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Effects of temperature, carrier composition and sample load in asymmetrical flow field-flow fractionation

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ABSTRACT

The use of asymmetrical flow field-flow fractionation for accurate sample characterization requires good control of side effects which can adversely influence sample retention. Some of these factors are identified and means to avoid or minimize them are given. The behaviour of a model protein and virus was investigated. Bad reproducibility of the retention times and peak deformations were traced to incomplete temperature control and adsorption to the accumulation wall membrane. Good thermostatic control gave constant retention times because it eliminated temperature variations caused by friction heat produced in the carrier stream. Rinsing of the channel removed adsorbed species. The mechanism involved in the adsorption is unknown but the pH of the carrier liquid appears to have an influence. Exceeding the sample load limit leads to a complex behaviour of peak deformations. This behaviour as well as the load limit is governed by the choice of buffer and ionic strength of the carrier liquid.

INTRODUCTION

Field-flow fractionation (FFF) is a family of fractionation techniques designed for macromolecular and particle separation and characterization [1]. The common characteristic for all of the FFF sub-techniques is the parabolic flow profile which is created by a carrier liquid being pumped through the open, thin flat channels. Retention is effected by a field applied perpendicular across the channel. A number of different fields such as sedimentation, thermal gradient, electrical and crossflow can be used [2].

Asymmetrical flow FFF is a variant of FFF utilizing a crossflow field [3]. The crossflow is created as the carrier liquid, that is pumped through the channel, exits through the porous accumulation wall as well as through the channel outlet. The

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accumulation wall is made up of an ultrafiltration membrane making it possible for the carrier liquid to permeate the wall while the sample molecules are retained. The separations obtained are basically size fractionations since they depend on differences in the diffusion coefficients.

Previous papers [4–6] have reported on improvements in the asymmetrical flow FFF technique leading to faster and more efficient separations. The improvements include a downstream central injection technique, thinner channels and a new trapezoidal channel geometry.

While the construction and design of channels has advanced there is still very limited knowledge of the behaviour of samples under varying conditions for the elution. An accurate characterization of a sample, *e.g.*, by its diffusion coefficient, or after transformation, by its molecular weight or its distribution, requires that sample retention conforms to retention theory in a predictable way under all conditions used.

Experience gained during the development of the method has indicated a number of potential error sources such as variations in the retention time and band broadening during an extended sequence of repeated fractionations. The objective of the present paper is therefore to describe these problems, identify their origin and possibly find means to avoid or minimize them. Careful studies revealed that the problems mentioned often originate in unsuitable experimental conditions which therefore should be corrected. Without such knowledge attempts to run flow FFF may result in failures.

The factors studied are temperature effects, adsorption to the accumulation wall membrane, the reproducibility of measured diffusion coefficients and the influence of the carrier liquid composition on sample loadability. Some general guidelines for the optimization of the system will also be given.

Since the diffusion coefficient directly governs the retention time it must be important to perform separations under constant temperature. This is necessary in order to keep the diffusion coefficients constant so as to obtain good accuracy and reproducibility in sample retention and characterization. An increase in temperature will increase the diffusion coefficient. Repeated sample fractionations performed with no thermostatic control will be shown to cause gradually decreasing retention times. This effect will be traced to an increasing channel temperature originating in frictional heat produced within the system. Good thermostatic control of the equipment is therefore necessary, at least when using high flow-rates.

One property of the FFF techniques that is often brought forward as an advantage is the non-existence of a stationary phase. It has been assumed that difficulties such as surface interactions often associated with the stationary phase in chromatography may therefore be avoided in FFF. There are, however, numerous opportunities for the solutes to interact with the channel walls, particularly the accumulation wall, and this has to be considered. This is becoming increasingly important as the channel thickness is reduced in order to shorten elution times and to improve efficiency. Such conditions will drive the solutes into more concentrated layers and closer to the accumulation wall. Thus the possibilities of undesired interactions with the wall, and also within the concentrated solute zone, are enhanced. Extended use of a channel will be shown to affect the retention time and peak symmetry, presumably by adsorption. A rinsing procedure is suggested.

Problems associated with sample mass overloading of FFF channels is one of the

major drawbacks of the FFF techniques. The effects have been shown to vary with sample components and carrier liquid composition. In sedimentation FFF collodial particles in solutions of ionic strength 10^{-3} M or lower have been reported to be eluted earlier than predicted with a tailing peak shape [7]. For polymers in organic solvents the opposite effect has been reported, *i.e.*, the overloaded peaks were fronting, emerging from the channel later than predicted [8]. In asymmetrical flow FFF a buffer of ionic strength 0.1 M resulted in plasmids and plasmid fragments being eluted with a fronting peak symmetry or with completely distorted peaks when the channel was overloaded [4]. At the same ionic strength water-soluble polymers were observed to emerge later than predicted when the load limit was exceeded [5]. All of the above-mentioned effects were observed in this study, *i.e.*, sample overloading can lead to either retained symmetry, fronting or tailing of the peaks accompanied by increased zone broadening, and increased or decreased retention times.

THEORY

The theory has been described in detail elsewhere [3,6] and only the more important equations will be defined below.

The retention ratio, R, is calculated from the ratio of the void time, t_0, to the retention time, t_R, or from the ratio of the sample zone velocity, V, to the channel flow velocity, $\langle v \rangle$ [3]

$$R = \frac{t_0}{t_R} = \frac{V}{\langle v \rangle} \tag{1}$$

The void time is calculated from [6]

$$t_0 = \frac{V^0}{\dot{V}_c} \ln \left[1 + \frac{\dot{V}_c}{\dot{V}_{out}} \left(1 - \frac{w\{b_0 z' - [(b_0 - b_L)/2L]z'^2 - y\}}{V^0} \right) \right] \tag{2}$$

where V^0 is the void volume, $\dot{V}_c$ is the crossflow-rate, $\dot{V}_{out}$ is the channel outlet flow-rate, z' is the distance from the inlet to the focusing point, and L is the channel length. b_0 and b_L are the breadths of the channel at the inlet and outlet, respectively, when the tapered ends are not considered, and y is the area cut off by the tapered inlet end (see Fig. 1). The void time equation is valid for $z' \geqslant z''$ where z'' is the length of the tapered end.

The retention ratio can be expressed in terms of the transversal distribution of the sample zone, *i.e.*, by λ, which is a dimensionless measure of the distance of a sample zone from the accumulation wall. λ is defined by the ratio l/w where l is the average distance of the zone from the wall and w is the channel thickness. The retention ratio is a complex function of λ [3] and a calculation of λ from experimental retention ratios requires numerical integration. However, at high retention levels the retention equation can be approximated by [3]

$$R \approx 6\,\lambda \tag{3}$$

and it is a simple matter to calculate λ from R values. The diffusion coefficient, D, of a sample can be calculated from the λ value

$$D = \frac{\lambda \dot{V}_c w^2}{V^0} \tag{4}$$

Alternatively, of course, if the flow conditions and the diffusion coefficient are known, the retention time can be predicted from

$$t_R = \frac{t_0 \dot{V}_c w^2}{6 D V^0} \tag{5}$$

The diffusion coefficient for a spherical particle with radius r can be calculated from Stokes' equation:

$$D = \frac{k T}{6 \pi \eta r} \tag{6}$$

where k is the Boltzmann constant, T the temperature and η the viscosity coefficient.

EXPERIMENTAL

Two different channels were used. The design was basically the same as described previously [3–6]. However, one of the channels was modified to allow for thermostatic control.

The channels differ only in the material and design of the upper wall. In one it was a glass plate and in the other it was made of Lucite. Both walls had a length of 36 cm and a breadth of 6 cm. The thickness was 1 cm for the glass wall and 2 cm for the Lucite wall. The latter was equipped with an internal channel to permit the passage of thermostated water. During operation thermostated water was continuously pumped through the wall to help control the temperature of the channel. In both walls PTFE tubing for the carrier inlet and outlet flows were positioned 28.5 cm apart. The injection inlet was positioned 2.0 cm downstream from the carrier flow inlet. The channel with the glass wall was only used for the initial studies documenting the temperature changes occurring and is referred to as the "non-thermostated channel". In all other studies the thermostated channel with the Lucite wall was used.

The ultrafiltration membrane was a cellulosic DDS membrane type RC70PP with a nominal cut-off of 10 000 dalton (De danske suckerfabrikker DDS RO-division, Nakskov, Denmark).

A PTFE spacer (fluorinated ethylene propylene, FEP) of thickness 0.013 cm was used to define the channel thickness. The channel geometry was trapezoidal (Fig. 1). The length was 28.5 cm, the breadth at the inlet, b_0, was 2.12 cm, and the breadth at the outlet, b_L, was 0.47 cm. The length of the tapered end at the inlet was 2.0 cm and at the outlet 0.5 cm. The area, y, cut off at the inlet, was 2.12 cm^2. The geometrical void volume of the channel was 0.45 ml.

Two pumps were used. A Beckman 114 M solvent delivery system (Beckman

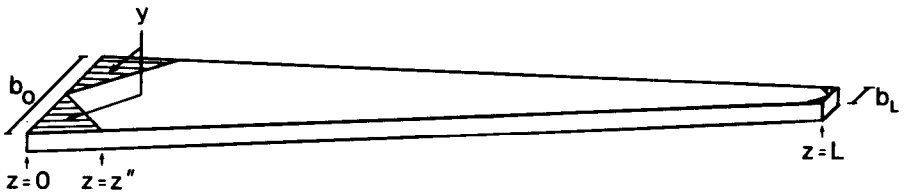

Fig. 1. Geometry of the trapezoidal channel.

Instruments, Berkeley, CA, USA) delivered the carrier flow, and the injection flow was delivered by an LKB 2150 HPLC pump (Pharmacia LKB Biotechnology, Bromma, Sweden). An LDC SpectroMonitor III spectrophotometric detector (LDC, Riviera Beach, FL, USA) set at 280 nm was used for the detection. A Rheodyne 9125 syringe injector (Cotati, CA, USA) was used with a sample loop of 20 μl. The system was designed according to a previous procedure [5] and the same valves were used. One additional three-way valve was inserted between the pressure gauge valve and the inlet. This valve was used during the backwards flushing and the rinsing procedures.

Temperature control was conducted by a Haake type F water-bath (Haake, Berlin, Germany). The water was pumped through a mantled carrier reservoir and the upper wall. The temperature was kept at 24.0°C. For the temperature study the entire channel was placed in a thermostated water bath (Heto, Birkeröd, Denmark).

Ferritin was obtained from a gel filtration calibration kit (Pharmacia, Uppsala, Sweden). Human serum albumin (HSA) Fraction V was from Sigma (St. Louis, MO, USA). The Cow Pea Mosaic Virus (CPMV) was a generous gift from Dr. P. Oxelfeldt at the Agricultural University (Uppsala, Sweden).

The retention times were measured from the peak apices and the void times were determined from eqn. 2. Calculations of the λ values from the retention ratios were made on a personal computer by numerical integration using Simpson's rule. To measure the asymmetry factor (asf) at 10% of the peak height, a perpendicular was drawn from the apex. The back part of the peak divided by the front part of the peak, both measured at 10% of the peak height, gives the asf.

The operation of the system followed previous procedures [3,5,6]. The relaxation/focusing procedure was carried out for 45 s using a pump flow-rate of 3 ml/min. The focusing point, z', was adjusted to 2.5 cm. Sample injection was made with a flow-rate of 0.1 ml/min for 30 s. The sample loop had a volume of 20 μl.

Channel rinsing between runs was effected by flushing backwards for 1–2 min. During the back flush the three-way valve at the inlet was switched to direct the liquid from the channel to waste. A more thorough cleaning of the membrane was obtained if the channel was filled with air before the back flush. In this case air was introduced into the channel from a Luer syringe attached to the additional three-way valve at the channel inlet. At the same time the channel outlet tubing was disconnected from the detector. The channel was then flushed with carrier liquid using the same Luer syringe. A very high flow-rate was obtained and this easily removed the air from the channel. This was done after fractionation of highly retained materials.

RESULTS AND DISCUSSION

The retention characteristics of the proteins albumin and ferritin, and the spherical virus CPMV were studied under different experimental conditions in a number of different carriers (Table I). The molecular weights of albumin, ferritin and CPMV are 67 000, 440 000 and $5.5 \cdot 10^6$, respectively. The isoelectric point for albumin is 4.7–4.9 [9], for ferritin 4.1–4.6 and for CPMV 3.7–4.5 [10].

Temperature effects

According to Stokes' equation the diffusivity of molecules will depend upon the temperature in two ways (eqn. 6). One is due to the increase in kinetic energy (kT) along with an increase in temperature. This effect is of relatively small magnitude for modest temperature changes. The other, and more important in this case, is due to the decrease in the viscosity of water which occurs at increasing temperatures. Calculations based on the known viscosity of pure water [11] show that an increase of the temperature by 1°C will increase the diffusion coefficient by almost 3% at the temperature levels used in this study. It is thus clear that good temperature control is mandatory in order to obtain consistent and reproducible retention data.

A good temperature control is even more important in the present apparatus when it is used for rapid separations requiring high flow-rates. Somewhat surprisingly it was found that in a non-thermostated channel having an upper glass wall the temperature increased by several tenths of a degree centigrade as soon as the relaxation flow was turned on. This was measured by a thermometer in contact with the glass wall and positioned next to the outlet tubing in the bore drilled through the upper Lucite block. The bore contained some water which improved the contact between the tip of the thermometer and the glass wall. During a day of operation a continuously increasing channel temperature was observed. Thus repeated fractionations of a sample led to shorter and shorter retention times caused by the increasing diffusion coefficients of the sample molecules. The temperature increase was apparently caused by frictional heat produced in the tubing.

Manipulation of the temperature can be made to an advantage. An increase in temperature will increase the diffusion coefficient. For ultra-high-molecular-weight materials, which, due the low levels of their diffusion coefficients, tend to give high zone broadening, a temperature increase may help in obtaining more efficient separations.

TABLE I

LIST OF CARRIER LIQUIDS USED FOR THE FRACTIONATIONS

Carrier liquid	pH	Ionic strength (M)
(I) 0.02% NaN_3	6.9	0.003
(II) 0.02% NaN_3, Tris–HNO_3 buffer	7.5	0.008
(III) 0.02% NaN_3, Tris–HNO_3 buffer, 1 mM glutamic acid	6.3	0.009
(IV) 0.02% NaN_3, Tris–HNO_3 buffer	7.5	0.1
(V) 0.02% NaN_3, 0.1 M NaCl	6.5	0.1
(VI) 0.02% NaN_3, citrate buffer	4.8	0.008

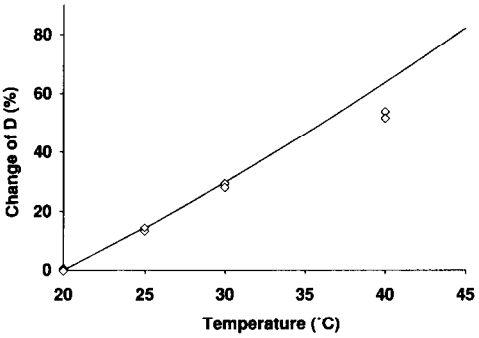

Fig. 2. Percentage increase of the diffusion coefficient, D, relative to D at 20°C. ——— = Prediction according to eqn. 6; ◇ = experimental determination. Sample: CPMV, 0.7 μg in 20 μl. Elution: $\dot{V}_{in}$ = 5.9 ml/min, $\dot{V}_c$ = 5.50–5.90 ml/min, $\dot{V}_{out}$ = 1.00–1.40 ml/min, where $\dot{V}_{in}$ is the channel inlet flow-rate. Carrier II.

A test of the effect of temperature on the experimentally determined diffusion coefficient of the virus CPMV was done in the temperature range 20–40°C. The relative increase in the diffusion coefficient was compared to that predicted by Stokes' equation, assuming that the viscosity of the carrier liquid varies with temperature as in pure water. During this study the channel was kept in thermostatically controlled water bath, since it is not sufficient to thermostat only the upper wall when working at elevated temperatures.

Fig. 2 shows that in the temperature interval from 20 to 30°C the increase in the experimentally determined diffusion coefficient agrees well with that predicted by Stokes' equation. The diffusion coefficient at 40°C was, however, somewhat lower than expected. In this case the temperature of the water bath was at 40°C but the temperature measured on the upper wall by the outlet tubing was only 37°C. Therefore the temperature within the channel was probably somewhere between 37 and 40°C, which might explain part of the deviation.

Membrane effects

Practical experience shows that the retention times gradually increase and the peak efficiencies decrease, followed by tailing, when the channel is used without any rinsing procedures. So far this applies to all channels that have been tested, and to all of the carrier liquids in Table I. In order to study this effect the virus CPMV was injected repeatedly at conditions of very high retention without using the rinsing procedure between each run. At very high retentions the relative concentration by the accumulation wall is high and thus the possibility of interactions with the wall is enhanced. Fig. 3 shows that the retention times increases, as expressed by the decrease in the observed diffusion coefficient, at the same time as the peak efficiency decreases for each consecutive sample injection. After using the rinsing procedure, *i.e.*, the channel is first flushed with air and then with the carrier liquid, the performance of the first injection could be reproduced (Fig. 3). The peak efficiency was expressed by the plate number defined in the usual way according to

$$N = \frac{t_R^2}{\sigma^2} \tag{7}$$

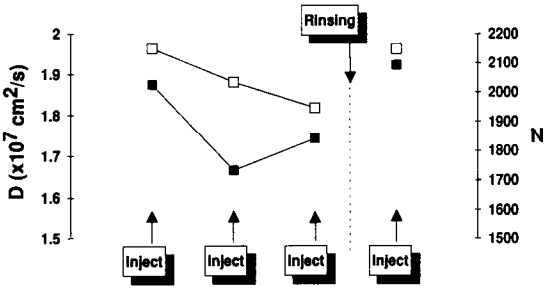

Fig. 3. Influence of channel rinsing on high retention fractionations. Sample: CPMV, 0.35 μg in 20 μl. Elution: $\dot{V}_{in}$ = 9.0 ml/min, $\dot{V}_c$ = 7.80–8.00 ml/min, $\dot{V}_{out}$ = 1.10–1.40 ml/min, t_0 = 0.11 min, R = 0.02. Carrier IV. After the third injection the rinsing procedure was applied. □ = Observed diffusion coefficient (D); ■ = efficiency (N).

where τ is the standard deviation of the peak in time units. Fig. 4 shows a CPMV peak obtained after the channel had been subjected to the rinsing procedure. The peak is symmetrical with no indication of tailing.

A simpler rinsing procedure, involving only a backwards flushing with the carrier liquid while the crossflow was shut off, did not give complete recovery of channel performance. Apparently the introduction of air in the channel is vital in order to obtain complete rinsing. Observations during the rinsing procedure revealed that after the air was pumped into the channel, so that the latter is emptied from carrier liquid, the carrier liquid penetrates the membrane from below and re-enters the channel when the excess pressure disappears. This may suggest that a backwards flushing of the membrane by reversing the crossflow could help in rinsing the membrane. Such a procedure was, however, impossible to perform because it displaced the membrane from the support causing it to contact the upper wall so that the flow was interrupted. It may well be possible that the introduction of air is the key step in

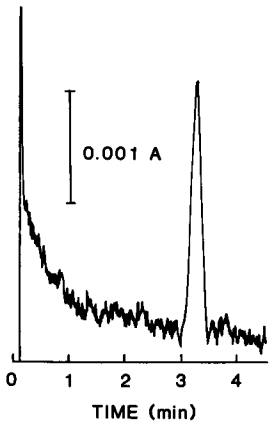

Fig. 4. Elution of CPMV. Load 0.28 μg in 20 μl. Relaxation/focusing: $\dot{V}'_c$ = 3 ml/min for 30 s and 7 ml/min for 15 s. Elution: $\dot{V}_c$ = 7.60 ml/min, $\dot{V}_{out}$ = 2.18 ml/min, t_0 = 0.085 min. Carrier IV.

recovering the membrane properties, perhaps by somehow changing the interaction between the solute and the membrane.

There are several possible causes for the peak distortion and excess retardation found in all carrier liquids when the channel is not rinsed regularly. First, the sample can be physically entrapped in the pores. This may potentially lead to increased flow resistance across the membrane. This is, however, contradicted by the fact that there are indications that the permeability of the membrane is increased by the presence of a sample in the channel. During a series of sample injections, without intermediate washing, the ratio $\dot{V}_c/\dot{V}_{out}$ continuously increases. Because the flow-rates $\dot{V}_c$ and $\dot{V}_{out}$ are controlled by the ratio of the restriction to flow in each flow-line [3] this effect can be interpreted as being due to an increased permeability of the membrane leading to an increase of $\dot{V}_c$ at the cost of $\dot{V}_{out}$. Second, higher-molecular-weight compounds of unknown origin may be left in the channel, interacting with the samples in the next injection. This is, however, unlikely because then it should be sufficient to flush the channel while the crossflow is shut off. The last statement suggests rather that the need

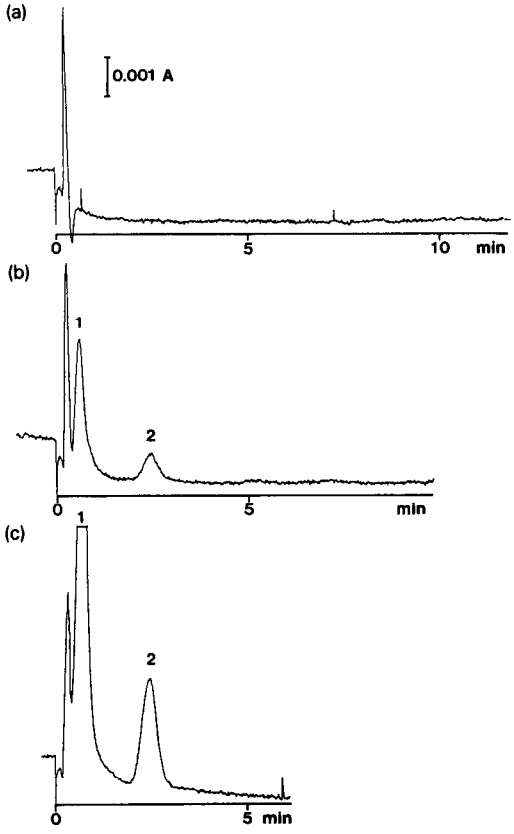

Fig. 5. Behaviour of CPMV and albumin in the citric acid buffer. (a) Sample: CPMV, 0.35 μg in 20 μl. (b) Sample: HSA, 5 μg in 20 μl. Peaks: 1 = HSA; 2 = CPMV. (c) Sample: HSA, 10 μg and CPMV, 0.7 μg in 20 μl. Peaks: 1 = HSA; 2 = CPMV. Elution: $\dot{V}_c$ = 4.7 ml/min, $\dot{V}_{out}$ = 1.2 ml/min, t_0 = 0.23 min. Carrier VI.

for the channel to be rinsed is caused by adsorption of solute to the accumulation wall membrane, and this third cause is indeed the more likely one. Adsorption may also explain the increase in membrane permeability discussed above. It may be a result of a change of a property such as the wettability of the membrane. Nevertheless, the washing procedure, which is easy to manage, eliminates most of the above-mentioned problems.

The suspicion that some solutes adsorb to the membrane was further confirmed when the behaviour of CPMV, ferritin and albumin was studied in a carrier liquid buffered by citric acid to a pH of 4.8 (carrier VI, Table I). In this carrier the performance of all three compounds changed for the worse as compared to the other carriers. The protein peaks were tailing also at loads well below the loads that gave symmetrical peaks in the other carriers. The virus sample did not elute at all, it remained within the channel during the entire elution, which was maintained for 11 min (Fig. 5a). After this sample albumin (HSA) was injected. This run not only gave an albumin peak, but also caused elution of the CPMV which had remained in the channel from the previous injection (Fig. 5b). The CPMV peak was symmetrical and was eluted at the retention time predicted from the results in the other carrier liquids. The narrow peak and the retention time indicate that the virus must have been retained at the focusing point. If it were distributed along a large portion of the channel one would have expected a broader peak eluted earlier. The introduction of albumin obviously released CPMV, apparently from the membrane. This was further confirmed by injection of CPMV in a mixture with albumin. The result was two peaks, both at the expected retention times for albumin and CPMV, respectively (Fig. 5c). Once albumin had been present in the channel a subsequent injection of CPMV caused a peak at the expected retention time for CPMV, although a further injection of albumin released yet more CPMV along with the elution of albumin.

The results from the citric acid buffer pH 4.8 may be caused by adsorption to the membrane. The pH of the buffer was close to the isoelectric points of the samples and several authors (*cf.*, refs. 12 and 13) have reported on an enhanced adsorptivity of proteins at pH values around their isoelectric point on several different materials. A similar situation may exist in the flow FFF channel. The elution of CPMV in the presence of albumin indicates that some kind of competitive adsorption takes place between these two samples.

Influence of ionic strength on sample loadability

Fig. 6a and b show the observed diffusion coefficients, calculated from the retention time measured at the peak maximum, as a function of the sample load of CPMV and ferritin, respectively. The data were obtained using carrier liquids differing in their component types and ionic strengths according to Table I. Even if only a limited range of composition types were tested it appears that the ionic strength has a strong influence on the retention behaviour. Carriers II and IV, containing identical component types but having different ionic strengths, result in opposing effects on the observed diffusion coefficient at increasing sample loads. At a low ionic strength, 0.008 *M* (carrier II), an increase in sample load causes an increase of the diffusion coefficient, *i.e.*, a decrease in the retention time. At high ionic strength, 0.1 *M* (carrier IV), the opposite result is obtained. The same tendencies are observed for ferritin and CPMV.

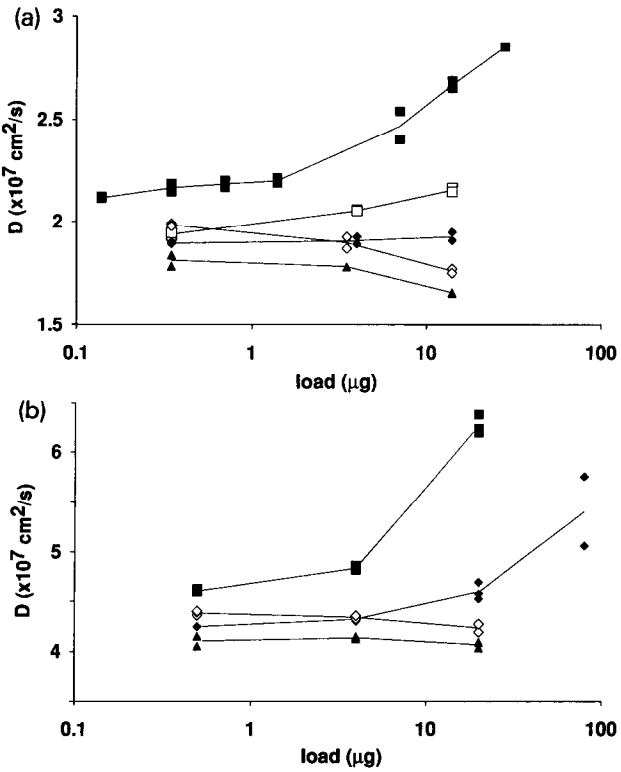

Fig. 6. Effects of sample load on observed diffusion coefficients, D, in different carriers. Carriers (see Table I): ■ = I; □ = II; ◆ = III; ◇ = IV; ▲ = V. (a) Sample: CPMV, 0.14–0.28 μg in 20 μl. Elution: $\dot{V}_{in}$ = 6.4 ml/min, $\dot{V}_c$ = 5.00–5.70 ml/min, $\dot{V}_{out}$ = 0.70–1.50 ml/min. (b) Sample: ferritin, 0.5–80 μg in 20 μl. Elution: $\dot{V}_{in}$ = 9.1 ml/min, $\dot{V}_c$ = 7.40–8.20 ml/min, $\dot{V}_{out}$ = 0.90–1.60 ml/min.

The change in retention time with increasing sample load is accompanied by a change in peak symmetry. This is demonstrated in Fig. 7a and b where the peak asymmetry factors, measured at 10% of the peak height, are given. Again, as with the data in Fig. 6, the ionic strength appears as an important factor. At low ionic strength increased sample loads causes peak tailing (asf >1). On the contrary, at high ionic strength large sample loads cause fronting (asf <1) and for the highest loads even a peak distortion. The appearances of the overloaded peaks in carriers II and IV are shown in Fig. 8. In carrier III neither fronting nor tailing was observed. Yet, the loadability was limited because there was a gradual increase in the peak width. This carrier differs from carrier II only by its content of 1 mM glutamic acid. Glutamic acid is known to reduce the adsorption of proteins to glass [14] and was therefore added as an attempt to affect the particle wall interactions.

Other authors [7,8] who have investigated overloading phenomena have reported on one or the other of the two effects discussed above. The origin of the phenomena is in both cases thought to be caused by too high a concentration in the migrating sample zone. In the case of collodial particles in an aqueous buffer (ionic strength below 10^{-3} M) the overloading was reported to originate in effective

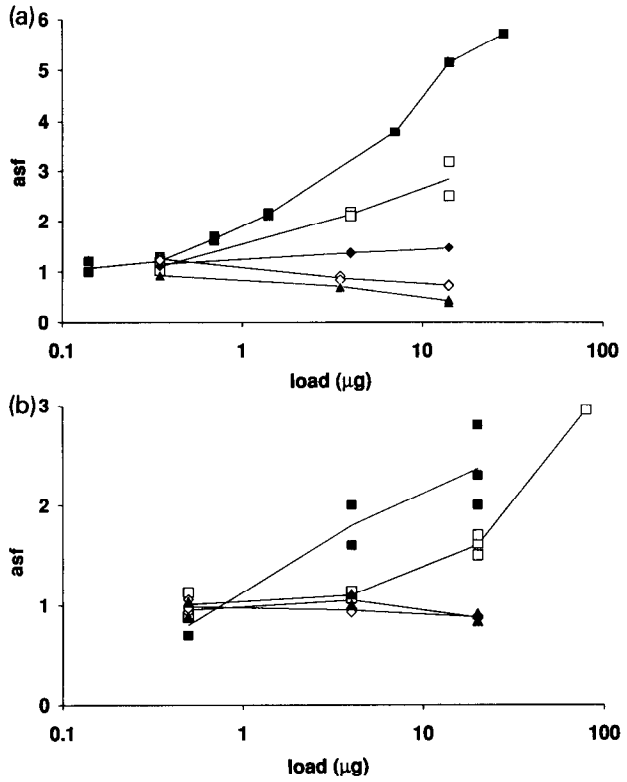

Fig. 7. Effects of sample load on asymmetry factors (asf) in different carriers. (a) Sample: CPMV. (b) Sample: ferritin. Conditions as in Fig. 6.

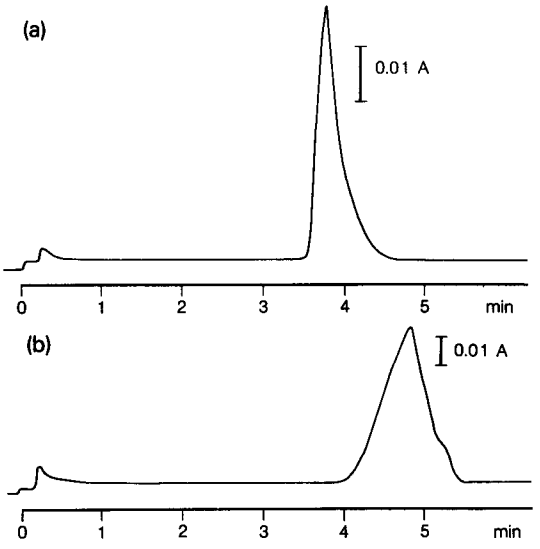

Fig. 8. Appearance of overloaded peaks in different carriers. Sample: CPMV, 14 μg in 20 μl. (a) Carrier II: $\dot{V}_c$ = 5.36 ml/min, $\dot{V}_{out}$ = 1.09 ml/min. (b) Carrier IV: $\dot{V}_c$ = 5.47 ml/min, $\dot{V}_{out}$ = 0.96 ml/min.

repulsion between particles caused by volume exclusion as well as other repulsive effects [7]. This effected the exponential distribution of the particles by the wall causing them to migrate at a larger distance from the accumulation wall than they would at low loads. Therefore the retention time decreased with increasing sample loads [7].

The different behaviour shown by CPMV and ferritin is probably related to the fact that these two samples are polyelectrolytes. This can lead to quite complex solution behaviour as a function of the electrolyte composition of the carrier. Also, a wider range of ionic strengths was studied than in the other reports. Yet in the buffers having the lowest ionic strengths the behaviour was very similar to that previously mentioned [7] and it is reasonable to assume that similar mechanisms are involved. In carrier I the ionic strength was only 0.003 M leading to a modest screening of the charged groups. Therefore the intermolecular interaction may be mainly repulsive. This will effect the particle distribution so that the samples migrate further away from the wall. When the ionic strength is increased the screening of the charged groups will increase and the net interaction may instead be attractive, thus explaining the change in behaviour. More studies are necessary to evaluate these effects in detail.

Guidelines for the choice of experimental conditions

It is clear that the choice of experimental conditions such as membrane material, type of carrier and temperature control are vital to obtain efficient, reproducible and accurate data. If fractionations are performed at very high retentions, where the possibilities for interactions between the solutes and the membrane are enhanced, the channel should be flushed with air and carrier liquid between each run. For proteinaceous samples it may be necessary to chose a pH outside the range of the isoelectric point in order to avoid adsorption tendencies.

To avoid conditions where concentration-dependent retention data are obtained it seems recommendable that the sample load be kept below the maximum load (defined as the highest load for which the diffusion coefficient is still constant and the peak shape symmetrical) which is obtained at conditions which give the highest retention level to be used (equal to the highest crossflow-rate to be used). Then, concentration dependencies should not be observed in experiments done at lower retention levels.

The flow-rate for the relaxation-focusing procedure can be chosen rather arbitrarily. Ideally the crossflow-rate during the relaxation should be set to the same value as the crossflow to be used during the elution. This is the prerequisite for the sample zones to form the correct steady-state distributions. It has, however, been found that the performance is not significantly effected even if the crossflow-rate during the relaxation is kept much lower than during the elution. When such a situation exists a secondary relaxation will occur once the elution is started [15]. Since this process is very rapid its contribution to the overall bandbroadening may be negligible.

In Table II results are shown from runs with CPMV using the same flow conditions during the elution but different conditions for the relaxation. The crossflow-rate during the elution was kept at 7.4 ml/min, corresponding to a λ value of 0.004. In case 1 the relaxation crossflow-rate was kept at 3 ml/min for 45 s. Thus the sample zone was subjected to less than half the force field acting upon the particles during the elution. In the second case the flow-rate was increased to 7.4 ml/min for the

TABLE II

INFLUENCE OF RELAXATION CONDITIONS ON THE ELUTION PERFORMANCE

Sample: CPMV, 0.35 μg in 20 μl. Elution: $\dot{V}_c$ = 7.8–7.9 ml/min; $\dot{V}_{out}$ = 1.3 ml/min. Carrier I.

Relaxation crossflow-rate (ml/min)	Duration (s)	Diffusion coefficient (cm^2/s)
3	45	$2.1 \cdot 10^{7}$
3 + 7.4	30 + 15	$2.1 \cdot 10^{7}$

last 15 s of the relaxation in order to allow for the molecules to adjust to the field strength that would act upon them during the elution. Even though the particles in the first run were subjected to a secondary relaxation after the initiation of the elution the agreement between the calculated diffusion coefficients was good.

For practical reasons it is preferable to use the same relaxation flows for most of the applications. It is also thought to be an advantage to keep the duration of the relaxation and the relaxation flow-rate as low as possible in order to minimize the opportunity for the sample to interact with the membrane. Experience shows that with the downstream central injection technique a relaxation time of 1 min at a flow-rate of 3 ml/min is usually sufficient to assure a complete relaxation.

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