Influence of the acid-base ionization of drugs in their retention in reversed-phase liquid chromatography

Sara Soriano-Meseguer[1], Elisabet Fuguet[1,2], Adriana Port[3], Martí Rosés[1,*]

[1] Departament de Química Analítica i Institut de Biomedicina, Universitat de Barcelona, Martí i Franquès 1-11, 08028 Barcelona, Spain

[2] Serra Húnter Programme, Generalitat de Catalunya, 08002 Barcelona, Spain

[3] ESTEVE Pharmaceuticals, Drug Discovery and Preclinical Development, Parc Científic de Barcelona, Baldiri Reixac, 4-8, 08028 Barcelona, Spain

*Address for correspondence: Prof. Martí Rosés
Departament de Química Analítica
Universitat de Barcelona
Martí i Franquès, 1-11
E-08028-Barcelona
Spain
Tel. +34 93 403 92 75
Fax +34 93 402 12 33
e-mail: marti.roses@ub.edu

Abstract

The effect of the ionization in the RP-HPLC retention of 66 acid-base compounds, most of them drugs of pharmaceutical interest, is studied. The retention time of the compounds can be related to the pH measured in the mobile phase ($^{s}_{w}$pH) through the sigmoidal equations derived from distribution of the neutral and ionic forms of the drug into the stationary and mobile phases. Fitting of the obtained retention vs. pH profiles provides the retention times of the ionic and neutral forms and the pK_a values of the drugs in the mobile phase ($^{s}_{w}$pK_a).

The obtained $^{s}_{w}$pK_a values are linearly correlated to the pK_a values in water ($^{w}_{w}$pK_a) with two different correlations, one for neutral acids and another for neutral bases that reflect the different influence of the dielectric constant of the medium in ionization of acids and bases. The retention of the neutral species is well correlated to the octanol-water partition coefficient of the drugs as measure of the lipophilicity of the drug, which affects chromatographic retention. Also, the retention time of the ionized forms is related to the retention time of the neutral forms by two different linear correlations, one for anions and the other for cations. These last correlations point out the different retention behavior of anions and cations: anions are less retained than cations of the same lipophilicity, as measured by the octanol-water partition coefficient of the neutral form.

The different retention behavior of anionic, cationic and neutral forms is confirmed by the hold-up times obtained from different approaches: pycnometry and retention times of anionic (KBr and KI) and neutral (DMSO) markers. Hold-up times obtained by pycnometric measurements agree with those obtained by retention of neutral markers (0.83-0.85 min), whereas hold-up time for anions is mobile phase pH dependent. At acidic pH it is similar to the hold-up time for neutral markers (0.83 min), but then it decreases with the increase of mobile phase pH to 0.65 min at pH 11. The decrease can be explained by the ionization of the silanols of the column and exclusion of anions by charge repulsion. Although not directly measured, the obtained retention data and

correlations indicate hold-up time for cations are similar or slightly lower than hold-up time for neutral compounds (0.77 – 0.83 min).

The model proposed and the correlations obtained can be very useful for its implementation in retention prediction algorithms for optimization of separation purposes.

Keywords: Chromatographic retention; Retention models; Acid-base ionization; Hold-up time; Mobile phase pH

1. Introduction

It is well-known that the retention of ionic species in reversed-phase high-performance liquid chromatography (RP-HPLC) is much lower than the retention of neutral ones. Thus, the retention of ionizable compounds, *i.e.* compounds with acid-base properties, is strongly dependent on its degree of ionization [1–6], which in turn depends on the pK_a of the compound and the pH of the mobile phase. The chromatographic retention of an analyte depends also on the concentration and type of organic modifier used, which in addition to the retention of the neutral and ionic species also modifies the proportion of these species because it modifies the pK_a of the compound and the pH of the mobile phase. When an organic modifier is added to an aqueous buffer to prepare the mobile phase there is a change in the pK_a of the buffer and consequently there is a variation in the pH of the hydroorganic mixture. The pH of the mobile phase is then a powerful optimization parameter, additional to mobile phase composition, that needs to be adequately measured and controlled by appropriate buffers.

The correct measurement of pH in HPLC mobile phases and their influence on retention of ionizable solutes have been previously studied by our group [7–10] and others [11–16]. It is clear the pH must be measured in the mobile phase in order to obtain good relationships between retention and pH. The pH electrode can be calibrated with buffers prepared in the same solvent composition used as mobile phase (${}^{s}_{s}$pH) or more easily with commercial aqueous buffers (${}^{s}_{w}$pH). The two pH scales are related by means of the δ parameter, constant for each mobile phase composition and electrode system [17,18]. Measurement of pH in the aqueous buffer before mixing it with the organic modifier (${}^{w}_{w}$pH) does not provide the ionization degree of the solute in the mobile phase.

The aim of this work is to provide a systematic study about the influence of ionization in retention for a wide range of acid-base compounds of different chemical nature: monoprotic and diprotic acids and bases, and amphoteric solutes, most of them drugs of pharmaceutical interest. The retention of the ionized species is specially studied

in order to determine its significance by comparison to the hold-up time of the column, which is also discussed.

2. Materials and methods

2.1. Instruments

Chromatographic measurements were performed with an Agilent Technologies (Santa Clara, CA, USA) 1200 Series instrument equipped with G1312B binary pump and G1367D autoinjector. In general, an UHD 6540 Accurate-Mass Q-TOF detector with electrospray ionization (ESI) was used for compound detection in most solutions. However, a G1315C DAD was also used at 254 nm for detection in the phosphate buffers. Instrument control and processing were performed by Masshunter software 4.0. A 100 mm, 4.6 mm i.d, 2.6 µm octadecylsilica Kinetex EVO C18 analytical column provided by Phenomenex (Torrance, CA, USA) with a core-shell Technology was used for all determinations. This material is stable within the pH range 1-12.

pH measurements of mobile phase were done with a combined Crison 5202 electrode in a Crison 2001 pH meter (Hach Lange Spain, L'Hospitalet de Llobregat, Spain). The electrode system was calibrated with ordinary aqueous buffers of pH 4.01, 7.00 and 9.21 (25 ºC).

2.2. Reagents

Acetonitrile LCMS grade was purchased from Fluka Analytical VWR (West Chester, PA, USA) and water was purified by Milli-Q deionizing system from Millipore (Billerica, MA, USA) with a resistivity of 18.2 MΩ cm. Reagents used to prepare the buffer solutions were sodium phosphate monobasic monohydrate (Sigma-Aldrich, ≥99.0%), formic acid (Scharlau, eluent additive for LC-MS), acetic acid (Fluka Analytical, eluent additive for LC-MS), ethylendiamine (Fluka Analytical, ≥ 99.5%) and 25% w/w ammonia solution Sharlau, extrapur). The 66 studied acid-base analytes were purchased from Sigma-

118 Aldrich (Steinheim, Germany), Fluka Analytical VWR (West Chester, PA, USA), Riedel-
119 de Haën (Seelze, Germany), Merck (Darmstadt, Germany), Carlo Erba (Milano, Italy),
120 Baker (Center Valley, PA, USA) or synthesized in ESTEVE (Barcelona, Spain).
121
122 **2.3. Procedure**
123 The acid-base solutes of different chemical nature were injected in a HPLC system at 6
124 different pH values, between 2 and 11, approximately (see Table 1 for the exact pH
125 values in the different pH scales). The mobile phase composition was 40% acetonitrile
126 and 60% aqueous buffer. The appropriate detection mode was used for each buffer
127 solution. For mass spectrometry detection the pH range was restricted to 3-11 because
128 only volatile buffers were compatible. At ${}^{w}_{w}$pH 2.0, the detection was performed by UV
129 because a mixture of phosphoric acid and sodium dihydrogenphosphate at concentration
130 50 mM was used as buffer. pH of this buffer was adjusted with diluted hydrochloric acid.
131 Formic acid, acetic acid and ammonia solution were used at ${}^{w}_{w}$pH 3.0, 5.0 and 9.0,
132 respectively. Ethylendiamine was used at ${}^{w}_{w}$pH 7.0 and 11.0. The buffer concentrations
133 at ${}^{w}_{w}$pH 3.0, 5.0, 7.0, 9.0 and 11.0 were 10 mM and were adjusted by addition of diluted
134 acetic acid or diluted ammonia. The pH of the aqueous HPLC buffers was measured
135 before and after mixing it with the organic modifier, obtaining the ${}^{w}_{w}$pH and the ${}^{s}_{w}$pH values
136 of Table 1. All experiments were done at 25 ºC.
137 Stock solutions of the compounds at 5 mg mL^{-1} were prepared by dissolving the
138 appropriate weight or volume in methanol. A more diluted solution at 0.1 mg mL^{-1} was
139 prepared by dissolving an aliquot of the previous stock solution in an ACN-H_2O mixture
140 (40:60). Isocratic conditions were used at flow rate of 1 mL min^{-1} and the injection volume
141 was 10 µL. The hold-up times and extra-column times were measured by injections of
142 aqueous solutions of potassium bromide, detected by UV at 200 nm, dimethyl sulfoxide,
143 detected by ESI+, and potassium iodide, detected by ESI-. The concentration of these

144 solutions was 0.1 mg mL^{-1}. All results were the average of triplicate injections at each pH
145 buffer (Table 1).
146 To measure the extra-column times, a chromatographic connection with
147 negligible hold-up volume was used.
148 The pycnometry measurements were performed filling the column successively
149 with pure water, methanol and acetonitrile at a constant temperature of 25 ºC. These
150 solvents were pumped through the column at a constant flow rate of 1 mL min^{-1} for an
151 hour. Immediately after, the pump was stopped, the inlet and outlet of the column were
152 sealed with screw caps and the column was weighted. This process was repeated three
153 times.
154

155 **2.4. Data treatment**

156 The nonlinear regressions of experimental retention with the pH were performed using
157 available commercial software TableCurve 3D 4.0.
158

159 **3. Theory**

160 Chromatographic retention for acid-base analytes can be described as a function of the
161 mobile phase pH and solute pK_a with a sigmoidal plot which has a pronounced jump
162 around the analyte pK_a [7,8,19]. The derivation of the function comes out from the
163 definition of the distribution constant (K_C) which is the ratio of the overall concentrations
164 of the compound in the stationary and mobile phases [7]. Since concentrations are
165 difficult to measure directly in HPLC, the equation is usually developed in terms of
166 retention factor (k), which is the ratio of the amounts of compound in stationary and
167 mobile phase. k is related to K_C through the phase ratio, *i.e.* the ratio between the
168 volumes of stationary (V_S) and mobile (V_M) phases:

169 $$K_C = k\frac{V_M}{V_S} \quad (1)$$

V_M (also called hold-up volume) can be directly calculated from the mobile phase flow and the hold-up time (t_M), *i.e.* the retention time measured for an unretained compound. However, the volume of stationary phase cannot be easily measured and then conversion of k to K_C is not feasible. Therefore, commonly HPLC retention is described in terms of retention factor, which in the case of an acid-base compound can be given as the sum of the retention factors (k_i) of all acid-base species present in solution averaged by the molar fraction of each species (α_i), *i.e.*

$$k = \sum k_i \alpha_i \quad (2)$$

k can be linearly related to the adjusted retention time (t'_R) and to the retention time (t_R) of the compound through the hold-up time.

$$k = \frac{t'_R}{t_M} = \frac{t_R - t_M}{t_M} \quad (3)$$

The general equation relating retention to pH can be equally written in terms of retention factor, adjusted retention time (or volume) or simple retention time (or volume) [7]. Because in many instances the exact hold-up time is not known, or may be different for the different forms of the analyte [6], it seems most practical to write the equation in terms of retention time, which is the quantity directly measured. In this case the main equation can be written as:

$$t_R = \frac{\sum_{r=0}^{n} t_{R_{H_{n-r}A}} 10^{rpH - \sum_{i=0}^{r} pK_{ai}}}{\sum_{r=0}^{n} 10^{rpH - \sum_{i=0}^{r} pK_{ai}}} \quad (4)$$

For a monoprotic solute, HA, Eq. (4) can be rewritten as:

$$t_R = \frac{t_{R_{HA}} + t_{R_A} 10^{(pH - pK_a)}}{1 + 10^{(pH - pK_a)}} \quad (5)$$

where $t_{R_{HA}}$ and t_{R_A} represent the retention time of the protonated and the unprotonated forms of the solute, respectively (charges of the subscripted forms are omitted for simplicity).

For a diprotic solute, H_2A, Eq. (4) can be expressed as:

$$t_R = \frac{t_{R_{H_2A}} + t_{R_{HA}} 10^{(pH - pK_{a_1})} + t_{R_A} 10^{(2pH - pK_{a_1} - pK_{a_2})}}{1 + 10^{(pH - pK_{a_1})} + 10^{(2pH - pK_{a_1} - pK_{a_2})}} \quad (6)$$

where $t_{R_{H_2A}}$, $t_{R_{HA}}$ and t_{R_A} represent the retention times of the diprotonated, monoprotonated and unprotonated solute, respectively.

Particular equations (5) and (6) are enough for all cases studied in this work which are representative of almost all cases encountered in RP-HPLC acid-base retention fundamental studies. Equation (5) can be applied to monoprotic acids and bases, being HA and A^- or HA^+ and A, respectively, the subscripted species. Similarly, equation (6) can be applied to diprotic acids (with H_2A, HA^- and A^{2-} species) and diprotic bases (with H_2A^{2+}, HA^+ and A species) and also to ampholytes (with H_2A^+, HA and A^- species).

It has been extensively probed that the fitting capability of these equations are guaranteed only when pH and pK_a correspond to the true pH and pK_a in the solvent used as particular mobile phase values (^s_spH or ^s_wpH scale) [9–12,17,20,21].

4. Results and discussion

4.1. Measurement of extra-column and hold-up times

For practical reasons, usually, the hold-up volume includes the mobile phase volume in the column but also in the injector, detector and connections. Thus, when the same column is used in different HPLC systems, such as in this work where different detection systems were used, the hold-up times and retention factors cannot be directly compared. The extra-column time (t_{ext}), which is the retention time contribution due to the injector, detector and connections, of each HPLC system has to be subtracted from all measured retention times, including hold-up time, for a good comparison. For this reason, the International Union of Pure and Applied Chemistry (IUPAC) proposes to calculate retention based on extra-column retention time correction [22].

This approach has been followed in this work and thus all measured retention times refer to the column solely. The obtained extra-column time values were 0.048 min for UV detection and 0.249 min for MS detection.

There is not a clear definition of hold-up time or volume [22–25] IUPAC defines the hold-up volume (time) in column chromatography as “the volume of the mobile phase (or the corresponding time) required to elute a component the concentration of which in the stationary phase is negligible compared to that in the mobile phase. In other words, this component is not retained at all by the stationary phase. However, it has been shown that eluant molecules are adsorbed onto the bonded phase surface or support, forming a stationary layer of mobile phase components, increasing the volume of stationary phase and thus decreasing the hold-up volume [24][. It is also known that for different kinds of molecules, the volume of stationary phase (adsorbed layer) is different. Molecular exclusion effect and ionic electrostatic interactions may also take place. Therefore, there are several methods to measure hold-up time which lead to different results and someones among them have been tested in this work.

Pycnometry is often used to determine the volume of mobile phase inside the column by weighting the column filled by two solvents of different density [24,25]. The results obtained for our column were 0.84$\pm$0.01 mL and 0.86$\pm$0.01 mL using the pairs of solvents water/methanol and water/acetonitrile, respectively. Density values of 0.9971, 0.7866 and 0.7766 g mL^{-1} at 25 ºC were used for water, methanol and acetonitrile, respectively [26,27]. Given the flow rate of 1 mL min^{-1} they would correspond to hold-up times of 0.84 and 0.86 min.

Several unretained markers were also tested, depending on their suitability for detection system. KBr, KI and DMSO were used for UV, MS-ESI- and MS-ESI+ detection, respectively. Results are presented in Table 1 and Figure 1 for the studied buffers.

The results show that the neutral marker DMSO gives a constant value of 0.83$\pm$0.01 min, regardless of the buffer employed. This value is very similar to the ones obtained by pycnometry and we assume that it can be taken as the hold-up time for neutral compounds.

The ionic markers KBr and KI show a very similar behaviour of variation of the retention time with the pH of the buffer. At acidic pH values ($^{s}_{w}$pH < 4 or $^{w}_{w}$pH <3), the retention time agrees with that obtained for DMSO, but later it decreases with the pH of the buffer. We attribute this behaviour to electronic repulsion between the anionic marker (Br^- or I^-) and the ionized silanols of the column. At acidic pH, silanols are protonated, there is no charge repulsion and the hold-up time is the same as the one of neutral markers. However, when pH increases, ionization of silanols and repulsion increase too and the hold-up time decreases [28]. A similar behaviour was previously observed by comparison of the retention times of KBr and 2-nitrobenzoate [6].

Therefore, we can expect the hold-up times (or available mobile phase volume) of the studied ionized acids to be lower that the hold-up time of the corresponding neutral species.

4.2 Variation of retention with the pH of the mobile phase

The obtained results are presented in Table 2 (monoprotic neutral acids), Table 3 (monoprotic neutral bases - or cationic acids -) and Table 4 (diprotic compounds: a diprotic neutral acid, amphiprotic acid-base compounds, and diprotic neutral bases). Quite good statistics are obtained in most cases. The Tables also present literature data in water that can be related to the expected fitting parameters, i.e. octanol/water partition coefficient (log $P_{o/w}$), an unspecific measure of compound polarity (or hydrophobicity) commonly related to retention parameters, and acid-base dissociation constant in water ($^{w}_{w}pK_a$) which should be related to the obtained acid-base dissociation constant in the mobile phase ($^{s}_{w}pK_a$). Additionally, the Abraham descriptor of solute hydrogen bond acidity (A) is also presented because of its clear relationship with log *k* and log $P_{o/w}$ [29]. Inside each table, the compounds are grouped according to dissociation group types because the expected relation between water and mobile phase (40% acetonitrile)

mainly depends of the acidic group type and solute charge [30]. Figures 2-4 present representative profiles of the acid-base drugs.

Table 2 shows the fitting parameters obtained for monoprotic neutral acids by application of equation (5). Compounds have been divided in three groups: phenols, carboxylic acids, and other ones that do not have carboxylic nor phenolic group (two barbituric acids, 5-fluorouracil and warfarin). As expected for neutral acid dissociation [7], the obtained pK_a values in the mobile phase ($^{s}_{w}pK_a$) are higher (0.8-1.5 pK_a units) than the pK_a values in pure water ($^{w}_{w}pK_a$). Also, in all cases the retention time of the anions is much lower than that of the neutral forms, although there are some small differences depending on the type of compounds.

The retention time of the neutral forms of the phenols goes from about 1 min for the most polar ones (log $P_{o/w}$ < 1) to more than 5 min for the most retained ones (thymol and capsaicin, log $P_{o/w}$ > 3). However, the retention time of ionic forms in all cases is the same than the hold-up time expected for anions at pH 11 (0.65±0.01, see Table 1). In fact, the retention time of phenolates could be calculated only for the most acidic phenols. We have not enough basic pH data to calculate retention of phenols with $^{w}_{w}pK_a$> 9 appropriately, but in all cases the observed retention-pH profile is coherent with a retention time of 0.65 min for the phenolates. In these cases, the retention time of the anion was fixed to 0.65 min and the rest of parameters were estimated by fitting the t_R data to $^{s}_{w}$pH. Some representative profiles are presented in Figure 2A.

Three benzoic acid derivatives and some nonsteroidal anti-inflammatory drugs were studied as representative compounds of carboxylic acids. They are more retained than phenols, with retention times of the neutral forms going from about 1.3 min for the most polar ones (aspirin and benzoic acid, log $P_{o/w}$ < 2) to more than 7 min the most hydrophobic (diclofenac, indomethacin and ibuprofen, log $P_{o/w}$ ≥ 3.5). The retention time of the anionic forms is also higher than that of phenolates and thus, higher than that of the hold-up time for anions (0.65 min), although the most polar aspirin and benzoic acid

present retention time only slightly higher. The retention of the anions is clearly related to the retention of the neutral forms, although not quantitatively. As commented, aspirin and benzoic acid show the minimum retentions (0.68 min) and diclofenac, indomethacin, and ibuprofen the largest ones (about 0.9-1.0 min). Hence, the data shows that these anions are significantly retained in the column, probably as ion pairs. Figure 2B presents the profiles of several representative carboxylic acids.

The last group of studied neutral acids (labeled as others in Table 2) is formed by very polar compounds (barbital, phenobarbital and specially 5-fluorouracil) and warfarin, which is less polar. The retention of the three most polar compounds is very low for both, the neutral (t_R close to 1 min) and anionic form (not different from the hold-up time for anions). Retention of warfarin is larger for both neutral and anionic forms, as expected from the larger log $P_{o/w}$ value. The profiles of the four compounds are presented in Figure 2C.

The results for neutral bases are given in Table 3 for pyridines and amines, and several representative profiles are presented in Figure 3A for pyridines and 3B for amines. Conversely to acids, the fitting pK_a for bases in the mobile phase ($^{s}_{w}pK_a$) are lower than the pK_a values in water ($^{w}_{w}pK_a$), as expected. The polarity range studied, as measured by the log $P_{o/w}$ value, goes from 0.15 for o-phenylenediamine, which show very low retention, to 3.95 for sufentanyl with the highest retention (16.52 min for the neutral form). Retention times of the cations are clearly larger than that of the hold-up time for anions (0.65 min). Retention time of protonated benzyl nicotinate, the least basic compound, could not be precisely determined because of the lack of retention data at $^{s}_{w}$pH values lower than the $^{s}_{w}pK_a$. In order to fit the data, the retention time of the cationic form has been fixed to 0.83 min (the hold-up time of the neutral compounds), as a consensus value, due to the high variability of retention times shown by cations.

Several diprotic solutes have been also studied and the results presented in Table 4 and Figure 4. The studied compounds include a diprotic neutral acid with a

carboxylic and a phenolic group (4-hydroxyphenylacetic acid), four ampholytes with an amino or pyridino and a phenolic group, and four diprotic neutral bases. All of them are very or quite hydrophilic (log $P_{o/w} < 2$) and the neutral forms are poorly retained (about 2 min or less), with the exception of chloropheniramine. Retention of the ionic forms is even lower. Retention of the monocharged anions is in the range 0.63-0.77 min, *i.e.* close to the expected hold-up time for anions (0.65 min). Retention of the dicharged anion of 4-hydroxyphenylacetic acid is in the same range (0.63 min) and slightly lower than that of the monocharged anion of the same acid (0.77 min). This fact suggests that the exclusion from the stationary phase of the dicharged anions is slightly larger than that of only monocharged anions. Retention of monoprotonated cations is in the range 0.83-1.10 min for ampholytes and 0.90-1.50 min for diprotic bases. The retention of fully protonated diprotic neutral bases (dicharged) cannot be well estimated because of the lack of enough data at very low pH values, but the retention profiles are consistent with a retention value close to the hold-up time estimated for neutrals (see profiles in Figure 4B). The profiles clearly show that retention of dicharged cations is slightly lower than that of monocharged cations. The fitting ${}^{s}_{w}pK_a$ obtained are quite reasonable. As expected, the fitting ${}^{s}_{w}pK_a$ values are in general higher for acid groups and lower for basic groups than the ${}^{w}_{w}pK_a$ ones. Numeric exceptions are the values of the first pK_a of 2-amino-4-nitrophenol and ranitidine and the two pK_a values of p-phenylenediamine, which show high uncertainties. Also, the value of the second ${}^{s}_{w}pK_a$ of 4-hydroxyphenylacetic acid is lower than the corresponding value in water, but the later value is an estimated value, not an experimental one.

4.3. Conjoint analysis of results

From the results discussed above, some common trends for the different types of studied compounds are clear.

On one hand, it is evident that the pK_a in the mobile phase ($^{s}_{w}pK_a$) is related to the pK_a in water ($^{w}_{w}pK_a$), but it increases for acid groups (loss of hydrogen ions) and decreases for basic groups (gain of hydrogen ions). Figure 5 plots the $^{s}_{w}pK_a$ values obtained from fitting equations (4)-(6) vs. the literature $^{w}_{w}pK_a$ values. Two straight lines, one for acids and another for bases can be observed. The correlations obtained are presented in Eqs. (7) for neutral acids and (8) for neutral bases:

$$^{s}_{w}pK_a = 0.978(\pm0.019)\,^{w}_{w}pK_a + 1.38(\pm0.14) \quad (7)$$

$R^2 = 0.9879$ SD = 0.27 F = 2702

$$^{s}_{w}pK_a = 0.938(\pm0.053)\,^{w}_{w}pK_a - 0.58(\pm0.38) \quad (8)$$

$R^2 = 0.9374$ SD = 0.55 F = 314

The slope value of the correlation measures the ‘‘resolution of acid strength’’ for the compounds in the mobile phase solvent as regards to water (slope unity), *i.e.* the ability of the solvent to differentiate between the acidities of the compound’s set [31]. The two slopes are close to 1, which means that the specific solvation interactions of the compounds with the studied mobile phase (40% acetonitrile) are similar to those with water [30]. As expected, the intercepts are positive for neutral acids and negative for cationic acids (protonated neutral bases). The intercept of the correlation depends on the differences in basicities, dielectric constants, and specific solvation interactions of the solute (*e.g.* hydrogen bonding) between mobile phase and pure water [30]. Dielectric constant interactions are only significant for dissociation of neutral or anionic acids because of the change in charges of the dissociation process: a neutral acid is uncharged but the dissociated anion plus the solvated hydrogen cation have one negative and one positive charge. Solvent dielectric constant practically does not affect

dissociation from a monocharged cationic acid (one positive charge) to a neutral base plus solvated hydrogen cation (one positive charge too). Since specific interactions are similar in 40% acetonitrile and water (slope close to unity), the negative intercept for bases should be attributed to a higher basicity of 40% acetonitrile in comparison with pure water. The dielectric constant of 40% acetonitrile is much lower than that of water and electrostatic interactions disfavor solvation of ions and increase the pK_a of neutral acids. This effect surpasses the negative basicity change effect and thus, neutral acids become weaker in 40% acetonitrile and the plot presents a positive intercept.

On the other hand, it has been long recognized that the retention in reversed-phase liquid chromatography is related to the hydrophobicity of the compound and thus shows good correlations to the octanol-water partition coefficient [32–34]. In fact, log $P_{o/w}$ is frequently used for prediction of retention [35] and log $P_{o/w}$ is often determined by HPLC measurements [29,33,36–38]. In order to test these correlations, log *k* vs. log $P_{o/w}$ has been plotted in Figure 6A for the neutral forms of the different acid-base compounds. log *k* was calculated from the retention times in Tables 2-4 and using the hold-up time determined for neutral compounds from DMSO measurements (0.83 min). There is a clear linear relationship between the two parameters according to the following correlation:

$$\log k = 0.410(\pm 0.025)\log P_{o/w} - 0.705(\pm 0.057) \quad (9)$$

$R^2 = 0.806$ SD = 0.24 F = 266

Some dispersion of the points according to the different types of compounds studied can be observed in the plot, which can be attributed to the different hydrogen bond capabilities of the functional groups. For instance, anilines show a higher retention than predicted whereas phenols and carboxylic acids are slightly less retained than expected. It has been pointed that reversed phase retention is affected by the hydrogen bond acidity of the solute, but this property has not a significant effect on the

409 octanol/water partition [29]. Taking into account the hydrogen bond acidity of the solute,
410 measured by the *A* descriptor of Abraham, as an additional descriptor, the correlation
411 obtained is presented in Eq. 10 and Figure 6B.
412
413 $\log k = 0.411(\pm 0.019)\log P_{o/w} - 0.529(\pm 0.076)A - 0.464(\pm 0.056)$ (10)
414 $R^2 = 0.890$ SD = 0.18 F = 255
415 The new correlation is slightly better than correlation (9) and more important, no
416 congeneric effect can be observed in the plot.
417 Relationship between retention of the ionic forms of the compounds and their
418 hydrophobicity is more troublesome. In principle we would expected the retention to
419 correlate to the octanol water partition coefficient of the ion. However, the availability of
420 $\log P_{o/w}$ data for ions is scarce and questionable. Ions seems to be mostly partitioned to
421 organic solvents as ion pairs and higher neutral aggregates than by ionic species, and
422 the partition is strongly dependent on the nature and concentration of the counter ion.
423 Despite this problem, it seems evident than the hydrophobicity of the ionized form must
424 be related to the hydrophobicity of the neutral form. Donovan and Pescatore assumed
425 this difference to be 3.15 $\log P_{o/w}$ unities on average, being the actual difference between
426 the $\log P_{o/w}$ values of neutral and ionized forms from 1.5 to 4.5 depending on structure
427 and ionic strength [38]. Hence, we expect the retention of the ionic forms to be related to
428 the retention of the corresponding neutral forms and to test this assumption we have
429 simply plotted the retention times of monocharged ions against the retention times of the
430 corresponding neutral forms in Figure 7. Although there is some scattering of the points
431 at low retention, two different lines close to linearity are clearly observed, one for anions
432 from neutral acids and another for cations from neutral bases. Only 4-
433 hydroxyphenylacetic, out of the 24 acids, and N,N-dimethylaniline and 2-amino-4-
434 nitrophenol, out of the 26 bases, deviate more than 2SD from the straight lines. The
435 correlation equations are as follows:
436

437 $t_{R_{A^-}} = 0.0430(\pm 0.0049) t_{R_{HA}} + 0.607(\pm 0.017)$ (11)

438 $R^2 = 0.775$ SD = 0.051 F = 76

439

440 $t_{R_{HA^+}} = 0.0845(\pm 0.0055) t_{R_A} + 0.740(\pm 0.026)$ (12)

441 $R^2 = 0.908$ SD = 0.097 F = 237

442

443 The slope and intercept for bases are higher than for acids and they show that
444 retention of cations is larger than retention of anions. This fact can be also directly seen
445 from the retentions of the ampholytes in Table 4.

446 Correlations (11) and (12) provide a further evidence of the different hold-up times
447 for anions, neutral forms and cations. We have taken hold-up times of 0.65, 0.83 and
448 0.83 min for the three forms, respectively. Replacing the retention time of the neutral
449 form by its hold-up time of 0.83 min in the two equations, we get hold-up times of 0.63
450 min for anions and 0.77 min for cations. The calculated hold-up time for anions is in very
451 good agreement with the taken one and the calculated one for cations is slightly lower
452 than the taken one. This later point suggests that the hold-up time for cations may be
453 slightly lower than the hold-up time for neutral compounds (0.83 min) but clearly higher
454 than that of anions (0.65 min). In fact, the most polar bases show fitting retention times
455 of their cations in the range 0.77-0.83 min (see Table 2).

456 Moreover, the two correlations provide a useful tool to estimate the retention time
457 of the ionic forms of the studied compounds too basic or too acid to estimate it directly
458 from the fitting to equation (5) or (6), for which we assumed 0.65 min for anions and 0.83
459 min for cations. Recalculation of the fittings to Eqs. (5) or (6) using these new estimations
460 give the results presented in Table 5. The effect of the correction in the fitting is very
461 small. The largest corrections are for thymol and capsaicin for which the retention of the
462 anion moves from 0.65 to 0.84-0.85 min. All other fitting parameters remain very similar
463 to the ones of Tables 2-4 within the standard error of the fittings.

5. Conclusions

The retention of ionizable acid-base compounds is strongly dependent of its degree of dissociation which depend on the mobile phase pH and the specific pK_a values of the compound in the mobile phase too. If the pH of the used buffers is measured in the mobile phase, fitting of the retention time to pH, provides the pK_a values of the compound in the mobile phase. These pK_a values are higher than pK_a values in water for neutral acids, but slightly lower for neutral bases. In both cases, the pK_a values in the mobile phase can be linearly related to the pK_a values in water, although with some dispersion of the points because of the slightly different specific interactions of the compounds with the two solvents (water and mobile phase). The correlations are good enough to provide an approximate pK_a of the compound in the mobile phase from the pK_a in water and thus, predict the degree of ionization in a specific buffer of measured pH.

The fits also provide the retention time of the anionic, neutral and cationic forms of the acid-base compounds. The retention of the neutral forms can be directly related to the hydrophobicity of the compound as measured by its octanol/water partition coefficient, widely available and easily estimated. The correlation can be improved if an additional term for hydrogen bond acidity is added. The retention times of the ionic forms can be directly related to the retention time of the neutral form according to different linear correlations, one for anions from neutral acids and another for cations for neutral bases. The results and correlations show that cations are more retained than anions and both much less retained than neutral forms (as expected in this last instance).

Measurement of hold-up time by different methods (pycnometry, ionic and neutral markers) shows that the column hold-up time for anions is at most pH values lower than that for neutral compounds (about 0.83 min in our system) and it decreases with the pH of the mobile phase (from about 0.80 min at pH 2 to 0.65 min at pH 11). The results from the correlations between retention times of ions and neutral forms confirm the lower hold-

491 up time of anions at basic pH (about 0.63 min) and suggest a hold-up time for cations
492 between that of anionic and neutral form (about 0.77 min).

493 Overall, the study shows the importance of the ionization in the retention of the
494 acid-base compounds and derives relationships of the pK_a and retention of the ionic and
495 neutral forms in the HPLC system to the usually available data in water (pK_a and log
496 $P_{o/w}$). These relationships can be very useful for prediction of retention, establishment of
497 retention models, and optimization of separations [19,39–41] if the pH of the buffer is
498 correctly measured in the mobile phase.

Acknowledgements: Financial support from the Ministerio de Economía y Competitividad from the Spanish Government (CTQ2017-88179-P) and the Catalan Government (2017 SGR 1074) is acknowledged.

REFERENCES

[1] C.F. Poole, The Essence of Chromatography, Elsevier, Amsterdam, 2003.

[2] C. Horvath, W. Melander, Liquid Chromatography with Hydrocarbonaceous Bonded Phases; Theory and Practice of Reversed Phase Chromatography, J. Chromatogr. Sci. 15 (1977) 393–404. doi:10.1093/chromsci/15.9.393.

[3] J.L.M. van de Venne, J.L.H.M. Hendrikx, R.S. Deelder, Retention behaviour of carboxylic acids in reversed-phase column liquid chromatography, J. Chromatogr. A. 167 (1978) 1–16. doi:10.1016/S0021-9673(00)91142-7.

[4] P.J. Schoenmakers, R. Tijssen, Modelling retention of ionogenic solutes in liquid chromatography as a function of pH for optimization purposes, J. Chromatogr. A. 656 (1993) 577–590. doi:10.1016/0021-9673(93)80820-X.

[5] J.E. Hardcastle, I. Jano, Determination of dissociation constants of polyprotic acids from chromatographic data, J. Chromatogr. B Biomed. Sci. Appl. 717 (1998) 39–56. doi:10.1016/S0378-4347(98)00256-4.

[6] M. Rosés, I. Canals, H. Allemann, K. Siigur, E. Bosch, Retention of Ionizable Compounds on HPLC. 2. Effect of pH, Ionic Strength, and Mobile Phase Composition on the Retention of Weak Acids., Anal. Chem. 68 (1996) 4094–4100. doi:10.1021/ac960105d.

[7] M. Rosés, E. Bosch, Influence of mobile phase acid-base equilibria on the chromatographic behaviour of protolytic compounds, J. Chromatogr. A. 982 (2002) 1–30. doi:10.1016/S0021-9673(02)01444-9.

[8] M. Rosés, Determination of the pH of binary mobile phases for reversed-phase liquid chromatography, J. Chromatogr. A. 1037 (2004) 283–298.

525 doi:10.1016/j.chroma.2003.12.063.

526 [9] I. Canals, J.A. Portal, E. Bosch, M. Rosés, Retention of ionizable compounds on
527 HPLC. 4. Mobile-phase pH measurement in methanol/water, Anal. Chem. 72
528 (2000) 1802–1809. doi:10.1021/ac990943i.

529 [10] S. Espinosa, E. Bosch, M. Rosés, Retention of Ionizable Compounds on HPLC.
530 5. pH Scales and the Retention of Acids and Bases with Acetonitrile−Water
531 Mobile Phases, Anal. Chem. 72 (2000) 5193–5200. doi:10.1021/ac000591b.

532 [11] J. Barbosa, V. Sanz-Nebot, Assignment of reference pH-values to primary
533 standard buffer solutions for standardization of potentiometric sensors in
534 acetonitrile-water mixtures, Fresenius. J. Anal. Chem. 353 (1995) 148–155.
535 doi:10.1007/BF00322949.

536 [12] D. Sýkora, E. Tesarová, M. Popl, Interactions of basic compounds in reversed-
537 phase high-performance liquid chromatography. Influence of sorbent character,
538 mobile phase composition, and pH on retention of basic compounds, J.
539 Chromatogr. A. 758 (1997) 37–51. doi:10.1016/S0021-9673(96)00691-7.

540 [13] R. Bergés, V. Sanz-Nebot, J. Barbosa, Modelling retention in liquid
541 chromatography as a function of solvent composition and pH of the mobile
542 phase, J. Chromatogr. A. 869 (2000) 27–39. doi:10.1016/S0021-9673(99)00915-
543 2.

544 [14] J.J. Baeza-Baeza, M.C. García-Alvarez-Coque, Some observations on the
545 prediction of retention in reversed-phase liquid chromatography using the pH as
546 main factor, Anal. Chim. Acta. 521 (2004) 61–68. doi:10.1016/j.aca.2004.05.035.

547 [15] N. Sanli, G. Fonrodona, J. Barbosa, G.A. Özkan, J.L. Beltran, Modelling
548 retention in liquid chromatography of polyphenolic acids: Prediction of solvent

composition and pH of the mobile phase, Anal. Chim. Acta. 537 (2005) 53–61. doi:10.1016/j.aca.2005.01.006.

[16] J.K. Törnblom, T.F.W. Bureyko, C.D. MacKinnon, Simulating phenol high-performance liquid chromatography retention times as the pH changes: Mobile phase pH versus buffer pH, J. Chromatogr. A. 1095 (2005) 68–73. doi:10.1016/j.chroma.2005.07.115.

[17] I. Canals, F.Z. Oumada, M. Rosés, E. Bosch, Retention of ionizable compounds on HPLC. 6. pH measurements with the glass electrode in methanol–water mixtures, J. Chromatogr. A. 911 (2001) 191–202. doi:10.1016/S0021-9673(00)01271-1.

[18] L.G. Gagliardi, C.B. Castells, C. Ràfols, M. Rosés, E. Bosch, δ conversion parameter between pH scales (WSpH and SSPH) in acetonitrile/water mixtures at various compositions and temperatures, Anal. Chem. 79 (2007) 3180–3187. doi:10.1021/ac062372h.

[19] M. Rosés, X. Subirats, E. Bosch, Retention models for ionizable compounds in reversed-phase liquid chromatography. Effect of variation of mobile phase composition and temperature, J. Chromatogr. A. 1216 (2009) 1756–1775. doi:10.1016/j.chroma.2008.12.042.

[20] E. Bosch, P. Bou, H. Allemann, M. Rosés, Retention of Ionizable Compounds on HPLC. pH Scale in Methanol−Water and the p K and pH Values of Buffers, Anal. Chem. 68 (1996) 3651–3657. doi:10.1021/ac960104l.

[21] S. Espinosa, E. Bosch, M. Rosés, Retention of ionizable compounds on high-performance liquid chromatography, J. Chromatogr. A. 945 (2002) 83–96. doi:10.1016/S0021-9673(01)01486-8.

[22] J.A.G. Domínguez, J.C. Díez-Masa, Part A. Hold-up volume concept in column chromatography, Pure Appl. Chem. 73 (2001) 969–981.

[23] Y. V. Kazakevich, H.M. McNair, Thermodynamic Definition of HPLC Dead Volume, J. Chromatogr. Sci. 31 (1993) 317–322. doi:10.1093/chromsci/31.8.317.

[24] R.M. McCormick, B.L. Karger, Distribution Phenomena Of Mobile-Phase Components And Determination Of Dead Volume In Reversed-Phase Liquid Chromatography, Anal. Chem. 52 (1980) 2249–2257. doi:10.1021/ac50064a005.

[25] C.A. Rimmer, C.R. Simmons, J.G. Dorsey, The measurement and meaning of void volumes in reversed-phase liquid chromatography, J. Chromatogr. A. 965 (2002) 219–232. doi:10.1016/S0021-9673(02)00730-6.

[26] M. Nakamura, K. Tamura, S. Murakami, Isotope effects on thermodynamic properties: mixtures of x(D2O or H2O) + (1 - x)CH3CN at 298.15 K, Thermochim. Acta. 253 (1995) 127–136. doi:10.1016/0040-6031(94)02086-4.

[27] J. V. Herráez, R. Belda, Refractive indices, densities and excess molar volumes of monoalcohols + water, J. Solution Chem. 35 (2006) 1315–1328. doi:10.1007/s10953-006-9059-4.

[28] A. Méndez, E. Bosch, M. Rosés, U.D. Neue, Comparison of the acidity of residual silanol groups in several liquid chromatography columns, J. Chromatogr. A. 986 (2003) 33–44. doi:10.1016/S0021-9673(02)01899-X.

[29] K. Valko, C. Du, C. Bevan, D. Reynolds, M. Abraham, Rapid Method for the Estimation of Octanol / Water Partition Coefficient (Log Poct) from Gradient RP-HPLC Retention and a Hydrogen Bond Acidity Term (Sigma alpha2H), Curr. Med. Chem. 8 (2001) 1137–1146. doi:10.2174/0929867013372643.

[30] S. Espinosa, E. Bosch, M. Rosés, Retention of ionizable compounds in high-performance liquid chromatography 14. Acid-base pK values in acetonitrile-water mobile phases, J. Chromatogr. A. 964 (2002) 55–66. doi:10.1016/S0021-9673(02)00558-7.

[31] M.K. Chantooni, I.M. Kolthoff, Resolution of acid strength in tert-butyl alcohol and isopropyl alcohol of substituted benzoic acids, phenols, and aliphatic carboxylic acids, Anal. Chem. 51 (1979) 133–140. doi:10.1021/ac50037a039.

[32] R. Kaliszan, M.A. van Straten, M. Markuszewski, C.A. Cramers, H.A. Claessens, Molecular mechanism of retention in reversed-phase high-performance liquid chromatography and classification of modern stationary phases by using quantitative structure–retention relationships, J. Chromatogr. A. 855 (1999) 455–486. doi:10.1016/S0021-9673(99)00742-6.

[33] K. Valkó, Application of high-performance liquid chromatography based measurements of lipophilicity to model biological distribution, J. Chromatogr. A. 1037 (2004) 299–310. doi:10.1016/j.chroma.2003.10.084.

[34] T. Baczek, R. Kaliszan, K. Novotná, P. Jandera, Comparative characteristics of HPLC columns based on quantitative structure-retention relationships (QSRR) and hydrophobic-subtraction model, J. Chromatogr. A. 1075 (2005) 109–115. doi:10.1016/j.chroma.2005.03.117.

[35] T. Baczek, R. Kaliszan, Predictive approaches to gradient retention based on analyte structural descriptors from calculation chemistry, J. Chromatogr. A. 987 (2003) 29–37. doi:10.1016/S0021-9673(02)01701-6.

[36] A. Pagliara, E. Khamis, A. Trinh, P.-A. Carrupt, R.-S. Tsai, B. Testa, Structural Properties Governing Retention Mechanisms on RP-HPLC Stationary Phases Used for Lipophilicity Measurements, J. Liq. Chromatogr. 18 (1995) 1721–1745.

622 doi:10.1080/10826079508010002.

623 [37] E.H. Kerns, High throughput physicochemical profiling for drug discovery, J.
624 Pharm. Sci. 90 (2001) 1838–1858. doi:10.1002/jps.1134.

625 [38] S.F. Donovan, M.C. Pescatore, Method for measuring the logarithm of the
626 octanol-water partition coefficient by using short octadecyl-poly(vinyl alcohol)
627 high-performance liquid chromatography columns, J. Chromatogr. A. 952 (2002)
628 47–61. doi:10.1016/S0021-9673(02)00064-X.

629 [39] S. Pous-Torres, J.R. Torres-Lapasió, J.J. Baeza-Baeza, M.C. García-Álvarez-
630 Coque, Combined effect of solvent content, temperature and pH on the
631 chromatographic behaviour of ionisable compounds, J. Chromatogr. A. 1163
632 (2007) 49–62. doi:10.1016/j.chroma.2007.06.004.

633 [40] P. Nikitas, A. Pappa-Louisi, Retention models for isocratic and gradient elution in
634 reversed-phase liquid chromatography, J. Chromatogr. A. 1216 (2009) 1737–
635 1755. doi:10.1016/j.chroma.2008.09.051.

636 [41] P. Wiczling, Analyzing chromatographic data using multilevel modeling, Anal.
637 Bioanal. Chem. 410 (2018) 3905–3915. doi:10.1007/s00216-018-1061-3.

638 [42] Bio-Loom, 2017. BioByte Corp. Claremont, CA, USA. www.biobyte.com, (n.d.).

639 [43] ACD/Percepta, www.acdlabs.com, Advanced Chemistry Development, Inc.,
640 Release (Build 2726, 5 359 May 2014), (n.d.).

641 [44] H. Wan, A.G. Holmén, Y. Wang, W. Lindberg, M. Englund, M.B. Någård, R.A.
642 Thompson, High-throughput screening of pKa values of pharmaceuticals by
643 pressure-assisted capillary electrophoresis and mass spectrometry, Rapid
644 Commun. Mass Spectrom. 17 (2003) 2639–2648. doi:10.1002/rcm.1229.

[45] J.M. Cabot, E. Fuguet, M. Rosés, Determination of acidity constants of sparingly soluble drugs in aqueous solution by the internal standard capillary electrophoresis method, Electrophoresis. 35 (2014) 3564–3569. doi:10.1002/elps.201400353.

[46] A. Port, M. Bordas, R. Enrech, R. Pascual, M. Rosés, C. Ràfols, X. Subirats, E. Bosch, Critical comparison of shake-flask, potentiometric and chromatographic methods for lipophilicity evaluation (log Po/w) of neutral, acidic, basic, amphoteric, and zwitterionic drugs, Eur. J. Pharm. Sci. 122 (2018) 331–340. doi:10.1016/j.ejps.2018.07.010.

[47] F. Rived, M. Rosés, E. Bosch, Dissociation constants of neutral and charged acids in methyl alcohol. The acid strength resolution, Anal. Chim. Acta. 374 (1998) 309–324. doi:10.1016/S0003-2670(98)00418-8.

[48] J.M. Cabot, E. Fuguet, C. Ràfols, M. Rosés, Determination of acidity constants by the capillary electrophoresis internal standard method. IV. Polyprotic compounds, J. Chromatogr. A. 1279 (2013) 108–116. doi:10.1016/j.chroma.2013.01.018.

FIGURE CAPTIONS

Figure 1. Variation of the retention time of neutral and ionic hold-up time markers with the pH of the mobile phase. (♦) DMSO, (▲) KBr, (■) KI.

Figure 2. Retention time vs mobile phase pH profiles of some representative monoprotic acids. **A (phenols):** (■) Thymol, (●) 2,4-Dichlorophenol, (▲) 3-Methylphenol, (♦) 4-Chloro-3-methylphenol, (□) Estrone, (○) 4-Nitrophenol, (×) 4-Hydroxybenzyl alcohol. **B (carboxylic acis):** (■) Ibuprofen, (●) Flurbiprofen, (▲) Ketorolac, (♦) Naproxen, (○) Aspirin, (×) Salycilic acid. **C (other acids):** (■) Phenobarbital, (●) Barbital, (▲) 5-Fluorouracil, (♦) Warfarin.

Figure 3. Retention time vs mobile phase pH profiles of some representative monoprotic bases. **A (pyridines):** (■) Isoquinoline, (●) Benzyl nicotinate, (▲) Pyridine. **B (amines):** (■) N,N-Dimethylaniline, (●) Fentanyl, (▲) o-Phenylenediamine, (♦) Sufentanyl, (×) Tramadol.

Figure 4. Retention time vs mobile phase pH profiles of diprotic drugs. **A (diprotic acids and amphoteric drugs):** (■) 2-Amino-4-nitrophenol, (●) 4-Hydroxyphenylacetic acid, (▲) 4-Amino-2-nitrophenol, (♦) Morphine, (×) Piroxicam. **B (diprotic bases):** (■) Nicotine, (●) Chlorpheniramine, (▲) p-Phenylenediamine, (♦) Ranitidine.

Figure 5. pK_a values in mobile phase ($^{s}_{w}pK_a$) vs literature ones in water ($^{w}_{w}pK_a$). Acids: (●) phenols, (■) carboxylic acids, (×) others, (○) phenols with estimated $^{w}_{w}pK_a$ values, (□) carboxylic acids with estimated $^{w}_{w}pK_a$ values. Bases: (♦) pyridines, (▲) amines, (◊) pyridines with estimated $^{w}_{w}pK_a$ values, (△) amines with estimated $^{w}_{w}pK_a$ values.

688 **Figure 6.** Relationships between chromatographic retention and octanol/water partition
689 for the neutral forms of the different acid-base compounds. **A:** Retention factor vs
690 octanol/water partition coefficient. **B:** Retention factor vs octanol/water partition
691 coefficient corrected by the hydrogen bond acidity of the solute. Symbols: (●) phenols,
692 (■) carboxylic acids, (×) other acids, (♦) pyridines, (▲) amines, (*) diprotic drugs.
693
694 **Figure 7.** Retention of monocharged ions vs retention times of the corresponding neutral
695 forms. Symbols as in Figure 6.

TABLES

Table 1. pH values in water ($^{w}_{w}$pH) and pH values in the mobile phase of 40% v/v ACN ($^{s}_{w}$pH) of the used buffers and retention times of ionic (KBr, KI) and neutral (DMSO) hold-up time markers.

	$^{w}_{w}$pH	$^{s}_{w}$pH	t_M (KBr)	t_M (KI)	t_M (DMSO)
NaH_2PO_4 50mM	1.83	2.16	0.801	-	-
HCOOH 10mM	2.96	3.55	0.830	0.835	0.828
CH_3COOH 10mM	5.09	5.86	0.732	0.764	0.821
$H_2NCH_2CH_2NH_2$ 10mM	6.98	6.84	0.750	0.758	0.832
NH_3 10mM	9.05	8.91	0.706	0.719	0.840
$H_2NCH_2CH_2NH_2$ 10mM	11.03	10.84	0.658	0.636	0.820
Average			0.746 ± 0.06	0.742 ± 0.07	0.828 ± 0.01

Table 2. Parameters (±sd) and statistics obtained in the fits of the retention time of monoprotic acids to mobile phase pH.

MONOPROTIC ACIDS	Fitting parameters			Statistics			Physico-chemical parameters		
	$t_{R_{HA}}$	$t_{R_{A^-}}$	${}^{s}_{w}pK_a$	R^2	SD	F	${}^{w}_{w}pK_a$	log $P_{o/w}$ [a]	A [b]
Phenols									
2,4-Dichlorophenol	3.15±0.02	0.67±0.03	9.19±0.03	0.999	0.03	2593	7.89[b]	3.06	0.53
2-Chlorophenol	1.92±0.01	0.63±0.06	10.01±0.13	0.999	0.02	1159	8.48[b]	2.15	0.32
2-Isopropyl-5-Methylphenol (Thymol)	5.41±0.02	0.65	11.73±0.05	0.952	0.05	80	10.50[b]	3.30	0.52
2-Naphtol	2.49±0.01	0.65	10.73±0.03	0.996	0.03	1037	9.57[b]	2.70	0.61
2-Nitrophenol	2.24±0.01	0.66±0.02	8.41±0.04	0.999	0.02	3080	7.23[b]	1.79	0.05
3-Methylphenol (m-Cresol)	1.74±0.01	0.65	11.24±0.03	0.992	0.01	478	10.00[c]	1.96	0.57
3-Nitrophenol	1.70±0.01	0.64±0.02	9.57±0.06	0.999	0.02	1327	8.35[b]	2.00	0.79
4-Bromophenol	2.29±0.01	0.65	10.33±0.04	0.997	0.03	1559	9.35[b]	2.59	0.67
4-Chloro-3-methylphenol	2.73±0.01	0.65	10.57±0.03	0.997	0.03	1484	9.27[b]	3.10	0.67
4-Chlorophenol	2.08±0.01	0.65	10.38±0.05	0.996	0.03	1088	9.38[b]	2.39	0.67
4-Ethylphenol	2.34±0.01	0.65	11.27±0.04	0.979	0.03	184	10.20[b]	2.47	0.55
4-Hydroxybenzyl alcohol	0.95±0.01	0.65	10.95±0.11	0.916	0.02	43	9.82[a]	0.25	0.86
4-Hydroxyphenylacetamide	0.94±0.01	0.65	10.74±0.12	0.932	0.02	55	9.99[d]	-0.09	0.86
4-Methylphenol (p-Cresol)	1.73±0.01	0.65	11.34±0.03	0.989	0.01	357	10.26[b]	1.94	0.57
4-Nitrophenol	1.60±0.01	0.66±0.03	8.52±0.08	0.997	0.03	562	7.15[b]	1.91	0.82
Capsaicin	5.61±0.03	0.65	10.93±0.02	0.997	0.06	1145	9.76[d]	3.04[b]	0.53[d]
Catechol	1.11±0.01	0.65	10.51±0.09	0.982	0.02	214	9.45[b]	0.88	0.88
Estradiol	3.02±0.02	0.65	11.37±0.04	0.976	0.04	161	10.27[d]	4.01	0.86
Estriol	1.22±0.01	0.65	11.49±0.08	0.921	0.01	47	10.25[d]	2.54	1.06
Estrone	4.04±0.02	0.65	11.28±0.04	0.983	0.05	228	10.25[d]	3.13	0.50
Methyl 4-hydroxybenzoate	1.44±0.01	0.68±0.02	9.65±0.06	0.999	0.01	1336	8.37[a]	1.96	0.69
Phenol	1.41±0.01	0.65	11.09±0.04	0.989	0.01	320	9.98[c]	1.47	0.60
Resorcinol	1.02±0.01	0.65	10.67±0.05	0.990	0.01	410	9.81[c]	0.80	1.09

Table 2. Continued

MONOPROTIC ACIDS	Fitting parameters			Statistics			Physico-chemical parameters		
	$t_{R_{HA}}$	$t_{R_{A^-}}$	${}^{s}_{w}pK_a$	R^2	SD	F	${}^{w}_{w}pK_a$	log $P_{o/w}$ [a]	A [b]
Carboxylic acids									
2-Hydroxybenzoic acid (Salicylic acid)	1.61±0.07	0.73±0.03	3.85±0.20	0.981	0.07	79	2.98[a]	2.26	0.71
Acetylsalicylic acid (Aspirin)	1.27±0.04	0.68±0.04	5.31±0.29	0.976	0.06	62	3.48[a]	1.19	0.49
Benzoic acid	1.38±0.05	0.69±0.04	5.40±0.24	0.979	0.06	69	4.20[a]	1.87	0.59
Diclofenac	7.36±0.09	0.98±0.07	5.34±0.05	0.999	0.12	1700	4.21[e]	4.50	0.63
Flurbiprofen	5.89±0.11	0.80±0.09	5.53±0.07	0.998	0.15	645	4.19[f]	4.16	0.57[d]
Ibuprofen	7.51±0.11	0.89±0.10	5.84±0.05	0.999	0.15	1034	4.43[e]	3.50	0.59
Indomethacin	7.45±0.09	0.99±0.08	5.46±0.04	0.999	0.13	1507	4.15[e]	4.27	0.57
Ketoprofen	3.08±0.08	0.77±0.07	5.57±0.11	0.994	0.11	244	4.29[a]	3.12	0.55
Ketorolac	2.07±0.07	0.76±0.06	5.15±0.28	0.986	0.1	108	3.50[b]	1.68	0.65
Naproxen	3.19±0.08	0.75±0.07	5.77±0.10	0.994	0.12	242	4.28[g]	3.34	0.60
Others									
5,5-Diethylbarbituric acid (Barbital)	1.05±0.01	0.64±0.01	9.40±0.06	0.998	0.01	728	7.97[b]	0.65	0.47
5-Ethyl-5-phenylbarbituric acid (Phenobarbital)	1.35±0.01	0.63±0.01	8.85±0.04	0.999	0.01	1454	7.44[b]	1.47	0.73
5-Fluorouracil	0.85±0.01	0.62±0.03	9.18±0.23	0.959	0.02	35	7.86[b]	-0.89	0.57
Warfarin	4.40±0.10	0.76±0.09	5.91±0.07	0.996	0.14	402	5.01[g]	2.70	0.35

[a] From reference [42]; [b] From reference [43]; [c] From reference [30]; [d] Estimated values from reference [43]; [e] From reference [44]; [f] From reference [45]; [g] From reference [46]

Table 3. Parameters (±sd) and statistics obtained in the fits of the retention time of monoprotic bases to mobile phase pH.

MONOPROTIC BASES	Fitting parameters			Statistics			Physico-chemical Parameters		
	$t_{R_{HA^+}}$	t_{R_A}	${}^{s}_{w}pK_a$	R^2	SD	F	${}^{w}_{w}pK_a$	log $P_{o/w}$ [a]	A [b]
Pyridines									
Benzyl nicotinate	0.83	3.15±0.02	2.14±0.03	0.993	0.04	588	3.16[c]	2.40	0.00
Isoquinoline	0.80±0.02	1.80±0.01	3.79±0.05	0.998	0.02	922	5.36[b]	2.08	0.00
Pyridine	0.77±0.01	1.12±0.003	3.70±0.04	0.999	0.01	1190	5.16[b]	0.65	0.00
Amines									
2-Nitro-p-phenylenediamine	0.80±0.01	1.14±0.01	3.42±0.08	0.995	0.01	302	4.36[c]	0.53	0.35
2-Toluidine	0.80±0.03	1.71±0.01	3.43±0.05	0.998	0.02	678	4.45[a]	1.32	0.23
Aminopyrine	0.81±0.01	1.32±0.01	4.10±0.08	0.998	0.01	910	5.00[a]	0.80	0.00
Aniline	0.79±0.02	1.40±0.01	3.54±0.05	0.998	0.02	636	4.60[d]	0.90	0.26
Atropine	1.00±0.05	2.98±0.09	8.24±0.17	0.994	0.09	267	9.60[b]	1.83	0.26
Codeine	0.93±0.07	1.54±0.08	7.19±0.43	0.918	0.11	17	8.21[a]	1.19	0.33
Diethylcarbamazine	0.89±0.05	1.27±0.06	6.93±0.4	0.902	0.08	14	7.15[c]	1.62[e]	0.00
Ephedrine	0.88±0.03	2.05±0.04	7.68±0.21	0.994	0.05	268	9.71[b]	0.93	0.21
Fentanyl	1.57±0.22	9.95±0.27	7.40±0.13	0.995	0.36	312	8.43[b]	3.89	0.00
Lidocaine	1.04±0.07	4.53±0.08	7.15±0.07	0.998	0.11	616	7.96[f]	2.21	0.12
N,N-dimethylaniline	0.81±0.04	4.04±0.02	4.04±0.04	0.999	0.04	3895	5.07[b]	2.31	0.00
o-Phenylenediamine	0.79±0.01	1.03±0.003	3.59±0.06	0.997	0.01	589	4.80[a]	0.15	0.24
Oxycodone	0.91±0.05	2.51±0.07	7.56±0.20	0.992	0.09	198	7.60[c]	1.01	0.23[c]
Propanolol	1.27±0.13	5.55±0.17	7.58±0.19	0.994	0.22	230	9.57[f]	2.98	0.17
Scopolamine	0.91±0.05	1.29±0.05	6.92±0.40	0.904	0.08	14	7.55[b]	0.55	0.30
Sufentanyl	2.15±0.41	16.52±0.48	7.19±0.11	0.995	0.66	273	8.01[a]	3.95	0.00
Tramadol	1.15±0.10	4.87±0.20	8.48±0.15	0.991	0.2	173	9.37[b]	2.63	0.31[c]

[a] From reference [42]; [b] From reference [43]; [c] Estimated values from reference [43]; [d] From reference [47]; [e] Estimated values from reference [42]; [f] From reference [44]

Table 4. Parameters (±sd) and statistics obtained in the fits of the retention time of diprotic compounds to mobile phase pH. z is the charge of the most dissociated form of the acid-base drug.

DIPROTIC SOLUTES	z	Fitting parameters					Statistics			Physico-chemical parameters			
		$t_{R_{H_2A^{z+2}}}$	$t_{R_{HA^{z+1}}}$	$t_{R_{A^z}}$	${}^{s}_{w}pK_{a_1}$	${}^{s}_{w}pK_{a_2}$	R^2	SD	F	${}^{w}_{w}pK_{a_1}$	${}^{w}_{w}pK_{a_2}$	log $P_{o/w}$ [a]	A [b]
4-Hydroxyphenylacetic acid	-2	**1.01±0.01**	0.77±0.01	0.63±0.01	4.91±0.21	8.99±0.12	0.999	0.01	540	4.50[c]	10.19[c]	0.75	0.97
2-Amino-4-nitrophenol	-1	1.10±0.10	**1.32±0.05**	0.64±0.07	3.07±0.99	8.44±0.30	0.989	0.07	22	2.62[c]	6.82[c]	1.53	1.01
4-Amino-2-nitrophenol	-1	0.83±0.05	**1.39±0.02**	0.63±0.02	2.82±0.20	9.29±0.07	0.999	0.02	223	3.60[b]	7.59[b]	0.96	0.30
Morphine	-1	0.86±0.04	**1.26±0.08**	0.65	7.53±0.61	10.30±0.30	0.933	0.06	9	8.18[b]	9.26[b]	0.89	0.50
Piroxicam	-1	0.83	**2.14±0.15**	0.76±0.08	1.57±0.37	5.40±0.26	0.981	0.13	34	2.33[b]	5.07[b]	1.78	0.55
Chloropheniramine	0	0.83	1.50±0.31	**7.60±0.31**	2.61±1.25	7.79±0.33	0.994	0.37	114	3.64[d]	9.27[d]	3.17	0.00
Nicotine	0	0.83	0.97±0.12	**1.45±0.08**	2.87±2.15	7.58±1.05	0.938	0.11	10	3.13[e]	8.24[e]	1.17	0.00
p-Phenylenediamine	0	0.83	0.90±0.08	**0.93±0.04**	3.69±1.60	6.91±4.66	0.731	0.06	2	2.89[b]	6.16[b]	-0.30	0.31
Ranitidine	0	0.83	0.91±0.09	**1.17±0.07**	2.90±2.98	7.52±1.43	0.879	0.09	5	2.18[f]	8.38[f]	1.03	0.25

[a] From reference [42]; [b] From reference [43]; [c] Estimated values from reference [43]; [d] From reference [48]; [e] From reference [44]; [f] From reference [46]

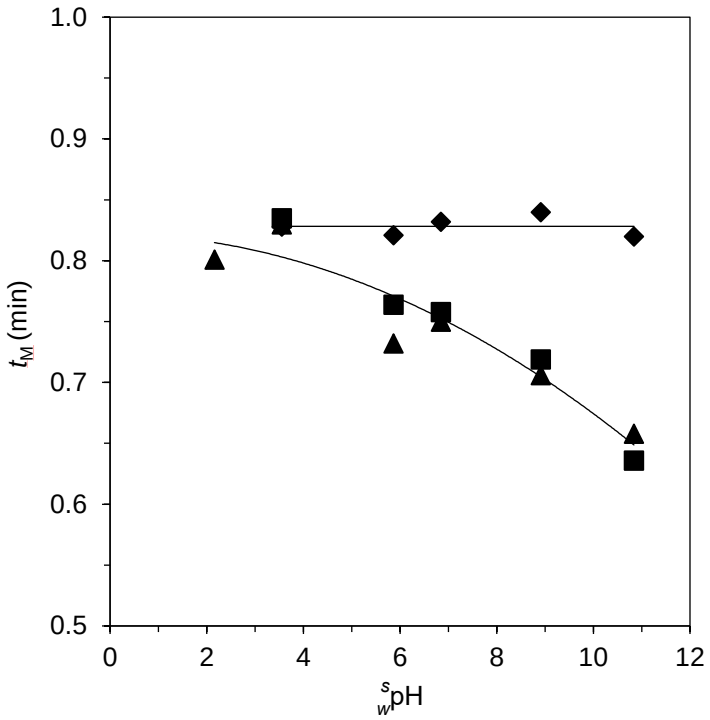
The retention time of the neutral form is highlighted in bold

Table 5. Parameters (±sd) and statistics obtained in the fits of the retention time to mobile phase pH of acids and bases with pK_a values too low or too high to estimate the retention of the ion from the fitting. Retention of the ion was estimated from correlation (11) or (12).

		Fitting parameters					Statistics		
	z	$t_{R_{H_2A^{z+2}}}$	$t_{R_{HA^{z+1}}}$	$t_{R_{A^z}}$	${}^{s}_{w}pK_{a_1}$	${}^{s}_{w}pK_{a_2}$	R^2	SD	F
Morphine	-1	0.86±0.04	1.26±0.08	0.66	7.53±0.61	10.29±0.30	0.933	0.06	9
Piroxicam	-1	0.92	2.14±0.15	0.76±0.08	1.61±0.38	5.40±0.26	0.981	0.13	34
2-Isopropyl-5-Methylphenol (Thymol)	-1	-	5.41±0.02	0.84	11.71±0.05	-	0.952	0.06	80
2-Naphtol	-1	-	2.49±0.01	0.71	10.70±0.03	-	0.996	0.03	1016
3-Methylphenol (m-Cresol)	-1	-	1.74±0.01	0.68	11.23±0.03	-	0.992	0.01	478
4-Bromophenol	-1	-	2.29±0.01	0.71	10.27±0.05	-	0.997	0.03	1357
4-Chloro-3-methylphenol	-1	-	2.73±0.01	0.72	10.52±0.03	-	0.997	0.03	1413
4-Chlorophenol	-1	-	2.08±0.01	0.70	10.34±0.05	-	0.996	0.03	995
4-Ethylphenol	-1	-	2.34±0.01	0.71	11.25±0.04	-	0.979	0.03	184
4-Hydroxybenzyl alcohol	-1	-	0.95±0.01	0.65	10.98±0.11	-	0.916	0.02	43
4-Hydroxyphenylacetamide	-1	-	0.94±0.01	0.65	10.77±0.12	-	0.933	0.02	55
4-Methylphenol (p-Cresol)	-1	-	1.73±0.01	0.68	11.33±0.03	-	0.989	0.01	357
Capsaicin	-1	-	5.61±0.03	0.85	10.90±0.02	-	0.996	0.06	1132
Catechol	-1	-	1.11±0.01	0.65	10.52±0.08	-	0.982	0.02	216
Estradiol	-1	-	3.02±0.02	0.74	11.35±0.04	-	0.976	0.04	161
Estriol	-1	-	1.22±0.01	0.66	11.49±0.08	-	0.921	0.01	47
Estrone	-1	-	4.04±0.02	0.78	11.26±0.04	-	0.983	0.05	228
Phenol	-1	-	1.41±0.01	0.67	11.09±0.04	-	0.988	0.01	320
Resorcinol	-1	-	1.02±0.01	0.65	10.69±0.05	-	0.990	0.01	413
Benzyl nicotinate	0	-	1.01	3.15±0.02	2.21±0.04	-	0.993	0.04	595

722 **Figure 1.**

723



724

725

726

Figure 2.

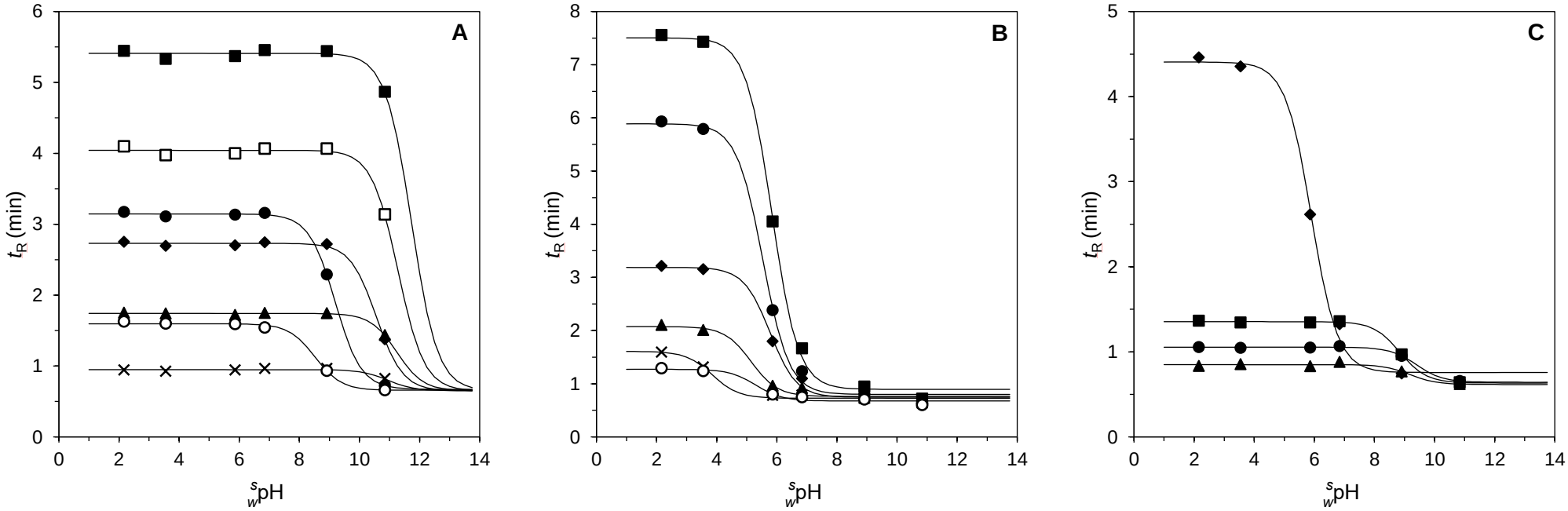
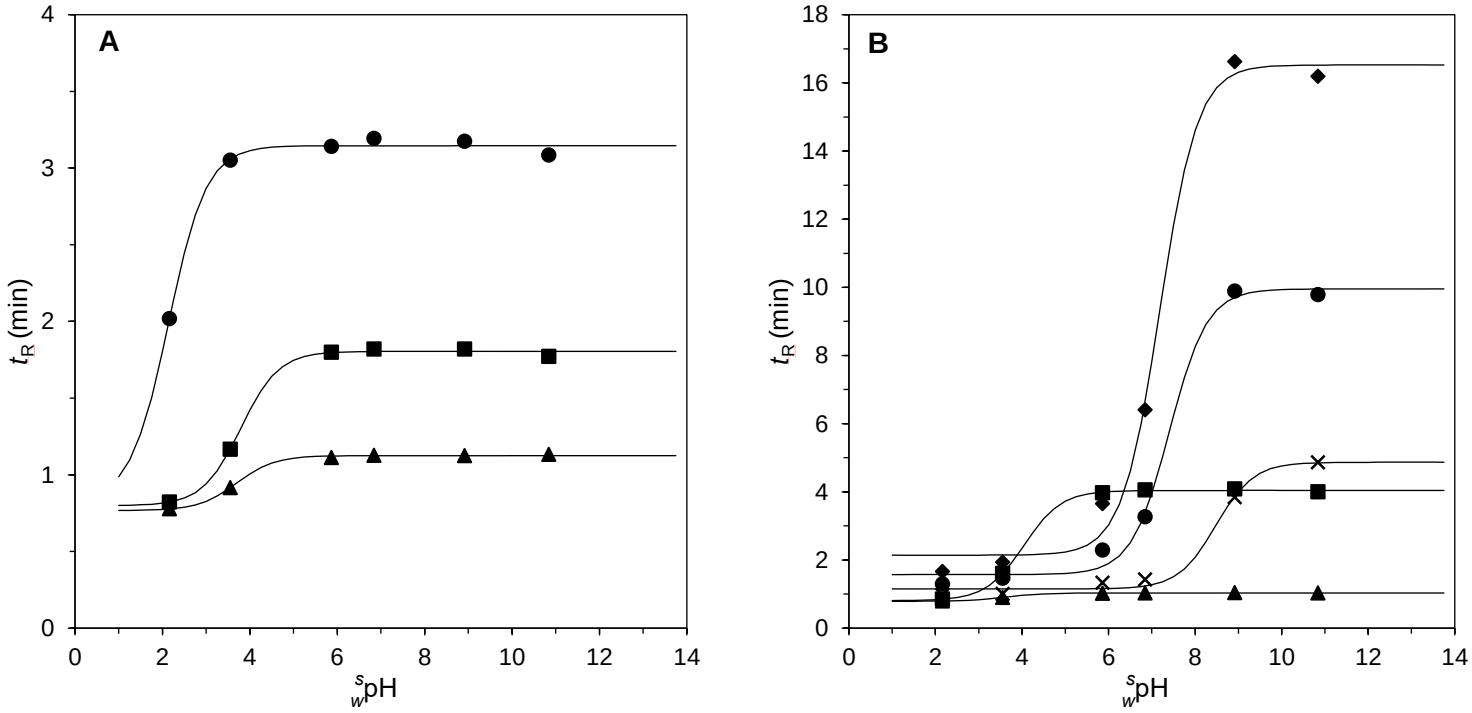
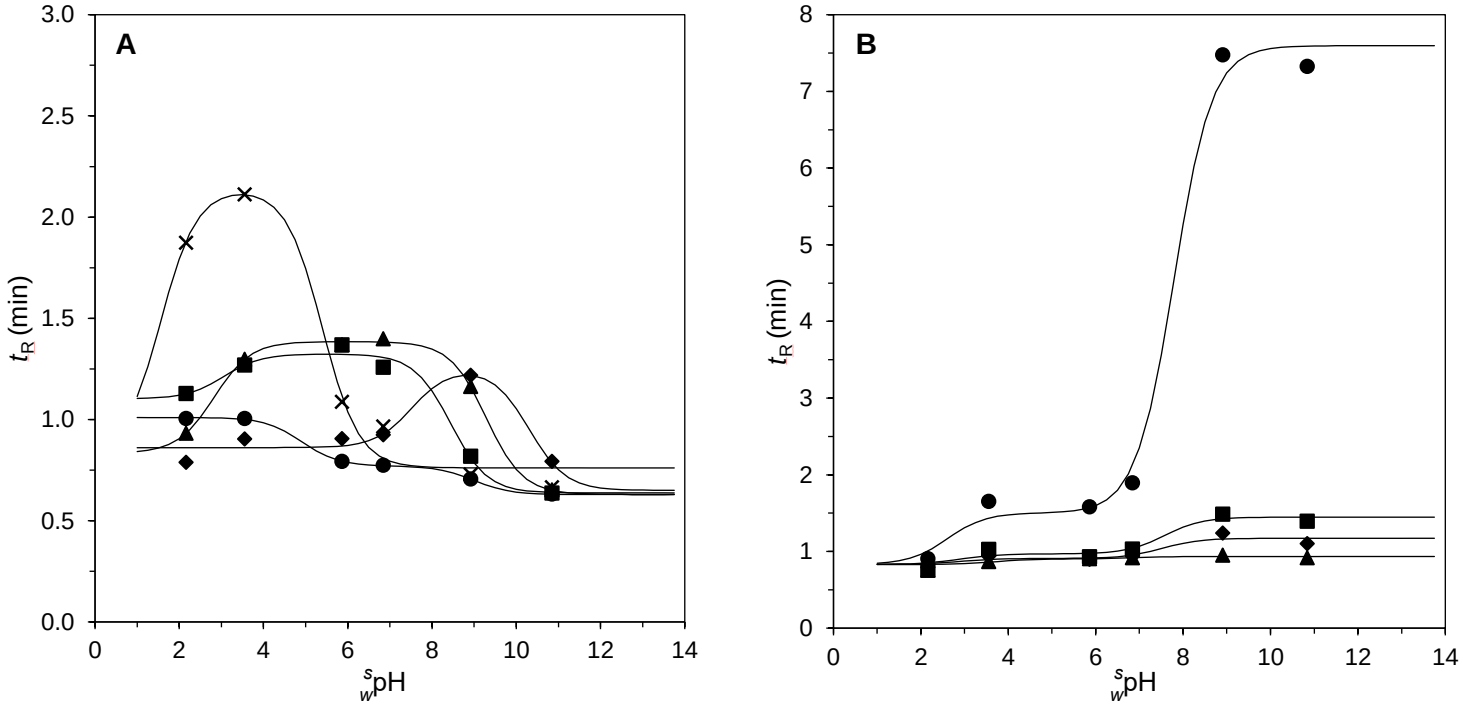


Figure 3.



740 **Figure 4.**

741



742

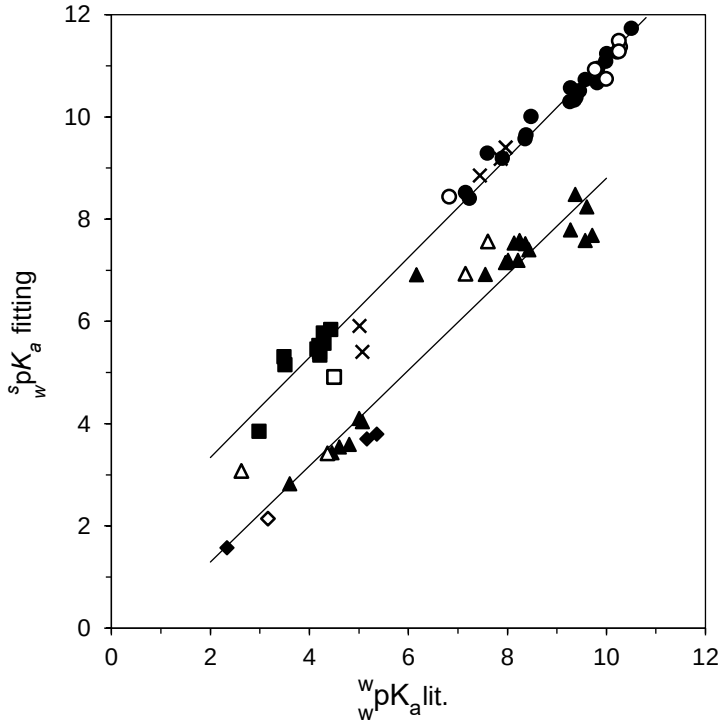
743

744

745

746 **Figure 5.**

747



748

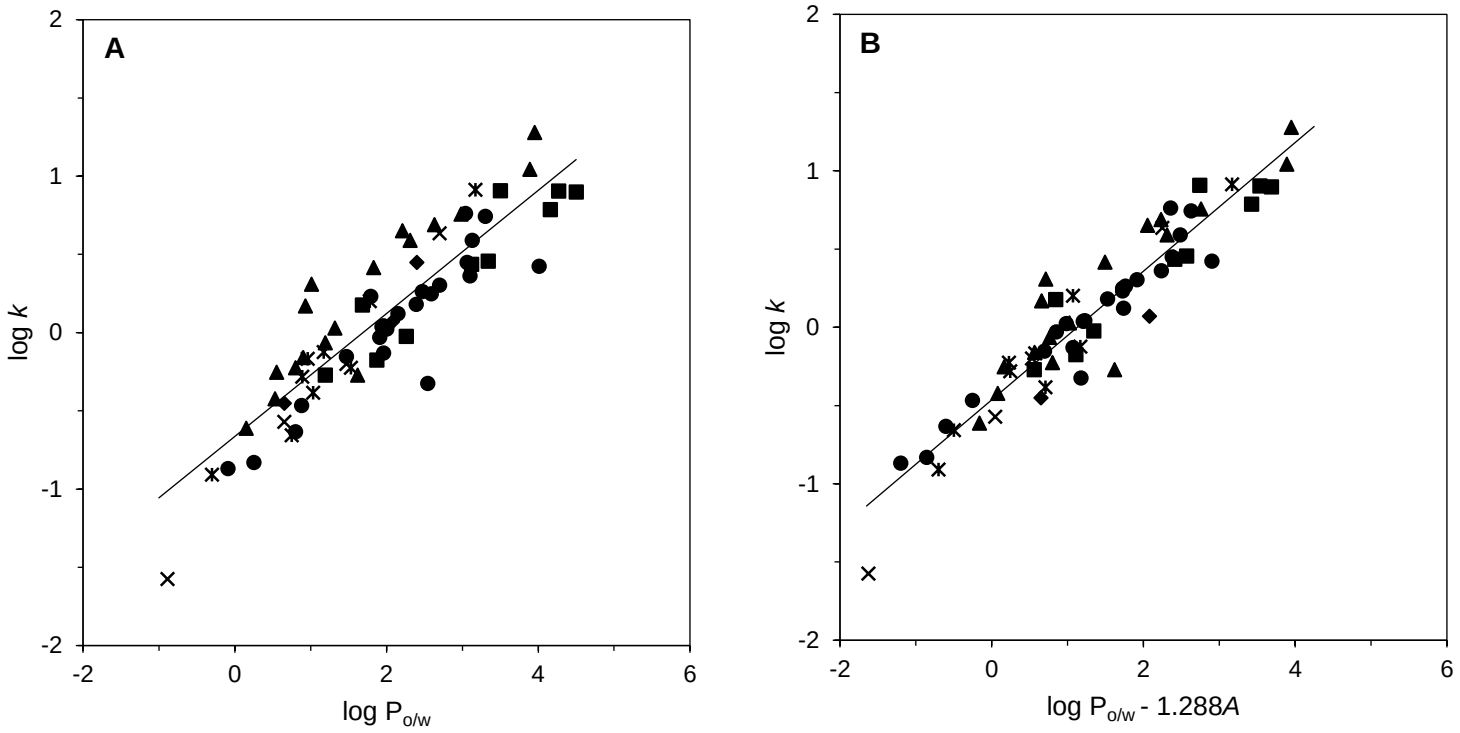
749

750

751

752 **Figure 6.**

753



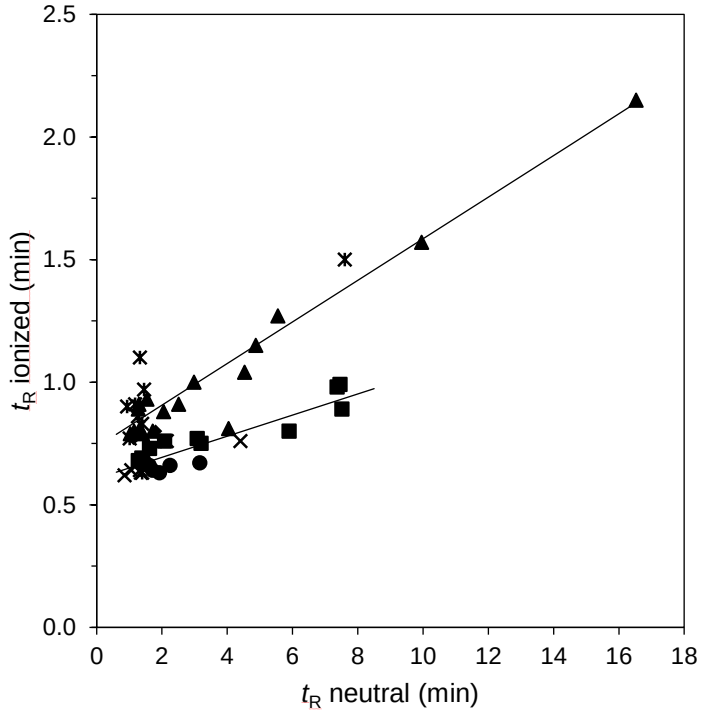
754

755

756

757

Figure 7.


2.5
2.0
1.5
1.0
0.5
0.0
t_R ionized (min)
0
2
4
6
8
10
12
14
16
18
t_R neutral (min)