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Simulating phenol high-performance liquid chromatography retention times as the pH changes Mobile phase pH versus buffer pH

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Abstract

The HPLC retention times of several substituted phenols have been measured and simulated using Advanced Chemistry Development's LC simulator, using 50% acetonitrile (ACN) as the mobile phase. For alkyl- and nitro-substituted phenols, the quality of the simulation improves when pH of the mobile phase is estimated and used in the simulation. Simply using the pH of the buffer gives simulation results that are not as close to the actual retention times. However, the opposite is the case for halogenated phenols. The pK_a values in 50% ACN for some of these phenols have also been determined, which tend to be one unit higher than the aqueous pK_a values reported in the literature.

Keywords: HPLC; Phenol; Acetonitrile–water mixture; Acid-dissociation constant; Computer simulation; pH variation

1. Introduction

The retention time of an analyte in a reversed-phase HPLC experiment will depend on the partition coefficient between the mobile and stationary phases, and on the volume phase ratio for the column employed. Separation of two analytes will depend on the differences in the partition coefficients of the analytes. When the species being separated are ionogenic (acidic and basic), there is the additional complication that the species will undergo an acid-base equilibrium, dependant on the pH, where both the acid and conjugate base will have different partition coefficients. Therefore, the retention times of the acid and its conjugate base will be different, and there will be a region of rapid change in the plot of t_R versus pH in the region where pH $\approx$ pK_a. Stable separations require pH values far from the pK_a of each ionogenic analyte. Unfortunately, identifying a region where the pH is far from the pK_a is not trivial in mixed organic-aqueous solvents because: (a) The pK_a values of weakly acidic or basic analytes (e.g., phenols, anilines, etc.) will change with the amount of organic solvent. They can be estimated using a Hammet equation, or some other semiempirical method [1]. Fortunately, similar species will have similar linear fitting parameters [2]. (b) The pK_a values of all *buffer* species will change, so the pH of the mobile phase will change when mixed with organic solvent. This is an area of current active research [3], and is the focus of this article. (Traditionally, pH is reported in the literature as the pH of the buffer *before* mixing with organic solvent [4].) (c) Matrix effects may change from pure to mixed solvents [5].

Automated experimental design for ionogenic species requires simulation software that can model the effect of pH on retention time, especially if using pH gradient separation [6]. A number of simulation programs are commercially available (e.g., DryLab, which was recently reviewed in this journal [7]). We have chosen to look at the LC simulator in the Method Development Suite of Advanced Chemistry Development, to test its performance in prediction of the retention times of ionogenic species as pH is varied.

In this paper, we report retention factors from RP-HPLC for several phenols, a commonly investigated family of organic acids [1,2,8–10]. Using our experimental data in the

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ACD prediction software, we will evaluate the quality of retention-time predictions as pH varies.

2. Theory

2.1. Calculation of mixed-solvent pK_a

For a generic acid HA,

$$HA + H_2O \rightarrow H_3O^+ + A^-,$$

k_{HA} is the retention factor for the acidic form and k_{A^-} for the basic form. Because the two forms are in equilibrium, there will be a time-averaged overall retention factor [11,12],

$$k = \alpha_{HA} k_{HA} + \alpha_{A^-} k_{A^-} \quad (1)$$

where α_{HA} and α_{A^-} are the mole fractions of the species in the acidic and conjugate basic forms, respectively. At low pH ($pH \ll pK_a$) the analyte will be predominately in the protonated form, so $k = k_{HA}$ ($\alpha_{HA} = 1$), while at high pH ($pH \gg pK_a$) the deprotonated form will dominate. When the pH is near the pK_a, k will change rapidly from k_{HA} to k_{A^-} (or vice versa).

This property of ionogenic species can be used to determine the pK_a from a LC experiment [13–16]. A plot of t_R versus pH will result in a curve similar in shape to a titration curve. At the inflection point, the analyte will be half in its acidic and half in its basic form ($\alpha_{HA} = \alpha_{A^-} = 50\%$; $c_b = c_a$), and by the Henderson–Hasselbach equation,

$$pH = pK_a + \log\left(\frac{c_b}{c_a}\right) \quad (2)$$

the pH will be equal to the pK_a. However, this number can only be accurate to the degree that the pH of the mixed solvent is known. There is also the limitation that most HPLC columns are limited to a pH range of 3–8, while many organic species will have pK_a values outside this range.

2.2. Calculation of mixed-solvent pH

A number of groups have measured buffer pH in mixed aqueous–acetonitrile solvent, and attempted to describe the change in pH mathematically [11,12,17,18]. For acetonitrile (and indeed for most organic solvents), pK_a varies linearly with percentage of acetonitrile added, through the range of about 0–70% acetonitrile. We have chosen the method of Rosés and coworkers to determine the pH of our phosphate buffer aqueous–acetonitrile mixed system [18].

The change in pK_a of the buffer components between pure water and mixed solvent is described by,

$$\Delta^s_w pK_a = m\varphi_{MeCN} \quad (3)$$

where m is the slope describing the straight-line variation in pK_a when organic solvent is added, φ_{MeCN} is the volume fraction of acetonitrile (50% in our experiment), and ${}^s_w pK_a$ is the pK_a in mixed solvent, referenced to an aqueous standard. Knowing the variation in pK_a allows calculation of the variation in pH. If we assume the Henderson–Hasselbach equation is valid through all our buffer pH ranges, i.e., throughout the range where the acid is acting as a buffer, then,

$$\Delta pH = \Delta^s_w pK_a = m_{pH}\varphi_{MeCN} \quad (4)$$

Using multiprotic acids requires the pK_a values of each ionisable proton. Luckily, multiprotic acids (and bases) behave roughly linearly through all their various equilibria (i.e., each pK_a changes the same amount), so an overall scaling value m can be estimated using,

$$m_{pH} = \frac{a_0 + \sum_{i=1}^{n} a_i 10^{s(ipH - b_i)} + a_{n+1} 10^{s((n+1)pH - b_{n+1})}}{1 + \sum_{i=1}^{n} 10^{s(ipH - b_i)} + 10^{s((n+1)pH - b_{n+1})}} \quad (5)$$

For the triprotic acid H_3PO_4, Rosés' group gives the following values: $s = 1.62$, $a_0 = 0$, $a_1 = 1.42$, $a_2 = 1.75$, $a_3 = 1.81$, $b_1 = 2.21$, $b_2 = 6.62$, and $b_3 = 15.16$. Table 1 gives the pH values used in this study, before and after mixing with organic solvent.

Table 1
Actual pH values of the buffers used, and the equivalent pH of the mobile phase calculated using Eqs. (4) and (5)

Buffers used for Set A (**1**–**6**) and **15**		Buffers used for Set B (**7**–**13**) and **16**	
pH of pure buffer	pH of mobile phase	pH of pure buffer	pH of mobile phase
2.0	2.22[a]	2.0	2.22[a]
2.8	3.44	3.0	3.67
3.8	4.52	4.1	4.83
4.8	5.62	5.05	5.90
6.0	6.87	6.05	6.92
6.9	7.87	7.0	7.87
7.9	8.88	8.05	8.93
8.9	9.78[a]	9.0	9.88[a]
10.0	10.89[a]	10.1	10.99[a]
11.0	11.90[a]	11.0	11.90[a]

[a] Invalid, as outside the buffering range of the buffer.

3. Experimental

3.1. Materials

Phenols were purchased from Aldrich Canada (Oakville, Canada), except **5** (BDH, Ottawa, Canada). Chemicals were used as received, injected as 1.0 mM solutions in methanol. Methanol and acetonitrile were HPLC grade, obtained from Anachemia (Winnipeg, Canada). Buffer solutions of pH 2–11 were prepared as solutions of $K_3PO_4 \cdot H_2O$ (J.T. Baker, Phillipsburg, NJ, USA) in water purified by the Barnstead Type 1 system (to 18 MΩ), then titrated with HCl (Caledon, Georgetown, Canada) to the desired pH. Final concentration of buffers were 0.01 M in phosphate. Buffers and

solvents were degassed and filtered through 0.1 μm nylon filters (Micron Separations) prior to use.

3.2. Apparatus

The HPLC system was a Varian Prostar, using the Prostar 410 autosampler, the Model 330 photodiode array detector (peak detection at 215 nm), and the Model 230 solvent delivery system (ternary gradient). The mobile phase was 50:50 buffer:acetonitrile (isocratic) with a flow rate of 1.0 mL/min. Injections were 6 μL onto 100 mm × 4.6 mm, 3.5 μm Agilent columns, after passing through a Varian ChromGuard RP 10 mm × 2 mm guard column. Three analytical columns were used: (i) Zorbax Extend-C18 column with an observed pressure of 80 atm, (ii) Zorbax SB-C18 with an observed pressure of 100 atm, and (iii) Zorbax Eclipse XDB-C18 with an observed pressure of 80 atm. Column temperature was maintained at a constant 30 °C.

3.3. Retention times—experimental and simulated

Experimental retention times t_R are uncorrected. The time of unretained mobile phase t_0 (taken as the peak from injected methanol sample solvent) is approximately 1.00 min on the Extend column, and 1.20 on the other two, with a variation up to 0.05 min. Predicted retention times were determined using Advanced Chemistry Development's LC simulator (version 6.05). Predictions were carried out twice per analyte: firstly by simulating the retention time at high pH using the experimental acidic retention time (buffer pH 3), and secondly by simulating the retention time at low pH using the experimental basic retention time (buffer pH 8). The simulator was run using the log *D* (partition coefficient) prediction routine with a t_0 value of 1.00 for comparison with experimental retention times on the Extend-C18 column.

4. Results and discussion

4.1. Experimentally determined retention factors

We chose to investigate sixteen phenols. Some of them have pK_a values within the buffering region of the phosphate buffers we used. These compounds should show variation in retention factor with pH. Others were chosen to be outside the buffering region, which would act as controls—their retention factors should not vary with pH. The compounds, along with the literature value of their pK_a values, are given in Table 2. Each compound was analysed by an isocratic HPLC run at a number of pH values. Mobile phase was 50% aqueous, 50% acetonitrile. Table 3 summarises the experimental results.

4.2. Simulation of retention times using buffer pH, and comparison with experimental values

The ACD simulation software requires input of a number of experimental values, including pH, solvent, and the analyte's molecular structure. It uses these values and the analyte's (aqueous) pK_a from ACD's extensive library to predict the analyte's behaviour as the pH is altered. If the pK_a is not in the ACD library, the software estimates it based on a structural similarity comparison with compounds of known pK_a; all the analytes simulated in this study were in the library (**15** is not in the library, a compound we could not model anyway,

Table 2
Experimentally determined pK_a values in 50% acetonitrile (ACN), using mobile phase pH (calculated using Eqs. (4) and (5))

Compound	pK_a in ACN/H_2O		Aqueous pK_a
	This work (50% ACN)	Literature value (30% ACN)[a]	
4-Propylphenol, **1**	–[b]	–	9.12[c] [1]
4-*tert*-Butylphenol, **2**	–[b]	10.67 [1]	10.39 [19]
2,4-Dimethylphenol, **3**	–[b]	10.69 [1]	10.60 [20]
2,4-Dibromophenol, **4**	9.18	8.20 [1]	–
4-Nitrophenol, **5**	7.82	7.23 [1]	7.01 [21]
4-Cyanophenol, **6**	8.93	–	7.97 [22]
2-Bromophenol, **7**	–[b]	8.90 [1]	–
3-Bromophenol, **8**	–[b]	10.80[d] [2], 9.67 [1]	9.03 [23]
4-Bromophenol, **9**	–[b]	10.22 [1]	9.37 [24]
4-Chlorophenol, **10**	–[b]	11.21[d] [2], 10.08 [1]	9.41 [24]
3-Methylphenol, **11**	–[b]	12.11[d] [2], 10.57 [1]	10.10 [20]
4-Methylphenol, **12**	–[b]	10.57 [1]	10.28 [20]
3-Nitrophenol, **13**	9.82[e]	8.56 [1]	8.35 [25]
2,4,6-Tribromophenol, **14**	6.87	–	6.10, 10.10[f] [26]
4-Octylphenol, **15**	9.08	–	–
2-Chlorophenol, **16**	10.02[e]	9.77 [1]	8.48 [25]

[a] Determined using an HPLC in 30% acetonitrile, but assuming pH (aq) = pH (mobile phase).
[b] The inflection point of the curve for these species is outside the pH range investigated.
[c] According to the ACD software database, this is the pK_a of 4-propylhydroxyben*zoate*, not 4-propylhydroxyben*zene*.
[d] In 50% acetonitrile.
[e] Strictly speaking, these values are outside the buffering range of the buffer (and therefore outside the valid range for Eqs. (4) and (5)).
[f] In 100% methanol.

Table 3
Summary of experimental results for compounds using the Zorbax Extend-C18 column

Compound	Retention factor (k) at pH									
	2	3	4	5	6	7	8	9	10	11
4-Propylphenol, **1**	2.34	2.43	2.34	2.31	2.27	2.30	2.30	2.26	2.22	1.58
4-*tert*-Butylphenol, **2**	2.86	2.99	2.88	2.83	2.82	2.81	2.81	2.79	2.71	1.87
2,4-Dimethylphenol, **3**	1.45	1.50	1.46	1.45	1.41	1.43	1.43	1.44	1.43	1.20
2,4-Dibromophenol, **4**	2.66	2.77	2.70	2.65	2.61	2.41	1.76	0.43	0	0
4-Nitrophenol, **5**	0.58	0.62	0.59	0.56	0.52	0.28	0	0	0	0
4-Cyanophenol, **6**	0.38	0.40	0.38	0.37	0.36	0.34	0.20	0	0	0
2-Bromophenol, **7**	0.92	0.94	0.94	0.97	0.96	0.94	0.91	0.77	0.42	0.08
3-Bromophenol, **8**	1.10	1.12	1.11	1.09	1.14	1.11	1.06	0.98	0.23	0
4-Bromophenol, **9**	1.04	1.06	1.07	1.04	1.09	1.06	1.03	1.00	0.61	0
4-Chlorophenol, **10**	0.89	0.88	0.89	0.88	0.92	0.90	0.87	0.86	–	–
3-Methylphenol, **11**	0.69	0.68	0.68	0.69	0.70	0.69	0.70	0.69	–	–
4-Methylphenol, **12**	0.70	0.69	0.68	0.68	0.70	0.69	0.70	0.71	–	–
3-Nitrophenol, **13**	0.56	0.56	0.57	0.57	0.60	0.55	0.42	0.24	0	0
2,4,6-Tribromophenol, **14**[a]	5.16	5.21	5.18	4.73	2.38	0.37	0.02	0	–	–
4-Octylphenol, **15**	2.67	2.85	2.68	2.67	2.30	2.43	1.71	0.32	0	0
2-Chlorophenol, **16**	0.82	0.81	0.80	0.82	0.83	0.79	0.72	0.56	0	0

Approximate buffer pH values are given; actual values are listed in Table 1. Mobile phase is 50% aqueous, 50% acetonitrile. The inflection point of the plots of k vs. pH is taken as the pK_a given in Table 2.

[a] Data collected on the SB-C18 column (actual buffer pH is given in the supplemental material).

as explained below). We have inputted chromatographic data at (buffer) pH 3 and then asked the simulator to predict the analyte's retention factor at higher (more basic) pH values. A second set of data used the experimental data at pH 8 to predict the retention factor as the pH was lowered (no simulation was done for **5** because its retention factor was 0 at pH 8).

Thirteen of the 16 phenols were divided into two "training groups" In our case, we ran compounds in the same training group if there was a linear correlation between their log D values and their initial retention times. No simulations are reported for **14**–**16** because they were consistently outliers in any grouping we tried. With its long octyl chain, the polarity of **15** is sufficiently different from phenols with smaller methyl groups. For **14**, the same argument can be made about the cumulative effect of three methyls. It is unclear why **16** is an outlier, although it turns out that all the halogen-substituted phenols behave badly in the simulations (see Fig. 1).

The simulations were done at pH 3 and 8 because they are at the limits of the effective buffering range of the phosphate buffer (and therefore the limits of the useful range of Eqs. (4) and (5)). By choosing to input the buffer pH values (not the mixed solvent pH), this simulation assumes the pH of the mixed mobile phase is equivalent to the starting pH of the buffer. Results for selected compounds are shown in Fig. 1.

4.3. Simulation of retention times using calculated, mixed-solvent pH, and comparison with experimental values

Simulations using the ACD prediction software were run using the mixed-solvent pH values as calculated using Eqs. (4) and (5) (Table 1). The results are shown in Fig. 1 to allow comparison with the results using buffer pH. Three compounds (**3**, **11**, **12**) are not shown in Fig. 1. They have pK_a values outside the buffering range and, as expected, they do not show variation in retention time with pH. For such compounds, the ACD simulation software accurately predicts the correct retention times whether buffer pH or calculated mixed-solvent pH is used.

Fig. 2 shows the results for compounds **4** and **7**–**10**. These five compounds all behave worse when the simulation is run with mobile-phase pH. They are all halogen-substituted phenols. We tried simulating these compounds in a number of different training groups, including one with only halogenated phenols, but they consistently showed anomalous behaviour in any grouping we tried.

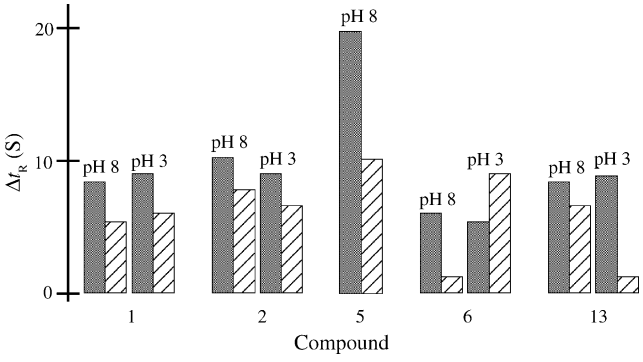

Fig. 1. Improvement in retention time prediction for selected phenols. Predictions of retention time at high pH (pH 8) from a low-pH model (pH 3) are labelled pH 8, and vice versa (labelled pH 3). The vertical axis is the absolute difference in retention time, $|t_R$ (predicted) $- t_R$ (actual)|. Shaded bars are predictions using buffer pH, striped bars are predictions using mobile-phase pH, calculated using Eqs. (4) and (5).

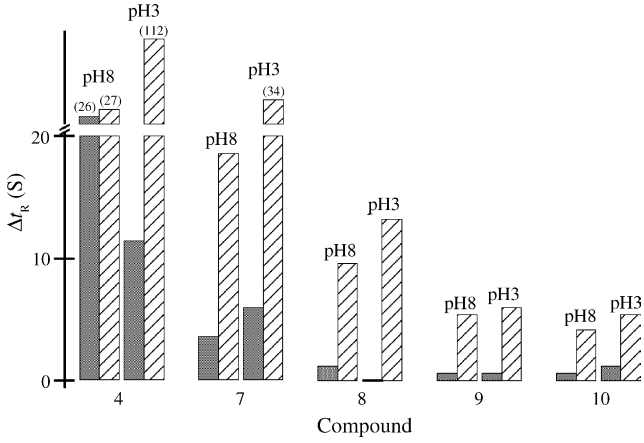

Fig. 2. Retention time predictions for halogenated phenols, with the same graphical representations as Fig. 1.

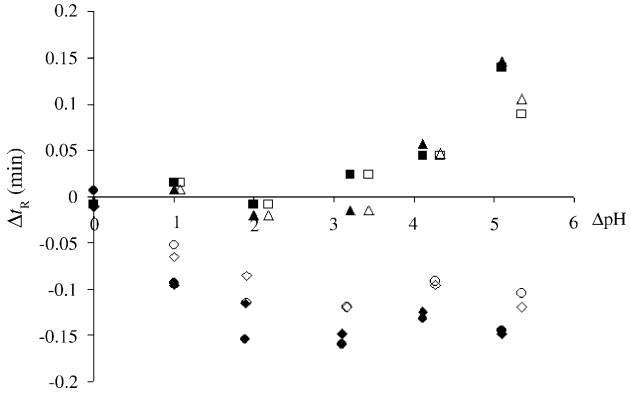

Fig. 3. Plot of the difference in retention time between predicted and actual values, $\Delta t_R = t_R$(predicted) − t_R(actual) vs. the pH difference between the simulation pH and the training pH, ΔpH. Solid symbols represent simulations using buffer pH, hollow symbols represent mobile-phase pH. For every pH, simulations using mobile-phase pH give results as good as or better than simulations using buffer pH. Legend: (■) **1** with training pH 3, (♦) **1** with training pH 8, (▲) **2** with training pH 3, and (●) **2** with training pH 8.

4.4. Additional RP columns

To verify the generality of our results, we tested the compounds on two additional columns. Selected experimental data and retention factors are given in Tables S1 and S2 in the Supplementary data. Retention times are similar, although there is a smaller change in retention times between the acidic and basic forms, so the effect for which we are testing is muted. Therefore, the chromatograms discussed in this paper are from the Agilent Extend-C18 column.

4.5. Determination of pK_a in 50% acetonitrile solution

We have plotted retention factor versus mobile-phase pH (calculated by Eqs. (4) and (5)) to determine the pK_a values of the analytes in mixed-solvent (50:50) acetonitrile:water. These values are presented in Table 2, to allow comparison to the literature values. We only give a pK_a value for those compounds that presented a clear titration-like curve. Even without running additional pH values in the region of rapid change, the values in Table 2 give a good indication that the analytes' pK_a values are significantly different under normal HPLC conditions than they are in water.

4.6. Discussion

Fig. 3 shows the variation in predicted pH as the simulation moves further from the pH used as the starting point of the simulation. Predictions from low pH appear in the positive part of the graph, indicating the simulator generally overestimates the retention time (predicted t_R > actual t_R). Alternatively, the negative part of the graph contains the data using a high-pH training set in the simulator, indicating a consistent underestimation of the retention time (predicted t_R < actual t_R). This is a general trend, 3-nitrobenzene **13** excepted (the simulator overestimates t_R when both a high- and a low-pH training set is used).

As shown in Figs. 1 and 3, compounds with normal behaviour show an improvement in predicted retention times when using mobile-phase pH as opposed to buffer pH. Improvements are small (a few seconds) when the analyte's pK_a is at the edge or outside the buffer range (compounds **1**–**3**, **11**, **12**). However, compound **5** improves by 10 s because its pK_a is in the range of pH values through which the simulation is passing. Likewise, **13** improves by 7 s.

Compound **6**, 4-cyanophenol, improves 5 s when using pH 3 as a training set, but the prediction is worse by 4 s when the training set is pH 8. It appears that the more polar **6** cannot be accurately simulated using less polar alkyl groups. This observation underlines the importance of choosing an appropriate training set when using the ACD (or any other) simulator program.

Unfortunately, halogenated phenols do not behave as expected, as shown in Fig. 2. A possible explanation is that we have only taken into account one of the three issues identified as affecting retention time—we have not investigated matrix effects nor pK_a variations in the analyte. This latter effect is the most probable cause for the poor performance—halogenated phenols could easily have different solubility and ionisation in aqueous solvents that are not properly modelled. As can be seen in Table 2, the mixed-solvent pK_a differs from the aqueous pK_a by about 1.0 for non-halogenated analytes. As shown by others, halogenated phenols have a greater change (relative to nitrophenols, at least) in pK_a as organic solvent is added [2]. Presumably, use of the true, mixed-solvent analyte pK_a in the simulation should lead to improvement in the predictions for the halogenated compounds.

5. Conclusion

We have shown that the ACD prediction software gives accurate predictions of retention times using buffer pH, as

long as the pK_a is outside the pH region investigated. If the pK_a is within the range of pH variation, improvements in the prediction times are obtained if mobile phase pH (calculated by the method of [2,18]) is used instead of the pH of the aqueous phase before mixing. A systematic exception is the halogenated phenols. We have also found some pK_a values for phenols in mixed 50:50 acetonitrile:aqueous solvent, using plots of t_R versus pH, which differ from the aqueous pK_a by a factor of 1.0.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2005.07.115.

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