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Differences in preparative loadability between the charged and uncharged forms of ionizable compounds

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Abstract

Ionizable compounds experience a drastic difference in preparative loadability as a function of pH. It can be shown that the preparative loadability of a compound in the ionic form is by a factor of 20 or more inferior to the loadability of the same compound in the unionized form. In this paper, we demonstrate the reason for this behavior, and show practical applications of the principle.

Keywords: Preparative chromatography; Adsorption isotherms; Ionic compounds

1. Introduction

In analytical chromatography, the separation and quantitation of a multitude of compounds is the primary goal of the separation. In preparative chromatography, the production of a single compound and its purity are often the principal concerns [1]. This is especially true for modern application methods of preparative chromatography in the purification of compounds from combinatorial synthesis [2]. In such a case, the primary goal is the identification of the compound of interest among the side products, together with an efficient and preferentially automated collection of this entity. Blind and automated methods are preferred that do not require the attendance of skilled personnel to maximize the load or throughput of the preparative separation. In addition, the throughput of the generic method is important, since the large number of samples to be purified requires often that the preparative HPLC instrument is in operation for 24 h a day. Of course, it is preferred if the entire sample can be purified in a single chromatographic separation instead of multiple runs.

In another important application of preparative chromatography, the impurities or degradation products of a parent compound are of interest. In such a case, the parent sample delivers a range of different chemical entities, for the most part of similar or related structure to the parent compound. A sufficient quantity of the impurities or degradation products is necessary for further identification of their chemical structure, or for toxicity tests. Once again, the most preferred approach is to maximize the preparative load without a compromise in the purity of the collected fractions.

In all preparative applications, a certain amount of purified material is desired. The cost of preparing this quantity depends strongly on the preparative loadability of the compound of interest. If the loadability is low, either a larger preparative bed is required to generate the necessary amount of purified compound, or multiple preparative runs are needed at the expense of time. Chromatographers have become accustomed to the fact that the preparative loadability may vary widely with the nature of the compound. Therefore, many preparative separations, especially those carried out blindly, are executed under conditions of analytical load and thus do not take advantage of the true loadability of the packings available. However, this is inefficient and expensive.

We have examined some of the parameters that are important for maximizing the load in preparative chromatography. In a previous publication [3], we looked at the effects caused by the way the sample is injected onto the preparative column. If a sample is loaded onto a reversed-phase column from an organic solvent such as dimethyl sulfoxide (DMSO), overload effects can be caused by the presence of the organic solvent rather than the true sample load. Such

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effects can be eliminated by using a special injection technique named at-column dilution [3]. Similar effects can be found when ionizable sample compounds are injected. The underlying factor of the latter phenomenon is the difference in preparative loadability between the ionic form and the non-ionic form of an ionizable sample. The adsorption of non-ionic samples can be described by simple isotherms such as the Langmuir isotherm [4]. On the other hand, the adsorption of ionic analytes is complicated by the mutual repulsion of the ions adsorbed on the surface of the packing. This effect can be described by a modification of the Langmuir isotherm [5] that takes account of this repulsion. In this paper, we examine the consequences of this effect for the preparative chromatography of ionizable compounds in more detail. First, we demonstrate the improvement in loadability for the non-ionic form of the compound. Then the focus is on simple practical solutions to the problem of maximizing load in the preparative chromatography of ionizable compounds.

Recently, Buckenmaier et al. [6] reported on the causes of the tailing of basic compounds on reversed-phase packings. They arrived also at the conclusion that the mutual repulsion of adsorbed ionic species on the surface of a packing is a major contributor to tailing. Our studies examine the theoretical framework, but more importantly expand the view of the phenomena to the difference in the behavior of ionized samples and non-ionized samples, including both acidic and basic analytes. In addition, the observations reported here do not rely on the existence or absence of surface silanols. The practical consequences of this understanding are not only important for preparative chromatography, but also for analytical chromatography.

2. Theory

The adsorption of a non-ionic molecule on a reversed-phase surface can often be described by a Langmuir isotherm [1]:

$$\frac{q}{q_\infty} = \frac{Kc}{1 + Kc} \tag{1}$$

where q is the adsorbed analyte concentration, q_∞ the concentration at saturation of the surface, c the concentration in the liquid phase, and K is the equilibrium constant.

In the case of an ionic compound, the Langmuir isotherm needs to be modified to include the effect of the mutual repulsion of the adsorbed ions [5]:

$$\frac{q}{q_\infty} = \frac{Kcf(q)}{1 + Kcf(q)} \tag{2}$$

The modification function $f(q)$ is given by Häglund and Ståhlberg [5] to be:

$$f(q) = \mathrm{e}^{-z^2Bq} \tag{3}$$

with z being the charge of the sample compound and with B as the following function:

$$B = \frac{F^2}{\kappa\varepsilon_0\varepsilon_\mathrm{r}RT}\frac{I_0(\kappa r)}{I_1(\kappa r)} \tag{4}$$

where F is the Faraday constant, ε_0 the permittivity in the vacuum, ε_r the dielectric constant of the liquid, and r is the pore radius of the packing and $1/\kappa$ is the Debye length, which is a measure of the thickness of the double layer. I_0 and I_1 are the modified Bessel functions of the first kind of order zero and one. Since we will not vary the temperature nor the pore diameter, it suffices to treat B as a constant for the purposes of our study here. It should be pointed out though that B is a function of the pore radius r accessible to the analyte.

For data analysis, it is necessary to convert Eq. (2) into an explicit form for the concentration of the sample in the mobile phase:

$$c = \frac{1}{K}\frac{q}{q_\infty - q}\mathrm{e}^{z^2Bq} \tag{5}$$

Note that in this equation the analyte concentration in the mobile phase has been expressed as a function of its concentration in the stationary phase, while the commonly used adsorption isotherms describe the concentration in the stationary phase as a function of the mobile phase concentration. This however does not impede the assessment of the equation, since it simply involves an inversion of the dependent and the independent parameters for curvefitting purposes.

All our compounds are singly charged, i.e. $z = 1$. The slope of the isotherm is related to the retention factor k_0 at low analyte concentration:

$$K = \frac{k_0}{\phi} \tag{6}$$

where ϕ is the phase ratio. Thus the equation used for curvefitting to the Ståhlberg equation was as follows:

$$c = \frac{\phi}{k_0}\frac{q}{q_\infty - q}\mathrm{e}^{B^*q} \tag{7}$$

Curvefitting to the Langmuir equation followed Eq. (1), from which the retention factor at infinite dilution was determined via:

$$k_0 = \phi K q_\infty \tag{8}$$

To illustrate the difference in the isotherms and in the preparative loadability, we have calculated the adsorption isotherm for a singly charged analyte on a packing with a pore size of 10 nm using the Ståhlberg equation. For comparison, a simple Langmuir isotherm with an equal distribution coefficient at infinitely low sample concentration (=equal retention factor) was chosen. Fig. 1 compares the sorption isotherms of the uncharged (Langmuir) and the charged form (Ståhlberg) for an equal concentration in the mobile phase and at an equal distribution coefficient between the mobile phase and the stationary phase. It is clear that the isotherm of the charged form flattens out at a lower concentration of

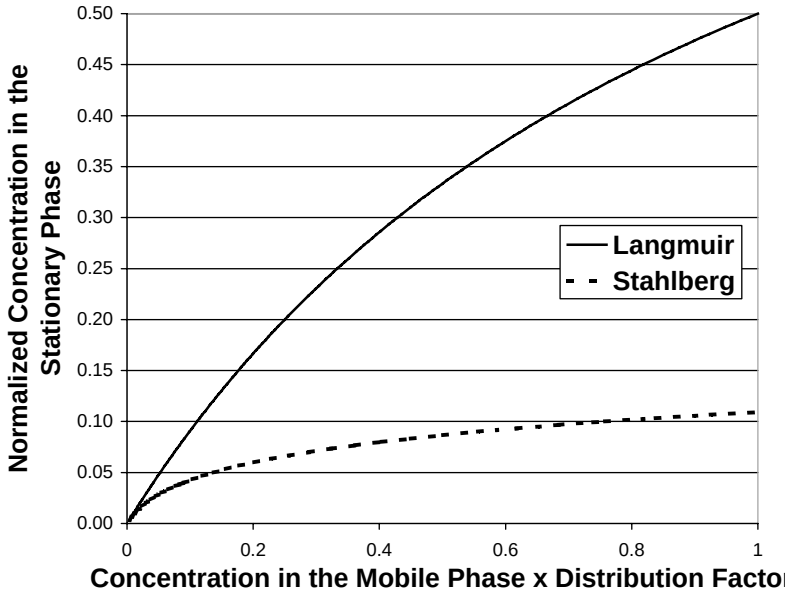


Fig. 1. Comparison of adsorption isotherms for a non-ionic (Langmuir) and an ionic sample (Ståhlberg). Note that the Ståhlberg isotherm flattens out at a much lower mobile phase concentration of the analyte!

sample in the stationary phase than the isotherm for the neutral form. This means fundamentally that the saturation of the stationary phase is reached at a lower concentration for a charged species compared to an uncharged species.

This can be seen even clearer in Fig. 2. Here, we have plotted the concentration in the mobile phase versus the derivative of the adsorption isotherm, dq/dc. This is in essence a representation of a peak shape in preparative chromatography [7]. The x-axis is proportional to the retention factor of a peak, or a slice of a peak in overload, while the y-axis is proportional to the detector response of a peak, and thus proportional to the concentration in the mobile phase. As the concentration of the compound in the mobile phase increases, the retention factor decreases. This decrease is much more pronounced for the charged species than for the uncharged species. As a matter of fact, for a roughly equal decrease in the retention factor as shown by the arrows, the isotherm of the uncharged species permits a roughly 20-fold higher load than the isotherm of the charged species. With other words, the preparative loadability is about 20-fold higher for a non-ionic sample than it is for an ionic sample. The remainder of the paper will demonstrate this effect in practice.

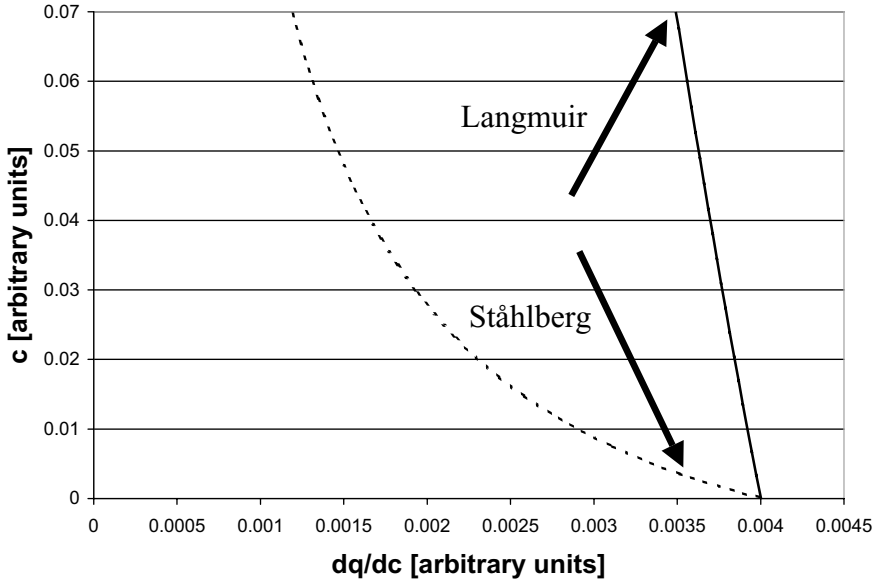


Fig. 2. Plot of the concentration versus the first derivative of the adsorption isotherm. This is a representation of the tail of a chromatographic peak under overload conditions. Note the larger peak distortion at lower load for the Ståhlberg isotherm, which represents an ionic sample!

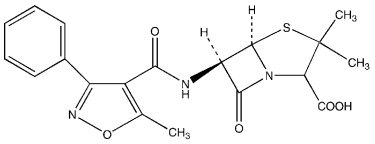
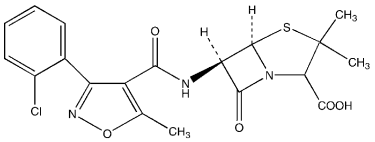
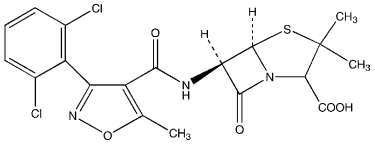
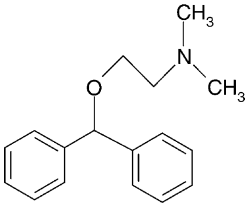
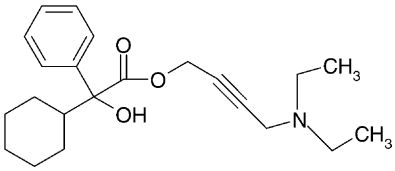
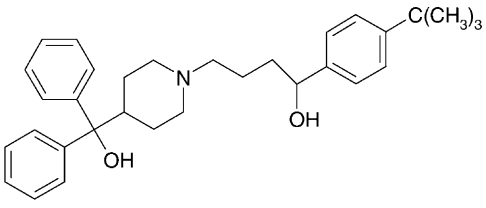
3. Experimental

During the studies reported here, several Waters instruments were used. We either utilized an Alliance system for the chromatography on small columns or a Waters Prep LC 4000 with 2700 Sample Manager and 2487 UV detector for the chromatography on large diameter columns.

The columns used were 19 mm i.d. XTerraPrep MS C_{18} columns or 4.6 mm i.d. XTerra MS C_{18} from Waters, Milford, MA, USA, with a length of 5 cm and packed with 5 μm particles. The particles have a specific pore volume of 0.63 ml/g and a specific surface area of 170 m^2/g before bonding. The surface coverage was 2.37 μmol/m^2. The phase ratio was determined from the information reported on the Certificate of Analysis supplied with the column and was 0.10, in units of ml of stationary phase per ml of mobile phase.

Oxacillin, cloxacillin, dicloxacillin, diphenhydramine, terfenadine and oxybutynin (Table 1) were obtained from Sigma. Literature pK_a values [9] were 2.7 for cloxacillin [10], 2.8 for dicloxacillin [11], and 9.0 for diphenhydramine [12]. The pK_a value for oxacillin was reported to be 2.7 [13], and 7.0 for oxybutynin [13]. High-purity water was generated with a Milli-Q system from Millipore, Bedford, MA, USA. The solvents used were all HPLC grade from J.T. Baker. For the studies of the adsorption isotherms of the six basic and acidic compounds, a constant concentration of 100 mM formic acid was used for the acidic pH and 100 mM ammonia for the basic pH. 1 mg of sample was applied to a 4.6 mm × 50 mm XTerra MS C_{18} column. Buffer pH measurements were performed using an Orion Model 720A pH meter calibrated just before the measurement. The column dead volume was measured using acetone. The value was corrected for the volume in the connection tubing

Table 1
Test compounds used in this study

Name	Structure
Oxacillin	
Cloxacillin	
Dicloxacillin	
Diphenhydramine	
Oxybutynin	
Terfenadine	

(100 μl). The studies were carried out at room temperature in a laboratory with central heating and cooling. The temperature does not vary by more than 1 °C around 21 °C.

Elution by characteristic point [4] was the method for the determination of the isotherms. Prostat from Polysoftware, Salt Lake City, was used for non-linear curvefitting to the various isotherms.

4. Discussion

In order to understand the impact of pH changes on the preparative loadability, we investigated the effects using both acidic and basic probes. For both types of compound, the pH was changed from acidic conditions around pH 3 to basic conditions around pH 10. As a consequence, acidic compounds are largely non-ionized under acidic conditions, and are completely ionized under basic conditions. For basic compounds, the opposite is true: at acidic pH they are protonated and ionized, while they are largely non-ionized under alkaline mobile phase conditions. Since there is a large difference in the retention factor of the ionized and the non-ionized form of the same sample, the organic concentration in the mobile phase needs to be adjusted to give roughly the same retention factors under acidic and basic conditions. The general rule of thumb says that there is roughly a factor of 10–30 difference in the retention of the ionized form and the non-ionized form of a compound, requiring an adjustment of the mobile phase composition by about 20% to achieve a similar retention factor.

A direct comparison of the preparative loadability for acidic and basic compounds under acidic and basic conditions is shown in Fig. 3a and b for an acidic compound and in Fig. 4a and b for a basic compound. Note that in all cases an equal load of sample was applied under acidic and basic conditions! We used a constant concentration of 100 mM formic acid for the acidic pH, and 100 mM ammonia for the alkaline pH. The organic solvent in the mobile phase was acetonitrile, and the different conditions used for the different analytes are shown in Table 2.

Fig. 3a shows the sorption isotherm for the acidic sample oxacillin under acidic conditions. The sample was therefore adsorbed in the neutral form. A mild overload has been achieved, but the departure of the isotherm from linearity is rather small. In contrast, a substantial departure from linearity can be seen in Fig. 3b for the ionized form of the same sample and the same load under basic mobile phase conditions. This departure from linearity resulted in a spreading of the sample over a broader range in the chromatogram, as can be seen from a comparison of the scales in Fig. 3a and b.

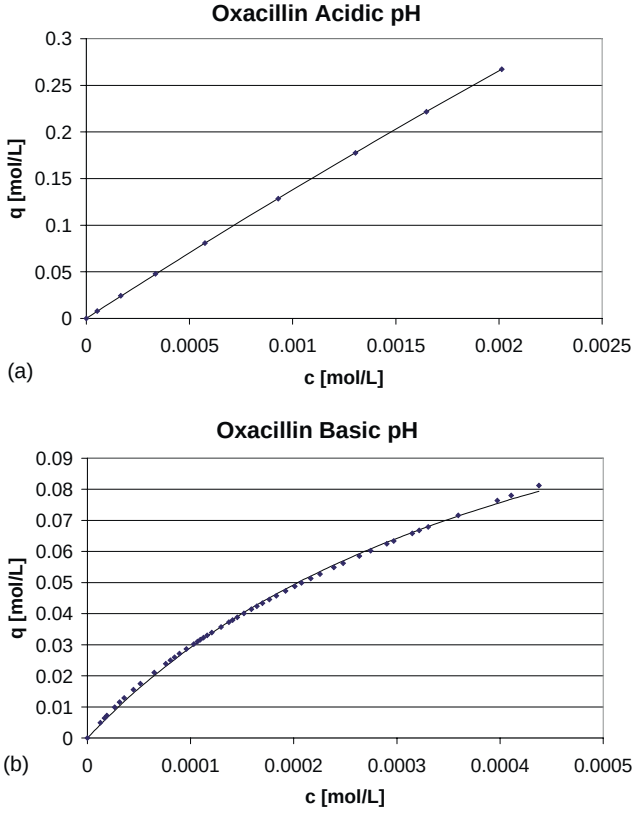


Fig. 3. Measured adsorption isotherms for an acidic sample, oxacillin: (a) acidic mobile phase; (b) basic mobile phase. Column: XTerra MS C_{18}, 4.6 mm × 50 mm. Mobile phases: see Table 2.

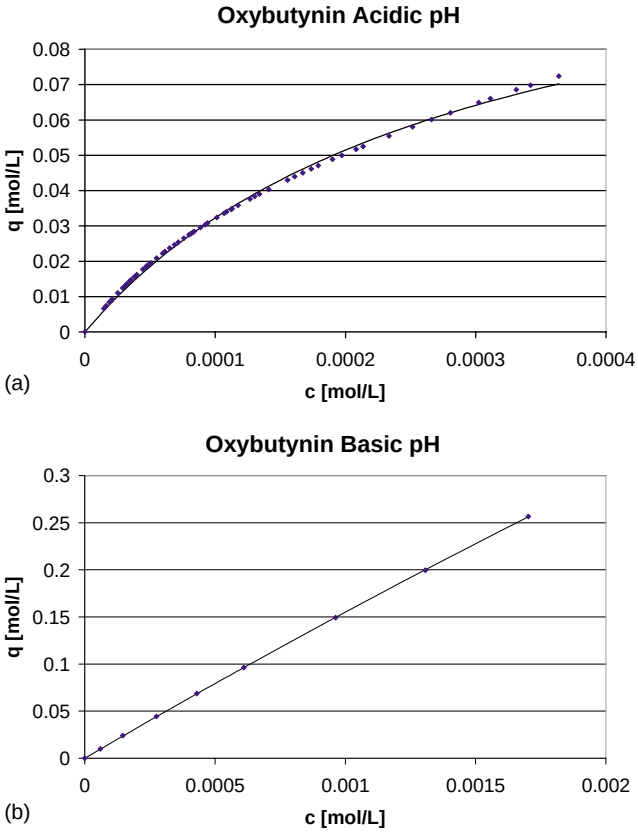


Fig. 4. Measured adsorption isotherms for a basic sample, oxybutynin: (a) acidic mobile phase; (b) basic mobile phase. Column: XTerra MS C_{18}, 50 mm × 4.6 mm. Mobile phases: see Table 2.

Table 2
Coefficients for the isotherms for acidic and basic compounds in charged and uncharged form

	Oxacillin	Cloxacillin	Dicloxacillin	Diphenhydramine	Oxybutynin	Terfenadine
Uncharged (fit to Langmuir equation)						
k_0	13.6	13.5	14.4	17.8	15.3	–
q_∞ (mol/l)	3.50	3.20	3.01	2.34	3.58	–
r^2	0.999998	0.999999	0.999999	0.999981	0.999995	–
Organic (%)	25.2	27.9	31.5	36.0	45.9	55.8
Charged (fit to Langmuir equation)						
k_0	33.4	29.3	31.3	38.0	40.9	38.8
q_∞ (mol/l)	0.163	0.145	0.156	0.275	0.127	0.0205
r^2	0.99953	0.99981	0.99962	0.99979	0.99913	0.99954
Organic (%)	7.2	9.9	13.5	6.3	15.3	27.9
Ratio q_∞	21.6	22.2	19.4	8.5	28.3	–
Charged (fit to Ståhlberg equation)						
k_0	35.5	30.6	33.1	39.7	44.3	41.3
B^* (l/mol)	7.82	8.27	8.04	3.91	11.0	69.7
r^2	0.99988	0.99995	0.99991	0.99996	0.99981	0.99992

Fig. 4a and b shows the equivalent results for a basic analyte, oxybutynin. As in Fig. 3, the acidic mobile phase conditions are shown in Fig. 4a, and the basic mobile phase conditions are depicted in Fig. 4b. The basic analyte is completely ionized under acidic conditions (Fig. 4a) and exhibits a curved isotherm, typical for preparative overload. Contrary to the behavior under acidic conditions, the isotherm is still rather linear under basic conditions (Fig. 4b), where the basic analyte is not ionized.

Figs. 3 and 4 thus demonstrate the same effect for both acidic and basic analytes. In both cases, the neutral form of the analyte exhibits a higher loadability than the non-ionized form. This demonstrates that the ionization of the analyte rather than a specific feature of the sample compound or the mobile phase conditions is responsible for the difference in loadability. To confirm this idea, we studied the phenomenon with other acidic and basic compounds.

Together with oxacillin, we investigated the loadability behavior of the closely related carboxylic acids cloxacillin and dicloxacillin. They differ from each other simply via the addition of one or two chlorine groups far away from the ionic center. Thus, the chlorine groups contribute to the hydrophobicity and the size of the molecule, but not to its ionization. All three compounds are completely ionized at alkaline pH, and at least largely non-ionized under acidic conditions.

For basic analytes, we also chose three compounds: diphenhydramine, oxybutynin and terfenadine. These three compounds vary widely in structure; their only common factor is that all three are tertiary amines. While the amino groups of diphenhydramine and oxybutynin are at the end of an alkyl chain, the amino group of terfenadine is part of a 6-membered aliphatic ring at the center of the molecule. All three compounds are completely ionized under our acidic mobile phase condition, and at least largely non-ionized under the basic conditions.

For the study, the concentration of the organic modifier was adjusted for each compound to move the peak into approximately the same retention window for each study. We adjusted the acetonitrile concentration to obtain a retention factor around 15 for the non-ionized forms and around 40 for the ionized forms. The results of the study are summarized in Table 2, which also contains the acetonitrile concentration for each compound and each condition.

The first lines of data in Table 2 are the experimental results obtained for all compounds but terfenadine under non-ionizing conditions. The solubility of terfenadine in the mobile phase limited the amount that could be injected with a reasonable injection volume. Thus, no overload was obtained for terfenadine under non-ionizing conditions. All other compounds exhibited a mild overload, sufficient to obtain the coefficients of the Langmuir isotherms. It should be noted that all five compounds followed a Langmuirian isotherm with high precision, at least within the concentration range tested. The correlation coefficients ranged from 0.999981 for diphenhydramine to 0.999999 for cloxacillin and dicloxacillin. It should be acknowledged though that the excellent correlation is partially due to the integration procedure used to generate the data for the stationary phase concentration.

The retention factors obtained from the curvefit varied from 13.5 for cloxacillin to 17.8 for diphenhydramine. The saturation capacity for the packing reached up to about 3.5 mol/l stationary phase. Since 1 l of stationary phase corresponds to about 3.6 mol of ligand, one can see that the limiting sorption capacity for these compounds was around one molecule for every ligand attached to the surface. This is not unreasonable. The saturation capacity decreases from oxacillin to cloxacillin to dicloxacillin, with an increase in the size of the hydrophobic area of the sample molecule. This is also in agreement with expectation. The loadability depends though to a significant extent on the structure of the

adsorbed molecule. This is demonstrated by the basic compounds, where the saturation capacity was notably different between diphenhydramine and oxybutynin.

The adsorption of the charged molecules is shown in the following rows of data in Table 2. First, the sorption isotherm was fitted to a Langmuir equation. This permits a direct comparison with the results obtained for the uncharged form of the same molecule. As we can see from Table 2, the saturation capacity is much lower for the charged form. The values hover around 0.15 mol/l for the oxacillin family. A rather high value was found for diphenhydramine, which also had the lowest value in the uncharged form. As a consequence, the ratio of the saturation capacity of the uncharged form to the saturation capacity of the charged form was lowest for diphenhydramine. The value was 8.5, compared to values around 21 for the oxacillin family and 28 for oxybutynin. The saturation capacity of the ionized form of terfenadine was rather low, 0.02 mol/l. This is an order of magnitude lower than the value for diphenhydramine. It is noteworthy that the charged group occupies the center of the molecule in terfenadine, while it is located on a side branch for diphenhydramine, which gave the best loadability in the charged form.

The correlation coefficients for all curvefits of the charged molecules to the Langmuir equation were better than 0.999. While this is quite satisfactory, it is not as good as the results obtained for the non-ionized form of the same analytes. In addition, a careful examination of the fitted curve showed a departure at high, low and central values, indicating that the association to the Langmuir equation is forced (see Figs. 3b and 4a).

We next investigated the curvefit to the Ståhlberg equation. The results are shown in the third data set in Table 2. In general, the Ståhlberg equation gave superior curvefitting results compared to the Langmuir equation, but admittedly the differences are small. The coefficient B^*, which expresses the ionic repulsion from the surface of the packing after adsorption of the compound, gave consistent results for the three oxacillins. For compounds that are structurally as similar in nature as the three oxacillins, consistent repulsion is expected. On the other hand, the differences in the coefficients for the three basic compounds are more difficult to explain, since the background theory of the Ståhlberg equation focuses on ionic charges but not on the structure or size of the charged molecule. Size exclusion effects are a possible explanation for the differences in the coefficients. It should be noted that all three basic compounds are tertiary amines, but that the location of the amino group in the molecule is quite different for the three compounds.

In Fig. 5, we are showing the isotherm of diphenhydramine at acidic pH to the Ståhlberg equation. Note the perfection of the curvefit to the experimental data! No systematic deviation is observed. Consequently, we can conclude that the Ståhlberg equation describes the experimental results very well, and is a good representation of the phenomenon observed.

A direct practical comparison of the peak shape of an acidic compound and a basic compound is shown in Fig. 6. This figure is equivalent to the theoretical results shown in Fig. 2. We have plotted the concentration in the mobile phase (i.e. the detector response) versus the normalized retention factor of the compound. To obtain the normalization, the measured retention factor was divided by the retention factor for a small amount of analyte injected, i.e. the value from the curvefit to the Langmuir equation in Table 2. The two compounds shown are dicloxacillin as the example for the acidic

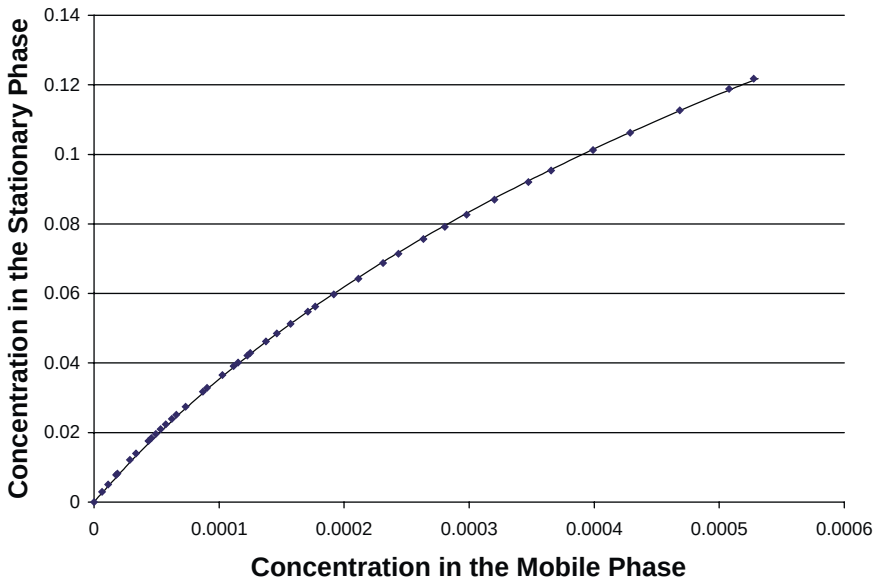


Fig. 5. Plot of the concentration of the sample in the stationary phase vs. the concentration in the mobile phase. Sample: diphenhydramine at acidic pH. The line is the curvefit to the Ståhlberg isotherm. Column: XTerra MS C_{18}, 50 mm × 4.6 mm. Mobile phases: see Table 2.

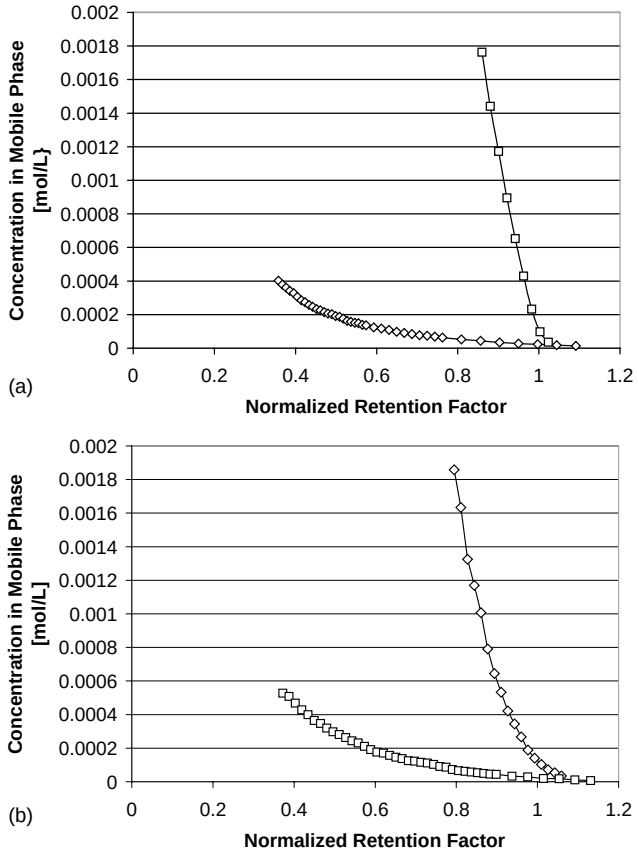


Fig. 6. Concentration in the mobile phase vs. normalized retention factor: (a) acidic compound (dicloxacillin); (b) basic compound (diphenhydramine); squares: acidic mobile phase; diamonds: basic mobile phase.

compound (Fig. 6a) and diphenhydramine as the example for the basic compound (Fig. 6b). The results under acidic conditions are shown as squares, and the results under basic conditions as diamonds. In both cases, the neutral form of the compound exhibits a much improved peak shape—and therefore a better loadability—than the ionized form of the same compound. This is the essence of our findings.

5. Applications

The principle demonstrated above is important in the routine purification of chemical entities. We can select a priori the best pH for the purification since we know the nature of the compound(s) to be purified. The roughly 50-fold difference in loadability between both pH values allows us to select a column with a roughly 7-fold smaller diameter for the separation, which drastically reduces the cost of the column as well as the cost of solvents. Also, simpler equipment may be selected.

In general, acidic compounds should be purified under acidic conditions, and compounds with a basic functional group should be purified under basic conditions. We have performed all of our separations using volatile

Table 3
pH of an ammonium bicarbonate buffer as a function of the organic solvent concentration

Organic (%)	$^{s}_{w}$pH measured	
	Methanol	Acetonitrile
0	9.81	9.80
10	9.80	9.71
20	9.75	9.70
30	9.71	9.72
40	9.65	9.65
50	9.59	9.62
60	9.55	9.62
70	9.49	9.68
80	9.48	9.89
90	9.51	10.01

mass-spectrometer-compatible mobile phases. Formic acid or ammonium formate are used for the control of the acidic pH range, while ammonia or ammonium bicarbonate allow the adjustment of the alkaline pH. Volatile mobile phases are of special importance in preparative chromatography, since the goal of a preparative separation is the preparation of a pure compound, uncontaminated by mobile phase constituents. Especially under the recommended conditions used here, the compounds are obtained in the molecular form, not in the salt form. Besides the high preparative loadability, this is an important side benefit of the recommended procedures.

Ammonium bicarbonate is a reasonably volatile buffer for the alkaline pH: it decomposes into ammonia, carbon dioxide and water above 60 °C. In addition, due to the use of a weak acid and a weak base as components of an ammonium bicarbonate buffer, its pH barely changes with the addition of organic solvents to the buffer. Table 3 shows the pH values [8] of an ammonium hydrogen carbonate buffer adjusted to pH 9.8 in water for both methanol and acetonitrile as the organic modifier. On the other hand, the p*K* values of basic analytes commonly shift to lower values in the presence of organic solvents. As a consequence, it is not difficult to convert most basic compounds to an unionized form to achieve maximum loadability in preparative chromatography.

Fig. 7 shows a loadability study under acidic and basic conditions. The basic analytes diphenhydramine, oxybutynin and terfenadine were injected onto an XTerraPrep MS C_{18} column with increasing sample concentration. The injection volume was fixed at 1.2 ml, and the samples were dissolved in DMSO. The total amount of sample injected varied by a factor of 2 from chromatogram to chromatogram, and the amount loaded is shown on the graph in units of mg total load per ml of packed bed. A gradient was used for the separation. Under these conditions, the separation pattern is similar at acidic and basic pH. Note that even at a low concentration, a larger peak distortion is observed at acidic pH! At high load, nearly 35 mg/ml for a total load of 500 mg on this column were injected under basic conditions, and the resolution was still largely preserved. Under acidic condi-

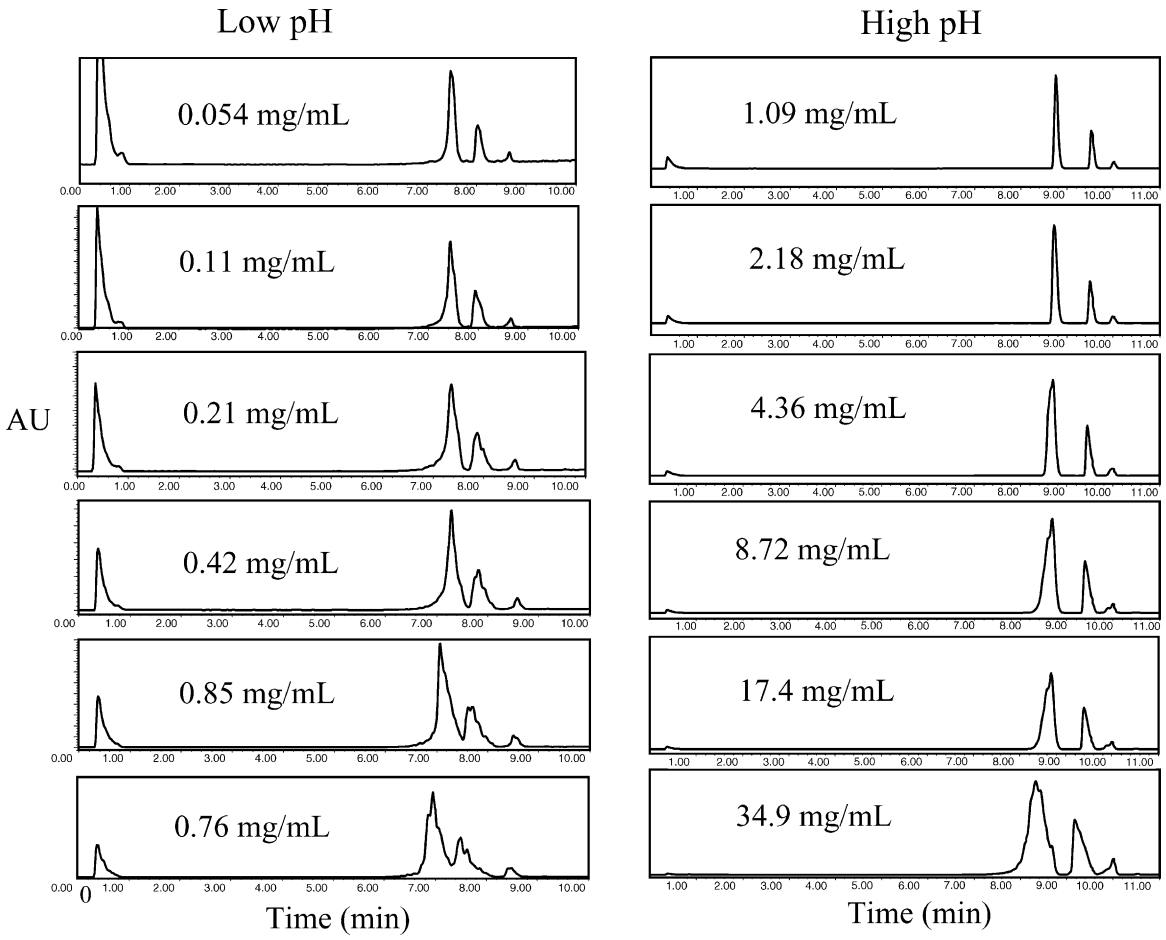


Fig. 7. Loading studies for diphenhydramine, oxybutynin and terfenadine at acidic and basic pH. Column: XTerraPrep MS C_{18}, 50 mm × 19 mm, 5 μm. Conditions: gradient from 5 to 95% acetonitrile over 5 min with a 7 min pre-equilibration in 5% acetonitrile. Buffers: 10 mM ammonium formate at pH 3.75 and 10 mM ammonium hydrogen carbonate at pH 10.0. The samples were dissolved in DMSO. Flow rate: 30 ml/min (=3.2 column volumes per minute). Detection: UV, 254 nm. Elution order: diphenhydramine, oxybutynin, terfenadine.

tions however, the resolution started to decline at a load of 0.85 mg/ml (total sample load of 12 mg) already, and became insufficient at a load of 1.76 mg/ml (total load 25 mg). This difference in loadability is in agreement with the theoretical expectations and confirms the practical importance of choosing the correct pH for the preparative separation of ionizable compounds.

If only a smaller total load is desired, a smaller column can be chosen. Fig. 8 shows the separation of the same three compounds at a load of 41.2 mg on an analytical 4.6 mm XTerra MS C_{18} column at alkaline pH. Excellent resolution is still maintained despite the high sample load. The resolution is better than the one obtained at the largest load shown in Fig. 5 for the 19 mm column at low pH. The flow rate was 1.4 ml/min compared to 30 ml/min for the 19 mm column. Overall, a better separation was obtained at a higher load and with a lower flow rate on the analytical column at high pH compared to the results obtained on the preparative column at low pH. This comparison demonstrates clearly the importance of pH in the preparative separation of ionizable compounds.

The examples above showed the difference in loadability under gradient conditions. In Fig. 9, an example is shown for a basic compound under isocratic conditions. The compound shown is terfenadine. The top chromatogram shows the chromatogram obtained with 40% acetonitrile under acidic conditions. Under these conditions, the compound is completely ionized. The bottom chromatogram is the same peak at alkaline pH, with a mobile phase of 62% acetonitrile. Under the second conditions the compound is unionized. As one can see, the peak distortion is roughly the same under both conditions. However, the load applied to the chromatogram on top was only 0.016 mg, while the load applied to the bottom chromatogram was slightly more than 1 mg. This means that a roughly 60-fold higher load was applied under alkaline conditions compared to acidic conditions. For this compound, the preparative loadability was therefore roughly 60 times larger in the unionized form compared to the ionized form. Such drastic differences in the loadability are the consequence of the principles outlined in Section 2.

An example of isocratic preparative chromatography of an acidic compound is shown in Fig. 10. The sample is

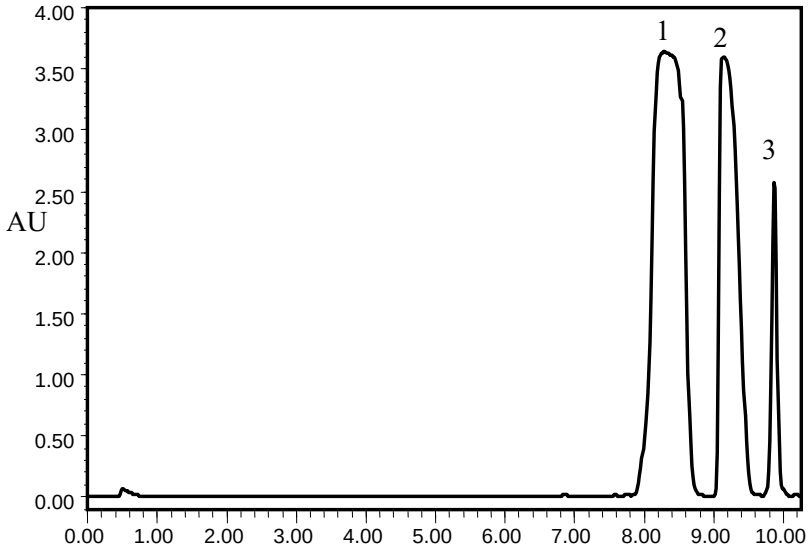


Fig. 8. Preparative separation of diphenhydramine (1), oxybutynin (2) and terfenadine (3) at high load on an analytical column at basic pH. Column: XTerra MS C_{18}, 50 mm × 4.6 mm. Load: 41.2 mg. Gradient from 5 to 90% acetonitrile over 5 min after an initial hold at 5% acetonitrile. Buffer: 10 mM ammonium bicarbonate at pH 10.0. Detection: UV, 254 nm.

cloxacillin, and the chromatography was monitored with a mass spectrometer. An acidic mobile phase was utilized, i.e. an ammonium formate buffer at a pH of 3.0. Therefore, the compound was partially unionized under the operating conditions. Besides the advantage pointed out in this article for the preparative loadability, this condition is also favorable for the use of a highly sensitive MS instrument for the monitoring of the preparative separation. The ionization of the compounds for preparative chromatography is suppressed under the conditions recommended here, which reduces the sensitivity for mass spectrometry as well. In our case, this is advantageous due to the large loads employed in preparative chromatography. The graph shows the peak distortion as a function of increased load. In agreement with the expectation, the preparative loadability for cloxacillin was comparable to the loadability of a neutral compound. At alkaline pH

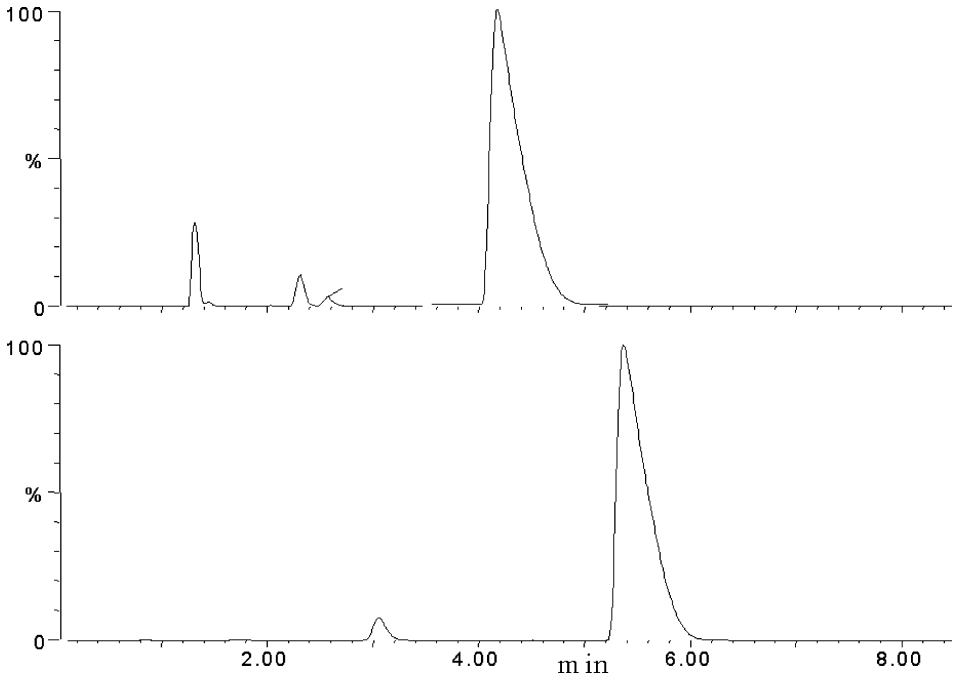


Fig. 9. Comparison of the isocratic preparative separation under acidic conditions (top) and basic conditions (bottom). Column: XTerra MS C_{18} 150 mm × 4.6 mm. Main peak: terfenadine. Top: 0.016 mg load; mobile phase: 40% acetonitrile, 50% water, 10% 1% TFA. Bottom: 1.024 mg; mobile phase: 62% acetonitrile, 28% water, 10% 0.1 M $(NH_4)_2CO_3$ buffer, pH 10.0. Flow rate: 1.75 ml/min. Detection: UV, 258 nm.

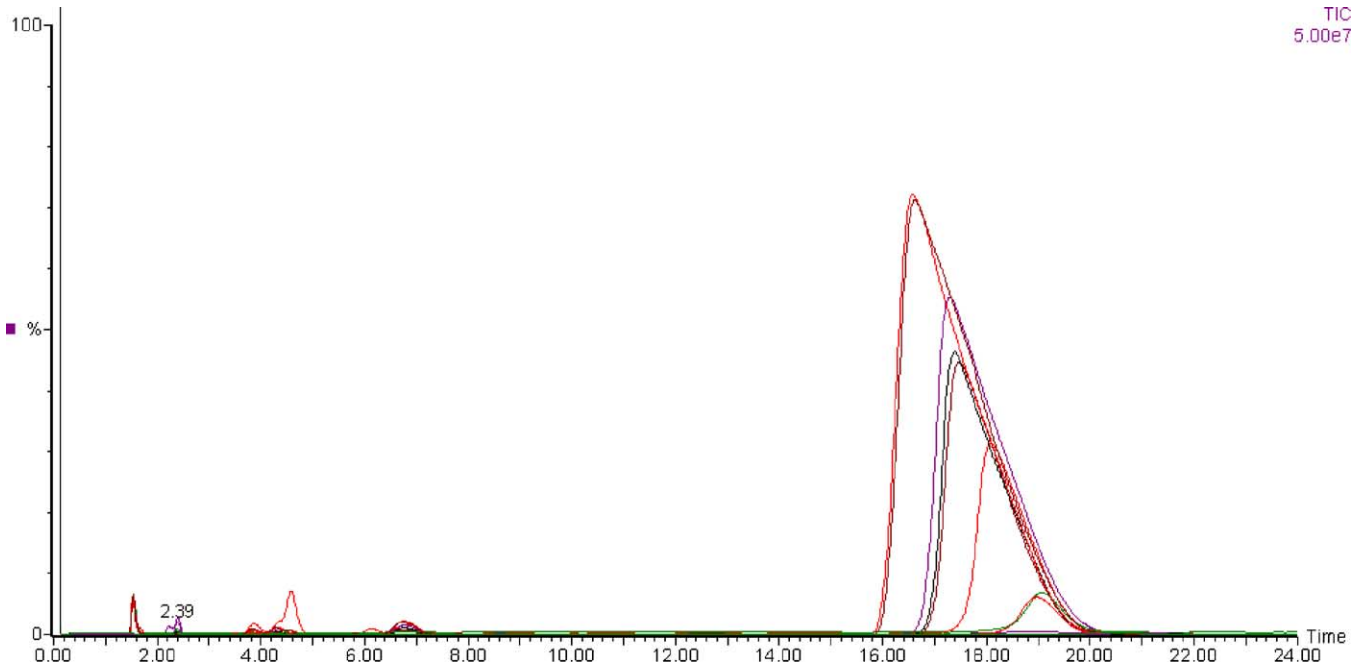


Fig. 10. Isocratic preparative separation of an acidic compound. Column: XTerra MS C_{18}, 50 mm × 4.6 mm. Mobile phase: 32% acetonitrile, 68% ammonium formate buffer at pH 3.0. Total buffer concentration: 50 mM. Sample concentration: 100 mg/ml, dissolved in water. Amounts injected: 23, 15, 10, 5 and 2 mg.

(not shown) only a 30-fold lower amount could be injected for roughly equal peak distortion.

It should be pointed out that the best preparative loadability is achieved when the sample is loaded onto the column in the non-ionized form. On the other hand, the best solubility is often obtained, when the sample is in the salt form. To obtain the best combination of maximum preparative loadability with good sample solubility, the sample should be dissolved in the ionic form and converted to the non-ionic form just in front of the chromatographic column. This technique, called at-column dilution, has been described in detail in a previous publication [3]. For optimum loadability, a combination of the technique described in this paper with the at-column dilution technique described earlier is recommended. Practical applications of both techniques have been shown in previous papers [14–16].

6. Conclusions

In this paper, we have demonstrated that the column loadability in preparative chromatography is a strong function of the ionization of the sample. Differences in loadability by at least a factor of 20, often a factor of 50 are observed between the ionic form of the sample and its non-ionic form. The best loadability is always obtained for the unionized form of the sample. Thus, the preparative chromatography of acidic compounds should be carried out at acidic pH. Basic compounds should be purified using mobile phases with an alkaline pH. Both the theoretical explanation as well as practical examples have been demonstrated in this paper. We expect that this paper encourages the practitioners of preparative chromatography to actively use pH as an important tool in the purification of compounds. We also hope to stimulate other researchers to further investigate the details of the theoretical background leading to the drastic influence of pH on preparative loadability described here, especially the influence of the compound structure and the preparative loadability in the transition range between the completely charged and the completely uncharged form of the compound.

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