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Measurement and simulation of tailing zones of a cationic dye in analytical-scale reversed phase chromatography

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Abstract

A quantitative physical description of tailing is reported here for analytical-scale reversed phase chromatography with Type B silica. Simulations of experimental chromatograms for a cationic dye, 1,1′-didodecyl-3,3,3′3′-tetramethylindocarbocyanine perchlorate (DiI) were performed as a function of DiI concentration and flow rate, revealing nonlinear tailing due to a bi-Langmuir adsorption isotherm. The strong site comprises less than 0.1% of the residual silanols, the desorption rate constant of DiI from this type of site is $(85 \pm 8\,\text{ms})^{-1}$, and the free energy of the silanophilic interaction is 16 kJ/mol, indicating hydrogen bonding or another strong electrostatic interaction.

Keywords: Tailing; Simulation; Desorption; Silanols; DiI; Dyes

1. Introduction

Tailing of reversed phase chromatographic zones is a widespread problem for the separation of pharmaceuticals, peptides and proteins due to the interactions of amino groups with residual silanols [1,2]. Experimental studies have described the extent of tailing for different bases [3], stationary phases [4,5], mobile phases [6], and pH values [7,8]. In addition, sets of test compounds have been developed for characterizing the silanophilic activity of chromatographic columns [9–13]. Tailing in reversed phase analytical-scale chromatography is known to be due to "mixed mode" retention, where the hydrophobic monolayer comprises one type of site and the "active" silanols comprise a second type of site [1,2]. The "active" silanols that give rise to tailing are reported to be isolated silanols [14,15]. The body of knowledge has provided tools to the practitioner for reducing tailing and guidance to manufacturers for designing improved silica gel.

Despite the large body of knowledge on tailing, and its continued importance, there is a dearth of quantitative information about tailing. To understand tailing quantitatively, one would need to know the physical parameters (equilibrium constants, surface coverages, and desorption rate constants) for the compound at the different types of sites. One would also need to have a model into which these parameters could be input to generate chromatograms that matched experiment. This combination of knowing the model and the parameters would constitute a quantitative understanding of the chromatographic phenomenon.

Significant progress has been made toward the quantitative understanding of chromatography. Lenhoff showed that simulations of chromatographic zones using reasonable physical parameters and a two-site model predicts tailing at the nominally non-overloading concentrations that one uses in analytical-scale reversed phase chromatography for organic bases [16]. Guiochon pioneered both methodology for determining the physical parameters experimentally and investigating appropriate models for using these physical parameters to simulate chromatograms for comparison with experiment. These efforts began in 1988 [17] and have recently been reviewed [18]. The main focus has been the nonlinear tailing zones of preparative and process-scale chromatography, but the approach is directly applicable and recently used to study tailing in analytical-scale chromatography [18]. The Guiochon group has shown the important distinction between tailing due to a nonlinear adsorption isotherm and tailing due to slow kinetics [19,20]. These advances have also been applied to gas chromatography, where the model in-

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cludes distributions of adsorption sites [21]. There has been one use of this approach, to our knowledge, to study tailing of an organic base in reversed phase analytical-scale chromatography [22]. The results indicate the presence of rare sites giving rise to tailing of *N*,*N*-dimethylaniline on Type A silica, although the simulations do not overlay the experimental data. It would be valuable to have a study of Type B silica, since this is much more commonly used in the pharmaceutical industry.

The purpose of this work is to investigate the chromatographic tailing of a compound on Type B silica by experimental chromatography and simulation. We chose the compound 1,1′-dioctadecyl-3,3,3′3′-tetramethylindocarbocyanine perchlorate (DiI) for two reasons: (1) it is cationic and hydrophobic, which are characteristic of compounds that tail significantly in reversed phase analytical-scale chromatography, and (2) it is amenable to ultrasensitive study by fluorescence spectroscopy, potentially enabling the results of spectroscopic and chromatographic experiments to be combined. Herein, we report on our investigations of the reproducibility of the DiI chromatograms, we determine whether the simple model of the bi-Langmuir adsorption isotherm allows quantitative agreement between simulation and experiment, and obtain estimates for equilibrium constants, surface coverages, and desorption rate constants within experimental error.

2. Experimental

The cationic dye, 1,1′-didodecyl-3,3,3′3′-tetramethylindocarbocyanine perchlorate, was obtained from Molecular Probes and was used as the analyte. The acetonitrile was obtained from Aldrich as HPLC grade. The water was purified to a resistance of 18 MΩ cm using a Barnstead E-pure system.

The chromatograph used in these experiments was an Agilent 1100 with an isocratic pump, vacuum degasser, thermostatted column, and diode-array absorbance detector. The stationary phase was Zorbax-RX-C8, with column dimensions of 8 cm × 4.6 mm, and was purchased from Agilent. This is a nonendcapped material that is prepared commercially by derivatizing a Zorbax RX silica substrate with chlorodiisopropyloctylsilane, which is called Stable Bond^{TM}. The mobile phase was 90% acetonitrile/water (v/v) with 0.01 M HCl added to the water portion. The temperature was set to 20 °C. For studying the concentration dependence, the injected volume was 10 μl and the flow rate was 1.0 ml/min. The injected concentrations were 0.9, 8.1, 26, 88, 260, 309 μM. For studying the flow rate dependence, the injected concentration was 8.1 μM. The injected volume was adjusted to keep a constant injection time of 0.01 min for each flow rate. The detector response time was set to 1 s, which was significantly faster than the peak widths for all flow rates studied. Three replicates were performed in every case to establish that the chromatograms were reproducible.

Fortran software for the simulations was kindly donated by Professor Georges Guiochon of the University of Tennessee. This code uses the bi-Langmuir model for adsorption of analyte onto a stationary phase containing two sites, and the equilibrium-dispersive model to account for the sources of zone broadening, which combines all sources of non-equilibrium broadening into an apparent axial dispersion term and assumes fast exchange of the analyte between stationary and mobile phases [23,24]. We translated the code for operation with the computer program Matlab v.6.5. To determine the best-fit parameters, the equilibrium constant for the strong site was systematically varied from 10,000 to 100,000 and the relative coverages of strong and weak sites were then optimized to determine the best fit. In order to minimize the number of independent parameters that needed to be determined from the simulation, the net column efficiency was measured experimentally using an 8.1 μM solution of DiI, and was input as a simulation parameter for the plate height. This parameter is used to calculate the apparent axial dispersion coefficient. The desorption times were then independently determined from the flow rate dependence of the zone broadening.

3. Results and discussion

Fig. 1a shows chromatograms for DiI at six concentrations, 0.9, 8.1, 26.2, 88, 260, 309 μM. The chromatograms shown are for three replicate runs, which virtually superimpose, demonstrating that a highly reproducible phenomenon is being studied. The column had been used periodically at low pH for 3 months, which likely had lead to some hydrolysis or reorganization of the hydrocarbon [25], therefore, the behavior described here is the stable behavior of an aged column. The chromatograms of Fig. 1a exhibit a shortening of the peak retention time with higher concentration, which is a known phenomenon that indicates nonlinear tailing is occurring. In analytical-scale reversed phase chromatography of organic bases, for which tailing is prevalent, injected concentrations would usually be in the range of 10–100 μM, and DiI exhibits tailing for this same range of concentrations. The chromatogram at the concentration of 0.9 μM is shown in Fig. 1b, establishing that the zone becomes symmetric at sufficiently low concentration. There is a small deviation near the baseline of the rising side of the peak, which is likely due to an impurity. The ability to observe chromatograms of DiI well below the concentration where nonlinear tailing occurs owes to DiI having an unusually large molar absorptivity, 160,000 in acetonitrile, in the visible part of the spectrum.

Fornstedt et al. have described two types of processes leading to tailing: nonlinear tailing and kinetic tailing [20]. Nonlinear tailing, as we have discussed, derives from the concentration reaching the nonlinear part of the adsorption isotherm, and it is characterized by an increase in tailing with concentration. Kinetic tailing derives from very slow

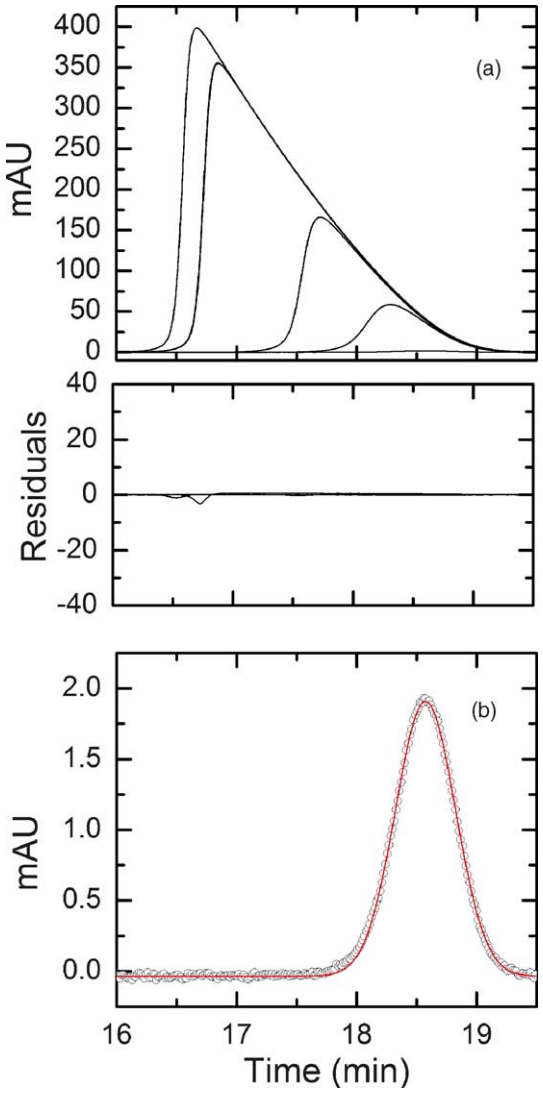

Fig. 1. (a) Chromatograms of DiI as a function of analyte concentration over the range of 0.9–309 μM. This graph overlays three replicate chromatograms at each concentration. A plot of the residuals between the replicate chromatograms is shown, which demonstrates high reproducibility of the experimental data. (b) Chromatogram (○) for the lowest concentration of DiI, 0.9 μM, plotted on a smaller scale to show that the chromatogram is symmetric. A Gaussian curve (—) is shown for reference.

desorption kinetics, and it is characterized by an increase in tailing with increased flow rate. As it is possible for both types of tailing to contribute to the shape of the zone, we varied the mobile phase flow rate to assess the possible onset of kinetic tailing. The chromatograms for an 8.1 μM solution of DiI at flow rates of 1.0 and 5.0 ml/min are shown in Fig. 2. These chromatograms show that the zone becomes slightly asymmetric at the highest flow rate, indicating that kinetic tailing just begins to contribute at the unusually high flow rate of 5 ml/min. For the chromatograms analyzed in this work, where a more typical flow rate of 1.0 ml/min was used, kinetic tailing is negligible, and only nonlinear tailing contributes to the zone shape.

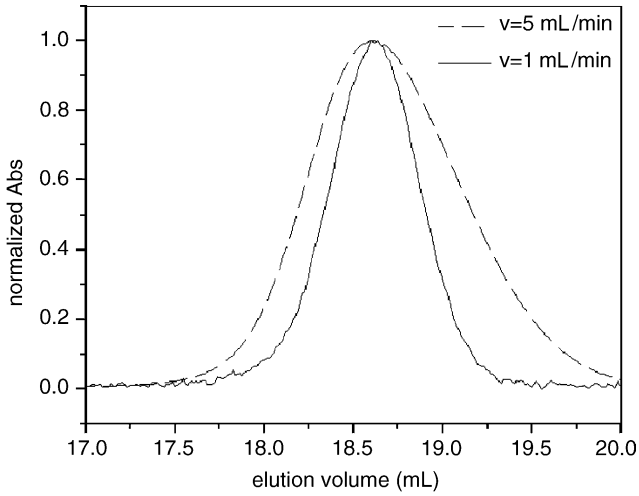

Fig. 2. Flow rate dependence of DiI chromatograms for the concentration of 8.1 μM: 1 ml/min (—) and 5 ml/min (– – –). The asymmetry of the zone at the higher flow rate shows that kinetic tailing begins to occur only at the highest flow rate.

The fact that the peak retention time begins to shorten between 10 and 30 μM indicates that the equilibrium constant for the site being saturated is between 1/(30 μM) and 1/(10 μM), i.e., between 30,000 and 100,000. This can be used to test whether it is feasible for a one site model to describe our data by comparing the expected retention time with a one site model with the actual retention time observed for the DiI chromatograms. In the limit of no saturation of adsorption sites, K has its well known relation to k' through the phase ratio, ϕ.

$$k'_{c\to 0\,\mathrm{lim}} = \frac{t_\mathrm{r} - t_0}{t_0} = \frac{\mathrm{mol_s}}{\mathrm{mol_m}} = K\phi \tag{1}$$

The equilibrium constant is inversely proportional to the surface coverage at saturation, Γ_{sat}.

$$K = \frac{\Gamma}{a}\frac{1}{\Gamma_{\mathrm{sat}}} \tag{2}$$

The phase ratio is related to the surface area, A, and the experimentally measurable volume of:

$$\phi = \frac{\Gamma_{\mathrm{sat}}}{1M}\frac{A}{V_\mathrm{m}} \tag{3}$$

the mobile phase, V_m, in the column. The value of Γ_{sat} can be determined from the adsorption isotherm. The concentration term in the denominator of Eq. (3) accounts for the use of the standard state in Eq. (2). For the material used in these separations, $V_\mathrm{m} = 0.6$ ml, $A = 180\,\mathrm{m^2/g} \times 0.75\,\mathrm{g}$, which is the dry weight of silica in the 8 cm column. Therefore, $\phi = \Gamma_{\mathrm{sat}}(2.3\times10^5)\,\mathrm{m^2/l}$. From Eqs. (1)–(3), for $K \geq 30{,}000$, and assuming $\Gamma_{\mathrm{sat}} \approx 1\,\mu\mathrm{mol/m^2}$, which is the highest concentration of DiI on the stationary phase limited only by steric constraints, one can estimate that $t_\mathrm{r} \geq 4140$ min or 69 h in the limit of low DiI concentration. This calculation demonstrates that there must be at least two sites because

the high equilibrium constant required for there to be nonlinear tailing in the micromolar range is simply not compatible with the early retention time of 18.5 min, unless the saturating site is very rare. Qualitatively, therefore, nonlinear tailing coupled with fast elution must be due to plentiful weak sites combined with rare strong sites, necessitating at least a two-component Langmuir adsorption isotherm.

Simulations of the chromatograms can be used to determine the equilibrium constant and coverages of the sites, an endeavor that has been pioneered by Guiochon [17,18]. Simulations require choice of a model—preferably the simplest model that can be applied to describe the data. As discussed previously, at least two sites are needed to account for the DiI chromatograms, therefore, the Langmuir adsorption isotherm is not a suitable model. The next simplest model is the bi-Langmuir adsorption isotherm, given in Eq. (4). K is the:

$$\Gamma = \Gamma_{1,\mathrm{sat}}\frac{K_1 c}{1+K_1 c} + \Gamma_{2,\mathrm{sat}}\frac{K_2 c}{1+K_2 c} \quad (4)$$

equilibrium constant and c is the solution concentration, which is approximately equal to the activity, Γ is the surface coverage, and $\Gamma_{1,\mathrm{sat}}$ and $\Gamma_{2,\mathrm{sat}}$ are the saturated surface coverages for sites 1 and 2, respectively. A more complex model might be needed. For example, one could add more types of adsorption sites, use a more complex adsorption isotherm, or include the possibility that each type of adsorption site has a range of equilibrium constants. Prior to invoking more complicated models, the bi-Langmuir model was tested to see if it could be used to describe the DiI chromatograms.

It was assumed as a starting point that the weak sites never saturate over the concentration range used, therefore, only the product $K_1\Gamma_{1,\mathrm{sat}}$ is an independent parameter. Under this condition, the zone shape and position are described fully from four independent parameters: k'_1, K_2, $\Gamma_{\mathrm{sat},2}/\Gamma_{\mathrm{sat}}$, and N, where N is the number of theoretical plates and Γ_{sat} is the total saturated surface coverage. The value of N was calculated from the zone variance for the second lowest concentration of DiI, which produced a symmetric zone. This left three independent parameters, k'_1, K_2, and $\Gamma_{\mathrm{sat},2}/\Gamma_{\mathrm{sat}}$, to be determined from the simulations of the six chromatograms, which gives an overdetermined fit.

The results from simulating the concentration dependence of the DiI chromatograms using the bi-Langmuir model are shown in Fig. 3a. The agreement between simulation and experiment is remarkable, demonstrating that the zones are described well by the bi-Langmuir model. There is a small but consistent disagreement between simulation and experiment at the beginning of each peak. This could be due to an impurity or to departure from Langmuir adsorption, e.g., fronting. The excellent agreement between experiment and simulation does not prove that other more complex models are wrong. The agreement merely indicates that no degree of complexity beyond the bi-Langmuir adsorption isotherm needs to be invoked for this chemical system and the experimental conditions used in this study.

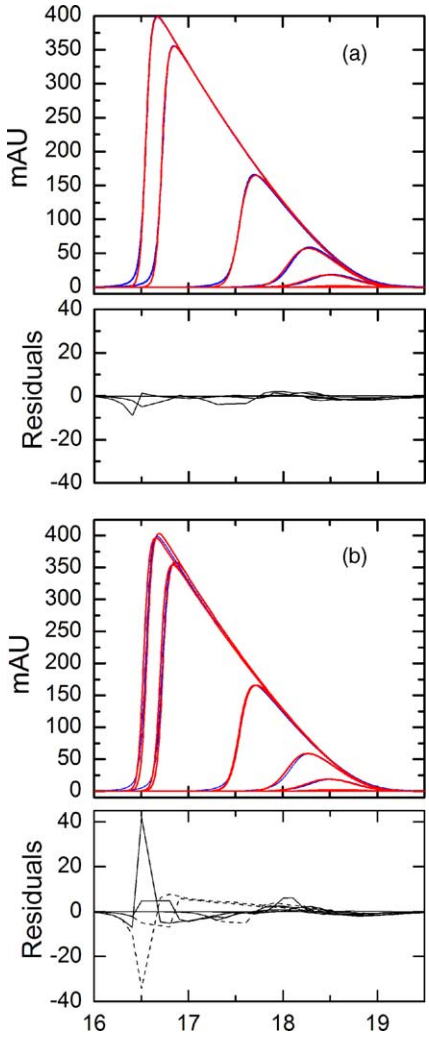

Fig. 3. Simulation (red) and experiment (blue) for the chromatograms of DiI over the six concentrations studied (a) for the best-fit parameters summarized in Table 1, and (b) for K values that are 10% higher and lower than the best-fit values. The difference between the experimental data and the results of the simulation are plotted below each set of chromatograms.

The best-fit parameters are listed in Table 1. The value for K_2 was found to be 51,000, which is consistent with the onset of nonlinear tailing being evident at the injected concentration of 26 μM. Compared to the negligible experimental spread in retention behavior that was demonstrated in Fig. 1a, it is established that variations of K_2 within 2% of the best-fit value still fit acceptably well, but a larger variation gave obvious disagreement. The latter is demonstrated by the simulated chromatograms in Fig. 3b, which corre-

Table 1
Results of simulations that reveal the best-fit values of the parameters k'_1, K_2, and ($\Gamma_{\mathrm{sat},2}/\Gamma_{\mathrm{sat}}$). The dependent parameter k'_2 is included, as well as the independently determined value of the number of theoretical plates, N

k'_1	k'_2	$\Gamma_{\mathrm{sat},2}/\Gamma_{\mathrm{sat}}$	K_2	N
14.1 ± 0.7	15.8 ± 0.7	0.0013	51000 ± 1000	5500 ± 550

spond to K_2 values of 46,000 and 56,000. One can determine the allowed energy range for a 2% spread in equilibrium constants to ascertain how much of an energy spread is allowed, this corresponds to $\Delta G_{ads} = 26.4 \pm 0.05$ kJ/mol. Given that the discrepancy in the simulations is less than 2%, the range of energies is less than 1%, pointing to a negligible distribution of energies for this system.

The k' values of Table 1 show that the strong adsorption sites contribute slightly more to retention than do the weak sites. The capacity factors are tabulated in the limit of $c \rightarrow 0$ because the relation in Eq. (1) is only valid at low concentration. Taking into account the steric restrictions for packing a full layer of DiI on the stationary phase, it can be assumed that the total saturated surface coverage (Γ_{sat}) of DiI is on the order of 1 μmol/m^2. This can be used to estimate the saturated surface coverage of DiI on the strong adsorption sites ($\Gamma_{sat,2}$), which is on the order of 1 nmol/m^2. This demonstrates that it is rare sites that allow nonlinear tailing to occur for moderately fast elution. The simulations thus illustrate that mixed-mode retention occurs, where tailing is due to the nonlinear adsorption isotherm for a rare, strong adsorption site. This rare site contributes significantly to the zone shape, despite the strong sites being low in abundance, because the high equilibrium constant compensates to populate the surface as much as the prevalent weak sites: $k'_1/k'_2 = 0.89$.

One value of knowing the coverage of active silanols is that it provides manufacturers with a physically insightful basis for assessing steps to improve chromatographic silica gel. On Zorbax RX with the Stablebond^{TM} monolayer, the residual silanols comprise an estimated 5.5 μmol/m^2, based on an average silanol surface density of 8 μmol/m^2 on the bare silica, and 2.5 μmol/m^2 of modified silanols in a typical silanization reaction [2,15]. Yet the strong adsorption sites are more than three orders of magnitude lower in coverage than the residual silanols. This underscores the notion that it is not the residual silanols that cause tailing, but a subpopulation of these that are especially active. These are reputed to be isolated silanols [14]. The number of these active silanols accessible to DiI could be different from that for other compounds, since McCalley has found that sterically bulkier analytes tail less severely [3]. DiI is bulkier than typical organic amines, therefore the coverages of active silanols might be higher for other amines. Studies of different structures would be interesting for determining how much the coverage of isolated silanols changes from one analyte to the next.

Using the simulation parameters, and some reasonable assumptions, one can obtain an estimate of the free energy of interaction of DiI with the active silanols. Assuming that the saturated coverage of DiI at weak sites is on the order of 1 μmol/m^2, then $K_1 = 60$. The free energy of interaction between DiI with the strong adsorption site (ΔG_2) will contain a contribution from the interaction of DiI with the nearby hydrocarbon component. If it is assumed that the free energy of interaction between DiI and the hydrocarbon is constant, regardless of whether DiI is adsorbed to a strong site or a weak site, then the free energy of interaction of DiI with the active silanols (ΔG_{SiOH}) can be computed by subtraction.

$$\Delta G_{SiOH} = \Delta G_2 - \Delta G_1 = RT \ln \left(\frac{K_2}{K_1} \right) \quad (5)$$

The logarithmic relation greatly reduces the impact from uncertainty in the assumptions. The resulting estimate is $\Delta G_{SiOH} \approx 16$ kJ/mol. This is the energy of a strong hydrogen bonding interaction or other strong electrostatic interaction. It is consistent with the idea of strongly hydrogen bonding, isolated silanols. Studies of the interaction energies with a suite of compounds, as well as the dependence on ionic strength, could provide further insight into the nature of these interactions.

Our conclusion of one type of strong adsorption site with a negligible distribution of energies does not refute studies that indicate a range of adsorption energies. Instead, the narrow distribution applies to this particular system and conditions. The fact that the DiI chromatograms at low analyte concentration fit to a Gaussian curve precludes a range of K values that are higher than those reported here. Gas chromatograms for methanol, diethyl ether, 1-chlorobutane, and dichloromethane on bare silica are described well by several types of sites, each with a distribution of adsorption energies [21]. FTIR studies have shown that pyridine adsorbs to bare and chemically modified silica at two types of sites, hydrogen bonded water and isolated or weakly hydrogen bonded silanols [26]. In our simulations, if the adsorption of DiI to surface water has a low enough equilibrium constant that it does not begin to saturate at the highest concentration of DiI studied, then it would only contribute to k' for the weak sites, and the simulations would fit well to the two-site model despite there being three sites. Nonetheless, our study is valuable in identifying the equilibrium constant and coverage of the most deleterious site with respect to tailing.

Kinetic data can also be obtained from the simulations. Chromatograms are broadened by multiple factors, one of which is slow desorption from the surface sites. The C term in the van Deemter equation contains information about the desorption time.

$$H = A + \frac{B}{\nu} + C\nu \quad (6)$$

Variation of the flow rate, ν, thus allows one to determine whether slow desorption contributes significantly to broadening of the chromatogram. Fig. 4 shows the van Deemter plot of experimental data for DiI, obtained by varying flow rate from 0.1 to 5 ml/min for an 8.1 μM solution of DiI. The best-fit parameters of A, B and C to the van Deemter equation are listed in Table 2, along with the 95% confidence intervals. The small number of data points at low flow rates corresponds to a large uncertainty associated with the analyte diffusion coefficient (B). The good correlation between the data and the best-fit curve at high flow rates provides a narrow error range for C. The plot shows that slow de-

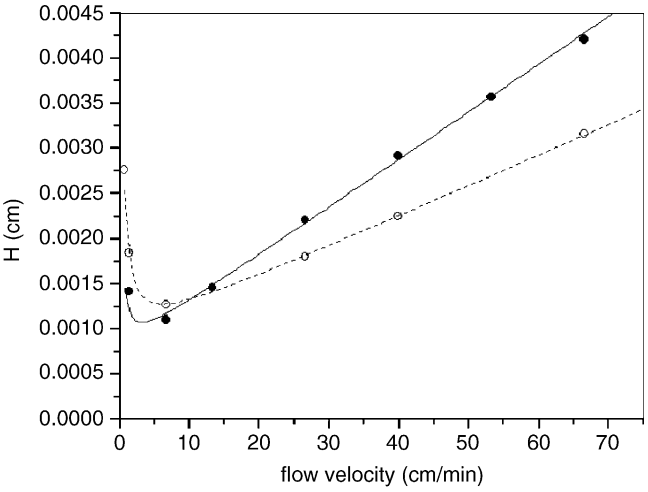

Fig. 4. van Deemter plots for DiI (●) and tetracene (○), each at 8.1 μM in concentration for the same separation conditions as in Fig. 1.

sorption begins to contribute more than other factors to zone broadening once the linear flow rate exceeds 10 cm/min. The volume flow rate of 1 ml/min corresponds to a linear flow rate of 13 cm/min. Under the conditions of the simulated chromatograms, it is evident that slow desorption from strong sites does not dominate zone broadening. This indicates that tailing is more of a problem than broadening for the silanophilic interactions of this chemical system.

Slow desorption from the strong sites contributes to the *C* term, but other factors are expected to contribute as well to *C*, such as desorption from weak sites, diffusion across the velocity distribution in the mobile phase, and intraparticle diffusion. All of these other contributions to the *C* term would affect a hydrophobic compound having no hydrogen bonding or charged group available to interact with silanols. To estimate these other contributions, tetracene was used. The van Deemter plot for tetracene is shown in Fig. 4 along with the plot for DiI, and the best-fit parameters to the tetracene data are shown in Table 2. These data were obtained for the same column, mobile phase conditions, and temperature as for DiI. The *C* term is clearly lower for tetracene, supporting the notion that slow desorption from strong sites contributes significantly to the *C* term for DiI.

To use the *C* term obtained from the tetracene data to isolate the mass transfer for DiI due only to strong adsorption, one has to derive an appropriate expression that accounts for multiple broadening processes. Giddings derived an expression for *C* using the random walk model when there is one type of adsorption site, where the desorption time, τ, is the inverse of the desorption rate constant [27]. *R* is the retention ratio, which is the ratio of velocities of the analyte and the mobile phase.

$$C = 2R(1-R)\tau \quad (7)$$

The random walk model is easily extended to multiple independent processes. One lumped time constant, τ_{sum}, will be used here to represent all processes that contribute to the *C* term other than slow desorption from the strong sites, which is represented by τ_2. Considering the two net types of processes, *C* is a linear combination of the time constants of the individual sites, where *f* is the fraction of the capacity factor due to the strong sites, in the limit of $c \to 0$.

$$C = 2R(1-R)\{\tau_{sum} + f\tau_2\} \quad (8)$$

The *C* term obtained for tetracene is used to estimate τ_{sum} since tetracene should be affected comparably by the other broadening processes while negligibly adsorbing to strong sites, which are presumed to be isolated silanols. The results for tetracene reveal $\tau_{sum} = 4.5$ ms. This value is on the order of what one would estimate for intraparticle diffusion: $r^2/2D = 3.1$ ms, where *r* is the particle radius and 2D the *B* term from the van Deemter plot for tetracene. Since the contributions of intraparticle diffusion and slow diffusion across the velocity distribution of the mobile phase both scale inversely with diffusion coefficient, the smaller diffusion coefficient of DiI would increase the value of τ_{sum} to 7.7 ms, as estimated from the *B* term in the van Deemter plot for DiI. Having a reasonable estimate for τ_{sum} now allows straightforward calculation of the desorption time from strong sites using Eq. (7). The result is that $\tau_2 = 85 \pm 5$ ms. The 5 ms spread represents a 2% spread of the K_2 values obtained from the simulations; there is potentially an additional error of a few ms due to the uncertainty in knowing the relative diffusion coefficients of DiI and tetracene. Despite the slowness of this desorption process, at the flow rate of 13 cm/min (1 ml/min), slow desorption contributes little to zone broadening, which is primarily contributed by the *A* term. The slow desorption does sharply limit the speed of the separation by virtue of the large value of *C*, and this is evident in the van Deemter plot.

We previously reported two much longer desorption processes of 7 s and 4 min for DiI using the same silica gel, and showed that these two processes occurred on fused silica [28]. These studies showed that the two very slow desorption processes were associated with very slow adsorption rates. The simulations of this present paper indicate that these very slow desorption processes contribute negligibly to the chromatographic zone, presumably due to the slow adsorption

Table 2
Summary of data from van Deemter plots and retention ratio (*R*) for DiI and tetracene as eluted under the same conditions

	A (cm)	*B* (cm²/s)	*C* (min)	*R*
DiI	$6.9 \times 10^{-4} \pm 1 \times 10^{-4}$	$1.4 \times 10^{-5} \pm 4 \times 10^{-6}$	$5.4 \times 10^{-5} \pm 2 \times 10^{-6}$	0.035
Tetracene	8.7×10^{-4}	2×10^{-5}	3.4×10^{-5}	0.65

rates. Sites populated after equilibration times much longer than those of HPLC are thus not necessarily relevant to tailing. They could, however, be relevant to other processes, such as memory effects or low recovery of sample.

A similar compound, which differs only in having longer alkyl chains, was studied by single molecule fluorescence spectroscopy on C_{18} modified fused silica. The molecule used for the fluorescence studies was DiI with C_{18} chains instead of C_{12} chains: 1,1′-dioctadecyl-3,3,3′3′-tetramethylindocarbocyanine perchlorate versus 1,1′-didodecyl-3,3,3′3′-tetramethylindocarbocyanine perchlorate. The general behavior was found to be similar for DiI-C_{18} on fused silica studied by single-molecule spectroscopy and DiI-C_{12} on Zorbax C_8 studied by chromatography. The single-molecule spectroscopy revealed strong and weak sites on fused silica with comparable populations of DiI [29], while chromatography reveals strong and weak sites with comparable capacity factors. The single-molecule spectroscopy showed that the desorption time was 68 ± 8 ms from the strong sites, which is on the order of the 85 ± 5 ms desorption time for the chromatographic experiment. The small difference could easily be accounted for by the higher temperature of several degrees centigrade for the spectroscopy experiment. The agreement between the single molecule fluorescence data that has been previously reported and the chromatographic simulations reported here supports the notion that fused silica is a good model for silica gel.

4. Conclusions

Simulations of the chromatograms of DiI have been used to achieve a quantitative understanding of the tailing behavior of this cationic probe in analytical-scale reversed phase chromatography. The simulations reveal that the chromatograms of DiI are described well by a bi-Langmuir adsorption isotherm with mixed-mode retention of strong and weak sites that have a negligible distribution of energies. The strong sites have an equilibrium constant of 51,000, which causes tailing due to non-linearity in the adsorption isotherm. These strong adsorption sites comprise less than 1:1000 of the residual silanols, and they are presumed to be isolated silanols. This supports the picture that most residual silanols do not cause tailing, and that active silanols are rare. Contributions to zone broadening by kinetic tailing were studied by varying the flow rate of the mobile phase; and tetracene was used to estimate the other contributions to zone broadening. These results reveals that slow desorption from strong sites (85 ± 5 ms) does not cause kinetic tailing, except for a small amount at the unusually high flow rate of 5 ml/min. At a flow rate of 1 ml/min the slow desorption process contributes only slight, symmetric broadening of the zone. These chromatographic findings parallel those of single-molecule spectroscopy experiments for similar systems that used fused silica rather than silica gel, which supports the notion that fused silica can be used as a model substrate for chromatographic silica gel.

Acknowledgements

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