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Overloading study of basic compounds with a positively charged C18 column in liquid chromatography

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ABSTRACT

While tailing and overloading of basic compounds remain problematic on most RP columns, a new kind of positively charged RP column named XCharge C18 was found to be superior good for the separation of alkaloids in our practical use. In this work, the surface charge property of the XCharge C18 column was evaluated by the retention of NO_3^- under different pH values and buffer concentrations. A considerable and pH-dependent positive charge was confirmed on the column. Then overloading behaviors of bases were systematically studied using amitriptyline as a basic probe. Good peak shapes (Tf < 1.5) and extra high loadability with a $C_{0.5}$ of about 30,000 mg/L were observed on the column, with commonly used 0.1% formic acid as mobile phase additive. However, increasing the ionic strength of buffer with phosphates led to tailing peaks at high sample amount and sharp decline in loadability ($C_{0.5}$ of 2000–3000 mg/L), although it brought higher column efficiency at low sample amount. Higher pH also induced worse performance and lower loadability. The overall results demonstrated the importance of an appropriate level of ionic repulsion for the XCharge C18 column to achieve the good performance for bases, which could be explained by the multiple-site adsorption theory as ionic repulsion would shield the solute from occupying high-energy sites deeper in C18 layer.

1. Introduction

Basic compounds take an important position in the pharmaceutical industry, as over 60% of pharmaceuticals are nitrogenous bases [1]. However, separation of such ionizable solutes remains challenging with reversed-phase liquid chromatography (RPLC), the most widely used HPLC mode so far [2]. Low efficiency and tailing peaks continue to be common phenomenon for basic compounds. Furthermore, quick overloading often occurs when increasing the load amount of basic analytes to only a few submicrograms on standard size analytical RP columns [3,4]. The loadability of bases is often much lower, comparing with that of neutral compounds. The problems make it especially miserable when large sample amounts are needed in HPLC applications, such as preparative use or impurity analysis.

The distinctive chromatographic behaviors of bases attract much attention and numerous investigations have been made for better understanding of the mechanisms [3–23]. One of the pioneers in this area, Snyder, in studies [5–7] performed more than 20 years ago with type A phases (made of rather impure silica, having a relatively high activity of silanols even at low pH), proposed that ionized silanols provided strong retention sites for ionized bases that are filled rapidly, prior to the filling of high-capacity weak sites furnished by the ODS ligands. According to this explanation, minimizing the amount or the activity of residual silanols would help to eliminate tailing and overloading problems of bases. Great efforts have been made in column technology development to achieve this goal, including the use of high purity silica (>99.99%, type B phases), extensive silanization and end-capping, etc. Although about 50% of the original silanol groups may still remain unreacted [24], the activity of silanols have been greatly reduced on modern type B phases, especially at low pH. As shown in a study made by Neue, there were negligible ionized silanols on the surface of some type B phases (Symmetry C18 and XTerra MS C18) at low or even neutral pH, as no retention of lithium ions was observed throughout the pH range of 3–7 [25]. However, peak tailing would still frequently take place on these modern columns even at very small sample amount [3]. It seems that the detrimental interaction between ionized basic solutes and oppositely charged silanol groups might not be the main cause for tailing and overloading of bases on modern columns, at least not the only one.

Later, Häglund and Ståhlberg proposed mutual repulsion of the same charged ions on the phase surface as another possible cause of quick overloading of ionized solutes [8]. To avoid the possibility of silanophilic interactions, they studied with a strong acid and

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found that anionic solutes would also display similar tailing and overloading behaviors with that of bases. This mutual repulsion mechanism was used by McCalley to rationalize the overloading observed on purely polymeric phases [9], which clearly contain no silanols but still showed typical tailing peak shapes as often obtained on silica-based columns. Neue also used this theory to demonstrate the idea that the best loadability is always obtained for the unionized form of the sample, that is to say, basic compounds should be separated at alkaline pH [11]. High-pH stable columns were developed and the strategy was successful to a great extent for weak and moderately strong bases. However, for stronger bases (p*K*a > 10) and quaternary amines, their ionization can hardly be depressed even at a extreme pH of 12, and the performance would be even worse, possibly due to the negative influence of increased column silanol ionization [16,19]. In addition, the solubility of basic compounds at high pH mobile phases might be another problem, especially for a preparative purpose. Meanwhile, the practical stability of silica-based columns at extreme high pH is still need to be improved [19]. Thus, the high pH way provides a good approach, but not a complete solution to the problems of bases.

In an extensive series of publications [10,12,13,17,20–22], Gritti and Guiochon studied the overloading of bases on many highly inert modern RP materials, through rigorous evaluation of the adsorption isotherms obtained with frontal analysis. With the data obtained, they raised a query on the mutual repulsion theory as it could not explain the similar total saturation capacity for neutral and ionized compounds. After calculating the adsorption energy distribution and matching the isotherm models, they proposed that the heterogeneity of the column surface, consisting of multiple (often two or three) adsorption sites, would be the reason for the unusual overloading behaviors of charged solutes. In this theory, the charged analytes would firstly occupy a very small amount of high-energy sites, and then begin to fill with the plentiful weak sites. As retentions are different on these two sites, the homogeneity of mass transfer would be destroyed and tailing occurs when sample mass is more than the saturation capacity of the high-energy sites. This interpretation sounds something like the Snyder's mechanism of secondary interactions caused by ionized silanols, but the strong sites herein are obviously not ionized silanols according to their measured adsorption energies and saturation capacity [10]. However, the physical identity of the high-energy sites is still obscure. One reasonable hypothesis is that the different sites are corresponded to different environments on the adsorbent surface, with a large number of weak sites at the interface of C18 layer while very limited strong sites located deeper inside the C18 layer. The mechanism of multiple-sites adsorption was also supported by the study of McCalley [18], in which it satisfactorily explained the overloading behavior of both charged cation and anion they observed.

According to the heterogeneity theory, minimizing the amount or the activity of residual silanols may not be able to solve the problems radically. Instead, a "shielding" layer that prevents the charged bases from filling to the strong sites would make sense. In polar-embedded (PEG) RP columns, the embedded polar groups (EPGs), like carbamate or amide, seem to have such a function. A tightly bound water layer around the polar group but below the reversed-phase layer has been hypothesized to prevent the interaction of the analytes with the silanol groups located below the water layer [26]. In addition, the shielding effect of some PEG columns may also be attributed to ionic repulsion caused by a degree of positive charge, which was possibly from the residue amino or carbamate functionality in two steps bonding of the PEG phases [27]. Improved peak shapes for basic compounds were often obtained by researcher using PEG columns. Although the reports still emphasize more on the silanol groups, it is also feasible that the polar group actually shield the strong sites as they might also located in deep C18 layer.

More recently, the impact of the surface charge on the chromatographic behaviors of ionized analytes has gained new attention of some column manufacturers. Based on their BEH technology (ethylene-bridged-hybrid), the Waters developed a new surface-modification process named "charged surface hybrid" (CSH), which allows the introduction of a reproducible, low-level positive surface charge in acidic mobile phases [28]. Conspicuous advantages in peak shape and loadability for basic compounds have been observed on CSH C18 when using low ionic-strength mobile phase additives [29,30]. A local column supplier in China also developed a so-called "XCharge" technology to provide solutions for basic compounds, including XCharge SCX [31] and XCharge C18 [32] serials, which were found to be superior good for the separation of alkaloids in natural products. Although different in technology, the idea of positively charged surface seems to be the same way for the two new unique columns to solve the chromatographic problems of bases. However, detailed explanations of the mechanism have not been offered yet.

In this work, the surface charge property of the XCharge C18 column was studied and then overloading behaviors of basic compounds were systematically investigated under different buffer concentrations and pH, trying to find out the reason for the good performance of the column in base separation.

2. Experimental

A Waters Alliance HPLC system comprising autosampler, 2695 binary pump, heated column thermostat, 2489 UV–vis detector (1 μL flow cell, 5-mm path length) and Empower chemstation was used in all experiments. A 0.01 cm i.d. connection tubing of minimum length was used in order to limit extra column volume. Column temperature was maintained at 30°C throughout using the instrument thermostat.

The XCharge C18 column (150 mm × 4.6 mm, 5 μm) was purchased from Acchrom Co., Ltd. (Beijing, China). Its surface chemistry was shown in Fig. 1 and the detailed properties were listed in Table 1. HPLC grade acetonitrile (ACN) and formic acid (FA) were obtained from J&K (Hebei, China). Sodium biphosphate (NaH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4) were obtained from Sinopharm (Shanghai, China). Phosphoric acid was purchased from

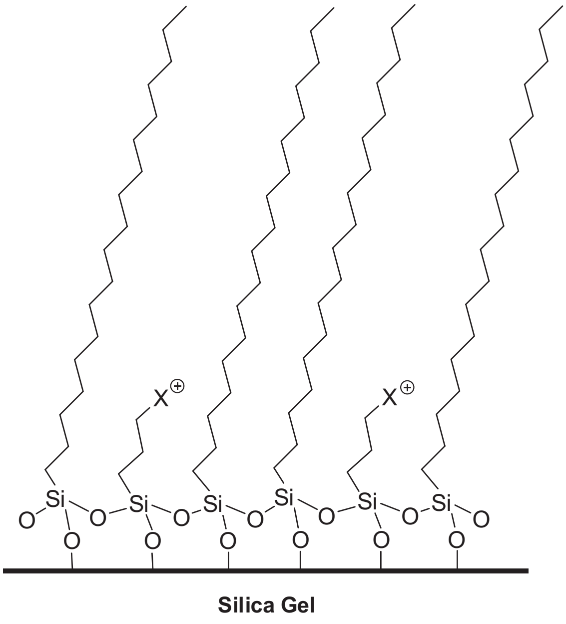

Fig. 1. Representative surface chemistry of the XCharge C18 column.

Table 1
Physico-chemical properties of the XCharge C18 column according to the manufacturer.

Serial number	Column dimension	Average partical size	Specific surface area[a]	Total carbon	Average pore size[a]	Average pore volume[a]
12081406C	4.6 × 150 mm	5 μm	300 m^2/g	11.5%	100 Å	0.75 mL/g

[a] Information of the neat silica material before C18 derivatization.

Tedia (Fairfield, USA). All of these reagents were analytical reagents. Water was prepared by a Milli-Q system (Billerica, MA, USA). Amitriptyline (p*K*a 8.9) was used as basic probe and it was purchased from Acros (NJ, USA). Its structure was shown in Fig. 2.

Phosphate buffers were prepared using NaH_2PO_4/Na_2HPO_4 or NaH_2PO_4 and adjusted to desired pH with H_3PO_4 solutions at corresponding concentrations before addition of organic solvent (pH values). The buffers were filtered using 0.22 μm membranes before use. Solute solutions of various concentrations were made up in the appropriate mobile phase and injected singly (5 μL).

The dead time (t_0) of the column was measured by injection of uracil and it was 1.58 min as the average of three repeated injections. For convenience of comparison of overloading from tailing factors and column efficiency measurements, and for further minimization of effects of extra-column volume, the organic modifier concentration were adjusted to give a similar $k \sim 6$ for each analysis. All results were the mean of three injections. Tailing factors were measured with 5% height method and column efficiencies were with 61% tangent method according to United States Pharmacopeia (USP). These data could be read out from the system suitability software of the chemstation.

In all experiments, the column was first equilibrated with the mobile phase for at least 30 min.

3. Results and discussion

3.1. Evaluating of the surface charge of the XCharge C18 column

While alkyl ligands are designed to contribute most of the retention on RP columns, surface charge properties may also play an important role, especially in the chromatographic behaviors of ionized solutes. On older, type A phases, the considerable negative charge, resulting from residual of silanols or metal ion, would significantly increase the retention of positively charged cation by electrostatic interaction, which has long been recognized as an important reason for peak tailing of bases. Nowadays, on modern, type B phases, the negative charge has been greatly reduced with the effort of high-purity silica, high-density bonding and exhausted end-capping, etc. and the peak shape of bases has been much more improved on analytical level. More recently, positive charge was observed on some polar-embedded C18 columns, and the ionic repulsion was considered to be benefit for the further improved peak shapes, comparing with traditional C18 columns [33]. Therefore, the property of surface charge should also be taken into consideration when choosing the right column for analysis of bases.

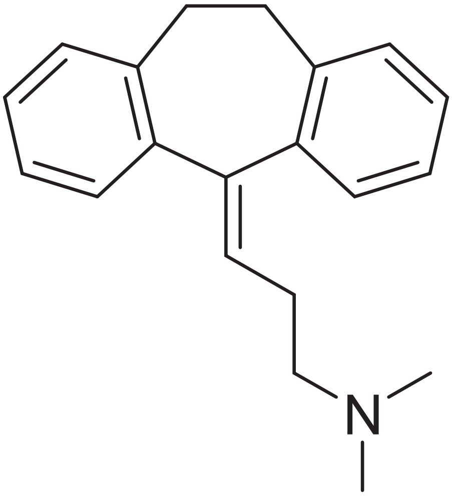

Fig. 2. Chemical structure of amitriptyline.

For the determination of charge property of solid surface, zeta potential measurement is often applied and the flow induced streaming potential has also been shown to be useful [24,34]. However, for a more intuitively evaluation, it's better to use a chromatographic method with an oppositely charged ion without hydrophobic affinity to the column. In the present work, a UV detectable inorganic ion, nitrate ion, was applied, as had been reported by others [25,35].

Fig. 3(a) presents the retention time (t_R) of NO_3^- on the XCharge C18 column with mobile phases buffered with 10 mM NaH_2PO_4/Na_2HPO_4 (1:1), which were adjusted to desired pH values with 10 mM H_3PO_4. Significant retention ($t_R = 19.5$ min) of NO_3^- was observed at pH 2.5, indicating a considerable degree of positive charge on the column. With the increasing of buffer pH, the retention of the anion quickly declined, and to the neutral pH of 7.0, it was near the dead time. As the ionization of NO_3^- is not effected by buffer pH, it is clear that the surface charge of the column is pH dependent.

Aside from the pH value, buffer concentration would also greatly influence electrostatic interaction. Fig. 3(b) shows the plot of retention of NO_3^- vs. buffer concentrations of phosphate at the constant pH. As expected, the retention displayed a decreasing trend with the increasing of buffer concentration. At the low buffer concentration of 5 mM, the retention reached to 26.2 min and it could still be much stronger if lower ionic strength buffer was used. In fact, the NO_3^- could not be eluted out within an hour with 0.1% formic acid, the widely used mass compatible buffer. As the buffer concentration increased, the retention declined quickly at the beginning and then slow down. However, to a relative high concentration of 60 mM, the retention of NO_3^- was still notable ($k \sim 2.7$) and much longer than those obtained with lower buffer concentrations on other positively charged C18 columns ($k \sim 2.2$ for Gemini C18 and $k \sim 1.7$ for Symmetry C18 with 1.36 mM phosphate buffer) [35], indicating a much higher degree of positive charge of the XCharge C18 column.

3.2. Overloading for XCharge C18 column with 0.1% formic acid

Overloading is the deterioration in column performance that often results from increasing sample mass. The capacity or loadability of a column is especially important in preparative chromatography or trace impurity detection that needs a high loading amount. For a quantitative comparison of column loadability, a simple empirical method was recently proposed by McCalley et al. [36]. This method involves the measurement of two parameters, the limiting plate count N_0, that is observed when the sample amount is so small that a linear isotherm pertains, and the sample amount $\omega_{0.5}$, that leads to a plate count half the value of N_0. The loadability can also be expressed as the concentration of sample

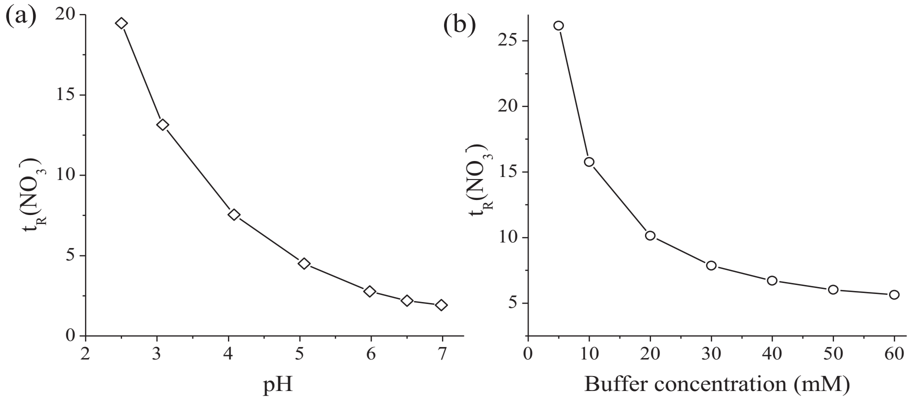

Fig. 3. Retention of NO_3^- ion on the XCharge C18 column at (a) different pH values buffered with 10 mM NaH_2PO_4 and (b) different concentrations of NaH_2PO_4 at pH 2.8 in 20% ACN. Flow rate = 1 mL/min. Detection wavelength: 220 nm.

$C_{0.5}$ that gives this reduction of efficiency for a specified injection volume [29]. In this paper, the later description was applied, which can provide intuitive information for comparing the different overloading behaviors of the column under different conditions. However, it should be stated clearly that the "loadability" in this paper corresponded to the empirical measurement of $C_{0.5}$ and for the calculation of saturation capacity, it's more rigorous to apply nonlinear chromatography methods like frontal analysis, equilibrium perturbation methods and inverse method, as extensively used and claimed by Gritti and Guiochon [37].

Fig. 4 shows overlaid chromatograms for 0.01–250 μg of amitriptyline on the XCharge C18 column using 0.1% formic acid as mobile phase additive, which is often favored in LC–MS analysis due to the compatibility for MS detection and also in preparative chromatography due to its easiness to be removed. Good peak shapes with little broadening were obtained throughout a sample mass scale of 0.01–150 μg, and only when the sample mass overloaded the upper detection limit (3 AU with injection sample higher than 150 μg), obvious peak broadening began to take place. The overloading behaviors were quite different from those obtained on regular RP columns, where peaks of bases often start tailing at less than 1 μg of sample loading and quickly frontad broaden with increasing sample mass, leading to continuously decreasing of the retention of the whole peak till sharp L-shaped peaks occur [18]. The conventional overloading phenomenon could be explained as the gradual total saturation of a small subset of high-energy sites and the subsequent occupation of the plentiful weakly retaining sites [18]. According to this multiple-site adsorption mechanism, it is possible that the sufficient positive charge on the surface of the XCharge C18 column effectively shielded the basic solute from reaching the high-energy site deeper in C18 layer and thus the solute would directly fill the abundant weak sites on the top of C18 layer, resulting in a considerable higher loading property. The value of $C_{0.5}$ with 0.1% FA reached to 30,000 mg/L (see Table 2), strikingly higher than the data for all the three RP columns including CSH C18 in McCalley's paper [29]. However, it should be mentioned that the literature data was obtained on finer particle (sub 2 or 3 μm) columns and a strict comparison is out of the scope of this work.

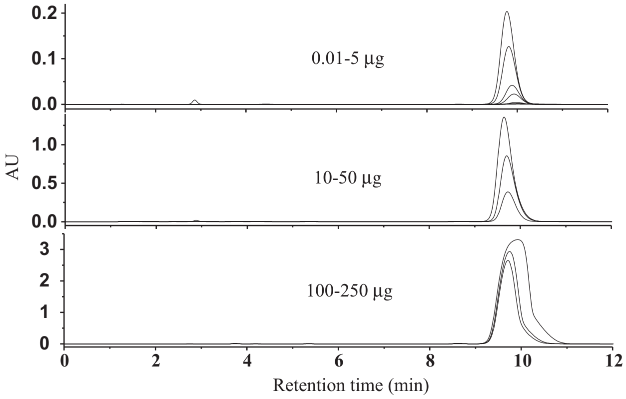

Fig. 4. Overlaid chromatograms of amitriptyline on the XCharge C18 column in 16% ACN with 0.1% formic acid additive. Sample concentrations: 2–50,000 mg/L. Injection volume: 5 μL. Detection wavelength: 260 nm. Column temperature: 30 °C. Flow rate = 1 mL/min.

3.3. Effect of buffer concentration on overloading for XCharge C18 column

On conventional RP columns, overloading can be reduced as the mobile-phase ionic strength is increased [18]. It was reported that capacity of the columns with even 5 mM ammonium formate buffers (ionic strength 6.1 mM/L) is superior to 0.1% formic acid solutions (ionic strength 2.2 mM/L) [23]. However, the situation was rather different on the XCharge C18 column. As shown in Fig. 5, at lower sample mass of 0.01–5 μg, good peak shapes could still be obtained with NaH_2PO_4 buffers range from 5 mM to 50 mM. But tailing and right-angled triangle shapes similar to the typical ones on regular RP columns occurred at higher sample mass of 10–250 μg. Plots of column efficiency and tailing factor vs. sample mass in Fig. 6 clearly showed a much more rapid decline in column performance with phosphate buffers, comparing with the low ionic strength buffer (0.1% FA). Correspondingly, the loadability expressed in $C_{0.5}$ also decreased by about one order of magnitude (see Table 2). This distinctive trend is most probably due to the weakening of the shielding effect by ionic repulsion with higher strength buffers. More acetonitrile was needed to elute the solute to a similar retention when higher buffer concentration was used (from 16% with 0.1% FA to 26% with 50 mM NaH_2PO_4), which clearly confirmed the inhibition of ionic repulsion.

Table 2
Overloading of amitriptyline with different buffers on the XCharge C18 column. Injection volume 5 μL.

Buffer	Tf at N_0	N_0	$C_{0.5}$ (mg/L)
0.1% formic acid	1.01	4700	30,000
5 mM NaH_2PO_4 pH 2.7	1.04	5400	~3000
20 mM NaH_2PO_4 pH 2.7	0.97	5800	2000
50 mM NaH_2PO_4 pH 2.7	1.05	5300	~2500
5 mM NaH_2PO_4 pH 4.1	1.28	4200	1000
5 mM NaH_2PO_4 pH 5.1	1.23	4000	500

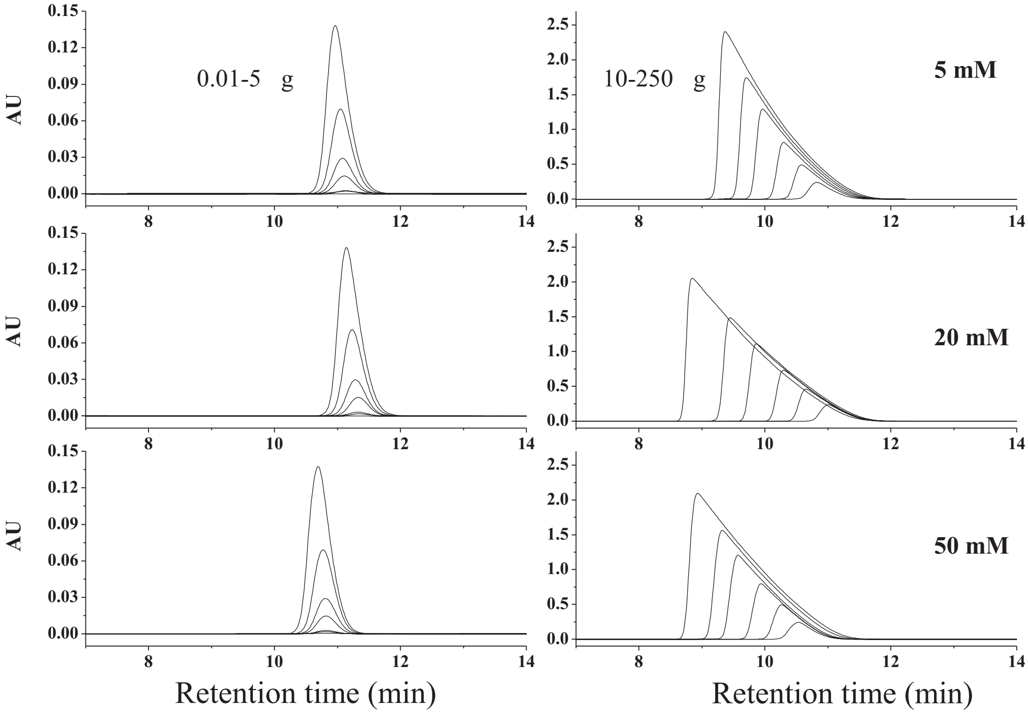

Fig. 5. Overlaid chromatograms of amitriptyline on the XCharge C18 column with different concentrations of NaH_2PO_4 buffers (pH adjusted to 2.8). ACN concentrations for 5 mM, 20 mM, 50 mM were 18%, 23%, 26%, respectively. Other conditions same as Fig. 4.

Nevertheless, the effect of buffer to the overloading on XCharge C18 column is not so straightforward. In spite of the adverse effect, buffers may also bring some benefits by decreasing the Donnan exclusion and the repulsion of the same charged ions [29]. As we can see in Fig. 6(a), higher column efficiency was obtained at lower level sample mass with phosphate buffer, comparing with the low ionic strength buffer. In addition, although the value of $C_{0.5}$ quickly decreased as the ionic strength increased from 0.1% FA to 5 mM NaH_2PO_4, further increasing the buffer concentration to 20 mM resulted in fewer declines and to 50 mM, a slight increase took place. It seems that the overall results were the compromise of the pros and cons effect. But in general, better loadability could be achieved on the charged column with low ionic strength buffer, which may bring lots of convenience in practical use.

3.4. Effect of mobile phase pH on overloading for XCharge C18 column

Besides the buffer concentration, the pH value also has great influence to the retention behaviors of ionizable solutes and thus is often interested in overloading studies. Fig. 7 shows the comparison of overlaid chromatograms of amitriptyline on the XCharge C18 column under different pH all buffered with 5 mM NaH_2PO_4. Much Wider and more tailing peaks were observed with higher pH, both at lower or higher level of sample mass. The plots of column efficiency and tailing factor vs. sample mass in Fig. 8 also revealed worse performance and more rapid depravation trends with higher pH. Consequently, the loadability was declined from ~3000 mg/L at pH 2.8 to 1000 mg/L at pH 4.1 and 500 mg/L at pH 5.1, respectively (see Table 2). As all samples were prepared with corresponding

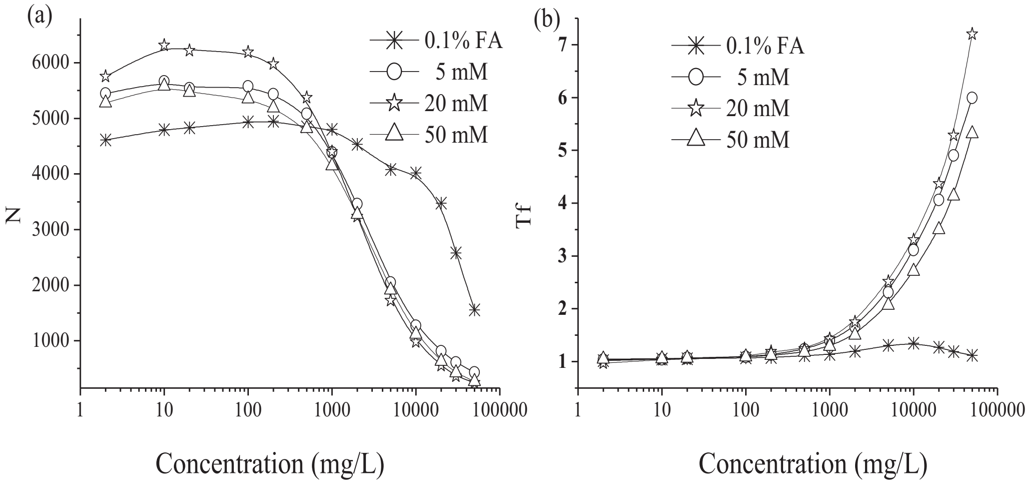

Fig. 6. Overloading plots of column efficiency (*N*) and tailing factor (Tf) vs. sample concentration on the XCharge C18 column using 0.1% formic acid and 5–50 mM phosphate buffers. Injection volume: 5 μL. Conditions for 0.1% FA same as Fig. 4 and for 5–50 mM phosphate buffers same as Fig. 5.

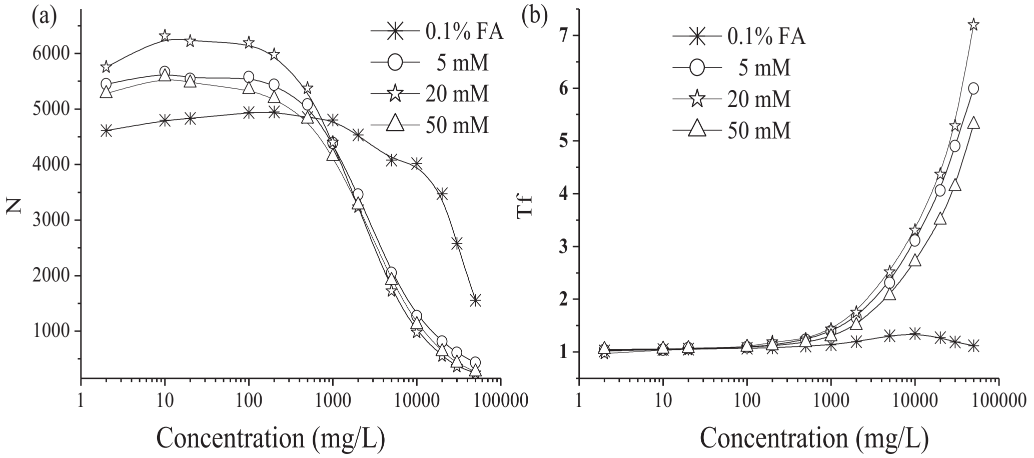

Fig. 7. Overlaid chromatograms of amitriptyline on the XCharge C18 column under different pH values buffered with 5 mM NaH_2PO_4. ACN concentrations for pH 2.8, 4.1, 5.1 were 18%, 21%, 24%, respectively. Other conditions same as Fig. 4 and injection volume was 5 μL.

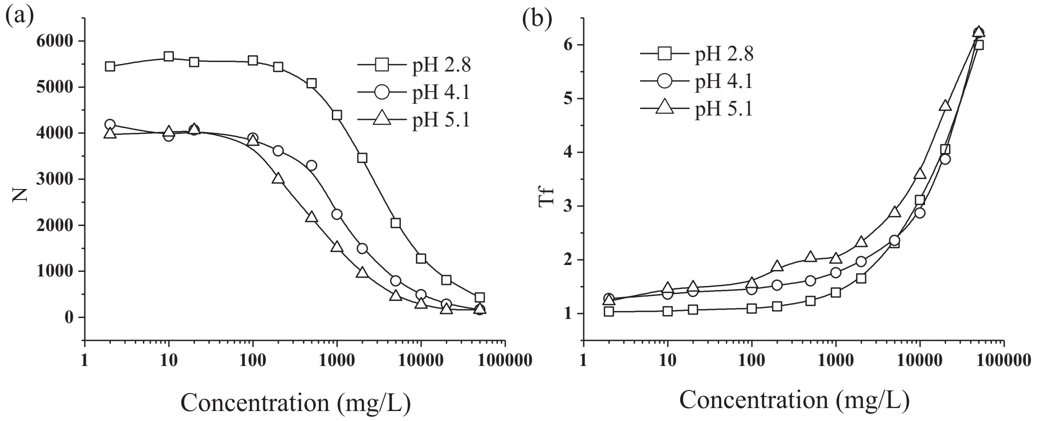

Fig. 8. Overloading plots of column efficiency and tailing factor (Tf) vs. sample concentration on the XCharge C18 column under different pH buffered with 5 mM NaH_2PO_4. Conditions same as Fig. 7 and injection volume was 5 μL.

mobile phases, the possibility of pH-mismatching to bring on the deterioration could be excluded. A more possible rationale could also be attributed to the sharp decline of the surface charge of the column with higher pH values, which had been demonstrated in Section 3.1. As the ionic repulsion was reduced with higher pH, more acetonitrile was again needed to elute the solute to a similar retention (from 18% at pH 2.8 to 21% at pH 4.1 and 24% at pH 5.1, respectively). In addition, the ionization of silanol groups at higher pH might also contribute to the decreasing of column performance as well as extending the retention.

Further increasing the pH value to be more closing to neutral conditions would lead to awful peak shapes, together with poorer reproducibility. It seems that the performance of the column would somewhat be compromised more quickly by the use of higher pH mobile phases. As a result, low pH conditions are often favored in the practical use of XCharge C18 column for bases.

4. Conclusions

In this work, a considerable level of positive charge was proved on the XCharge C18 column under conditions of low pH and low ionic strength buffer, using the retention of NO_3^- a simple but direct method. The charge or the electrostatic interaction caused by the charge can be greatly influenced by changing the pH as well as the ionic strength of the buffer, which was then confirmed to be of significant impact to the overloading behavior of the probe base on the column. In general, better loadability was obtained with lower ionic strength buffer, which was quite different from reported situations on regular RP columns without such notable positive charge. According to the multiple-site adsorption theory for overloading, it is reasonable that ionic repulsion between charged column surface and solutes would shield the solute from filling into high-energy sites deeper in C18 layer and thus levels higher loadability for bases could be achieved on the positive charged column. Nevertheless, the surface charge should be adjusted to an appropriate level with suitable mobile phase to get a satisfactory result.

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