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Band splitting in overloaded isocratic elution chromatography I. The experimental evidence

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Abstract

A series of samples of increasing volume (from 0.001 to 4.0 cm^3), containing the same constant concentration (40 g/l) of two simple compounds, ethylbenzoate and 4-*tert*.-butylphenol were injected on a Kromasil-C_{18} column with methanol–water (62:38, v/v) as the mobile phase. Complex band profiles were observed when the volume of the sample became large enough and strong band interference took place. The analysis of the fractions collected during the elution of the mixed band demonstrates that, for samples larger than 2 cm^3, the band of 4-*tert*.-butylphenol is split into two separate bands, one eluted before and the other eluted after the band of ethylbenzoate. Such a phenomenon has never been observed yet in RPLC, under isocratic elution conditions.

Keywords: Band splitting; Band profile; Fraction collection; Phenol

1. Introduction

Concentration profiles at the outlet of chromatographic columns depend essentially on the nature of the changes made at the column inlet and on the thermodynamics of phase equilibrium involved, particularly at high concentrations [1–3]. The influence of the mass transfer kinetics is less important unless this kinetics is slow and it can be accounted for. The agreement between experimental band profiles and profiles calculated from the set of competitive isotherms is so satisfactory that computer-assisted optimization of newly developed method is widely used [1,4]. Because competitive isotherms are difficult to measure, as often as possible, they are derived from the single-component isotherms of the compounds involved [1,5–8]. Accordingly, various competitive isotherm models have been proposed to account for the behavior of binary mixtures. For most compounds whose single-component isotherms are convex upward, competitive isotherm models like the competitive Langmuir [8,9], the competitive Bi-Langmuir [10], the competitive Toth or Bi-Toth [11] models have been successfully applied to calculate individual band profiles. More recently, a new competitive isotherm model was derived for compounds that exhibit convex downward single-component isotherms such as the liquid–solid extended BET isotherm. This model was successfully used to calculate the individual band profiles of toluene and

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ethylbenzene during the elution of their mixture on a RPLC column [12].

Most generally, the elution of a large sample of a mixture, under nonlinear conditions, consists in two bands that interfere more or less strongly but follow the same pattern, their elution profiles are both unimodal concentration distributions. It is exceedingly rare that the individual band profile of a component is a bimodal concentration distribution. No cases have been reported, yet, to the best of our knowledge. The most unusual case is the one encountered with the separation of *cis*- and *trans*-androsterone on silica, in normal-phase chromatography [13]. When pure, each of these two compounds follows Langmuir isotherm behavior. However, their saturation capacities are markedly different and their isotherms intersect at some concentration. Then, at high concentrations, the band of the more retained *cis*-androsterone begins first and ends last [13]. Its concentration distribution is nearly bimodal but not quite. This phenomenon was independently confirmed by Antia and Horvath [14]. Band splitting was also reported under gradient conditions [15].

In this paper, we report the unusual concentration distribution observed in the investigation of the competitive behavior of two compounds, ethylbenzoate and 4-*tert*.-butylphenol, that we knew for having, respectively, single-component BET and Langmuir equilibrium behavior. The distribution of the latter is bimodal with a complete separation of the two modes. This behavior can be explained only by a peculiar competitive equilibrium isotherm.

2. Experimental

2.1. Chemicals

The mobile phase used in this work was a mixture of HPLC-grade water and methanol (62% methanol, 38% water, v/v), both purchased from Fisher Scientific (Fair Lawn, NJ, USA). The solvents used to prepare the mobile phase were filtered before use on an SFCA filter membrane, 0.2-μm pore size (Suwannee, GA, USA).

The solutes used were 4-*tert*.-butylphenol and ethylbenzoate. These two compounds and uracil, used as a nonretained tracer, to measure the hold-up volume, were obtained from Aldrich (Milwaukee, WI, USA). A 1:1 mixture of these two compounds was prepared and diluted into the mobile phase, to concentrations of 40 g/l for each compound.

2.2. Materials

A manufacturer-packed, 250×4.6 mm Kromasil column was used (Eka Nobel, Bohus, Sweden). This column was packed with a C_{18}-bonded, endcapped, porous silica. This column (Column #E6021) was one of the lot of 10 columns that were previously used by Kele [16] and Gritti [17] (Column E6019, #E6103 to E6106, #E6021 to E6024 and E6436) for their study of the reproducibility of the chromatographic properties of RPLC columns under linear and non-linear conditions, respectively. The main characteristics of the bare porous silica and of the packing material used are summarized in Table 1. With the mobile phase composition used in this work, the column hold-up volume was 2.40 ml, as derived from the retention volume measured for uracil.

2.3. Apparatus

The different chromatographic band profiles were acquired using a Hewlett-Packard (Palo Alto, CA, USA) HP 1090 liquid chromatograph. This instrument includes a multi-solvent delivery system (three tanks, volume 1 l each), an auto-sampler with a 250-μl syringe, a diode-array UV-detector, a column thermostat and a computer data acquisition station. Compressed nitrogen and helium bottles (National

Table 1
Physico-chemical properties of the packed Kromasil-C_{18} (Eka) #E6021 column

Particle size	5.98 μm
Particle size distribution (90:10, % ratio)	1.44
Pore size	112 Å
Pore volume	0.88 ml/g
Surface area	314 m^2/g
Na, Al, Fe content	11; <10; <10 ppm
Particle shape	Spherical
Total carbon	20.0%
Surface coverage	3.59 μmol/m^2
Endcapping	Yes

Welders, Charlotte, NC, USA) are connected to the instrument to allow the continuous operation of the pump and the auto-sampler and solvent sparging of the mobile phase. The extra-column volumes are 0.058 and 0.900 ml, as measured from the auto-sampler and from the pump system, respectively, to the column inlet. All the retention data were corrected for this contribution. The flow-rate accuracy was controlled by pumping the pure mobile phase at 23 °C and 1 ml/min during 50 min, from each pump head successively, into a volumetric glass of 50 ml. A relative error of less than 0.4% was measured, so we can estimate the long-term accuracy of the flow-rate at 4 μl/min at flow-rates around 1 ml/min. All measurements were carried out at a constant temperature of 23 °C, fixed by the laboratory air-conditioning system. The daily variation of the ambient temperature never exceeded 1 °C.

Injections of the prepared solution of the 1:1 mixture of 4-*tert*.-butylphenol and ethylbenzoate were carried out using either one of the delivery pumps of the HPLC instrument (to inject large-volume samples, ranging from 0.2 to 4.0 ml) or the auto-sampler (to inject small-volume samples, ranging from 1.0 to 250 μl). The band profiles of samples of increasing volumes were recorded successively, all at a flow-rate of 1 ml min^{-1}, with a sufficiently long time delay between each injection to allow for the complete reequilibration of the column with the pure mobile phase. To avoid any UV-absorbance superior to 1500 mAU and the corresponding increase in signal noise for each individual solute, the signals of 4-*tert*.-butyl phenol and ethylbenzoate were both detected with the UV detector set at 295 nm.

2.4. Determination of the individual band profiles of the two components

A 2-ml sample of the solution of a 1:1 mixture of 4-*tert*.-butyl phenol and ethylbenzoate (40 g/l each) was injected into the column, using the solvent delivery pump. The time of injection (120 s) was long enough to achieve strong interference between the two compounds during their entire elution and a large mixed band in the elution profile. This band profile was investigated by collecting 43 fractions of 300 μl (i.e., 22 droplets) each, at the flow-rate of 1 ml/min, from the elution time of 11 min to that of 24 min. Ten-μl aliquots of each one of the 43 fractions were injected into the same column, using as the eluent a 70:30 (v/v) methanol–water solution. The stronger mobile phase allows a faster analysis of the collected fractions. After preliminary calibration, the measurement of the areas of the two separated peaks allowed the determination of the concentration of each individual component in the analyzed fraction. The individual elution band profiles of each component can then be reconstituted, knowing the concentration of each fraction and its elution time. This time is derived from the average collection time $(t_i + t_{i+1})/2$ (t_i is the time when the ith fraction starts to be collected), minus the sum of the time needed for the mobile phase to percolate from the pump mixer to the column inlet (54 s at 1 ml/min) and the time necessary for the mobile phase to percolate through the capillary joining the detector cell and the collector vials (10 s at 1 ml/min).

3. Results and discussion

3.1. Evolution of the shape of the overall band profiles versus the volume injected

The sample solution was first injected using the auto-sampler of the HPLC instrument, which is equipped with a syringe the maximum delivery volume of which is 250 μl. Volumes of 1, 20, 50, 80, 110, 160, and 250 μl were injected successively. The speed of injection was constant, at 833 μl/min. Making sample injections with the auto-sampler presents a major advantage. The hold-up volume between the outlet of the injection loop and the inlet of the column is very small (approximately 68 μl, hold-up time, 4 s). Hence, the injection profile is essentially a rectangle which is only slightly smoothed and dispersed before entering the column. The outlet band profiles recorded under these conditions are nearly free of the band broadening effects originating from the extra-column volumes due to the connecting tubes. However, no more than 250 μl can be injected with the auto-sampler.

The band profiles obtained as the results of these seven injections are shown in Fig. 1. Because the concentration of the sample solution is high (40 g/l),

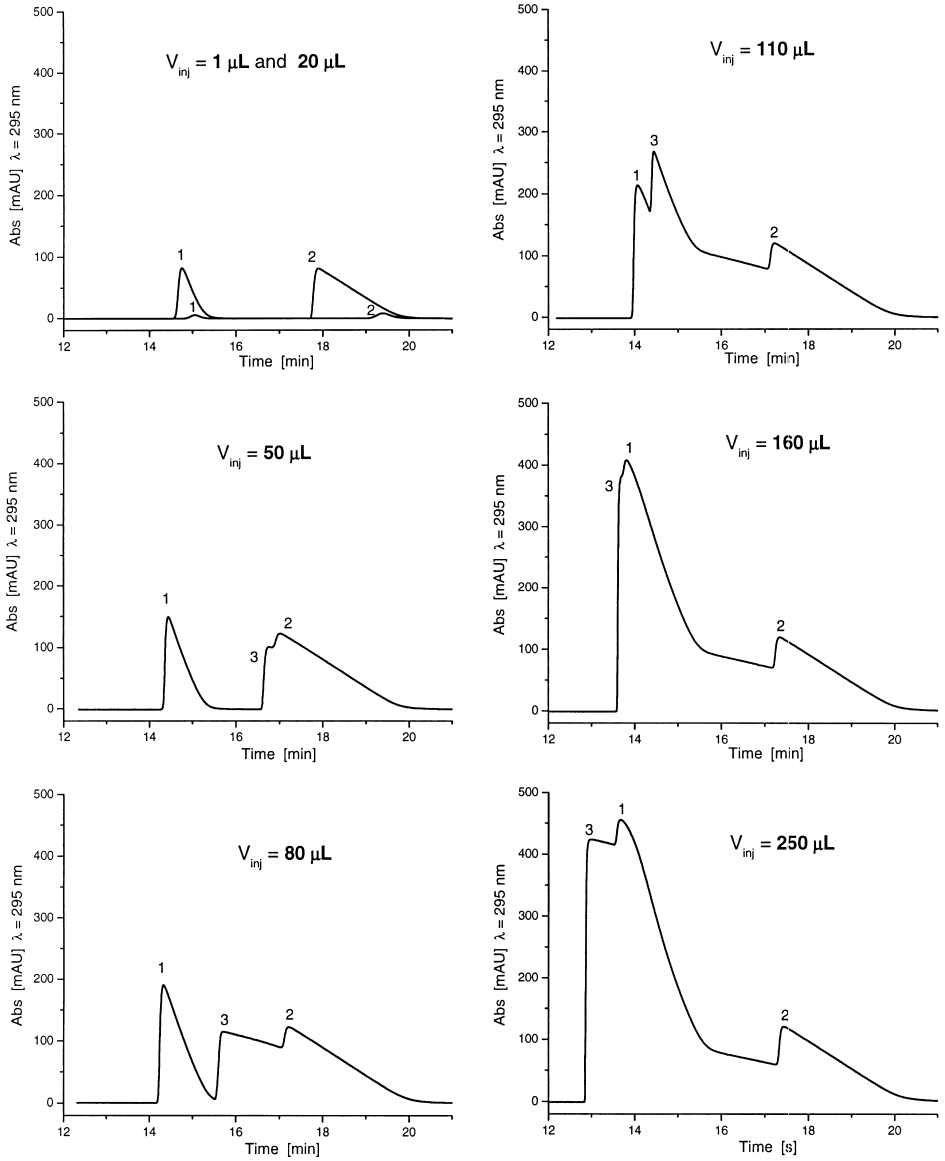

Fig. 1. Progressive changes of the chromatogram of a binary mixture of ethylbenzoate and 4-*tert*.-butylphenol with increasing sample size. Sample volume as indicated on each chromatogram. Sample concentration, 40 g/l of each solute. Injection with the autosampler. C_{18}-Kromasil column (250×4.6 mm), methanol–water mobile phase (62:38, v/v), flow-rate 1 ml/min, $T=295$ K. Note the nearly constant size of band # 2 once band # 3 has appeared.

it is only with the smallest injection volume illustrated here (1 μl) that the separation of ethylbenzoate (peak # 1) and 4-*tert*.-butylphenol (peak # 2) takes place under linear conditions and that the profiles of the two bands are nearly Gaussian. This chromatogram shows that the Henry constant of 4-*tert*.-butylphenol ($H=5.1$) is larger than that of ethylbenzoate ($H=3.8$). For larger injection volumes, the band profiles are no longer Gaussian but skewed. For a 20-μl injection volume, both peaks are still well resolved but both exhibit the classical Langmuirian overloaded profiles, suggesting that the

single-component isotherms of the two compounds are convex upward. For all larger injection volumes, anomalous band profiles are recorded, exhibiting three peaks although the sample remains a binary mixture. For an injected volume of 50 μl, a bump appears on the front of the profile of 4-*tert*.-butylphenol. Two shock layers are now observed, the first one corresponds to the peak referred in further chromatograms as peak # 3, for reasons that will be made clear later. The existence of this third shock layer is confirmed in the following chromatogram, obtained for an injection volume of 80 μl. It is worth noting that, in the following chromatograms, the retention times of the shock layer of peak # 2, the time of the end of this peak and its height (hence, its size) remain nearly constant. The increase in sample size contributes only to enlarge the bands # 1 and 3 and to move forward the shock layer of peak # 3. This effect is clearly illustrated by the last chromatograms of this first series, corresponding to injections volumes of 110, 160, and 250 μl.

We have never yet encountered such a phenomenon, nor do we know any report of it. Usually, during the elution of a binary mixture, only two more or less overlapping bands are recorded, never three. In the case of mixtures of ethylbenzoate and 4-*tert*.-butylphenol, under conventional RPLC conditions, three distinct bands are observed and the progressive evolution of the respective retention time and size is exceptional.

This observation is confirmed by the chromatograms obtained upon the injection of larger volumes of the same mixture. These injections were made using the pumps of the solvent delivery system and the step gradient function of its programmer instead of the autosampler. The extra-column hold-up volume is now larger since it includes all the volume from the pump mixer to the column inlet. It is 0.90 ml (extra-column hold-up time, 54 s). As a consequence, a significant degree of dispersion takes place during the migration of the sample along these tubings. The chromatograms recorded for sample volumes between 0.20 and 4.0 ml are shown in Fig. 2. The comparison of the chromatograms obtained with 250 μl in Fig. 1 and with 0.20 ml in Fig. 2 illustrates the influence of the increased degree of axial dispersion. There should be two distinct shock layers in the latter of these two chromatograms but they are not resolved (the shock layer # 3 passes the shock layer # 1 for a sample size of approximately 150 μl, compare the chromatograms for 110 and 160 μl). Also, the resolution between the band 1 + 3 and the band 2 is significantly lower in Fig. 2 (0.20 ml) than in Fig. 1 (250 μl).

Nevertheless, the chromatograms in Fig. 2 are clearly similar to those in Fig. 1 and the continuation of same trends is observed. The third band at the beginning of the chromatogram reappears for an injection volume of 0.50 ml, the retention times of the shock layers of peaks # 1 and 3 keeps decreasing with increasing sample size, the retention time of the first shock layer decreasing faster than that of the second. Finally, the size and position of the peak # 2 remain unchanged. Since we keep dealing with a binary mixture, it is necessary to elucidate the individual elution profiles of the two components and find out what can be the composition of this strange third band.

3.2. Composition of the overall band profile

Forty-three fractions were collected, one every 0.3 min, during the elution of the band set corresponding to the injection of a 2-ml sample, between elution times of 11 and 23.9 min, the time origin between the time when the pump starts to deliver the sample. This interval of time encompasses the entire mixed zone. Taking into account the time delays introduced by the pump delivery system (54 s) and the capillary tube joining the UV cell and the collector vials (10 s), the first vial is actually collected between true retention times of 9.9 and 10.2 min. We assigned it the collection time t_1 of 10.05 min. Accordingly, vial i was assigned the collection time t_i given by:

$$t_i = 10.05 + 0.3\,(i - 1) \quad i \in [1;43] \tag{1}$$

The results of the analysis of these fractions are so strange and unexpected that we give in Fig. 3a–c the chromatograms obtained. Fractions 1–4 contain only pure *tert*.-butylphenol. So do also all the fractions beyond 32. Fractions 23–28 contain less than 0.15 g/l of *tert*.-butylphenol and between 6 and 1 g/l of ethylbenzoate. Fractions 5–22 and fractions 29 and 30 constitute the mixed zone. For times shorter than 9.9 min or longer than 22.8 min, the band profile is

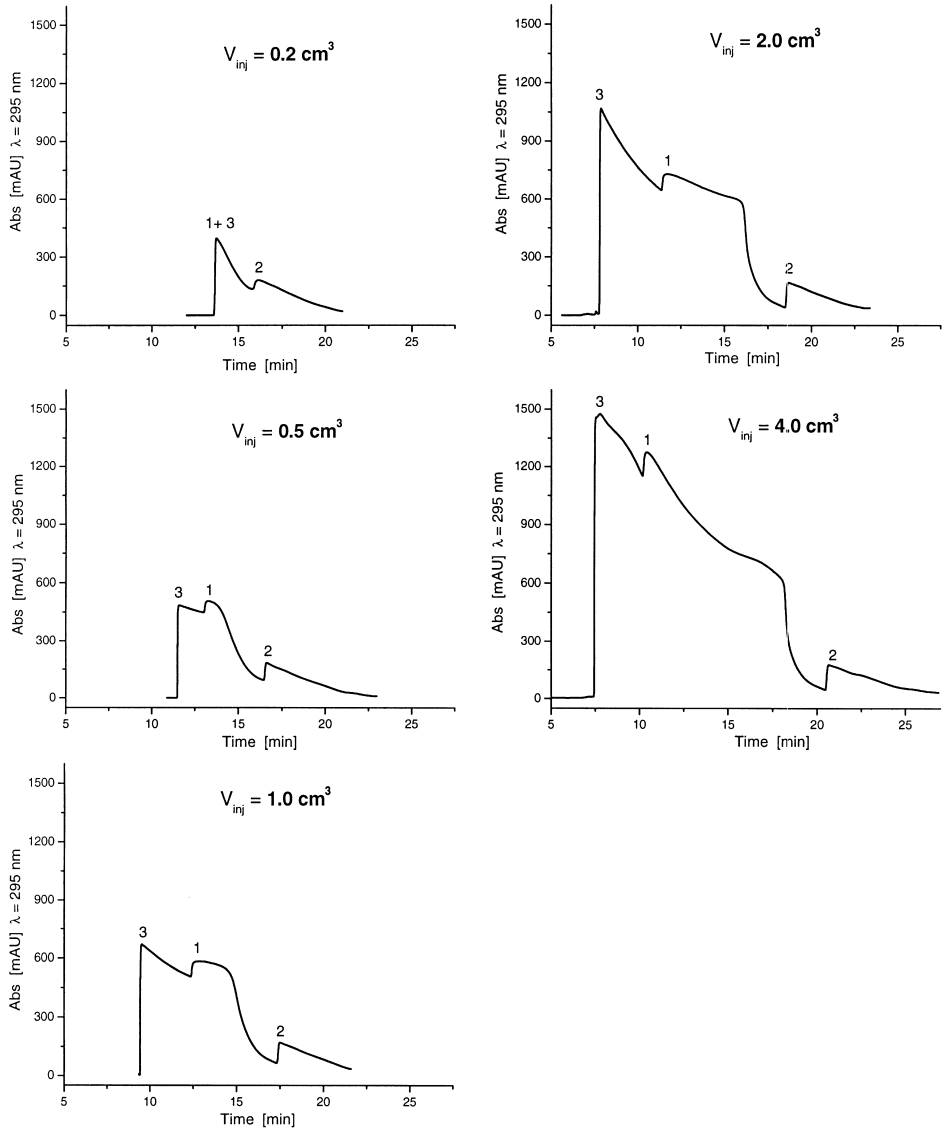

Fig. 2. Progressive changes of the chromatogram of a binary mixture of ethylbenzoate and 4-*tert*.-butylphenol with increasing sample size. Sample volume as indicated on each chromatogram. Same chromatographic condition as in Fig. 1. Injection with the step gradient system. Note the constant size of band # 2 when the injection volume increases.

actually a single-component band profile. Both segments are actually parts of the band of 4-*tert*.-butylphenol. The individual concentration profiles of the two compounds, as they result from these analyses, are shown in Fig. 4. The concentration of 4-*tert*.-butylphenol before collection of fraction 1 and after collection of fraction 43 were calculated from the UV-absorbance data signal, by averaging the concentration over successive periods of 0.3 min, using the calibration curve of 4-*tert*.-butylphenol.

Fig. 4 demonstrates that, under the experimental conditions selected, the chromatographic band profile of 4-*tert*.-butylphenol is split into two separate bands. One band elutes before the band of the other component of the binary mixture, the other one, after. The experimental conditions correspond to the

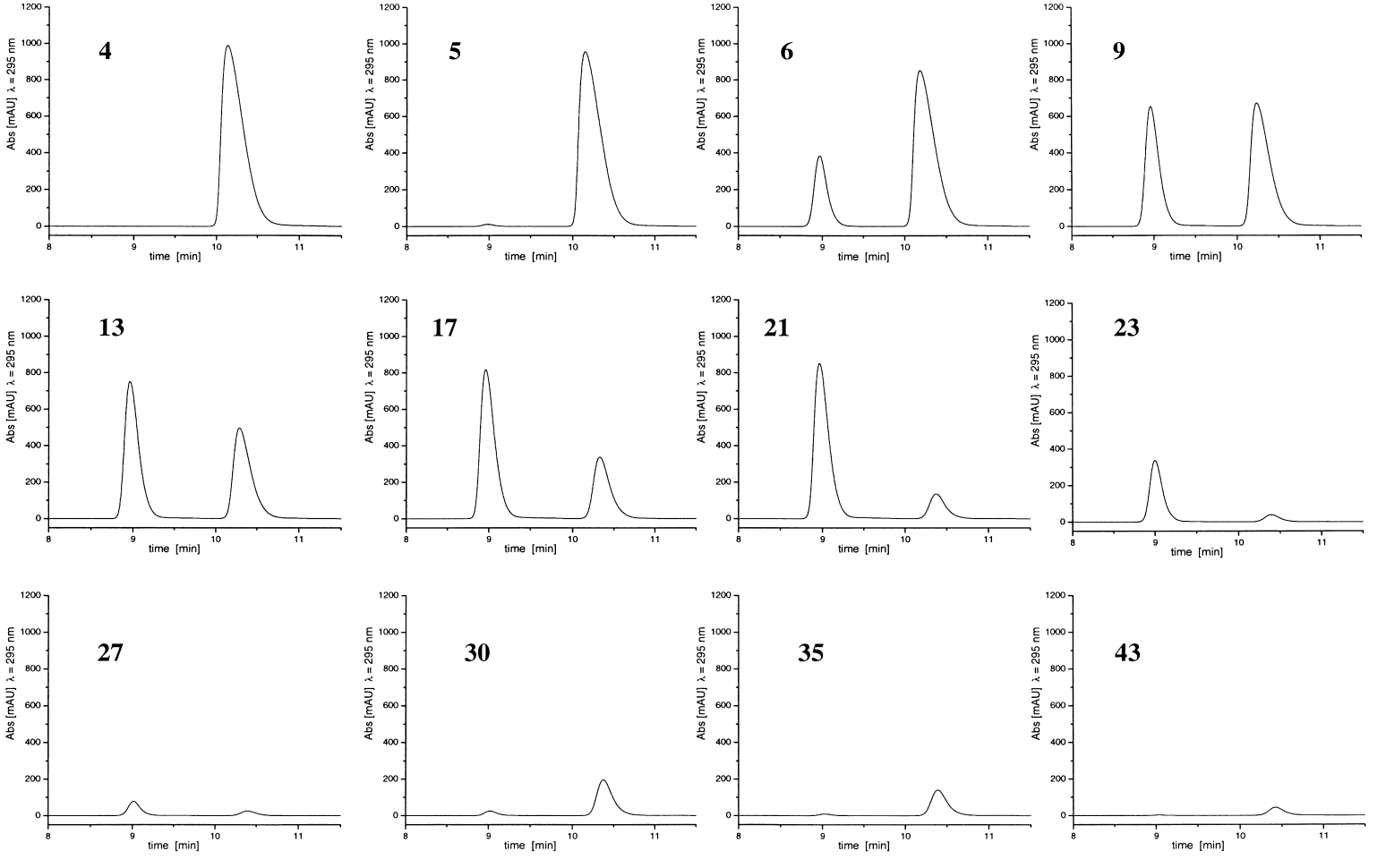

Fig. 3. (a,b,c) Main chromatograms obtained in the analysis of the 43 fractions collected during the elution of a 2-ml sample of the mixture of ethylbenzoate and 4-*tert*.-butylphenol (Fig. 2). The number on each chromatogram is the fraction rank. Same C_{18}-Kromasil column as for Figs. 1 and 2. Sample size, 10 μl of each collected fraction. Mobile phase, methanol–water (70:30, v/v), flow-rate 1 ml/min, $T = 295$ K.

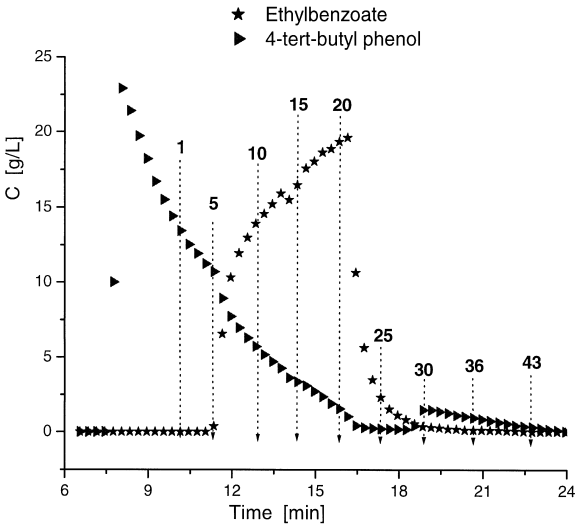

Fig. 4. Individual band profiles of ethylbenzoate and 4-*tert*.-butylphenol in the chromatogram of a 2-ml sample (Fig. 2), reconstituted from the analysis of the 43 fractions collected (Fig. 3a–c) during this sample. The data before the first and after the last collected fractions, corresponding to the elution of pure 4-*tert*.-butylphenol were calculated from the UV signal (see text).

injection of a binary mixture with ethylbenzoate, and to the use of a relatively concentrated solutions. The mass corresponding to the second band of *tert*.-butylphenol accounts for 4 mg, i.e., 5% of the total mass of this compound in this sample and amounts to approximately 1.4% of the monolayer saturation capacity of the column ($q_S = 164$ g/l [18]). This amount is nearly constant, independently of the mass of sample (see Figs. 1 and 2). It is eluted after the elution of ethylbenzoate is nearly complete, as if were trapped in the column. Such a behavior points to an unusual competitive equilibrium isotherm behavior. Note, for example, that the profile of the rear diffuse boundary of the first 4-*tert*.-butylphenol band has a sudden inflection point and becomes convex upward instead of downward during the collection of fraction 13 and 14, precisely when the profile of ethylbenzoate exhibits a clear anomaly.

In a companion paper [18], we show that the experimental isotherm data for these two compounds are well modeled by a Langmuir model for 4-*tert*.-butylphenol and a multilayer BET model for ethylbenzoate, similar to the one observed for propyl- and butyl-benzoate. From these single-component isotherms, we show how to derive a new model of competitive equilibrium isotherms. This model explains the strange adsorption behavior observed and reported in this work.

4. Conclusion

Our experimental results demonstrate that, under certain experimental conditions that are not extreme, the elution band profile of 4-*tert*.-butylphenol is split into two separate bands, one eluted before, the other after the band of ethylbenzoate. Between these two modes, the concentration of 4-*tert*.-butylphenol is very small, approximately 150 times less than the maximum concentration of the first mode, less than one-tenth that of the second mode. The experimental conditions used are that large volumes of a rather concentrated 1:1 binary mixture are injected on a conventional RPLC stationary phase and eluted with an aqueous solution of methanol. The loading factors investigated range from 0.014 to 56%. The unusual behavior reported takes place for values of the loading factor larger than approximately 0.5%. At higher loading factors, the mass of 4-*tert*.-butylphenol eluted in the second band remains nearly constant, independent of the injected amount, and is approximately equal to 1.4% of the monolayer saturation capacity of the column. This phenomenon was not reported previously in the literature, to the best of our knowledge. It must be considered most seriously because preparative applications of chromatography would be difficult to carry out when it takes place.

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