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SAMPLE SIZE AND RETENTION VALUES IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF BIOLOGICAL AND SYNTHETIC POLYMERS

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SUMMARY

In the chromatographic separation of natural and synthetic polymers, the retention and peak broadening are strongly dependent on the sample size. Contrary to chromatography of low-molecular-weight solutes, no linear region was observed where both factors are independent of sample size. Even in gradient elution, proteins are eluted approximately 3% of the gradient time earlier when the sample size is increased by a factor of ten. With non-porous materials of small particle diameters and small surface areas, the peak broadening is markedly affected by sample size. Their advantage in efficiency compared to standard porous materials is lost at sample sizes around 100 μg. Generally, retention decreases with increasing sample size, but also the contrary has been observed. In precipitation chromatography of polystyrenes similar behaviour has been observed. Consequently, one has to be careful in qualitative identifications of polymers in mixtures of unknown concentrations and in the transfer of analytical results to preparative-scale work.

INTRODUCTION

A prerequisite for peak identification in high-performance liquid chromatography (HPLC) is the independence of retention time on the sample size. It has been shown that in LC as long as the sorption isotherm is linear, the amount adsorbed increases linearly with the amount of sample in solution. In this case the retention time and peak broadening are independent of the sample size[1]. According to Snyder[1,2], this linear range can be determined experimentally and summarized conveniently as a plot of sample retention values (capacity factor, k', or retention volume per gram stationary phase) *versus* sample size, usually on a logarithmic scale. The maximum sample size can be obtained from these semi-logarithmic plots in two ways: the maximum sample size is the one where the deviation of k' or peak width exceeds 10% of the value measured at low sample sizes (near the detection limit of the detector), or the one where the tangents to both branches of the curves cross. Consideration of preparative scale LC leads to more optimistic definitions of the maximum sample size[3], where a double logarithmic plot of plate number *versus* sample size has been proposed. The maximum sample size is designated as the one at which the slope of

TABLE I

STATIONARY PHASES AND COLUMNS USED

Phase	Surface area (m^2/cm^3)	Pore diameter (nm)	Column dimensions (mm)	Origin
Monospher RP-8	3	-	50 × 4	Merck, Darmstadt, F.R.G.
Monospher RP-18	3	-	50 × 4	Merck, Darmstadt, F.R.G.
LiChrospher Si 1000 RP-18	15	100	50 × 4	Merck, Darmstadt, F.R.G.
LiChrospher Si 500 RP-18	30	50	50 × 4	Merck, Darmstadt, F.R.G.
Grace 250 Acetamide	150	25	50 × 4	Silica: Grace, Worms, F.R.G. Modification[14]
Nucleosil 300 acetamide/carbamate	45	30	50 × 4	Silica: Machery-Nagel, Düren, F.R.G.
Nucleosil 300 SAX	45	30	125 × 4	Macherey-Nagel, Düren, F.R.G.
Waters Prot PAK DEAE-5PW	-	50	75 × 8	Millipore Waters, Eschborn, F.R.G.
LiChrosorb Si 100 RP-18	160	10	250 × 4.1	Merck, Darmstadt, F.R.G.

this plot is equal to -1. Up to that point, the decrease in plate number is compensated by the increase in sample size, whereas at higher sample sizes the loss in efficiency is much more rapid. Other definitions are more or less of theoretical importance, *e.g.*, the statement that "the extra peak broadening is dependent only on the total mass of solute per gram of stationary phase contained in one plate"[4].

Volume overloading may be of importance in isocratic preparative chromatography. Since proteins are usually separated with gradient elution, its influence on H and retention times can be neglected here. Sampling is done under extremely weak elution conditions and the solutes are enriched in the first zone of the stationary phase and do not migrate under these initial conditions.

The maximum sample capacity in protein analysis has not been considered so far. In some papers[5–10] one can find, in cautious wording, that in gradient elution of proteins the retention decreases slightly and continuously as the sample size is increased, but no hints of a limiting value are given. In one paper[5] independence of retention time in the range of 100 µg to 3 mg protein per gram of stationary phase was found for hydrophobic interaction chromatography, whereas a strong dependence was claimed for reversed-phase chromatography. More often the influence of the sample size on peak broadening is considered for the determination of loading capacity: here, a 75% increase in peak broadening (corresponding to a doubling of peak width) has been proposed to determine the loading capacity[11]. Semilogarithmic plots have also been used in this case.

The exact knowledge of the dependence of retention on sample size is of paramount importance for verifying chromatographic identification. The advent of nonporous materials with extremely small specific surface areas and, hence, probably reduced loading capacity, as well as the discrepancies in literature discussed above, induced us to study the sample capacity in HPLC of proteins.

EXPERIMENTAL

A Waters liquid chromatograph (gradient system 600, diode array detector 490) (Millipore, Waters Chromatographie, Eschborn, F.R.G.) with a Rheodyne 7120 sample injector (ERC, Alteglofsheim, F.R.G.) was used. For reversed-phase separations, gradients from water (containing 0.01 M trifluoroacetic acid, TFA) to acetonitrile (also with 0.1 M TFA) ranging from 20 to 75% B in 10 min were applied, and for hydrophobic interaction chromatography, gradients from 2.5 M ammonium sulphate in 0.1 M phosphate buffer pH 7 to pure phosphate buffer in 10 min. Isocratic elution was also studied (for conditions see figure legends). The flow-rate was 2 ml/min in all cases. Buffer salts and organic eluent components were obtained from various suppliers, and were at least of pro analysis quality. The columns and stationary phases are summarized in Table I together with their sources. The proteins were obtained from Sigma Chemie (Deisenhofen, F.R.G.). The solutions were prepared fresh daily and stored in ice–water.

Constant volumes (10 µl) of protein solutions of defined concentration were injected to cover the range between 1 µg and 1 mg protein per injection. To compensate for injection errors, the amounts stated have been correlated via peak areas, determined at 250 nm, to a standard solution containing 500 µg protein/10 µl. The columns were equilibrated with the corresponding protein by injection of solutions

until the peak area remained constant. The peak width at half height and retention times were determined from diode-array detector data.

RESULTS AND DISCUSSION

Dependence of peak width on sample size

For protein separations, primarily gradient elution conditions are of interest. Because H values are not defined in gradient elution, the dependence of the peak width on sample size was determined with different columns under standardized gradient conditions. Fig. 1 shows the dependence of the peak width at half height on the sample size. At low sample sizes, these values can be correlated to the particle diameters, which are 1.5 μm (Monospher), 5 μm (Acetamide) and 10 μm (Si 100, RP-18) respectively. With all columns, the peak width increased continuously with sample size, starting at very low concentrations. Over the whole concentration range, the peak width on sample size was especially pronounced with the Monospher column. This is not surprising, because with around 3 m^2 per ml of column volume this stationary phase has by far the smallest surface area. The efficiencies one may expect from these small particles (particle diameter *ca.* 1.5 μm) are soon lost with increasing sample size; at loadings above 100 μg the efficiencies of classical porous materials with particle diameters of around 5 μm are approached. The small differences in the behaviour found for the reversed-phase material (pore diameter 1000 Å) and the acetamide hydrophobic interaction material (250 Å), differing by a factor of ten in surface area, cannot easily be explained. Perhaps the kinetics of the two different sorption mechanisms plays a role in this case.

In Fig. 2 the same data are plotted on the usual semi-logarithmic scale. Here, the typical and expected curve shape was obtained. However, this plot is just a result of mathematical data transformation. Even a linear dependence of the retention or peak width on the sample size will give this typical curve when transformed to a
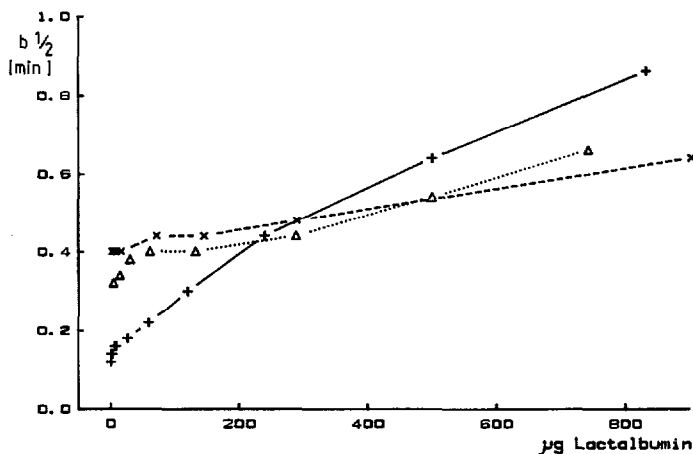

Fig. 1. Dependence of the peak width at half height on the sample size. Sample: lactalbumin. Columns: + = Monospher RP-8; $\triangle$ = Grace 250 Acetamide; $\times$ = LiChrospher Si 1000 RP-18. For gradient conditions, see Experimental section.
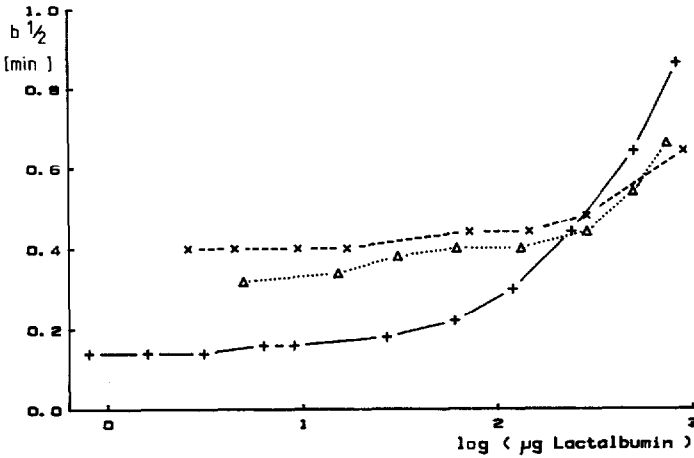
Fig. 2. Semilogarithmic plot of the dependence of peak width on sample size. Columns and conditions as in Fig. 1.

semi-logarithmic scale. The position of the bend in the curve depends only on the range of the *x*-axis. It is, therefore, dangerous to deduce any scientific meaning from this plot, and the place of the bend.

Dependence of retention on sample size

Under standard gradient conditions, the dependence of the elution time in the gradient on the sample size can also be used to compare stationary phases. In Fig. 3 the change in retention time with sample size is demonstrated for different stationary phases. The retention time decreases in every case with increasing sample size. Even at low sample sizes, close to the detection limit, no range of constant retention time was observed. Retention times in the standard gradient are clearly a function of the surface area per unit column volume.
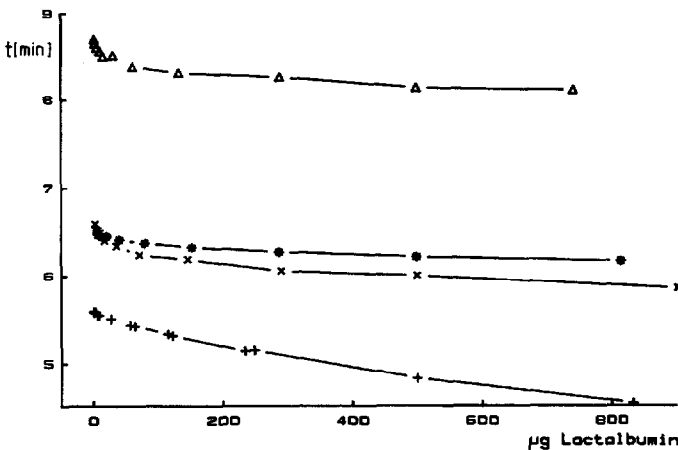

Fig. 3. Dependence of the elution time (*t*) in gradient elution on sample size. * = LiChrospher Si 500 RP-18; other columns and conditions as in Fig. 1.
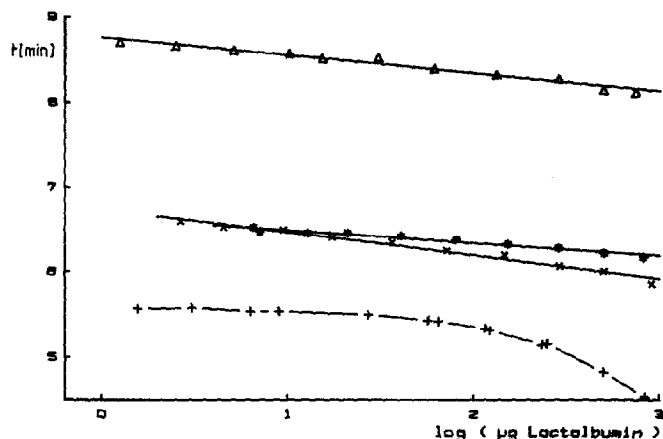
Fig. 4. Semilogarithmic plot of the dependence of elution time on sample size. Columns and conditions as in Fig. 3.

When these data are plotted in a semi-logarithmic plot, straight lines with different slopes are obtained, as shown in Fig. 4. This indicates that retention times in gradient elution always decrease by a constant value upon doubling the sample size. This means that with the exception of the Monospher column, the retention time is affected by the same factor when changing the sample size whether working in the concentration range of 1–10 μg or 0.1–1 mg. In this case, a displacement between 0.15 and 0.25 min was observed when changing the sample size by a factor of ten. Consequently, in gradient elution, variations in retention up to 3% of the gradient program time are expected. The slopes of these curves correspond to the surface area of the stationary phases and can be taken as a measure of the adsorption capacity.

Although the surface area of the Grace 250 acetamide phase is larger by a factor of 10, compared to LiChrosorb Si 1000 RP-18, the loading capacity was quite similar in both cases. This may be due to different separation mechanisms. In hydrophobic interaction chromatography (HIC), the S values (slope of log k' vs. eluent composition)[12,13] are lower than in reversed-phase (RP) chromatography. Consequently, the elution conditions are closer to those of isocratic chromatography, where the sample size influences retention much more strongly.

Isocratic elution

Gradient elution is the standard mode in protein chromatography. However, it is common practice, especially in HIC, to characterize the system by the slopes, S, of the plot of log k' vs. salt concentration in the eluent. These S values can be calculated —with some restrictions— from retention in two different gradient experiments. On the other hand, it is also possible to measure retention values isocratically. In this case, the influence of the sample size must be known. In order to evaluate this influence and to obtain some insight in the problems discussed so far, isocratic measurements were made in both HIC and RP systems. In the latter case, extreme precautions were taken to keep the eluent composition constant within a few fractions of a percent.
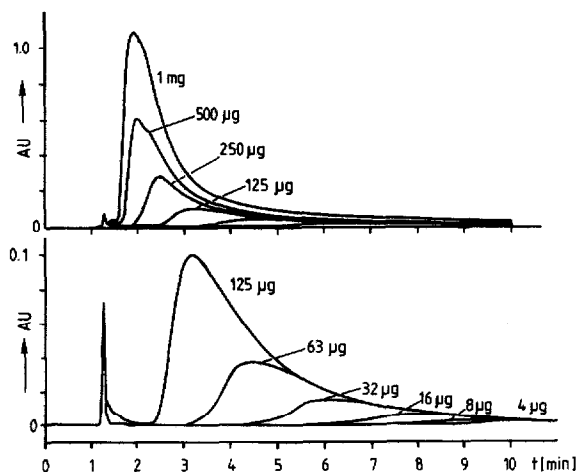
Fig. 5. Elution profiles of lactalbumin in reversed-phase chromatography. Range of sample sizes: 4 μg to 1 mg. Column: Si 500 RP-18. Eluent: 30% (v/v) acetonitrile in 0.01 M aqueous TFA. Lower curves, UV range 0–0.1 a.u.f.s.; upper curves, 0–1 a.u.f.s.

In Fig. 5 the change in peak shape and retention with increasing sample size is demonstrated for lactalbumin. A RP-18 column (Si 500) and 30% acetonitrile in 0.01 M TFA was used. If the sample sizes are increased from 4 to 125 μg, the retention times decrease from more than 10 min ($k' = 6.5$) to ca. 3 min ($k' = 1.5$). As expected for overloading curves, the peaks eluted for smaller sample sizes fit in the tail end of the peaks for higher sample sizes. This can be explained by a strong curvature of the adsorption isotherm, caused by inhomogeneous surface properties at different sites and with different energies of interaction. In the upper part, at lower detection sensitivity, it is quite obvious that with 1 mg sample the surface area is almost totally covered with solute and the sample breaks through ($k' = 0.5$).

Similar behaviour was observed for other stationary phases, eluent compositions and proteins. As expected, the decrease in retention time is extremely pronounced for the Monospher column. Here, the k' values decrease from ca. 70 for 1 μg to zero for 100 μg lactalbumin. The strongly basic lysozyme shows a behaviour similar to that of the acidic lactalbumin both in RPC and HIC. This demonstrates that this effect may not be due exclusively to overloading of residual silanol groups on the surface.

If the k' values, as determined from the peak maximum and not—as would be more correct—from the centre of mass, are plotted in the typical semi-logarithmic plot, a linear decrease of k' with sample size is observed. Fig. 6 shows the two different curves obtained for isocratic measurements with two different eluent compositions. The lower curve corresponds to measurements taken with 31% acetonitrile, the upper curve with 30% acetonitrile. The slopes are a function of the k' values. The linear sections of both curves cross at a retention value close to zero. Dividing the slopes by their corresponding ordinate intersections leads to a similar value of $-0.4 \, k'$ units per decade of concentration change at normalized retention. Increasing the sample size by a factor of 10 from 1 to 10 μg gave a decrease in the k' value from 1 to 0.6 or from 10 to 6.
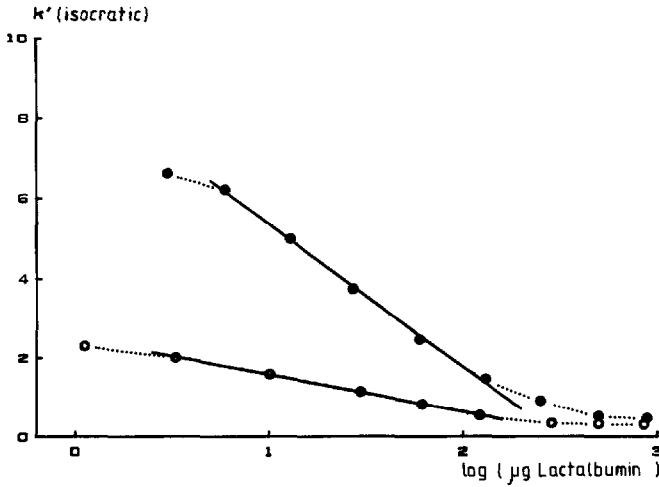
Fig. 6. Decrease in retention time for lactalbumin with sample size in reversed-phase chromatography. Isocratic elution; column and eluent as in Fig. 5. Reduced slopes: 30% acetonitrile; −0.40 (upper); 31% acetonitrile, −0.38 (lower).

Similar to Fig. 6 which shows the dependence of k' on sample size for one protein at different eluent compositions, similar reduced slopes were also obtained for different proteins on the same stationary phase. In Fig. 7 the dependence of k' on sample size is shown for three different proteins of similar molecular weight in an HIC system. The normalized slopes are very similar. The three curves do not cross close to zero but at a k' value of 1.1. If this k' value is deducted from the extrapolated one, the reduced slope is identical, −0.26, for all three proteins. This means that the slope is
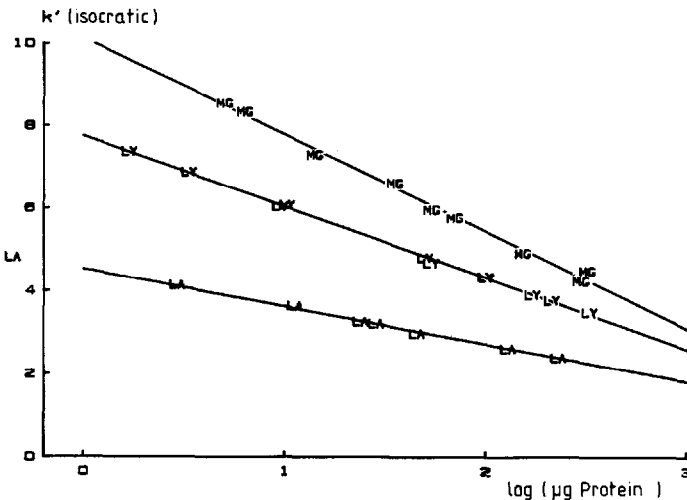

Fig. 7. Dependance of k' on sample size in HIC. Isocratic elution. Column: Nucleosil 300, 0.9 $\mu mol/m^2$ carbamate. Eluent: phosphate buffer, pH 7, with different concentrations of ammonium sulphate. Samples: MG = myoglobin, reduced slope −0.33; LY = lysozyme, reduced slope −0.22; LA = lactalbumin, reduced slope −0.20.

not only proportional to k', but increases linearily with k'. It might be possible to deduce from this slope a characteristic figure for each stationary phase.

The knowledge of this normalized dependence of k' on sample size is also important because it would be extremely difficult to compare various stationary phases under identical retention conditions. Achieving the desired k' value would require a tedious adjustment of eluent conditions. The advantage of the use of this reduced slope for stationary phase comparison is demonstrated in Fig. 8. Here, the dependence of k' on sample size is shown for four HIC columns, packed with stationary phases of different hydrophobicities. The most polar column is an acetamide column. By stepwise reaction with increasing amounts of carbamate the hydrophobicity was successively increased[14]. Stationary phase 3 was the most hydrophobic one, containing 2.2 μmol of carbamate per m^2. The relative location of the three curves in this diagram depends on the individual eluent compositions which were adjusted to get reasonable k' values.

As is seen, the normalized slope decreases with increasing hydrophobicity. This indicates that the loadability increases and that the k' values are consequently less influenced by sample size when the stationary phase hydrophobicity is increased. Increasing hydrophobicity means in this case also increasing density of carbamate groups at the surface. For proteins, this may also mean increased surface homogeneity. Surface homogeneity may be the reason why the normalized slope for the pure acetamide column—homogeneous surface coverage with a single functional group—is also relatively low (-0.17). However, this comparison should be made with some caution. The surface area of the silica support was three times as large as in the case of the mixed stationary phases. On the other hand, the column length was only one third of that for the mixed stationary phases. The surface area available for sorption in each column was, therefore, approximately the same. The slope for the acetamide phase was also only half of that for the reversed-phase systems discussed above. Consequently, the normalized slope of k' vs. log sample size may be a measure of the
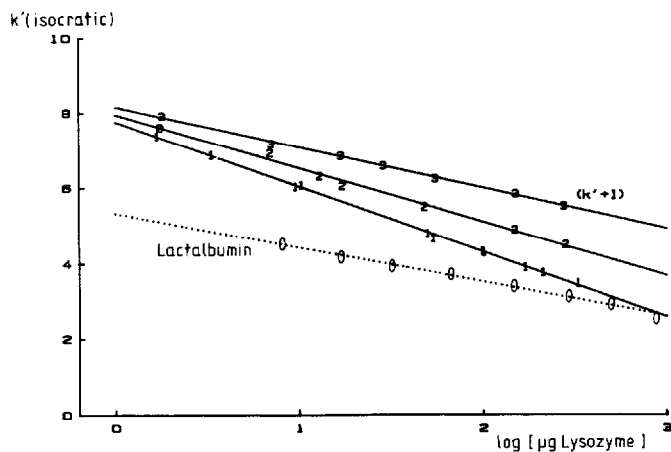

Fig. 8. Dependence of k' on sample size with different stationary phases for HIC. Curves: 1–3, lysozyme with acetamide–carbamate phases, 0.9, 1.8 and 2.2 μmol/m^2 carbamate; 0, lactalbumin with acetamide phase. Phosphate buffer, pH 7, retentions adjusted by addition of ammonium sulphate. Reduced slopes: 0 = -0.17; 1 = -0.22; 2 = -0.18; 3 = -0.17.

stationary phase hydrophobicity and homogeneity. However, further experiments are necessary to prove this.

Other separation systems

So far, only separation systems have been discussed in which hydrophobic and solvophobic interactions contribute to the retention. One of the main techniques for protein separation is ion-exchange chromotography. With the silica-based strong anion exchangers studied, the peak shapes and retention times also depend strongly on the sample size, as demonstrated in Fig. 9 for lactalbumin in the concentration range between 10 and 200 μg. The peaks are plotted in a manner similar to that used in Fig. 5, but in this case taken from gradient elution experiments[15]. Up to 50 μg lactalbumin the retention seems to be almost independent of sample size, but at higher concentrations a decrease in elution time is noticeable. The only difference between this plot and that in Fig. 5 is that here the tailing ends of the peaks do not fit in a common curve. This may indicate that retention of the protein may occur not only by electrostatic interactions. It is conceivable that, with this stationary phase, at least three different interactions may occur at different locations: the ion-exchange groups, the polar functionalities of the bonded groups and—last but not least—silanophilic interactions with residual surface silanol groups.

Proteins are always good for surprises. With polymer-based stationary phases, *e.g.*, a Waters Protein PAK DEAE-5PW column or polymer-coated phases, *e.g.*, a Baker PEI protein column the retention time of lactalbumin increases with increasing sample size, as demonstrated in Fig. 10 for the polymer-based anion exchanger. Here, the separation mechanism is also anion exchange, but no additional interaction with the matrix seems to contribute to retention. However, this does not explain why the retention increases with increasing sample size. Aggregate formation of the protein at the surface may be one explanation.
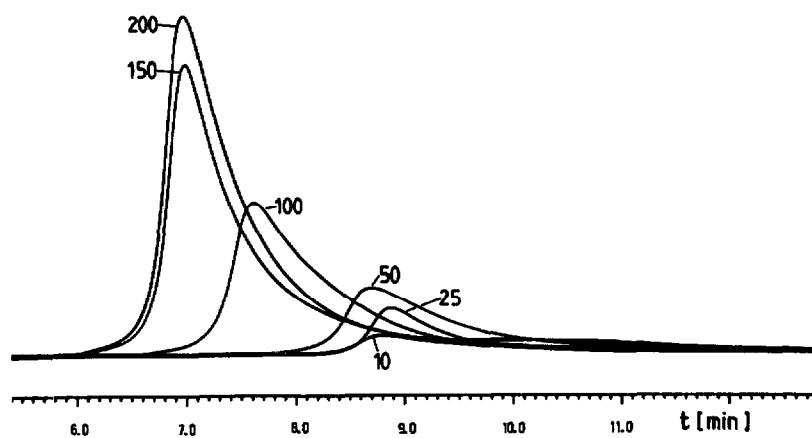

Fig. 9. Retention and sample size in ion-exchange chromatography. Stationary phase: SAX on Nucleosil 300. Eluent: 0.02 *M* phosphate buffer, pH 7; from 0 to 0.6 *M* sodium chloride in 30 min. Sample size range: 10–200 μg lactalbumin, as indicated.

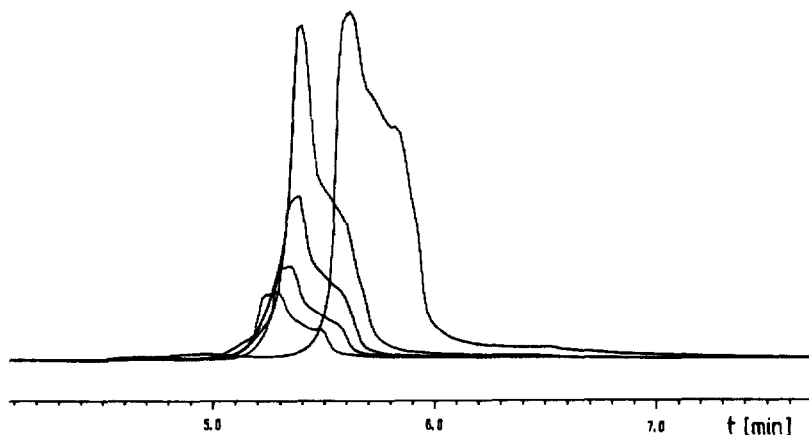Fig. 10. Retention and sample size in ion-exchange chromatography. Stationary phase: Waters Protein PAK DEAE-5PW; eluent and samples as in Fig. 9.

Studies with polystyrenes

In the course of our studies[16] on precipitation chromatography of synthetic polymers[17] a similar influence of sample size on retention was observed for polystyrenes. Usually, in precipitation chromatography a solution of a synthetic polymer is injected into a chromatographic system with an eluent in which the polymer is insoluble. For polystyrenes, this may either be an alkane, like heptane, or an alcohol, like methanol. The polystyrene is precipitated on the stationary phase and elution is achieved in both cases by a gradient to a dissolving eluent which can be dichloromethane. In the system, where heptane is the precipitating agent and dichloromethane is the dissolving eluent, retention times decreased with increasing sample size, Fig. 11. An additional contribution of adsorption to polymer retention was observed[18]. This has been confirmed by comparing the dichloromethane concentration required for elution and the results obtained by turbidimetric titrations of the polystyrenes. In turbidimetric titrations, if polystyrenes have been precipitated by the "weaker" agent
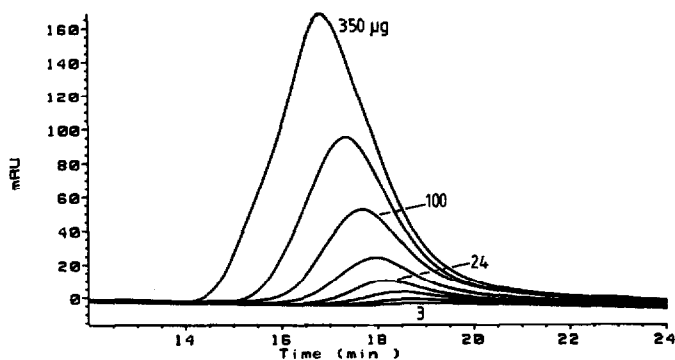

Fig. 11. Precipitation chromatography of polystyrene (mol. wt. 200 000 daltons). column: RP-18, Si 100; gradient, n-heptane to dichloromethane in 20 min.

heptane for redissolution of the polymer, usually lower concentrations of dichloro-methane are required compared to the cases where the "harder" precipitation agent methanol has been used. In those cases, where solubility is the only mechanism and the polymers are not additionally retarded by adsorption on the stationary phase surface, the eluent composition, at which the polymer is eluted, should be identical with the solvent composition at which the polymer is precipitated in turbidimetric titrations. In Fig. 11, for elution a higher dichloromethane concentration is required than expected from turbidimetric titrations. Although a typical normal phase gra-dient (heptane to dichloromethane) was used, adsorption occurred even on alkyl bonded stationary phases, due to interactions with the residual silanol groups. Conse-quently, overloading and, hence, a decrease in retention time with increasing sample size is feasible. This corresponds to the cases in protein chromatography demon-strated in Figs. 5 and 9. The influence of sample size on retention time was much less pronounced with normal phases, *e.g.*, silica columns, due to higher loading capacities. Additionally, higher concentrations of dichloromethane were required with these columns.

Exactly the opposite influence of sample size on retention times was observed when the stronger precipitating agent methanol was used. As demonstrated in Fig. 12, the retention times increased with increasing sample size. Interaction with the surface area is not expected in this case. The eluent composition (methanol–dichloro-methane) at which the polymer is eluted is, over a wide molecular weight range, identical with the solvent composition at which the polymer is precipitated in turbidi-metric titrations[19]. No influence of the type of the stationary phase used was ob-served. As a consequence, polymer solubility must be the only separation mechanism and no additional adsorption on the stationary phase surface contributes to retention. One plausible explanation for this behaviour is that adsorption effects are diminishing at the high concentrations of the strongly eluting agent dichloromethane that are required for dissolution. The increase in retention times, and hence dichloromethane content, with sample size might be due to polymer–polymer interactions and corre-sponds to the results of the influence of the amount of polystyrenes in turbidimetric titrations[20]. Additionally, the polymers were eluted as colloidal suspensions[21]. This was evidenced by the fact that the peak areas were larger in this elution system than
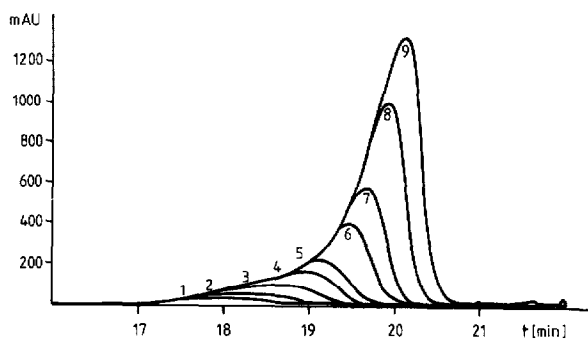

Fig. 12. Precipitation chromatography of polystyrene (MW 110 000 daltons). Influence of sample size. Column as in Fig. 11. Gradient: methanol to dichloromethane in 20 min. Sample sizes: 12.5 (1) to 400 μg (9).

when the same amount was injected in size-exclusion chromatography (SEC), where true solutions are eluted. Also the polystyrenes were detected in this case even in the visible region: the "absorption" is caused by light scattering by this colloidal solution.

CONCLUSIONS

For proteins and for synthetic polymers it was observed that the chromatographic resolution is strongly dependent on the sample size. The retention time and peak width are both affected by increasing sample size. No range was found where the retention is independent of the sample size. Even in gradient elution, variations in elution time by several percent must be dealt with. For the identification of solutes from their chromatographic retention, one must define a retention window and a corresponding concentration range in which one can work with a given system. Because of the linear dependence of retention on sample size in a semi-logarithmic plot, it is possible to calculate for each given system the change in retention time when the sample size is increased ten-fold. The change in retention is identical whether one works at low or at high concentrations.

The peak broadening and, hence, chromatographic resolution is also affected by sample size. For stationary phases with small surface areas, like the new nonporous materials with particle diameters of *ca.* 1.5 μm, the efficiencies one might expect from these materials are soon lost with increasing sample size. At a load above 100 μg the efficiencies of classical porous materials with particle diameters of *ca.* 5 μm are approached.

The dependence of retention on sample size is a function of the surface inhomogeneity. The strongest dependences on sample size are observed with reversed-phase and HIC systems. However, it is not the surface coverage that is important, but the homogeneity of the surface with which the proteins interact.

For stationary phases, where either the silica matrix has been covered or organic polymer matrices are used, surprisingly an increase in retention with sample size has been observed. Comparison of this behaviour with similar observations made in the chromatographic separation of polystyrenes leads to the assumption that proteins are also eluted as aggregates at high sample concentrations. In chromatography of proteins aggregate formation has been observed[22]. In the other cases, where multiple interaction with different active sorption sites is expected with both groups of polymers studied (synthetic and natural), the retention decreases with increasing sample size.

Further studies may lead to new types of stationary phases on which retention of polymers is, at least for a given concentration range, independent of sample size. In the meantime one must be cautious in the qualitative identification of proteins in unknown mixtures and in preparative scale work. Knowledge of the influence of sample size on retention is paramount and must be determined for each individual separation system.

ACKNOWLEDGEMENTS

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