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QUANTITATIVE COMPARISON BETWEEN THE EXPERIMENTAL BAND PROFILES OF BINARY MIXTURES IN OVERLOADED ELUTION CHROMATOGRAPHY AND THEIR PROFILES PREDICTED BY THE SEMI-IDEAL MODEL[a]

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SUMMARY

The experimental elution profiles of the components of a binary mixture were determined under conditions where the column is strongly overloaded and the two bands interfere markedly. These profiles were derived from the analysis of approximately 100 fractions collected during the elution of large samples of synthetic mixtures of two similar compounds having different compositions. The system investigated was the separation of 2-phenylethanol and 3-phenylpropanol on Vydac ODS silica eluted isocratically with methanol–water (50:50).

The results demonstrate the displacement and the "tag-along" effects previously predicted on the basis of theoretical investigations of the elution behavior of binary mixtures in non-linear chromatography. The displacement of the first band by the second takes place essentially when the concentration of the second eluted component is the larger. The tag-along effect of the second component is observed when the major component of the mixture is the first eluted.

Comparison between the experimental profiles and the profiles predicted by the semi-ideal model was performed using the competitive Langmuir isotherm model. For numerical calculations, the parameters of these isotherms were determined by measurement of the single-component isotherms of each of the two compounds by frontal analysis. The results of the comparison show that the competitive Langmuir isotherm gives only a fair approximation of the multi-component adsorption behavior of the two compounds studied. Competition between them for adsorption seems to be stronger than predicted by the competitive Langmuir model.

INTRODUCTION

Recent investigations on the theory of non-linear chromatography have led to the prediction of the profiles of incompletely separated bands, such as those which are often observed in preparative liquid chromatography[1,2]. These predictions are based on simple assumptions and straightforward derivations, which gives them high credibility[3]. Comparison between these predictions and experimental results must be performed systematically, because a detailed understanding of the processes involved in preparative chromatography can only come from a fruitful combination of sound theory and carefully planned and executed experiments. This understanding is required in order to improve the methods used in the design and operation of preparative chromatographs in laboratories and in plants.

Few experimental results are available at this stage that could permit a proper investigation of the important new phenomena in overloaded elution chromatography predicted by the theoretical models, the displacement and the "tag-along" effects[4]. Both effects result from the competition of the components of a mixture for the stationary phase due to adsorption, exchange, complexation or reaction. The displacement effect results from stronger interaction of the more retained compound with the stationary phase. The higher the concentration of the second eluted component and the larger the separation factor of the two components, the stronger is the displacement effect. The tag-along effect results from the crowding of the stationary phase by the molecules of the less retained component in the front part of the mixed band between the two interfering components, when they are not completely resolved. This crowding increases with increasing relative concentration of the first component in the sample and with decreasing value of the separation factor. Accordingly, the tag-along effect is most prominent when the relative concentration of the second component is lower than that of the first and their separation factor is close to unity.

The displacement effect is more common since displacement chromatography is a well known process and an established technique of separation. Moreover, this effect proceeds from the same phenomenon, but under slightly different conditions (there is no way an isotachic train can develop under elution conditions, as a plateau does not form). This effect has been reported experimentally, on a qualitative basis, by Newburger and co-workers[5,6] and Hodges[7], among others[8]. The tag-along effect has not yet been documented experimentally.

Comparison between the band profiles predicted by the theory of non-linear chromatography for a binary mixture and the experimental band profiles raises the difficult problem of determining the two individual profiles. Although some tests can be made under conditions when the elution bands are totally or nearly completely resolved, the most definitive experiments will obviously involve the investigation of bands which are incompletely separated. The use of the signal of conventional LC detectors is unsuitable. Although pairs of solutes can be found such that one of them absorbs at a UV wavelength where the other is transparent, there are several major drawbacks to this approach. First, this scheme permits the detection of only one component and not the other, which leaves half the problem unsolved. Second, the two compounds selected will necessarily have markedly different chemical structures. Their chromatographic separation is then a trivial problem. Moreover, few relevant

conclusions can be derived from the results regarding the competitive adsorption isotherms of chemically unrelated compounds. The important separation problems in both preparative and analytical chromatography are those which involve closely related compounds. Finally, the calibration of UV detectors under the conditions where they must be used is a difficult task, complicated by the need to avoid any response contribution due to the other component.

Accordingly, we have used a more direct approach. Bands are generated by injecting large samples of binary mixtures of various compositions, where a certain degree of band interference occurs. A large number of fractions are collected during the elution of these bands and they are analysed by liquid chromatography under conditions where total separation of these analytical samples occurs. From these quantitative analyses, the desired elution profiles of each component are calculated. The method is simple and powerful. This procedure is not very time consuming if an automatic fraction collector and an automatic sampling system are used, especially in overnight runs.

The second difficulty encountered in the systematic investigation of the validity of theoretical predictions is the prerequisite for a suitable set of equations to describe the equilibrium isotherms of the compounds used. The studies of phase equilibria made so far have focused on single-compound equilibria and they are of little interest in preparative chromatography. Of major concern, in contrast, is the competitive isotherm behavior of the two components of the mixtures considered. In practice, there is only one workable model available, the set of competitive Langmuir isotherms. The validity of this model has not been investigated systematically in order to determine its limitations. It has been shown that it cannot be used to model crossed isotherms[9,10]. The Langmuir model assumes ideal behavior of both the mobile phase and the adsorbed layer, whereas we know that molecular interactions are pervasive in condensed phases. Deviations between the predictions of the semi-ideal model and the experimental results are important to determine, as they will give important clues regarding the extent of the deviations between the actual adsorption behavior of the compounds investigated and the predictions of the competitive Langmuir model.

This paper presents the first results obtained in a long series of experiments and reports on the first experimental demonstration of the tag-along effect. Additionally, it reports on the degree of agreement observed between the theoretical predictions and the experimental profiles derived from the experiment. This leads to important conclusions regarding the validity of the competitive Langmuir isotherm model.

THEORY

Band profiles in this paper are predicted by numerically solving the system of partial differential equations that describes the band migration in liquid chromatography[2]. This system of equations states the conservation of mass for each component of the mixture in a column slice. For a binary mixture, assuming plug flow and equilibrium for both components between the mobile and the stationary phases (ideal model) at all points along the column, the mass balance equations are written as follows for each component i in the mixture:

$$F\mathrm{d}Q_i/\mathrm{d}t + \mathrm{d}C_i/\mathrm{d}t + u_0\mathrm{d}C_i/\mathrm{d}z = 0 \tag{1}$$

A solution of this system of equations requires a relationship between the Q_i and the C_i values (*i.e.*, the composition of the stationary phase in equilibrium with the mobile phase). One of the simplest relationships that describes this equilibrium is the Langmuir competitive equilibrium isotherm:

$$Q_i = a_i C_i / (1 + \Sigma b_i C_i) \tag{2}$$

The parameters of these two equations are obtained by determining successively the single-component isotherms of each of the pure two components. This very simple procedure and the functional simplicity of eqn. 2 explain the great interest for the competitive Langmuir isotherm in practical applications of the theory of non-linear chromatography.

Given the concentration–time functionality at the entrance of the column (injection profile) and the values for the various parameters, the system of eqns. 1 and 2 can be solved numerically[2,3]. The band-broadening effects of the axial dispersion and the deviation of the system from thermodynamic equilibrium, which are the basic reasons for the finite column efficiency, can be accounted for by employing a finite difference algorithm for the calculations. The column is divided into a linear grid, *nh*, and the concentrations of the two compounds are calculated at each summit of the grid, at increasing times, *lτ*. By using carefully chosen space (*h*) and time (τ) increments[11,12], we can obtain a solution where the column height equivalent to a theoretical plate (HETP) is based on the average variance of the two components eluting under linear conditions. Thus, the finite nature of the column efficiency is taken into account numerically[13].

EXPERIMENTAL

Equipment

The modular liquid chromatograph used for the preparative separations was assembled from two Waters Assoc. (Milford, MA, U.S.A.) Model 510 pumps, a Valco (Houston, TX, U.S.A.) electrically actuated six-port valve fitted with a 100-μl sample loop, a Kratos (Ramsey, NJ, U.S.A.) Spectroflow 757 variable-wavelength detector operated at 272 nm and a Gilson (Middleton, WI, U.S.A.) Model 232/401 automatic sample processor and injector with a Model 401 diluter operated in the fraction collection mode. The pumps were controlled and the detector signal was monitored via a Waters Assoc. system interface module using the Waters Assoc. Maxima 860 Dynamic Solutions (Ventura, CA, U.S.A.) software installed on an NEC APCIV Powermate 2 computer. The collected fractions were then diluted using the Model 232/401 sample processor in the dilution mode.

The modular chromatograph used for the analysis of the collected fractions was assembled by connecting the Model 510 pump outlet directly into the Model 232/401 sample processor in the autosampler operation mode, fitted with a 20-μl loop. The column effluent was monitored at 254 nm with the Spectroflow 757 detector. Quantitative determinations of the unknown concentrations of the two components in each of the collected fractions was accomplished using the Maxima 860 software.

Columns and chemicals

The 25 cm × 4.5 mm I.D. preparative column and the 5 cm × 4.5 mm I.D. analytical column were packed in our laboratory at 9000 p.s.i. with 10-μm Vydac ODS stationary phase (The Separation Group, Hesperia, CA, U.S.A.), generously donated to us.

3-Phenylethanol and 2-phenylpropanol were purchased from Fluka (Ronkonkoma, NY, U.S.A.) and used without further purification. High-performance liquid chromatographic grade methanol and water (Burdick and Jackson, Baxter Chemical, Muskegon, MI, U.S.A.) were used.

Procedure

The experiments were carried out by first injecting 100 μl of the selected mixture at a concentration at which the preparative separation is incomplete. During the elution of the partially separated mixture, fractions are collected on a time basis, at a rate of one vial every 3 s (*i.e.*, the fractions collected were *ca.* 50 μl for a mobile phase flow-rate of 1 ml/min). Then, a dilution was performed on each fraction by adding 300 μl of solvent to each vial at a rate of *ca.* 3 μl/s. Finally, the automatic sampler was employed to aspirate 100 μl of the sample at a rate of 6 μl/s, dispense these 100-μl aliquots into the 20-μl loop at the same rate and then inject the sample. After 5 min the run was complete. The apparatus rinsed the syringe needle with solvent and the whole process was repeated until the contents of each vial had been analyzed.

On switching the sampling valve from load to inject, the sampling system sent an electric signal to the interface box to initiate data acquisition. Chromatograms were obtained automatically and sequentially. The detector signal was acquired by the software, which opens a file for each fraction analyzed, acquires data for 5 min at a rate of 2 points/s, closes and stores the file and repeats this process for each sample. The peak area of the two components was obtained by running an analysis program on each of the stored files. The concentration of each component in each fraction was calculated from a linear calibration graph previously determined for the two compounds and adjusted according to the dilution factor.

All experiments were conducted at ambient temperature. The preparative column was operated at 1 ml/min with methanol–water (50:50) as the mobile phase and with detection at 272 nm. The analytical column was operated at 1 ml/min with methanol–water (35:65) as the mobile phase and with detection at 254 nm.

Detector calibration

This is a most delicate problem, as the UV detector gives an optical absorbance profile of the eluent, whereas we are interested in the concentration profile. As the concentration of the solutes in the eluent is high when the column is overloaded, the detector response is not linear and thus the calibration is more difficult.

Depending on whether we are dealing with the single-component elution profiles or with those of binary mixtures, the problem is different. For the single-component band profiles, the use of a non-linear calibration graph is possible. For binary mixtures, it is not possible and fractions have to be collected and analyzed. Then a conventional linear calibration graph is employed in quantitation.

Single component. Conversion of the single-component overloaded chromatograms (*i.e.*, the elution profiles supplied by the detector response) into concentration

profiles of each of the corresponding compounds (2-phenylethanol and 3-phenyl-propanol) was accomplished by using a non-linear concentration *versus* response calibration graph. This graph was derived from experimental absorbances measured for standard solutions having concentrations between 0 and 80 mg/ml of each compound studied. However, the curve fitting was done only over the concentration range required for prediction of the band profiles. The concentration *versus* absorbance data were fitted to a polynomial. In fitting the calibration data for 2-phenylethanol, oscillations were found when using a third- or a fifth-degree polynomial. These oscillations lead to artifactual humps on the concentration profiles. This problem was avoided by eliminating the odd power terms greater than 1 (ref. 14). The 3-phenylpropanol response calibration was fitted to a fifth-degree polynomial.

    In practice, the experimental data for the calibration graphs were derived from the detector responses obtained for each concentration plateau recorded in frontal analysis during the determination of the equilibrium isotherm for each of the compounds studied[15].

    Fig. 1 shows the non-linear calibration graph at 272 nm of 2-phenylethanol and 3-phenylpropanol used for quantitation of the single-component band profiles. Note that 3-phenylpropanol absorbs more strongly than 2-phenylethanol.

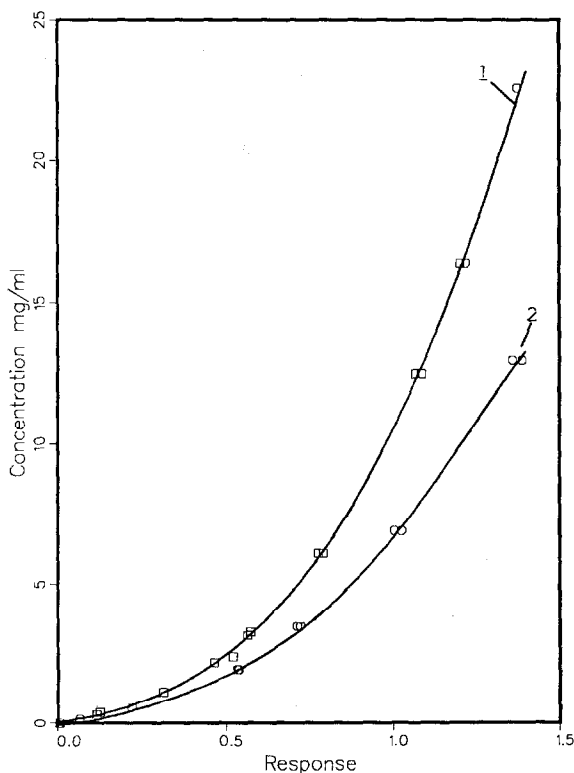   *Binary mixtures.* The concentration of each component in each fraction was

Fig. 1. Calibration graphs of the response (absorbance) of a UV spectrophotometric detector for (1, □) 2-phenylethanol and (2, ○) 3-phenylpropanol at 272 nm. Experimental points and polynomial curve fit.

determined by using standard analytical techniques. The fractions are small and dilute enough that their analysis can be carried out under linear conditions. Twelve standards were prepared and samples were injected in the same way as the fraction aliquots. The peak areas were calculated from the detector responses monitored at 254 nm, where the absorbance is much stronger than at 272 nm. A linear calibration graph of concentration *versus* peak area was determined by a least-squares fit of the data to the equation of a straight line for each of the two components. The standard deviation was 0.99991 and 0.999992 for the 2-phenylethanol and 3-phenylpropanol fits, respectively.

RESULTS AND DISCUSSION

The retention times at infinite dilution for 2-phenylethanol and 3-phenyl-propanol were measured on the preparative column by injection of several samples at decreasing concentrations. This procedure insures that the retention times obtained correspond to the slope of the tangent to the isotherm at the origin of coordinates. The dead time was measured from the solvent peak. The dead time was 3 min, which corresponded to a mobile phase volume of 3.0 ml and a column porosity of 0.755. In the methanol–water (50:50) mobile phase, the capacity factors of the two compounds were 0.68 and 1.25, respectively. The separation factor was 1.84.

The single-component isotherms of the two compounds were measured by frontal analysis[16,17]. The parameters were obtained by a least-squares fit of the data on the linearized equation ($1/Q_i = 1/a_iC_i + a_i/b_i$) or by a non-linear least-squares fit. The values obtained were a_E = 1.9239 ml/ml, b_E = 1.8092 ml/mmole, a_p = 3.554 ml/ml and b_p = 3.479 ml/mmole, respectively (the subscripts E and P represent 2-phenylethanol and 3-phenylpropanol, respectively). The values of the phase ratio derived from the porosity (3.09) and from the ratio of the isotherm slope to the column capacity factor (2.84) were in good agreement.

The total column saturation capacity corresponding to monolayer coverage is 140 mg for the first and 150 mg for the second component. The saturation capacities differ by 7% (whereas the total number of carbon and oxygen atoms in their molecules differ by 10%), which suggests that Langmuirian behavior for the competitive isotherms is a possibility. However, we know that the Langmuir competitive isotherms are consistent with thermodynamics only if the column saturation capacity is the same for the two compounds (otherwise they do not satisfy the Gibbs–Duhem equation). Further, Cox *et al.*[18] have shown that the profiles of overloaded interfering bands depend on the ratio of the column saturation capacities of the two components.

For the calculation of the amount loaded onto the column in terms of the percentage of the column saturation capacity for monolayer coverage,

$$\text{Column saturation capacity} = 1/2\Sigma a_i V_{sp}/b_i \qquad (3)$$

an arbitrary average value of 145 mg was used in all the calculations. This equation gives an approximate value of the degree of column overloading.

Two experimental single-component overloaded elution chromatograms were measured by injecting successively amounts corresponding to 5% of the column saturation capacity for 2-phenylethanol and 3-phenylpropanol. The detector response profiles were converted into concentration profiles by using a polynomial fit to
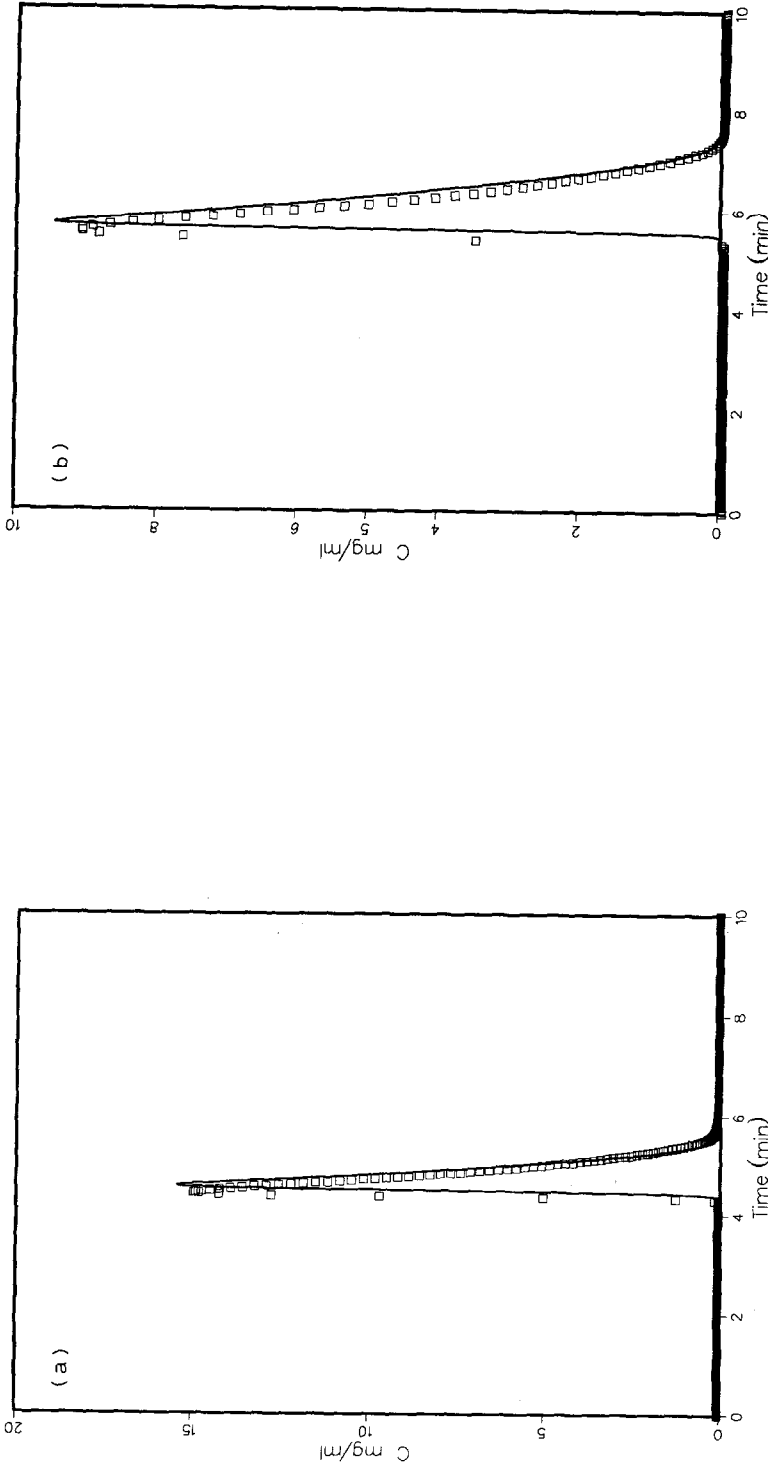
Fig. 2. Comparison of single-component overloaded elution profiles with the prediction of the semi-ideal model of chromatography using the single-component Langmuir equilibrium model. Symbols: concentration of each collected fraction. Solid line: profile calculated with the semi-ideal model program using the isotherm parameters determined by frontal analysis. Column, 25 cm long, packed with 10-μm Vydac ODS silica; mobile phase, methanol–water (50:50); flow-rate, 1 ml/min; sample sizer, 5% of the column saturation capacity. (a) 2-Phenylethanol (7.1 mg); (b) 3-phenylpropanol (7.1 mg).

a non-linear calibration graph as described above. The symbols in Fig. 2a and b show the concentration–time profiles for 2-phenylethanol and 3-phenylpropanol, respectively, determined experimentally. The solid line represents the theoretical prediction based on the fit of the single-component adsorption isotherm data to the single-component Langmuir isotherm and calculated using the program for the single-compound semi-ideal model[3]. The excellent agreement suggests that the Langmuir expression is a good model for the equilibrium data. Additionally, the validity of the assumptions of the semi-ideal model is confirmed[1,3]. This model can give a very accurate quantitative prediction of the band profiles provided accurate isotherms are determined, which classical frontal analysis permits[19].

For binary mixtures, the individual band profiles were determined by analysis of collected fractions. For the purpose of evaluation of this technique, a comparison was made with the profiles obtained for 2-phenylethanol by direct calibration of the detector. The results (Fig. 3) are in excellent agreement and demonstrate that the fraction collection system contributes very little to band broadening.

A comparison was made between experimental and theoretical profiles for 1:3 and 3:1 mixtures of 2-phenylethanol and 3-phenylpropanol. The theoretical profiles were calculated using the program for the two-component semi-ideal model[2]. The competitive Langmuir isotherms were derived from the parameters of the single-component Langmuir isotherms measured by frontal analysis (see Theory section). The experimental band profiles for the two-component mixtures were measured using the technique described above.

Fig. 4 shows the comparison between theory and experiment for the 1:3 mixture. The sample size used corresponds to 5% of the column saturation capacity for
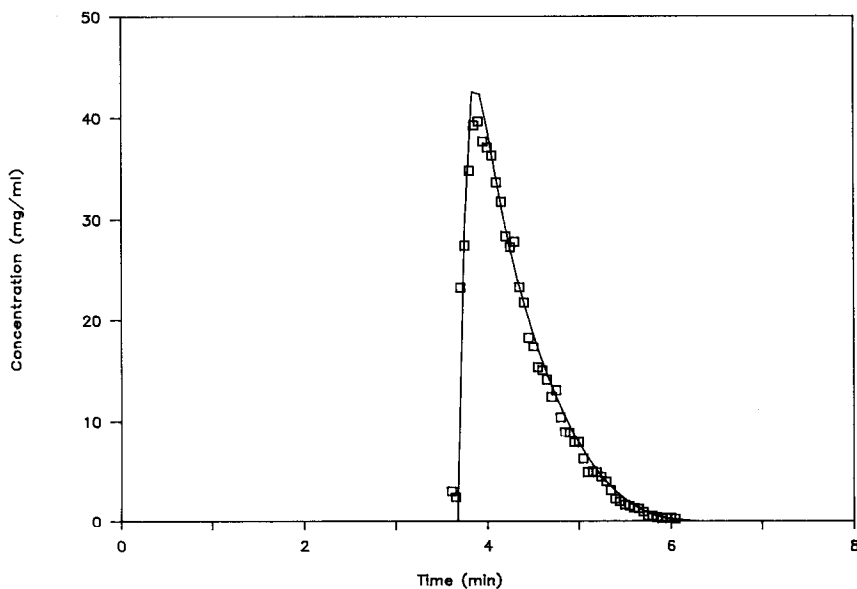

Fig. 3. Evaluation of the fraction collector. Solid line: concentration profile derived from the detector signal and a calibration graph. Symbols: composition of the collected fractions. Experimental conditions as in Fig. 2a, except amount injected (30 mg).
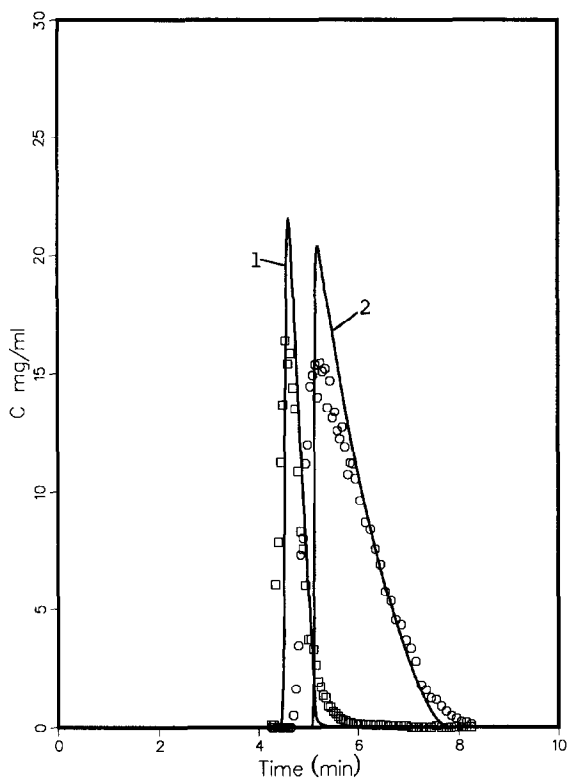
Fig. 4. Comparison of the experimental and theoretical band profiles of the two components for a 1:3 mixture of (1, □) 2-phenylethanol and (2, ○) 3-phenylpropanol using a competitive Langmuir adsorption isotherm (eqn. 2). Column as in Fig. 2. Sample sizes: 7.6 mg of 2-phenylethanol and 21.1 mg of 3-phenylpropanol. Symbols: experimental results. Solid lines: prediction of the semi-ideal model of chromatography using the competitive Langmuir isotherm (eqn. 2) with the coefficients derived from the single-component adsorption isotherms determined by frontal analysis.

component 1 and 15% of that for component 2. This is a significant degree of overloading, but the relative retention of the two components is high so partial resolution of the band is expected. The experimental profile shown includes the effect of extra-column and post-detector dead volume whereas the theoretical profile assumes no dispersion as the effluent elutes through these volumes. The experimental profiles demonstrate the displacement effect encountered in the elution of high concentration bands of a binary mixture.

The agreement observed in Fig. 4 between experimental results and theory is, however, of an approximate nature, within 10%. The rear boundary of the second component coincides with the predicted boundary, which is an important test of the applicability of the semi-ideal model. The rear boundary of the second component band profile should be identical with the band profile of the same amount of the second component when it is pure, a profile which is predicted by the single-component isotherm data. It appears that there is slightly more dispersion at the very end of the tail than predicted, probably the result of some additional and unavoidable diffusion in the tubing of the fraction collector.
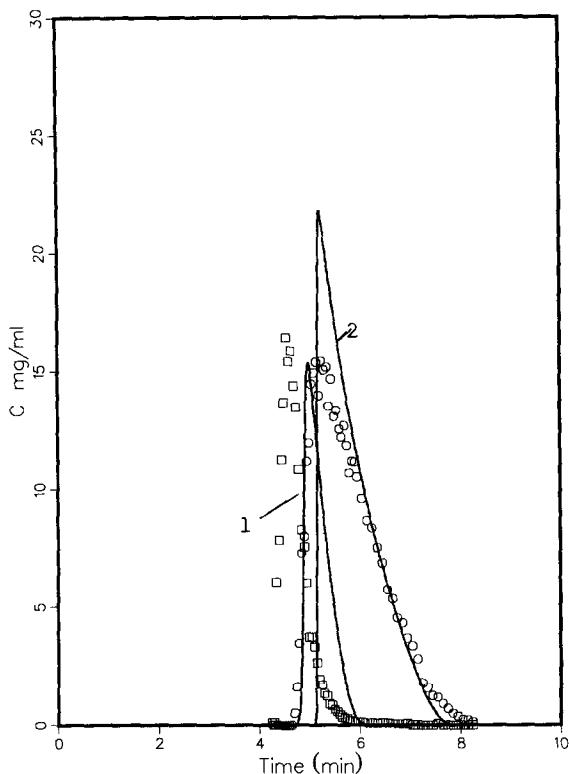
Fig. 5. Comparison of the experimental and theoretical band profiles calculated for the elution of the 1:3 mixture in Fig. 3, using a non-interacting (single-component) Langmuir isotherm model for each compound (solid lines). (1, □) 2-Phenylethanol; (2, ○) 3-phenylpropanol.

Fig. 5 shows a comparison of the experimental data for the mixture and the band profiles predicted as if the two components did not interact. The solid line represents the band profile predicted for two components having as their competitive isotherm their respective single-component isotherms (or, alternately, the profiles obtained when each component is injected alone). The symbols represent the experimental data which were also shown in Fig. 4. The presence of a displacement effect of the second component on the first is clearly seen in the experimental results, because the first component elutes faster in the experimental mixture than if the two bands were not interacting. The band obtained for the first component is taller and narrower than it would be if it were injected alone. In conclusion, Fig. 5 demonstrates that a non-interacting isotherm model (i.e., two single-component isotherms) gives a poor representation of the chromatographic behavior of the two components of interfering bands. Together, Figs. 4 and 5 show the existence and potential importance of the displacement effect and the necessity for modelling accurately the competitive adsorption behavior in the chromatography of binary mixtures at high concentration.

Fig. 6 illustrates a comparison between theory and experiment for a 3:1 mixture for the same compounds. The experimental profiles are shown by the symbols and the predicted profiles are the solid lines. Here the major component of the mixture is eluted
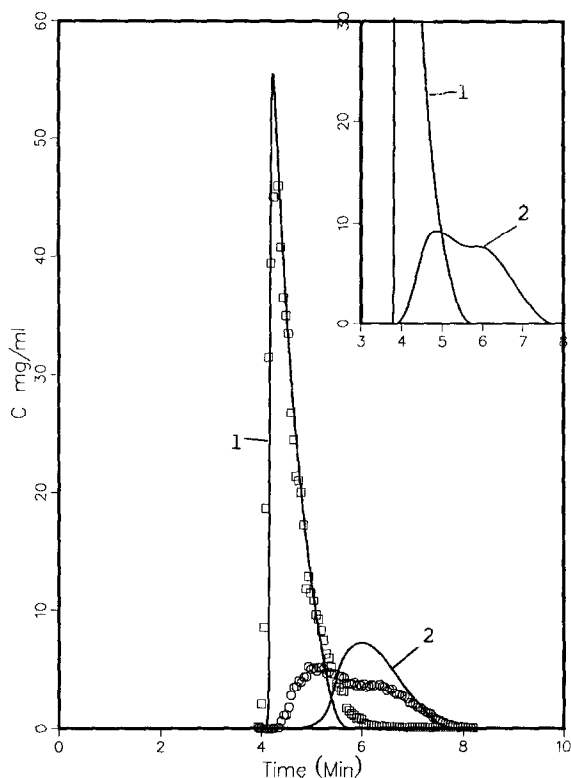
Fig. 6. Comparison of the experimental and theoretical band profiles of the two compounds for a 3:1 mixture of (1, □) 2-phenylethanol and (2, ○) 3-phenylpropanol, using a competitive Langmuir adsorption isotherm (eqn. 2). Main figure: same conditions as in Fig. 4, except sample composition (3:1) and size (30.1 mg of 2-phenylethanol and 10.2 mg of 3-phenylpropanol). Symbols: composition of the collected fractions. Solid lines: prediction of the semi-ideal model of chromatography using the competitive Langmuir isotherm (eqn. 2) with the coefficients derived from the single-component adsorption isotherms determined by frontal analysis. Inset: theoretical band profile calculated as for the main figure, but for twice the sample size.

first, not second. The column is loaded to 21% and 7% of the column saturation capacity for the first and second components, respectively. The tag-along effect predicted on the basis of computational results[2,4] is demonstrated for the first time. As observed previously[4], this effect is a consequence of the first component being in large excess in the front of the zone where the two components coexist and thus covering a large fraction of the adsorption sites. As there is near equilibrium between the mobile and the stationary phases, adsorption of the second component follows according to eqn. 2. The second component must move faster than it would if it were alone, because fewer sites are available for adsorption of its molecules, and this gives rise to the profiles shown in Fig. 6. Experimentally, the bands overlap to a greater extent than predicted, which means that the exclusion of 2-phenylethanol molecules from the surface by 3-phenylpropanol molecules is not as strong as predicted by the competitive Langmuir isotherm.

The inset in Fig. 6 shows the calculated elution profiles of the two components of the same 3:1 mixture at twice the loading factor and at the same composition. This
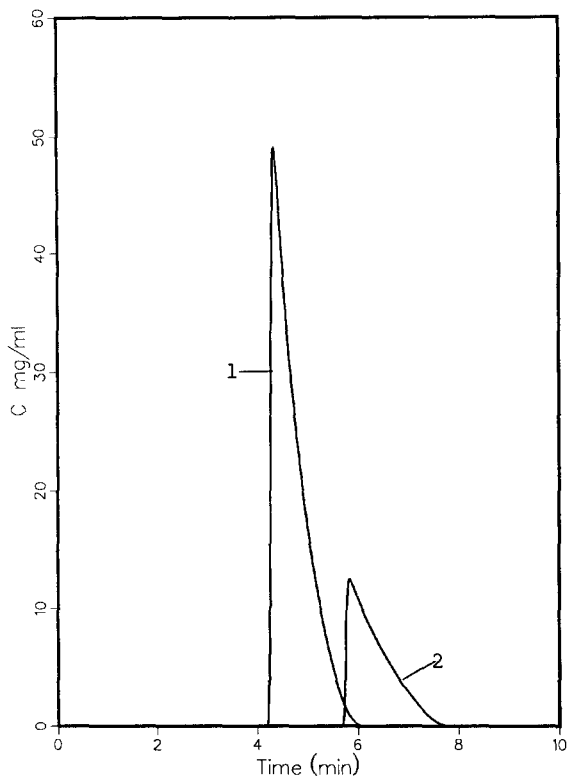
Fig. 7. Theoretical band profile calculated for the 3:1 mixture at the same loading as in Fig. 6, but using a non-interacting (single-component) Langmuir isotherm model (same as for Fig. 5).

permits a qualitative comparison of the second component profile with the experimental profile in the main part of Fig. 6. The inset shows that the semi-ideal model of chromatography can predict the strange shape of the elution profile of the later eluting component. This comparison suggests that the difficulty in predicting the interference profile observed experimentally is in the proper representation of the competitive adsorption isotherm and not in the assumptions made in the semi-ideal model.

The band profiles of the two components, predicted by the semi-ideal model in the assumption that they are not competing for access to the stationary phase, are shown in Fig. 7. Comparison of the band profiles in the interacting and non-interacting cases shows that there is no significant difference in the profiles predicted for the first component. The experimental profile is very slightly wider than the predicted profiles. In contrast, large deviations are predicted and seen for the front part of the profile of the second component. The profile front is no longer a sharp boundary; it is very flat and the plateau predicted by the theory of non-linear chromatography[1] can be observed in Fig. 6. The rear boundaries of the profiles predicted with and without interaction and of the profile determined experimentally for the second component coincide. Experiments are in progress[19] to determine whether the slope of this front is related to the column efficiency, as predicted by the semi-ideal model[1].

CONCLUSION

    While the agreement between the experimental results and the calculations derived from the semi-ideal model of non-linear chromatography show excellent agreement for the single-component elution profiles under all the experimental conditions investigated, substantial differences are observed for the elution profiles of binary mixtures. The reason for this disagreement stems from the fact that, although the single-component Langmuir isotherm offers most often a very good description of the adsorption equilibrium of a pure compound in a chromatographic system, the competitive Langmuir isotherms offer only a first-order approximation to the adsorption behavior of the components of a binary mixture. Owing to the lack of a suitable set of equations describing the competitive equilibrium of the components of a mixture in a biphasic system, it is not yet possible to predict more accurately the individual component profiles of the interfering zones in overloaded elution chromatography of binary mixtures.

    In the system investigated here, which seems fairly simple with its two homologous components, we note that the exclusion of the lower molecular weight component by the heavier one is less than predicted on the basis of the Langmuir model. This lower extent of competition results in the second component travelling faster than predicted in the presence of the first component. A more detailed understanding of the competitive adsorption behavior must wait until the competitive isotherms are determined experimentally[20].

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