Effect of buffer nature and concentration on the chromatographic performance of basic compounds in the absence and presence of 1-hexyl-3-methylimidazolium chloride

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Abstract

In reversed-phase liquid chromatography, the performance for basic compounds is affected by the interaction of the protonated (cationic) species with the anionic free silanols on the alkyl-bonded stationary phases. Using aqueous-organic mobile phases in the absence of additives, the retention may be too high, and the peaks be broad and asymmetric. The performance is improved by addition to the mobile phase of ionic liquids, from which 1-hexyl-3-methylimidazolium chloride ([C_6MIm][Cl]) has especially good characteristics. A recent report has also revealed that the use of the phosphate system as buffer, at varying concentration and pH, may have a significant role in the chromatographic performance of basic compounds, with effects on both retention and peak shape. In this work, this study has been extended to other three buffer systems (acetate, citrate, and formate), at increasing concentrations and pH 3 and 7, in the presence and absence of [C_6MIm][Cl]. The results have been compared with those obtained with the phosphate system. The retention increases by addition of larger concentration of all buffers, in both absence and presence of [C_6MIm][Cl]. Without additive, peak performance is also enhanced significantly. This effect is minimal in the presence of [C_6MIm][Cl], which yields highly symmetrical peaks at all buffer concentrations, due to an effective blocking of the silanol activity.

Keywords: Reversed-phase liquid chromatography; Silanol effect; β-Adrenoceptor antagonists; Ionic liquids; Buffer systems

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1. Introduction

Ionic liquids (ILs) can be considered as salts or solvents. By essence, they are made of cations and anions, and possess as special feature low melting temperature (usually below 100 ^{o}C). As solvents, ILs have non-molecular nature, and show mainly low volatility and flammability, together with high thermal stability [1–3]. Due to these properties, ILs started to be used as promising benign or green solvents that could replace pollutant organic solvents in the laboratories. Nevertheless, several reports have specified in the last years certain reticence to the term "green" [4,5], since some ILs are not as safe and non-toxic as it was thought, especially during their synthesis.

In the field of chromatographic analysis, ILs have been used in reversed-phase liquid chromatography (RPLC) as mobile phase additives to successfully enhance peak performance in the analysis of basic compounds, using conventional stationary phases [6–18]. It should be noted that in RPLC, basic compounds may experience additional ion-exchange interactions with residual anionic silanols on silica packings, which increase their retention and result in broad and asymmetrical peaks [19–22]. In this regard, ILs have become a serious alternative to the common reagents traditionally used to enhance chromatographic performance (e.g. amines and surfactants).

Ionic liquids of different nature have been considered in RPLC, starting with the alkylammonium-based ILs associated to nitrate and thiocyanate [23,24], or acetate and formate [25–29]. However, the use of these ILs was problematic due to their high viscosity, production of corrosion of the metallic parts of the HPLC systems after extensive use, poor baseline stability, weaker elution strength compared to common organic solvents, rapid deterioration of the silica-based packing, and poor efficiencies. For this reason, in the 90's, imidazolium-based ILs associated to chloride, bromide, tetrafluoroborate or

hexafluorophosphate, were proposed as the best option to combine with acetonitrile-water mixtures, since they were able to overcome these initial drawbacks [6–18].

The interaction mechanism of ILs with RPLC stationary phases is more complex compared to the behaviour of other additives, such as amines or the anionic surfactant sodium dodecyl sulfate (which is also a good silanol suppressor [30,31]). Both cation and anion in the IL may be able to interact with the stationary phase and create an asymmetrical bilayer, positively or negatively charged, depending on the relative adsorption of the cation and the anion [32,33]. This means that these additives are dual modifiers, which enrich the nature of the interactions taking place simultaneously inside the column.

In order to gain more insight in the behaviour of ILs as silanol suppressors, a series of comprehensive studies on the silanol suppression effect of imidazolium-based ILs with long side chains was carried out, based on modelling the changes of retention and peak shape using increasing amounts of the ILs in the mobile phase [33–36]. The effect of ILs associated to tetrafluoroborate and hexafluorophosphate anions was also compared to that achieved with different amines [32,33]. Since the silanol suppression effect of imidazolium cations was dominant with respect to the interactions with the associated anion, the research was then extended to ILs associated to chloride (without affinity towards the stationary phase) [36]. 1-Hexyl-3-methylimidazolium chloride ([C_6MIm][Cl]) was revealed as an efficient peak profile enhancer of basic compounds in RPLC, using conventional stationary phases, since it produced narrower chromatographic peaks, combined with smaller retention and consumption of organic solvent.

Acetonitrile-water mixtures containing an ionic liquid as modifier in RPLC are usually buffered at fixed pH. It should be noted that changes in organic solvent concentration may affect the pH and pK_a of the acid-base buffer and solute [37], which would have influence on solute retention. However, the selection of the buffer nature and concentration, which may be

critical, is not usually considered. A recent report with basic analytes revealed that the addition of phosphate buffer to the mobile phase at variable concentration had a significant role in the separation process, with effects on both the retention mechanism and peak shape [38]. Therefore, the performance of basic analytes under the addition of a specific buffer system deserves a more deep study. In this work, the study was extended to buffers of different nature, in the absence and presence of the ionic liquid [C_6MIm][Cl] in acetonitrile-water mobile phases. The variation of retention times, and eventual changes in peak shape and selectivity, of a group of basic β-adrenoceptor antagonists were investigated with mobile phases containing four different buffer systems (acetic acid/acetate, citric acid/dihydrogen citrate, formic acid/formate, and phosphoric acid/dihydrogen phosphate) at increasing concentrations at pH 3. Buffer solutions composed of hydrogen citrate/citrate and dihydrogen phosphate/hydrogen phosphate were also compared at pH 7. Conclusions about the effect of the buffer systems on the suppression of silanol activity, in the presence and absence of [C_6MIm][Cl] are addressed.

2. Experimental

2.1. Reagents

β-Adrenoceptor antagonists are commercialised and used for the treatment of various cardiac diseases [39]. Six of these drugs were used in this work as probe compounds: acebutolol, atenolol, metoprolol, nadolol, oxprenolol and timolol (all from Sigma, St. Louis, MO, USA). The drugs were dissolved in a small amount of acetonitrile, with the aid of an Elmasonic IT-H ultrasonic bath from Elma (Singen, Germany), and diluted with water up to a concentration of approximately 100 μg/mL. The solutions were stored at 4 ^{o}C, remaining stable during at least two months. These were diluted to obtain solutions of approximately 20

103 μg/mL, which were injected into the chromatograph. Uracil (Acros Organics, Geel, Belgium)
104 was selected as dead time marker.

105 Mobile phases were prepared with acetonitrile (Scharlab, Barcelona, Spain) in the
106 absence and presence of the ionic liquid [C_6MIm][Cl] (Sigma). Based on previous experience
107 [33,36], the concentration of acetonitrile was fixed at 10 % (*v*/*v*), except when the citrate
108 buffer was added, for which 15% acetonitrile was used.

109 The mobile phases were buffered at pH 3 with ammonium formate (Sigma), citric acid
110 (Scharlab), sodium acetate (Sigma) and sodium dihydrogen phosphate (Sigma), and at pH 7
111 with citric acid and sodium dihydrogen phosphate, in both cases at concentrations in the range
112 from 5 to 30 mM, to which HCl (Scharlab) or sodium hydroxide (Panreac, Barcelona) was
113 added to get the desired pH. Measurement of pH was made with a Hach (Loveland, CO,
114 USA) combination electrode 5202 (glass electrode and reference electrode containing 3.0 M
115 KCl solution in water as salt bridge), and a Crison micropH 2002 potentiometer with a
116 precision of 0.1 mV. The standardisation of the pH-meter was always carried out using
117 aqueous buffers. The pH was measured in the aqueous solution before adding the organic
118 solvent to the mobile phase, and afterwards, in the aqueous-organic mixture to get the final
119 pH value [40].

120 Nylon membranes of 0.45 μm (Micron Separations, Westboro, MA, USA) were used to
121 filter the drug solutions and mobile phases, which were also degassed in an ultrasonic bath.
122 Nanopure water (Barnstead, Sybron, Boston, MA, USA) was used throughout.

123

124 *2.2. Apparatus and column*

125 An Agilent chromatograph (Waldbronn, Germany), equipped with an isocratic pump
126 (Series 1260), an autosampler (Series 1260), a thermostated column compartment (Series
127 1260) set at 25 ºC, a UV-visible wavelength detector (Series 1100), and an HPChemStation

(Agilent, B.02.01) for data acquisition was used. β-Adrenoceptor antagonists were monitored at 254 nm, except timolol, for which the signal was measured at 300 nm. The mathematical treatment was carried out with Excel (Microsoft Office 2010, Redmond, WA, USA). The chromatographic peaks were integrated with MICHROM [41].

The chromatographic column was a Zorbax Eclipse XDB C18 (Agilent), with the following characteristics: 150 mm × 4.6 mm i.d., 5 μm particle size, 10 % carbon load, 180 m^2/g surface area, and 80 Å pore size. A similar 30 mm pre-column was connected to the analytical column for protection. The flow-rate was 1 mL/min. Duplicate injections of 20 μL were made.

3. Results and discussion

3.1. pK_a and pH variation of the selected buffers with solvent composition

β-Adrenoceptor antagonists are ionisable compounds that can exist in the ionised (cationic) or non-ionised forms depending on the mobile phase pH. These compounds are basic (pK_a = 9–10), due to the presence of an aromatic ring attached to a side alkyl chain possessing secondary hydroxyl and amine functional groups [42]. This means that at the working pH range of conventional silica-based C18 stationary phases (2–8) they are positively charged. When fixing the pH, it should be considered that the mobile phase pH and also the pK_a for a specific acid-base buffer may vary with solvent composition. Methanol and acetonitrile are the most common organic solvents used for the preparation of mobile phases in RPLC. In a series of reports by Bosch and co-workers [37,40,43–47], it was found that buffered solutions prepared from anionic and neutral (uncharged) acids, such as those used in our study (e.g. acetic acid/acetate), increased their pH value when acetonitrile or methanol were added, whereas buffers prepared with cationic acids (e.g. ammonium/ammonia) showed

the reverse trend. The pH changes were induced by the variation of the pK_a values of buffer components with solvent composition changes.

In Table S1 in the Supplementary material, the ${}^{s}_{w}\mathrm{p}K_a$ values obtained in aqueous-organic solutions containing acetonitrile in the 10–15% range are compared with the pK_a values in water [40]. Note that the ${}^{s}_{w}\mathrm{p}K_a$ values were obtained in the ${}^{s}_{w}\mathrm{pH}$ scale (i.e. the electrode system was calibrated with aqueous buffers, and the pH measured in the mobile phase after mixing the aqueous buffer with the organic modifier). The ${}^{s}_{w}\mathrm{pH}$ scale is especially convenient for its simplicity of measurement, since it does not require pH standards for each aqueous-organic composition. As observed, the pK_a values increased with a mean change of $({}^{s}_{w}\mathrm{p}K_a - \mathrm{p}K_a) = +0.18$ pH unities.

The pH of the mobile phases used throughout the study was fixed at 3, using the four buffer systems (acetate, citrate, formate and phosphate), and at pH 7 (buffered with the citrate and phosphate systems). Table 1 shows the difference in pH between aqueous and aqueous-organic solutions containing 10 or 15% acetonitrile, at three buffer concentrations (5, 10 and 30 mM). The measurements were made in the absence and presence of 10 mM [C_6MIm][Cl]. As observed, in the studied range, the pH variation seemed not be affected by the buffer concentration (only a slight variation was observed for the formate system), or by the buffer nature, being hydrogen citrate/citrate the system showing greater differences: 0.56, 0.61 and 0.58 at 5, 10 and 30 mM, respectively. The addition of 10 mM [C_6MIm][Cl] to the mobile phases did not change these trends.

3.2. Dominant acid-base species at the pH of the assayed mobile phases

According to the information obtained from Table S1, acetate and formate buffers exist mainly in solution in their protonated neutral forms at pH 3, whereas for the citrate and

phosphate buffers (especially for the latter), the concentration of the anionic species is dominant. Close to pH 7, the citrate and phosphate systems loss an additional hydrogen ion, which means that the anionic species of the buffer systems are clearly dominant in the aqueous-organic solutions.

Meanwhile, in theory, the described changes in pK_a and pH values of the buffer systems should not affect the retention behaviour of the β-adrenoceptor antagonists. The protonated cationic species of these compounds will be still dominant at both pH 3 and 7, since they have a strong basic character with p$K_a \geq 9$ [42]. The organic solvent is likely to cause a reduction in the pK_a of these compounds, but not enough to change their ionisation state.

Therefore, the only relevant acid-base equilibria at the working pH values (3 and 7) will be that of silanols and buffers. Silanols are weakly acidic, with average acid-base constants ranging from ca. pK_a = 4.5 to ca. 7, depending on the type of silica [48]. This means that their degree of ionisation (from neutral to anionic) varies with the mobile phase pH, inducing changes in the cationic solute/anionic silanol slow interaction that is the reason of the broad and asymmetrical peaks of basic compounds. This interaction may be blocked by the addition of different types of reagents, such as ILs and buffers. A detailed study of the changes in retention and peak shape of β-adrenoceptor antagonists at pH 3 and 7, in the absence and presence of [C_6MIm][Cl] at varying buffer concentration, is described in Sections 3.3 to 3.5, which will confirm these observations.

3.3. Effect of ionic liquid on retention

In previous work [36], the chromatographic retention of a set of β-adrenoceptor antagonists on a C18 Kromasil column was compared upon addition, to mobile phases containing a fixed concentration of 15% acetonitrile, of the ILs 1-ethyl, 1-butyl and 1-hexyl-3-methylimidazolium associated to chloride as anion ([C_2MIm][Cl], [C_4MIm][Cl] and

201 [C_6MIm][Cl], respectively), at concentrations ranging between 10 and 40 mM. It was
202 observed that solute retention decreased dramatically after the first addition (10 mM) of the
203 ILs, the effect with [C_6MIm][Cl] being larger. This IL also gave rise to better peak shape.
204 Further addition of the ILs in the mobile phase resulted in minimal changes of the retention
205 factors in all cases.

206 The larger effect induced by [C_6MIm][Cl] was explained by the more intense adsorption
207 of the IL cation ([C_6MIm]$^+$) on the alkyl-bonded stationary phase with respect to [C_2MIm]$^+$
208 and [C_4MIm]$^+$. Also, in these experiments, the influence of the anion was minimised since in
209 aqueous solution, chloride is strongly hydrated and has low affinity towards the stationary
210 phase. Therefore, chloride is not (or is only scarcely) involved in the chromatographic
211 separation, allowing the isolation of the effect of the imidazolium cation. The low retention
212 times and efficient peak profile obtained with [C_6MIm][Cl] make this IL a convenient
213 additive to enhance the chromatographic performance of β-adrenoceptor antagonists. For this
214 reason, it was selected for this work.

215 To investigate the effect of [C_6MIm][Cl] on the Zorbax Eclipse C18 column, a similar
216 experiment at increasing concentrations of this IL, ranging between 10 and 40 mM was
217 developed. In previous work [49], it was checked that this column yielded shorter retention
218 times with respect to the Kromasil C18 column. For this reason, the concentration of
219 acetonitrile was fixed mainly at 10% instead of 15%. In this study, 10 mM phosphoric
220 acid/dihydrogen phosphate was added to buffer the pH at 3. The retention behaviour is shown
221 in Fig. S1 (Supplementary material). As before with the Kromasil column, the addition of a
222 bulky imidazolium cation to the mobile phase, such as [C_6MIm]$^+$, gave rise to a significant
223 decrease in the retention of the β-adrenoceptor antagonists, reaching quickly column
224 saturation where changes in retention are minimal. For this reason, [C_6MIm][Cl] was added to
225 the mobile phase at a fixed concentration of 10 mM in further studies. The selected

226 concentrations for [C_6MIm][Cl] and acetonitrile guaranteed the elution of the assayed drugs in
227 practical analysis times.
228
229 *3.4. Effect of buffer on retention*

230 In previous work [38], it was observed that phosphate buffer may have an important role
231 in the separation process. The results obtained showed that increasing amounts of this buffer
232 system at low pH (pH 3), in the absence of any mobile phase additive, increased the retention
233 times. Also, the peak shape was improved. The change in retention and peak shape suggested
234 a possible adsorption of the phosphate anion on the stationary phase, which attracts the
235 cationic solutes and blocks the activity of residual silanols in the C18 column. However, the
236 effect of increasing the concentration of phosphate buffer on the chromatographic
237 performance was minimal when an IL was added to the mobile phase.

238 In order to extend this previous research, four buffer systems composed of acetic
239 acid/acetate, formic acid/formate, citric acid/dihydrogen citrate, and phosphoric
240 acid/dihydrogen phosphate, were added at increasing concentrations to mobile phases
241 containing 10% acetonitrile at pH 3, in the absence and presence of 10 mM [C_6MIm][Cl]. In
242 another series of experiments with hydrogen citrate/citrate and dihydrogen
243 phosphate/hydrogen phosphate the pH was fixed at 7. It should be noted that citrate buffers
244 gave rise to longer retention times for all compounds (e.g. > 120 min for oxprenolol at pH 3
245 and 10% acetonitrile), which forced the use of 15% acetonitrile in the mobile phases to reduce
246 the retention times to more practical values.

247 The effect of buffer concentration on retention, in the absence of [C_6MIm][Cl], can be
248 observed in Fig. 1. As observed, phosphate buffer (Fig. 1b) gave rise to somewhat lower
249 retention. The effect of formate and acetate on retention was similar. The retention factors in
250 the presence of increasing concentration of the buffers showed an increasing trend at pH 3,

whereas the retention decreased for 30 mM phosphate and citrate buffers at pH 7 (Figs. 1c and f). In previous work [38], the changes in retention with buffer concentration was attributed to a combination of interactions that included hydrophobic association of solutes with the alkyl-bonded stationary phase, and competitive attraction of the cationic solutes to the anionic silanols and phosphate ion weakly adsorbed to the stationary phase. It should be noted, that although the dominant species for the acetate and formate systems in the mobile phase, at pH 3, are not mainly the basic (anionic) species, the change in retention was more pronounced at increasing concentrations of these buffers. At pH 7, using the phosphate and citrate systems as buffers (Figs. 1c and f), the retention factors were larger with respect to pH 3, probably due to the interaction of the cationic solutes with the anionic silanols more strongly ionised at pH 7.

Fig. 2 shows that in the presence of 10 mM [C_6MIm][Cl], the chromatographic behaviour of the cationic solutes upon addition of the buffers to the mobile phase differs from the behaviour observed without IL. The retention factors increased almost linearly at increasing buffer concentration, except for citrate at pH 7. The changes were more significant at pH 3. Note that the retention factors were significantly shorter in the presence of [C_6MIm][Cl], which is a consequence of the repulsion of the basic compounds by the $[C_6MIm]^+$ cation adsorbed on the alkyl chains of the stationary phase. At constant concentration of this IL, ionic interactions derived from the adsorption of buffer anion on the stationary phase may be primarily responsible of the observed changes in retention.

3.5. Effect of ionic liquid and buffer on peak shape

The plots of the left and right half-widths at 10% peak height versus the retention time have demonstrated to be a practical tool to evaluate the effect on the peak shape of basic drugs of the silanol activity inside a chromatographic column [30,31]. The plots can be assimilated to linear trends:

$$A = m_A t_R + A_0 \quad (1)$$

$$B = m_B t_R + B_0 \quad (2)$$

In these equations, A and B are the left and right peak half-widths, m_A and m_B are the slopes of the linear correlations, and A_0 and B_0 represent the extra-column contributions to the peak broadening. The peak broadening rate inside the column is given by the sum of slopes ($m_A + m_B$), whereas the ratio (m_B/m_A) indicates the asymmetry of peaks eluting at a time where the extra-column contributions are non-significant. The half-width plots can be built from the data for: (i) all analytes eluted at each mobile phase composition, (ii) each analyte eluted at several mobile phase compositions, or (iii) all analytes eluted at all assayed mobile phase compositions (global plots).

Figs. 3 and 4 depict the half-widths plots obtained for the set of β-adrenoceptor antagonists, in the absence and presence of [C_6MIm][Cl], respectively, with 10 mM buffer concentration at pH 3 and 7. Similar plots were obtained for 5 and 30 mM buffer system. As observed, the correlations for the individual plots were highly satisfactory in all cases ($r^2 > 0.99$). In the absence of additive (Fig. 3), the slope of the right half-width (B) was significantly larger than for the left half-width (A), which was indicative of asymmetrical peaks seemingly produced by the slow interaction equilibrium between solutes and silanols in the stationary phase, especially at pH 7 (Figs. 3c and f). When [C_6MIm][Cl] was added, the angle between the straight-lines for the half-widths was smaller (i.e. the peaks reduced significantly their asymmetry), both at pH 3 (Figs. 4a to d) and 7 (Figs. 4c to f).

The parameters obtained from the fitting of the half-width plots in the absence of [C_6MIm][Cl] are shown in Table 2 (the trends for the width and asymmetry of the peaks of β-adrenoceptor antagonists, are shown in Figs. S2 and S3, respectively). As observed, an increasing buffer concentration in the range 5 to 30 mM resulted in narrower and more symmetrical peaks for the basic compounds, in the presence of all buffer systems. The changes in peak shape were especially notorious for the formate and citrate buffers (m_B/m_A values changed from 4.7 to 1.7, and 6.0 to 2.4, respectively). This again suggests that, in the absence of IL, the access of the cationic basic compounds to the silanols on the column is more efficiently hindered at higher buffer concentration, due to the increasing amount of the anionic species of the buffer adsorbed on the stationary phase.

Table 3 shows the parameters for the half-widths plots obtained at increasing buffer concentration, in the presence of [C_6MIm][Cl] in the mobile phase. Note that upon addition of the IL, the peak symmetry was significantly improved (with slope ratios, m_B/m_A, mostly close to one) and the peaks were narrower, even compared to those obtained with 30 mM buffer systems in the mobile phase without IL. Also, in the presence of IL, there is no apparent effect of the buffer concentration on both peak width and symmetry for all the assayed systems. Thus, at increasing buffer concentration, the peaks varied only slightly their slopes ratio between 1.3 and 1.1. The more symmetrical peaks, obtained in the presence of [C_6MIm][Cl] at any buffer concentration, indicate an effective column protection of the IL, which coat the surface of the C18 stationary phase.

At pH 7, silanols are significantly deprotonated, which is the reason of the poorer peak shape, especially at low buffer concentration (Table 2). However, at increasing buffer concentration, the peak shape improves. Again, the addition of [C_6MIm][Cl] at this pH gave rise to better performance, although it was slightly poorer compared to pH 3 (Table 3). The efficiency values (number of theoretical plates), obtained for the β-adrenoceptor antagonists

using all assayed buffer systems, in the absence and presence of [C_6MIm][Cl] are given in Tables S2 and S3 in the Supplementary material. Note that the information given by the efficiencies do not reveal the trends in the behavior of chromatographic peaks as clearly as the half-widths plots do, owing to the contribution of the extra-column tubing to peak broadening which is more significant for the less retained compounds.

3.6. Selectivity and resolution

In Sections 3.3. and 3.4, the effect of the addition of IL, and of the nature and concentration of the four buffer systems, on the retention is commented. The addition of IL gives rise to a significant reduction in retention. In contrast, in the presence and absence of IL, retention increases when larger concentration of buffer is added, especially in the case of the citrate system. Besides these changes in retention, it is convenient to check possible changes in selectivity (i.e. relative retention). In order to explore this, the retention factors of the β-adrenoceptor antagonists were correlated for different conditions. Figs. S4 and S5 depict the correlations achieved by comparing the retention factors with 30 mM buffer, in the absence and presence of IL, and without IL in the presence of 5 and 30 mM buffer, respectively. In all examined situations, and for all buffer systems, a high correlation was observed between the retention factors in the different conditions. This suggests similar selectivity when either IL or a larger concentration of the buffers is added to the mobile phase, although the retention times are different.

Chromatograms monitored at 254 nm for mixtures of the probe compounds eluted with mobiles phases containing 10% or 15% acetonitrile, and 30 mM buffer concentration in the absence and presence of 10 mM [C_6MIm][Cl], are shown in Figs. 5 and 6. The peaks obtained for timolol does not appear in the chromatograms, owing to its very low absorption at 254 nm (its absorption maximum is at 300 nm). The retention times for timolol in the absence and

presence of 10 mM [C_6MIm][Cl] were 38.40/8.67 (formate at pH 3), 38.42/8.56 (acetate at pH 3), 33.57/7.84 (phosphate at pH 3), 41.29/18.45 (phosphate at pH 7), 11.26/3.88 (citrate at pH 3) and 12.67/8.19 (citrate at pH 7). Note that for citrate the acetonitrile content was 15% instead of 10%.

The elution order of the mixture of β-adrenoceptor antagonists did not change by addition of the different buffer systems assayed in this work. As commented above, the retention times were significantly shorter and the behaviour of the peaks was enhanced in the presence of [C_6MIm][Cl]. The resolution was quite satisfactory, with baseline resolved peaks, except for the partial overlapping between metoprolol and acebutolol with the dihydrogen phosphate/hydrogen phosphate system at pH 7 (Fig. 6c), and atenolol and nadolol with the citric/dihydrogen citrate system at pH 3 (Fig. 6e). In Fig. 6c, the peak of nadolol (peak 2) appears as a double peak.

4. Conclusions

Protonated (cationic) basic compounds give rise to broad and asymmetrical peaks, due to their slow sorption-desorption kinetics on silanols. The adsorption on the stationary phase of different types of additives in the aqueous-organic mobile phase may avoid this interaction, but the retention behaviour is also affected. Thus, depending on the charge of the adsorbed additive, the retention may decrease (repelled by a cationic additive), or increase (attracted by an anionic additive).

The positive effect on the chromatographic performance of basic compounds when using ILs as mobile phase additives is well-known. However, these studies normally perform without considering in-depth the effect that the buffer can be also exerting. The $[C_6MIm]^+$ cation is highly adsorbed on these columns, whereas the chloride anion shows scarce adsorption. This gives rise to a significant reduction in the retention times of basic

compounds. Also, the adsorption of $[C_6MIm]^+$ is able to suppress the silanol activity giving rise to highly symmetrical peaks. On the other hand, the retention times increased when larger concentrations of the buffers acetic acid/acetate, citric acid/dihydrogen citrate, formic acid/formate and phosphoric acid/dihydrogen phosphate were added to the mobile phase, both in the absence and presence of $[C_6MIm][Cl]$. In spite of the different nature of the four acid-base systems, they have the common feature of forming anionic species at the working pH values (3 and 7).

At increasing buffer concentration, the retention factors followed a concave trend in the absence of IL for all acid-base systems, whereas the trend was almost linear in the presence of $[C_6MIm][Cl]$ at both pH 3 and 7. Although the change in retention was larger in the absence of IL, it was relatively more significant in the presence of the additive. The observed behaviour for the retention reveals a small adsorption of the buffer anions on the stationary phase. The adsorbed buffer anions attract the cationic basic compounds to the stationary phase, giving rise to larger retention times at increasing buffer concentration. In the series without IL, the buffer anions are directly adsorbed on the alkyl bonded chains on the stationary phase, and the observed concave trend in the retention indicates column saturation for the buffer anions. In the presence of IL, the buffer anions are probably attracted to the IL cation. The linear trends observed for the retention of the cationic compounds, at increasing concentration of buffer anions, indicate their increasing interaction by electrostatic attraction to the stationary phase covered by the cationic IL. Finally, the similar selectivity observed in the presence and absence of IL, and at varying concentration of the buffer anions, indicates that the observed effects should be explained mainly by the electrostatic interaction of the cationic solutes with the cationic IL and/or the anionic species of the buffer, adsorbed on the stationary phase.

On the other hand, in the absence of IL, the peak shape for basic compounds was improved (the peaks were narrower and more symmetrical) at larger concentration of the buffers. This also suggests the adsorption of the buffer anions on the stationary phase and their blocking effect on the activity of residual silanols in the C18 column, although this effect is weaker compared to that observed for $[C_6MIm]^+$. The kinetics of the electrostatic association of the basic compounds with the buffer anions seems to be faster than the kinetics for the ion-exchange processes involving the anionic silanols on the silica surface. In contrast to this behaviour, in the presence of $[C_6MIm][Cl]$, the effect on the peak shape of increasing concentrations of the buffers was non-significant, since the adsorbed IL already produces an effective blocking of the silanol activity with highly symmetrical peaks at all buffer concentrations.

Among the studied buffers, the citrate system seemed to show more affinity for the stationary phase, based on the larger retention times and better peak symmetries. Phosphate buffer yielded somewhat smaller retention than the formate and acetate buffers (which are only partially deprotonated in solution), whose effect on retention was similar. The increased retention observed with these two buffers suggests an induced deprotonation of the monoprotic acid-base systems due to anion adsorption, which would give rise to a larger concentration of the anionic species in the aqueous-organic mobile phases. In any case, in the absence and presence of additive, the studies in this work make attention on the relevance of controlling the buffer concentration on the analytical procedures.

Finally, it should be noted that due to the need to develop more biodegradable and safer ILs [50], recently, cations derived from natural sources, such as cholinium, morpholinium, or guanidinium are being used [51], in order to reduce the toxicity of common ILs based on imidazolium.

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FIGURE CAPTIONS

Fig. 1. Retention behavior of the β-adrenoceptor antagonists eluted from a C18 column with mobile phases containing a fixed concentration of acetonitrile and different concentrations of: (a) formate, (b) phosphate at pH 3, (c) phosphate at pH 7, (d) acetate, (e) citrate at pH 3, and (f) citrate at pH 7. Mobile phase composition was 10% acetonitrile, except for citrate buffer (15%). Solute identities: atenolol (●), nadolol (○), timolol (■), metoprolol (□), acebutolol (♦), and oxprenolol (◇).

Fig. 2. Retention behavior of the β-adrenoceptor antagonists, in the presence of 10 mM $[C_6MIm][Cl]$. Other conditions and compound identities are given in Fig. 1.

Fig. 3. Plots depicting the left (●) and right (○) peak half-widths versus the retention times for the assayed β-adrenoceptor antagonists, in the absence of ionic liquid. Other conditions are given in Fig. 1.

Fig. 4. Plots depicting the left (●) and right (○) peak half-widths versus the retention times for the assayed β-adrenoceptor antagonists, in the presence of 10 mM $[C_6MIm][Cl]$. Other conditions are given in Fig. 1.

Fig. 5. Chromatograms for a mixture of β-adrenoceptor antagonists detected at 254 nm, eluted with mobile phases containing a fixed concentration of acetonitrile and 30 mM buffer: (a) formate, (b) phosphate at pH 3, (c) phosphate at pH 7, (d) acetate, (e) citrate at pH 3, and (f) citrate at pH 7. Mobile phase composition was 10% acetonitrile, except for citrate buffer (15%). Solute identity: (1) atenolol, (2) nadolol (3) metoprolol, (4) acebutolol, and (5) oxprenolol.

Fig. 6. Chromatograms for a mixture of β-adrenoceptor antagonists detected at 254 nm, in the presence of 10 mM [C_6MIm][Cl]. Other details are given in Fig. 5.

Figure 1

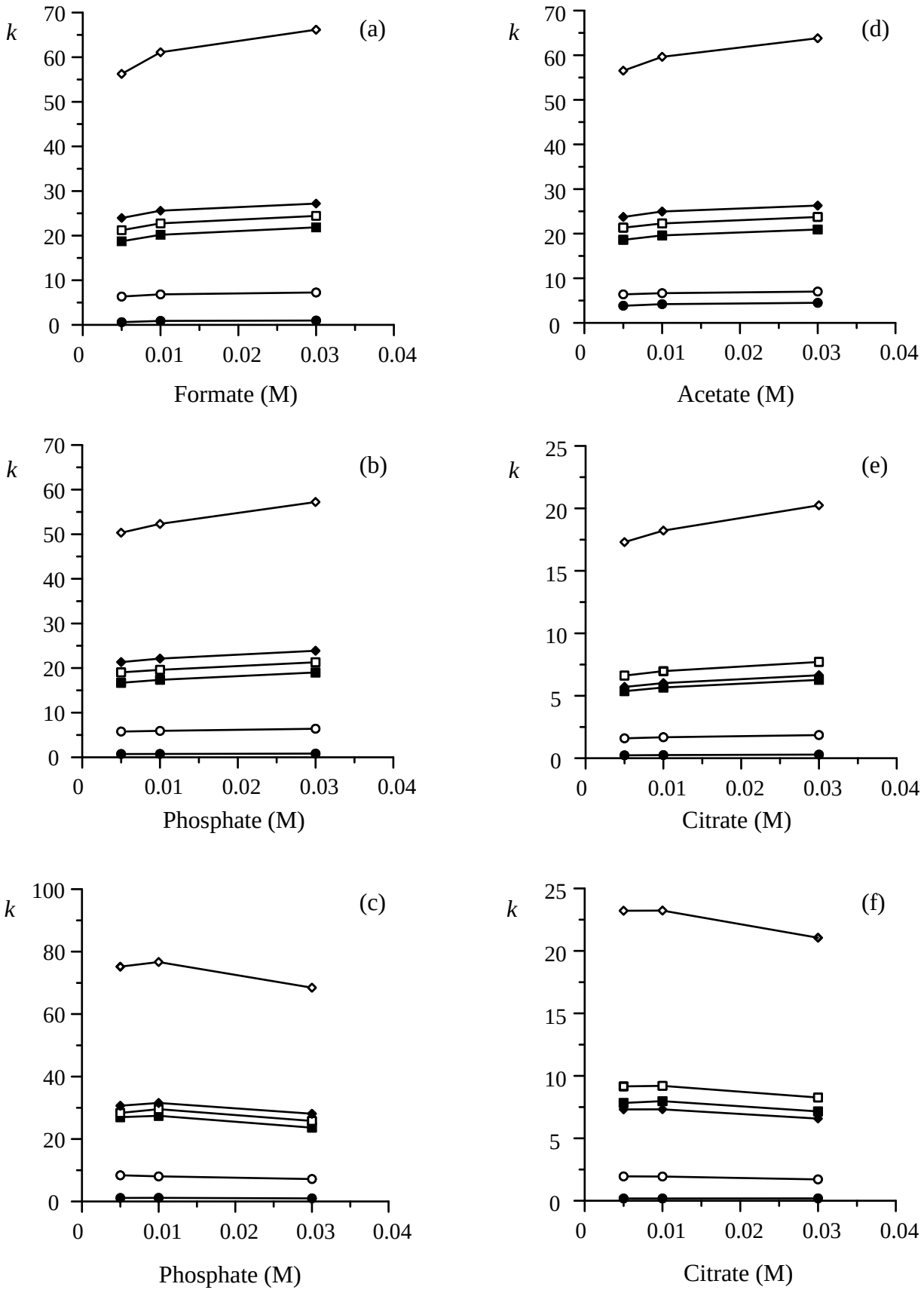

Figure 1 Peris et al.

Figure 2

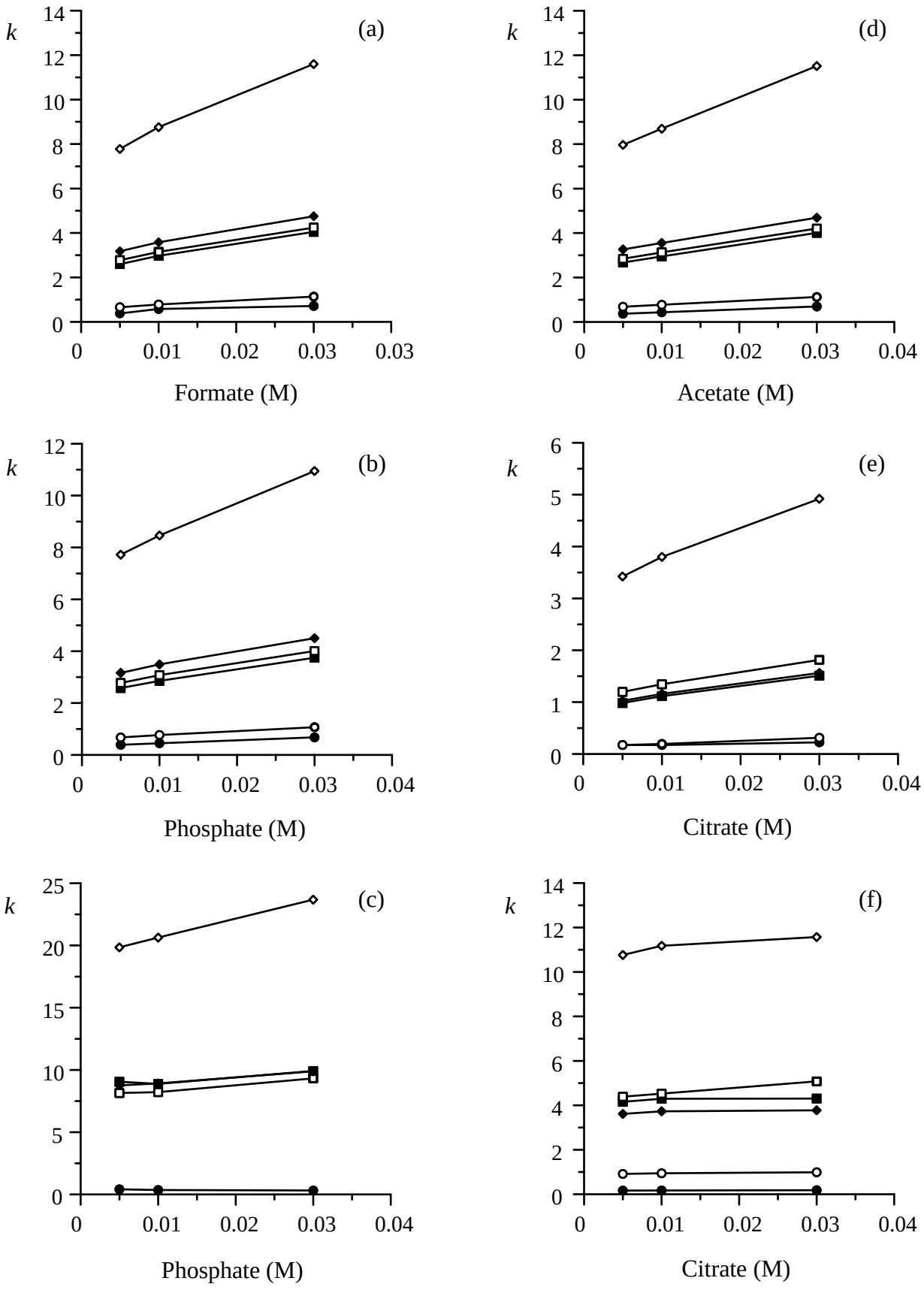

Figure 2 Peris et al.

Figure 3

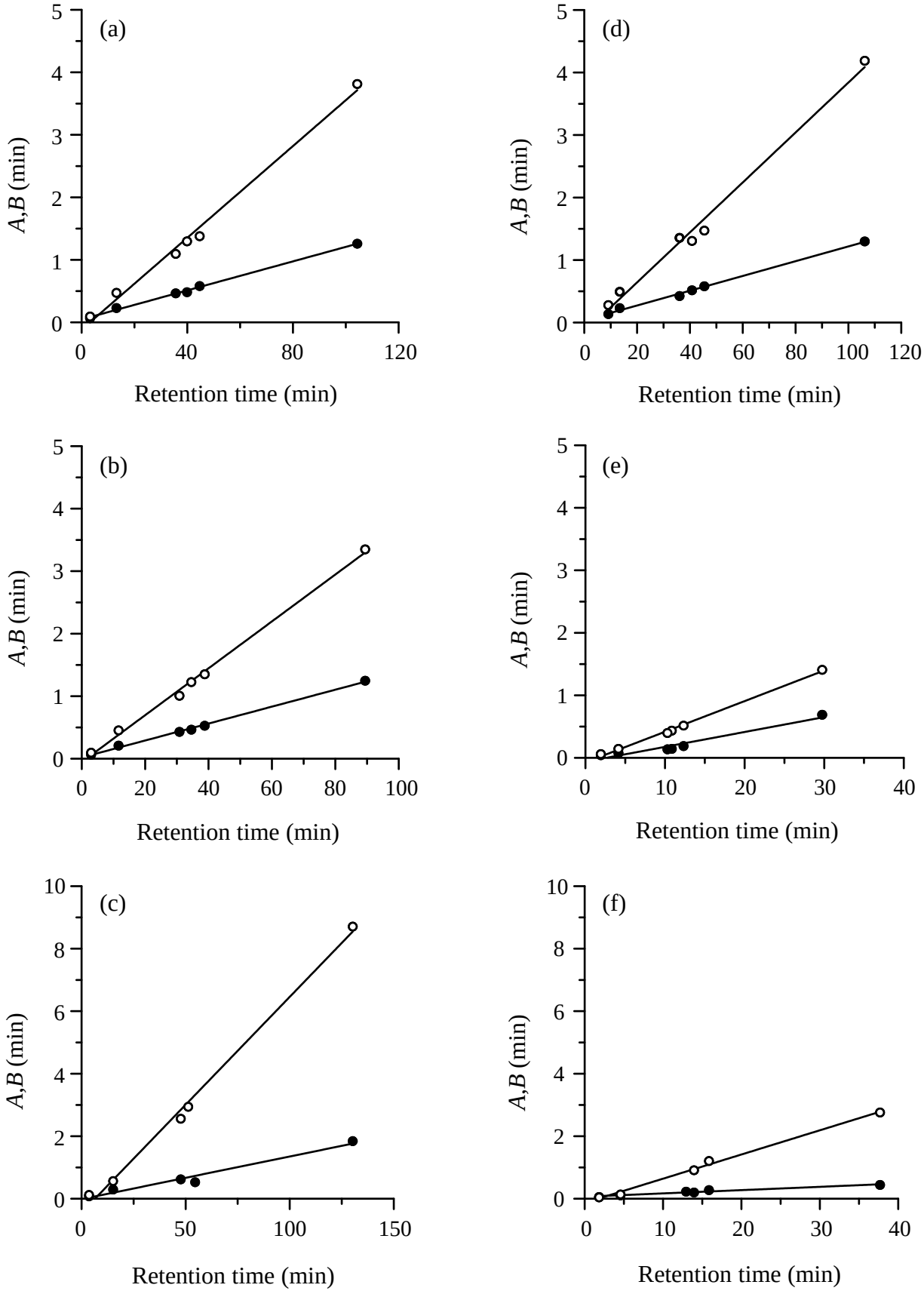

Figure 3 Peris et al.

Figure 4

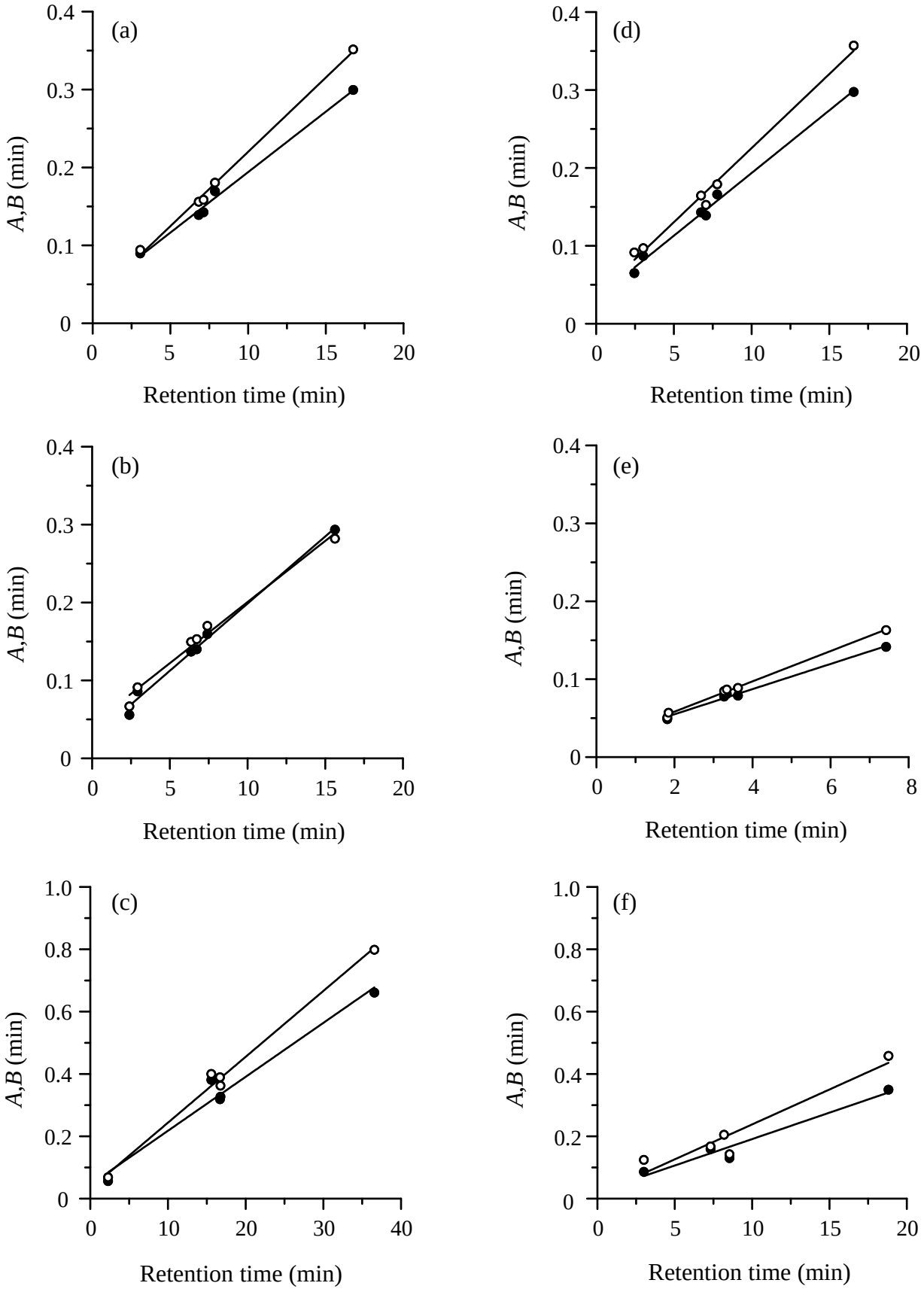

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Figure 5

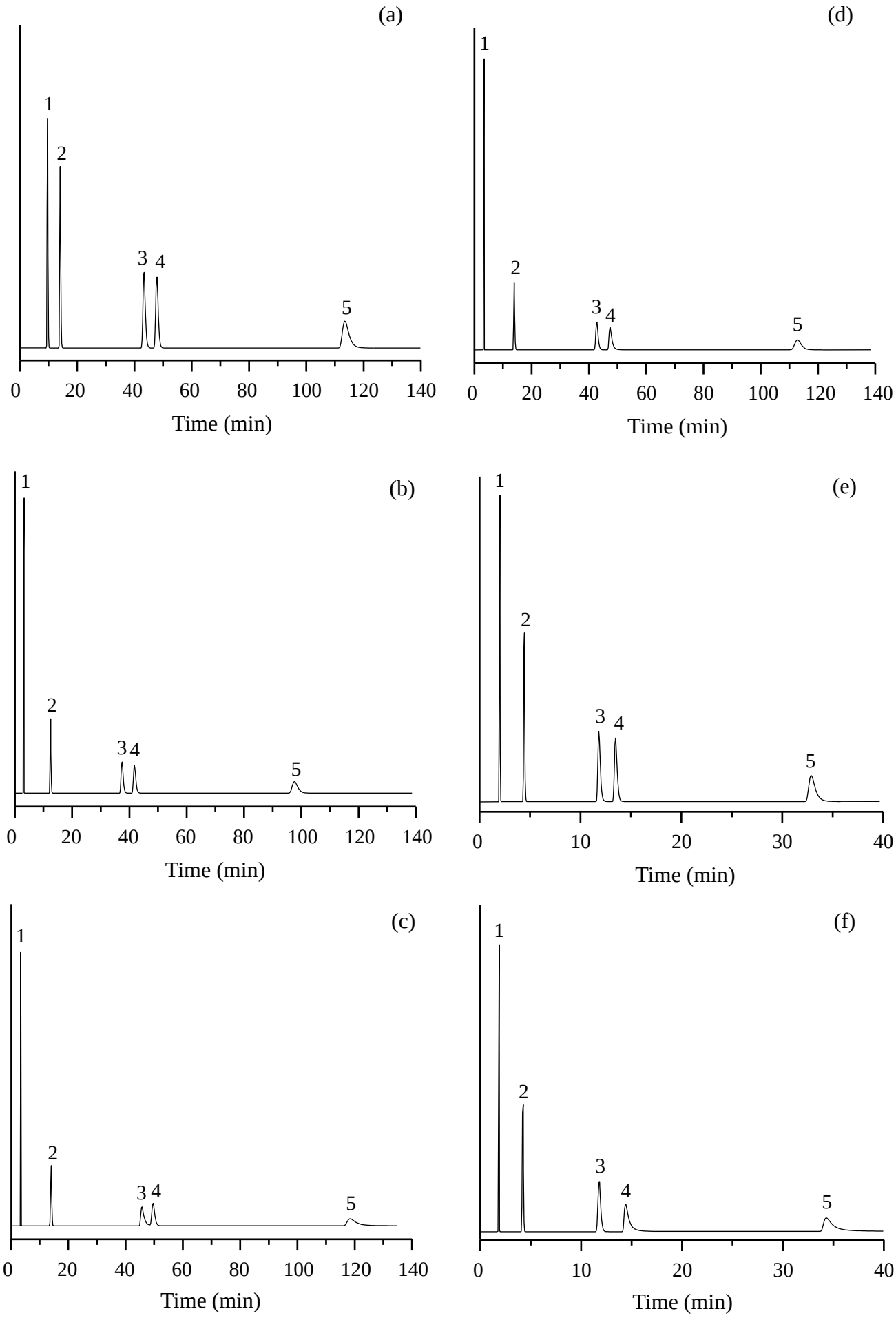

Figure 5 Peris et al.

Figure 6

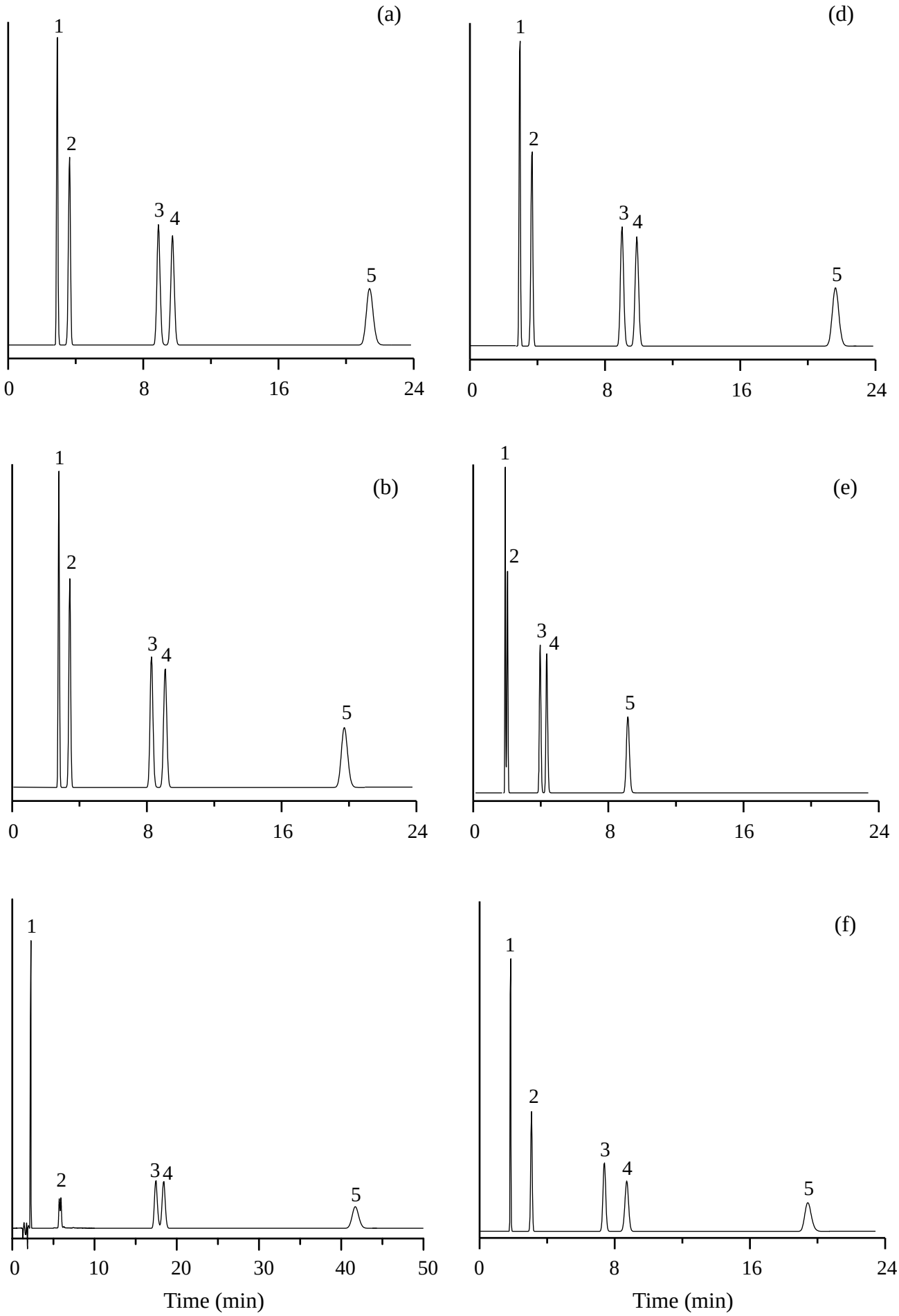

Figure 6 Peris et al.

Table 1. Experimental ΔpH values between aqueous and acetonitrile-water solutions at different buffer concentrations, in the absence and presence of 10 mM [C_6MIm][Cl].

Buffer	5 mM		10 mM		30 mM	
	No additive	[C_6MIm][Cl]	No additive	[C_6MIm][Cl]	No additive	[C_6MIm][Cl]
Acetic / acetate[a] (pH = 3)	0.47	0.48	0.44	0.42	0.35	0.35
Citric / dihydrogen citrate[b] (pH = 3)	0.31	0.34	0.30	0.34	0.30	0.35
Hydrogen citrate / citrate[b] (pH = 7)	0.56	0.58	0.61	0.60	0.58	0.64
Formic / formiate[a] (pH = 3)	0.33	0.30	0.23	0.25	0.19	0.23
Phosphoric / dihydrogen phosphate[a] (pH = 3)	0.45	0.46	0.36	0.38	0.30	0.31
Dihydrogen phosphate / hydrogen phosphate[a] (pH = 7)	0.32	0.35	0.39	0.37	0.37	0.39

[a]Acetonitrile-water 10:90 (v/v)

[b]Acetonitrile-water 15:85 (v/v)

Table 2. Half-widths plots parameters for the assayed β-adrenoceptor antagonists at increasing concentration of the buffer systems, at 10% or 15% acetonitrile without additive.[a,b]

		Acetate		Formate		Citrate		Phosphate	
	Buffer (mM)	$m_A + m_B$	m_B/m_A	$m_A + m_B$	m_B/m_A	$m_A + m_B$	m_B/m_A	$m_A + m_B$	m_B/m_A
pH 3	5	0.061	3.99	0.064	4.70	0.077	5.99	0.066	2.98
	10	0.052	3.39	0.048	3.16	0.073	2.06	0.051	2.78
	30	0.044	2.20	0.042	1.70	0.042	2.45	0.043	1.94
pH 7	5	–	–	–	–	0.106	7.69	0.065	3.13
	10	–	–	–	–	0.088	7.37	0.083	5.07
	30	–	–	–	–	0.067	4.56	0.064	3.53

[a] m_A and m_B are the slopes of the half-widths plots (see Eqs. (1) and (2)).

[b] See Table 1.

Table 3. Half-widths plots parameters for the assayed β-adrenoceptor antagonists at increasing concentration of the buffer systems, in the presence of 10 mM [C_6MIm][Cl] at 10% or 15% acetonitrile (see Table 1).[a,b]

		Acetate		Formate		Citrate		Phosphate	
	Buffer (mM)	$m_A + m_B$	m_B/m_A	$m_A + m_B$	m_B/m_A	$m_A + m_B$	m_B/m_A	$m_A + m_B$	m_B/m_A
pH 3	5	0.034	1.13	0.036	1.17	0.037	1.26	0.036	1.16
	10	0.035	1.18	0.035	1.23	0.036	1.20	0.033	0.91
	30	0.034	1.21	0.032	1.13	0.036	1.14	0.035	1.17
pH 7	5	–	–	–	–	0.042	1.40	0.045	1.56
	10	–	–	–	–	0.039	1.32	0.040	1.20
	30	–	–	–	–	0.039	1.31	0.039	1.13

[a] m_A and m_B are the slopes of the half-widths plots (see Eqs. (1) and (2)).

[b] See Table 1.

Table S1. Values of acid-base dissociation constants in water and acetonitrile-water for the buffers used in this work.

	pK_a [a]	${}^{s}_{w}pK_a$ [b]
Buffer	Water	10 – 15% (*v*/*v*) acetonitrile
Acetic acid / acetate	4.74	4.93
Citric acid /dihydrogen citrate	3.16	3.30
Hydrogen citrate/citrate	6.42	6.61
Formic acid / formiate	3.72	3.93
Phosphoric acid / dihydrogen phosphate	2.21	2.38
Dihydrogen phosphate/ hydrogen phosphate	7.23	7.39

[a] Ref. [40].

[b] ${}^{s}_{w}pK_a$ was obtained by calibrating the electrode system with aqueous buffers, and measuring the pH in the mobile phase after mixing the aqueous buffer with the organic modifier.

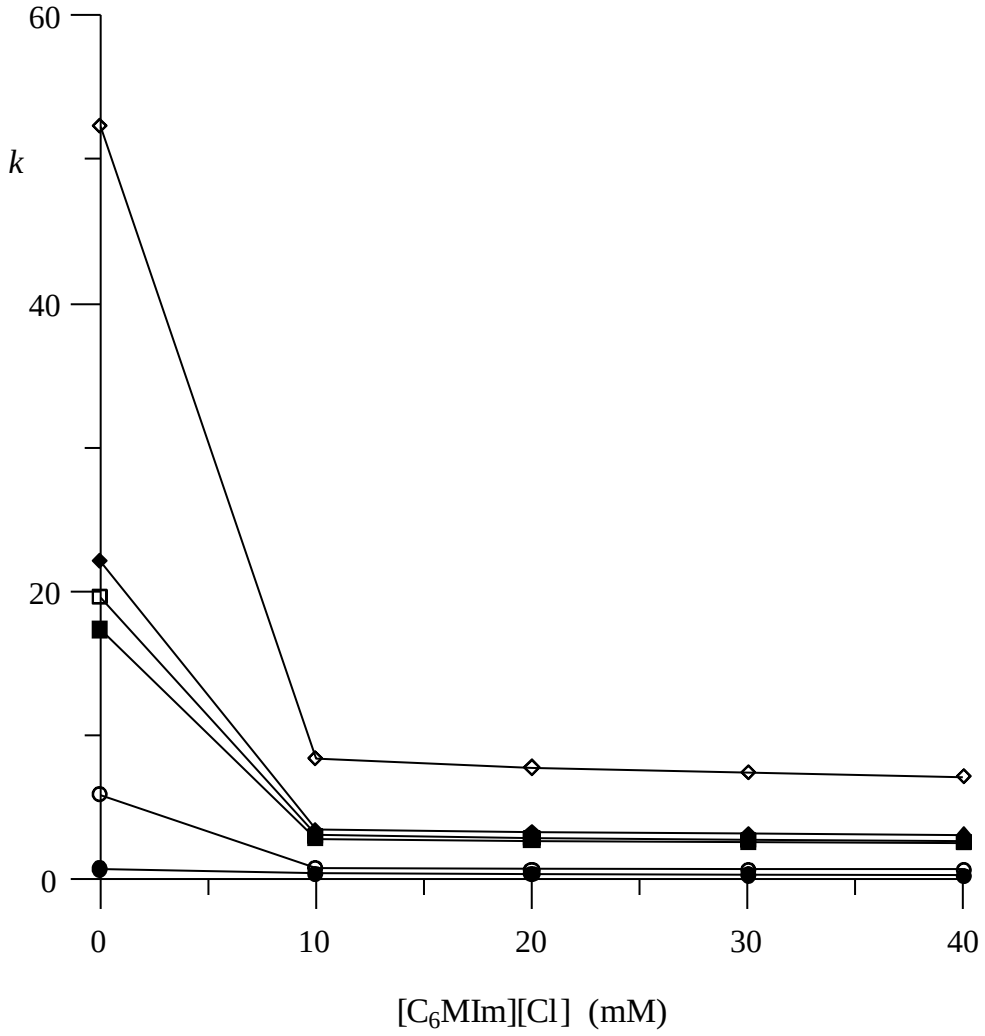

Fig. S1. Retention behavior of the assayed β-adrenoceptor antagonists eluted from a C18 column with mobile phases containing 10% acetonitrile, 10 mM phosphate at pH 3, and increasing concentrations of 1-hexyl-3-methylimidazolium chloride. Solute identities: atenolol (●), nadolol (○), timolol (■), metoprolol (□), acebutolol (♦), and oxprenolol (◇).

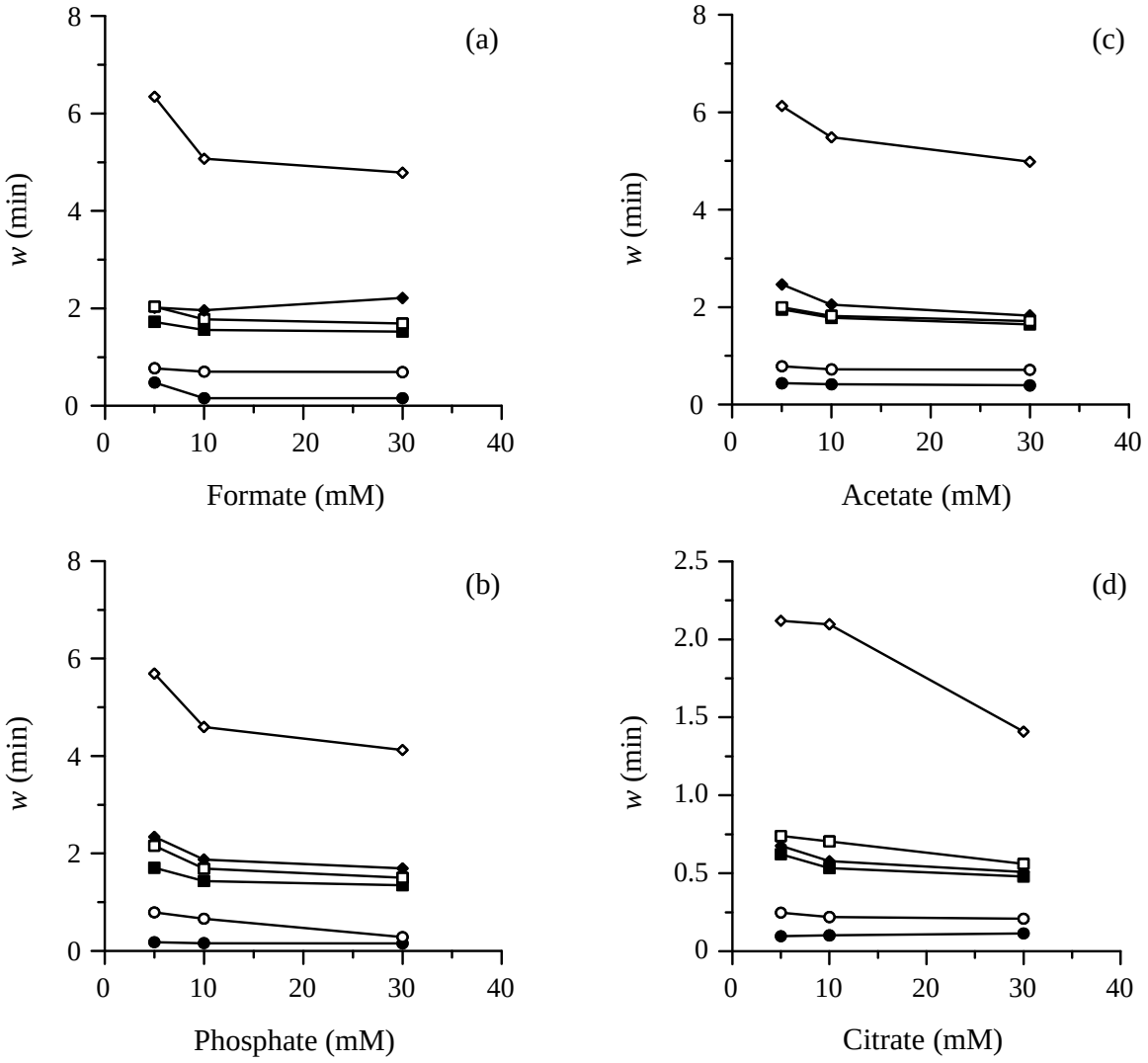

Fig. S2. Peak widths for the probe compounds eluted from a C18 column with mobile phases containing a fixed concentration of acetonitrile and different concentrations of: (a) formate, (b) phosphate at pH 3, (c) acetate, and (d) citrate at pH 3. Mobile phase composition was 10% acetonitrile, except for citrate buffer (15%). Solute identities are given in Fig. S1.

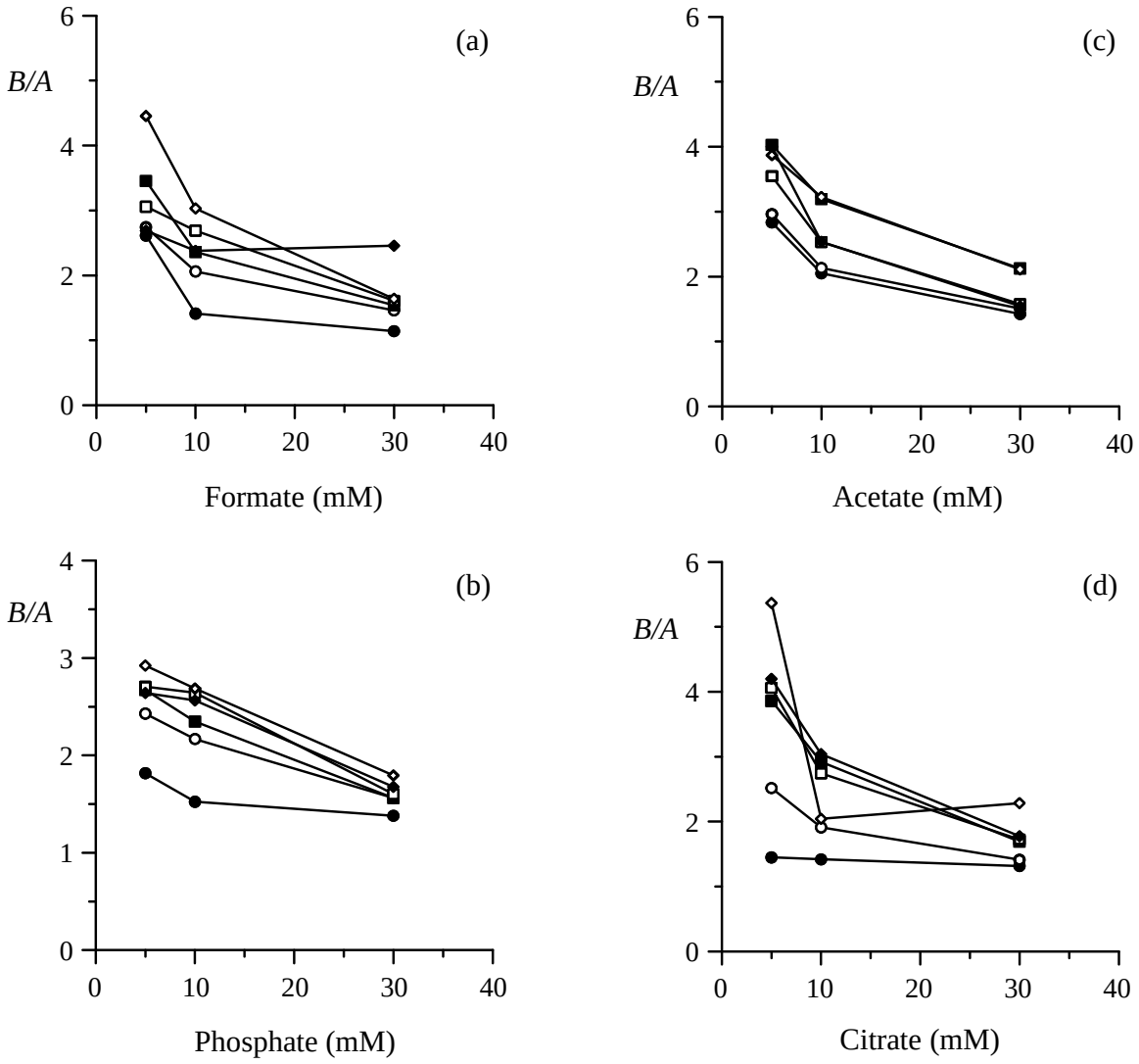

Fig. S3. Peak asymmetries for the probe compounds eluted from a C18 column with mobile phases containing a fixed concentration of acetonitrile and different concentrations of the buffer systems. Other details are described in Figs. S1 and S2.

Table S2. Peak efficiencies (*N*, theoretical plates) observed for the β-adrenoceptor antagonists in acetonitrile-water mixtures at pH 3, without ionic liquid, at several buffer concentrations.

	Acetic acid / acetate (mM)			Formic acid / Formate (mM)			Phosphoric acid / dihydrogen phosphate (mM)			Citric acid / dihydrogen citrate (mM)		
Compound	5	10	30	5	10	30	5	10	30	5	10	30
Atenolol	3800	6000	4600	3600	6300	7600	3500	5200	6000	6000	5500	4900
Nadolol	2700	4200	5900	2700	4400	6100	2300	3800	5900	2900	4700	7000
Timolol	2500	3800	6700	3300	6000	9500	3200	5300	9100	2000	3700	7800
Metoprolol	3300	5500	9400	3300	5300	9300	2500	4500	9000	2000	3200	8000
Acebutolol	2500	5400	10200	4600	6000	5200	2700	4600	8600	1900	3400	7400
Oxprenolol	2200	3500	6400	1700	4100	8000	2300	4000	7700	1100	2500	6400

Table S3. Peak efficiencies (*N*, theoretical plates) observed for the β-adrenoceptor antagonists in acetonitrile-water mixtures at pH 3, containing 10 mM [C_6MIm][Cl], at several buffer concentrations.

	Acetic acid / acetate (mM)			Formic acid / Formate (mM)			Phosphoric acid / dihydrogen phosphate (mM)			Citric acid / dihydrogen citrate (mM)		
Compound	5	10	30	5	10	30	5	10	30	5	10	30
Atenolol	6000	3800	6300	6200	9100	6600	6100	6500	6300	7100	6000	7900
Nadolol	4500	4800	4200	5400	5000	4600	4800	4900	4500	6600	3600	4900
Timolol	8500	8400	9100	8600	9400	9500	8500	8700	9500	7000	7200	7800
Metoprolol	9400	10400	9300	8300	9900	9400	9300	9400	9500	6400	8200	7800
Acebutolol	8700	9100	9500	9000	9100	10000	9000	9100	9800	6900	7100	7600
Oxprenolol	11400	10900	11900	11000	11400	13300	10800	11100	11500	10000	10300	10800

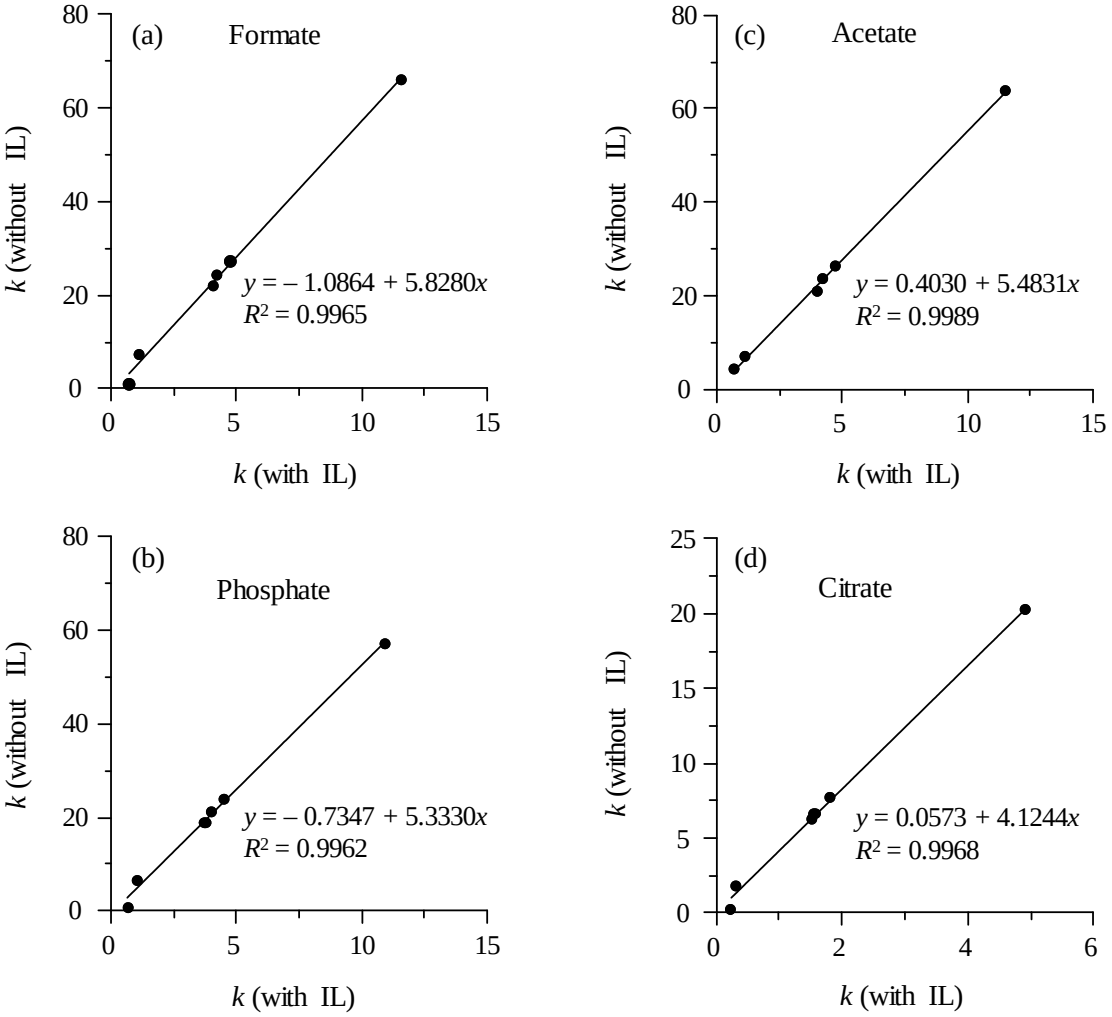

Fig. S4. Selectivity comparison for the probe compounds eluted from a C18 column with mobile phases containing a fixed concentration of acetonitrile and 30 mM buffer system at pH 3. Mobile phase composition was 10% acetonitrile, except for citrate buffer (15%). The plotted data are the retention factors in the absence and presence of [C_6MIm][Cl] for the six probe compounds.

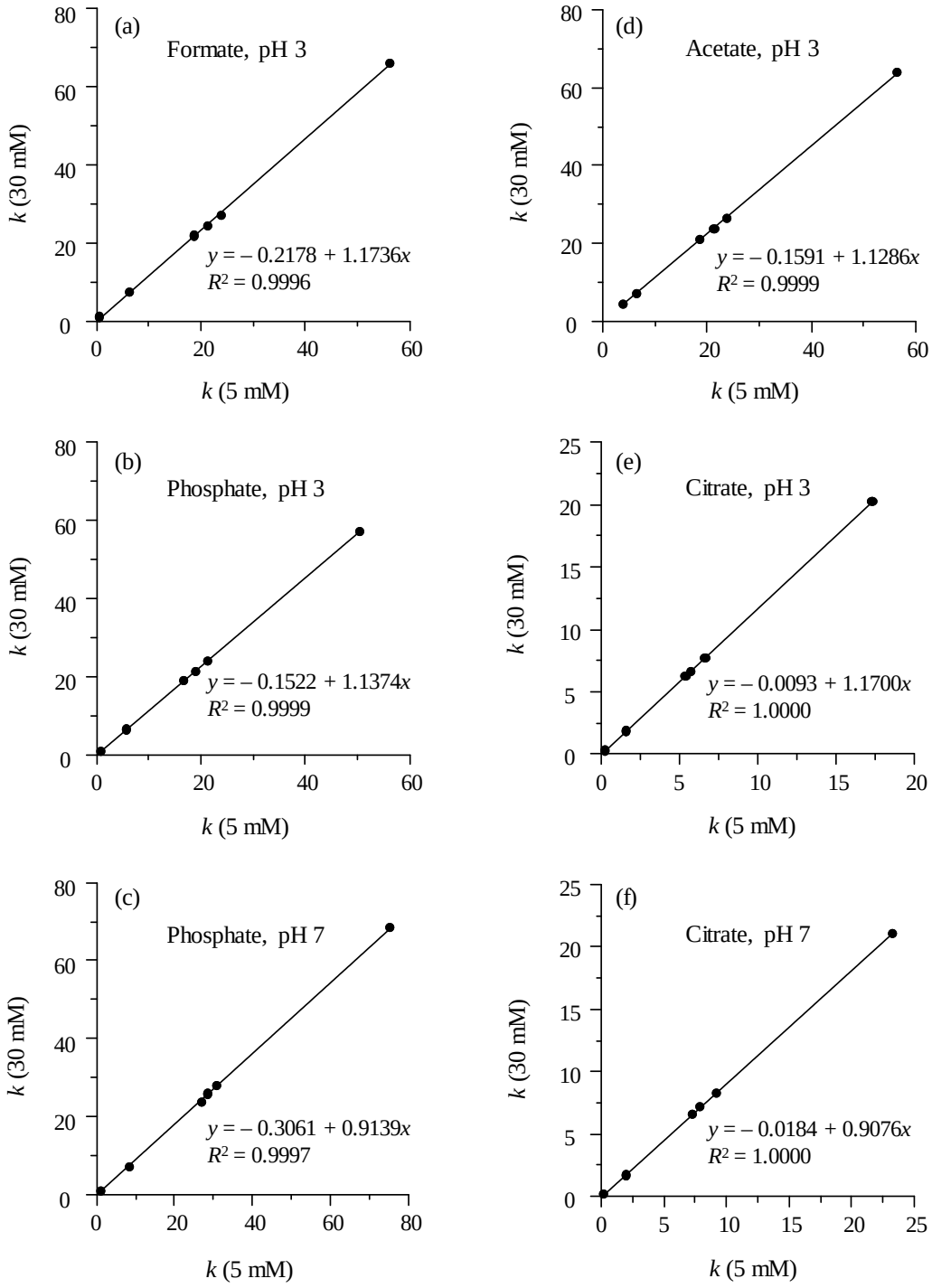

Fig. S5. Selectivity comparison for the probe compounds eluted from a C18 column with mobile phases containing a fixed concentration of acetonitrile in the absence of ionic liquid. Mobile phase composition was 10% acetonitrile, except for citrate buffer (15%). The plotted data are the retention factors for the six probe compounds, with mobile phases that contained 5 and 30 mM buffer systems.