Separation Science and Technology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713708471

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Online publication date: 29 May 2002

To cite this Article Böcker, Sebastian , Mazzotti, Marco , Morbidelli, Massimo , Fleury, Christian and Schenkel, Berthold(2002) 'Design of chromatographic separations on reversed phase', Separation Science and Technology, 37: 7, 1725 — 1745
To link to this Article: DOI: 10.1081/SS-120002746
URL: http://dx.doi.org/10.1081/SS-120002746

SEPARATION SCIENCE AND TECHNOLOGY, 37(7), 1725–1745 (2002)

DESIGN OF CHROMATOGRAPHIC SEPARATIONS ON REVERSED PHASE

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ABSTRACT

The accurate determination of adsorption isotherms is of key importance in the design and optimization of preparative chromatographic bulk separations and purifications.

Issues to be considered involve other things: limited amount of test material, particularly in the case of the development of drugs; multicomponent mixture with widely different concentration levels of the main product and of the impurities. In this work, a rational procedure for the characterization of adsorption equilibria in reversed phase chromatography is proposed. This is applied to two different model separations, both involving an ascomycin derivative of industrial interest.

The procedure is based on the peak fitting method and the use of a lumped pore diffusion model to simulate column dynamics. The accuracy of the obtained results is assessed thoroughly.

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INTRODUCTION

The knowledge of adsorption equilibria is essential for the proper design of batch and continuous chromatographic separation processes. In particular, we need mathematical models, which provide the concentration in the stationary phase as a function of the concentration in the fluid phase under equilibrium conditions.

Several experimental procedures to measure adsorption equilibria are described in the literature (1,2). Here we focus on the problem of determining adsorption isotherms to be used in designing chromatographic separations at the early stage of new product developments. This situation, which is typical for the pharmaceutical industry, poses some important constraints to the procedure to be adopted to measure adsorption equilibrium in multicomponent mixtures. In particular, these involve the availability of only small amounts of the mixture to be separated, and in addition, no pure species are available. So for example, in this context the typical approach of describing first single-component equilibria and then extending the model to multicomponent equilibria through the introduction of some interaction parameters is not feasible. In addition, these isotherms have to be studied on the same packing materials used in the preparative or industrial process, which exhibit usually lower efficiencies than the corresponding ones used for analytical purposes, characterized by much lower particle size. These constraints prevent the utilization of the classical methods for measuring multicomponent equilibria.

In this work we use the peak fitting method (3,4), whereby the parameters of a predefined equilibrium isotherm are estimated by fitting directly the composition values at the outlet of an analytical column packed with preparative packing material and fed with a pulse of the mixture to be separated, and operated in the isocratic elution mode. During the first screening phases, a single experiment is sufficient for a first estimation of the adsorption isotherm in a multicomponent mixture. By increasing the number of experiments in a later stage of the product development process, the precision of the model can be improved.

The experimental peak profiles are simulated using a detailed column model, which requires the correct knowledge of various parameters, which affect the column behavior. These involve the stationary phase porosities, axial dispersion, and mass transfer parameters. Each of these has to be estimated carefully before estimating the equilibrium parameters, since any error in these parameters would lead to a corresponding error in the equilibrium parameters.

In the following, for illustrative purposes, we apply this procedure to a separation and a purification process, based on reversed phase chromatography, involved in the development of a new drug. The obtained conclusions have a rather general validity and can be regarded as guidelines for the measurement procedure of adsorption isotherms on the reversed phase.

THE MODEL SEPARATION PROBLEM

Drug therapy for the major inflammatory skin diseases, which includes atopic dermatitis, psoriasis, and allergic contact dermatitis, is often inadequate due to poor efficacy, toxicity, or both. Much research has focused on the macrolactam T-cell inhibitors as a promising new class of agents for immunotherapy, and medicinal chemistry efforts to design novel ascomycin analogues have produced clinically promising agents. One example is the ascomycin derivative shown in Fig. 1, which will be referred to as AD in this paper. Several undesired products are formed during the manufacture of AD. In particular, the synthesis of AD produces among others the 9-epimer of AD shown in Fig. 2 and referred to in the following as 9-EPI. Ascomycin derivative and 9-EPI are diastereoisomers, differing only for the inversion of the ethylgroup in position 9. On the other hand two nonpolar ascomycin analogues produced in the fermentation process, are converted to nonpolar structurally closely related homologues of AD, referred to as NP1 and NP2. In the following, we illustrate the procedure for the determination of the multicomponent adsorption isotherms with reference to the two chromatographic processes used to separate such undesired products from AD.

The first one is actually a purification process involving a ternary mixture, where the mass fraction of AD is 96% while that of NP1 and NP2 is only 2%. The second is a binary separation process since the mass fraction of AD is 70% and

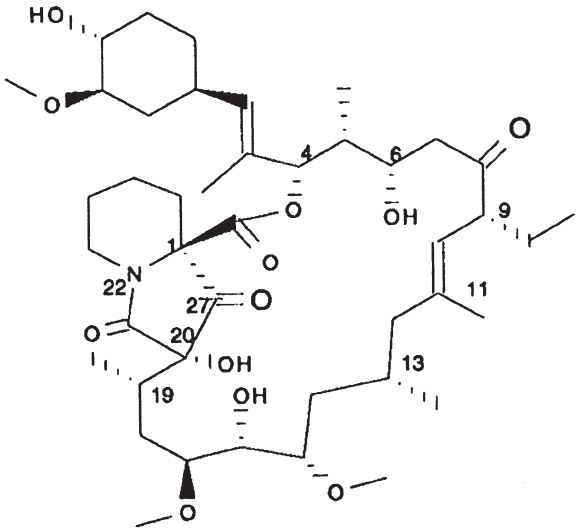

Figure 1. Structural formula of AD, $C_{43}H_{69}NO_{12}$, $M_w = 792.02\,g/mol$.

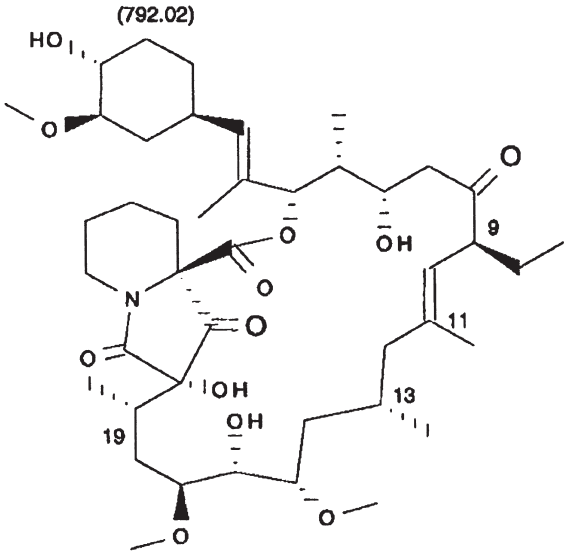

Figure 2. Structural formula of 9-EPI, i.e., the 9-epimer of AD.

that of 9-EPI is 30%. In both processes, the reversed phase preparative packing material MN RP C18 (Macherey-Nagel GmbH, Düren, Germany) has been used. At 25°C the by-products NP1 and NP2 exhibit a selectivity with respect to AD equal to 1.24 and 1.46, respectively, while for 9-EPI this is equal to 1.14. The packing is characterized by a pore width of 120Å and a particle diameter of 30 μm. The surface is covered partially with a C18 ligand leaving about 10% of the SiOH groups free. The used column is 250 mm long and has a diameter of 4 mm. All experiments have been carried out on a HP 1090 high-performance liquid chromatography (HPLC) station. The eluent is composed of a methanol/water mixture with 80/20 v/v%. It is worth noting that the possibility of performing the second separation process on a different stationary phase with a simulated moving bed has been studied recently by Küsters et al. (5).

THE PEAK FITTING METHOD

The peak fitting method involves estimating the adsorption isotherms by fitting directly the composition values at the outlet of a column fed with a pulse of the mixture to be separated. The pulse should be sufficiently large to involve the nonlinear portions of the isotherm that has to be estimated, i.e., the column should

operate under overloaded conditions, where chromatographic peaks are not symmetric any more due to the large amount injected.

For this we need an accurate simulation model to predict the behavior of chromatographic columns. The model used in this work consists of a set of mass balance equations for both the fluid and the stationary phase. The model accounts for axial dispersion and mass transfer resistance. In particular, since solid diffusion does not occur here and pore diffusion determines the rate of the transport process from the liquid bulk to the solid surface, the so-called lumped pore diffusion model has been used to describe mass transfer (6–8). The differential mass balances in the liquid and solid phase are given by:

$$\epsilon_b \frac{\partial c_i}{\partial t} + (1 - \epsilon_b)\epsilon_p \frac{\partial c_i^p}{\partial t} + (1 - \epsilon_b)(1 - \epsilon_p)\frac{\partial q_i}{\partial t} + u\frac{\partial c_i}{\partial x} = \epsilon_b D_{ax,i}\frac{\partial^2 c_i}{\partial x^2} \quad (1)$$

$$\epsilon_p \frac{\partial c_i^p}{\partial t} + (1 - \epsilon_p)\frac{\partial q_i}{\partial t} = k_{m,i}(c_i - c_i^p) \quad (2)$$

where c, c^p, and q represent the concentrations in the liquid, pore, and solid phase, respectively, D_{ax} is the axial dispersion coefficient, and k_m the lumped mass transfer coefficient.

In addition to Eqs. (1) and (2), the model includes the adsorption isotherm, which relates directly to the compositions of the pore and the solid phases, which are assumed to be in equilibrium always.

The model equations are solved numerically using the solver DDASSL (9) from the IMSL library. A critical point for the success of the developed procedure is to evaluate accurately all the parameters in the model, before proceeding with the estimation of the equilibrium isotherm. Accordingly, we proceed through the following steps, operating first on diluted systems with the aim of determining the linear portion of the isotherm, and then with overloaded systems to estimate its nonlinear, competitive part:

Diluted experiments:

1. Measurement of external and total porosity of the packing.
2. Measurement of the Henry coefficient of each component.
3. Measurement of the van-Deemter plot and evaluation of axial dispersion and mass transfer coefficients.

Overloaded experiments:

4. Measurement of a series of chromatographic peak profiles with increase in loading.
5. Estimation of the remaining parameters of the isotherm by fitting the experimental peak profiles.

An important aspect in the development of all the above-mentioned steps is that we have to account for the limitations typical of the early-stage development of new products.

As discussed earlier, this implies that no pure components but only the mixtures to be separated are available, and in very limited amount. This last constraint forces us to operate with very small amounts of stationary phase, i.e., with analytical columns. On the other hand, in order to guarantee the scale up of the estimated isotherm at the preparative scale, we need to operate directly on preparative stationary phases. These are characterized by particle dimensions, which are about one order of magnitude larger than those of analytical stationary phases. The conclusion is that we have to operate with analytical columns packed with preparative stationary phases, which exhibit typically very low efficiency and therefore often do not provide complete resolution of the peaks of the various components of the mixture to be separated.

In the following sections we analyze in detail each of the above-mentioned steps with reference to the selected model separations.

TOTAL POROSITY OF THE PACKED COLUMN

The total porosity ϵ of a packed column includes all the volumes available to the fluid phase, both inside and outside the particles constituting the packing material. In principle, this can be estimated from the retention time of an inert tracer as follows:

$$\epsilon = t_{r,tracer} Q/V \tag{3}$$

where, Q is the volumetric flow-rate and V the total column volume.

In practice, the problem is to identify such an inert tracer, i.e., a chemical species, which does not adsorb on the stationary phase, since even a small adsorptivity would lead to significant errors in the determination of the porosity. It is common practice to select a tracer with opposite polarity as the stationary phase under examination. For example, in the case of normal-phase silica gel, the use of nonpolar species such as heptane or hexane is appropriate due to their strong repulsion with the SiOH groups covering the adsorbent surface. The situation is more complicated in the case of the reversed phase considered in this work, since the C18 ligands do not cover completely the adsorbent surface thus leaving some free SiOH groups. Therefore, the surface exhibits portions with polar and portions with nonpolar character, which make difficult the selection of a species, which does not adsorb in either one of them. Another important aspect in selecting a suitable tracer arises from the complex nature of the intraparticle porosity structure of the stationary phase, which makes it difficult to determine

the smallest pore dimension that should be accounted for in the intraparticle porosity. A practical answer to this question, particularly valuable when considering large molecules such as those typically of interest in the pharmaceutical industry, is that the intraparticle porosity to be considered is the one accessible to the molecules to be separated in the particular system under examination. With respect to this the best tracer would be obviously the species to be separated, i.e., AD in the case considered in this work.

On the basis of the above considerations we can now proceed to consider the pulse tracer experiments, which provide an accurate estimate of the total porosity. According to the solvophobic theory (10), the interaction between the ligand and the solute, i.e., the tracer, can be controlled by changing the polar character of the eluent. For example, using an eluent with increasing polarity would increase the adsorptivity of the nonpolar solutes on nonpolar surfaces. Accordingly, by using nonpolar solutes and eluents on a reversed-phase stationary phase we can minimize the interaction of the solute with the polar portion of the surface. On the other hand, both the solute and eluent compete for adsorbing on the nonpolar portion. This can be described using the competitive Langmuir isotherm as follows:

$$q_s = \frac{q_s^s K_s c_s}{1 + K_s c_s + K_e c_e}; \quad K_i = \frac{H_i}{q_i^s} \tag{4}$$

where the subcripts s and e refer to the solute and the eluent, respectively, while the superscript s indicates saturation conditions. If we are in the situation where solute and eluent have comparable adsorptivity, since the concentration of the eluent is obviously much larger, it follows that $K_e c_e \gg K_s c_s$, so that Eq. (4) reduces to:

$$q_s = \frac{q_s^s K_s}{1 + K_e c_e} c_s = H_s^* c_s \tag{5}$$

where H_s^* is a constant representing the Henry constant of the solute modified by the presence of the eluent. Therefore, the solute behaves as in the linear adsorption regime but with a decreased adsorptivity. Accordingly, its retention time is given by:

$$t_{r,s} = t_0 \left(1 + \frac{1 - \epsilon}{\epsilon} H_s^* \right) \tag{6}$$

From this relation it is seen that if we reduce H_s^* to values well below unity, the measured retention time of the solute approaches that of an ideal tracer. For this, we need to select an eluent, which exhibits sufficiently larger adsorptivity than that of the solute. In the first part of Table 1 are reported the total porosities measured using pulses of hexane in pentane at three different temperature values.

The observed value of $\epsilon = 0.70$ is found to be temperature independent, thus indicating that the effect of hexane adsorption has been eliminated, i.e., $H_s^* \ll 1$ in Eq. (6). All data in Table 1 have been shown to the well reproducible, with an estimated error of about 0.5%.

The porosity measurement obtained above is however not satisfactory since hexane and AD have quite different molecular size and therefore they access most likely a different network of internal pores. To avoid this problem we have used AD as a tracer and various alcohols as eluents. The porosity values measured with the different alcohols and at various temperatures are summarized in Table 1. Note

Table 1. Measurement of the Total Porosity ϵ, Through Eq. (3); $V = 3.14\,\text{mL}$, $Q = 0.5\,\text{mL/min}$; $\underline{\epsilon}$ Represents the Value of ϵ Estimated Without Density Correction for Temperature Changes

Tracer/Eluent	T [°C]	$\underline{\epsilon}$	ϵ
Hexane/Pentane	30	0.70	0.70
	35	0.70	0.70
	40	0.69	0.71
AD/Methanol	25	0.73	0.73
	35	0.73	0.74
	45	0.72	0.74
	65	0.70	0.74
	75	0.69	0.73
AD/Ethanol	25	0.69	0.69
	45	0.68	0.69
	65	0.66	0.70
	75	0.66	0.70
	80	0.65	0.70
	85	0.65	0.70
AD/Isopropanol	25	0.68	0.68
	45	0.67	0.68
	65	0.66	0.69
	85	0.64	0.69
AD/2-Butanol	25	0.68	0.68
	45	0.67	0.68
	65	0.65	0.68
	75	0.64	0.68
	85	0.63	0.68

that two sets of porosity values, $\underline{\epsilon}$ and ϵ, are reported in Table 1, these have been obtained before and after correcting the volumetric flow-rate for the density change as a function of temperature, respectively. The density has been corrected using the Francis equation (11). It is seen that this effect is not negligible at all and could lead to misleading conclusions when not properly accounted for.

From the data in Table 1 it is seen that the obtained porosity values are temperature independent, but differ depending upon the alcohol used. In particular, larger porosities are estimated using smaller alcohol molecules. This indicates that a residual effect of AD adsorption, which is not sufficiently strong to be detected by changing the temperature, is actually present. This is explained by considering that when decreasing the size of the alcohol molecule its polarity increases. This leads on one hand to stronger interactions of AD with the solid surface, i.e., larger K_s, and on the other hand to lower values of the eluent adsorptivity on the reversed phase, i.e., lower K_e, which both imply larger values of H_s^* in Eq. (6). When considering that the porosity values measured with 2-butanol are very close to those measured with isopropanol, we can conclude that 0.68 is the correct value of intraparticle porosity to be used when considering chromatographic processes involving AD. Eluents with polarity lower than 2-butanol cannot be used, since it would then be very difficult to regenerate again the reversed-phase stationary phase.

Finally, it is worth noting that the porosity value measured above, i.e., 0.68, is smaller than the value of 0.70 estimated using hexane as a tracer; such a difference is significant since it is larger than the experimental error. This is consistent with the observation that AD is a much larger molecule and therefore it can access a smaller fraction of the intraparticle porosity.

INTERPARTICLE POROSITY OF THE PACKED COLUMN

The interparticle porosity can be measured on reversed phase using again a pulse tracer technique, but now the tracer should be a chemical species that does not enter the pores of the stationary phase. Relatively large nonpolar substances such as sugars or polymers are used typically for normal phases. But they cannot be used for reversed phases, because they interact with the nonpolar external surface of the particles. In the case of the reversed phases, it is convenient to use potassium nitrate in an aqueous solution, since ionic substances are excluded from the pores due to electrostatic interactions. For the model system considered in this work, an external porosity of 0.41 has been measured using potassium nitrate as a tracer. From the interparticle porosity, ϵ_b, and the definition of total porosity:

$$\epsilon = \epsilon_b + (1 - \epsilon_b)\epsilon_p \tag{7}$$

the intraparticle porosity, ϵ_p, can be estimated as $\epsilon_p = 0.46$.

HENRY COEFFICIENTS OF THE MAIN- AND THE UNDESIRED PRODUCTS

The Henry coefficient, H, represents the slope of the adsorption isotherm of a pure component at infinite dilution. For the generic ith component of the mixture to be separated, H can be estimated using a pulse of the pure component in the same eluent used in the separation process, and measuring the corresponding retention time $t_{r,i}$ from the relation:

$$t_{r,i} = t_0\left(1 + \frac{1-\epsilon}{\epsilon} H_i\right) \tag{8}$$

However, as discussed earlier, we have the constraint of using only mixtures (no pure components) and preparative stationary phases in analytical columns. Therefore, in order to measure the Henry coefficients we need to achieve a reasonable resolution of the components in the mixture to be separated, which is not always possible due to the poor efficiency of these columns compared to the corresponding ones packed with analytical stationary phases. In addition, we deal often with rather low selectivity values, particularly in the case of separations to be performed by SMB chromatography, which can be operated also with very small selectivities conveniently. The conclusion is that often, particularly in the case of purification processes where some of the components to be separated are present only in a very small amount, the peaks of some components cannot be seen and then their Henry coefficients cannot be measured, while the estimation of the Henry coefficient of the main component is instead straightforward.

Therefore, we suggest the following procedure to estimate the Henry coefficients of all components involved in the separation. The first step is to try to achieve sufficient resolution with the preparative stationary phase by reducing the flow-rate in order to increase the column efficiency. If this is not sufficient, one can use the corresponding analytical stationary phase, which would yield complete resolution definitely. However, in this case care must be taken in selecting the analytical stationary phase, since this should differ from the preparative one only in size and not in chemical composition. This is often not true for commercial phases even characterized by the same denomination. In the case of purification processes, a good check can be obtained by comparing the Henry coefficients of the main component measured on the two phases. If this alternative is also not successful, then we should postpone the evaluation of the Henry coefficients, at least for the components whose maxima cannot be identified, to a later step in this procedure as discussed in sections “Dissipative Phenomena” and “Estimation of the Adsorption Isotherms”.

For the separation systems considered in this work the Henry coefficients values reported in Table 2 have been measured. For AD, being the main component, the evaluation using the preparative material was straightforward, while for NP1 and NP2 as well as for 9-EPI a significant decrease in the flow-rate in order to increase the column efficiency was required. Figure 3 shows a chromatogram of the ternary system AD, NP1, and NP2 obtained at a very low flow-rate of 0.1 mL/min. A complete resolution of the by-products from the main component could be achieved, and the Henry coefficients could be calculated from the retention times of the single peaks using Eq. (8).

DISSIPATIVE PHENOMENA

In the frame of the simulation model described above, the dissipative phenomena are described in terms of axial dispersion and mass transfer resistance between the bulk and the solid phase. The corresponding parameters, i.e., D_{ax} and k_m, are evaluated typically through the van-Deemter plot (12), which represents the HETP (height equivalent to a theoretical plate) as a function of the superficial velocity:

$$\text{HETP} = \frac{2\epsilon_b D_{ax}}{u} + \frac{2u}{1-\epsilon_b}\left[1 + \frac{\epsilon_b}{K_i(1-\epsilon_b)}\right]^{-2} \frac{1}{k_{m,i}} \tag{9}$$

with $K_i = \epsilon_p + (1-\epsilon_p)H_i$ and $D_{ax} = \gamma D_m + \beta u d_p$ (13).

The HETP is evaluated from the same outlet concentration profiles obtained by feeding a pulse of the mixture to be separated, that we have used in the previous section to estimate the Henry coefficients, using the following equation (14):

$$\text{HETP} = L/(5.54(t_r/w_{0.5})^2) \tag{10}$$

where $w_{0.5}$ is the peak width at half height of the peak concentration and t_r its retention time.

Typically, in the range of superficial velocity values of interest in chromatographic separations the contribution of molecular diffusion is negligible

Table 2. Henry Coefficient and Selectivity Values with Respect to AD

	AD	NP1	NP2	9-EPI
Henry coefficient	1.89	2.42	2.76	2.16
Selectivity	1.00	1.24	1.46	1.14

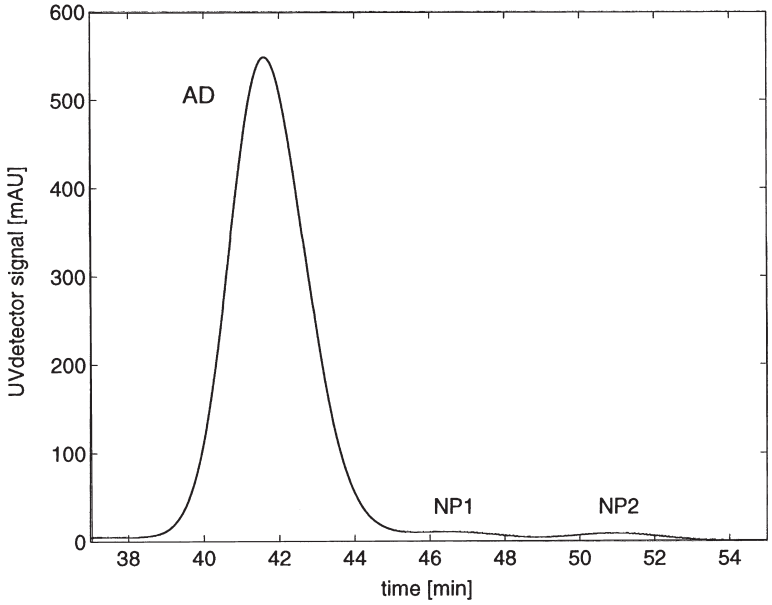

Figure 3. Measurements of the Henry coefficients of AD, NP1, and NP2. Plot of the on-line UV measurement.

compared to that of eddy diffusion. In this case, the van-Deemter curve reduces to a straight line. This was indeed the case for the purification process considered in this work, for which the corresponding van-Deemter plot is shown in Fig. 4. Note that these values have been obtained using directly the pulses of the mixtures to be separated and ignoring the presence of NP1 and NP2 due to their low concentration. From the intercept with the y-axis and the slope of the straight line in Fig. 4, the axial dispersion and the mass transfer coefficient can be estimated as $D_{ax} = 9.27 \times 10^{-3} u$, where u is in cm/sec and D_{ax} is obtained in cm^2/sec, and $k_m = 3.42\,sec^{-1}$, respectively.

ESTIMATION OF THE ADSORPTION ISOTHERMS

According to the peak fitting method, the parameters of the adsorption isotherms are estimated by fitting directly the concentration values at the outlet of the chromatographic column, when a sufficiently large pulse of the mixture to be separated has been fed. Actually, since the linear part of the isotherm given by the

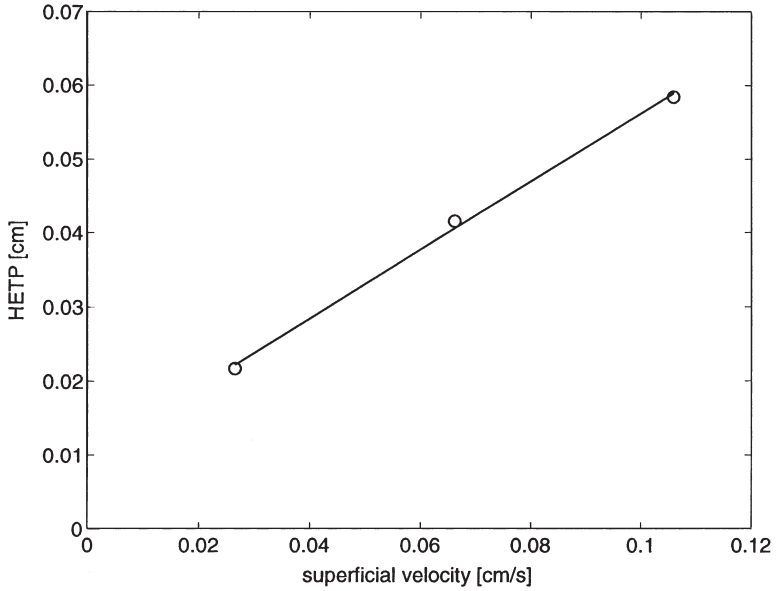

Figure 4. Van-Deemter plot of AD on MN RP C18 30 μm in MeOH/H_2O: 80/20 v/v%; (○) experimental data, (—) linear regression.

Henry coefficient has been estimated already in previous steps of this procedure, here we focus on the nonlinear portion of the isotherm, which describes the competition among the different species for adsorption. For this we need to feed to the column a pulse, which is sufficiently large, i.e., to operate the column under overloaded conditions.

In order to apply successfully this procedure it is necessary that the model is sufficiently accurate, since all possible errors reflect on the estimated isotherms eventually. For this we use the lumped pore diffusion model described above, whose parameters have been estimated carefully in the previous sections. In addition, we need to measure the concentration values of all components in the outlet stream as a function of time. Typically, chromatographic columns are equipped with a single online detector, and therefore the complete composition of the outlet stream can be measured only if complete resolution of all components is achieved in the column. As discussed above, this is unlikely when using preparative stationary phases in analytical columns, particularly when operating under overloaded conditions. Therefore, it is necessary to take samples of the outlet streams in time and to analyze them separately using some other analytical technique. In the following we discuss the application of this procedure separately for the two systems under examination.

Ternary System: AD, NP1, and NP2

A pulse of 250 μL with concentration of 100 g/L of the mixture to be separated (AD 96%, NP1 and NP2 2% each) was eluted at a flow-rate of 0.5 mL/min. Fractions were collected every 30 sec, and the entire peak was separated in 11 fractions, each with a volume of 250 μL. Each of these fractions was analyzed by HPLC to determine its composition. Figure 5 shows the results of the fraction collection method for the overloaded AD peak profile and, as a comparison, the peak profile measured on line by the ultraviolet (UV) detector at high wavelength. Symbols indicate the concentration in the collected sample and the corresponding horizontal segment indicates the corresponding sampling interval. Off-line analysis of the samples and on-line UV signal are in good agreement, again due to the negligible concentration level of the impurities NP1 and NP2.

For the system under consideration the adsorption equilibria have been described using the multicomponent Langmuir model:

$$q_i = \frac{H_i c_i}{1 + \frac{H_{\mathrm{AD}} c_{\mathrm{AD}}}{q^{\mathrm{s}}_{\mathrm{AD}}} + \frac{H_{\mathrm{NP1}} c_{\mathrm{NP1}}}{q^{\mathrm{s}}_{\mathrm{NP1}}} + \frac{H_{\mathrm{NP2}} c_{\mathrm{NP2}}}{q^{\mathrm{s}}_{\mathrm{NP2}}}} \quad (i = \mathrm{AD}, \mathrm{NP1}, \mathrm{NP2}) \tag{11}$$

where q^{s}_i represents the adsorbed phase concentration at saturation for the ith component. As happens typically in the purification processes, the concentration of the main component is much larger than that of the impurities, i.e., in this case $c_{\mathrm{AD}} \gg c_{\mathrm{NP1}}, c_{\mathrm{NP2}}$, so that the above relation reduces to:

$$q_i = \frac{H_i c_i}{1 + \frac{H_{\mathrm{AD}} c_{\mathrm{AD}}}{q^{\mathrm{s}}_{\mathrm{AD}}}}; \quad (i = \mathrm{AD}, \mathrm{NP1}, \mathrm{NP2}) \tag{12}$$

under the reasonable assumption that $q^{\mathrm{s}}_{\mathrm{AD}} \cong q^{\mathrm{s}}_{\mathrm{NP1}} \cong q^{\mathrm{s}}_{\mathrm{NP2}}$ due to the very similar structure of the three molecules.

Although not essential, this observation allows to apply the peak fitting procedure first to the main component alone and then to the two impurities, thus reducing the computational effort. In particular, since the value of $H_{\mathrm{AD}} = 1.89$ has been obtained earlier from experimental data in the diluted region, the only parameter left is the saturation concentration $q^{\mathrm{s}}_{\mathrm{AD}}$. This has been estimated as $q^{\mathrm{s}}_{\mathrm{AD}} = 175\,\mathrm{g/L}$ by fitting the experimental concentration data as shown in Fig. 6.

It is seen that the obtained agreement is reasonable, although the concentration values in the tail of the peak are somehow underestimated. A second curve in Fig. 6 shows that a better agreement with the experimental data could be obtained by changing the Henry coefficient value slightly from 1.89 to 2.03, i.e., by about 7%. This indicates the good sensitivity of the peak profile to

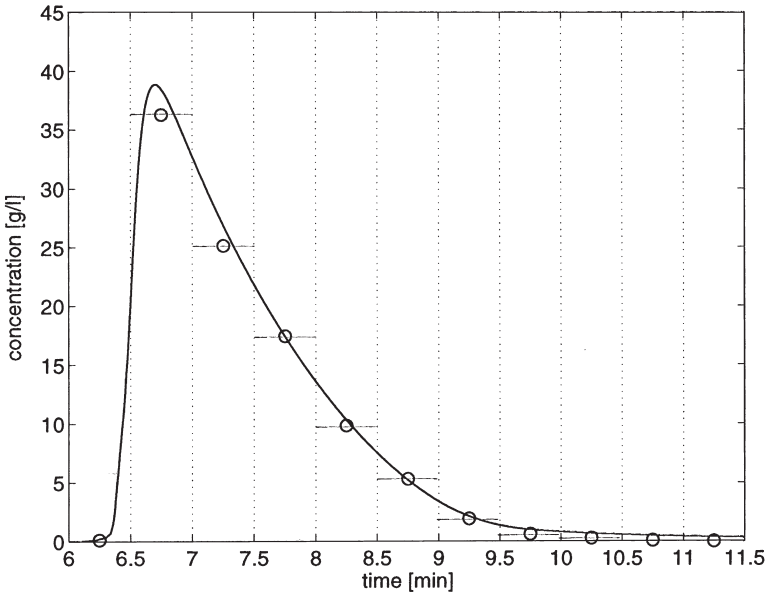

Figure 5. Plot of the calibrated on-line UV signal and the off-line analysis of the fractionated samples of AD; (○) concentration in the fractionated sample, (—) on-line UV measurement.

the value of the equilibrium parameters, which is very helpful in providing reliable estimates. The above fitting procedure has been repeated using the bi-Langmuir equilibrium isotherm. Since the improvement is only marginal we kept it as the final isotherm for AD the Langmuir model with $H_{AD} = 1.89$ and $q^s_{AD} = 175\,\mathrm{g/L}$.

Let us now apply the peak fitting procedure to the two impurities NP1 and NP2. From the adsorption isotherm in Eq. (12) it is seen that their behavior is not affected by their own saturation concentration in the adsorbed phase but only by the corresponding Henry constants that have already been measured (see section "Henry Coefficients of the Main- and the Undesired Products," Table 2). This is consistent with the fact that these components are present only in small concentrations, and therefore the competition to adsorption comes really only from the main component. Accordingly, there is no free parameter left for these components and we can use the comparison between measured and predicted peak profiles shown in Fig. 7 as a check of the reliability of the entire procedure.

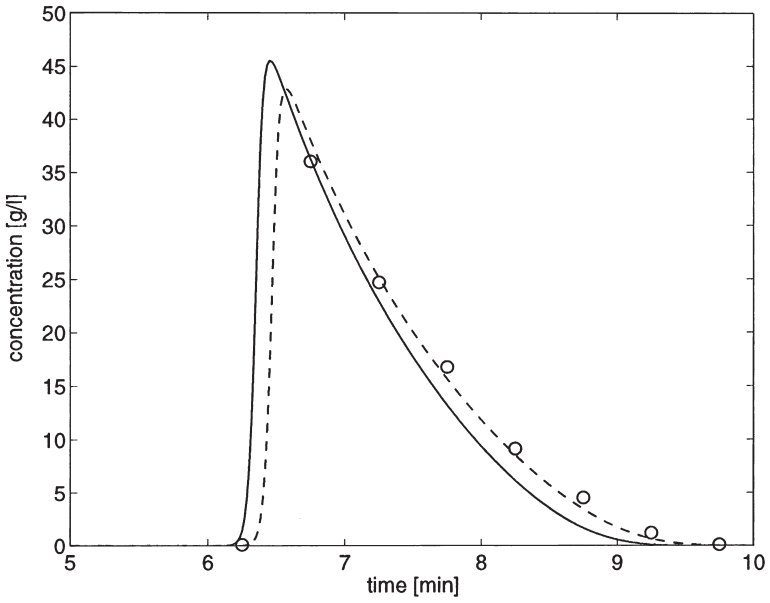

Figure 6. Simulated and experimental peak profile of AD on reversed phase; (○) experimental data; model simulations: (– –) $H_{AD} = 2.03$, (—) $H_{AD} = 1.89$.

Binary System: AD and 9-EPI

A pulse of 100 μL of a 100 g/L feed solution (AD 70%, and 9-EPI 30%) was eluted at a flow-rate of 0.5 mL/min. Fractions were collected every 30 sec, and the peak was fractionated in 10 samples, which have been analyzed separately by HPLC. In this case, the peak fitting method is applied using the complete binary Langmuir isotherm:

$$q_i = \frac{H_i c_i}{1 + \frac{H_{AD} c_{AD}}{q^s_{AD}} + \frac{H_{9\text{-EPI}} c_{9\text{-EPI}}}{q^s_{9\text{-EPI}}}} \quad (i = \text{AD}, 9\text{-EPI}) \tag{13}$$

Since both Henry coefficients were estimated in previous steps of the procedure (i.e., section "Henry Coefficients of the Main- and the Undesired Products"), only the two saturation concentrations in the adsorbed phase have been used as adjustable parameters in the fitting procedure since the value of $q^s_{AD} = 175$ g/L from the previous section has been used, only one parameter, i.e., $q^s_{9\text{-EPI}}$, has been varied. As shown in Fig. 8, it is found that using for both

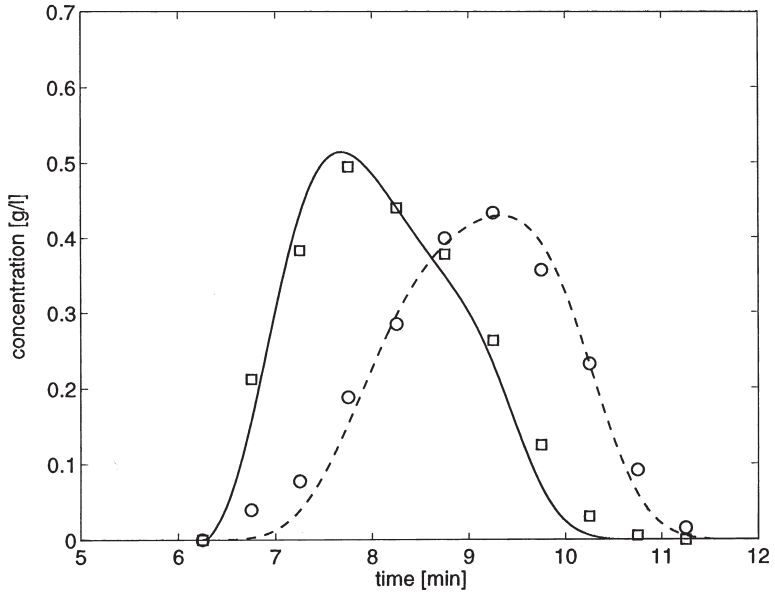

Figure 7. Simulated and experimental peak profiles of the byproducts NP1 and NP2 in an overloaded peak; experimental data: (□) NP1, (○) NP2; model simulations: (—) NP1, (– –) NP2.

components the same value obtained previously for AD, i.e., $q^{s}_{AD} = q^{s}_{9\text{-EPI}} = 175\,g/L$, a rather good agreement between the experimental and calculated concentration values is obtained. In addition, when slightly increasing the Henry coefficient of AD to the value of 2.03, similarly as for the separation system examined above, the quality of the fitting improves further. Note that in this case the Henry coefficient for 9-EPI has also been increased, in order to maintain the same selectivity of 1.14 measured in the diluted experiments. On the whole, the obtained agreement with the experimental data and the consistence with the data obtained with the separation system considered earlier, indicate the reliability of the developed procedure for estimating the equilibrium parameters.

Validation of the Procedure with Independent Equilibrium Data

In order to further support the developed procedure, the obtained equilibrium isotherms were compared to equilibrium data obtained through a

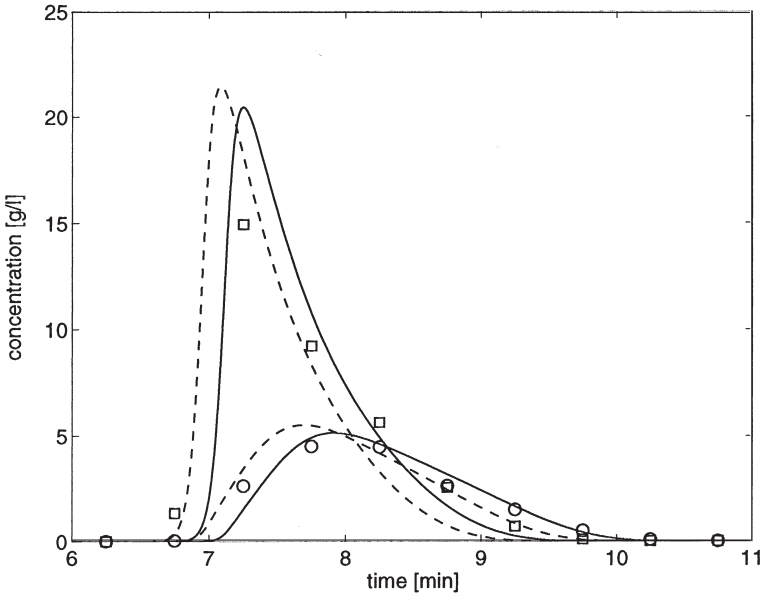

Figure 8. Simulated and experimental peak profiles of AD and 9-EPI; experimental data: (□) AD, (○) 9-EPI; model simulations: (– –) $H_{AD} = 2.03$ and $H_{9\text{-EPI}} = 2.31$, (—) $H_{AD} = 1.89$ and $H_{9\text{-EPI}} = 2.16$.

different technique. In particular, the perturbation method (15) was applied to the same purification process considered in section "Ternary System: AD, NP1, and NP2." In the following, we neglect the presence of the two impurities NP1 and NP2 and we concentrate on measuring the equilibrium isotherm of AD. In the frame of the perturbation method various experimental runs were performed using different background values for the concentration of AD ranging from 0 to 80 g/L. In each run the value of the slope of the equilibrium isotherm, i.e., dq_{AD}/dc_{AD}, at the considered background value of C_{AD} is estimated. The obtained values are compared in Fig. 9 with the curve obtained by deriving the AD isotherm (12), with $H_{AD} = 1.89$ and $q^s_{AD} = 175\,\text{g/L}$, estimated in section "Ternary System: AD, NP1, and NP2" using the peak fitting method. The obtained agreement indicates that the isotherm produced by the peak fitting method is consistent also with the equilibrium data measured with an independent technique, i.e., the perturbation method.

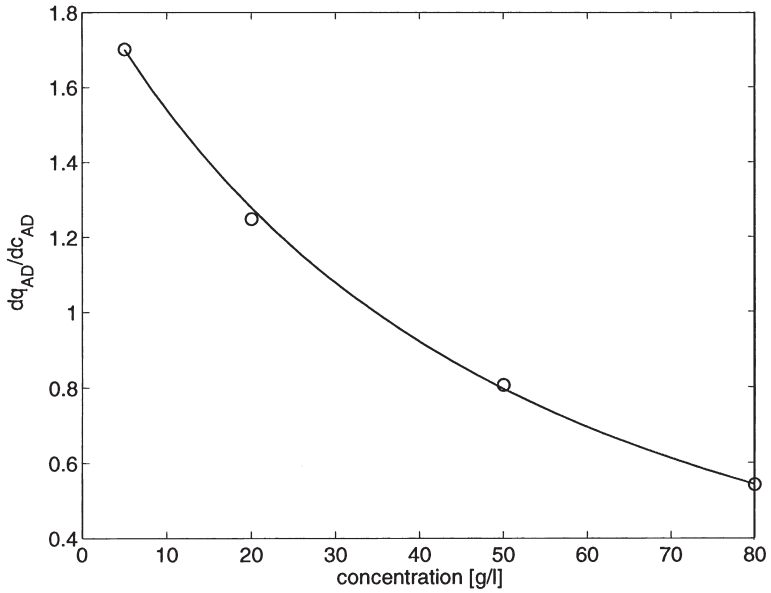

Figure 9. Derivative of the adsorption isotherm of AD. Comparison between data measured using the perturbation method (○), and values computed using the isotherm (12), with the parameters obtained by the peak fitting method, i.e., $H_{AD} = 1.89$ and $q^{s}_{AD} = 175\,g/L$ (—).

CONCLUSION

A general procedure for measuring the equilibrium isotherms using mixtures available only in limited amounts (and no pure substances) and preparative stationary phases has been developed. The procedure is based on the peak fitting method, which implies the estimation of the equilibrium parameters through the fitting of overloaded peaks of the mixture to be separated. The procedure requires the preliminary accurate estimation of all the other parameters, which affect the behavior of the chromatographic column, such as porosities, axial dispersion, and resistances to mass transfer.

In order to test the procedure in a realistic industrial context, the production process of the ascomycin derivative AD has been considered. In particular, the purification process of AD from NP1 and NP2, and the separation process between AD and 9-EPI, both based on reversed-phase chromatography, have been investigated. These two processes involve largely different concentration ranges of the mixture to be separated, which lead to specific problems with

respect to the estimation of the corresponding equilibrium parameters. It is found that in both cases the developed procedure provides reliable estimates of the equilibrium isotherms, which have also been verified through independent measurements obtained using a different technique. An important aspect of the procedure is the possibility of collecting individual fractions of the stream leaving the column and to analyze it individually. This allows overcoming the problem of incomplete peak resolution, which derives from the use of preparative stationary phases, i.e., of chromatographic columns with low efficiency. This procedure allows to reduce, with respect to the perturbation method, the amount of mixtures to be separated of about one order of magnitude.

NOTATION

c_i	Concentration of component i in the fluid phase (g/L)
c_i^{p}	Concentration of component i in the pores of the particles (g/L)
D_{ax}	Axial dispersion coefficient (cm^2/sec)
D_{P}	Pore diffusion coefficient (cm^2/sec)
d_{p}	Diameter of the particle (cm)
H_i	Henry coefficient of component i
H_i^*	Effective Henry coefficient of component i defined by Eq. (5)
$k_{\mathrm{m},i}$	Lumped mass transfer coefficient of component i (sec^{-1})
L	Column length (cm)
Q	Flow-rate (mL/s)
q_i	Concentration of component i in the stationary phase in (g/L)
q_i^{s}	Saturation capacity of component i in the stationary phase (g/L)
R_{p}	Radius of the particle (cm)
t	Time (sec)
$t_{\mathrm{r},i}$	Retention time of component i (sec)
t_{o}	Retention time of an inert component (sec)
u	Superficial velocity ($cm\,sec^{-1}$)
V	Volume of the empty column (cm^3)
$w_{0.5}$	Width of the peak at 50% of its height (cm)
x	Axial coordinate in the column (cm)

Greek Symbols

α	Selectivity
β	Packing specific constant in Eq. (9)
ϵ	Total porosity
ϵ_{b}	Interparticle porosity
ϵ_{p}	Intraparticle porosity
γ	Packing specific constant in Eq. (9)

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