Simultaneous Concentration and Purification through Gradient Deformation Chromatography

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Mobile-phase additives, commonly used to modulate absorbate retention in gradient elution chromatography, are usually assumed to be either linearly retained or unretained. Previous theoretical work from our laboratory has shown that these modulators, such as salts in ion-exchange and hydrophobic interaction chromatography and organic modifiers in reversed-phase chromatography, can absorb nonlinearly, giving rise to gradient deformation. Consequently, absorbate peaks that elute in the vicinity of the head of the deformed gradient may exhibit unusual shapes, form shoulders, and/or be concentrated. These effects for a reversed-phase sorbent with aqueous acetone triole (ACN) as the modulator are verified experimentally. Gradient deformation is demonstrated experimentally and agrees with simulations based on ACN isotherm parameters that are independently determined from batch equilibrium studies using the layer model. Unusual absorbate peak shapes were found experimentally for single-component injections of phenylalanine, similar to those calculated by the simulations. A binary mixture of tryptophan and phenylalanine is used to demonstrate simultaneous concentration and separation, again in agreement with simulations. The possibility of gradient deformation in ion-exchange and hydrophobic interaction chromatography is discussed.

Introduction

Gradient elution chromatography, in which the mobile phase composition at any point in the chromatographic column is a function of time, is widely used in the analysis and purification of both large and small compounds. Mobile phase additives, or modulators, are used to decrease absorbate retention and thereby achieve separation (for macromolecules) or decrease separation time (for small molecules). Typical modulators are salts in ion-exchange and hydrophobic interaction chromatography, and organic modifiers such as methanol and aqueous acetone-tri (ACN) in reversed-phase chromatography.

Adsorbate retention in gradient elution has been summarized in Snyder (1980), Snyder and Studius (1966), Jander and Churaček (1985), and Yamamoto et al. (1980), all of whom consider the mobile phase modulator itself to be either linearly retained or unretained. Modulator adsorption, however, is frequently appreciable and can be nonlinear. Their different concentrations of the modulator will move with different velocities, giving rise to gradient deformation. An analogous, but not identical, effect that has been discussed qualitatively in the chromatographic literature is “solvent demixing,” which is based on the different velocities with which different components of a multicomponent mobile phase travel. This article is concerned with the consequences of different portions of a single component moving with different velocities, due to nonlinear sorption.

Recent work in our laboratory (Velayudhan and Ladisch, 1991, 1992, 1993) has quantified conditions leading to gradient deformation and described how this could be exploited to obtain simultaneous concentration and purification of moderately retained components. This is achieved by positioning the desired product just behind the head of the gradient, where maximum deformation occurs, so that it will be concentrated while the normal mechanism of gradient elution...
causes it to be separated from its impurities. In this article, experimental validation of this concept is presented using reverse-phase chromatography of phenylalanine (Phe) and tryptophan (Trp) in an aqueous ACN gradient as a case study. Batch experiments were carried out to establish the excess isotherm for the ACN-water system on the chosen stationary phase, and the layer model was used to extract the individual isotherm for ACN. A series of gradient elution runs was then carried out, both for Phe alone and for a mixture of Phe and Trp, and shown to give profiles anticipated by the numerical predictions. Gradient deconvolution, based on nonlinear modulator adsorption, as well as simultaneous concentration and separation, is demonstrated.

Materials and Methods

Liquid chromatography system

A Rainin model HPX-10 (Woburn, MA) gradient system with a flow range of 0.1-10 mL/min was used, along with a Rheodyne model 7125 injector (Cotati, CA). Eluting amino-acid peaks were detected by UV absorbance at 254 nm using an Isco model V4 variable wavelength detector (Lincoln, NE). Composition of the ACN-water gradient was measured by a Waters 401 differential refractometer (Milford, MA). Chromatographic elution histories were recorded on a Linear model 1200 strip chart recorder (Houston, TX). Analytical results were quantitated using a Hewlett-Packard model 3390A reporting integrator (Avondale, PA).

All gradient experiments were run on a 0.8×30-cm stainless-steel column capped with stainless-steel end fittings with 10 μl syringes (Indianapolis Valve and Fitting, Indianapolis, IN). The reverse-phase adsorbed Amberchrom CG-71D (Rohm and Haas, Spring House, PA), a polymeric methylacyl material, was used. A mean particle size of 30 μm was used in deionized water using a Malvern Series 20000 Droglet and Particle Sizer (Malvern, England). The same solvent can be obtained in the 20-50 μm range from Tosoh Haas (Northridgeville, PA). The CG-71D was packed as a slurry at a flow rate of approximately 9 mL/min using a technique developed in our laboratory (Lin et al., 1988). Gradients were formed using 0.45 μl filtered, degassed, deionized water and 99.9% high-performance liquid chromatography (HPLC) grade ACN. The adsorbates used were 1-phenylalanine (Serva, Heidelberg, Germany) and L-tryptophan, Sigma Grade (Sigma, St. Louis, MO).

Analytical chromatography runs were carried out on a 4-mm I.D.×25-cm Rainin Dynamax C-18 column with a particle size of 5 μm and an average pore size of 300 Å. Fractions collected from the gradient runs were analyzed twice, once for ACN and again for the adsorbates (Phe and Trp). ACN samples were run with 0.45 μl filtered, degassed water as the mobile phase at a flow rate of 0.7 mL/min. Peak areas resulting from 20 μL injections were calculated using the recording integrator. Analytical runs for the adsorbates were carried out using a mobile phase of 25% ACN in deionized water at a flow rate of 0.7 mL/min. Sample injections of 20 μL were analyzed on the UV detector at 254 nm.

Isotherm determination

Batch experiments were run in which a fixed amount of the CG-71D adsorbent in an air-dry state at 16.9% moisture was equilibrated with ACN-water mixtures of different composition. A typical isotherm measurement consisted of weighing 2 g of CG-71D into a 30-mL Teflon solid-core vial (Anspec Co., Ann Arbor, MI). A volume of 15 mL of aqueous 49% ACN was added to the vial, which was then capped and placed into a Burrell wrist action shaker (Burrell Co., Pittsburgh, PA). The mixture was gently shaken at room temperature (25°C) for 24 hours. An aliquot of the supernatant was withdrawn and placed in a microcentrifuge tube. Prior to injection, all isotherm samples were centrifuged to remove fine adsorbent particles suspended in solution. The final equilibrium ACN concentration was determined by analyzing the supernatant liquid from each batch experiment by HPLC, as described earlier.

The surface molar excess isotherms in Figure 1a were calculated from the batch equilibrium data under the convention that nothing is adsorbed, in moles. Excess isotherm data for ACN-water on octadecyl reversed-phase silica with linear or branched C-18 chains, taken from the literature, are also shown in Figure 1a. Remarkable similarity between these C-18 data is evident. The polymeric sorbent used in this study shows significantly greater ACN retention, and the presence of an adsorption isostere. Some C-18 silica also show azoetroping, making it necessary to account for the adsorption of water (the lesser retained component) in constructing an individual isotherm for ACN from the excess isotherm (Slaats et al., 1981). Otherwise, an individual isotherm for ACN that exhibits a maximum would result, which is physically unreasonable.

We therefore use the layer model (Kipling and Tester, 1952; Everett, 1981), in which it is assumed that the stationary phase is always saturate. As the mobile phase composition of the ACN-water mixture changes, so does the stationary phase composition in the saturated layer associated with the stationary phase. The number of layers in this saturated region is found from Rusnok's criteria (Rusnok, 1967): the minimum number of layers is used, provided that the resulting stationary phase mole fraction of ACN and water lie between and 1, and the individual isotherms are monotonic. The individual isotherm for ACN is then obtained as (Everett, 1981)

\[
x^* = \frac{1}{t_1} \left( \frac{a_1 - a^*}{a_1 - a_0} \right)^
u
\]

where \(t_1\) is the minimum number of layers consistent with Rusnok's criteria. This is a trial-and-error method: increasing values of \(t_1\), are tried, starting with 1, and the first value that satisfies Rusnok's criteria is chosen. The calculation leads to \(t_1 = 10\).

An alternative direct, that is, noniterative, calculation based on the specific surface area of the adsorbent provides a consistency check. In this method, the areas associated with ACN and water on adsorption can be approximated by a simple packing calculation (Emmett and Brunauer, 1937; Tani and Suzuki, 1969). The theoretical saturation levels of ACN and water are calculated by the method of Schay and Nagy (1961), but allowing for multilayer adsorption. The linear portion of the excess isotherm is extrapolated to the ordinates corresponding to pure ACN and pure water, respectively. Then
the following independent estimation of the number of layers is available:

\[ \ln l_i = \frac{n_{ACN}^0 a_{ACN}^0 + n_{H_2O}^0 a_{H_2O}^0}{A_i}. \]  

For the present system, the specific surface area of the sorbent is 500 m²/g (manufacturer's data), and the adsorption areas are 130 m²/mmol for ACN and 83 m²/mmol for water. The saturation concentrations are 27 mmol/g for ACN and 22 mmol/g for water. Substituting these values in Eq. 2, we obtain the following estimate

\[ l_i = \frac{4.896 \text{ m}^2/\text{g}}{500 \text{ m}^2/\text{g}} = 9.8 \times 10^{-4}. \]

This is consistent with our earlier trial-and-error calculation based on Rusano's criteria. The latter method requires a linear region in the excess isotherm, so that the Schay–Nagy calculation becomes possible. The method is therefore not always applicable, but seems useful for chromatographic systems exhibiting adsorption zeotropy.

The data points for the individual isotherm for ACN, calculated from excess isotherm data as previously described, are shown in Figure 1b. As expected, the individual isotherm is sigmoidal. A quaternary polynomial was fitted to the data by nonlinear regression, and Figure 1b shows that the fit is reasonable. Practical uses of ACN gradients, however, involve concentration well below the extremely high mobile phase concentrations (around 14–15 M) at which the plateau is reached. Consequently, fitting the monotonic data before this plateau to simple isotherm forms such as the Langmuir,

\[ q_U = \frac{\alpha_U C_M}{1 + \beta_U C_M} \]

and the Jovanovic,

\[ q_U = \Lambda_U (1 - e^{-k_U C_M}) \]

can be useful. These fits are also shown in Figure 1b, with parameter values for all fits given in the figure legend.

### Gradient deformation runs

All gradient runs were carried out on the same Amberchrom CG-71s methacrylate material used earlier in the batch isotherm studies. The gradient system was described earlier; operating conditions are listed in Table 1. Fractions were collected every 15 s during a gradient run for analysis by HPLC of ACN and adsorbate concentrations, as described earlier.

A linear gradient of 0–100% ACN was run in 40 min. This gradient slope, of 0.47 M/min, was chosen as a representative value for large-scale chromatography, although steeper slopes are frequently used at bench scale. Theory (Velayutham and Lastich, 1972) predicts that, under ideal conditions, significant gradient deformation would not occur but a true shock wave would not form in the column. Nevertheless, even in the absence of a shock, considerable gradient deformation occurs, as can be seen from the replicates experimental runs.

**Table 1. Operating Conditions**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow rate</td>
<td>1 ml/min</td>
</tr>
<tr>
<td>Gradient</td>
<td>0%–100% ACN in 40 min</td>
</tr>
<tr>
<td>Temperature</td>
<td>Ambient</td>
</tr>
<tr>
<td>Feed volume</td>
<td>2 ml</td>
</tr>
<tr>
<td>Feed concentration</td>
<td>0.5 mg/mL (Phc)</td>
</tr>
<tr>
<td>Column dimensions</td>
<td>0.76 I.D. × 70 cm length</td>
</tr>
</tbody>
</table>

*Applicable to Figure 2 through 5.*
in Figure 2a. At the head of the gradient, the slope steepens considerably between 34 and 35 min; the gradient goes from 6.4 to 16.8 M ACN, corresponding to a linear slope of 10.4 M/min; this incipient shock layer has a slope far higher than the initial slope of 0.47 M/min. The corresponding simulation shows a comparable steepening. Since the simulation does not account for bandsaxing, it does not capture the shallow region at the head of the experimental gradient. After the experiments were completed, the data suggested that a longer column would be needed for a shock to form under these operating conditions. This is consistent with the previously developed theory, which indicates that a shock would occur just at the outlet of a column that was 16% longer (that is, a 58-cm volume).

The relatively sharp change in slope in the experimental data around 50% v/v ACN could be due to the different extents of swelling undergone by the stationary phase as the mobile phase composition is varied, although this effect is likely to be mitigated in a tightly packed column. Swelling in batch systems was found to increase abruptly at around 50% ACN for the polymeric sorbent. Since the simulation did not incorporate swelling effects, or the possibility of the adsorbent swelling to different degrees as it sees different mobile phase compositions (Marra and Cooney, 1978; Durão et al., 1992), the calculated profile varies smoothly.

Numerical method

A lumped model incorporating overall mass-transfer coefficients is used to describe column dynamics. We first derive it from the following well-known general model for chromatography (Schoeder and Smith, 1966; Furusawa et al., 1976; Arnold et al., 1985a).

The mass balance over the interstitial fluid is

$$\frac{\partial C}{\partial t} + \varepsilon_{m \varepsilon} \frac{\partial C}{\partial x} + (1 - \varepsilon_{m \varepsilon}) \frac{\partial C}{\partial t} = E \frac{\partial^2 C}{\partial x^2}. \quad (6)$$

The mass balance over the sorbent particles is

$$\varepsilon_{p \varepsilon} \frac{\partial C_p}{\partial t} + \varepsilon_{m \varepsilon} \frac{\partial C}{\partial t} = D_p \left( \frac{\partial^2 C_p}{\partial r^2} + \frac{2}{r} \frac{\partial C_p}{\partial r} \right). \quad (7)$$

These mass balances are coupled by

$$\frac{dC_p}{dt} = \frac{6(1 - \varepsilon_p)}{d_p} D_p \frac{\partial C}{\partial r} \bigg|_{r = \delta_{p,2}} = \frac{6(1 - \varepsilon_p)}{d_p} k_f (C - C_p) \bigg|_{r = \delta_{p,2}}. \quad (8)$$

Finite sorption kinetics as a first-order process is expressed by

$$\frac{\partial q_f}{\partial t} = \mu_q (K_{st} C_p - q_f). \quad (9)$$

Equations 6 through 9, along with standard initial and boundary conditions, account for bandshaping contributions from axial dispersion, film mass transfer, pore diffusion, and finite sorption kinetics. From this general model we first derive the limiting case where only finite sorption kinetics are important; the other sources of bandsaxing are considered negligible. Then the following limits are obtained:

$$E \rightarrow 0; \quad \frac{\partial^2 C}{\partial x^2} \rightarrow 0; \quad D_p \rightarrow \infty; \quad \frac{\partial C_p}{\partial r} \rightarrow 0; \quad \frac{\partial^2 C_p}{\partial r^2} \rightarrow 0; \quad k_f \rightarrow \infty; \quad C_s \rightarrow C. \quad (10)$$

Note that the particle concentration $\bar{C}$ is then given by

$$\bar{C} = \varepsilon_p C + \rho_p q \quad (11)$$

and the system of Eqs. 6 through 9 reduces to

$$\varepsilon_{p \varepsilon} \frac{\partial \bar{C}}{\partial t} + \varepsilon_{m \varepsilon} \frac{\partial C}{\partial t} + (1 - \varepsilon_{m \varepsilon}) \frac{\partial \bar{C}}{\partial t} = 0 \quad (12)$$

$$\varepsilon_{p \varepsilon} \frac{\partial \bar{C}}{\partial t} + \varepsilon_{m \varepsilon} \frac{\partial C}{\partial t} \bigg|_{r = \delta_{p,2}} = \frac{\partial q_f}{\partial t} = \mu_q (K_{st} C_p - q_f). \quad (13)$$

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Combining Eqs. 11 and 12 results in
\[t_e = \rho + (1 - \rho)\epsilon_p + n_{w}P_{\text{H}} + \frac{L}{\epsilon_p + (1 - \rho)}k_{\text{H}} + \frac{L}{\epsilon_p + (1 - \rho)}K_{\text{H}} = 0.\] (14)

Equations 13 and 14 therefore represent the desired limiting case of finite sorption kinetics as the only source of band-sprading. In order to use this limiting case as a lumped model of the entire bandsprading process, we replace \(\rho_e\) in Eq. 13 by an overall lumped rate \(k^\rho\), and choose \(k^\rho\) so that the band-sprading generated by the general model, Eqs. 6 through 9, will be captured by the lumped model, Eqs. 13 and 14. For this purpose, the retention time and variance of the two models are matched.

The retention time and variance of the general model are available in the literature (Furusawa et al., 1986b):

\[t_e = \frac{L}{v_{\text{up}}(\epsilon_e + (1 - \epsilon_e)\epsilon_p + \rho_e K_{\text{H}}) + \frac{t_{\text{H}}}{2}}\] (15)

\[\sigma^2 = \frac{2L}{v_{\text{up}}(\epsilon_e + (1 - \epsilon_e)\epsilon_p + (1 - \epsilon_e)\rho_e K_{\text{H}}) + \frac{t_{\text{H}}}{2}} \times \left( \frac{\rho_e K_{\text{H}}^2}{\rho_e} + \frac{d^2}{\rho_e} + 1 + \frac{1}{\rho_e K_{\text{H}}(1 + \frac{1}{\rho_e K_{\text{H}}})} \right) \left( \frac{1}{\rho_e K_{\text{H}}} \right)^2 \] (16)

Equations 13 and 14 can be rewritten as
\[\frac{\partial C}{\partial t} + v_{\text{denom}} \frac{\partial C}{\partial x} + \frac{\partial q}{\partial t} = 0\] (17)

\[\frac{\partial q}{\partial t} + \frac{\partial q}{\partial x} + k^\rho (C_{\text{H}} - q) = 0\] (18)

where
\[v_{\text{denom}} = \frac{v_{\text{denom}}}{\epsilon_e + (1 - \epsilon_e)\epsilon_p}\] (19)

and
\[\phi = \frac{(1 - \rho)\rho_e}{\epsilon_e + (1 - \rho)\epsilon_p}\] (20)

In the form given by Eqs. 17 and 18, the lumped model is identical in form to one given in Ruthven (1984), with \(v_{\text{up}}\) and \(\phi\) replacing \(v_{\text{denom}}\) and \(1 - \rho\). The physical picture in Ruthven's model is that of external mass transfer being the single source of band-sprading, and consequently his adsorption constant \(K_{\text{H}}\) includes holdup within the pores of the particle, while \(K_{\text{H}}\) in Eq. 18 accounts solely for sorbed concentration. Nevertheless, the formal analogy can be used to apply Ruthven's results for retention time and variance to the present lumped model in Eqs. 17 and 18.

The results for retention time (first moment) is
\[t_e = \frac{L}{v_{\text{up}}(1 + (1 - \epsilon_e)\epsilon_p + (1 - \rho)K_{\text{H}}) + \frac{t_{\text{H}}}{2}}\] (21)

This can be shown to be equivalent to Eq. 15 by noticing that \(K_{\text{H}}\) and \(K_{\text{H}}\) have different units and are related through
\[\rho_e K_{\text{H}} = (1 - \rho)K_{\text{H}}\] (22)

Ruthven's result for variance (second central moment) is
\[\sigma^2 = \frac{2L}{v_{\text{up}}(1 + (1 - \epsilon_e)\epsilon_p + \frac{1}{k^\rho} (\epsilon_e + (1 - \rho)K_{\text{H}})) \frac{t_{\text{H}}}{12}}\] (23)

Equation 23 can be compared to Eq. 16 to obtain the appropriate value of \(k^\rho\).

**Column simulations**

The lumped system just given, Eqs. 17 and 18, are now applied to the gradient system with ACN as the modulator and Phe and Trp as the feed components. The governing equations are therefore
\[\frac{\partial C}{\partial t} + \frac{\partial C}{\partial x} + \frac{\partial q}{\partial t} = 0, \quad i = \text{ACN, Phe, Trp}\] (24)

\[\frac{\partial q}{\partial t} + k^\rho (q^\rho - q), \quad i = \text{Phe, Trp}\] (25)

\[q^\rho_{\text{ACN}} = \frac{k_{\text{ACN}}}{1 + 0.0637k_{\text{ACN}}}\] (26)

\[\frac{\phi q^{\rho}_{\text{ACN}}}{C_{\text{Phe}}} = (C_{\text{ACN}} + 1.07) \frac{0.31}{0.41}\] (27)

\[\frac{\phi q^{\rho}_{\text{ACN}}}{C_{\text{Trp}}} = \frac{C_{\text{ACN}}}{2.80} - 0.243\] (28)

Here \(k^\rho\) is the overall mass-transfer coefficient for the ith component, and is calculated as described earlier by comparing Eqs. 16 and 23. Standard correlations and equations from the literature are used to estimate the quantities in Eq. 16. The molecular diffusivity for Phe is available experimentally (Conway, 1952), while that for Trp is calculated from a version of the Wilke-Chang expression due to Snyder and Starbuck (1986). This allows the pore or intraparticle diffusivities to be calculated in the usual way (Satterfield, 1969), accounting for the tortuosity. Wilson and Grencikop's correlation (1966) is used to estimate the mass-transfer coefficient. Axial dispersion is relatively unimportant for liquid chromatography using small or moderate particles (Ruthven, 1984) and is therefore neglected in Eq. 16. The values of the various parameters are given in Table 2.

Equation 26 represents the Langmuirian form of the ACN individual isotherm calculated earlier. The equilibrium model is used for ACN, hence Eq. 25 does not apply to ACN. The retention of the feed components, as shown in Eqs. 27 and 28, followed usual reversed-phase patterns and were calculated from separate isocratic runs. The adsorbates are assumed to be at concentrations low enough to prevent appreciable interference, and are therefore regarded as moving independently of each other. Single-component isotherm measurements carried out in our laboratory for both Phe and Trp.
Table 2. Parameters Used in the Simulations in Figures 2 to 6

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle diameter (d_p)</td>
<td>60 μm</td>
</tr>
<tr>
<td>Particle density (ρ_p)</td>
<td>0.3 g/cm³</td>
</tr>
<tr>
<td>Torricelli number (R_t)</td>
<td>4</td>
</tr>
<tr>
<td>Red blood cell count (ρ_b)</td>
<td>0.30</td>
</tr>
<tr>
<td>Specific surface area (A_s)</td>
<td>500 m²/g</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Phenol (Phe)</th>
<th>Trp</th>
</tr>
</thead>
<tbody>
<tr>
<td>k_f (cm²/s)</td>
<td>9.47 × 10⁻³</td>
</tr>
<tr>
<td>k_d (cm²/s)</td>
<td>9.12 × 10⁻³</td>
</tr>
<tr>
<td>Diffusivity (cm²/s)</td>
<td>1.08 × 10⁻⁵</td>
</tr>
<tr>
<td>Overall lumped mass-transfer coefficient</td>
<td>35.5</td>
</tr>
</tbody>
</table>

*From the correlation 5A = (1.09ρ_b d P/ε)¹.¹
†From D_t = (ε/Φ_{a,v})²;
‡From Eq. 11.

were found to be effectively linear in the concentration range of interest.

The column is initially pre-equilibrated with mobile phase and is free of any feed component. Thus the initial conditions corresponding to the hyperbolic partial differential equation system. Eqs. 24 through 28, are

\[
\begin{align*}
&\text{At } t = 0, \quad C_i = \begin{cases} C_{i,0} & i = 1, \ldots , N-1 \\ C_{N} = C_{N,0} & \text{(modulator)} \end{cases} \\
&\text{At } t = 0, \quad q_i = \begin{cases} q_{i,0} & i = 1, \ldots , N-1 \\ q_{N} = q_{N,0} & \text{(Phe, Trp)} \end{cases}
\end{align*}
\]

The boundary conditions are

\[
\begin{align*}
&\text{At } x = 0, \quad C_i = \begin{cases} C_{i,0} & 0 \leq t \leq t_{eq} \\ C_{i,\infty} & t_{eq} < t \end{cases} \quad i = 1, \ldots , N-1 \\
&\text{At } x = 0, \quad C_N = C_{N,0} + \alpha (t - t_{eq}) \\
&\text{At } x = 0, \quad q_i = \begin{cases} q_{i,0} & 0 \leq t \leq t_{eq} \\ q_{i,\infty} & t_{eq} < t \\ q_{i,\infty} + \alpha (t - t_{eq}) \end{cases}
\end{align*}
\]

The feed components are injected into the column as a rectangular pulse over a time \( t_{eq} \), the associated mobile phase composition being identical to the pre-equilibrated state of the column. Then the gradient is fed into the column; here a linear gradient is used, at the inlet, with slope \( \alpha \).

These simple boundary conditions are used instead of the more rigorous Danckwerts' boundary conditions (1953), since Eq. 24 does not account for axial dispersion. (However, as described earlier, the contribution of axial dispersion to adsorbate bandspeeding can be incorporated into Eq. 16 when needed.) The hyperbolic system was then solved by the method of characteristics (Aebeiss, 1956; Courant and Hilbert, 1962) on a Sun SPARC 2 workstation.

Results

Modulator behavior

The predicted effluent ACN history of Figure 2 is bounded by the results from the two classic assumptions—that ACN is unretained, and that it is linearly retained. Under both of these assumptions, since the gradient was linear at the inlet, it remains linear at the outlet. These limiting cases diverge from the experimental results, as expected, since the ACN solute is highly nonlinear (cf. Figure 1). An unretained modulator gives rise to a gradient that moves at the mobile phase velocity. Since this is the maximum possible velocity for any component in the given column (neglecting size exclusion effects, and ion exclusion effects in ion-exchange chromatography), the actual gradient cannot emerge from the column earlier than the unretained prediction. Linear retention of the modulator corresponds to the slowest speed the gradient can assume, and therefore represents the upper limit for the actual gradient effluent. The physical basis for these upper and lower bounds is the self-interference (Helfferich and Klein, 1970; DeVault, 1963) of the modulator, and has been described previously (Venugopalan and Laidisch, 1993).

In fact, the experimental gradient can be seen to reach these two limiting results at its extrema. At very low ACN concentrations (< 3-4% v/v), the experimental gradient is close to the linearly retained prediction. At high concentrations (beyond 70% v/v), the experimental gradient agrees well with the unretained prediction.

Single-component runs

Adsorbable peak shape is strongly dependent on gradient delay. The effect of delay on peak shape is readily demonstrated, as shown for a series of Phe runs in Figures 3 to 5. In all these figures, the experimental result is shown on the left, and the simulation is shown on the right. In Figure 3a, the part of the Phe peak that emerges before the head of the gradient (before 38 min) elutes anaerobically and emerges as a broad front, while the region behind the incept shock is concentrated to more than twice its feed value. The inlet gradient slope and timing should thus be manipulated to place the desired product just behind the gradient to achieve peak concentration. The formation of a split peak from a single compound is noteworthy, since such a profile might easily be erroneously attributed to an impurity, or to the presence of an on-column reaction. The corresponding simulation, Figure 3b, is for a delay of 4 min, rather than 6 min, since it is for a delay of 4 min that the simulation captures the experimental behavior shown in Figure 3a. This discrepancy is at least partially attributable to the weakly retained impurity present in commercially available Phe. The impurity can be seen more clearly in Figures 4a, and 5a. Since the impurity elutes anaerobically in all three figures, it is not the cause of the split peak, which is due to Phe straddling the gradient. The analytical column, on which the capacity factor of Phe as a function of ACN concentration was measured, is only 25 cm long, and did not resolve this impurity from the main Phe peak. Since the dependence of Phe on ACN in the simulation is based on analytical data, the column simulations do not show the impurity. The retention shown in the simulation is therefore an average between the experimental retentions of the weakly retained impurity and the more strongly retained Phe. Thus the Phe in the experiments is more retained than in the simulations, and a given experimental effluent history is only attainable by a simulation with a smaller gradient delay.

In Figure 4a, the gradient is delayed by 7 min, one minute...
more than in Figure 3a. This allows more of the Phe to elute before it is overtaken by the gradient. A split peak is again seen, but the concentration effect behind the gradient is less than in part (a), since there is relatively less Phe in this region. The corresponding simulation, Figure 4b, is for a delay of 5 min.

Figure 5a shows a gradient delay of 8 min. Here almost all of the Phe elutes ahead of the gradient; the very small amount of Phe caught by the gradient is seen as a small shoulder starting at 40 min. Since the bulk of the Phe peak elutes isotropically, it is diluted with respect to the feed. The corresponding simulation, Figure 5b, has a delay of 7 min.

Qualitative agreement between simulation and experiments is evident: the same sequence of split peaks is seen, and the Phe found behind the modulator is concentrated. The discrepancy in gradient delay between experiment and corresponding simulation is further described in the Discussion section.

It should be emphasized that there are no fitted parameters in the simulations; all the parameters used are either directly from the manufacturer (such as the specific surface area of the stationary phase) or measured in independent experiments (such as the ACN isotherm, obtained by batch studies, and the adsorbate retention as a function of mobile
phase composition, obtained by separate isocratic runs. In this context, the agreement between experiment and simulation is reasonable.

**Separation of binary feed mixtures**

Mixtures of Phe and Trp were used to test the effect of gradient deformation on separation quality. Figure 6 corresponds to a gradient delay of 11 min. Phe has fully eluted under isocratic conditions, while the Trp is found just behind the ACN breakthrough and is therefore concentrated. Reasonable agreement is seen between experiment and simulation. This run shows how gradient deformation can be used to advantage: the operating conditions were manipulated such that the desired product (Trp) was located just behind the gradient and thereby concentrated, while being separated from Phe.

**Discussion**

Since peak concentration occurs in the vicinity of the shock layer, the head of the gradient must be modeled accurately for the simulations to give reasonable agreement with experimental results. There are two factors, not accounted for by the present simulations, that could strongly affect gradient deformation. The first is nonideal mixing of ACN and water. This would tend to increase the ACN concentration and thus

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**Figure 5. Single-component Phe run.**

(a) Shows the experimental result for a delay of 8 min, while (b) shows the simulation for a delay of 7 min (with respect to \( t = 0 \), when feed introduction begins. Operating conditions as in Table 1.

**Figure 6. Binary mixture run.**

Separation of Phe and Trp under conditions of gradient deformation. Gradient delay of 11 min with respect to \( t = 0 \). Operating conditions as in Table 1. (a) Shows the experimental result, and (b) the corresponding simulation.
to increase the shock strength. The shock layer would then move faster, which would account for the time difference between Figures 3 and 4. The second factor, which is harder to assess, is the degree of well-mixedness achieved in the gradient former (the part of the gradient delivery system that mixes the ACN and water together in appropriate quantities so as to generate the desired gradient). At the beginning of the process, when the ACN is just introduced into the former, the possibility for error is high; this corresponds to the head of the gradient.

The exact process is therefore difficult to model accurately. Further, small changes in the position or strength of the modulator shock can result in large changes in adsorbate peak shapes (cf. Figures 3-5). For example, if the shock strength were increased by around 5% (for example, due to nonideal mixing of ACN and water), the discrepancy of 2 min between experiment and simulation for the Phe runs in Figure 3 would be removed. The agreement between experiment and theory in Figure 2b, at the head of the gradient, is also not exact, and such small differences could affect the separation appreciably. However, these gradient separations involving shock formation are experimentally reproducible. Once the gradient system has been set up for a given separation, an experienced practitioner can rapidly arrive at the optimum conditions of gradient slope and delay.

In addition, accounting even approximately for nonlinear modulator adsorption can provide better results than the classic theories. El Fallah and Guiochon (1991) have applied our theory of nonlinear modulator adsorption to gradient elution on an octadecyl silica solvent, thereby improving agreement with experiment, even though the operating conditions were such that no shock layer was formed.

Other modes of chromatography

Nonlinear modulator adsorption is not restricted to reversed-phase chromatography, and we briefly discuss its implications for two other commonly used chromatographic techniques: ion-exchange and hydrophobic interaction chromatography.

In hydrophobic interaction chromatography, the salts that are used as modulators usually do not bind strongly to the stationary phase. For example, when ion-exchange adsorbers are used at very high salt levels as hydrophobic interaction supports, all the electrostatic binding sites on the stationary phase have already been occupied by salt counterions. This is why hydrophobic interactions, which are weaker than electrostatic interactions, become dominant at high salt levels in the first place. Thus the decreasing salt gradient used in hydrophobic interaction does not generally exhibit appreciable adsorption, and the classic theory of unresolved modulators is appropriate. Hydrophobic interaction chromatography is also carried out on uncharged sorbents. Again, in the absence of considerable hydrophobic patches on the sorbent, the salt is unable to bind appreciably and the classic theory is appropriate.

Ion-exchange chromatography involves the complication of electroneutrality, which requires that the stationary phase always be saturated. If the column is preequilibrated with the same salt (buffer) that is to be used as the modulator, the salt will already have saturated the stationary phase, regardless of its initial concentration. Thus the modulator, when it enters the column in the gradient, cannot adsorb, and will travel as an unretained component. If, on the other hand, the column is initially equilibrated with a buffer salt different from the modulator, the modulator, on entering the column will adsorb competitively. In this case, nonlinear modulator adsorption again becomes possible, and so consequently does gradient deformation. These possibilities deserve further study.

Conclusions

The experiments discussed in this article have experimentally verified that nonlinear modulator adsorption gives rise to gradient deformation. The isotherm formulism used worked well in capturing the multicomponent adsorption of acetonic acid and water, as seen by the agreement between theory and experimental results in Figure 2a. The single-component runs for Phe showed that unusual behavior occurs when an adsorbate peak is found in the vicinity of the strongly deformed gradient. Both the single-component Phe runs and the binary separation showed that adsorbate peaks can be compensated by an improved separation of Tep and Phe, exemplifying the possibility of simultaneous concentration and separation.

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Notation

- $k_g$: Langmuir isotherm parameter, ml/g
- $A^*$: specific surface area of adsorbent, m$^2$/g
- $b_g$: Langmuir isotherm parameter, M$^{-1}$
- $C_{eq}$: mobile phase concentration, mg/ml
- $C_{eq}^*$: mobile phase concentration of modulator (ACN), M
- $C_{eq}^*$: initial mobile phase concentration of modulator (ACN), M
- $d_p$: particle diameter, m
- $D_p$: molecular diffusivity, cm$^2$/s
- $L_0$: particle diffusivity, cm$^2$/s
- $E$: axial dispersion coefficient, cm$^2$/s
- $F$: association factor of water ($>2.6$), dimensionless
- $f$: volumetric flow rate, ml/min
- $L$: liquid penetration depth, cm
- $K_m$: distribution coefficient (Henry's law constant for adsorption), ml/g
- $K_m^*$: distribution coefficient (Henry's law constant for adsorption), ml/g
- $K_m^*$: distribution coefficient (Henry's law constant for adsorption), ml/g
- $M_A$: molecular weight of adsorbate, g/mol
- $n^*$: saturation concentration on the stationary phase, mmol/g
- $P$: Peclet number ($=v_s d_p/L$), dimensionless
- $q$: stationary phase concentration, mg/g (Phe and Tp) or mmol/g (ACN)
- $q^*$: equilibrium stationary phase concentration, mg/g (Phe and Tp) or mmol/g (ACN)
- $r$: radial distance into particle, cm
- $S$: liquid flow rate ($=K_m^* p_{d_2}/D_2$), dimensionless
- $T$: absolute temperature, K