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The adsorption mechanism of nortryptiline on C_{18}-bonded Discovery

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Abstract

The adsorption isotherms of an ionizable compound, nortriptyline, were accurately measured by frontal analysis (FA) on a C_{18}-Discovery column, first without buffer (in an aqueous solution of acetonitrile at 15%, v/v of ACN), then with a buffer (in 28%, v/v ACN solution). The buffers were aqueous solutions containing 20 mM of formic acid or a phosphate buffer at pH 2.70. The linear range of the isotherm could not be reached with the non-buffered mobile phase using a dynamic range larger than 40 000 (from 1.2×10^{-3} g/L to 50 g/L). With a 20 mM buffer in the liquid phase, the isotherm is linear for concentrations of nortriptyline inferior to 10^{-3} g/L (or 3 μmol/L). The adsorption energy distribution (AED) was calculated to determine the heterogeneity of the adsorption process. AED and FA were consistent and lead to a trimodal distribution. A tri-Moreau and a tri-Langmuir isotherm models accounted the best for the adsorption of nortriptyline without and with buffer, respectively. The nature of the buffer affects significantly the middle-energy sites while the properties of the lowest and highest of the three types of energy sites are almost unchanged. The desorption profiles of nortriptyline show some anomalies in relation with the formation of a complex multilayer adsorbed phase of acetonitrile whose excess isotherm was measured by the minor disturbance method. The C_{18}-Discovery column has about the same total saturation capacity, around 200 g of nortriptyline per liter of adsorbent (or 116 mg/g), with or without buffer. About 98–99% of the available surface consists in low energy sites. The coexistence of these different types of sites on the surface solves the McCalley's *enigma*, that the column efficiency begins to drop rapidly when the analyte concentration reaches values that are almost one hundred times lower than those that could be predicted from the isotherm data acquired under the same experimental conditions. Due to the presence of some relatively rare high energy sites, the largest part of the saturation capacity is not practically useful.

Keywords: Adsorption equilibrium; Frontal analysis; Column heterogeneity; Affinity energy distribution; Retention mechanism; Analytical and preparative chromatography; Peak tailing; Silica; Discovery-C_{18}; Nortriptyline; Acetonitrile; Excess isotherm

1. Introduction

The understanding of the retention mechanism of ionizable compounds in RP-HPLC is a field of large interest which find applications in most of the analytical separations performed in pharmaceutical, biological, alimentary, and environmental industries. Most of the studies published so far concern the retention behavior of ionizable compounds under linear conditions, evaluating the effect of the mobile phase pH, its buffer concentrations, and its organic modifier content [1–9]. For preparative applications, another crucial experimental parameter, the saturation capacity of the chromatographic bed, must be known because it will allow a good estimation of the production rate of the investigated process. While the measurement of the saturation capacities of neutral compounds has been largely investigated, it should also be measured accurately for ionizable compounds. The determination of the saturation capacity of HPLC columns is not easy because the range of concentrations that are accessible in the liquid phase is most often limited upward by the solubility of the compound studied. So, only an extrapolation of the amount adsorbed as a function of the mobile phase concentration to an infinite value of this concentration allows the determination of an estimate of the column saturation capacity. The shape of the adsorption isotherm can preclude any guess of the saturation capacity if its curvature is anti-langmuirian at the solubility concentration. Only for convex upward isotherm (langmuirian) it becomes possible to extrapolate the isotherm at infinite so-

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lute concentration and estimate the saturation capacity of the column.

The question of the quantitative measurement of the saturation capacities of ionizable compounds on commercially available RPLC columns has recently raised the attention of a few research groups [10–13]. Up to now, no systematic method has been proposed to fairly assess the column saturation capacity. Based on measurements made on the profiles of the peaks recorded for simple analytical or slightly overloaded injections, it was found that the saturation capacity for ionizable compounds was much lower that those currently measured for neutral ones [10–13]. However, the saturation capacity of an adsorbent can be estimated only if this latter is actually "saturated", e.g. when very large concentrations of the analytes are injected so that even the lowest energy sites are mostly occupied. When measurements are made under such conditions, values nearly a hundred times larger are found [14].

The fundamental and consistent reason blamed for the low adsorbent capacity derived from measurements of the column efficiency as a function of the sample size was the occurrence of repulsion between the charged adsorbent surface and the analyte. One molecule of analyte would then occupy a larger surface area on the adsorbent at saturation. Most often, analysts who use chromatography have failed to realize that commercial RPLC adsorbents are definitely heterogeneous [15]. The concentration range of analytes with which they are concerned is usually very low, hence, only a small fraction of the adsorption sites is being occupied during the elution of the propagating band profile. Stählberg and co-worker [10,11], McCalley [12], and Neue et al. [13] have observed asymmetrical band profiles (characteristic of nonlinear adsorption isotherms) with ionizable compounds at very low concentrations for which neutral compounds elutes as symmetrical gaussian peaks (corresponding to linear adsorption isotherms). They attribute this "early" deviation of the isotherm from linear behavior to electrostatic interactions that would take place between a charged surface and the ionizable analyte in the liquid phase. The analytes would be more and more repulsed from the stationary phase when the adsorbed amount increases (because the surface potential increases too).

The conclusion of repulsive interactions between the surface of the RPLC adsorbent and the ionizable analyte which could explain the nonlinear behavior of the isotherm at very low concentrations appears to be hasty and hazardous for several reasons: (1) no evidence was ever presented that the ionizable compounds adsorbed on the RPLC stationary phases as a free charged species. Instead, recent observations have shown that these organic ions adsorb rather as ion-pairs on the hydrophobic surface, with adsorbate-adsorbate interactions [16]; (2) the maximum peak concentrations corresponding to the amounts injected are too low (a few μg injected by McCalley on the C_{18}-Discovery column [12], maximum concentration of 0.5 mM for the samples injected by Neue on XTerra MS C_{18} [13] and of 1.2 mol/m^3 for those injected by Stählberg and co-worker [10] on C_{18}-LiChrospher) accurately to measure the overall saturation capacity of the column. On the other hand, it was shown that the saturation capacities are typically in the range between 100 and 250 g/L or between 1 and 2.5 mol/L for low-molecular-weight compounds (MW of ca. 100 g/mol) [17]; and (3) since the surfaces of the adsorbents packing commercial columns are likely to be heterogeneous, only the high-energy sites may be occupied at low concentrations, hence only the saturation capacity of the high-energy sites can be estimated from plots of the column efficiency versus the sample size. The saturation capacity of the low-energy sites (the largest contribution) is likely to be experimentally omitted. Snyder showed that there was a considerable difference between what he called the apparent and the maximum column capacities (1 and 60 mg for angiotensin on a RPLC silica, respectively) [18,19]. However, the obvious consequences of this result have not been accepted nor even understood. This was difficult to do as long as there was no accurate method for the study of the surface heterogeneity of RPLC stationary phases nor for the characterization of the different types of sites identified on these surfaces.

This situation has lead to what we have called the "McCalley's *enigma*" because this author was the first to point it to us. This enigma arises from the striking inconsistency between the maximum amounts of an ionizable compound that can be adsorbed by RPLC adsorbents, whether determined from the peak efficiency of low sample injections or by conventional frontal analysis (FA) measurements. The former method leads to saturation capacities of the order of 1 mg/g of adsorbent while the second gives values around 100 mg/g. The factor 100 found between the two methods is very large. Some rational must be found to reconcile them and solve the *enigma*. In this work, we performed strictly the same experiments as those made by McCalley [12] so that the comparison will be straightforward. The ionic compound analyzed was nortriptyline ($pK_a = 9.7$), the RPLC column was a C_{18}-bonded Discovery column, and the mobile phase a mixture of acetonitrile and water (28/72, v/v) buffered at pH 2.70 with either 20 mM of a phosphate or a formic acid buffer. The isotherms of nortriptyline were measured by FA and the isotherm parameters (the saturation capacities and the binding constants) will be compared to the parameters obtained by following the McCalley procedure and those of others. It will be demonstrated that the present measurements and those of McCalley are consistent and fully explain the contradiction between the interpretations of the results afforded by FA and by the low sample loading method.

2. Theory

2.1. Determination of the adsorption isotherms

The adsorption data were acquired by the dynamic frontal analysis method. The experimental details of this

method are given in the experimental section. The method for calculating the amount adsorbed per unit volume of the stationary phase were given in a previous publication [20].

2.2. Models of isotherm

The adsorption isotherm data obtained by FA for nortriptyline on C_{18}-Discovery, from an unbuffered aqueous solution of acetonitrile were best accounted for by a tri-Moreau isotherm model, an extension of the Moreau model [21] to heterogeneous surfaces (similar to the bi-Moreau that was successfully used to describe the adsorption of propranolol on different commercial RPLC columns from methanol:water solutions [16]). The tri-Moreau Isotherm model is written:

$$q^* = q_{s,1}\frac{b_1C + I_1b_1^2C^2}{1 + 2b_1C + I_1b_1^2C^2} + q_{s,2}\frac{b_2C + I_2b_2^2C^2}{1 + 2b_2C + I_2b_2^2C^2} + q_{s,3}\frac{b_3C + I_3b_3^2C^2}{1 + 2b_3C + I_3b_3^2C^2} \quad (1)$$

where $q_{s,1}$, $q_{s,2}$, $q_{s,3}$, b_1, b_2, b_3 are the monolayer saturation capacities and equilibrium constants on the sites of types 1, 2, and 3, respectively, and I_i is the adsorbate–adsorbate interaction parameter in the monolayer on sites of type i.

The same isotherm model was used to account for the FA adsorption data obtained for nortriptyline on the Discovery column in a buffered aqueous solution of acetonitrile (buffered with either a formic acid of a phosphate buffer, at pH 2.70), in which case there are no adsorbate–adsorbate interactions (hence, $I_i = 0$). Eq. (1) then becomes that of the tri-Langmuir isotherm model and corresponds to the adsorption on a heterogeneous surface covered with sites of three different, independent types. The model assumes that the surface is paved with three different types of homogeneous chemical domains which behave independently. The equilibrium isotherm results from the addition of three independent local Langmuir isotherms:

$$q^* = q_{s,1}\frac{b_1C}{1 + b_1C} + q_{s,2}\frac{b_2C}{1 + b_2C} + q_{s,3}\frac{b_3C}{1 + b_3C} \quad (2)$$

The equilibrium constants b_1, b_2 and b_3 are associated with the adsorption energies $\epsilon_{a,1}$, $\epsilon_{a,2}$, and $\epsilon_{a,3}$, through the following equation [22]:

$$b_i = b_0 e^{\frac{\epsilon_{a,i}}{RT}} \quad (3)$$

where $\epsilon_{a,i}$ is the energy of adsorption on the sites of type i, R is the universal gas constant, T is the absolute temperature, and b_0 is a preexponential factor that could be derived from the molecular partition functions in both the bulk and the adsorbed phases. b_0 is often considered to be independent of the adsorption energy $\epsilon_{a,i}$ [22].

The adsorption energy distribution (AED) functions of a tri-Langmuir is the sum of three Dirac functions:

$$F(\epsilon) = q_{s,1}\delta(\epsilon - \epsilon_{a,1}) + q_{s,2}\delta(\epsilon - \epsilon_{a,2}) + q_{s,3}\delta(\epsilon - \epsilon_{a,3}) \quad (4)$$

This energy distribution is trimodal, all these modes having a width equal to 0.

2.3. Calculation of the adsorption energy distributions

The calculation of the adsorption energy distribution (AED) was performed by using the expectation-maximization (EM) method [23]. The details of the algorithm applicable for any local isotherm (Langmuir, Jovanovic, Moreau or BET) were given in a previous publication [16].

2.4. Modeling of desorption-band profiles in HPLC

The breakthrough curves of nortriptyline were calculated, using the equilibrium-dispersive model (ED) of chromatography [24–26]. The ED model assumes instantaneous equilibrium between mobile and stationary phases and a finite column efficiency originating from an apparent axial dispersion coefficient, D_a that accounts for all the mass-transfer resistances in the chromatographic column. This model has been used successfully to describe the overloaded elution band of small compounds in RPLC [27,28] or, more generally, when the mass transfer steps are sufficiently fast and do not affect much the shape of the band profiles, simply smoothing the ideal band profiles predicted by pure thermodynamics. The ED model has the advantage of requiring only one parameter, the axial dispersion coefficient, and short calculation times, as compared to more elaborated models like the lumped pore diffusion model or the general rate model [24] which are useful only for detailed investigations of the mass transfer mechanisms.

Table 1
Physico-chemical properties of the Discovery-C_{18} adsorbent material packed in a stainless steel tube (150 mm × 4.0 mm)

Particle shape	Spherical
Particle size (μm)	5
Pore size (Å)	180
Specific surface (m^2/g) (before derivatization)	200
Total carbon (%)	12
Surface coverage (μmol/m^2)	3.0
Endcapping	Yes
Void volume measurements	1.363[a]
	1.378[b]
	1.349[c]

[a] Elution of unretained compound method.
[b] Minor disturbance method.
[c] Pycnometry method (ACN-CH_2Cl_2).

3. Experimental

3.1. Chemicals

The mobile phase used in this work was a mixture of acetonitrile and water (28:72, v/v), both HPLC grade, purchased from Fisher Scientific (Fair Lawn, NJ, USA). The solvents used to prepare the mobile phase were filtered before use on an SFCA filter membrane, 0.2 μm pore size (Suwannee, GA, USA). Thiourea was chosen to measure the column hold-up volume. Nortriptyline hydrochloride was used because it was one of the solutes used by McCalley in his previous work [12]. Thiourea and nortriptyline hydrochloride were obtained from Aldrich (Milwaukee, WI, USA). Formic acid (96%), phosphoric acid (85%), sodium formate and sodium dihydrogenophosphate, used to prepare the buffer solutions at pH 2.70, were also from Aldrich. The buffer pH was fixed at 2.70 before the addition of the organic modifier by addition of the buffer acidic solution (formic or phosphoric acid) to the buffer basic solution (sodium formate or dihydrogenophosphate) both at a concentration of 20 mM. The final total buffer concentration in the mobile phase, af-

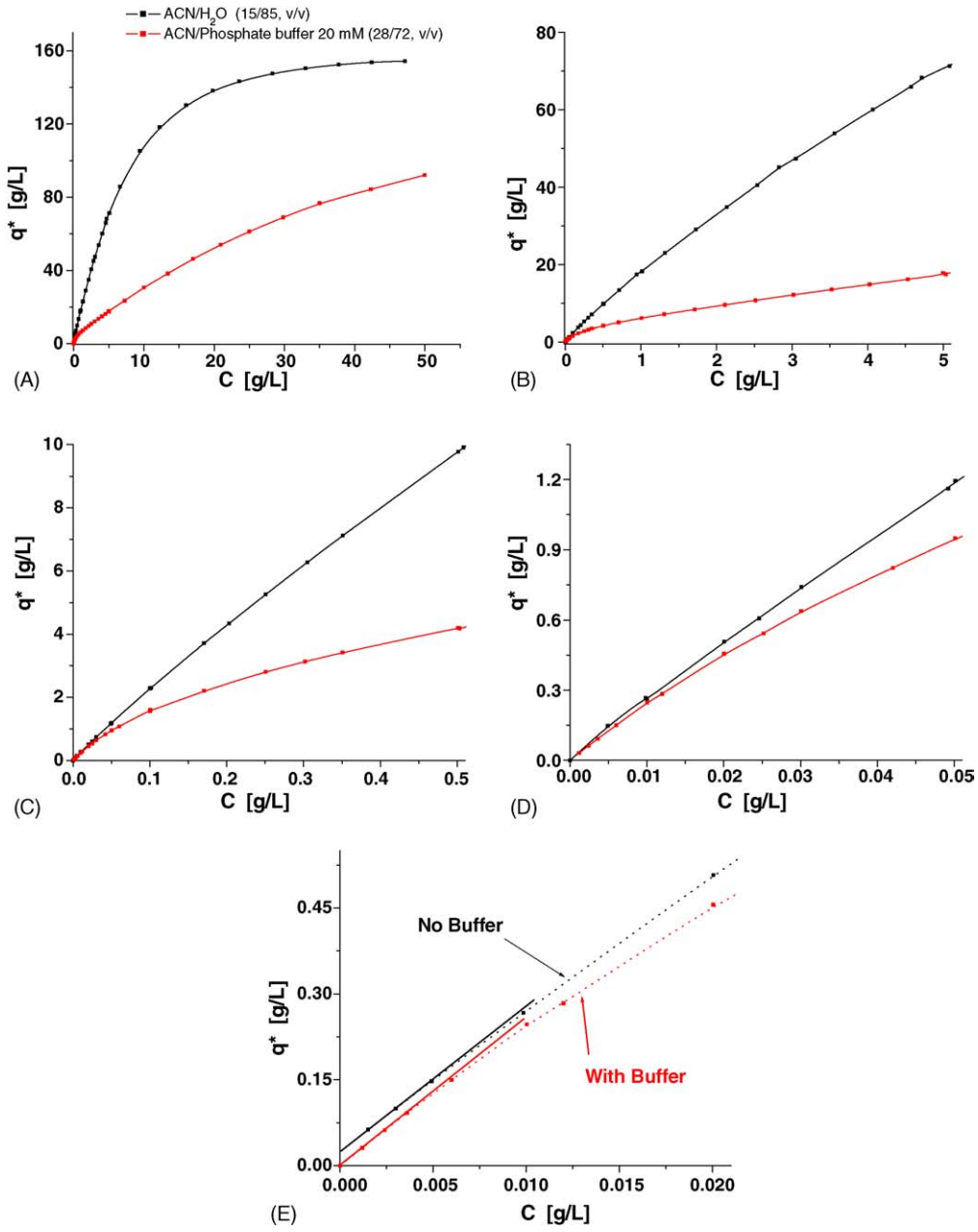

Fig. 1. Adsorption isotherm data of nortriptyline with (upper plot, 15% acetonitrile in water, v/v) and without (lower plot, 28% acetonitrile in phosphate buffered water, 20 mM, pH 2.70. $T = 295$ K). (A) 0–50 g/L concentration range in the mobile phase, (B) 0–5 g/L, (C) 0–0.5 g/L, (D) 0–0.05 g/L, (E) 0–0.02 g/L. Note that the linear range of the isotherm measured without buffer is not reached for concentration around 1 mg/L.

ter addition of the needed volume of organic modifier, is 14.4 mM.

3.2. Columns

The column used was a previously unused Discovery C_{18} column, the same brand as the one used by McCalley in his studies on the retention and the overloading behavior of basic compounds in RPLC. It was purchased from Supelco (Supelco Park, Bellefonte, PA, USA). It has the dimensions of 150 mm × 4.0 mm (different from those of the column used by McCalley [12]). The main characteristics of the bare porous silica and of the packing material used are summarized in Table 1. The hold-up volume of this column was measured by three independent methods, the elution of a compound assumed to be unretained (thiourea), the minor disturbance method, and pycnometry measurements.

3.3. Apparatus

The isotherm data were acquired using a Hewlett-Packard (Palo Alto, CA, USA) HP 1090 liquid chromatograph. This instrument includes a multi-solvent delivery system (tank volumes, 1 L each), an auto-sampler with a 25 μL sample loop, a diode-array UV-detector, a column thermostat and a data station. Compressed nitrogen and helium bottles (National Welders, Charlotte, NC, USA) are connected to the instrument to allow the continuous operations of the pump, the auto-sampler, and the solvent sparging. The extra-column volumes are 0.035 ml and 0.29 ml as measured from the auto-sampler and from the pump system, respectively, to the column inlet. All the retention data were corrected for these contributions. The flow-rate accuracy was controlled by pumping the pure mobile phase at 23 °C and 1 mL/min during 50 min, from each pump head, successively, into a volumetric glass of 50 mL. The relative error was less than 0.4%, so that we can estimate the long-term accuracy of the flow-rate at 4 μL/min at flow rates around 1 mL/min. All measurements were carried out at a constant temperature of 21 °C, fixed by the laboratory air-conditioner. The daily variation of the ambient temperature never exceeded ±1 °C.

3.4. Measurements of the adsorption isotherms by FA

The solubility of nortryptiline in aqueous solutions of acetonitrile containing between 15 and 30% (v/v) ACN is largely superior to 100 g/L. The maximum concentration used in FA was fixed at 50 g/L. Measurements were carried out at lower and lower concentrations until the linear regime of the adsorption isotherm was reached (the linear regime was defined as the concentration range within which symmetrical breakthrough curves were observed). Successive solutions of nortryptiline were prepared at 50, 5, 0.5, 0.05 g/L and 0.005 g/L. The UV detection limit was reached when 10% of the last concentration was injected ($\lambda_{max} = 208$ nm, $C = 0.0005$ g/L, ≤ 1.7 μmol). Consecutive FA runs were

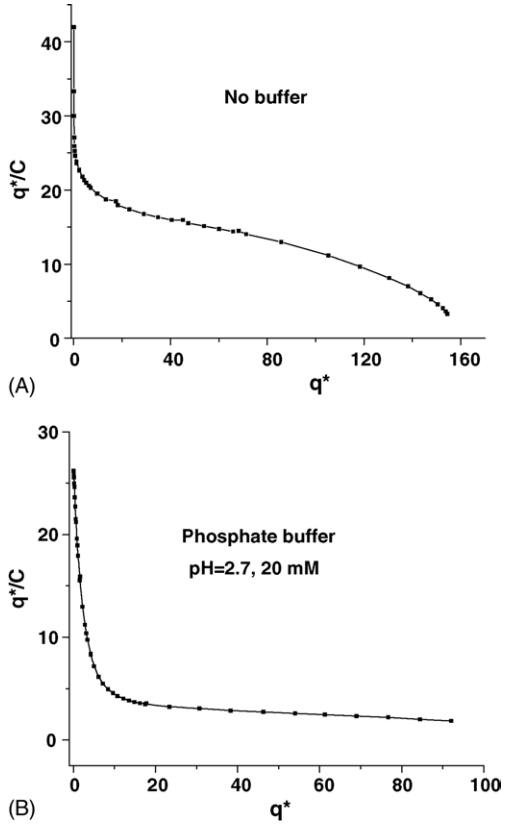

Fig. 2. Scatchard plot representation of the same data shown in Fig. 2. (A) no buffer, (B) Phosphate buffer, 20 mM, pH 2.70. Note the change of curvature of the Sactachard plot measured without buffer inconsistent with a multi-Langmuir isotherm model.

then performed starting from the highest to the lowest concentrations until the linear regime was reached. For each FA run, one pump (A) of the HPLC instrument was used to deliver a stream of the pure mobile phase (acetonitrile:water, 28:72, v/v, non-buffered or buffered at pH 2.70), the second pump (B, for the mother solutions) a stream of the sample solution in the same mobile phase. The concentration of nortryptiline in the FA stream is determined by the concentration of the mother sample solution and the flow rate fractions delivered

Table 2
Comparison between the best fit of the adsorption data of nortriptyline (C_{18}-Discovery, acetonitrile-water, 15/85, v/v) using three different isotherm models, the Moreau, bi-Morerau and Tri-Moreau isotherms

	Moreau	Bi-Moreau	Tri-Moreau
Fisher	203	438	14979
$q_{s,1}$ (g/l)	207.6	190.2	155.3
b_1 (l/g)	0.1119	0.1083	0.0635
I_1	0.64	1.14	5.11
$q_{s,2}$ (g/l)	–	0.13	25.4
b_2 (l/g)	–	242	0.527
I_2	–	0.20	0
$q_{s,3}$ (g/l)	–	–	0.058
b_3 (l/g)	–	–	3998
I_3	–	–	0.02

by the two pumps. The breakthrough curves were recorded at a flow rate of 1 mL min^{-1}, with a sufficiently long time delay between each breakthrough curve to allow for the complete reequilibration of the column with the pure mobile phase. The injection time of the sample was fixed between 6 and 12 min in order to reach a stable plateau at the column outlet. The signal was recorded at 299, 290, 275, 245 and 208 nm for mother solutions at concentrations of 50, 5, 0.5, 0.05, and 0.005 g/L, respectively.

This procedure of measurements of adsorption data of nortryptiline is not strictly rigorous. The values measured for the amounts of nortryptiline adsorbed at low and at high concentrations are not quite comparable. During these measurements, from one solution to the next, the relative concentrations of the co-ions (chloride) and of the buffer ions (formate or phosphate) vary continuously with increasing concentration of nortryptiline. The adsorption isotherm is considered to be measured under constant thermodynamic conditions when the buffer concentration is about 10 times that of the co-ion. The isotherm measurements reported here are thus correct in the low-concentration range but only approximate in the high-concentration range. However, assuming that chloride, formate, and phosphate form ion-pairs that have about the same molecular size, the isotherm parameters derived for the low-energy type of adsorption energy sites will not be affected much and remain fair estimates of these parameters.

4. Results and discussion

4.1. Adsorption of nortryptiline on Discovery-C_{18} column

4.1.1. No buffer: acetonitrile:water, 15/85, v/v

The adsorption data of nortryptiline were acquired first without any buffer in the mobile phase, in order to check whether our recent finding that there is little correlation between the saturation capacity of the column and the presence or absence of any buffer in the mobile phase [28]. For instance, the saturation capacities of Kromasil-C_{18} for propranololium chloride are 180 and 140 g/L without and with a buffer solution, respectively. The rational often advanced for explaining a low adsorbent saturation capacity, that analyte-analyte repulsion takes place in the adsorbed phase, does not seem to apply because the charged analytes (cations here) are always accompanied by a counter anion (e.g., the chloride anion of the salt when no buffer is used or the counter anions brought by the buffer).

In order to obtain a sufficient retention of the breakthrough curves of nortryptiline on Discovery with no buffer added to the mobile phase, hence accurate measurements of the retention times of these curves, the acetonitrile concentration of the mobile phase was fixed at 15%. The retention time of the curve front was too low with 28% acetonitrile, and the

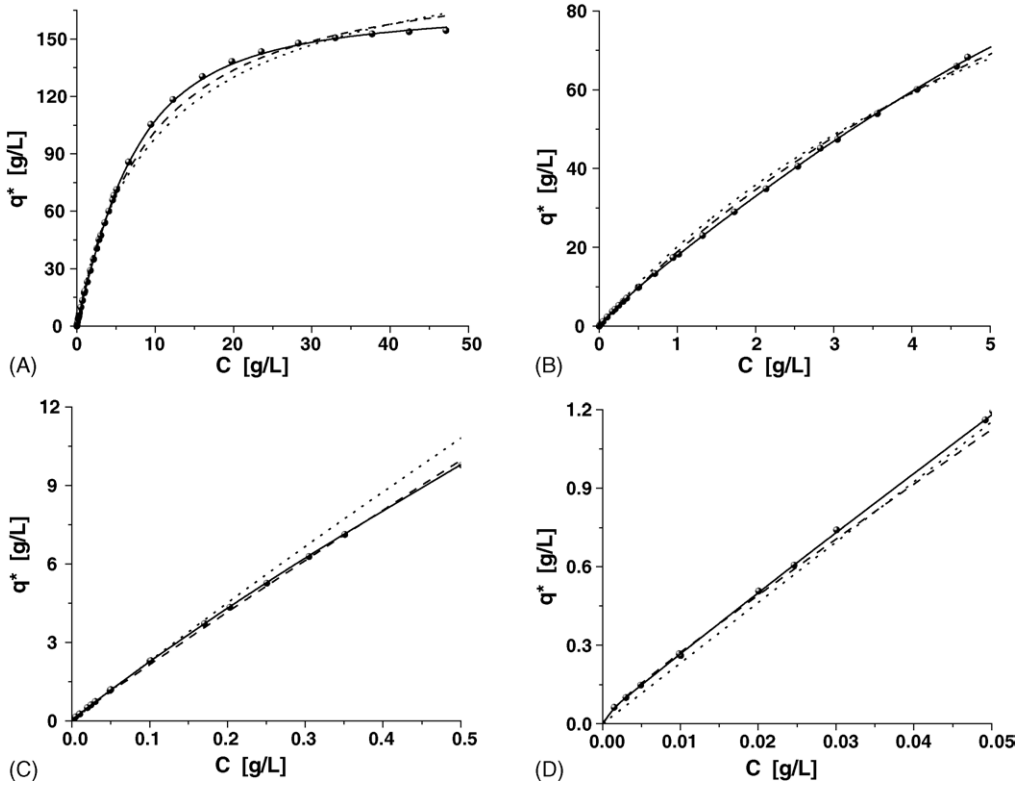

Fig. 3. Comparison between the adsorption data measured without buffer (spheres) and the best isotherm models (solid line: Tri-Moreau model, dashed line: Bi-Moreau model, dotted line: Moreau) obtained by MLRA. Acetonitrile:water (15/85, v/v), $T = 295$ K. (A) 0–50 g/L, (B) 0–5 g/L, (C) 0–0.5 g/L, (D) 0–0.05 g/L. Note that neither the Moreau nor the Bi-Moreau isotherm model correctly fit the data at low and high concentrations.

isotherm measurement would have been poorly accurate, especially at high concentration for which the retention time of the front shock becomes close to the hold-up time. Sajonz [29] has recently discussed this issue cogently. The adsorption isotherm and the corresponding Scatchard plot are shown in Figs. 1 and 2, respectively. The Scatchard plot is not consistent with Langmuir adsorption behavior of nortryptiline nor with any combination of several Langmuir isotherm models. The plot would be linear in the first case, strictly convex downward in the second. The experimental Scatchard plot exhibits clearly an inflection point for an adsorbed amount around 60 g/L. Accordingly, the experimental data were fitted to a Moreau, a bi-Moreau and a tri-Moreau isotherm model. These models are extension of the Langmuir and multi-Langmuir models in cases when adsorbate-adsorbate interactions take place. The bi-Moreau model had been successfully used to describe the adsorption of propranololium chloride on some classical commercial C_{18}-bonded phases (Kromasil [30], Symmetry [31], XTerra [32]) with methanol-water mixtures as the mobile phase. Table 2 summarizes the results of the multi-linear regression analysis (MLRA) obtained with the adsorption data of nortryptiline. Surprisingly, nor the Moreau nor the bi-Moreau can account for the FA data, it takes the tri-Moreau to obtain a really good fit of the data (see Fig 3).

The numerical values of the nine parameters found for this model make physical sense: the total saturation capacity ($155.3 + 12.7 + 0.06 = 168.06$ g/L) is typical of what was found for the total saturation capacity of low-molecular-weight compounds on other conventional RPLC packing materials [17,27]. The coefficient of adsorbate-adsorbate interactions is significant only for the most abundant low-energy sites ($I_1 = 5.11$, i.e., adsorbate-adsorbate interactions of 4 kJ/mol. For the high-energy sites, there are no adsorbate-adsorbate interactions ($I_2 = 0$, $I_3 = 0.02$), which suggests that these sites are rather isolated and can be viewed as spots on the surface which are remote from each other, with average distances larger than the molecular size of the analyte. The very large value of the equilibrium constant $b_3 = 3996$ L/g is consistent with the very long tailing observed for the breakthrough curves of nortryptiline. Fig. 4A shows that the breakthrough curve measured upon the injection of a 0.005 g/L solution is still asymmetrical, showing that the isotherm is nonlinear at this concentration (see Fig. 1E). Fig. 4B shows the breakthrough curve obtained with a 0.002 g/L solution of nortryptiline injected for 12 min. The tailing is so important that the plateau is eroded and cannot be seen. Therefore, longer plateau injections had to be performed. At this point, the acquisition of experimental adsorption data was becoming too time and solvent consuming and attempts to reach the linear range of the isotherm of nortryptiline with no buffer in the mobile phase were discontinued. From the value of b_3 obtained from the MLRA, it was calculated that nortryptiline would elute as a gaussian peak after 134 min. Unfortunately, the sensitivity of the UV detector at λ_{max} was insufficient to detect the nortryptiline peak after such a long time.

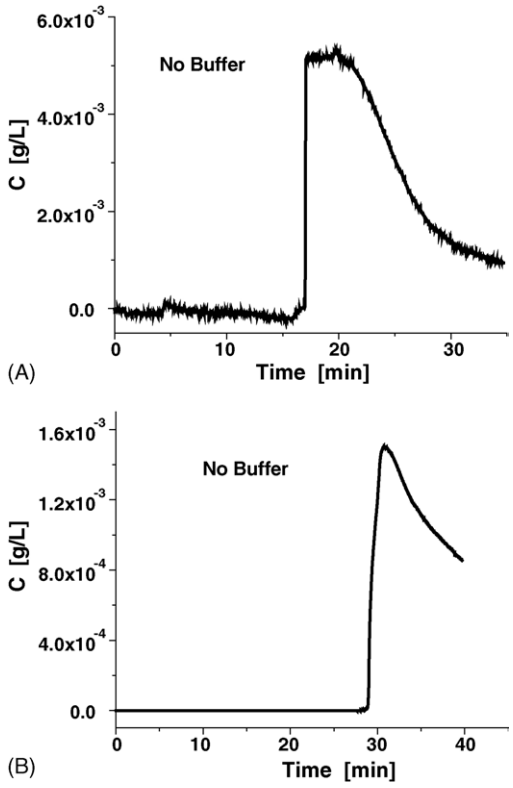

Fig. 4. Breakthrough curves recorded with no buffer in the mobile phase (acetonitrile:water, 15/85, v/v). C_{18}-Discovery column, $T = 295$ K. Flow rate 1 mL/min (A) 12 min injection of a 5 mg/L nortriptyline solution. (B) Same as (A) except 2 mg/L injection.

This result confirms our previous results with propranolol. The choice of a multi-Moreau isotherm model accounts well for the adsorption of an ionizable compound without buffer in the mobile phase. A physical interpretation of the three different types of sites observed can be suggested. It is highly probable that, as suggested in previous investigations of RPLC under nonlinear conditions, the sites of type 1, the most numerous sites, are located at the interface between the C_{18}-bonded layer and the bulk mobile phase. This layer has been shown by NMR studies to consist in ordered and disordered clusters. Sites ot type 3 involve strong electrostatic interactions. The difference in the adsorption energies on sites of types 3 and 1, $\epsilon_3 - \epsilon_1$ is about 27 kJ/mol, a value consistent with ionic exchange interactions (e.g., of nortryptiline with residual silanols). On the other hand, the difference in adsorption energies between the sites of types 2 and 1 is of the order of only 5 kJ/mol. So, the sites of type 2 are most probably located inside the bonded layer [15] and the increase of adsorption energy would be explained by the effect of dispersive interactions.

4.1.2. With buffer: acetonitrile:water, 28/72, v/v, phosphate or formate buffer, 20 mM, pH 2.70

Adsorption data of nortryptiline were then measured with a mobile phase containing a buffer. We reported earlier for propranolol the “langmuirization” of the bi-Moreau isotherm

(no buffer) toward a bi-Langmuir isotherm. However, in this study, we followed exactly the same experimental procedure as chosen by McCalley [12], in order to understand how a different operation procedure could lead to such a different value of the saturation capacity of the C_{18}-Discovery column. Two buffers were used, a phosphate and a formate buffer.

Fig. 5A–E show the experimental isotherms obtained with these two buffers. A striking difference is observed between them. The pH of the mobile phase alone cannot explain it. The adsorption mechanism of nortryptiline does not depend only on the pH. The Scatchard plots (Fig. 6) do not show any obvious inflection points and are convex downward, so a simple multi-Langmuir model should account for these adsorption data. An attempt to fit the data to the tri-Moreau isotherm gave values $I_1 = I_2 = I_3 = 0$. The adsorption data were then fitted to a Langmuir, bi-Langmuir and a tri-Langmuir isotherm models. The values of the best parameters obtained are listed in Table 2. The Fisher parameter of the tri-Langmuir isotherm for the phosphate buffer is not the largest, because a large importance is given to the high concentration points acquired (between 15 and 50 g/L) a region in which the tri-Langmuir model does not fit the data so well (Fig. 7A). However, the

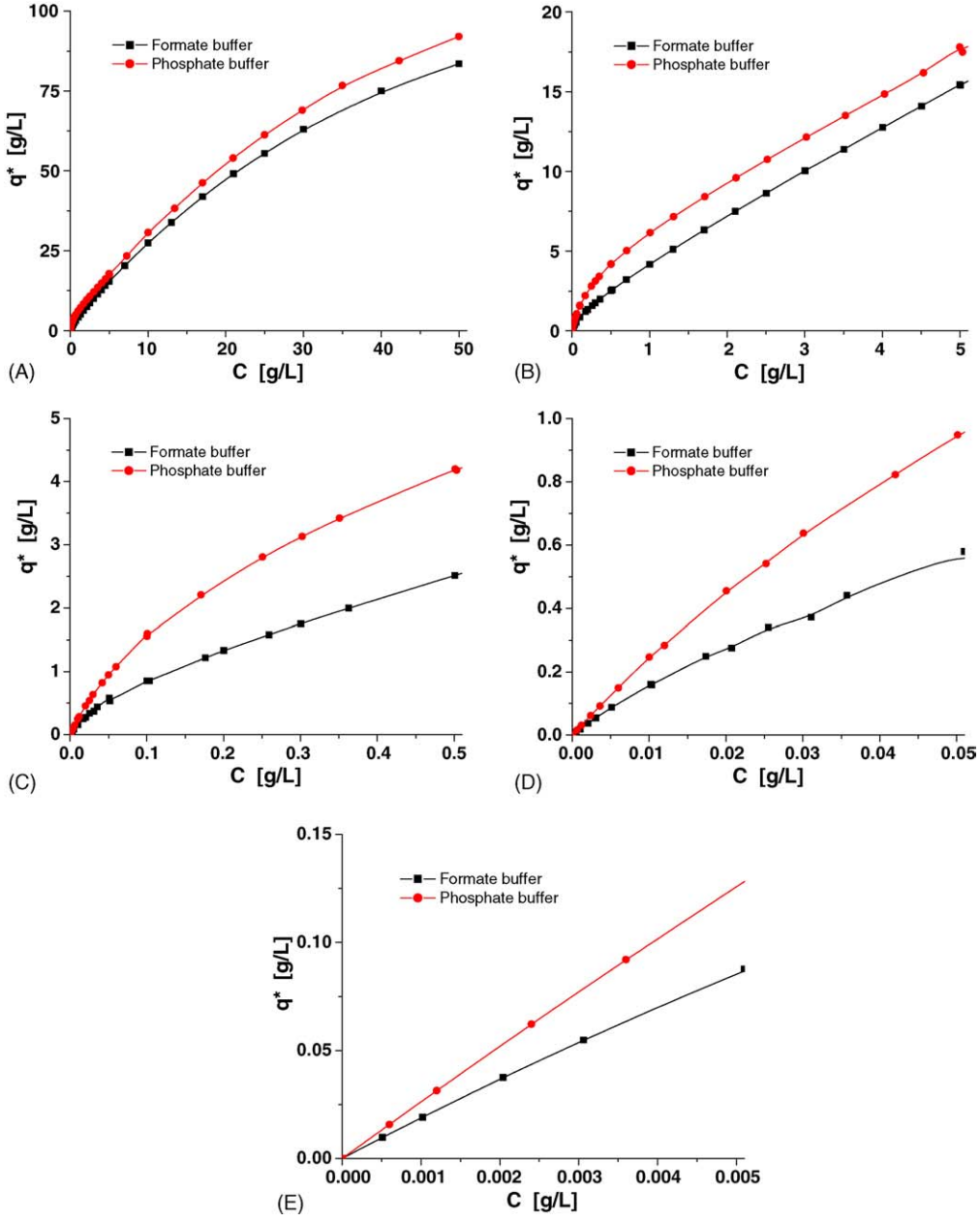

Fig. 5. Comparison between the experimental adsorption isotherms of nortriptyline on the C−18-Discovery adsorbent using formic and phosphate buffers (both 20 mM, pH 2.70) in the mobile phase (acteonitrile/water, 28/72, v/v). $T = 295$ K. (A) 0–50 g/L concentration range in the mobile phase, (B) 0–5 g/L, (C) 0–0.5 g/L, (D) 0–0.05 g/L, (E) 0–0.005 g/L. Note the higher amount of nortriptyline adsorbed with the phosphate buffer.

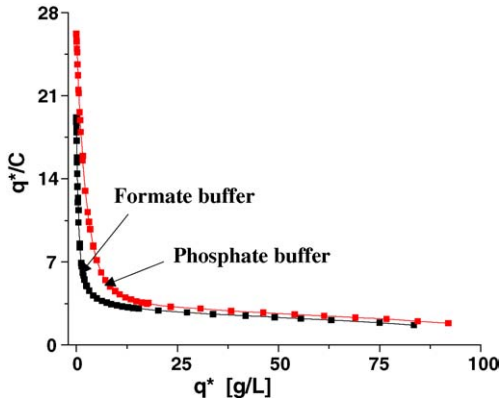

Fig. 6. Same as Fig. 6 except for the Scatchard plot.

agreement is much better at low concentrations as shown in Fig. 7B–E. Accordingly, it is likely that the adsorption energy distribution of nortryptiline is also trimodal with a buffer in the mobile phase. The contribution to the overall Henry constants of each site are $H_1 = 3.03$, $H_2 = 2.69$, $H_3 = 13.34$ and $H_1 = 2.98$, $H_2 = 13.5$, $H_3 = 10.0$ with the formate and the phosphate buffers, respectively. The retention factors of nortryptiline are $k_{\text{formate}} = 7.3$ and $k_{\text{phosphate}} = 10.1$ (with a phase ratio $F = 0.3829$), in good agreement with the earlier results of McCalley, who found values of $k_{\text{formate}} = 6.7$ and $k_{\text{phosphate}} = 10.1$. This agreement makes possible an attempt at formulating consistent interpretations of the two sets of results.

The validity of the choice of the tri-Langmuir isotherm model to account for the FA adsorption data measured with a buffered mobile phase is confirmed by the independent results of the calculations of the AED of nortryptiline in the two systems, assuming a local Langmuir isotherm model. In both cases, the AEDs converge toward a trimodal distribution as shown in Fig. 8.

From the modeling of the adsorption data, it appears that the nature of the buffer selected to fix the mobile phase pH affects only some of the adsorbent properties. While the parameters for the lowest (1) and highest (3) types of adsorption sites remain essentially constant, those of the sites of type 2 are seriously changed. The density of the sites is three times higher with the phosphate than with the formate buffer. The adsorption energy is higher with the phosphate buffer (by +1.1 kJ/mol). This difference explains also why the retention factors of nortryptiline are different in the two buffers. Analytical data cannot explain this. At this stage, unfortunately, too little is known regarding the properties of the sites of type 2 to venture a plausible physical interpretation of this result.

4.2. On the McCalley enigma

The presence of a buffer in the mobile phase enlarges markedly the concentration range within which the isotherm remains linear. With a buffer the breakthrough curves are symmetrical for concentrations up to approximately 0.001 g/L (or ca. 3.3 μmol/L). Using a low pH buffer (pH 2.7) certainly eliminates the ionized silanols from the surface, reduces the intensity of the strong electrostatic interactions between these silanol groups and the positively charged compound, which caused the very long band tailing observed without buffer and the high values of the equilibrium constant b_3. The breakthrough curves tail significantly at low concentrations (3 μmol/L) but the linear range can be experimentally reached (see Fig. 9).

The problem consists now in finding an estimate of the column saturation capacity and explaining why the two methods give different values. Recently, McCalley [12] derived the saturation capacity w_s of the same kind of Discovery-C_{18} column, using the same mobile phases (a formate and a phosphate buffer, at 20 mM and pH 2.7) by considering two injections, the first with a low sample size (such that a lower size would not give a more symmetrical peak) and the second with a sample size giving a heavily overloaded peak. He applied the method of Snyder et al. to estimate the column efficiency, using the "right-angle triangle shaped band profiles" method [30]. The result is a capacity of the Discovery column for nortriptyline of 5.8 mg and 0.7 mg in the phosphate and the formic buffers, respectively (Table 4 in Ref [12]). Note that McCalley used a column having different dimensions (25 cm × 0.46 cm while ours is 15 cm × 0.40 cm). However, both columns were packed with particles of the same size (5 μm). Thus, McCalley's column contained 2.2 times more packing material than ours. Normalized to our column size, the column saturation capacities become 2.63 and 0.32 mg, respectively. The volume of adsorbent material in our column was determined by pycnometry, the column being filled successively filled with two different solvents, e.g., acetonitrile ($\rho_{\text{ACN}} = 0.782\ \text{g/cm}^3$) and dichloromethane ($\rho_{\text{CH}_2\text{Cl}_2} = 1.326\ \text{g/cm}^3$), and weighted ($m_{\text{ACN}} = 63.54345$ g and $m_{\text{CH}_2\text{Cl}_2} = 64.27740$ g). Assuming that both solvents have access to the same free volume V_M,

$$V_M = \frac{m_{\text{ACN}} - m_{\text{CH}_2\text{Cl}_2}}{\rho_{\text{ACN}} - \rho_{\text{CH}_2\text{Cl}_2}} = 1.349\ \text{cm}^3 \quad (5)$$

This volume is slightly smaller than that found by injecting the "unretained" compound, thiourea (1.363 cm^3). Thiourea is a good hold-up volume marker but is significantly retained in RPLC ($k'_{\text{thiourea}} = 0.0104$). The volume of adsorbent material in our column is then 0.536 cm^3. According to our results (Table 3), the maximum amount of nortriptyline which the Discovery-C_{18} adsorbent can adsorb are:

$$w_s = (184.6 + 1.06 + 0.436) \times 0.000536 = 99.7\ \text{mg} \quad (6)$$

with the mobile phase buffered with formic acid (versus McCalley 0.32 mg), and

$$w_s = (231.3 + 3.39 + 0.440) \times 0.000536 = 126.0\ \text{mg} \quad (7)$$

with the phosphate buffer (versus McCalley 2.63 mg).

There is only one possible conclusion, the two methods do not measure the same thing. Note now that the results

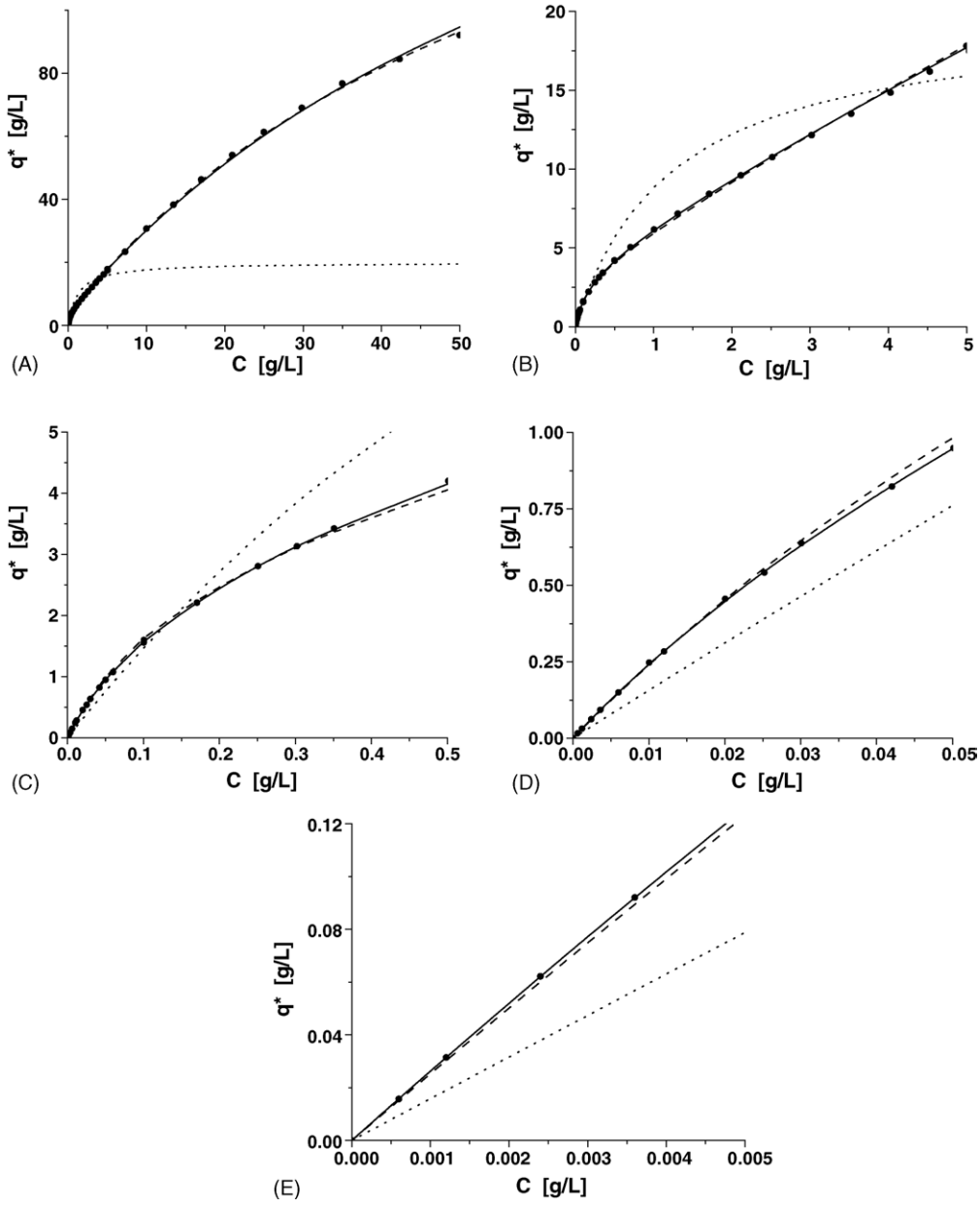

Fig. 7. Same as in Fig. 4 except with the adsorption data measured with a phosphate buffer (spheres) and with Tri-langmuir (solid line), Bi-Langmuir (dashed line) and Langmuir (dotted line) isotherm models. (A) 0–50 g/L, (B) 0-5 g/L, (C) 0–0.5 g/L, (D) 0–0.05 g/L, (E) 0–0.005 g/L. Again, note the better agreement with the Trimodal isotherm model.

are quite consistent if we omit the low-energy sites of type 1 and calculate the saturation capacity of the sites of types 2 and 3. The values are 0.80 and 2.05 mg in the formic and the phosphate buffers, respectively. In the method used by McCalley (who injected less than 1 μg of nortriptyline), the low-energy sites remain practically unoccupied, which results in his method determining the saturation capacity of the high-energy types of sites only. If we assume the injection of a 5 μL sample of a 1 g/L solution of nortrypline, corresponding to a sample size of 5 μg, the product b_1C is smaller than 0.02 with both buffers at the column inlet and it keeps decreasing along the column (peaks spread, diffuse and their heights decay). The sites of type 1 are clearly unoccupied (<2%) and their saturation capacity does not contribute to the measurements.

For analysts, the very low column saturation capacities found by McCalley are the correct estimates of the saturation capacities under linear conditions and the only values of interest for them. These values make sense because they represent the very small fraction of the adsorbent surface area with which analytes can interact, to which they have access, the sites having the strongest affinity. At higher concentrations, peak begin to experience a strong tailing of thermodynamic origin.

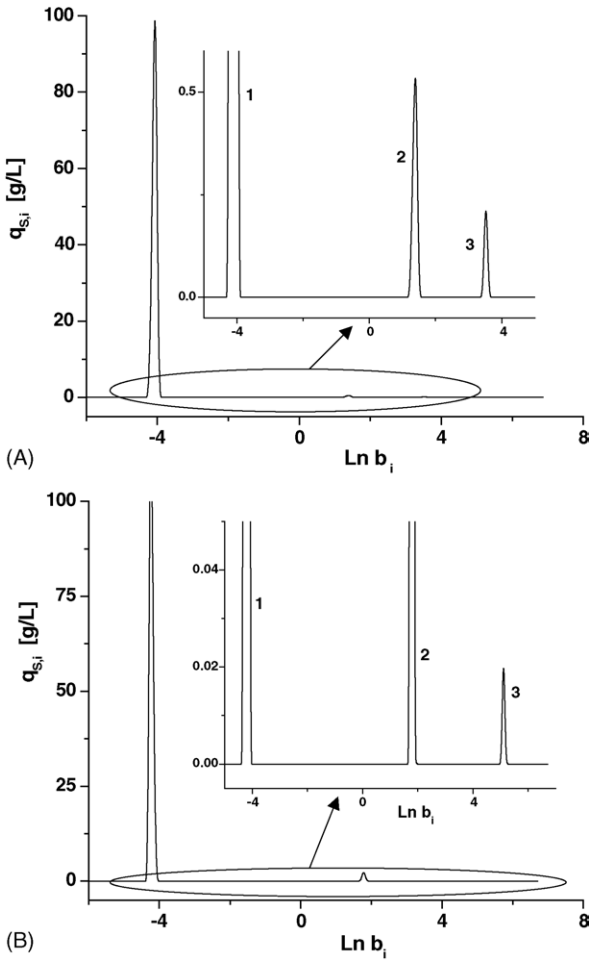

Fig. 8. Adsorption energy distributions (AEDs) calculated from the experimental adsorption data of nortriptyline on the C_{18}-Discovery column using formic (A) and phosphate (B) buffers. Note that, in both cases, the distribution is trimodal.

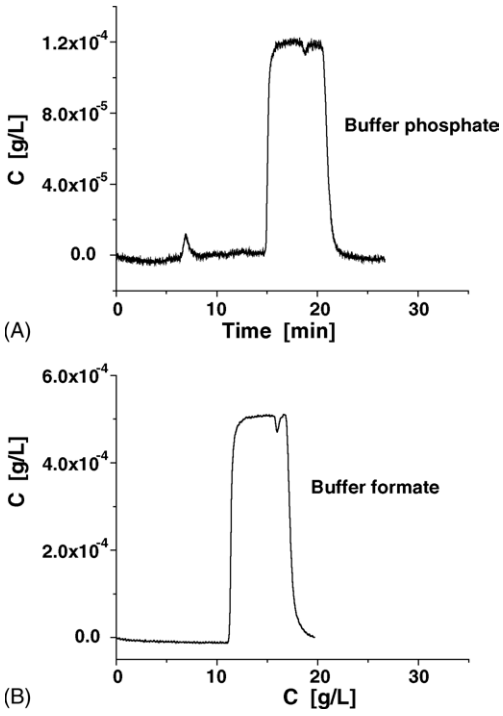

Fig. 9. Breakthrough curves of nortriptyline on the C_{18}-Discovery column. (A) Injection during 6 min of a 0.12 mg/L solution of nortriptyline with phosphate buffer. (B) Injection during 6 min of a 0.5 mg/L solution of nortriptyline with formic buffer. By contrast to Fig. 5, note the absence of asymmetry suggesting that the linearity of the isotherm is reached at such concencentrations.

For separation scientists, however, the low capacity values ignore the contributions of the adsorption of the eluites on the low energy type of sites to their retention at high concentrations. These low-energy sites are of major importance in preparative chromatography. Fig. 10 shows the variations with the concentration C of nortriptyline, of the elution time of this concentration, $t_R(C)$. Fig. 10A and B correspond to mobile phases buffered with formic and phosphate buffers, respectively. The times $t_R(C)$ were derived from the profiles of the rear diffuse boundaries of the breakthrough curves, corrected from the duration of the injection. From 0.001 to about 0.3 g/L, $t_R(C)$ decreases rapidly with increasing concentration because the sites of types 2 and 3 reach progressively their saturation. Beyond 0.4 g/L, the retention time remains constant, indicating that the isotherm is nearly linear. The sites of types 2 and 3 are almost saturated while those of type 1 begin to fill up but have a large saturation capacity. Fig. 11 shows the progressive evolution of the overloaded elu-

Table 3
Comparison between the best fit of the adsorption data of nortriptyline (C_{18}-Discovery, acetonitrile-water, 28/72, v/v, 20 mM buffer, pH 2.70) using three different isotherm models, the Langmuir, bi-Langmuir and Tri-Langmuir isotherms

Buffer	Langmuir		Bi-Langmuir		Tri-Langmuir	
	Formate	Phosphate	Formate	Phosphate	Formate	Phosphate
Fisher	<4	<2	2 077	5 179	7 703	1 932
$q_{s,1}$ (g/l)	37.0	19.9	160.1	207.4	184.6	231.3
b_1 (l/g)	0.1803	0.795	0.0206	0.0153	0.0164	0.0129
$q_{s,2}$ (g/l)	–	–	0.84	3.18	1.06	3.39
b_2 (l/g)	–	–	17.8	7.0	2.54	3.99
$q_{s,3}$ (g/l)	–	–	–	–	0.436	0.44
b_3 (l/g)	–	–	–	–	30.6	22.8

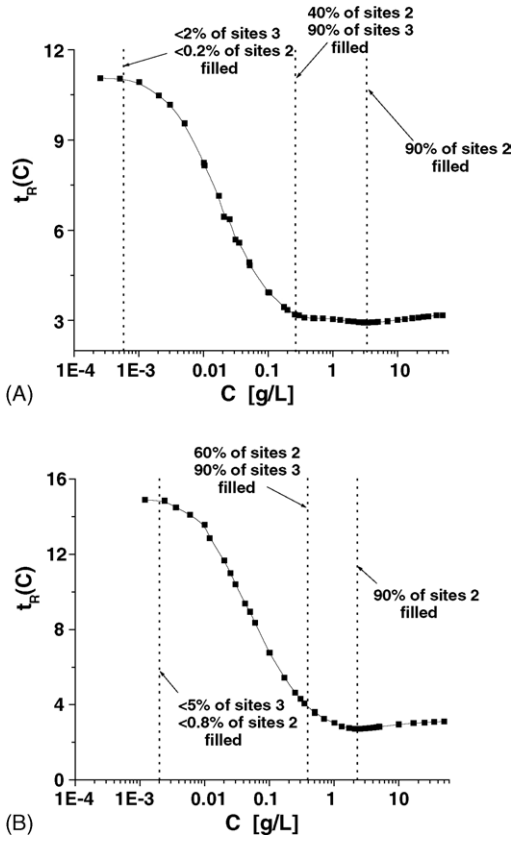

Fig. 10. Variation of the elution time of a concentration C on the C_{18}-Discovery column using formate (A) and phosphate (B) buffers. Note the initial strong decreasing of the retention (saturation of the high energy sites) followed by a quasi constant elution time (beginning of the occupancy of the low energy sites). Note these slight final increase due to the breakthrough curves anomalies mentionned in Section 4.3.

tion band profile with increasing sample loading. The rapid decrease of the retention time of the apex of the band illustrates the strongly nonlinear behavior of the isotherm in the intermediate concentration range. However, although there is a second range of concentrations within which the isotherm behaves linearly, this immense saturation capacity can hardly be used because of the large difference between the retention times associated with the two linear ranges and the intense band tailing which takes place at high concentrations, causing serious potential interference between neighbor bands.

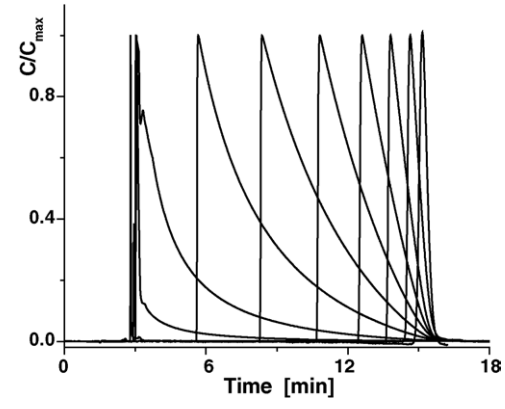

Fig. 11. Evolution of the peak profile of nortriptyline with increasing amount injected (0.21, 1.98, 5.95, 17.8, 53.5, 160, and 475 μg, 1.651, 3.85, and 8.80 mg). C_{18}-Discovery column, acetonitrile:water mixtures (28/72, v/v), phosphate buffer 920 mM, pH 2.70), $T = 295$ K. Normalized profiles. Note the almost constant elution times for the highest loads corresponding to the saturation of sites 2 and 3.

5. Conclusion

This work demonstrates the complexity of the retention mechanisms on RPLC packing materials, the difficulties encountered in the correct interpretation of the results of accurate measurements of isotherms, and the ambiguity of the definition of the actual saturation capacity of RPLC columns. The acquisition of equilibrium isotherm data has to be done following proper experimental procedures. The modeling of these data informs on the retention mechanisms. Simple extrapolations from these data lead to estimates of the saturation capacities that are tantalizingly large but are, unfortunately, useless at the present time. The use of empirical rules based on the extrapolation of the dependence of the low-concentration band profiles on increasing concentration leads to estimates of the saturation capacities that are disappointingly low and, unfortunately, realistic at the present time. The fundamental reason for this sorry state of affairs is that all alkyl-bonded RPLC adsorbents are heterogeneous. There is one high-energy site of type 3 for approximately four intermediate-energy sites of type 2 and 400 low-energy sites of type 1. The isotherm curvature takes place at very low concentrations, due to the rapid filling of the high-energy sites at low concentrations, when the low energy sites remain barely occupied. The coexistence on the surface of the packing materials of a few high-energy sites and an immense number of low-energy sites renders the practical use of the latter impossible.

The existence of the very high-energy sites that we have identified was suspected for forty years as the popular active sites on which tailing has long been blamed [33]. The main result of our work so far is to provide straightforward methods for the determination of the number of the types of sites, their respective densities and the differences between their adsorption energies. Consequently, this work provides the possibility of comparing different packing materials and of assessing the progress made in preparing new adsorbents. It gives a new, challenging goal to the chemists that try and synthesize advanced RPLC stationary phases.

Finally, this work answers the McCalley *enigma* and explains why analysts measure very small values for the saturation capacity of ionizable compounds on RPLC adsorbents, typically 100 times less than that derived from the direct consideration of the isotherm data. It also gives clues as to the origin of the saturation capacity observed with ions being much lower than that of neutral compounds. We still do not know what they are, but we have identified a type of super high-energy sites that interact very strongly with ions and not with

neutrals. Thus, our work open new areas of research, identifying the chemical nature of active sites and trying either to eradicate them or, if it is impossible entirely to eliminate them, to multiply them to increase the effective saturation capacity of the packing materials.

Acknowledgments

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