Determination of the retention factor of ionizable compounds in microemulsion electrokinetic chromatography

Alejandro Fernández-Pumarega[1], Susana Amézqueta[1], Elisabet Fuguet[1,2,*], Martí Rosés[1].

[1] Departament d'Enginyeria Química i Química Analítica and Institut de Biomedicina (IBUB), Facultat de Química, Universitat de Barcelona, Martí i Franquès 1-11, 08028, Barcelona, Spain.

[2] Serra Húnter Programme. Generalitat de Catalunya. Spain

* Corresponding author:

E-mail: elifuguetj@ub.edu

Departament d'Enginyeria Química i Química Analítica and Institut de Biomedicina, Facultat de Química, Universitat de Barcelona

c/ Martí i Franquès 1-11, 08028, Barcelona, Spain

Phone: (+34) 934039119

Fax: (+34) 934021233

Abstract

Determination of the retention factor of ionized compounds in microemulsion electrokinetic chromatography requires two mobility measurements at the same pH: one in the presence of the microemulsion and another in plain buffer. However, it has been observed that in some cases subtracting one mobility from another determined in a different medium leads to negative retention factors, which makes no sense from a chemical point of view. This indicates that there is some error in the process which has a direct impact when retention factors are used for further applications.

Here, we evaluate how the components of the microemulsion confer different properties to the buffer medium, particularly varying the viscosity parameter (which is inversely related to mobility). Whereas sodium dodecyl sulfate, the surfactant used in the microemulsion, has little effect on the medium viscosity (only an increase of 5%-6%), the presence of 1-butanol, used as a stabilizer, increases it by around 30%. Meanwhile, heptane, which is used as an oil, provokes a slight decrease. Consequently, the mobilities obtained in the microemulsion system are shifted to higher values (less negative mobilities) compared to mobilities obtained in the aqueous buffer, and so one cannot be directly subtracted from the other. Since the microemulsion-buffer medium cannot be directly reproduced, we propose a correction that takes into account the variation of viscosities. This is determined from the electrophoretic mobility of the benzoate ion. As this ion does not interact with the microemulsion, the ratio of its mobilities (measured in plain buffer and microemulsion) is equivalent to the ratio of viscosities, and can be used as the correction factor for other measurements. Thus, mobilities in buffer and microemulsion media are placed on the same scale, overcoming the errors in retention factor determination.

Abbreviations

CMC: critical micelle concentration

CZE: capillary zone electrophoresis

DMSO: dimethyl sulfoxide

EOF: electroosmotic flow

F: Fisher's F parameter

I: ionic strength

k: retention factor

λ: wavelength

ME: microemulsion

MEKC: micellar electrokinetic chromatography

MEEKC: microemulsion electrokinetic chromatography

R^2: determination coefficient

SD: standard deviation

SDS: sodium dodecyl sulfate

UV-vis: ultraviolet-visible

1. Introduction

Capillary electrophoresis is a widely used technique that separates different solutes depending on their charge/size ratio. Although this technique cannot separate non-charged compounds, over the last few decades other modalities of the technique that can separate neutral compounds, such as micellar electrokinetic chromatography (MEKC) [1,2] and microemulsion electrokinetic chromatography (MEEKC) [3], have been developed. In these latter techniques, the solutes become distributed between an aqueous buffer and a pseudo-stationary phase. In MEEKC, the pseudo-stationary phase is a microemulsion (ME) formed by an ionic surfactant, a cosurfactant, and an oil that are mixed together in an aqueous solution at specific concentrations. The surfactant and the cosurfactant act as stabilizers, reducing the surface tension that exists between the oil droplets and water, and allowing the creation of the ME [4]. MEEKC has been used in different applications over recent years, as a separation technique for highly hydrophobic compounds [5–7], or as a method to predict biopartitioning properties, such as lipophilicity, which can be estimated from the retention factor of compounds in the ME media [8–11], among others.

The determination of the retention factor (k) of neutral substances is not a complex issue in MEEKC, as the migration of the compound is affected only by its partition between the buffer and the charged ME. For neutral solutes, k can be calculated from the mobilities of the compounds and the ME. However, the mobility of partly ionized compounds depends on the partition of the neutral form that is within the ME and also on the electrophoretic mobility of the charged forms [12,13].

In order to evaluate how ionized compounds are partitioned between the aqueous buffer and ME phase, the contribution of the electrophoretic mobility of the compound (i.e. the ionic mobility of the compound caused by the application of an electric field) must be

subtracted from the observed mobility. Therefore, two different analyses of the compounds are required: one under MEEKC conditions, in which observed mobility is measured; the other only in the plain buffer (capillary zone electrophoresis (CZE) mode), in which the electrophoretic mobility of the compound is measured [12]. In addition, the acid-base compound has to be equally ionized in both media, i.e. both solutions must be at the same pH.

The use of two different media can sometimes be an important handicap in the accurate determination of *k* values. For instance, some works report negative MEKC retention factors for ionizable compounds, especially for quite polar ones [14,15]. In a strict sense, the two media in which we determine the values that are subtracted one from the other should be the same, except for the presence of the ME. Therefore, some attempts have been made to emulate the aqueous composition of the solutions that contain micelles (MEKC) or microemulsions (MEEKC). Muijselaar *et al.* [16] pointed out an increase in the absolute mobility value of ionized acids when surfactant monomers below the critical micelle concentration (CMC) were present in the CZE buffer, compared to the value obtained just in plain buffer. This difference was greater for the most hydrophobic compounds. Other authors have proposed other approximations, such as adding sodium chloride to the CZE buffer in order to compensate for the difference in ion composition between solutions [17], or adding the cosurfactant (1-butanol) to the buffer used in CZE measurements [18,19]. The addition of the cosurfactant produced important differences between the mobility values obtained with or without it, although the reason for these differences has not been systematically studied. Taking into account that the retention factor of a substance in a given system is often used to estimate other of its properties, such as $\log P_{o/w}$ [8–11] or biopartitioning parameters [20–24], it is very important to ensure it is determined correctly.

In the present work we evaluate the effect of the medium on the electrophoretic mobility of ionizable compounds, which in turn is directly related to the retention factors obtained. To this end, we determine the retention factor vs. pH profiles of six monoprotic acids selected as test compounds. Then, the influence of the different components of the ME on the electrophoretic mobilities used to calculate the retention factors is evaluated, and finally we propose a correction of the medium effect.

2. Theory

Due to the similarity between the retention mechanisms involved, we indistinctly apply equations developed for MEKC [12] to MEEKC in the present study. The retention factor of an acid is defined as the weighted average of the retention factor of the ionized (A^-) and the neutral (HA) species (Eq. 1):

$$k = \alpha_{(\mathrm{HA})} k_{(HA)} + \alpha_{(\mathrm{A}-)} k_{(A-)} \qquad \text{Eq. 1}$$

where $k_{(\mathrm{HA})}$ and $k_{(\mathrm{A}^-)}$ are the retention factor of the fully protonated and the totally ionized forms of the acid, respectively, and $\alpha_{(\mathrm{HA})}$ and $\alpha_{(\mathrm{A}^-)}$ are their mole fractions. If the acidity constant of the compound is known, the mole fractions of both species can be calculated for any pH value using the following equations:

$$\alpha_{(\mathrm{HA})} = \frac{[\mathrm{H}^+]}{[\mathrm{H}^+]+K_a'} \qquad \text{Eq. 2}$$

$$\alpha_{(\mathrm{A}-)} = \frac{K_a'}{[\mathrm{H}^+]+K_a'} = 1 - \alpha_{(HA)} \qquad \text{Eq. 3}$$

where K_a' is the apparent acidity constant of the acid. Substituting Eqs. 2 and 3 into Eq. 1, and reorganizing terms, we obtain:

$$k = \frac{k_{(HA)} + k_{(A-)} \cdot 10^{\mathrm{pH}-\mathrm{p}K'_a}}{1 + 10^{\mathrm{pH}-\mathrm{p}K'_a}} \qquad \text{Eq. 4}$$

Eq. 4 relates the retention factor of a monoprotic acid with the pH of the media. This expression has been used by several authors [12,15,16] to model the retention behavior of ionizable acids in micellar systems. Nevertheless, there is a lack of studies based on the retention of ionizable compounds in ME-based systems.

As mentioned in the introduction, to calculate the retention factor of an acid, its electrophoretic mobility has to be subtracted from the overall observed mobility. Khaledi *et al.* [12] proposed an equation for MEKC in which the overall observed mobility of acidic compounds is expressed as a weighted average of their mobilities in the aqueous phase in absence of micelles and in the micellar phase. This equation can be adapted to MEEKC:

$$\mu = \left[\frac{k}{k+1}\right] \mu_{\mathrm{ME}} + \left[\frac{1}{k+1}\right] \mu_0 \qquad \text{Eq. 5}$$

where μ is the overall observed mobility, μ_{ME} the mobility of the ME phase, and μ_0 the mobility of the compound in an aqueous buffer without ME. Rearranging Eq. 5, the following expression is obtained:

$$k = \frac{\mu - \mu_0}{\mu_{\mathrm{ME}} - \mu} \qquad \text{Eq. 6}$$

The mobility of a compound can be calculated from its retention time as follows:

$$\mu = \left[\frac{1}{t_r} - \frac{1}{t_0}\right] \cdot \left[\frac{L_T L_D}{V}\right] \qquad \text{Eq. 7}$$

In this expression, t_r is the retention time of the compound of interest, t_0 the retention time of the electroosmotic flow (EOF) marker, L_T the total length of the capillary, L_D the effective length of the capillary, that is, the portion from the inlet to the detector, and V the voltage applied.

3. Experimental

3.1 Apparatus and conditions

A capillary electrophoresis system equipped with a diode array from Agilent Technologies (Santa Clara, CA, USA) was used to obtain the MEEKC measurements. The effective length of the capillary was 25 cm or 30 cm, depending on pH.

A GLP 22 pH-meter from Crison (Barcelona, Spain) was used to measure the pH of the buffer solutions.

For the analysis, fused-silica capillaries from Polymicro Technologies (Lisle, IL, USA) were used. The effective length of the capillaries was 25 cm (pH 2.0 and pH 3.0) or 30 cm (other pH values studied), with the total length of the capillaries being, respectively, 33.5 and 38.5 cm. Different conditions (pressure and voltage) were used at each pH in order to obtain the best possible electrophoretic window. The applied voltage ranged between 8 and 15 kV, and the pressure applied during separation between 0 and 50 mbar. In all cases, the temperature was set at 25ºC. The solutes were injected applying a pressure of 50 mbar for 5s, and detected at λ = 200, 214 or 254 nm (depending on the solute). A minimum of 3 replicate measurements were performed for each determination.

3.2 Reagents and materials

190 Sodium dihydrogen phosphate monohydrate (≥99%), dimethyl sulfoxide (DMSO,
191 ≥99.9%), hydrochloric acid (1 N Titrisol™), and sodium hydroxide (0.5 N Titrisol™)
192 were from Merck (Darmstadt, Germany). Methanol (HPLC grade) was obtained from
193 Thermo Fisher Scientific (Waltham, MA, USA). Sodium dodecyl sulfate (SDS, ≥99%),
194 1-butanol (≥99.7%), heptane (99%), sodium phosphate dodecahydrate (>98%), and
195 dodecanophenone (98%) were from Sigma-Aldrich (St. Louis, MO, USA). Disodium
196 hydrogen phosphate (99.5%) and sodium acetate anhydrous (99.6%) were from Baker
197 (Center Valley, PA, USA). Water was purified using a Milli-Q plus system from
198 Millipore (Bedford, MA, USA), with a resistivity of 18.2 MΩ cm.
199 The test compounds were ibuprofen (≥98%), 2,4,6-trichlorophenol (98%), ketoprofen
200 (≥98%), and naproxen (≥98%) from Sigma-Aldrich; benzoic acid (99.99%) from Baker;
201 and 3-bromobenzoic acid (98%) from Merck.
202
203 *3.3 Preparation of solutions*
204 *3.3.1 Buffer preparation*
205 Different buffer solutions in the pH range between 2.0 and 8.0 were prepared. Aliquots
206 of a 0.2 M sodium dihydrogen phosphate stock solution were adjusted with 1 M
207 hydrochloric acid to prepare the buffer solutions at pH 2.0 and pH 3.0. The pH 4.0 and
208 pH 5.0 buffers were prepared also by addition of 1 M hydrochloric acid to aliquots of a
209 0.2 M anhydrous sodium acetate stock solution. Finally, the other buffer solutions used
210 (pH 6.0, 7.0, and 8.0) were prepared by mixing different amounts of 0.2 M sodium
211 dihydrogen phosphate and 0.2 M disodium hydrogen phosphate stock solutions. A buffer
212 solution at pH 11.0 was prepared by mixing different amounts of 0.2 M disodium
213 hydrogen phosphate and 0.2 M sodium phosphate dodecahydrate. All the buffer solutions

were prepared maintaining the ionic strength (I) at 0.05 M. Table 1 shows the final concentration of individual buffer components.

Additionally, another full set of buffer solutions in which SDS was added at a concentration of 2 mM (just below the CMC) was prepared.

3.3.2 Microemulsion preparation

In the present work, the ME was composed of SDS (surfactant), 1-butanol (cosurfactant), and heptane (oil). The ME was prepared by first dissolving 1.30 g of SDS in 70 mL of the aqueous buffer. Then 8.15 mL of 1-butanol and 1.15 mL of heptane were added. The additions were performed at room temperature, employing a burette and under continuous magnetic stirring. If after stirring the ME remained turbid, it was sonicated until it clarified [8]. Finally, more buffer solution was added up to 100 mL (total final volume). The final concentrations of each component with respect to the total volume of the ME were: 1.30% w/v of SDS, 8.15% v/v of 1-butanol, and 1.15% v/v of heptane.

3.3.3 Sample preparation

For the MEEKC analysis, the test compounds were dissolved at a concentration of 200 mg L^{-1} in a microemulsion:methanol mixture (9:1, v:v). Similarly, in the CZE analysis, they were dissolved at 200 mg L^{-1} in a water:methanol mixture, also at a 9:1 (v:v) ratio. The ME marker was dodecanophenone (200 mg L^{-1}), and the EOF marker was DMSO (0.2% v/v) [25].

3.4 Data analysis

237 Retention profiles were adjusted with TableCurve 2D v5.01 from Systat Software Inc.
238 (San Jose, CA, USA). Data calculations were performed using Excel 2010 from Microsoft
239 (Redmond, WA, USA).
240 The pK_a and the logarithm of the octanol-water partition coefficient ($logP_{o/w}$) of the test
241 compounds were obtained from Bio-Loom database v1.7 from BioByte Corporation
242 (Claremont, CA, USA).
243
244 **4. Results and discussion**
245 We selected 6 compounds with different acidity and lipophilicity values to study their
246 behavior in MEEKC. They all have pK_a values in the electrophoretic working pH range
247 (2.0-12.0), and contain chromophore groups in their structure (in order to be detected by
248 UV-vis). Moreover, their different lipophilicity values allowed us to test their different
249 degrees of partition with the ME. The six compounds selected were: naproxen,
250 ketoprofen, and ibuprofen (non-steroidal anti-inflammatory drugs); 2,4,6-trichlorophenol
251 (a potentially polluting substance used as a fungicide, insecticide and preservative);
252 benzoic acid (an important chemical precursor); and 3-bromobenzoic acid (a derivate of
253 benzoic acid). Their physicochemical properties (pK_a and $logP_{o/w}$) are presented in Table
254 2 [26–38]. The most acidic of the compounds studied was 3-bromobenzoic acid, and
255 benzoic acid was the least lipophilic. Meanwhile, 2,4,6-trichlorophenol and ibuprofen
256 were the solutes with the highest $logP_{o/w}$ value, so they are supposed to be those that
257 interact most with the inner hydrophobic core of the ME.
258
259 4.1 Determination of the retention factor vs. pH profiles
260 The retention factor of the selected compounds was calculated at each pH value between
261 2.0 and 8.0. To this end, mobility was measured under MEEKC conditions, and also in

CZE using the 50 mM constant-ionic-strength buffers. Eq. 7 was used to obtain the mobility values from the migration time, and then the retention factor was calculated from these mobilities according to Eq. 6. Figure 1 shows the experimental *k* vs. pH profiles (circles), and also the fitting profile (dashed line). Table 3 shows the results of the fitting together with statistics (determination coefficient, R^2; Fisher's F parameter, F; and standard deviation, SD) of the fit. The *k* and pH values were the input data and pK_a', $k_{(A)}$, and $k_{(HA)}$ were obtained from the fit.

The profile was similar in all cases and, as expected, the neutral form of the compounds had a stronger interaction with the ME than the ionic form. The point of inflection of the curve corresponds to the pK_a' value. If the pK_a' values obtained (Table 3) are compared to those in Table 2, quite good agreement is observed. This indicates that the presence of the ME seems to have only a minor effect on the acidity of the compounds. As regards the interaction with the ME, the neutral form of ibuprofen and 2,4,6-trichlorophenol are those that show the greatest retention factors, whereas benzoic acid is the compound that shows a weakest interaction with the ME. This behavior is in agreement with the log $P_{o/w}$ values shown in Table 2, which indicates the correlation between retention in the ME system studied and the hydrophobicity of the compounds. Anyway, the results obtained for ibuprofen must be treated with caution, as this compound demonstrated a very strong interaction with the ME at pH values below 4.0, always co-eluting with the ME marker. As a consequence, *k* could not be determined experimentally at low pH values, and considerable extrapolation was necessary in the fit. Therefore, it is quite likely that the ibuprofen pK_a' and $k_{(HA)}$ values are not properly estimated and therefore present a high uncertainty.

The most notable fact derived from Figure 1 and Table 3 are the negative values obtained for some of the retention factors of the ionic form of the compounds; this cannot be

287 realistic but is also seen in other studies [14,15]. As explained in the introduction,
288 subtracting mobilities obtained in two different media to calculate *k* can influence the
289 value obtained if the two systems (one with and one without ME) are not really
290 equivalent.
291
292 4.2 Effect of SDS monomers on electrophoretic mobility
293 In MEEKC and MEKC the aqueous phase is saturated with SDS monomers, whereas the
294 CZE buffer solution is not. Some authors [16] point out that determination of the *k* value
295 in MEKC should take into account the presence of SDS monomers in the aqueous phase
296 in order to make them fully comparable. That is, the buffer for CZE analysis should also
297 contain the surfactant monomers at a concentration corresponding to that of the CMC.
298 Fuguet *et al.* [39] observed that the CMC of a surfactant is related to the concentration of
299 counter-ions (C) in the electrolyte used to prepare the electrophoretic buffer. The equation
300 obtained for SDS, with the sodium ion as counter-ions was:
301
302 $\log CMC = -3.230 - 0.486 \log C$ Eq. 8
303
304 The concentration of sodium present in the buffers in this study was never above 50 mM,
305 so the CMC, according to Eq. 8, cannot be lower than 2.5 mM. Thus, in order to test the
306 effect of the free monomers on the *k* values obtained, μ-pH profiles were performed with
307 plain buffer and using buffers with a concentration of SDS just below the CMC (2 mM)
308 to avoid the formation of micelles.
309 Similarly to Eq. 4 for *k*, μ–pH profiles can be fitted to Eq. 9:
310
311 $$\mu = \frac{\mu_{(HA)} + \mu_{(A-)} \cdot 10^{pH - pK'_a}}{1 + 10^{pH - pK'_a}}$$ Eq. 9

where, μ is the mobility of the acid at a specific pH value, and $\mu_{(HA)}$ and $\mu_{(A-)}$ are, respectively, the electrophoretic mobility of the neutral and the fully ionized acid. As $\mu_{(HA)}$ is referred to a neutral compound, its value is equal to 0, simplifying the equation to:

$$\mu = \frac{\mu_{(A-)} \cdot 10^{pH-pK'_a}}{1+10^{pH-pK'_a}} \qquad \text{Eq. 10}$$

Figure 2 shows the results for comparison. No differences are apparent between the experimental conditions since the mobilities are practically the same in both media, for all the compounds. Thus, the presence of monomers of SDS in the CZE buffer does not have a direct effect on *k* calculation, at least not for compounds such as those used in this study. Nonetheless, it becomes evident that some other phenomenon, mostly related with the different nature of the solutions used in MEEKC and CZE analysis, is present.

4.3 Evaluation of the different mobility contributions in retention factor determination

In order to understand the reason behind the negative retention factors obtained, we analyzed the different mobility values involved in the calculation of the parameter (Eq. 6). Figure 3 shows the variation of the mobilities for benzoic acid (the compound with the largest negative *k* values) with pH. Thus, three profiles are presented: benzoic acid in CZE (circles); benzoic acid in MEEKC (squares); and dodecanophenone in MEEKC, which acts as the ME marker (triangles). All the mobilities are negative as they correspond to anionic compounds. Moreover, in MEEKC a compound not interacting with the ME would have μ=0. As can be observed, the mobility of the ME is not pH dependent. In MEEKC, benzoic acid elutes between the EOF (μ=0) and the ME marker

($\mu=\mu_{ME}$). Thus, the denominator of Eq. 6 is always negative ($\mu_{ME}-\mu < 0$), and the numerator has to be negative to obtain $k > 0$, *i.e.* $\mu_0 > \mu$. The mobility plot for benzoic acid in CZE (μ_0) always has to be between 0 (for neutral benzoic acid) and μ (for benzoate), and this is not the case for pH > 4.0. The reason for this disagreement must lie in the different natures of the running buffers in CZE and MEEKC.

4.4 Effect of the microemulsion components on medium viscosity and electrophoretic mobility

To test how different the mobilities of the same compound are in the two media, the mobility of benzoic acid was determined at pH 11.0 in solutions with different concentration of SDS. At this pH value, benzoic acid is totally ionized, so it is expected to have the same mobility in the different media (Figure 4, squares). In the plot, 0% corresponds to measurements in plain buffer solution, 100% to measurements in a solution with 1.30% w/v of SDS (the amount of SDS equivalent to that in the ME), and the other percentages are measurements in electrophoretic buffers which are mixtures (v/v) of these two solutions. The mobility of the benzoate ion in CZE is $-29.9\cdot10^{-5}$ $cm^2s^{-1}V^{-1}$, meanwhile a solution of SDS at 1.30% w/v shows a mobility of $-28.1\cdot10^{-5}$ $cm^2s^{-1}V^{-1}$. This indicates that SDS decreases the mobility of benzoate by around 6% in absolute value.

The reason for the change of ionic mobilities when the SDS at 1.30% w/v is added to the buffer is presumably the change in viscosity caused by the SDS. According to Eq. 11 [40], the viscosity (η) of the electrophoretic solution is inversely related to the mobility of the compounds. Provided that at a given pH the charge (q) of a compound is the same in MEEKC, MEKC, and CZE, and assuming that the hydrated radius (r) does not change,

differences of mobility for a given compound in two different solutions could be attributed to the differences in viscosity between the solutions.

$$\mu = \frac{q}{6\pi\eta r} \qquad \text{Eq. 11}$$

Some works [41–43] show that the addition of a surfactant to an aqueous solution causes an increase in the viscosity of the solution. Kushner *et al.* [41] measured the viscosity of aqueous solutions containing different SDS concentrations at 25ºC. Their results show an increase in the viscosity of the solution of more than 3% from 0% to 0.8% (w/v) of SDS content (Figure 5a). In the present work, the SDS content in the ME is 1.3% (w/v) which, if we assume linear behavior, would imply around a 5% difference in viscosity between the aqueous buffer and the SDS solution. This percentage matches the difference in mobility between CZE and MEKC shown in Figure 4. Muijselaar *et al.* [16] already pointed out that the differences in viscosity may have an effect on the calculation of retention factors from MEKC measurements; although they concluded that this difference was small enough to be considered negligible. Note that typical SDS concentrations in MEKC are around 50 mM, which corresponds to 1.44% (w/v); so viscosity differences should be close to 5%-6%. This variation agrees with that expected from Figure 4, but it is not enough to explain the variation in the mobility plots in Figure 3, which is about 20%-30%. Thus, we also investigated the effect of the other ME components.

The literature indicates that the viscosity of 1-butanol and heptane, the cosurfactant and oil used in the ME respectively, are quite different from that of water [44–47]. Thus, the overall viscosity of the ME-buffer medium may be significantly altered. As the proportion of 1-butanol in the ME is much greater than that of heptane, the differences in mobilities due to the change in medium should mostly be attributed to the former. In fact, 8.15%

(v/v) of 1-butanol increases the viscosity of an aqueous solution by more than 30%, according to Figure 5b [44–46]. Figure 5c is a plot of the dynamic viscosity of 1-butanol/heptane mixtures at different mole fractions. As can be seen in the figure, an increase of heptane in the mixture leads to a reduction of the overall dynamic viscosity [47]. A heptane mole fraction of 0.08 (that in the ME, taking into account only 1-butanol and heptane as components), implies a decrease of dynamic viscosity of around 15%, compared with pure 1-butanol. This suggests that the heptane present in the ME will slightly diminish (by around 15%) the increment of the viscosity due to the 1-butanol also present. Unfortunately, it is not possible to evaluate the individual effect of 1-butanol in the CZE buffer directly because, although it is miscible with water, it is not miscible with the buffer due to the presence of salts that increase the polarity of the aqueous phase. Furthermore, heptane is not miscible with water and so could not be tested either.

As it was not possible to evaluate the effect of each compound independently, we studied the effect of the overall ME on mobilities. Figure 4 shows the electrophoretic mobility of the benzoate ion at different proportions of aqueous buffer and ME (circles). As before, the point at 0% shows the mobility of the benzoate ion in CZE (μ_0 = -30.2·10^{-5} cm^{2}s^{-1}V^{-1}), with no ME; and the point at 100% shows its mobility in the MEEKC conditions used in this work (μ = -22.9·10^{-5} cm^{2}s^{-1}V^{-1}). There is an important difference in mobilities when comparing the CZE and MEEKC values (around a 24% decrease in absolute value). Taking into account all contributions, and being aware that the viscosities of the different components of a mixture are not additive at all, the ME is expected to have a viscosity some 20%-30% higher than that of the buffer solution (according to Figure 5). This matches the shifted mobilities obtained from Figure 3 and Figure 4. Therefore, we can conclude that differences in mobility due to the different solutions can mostly be attributed to differences in viscosity between the media involved.

As can be observed in the profiles in Figure 1, this situation has an important impact on calculation of *k*, since mobilities in MEEKC and CZE will be different, not only due to the retention of the compound in the ME, but also due to the considerable difference in viscosity between the solutions. As a consequence, mobility in CZE becomes more negative than mobility in MEEKC, especially for those compounds that show a weak interaction with the ME, leading to negative retention factors when direct subtraction of mobilities in MEEKC and CZE is performed for the numerator of Eq. 6. This error may be negligible in MEKC because the increase of viscosity caused by the addition of surfactant is not very high, but it becomes much more important in MEEKC.

4.5 Determination of corrected retention factors

Since it is not possible to measure the mobility in the exact ME-buffer medium, we propose a mobility correction based on the difference of viscosity of the 2 solutions (that with ME and the plain buffer), which can be calculated very easily. We selected benzoic acid to do the correction, because it is a relatively small compound, quite polar, with absorbance in the UV range, and it is not supposed to interact with ME when it is fully ionized ($\log P_{o/w(benzoate)} \approx -1.3$ [48]).

Eq. 11 gives the relation between μ of a compound and the viscosity of the electrophoretic solution. If constant terms are grouped together (c), we obtain:

$$\mu = \frac{c}{\eta} \qquad \text{Eq. 12}$$

In this way, the difference in viscosities and mobilities between the ME and CZE conditions can be related thus:

$$\frac{\mu}{\mu_0} = \frac{\eta_0}{\eta} \qquad \text{Eq. 13}$$

So, the calculation of the retention factor for any compound can be corrected by the difference of viscosity according to the expression:

$$k = \frac{\mu - \left(\frac{\eta_0}{\eta}\right) \cdot \mu_0}{\mu_{ME} - \mu} \qquad \text{Eq. 14}$$

Viscosities are not directly measured, but since the ratio of viscosities is the inverse of the ratio of mobilities of the benzoate ion (Eq. 13), for any compound the correction is given by:

$$k = \frac{\mu - \left(\frac{\mu}{\mu_0}\right)_{benzoate} \cdot \mu_0}{\mu_{ME} - \mu} \qquad \text{Eq. 15}$$

We then recalculated retention factors of the test compounds in accordance with this correction (the viscosity correction $(\frac{\mu}{\mu_0})_{benzoate}$ has a value of 0.76). The profiles obtained (squares) and results of the fit (solid line) are shown in Figure 1 and Table 4, respectively. No significant differences are observed for pK_a' and $k_{(HA)}$ values compared to those in Table 3. Notwithstanding, values of $k_{(A^-)}$ are now all positive, being zero or close to zero for benzoate and 3-bromobenzoic acid, a bit higher for naproxen and ketoprofen (which indicates a slight interaction of their anionic form with the ME), and relatively high for ibuprofen and 2,4,6-trichlorophenol (the two most hydrophobic compounds). In the last two cases, there is a clear interaction between the anionic forms of the compounds and the ME. The plots in Figure 1 demonstrate that differences in the

nature of the solutions needed for the calculation of *k* can be compensated by a correction using a compound that does not interact with the ME.

5. Conclusions

This work demonstrates that the nature of the solutions used for the calculation of retention factors of ionizable compounds in MEKC and MEEKC can have a considerable effect on the values obtained. This is especially so when the viscosities of the aqueous buffer and the micellar or ME solutions are very different. This effect is not so important in MEKC measurements, since the presence of surfactant micelles does not increase the viscosity of the aqueous buffer to a great extent. However, it can make an important contribution to MEEKC retention factors, as the viscosity of some of the components of microemulsions can be very different from that of water (mainly that of 1-butanol in this case). As the viscosity of different microemulsions can change to a greater or lesser extent depending on the proportion and viscosity of the components used in their formation, a viscosity correction has to be introduced. In the present work, we propose a calculation of this viscosity correction using the ratio of mobilities (in MEEKC and CZE) of a compound that does not interact with the pseudo-stationary phase, such as the benzoate ion.

With the proposed correction, the error introduced into the determination of the retention factor in MEKC and MEEKC due to the different viscosities of the media is removed. It has been demonstrated that such an error is especially important for quite polar ionizable compounds, and the correction should always be performed when the retention factor is used for further applications, such as the optimization of analytical separations or the estimation of biological or physicochemical parameters of compounds through quantitative structure-activity relationships.

ACKNOWLEDGEMENTS

Financial support from the Spanish *Ministerio de Economía y Competitividad* (award: CTQ2017-88179-P) and the Catalan Government (award: 2017SGR1074) is acknowledged. AFP wishes to thank the University of Barcelona for his APIF PhD fellowship.

CONFLICT OF INTEREST

The authors declare no competing financial interests.

REFERENCES

[1] S. Terabe, K. Otsuka, T. Ando, Electrokinetic chromatography with micellar solution and open-tubular capillary, Anal. Chem. 57 (1985) 834–841.

[2] S. Terabe, K. Otsuka, K. Ichikawa, A. Tsuchiya, T. Ando, Electrokinetic separations with micellar solutions and open-tubular capillaries, Anal. Chem. 56 (1984) 111–113.

[3] H. Watarai, Microemulsion capillary electrophoresis, Chem. Lett. 231 (1991) 391–394.

[4] R. Ryan, K. Altria, E. McEvoy, S. Donegan, J. Power, A review of developments in the methodology and application of microemulsion electrokinetic chromatography, Electrophoresis. 34 (2013) 159–177.

[5] C.-W. Chang, Y.-C. Chen, C.-Y. Liu, Separation and on-line preconcentration of nonsteroidal anti-inflammatory drugs by microemulsion electrokinetic chromatography, Electrophoresis. 36 (2015) 2745–2753.

[6] W. Xiao, Q. Zhang, C. Chen, Q.-H. Zhang, Y.-J. Hu, Z.-N. Xia, F.-Q. Yang, Analysis of eight isoflavones in Radix Puerariae by MEEKC: comparison on three different oil phases, J. Chromatogr. Sci. 54 (2016) 1678–1686.

[7] Q. Zhou, J. Mao, G. Xie, J. Xiao, Separation and sensitive analysis of chlorophenols by MEEKC, Chromatographia. 71 (2010) 875–880.

[8] X. Subirats, H.P. Yuan, V. Chaves, N. Marzal, M. Rosés, Microemulsion electrokinetic chromatography as a suitable tool for lipophilicity determination of acidic, neutral, and basic compounds, Electrophoresis. 37 (2016) 2010–2016.

[9] M.H. Abraham, C. Treiner, M. Roses, C. Rafols, Y. Ishihama, Linear free energy relationship analysis of microemulsion electrokinetic chromatographic determination of lipophilicity, J. Chromatogr. A. 752 (1996) 243–249.

[10] S.K. Poole, D. Durham, C. Kibbey, Rapid method for estimating the octanol – water partition coefficient (log Pow) by microemulsion electrokinetic chromatography, J. Chromatogr. B Biomed. Sci. Appl. 745 (2000) 117–126.

[11] A. Fernández, S. Amézqueta, E. Fuguet, M. Rosés, Feasibility of the estimation of octanol-water distribution coefficients of acidic drugs by microemulsion electrokinetic chromatography, ADMET DMPK. 6 (2018) 55–60.

[12] M.G. Khaledi, S.C. Smith, J.K. Strasters, Micellar electrokinetic capillary chromatography of acidic solutes: migration behavior and optimization strategies, Anal. Chem. 63 (1991) 1820–1830.

[13] J.K. Strasters, M.G. Khaledi, Migration behavior of cationic solutes in micellar electrokinetic capillary chromatography, Anal. Chem. 63 (1991) 2503–2508.

[14] C.-E. Lin, W.-C. Lin, W.-C. Chiou, Migration behaviour and selectivity of dichlorophenols in micellar electrokinetic capillary chromatography influence of micelle concentration and buffer pH, J. Chromatogr. A. 722 (1996) 333–343.

[15] A. Téllez, E. Fuguet, M. Rosés, Comparison of migration models for acidic solutes in micellar electrokinetic chromatography, J. Chromatogr. A. 1139 (2007) 143–151.

[16] P.G. Muijselaar, H.A. Claessens, C.A. Cramers, Migration behaviour of monovalent weak acids in micellar electrokinetic chromatography mobility model versus retention model, J. Chromatogr. A. 765 (1997) 295–306.

[17] D.J. Bailey, J.G. Dorsey, pH effects on micelle-water partitioning determined by micellar electrokinetic chromatography, J. Chromatogr. A. 852 (1999) 559–571.

[18] Y. Ishihama, Y. Oda, N. Asakawa, A hydrophobicity scale based on the migration index from microemulsion electrokinetic chromatography of anionic solutes, Anal. Chem. 68 (1996) 1028–1032.

[19] Y. Ishihama, Y. Oda, N. Asakawa, Hydrophobicity of cationic solutes measured by electrokinetic chromatography with cationic microemulsions, Anal. Chem. 68 (1996) 4281–4284.

[20] A. Fernández-Pumarega, S. Amézqueta, S. Farré, L. Muñoz-Pascual, M.H. Abraham, E. Fuguet, M. Rosés, Modeling aquatic toxicity through chromatographic systems, Anal. Chem. 89 (2017) 7996–8003.

[21] A. Fernández-Pumarega, S. Amézqueta, E. Fuguet, M. Rosés, Tadpole toxicity prediction using chromatographic systems, J. Chromatogr. A. 1418 (2015) 167–176.

[22] S. Soriano-Meseguer, E. Fuguet, A. Port, M. Rosés, Estimation of skin permeation by liquid chromatography, ADMET DMPK. 6 (2018) 140–152.

[23] M. Hidalgo-Rodríguez, S. Soriano-Meseguer, E. Fuguet, C. Ràfols, M. Rosés, Evaluation of the suitability of chromatographic systems to predict human skin permeation of neutral compounds, Eur. J. Pharm. Sci. 50 (2013) 557–568.

[24] M. Hidalgo-Rodríguez, E. Fuguet, C. Ràfols, M. Rosés, Modeling nonspecific toxicity of organic compounds to the fathead minnow fish by means of chromatographic systems, Anal. Chem. 84 (2012) 3446–3452.

[25] E. Fuguet, C. Ràfols, E. Bosch, M. Rosés, Solute-solvent interactions in micellar electrokinetic chromatography: IV. Characterization of electroosmotic flow and micellar markers, Electrophoresis. 23 (2002) 56–66.

[26] E. Grunwald, A differential potentiometric method of measuring acid and base dissociation constants, J. Am. Chem. Soc. 73 (1951) 4934-4938.

[27] B. Saxton, H.F. Meier, The ionization constants of benzoic acid and of the three monochlorobenzoic acids, at 25°, from conductance measurements, J. Am. Chem. Soc. 56 (1934) 1918–1921.

568 [28] J.A. Cleveland Jr, M.H. Benko, S.J. Gluck, Y.M. Walbroehl, Automated pKa
569 determination at low solute concentratrions by capillary electrophoresis, J.
570 Chromatogr. A. 652 (1993) 301–308.
571 [29] F.G. Brockman, M. Kilpatrick, The thermodynamic dissociation constant of
572 benzoic acid from conductance measurements, J. Am. Chem. Soc. 56 (1934) 1483–
573 1486.
574 [30] F.H. Clarke, N.M. Cahoon, Ionization constants by curve fitting: Determination of
575 partition and distribution coefficients of acids and bases and their ions, J. Pharm.
576 Sci. 76 (1987) 611–620.
577 [31] B. Slater, A. McCormack, A. Avdeef, J.E.A. Comer, pH-metric log P. 4.
578 Comparison of partition coefficients determined by HPLC and potentiometric
579 methods to literature values, J. Pharm. Sci. 83 (1994) 1280–1283.
580 [32] J. F. J. Dippy, R.H. Lewis, Chemical constitution and the dissociation constants of
581 monocarboxylic acids. Part V. Further substituted benzoic and phenylacetic acids,
582 J. Chem. Soc. (1936) 644–649.
583 [33] T. Degim, V. Zaimoglu, C. Akay, Z. Degim, pH-Metric logK calculations of
584 famotidine, naproxen, nizatidine, ranitidine and salicylic acid, Farmaco. 56 (2001)
585 659–663.
586 [34] S. Winiwarter, N.M. Bonham, F. Ax, A. Hallberg, H. Lennernäs, A. Karlén,
587 Correlation of human jejunal permeability (in vivo) of drugs with experimentally
588 and theoretically derived parameters. A multivariate data analysis approach, J.
589 Med. Chem. 41 (1998) 4939–4949.
590 [35] Y. Ishihama, M. Nakamura, T. Miwa, T. Kajima, N. Asakawa, A rapid method for
591 pKa determination of drugs using pressure-assited capillary electrophoresis with
592 photodiode array detection in drug discovery, J. Pharm. Sci. 91 (2002) 933–942.

[36] M. Shalaeva, J. Kenseth, F. Lombardo, A. Bastin, Measurement of dissociation constants (pKa values) of organic compounds by multiplexed capillary electrophoresis using aqueous and cosolvent buffers, J. Pharm. Sci. 97 (2008) 2581–2606.

[37] M. Meloun, S. Bordovská, L. Galla, The thermodynamic dissociation constants of four non-steroidal anti-inflammatory drugs by the least-squares nonlinear regression of multiwavelength spectrophotometric pH-titration data, J. Pharm. Biomed. Anal. 45 (2007) 552–564.

[38] K. Schellenberg, C. Leuenberger, R.P. Schwarzenbach, Sorption of chlorinated phenols by natural sediments and aquifer materials, Environ. Sci. Technol. 18 (1984) 652–657.

[39] E. Fuguet, C. Ràfols, M. Rosés, E. Bosch, Critical micelle concentration of surfactants in aqueous buffered and unbuffered systems, Anal. Chim. Acta. 548 (2005) 95–100.

[40] D.R. Baker, Capillary electrophoresis, Wiley, New York (USA), 1995.

[41] L.M. Kushner, B.C. Duncan, J.I. Hoffman, A viscometric study of the micelles of sodium dodecyl sulfate in dilute solutions, J. Res. Natl. Bur. Stand. 49 (1952) 85–90.

[42] M.A. Motin, M.A. Hafiz Mia, A.K.M. Nasimul Islam, K.M. Salim Reza, M.A. Yousuf, Effect of sodium dodecyl sulfate on viscometric properties of methanol, ethanol, n-propanol, and iso-propanol at different temperatures, J. Bangladesh Chem. Soc. 25 (2012) 110–123.

[43] T. Ito, H. Mizutani, The Viscosity of Aqueous Sodium Dodecyl sulfate solution and the effect of ionorganic electrolytes on it, J. Japan Oil Chem. Soc. 17 (1968) 246–248.

[44] M.V. Ionin, T. V. Sherstneva, G.N. Koleboshin, Phase equilibria in the system butanol-1-water-dioxane, Zh. Obs. Khim. 39 (1969) 23–25.

[45] J. Gregorowicz, A. Bald, A. Szejgis, A. Chmielewska, Gibbs energy of transfer and conductivity properties of NaI solutions in mixtures of water with butan-1-ol at 298.15 K, and some physicochemical properties of mixed solvent, J. Mol. Liq. 84 (2000) 149–160.

[46] C.M. Romero, E. Moreno, J.L. Rojas, Apparent molal volumes and viscosities of DL-α-alanine in water-alcohol mixtures, Thermochim. Acta. 328 (1999) 33–38.

[47] C. Magallanes, A. Catenaccio, H. Mechetti, Relaxation time and viscosity of several n-alcohol/heptane systems, J. Mol. Liq. 40 (1989) 53–63.

[48] N. Gulyaeva, A. Zaslavsky, P. Lechner, A. Chait, B. Zaslavsky, pH dependence of the relative hydrophobicity and lipophilicity of amino acids and peptides measured by aqueous two-phase and octanol-buffer partitioning, J. Pept. Res. 61 (2003) 71–79.

634 **Figure captions**

635

636 **Figure 1:** Retention factor vs. pH profiles of the six test compounds in MEEKC, before
637 (•) and after (▪) viscosity correction. The dotted and solid lines show the result of the fit
638 of Eq. 4 to the experimental points, respectively, before and after viscosity correction. a)
639 benzoic acid, b) 3-bromobenzoic acid, c) naproxen, d) ketoprofen, e) ibuprofen, and f)
640 2,4,6-trichlorophenol.

641

642 **Figure 2:** Mobility profiles of the six test compounds in CZE, in plain buffers (○), and in
643 buffers containing 2 mM SDS (x). The lines are the fit of Eq. 10 to the experimental points
644 in plain buffers (dotted red line), and in the buffers containing 2 mM SDS (dashed black
645 line). a) benzoic acid, b) 3-bromobenzoic acid, c) naproxen, d) ketoprofen, e) ibuprofen,
646 and f) 2,4,6-trichlorophenol.

647

648 **Figure 3:** Effect of pH on the mobility of benzoic acid in CZE (●), and in MEEKC (■).
649 Effect of pH on the mobility of the ME (▲).

650

651 **Figure 4:** Effect of amount of SDS (■), and ME (●) on the mobility of benzoate ion. The
652 analysis was performed applying a voltage of 12 kV and with no additional pressure, in a
653 buffer at pH 11.

654

655 **Figure 5:** Effect of individual ME components on dynamic viscosity. a) Mixtures of
656 SDS:water, according to the percentage (w/v) of SDS [41]. b) Mixtures of 1-
657 butanol:water, according to the percentage (v/v) of 1-butanol: (●) from [44], (▲) from
658 [45], and (■) from [46]. c) Mixtures of 1-butanol:heptane, according to the mole fraction

659 (X) of heptane [47].

660

661 **Table 1.** Final concentration of individual buffer components expressed in molarity (M)

pH	$[Na^+]$	$[Cl^-]$	$[H_3PO_4]$	$[H_2PO_4^-]$	$[HPO_4^{2-}]$	$[PO_4^{3-}]$	[HAc]	$[Ac^-]$
2.0	0.040	0.033	0.023	0.017	-	-	-	-
3.0	0.049	0.007	0.006	0.043	-	-	-	-
4.0	0.050	0.042	-	-	-	-	0.042	0.008
5.0	0.050	0.018	-	-	-	-	0.018	0.032
6.0	0.047	-	-	0.042	0.003	-	-	-
7.0	0.039	-	-	0.018	0.011	-	-	-
8.0	0.034	-	-	0.003	0.016	-	-	-
11.0	0.033	-	-	-	0.015	0.001	-	-

662

663 **Table 2.** Physicochemical properties (pK_a and log$P_{o/w}$) of the compounds tested

Compound	**pK_a (SD)[a]**	**pK_a' (SD)[b]**	**log$P_{o/w}$[c]**	**Ref.[d]**
benzoic acid	4.19 (±0.02)	4.11 (±0.02)	1.87	[26–31]
3-bromobenzoic acid	3.81	3.72	2.75	[32]
naproxen	4.24 (±0.10)	4.16 (±0.10)	3.18	[33–36]
ketoprofen	4.13 (±0.12)	4.04 (±0.12)	3.12	[31,34,35,37]
ibuprofen	4.36 (±0.08)	4.28 (±0.08)	3.50	[35–37]
2,4,6-trichlorophenol	6.17 (±0.04)	6.08 (±0.04)	3.69	[38]

664 [a]Average of the thermodynamic pK_a reported in the literature

665 [b] pK_a' at 0.05 M ionic strength

666 [c] from Bio-Loom database v1.7

667 [d] references for pK_a values

668 **Table 3.** Parameters and statistics for fitting the retention factor to pH (Eq. 4). Standard
669 deviations are shown in brackets

Compound	**pK_a'**	**$k_{(A^-)}$**	**$k_{(HA)}$**	**R^2**	**F**	**SD**
benzoic acid	4.37 (0.16)	-1.79 (0.14)	0.98 (0.16)	0.985	99	0.20
3-bromobenzoic acid	3.61 (0.07)	-1.19 (0.13)	5.81 (0.23)	0.997	478	0.21
naproxen	4.16 (0.07)	-0.18 (0.19)	8.52 (0.24)	0.997	479	0.28
ketoprofen	3.99 (0.04)	-0.08 (0.07)	6.29 (0.09)	0.999	1810	0.11
ibuprofen	3.99 (0.04)	0.54 (0.11)	76.79 (3.60)	1.000	17178	0.15
2,4,6-trichlorophenol	6.01 (0.12)	-0.58 (0.78)	14.88 (0.51)	0.988	160	0.94

670

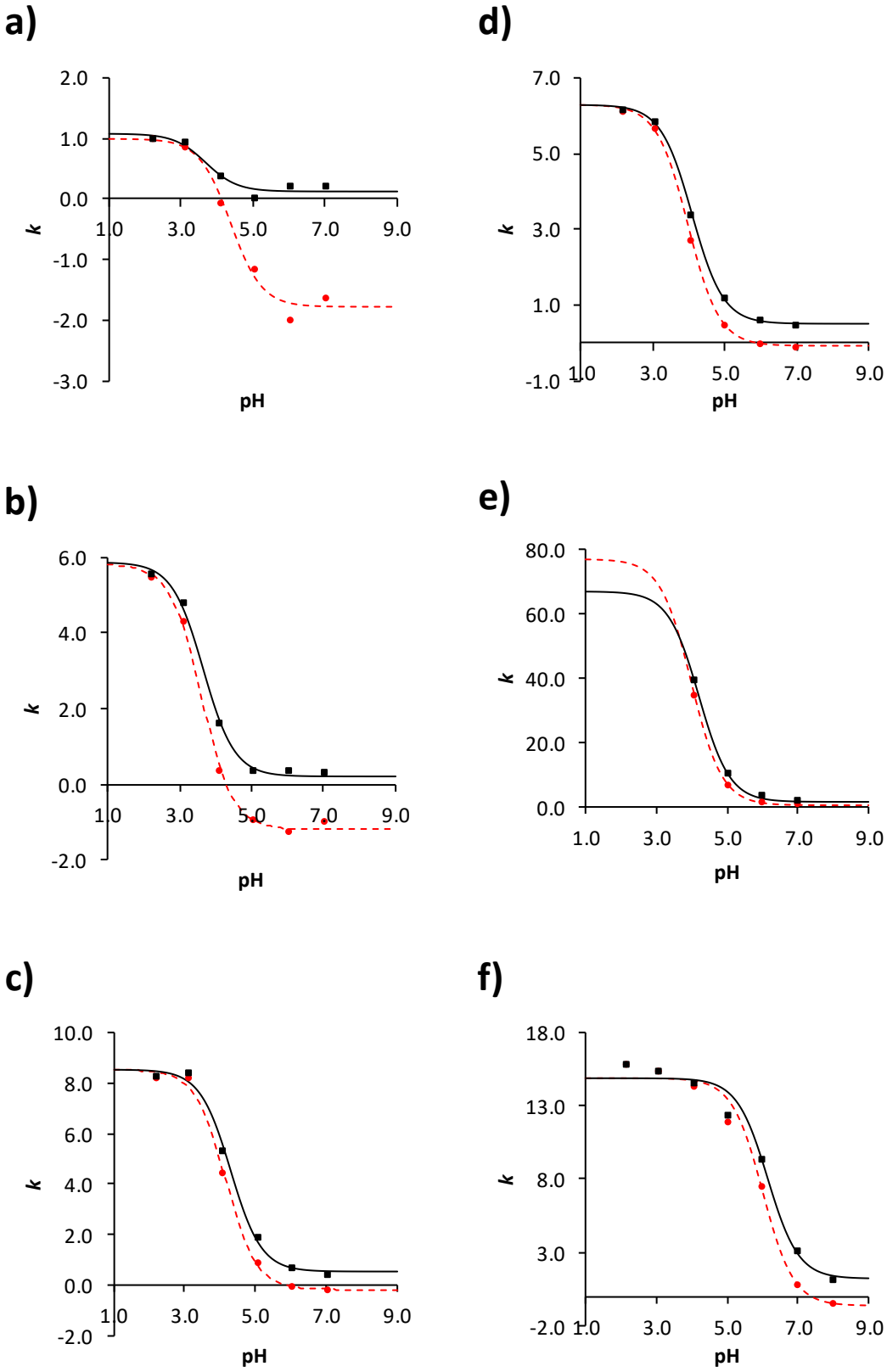
671

672 **Table 4.** Parameters and statistics for fitting the retention factor to pH (Eq. 4) after
673 viscosity correction. Standard deviations are shown in brackets

Compound	pK_a'	$k_{(A^-)}$	$k_{(HA)}$	R^2	F	SD
benzoic acid	3.68 (0.30)	0.13 (0.07)	1.07 (0.13)	0.948	28	0.12
3-bromobenzoic acid	3.66 (0.08)	0.23 (0.11)	5.84 (0.19)	0.996	415	0.19
naproxen	4.29 (0.08)	0.50 (0.21)	8.54 (0.25)	0.996	360	0.30
ketoprofen	4.10 (0.04)	0.52 (0.07)	6.30 (0.09)	0.999	1424	0.11
ibuprofen	4.21 (0.05)	1.79 (0.28)	67.04 (3.55)	1.000	3448	0.36
2,4,6-trichlorophenol	6.15 (0.14)	1.24 (0.82)	14.87 (0.51)	0.983	119	0.94

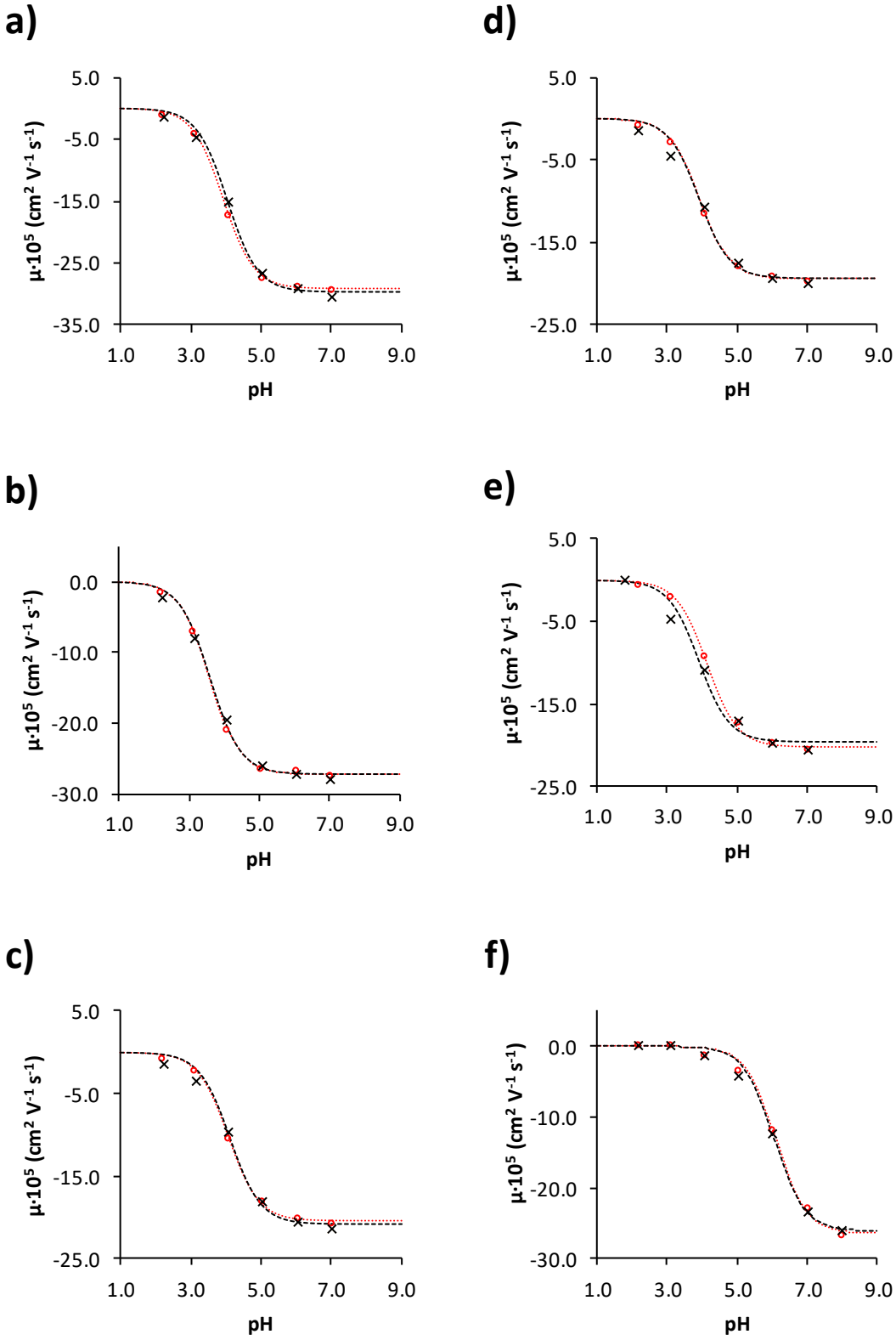
674
675

676 **Figure 1**



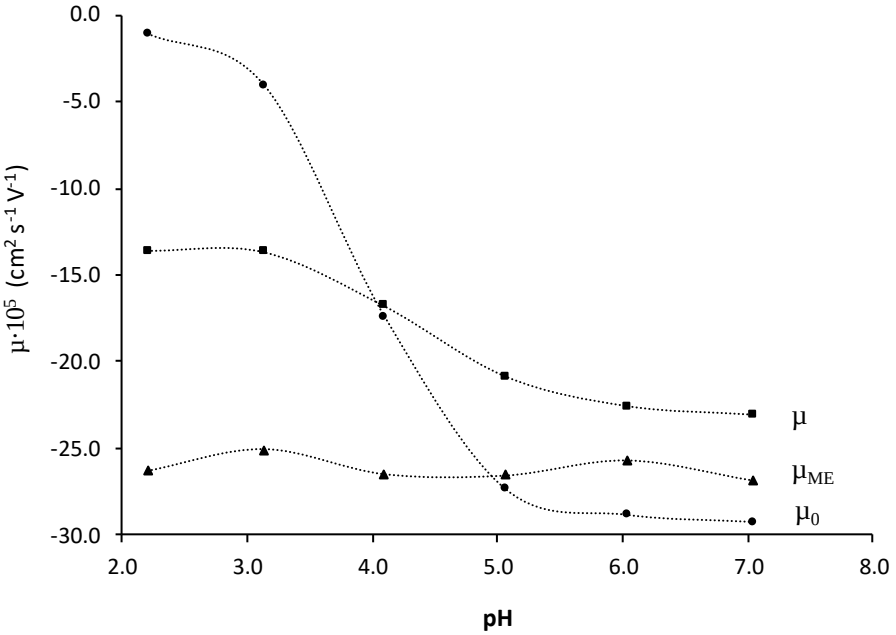
677
678

679 **Figure 2**



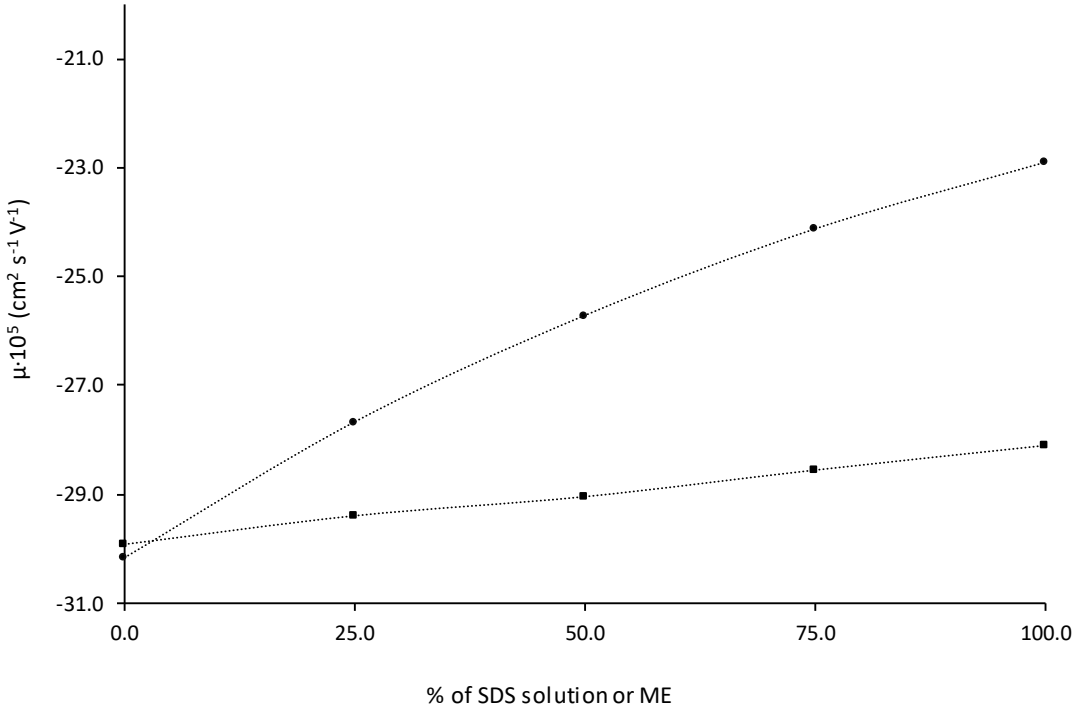
680
681

682 **Figure 3**



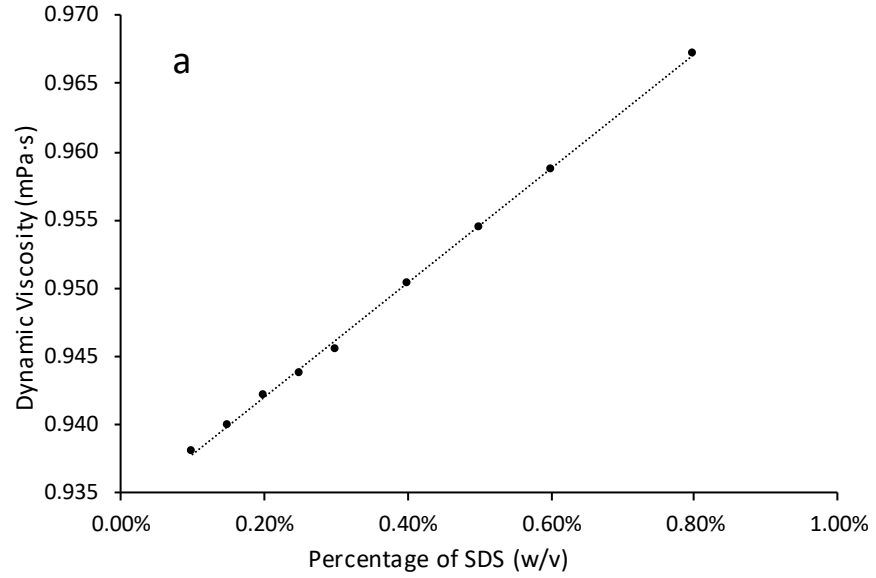
683
684

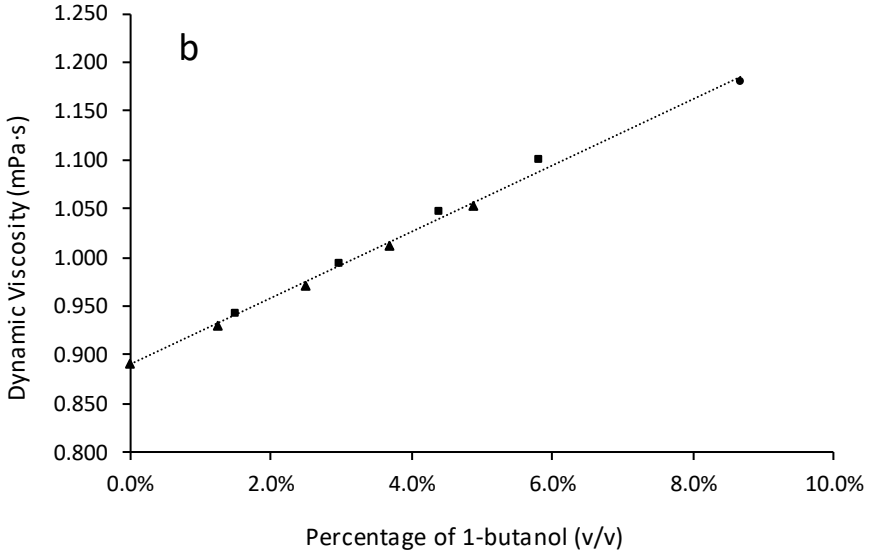
685 **Figure 4**

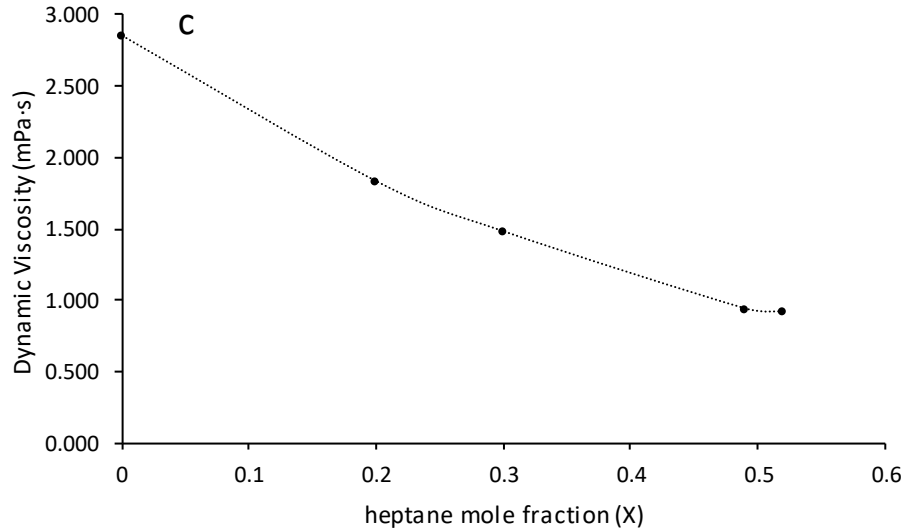


686
687

688 **Figure 5**







689
690