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# PREPARATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY UNDER ISOCRATIC CONDITIONS

## III. THE CONSEQUENCES OF TWO ADJACENT BANDS HAVING UNEQUAL COLUMN CAPACITIES

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SUMMARY

Previous treatments of preparative high-performance liquid chromatography (HPLC) have generally assumed Langmuir isotherms, a fixed number of surface sites for the sample compounds being separated, and equal molecular weights for these compounds. This is consistent with assuming that values of the column saturation capacity ($w_s$) are equal for the various sample components. However, data summarized in this paper indicate that values of $w_s$ can vary widely for small molecules. The possibility of a similar variation of $w_s$ values for larger molecules such as peptides and proteins may be even more likely.

The practical consequences of such variations in solute column capacity can be striking in heavily overloaded HPLC separations. The separation can improve dramatically when the second-eluted component has a larger $w_s$ value than the first-eluted compound. Conversely, the separation can be much poorer when the $w_s$ values are reversed. When developing a procedure for preparative HPLC under conditions of heavy overloading (overlapping bands), it is therefore important to know the $w_s$ values of the compounds being separated.

## INTRODUCTION

Studies by Guiochon and co-workers[1–3] and Part I of this series[4] suggest that the rate of production of a purified product ($P_R$, g/h) in preparative high-performance liquid chromatography (HPLC) can be significantly increased by operating under conditions of heavy overloading (overlapping bands). Practical workers often find, however, that this is not always the case. Sometimes a separation changes in a predictable manner as sample size is increased, and sometimes not. In the course of experimental studies aimed at verifying previous theories[1–4] of heavily overloaded preparative HPLC, we also encountered some apparently puzzling results. This in turn

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led to a re-examination of the role of the column saturation capacity $w_s$ and its effect on preparative separations under overlapping-band conditions.

## THEORY[a]

The effect of solute $w_s$ values on preparative separations is illustrated in the isotherm plots in Fig. 1; isotherms are shown for two sample components which are adjacent in the chromatogram, and which it is desired to separate by preparative HPLC. Prior treatments of preparative HPLC have assumed equal $w_s$ values for each compound, as illustrated in Fig. 1A. Under conditions of heavy overloading, the sample concentration will be high enough for the stationary phase to be effectively saturated by the sample. In this case, we can write for a two-component sample $X$–$Y$

$$Y_m + X_s \rightleftharpoons Y_s + X_m \tag{1}$$

That is, sorption of a molecule of $Y$ from the mobile phase (m) into the stationary phase (s) results in the displacement of a molecule of $X$ from the stationary phase into the mobile phase. The resulting equilibrium can be described by

$$\theta_y C_x / \theta_x C_y = K$$

or

$$(\theta_y / C_y)/(\theta_x / C_x) = K \tag{2}$$

where $\theta_i$ refers to the mole fraction of compound $i$ in the stationary phase and $C_i$ refers to its concentration in the mobile phase. The ratio $\theta_i / C_i$ is proportional to the capacity factor $k'$ for compound $i$, so we can write

$$k_y / k_x = \alpha = K \tag{3}$$

where $\alpha$ is the usual separation factor for compounds $X$ and $Y$. Eqn. 3 indicates that $\alpha$ does not change as the sample size is varied.

The case of unequal $w_s$ values is illustrated in Fig. 1B and C. In Fig. 1B, the more strongly retained sample compound $Y$ has a lower value of $w_s$. Consequently, its isotherm levels off at a lower stationary-phase concentration relative to component $X$, so that the isotherms of $X$ and $Y$ cross. It would appear from Fig. 1B that the separation becomes poorer with increasing sample size, *i.e.*, the value of $\alpha$ should decreases from its initial value for a small sample (thus $\alpha = 1$ when the isotherms cross, and initially $\alpha > 1$). Previous work[4,5] has established that the preparative separation of two compounds is strongly dependent on their value of $\alpha$. It might therefore be expected that HPLC separation for larger sample weights will be poorer for the example in Fig. 1B then that in Fig. 1A. This has been demonstrated experimentally by Gonzalez *et al.*[6] and examined theoretically by Lin *et al.*[7].

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[a] A list of all symbols used in Parts I–III is included in ref. 4.

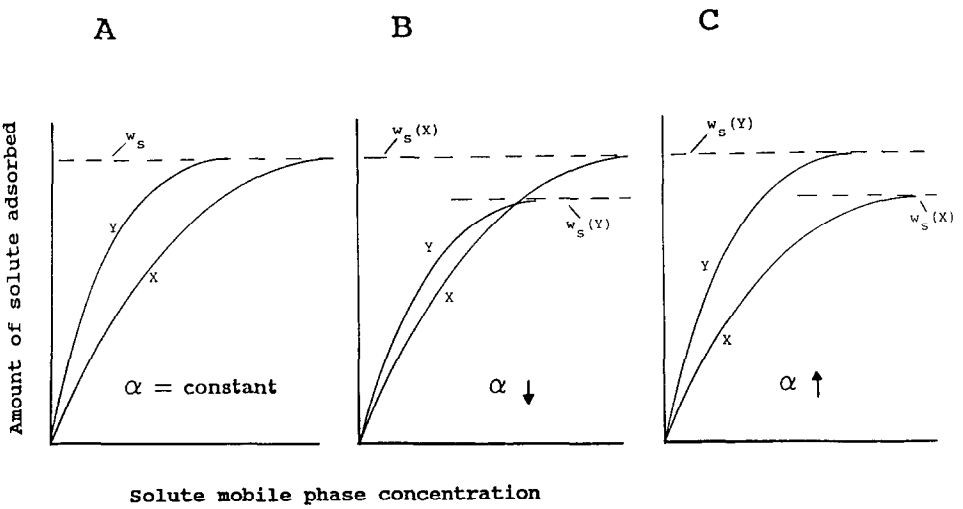


Fig. 1. Illustration of isotherms for two compounds having (A) equal column capacities $w_s$, (B) a larger value of $w_s$ for the earlier eluting compound and (C) a smaller value of $w_s$ for the earlier-eluting compound ($\alpha$ is the separation factor).

The case where the first-eluted compound has a lower $w_s$ value than the second is illustrated in Fig. 1C. In this instance values of $\alpha$ increase with increasing sample size, which should therefore result in a much better separation under conditions of heavy overloading.

The preceding discussion concerning Fig. 1 requires some qualification, as was pointed out by a referee of this paper. The argument proceeds as follows. For the case of competitive Langmuir isotherms (as assumed in Fig. 1), we can write for the two solutes $X$ and $Y$

$$\theta_x = w_x/w_{sx} = b_xC_x/(1+b_xC_x+b_yC_y) \tag{4}$$

and

$$\theta_y = w_y/w_{sy} = b_yC_y/(1+b_xC_x+b_yC_y) \tag{5}$$

where $w_x$ and $w_y$ are the weights of $X$ and $Y$ taken up by the stationary phase, $w_{sx}$ and $w_{sy}$ are the saturation capacities of the stationary phase for $X$ and $Y$ and $C_x$ and $C_y$ are the concentrations of $X$ and $Y$ in the mobile phase and $b_x$ and $b_y$ are constants for a particular Langmuir system.

The separation factor for any concentrations of $X$ or $Y$ is given by

$$\alpha = (w_y/C_y)/(w_x/C_x) \tag{6}$$

(ignoring any differences in the phase ratios for $X$ and $Y$). Combining eqns. 4–6 then yields

$$\alpha = (b_yw_{sy}/b_xw_{sx}) \tag{7}$$

Eqn. 7 predicts that $\alpha$ will remain constant for all concentrations of $X$ and $Y$, *i.e.*, it contradicts the conclusions above with respect to Fig. 1.

Which point of view is correct? In our opinion, both arguments have some validity. The derivation of eqn. 7 is based on the two component Langmuir isotherm (eqns. 4 and 5), which in turn assumes that there are an equal number of adsorption sites for either $X$ or $Y$ in the stationary phase, *i.e.*, if $w_s$ is expressed in terms of moles, $w_{sx} = w_{sy}$. However, as discussed below (see the following section and Fig. 2), different values of $w_s$ for two solutes may reflect different configurations of the adsorbed molecules, such that a larger number of molecules of one than the other is taken up at saturation. In this instance eqns. 4 and 5 are no longer applicable, and the conclusions regarding Fig. 1 should apply (see the further discussion in the Appendix).

Final answers to this question are not yet available. Our own opinion is that the logic of Fig. 1 will prove applicable more often than not for separations of practical interest. In these instances, solute molecules of similar size and structure will generally be involved, and changes in molecular configuration or conformation will usually be responsible for changes in $w_s$. In this situation, the number of solute molecules $n$ corresponding to stationary-phase saturation will be proportional to $w_s$, *i.e.*, when $w_s$ values are unequal for two solutes, values of $n$ will also be unequal.

*Variation in values of* $w_s$

We previously summarized $w_s$ values for a number of reversed-phase HPLC systems[8], based on our own work and that reported by Jacobson *et al.*[9]. These data plus additional results reported in this paper confirm that $w_s$ can vary widely for different compounds in the same reversed-phase system (same column, same or similar mobile phases). In this section we review some of the reasons for this variation in $w_s$ among different compounds.

When sample retention is governed by a displacement process (*i.e.*, the basis of the Langmuir isotherm), it is assumed that the stationary phase can be represented by a surface on which sample (and solvent) molecules are adsorbed. If a sample molecule requires some area $A_s$ on the surface in the adsorbed state, if the weight of the sample molecule is $M$ (daltons) and if the area of the stationary phase surface (total column) is $SA$, then it follows that

$$w_s = \text{constant} \cdot SA \cdot M/A_s \tag{4}$$

or

$$w_s/SA = \text{constant} \cdot M/A_s \tag{4a}$$

That is, the smaller is the area required by the adsorbing sample molecule, the larger is $w_s$. The area $A_s$ in turn depends on the configuration and conformation of the sample molecule, as illustrated in Fig. 2 for *n*-butylphenol. The variety of surface configurations shown here for a flat surface is further multiplied for reversed-phase packings, where the retained sample molecule is probably (to some extent) intercalated between the alkyl chains[10].

For flatwise adsorption (with all of the solute atoms close to the surface), it can be estimated[11] that $w_s/SA \approx 0.3$–$0.4$ mg/m$^2$. As summarized in ref. 8, actual values of

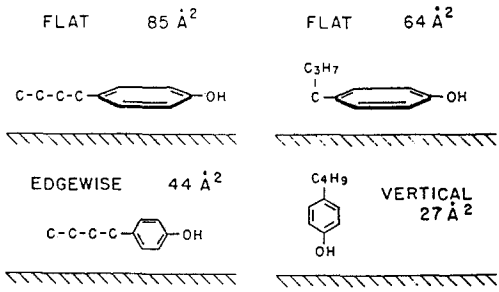


Fig. 2. Change in the area (27–85 Å) required by an adsorbing molecule ($A_s$) as a function of molecular configuration in the stationary phase surface.

$w_s/SA$ in reversed-phase systems can vary from 0.02 to 0.6 mg/m$^2$. For the sorption of the protein lysozyme on a wide-pore surface[12], it was noted that $w_s/SA = 0.7$ mg/m$^2$. Some possible causes of this considerable variation in sample $w_s$ values are summarized in Table I for both reversed-phase and ion-exchange systems.

## EXPERIMENTAL

### *LC system*

An HP 1090 M liquid chromatograph (Hewlett-Packard, Avondale, PA, U.S.A.), fitted with a preparative autoinjector (250 $\mu$l) was used for this work. A second system (HP 1090 L) was used for the analysis of the fractions collected from the preparative experiments.

### *Data system.*

A Nelson Analytical (Cupertino, CA, U.S.A.) data system, based on an HP 200 computer, was used for data collection and storage in addition to the system built into the HP 1090 M. The Nelson software was modified in-house to incorporate the calculation of capacity factors, efficiencies and skews of detected peaks.

TABLE I

POSSIBLE REASONS FOR THE VARIATION OF SAMPLE $w_s$ VALUES WITH MOLECULAR STRUCTURE AND SEPARATION CONDITIONS

| *Cause* | *Examples* |
|---|---|
| Changes in configuration of the adsorbed molecule | See Fig. 2 |
| Changes in conformation of the adsorbed molecule | Retention of native (globular) proteins which have a three-dimensional structure |
| Repulsion between adsorbed molecules | Sample molecules bearing a negative charge; see discussion of ref. 8 for ionized carboxylic acids where $w_s/SA \approx 0.05$ mg/m$^2$ |
| Limited number (low density) of sorption sites | Retention of basic or cationic of compounds on silanols[8] ($w_s/SA \approx 0.02$ mg/m$^2$); ion-exchange column with very low capacity |

*Chromatography*

All columns were constructed from stainless steel and were packed by a downward slurry technique. Chromatography of mixtures of phenol and benzyl alcohol and of *p*-cresol and 2-phenylethanol was performed using a 15 × 0.46 cm I.D. column packed with Zorbax ODS (5-$\mu$m particle size). Detector wavelengths of 240 and 290 nm were employed. Mobile phases were 30% aqueous methanol for the benzyl alcohol–phenol mixture and 35% aqueous methanol for the *p*-cresol–2-phenylethanol sample. Separations of caffeine and 7-$\beta$-hydroxypropyltheophylline were carried out on a 25 × 6.2 cm I.D. column packed with Zorbax Pro-10 $C_8$ process-grade material (10 $\mu$m), using methanol–0.1 *M* sodium dihydrogenphosphate (pH 4.6) (15:85) as mobile phase at a flow-rate of 2 ml/min. A detector wavelength of 300 nm was employed. Fractions (0.5 ml) from this separation were collected manually every 15 s and were subsequently analysed using a 15 × 4.6 cm I.D. column packed with Zorbax ODS using methanol–0.05 *M* sodium dihydrogenphosphate (pH 4.6) (50:50) as mobile phase at a flow-rate of 1 ml/min.

*Chemicals and mobile phases*

Methanol of HPLC grade and sodium dihydrogenphosphate of Baker Analyzed grade were obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.). Caffeine, benzyl alcohol, *p*-cresol, 2-phenylethanol and phenol were purchased from Aldrich (Milwaukee, WI, U.S.A.) and 7-$\beta$-hydroxypropyltheophylline from Sigma (St. Louis, MO, U.S.A.). All compounds were used as received.

*Determination of isotherms*

The various compounds studied were selected (in part) for their established Langmuir isotherm behaviour in reversed-phase systems. In this instance, the individual solute isotherms can be determined from a knowledge of the capacity factor $k_0$ (small sample) and the $w_s$ value for the solute and column of interest. We used the relationship for the Langmuir isotherm derived in ref. 3 (eqns. 2, 3 and 4). Rearrangement into a more familiar form yields

$$w_{sx} = (V_m k_0 C_m)/[1 + (V_m k_0/w_s)C_m] \quad (5)$$

Given a value of the column void volume $V_m$, the saturation capacity $w_s$ and the capacity factor $k_0$, the isotherm can be constructed by calculating values of the weight of solute in the stationary phase ($w_{xs}$) for values of its concentration in the mobile phase ($C_m$).

It is also of interest to note that the measurement of values of $w_s$ for a wide range in sample size allows verification that the solute follows Langmuir sorption. If the $w_s$ values so determined are all equal, then the solute can be assumed to follow the Langmuir isotherm. Here we have measured values of $w_s$ from chromatographic runs in which sample size is varied (see below).

## RESULTS AND DISCUSSION

*Overlapping-band separations*

On the basis of $w_s$ values for various solutes (see the following section), it was

possible to select samples (two closely eluting compounds) and conditions that match each of the isotherm possibilities in Fig. 1. Separations for each sample were then carried out as a function of sample size. In each instance it was possible to monitor the separate elution of each compound in the sample, either by collecting small fractions for HPLC analysis or by using dual-wavelength detection with subtraction of one compound's response from the total elution profile.

*Case of equal $w_s$ values.* An example of the separation to be expected (overlapping bands) for this case is shown in Fig. 3A for a computer simulation from the paper of Ghodbane and Guiochon[2]. Here, the separation of two components is modeled [$w/w_s = 0.02$, $N_0 = 5600$, $k_x = 5.75$ and $k_y$ 6.27 ($\alpha = 1.09$)]. A characteristic pattern is seen, with a more or less sharp boundary beginning to form between the two bands. Note the tailing of the first band ($X$) under band $Y$ (arrows), which limits the recovery of pure $Y$ in this separation.

For the experimental illustration of a similar situation, the separation of caffeine and 7-$\beta$-hydroxypropyltheophylline (HPT) was chosen. These compounds were selected because they (a) exhibit Langmuir isotherm behaviour[13,14] and (b) have very similar $w_s$ values. A 25 × 0.62 cm I.D. Zorbax Pro-10 $C_8$ (10 $\mu$m) column was used, with the chromatographic parameters shown in Table II. The selectivity ($\alpha = 1.14$) and plate number (average $N_0 = 5100$) were similar to those chosen for the simulation in Fig. 3A ($\alpha = 1.09$, $N_0 = 5600$). The capacity factors are about twice as large, caffeine (the first eluted peak) being eluted with $k_0 = 11.7$ (*vs.* $k_0 = 5.7$ in Fig. 3A).

The column exhibited a saturation capacity of 282 mg for caffeine and 286 mg for HPT (Table II); these values are equal within experimental error. A mixture of 10

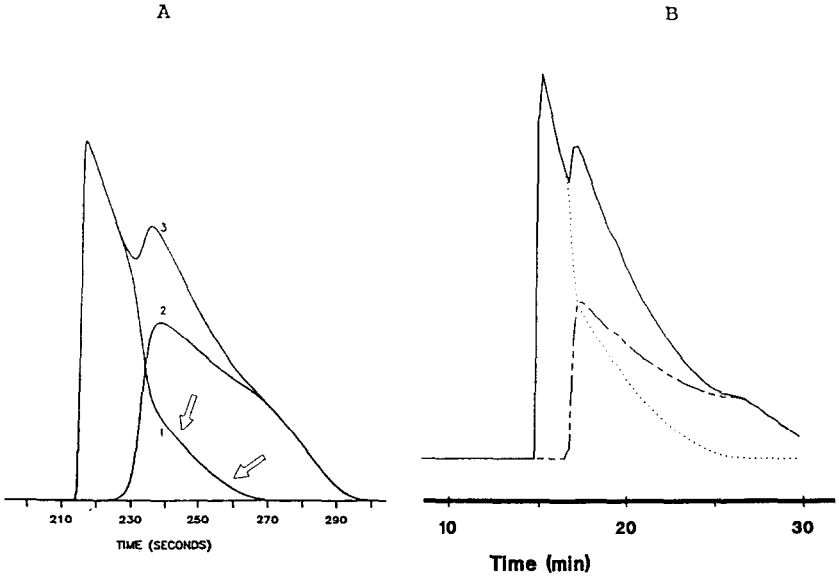


Fig. 3. Overlapping-band separations of two compounds having equal values of $w_s$. (A) computer simulation from ref. 2 (see text for details); (B) experimental separation of caffeine and hydroxypropyltheophylline (HPT) (see text and Table II for details).

mg each of the two components was then injected ($w/w_s = 0.070$[a]), and the resulting chromatogram is shown in Fig. 3B (solid curve). Sixty fractions (0.5 ml) were also collected from the beginning of the first peak through the overall peak envelope. Each fraction was analysed in duplicate by analytical HPLC using a 15 × 4.6 cm I.D. Zorbax ODS column with methanol–phosphate buffer (50:50) as the mobile phase, and the peak-area data were used to reconstruct the profiles of the two sample components (dashed and dotted curves in Fig. 3B).

While the separations of Fig. 3A and B are not identical, they are similar enough (values of $w/w_s$, $N_0$ and $\alpha$) to merit comparison. In general, the same features noted for the simulation in Fig. 3A are duplicated in Fig. 3B, *i.e.*, a sharp boundary between parts of the two bands and a pronounced tailing of the first band into the second. It will be of interest to compare these results with experimental separations (see below) where the $w_s$ values for the two sample components are different.

*Case of smaller $w_s$ value for first-eluting compound.* For this illustration, the compounds chosen were phenol ($k_0 = 4.03$, $w_s = 203$ mg) and benzyl alcohol ($k_0 = 4.52$, $w_s = 381$ mg/m$^2$); $\alpha = 1.12$ and the average value of $N_0 = 8400$ (data in Table II). Fig. 4 shows the isotherms for these two compounds (conditions in Table II), as determined from values of $w_s$ and $k_0$ (Langmuir isotherm assumed)[b]. Because the ratio of $w_s$ values (phenol/benzyl alcohol) is only 203/381 = 0.5, it can be expected that the resulting separation will be much better (for similar sample sizes) than the separations in Fig. 3. The choice of these two compounds was also influenced by their different UV spectra, allowing the approximate determination of each compound in their mixtures by bichromatic analysis.

The separation (conditions in Table II) was carried out on a 15 × 0.46 cm I.D. Zorbax ODS column with methanol–water (35:65) as the mobile phase and detection at 240 and 290 nm. On increasing the sample weight beyond that required for touching bands (0.5 mg of benzyl alcohol), progressive peak overlap was seen. Composite chromatograms are shown in Fig. 5A for samples containing 3 mg of phenol and 0.05–1.0 mg of benzyl alcohol. As the weight of benzyl alcohol is increased, the peak maximum of the phenol elutes at progressively lower retention time. The corresponding chromatograms of the phenol peak viewed alone (*i.e.*, at 290 nm) are shown in Fig. 5B. By comparison with Fig. 3, it is apparent that there is much less tailing of the initial (phenol) band into the following (benzyl alcohol) band[c]. Rather, there is a very sharp boundary between the two bands, which is the result of strong sample displacement (due to larger values of $\alpha$ for larger samples; see Fig. 1C).

Separations were also carried out with samples containing 3 mg of benzyl alcohol and various amounts of phenol. Fig. 6A shows the separation (240 nm) of a sample containing 1 mg of phenol and 3 mg of benzyl alcohol; Fig. 6B shows the corrected (bichromatic analysis), superimposed tracings for each band in the separation in Fig. 6A. Again, a very sharp boundary between the two bands is observed, with very little tailing of the phenol band into the benzyl alcohol peak.

Fig. 7A is the chromatogram for the similar separation of a sample containing

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[a] A larger value of $w/w_s$ was used than in the example in ref. 2 ($w/w_s = 0.05$) in order to compensate for the larger values of $\alpha$ and $k_0$.

[b] For sample sizes (individual compounds) of 0.1, 0.3, 0.5, 1.0 and 3.0 mg, values of $w_s$ were 223, 186, 195, 229 and 182 = 203 ± 18 mg (phenol) and 406, 381, 386 and 351 = 381 ± 25 mg (benzyl alcohol).

[c] The minor tailing of the phenol bands in Fig. 5B is mainly due to residual absorption from the benzyl alcohol at 290 nm.

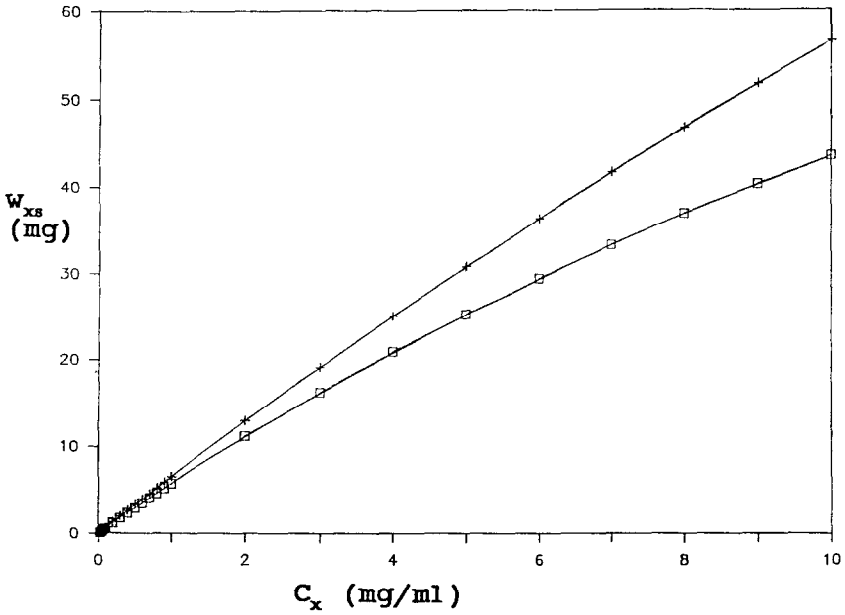


Fig. 4. Calculated isotherms for (□) phenol and (+) benzyl alcohol in the system of Table II.

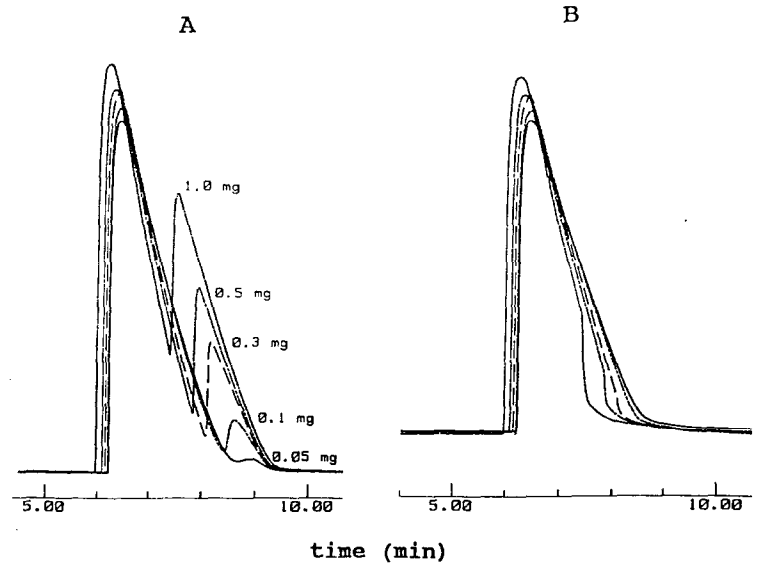


Fig. 5. Separation of mixtures of phenol and benzyl alcohol (system in Table II). Sample: mixture of 3 mg of phenol plus indicated weights of benzyl alcohol. Chromatograms determined at (A) 240 nm and (B) 290 nm (mainly phenol).

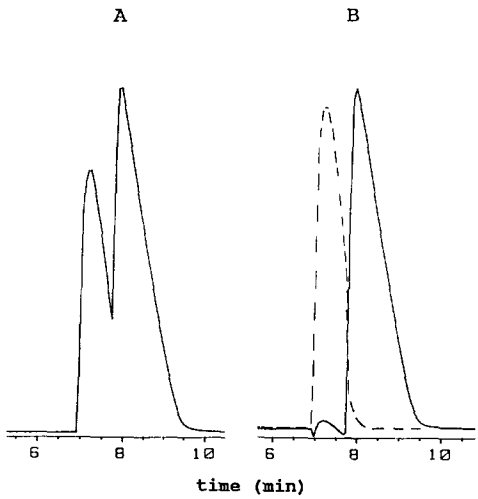


Fig. 6. Separation of mixture of phenol and benzyl alcohol (system in Table II). Sample: mixture of 1 mg of phenol plus 3 mg of benzyl alcohol. (A) Detection at 240 nm; (B) corrected chromatograms (bichromatic analysis) for each compound in the separation in (A).

0.05 mg of phenol and 3 mg of benzyl alcohol. Fig. 7B is the chromatogram of this mixture at 290 nm (with correction for benzyl alcohol absorbance), showing the phenol band. The phenol peak is one third as wide as an equal weight of phenol injected alone. Simply shaving the leading edge of the band in Fig. 7A would be sufficient to allow the collection of the remainder (benzyl alcohol) in $\geqslant 99\%$ purity and with $\geqslant 95\%$ recovery, even for this large sample.

The column efficiency for the runs in Figs. 6 and 7 ($N_0 = 8400$) is greater than

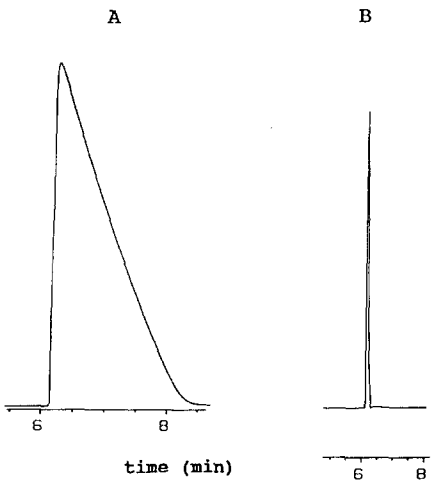


Fig. 7. Separation of mixture of phenol and benzyl alcohol (system in Table II). Sample: mixture of 0.05 mg of phenol plus 3 mg of benzyl alcohol. (A) Detection at 240 nm; (B) corrected chromatograms (bichromatic analysis) for phenol band in the separation in (A).

bthat for the runs in Fig. 3 ($N_0$ = 5600). To see if this affected the above comparisons of these runs, the separation in Fig. 6 was repeated with the flow-rate increased from 1 ml/min (Fig. 6) to 2.6 ml/min. The average value of $N_0$ was thereby decreased to 5800 plates, but no visible change in the chromatogram resulted; that is, any differences in these separations cannot be accounted for by differences in $N_0$.

*Case of larger $w_s$ value for first-eluting compound.* For this study, we chose as sample compounds 2-phenylethanol and *p*-cresol (data in Table II). The same column was used for this experiment as for the separation of phenol and benzyl alcohol (see above). The mobile phase was adjusted to give comparable selectivity ($\alpha$ = 1.12) and retention ($k_0$ = 5.7 and 6.4). Measurement of $w_s$ as a function of sample size indicated that both solutes exhibit Langmuir isotherm behaviour; $w_s$ is 393 mg for 2-phenylethanol and 218 mg for *p*-cresol. As 2-phenylethanol elutes first, the isotherms (Fig. 8) resemble those in Fig. 1B. The isotherms cross ($\alpha$ = 1.00) for intermediate sample sizes, and this should lead to much poorer separations than in the examples in Figs. 4, 6 and 7.

Chromatograms for the injection of samples containing 3 mg of 2-phenylethanol plus 0–1 mg of *p*-cresol are shown in Fig. 9A. Corresponding traces at 290 nm (for the *p*-cresol peak) are shown in Fig. 9B. The *p*-cresol peak is undistorted by the presence of 2-phenylethanol in the sample, but the bands are much wider than for injection of *p*-cresol alone (in similar concentrations). Hence the apparent $w_s$ value for *p*-cresol in the presence of 3 mg of 2-phenylethanol decreases by 70% ($w_s$ = 55 mg).

The 2-phenylethanol band in the presence of *p*-cresol was also studied. Correction of the chromatogram at 240 nm (bichromatic analysis at 240 and 290 nm) gave a residual chromatogram that can be ascribed to 2-phenylethanol alone. This is

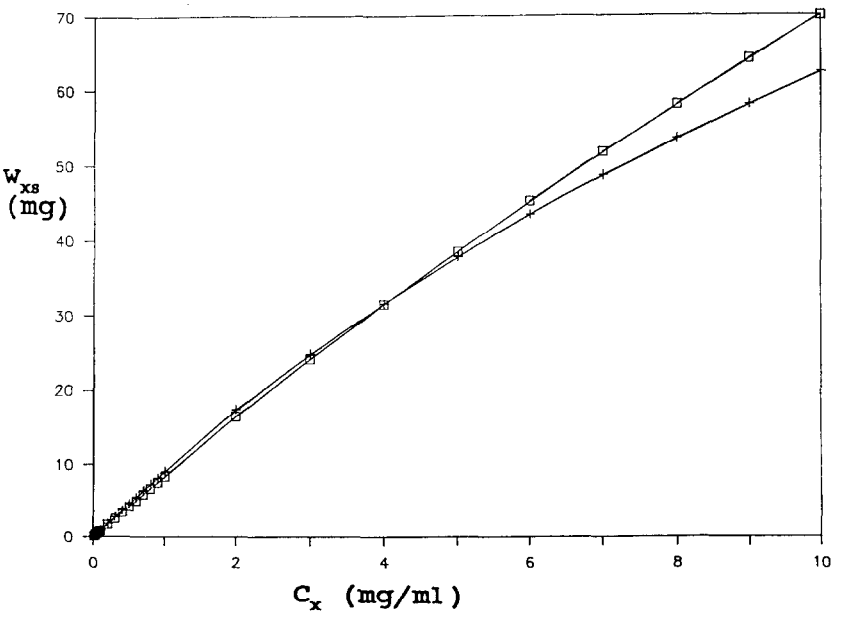


Fig. 8. Calculated isotherms for (□) 2-phenylethanol and (+) *p*-cresol in the system in Table II.

TABLE II
CHROMATOGRAPHIC CONDITIONS AND DATA FOR THE SEPARATION OF VARIOUS DAMPLES
See text and figures.

| *Conditions*[a] | *Solutes* | *Column* | *Mobile phase* | *k'* | *N* | $w_s$ *(mg)* |
|---|---|---|---|---|---|---|
| $w_s(X) = w_s(Y)$ | Caffeine | Zorbax | Methanol– | 11.7 | 4144 | 282 |
| | HPT | Pro-10 $C_8$ | 0.1 *M* sodium | 13.3 | 6148 | 286 |
| | | (25 × 0.62 cm I.D.) | phosphate (pH 4.6) (15:85) | | | |
| $w_s(X) < w_s(Y)$ | Phenol | Zorbax ODS | Methanol– | 4.03 | 8039 | 203 |
| | Benzyl alcohol | (15 × 0.46 cm I.D.) | water (30:70) | 4.52 | 8879 | 381 |
| $w_s(X) > w_s(Y)$ | 2-Phenylethanol | Zorbax ODS | Methanol– | 5.73 | 7100 | 393 |
| | *p*-Cresol | (15 × 0.46 cm I.D.) | water (35:65) | 6.42 | 7200 | 218 |

[a] $w_s(X) = w_s$ for solute $X$; $w_s(Y) = w_s$ for solute $Y$.

shown for the co-injection of 3 mg of 2-phenylethanol and 0.5 mg of *p*-cresol in Fig. 10 (solid curve). Comparison of this band with that for 3 mg of 2-phenylethanol injected alone (dashed curve in Fig. 10) shows that the 2-phenylethanol peak is not significantly affected by the presence of the *p*-cresol, in contrast to the development of a sharp boundary and truncated tail as in Fig. 3 and especially Fig. 5. Hence the separation of large samples containing these two compounds [where $w_s(X) > w_s(Y)$] results in no change in the width or retention of the first band, but a very rapid broadening of the second band. The result is extensive band overlap (and poor separation) for larger samples, as seen in Fig. 9 (compare equivalent separations of Figs. 5A and 9A).

*Values of $w_s/SA$ for different compounds*

The column capacity per square meter of stationary phase surface area ($w_s/SA$) for different compounds determines their $w_s$ values and thus markedly affects the separation (see the above discussion and examples in Fig. 1). It is therefore of interest

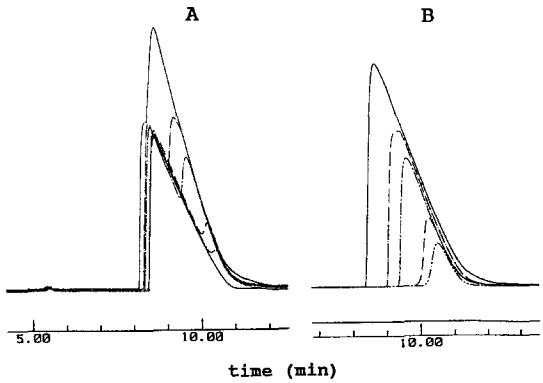


Fig. 9. Separation of mixtures of 2-phenylethanol and *p*-cresol (system in Table II). Sample: mixture of 3 mg of 2-phenylethanol plus 0.0, 0.05, 0.1, 0.3, 0.5 and 1.0 mg of *p*-cresol. Chromatograms determined at (A) 240 nm and (B) 290 nm (mainly *p*-cresol).

to compare values of $w_s/SA$ with the molecular structures of different compounds. This may allow us to anticipate how often actual samples will deviate from the "ideal" behavior modeled by ourselves[4] and others[2,3]. It might also eventually allow us to better control the overlapping-band separation of different samples. We summarized some data of this type in a previous paper[8]. There it was shown that ionized compounds will generally exhibit lower values of $w_s/SA$ because of either (a) ionic repulsion between sorbed molecules or (b) secondary retention on a small population of active sites (*e.g.*, ion exchange of protonated bases on silanols). Presumably such effects can be minimized (with a resulting increase in $w_s/SA$ and production rate) by changing the pH to avoid sample ionization, increasing the mobile-phase ionic strength, using columns with minimal silanol effects or adding competetive agent (*e.g.*, amines) to block active sites (silanols).

However, it also appears that neutral molecules can exhibit significant differences in values of $w_s/SA$, and it is this situation which we wish to examine further. For example, Gonzalez *et al.*[6] reported significant differences in values of $w_s$ for two non-ionic steroids of similar structure, with the result that crossing isotherms (as in Fig. 1B) and poor separation resulted. Table III gives values of $w_s/SA$ for various compounds in reversed-phase separations, as reported earlier or since determined by us.

Compounds 1 and 2 in Table III are dihydroxybenzenes and exhibit very low values of $w_s/SA$ of 0.03–0.09 mg/m$^2$; 3–5 are phenols with no or one methyl groups and their $w_s/SA$ values are higher, 0.21–0.33 mg/m$^2$ (excluding the out-of-line value of 0.47 for *p*-cresol); 6–9 are $C_1$–$C_4$ alkyl hydroxybenzoates with $w_s/SA$ = 0.38–0.46 mg/m$^2$; 10 and 11 are methylanilines with $w_s/SA$ = 0.5–0.6 mg/m$^2$; 12 and 13 are phenylalkanols with $w_s/SA$ = 0.53–0.56 mg/m$^2$; and 14–17 are xanthines substituted by various numbers of methyl or hydroxyalkyl groups with $w_s/SA$ = 0.25–0.32 mg/m$^2$ (averages of data in Table III).

The data of Table III are limited, but the following tentative conclusions seem warranted:

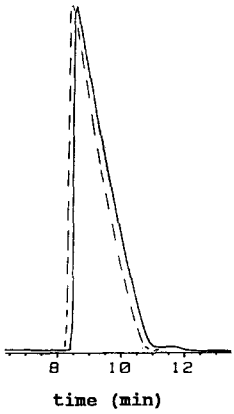


Fig. 10. Separation of a mixture of 3 mg of 2-phenylethanol plus 0.5 mg of *p*-cresol, with display of 2-phenylethanol band alone (bichromatic analysis). Solid line, 2-phenylethanol in mixture; dashed line, 2-phenylethanol injected alone.

TABLE III

EXPERIMENTAL VALUES OF COLUMN CAPACITY PER SQUARE METER ($w_s/SA$) FOR NON-IONIZED COMPOUNDS IN REVERSED-PHASE SEPARATIONS

| *No.* | *Compound* | *Ref.* | $w_s/SA$ |
|---|---|---|---|
| 1 | Hydroquinone | 9 | 0.03 |
| 2 | Resorcinol | 9 | 0.09 |
| 3 | Phenol | 9 | 0.21, 0.29[a] |
| 4 | *m*-Cresol | 9 | 0.33 |
| 5 | *p*-Cresol | 9 | 0.47, 0.25[a] |
| 6 | Methyl *p*-hydroxybenzoate | 14 | 0.46 |
| 7 | Ethyl *p*-hydroxybenzoate | 14 | 0.41 |
| 8 | Propyl *p*-hydroxybenzoate | 14 | 0.39 |
| 9 | Butyl *p*-hydroxybenzoate | 14 | 0.38 |
| 10 | *o*-Toluidine[b] | 9 | 0.46 |
| 11 | *p*-Toluidine[b] | 9 | 0.65 |
| 12 | Benzyl alcohol | 8 | 0.55, 0.56[a] |
| 13 | 2-Phenylethanol | | 0.53[a] |
| 14 | Theophylline | 14 | 0.27, 0.28[a] |
| 15 | Caffeine | 14 | 0.22, 0.28[a] |
| 16 | β-Hydroxyethyltheophylline | 14 | 0.33, 0.28[a] |
| 17 | 7-β-Hydroxypropyltheophylline | 8, 18 | 0.35, 0.28[a] |

[a] Data from this study.
[b] Unprotonated in this system.

(1) the degree of alkyl substitution in the parent compound has only a limited effect on $w_s/SA$; therefore homologs tend to have similar $w_s/SA$ values;

(2) substitution of a hydroxyl group on an aromatic ring lowers $w_s/SA$ markedly;

(3) substitution of a hydroxyalkyl or carboxyalkyl group on an aromatic ring does not have much effect on $w_s/SA$;

(4) values of $w_s/SA$ for neutral molecules can vary by an order of magnitude.

Other generalizations may be possible, but more data of this type are required before any real understanding of $w_s$ as a function of molecular structure can become possible.

It should be noted that different values of $w_s$ can arise in different ways. First, the sample molecules may cover the surface completely, but have different values of $M/A_s$ (eqn. 4a). Second, the apparent value of $w_s$ may change as a function of sample size (see Fig. 4 in ref. 8), reflecting non-Langmuir sorption over a wide range of sample size. Finally, the chromatographic process may be such that some compounds do not access all of the available surface during separation, as illustrated by the protein lysozyme (Table I in ref. 12).

## CONCLUSIONS

The choice of appropriate conditions (including sample size) for overlapping-band separations is more complex than suggested by previous workers[2,3] and in Part I[4], where equal $w_s$ values were assumed for the separation of two adjacent bands. It is now apparent that values of $w_s$ can vary widely with molecular structure, which suggests that unequal $w_s$ values may be more common than was previously assumed.

When $w_s$ for the first-eluting band is larger than that for the following band, sample-displacement effects are minimal, and there may be little improvement in production rate for sample sizes larger than for touching bands. When this situation is reversed ($w_s$ for the second band larger), sample displacement effects are more pronounced, larger samples can be injected and the potential production rate is much greater than normal.

These observations suggest that in the design of overlapping-band separations it is of critical importance to measure values of $w_s$ for the two (or more) compounds being separated. With this information, it should be possible to estimate better the optimum sample size (and production rate) for a given separation on the basis of a few exploratory runs. A knowledge of these $w_s$ values should also prove useful when it is known that band position can be changed by a change in the separation conditions. In the latter instance, conditions that position the large-$w_s$ compound after the other compound will be strongly preferred.

## APPENDIX

For the case where the saturation capacity $w_s$ of two solutes $X$ and $Y$ corresponds to a different number of molecules, Langmuir-type sorption implies the following process:

$$Y_m + nX_s \rightleftharpoons Y_s + nX_m \tag{A1}$$

for the displacement of an adsorbed molecule $X$ by an adsorbing molecule $Y$ (when the surface is covered by a monolayer of $X$ plus $Y$). Here, m and s refer, respectively, to a molecule of $X$ or $Y$ in the mobile or in the stationary phase. For the case under discussion, $n \neq 1$.

The equilibrium constant for the process of eqn. A1 can be written as

$$K = [Y]_s[X]_m^n/[Y]_m[X]_s^n \tag{A2}$$

It will be seen that $\alpha$ cannot remain constant for this case ($n \neq 1$) as the sample concentration is changed. Thus, $\alpha$ is given by

$$\alpha = ([Y]_s/[X]_s)/([Y]_m/[X]_m) \tag{A3}$$

Eqns. A2 and A3 then yield

$$\alpha = K\,([X]_s/[X]_m)^n \cdot ([X]_m/[X]_s)$$

$$\alpha = K\,([X]_s/[X]_m)^{n-1} \tag{A4}$$

As $[X]_s$ does not increase as fast as $[X]_m$ (*e.g.*, Fig. 1), $\alpha$ must decrease as $[X]_m$ increases.

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