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EFFECT OF SAMPLE SIZE ON RETENTION IN PACKED COLUMN SUPERCRITICAL FLUID CHROMATOGRAPHY

A METHOD FOR CHARACTERIZING STATIONARY PHASE HOMOGENEITY

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SUMMARY

Retention was measured as a function of the amount of solute injected in packed column supercritical fluid chromatography with three different chemically bonded stationary phases and with carbon dioxide as the mobile phase. The retention was found to decrease with increasing sample size. The effect was more pronounced for polar solutes.

A mixed retention mechanism is postulated to explain the results. Both the chemically bonded groups on the surface and the residual silanol groups are assumed to contribute to the retention. The former type of interaction is assumed to be independent of the solute concentration (*i.e.*, a constant distribution coefficient). The adsorption on silanol groups is described by a Langmuir isotherm. By assuming that the observed capacity factor is proportional to the distribution coefficient, an equation can be fitted to the experimental data. The resulting coefficients can be used to characterize the extent to which residual silanols contribute to the retention process. When methanol is added as a modifier to the mobile phase, the effect of sample size on retention disappears, suggesting that preferential adsorption of modifier deactivates the silanol groups on the surface.

INTRODUCTION

There are many good reasons for preferring carbon dioxide as the mobile phase for supercritical fluid chromatography (SFC)[1], including its favourable critical properties ($T_c \approx 31$°C and $p_c \approx 73$ atm) and its excellent compatibility with detection devices, such as flame ionization and UV detectors. The fact that carbon dioxide is non-toxic and non-flammable also needs to be stressed. The use of carbon dioxide as a cheap, clean and safe solvent is one of the potential advantages of SFC over contemporary liquid chromatography.

However, if carbon dioxide is used as the mobile phase in combination with chemically bonded stationary phases (*e.g.*, octadecyl-modified silica) in packed column SFC, poorly shaped peaks are often observed for hydrogen-bonding solutes.

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For example, in a study concerning the applicability of SFC for the analysis of liquid crystal mixtures[2] we found sharp, symmetrical peaks for solutes containing hydrocarbon (alkyl, cyclohexyl, phenyl) and cyano groups, but strongly asymmetric peaks for solutes containing ether or ester groups in the molecular structure. The tentative explanation for these observations is a heterogeneous surface, on which the solute molecules interact with active sites. Chemically bonded stationary phases (CBPs) are prepared by a reaction of the surface of silica particles with a bonding agent. In this reaction silanol (SiOH) groups on the surface are replaced by other functional groups (polar or non-polar). If only because of the size of the bonding agent, not more than about half of the silanol groups can react, so that residual silanols will always be present on the surface. The presence of these silanol groups, the extent to which they are accessible to solute molecules and their actual state at the surface determine the nature and magnitude of their effect on retention and selectivity in chromatography.

If the number of silanol groups at the surface is reduced as much as possible by a prolonged reaction with small silylating agents (so-called "end-capping"), better peak shapes and shorter retention times can be observed for solutes of intermediate polarity[3]. However, in our opinion, chemically bonded stationary phases formed by the reaction of small molecules with the silica surface may differ in degree rather than in kind. In other words, the effect of residual silanol groups may be reduced, but not eliminated, by better bonding procedures.

The actual state of the silanol groups at the surface is determined by their location and by their environment. The environment of the silanol groups may be changed by the presence of adsorbed molecules, such as water or other polar molecules. This latter effect is frequently employed in packed column SFC when polar modifiers are added to the mobile phase (*e.g.*, ref. 3). If silanol groups and bonded groups contribute independently to retention in packed column SFC, a mixed retention mechanism will prevail. This will not by definition result in poor peak shapes, but it will (i) if the two mechanisms have very different kinetics, for example if one of them is a slow exchange between adsorbed molecules on the surface, or (ii) if the two mechanisms result in different types of adsorption isotherms. Because packed column SFC with carbon dioxide as the mobile phase can be performed on chemically bonded stationary phases as well as on bare silica[4], we consider the first possibility to be unlikely. We have assumed the second possibility to be valid. This hypothesis is supported by our experimental results (see below).

The surface heterogeneity can be studied from the shapes of the experimentally obtained peaks. This is difficult in SFC, because of (i) the lack of rigorous theories for the dispersion in ideal situations (on homogeneous surfaces) and (ii) the exceptionally bad peak shapes that may be observed under some conditions in non-ideal situations. The purpose of this work was to establish a much simpler pragmatic method for characterizing the homogeneity of stationary phases, based on the observed variation of retention with sample size. Developing homogeneous stationary phases may be a general goal for packed column SFC, but it was not one of the aims of this work.

Adsorption isotherms

In this paper we present a model for the adsorption isotherm in SFC based on a mixed retention mechanism, to which the bonded groups and the silanol groups contribute independently. The adsorption isotherm is assumed to be linear for the first interaction, because of the relatively large sample capacity of chemically bonded phases[5]. Because of the number of accessible silanol groups on the surface is limited, we assume a Langmuir-type isotherm for the contribution of silanol groups to solute retention. This model is then assumed to apply to observed capacity factors in addition to distribution coefficients.

The distribution coefficient in terms of concentrations (K_c) is defined by

$$K_c = c_s/c_m \tag{1}$$

where c_s and c_m are the concentrations of the solute in the stationary and the mobile phase, respectively. If K_c is independent of c_m, we speak of a linear distribution isotherm. In this case, the capacity factor (k) of the solute in a chromatographic experiment is related to the distribution coefficient by

$$k = Q_s/Q_m = (c_s V_s)/(c_m V_m) = K_c(V_s/V_m) \tag{2}$$

where Q is the total mass of solute in either phase and V is the total volume of the indicated phase in the column. In chromatography the distribution isotherm is ideally linear. For very small sample sizes this is usually a reasonable approximation. A non-linear adsorption isotherm causes the distribution coefficient to vary with the concentration. This will affect the shape of chromatographic peaks. If the distribution coefficient is lower at higher solute concentrations (concave isotherm), the top of the peak will move more quickly than the edges, so that the top will tend to catch up with the front and tailing results. Such concave isotherms can be interpreted as an overloading of the stationary phase. The reverse effect, convex isotherms, caused (for example) by mobile-phase overloading, leads to fronting.

Yonker *et al.*[6] studied the effect of solute concentration on retention in capillary SFC. The results obtained with large amounts of sample (up to 8 μg) may be interpreted as mobile phase overloading. In packed column SFC the solubility of the solute in the mobile phase is unlikely to cause problems, because of the much higher volumetric flow-rates. We can estimate the concentration at the top of the column by assuming that the solute is introduced on the first theoretical plate. For a plate height of 0.5 mm in a 50-μm I.D. capillary column (a reduced plate height of 10), the plate volume is about 1 nl. For a packed column of 4.6 mm I.D. and a plate height of 15 μm (reduced plate height of 3 with 5-μm particles) the mobile phase volume per plate is about 150 nl. We consider amounts of solute exceeding 1 μg to be large, even for packed column SFC on 4.6 mm I.D. columns. The study of Yonker *et al.* was conducted at a much higher range of C_m values than in this work.

THEORY

If we consider the adsorption of solute molecules at active sites (silanol groups) on the surface and if we assume that only one solute molecule can be adsorbed on a given site, and that the adsorbed molecules do not affect each other (constant adsorption energy), then a Langmuir-type equation can be used to describe the distribution isotherm, *i.e.*,

$$c_{s,\mathrm{SiOH}} = ac_m/(bc_m + 1) \tag{3}$$

where $c_{s,SiOH}$ is the concentration of the solute on the silanols (mol/m^2) and a and b are coefficients that depend on the solute and the stationary phase. Also, different values for a and b may be found with different mobile phases or at different pressures and temperatures. The distribution coefficient, in terms of concentrations, for the distribution of the solute between the silanol groups and the mobile phase ($K_{c,SiOH}$) follows from

$$K_{c,SiOH} = c_{s,SiOH}/c_m = a/(bc_m + 1) \tag{4}$$

For the distribution of the solute between the chemically bonded groups and the mobile phase we assume a linear distribution isotherm, *i.e.*, $K_{c,CBP}$ is independent of the concentration. The effective distribution coefficient, $K_{c,Tot}$, is the sum of the two individual contributions:

$$K_{c,Tot} = (c_{s,SiOH} + c_{s,CBP})/c_m = K_{c,SiOH} + K_{c,CBP} \tag{5}$$

or, in terms of the (dimensionless) chromatographic capacity factor (k)[7,8],

$$k = \varphi_{SiOH}K_{c,SiOH} + \varphi_{CBP}K_{c,CBP} \tag{6}$$

where φ_{SiOH} and φ_{CBP} are phase ratios for the two distribution processes. The dimension and, therefore, the value of φ depends on the definition of c_s. In the present situation we shall define c_s as the adsorbed amount per unit area of stationary phase (mol/m^2), in which case

$$\varphi_{SiOH} = \varphi_{CBP} = A_s/V_m \tag{7}$$

where A_s is the total surface area of stationary phase in the column (specific surface area × weight of stationary phase in the column) and V_m the total volume of mobile phase in the column.

Eqn. 5 describes the distribution coefficient for the adsorption of the solute on two different sites. If the assumed model is correct, the capacity factor at any time and place in the column will follow eqn. 6. However, in chromatography we do not measure a single ("static") value of the distribution coefficient, but rather a capacity factor, which corresponds to an integrated ("dynamic") value of the distribution coefficient. The capacity factor is determined by the entire process in the column. If the distribution coefficient is independent of time and place in the column, the static and dynamic values are identical. In the present situation (p = 255 bar and T = 35°C), the density does not decrease significantly along the column[9]. However, the capacity factor does vary with the concentration of the solute. We shall therefore write the observed capacity factor k_{obs} as

$$k_{obs} = \varphi_{SiOH}\bar{K}_{c,SiOH} + \varphi_{CBP}\bar{K}_{c,CBP} = \frac{aA_s}{V_m(b\bar{c}_m + 1)} + \frac{\bar{K}_{c,CBP}A_s}{V_m} \tag{8}$$

In eqn. 8 $\bar{c}_m$ is an effective average value of the concentration of the solute in the mobile phase and $\bar{K}$ denotes a corresponding average distribution coefficient. The actual concentration will vary both along the column (due to dilution of the chromatographic peak) and along the profile of the peak. $\bar{c}_m$ will be related to the injected amount of solute (Q_i, mol) by

$$\bar{c}_m = \beta(Q_i)Q_i \tag{9}$$

where the proportionality factor, β, is a function of Q_i and has a dimension of l^{-1}. Combining eqns. 8 and 9 yields

$$k_{obs} = \frac{aA_s}{V_m(b\beta(Q_i)Q_i + 1)} + \frac{\bar{K}_{c,CBP}A_s}{V_m} \tag{10}$$

We shall now make the most rigorous approximation in the present model by assuming "quasi-static" behaviour, *i.e.*, β does not vary with Q_i. If k values and peak shapes vary with Q_i, an effect of Q_i on the dilution of the peak during its migration through the column may also be anticipated. According to eqn. 10, increasing amounts of solute may lead to reduced capacity factors and, hence, to less dilution. Limiting ourselves to first-order effects, eqn. 10 assumes the form

$$k_{obs} = a'/(b'Q_i + 1) + d' \tag{11}$$

with

$$a' = aA_s/V_m \tag{12}$$

$$b' = \beta b \tag{13}$$

and

$$d' = K_{c,CBP}A_s/V_m = k_{CBP} \tag{14}$$

where k_{CBP} is the capacity factor that would be observed if the contribution of the silanol groups to the retenton were negligible.

In eqn. 3 the ratio a/b is the number of silanol groups available for interaction. From eqns. 12 and 13 we find

$$a/b = (a'/b')(V_m\beta/A_s) \tag{15}$$

Also, b in eqn. 3 and therefore b' in eqn. 11 are a measure of the strength of the interaction. Because the parameter β is not known, we cannot obtain estimates for a/b and b, but we may use the values of a/b' and b' as indications for the *relative* numbers of accessible silanol groups on different stationary phases and for the *relative* strength of the interactions, respectively.

EXPERIMENTAL

The SFC instrument used in this study has been described previously[10–12]. Carbon dioxide (99.8%) (Philips Gasfabriek, Eindhoven, The Netherlands) was used as the mobile phase. The purity was judged to be sufficient in combination with UV detection. When methanol (analytical-reagent grade; Merck, Darmstadt, F.R.G.) was added as a modifier to the mobile phase, this was done by means of a second pump (PU 4015; Philips Scientific, Cambridge, U.K.). The proportional flow-rates of the two pumps were kept constant, while the total flow-rate was controlled by a constant-pressure feedback circuit. The effluent streams of the two pumps were combined in a standard T-piece and fed into a 4100-type cyclonic mixing chamber (Philips Scientific) at low temperatures (liquid state) and the combined stream was led into a temperature equilibration coil in the oven prior to injection.

Retention times were measured from the tops of the peaks. Chromatograms were recorded at constant inlet and outlet pressures of 255 and 240 bar, respectively, in order to avoid a bias due to pressure-drop effects[11]. The retention time of dichloromethane was used as t_0.

Four different liquid-crystal components were used as the test solutes. Their structures are shown in Fig. 1. These solutes were selected on the basis of our own practical experience with packed column SFC. It was not our intention to develop a coherent set of test solutes. Solutes were injected as individual standard solutions of varying concentrations. By simultaneously varying the injection volume and the dilution factor, it was verified that identical results were obtained for identical amounts (mass units) of solute injected. Injection of a mixture of solutes led to different results. This, we believe, is due to competition for the active sites between the different solutes.

Stationary phases

A commercial octadecylsilyl column (Chromspher C_{18}, 5-μm particles) (Chrompack, Middelburg, The Netherlands) of 150 mm × 4.6 mm I.D. was used.

Two additional stationary phases were synthesized in our laboratory. A perfluorooctyl phase ("Fluoro column") was prepared by "chemically drying" the surface of Chromspher silica (Chrompack, particle size 5 μm) with thionyl chloride[13], followed by a reaction with (heptadecafluoro-1,1,2,2-tetrahydrodecyl)-1-trichlorosilane, refluxing in dry toluene under an argon atmosphere for about 15 h with pyridine as a scavenger (for HCl). A trimethylsilyl phase ("TMS column") was prepared by the reaction of a sample of the same silica with diethyl-1,1,1-trimethylsilylamine for 2 h in dry toluene. The structures of the bonded groups are listed in Table I. Columns (100 mm × 4.6 mm I.D.) were packed with the two synthesized phases using slurries in 2-propanol.

For each of the three phases the carbon percentage was determined by elemental

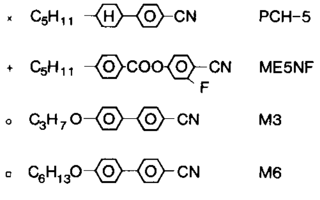

Fig. 1. Molecular structure and identification codes of solutes used in this study.

TABLE I

STRUCTURES AND COVERAGES OF THE STATIONARY PHASES USED

Specific surface area of the silica, 176 m^2/g.

Column	*Bonded group*	*Coverage*		
		C (%)	*Å²/group*	*μmol/m²*
C_{18}	$-Si(CH_3)_2C_{18}H_{37}$	13.3	43	3.86
Fluoro	$\equiv Si(CH_2)_2C_8F_{17}$	3.6	82	2.02
TMS	$-Si(CH_3)_3$	1.9	53	3.10

analysis.Together with the specific surface area obtained from nitrogen-adsorption measurements, these figures were used to calculate the coverage according to ref. 14 (Table I). The coverage of the C_{18} column of 3.86 $\mu mol/m^2$ implies that about half of the silanol groups present on the surface (*ca.* 8 $\mu mol/m^2$)[14] had reacted. A secondary ("end-capping") reaction carried out by the manufacturer may cause the actual number of reacted silanol groups to be slightly higher than the calculated coverage.

The Fluoro column was included in the study as an example of a stationary phase with a low surface coverage. Therefore, a much larger number of silanol groups remains present on the surface. The TMS column has a fairly high surface coverage, but the small size of the bonded group (methyl rather than octadecyl groups) may imply that the remaining silanols can be more easily accessible to solute molecules.

The three stationary phases used in this study were selected to demonstrate the variation of retention with sample size as a means of characterizing surface homogeneity. Batch-to-batch variability was not tested, as it was beyond the scope of this study.

RESULTS

Fig. 2 shows the variation of the observed capacity factor with the amount of solute injected for the four solutes shown in Fig. 1 on the C_{18} column. Here and in subsequent figures, the drawn lines represent the best fit according to eqn. 11. A significant variation in k_{obs} with varying Q_i was found for all solutes, but the effect was largest for the two ethers M3 and M6 (see Fig. 1). This is not simply because of the higher capacity factors of these two solutes, because at high concentrations the capacity factors of M3 and PCH-5 are about equal. The estimates of the limiting capacity factors at very high concentrations (d' in eqn. 11) for M3 and M6 are indicated in Fig. 2. For the two other solutes d' is virtually reached within the experimental range.

Fig. 3 shows the effect of sample size on the observed capacity factor for two solutes on the Fluoro column and Fig. 4 that for the same two solutes on the TMS columns. A very substantial variation of k with Q_i is observed in all four instances. Especially for PCH-5, the magnitudes of the observed differences in k are greater on the Fluoro and TMS columns than on the C_{18} column.

The magnitude of the effect of the sample size on the capacity factor can be quantified by use of the data in Table II, which gives the coefficients obtained by fitting

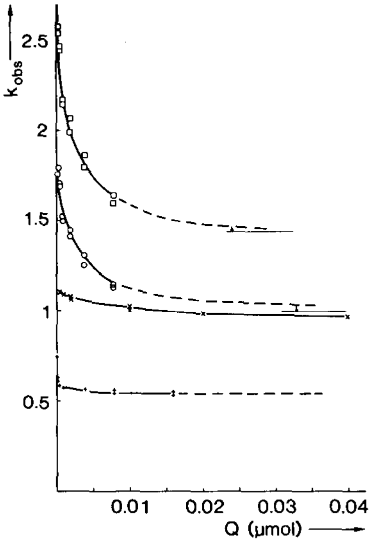

Fig. 2. Variation of the observed capacity factor with the amount of solute injected for the four solutes shown in Fig. 1 on the C_{18} column. The drawn lines represent the best fit according to eqn. 11. Solutes: (□) M6; (○) M3; (×) PCH-5; (+) ME5NF. Mobile phase, CO_2; column inlet pressure, 255 bar; outlet pressure, 240 bar; temperature, 35°C.

eqn. 11 to the data shown in Figs. 2–5. The highest capacity factor for ($Q \downarrow 0$) corresponds to $a' + d'$, whereas d' is the lowest possible value (for $Q_i \rightarrow \infty$). According to the present model, the ratio a'/d' is representative of the possible variation in k due to the silanol-group effect relative to the retention caused by the chemically bonded groups. The coefficient b' is an indication of the interaction of the solute molecules with the active sites on the surface (silanol groups). In addition to a', b' and d', Table II also lists a calculated value of a/b', which may serve as an indication of the number of silanol groups accessible to the solute molecules.

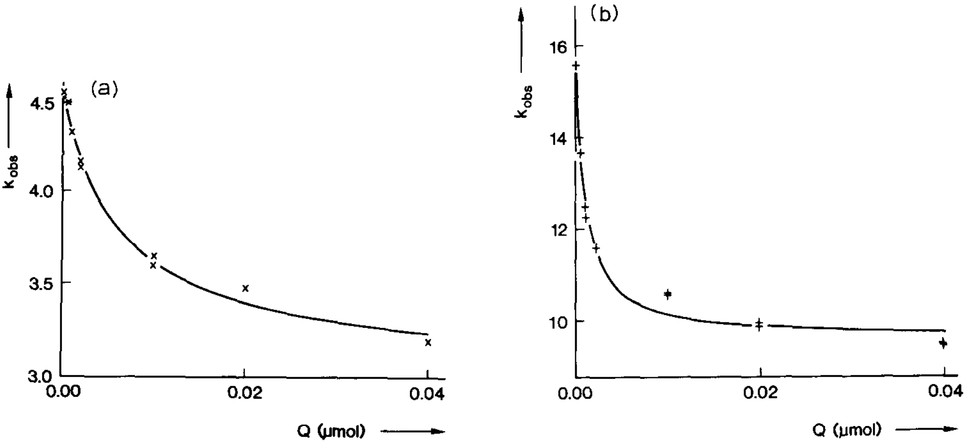

Fig. 3. As Fig. 2 for (a) PCH-5 and (b) ME5NF on the Fluoro column.

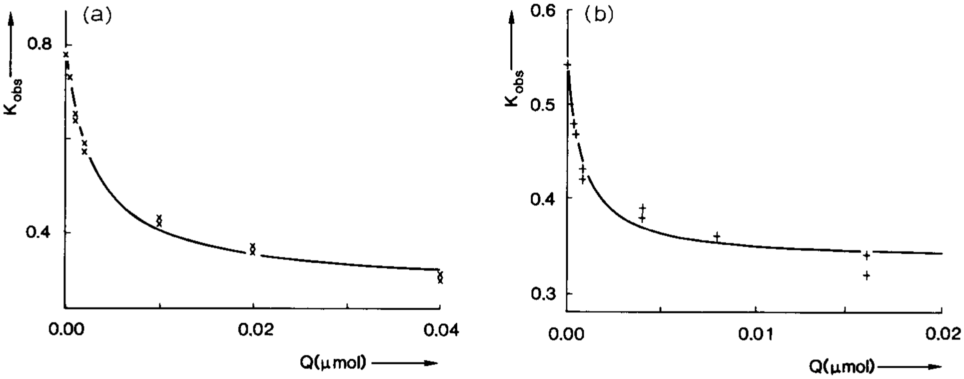

Fig. 4. As Fig. 2 for (a) PCH-5 and (b) ME5NF on the TMS column.

Fig. 5 illustrates the effect of the addition of about 5% (v/v) of methanol to the mobile phase. With the polar modifier present in the mobile phase, no effect of sample size on capacity factors can be measured. Also, a considerable reduction in the observed capacity factors (see d' values in Table II) and an improved peak shape and

TABLE II

COEFFICIENTS FOUND BY FITTING EQN. 11 TO THE DATA SHOWN IN FIGS. 2–5

Column inlet pressure, 255 bar; outlet pressure, 240 bar; temperature, 35°C, except for mobile phases containing methanol (50°C). Figures in parentheses indicate the precision of the parameter estimates [relative standard deviation (R.S.D.), %].

Stationary phase	*Mobile phase*	*Solute*	a'	b' *(mol^{-1})*	d'	a/b'* *(lmol/m^2)*
C_{18}	CO_2	PCH-5	0.17	2.0×10^8	0.95	7.3×10^{-15}
			(4)	(21)	(0.7)	(23)**
C_{18}	CO_2	ME5NF	0.16	6.0×10^9	0.54	2.3×10^{-16}
			(46)	(80)	(0.8)	(36)
C_{18}	CO_2	M3	0.89	6.0×10^8	1.00	1.3×10^{-14}
			(4)	(22)	(5)	(23)
C_{18}	CO_2	M6	1.31	6.6×10^8	1.44	1.7×10^{-14}
			(4)	(23)	(5)	(24)
C_{18}	CO_2–methanol	PCH-5	0	–	0.40	–
C_{18}	CO_2–methanol	ME5NF	0	–	0.21	–
Fluoro	CO_2	PCH-5	1.51	1.6×10^8	3.04	8.4×10^{-14}
			(4)	(21)	(2)	(23)
Fluoro	CO_2	ME5NF	5.84	1.0×10^9	9.65	5.0×10^{-14}
			(13)	(34)	(2)	(23)
TMS	CO_2	PCH-5	0.49	3.2×10^8	0.29	1.3×10^{-14}
			(4)	(23)	(6)	(21)
TMS	CO_2	ME5NF	0.20	1.2×10^9	0.34	1.4×10^{-14}
			(10)	(40)	(3)	(34)

* Calulated by using eqn. 12. $A_s = 146$ m^2 was calculated from a specific surface area of 176 m^2/g, a packing density of 0.5 g/ml and a column volume of 1.66 ml. $V_m = 1.3$ ml.

** Calculated from the equation

$$\text{R.S.D.}_{a/b} = (b/a)\sqrt{(1/b^2)\text{var}_a + (a^2/b^4)\text{var}_b - (2a/b^3)\text{cov}_{ab}}$$

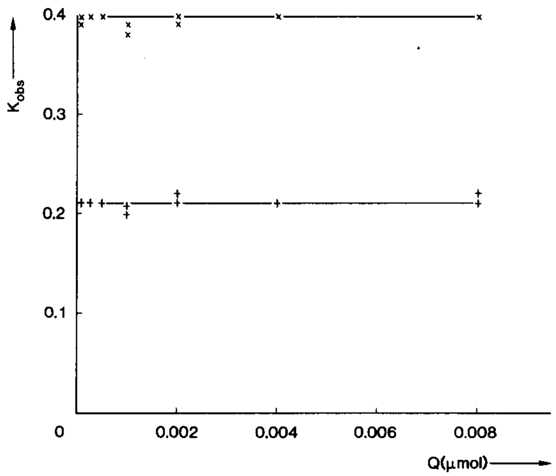

Fig. 5. As Fig. 2 for (×) PCH-5 and (+) ME5NF. Mobile phase, CO_2, with *ca.* 5% (v/v) of methanol; temperature, 50°C.

efficiency were obtained. Both of these effects are well documented (*e.g.*, ref. 3) and will not be discussed further in this paper.

DISCUSSION

Eqn. 11 can be seen to provide a good representation of the experimental data on the variaton of retention with sample size. This suggests that the shape of the distribution term may be described by eqn. 8, and that our hypothesis of a mixed retention mechanism is not unreasonable. On the C_{18} column the differences between the model equation and the experimental data are smaller than on the Fluoro and TMS columns. In the range of large amounts of sample on the latter two columns the capacity factor varies more strongly with Q_i than suggested by the model. A possible explanation is that a constant distribution coefficient (linear isotherm) is a valid assumption for the retention on the C_{18} phase (featuring a relatively large number of long chains on the surface), but is not rigorously correct for either the Fluoro column (small number of chains) or for the TMS column (short chains). Nevertheless, eqn. 11 does provide a reasonable description of the experimental data.

An indication of the precision of the coefficients in Table II is provided by the relative standard deviations (shown in parentheses). These figures show that when b' is large both a' and b' cannot be determined with a high precision. However, the observed variations in b' are definitely significant within the constraints of the model. Because inprecise values for a' and b' tend to coincide with large values for the covariance, the precision of the ratio a/b' is always between 20 and 40%. Therefore, differences between a/b' values larger than about a factor of two are significant.

The coefficients in Table II can be used to characterize the suitability of stationary phases for packed column SFC. The parameters a' and b' represent the extent to which k_{obs} varies with Q_i. By differentiating eqn. 11 we find that at small sample amounts (for $Q_i \downarrow 0$) the variation of k with Q_i (dk/dQ_i) equals $-a'b'$, whereas at high concentrations (for $Q_i \rightarrow \infty$) dk/dQ_i approaches $-a'/b'$. The extent to which k varies with Q_i will determine the shape of the chromatographic peaks. For example, on the C_{18} column ME5NF yielded sharp peaks (calculated plate counts between 6000 and 9000), despite a large value for b' (see Table II), owing to the low value for a'. The ethers M3 and M6 show lower values for b', but much higher values for a', leading to poor peak shapes, especially at higher concentrations.

The relatively strong interaction of the ME5NF ester with the silanol groups on the surface (high b' value) is coupled to a (relatively) low number of accessible silanol groups (low value for a/b'). This may be due to the molecular structure (Fig. 1), which shows the ester group to be "hidden" between two phenyl rings. In contrast, the two ethers (M3 and M6), in which the ether group may be more easily accessible, appear to interact with a much larger number of silanols on the surface. The magnitude of the interaction (b') and the number of accessible silanol groups are approximately the same for the two ethers. The cyano compound (PCH-5) showed the weakest interaction with the silanol groups. This is in agreement with our previous observations of good peak shapes for these compounds in packed column SFC with carbon dioxide as the mobile phase[1].

The Fluoro column, which has the lowest surface coverage in terms of μmol/m^2, yields much higher values for the accessible number of silanol groups (a/b' in Table II). The magnitude of the interaction is similar to that on the C_{18} column for PCH-5, but is a factor of six lower for ME5NF. This suggests that the interaction is different (and weaker) with the more readily accessible silanol groups in between the relatively scarce perfluorooctyl chains than with the isolated silanol groups surrounded by long octadecyl chains.

The values for the accessible number of silanol groups on the TMS column are between those for the other two columns. This is a reasonable result, considering that the surface coverage is higher than on the Fluoro column, but that the chain length and, hence, the extent of shielding per bonded group is less than on the C_{18} column. The magnitude of the interaction with the silanol groups is similar to that on the Fluoro column for ME5NF, but larger by a factor of two for PCH-5.

The values of a' and d' in Table II give an indication of the extent to which k varies with sample size. For the first two solutes on the C_{18} column k may increase by 20% (for PCH-5) to 30% (for ME5NF) over the value obtained with very large samples (d'). For the same two solutes on the Fluoro column the increase is about 50%, whereas on the TMS column the respective values are about 170 and 60%. This also suggests that of the three columns considered, the C_{18} column is the most suitable for these solutes. However, even on this column, the two ethers (M3 and M6) show increases in k of almost 100% on going from very large amounts of sample ($k = d'$) to very small amounts ($k = a' + d'$). The capacity factors at high concentrations (d') are much higher on the Fluoro column than on the C_{18} column, wich in turn shows more retention than the TMS column. The difference in selectivity ($\alpha_\infty = d'_{\text{PCH-5}}/d'_{\text{ME5NF}}$) between the three different phases is remarkable, with values of 1.76, 0.32, and 0.85 for the C_{18}, Fluoro and TMS columns, respectively. However, stationary phase selectivity is not the subject of this paper and we plan to discuss it in a future publication.

The addition of about 5% (v/v) of methanol to the mobile phase caused the effect of the sample size on the observed capacity factor to disappear completely. Apparently, the stationary phase behaves as a homogeneous surface (*i.e.*, $a' = 0$). This supports the assumption that polar modifiers, such as methanol, provide a dynamic coating of surface silanol groups. The retention is much lower than with pure carbon dioxide as the mobile phase, even when compared with the (extrapolated) capacity factors for very large sample sizes (*d'*). The experiments with methanol in the mobile phase were performed at a higher temperature (50 rather than 35°C) in order to stay above the critical temperature of the mixture. At a temperature of 35°C the capacity factors would have been even lower with the methanol–carbon dioxide mixture. This illustrates that the effect of the addition of methanol on retention is not due exclusively to a dynamic coverage of the silanol groups, because in that event we would expect a capacity factor close to the *d'* value observed with pure carbon dioxide. A change in the nature of the mobile phase also appears to contribute to a reduction of the observed capacity factors. This conclusion can be supported by spectroscopic data[15,16].

CONCLUSIONS

(1) Retention in packed column SFC with carbon dioxide as the mobile phase and silica-based chemically bonded stationary phases may be strongly dependent on the amount of sample injected.

(2) The variation of the observed capacity factor with the sample size can be described by a model assuming a mixed retention mechanism. In this model, silanol groups are assumed to contribute to retention according to a Langmuir isotherm, whereas the chemically bonded groups on the surface are assumed to give rise to a linear isotherm.

(3) The coefficients found from the above model give an indication of the relative number of accessible silanol groups on the surface of different stationary phases and of the relative strength of the interaction between these groups and the solute molecules. Therefore, the variation of the observed capacity factor with the amount of sample injected can be used to characterize the homogeneity of stationary phases.

(4) The hydrogen-bonding properties of the solute, the surface coverage and the accessibility of active sites are all reflected in the coefficients of the above model.

(5) On the basis of conclusions 3 and 4, the variation of the observed capacity factor with the amount of sample injected can be used to characterize the homogeneity of stationary phases.

(6) The addition of *ca.* 5% (v/v) of methanol to the mobile phase caused the effect of sample size on retention to vanish completely.

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