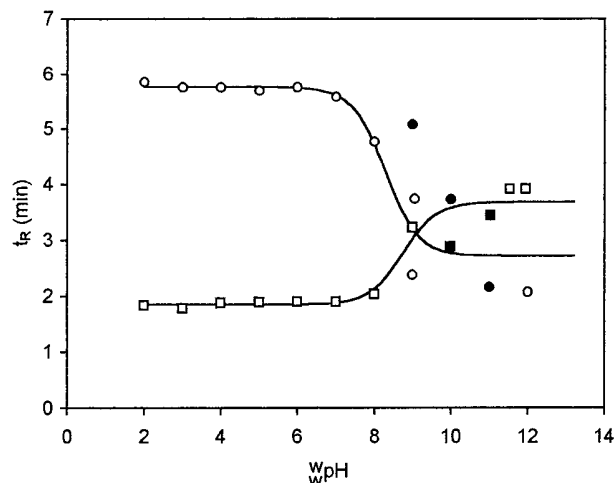

Figure 4. Variation of the retention time of 3-nitrophenol and triethylamine in the polymeric column with the 40% acetonitrile mobile-phase pH measured before mixing the aqueous buffer with the organic modifier ($_w^w$pH scale). Symbols as in Figure 3.

and borates) show $_w^s$pH values lower than the $_s^s$pH values. Since, they are in the pH range of variation of retention, where 3-nitrophenol and triethylamine are partially ionized, the positive shifts of ammonia and butylamine buffers and the negative shifts of the other buffers combine to produce poor fits of the data to the theoretical eq 7.

Figures 3 and 4 present a clear example of this effect for 3-nitrophenol. We have prepared three different buffers (from NH_4^+/NH_3, $H_2PO_4^-/HPO_4^{2-}$, and $H_3BO_3/H_2BO_3^-$) at the same $_w^w$pH value of 9.00. However, the retention of 3-nitrophenol in

these three buffers is very different: t_R is 5.08 min with NH_4^+/NH_3, 3.74 min with $H_2PO_4^-/HPO_4^{2-}$, and 2.38 min with $H_3BO_3/H_2BO_3^-$. It is impossible that the line fitted through eq 7 for $_w^w$pH crosses these three points (Figure 4). However, the $_s^s$pH values of NH_4^+/NH_3, $H_2PO_4^-/HPO_4^{2-}$, and $H_3BO_3/H_2BO_3^-$ buffers are 8.86, 9.60, and 10.39, respectively, and the fitting line crosses these three points very nicely (Figure 3). The same effect is observed for triethylamine, which is more retained with a borate buffer of $_w^w$pH = 9.00 (but $_s^s$pH = 10.30) than with a butylamine buffer of $_w^w$pH = 10.00 (but $_s^s$pH = 9.60).

We must recognize that combined acid- and base-prepared buffers are almost never used in HPLC retention−pH studies, but we believe that the reason is just the poor precision obtained because almost invariably the pH is measured in the aqueous buffer ($_w^w$pH scale). The examples presented in this work demonstrate that very good HPLC retention−pH relationships can be obtained for any combination of different types of buffers if the pH is measured in the rigorous $_w^s$pH and $_s^s$pH scales, i.e., after mixing the aqueous buffer with the organic modifier. In addition, the pK_a parameters obtained from retention are, or show a direct relation with, the thermodynamic acid−base constants of the solute, which allow one to make quantitative predictions from the thermodynamic data (e.g., to predict the degree of protonation of a base in a given mobile phase).

Therefore, we must recommend, in agreement with IUPAC rules and suggestions, measurement of the pH of the mobile phase after mixing the aqueous buffer and the organic modifier. The electrode system used can be calibrated with standards of high buffer capacity prepared in the same solvent mixture used as mobile phase, if the $_s^s$pH values of these standards are accurately known ($_s^s$pH scale). However, it is equally rigorous and more easy to calibrate the electrode system with the common aqueous buffers and work in the $_w^s$pH scale, which, if necessary, can be converted to the $_s^s$pH scale through the δ values determined in this work.

SUPPORTING INFORMATION AVAILABLE

Tables with the $_w^w$pH, $_w^s$pH, and $_s^s$pH values of the HPLC buffers and the retention times of the test solutes at the different mobile phases. This material is available free of charge via the Internet at http://pubs.acs.org.