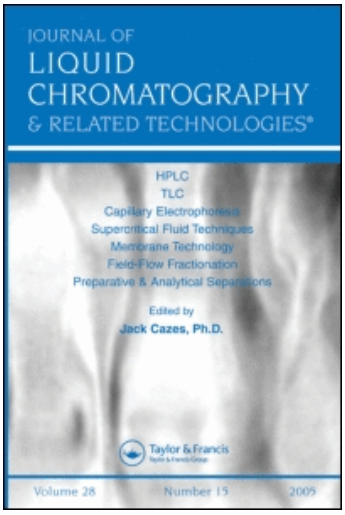

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The Fractional Charge Approach in Ion-Interaction Chromatography of Zwitterions: Influence of the Stationary Phase Concentration of the Ion Interaction Reagent and pH

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Abstract: The fractional charge approach to Ion-interaction chromatography (IIC) of zwitterions was used to quantitatively explain their retention behavior as a function of the Ion Interaction Reagent (H) concentration in the stationary phase and also to account for the influence of the zwitterion ionization degree, according to the mobile phase pH.

The theory was validated using two Hs and two reversed phase columns. The good predictive abilities of the retention equations lend strong support to the hypotheses made by the fractional charge approach in the IIC of zwitterions. For the investigated homologous series the increase in retention upon H addition can be quantitatively related to the estimated fractional charge. The estimates of the operative charges and bonded phase coverages are very reasonable since they make sense physically. This way, they offer a cross validation of the fractional charge approach, since they compare well with those obtained from the fitting of parallel retention data, obtained as a function of the mobile phase concentration of H. This confirms that retention modelling may instruct the chromatographer through the optimization procedure in the parameter space.

Keywords: Reversed-phase ion-interaction chromatography, Zwitterion, Retention model, Ion interaction reagent surface concentration

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INTRODUCTION

The most important and representative zwitterionic analytes are amino acids and peptides. The importance of these biomolecules in food chemistry biomedical research and therapeutical applications[1–3] is now widely recognized. The qualitative and quantitative analysis of zwitterionic analytes is essential in the proteomic field but poses a complex problem. HPLC separations may form an important stage in the analytical scheme, because CE often lacks selectivity.

It was already experimentally observed,[4,5] and it will be hereby confirmed that the reversed phase retention of zwitterions decreases when the fraction of the dipolar form of the molecule increases according to the mobile phase pH, and is at the minimum at the isoelectric point. A reasonable explanation is the following: if both the head and the tail of the analyte are charged, the reversed phase interactions with the stationary phase are prevented in both sides of the molecule; its hydrophilicity is higher, hence its retention is lower. Since in life science, it is of utmost importance to keep the analyte at its isoelectric point, our goal was to devise a method to increase retention of the fully charged zwitterion. This issue was already addressed for amino acids and peptides via their derivatization, but the procedure is obviously time consuming.[6] Hydrophilic stationary phases[7] were also tested. The IIC strategy[8] offers the possibility to use a non-dedicated reversed phase column; hence it is the focus of our interest with the aim to increase zwitterion retention.

The development of most HPLC methods starts with the optimization of the mobile phase composition after an appropriate column has been selected. The theory may help the chromatographer to perform an educated guess and to limit trial-and-error procedures. Recently, we developed an extended thermodynamic retention model for IIC,[9–17] which is able to predict experimental evidence that was not previously rationalized. We focused this retention model on zwitterions retention in a series of papers,[18–20] since their behaviour in IIC was not widely and quantitatively investigated. The theory was put forward[18] by taking into account the electrical interaction between the zwitterion electrical dipole with the non-homogeneous electrical field that results from the adsorption of H onto the stationary phase surface. According to the subsequent fractional charge approach,[20] the molecular dipole may be considered to be equivalent to an "effective charge", whose sign is opposite to that one of the stationary phase surface, because the electrical torque moment arranges the analyte dipole with the tail similarly charged to the stationary phase as far as possible from it and with the head oppositely charged to the stationary phase closer to it. According to the Coulomb's law, a neat attractive force results; since its modulus is lower than that corresponding to the attractive force on the analyte head, whose charge is z_E; it follows that this operative charge can be considered a fraction (χ) of z_E. Retention equations were obtained, including the work necessary to bring this fractional charge from the electroneutral bulk

solution to the charged stationary phase in the electrochemical potential of the zwitterionic analyte. This fractional charge approach proved to be more operative than the dipole approach. It was able to predict very different zwitterions retention patterns as a function of the mobile phase concentration of the H. It is the purpose of the present contribution to present and model the zwitterions retention as a function of the surface concentration of H, to offer a cross validation of the model.

THEORY

We have demonstrated that:[20]

$$k = \phi K_{LE} \frac{\gamma_L \gamma_E}{\gamma_{LE}} \exp(|\chi \cdot z_E F \Psi^\circ| / RT)([L]_T - [LH]) \quad (1)$$

where ϕ is the phase ration of the column, K_{LE} is the thermodynamic equilibrium constant for the adsorption of the analyte E, γ is the activity coefficient for each species, F and R are, respectively, the Faraday and the gas constants, $[L]_T$ is the total hydrocarbonaceous ligand concentration, [LH] is the surface concentration of the H, Ψ° is the surface potential of the stationary phase, which it develops because the inorganic counterion of the lipophilic H is not prone to adsorb onto the stationary phase; hence a net surface charge density and electrostatic potential are established.

The exponential term describes how the electrostatic interaction between E and the surface potential increases retention of zwitterions. Equation (1) predicts what Knox and Hartwick[21] already noticed: both cationic and anionic Hs could increase the retention of zwitterions via electrostatic interaction.

The right hand factor of Eq. (1) accounts for adsorption competition phenomena for the available ligand sites between the analyte and H. These exclusion phenomena, due to the limited adsorption capacity of the column, decrease analyte retention at high [LH], since, in this case, free ligand sites are expected to be a smaller fraction of the total ligand sites. The course of k vs. [LH] results from a compromise between these two opposite factors.

If adsorption competitions does not apply, the plot of k vs. [LH] will not show a fold-over, because the number free ligand sites approaches $[L]_T$ and Eq. (1) can be approximated by the following equation:[20]

$$K = \phi K_{LE} \frac{\gamma_L \gamma_E}{\gamma_{LE}} \exp(|\chi \cdot z_E F \Psi^\circ| / RT) \cdot [L]_T \quad (2)$$

k as a function of [LH]

By inserting the rigorous solution of the Poisson Boltzman expression,[9] which gives the surface potential as a function of [LH], into Eq. (1), if we

assume that the activity coefficient ratios are almost constant (experiments with constant ionic strength will be performed to fulfill this condition), we have the following expression that describes the zwitterionic analytes retention as a function of [LH]:

$$k = d_1\left([\mathrm{LH}]f + (([\mathrm{LH}]f)^2 + 1)^{1/2}\right)^{2d_2}(d_3 - [\mathrm{LH}]) \tag{3}$$

where:

$$f = \frac{z_{\mathrm{H}}\mathrm{F}}{(8\varepsilon_0\varepsilon_{\mathrm{r}}\mathrm{R}T\sum_i c_{0i})^{1/2}} \tag{4}$$

and ε_{o} is the electrical permittivity in vacuum, ε_r is the dielectric constant of the mobile phase, and Σc_{0i} is the mobile phase concentration ($\mathrm{mol\,m^{-3}}$) of singly charged electrolyte ions; the constant f ($\mathrm{m^2\,mol^{-1}}$), can be evaluated from the mobile phase composition, we also have:

$$d_1 = \phi K_{\mathrm{LE}}\frac{\gamma_{\mathrm{L}}\gamma_{\mathrm{E}}}{\gamma_{\mathrm{LE}}} \tag{5}$$

$$d_2 = |\chi \cdot z_{\mathrm{E}}| \tag{6}$$

$$d_3 = [\mathrm{L}]_{\mathrm{T}} \tag{7}$$

It follows that[20] d_1 is equal to (k_o/d_3): hence, it is not an additional fitting parameter, if k_o that represents the retention factor when H is not present in the eluent is known.

If adsorption competitions are missing from Eq. (2) we have:

$$k = d_1\left([\mathrm{LH}]f + \left(([\mathrm{LH}]f)^2+1\right)^{1/2}\right)^{2d_2}d_3 \tag{8}$$

The fitting of Eqs. (3) and (8), if k_o is known, requires the optimization of only two parameters. We may consider Eq. (8) a particular case of Eq. (3).

Equation (3) of the present work parallels Eq. (24) of our recent dipole-based retention model for IIC of zwitterions,[18] except the d_2 parameter is not related anymore to the dipole moment of the molecule, but to an operative, effective charge.

Equation (3) can also be obtained from Eq. (34) of reference [9] concerning IIC of charged analytes, if the analyte is considered fractionally charged and ion pair equilibria are neglected. This confirms how wide in scope and how versatile is the general extended thermodynamic approach to IIC.

k as a Function of [LH] and pH

Under the hypothesis that adsorption competitions are not operating, we wish to modify the retention equation as a function of [LH] to take into account that only a fraction of the analyte is in the zwitterionic form.

For an aminocarboxylic acid at acidic pH,[22] k may be described by the following expression:

$$k = \alpha_+ k_+ + \alpha_\pm k_\pm \tag{9}$$

where α_+ and $\alpha_\pm$ are the molar fraction of the protonated and the zwitterionic form of the aminocarboxylic acid and k_+ and $k_\pm$ are, respectively, retention factors of the positive ion and of the zwitterionic form of the analyte. The dependence of k_+ on [LH] is described by Eq. (34) of reference 9 (the right hand factor in the denominator is omitted under the assumption that adsorption competitions are not operating). Under the same hypothesis the dependence of $k_\pm$ on [LH] is described by the above Eq. (8). By inserting these relationships into Eq. (9) we obtain the following expression that accounts for the simultaneous effect of pH and [LH]:

$$k = \alpha_+ \frac{d_{1+}\left([\mathrm{LH}]f + \left(([\mathrm{LH}]f)^2+1\right)^{1/2}\right)^2 + d'_2[\mathrm{LH}]^{1/b}}{(1 + d'_3[\mathrm{LH}]^{1/b})} + \alpha_\pm d_{1\pm}\left([\mathrm{LH}]f + \left(([\mathrm{LH}]f)^2+1\right)^{1/2}\right)^{2d_2} d_{3\pm} \tag{10}$$

where d_{1+} and $d_{1\pm} \cdot d_{3\pm}$, respectively, correspond to k_{0+} and $k_{0\pm}$ which, respectively, represent k_+ and $k_\pm$ when H is not present in the eluent. They are experimentally obtained at suitable mobile phase pHs to control the charge status of the analyte; d_2 is given by Eq. (6), and d'_2 and d'_3 are given by Eqs. (36) and (37) of reference 9. Noteworthy, Eq. (10) requires only the optimization of three fitting parameters.

EXPERIMENTAL

The instrumentation, chemicals, procedures, registration of the adsorption isotherms, and software have been described elsewere.[20] Detailed specific information is detailed in the figure captions.

RESULTS AND DISCUSSION

A judicious choice of model compound was devised. Most solutes belong to a homologous series varying in the length of the spacer unit separating the two oppositely charge centres of the molecule.

Two different H's were used: the first is a typical reagent, sodium 1-hexanesulfonate ($NaHexSO_3$), while the second is an unusual reagent, [S-(R,R)]-(-)-Bis(-alpha-methylbenzyl)amine HCl (MBA). Noteworthy, for the latter, the retention equations hold true, thereby demonstrating how comprehensive they are. H concentrations were always below the critical micelle concentration to rule out the possibility that retention maxima could be related to exceedingly high [LH]. The ionic strength, while changing H concentrations,

was kept constant via the addition of a compensatory electrolyte to always give the same counter-ion concentration. This assures that f in Eq. (4) is actually constant, and salting effects on the activity coefficients are absent.

Figure 1 of reference 20 shows the experimental adsorption isotherms of $NaHexSO_3$ and MBA fitted by the Freundlich equation, which gives [LH] as a function of the mobile phase concentration of H.

Figures 1–4 show retention data obtained with $NaHexSO_3$ in its standard eluent, with the Synergi Hydro-RP column, as a function of [LH], and their fitting according to Eqs (3) and (8), as detailed in the captions.

Figure 5 illustrates retention data obtained with $NaHexSO_3$ in its standard eluent, with the Synergi Max-RP column, as a function of [LH], and their fitting according to Eq. (10), as detailed in the captions. Equation (10) was used, since the ionization of the carboxylic group could not be considered complete, according to the mobile phase pH and the pK_a of each analyte.

Figure 6 details retention data obtained with MBA in its standard eluent, with the Synergy Max-RP column, as a function of [LH], and their fitting according to Eq. (3), as detailed in the captions.

The parameter estimates, correlation coefficients, standard deviations, and sum of square errors are detailed in Tables 1–3, respectively.

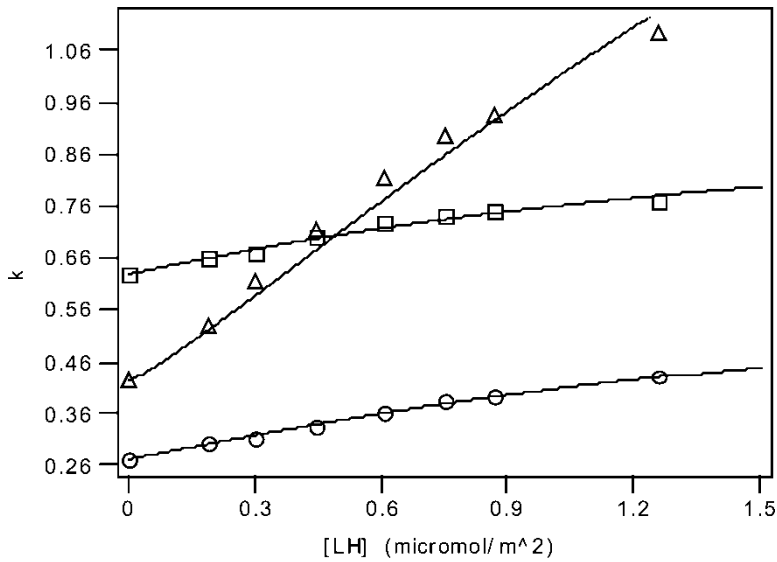

Figure 1. Dependence of k for 2-aminovaleric acid (squares), 4-aminobutyric acid (circles), and 5-aminovaleric acid (triangles) upon stationary phase concentration of $NaHexSO_3$ in its standard eluent; phosphate buffer 37.10 mM KH_2PO_4 and 4.29 mM Na_2HPO_4; total concentration IIR plus NaCl: 50 mM (constant ionic strength); final pH: 5.83. Column: Synergi Hydro-RP (150 mm × 4.6 mm I.D., 4 μm particle size, 80Å pore size). Temperature: 25.0 ± 0.1°C. Experimental data were fitted by Eq. (8).

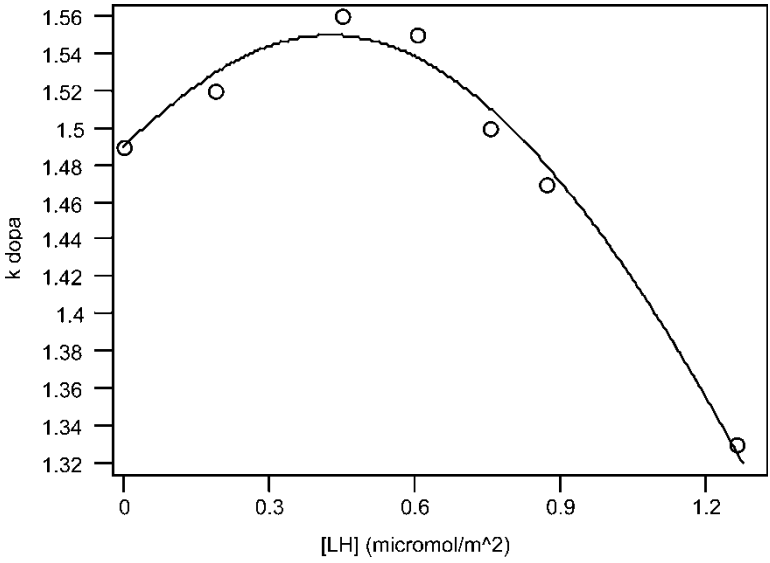

Figure 2. Dependence of k for dopa upon stationary phase concentration of $NaHexSO_3$ in its standard eluent; phase system as in Fig. 1. Column: Synergi Hydro-RP. Temperature: 25.0 $\pm$ 0.1°C. Experimental data were fitted by Eq. (3).

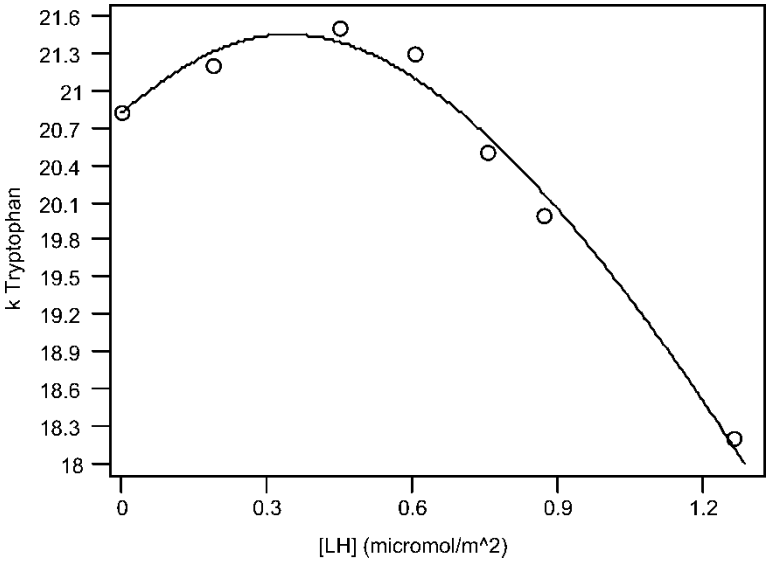

Figure 3. Dependence of k for tryptophan upon stationary phase concentration of $NaHexSO_3$ in its standard eluent; phase system as in Fig. 1. Column: Synergi Hydro-RP; Temperature: 25.0 $\pm$ 0.1°C. Experimental data were fitted by Eq. (3).

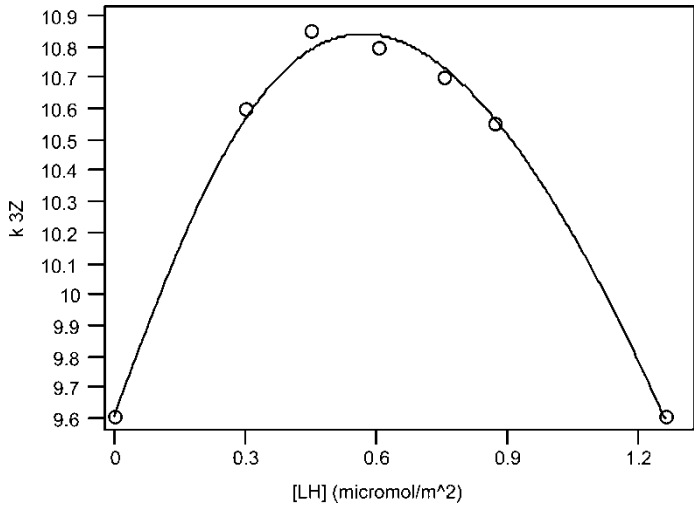

Figure 4. Dependence of k for the enantiomeric dipeptides H-Gly-Phe-OH, and H-Gly-D-Phe-OH upon stationary phase concentration of $NaHexSO_3$ in its standard eluent; phase system as in Fig. 1. Column: Synergi Hydro-RP. Temperature: $25.0 \pm 0.1^{\circ}C$. Experimental data were fitted by Eq. (3).

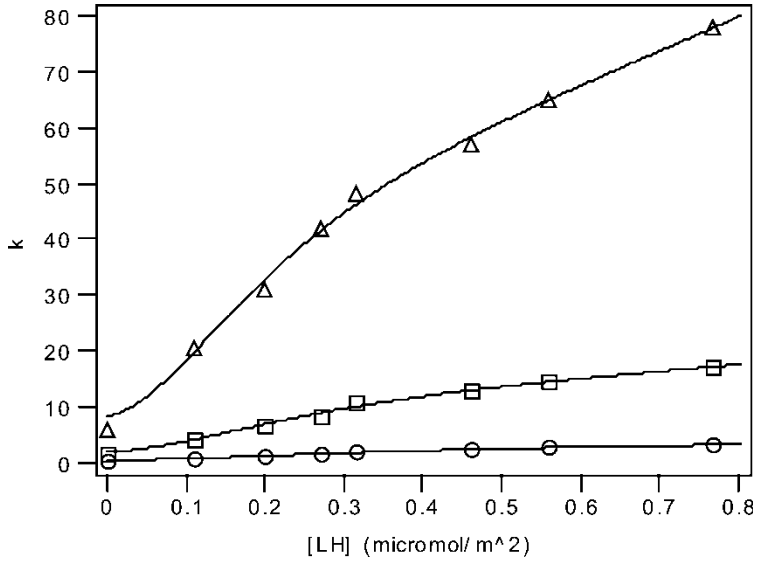

Figure 5. Dependence of k for 6-aminocaproic acid (circles) for 7-aminoheptanoic acid (squares), and 8-aminocaprylic acid (triangles) upon stationary phase concentration of $NaHexSO_3$ in its standard eluent; phase system as in Fig. 1. Column: Synergi Max-RP: (150 mm $\times$ 4.6 mm I.D., 4 μm particle size, 80Å pore size). Temperature: $25.0 \pm 0.1^{\circ}C$. Experimental data were fitted by Eq. (10).

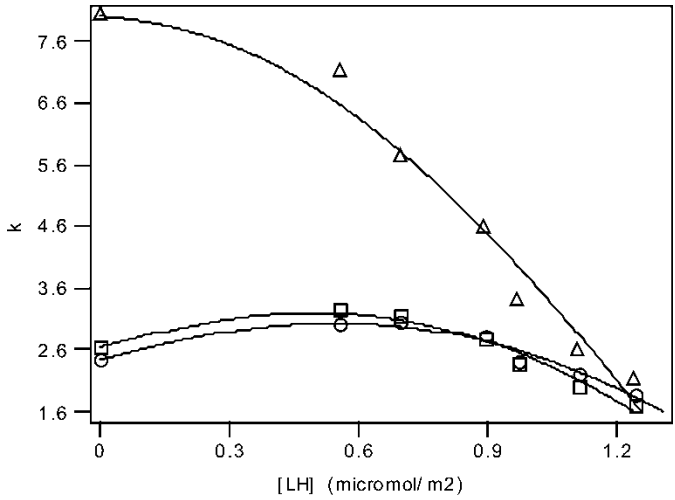

Figure 6. Dependence of k for the following enantiomeric dipeptides H-Gly-Phe-OH, and H-Gly-D-Phe-OH (circles); H-Ala-Phe-OH, and H-D-Ala-D-Phe-OH $*$ H_2O (squares); H-Ala-D-Phe-OH, and H-D-Ala-Phe-OH (triangles), upon stationary phase concentration of MBA in its standard eluent: phosphate buffer 37.10 mM KH_2PO_4 and 4.29 mM Na_2HPO_4–methanol (90 : 10); total concentration IIR plus NaCl: 50 mM (constant ionic strength); final pH: 6.00 Column: Synergi Max-RP. Temperature: 25.0 $\pm$ 0.1°C. Experimental data were fitted by Eq. (3).

The practicality of retention equations is supported by their good fitting capabilities of very variable retention data, as may be observed in Figs. 1–6. The good correlation coefficients in Tables 1–3 confirm their usefulness.

The plot of analyte retention as a function of [LH] reveals noteworthy facets of the phenomenon that cannot be observed when retention is plotted as a function of the eluent concentration of H. It is interesting to observe

Table 1. Summary of parameter estimates, standard deviations (σ), correlation coefficient (r), and sum of square errors (SSE) for the best fit of experimental data in Figs. 1–4 by Eqs. (3) and (8)

Analyte	d_2	σd_2	d_3 (μmol/m^2)	σd_3	r	SSE
2-Aminovaleric acid	5.33E−02	1.26E−03			0.9914	0.0003
4-Aminobutyric acid	1.15E−01	2.54E−03	—	—	0.9919	0.0003
5-Aminovaleric acid	2.50E−01	2.10E−03	—		0.9982	0.0013
Dopa	8.92E−02	4.30E−03	3.42E+00	1.30E−01	0.9922	0.0006
Tryptophan	8.07E−02	4.71E−03	3.47E+00	1.49E−01	0.9920	0.1228
H-Gly-Phe-OH/ H-Gly-D-Phe-OH	1.32E−01	2.08E−03	3.14E+00	5.14E−02	0.9978	0.0078

Table 2. Summary of parameter estimates, standard deviations (σ), correlation coefficient (r), and sum of square errors (SSE) for the best fit of experimental data in Fig. 5 by Eq. (10). See the text for the explanation of the terms $c'_2/(a^{1/b})$ and $c'_3/(a^{1/b})$

Analyte	d_2	σd_2	d'_2	$\sigma d'_2$	$c'_2/(a^{1/b})$	d'_3	$\sigma d'_3$	$c'_3/(a^{1/b})$	r	SSE
6-Aminocaproic acid	2.84E−01	9.85E−02	8.49E+01	3.64E+02	3.56E+02	6.47E+00	2.10E+00	7.11E+00	0.9965	0.0478
7-Aminoheptanioc acid	5.73E−01	1.02E−01	4.12E+02	2.31E+03	2.26E+03	1.14E+01	3.74E+00	1.14E+01	0.9976	0.9326
8-Aminocaprylic acid	6.97E−01	5.35E−02	2.63E+03	1.16E+04	1.16E+04	2.05E+01	6.45E+00	2.06E+01	0.9976	18.5506

Table 3. Summary of parameter estimates, standard deviations (σ), correlation coefficient (r), and sum of square errors (SSE) for the best fit of experimental data in Fig. 6 by Eq. (3).

Analyte	d_2	σd_2	d_3	σd_3	r	SSE
H-Gly-Phe-OH/ H-Gly-D-Phe-OH	2.50E−01	1.31E−02	3.42E+00	1.30E−01	0.9793	0.0488
H-Ala-Phe-OH/ H-D-Ala-D-Phe-OH $*$ H_2O	2.54E−01	1.13E−02	3.47E+00	1.49E−01	0.9886	0.0463
H-Ala-D-Phe-OH/ H-D-Ala-Phe-OH	1.20E−01	2.26E−02	3.14E+00	5.14E−02	0.9866	0.8038

that, for both the Hs and for both the columns, the surface concentration of H at which the retention fold-over eventually shows is *ca.* 0.5 $\mu mol/m^2$, even if, according to the specific adsorption isotherm, this surface concentration corresponds to different mobile phase concentration of the two different Hs. It is rewarding to observe that this surface concentration perfectly compares to the one (0.58 $\mu mol/m^2$) at which Stahlberg and Bartha[23] noticed adsorption competitions for $NaHexSO_3$ (taking into account that the surface area was 173 m^2/g for their stationary phase).

It has to be remarked, in Tables 1 and 3, that $[L]_T$ estimated by the d_3 parameter matches well with the bonded phase coverage of the columns. The mean d_3 in Table 1 is 3.34 $\mu mol/m^2$ while the calculated bonded phase coverage of the Synergi Hydro-RP column is 4.05 $\mu mol/m^2$. The mean d_3 in Table 3 is 1.58 $\mu mol/m^2$ while the calculated bonded phase coverage of the Synergi Max-RP column is 3.21 $mmol/m^2$. The order of magnitude is correct and the estimates make sense physically.

As expected, there is a good agreement, as regards the effective charge of a particular analyte, between its estimates obtained, respectively, from the fitting of retention data plotted against the mobile phase (see Tables 2–4 of reference 20) and the stationary phase concentrations of H (Tables 1–3). The same can be said as regards the estimated total ligand concentration. It is noteworthy that, as predicted by the theory,[9] the d'_2 and d'_3 parameter estimates in Table 2 compare well with $c'_2/(a^{1/b})$ and $c'_3/(a^{1/b})$ that were calculated from the c'_2 and c'_3 values in Table 3 of reference[20] and the Freundlich constants for the adsorption isotherm of $NaHexSO_3$ (a = 1.10E−01 - $\mu mol\, m^{-2}\, mmol^{-b}\, dm^{3b}$, b = 5.20E−01). It is also rewarding to notice that both d'_2 and d'_3 increase with increasing analyte lipophilicity.

Estimated magnitudes of the effective charges fit in the picture of the system given by this fractional charge approach, since they are always lower than the unit charge. It is noteworthy that their estimates show the same trend as that of the molecular dipole moments for the homologous series

4-aminobutyric-, 5-aminovaleric-, 6-aminocaproic-, 7-aminoheptanoic-, 8-aminocaprylic acid, always increasing with increasing bridge-chain length (that is the length of methylene-chain that connect the two ionic centers of the zwitterion) (see Tables 1 and 2). A longer chain length implies that the molecular tail, similarly charged as the stationary phase, is farther from the latter; hence, the electrostatic repulsive force is lower and the modulus of the neat attractive force is higher. It follows that the fractional charge estimated by the model (d_2) is consistently higher. We have demonstrated that retention of a neutral analyte decreases with increasing H concentration because it experiences adsorption competitions. We may push forward our predictions and hypothesize that the retention patterns of strongly polar neutral solutes could resemble those hereby presented for zwitterionic analytes. A retention increase upon the addition of H should not, a priori, be excluded because of the attractive influence of the electrostatic surface potential on the molecular dipole.

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