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Chromatographic peak profile of ionogenic analytes upon elution with unbuffered eluents

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Abstract

Experimental proof of the model was carried out for the separation of benzoic acid on a LiChrospher RP-18 column. The observed chromatographic peak profile coincides with the peak profile predicted by the model. The retention times of the dissociated and non-dissociated species determined by using an appropriate fitting procedure were 2.2 and 4.3 min, respectively. Based on these values, a theoretical peak profile was calculated, demonstrating a close agreement with the experimentally observed peak profile. An unexpectedly large difference was found for the retention times of the dissociated and non-dissociated species calculated by a fitting procedure and those experimentally measured on the elution of benzoic acid with buffered eluents. A possible explanation for this difference is a contribution of the ion-exchange adsorption on the silica matrix. © 1998 Elsevier Science B.V.

Keywords: Peak profiles; Adsorption isotherms; Benzoic acid

1. Introduction

Chromatographic separation of ionogenic compounds has been the subject of extensive investigation since the mid-1970s [1]. In contrast to separation of non-dissociable solutes, ionogenic compounds can dissociate and their separation, therefore, becomes more complex.

According to Horváth et al. [1], the dissociation of an ionogenic analyte can be defined in terms of secondary equilibria that accompany the main adsorption/desorption process in the column. The dissociation equilibrium:

$$HA \leftrightarrow A^- + H^+ \quad (1)$$

can be simplified in chromatography to:

$$HA \leftrightarrow A^- \quad (2)$$

when the eluent contains a constant concentration of protons, H^+ [1]. Analyte retention is described by [1–3]:

$$k' = \varphi_A k'_A + \varphi_{HA} k'_{HA} = \phi([A^-]^s + [AH]^s)/([A^-]^m + [AH]^m) \quad (3)$$

where k'_A and k'_B are the retention factors of the dissociated and non-dissociated analyte species, respectively; φ_A and φ_{HA} are the molar fractions of A^- and HA in the solution at equilibrium, ϕ is the phase ratio and superscripts s and m denote the analyte in the stationary phase and the mobile phase, respectively.

Eq. (3) was obtained under the assumption that (a) the dissociation process (1) is quick enough on a chromatographic time scale and the system, therefore, is considered to be at thermodynamic equilibrium and (b) Henry's law for the distribution of individual species, A^- and HA is valid:

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$$[A^-]^s = k_A[A]^m \quad \text{and} \quad [HA]^s = k_{HA}[HA]^m.$$

According to Eq. (3), the apparent adsorption isotherm of the equilibrium according to Eq. (2) obeys Henry's law and the corresponding constant is given by:

$$k = \varphi_A k_A + \varphi_{HA} k_{HA} \quad (4)$$

which is similar to Eq. (3).

Eq. (3) has been used extensively to describe the chromatographic behavior of ionogenic compounds and for the optimization of separations of these types of analytes [4,5]. Hägglund and Ståhlberg [6] have recently pointed out that the adsorption of a charged solute on the stationary phase is accompanied by the formation of a charged layer on the surface. This affects the overall adsorption process and, consequently, the chromatographic peak profile. Using the ideal model of chromatography, Hägglund and Ståhlberg calculated the chromatographic peak profile of toluene sulfonic acid on a reversed-phase (RP) packing and showed that peak tailing occurs as a result of a non-linear adsorption isotherm of the charged analytes. The extent to which this process affects the adsorption of weak electrolytes, like organic acids and bases, remains unclear. It certainly depends on the value of the dissociation constant and on the adsorption properties of the charged species. In any case, the results of Hägglund and Ståhlberg [6] predict the non-linear behavior of ionogenic analytes and a tailing peak profile when adsorption of the charged species becomes significant. Therefore Eqs. (3) and (4) should be used with caution when the adsorption properties of the analyte are not known.

Another deviation from the system described by Horváth et al. [1] was observed during the synthesis and preparation of the RP stationary phases. Manufacturers have attempted to produce stationary phases for the separation of ionogenic compounds, particularly basic compounds, which can be used with non-buffered eluents. A suitable testing procedure, described by Engelhardt and Jungheim [7] has been used by a number of firms. According to Engelhardt and Jungheim [7], basic compounds can be separated on a suitable RP-packing using non-buffered eluents, such as methanol–water or acetonitrile–water mixtures. Using Engelhardt's testing procedure, it became clear that, for these kinds of separations, the assumptions made by Horváth et al. [1] are no longer valid and the retention behavior and resulting peak profile cannot be adequately described by Eqs. (3) and (4).

In this paper, we will describe how the dissociation process of the solute affects the apparent adsorption isotherm of the system and the chromatographic peak profile of the analyte when the separation is carried out using non-buffered eluents. The ideal model of chromatography is used to simulate chromatographic peak profiles and the results are compared with the chromatographic behavior of benzoic acid.

2. Theory

One should consider the fact that, for the separation of ionogenic compounds, the detector signal provides information on the concentration of both the dissociated and the non-dissociated analyte species (while protons do not absorb light in the near UV range). Therefore, the apparent adsorption isotherm of the system containing an ionogenic analyte, HA, should combine the total concentration of the analyte in the stationary phase:

$$A_t^s = [A^-]^s + [HA]^s \quad (5)$$

with the total concentration of the analyte in the mobile phase:

$$A_t^m = [A^-]^m + [HA]^m \quad (6)$$

As in the previous models, we assume fast kinetics of the dissociation process and Henry's distribution of the individual species between the mobile and the stationary phases:

$$[A^-]^s = k_A[A^-]^m \quad \text{and} \quad [HA]^s = k_{HA}[HA]^m \quad (7)$$

Combining Eqs. (5)–(7) with the equation of the stability constant of the protonated species of the analyte

$$K = [HA]/[A^-][H^+] \quad (8)$$

and accounting for the fact that the proton concentration in the solution of any monoprotic analyte is always equal to the concentration of the deproto-

nated species, i.e. $[H^+]=[A^-]$, we obtain an expression of the apparent adsorption isotherm [8]:

$$A^s = k_A \frac{-1+\sqrt{1+4KA^m}}{2K} + k_{HA} \frac{(-1+\sqrt{1+4KA^m})^2}{4K} \quad (9)$$

It is not difficult to see that the apparent adsorption isotherm is non-linear in spite of the fact that Henry's adsorption isotherm is valid for both the dissociated and the non-dissociated species. This situation is similar to the one we described earlier for the dimerization process in the chromatographic column [8]. As expected, the apparent adsorption isotherm has two limiting values: It is reduced to the Henry's adsorption isotherm of the deprotonated species at $K\to 0$ and to the Henry's adsorption isotherm of the protonated species at $K\to\infty$.

Chromatographic peak profiles of the analyte with a non-linear adsorption isotherm were widely investigated in preparative chromatography and have been reviewed [9]. Commonly, in non-linear chromatography, the peak is considered to consist of two parts, the so-called diffuse part, where the concentration of the solute gradually changes with the elution time and the so-called shock position, where the concentration drops from a particular value to zero within an indefinitely small time window. The profile of the diffuse part of the peak can be calculated by means of an equation derived by Helfferich and Klein [10]:

$$t_R = t_o \left(1 + \phi \frac{dF(A^m)}{dA^m}\right) \quad (10)$$

where t_R is the elution time at which the concentration A^m of the analyte is eluted from the column; t_o is the hold-up time of the column and $F(A^m)$ is the adsorption isotherm of the analyte. The shock position of the peak can be determined from the mass conservation integral, as pointed out by Goldshan-Shirazi and Guiochon [11]:

$$F \int_{t_{R0}}^{t_{sh}} A^m(t)dt = n \quad (11)$$

where F is the volumetric flow-rate, in l min^{-1}; t_{sh} and t_{R0} are the times of the shock position and the end of the peak, in min; $A^m(t)$ is the equation of the diffuse part of the peak from Eq. (10) and n is the injected sample amount, in moles.

Substituting the derivative of the adsorption isotherm in Eq. (10) by its value taken from Eq. (9), we obtain the profile of the diffuse part of the peak:

$$A^m = \frac{1}{4K}\left[\left(\frac{t_A - t_{HA}}{t - t_{HA}}\right)^2 - 1\right] \quad (12)$$

where t_A and t_{HA} are the retention times of the deprotonated and the protonated species of the analyte. Combining Eqs. (11) and (12) and solving for the shock time, we obtain the expressions:

$$t_{sh} = t_A - \frac{C}{2} + \frac{\sqrt{C^2 + 4C|t_{HA} - t_A|}}{2} \quad \text{when } t_A < t_{HA} \quad (13a)$$

$$t_{sh} = t_A - \frac{C}{2} + \frac{\sqrt{C^2 + 4C|t_A - t_{HA}|}}{2} \quad \text{when } t_A > t_{HA} \quad (13b)$$

where $C=4Kn/F$.

From Eq. (12), it can be seen that the diffuse concentration profile of an ionogenic analyte always drops to zero at t_A, i.e. at the retention time of the dissociated species. The shock position of the peak stretches towards the retention time of the protonated species. Consequently, when the protonated species is retained more than the deprotonated species, the peak will tail. Fronting is observed when the protonated species is retained less than the deprotonated species. The shock position of the peak can, theoretically, never reach the retention time of the protonated species and the peak always stretches between the two limiting values, i.e. the retention times of the protonated and the deprotonated species. Fig. 1 illustrates the chromatographic behavior of an ionogenic analyte for the case where the retention of the protonated species is higher than the retention of the deprotonated one.

3. Experimental

The chromatographic system consisted of a Merck LC-pump 655A (Merck, Darmstadt, Germany), a variable-wavelength UV detector (Shimadzu, Japan)

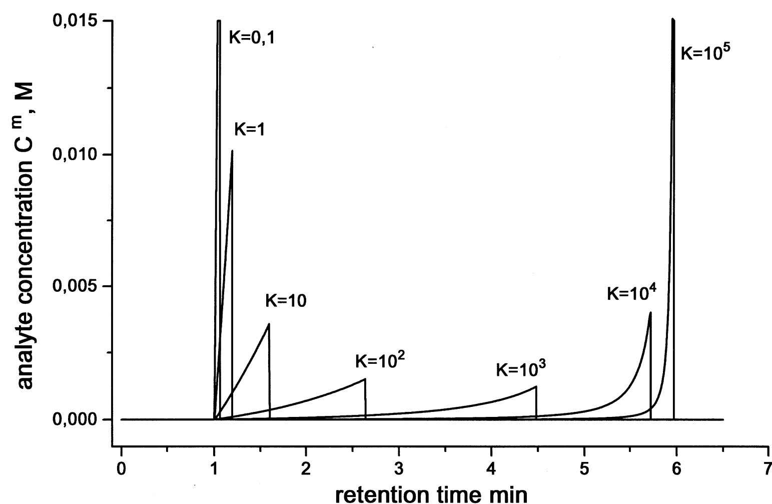

Fig. 1. Simulated peak profile of an ionogenic analyte for different values of the stability constant. Parameters used in simulation procedure: Retention time of deprotonated species, 1 min; retention time of the protonated species, 6 min; flow-rate, 1 ml/min; amount of sample, 10^{-6} mole. Values of the stability constant are given in the figure.

operated at 250 nm, a Rheodyne 7125 injection valve equipped with a 20-μl sample loop NI (Rheodyne, USA). Data were collected and evaluated using a Chromstar data system (SCPA, Germany). A 125×4 mm LiChrosper RP-18 cartridge, particle size, 5 μm (Merck) was used. The efficiency of the column, determined for the separation of a mixture of alkylbenzenes using acetonitrile–water (70:30, v/v), was 6000 theoretical plates, which has been calculated according to the standard procedure from the peak width at half height. A three times lower value was found for benzoic acid eluted with acetonitrile–phosphate buffer, pH 2 (20:80, v/v).

Milli-Q quality water was used to prepare the eluent. The acetonitrile used was of HPLC gradient quality and benzoic acid was of analytical grade quality (both from Merck). Both eluents were prepared from acetonitrile and 0.1 *M* phosphate buffer, adjusted to pH values of 2 and 8, respectively, at a ratio of 20:80 (v/v). All measurements were performed at room temperature. The detector signal was converted into concentration values using a calibration curve and accounting for almost equal absorption of protonated and deprotonated species at 250 nm.

The hold-up volume of the system was determined using thiourea, which is considered to be a marker of the dead volume.

4. Results and discussion

Considering the chromatographic behavior of benzoic acid on Superspher RP-18 packing, it is logical to expect that the negatively charged deprotonated form of the solute will be more weakly retained than the neutral protonated form. According to the model presented above, analyte peak tailing is expected in the analytical concentration range. Our model is thus clearly in contrast to the conclusions drawn from the model proposed by Horváth et al. [1] as well as that by Häggelund and Ståhlberg [6]. Horváth et al.'s model assumes an ideal chromatographic system and predicts a symmetrical peak, as it does not account for complex equilibria. The model proposed by Häggelund and Ståhlberg predicts peak tailing of the charged analyte as a result of the formation of a charged adsorbed layer on the adsorbent surface. The experimentally observed peak profile for different sample amounts is shown in Fig. 2. The observed peak profile clearly shows that the dissociation process itself contributes to a large extent to peak distortion.

To calculate the theoretical peak profile of benzoic acid, the values of the dissociation constant and the retention times of the deprotonated and protonated species must be known. The dissociation constant of benzoic acid has been reported in numerous publi-

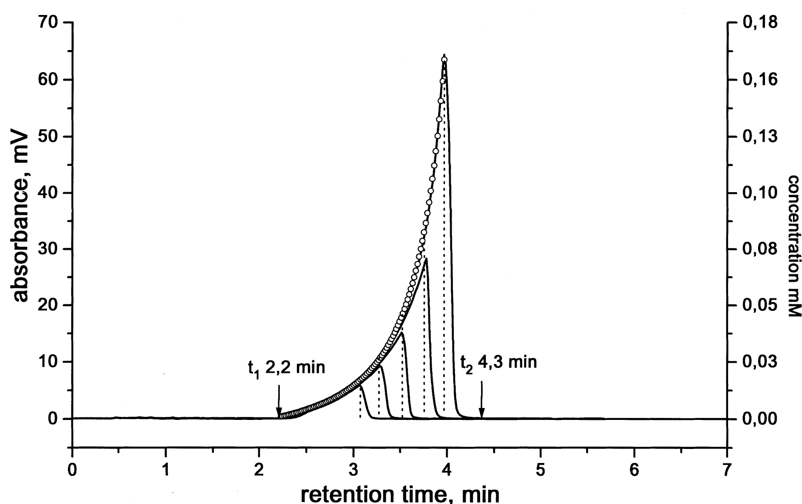

Fig. 2. Experimentally observed (line) and simulated (dots) chromatographic peak profile of benzoic acid on a LiChrospher RP-18 column. Column size, 125×4 mm; eluent, water–acetonitrile (80:20, v/v); flow-rate, 1 ml/min; amount of sample, 10^{-8} mol; 0.169; 0.339; 0.678; 1.356; 2.712; sample volume, 20 μl.

cations for a broad range of conditions and the data are collected in two sources [12,13]. Unfortunately, these data do not contain values for water–acetonitrile mixtures and, as an approximation, we used the pK value of benzoic acid in a water–dioxane mixture (pK=4.84 [12]). To calculate the retention of the dissociated and non-dissociated species, the retention times of the shock measured for different sample amounts were fitted using Eq. (13a) (Fig. 3) using the dissociation constant of benzoic acid in water–dioxane. The retention times of the individual species were 2.2 and 4.3 min for the deprotonated and the protonated species, respectively. Substituting these values for t_A and t_{HA} into Eq. (12), the profile of the diffuse part of the peak is obtained, which correlates well with the peak profile that was observed experimentally (Fig. 2).

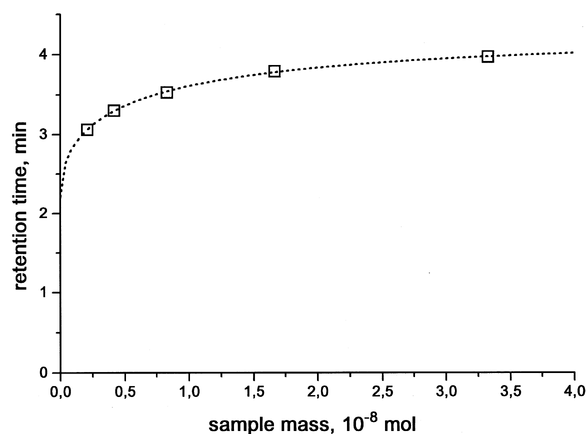

Fig. 3. Dependence of the shock retention of benzoic acid on the amount of sample on a LiChrospher RP-18 column. Chromatographic conditions as in Fig. 2.

Another possibility for determining the retention of the dissociated and non-dissociated species is to elute the analyte with buffered eluents. Chromatographic separations of ionogenic analytes with eluents at constant pH have been studied in depth by Horváth et al. [1]. Eq. (3) shows that the retention factor for benzoic acid, eluted using buffered eluents at a constant pH, corresponds to the retention factor of the protonated and deprotonated species corrected for their molar fractions in the equilibrium solution. The equilibrium solution of benzoic acid contains nearly 100% non-dissociated species at pH=2 and almost 100% of the dissociated species at pH=8.

Therefore, it should be possible to measure the retention of the individual benzoic acid species when using these eluents. The corresponding peak profiles are shown in the inserts in Fig. 4. In agreement with Horváth's model, both peaks are quite symmetrical and the retention does not depend on the amount of sample, up to 3 μg of benzoic acid (Fig. 5). This is an indication that the adsorption of the charged

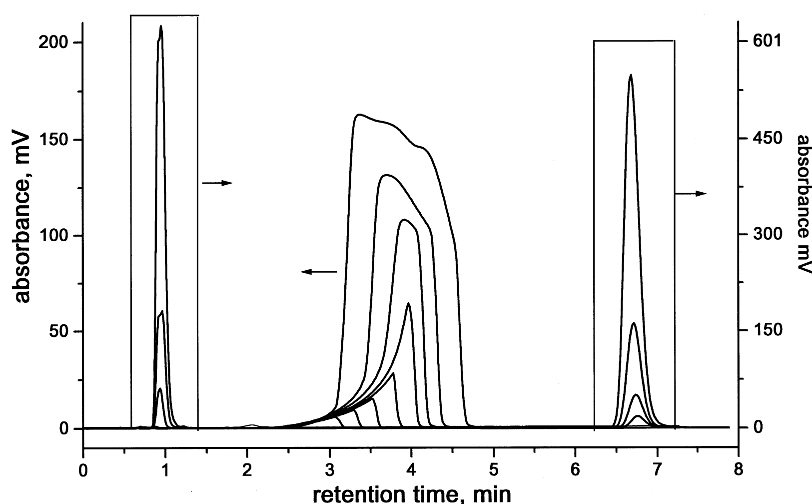

Fig. 4. Changes of the chromatographic peak profile of benzoic acid on a LiChrospher RP-18 column with mass load. Chromatographic conditions as in Fig. 2 except that the amount of sample ($\times 10^{-8}$ mol) was 0.169; 0.339; 0.678; 1.356; 2.712; 5.425; 10.85; 21.7. Insertions show the peak profile of benzoic acid on elution with buffered eluents with pH values of 2 and 8, respectively. The amount of sample injected was 0.169; 0.678; 2.712; 10.58.

species onto the surface does not significantly affect the adsorption process in the system studied. The retention time of the deprotonated form of benzoic acid on the LiChrospher RP-18 packing (Fig. 5) was 0.94 min, while the retention time of the non-dissociated species was 6.7 min. It is interesting to note

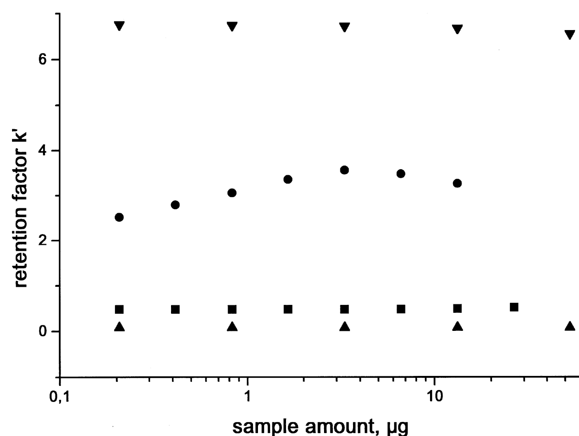

Fig. 5. Dependence of the retention factor of benzoic acid on the amount of sample. Chromatographic conditions as in Fig. 2. Eluents: ●, water–acetonitrile (80:20, v/v); ◇, 50 m*M* NaCl in water–acetonitrile (80:20, v/v); ▼, 50 m*M* phosphate buffer, pH 2–acetonitrile (80:20, v/v); ▲, 50 m*M* phosphate buffer, pH 8–acetonitrile (80:20, v/v).

that the peak shape of benzoic acid on elution with buffered eluents remains unaffected by an increase in the amount of sample in the range of concentrations where elution with non-buffered eluents would clearly cause a significant distortion of the peak shape (Fig. 4). The peak distortion observed reveals an inflection point on the adsorption isotherm of benzoic acid for an acetonitrile–water eluent. One may suspect that the linear range of the adsorption of the individual species required by the model is exceeded for amounts of sample higher than 4 μg. As a result, the apparent adsorption isotherm changes to another type, which is not represented by the model developed in this paper. Unfortunately, this explanation is not consistent. It was shown above that either dissociated or non-dissociated species alone do not cause column overloading, up to a sample amount of 30 μg. Therefore, it would not be logical to assume that a mixture of both species of the same total concentration can cause column overloading. It is possible that some other equilibria can occur in the column for high mass loading. Whether or not such processes do occur is unclear and they are not accounted for by the model presented here. The amounts of benzoic acid, for which the theoretical peak profile was simulated, therefore did not exceed 4 μg (Fig. 2).

Even with such precautions, the retention times of dissociated and non-dissociated species, measured with buffered eluents, differ strongly from those obtained by the fitting procedure: 0.94 and 2.2 min for dissociated species and 6.7 and 4.3 min for protonated species, respectively. For a possible explanation of these differences, one should recall the chromatographic conditions under which these retention times were obtained. Whereas the condition of the stationary phase is uncertain when non-buffered eluents are used, the silanol groups of the silica matrix are completely ionized when an alkali buffer of pH 8 is used and they are completely protonated when an acidic buffer of pH 2 is used. Benzoic acid is also completely deprotonated at pH 8. Therefore, the retention time can be expected to decrease as a result of repulsion between the negatively charged solute molecules and the negatively charged stationary phase. The situation at acidic pH values is less clear. Benzoic acid molecules are almost completely protonated and uncharged at pH 2. In additional experiments, however, we have shown that RP packing materials can be positively charged at acidic pH, as was proved by additional experiments performed by us [14]. As a result, even small amounts of dissociated acid can markedly increase retention. It seems that ion-exchange properties of the RP stationary phase can have a more pronounced effect on analyte retention than was previously considered. We believe that it is worthwhile to pursue more detailed investigations on the ion-exchange properties of RP packings. This work is in progress now and the results will be published elsewhere.

5. Conclusions

The theoretical model presented in this paper clearly shows that the peak profile of ionogenic compounds using non-buffered eluents differs markedly from peak profiles obtained when using eluents of constant pH. Instead of the symmetrical peaks produced when a buffered eluent is used, non-buffered eluents produce tailing or fronting peaks as a result of the non-linear adsorption isotherm. A distortion of the rear part of the peak appears when the protonated species are more strongly retained than the dissociated species. Conversely, a distortion at the front part of the peak is observed when the dissociated species are more strongly retained. The diffuse part of the peak always drops to zero at the retention time of the dissociated species and rises up towards the retention time of the protonated species. The greater the difference in retention is between protonated and deprotonated species, the stronger will be the peak distortion. The retention of analyte, which is actually the time of the shock, depends on the amount of sample and a symmetrical peak cannot be achieved even for very small amounts of solute. A symmetrical undistorted peak can theoretically only be expected when the retention times of both dissociated and non-dissociated species are equal.

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